Spellbinding Effects of the Acidic COOH-Terminus of Factor Va Heavy Chain on Prothrombinase Activity and Function

Jamila Hirbawi† and Michael Kalafatis*‡,‡,1

†Department of Chemistry and Center for Gene Regulation in Health and Disease (GRHD), Cleveland State University, Cleveland, Ohio 44115, United States
‡Department of Molecular Cardiology, Lerner Research Institute, The Cleveland Clinic, Cleveland, Ohio 44195, United States

ABSTRACT: Human factor Va (hfVa) is the important regulatory subunit of prothrombinase. Recent modeling data have suggested a critical role for amino acid Arg701 of hfVa for human prothrombin (hPro) activation by prothrombinase. Furthermore, it has also been demonstrated that hfVa has a different effect than that of bovine fVa on prothrombin-1 activation by prothrombinase. The difference between the two cofactor molecules was also found within the Asn700–Arg701 dipeptide in the human factor V (hfV) molecule, which is replaced by the Asp–Glu sequence in hfV. As a consequence, we produced a recombinant hfV (rhfV) molecule with the substitution 700NR→DE. rhfV700NR→DE together with the wild-type molecule (rhfVWT) were expressed in COS7 cells, purified, and tested for their capability to function within prothrombinase. Kinetic studies showed that the $K_{cat}$ of rhfV700NR→DE for human fXa as well as the $K_m$ of prothrombinase made with rhfV700NR→DE for hPro activation were similar to the values obtained following hPro activation by prothrombinase made with rhfVWT. Remarkably, sodium dodecyl sulfate polyacrylamide gel electrophoresis analyses of hPro activation time courses demonstrated that the rate of cleavage of hPro by prothrombinase reconstituted with rhfV700NR→DE was significantly delayed with substantial accumulation of meizothrombin, and delayed thrombin generation, when compared to activation of hPro by prothrombinase made with rhfVWT. These unanticipated results provide significant insights on the role of the carboxyl-terminal end of the heavy chain of hfV for hPro cleavage and activation by prothrombinase and show that residues 700NR701 regulate at least in part the enzyme–substrate/product interaction during fibrin clot formation.

INTRODUCTION

Human factor Va (hfVa) is the important regulatory subunit of prothrombinase that controls the rate of human prothrombin (hPro) activation by prothrombinase during hemostasis.1 This process is a highly regulated event, which involves various enzymatic entities that participate in a series of reactions. Prothrombinase activation of hPro is the penultimate step in the coagulation cascade implemented after any event that exposes a procoagulant membrane surface.2 In vivo, the procoagulant membrane surface is usually delivered by activated platelets and/or endothelial cells.3,4 Human factor Xa (hfXa), the enzymatic subunit of prothrombinase, can itself activate hPro after two consecutive cleavages at Arg271 and Arg1545 (Figure 1).10 The mature hPro is an inactive zymogen with a $M_r$ of 330 000. It is composed of three protein domains (A–C) that will produce a two-subunit protein made of light and heavy chains following cleavage and activation by thrombin at Arg709, Arg1018, and Arg1545 (Figure 1).10 The 105 000 heavy chain is bound to the 74 000 light chain through hydrophobic interactions. These hydrophobic interactions are only exposed on the hfV/hfVa molecule following its interaction with calcium ions (Figure 1).14

Human factor V (hfV) is present in blood at 20 nM as a single chain protein with a high molecular weight ($M_r$ of 330 000). It is composed of three protein domains (A–C) that will produce a two-subunit protein made of light and heavy chains following cleavage and activation by thrombin at Arg709, Arg1018, and Arg1545 (Figure 1).10 The 105 000 heavy chain is bound to the 74 000 light chain through hydrophobic interactions. These hydrophobic interactions are only exposed on the hfV/hfVa molecule following its interaction with calcium ions (Figure 1).14

Mature hPro is an inactive zymogen with a $M_r$ of 72 000 that has undergone substantial post-translational modifications before being secreted by the liver. Included in these modifications is the carboxylation of several glutamic acids at the NH2-terminus, a process that is vitamin K-dependent and is essential for proper binding of hPro to the procoagulant surface, which in turn is required for timely and efficient activation by prothrombinase. Thrombin has two charged regions (anion binding exosite I (ABE-I) and anion binding exosite II (ABE-II)), which are vital for its function. ABE-I is responsible for

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interacting with a plethora of proteins participating in the coagulation cascade like hfV, hfVa, and fibrinogen. ABE-II is the heparin-binding site.\textsuperscript{15} The $K_d$ value of the hfVa–hPro interaction is 1 $\mu$M.\textsuperscript{21,22} hfVa provides binding sites for proexosite 1 and the Gla domain of hPro, explaining one of the possible mechanisms by which the cofactor functions to increase enzyme efficiency.\textsuperscript{23} It has been proven that several acidic amino acids at the very end of the heavy chain of hfVa are vital for hfVa cofactor activity\textsuperscript{24} and participate in the interaction of hPro with prothrombinase. It has been proposed that amino acids from the region 680–709 are required for proper interaction and catalysis of hPro by hfVa. hfV is composed of three A domains (red), two C domains (light blue), and a B region (yellow). hfV is activated following three sequential cleavages by $\alpha$-thrombin at Arg\textsuperscript{209}, Arg\textsuperscript{263}, and Arg\textsuperscript{346}. These cleavages are required to release the active cofactor composed of heavy (amino acids 1–709) and light (amino acids 1546–2196) chains noncovalently associated in the presence of divalent metal ions, and two activation fragments. The carboxyl-terminal portion of the heavy chain contains an acidic hirudin-like amino acid region that is important for cofactor function.\textsuperscript{26} Adjacent to this region are also the amino acids Asp\textsuperscript{553} and Arg\textsuperscript{553}, which are important for cofactor activity.\textsuperscript{33} The amino acid substitutions within the heavy chain are indicated together with the designation for the recombinant mutant hfV molecule created and used throughout the article. A substantial difference was demonstrated when bovine factor Va (bfVa) or hfVa were used to activate prothrombin-1 (Pre1) by prothrombinase.\textsuperscript{35} The reason for the different effect of the cofactor on Pre1 was shown to be confined within the very last portion of the COOH-terminus of the hfVa heavy chain. A prominent difference in amino acid between the two cofactor molecules in that region is restricted to positions 700–701 where an Asn–Arg dipeptide in hfVa is replaced by the Asp–Glu sequence in bfVa,\textsuperscript{35–36} resulting in a total replacement of the net positive charge with two negative charges. Interestingly, very recently modeling data have shown that Arg\textsuperscript{701} of hfVa interacts with Glu\textsuperscript{345} of hPro.\textsuperscript{32} The work undertaken herein proposes to evaluate the significance of the 700Asn–Arg\textsuperscript{701} dipeptide of the hfVa heavy chain for enzyme–substrate recognition/interaction during hPro activation by prothrombinase. A substantial amino acid change, and a change in charge, in this portion of the cofactor will allow for an in-depth study of this very important contributor of prothrombinase. We directly tested the function of this portion of the heavy chain by using a recombinant molecule and several specific assays. The remarkable and unpredicted results presented herein solidify the notion that the acidic carboxyl-terminus of the heavy chain of hfVa is critical for proper prothrombinase function and dictates the timely interaction of hFVAs with the substrate/product, as recently suggested by modeling studies of prothrombinase.\textsuperscript{32}

**RESULTS AND DISCUSSION**

**Transient Expression and Analysis of rhfVNR–DE.** To ascertain the significance of the amino acid region 700–701 of

![Image](https://example.com/image.png)

**Figure 1.** hfV. Left panel, hfV structure; hfV is composed of three A domains (red), two C domains (light blue), and a B region (yellow). hfV is activated following three sequential cleavages by $\alpha$-thrombin at Arg\textsuperscript{209}, Arg\textsuperscript{263}, and Arg\textsuperscript{346}. These cleavages are required to release the active cofactor composed of heavy (amino acids 1–709) and light (amino acids 1546–2196) chains noncovalently associated in the presence of divalent metal ions, and two activation fragments. The carboxyl-terminal portion of the heavy chain contains an acidic hirudin-like amino acid region that is important for cofactor function.\textsuperscript{26} Adjacent to this region are also the amino acids Asp\textsuperscript{553} and Arg\textsuperscript{553}, which are important for cofactor activity.\textsuperscript{33} The amino acid substitutions within the heavy chain are indicated together with the designation for the recombinant mutant hfV molecule created and used throughout the article. Right panel; electrophoretic analyses of rhfVNR–DE and rhfVNR–DE molecules. rhfVNR–DE was activated with thrombin as described in the Experimental Section and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following transfer to a poly(vinylidene difluoride) (PVDF) membrane, immunoreactive fragments were detected with the monoclonal antibodies HFVaHC9 (recognizing the light chain) and HFVaHC17 (recognizing an epitope on the heavy chain).

| Table 1. Cofactor Properties of rhfVa Molecules |
|-----------------------------------------------|
| rhfVa species | clotting activity (U/mg) | decrease (-fold) | $K_{app}$ (nM) | $K_m$ (μM) | $k_{cat}$ (min$^{-1}$)$^a$ | $k_{cat}/K_m$ (M$^{-1}$·s$^{-1}$) | decrease (-fold) |
|----------------|--------------------------|-----------------|----------------|------------|----------------|-------------------|-----------------|
| rhfVaWT         | 3631 ± 237               |                 | 0.48 ± 0.10   | 0.207 ± 0.01 | 2688 ± 47      | 2.1               |                 |
| rhfVaNR–DE      | 210 ± 35                 | 17.3            | 0.75 ± 0.30   | 0.214 ± 0.03 | 2641 ± 87      | 2.0               | 1.05            |

$^a k_{cat} = V_{max}/[enzyme]$ (in the presence of fVa); the enzyme concentration of prothrombinase was 10 pM for all experiments.
hFVa, we made a recombinant mutant factor V molecule (rhFV) with the substitution NR→DE (Figure 1, left panel). Figure 1, right panel, shows a typical quality control practice. The data show the makeup of rhFVNR→DE before (right panel, lane 1) and after (right panel, lane 2) activation by thrombin. After treatment with thrombin, no rhFVNR→DE was apparent on the gel, whereas fragments with the molecular weights of the two subunits were visible (a fragment of M, 105 000 representing the heavy chain and a fragment of M, 74 000 representing the light chain, both identified with monoclonal antibodies specifically made against each subunit). Clotting activity assays illustrated the fact that activation of rhFVWT gave rise to a molecule with similar activity to that of the plasma-derived molecule (Table 1).26,36 In contrast, using analogous experimental conditions, rhFVaNR→DE had ~17-fold less clotting activity (Table 1). These results are unexpected, and they show that amino acids 700–701 are essential for expression of rhFVa clotting activity.

Characterization of rhFVa Molecules through Kinetic Experiments. The kinetic effect that hFVa has on the prothrombinase-mediated activation of hPro has been well-studied over the past 50 years, but it is still not properly understood, and no specific molecular role has yet been assigned to the cofactor. Research with discontinuous assays using a chromogenic substrate for thrombin revealed that when hFVa is combined into the prothrombinase complex, the overall increase in activity of the enzymatic complex for the activation of hPro, compared to cleavage and activation of hPro by FXa alone, is substantially increased, making the two-subunit enzyme one of the most proficient catalysts known in the human body and similar to several other enzymes required for survival such as superoxide dismutase, catalase, and carbonic anhydrase. This significant increase in affinity of prothrombinase toward its substrate is attributed to tighter binding of the enzyme complex to hPro because of its localization on the membrane surface by hFVa. Thus, hFVa enhances thrombin generation by facilitating initial cleavage at Arg220 on hPro by FXa that provides for the generation of an important enzymatic intermediate, MzT, with demonstrated anticoagulant activity.

We next evaluated the ability of rhFVNR→DE to bind FXa and form prothrombinase (Table 1). The data show that rhFVWT has similar affinity for FXa as its plasma counterpart.26,36 Similarly, rhFVNR→DE has comparable affinity for plasma-derived FXa, which is approximately the same as the affinity of rhFVWT for FXa. These data demonstrate that residues 700–701 from the heavy chain of FVa, while being a major contributor for optimal expression of FVa clotting activity, do not contribute to the interaction between the cofactor and FXa. We subsequently assessed the consequence of the mutation on the Km and kcat of the enzyme. The resulting kinetic graphs are shown in Figure 2, and the constants of the enzyme made with either rhFVWT or rhFVNR→DE are provided in Table 1. The results surprisingly demonstrate that the amino acid substitutions had no substantial effect on either the Km of the reaction or on the catalytic efficiency of prothrombinase made with rhFVNR→DE (Figure 2, Table 1). Compared to the data using the clotting assays, these unexpected results are somehow puzzling, and thus far demonstrate that the NR→DE substitution only affects the clotting activity of rhFVNR→DE. The kinetic data alone do not provide an explanation for the impaired clotting activity of rhFVNR→DE.

Visualization of the Activation Pathway. Because of the unexpected and contradictory results obtained in the activity assays (clotting vs kinetic assay), and to better appreciate the reason for the dearth in clotting activity of rhFVNR→DE, we evaluated hPro activation by SDS-PAGE. The results display a considerable and significant delay in hPro activation by prothrombinase made with rhFVNR→DE when related to cleavage of hPro by prothrombinase reconstituted with rhFVWT with MzT lingering very late in the reaction and with little visible thrombin formation (Figure 3). Densitometry scanning of the SDS-PAGE results presented in Figure 3 established a 10-fold delay in hPro cleavage by prothrombinase made with rhFVNR→DE when matched to the consumption of hPro with prothrombinase assembled with rhFVWT (Figure 4, Table 2). Furthermore, it is clearly visible on the gels that when hPro is cleaved by prothrombinase reconstituted with rhFVNR→DE, there is lingering of MzT during the activation time course. A peak for MzT is noticeable at 60 s when hPro is cleaved by prothrombinase made with rhFVWT, and a peak for MzT is present at ~600 s when hPro is processed by prothrombinase made with rhFVNR→DE (Figure 4). These data can explain the paradoxical findings above and the apparent discrepancy between the clotting and kinetic assays, and they undeniably suggest that the slow accumulation of MzT is responsible for the poor clotting activity observed with rhFVNR→DE. These data also verify our previous findings,26 and demonstrate that MzT can counterbalance the dearth of thrombin activity in the chromogenic test because its catalytic activity toward the chromogenic substrate used in our study is much higher than that of thrombin, as previously demonstrated.26,37,38

Our data clearly show that although prothrombinase assembled with rhFVNR→DE has similar Km and kcat as prothrombinase assembled with rhFVWT, the clotting activity of rhFVNR→DE is severely impaired. In addition, visualization of the cleavage pattern of hPro activation by prothrombinase assembled with rhFVNR→DE demonstrated a significantly different pattern of activation from the gels analyzing hPro activation.
by prothrombinase assembled with rhFVaWT. Thus, if we had limited our initial analysis of the mutant molecule to prothrombinase assays using purified proteins and a chromogenic substrate without using clotting assays and gel electrophoresis analysis, as is the case in the majority of the studies assessing mutations in the hFVa molecule, we would have missed and dismissed the particular critical regulatory function of this region of the molecule, as was the case on multiple occasions in the past.39

Activation of Recombinant Mutant hPro, FPR-MzT, and Pre1 by Prothrombinase Assembled with rhFVaNR→DE. The data obtained thus far show that both cleavages at Arg320 and Arg271 in hPro appeared to be impaired when using prothrombinase made with rhFVaNR→DE. To quantify the level of impairment of each of the cleavages separately in hPro that were specifically affected by the700NR→DE701 substitution, we

Table 2. Rate of Various Substrate Cleavage by Prothrombinase

| substrate                  | prothrombinase assembled with rhFVaWT (moles consumed·s⁻¹·(mole factor Xa)⁻¹) | prothrombinase assembled with rhFVaNR→DE (moles consumed·s⁻¹·(mole factor Xa)⁻¹) |
|----------------------------|--------------------------------------------------------------------------------|---------------------------------------------------------------------------------|
| plasma-derived prothrombin | 26.7 ± 2.5 (0.99)                                                              | 2.6 ± 0.17 (0.99)                                                               |
| rMZ-II                     | 11.8 ± 0.9 (0.99)                                                              | 1.7 ± 0.2 (0.99)                                                                |
| rP2-II                     | 3.0 ± 0.1 (0.86)                                                               | 1.5 ± 0.4 (0.97)                                                                |
| FPR-mezothrombin           | 56.8 ± 0.8 (0.99)                                                              | 19.4 ± 1.7 (0.99)                                                               |
| prethrombin-1              | 46.7 ± 3.3 (0.99)                                                              | 12.1 ± 1.2 (0.77)                                                               |

* The numbers in parentheses represent the goodness of fit for the fitting of the data to a first-order exponential decay.

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Activation of Recombinant Mutant hPro, FPR-MzT, and Pre1 by Prothrombinase Assembled with rhFVaNR→DE. The data obtained thus far show that both cleavages at Arg320 and Arg271 in hPro appeared to be impaired when using prothrombinase made with rhFVaNR→DE. To quantify the level of impairment of each of the cleavages separately in hPro that were specifically affected by the700NR→DE701 substitution, we
employed recombinant hPro molecules with only one site specific for prothrombinase cleavage (Arg$^{320}$ for rMZ-II and Arg$^{271}$ for rP2-II).40

The results provided in Figure 5A illustrate a substantial delay in the rate of cleavage of rMZ-II by prothrombinase reconstituted with rhfVa molecules. The gel shown in Figure 5 (A) was scanned and hPro consumption was recorded as described in the Experimental Section. Following scanning densitometry, the data representing recombinant mutant hPro consumption as a function of time (s) were plotted using nonlinear regression analysis according to the equation representing a first-order exponential decay using the software Prizm (GraphPad, San Diego, CA), as described in the Experimental Section. Prothrombinase was assembled with rhfVaWT (filled circles) or rhfVaNR→DE (open circles). The apparent first-order rate constant, $k$ (s$^{-1}$) was obtained directly from the fitted data, and the resulting numbers representing recombinant mutant hPro consumption are reported in Table 2.

![Figure 5. Analysis of the activation of rMZ-II and rP2-II. rMZ-II (1.4 μM, panel A) and rP2-II (1.4 μM, panel B) were incubated in different mixtures with PCPS vesicles (20 μM), DAPA (3 μM), and rhfVaWT (20 nM) or rhfVaNR→DE (20 nM). The reaction was started by the addition of fXa, and the samples were treated as detailed in the Experimental Section. Lanes 1–9 represent samples of the reaction mixture following incubation of prothrombinase assembled with rhfVaWT with rMZ-II or rP2-II before (lane 1) or following 1, 3, 5, 10, 20, 45, 60, and 120 min of incubation with fXa, respectively. Lanes 10–18 represent samples of the reaction mixture following incubation of prothrombinase assembled with rhfVaNR→DE with rMZ-II or rP2-II before (lane 10) or following 1, 3, 5, 10, 20, 45, 60, and 120 min of incubation with fXa, respectively. Positions of hPro-derived fragments are indicated to the right, as detailed in the legend of Figure 3. For easy reading of the article, the rhfVa species used for the reconstitution of prothrombinase are also shown.

![Figure 6. Analysis of rMZ-II consumption by prothrombinase reconstituted with rhfVa molecules. The gel shown in Figure 5 (A) was scanned and hPro consumption was recorded as described in the Experimental Section. Following scanning densitometry, the data representing recombinant mutant hPro consumption as a function of time (s) were plotted using nonlinear regression analysis according to the equation representing a first-order exponential decay using the software Prizm (GraphPad, San Diego, CA), as described in the Experimental Section. Prothrombinase was assembled with rhfVaWT (filled circles) or rhfVaNR→DE (open circles). The apparent first-order rate constant, $k$ (s$^{-1}$) was obtained directly from the fitted data, and the resulting numbers representing recombinant mutant hPro consumption are reported in Table 2.

with rhfVaNR→DE (lanes 10–18) when compared to the rate of activation of rMZ-II by prothrombinase composed with rhfVaWT (lanes 1–9). Scanning densitometry showed that activation of rMZ-II is slower by ~10-fold when prothrombinase is made with rhfVaNR→DE compared to activation of rMZ-II by prothrombinase composed with rhfVaWT (Table 2, Figure 6). A 2-fold slower hPro rate of activation was also detected when rP2-II was cleaved by prothrombinase reconstituted with rhfVaNR→DE when matched to the activation of rP2-II by prothrombinase made with rhfVaWT (Figure 5B, Table 2). These results support our data acquired with hPro. Altogether, the data suggest that prothrombininase-mediated cleavages at Arg$^{320}$/Arg$^{271}$ in hPro are significantly delayed when prothrombinase is made with rhfVaNR→DE. It is also clear, however, that cleavage at Arg$^{320}$ is more affected by the substitution in hPro than cleavage at Arg$^{271}$.

Examination of the results acquired up until now with plasma-derived and recombinant hPro demonstrates that the rate of activation of hPro or rMZ-II following cleavage at Arg$^{320}$ is more sensitive to the mutation in rhfVa than the rate of activation of hPro or rP2-II following cleavage at Arg$^{271}$. To understand the effect of the 700NR701→DE mutations on the cleavage of hPro at Arg$^{271}$ alone after the conformational alteration occurring in hPro following cleavage at Arg$^{320}$, we assessed the change in the rate of cleavage of FPR-meizothrombin (FPR-MzT) by prothrombinase reconstituted with rhfVaWT or rhfVaNR→DE (Figure 7). The results demonstrate a considerable delay for cleavage of FPR-MzT at Arg$^{271}$ by prothrombinase made with rhfVaNR→DE (panel B) when related to the reaction that is catalyzed by prothrombinase reconstituted with rhfVaWT (panel A). Densitometry scanning analysis of the concentration of fragment 1•2-De. It is also clear, however, that cleavage at Arg$^{271}$ is more affected by the substitution in hPro than cleavage at Arg$^{321}$.
hFvαWT (panel A). Scanning of Pre1 from the gels depicted in Figure 9 confirmed an ∼4-fold delay in activation of Pre1 at Arg320 by prothrombinase made with rhFvαWT-DE (Figure 10, Table 2). As a consequence, the 700NR701→DE mutations in the hFvα heavy chain substantially impair activation of Pre1 by prothrombinase because of an impaired capability for cleavage at Arg320 even when the substrate (Pre1) is not associated with a membrane surface.

Thus, because we have studied the consequence of the mutations on the clotting activity and on the rate of each cleavage individually, by analyzing activation of rMZ-II, rP2-II, FPR-MzT, and Pre1 by SDS-PAGE using prothrombinase made with rhFvαNR→DE, we can determine that both cleavages at Arg320 and Arg701 are impaired, resulting in MzT being present throughout the time course, while lacking acidic carboxyl-terminus of the hFvα heavy chain and several residues adjacent or nearby to the crucial cleavage site at Arg320 of hPro.32 We show that replacement of Arg701 in hFvα by a Glu, and Arg320 by prothrombinase because of an impaired capability for cleavage at Arg320 even when the substrate (Pre1) is not associated with a membrane surface.

Figure 7. Gel electrophoresis analyses for cleavage of FPR-MzT. FPR-MzT (1.4 μM) was incubated in different mixtures with PCS vesicles (20 μM) and rhFvα as described in the legend to Figure 3. The reactions were started by the addition of FXa, and the samples were further treated, scanned, and quantified as detailed in the Experimental Section. Panel A, prothrombinase assembled with rhFvαWT; panel B, prothrombinase assembled with rhFvαNR→DE. M represents the lane with the molecular weight markers (from top to bottom): M, 50 000, M, 36 000, M, 22 000. Lanes 1–17 represent samples from the reaction mixture before (0 min) the addition of FXa and 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, and 240 s, 5, 6, 10, and 20 min, respectively, following the addition of FXa. The hPro-derived fragments are shown as detailed in the legend to Figure 3. The recombinant hFvα species used for the reconstitution of prothrombinase is also shown under each panel.

Figure 8. Analysis of FPR-MzT consumption by prothrombinase assembled with rhFvα molecules. The gels shown in Figure 7 were scanned and FPR-MzT consumption was recorded as described in the Experimental Section. Following scanning densitometry, the data representing FPR-MzT consumption as a function of time (s) were plotted using nonlinear regression analysis according to the equation representing a first-order exponential decay using the software Prizm (GraphPad, San Diego, CA), as described in the Experimental Section. The apparent first-order rate constant $k$ (s$^{-1}$) was obtained directly from the fitted data. Prothrombinase was assembled with rhFvαWT (filled circles) or rhFvαNR→DE (open circles); factor FXa alone cleavage of FPR-MzT is shown by the open squares. The resulting numbers representing FPR-MzT consumption are reported in Table 2.

Figure 9. Analysis of FPR-MzT consumption by prothrombinase assembled with rhFvα molecules. The gels shown in Figure 7 were scanned and FPR-MzT consumption was recorded as described in the Experimental Section. Following scanning densitometry, the data representing FPR-MzT consumption as a function of time (s) were plotted using nonlinear regression analysis according to the equation representing a first-order exponential decay using the software Prizm (GraphPad, San Diego, CA), as described in the Experimental Section. The apparent first-order rate constant $k$ (s$^{-1}$) was obtained directly from the fitted data. Prothrombinase was assembled with rhFvαWT (filled circles) or rhFvαNR→DE (open circles); factor FXa alone cleavage of FPR-MzT is shown by the open squares. The resulting numbers representing FPR-MzT consumption are reported in Table 2.

generating the wrong conclusion that the NR→DE substitution has negligible or no effect on prothrombinase activity.

The hypothesis that Fvα confines and places hPro in an optimum position for efficient catalysis by FXa was confirmed by computational studies with prothrombinase by Shim et al.,32 who demonstrated that the acidic end of the heavy chain of hFvα is essential in its ability to capture the serine protease domain of hPro and reposition the Arg320 cleavage site at an optimal position for well-timed cleavage by FXa and hPro activation.32 These productive interactions between the acidic amino acids from the carboxyl-terminal portion of the hFvα heavy chain and hPro have been repeatedly suggested following experiments with synthetic peptides and recombinant hFvα molecules.26–28 In particular, Shim et al. proposed a direct interaction between Arg320 of Fvα and Glu345 of hPro, the latter being only 24-amino acids away from the crucial activating hPro cleavage site at Arg320.32 They also proposed a salt bridge between Asp695 of Fvα and Lys340 of hPro, and between Tyr698 of Fvα and Lys474 of hPro.32 We show that replacement of Arg320 in hFvα by a Glu, and the subsequent loss of the positive charge, results in a considerable delay in activating hPro following cleavage at Arg320.32 We have also shown repeatedly that Asp695 and Tyr700 are a part of a peptide portion of the hFvα heavy chain that represents a control switch for the activity of prothrombinase.27,28 Overall our experimental data together with the recent computational model of hFvα clearly establish a prolific interaction between the acidic carboxyl-terminus of the hFvα heavy chain and several residues adjacent or nearby to the crucial cleavage site at Arg320 of hPro for timely thrombin production.
CONCLUSIONS

The specific amino acid sequence 700−701 from hfVa is not strictly conserved among species36 (indicated by the vertical arrow in Figure 11). Although hfVa has the identical sequence as higher primates and fVa from horse, all other species shown in Figure 11 have different amino acids at these two positions, suggesting an important role of these two residues within fVa. Although there is no effect of the 700NR701 → DE mutation in hfVa on the direct binding of hfVa to hfXa, there is a significant effect of the mutation on hPro activation by prothrombinase reconstituted with the mutated cofactor molecule, which is translated by hindered cleavage at both Arg320/Arg271. Consequently, our results strongly suggest that these amino acids are part of an important sequence that is accountable for the recognition and interaction of the substrate (Pro) with prothrombinase within different species.36 Moreover, the data presented herein support the notion that Arg701 of the hfVa heavy chain makes a salt bridge with Glu345 of hPro, thus facilitating and promoting initial cleavage of hPro at Arg320, which was suggested by a recent computational model of prothrombinase.32 Collectively, the results strongly suggest that hfVa, the regulatory subunit of prothrombinase, undeniably controls the activity of hfXa within the enzymatic complex by directing the enzymatic subunit toward cleavage at Arg320, and thus actively participates in the cleavage and activation of hPro by prothrombinase. We must also conclude that all assays used herein to characterize rhfVaNR → DE are not redundant, but rather they are complementary and a procedural requirement to understand the structural intricacies of the cofactor and its contributions to the activity of prothrombinase.

EXPERIMENTAL SECTION

Materials and Reagents. Human fV cDNA was obtained from American Type Tissue Collection (ATCC# 40515 pMT2-V, Manassas, VA). The origin of all other chemicals, reagents, and proteins used by our laboratory is provided elsewhere.36

Figure 9. Gel electrophoresis analyses for cleavage of Prethrombin-1. Prethrombin-1 was incubated in different mixtures with PCPS vesicles and rhfVa as described previously in detail.20 The reaction and the samples were further treated, scanned, and quantified as detailed in the Experimental Section. Panel A, control, rhfVaWT; panel B, rhfVaNR → DE. M represents the lane with the molecular weight markers (from top to bottom): M, 50 000, M, 36 000, M, 22 000. Lanes 1−19 represent samples from the reaction mixture before and after the addition of fXa as previously described.20 The hPro-derived fragments are shown as detailed in the legend to Figure 3. The fragment denoted as P2′ depicts Pre2 cleaved at Arg284. For easy reading of the article, the rhfVa species used for the reconstitution of prothrombinase is also shown under each panel.

Figure 10. Analysis of Prethrombin-1 consumption by prothrombinase assembled with rhfVa molecules. The gels shown in Figure 9 were scanned and Pre1 consumption was recorded as described in the Experimental Section. Following scanning densitometry, the data representing Pre1 consumption as a function of time (s) were plotted using nonlinear regression analysis according to the equation representing a first-order exponential decay using the software Prism (GraphPad, San Diego, CA), as described in the Experimental Section. The apparent first-order rate constant k (s−1) was obtained directly from the fitted data. Prothrombinase was assembled with rhfVaWT (filled circles), rhfVaNR → DE (open circles); cleavage by fXa alone is shown by open squares. The resulting numbers representing Pre1 consumption are reported in Table 2.

Figure 11. Comparison of the last 20 amino acids from the acidic carboxyl-terminal portion of the fVa heavy chain. The acidic sequence from 20 species is illustrated as adapted from ref 36. The special nomenclature of all species was previously described.36
Recombinant hPro rMZ-II with only one site for hFXa (i.e., Arg\(^20\)) and hPro rP2-II with only one site for hFXa (i.e., Arg\(^271\)) were obtained as detailed previously.\(^{10}\)

**Mutagenesis and Transient Expression of Recombinant FV Molecules.** A mutant hFV molecule mutated at the COOH-terminus of the heavy chain was produced using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), and was constructed as previously detailed\(^{26}\) with the mutagenic primers fVNR\(^\text{DE}\) mutant: 5′-GATTGCTGACTATGATTACGAGCAACTGGCTGAGCATT-3′ (sense) and 5′-GATTCTAATGCTGCAGCCATCGTCTGGTAATCATG-3′ (antisense).

Expression of Recombinant Wild-Type and Mutant FV in Mammalian Cells. Expression in COS-7L cells was performed as previously detailed,\(^{26}\) and the concentration of recombinant proteins was obtained by enzyme-linked immunosorbent assay as previously shown.\(^{43}\) The activity of the recombinant proteins was obtained by enzyme-linked immunosorbent assay as previously shown.\(^{43}\) The activity of the recombinant molecules was verified by clotting assays using FV-deficient plasma.\(^{36}\)

**Gel Electrophoresis and Western Blotting.** SDS-PAGE analyses were performed using the method of Laemmli.\(^{44}\) Western blotting using poly(vinylidene difluoride) (PVDF) membranes was performed according to Towbin et al.\(^{45}\) Following transfer to PVDF, FV heavy and light chain(s) were identified using the suitable monoclonal and polyclonal antibodies\(^{5,6,44}\) and chemiluminescence.

**Analysis of hPro Activation by Gel Electrophoresis.** hPro molecules (1.4 μM) were incubated with PCPS vesicles (20 μM), DAPA (50 μM), and IVa, and analysis was performed as previously detailed,\(^{26,28,36}\) following transfer to PVDF.

**Measurement of Rates of hPro Activation.** All rhFVa molecules were activated with human thrombin and tested as previously detailed using a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA).\(^{31,50}\)

**Scanning Densitometry of SDS-PAGE and Calculation of the Rate of hPro Consumption.** Scanning densitometry of the gels was performed as described in detail elsewhere.\(^{30}\)

## AUTHOR INFORMATION

**Corresponding Author**
*E-mail: m.kalafatis@csuohio.edu.* Tel.: (216) 687-2460. Fax: (216) 687-9298.

**ORCID**
Michael Kalafatis: 0000-0002-3254-3101

**Author Contributions**
J.H. designed, performed, and analyzed the experiments and participated in the writing of the paper. M.K. conceived and coordinated the study, designed the experiments, and wrote the paper. Both authors reviewed the results and approved the final version of the manuscript.

**Notes**
The authors declare no competing financial interest.

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**ABBREVIATIONS**
PS, l-α-phosphatidylserine
PC, l-α-phosphatidylcholine
PCPS, small unilamellar phospholipids vesicles composed of 75% PC and 25% PS (w/w)
SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
rhFVNR\(^\text{DE}\), wild-type recombinant human FV
rhFVWT, wild-type recombinant human FV activated with thrombin
rhFVNR\(^\text{DE}\), recombinant human FV with the mutations 706NR\(^\text{DE}\), recombinant human FV with the mutations 705NR\(^\text{DE}\), recombinant human FV with the mutations 706NR\(^\text{DE}\), recombinant human FV with the mutations rhFVNR\(^\text{DE}\), recombinant human FV with the mutations
rhFVWT, recombinant human FV with the mutations
rF, recombinant human FV
rF WT, recombinant human FV wild type
hF, human factor F
hFa, human factor Ea
MrT, meizothrombin
Pre1, prothrombin-1
FPR, Phe-Pro-Arg-Chloromethylketone

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