Thrombin-Catalyzed Activation of Recombinant Human Factor V†

Frank G. Keller,‡ Thomas L. Ortel, Mary Ann Quinn-Allen, and William H. Kane*

Division of Hematology−Oncology, Departments of Medicine, Pediatrics, and Pathology, Duke University Medical Center, Durham, North Carolina 27710

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ABSTRACT: Proteolytic activation of human factor V by thrombin results from the cleavage of three peptide bonds at Arg_{709}, Arg_{1018}, and Arg_{1545}. In order to define the functional importance of these sites, mutants with isoleucine substitutions blocking thrombin cleavage at one, two, or all three activation sites were expressed in COS-7 cells. The wild type protein is activated ~10-fold by thrombin or Russell's viper venom (RVV-V). Thrombin cleavage at Arg_{709} alone did not result in an increase in procoagulant activity. Cleavage at both Arg_{709} and Arg_{1018} resulted in an ~3.4-fold increase in activity. Cleavage at these sites was required for rapid cleavage by thrombin at Arg_{1545}, however, which resulted in maximal activation of the factor V molecule. In contrast, isolated cleavage at Arg_{1545} by RVV-V was sufficient for efficient and complete activation of factor V. The effect of isoleucine substitutions at one or both thrombin cleavage sites in a B-domain deletion mutant lacking amino acids 811−1491 was also investigated. The specific activity of all four mutants was ~30% compared to thrombin activated factor V, indicating that these isoleucine substitutions do not drastically alter the structure of the protein and that cleavage at these sites is not required for the expression of partial procoagulant activity.

Proteolytic activation of coagulation factor V is a critical step leading to the formation of the prothrombinase complex and the generation of thrombin during hemostasis and thrombosis (Esmon, 1979; Nesheim & Mann, 1979; Kane & Majerus, 1981; Suzuki et al., 1982). Factor V is an essential component of this complex which consists of factors Va and Xa, calcium ions, and a phospholipid surface (Ortel et al., 1995). Assembly of the prothrombinase complex results in a 300 000-fold increase in the rate of prothrombin activation (Mann et al., 1990). Factor V circulates in plasma as an inactive single chain glycoprotein comprised of repetitive homologous domains with a structure of A1-A2-B-A3-C1-C2 (Figure 1) (Kane & Davie, 1986; Jenny et al., 1987; Kane et al., 1987; Kane and Davie, 1988). The amino terminal heavy chain region contains the first two A-domains whereas the carboxyl terminal light chain region contains the third A-domain and the two C-domains. The heavy chain and light chain regions are separated by a heavily glycosylated and unique B-domain or connecting region.

Limited proteolysis within the B-domain of factor V exposes binding sites for factor Xa (Suzuki et al., 1982) and prothrombin (Esmon et al., 1973), which allows formation of the prothrombinase complex. Thrombin appears to be the predominant activator of factor V, since factor Va formation is blocked by the thrombin inhibitor hirudin in an *in vitro* plasma based system (Pieters et al., 1989). Feedback activation of factor V by thrombin results from cleavage following arginine residues at positions 709, 1018, and 1545 (Jenny et al., 1987). Thrombin activated factor Va is a calcium dependent heterodimer consisting of the heavy chain and light chain fragments (Esmon, 1979; Suzuki et al., 1982). During activation by thrombin most of the heavily glycosylated B-domain is released as two large activation fragments (Esmon, 1980; Kane & Majerus, 1982). Ultrastructural studies of factor V and thrombin activated factor Va indicate that the heavy chain and light chain regions form a 10−12 nm diameter globular domain while the B-domain forms a rod-like tail extending up to 34 nm in length (Mosesson et al., 1990; Fowler et al., 1990).

Factor Xa also has been reported to fully activate human factor V in a reaction that requires calcium and a phospholipid surface (Monkovic & Tracy, 1990a). Factor Xa cleaves factor V at or near Arg_{709} and at Arg_{1018}, resulting in release of the amino terminal portion of the B-domain (Monkovic & Tracy, 1990a). In contrast, a factor V activator isolated from Russell's viper venom (RVV-V)[1] activates factor V by a single proteolytic cleavage following Arg_{1545}, resulting in a fully active heterodimer retaining the entire B-domain (Esmon, 1980; Kane & Majerus, 1981). Processing by other proteases including chymotrypsin (Smith & Hanahan, 1976), calpain (Bradford et al., 1988; Rodgers et al., 1987), plasmin (Lee & Mann, 1989), and a platelet protease (Kane et al., 1982) results in partial activation of factor V; however, the cleavage sites for these enzymes have not yet been identified.

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* To whom correspondence should be addressed. Tel: 919-684-8952; Fax: 919-681-6160; Internet: whk@galactose.mc.duke.edu.

‡ Present address: Division of Pediatric Hematology/Oncology, West Virginia University School of Medicine, Morgantown, WV.

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[1] Abbreviations: RVV-V, factor V activator from Russell's viper venom; rHFV, recombinant human factor V; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ELISA, enzyme linked immunosorbent assay; PPACK, D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone; DAPA, dansylarginine 4-ethylpiperidine amide.

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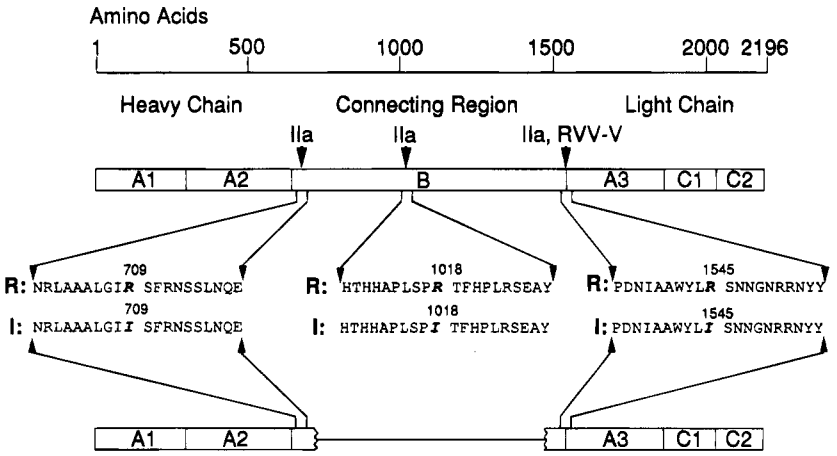

FIGURE 1: Mutation of the thrombin cleavage sites in human factor V. The domain structure of human factor V and the deletion mutant lacking a large portion of the B-domain are shown by the labeled boxes (Kane & Davie, 1986; Kane et al., 1990). The thin line indicates the B-domain sequences deleted in the mutant factor V des $B_{811-1491}$. The locations of the heavy chain, connecting region, and light chain are indicated along with the scale in amino acids at the top of the figure. The locations of the sites cleaved by thrombin (IIa) and the factor V activating enzyme from Russell's viper venom (RVV-V) are indicated by the short arrows. The sequences flanking the cleavage sites at arginines-709, -1018, and -1545 are shown at the center of the figure. The sequences labeled "R" indicate the wild type sequence. The sequences labeled "I" have been modified by site-directed mutagenesis to substitute isoleucine for arginine. Mutants are named according to the presence of either arginine (R) or isoleucine (I) at each cleavage site. Recombinant proteins expressed included the native sequence (RRR); single site mutants (IRR, RIR, RRI); double site mutants (IIR, IRI, RII); and the triple mutant (III). Arginine-1018 is deleted in the factor V B-domain deletion mutant factor V des $B_{811-1491}$ (RR). Factor V B-domain deletion mutants with single (IR, RI) and double (II) thrombin cleavage site mutations were also expressed.

The molecular events leading to activation of factor V and the role of the B-domain in regulating procoagulant activity remain poorly understood. Kinetic studies of the production of proteolytic fragments during the activation of factor V by thrombin have yielded conflicting results, suggesting that cleavage at either Arg_{709} (Nesheim & Mann, 1979), Arg_{1018} (Suzuki et al., 1982), or Arg_{1545} (Kane & Majerus, 1981) results in activation of the molecule. Activation of human factor V by factor Xa has been reported to correlate with cleavage following Arg_{1018} (Monkovic & Tracy, 1990a). We have recently used site-directed mutagenesis to investigate the function of the B-domain in factor V (Kane et al., 1990). Deletion of amino acids 811−1491 from the B-domain of factor V results in the expression of a single chain molecule that is efficiently processed by thrombin, resulting in formation of the heavy and light chains (Figure 1). The specific activity of the activated mutant is identical to thrombin activated factor V; however, in contrast to recombinant factor V, the single chain mutant expressed significant constitutive procoagulant activity. These results suggested that one function for the large B-domain in factor V is to inhibit constitutive procoagulant activity.

The purpose of the present study was to determine which of the three cleavage sites were necessary or sufficient for the activation of factor V by thrombin. We find that cleavage at Arg_{709} by thrombin results in little or no increase in procoagulant activity, but that this cleavage is required for rapid thrombin cleavage at Arg_{1545} and maximal activation by thrombin. Mutation of the thrombin cleavage sites in the B-domain deletion mutant indicates that neither cleavage site is necessary for the expression of partial activity; however, maximal activation requires cleavage at Arg_{1545}. These studies provide new insights into the molecular mechanisms regulating the activity of factor V and the prothrombinase complex. They also provide the groundwork for characterization of other factor V activators that may be important in the early phases of hemostasis prior to thrombin formation.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes, vectors, and T4 DNA ligase were obtained from Bethesda Research Laboratories (Gaithersburg, MD). Kits for DNA sequencing (Sequenase Version 2.0) and site-directed mutagenesis (T7-GEN) were from U.S. Biochemical Corp. (Cleveland, OH). Purified human plasma factor V, immunopurified rabbit polyclonal anti-human factor V antibodies, and the factor V activator from Russell's viper venom were prepared as previously described (Kane & Majerus, 1981; Kane & Davie, 1986; Kisiel, 1979). Human thrombin, prothrombin, and factor Xa were obtained from Haemotologic Technologies Inc. (Essex Junction, VT). The chromogenic substrate S-2238 was obtained from Kabi Pharmacia (Piscataway, NJ). Rainbow [^{14}C]-labeled molecular weight markers and [^{35}S]methionine were obtained from Amersham (Arlington Heights, IL). All other reagents were from Sigma (St. Louis, MO).

Construction and Expression of Factor V Mutants. Codons encoding arginine residues at positions 709, 1018, and 1545 were mutated to encode isoleucine using standard techniques (Sambrook et al., 1989). Mutations were verified by DNA sequencing and inserted into the full-length factor V cDNA and into a mutant lacking residues 811−1491 in the B-domain (Kane et al., 1990). Mutants were named according to the presence of either arginine or isoleucine at each cleavage site. Thus the wild type protein is named RRR while the mutant containing isoleucine at all three cleavage sites is named III. Mutants containing a single isoleucine residue at either position 709, 1018, or 1545 are named IRR, RIR, and RRI, respectively. Mutants containing isoleucine residues at two of the three cleavage sites with a single arginine residue at position 709, 1018, or 1545 are named RII, IRI, and IIR, respectively. Similarly, the B-domain deletion mutant which lacks the cleavage site at position 1018 is named RR, while the B-domain deletion mutants containing isoleucine at position 709 or 1545 or both positions are named IR, RI, and II, respectively. Cleavage sites in the B-domain deletion mutants are labeled by their corresponding

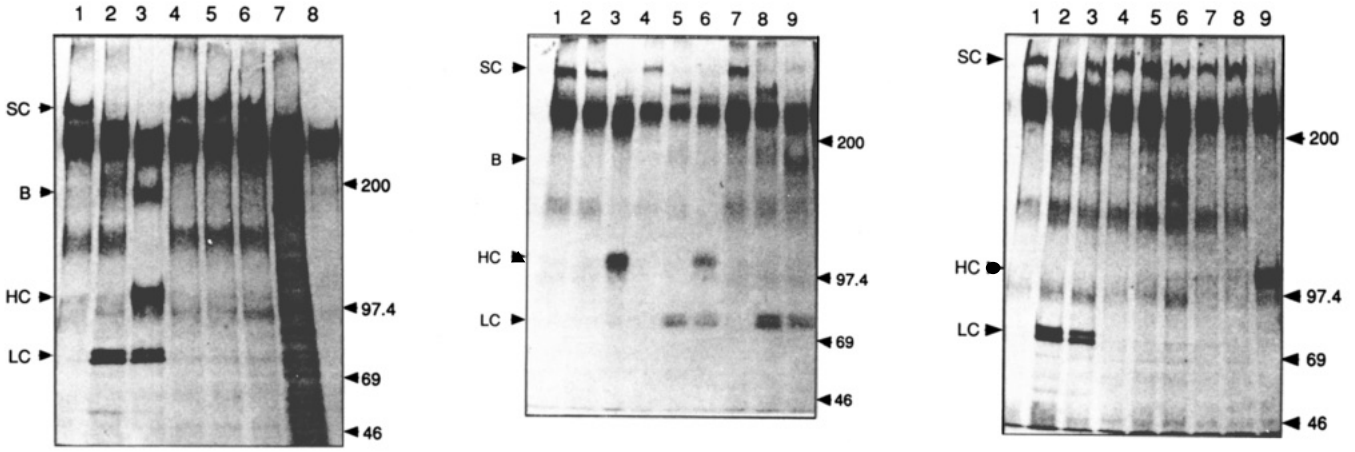

FIGURE 2: Proteolysis of factor V thrombin cleavage site mutants. COS-7 cells were transfected with an expression vector encoding the indicated mutant and were then labeled with [^{35}S]methionine. Recombinant factor V was immunoprecipitated using a rabbit polyclonal anti-human factor V antibody before and following activation with RVV-V or thrombin, as described in the Experimental Procedures. The immunoprecipitates were analyzed by SDS−polyacrylamide gel electrophoresis on 5% gels followed by autoradiography. The location of single chain factor V is indicated (SC). Thrombin activated factor V consists of a heavy chain (HC) and a light chain (LC). One radiolabeled B-domain fragment (B, amino acids 1019−1545) is released during thrombin activation. A second B-domain fragment (amino acids 710−1018) is also released during thrombin activation, but is not visualized because of its low methionine content. Apparent molecular masses, in kilodaltons, are indicated at the right of each panel. Left panel: rHFV (lanes 1−3) and III (lanes 4−6) without activation (lanes 1, 4) and following incubation with RVV-V (lanes 2, 5) or thrombin (lanes 3, 6). Controls shown include gelatin agarose binding fraction (lane 7) and preimmune rabbit IgG (lane 8). Middle panel: RRI (lanes 1−3), RIR (lanes 4−6), and IRR (lanes 7−9) without activation (lanes 1, 4, 7) and following incubation with RVV-V (lanes 2, 5, 8) or thrombin (lanes 3, 6, 9). Right panel: IIR (lanes 1−3), IRI (lanes 4−6), and RII (lanes 7−9) without activation (lanes 1, 4, 7) and following incubation with RVV-V (lanes 2, 5, 8) or thrombin (lanes 3, 6, 9).

position in the wild type protein. These mutants were subcloned into the expression vector pDX and expressed in COS-7 cells as previously described (Kane et al., 1990). Serum free conditioned media containing 5 mg/mL bovine serum albumin and 2.5 mM $CaCl_2$ were harvested 48 h following transfection and were stored at −70 °C until use.

Characterization of Recombinant Factor V. Factor V antigen was quantitated by ELISA using an affinity purified rabbit polyclonal antibody as previously described (Kane et al., 1990; Ortel et al., 1992). For recombinant proteins lacking the B-domain, factor V antigen was quantitated using a monoclonal antibody based ELISA, with monoclonal antibodies HV-1 and 6A5 being used for capture and detection, respectively (Ortel et al., 1994). Factor Va activity present in conditioned media was quantitated using a chromogenic assay utilizing purified prothrombin and factor Xa, as previously described (Kane et al., 1990; Ortel et al., 1992). Samples were assayed before and after incubation at 37 °C with either 3.3 nM RVV-V for 3 min or 2 nM thrombin for 5 min. The concentrations of thrombin used in these experiments did not interfere with the factor Va chromogenic assay. Furthermore, the concentrations of factor Xa (20 pM) and factor V (0−20 pM) used in the prothrombinase assay were not sufficient for significant factor Xa-catalyzed activation of factor V during the 2 min assay incubation, as evidenced by the fact that single chain plasma factor V has 1−2% of the activity of thrombin activated factor Va (Kane et al., 1990). One unit is defined as the amount of factor V activity present in 1 mL of RVV-V activated pooled human plasma. COS-7 cells expressing recombinant factor V were metabolically labeled with [^{35}S]-methionine, as previously described (Kane et al., 1990). Samples were immunoprecipitated using an affinity purified rabbit polyclonal antibody before and after incubation at 37 °C with either 3.3 nM RVV-V for 3 min or 2 nM thrombin for 5 min (Kane et al., 1990; Ortel et al., 1992). Thrombin activation was terminated by the addition of 4 μM D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK). The immunoprecipitated recombinant factor V was then denatured in the presence of SDS and β-mercaptoethanol and analyzed by SDS−polyacrylamide gel electrophoresis followed by autoradiography (Kane et al., 1990). The conditions for assay of procoagulant activity and proteolytic cleavage of recombinant factor V were identical.

RESULTS

Construction and Expression of Factor V Thrombin Cleavage Site Mutants. In order to prevent thrombin activation of factor V, the codons encoding the P1 arginine residues for the thrombin cleavage sites at positions 709, 1018, and 1545 of factor V were mutated to encode isoleucine residues (Figure 1). Restriction fragments containing these mutations were subcloned into the full-length factor V cDNA (Kane et al., 1990), resulting in a family of mutants in which one, two, or all three of the cleavage sites were mutated. These constructs were subcloned into the expression vector pDX and expressed in COS cells as described previously (Kane et al., 1990; Ortel et al., 1992). The concentration of factor V antigen in the conditioned media for wild type and mutant proteins was similar (40−200 ng/mL). The isoleucine mutations at positions 709 and 1545 were also subcloned into a factor V construct in which the sequences encoding residues 811−1491 of the B-domain were deleted (Figure 1). Mutants containing isoleucine at one or both sites were secreted into the conditioned media at levels comparable to the truncated protein containing arginine at both sites (150−950 ng/mL).

Characterization of the Triple Mutant. In order to demonstrate that the isoleucine substitutions blocked proteolytic processing of factor V, we compared the processing of [^{35}S]-labeled wild type factor V to the triple mutant III (Figure 2, left panel). Recombinant factor V is expressed predominantly as a single chain 330 kDa precursor (Figure 2, left panel, lane 1). A >200 kDa contaminant protein is also seen in these immunoprecipitations that is not cleaved

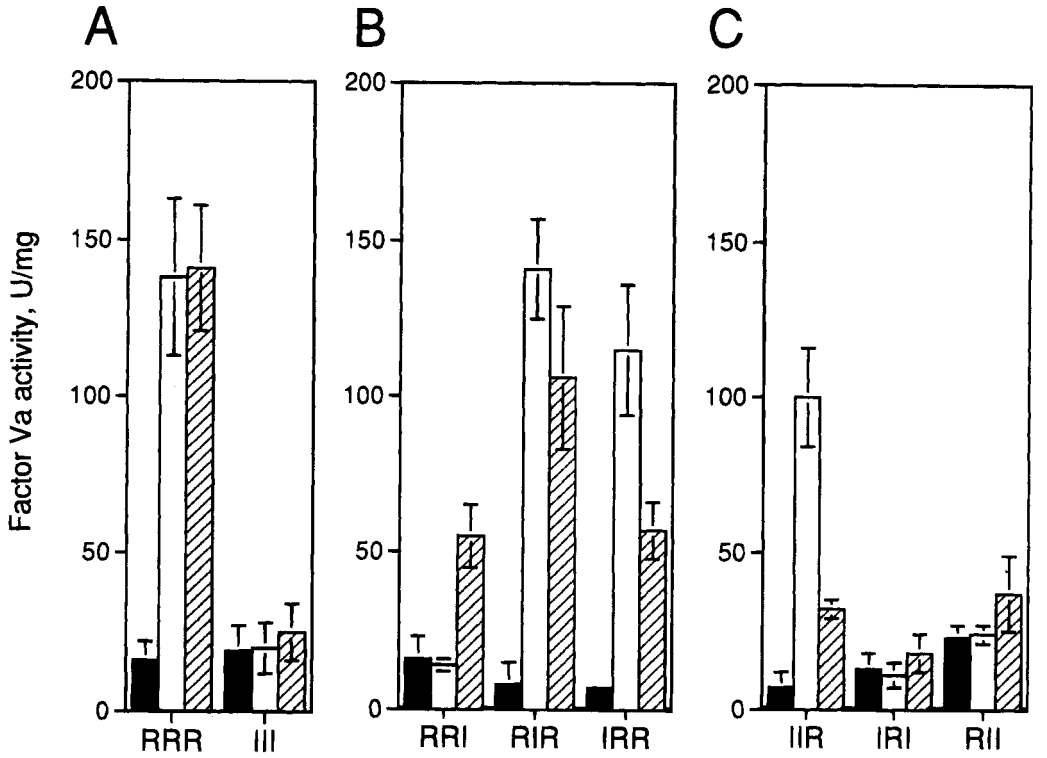

FIGURE 3: Procoagulant activity of factor V thrombin cleavage site mutants. Conditioned media containing either wild type or mutant recombinant factor V was assayed for factor V procoagulant activity using a chromogenic assay employing purified human coagulation proteins. The concentration of the recombinant factor V ranged from 40 to 200 ng/ml. Samples were assayed without activation (black bars), or after incubation with either RVV-V (white bars) or thrombin (hatched bars) as described in the Experimental Procedures. Error bars represent the standard error of the mean obtained from a minimum of four assays performed on conditioned media obtained from at least three separate transfections.

by thrombin or RVV-V (Kane et al., 1990). Incubation of factor V with RVV-V results in generation of the light chain with disappearance of the single chain precursor (Figure 2, left panel, lane 2). Incubation of factor V with thrombin results in formation of both the heavy and light chains as well as the 150 kDa activation fragment (Figure 2, left panel, lane 3). The 70 kDa activation fragment is not visualized in these studies because it contains relatively few methionine residues. As predicted, the triple mutant is not cleaved by either thrombin or RVV-V. The procoagulant activity of these proteins is shown in Figure 3A. Both factor V and the triple mutant (III) express a similar small amount of procoagulant activity. Following activation with RVV-V or thrombin, the activity of factor V increases approximately 10-fold to a specific activity of 140 units/mg. In contrast, the activity of the triple mutant (III) does not increase following treatment with RVV-V or thrombin. In the present study, the apparent specific activity of recombinant factor Va is slightly higher than the specific activity of purified plasma factor Va (~100 units/mg) (data not shown). In these experiments factor V procoagulant activity is expressed as an apparent specific activity based on measured procoagulant activity and antigen present in the conditioned media. These specific activities differ from previously published specific activities (Kane & Majerus, 1981; Katzmann et al., 1981; Suzuki et al., 1982) because in the present study 1 unit of activity is defined as the amount present in 1 mL of activated pooled human plasma whereas in previous studies 1 unit was defined as the amount of activity present in 1 mL of *unactivated* pooled human plasma.

Analysis of the Single Site Mutants. The individual isoleucine substitutions have the predicted effect on proteolysis by RVV-V and thrombin (Figure 2, middle panel). For example, release of the light chain is blocked in mutant RRI, thrombin releases both the heavy and light chains from mutant RIR, and release of the heavy chain by thrombin is blocked in mutant IRR. The procoagulant activity of mutant RRI increases approximately 3.4-fold following treatment with thrombin to a specific activity of ~50 units/mg (Figure 3B). The procoagulant activity of mutant RIR following activation with RVV-V is identical to that of wild type factor V; however, the activity of mutant RIR following activation with thrombin is slightly decreased to ~106 units/mg. The activity of mutant IRR following activation with RVV-V is ~115 units/mg, which is also slightly decreased compared to that of the wild type protein. However, the activity of this mutant following activation with thrombin is significantly reduced, to approximately 30% of that of wild type. Incubation of thrombin activated mutant IRR with RVV-V resulted in a further increase in specific activity to a level identical to that of the RVV-V activated mutant (data not shown). Therefore, the discrepancy between the activity of RVV-V and thrombin activated mutant IRR appears to be due to incomplete processing of the mutant by thrombin. Thus, the reported specific activities reflect the efficiency of proteolytic processing by thrombin as well as the intrinsic activity of the fully processed molecule.

Analysis of the Double Site Mutants. Thrombin treatment of the double site mutants produced several unexpected results. First, although thrombin treatment of mutant IIR releases the light chain, cleavage is not quantitative and substantial amounts of single chain factor V remain at the end of the 5 min incubation (Figure 2, right panel, lane 3). Similarly, the mutant IRI is also not quantitatively cleaved by thrombin (Figure 2, right panel, lane 6). In contrast, thrombin treatment of mutant RII does result in efficient

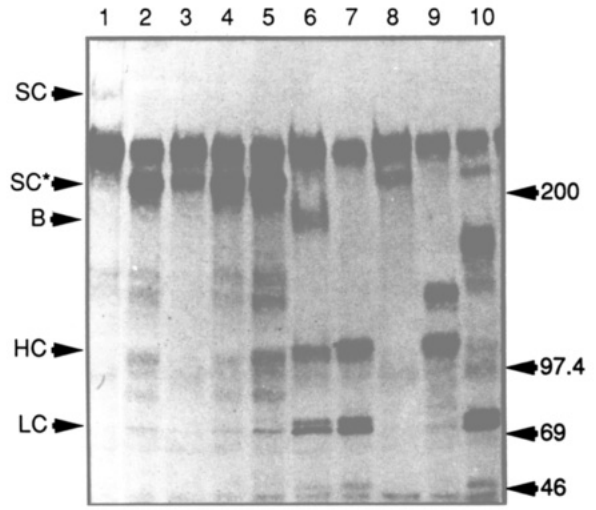

FIGURE 4: Proteolysis of factor V thrombin cleavage site mutants lacking a major portion of the B-domain. COS-7 cells were transfected with an expression vector encoding the indicated mutant and were then labeled with [^{35}S]methionine. Recombinant factor V was immunoprecipitated using a rabbit polyclonal anti-human factor V antibody before and following activation with thrombin as described in the Experimental Procedures. The immunoprecipitates were analyzed by SDS−polyacrylamide gel electrophoresis on 5% gels followed by autoradiography. The locations of single chain factor V (SC), B-domain deletion mutants (SC*), B-domain activation fragment (B), factor Va heavy chain (HC), and factor Va light chain (LC) are indicated. Apparent molecular masses, in kilodaltons, are indicated at the right of the figure. The samples are wild type factor V (RRR) (lanes 1, 6), rHFV des B (RR) (lanes 2, 7), mutant II (lanes 3, 8), mutant RI (lanes 4, 9), mutant IR (lanes 5, 10) before (lanes 1−5) and following (lanes 6−10) activation with thrombin.

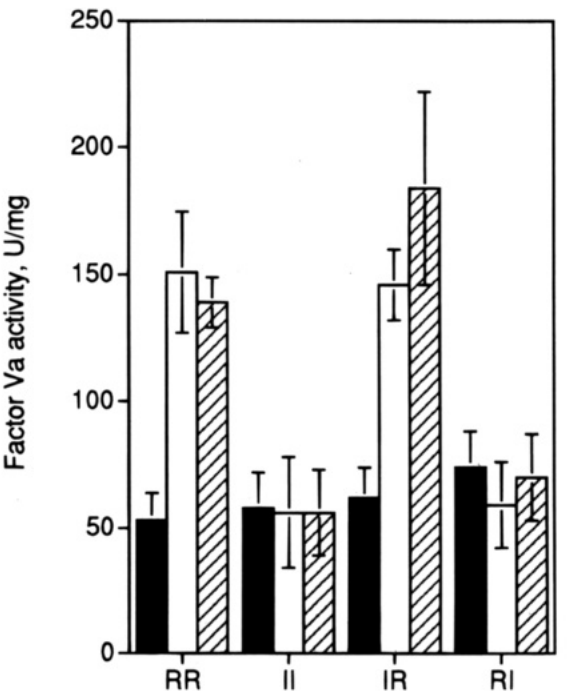

FIGURE 5: Procoagulant activity of factor V thrombin cleavage site mutants lacking a major portion of the B-domain. Conditioned media containing recombinant factor V were assayed for factor V procoagulant activity using a chromogenic assay employing purified human coagulation proteins as described in the Experimental Procedures. Samples were assayed without activation (black bars) or after incubation with either RVV-V (white bars) or thrombin (hatched bars) as described in the Experimental Procedures. Error bars represent the standard error of the mean obtained from a minimum of three assays performed on conditioned media obtained from at least two separate transfections.

proteolytic processing and release of the heavy chain (Figure 2, right panel, lane 9). The procoagulant activity of mutant IIR increases 14-fold following treatment with RVV-V, to approximately 100 units/mg, which is approximately 70% of that of wild type. In contrast, the activity of thrombin activated mutant IIR is only 30 units/mg (Figure 3C). Incubation of thrombin activated mutant IIR with RVV-V resulted in a further increase in specific activity to a level identical to that of the RVV-V activated mutant (data not shown), indicating that this mutant was also incompletely processed by thrombin. Treatment of mutants IRI or RII with thrombin or RVV-V is not accompanied by a significant increase in procoagulant activity. In contrast to the 3.4-fold increase in procoagulant activity seen following activation of mutant RRI with thrombin, the activity of mutant RII increased only 1.7-fold.

Mutagenesis of Thrombin Cleavage Sites in a B-Domain Deletion Mutant. We have previously demonstrated that deletion of amino acids 811−1491 in the factor V B-domain appears to result in a single chain molecule with constitutive procoagulant activity (Kane et al., 1990). Isoleucine substitutions were introduced into the two thrombin cleavage sites present in this mutant, yielding mutants II, RI, and IR. These B-domain deletion mutants are expressed as single chain proteins (Figure 4, lanes 2−5). Following incubation with thrombin, the heavy chain is released from mutants RR and RI, the light chain is released from mutants RR and IR, and mutant II is not cleaved (Figure 4, lanes 7−10). All four of the B-domain deletion mutants express partial procoagulant activity with a specific activity of approximately 50 units/mg (Figure 5). Only mutants containing arginine at position 1545 (mutants RR and IR) can be fully activated with RVV-V or thrombin, however, indicating that light chain cleavage is required for maximal activity (Figure 5).

DISCUSSION

The proteolytic activation of factor V by thrombin is a critical step leading to formation of the prothrombinase complex and the explosive generation of thrombin required for formation of a fibrin clot (Pieters et al., 1989). Although three thrombin cleavage sites in human factor V have been identified (Jenny et al., 1987), the precise nature of the molecular events leading to generation of procoagulant activity has remained poorly understood. Previous studies have attempted to determine structure−activity relationships during the activation of factor V based on the kinetic appearance of proteolytic fragments during the activation of factor V (Esmon, 1979; Nesheim & Mann, 1979; Kane & Majerus, 1981; Suzuki et al., 1982) or the activity of heterodimers reconstituted from isolated factor V activation products (Esmon, 1979; Nesheim et al., 1984; Suzuki et al., 1982). Interpretation of these studies is complicated by the fact that thrombin, the most potent activator of factor V, is generated during all assays of factor V function. Nesheim and co-workers (Nesheim et al., 1979) have used the reversible thrombin inhibitor dansylarginine 4-ethylpiperidine amide (DAPA) to both measure thrombin formation and inhibit thrombin action during the assay of factor V procoagulant activity. Their results indicated that single chain bovine factor V expressed less than 0.27% of the activity of the thrombin activated protein (Nesheim et al., 1979). This suggested that the procoagulant activity measured during assay of factor V preparations was either due to contaminat-

ing amounts of factor Va or due to *in situ* activation of factor V occurring during assay of procoagulant activity. In the present study we have blocked thrombin cleavage of factor V by substituting isoleucine for arginine residues at positions 709, 1018, and 1545. A similar approach has been used successfully to study the thrombin cleavage sites in coagulation factor VIII (Toole et al., 1986). Loss of function point mutations provides little insight into structure−function relationships since these mutations may drastically affect protein folding. However, in the present study, isoleucine substitutions at positions 709 and 1018 have only a minimal effect on factor V procoagulant activity following activation with RVV-V. Furthermore, the significant base-line activity of the factor V des B constructs containing isoleucine at position 1545 is identical to constructs containing the native arginine residue (Figure 5). These results indicate that these isoleucine substitutions have not drastically altered the structure of factor V. This has allowed us to study the functional consequences of thrombin cleavage at each of these sites and has provided new insights into the molecular events required for expression of procoagulant activity.

Both recombinant factor V and the full-length mutants containing isoleucine substitutions express a small but significant amount of procoagulant activity that is <10% of the activity of fully activated factor V. The basal activity of these recombinant proteins does not appear to be due to *in situ* activation by thrombin during assay of procoagulant activity. However, this activity may result from proteolysis of small amounts of recombinant factor V prior to harvesting the conditioned media. An ~140 kDa fragment is seen in these preparations, and this fragment is further processed by thrombin in conditioned media containing recombinant factor V but not mutants IRR, IIR, IRI, nor III (Figure 2). The location of this proteolytic cleavage site is unknown, but is most likely in the amino terminal region of the B-domain. Similar heterogeneity is also occasionally seen in purified factor V preparations (Dahlback, 1980), and in these cases the base-line activity and activation quotients are similar to those seen in the present studies. We also cannot exclude the possibility that the basal activity of recombinant factor V and mutant III may require factor Xa mediated cleavage of the recombinant proteins during the prothrombinase assay. The basal activity of mutant III is important since it is the first demonstration that factor V procoagulant activity can be expressed in the absence of proteolytic cleavage at Arg_{709}, Arg_{1018}, or Arg_{1545}. Purification of mutant III should facilitate identification of alternate cleavages and the enzymes responsible for them.

This study has helped to define structure−function relationships for the individual thrombin cleavage sites in human factor V. First, release of the heavy chain by thrombin cleavage at Arg_{709} does not result in a significant increase in procoagulant activity (mutant RII). This conclusion is consistent with previous kinetic studies of the activation of purified plasma factor V by thrombin in which all three cleavages occurred simultaneously (Kane & Majerus, 1981; Suzuki et al., 1982). We cannot exclude the possibility that thrombin cleavage at Arg_{709} might lead to a small increase in procoagulant activity not detected in the present studies. Second, thrombin cleavage at both Arg_{709} and Arg_{1018} releases a portion of the B-domain and results in a species with ~30% activity (mutant RRI). Qualitatively, this is consistent with the observations of Suzuki et al. (1982) who concluded that a major increase in biological activity seemed to parallel the release of the amino terminal portion of the B-domain following thrombin cleavage at Arg_{1018}. Finally, the present studies suggest that cleavage of recombinant factor V at Arg_{1545} and release of the carboxyl terminal portion of the B-domain are essential for maximal activation by thrombin. This observation is consistent with our previous kinetic studies of the activation of human plasma derived factor V by thrombin (Kane & Majerus, 1981).

Studies of the activation of bovine factor V by thrombin also indicate that release of the light chain is required for maximal activation of the cofactor. The final products observed following the activation of human and bovine factor V by thrombin are similar; however, the transient activation intermediates are different. In the bovine protein the initial thrombin cleavage occurs at Arg_{1006} (corresponding to Arg_{1018} in the human protein) (Guinto et al., 1991; Jenny et al., 1987; Kane et al., 1987). Nesheim et al. (1984) isolated the two peptides produced by this cleavage in the bovine protein and found that the reconstituted heterodimer could be activated 10-fold following incubation with thrombin. Esmon (1979) also prepared a heterodimer consisting of the bovine factor Va heavy chain and the bovine 220 kDa thrombin activation intermediate. Activation of this reconstituted heterodimer with thrombin resulted in release of the factor Va light chain and a 2.2-fold increase in specific activity.

In contrast to the preceding observations, Monkovic and Tracy (1990a) have suggested that release of the light chain is not required for maximal activation of human factor V. These investigators studied the activation of human factor V by factor Xa and concluded that cleavage at Arg_{1018} was sufficient for complete activation of factor V. The present studies have not completely defined the effect of cleavage at Arg_{1018} alone on the expression of human factor V procoagulant activity since cleavage of mutant IRI by thrombin is not quantitative, even following prolonged incubations (data not shown). Partial cleavage of mutant IRI was not accompanied by a detectable increase in procoagulant activity (Figure 3); however, we cannot exclude the possibility that quantitative cleavage of this mutant might result in partial activation.

An important methodological difference between our study and that of Monkovic and Tracy (1990a) is that their conclusions are based upon the activation of human factor V by factor Xa. Factor Xa has been shown to cleave human factor V at Arg_{1018}; however, the location of the heavy chain cleavage has not been identified. Following activation with factor Xa, Monkovic and Tracy (1990a) purified a heterodimer consisting of a 110 kDa heavy chain and a 220 kDa fragment containing the light chain region. The procoagulant activity of this heterodimer was indistinguishable from that of thrombin activated factor Va and was not further increased following treatment with thrombin. Measurements of factor V procoagulant activity can be complicated by *in situ* activation of factor V by thrombin during the assay. Monkovic and Tracy (1990a) included the thrombin inhibitor DAPA in their assays to block further proteolysis by thrombin. Release of the light chain was not detected by Western blot analysis; however, the relative amount of the 220 kDa precursor fragment clearly decreased, suggesting that further proteolytic processing occurred during the prothrombinase assay. This possibility is consistent with the observation that factor Xa catalyzed activation of platelet factor V results in quantitative release of the light chain (Monkovic & Tracy, 1990b). In contrast to the findings of

Monkovic and Tracy (1990a), we have also observed production of the light chain fragment during activation of purified plasma factor V by factor Xa.[2] The reasons for these discrepancies are currently under investigation. These results suggest that *in situ* activation of factor V by factor Xa may also complicate prothrombinase assays. A potential pitfall in our studies using recombinant proteins is that the isoleucine substitution at position 1545 could affect factor V procoagulant activity in a way unrelated to proteolytic cleavage at this site. The observation that this isoleucine substitution does not affect the basal activity of full-length or B-domain deletion mutants makes this possibility less likely.

Although cleavage of Arg_{709} is not sufficient for activation of factor V by thrombin, cleavage at this site is required for rapid activation by thrombin. The decreased specific activity of thrombin activated IRR and IIR compared to that of the RVV-V activated protein (Figure 3) results from incomplete release of the light chain. These data suggest that cleavage at Arg_{709} enhances the rate of cleavage at Arg_{1545}. In addition, the activity of thrombin activated RIR is slightly reduced compared to that of the RVV-V activated protein although there is no detectable difference in the amount of light chain released. Recently, Pittman et al. (1993) characterized a factor V mutant identical to mutant RIR and were able to detect delayed activation and release of the light chain by thrombin compared to the wild type protein.

These studies have identified two functions for the ~900 amino acid B-domain of factor V. First, the entire B-domain is required to prevent expression of procoagulant activity prior to proteolytic activation. Release of the first 309 amino acids (residues 710−1018) of the B-domain by thrombin results in a species with partial procoagulant activity. Similarly, deletion of residues 811−1491 in the B-domain results in a single chain protein also possessing approximately 30% activity (Kane et al., 1990). The activity of the B-domain deletion construct is not affected by isoleucine substitutions at Arg_{709} and Arg_{1545}, indicating that these mutations do not drastically alter the structure of the protein and that cleavage at these sites is not required for expression of partial procoagulant activity (Figure 5). The second function for the B-domain of factor V is to inhibit thrombin activation of factor V by preventing rapid cleavage at Arg_{1545}. In both full-length and B-domain deletion mutants, release of the light chain leads to a further increase in procoagulant activity, whereas release of the heavy chain does not. However, in contrast to mutant IIR, the B-domain deletion mutant IR is rapidly cleaved and activated by thrombin. These results indicate that the requirement for initial cleavage at Arg_{709} in factor V is dependent on sequences within residues 811−1491 of the B-domain. These observations will necessitate further refinement of the proposed ultrastructural models for factor V (Mosesson et al., 1990; Fowler et al., 1990) since the models do not explain how truncation or cleavage of an extended rod-like B-domain would lead to generation of procoagulant activity or facilitate thrombin activation of the molecule.

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[2] L. A. Worfolk and W. H. Kane, unpublished observation.

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