The proteolytic conversion of factor V to factor Va is central for amplified flux through the blood coagulation cascade. Heterodimeric factor Va is produced by cleavage at three sites in the middle of factor V by thrombin, yielding an N terminus–derived heavy chain and a C terminus–derived light chain. Here, we show that light chain formation resulting from the C-terminal cleavage is the rate-limiting step in the formation of fully cleaved Va. This rate-limiting step also corresponded to and was sufficient for the ability of cleaved factor V to bind Xa and assemble into the prothrombinase complex. Meizothrombin, the proteinase intermediate in thrombin formation, cleaves factor V more slowly than does thrombin, resulting in a pronounced defect in the formation of the light chain. A ~100-fold reduced rate of meizothrombin-mediated light chain formation by meizothrombin corresponded to equally slow production of active cofactor and an impaired ability to amplify flux through the coagulation cascade initiated in plasma. We show that this defect arises from the occlusion of anion-binding exosite 2 in the catalytic domain by the covalently retained propiece in meizothrombin. Our findings provide structural insights into the catalytic domain by the covalently retained propiece in meizothrombin.

The proteolytic activation of factor V to factor Va is required for the amplified response of the blood coagulation cascade to vascular damage (1, 2). Factor Va, but not factor V, serves as a cofactor for thrombin formation by binding its cognate serine proteinase, factor Xa, on membranes containing phosphatidylserine to form prothrombinase (1, 3). The assembly of factor Xa into prothrombinase greatly increases (by several logs) the rate at which Xa acts on prothrombin to form thrombin (1). Consequently, activation of factor V and the assembly of prothrombinase results in an explosive increase in the rate of thrombin production necessary to catalyze formation of the clot.

Although several proteinases have been implicated as potential activators of factor V, it is generally agreed that thrombin (IIa) and factor Xa likely play primary roles in factor Va formation (4). In the case of IIa, the accepted explanation is that Xa formed following the initiation of coagulation yields low concentrations of IIa sufficient for the feedback activation of factor V, which then leads to accelerated IIa formation (4–6). Two exosites in IIa, anion-binding exosite 1 (ABE1)2 and anion-binding exosite 2 (ABE2), play a pronounced role in the various functions of the proteinase (7). Both exosites have been implicated in the activation of factor V by IIa (8–11).

Alternately, Xa can directly act to proteolyze factor V through interactions that obviously differ from those responsible for the Xa–Va interaction within prothrombinase. However, cleavage by Xa requires membranes containing phosphatidylserine, which would require thrombin production for the activation of blood cells (5). In addition, cleavage products of the action of Xa on factor V as well as its functional properties are proposed to differ from those produced by thrombin (6, 12, 13). Thus, there is uncertainty as to the exact disposition of factor V activation products and activated cofactor species in the initiation phase of coagulation.

Additional light on the factor V activation paradox draws from the body of literature showing efficient activation of factor V by meizothrombin (mIIa) produced as an intermediate during prothrombin activation (14–16). Meizothrombin is a serine proteinase but retains covalent linkage to the N-terminal pro-piece that confers membrane binding, which is then lost upon its further processing to IIa (1). Accordingly, the literature contains reports variably showing that membrane-dependent factor V activation by mIIa occurs at a rate that is superior to or comparable with its activation by IIa (14–17). Because mIIa is produced before IIa during prothrombin activation, the initial activation of factor V by mIIa has been proposed as a solution to the paradoxical relationship between factor V activation and amplified IIa formation (16). This proposal is logically weak because the formation and accumulation of mIIa is minimal in

2 The abbreviations used are: ABE1 and ABE2, anion-binding exosites 1 and 2, respectively; EGRck, l-glutamyl-glycyll-arginine chloromethylketone; acetothioacetyl-EGRck, acetothioacetyl derivative of EGRck; AR1 and AR2, acidic regions 1 and 2, respectively; BR, basic region; mIIa, meizothrombin; desGla-mIIa, mIIa lacking γ-carboxyglutamic acid modifications; mIIa\textsubscript{NTR}, mIIa lacking the N-terminal fragment 1 domain; OG\textsubscript{desGla}\textsubscript{Xa}, factor Xa inactivated with acetothioacetyl-EGRck and then modified with Oregan Green\textsubscript{maleimide} following thioester hydrolysis; PCPS, small unilamellar vesicles containing 75% (w/w) l-α-phosphatidylcholine and 25% (w/w) l-α-phosphatidylserine; RVV\textsubscript{α}, factor V–activating proteinase from Russell’s viper venom; Z, benzyloxycarbonyl; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
Despite being processed at the cleavage site required for serine in mIIa (19, 20). It also does not agree with recent findings that when ABE2 is occluded by the covalent linkage of the propiece mIIa also makes little sense when taken in the context of the absence of factor Va (18). Efficient factor V activation by mIIa is expected to bind Xa with high affinity in a membrane-dependent fashion is the major functional feature that distinguishes procofactor from active cofactor. Whereas partially cleaved forms or factor V variants cleaved at different sites may well exhibit differential cofactor function as has been proposed, the ability to bind Xa with high affinity is an absolute prerequisite for the expression of cofactor activity.

We now compare the action of Ila and mIIa on factor V with these new perspectives on factor V activation and employ an unambiguous quantitative Western blotting approach to assign the possible cleavage products. In agreement with the recent biochemical conclusions regarding mIIa, we show that it is a much poorer catalyst for factor V cleavage than is Ila. The basis for this difference lies in the confluence of two findings. The first is that a single cleavage reaction constitutes the rate-limiting step in cofactor formation. The second is that ABE2 of the proteinase plays a markedly significant role in the rate-limiting step. Consequently, mIIa and its variants that contain the propiece covalently bound to ABE2 produce factor Va at a much slower rate than Ila in which ABE2 is fully functional.

**Results**

**Factor V cleavage by thrombin**

Initial studies examined the fate of factor V following the addition of Ila and analysis by SDS-PAGE followed by staining with colloidal Coomassie staining and fluorescence imaging (Fig. 1). The consumption of factor V was accompanied by the appearance of several bands representing cleavage intermediates, B-domain fragments as well as the terminal subunits of factor Va corresponding to the A1-A2-AR1 heavy chain and the A3-C1-C2 light chain (Scheme 1 and Fig. 1). Whereas the findings are in line with those published previously, interpretations from this approach are not clear-cut. The intermediates and terminal products of the cleavage reaction exhibit major differences in staining intensity and deviation from expected electrophoretic mobility. The identity of the higher-molecular weight products that persist even at long times is not always obvious. Even the best factor V preparations inevitably contain trace amounts of partially cleaved species that are further processed to confound the source of some of the evolving species. Whereas these issues have been addressed by protein sequencing of bands and by Western blotting approaches, kinetic conclusions are still far from problem-free.
Factor V activation by thrombin and meizothrombin

Figure 1. Cleavage of factor V by thrombin analyzed by protein staining. Factor V (FV; 500 nm) was digested with Ila (1 nm) in Assay Buffer at 25 °C. Aliquots withdrawn, before the addition of Ila (0 min) and at the indicated times following its addition, were quenched with SDS sample preparation buffer containing DTT and EDTA and analyzed by SDS-PAGE (~4 μg/lane). The gel was stained using colloidal Coomassie Blue G-250, destained in water, and imaged with an infrared fluorescence scanner. The migration positions of factor V, the heavy chain (HC; A1-A2-AR1; Scheme 1), and light chain (LC; A3-C1-C2; Scheme 1) are indicated in the margins.

Dual-color Western blotting

We addressed these limitations by developing a quantitative Western blotting approach with simultaneous infrared fluorescence imaging of two antibodies targeting the A2 domain and the light chain labeled with different fluorophores (Fig. 2A). This approach allows unambiguous assignment of all possible polypeptide products containing the heavy or light chains with a linear response in integrated intensity as a function of a large variation in loaded protein. The assignment of bands based on staining color and molecular weight is informed by the large body of evidence in which the identity of cleavage products has been established by N-terminal sequence, mutational analysis, and Western blotting (9, 17, 26, 27).

A clearer picture of factor V cleavage by Ila emerges when assessed by the two-color blotting approach (Fig. 2B). Factor V consumption is matched by the appearance of the mature heavy chain (A1-A2-AR1). Trace contaminants in the starting material correspond to the N- and C-terminal halves resulting from cleavage at Arg1018, which also further contribute to product evolution. The light chain (A3-C1-C2) appears slowly with a significant fraction still present as a higher-molecular weight form even after 10 min following initiation. This reflects markedly slow proteolysis at the Arg1545 site rather than some peculiar aspect of the reaction that leads to self-limiting cleavage (see below).

Kinetics of factor V cleavage by thrombin

The wider applicability of the findings was established in multiple experiments done with different protein preparations and by varying the concentrations of both enzyme and substrate (not shown). Quantitative analysis of the consumption of factor V and the appearance of the terminal products of its activation across a 10-fold change in Ila bear out the initial observations (Fig. 3). In either set, accumulation of the heavy chain (A1-A2-AR1) occurred with approximately the same time constant as the consumption of factor V. Light chain (A3-C1-C2) production lagged behind heavy chain formation with an initial rate that was ~10-fold slower. Because light chain formation requires cleavage at Arg1545, it follows that the action of Ila at this site is consistently slower than cleavage at the other two sites over a wide range of concentrations. Taken with the cleavage patterns (Fig. 2B), the data provide the tentative conclusion that there is a pronounced gradation in the rate of cleavage by Ila at the three sites in factor V, such that $v_{1018} > v_{1545} > v_{709}$. It remains to be established whether this is solely a result of the kinetic constants for the independent action of Ila at these sites or whether it arises from ordered cleavage, wherein the action of Ila at one site impacts cleavage at the next.

Kinetics of factor V cleavage by meizothrombin

We prepared mIIa from a prothrombin derivative (IIQQQ) bearing Gln substitutions for Arg at positions 155, 271, and 284 susceptible to either autolysis or cleavage by Xa (21). The action of ecarin on this variant results in cleavage at position Arg320 and yields stable mIIa that is not susceptible to further cleavage even without the use of proteinase inhibitors that are known to perturb its equilibrium distribution between zymogen-like and proteinase-like forms (21). Initial studies done in the absence of added membranes revealed that mIIa was a profoundly poor catalyst for cleaving factor V. At conditions equivalent to those used in Fig. 2B, most of the factor V remained uncleaved even at 10 min. Very small amounts of heavy chain were produced, whereas the light chain was undetectable (not shown). Judging by heavy chain formation, the reaction catalyzed by mIIa was at least 50-fold slower than that seen with Ila.

Previous studies have stressed the efficient cleavage of factor V by mIIa in the presence of membranes (14, 15). We compared factor V cleavage by equal concentrations of Ila and mIIa in the presence of membranes and over an extended time course to drive the reaction further to completion (Fig. 4). Cleavage by Ila was modestly inhibited (by ~20%) in the presence of membrane vesicles containing 75% (w/w) l-α-phosphatidylcholine and 25% (w/w) l-α-phosphatidylserine but without a noticeable change in the pattern of cleavage (Fig. 4A). Whereas membranes significantly enhanced factor V cleavage by mIIa, the reaction still proceeded more slowly than observed with Ila and without a change in the intermediates produced (Fig. 4B). Incomplete cleavage seen at the 90-min point resembled the distribution of substrates, intermediates, and products seen after 5 min with Ila (Fig. 4). The images and associated quantitative densitometry imply that mIIa is a poor catalyst for factor V cleavage compared with Ila. This defect arises from a disproportionately slower production of light chain compared with heavy chain (not shown). This point is more reliably clear from a quantitative analysis done over a shorter time interval (see below).

Membrane binding, zymogen-like character, and occlusion of ABE2

Despite the fact that mIIa is a serine proteinase, it differs from Ila in its ability to bind PCPS membranes, its skewed distribution toward a zymogen-like form, and the occlusion of ABE2 by the covalently linked propiece (21). Some or all of these properties could account for the differences in factor V cleavage by Ila and mIIa. Our previous work has established variants or proteolytic derivatives of mIIa that differ from one
another in one or more of these features (Table 1). Preparation of mIIa from IIQQQ expressed in the absence of vitamin K yields desGla-mIIa devoid of 4-carboxyglutamic acid modifications at its N terminus. This form does not bind membranes but retains the covalently linked propiece occluding ABE2 and possesses zymogen-like character equivalent to mIIa (21). The N-terminal fragment 1 domain can be proteolytically removed from prothrombin (IIQQQ) containing Gln substitutions at Arg271 and

Figure 2. Cleavage of factor V by thrombin analyzed by dual-color Western blotting. A, schematic illustration of all possible cleavage products of the action of thrombin on factor V and their detection following Western blotting using a labeled anti-heavy chain antibody detected in the 700-nm fluorescence channel and a labeled anti-light chain antibody detected in the 800-nm fluorescence channel. The possible cleavage products separated by SDS-PAGE and their corresponding sequence numbers are illustrated. B, the initial cleavage conditions are identical to those in Fig. 1. Samples (200 ng/lane) were analyzed by Western blotting and detected simultaneously with the two antibodies. The various species are illustrated in the margins keyed to those illustrated schematically in A. The band appears yellow when both antibodies are simultaneously bound.
Arg284 (21). Subsequent cleavage at Arg320 yields mIIa/H9004F1, which lacks the membrane-binding domain and also has lost zymogen-like character even though ABE2 is covalently occluded by the truncated propiece (21).

We compared the kinetics of heavy chain and light chain formation in the presence of membranes after treatment of factor V by this array of proteinase variants (Fig. 5). This simplified approach was chosen to focus on the fastest and slowest steps in the formation of heterodimeric factor Va without distraction from the complexities of how the intermediates might be differentially processed by these variants. The rate of formation of the heavy chain was most rapid with IIa and was modestly reduced by a factor of ~6 for mIIa (Table 1). The further ~6-fold reduction in rate with desGla-mIIa, which cannot bind membranes, illustrates the contribution of membrane binding to the action of mIIa on heavy chain formation from factor V. mIIa/H9004F1 lacks the membrane-binding domain and has lost the zymogen-like character of the other two mIIa variants. In this case, the rate of heavy chain production was partially rescued. Because all three variants have a covalently occluded ABE2, the data suggest modest and complex effects of membrane binding, zymogen-like character and the availability of ABE2 in contributing to the reduced rate of heavy chain formation by mIIa compared with IIa.

The findings with light chain formation were more obvious. All three mIIa variants regardless of whether they could bind membranes yielded light chain at about one-one hundredth the rate seen with IIa. Because the common feature of all three variants is that they lack the ability to ligate ABE2 ligands, it follows that interactions with ABE2 play a major role in the ability of these proteinases to produce the light chain of factor V.

**Table 1**

<table>
<thead>
<tr>
<th>Proteinase</th>
<th>Properties</th>
<th>( V_{HC} )</th>
<th>( V_{LC} )</th>
<th>( V_{LC}/V_{HC} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>mIIa</td>
<td>Membrane binding</td>
<td>22.5</td>
<td>0.15</td>
<td>0.007</td>
</tr>
<tr>
<td>desGla-mIIa</td>
<td>No membrane binding</td>
<td>3.0</td>
<td>0.16</td>
<td>0.053</td>
</tr>
<tr>
<td>mIIaΔA1</td>
<td>No membrane binding</td>
<td>19.9</td>
<td>0.15</td>
<td>0.008</td>
</tr>
<tr>
<td>IIa</td>
<td>No membrane binding</td>
<td>135.1</td>
<td>15.4</td>
<td>0.11</td>
</tr>
</tbody>
</table>

* Relevant properties for the indicated proteinase variants are taken from those established in prior studies (21, 50).

* Initial velocities for A1-A2-AR1 heavy chain (\( V_{HC} \)) or A3-C1-C2 light chain (\( V_{LC} \)) formation are expressed as percentage formed/min and determined using the logarithmic approximation (54).
Va. Whereas it is possible that the low rates obscure more subtle contributions from membrane-binding and zymogen-like character, the data are consistent with a major requirement for ABE2 interactions in the cleavage at Arg1545 in factor V. For IIa and all mIIa variants, cleavage yielding light chain was between 9- and 140-fold slower than the formation of the heavy chain (Table 1). Thus, the rate-limiting step in the formation of heterodimeric Va is the cleavage at Arg1545 with an unexpectedly important contribution from ABE2 of the proteinase. This requirement provides an explanation for the poor ability of mIIa to cleave factor V.

**Factor V cleavage and the evolution of cofactor function**

Notwithstanding the large differences in the rate of factor V cleavage by IIa and mIIa, the existing literature would suggest that partially cleaved forms of factor V could exhibit varying levels of factor Va-like activity (9, 17, 28). If so, mIIa could readily be comparable with IIa in producing active cofactor, as has been suggested previously.

The evolution of cofactor function is typically tested by sampling the factor V cleavage reaction mixture and mixing with an excess of Xa, membranes, and prothrombin for measurements of the initial rate of IIa formation. The rate of IIa formation, under these conditions, is proportional to the concentration of active cofactor. We tested whether the addition of Xa and membranes to partially cleaved factor V could affect further proteolysis and compromise interpretation. Quantitative Western blotting was used following the initiation of relatively slow factor V cleavage with membranes and mIIa (Fig. 6). Following partial cleavage, an equimolar concentration of Xa was added to simulate sampling for functional measurements. Even without IIa generation, Xa caused rapid proteolysis leading to the consumption of factor V and quantitative formation of the light chain (Fig. 6). We specifically focus on light chain here because its formation reflects the rate-limiting step for the formation of heterodimeric Va. Whereas the current conditions may be extreme for the typical activity measurement, they were selected for technical reasons of detection of cleavage. They nevertheless point to the possibility of a major potential complication in relating cleavage to the formation of functional cofactor.

We instead chose to monitor the evolution of membrane-dependent Xa binding during factor V cleavage, utilizing inactivated factor Xa containing a fluorescent reporter group tethered to the active site with a peptidyl chloromethylketone. This allows binding to be inferred from a change in fluorescence anisotropy of the probe in the absence of added active proteinase. Whereas high-affinity Xa binding may not exactly correlate with cofactor function assessed by prothrombin activation, the rationale here is that the ability to bind Xa with high affinity is a prerequisite for cleaved factor V species to function as a cofactor.

Time courses reporting the development of Xa binding following the cleavage of factor V by either IIa or mIIa in the presence of membranes were compared with quantitative densitometry of the appearance of the heavy chain or light chain done contemporaneously (Fig. 7). We were surprised to find that Xa binding correlated with the slow appearance of light
Factor V activation by thrombin and meizothrombin

Figure 7. Correlation of factor V cleavage with the ability to assemble prothrombinase. Reaction mixtures containing 40 nm factor V, 30 μM PCPS, and 40 nm OG$_{45}$-Xa, were initiated with either 0.05 nm IIa (A) or 0.05 nm mIIa (B). Fluorescence anisotropy was measured and normalized (black) as described under "Experimental procedures." Alternatively, samples were contemporaneously withdrawn, quenched, and analyzed by densitometry following two-color Western blotting. The time course for the evolution of heavy chain (HC; red) and light chain (LC; blue) is shown in each case. The lines were arbitrarily drawn.

A single cleavage to yield light chain is sufficient for prothrombinase assembly

The RVV$_v$ proteinase from the venom of Russell’s viper is an established activator of factor V (29, 30). This proteinase primarily cleaves factor V at a single site to yield the light chain and cofactor activity (30). However, small amounts of cleavage at other sites are also possible and cannot be ruled out as contributors to resultant factor Va-like function. A recent X-ray structure indicates that it is a thrombin-like serine proteinase lacking the distinctive features of ABE1 but with an intact ABE2 (31). Given our findings with the role of ABE2 in light chain formation, it also follows that mIIa and variants that contain an occluded ABE2 are extremely poor activators of factor V because of their impaired ability to cleave at Arg$^{1545}$.

Feedback amplification of coagulation in plasma

Conclusions regarding mIIa in purified systems may greatly underestimate its contribution to the amplification of coagulation in the plasma milieu. This possibility derives from the combined effects of several proteinases in the explosive activation of factor V once a small amount of factor Va has been produced and from the known resistance of mIIa to inactivation by antithrombin (32).

We measured the evolution of the proteinase product of the action of prothrombinase on prothrombin in plasma where the activation of the cascade was initiated with tissue factor (33). These assays were performed using prothrombin-depleted plasma reconstituted either with the physiologic concentration of WT prothrombin (II$_{WT}$); II$_{QQQ}$, which yields mIIa stable to further autolysis; or II$_{Q271}$, which is initially cleaved to yield mIIa but is then subject to autolysis at Arg$^{584}$ to yield a IIa-like form lacking 13 residues in the A chain (34). Identical kinetic constants characterize the action of prothrombinase at the Arg$^{320}$ site for all three species (35).

As expected, deficient plasma reconstituted with II$_{WT}$ yielded a IIa profile comparable with that observed in normal plasma (Fig. 9). Thrombin accumulated rapidly following a lag of a few minutes resulting from the cumulative effects of feedback activation reactions following initiation with tissue factor. The occurrence of a peak followed by its decline to zero results from the inhibition of the proteinases formed by serine proteinase inhibitors. For the plasma sample containing II$_{QQQ}$, the lag phase was greatly extended to ≥30 min, diagnostic of impaired feedback activation by the proteinase product. A similar result but with a greater peak amount of proteinase was obtained in the plasma reconstituted with II$_{Q271}$ (Fig. 9). With purified proteins, the rate of cleavage of the indicator substrate at the concentration present in the plasma assay is 4-fold lower with mIIa than with IIa. This could partly account for the reduced amplitudes of proteinase formation seen with II$_{QQQ}$. The larger amplitude seen with II$_{Q271}$ likely reflects the further autolysis of mIIa produced from this form to a IIa-like species. Because of these complexities and our inability to completely account for

chain, well-separated in time from the rapid formation of the heavy chain (Fig. 7). There was some systematic deviation between light chain formation and Xa binding. This could reflect a small contribution to binding from other partially proteolyzed species at the concentrations used. However, it is at least partially related to the technical complexity of obtaining reliable fluorescence measurements under conditions relevant to the cleavage reactions.

The findings imply that light chain formation is the rate-limiting step in both the formation of heterodimeric Va and the development of Xa binding, a prerequisite for cofactor activity. Given the inferred importance of ABE2 in light chain formation, it also follows that mIIa and variants that contain an occluded ABE2 are extremely poor activators of factor V because of their impaired ability to cleave at Arg$^{1545}$. The findings imply that light chain formation is the rate-limiting step in both the formation of heterodimeric Va and the development of Xa binding, a prerequisite for cofactor activity. Given the inferred importance of ABE2 in light chain formation, it also follows that mIIa and variants that contain an occluded ABE2 are extremely poor activators of factor V because of their impaired ability to cleave at Arg$^{1545}$.
the fate of these proteinase products in plasma, we have not corrected the traces for differences in rates of indicator substrate cleavage. However, it is the greatly prolonged lags in these cases that are most relevant to the feedback activation steps. Although this is a contrived experimental system, its value here lies in the ability to document that when only mIIa is produced, amplification of the cascade is greatly impaired, consistent with its documented defect in catalyzing the activation of factor V.

**Discussion**

Proteolytic activation lies at the heart of the hemostatic response. In the case of the serine proteinase zymogens of coagulation, a single cleavage corresponding to the Arg15-Ile16 site in ancestral chymotrypsinogen is necessary to convert the inactive precursor to the active proteinase (36). This absolute requirement, predicated on structural information, holds true despite additional cleavages in some of the zymogens and facilitates the consideration of proteinase formation in precise terms. Until recently, the findings have not supported the consideration of the activation of factor V with analogous precision.

Part of the difficulty stems from the uncertain relationship between cleavage at specific sites and development of cofactor function. It is compounded by the confounding effects of further proteolysis, which can occur rapidly when cofactor function is assessed. Consequently, the literature is replete with the
Factor V activation by thrombin and meizothrombin

Figure 9. Measurement of the proteinase product of prothrombinase after initiation of the coagulation cascade in plasma with tissue factor. Thrombin or the proteinase product of prothrombinase was detected by cleavage of a fluorescent substrate following initiation of plasma with tissue factor and Ca\(^{2+}\). Shown are curves obtained with pooled normal plasma (black) and plasma immunodepleted of prothrombin and mixed with 1.4 \(\mu\)M IIWT (blue), IIQQQ (red), IIQ271 (cyan), or with the appropriate volume of buffer (green). The solid lines are averages of six replicates, and the 67% confidence limit bands are shown. For the IIQ271 experiment, one of the replicates was eliminated from the mean as an obvious outlier.

is readily cleaved by RVV\(_V\) without prior cleavage at the other sites (30). Conversely, potential support for the lack of ordered cleavage derives from the ability of IIa to cleave factor V variants wherein the three sites have been rendered uncleavable either singly or in combination (9).

Prior studies have implicated both ABE1 and ABE2 of IIa in factor V cleavage (8–11). Others have suggested that it is all ABE1 (17). Binding and structural studies with IIa and peptides surrounding the Arg\(^{709}\) and Arg\(^{1545}\) cleavage sites have indicated that elements surrounding Arg\(^{709}\) engage ABE1. Interactions with ABE2 and analogous but not identical interactions with ABE1 have been proposed from modeling studies with structures surrounding the Arg\(^{1545}\) site (10). The functional correlates for these ideas lie in the ability of ABE1- and ABE2-specific aptamers to differentially block peptide binding to IIa and affect cleavage of the three sites in the activation of factor V (9). These approaches have led to the conclusion that ABE1 is important for cleavage at Arg\(^{709}\), whereas both exosites are likely required for cleavage at Arg\(^{1545}\).

Instead, our findings portray a more complex picture of the role of these exosites in factor V cleavage. There is little doubt that ABE1 plays a significant role in cleavage at Arg\(^{709}\) and Arg\(^{1018}\) (Scheme 1) as has been proposed previously (9–11, 17). However, a role for ABE2 cannot be excluded, as mIIa variants in which this exosite is occluded are noticeably slower, albeit by a modest 6-fold, in producing the heavy chain (Table 1). Perhaps this modest effect, potentially attributable to ABE2, arises from steric effects of the propiece on the proposed looping of extended structures surrounding the Arg\(^{709}\) cleavage site around IIa (10). In contrast, ABE2 occlusion in the mIIa variants produces a ~100-fold reduction in the rate of cleavage at Arg\(^{1545}\), suggesting a primary contribution of ABE2 to binding and cleavage at this site. This conclusion is consistent with the known specificity of the action of RVV\(_V\) on factor V. It is also consistent with the recent X-ray structure of RVV\(_V\) both free and in complex with a factor V peptide encompassing the Arg\(^{1545}\) cleavage site (31). Obviously, we are unable to rule out some contribution of ABE1 to this cleavage step despite the fact that RVV\(_V\) is thrombin-like but lacks the features of ABE1 (31). However, experimental tests of such a contribution are effectively impossible, given the suggestion of ordered cleavage when the reactions preceding cleavage at Arg\(^{1545}\) are mediated by ABE1. Accordingly, all cleavage of factor V by thrombin was effectively eliminated by the addition of a strong ABE1-binding ligand that does not engage the active site (not shown).

The preparation of a stable mIIa product with multiple substitutions of Arg cleavage sites with Gln provides the added liability of having altered the intrinsic properties of mIIa. Although we cannot unequivocally rule this possibility out, an extensive body of work supports the conclusion that these mutations do not change its properties as a substrate (35) or affect active site function compared with IIa when occlusion of ABE2 by the propiece is accounted for (21). The latter includes the rate of protein C activation in the presence of saturating thrombomodulin (not shown).

The most unexpected finding derives from the observation that the evolution of high-affinity Xa binding correlates most closely with the slow cleavage at Arg\(^{1545}\) and the appearance of
the A3-C1-C2 light chain. Others have previously reported on the observation that cleavage at Arg$^{1545}$ by IIa is slow relative to cleavage at the other two sites (9, 17, 30). This slow cleavage has been proposed to be required for "optimal" cofactor activity with partial cofactor function attributed to other species generated following the action of IIa at one or both of the other sites (4, 30). The two new findings here relate to the fact that cleavage at Arg$^{1545}$ is at least 9-fold slower than the other cleavage reactions and is also the rate-limiting step in the ability of the cleaved species to bind Xa and assemble into prothrombinase. The membrane-dependent interaction with Xa is a prerequisite for cofactor function, and there is no further cleavage following the formation of the light chain. It follows that slow cleavage at Arg$^{1545}$ represents the rate-limiting step for the assembly of prothrombinase and the ability of the cleaved factor V to function as a cofactor. The explanation for the stark contrast between our findings and numerous previous studies likely lies in the documented potential for functional measurements of IIa formation in the presence of Xa added in excess to cause rapid proteolysis of partially cleaved forms of factor V. We nevertheless see some small but systematic deviation between the fluorescence measurements for Xa binding and cleavage at Arg$^{1545}$ (Fig. 7). The source of this deviation at least partly lies in the complexity of the experimental system and making time-dependent measurements of steady-state anisotropy. Nevertheless, we are unable to conclusively rule out some minor contribution of weak Xa binding by a partially cleaved factor V form, although which one that might be is not obvious from the cleavage patterns observed.

Meizothrombin and its variants are particularly poor at liberating the light chain and yielding a species that can bind Xa with high affinity. In contrast to several previous studies extending into the 1980s, it now follows that mIIa is a very poor activator of factor V even in the presence of membranes. This conclusion is in line with the properties of mIIa and its variants established in recent years (21). The discrepancy with prior findings lies in the problems of interpretation from functional measurements, the use of mIIa produced from prothrombin variants that are not completely resistant to autolytic attack to form a Ila-like species, or the use of light-scattering measurements to infer factor V cleavage with an unclear relationship between molecular weight changes and progressive cleavage (16). Comparisons with IIa also suffer from the difficulty in ascribing significance to a single rate constant assessed for factor V activation when there are numerous cleavage reactions involved (6, 16).

Several individuals with bleeding disorders have been identified with the disease attributable to a homozygous substitution at Arg$^{271}$ in prothrombin, which would render the site uncleavable and yield mIIa (37, 38). Whereas hemorrhagic disease was moderate to severe, the mutation was compatible with life (37, 38). This is rationalizable with the finding that although feedback activation steps would be impaired for the resultant mIIa, the proteinase product is actually produced after a long delay. An ameliorating factor for these patients is that mIIa produced would be autolysed to a Ila-like species with Ila-like activities including the ability to cleave fibrinogen to fibrin.

Analogous findings have been made with mice genetically modified to limit prothrombin activation to a stable mIIa, resistant to further autolysis (39). Although the mice exhibited severe bleeding and long lags in the thrombin generation assay, the animals that survived weaning had a normal lifespan and could reproduce (39). This again points to the adequate hemostatic potential despite the poor activities of mIIa in activating factor V and participating in the other reactions important for the regulation of the coagulation cascade.

Our findings add to the understanding of factor V activation and how the BR within the B domain regulates the transition of factor V to the active cofactor (Scheme 1). If cleavage at Arg$^{1545}$ and the release of the light chain is both required and sufficient for cofactor formation, then the data indicate that the BR is unable to restrict Xa binding following cleavage after AR2. This inhibitory role of the BR is considered to arise from its tight binding interactions with AR2 and likely also AR1, which bears sequences important for Xa binding (12, 24, 25). It follows that cleavage after AR2 at Arg$^{1545}$ is sufficient to disrupt the inhibitory structure and facilitate Xa binding despite the fact that AR1, BR, and AR2 are still contained in a polypeptide that remains linked to the heavy chain (Scheme 1).

In summary, our work addresses longstanding questions of the relative contributions of IIa and mIIa to factor V activation and the amplification of flux through the coagulation cascade. It also provides insights into factor V activation wherein a single cleavage reaction represents the rate-limiting step in the conversion of the procofactor to a cofactor that can assemble into prothrombinase. These findings shed new light on the mechanism of cofactor activation with important implications for the further biochemical and structural consideration of the process.

**Experimental procedures**

**Reagents**

Human plasma for the isolation of coagulation proteins was obtained as a gift from the plasmapheresis unit of the Hospital of the University of Pennsylvania. Crude venom from Russell’s viper (Daboia russelli) was from Latoxan (Portes-lès-Valence, France). The factor V antibody column and the blotting antibody directed toward the heavy chain (AHV-5146) and light chain (AHV-5112) were from Hematologic Technologies. L-Glutamyl-glycyl-L-arginine chloromethyl ketone (EGRck; Calbiochem), N-hydroxysuccinimidyl acetothioacetate, Oregon Green$_{488}$ maleimide (Thermo Fisher Scientific), and N-hydroxysuccinimidyl esters of 680LT and 800CW (Rockland Laboratories) were from the indicated suppliers. Acetoxyacetyl-EGRck was prepared and purified as described (40). Reagents for the IIa assays in plasma were pooled normal human plasma (George King), human plasma immunodepleted of prothrombin (Affinity Biologicals), RB tissue factor/phospholipid reagent (Technoclone), and Z-glycyl-glycyl-arginine-aminomethyl coumarin (Bachem). Small unilamellar phospholipid vesicles (PCPS) composed of 75% (w/w) hen egg l-α-phosphatidylcholine and 25% (w/w) porcine brain l-α-phosphatidylserine (Avanti) were prepared and quality-controlled as described (35). Unless specified, all measurements were conducted in 20
Factor V activation by thrombin and meizothrombin

**Proteins**

The factor X-activating proteinase and the factor V-activating proteinase (RVV\(\lambda\)) were purified from Russell’s viper venom using established procedures (29). Prothrombin and factor X were isolated using procedures described previously (41, 42). Prothrombin was purified free of trace contamination by factor X, and factor X was freed of contaminating protein C using immunoaffinity chromatography with immobilized HPC4 and 4G3 (43, 44). Factor X was activated using the purified factor X activator from Russell’s viper venom, and resulting Xa was purified free from the venom enzyme and factor X as described (42). Thrombin was produced by preparative activation of prothrombin using a mixture of Xa, Va, and PCPS and purified as described (45). Factor Xa was inactivated with acetoxyacetyl-EGRck and then modified with Oregon Green\(_{\text{abs}}\) maleimide following thioester hydrolysis using procedures detailed elsewhere to yield OG\(_{\text{abs}}\)-Xa (40).

Factor V was isolated from 3 liters of plasma by immunaffinity chromatography as described (46). Further purification was done by modifications to the ion-exchange chromatography strategy published previously (46). Pooled fractions from the antibody step were dialyzed overnight against 20 mM HEPES, 0.05 M NaCl, 10 mM benzamidine, and 5 mM CaCl\(_2\), pH 7.45 (20 liters). The dialyzed pool (0.75 liters) was adjusted to 2 liters with 20 mM HEPES and 5 mM CaCl\(_2\), pH 7.45, applied (15 ml/min) to a Poros HQ/M 10 × 100-mm column (Thermo Fisher Scientific), and eluted with a linear gradient of increasing NaCl (0.15–1.0 M, 5 ml/min, 40 ml) in the same buffer. The major protein peak was pooled, treated with 10 \(\mu\)M 4-amidino-phenylmethanesulfonyl fluoride hydrochloride, dialyzed overnight against 20 mM HEPES and 5 mM CaCl\(_2\), pH 7.4 (8 liters), and applied (5 ml/min) to a Poros HS/20 4.6 × 100-mm column (Thermo Fisher Scientific). Bound protein was eluted with a linear gradient of increasing NaCl (0.15–1.0 M, 5 ml/min, 40 ml) in the same buffer. The major protein peak was pooled, treated with 10 \(\mu\)M 4-amidino-phenylmethanesulfonyl fluoride hydrochloride, dialyzed overnight against 20 mM HEPES and 5 mM CaCl\(_2\), pH 7.4 (8 liters), and applied (5 ml/min) to a Poros HS/20 4.6 × 100-mm column (Thermo Fisher Scientific). Bound protein was eluted with a linear gradient of increasing NaCl (0.05–1.0 M, 5 ml/min, 40 ml). Fractions containing factor V were selected to minimize contamination by fragments, exchanged into Assay Buffer lacking PEG 8000, concentrated, and stored at −80 °C.

Recombinant WT prothrombin (II\(_{\text{WT}}\)); prothrombin with Gln substitutions at Arg\(_{155}\), Arg\(_{271}\), and Arg\(_{284}\) with either the full complement of 4-carboxyglutamate or lacking the post-translational modification (II\(_{\text{QQQ}}\), desGla-II\(_{\text{QQQ}}\); and prothrombin with Gln substitutions at Arg\(_{271}\) and Arg\(_{284}\) (II\(_{\text{QQ}}\)) were expressed in mammalian cells, purified, and quality-controlled as described (21). The production and purification of recombinant ecarnin has been described (21). Meizothrombin was produced from II\(_{\text{QQQ}}\) or desGla-II\(_{\text{QQQ}}\) by treating either protein (5 \(\mu\)M) in Assay Buffer containing 5 \(\mu\)M CoCl\(_2\), with 0.1 \(\mu\)M ecarnin for 10 min at 25 °C. The reaction mixture was diluted 3-fold with 20 mM HEPES, 5 mM CaCl\(_2\), pH 7.4, and applied (2 ml/min) to a Poros HS/M 4.6 × 100-mm column (Thermo Fisher Scientific). The desired stable products (mIIa or desGla-mIIa), free of ecarnin and uncleaved substrate, appearing in the flow-through were exchanged into Assay Buffer lacking PEG 8000 by repeated centrifugal ultrafiltration, concentrated, reconstituted with 0.1% (w/v) PEG 8000, and stored at −80 °C in aliquots.

Established procedures were used for the preparative proteolysis of II\(_{\text{QQQ}}\) at Arg\(_{155}\) and the purification of the resultant product (21). Thezymogen lacking the F1 domain was cleaved by ecarnin to yield mII\(_{\text{AQ1}}\) using the digestion conditions described above. Following cleavage for 10 min, the reaction mixture was applied (2 ml/min) to a Poros HQ/M 10 × 100-mm column. Bound protein was eluted by a gradient of increasing NaCl (0–1.0 M, 10 ml/min, 24 min) in 20 mM HEPES and 5 mM CaCl\(_2\), pH 7.45. Fractions containing mII\(_{\text{AQ1}}\) were exchanged into Assay Buffer lacking PEG 8000, concentrated by centrifugal ultrafiltration, reconstituted with 0.1% (w/v) PEG 8000, aliquoted, and stored at −80 °C.

The antibody directed against the heavy chain of factor V (AHV-5146) was labeled with 680LT N-hydroxysuccinimidyester, and the antibody reactive with the light chain of factor V (AHV-5112) was labeled with 800CW N-hydroxysuccinimidyester. For conjugation, the antibodies were exchanged into 20 mM HEPES, 0.15 M NaCl, pH 7.8, by centrifugal gel filtration using Sephadex G-25. The labeling reactions (0.3 ml) in the same buffer, contained 10 \(\mu\)M antibody and 50 \(\mu\)M probe. Following incubation for 3 h at 22 °C, reactions were quenched by the addition of 10 mM Tris, pH 8.0, and labeled antibody was purified from free probe by centrifugal gel filtration using P6-DG (Bio-Rad) equilibrated in 20 mM HEPES, 0.15 M NaCl, pH 7.5. Labeled antibodies were concentrated by centrifugal ultrafiltration, aliquoted, and stored at −80 °C. Labeling efficiency was ≈0.8 mol of dye/mol of IgG.

Protein concentrations were determined using the following molecular weights and extinction coefficients (\(E_{280}\)): factor V, 330,000, 0.96 (47); factor Xa, 45,300, 1.16 (48); all full length prothrombin variants, mIIa, and desGla-mIIa, 72,000, 1.47 (49); mII\(_{\text{AQ1}}\), 50,000, 1.78 (21, 49); Ila, 37,500, 1.89 (45); IgG, 150,000, 1.4; ecarnin, 88,000, 1.0; and RVV\(_{\lambda}\), 28,000, 1.56 (51).

**Factor V cleavage**

Cleavage of factor V was performed in Assay Buffer using concentrations of factor V ranging from 50 to 500 nm. Some reactions also contained 30 \(\mu\)M PCPS. After subsampling a reference point, cleavage was initiated by the addition of thrombin or mIIa variants to a final concentration ranging between 0.05 and 1 nm. Aliquots (15 \(\mu\)l) withdrawn at the indicated times were mixed with 15 \(\mu\)l of SDS quench containing 20 mM Tris, pH 6.8, 6.4 (w/v) SDS, 50 mM DTT, 50 mM EDTA, 0.04% (w/v) bromophenyl blue, and 32% (v/v) glycerol.

Quenched samples were subjected to electrophoresis (200 ng/lane) with commercial 4–12% BisTris gradient gels using the MOPS buffer system (Invitrogen). Proteins were electrophoretically transferred to 0.2-\(\mu\)m nitrocellulose membranes (Bio-Rad) for 2 h followed by blocking and washing steps that have been described in detail (52). The blots were then probed with a mixture of the labeled antibodies against the heavy and light chains (0.75 \(\mu\)g/ml each) in 20 mM HEPES, 0.15 M NaCl, 0.1% (v/v) Tween 20, pH 7.4, supplemented with 1% (v/v) Bovine Irish Cream for 1 h. Blots were washed three times with the
same buffer followed by two washes with 20 mM HEPES, 0.15 mM NaCl, pH 7.4, and air-dried. All blocking, washing, and probing steps were performed using an automated Freedom Rocker Blot Bot (Next Advance). Bound antibodies were detected by scanning infrared fluorescence in two channels (700 nm (heavy chain antibody) and 800 nm (light chain antibody)) using an Odyssey scanner (LI-COR) at the highest spatial resolution setting. Sensitivity in each channel was set to maximize the signal with a minimal observable background. The resulting raw TIFF files from each channel were inverted and saved in unscaled 16-bit TIFF format using Kodak molecular imaging software version 4.0.4 (Eastman Kodak Co.). Quantitation for each band was performed by volume integration using TotalLab Quant (Total Lab Ltd.).

**Fluorescence measurements**

Anisotropy measurements of the binding of OG$_{188}$-Xa$_8$ to Va in the presence of saturating concentrations of PCPS were performed using experimental considerations described previously in detail (46, 53). In the present study, the time-dependent increase in fluorescence anisotropy of OG$_{188}$-Xa$_8$ was measured during the activation of factor V in the presence of saturating PCPS. The concentrations of factor V and OG$_{188}$-Xa$_8$ were selected to provide an approximately linear dependence of anisotropy as factor V was activated up to the maximum concentration that could be achieved, established separately from equilibrium binding titrations with increasing concentrations of Va.

Reaction mixtures (2.5 ml) in Assay Buffer, maintained at 25 °C in stirred cuvettes, contained 40 nM OG$_{188}$-Xa$_8$ and 30 µM PCPS. Following the measurement of anisotropy to establish a baseline, a small volume of factor V was added to achieve a final concentration of 40 nM followed by anisotropy measurements to establish a new baseline. In all cases, the second measurement yielded a very small increase over that measured in the absence of factor V and likely reflects the variable traces of cleaved material in the factor V preparations. Factor V cleavage was initiated by the addition of 0.05 nM thrombin, 0.05 nM mIIa, or 4 nM RVVV. The time-dependent increase in anisotropy was measured from the G-factor and by averaging six 30-s readings alternating parallel and perpendicular orientations of the excitation polarizer. The corresponding time for the resulting mean anisotropy value was assigned to the midpoint of the 3-min measurement period. Samples (15 µl) were also withdrawn at the indicated times from parallel reaction mixtures or from the cuvette used for fluorescence measurements, quenched, and processed for analysis by Western blotting as described above. At the end of the time course, 5 nM IIa was added to the cuvette to cause quantitative conversion to factor Va. The achievement of a stable limiting value for anisotropy was established in three sets of measurements repeated ~15 min apart. The increase in anisotropy from baseline to this limiting value was used to normalize the time course and express the measurements as fractional Δ$r_{\text{max}}$.

**Measurements with plasma**

Thrombin generation was measured with a fluorometric assay in plasma immunodepleted of prothrombin by reconstituting II$_{WT}$, II$_{QQQ}$, and II$_{Q271}$ to 1.4 µM. Normal pooled plasma was also used as a positive control in comparisons. Plasma (40 µl) was mixed with 50 µl of 1 mM L-glycyl-glycyl-arginine-aminomethyl coumarin in 15 mM CaCl$_2$. Thrombin formation was initiated with RB tissue factor phospholipid reagent. Fluorescence was measured using $\lambda_{ex} = 360$ nm and $\lambda_{em} = 460$ nm for 90 min at 1-min intervals using a Molecular Devices Spectromax M2 at 37 °C. The first derivative was calculated from six replicates, averaged, and converted to thrombin concentration using a calibrator reagent.

**Analysis and replicates**

Normalization and validation of the linear response of the quantitative Western blotting signal over a large concentration range was done using considerations described previously (52). Initial velocities of heavy and light chain formation from densitometry data were obtained using the logarithmic approximation (54). For measurements of IIa formation in plasma, the final figure represents mean traces with 67% confidence limit bands from six replicates done simultaneously. A three-point sliding median filter was employed to dampen excursions in the first derivative at the longer times where the derivative is small and calculated in the face of a large fluorescence signal. For the mean trace with II$_{Q271}$, one replicate trace was eliminated as an obvious outlier. In one figure, densitometry data from three or four experiments have been averaged to provide means and S.D. values to illustrate the reproducibility of the measurements. All other cases illustrate representative plots and/or gel images from at least two experiments performed at an equal level of detail and frequently with different protein preparations.

**Author contributions**—H. N. B. did the work. H. N. B. and S. K. designed experiments, analyzed data, and wrote the paper.

**References**

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