DIFFERENCES IN PRETHROMBIN-1 ACTIVATION WITH HUMAN OR BOVINE FACTOR VA CAN BE ATTRIBUTED TO THE HEAVY CHAIN[*]

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SUMMARY

Human and bovine factor Va (FVa) function similarly in the activation of prothrombin but differently in the activation of prethrombin-1 (Pre-1). Pre-1 activation with human FVa proceeds at about 22 percent of the rate with bovine FVa. The dependencies of initial rates on the FVa and Pre-1 concentrations indicate that the differential activity is expressed in kcat differences, rather than differences in the assembly of prothrombinase or the K_m value of the substrate. The heavy and light chains of both species of FVa were separated and interspecies hybrids were constructed in the presence of Ca^{++}. Studies of the activation of Pre-1 with these hybrids indicate that the species difference can be attributed specifically to the heavy chain of FVa. Analyses of the reactions by SDS-PAGE indicated that cleavage at Arg271 occurs at about the same rate with both species of FVa, but cleavage at Arg320 with human FVa is specifically retarded. A major difference in primary structure between the human and bovine FVa heavy chains comprises 10 residues at COOH-terminus, adjacent to the negatively charged hirudin-like DYDYQ sequence. These residues have pI values of 12.5 and 4.26 in human and bovine FVa, respectively. The lower value would complement the negatively charged DYDYQ sequence but the higher value would counteract it. Thus, we suggest that the differences in the COOH-terminus of the heavy chain are responsible for the differences in Pre-1 activation, and that it specifically influences cleavage at Arg320 in Pre-1.

INTRODUCTION

Factor Va (FVa) is a two subunit protein that functions as a non-enzymatic cofactor in prothrombin activation (1,2). It is derived factor V by limited proteolysis, catalyzed by thrombin or FXa (3–6). The subunits of FVa are designated the heavy chain (105 kDa) and the light chain (74 kDa) (7,8). These come, respectively, from the amino (NH_2-) and carboxy (COOH-) termini of factor V (7–9). The heavy chain of human FVa spans residues 1–709 and contains an A1 and an A2 domain. The light chain of human FVa spans residues 1546–2196 and contains an A3, a C1 and a C2 domain (10). The heavy and light chains can be dissociated by Ca^{++} chelators and the individual chains can be isolated (3,7). Neither chain alone is functional, but activity can be regained by recombining the chains with Ca^{++} (3,7,11).

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Prothrombin is a 72 kDa vitamin K-dependent protein (12). It has 3 domains that are designated, from the NH_2-terminus, fragment-1 (22 kDa), fragment-2 (13 kDa) and prethrombin-2 (Pre-2) (38 kDa). Two respective cleavages catalyzed by FXa between fragment-2 and Pre-2 (Arg271) and within a disulfide loop in the Pre-2 domain (Arg320) generate thrombin (12,13). In addition, thrombin catalyzes cleavages of the bond joining fragment-1 and fragment-2. After activation, fragment-1 and fragment-2 are liberated as activation fragments. Fragment-1 confers upon prothrombin Ca^{++} and procoagulant phospholipid binding properties (12,14). These interactions are mediated by the ten gamma carboxyglutamic acid residues within fragment-1. Fragment-2 comprises a triple disulfide bonded structure referred to as a kringle. It mediates an interaction between prothrombin and FVa (13). Prethrombin-1 (Pre-1) is a truncated form of prothrombin lacking fragment-1 (12).

Prothrombinase consists of a 1:1 complex of FXa (serine protease) and FVa on a procoagulant surface (1,2,15). These components catalyze prothrombin activation 300,000-fold faster than FXa alone (16). In model systems, the surface can be provided by negatively charged phospholipid vesicles, whereas the physiologic surface is provided by activated platelets or monocytes and lymphocytes (17,18). FVa binds to both the surface and FXa, and thereby concentrates the protease with the substrate at the catalytic surface (19). This effect enhances the rate by approximately 100-fold (16,20). In addition, FVa, through an unknown mechanism, enhances the turnover number of the reaction by 3000-fold (16,20). These effects together account for the 300,000-fold increase in rate that occurs with the combination of the surface and FVa (20).

Studies of the kinetics of activation of prothrombin in the absence of phospholipid showed that the process conforms to a mechanism in which FVa forms a binary complex with either FXa or prothrombin, through interactions mediated by both the light and heavy chains of FVa (15,21–25). Either of these binary complexes can interact with the third component (FXa or prothrombin) to form a ternary FXa·FVa·prothrombin complex (23). Thrombin is then generated from prothrombin within this complex.

The heavy chain of FVa contains a hirudin-like sequence near the COOH-terminus, DYDYQ (695–699), that has been identified as a potential prothrombin interaction site and a determinant of the prothrombin activation pathway (26,27). Gerads *et al.* showed that cleavage of the heavy chain with an enzyme purified from the venom of the snake *Naja naja oxiana* resulted in a >9-fold increase in K_m for prothrombin activation (28). Subsequently, Bakker *et al.* showed that the removal of residues 683–709 of the heavy chain by the same enzyme caused a 9-fold or greater increase in the dissociation constant for the FXa-FVa complex on a phospholipid membrane surface, regardless of whether FXa or FVa were limiting (29). Camire *et al.* used cathepsin G or human neutrophil elastase to remove the COOH terminal of the heavy chain. They found similar increases in the dissociation constant for the interaction between FVa and FXa on the membrane surface (30). Hirbawi *et al.* recently utilized two mutants of FVa, one missing residues 680–709 of the COOH-terminus of the heavy chain, and the other having DYDY (695–698) substituted with four alanine residues (31). Both mutants showed significant decreases in the clotting activity. These mutants, however, showed increases in the apparent kcat values for prothrombin activation when assessed in assays using purified reagents and thrombin-specific chromogenic substrates. A similar increase in kcat also was reported by Gerads *et al.* (28) and Camire *et al.* (30). Hirbawi *et al.* also showed that the mutants promoted meizothrombin formation normally, but were specifically defective in conversion of meizothrombin to thrombin (31). These observations, however, are at odds with a study by Toso and Camire in which various mutants of human FVa with truncated COOH-termini of the heavy chain,

while defective in Pre-1 activation, were as functional as wild-type FVa in prothrombin activation (32).

The present studies were motivated by unexpected observations made when measuring thrombin generation in mixtures of bovine and human FXa, FVa, prothrombin and Pre-1. The results indicated that prothrombin is a good substrate for prothrombinase regardless of the species of the proteins. Pre-1, however, was efficiently converted only when bovine FVa was included in the prothrombinase complex, regardless of the species of FXa or Pre-1. These results were provocative since the sequences of the human and bovine FVa heavy and light chains, respectively, are 84 and 86 percent identical (8,9) and, therefore, similar if not identical function was expected. The studies below were undertaken to determine whether the differences in function between bovine and human FVa with respect to Pre-1 activation can be attributed to differences in K_m, kcat or prothrombinase assembly, whether the differences can be specifically localized to the heavy or light chains of FVa, and whether they rationally correlate with differences in primary structure.

MATERIALS AND METHODS

Proteins and Reagents

Human and bovine coagulation factor V (4), prothrombin and factor X were isolated as described previously (33) with modifications (34). FXa (35) and thrombin (36) were prepared as described previously. The fluorescent thrombin inhibitor dansylarginine N-(3-ethyl-1,5-pentanediyl)amide (DAPA) was prepared according to Nesheim, *et al.* (37). Vesicles of phosphatidyl-L-choline: phosphatidyl-L-serine (75% PC/25% PS (PCPS)) were prepared as described (38). Human Pre-1 was prepared by a modification of the procedure of Mann (12), as follows. Prothrombin (5.0mg/ml, 69μM, 12.0ml) in 0.017 imidazole, 0.144M NaCl, pH7.4 was incubated at 22°C with thrombin (5.0 NIH units/ml, 48nM) for 7.5 hours. The thrombin inhibitor D-phenylanyl-prolyl-arginyl chloromethyl ketone (PPAck, Calbiochem) then was added (50nM final) and the solution was dialyzed against 0.02M Tris-HCl, 0.15M NaCl, pH 7.4 at 4°C overnight (4 liters × 3). The dialyzed sample then was added at 22°C to a column of DEAE cellulose (20ml bed volume, 2 × 10cm) equilibrated in the sample buffer. The column was washed with the starting buffer and 5 ml fractions were collected. Pre-1 and residual inactive thrombin were recovered in the wash, which was diluted 2:3 with water and added to a column of SPC-50 Sephadex (40ml bed volume, 3 × 12cm) equilibrated in the sample buffer. Pre-1 was obtained in the flow through and was precipitated by dialysis against ammonium sulfate (80% saturation). A Pre-1 pellet, obtained by centrifugation, was resuspended in 1–1.5ml of the supernatant and stored at 4°C. Prior to use an aliquot of the suspension was centrifuged, the supernatant was discarded, and the pellet was dissolved in the appropriate buffer and dialyzed. The extinction coefficients and molecular weights were: FVa 1.74, 150,000 (11); FVa light chain 2.23, 74,000 (11); FVa heavy chain 1.24, 94,000 (11); FXa 1.16, 43,000 (39); human prothrombin 1.38, 72,000 (12); human Pre-1 1.64, 50,000 (12); human thrombin 1.83, 37,000 (40,41).

Conversion of human prothrombin and prethrombin-1 to thrombin with human or bovine factor Va

Human or bovine FVa (10nM), human FXa (1.0nM), CaCl$_2$ (2.0mM), PCPS vesicles (20μM phospholipid) and DAPA (3.0μM) were prepared in 0.02M Tris-HCl, 0.15M NaCl, 1% polyethylene glycol 8000 or 0.01% Tween-80 to minimize protein adsorption (1.6ml total volume). The solution then was placed in a cuvette thermostated at 22°C. Substrate conversion was initiated by adding a 30μL aliquot of concentrated prothrombin in Ca^{++} (2.0mM). Thrombin formation was monitored continuously by the enhanced fluorescence of the DAPA-thrombin complex using a Perkin Elmer Model MPF-66 spectrofluorometer. The

excitation and emission wavelengths were 335nm and 545nm, respectively, and a 430nm filter was used in the emission beam. The signal was calibrated by monitoring some of the reactions to completion. The fluorescence intensity of the human meizothrombin/DAPA complex is about 70 percent greater than that of the thrombin/DAPA complex (42). SDS-PAGE results (see below) indicated that at maximum, meizothrombin was 10 percent of thrombin in the initial parts of the reactions. Therefore, the rate of thrombin formation might be overestimated by as much as 7 percent. Because this is a relatively small effect, no efforts were made to correct signals for small amounts of meizothrombin (35,42,43) present transiently during activation reactions.

Similar experiments were performed using human Pre-1 (1.4μM) as the substrate, in the presence of human or bovine FVa (10nM), $CaCl_2$ (2.0mM), PCPS vesicles (20μM phospholipid) and DAPA (3.0μM) prepared in 0.02M Tris-HCl, 0.15M NaCl, 0.01% Tween-80, pH 7.4. The excitation and emission wavelengths were 280nm and 545nm, respectively, and a 515nm cutoff filter was used in the emission beam. Substrate conversion was initiated by the addition of 2.5nM FXa. Prothrombin at various concentrations, fully activated to thrombin, was used as the standard to quantify the thrombin/DAPA complex generated in the reactions.

Separation and reconstitution of the heavy and light chains of bovine and human factor Va

Human factor V (0.1mg/ml) in 10.0ml of 0.02M Tris-HCl, 0.15M NaCl, 5mM $CaCl_2$, pH 7.4 and was incubated at 37°C with thrombin (19nM) for 20 minutes. PPAck then was added (1μM final). The sample was diluted with an equal volume of water and was applied to a 1.0 × 2.5cm all glass column filled with QAE-cellulose (diethyl-(2-hydroxypropyl)-aminoethyl Cellulose, Sigma Chemical Co., St. Louis, MO) equilibrated at 22°C with the sample buffer. The contents of the column were washed with the same buffer. Then one bed volume of the buffer with EDTA (5mM) rather than Ca^{++} was added and the flow was stopped for 2.0 hours to dissociate the heavy and light chains of FVa on the column. Flow then was resumed with the same buffer and 10, 1.5ml fractions were collected in glass tubes. Elution then was continued in the same buffer containing 0.3M NaCl. Two peaks were identified by absorbance at 280nm. The first was obtained upon resumption of flow and contained the light chain. The second was obtained upon elution with 0.3M NaCl and contained the heavy chain. The bovine heavy and light chains were similarly isolated except prior chromatography of factor V on QAE-cellulose was used to remove minor contaminants. Bovine factor V (10mg) was dissolved in 10.0ml of 0.02M imidazole, 5mM $CaCl_2$, pH 6.5. This solution was added to QAE cellulose equilibrated at 22°C in the same imidazole buffer in an all glass column (1.1 × 5.0cm). The contents of the column were washed with the equilibration buffer and factor V was eluted in a gradient of (0 to 0.3M) NaCl in the same buffer. The total volume of the gradient was 45ml and 1.5ml fractions were collected. The ten peak fractions, identified by absorbance at 280nm, were pooled and incubated at 37°C. Thrombin was added (19nM) and incubation was continued for 20 minutes. PPAck then was added (1.0μM). Three volumes of 0.02M imidazole, 5.0mM $CaCl_2$, pH 6.5 then were added. The QAE-cellulose in the column used for the chromatography of factor V was washed with 0.02M imidazole, 5.0mM $CaCl_2$, 1.0M NaCl, pH 6.5 and was re-equilibrated 0.02M imidazole, 5.0mM $CaCl_2$, pH 6.5. The activated, diluted sample of factor V was applied to the column at 22°C and a wash with the equilibration buffer was applied. The buffer then was changed to 0.01M Tris-HCl, 0.075M NaCl, 2.5mM $CaCl_2$, pH 7.5 (5–10 column volumes were added). One bed volume of the same buffer with EDTA (5.0mM) rather than Ca^{++} then was added and flow was stopped for 2 hours in order to dissociate the heavy and light chains of FVa on the column. Flow was then resumed and the heavy and light chains were recovered exactly as described above for their human counterparts. The fractions eluted from the QAE-cellulose containing human or bovine heavy and light chains were

stored at 4°C. The isolated chains thus were stored in 0.01M Tris-HCl, 5.0mM EDTA, pH7.5 with NaCl at 0.075M (light chain) and 0.3M (heavy chain). The concentrations of stock solutions of the human chains were typically 0.5μM (light chain) and 0.9μM (heavy chain). The corresponding concentrations of the stock solutions of the bovine chains were 3.9μM (light chain) and 9.1μM (heavy chain). Specimens of FVa were prepared by diluting aliquots of the stock solutions containing heavy and light chains from both species in 0.02M Tris-HCl, 0.15M NaCl, pH 7.4 at 37°C and adding Ca^{++} (10mM). Clotting assays in factor V-deficient human plasma (4) were used to monitor reassembly. Clotting times decreased over the interval 0–20 minutes after the addition of Ca^{++} and were stable for at least 3 hours thereafter. The final concentrations (μM) of the heavy and light chains of the reconstituted species of FVa were: (human heavy, human light; 0.55, 0.24), (bovine heavy, bovine light; 0.39, 0.5); (human heavy, bovine light; 0.55, 0.55) and (bovine heavy, human light; 0.50, 0.18). The nominal FVa concentration was equated with the less concentrated chain in all cases.

Determination of K_m and kcat values for the activation of human prethrombin-1 by prothrombinase containing either human or bovine factor Va

Pre-1 (0 to 25μM) was incubated with human or bovine FVa (10nM), $CaCl_2$ (2.0mM), PCPS vesicles (20μM phospholipid) and DAPA (3.0μM) prepared in 0.02M Tris-HCl, 0.15M NaCl, 0.01% Tween-80, pH 7.4. Each reaction mixture was equilibrated in a microcuvette in a Perkin-Elmer lambda 50B spectrofluorimeter. The reactions were started by adding FXa (2.5nM), and thrombin generation was monitored by the fluorescence of the DAPA-thrombin complex. The excitation and emission wavelengths were 350nm and 545nm, respectively. Excitation at 350nm was used in order to minimize the internal filter effect at high Pre-1 concentrations. Slit widths of 5nm and 10nm were used in the excitation and emission paths, respectively, and a 430nm cut off filter was used in the emission beam. Initial rates of thrombin formation were fitted to the Michaelis-Menten equation by non-linear regression analyses using the NONLIN module of SYSTAT (SYSTAT, Evanston, IL).

Analysis of human prothrombin or prethrombin-1 activation by prothrombinase containing human or bovine factor Va by SDS-PAGE

Human prothrombin or Pre-1 (1.4μM) with either human or bovine FVa (10nM), $CaCl_2$ (2.0mM), PCPS vesicles (20μM) and DAPA (3.0μM) in 0.02M HEPES, 0.15M NaCl, pH 7.4, 0.01% (v/v) Tween-80 was activated by adding FXa (2.5nM with Pre-1 or 1.0nM with prothrombin) (14). Aliquots were removed at various times and added to acetic acid (final concentration 0.134N). The solvent was removed with a SpeedVac, the dried samples were dissolved in gel sample buffer, and the components were resolved by 10–14.5% SDS-PAGE. The gels were fixed in 50% methanol, 20% ethanol, and 6% trichloroacetic acid for at least 2 hours and were stained with Coomassie Blue and destained. The gels then were dried using BioDesign GelWrap (BioDesign Inc., New York) and scanned using a CanoScan model 5000F (Canon Canada, Mississauga, Ontario). Densitometry was carried out to determine the concentration of each band.

RESULTS

Comparison of the activation of human prothrombin and prethrombin-1 by the prothrombinase complex with either human or bovine factor Va

Time courses of prothrombin activation with human or bovine FVa are shown in Figure 1a. The initial rate in both instances was 26 mol thrombin/sec/mol FXa. The full time courses exhibited differences, however, in the amplitudes and durations of the transient meizothrombin peaks (35,42), which suggests species differences in the kinetics

meizothrombin processing. In contrast, substantial species differences are evident with Pre-1 activation (Fig. 1b). With bovine FVa, thrombin formation occurred at an initial rate of 5.7 mol thrombin/sec/mol FXa and the reaction was complete within 5 minutes. With human FVa, however, the initial rate of the reaction was only 1.3 mol thrombin/sec/mol FXa. Although the data are not shown, the Pre-1 activation was rapid with bovine FVa and slow with human FVa, regardless of the species of FXa or Pre-1. Thus, both the similarities of human prothrombin conversion and the differences in human Pre-1 conversion exhibited by human or bovine FVa can be attributed specifically to the two species of FVa.

Prethrombin-1 activation with preparations of factor Va reassembled from various combinations of human and bovine heavy and light chains

The isolated heavy and light chain preparations were analyzed by SDS-PAGE, and the bands were detected by silver staining. The heavy chains showed a single band while the light chains displayed the characteristic doublet (data not shown). The results indicated that the procedure yielded electrophoretically homogeneous chains from both species. The isolated chains then were recombined to produce preparations of FVa comprising various combinations of human and bovine heavy and light chains. Their contributions to the activation of both prothrombin and Pre-1 were investigated by measuring the initial rate of thrombin generation at various concentrations of the preparations of FVa. With prothrombin, very similar results were obtained with human FVa, bovine FVa, and human FVa reassembled from the heavy and light chains (Fig. 2). In each case, saturation in rates was exhibited with respect to the concentration of FVa. In contrast, with Pre-1, a clear distinction exists between those preparations that contained heavy chain of bovine FVa compared to those that contained the heavy chain of human FVa (Fig. 3). Bovine FVa and a preparation comprising reassembled bovine heavy and light chains yielded very similar concentration-*versus*-rate profiles and a rate at saturation of 7–8 mol thrombin/sec/mol FXa. A preparation comprising the bovine heavy chain and the human light chain yielded the same rate at saturation, although the concentration dependence suggests that a slightly higher concentration of this FVa is required to achieve saturation. The titration with human FVa also exhibited saturation, with a half-maximal concentration similar to that of bovine FVa. The maximum rate, however, was only one tenth of that obtained with bovine FVa. A preparation comprising the human heavy chain and the bovine light chain also showed saturation, but the maximum rate was only about 25 percent of that exhibited by human FVa. All data were analyzed by non-linear regression analyses for their ability to fit the binding equation $r = r(max) * [Va]/(K_{1/2} + [Va])$, where r = initial rate, $K_{1/2}$ = apparent dissociation constant for the assembly of FVa in the prothrombinase complex and r(max) is the maximum rate at saturation with FVa. The results of these analyses are listed in Table 1. Very similar results were obtained with all preparations of FVa when prothrombin was the substrate. The apparent $K_{1/2}$ values ranged from 0.5 to 0.9nM, and the rates at saturation ranged from 51 to 67 mol thrombin/sec/mol FXa. With Pre-1, saturation also was observed and similar values of $K_{1/2}$, ranging from 0.5 to 2.9nM, were obtained with all forms of FVa. Bovine FVa, reconstituted bovine heavy and light chains, and the combination of the bovine heavy chain and the human light chain exhibited saturation rates of 8.6, 8.7 and 9.7 mol thrombin/sec/mol FXa, respectively. In contrast, human FVa and the combination of the human heavy chain and the bovine light chain yielded, respectively, saturation rates of 1.2 and 0.3 mol thrombin/sec/mol FXa. Although rates of Pre-1 activation exhibited saturation at similar concentrations of both species of FVa, the rates at saturation were 7.2-fold higher with bovine FVa compared to those with human FVa. In addition, the differential in the rate at saturation can be attributed specifically to the heavy chain of FVa.

Kinetics of prothrombin and prethrombin-1 activation by the prothrombinase complex with human or bovine factor Va

The kinetics of prothrombin activation were very similar with bovine and human FVa (data not shown). With human FVa the K_m and kcat values were $0.53\pm0.05\mu M$, and 92 ± 3 s^{-1}, respectively; with bovine FVa the corresponding values were $0.66\pm0.07\mu M$ and 88 ± 4 s^{-1}. Results with Pre-1 are shown in Figure 4. As these data indicate, saturation of rates is obtained with respect to concentration of Pre-1 with both bovine and human FVa. The K_m values were $5.7\pm1.9\mu M$ and $3.4\pm0.7\mu M$ with human or bovine FVa, respectively. The kcat values were 4.2 ± 0.1 and 27.0 ± 1.5 (mol thrombin/sec/mol FXa) with human or bovine FVa, respectively. The catalytic efficiencies (kcat/K_m) for thrombin generation from Pre-1 by prothrombinase were $7.9\pm0.5\mu M^{-1}s^{-1}$ with bovine FVa and $0.7\pm0.2\mu M^{-1}s^{-1}$ with human FVa. Thus, these data indicate an approximate 11-fold decrease in catalytic efficiency for Pre-1 activation with human FVa compared to bovine FVa. Furthermore, the data suggest that prothrombinase complexes with human or bovine FVa bind Pre-1 similarly, but convert bound substrate to product very differently.

Analysis by SDS-PAGE of human prothrombin or prethrombin-1 activation in the presence of human or bovine factor Va

The data of Table 1 and Figure 4 indicate that the relatively slow rates of Pre-1 activation in the presence of human FVa are expressed through a reduction in kcat for thrombin formation and not through deficient substrate binding or prothrombinase assembly. Because two cleavages of Pre-1 (and prothrombin) are required to form thrombin, the measurements of the generation of thrombin do not indicate whether the reduced rate of catalysis is reflected in the cleavage of the substrate Pre-1 to form the intermediate Pre-2 or in cleavage of the latter to form thrombin. Thus, the progress of the reaction was monitored by SDS-PAGE, which can distinguish Pre-1, Pre-2, and thrombin.

With prothrombin as the substrate, the time courses of prothrombin, Pre-2, meizothrombin, and the B-chain were similar, in the presence of either human or bovine FVa (Fig. 5). Prothrombin was consumed at initial rates of 20.6s^{-1} and 19.8s^{-1} with human or bovine FVa, respectively. The initial rates of B-chain accumulation were 20.1s^{-1} and 19.0s^{-1} for human or bovine FVa, respectively. The rates of B-chain accumulation for both human and bovine FVa match their respective prothrombin consumption rates, suggesting that the majority of prothrombin consumption resulted from the initial cleavage at Arg320. With human FVa, a 0.088μM meizothrombin peak occurred at 30 seconds, whereas with bovine FVa, a 0.117μM peak occurred at 1.0 minute. Pre-2 time courses with human or bovine FVa were similar, in that they both peaked at 2 minutes at concentrations of 0.052μM and 0.071μM, respectively, and both were nearly consumed by 5 minutes.

The initial rate of Pre-1 consumption with human FVa (2.32s^{-1}) was 3.8-fold lower than with bovine FVa (8.81s^{-1}) (Fig. 6). With bovine FVa, the Pre-1 level approached a plateau comprising 85 percent total consumption at 3 minutes, whereas with human FVa, it approached a plateau comprising 86 percent total consumption at 7 minutes. With human FVa, Pre-2 accumulated at an initial rate of 1.50s^{-1} and approached the plateau of 0.62μM at 7 minutes. With bovine FVa, Pre-2 accumulated at an initial rate of 1.84s^{-1}, peaked at 0.24μM at 2 minutes, and slowly declined thereafter. With human FVa, B-chain accumulated at an initial rate of 0.99s^{-1} for the first three minutes, and continued to accumulate at a slower rate for the remainder of the time. With bovine FVa, B-chain accumulated at an initial rate of 8.61s^{-1}, and approached a plateau of 1.2μM at 5 minutes. Accumulation of fragment-2, which indicates the cleavage at Arg271 in either Pre-1 or meizothrombin-des-fragment-1, also was quantified. The initial rates of fragment-2 accumulation were 1.71s^{-1} and 7.09s^{-1} for human or bovine FVa, respectively.

The rate of Pre-2 consumption at the end of the reaction with bovine FVa was only $0.077s^{-1}$, which is very small relative to the initial rate of Pre-1 consumption ($8.81s^{-1}$). Therefore, one can assume that the rate of Pre-2 accumulation is, to a very good approximation, equal to the rate of cleavage of Pre-1 at Arg271. Accordingly, the initial rates of cleavage of Pre-1 at Arg271 were $1.50s^{-1}$ and $1.84s^{-1}$ for human and bovine FVa, respectively. The initial rates of cleavage at Arg320 were calculated by subtracting the rates of Pre-2 accumulation from rates of Pre-1 consumption, and values of $0.82s^{-1}$ and $6.97s^{-1}$ were found for human and bovine FVa, respectively. These calculations indicate that human and bovine FVa process cleavage at Arg271 very similarly, but cleavage at Arg320 very differently when Pre-1 is the substrate. All of the rates are summarized in Table 2.

DISCUSSION

These studies show that bovine and human FVa function very similarly in prothrombin activation but very differently Pre-1. The differences can be attributed specifically to the heavy chains of the two species. The effect is exhibited primarily in kcat rather than K_m or K_d for prothrombinase assembly and is specifically due to deficient catalysis of Arg320 cleavage.

As shown in Figure 7, the heavy chains of bovine and human FVa consist of two homologous repeated domains, designated A1 and A2, that comprise the bulk of the primary structure, plus two other regions of sequence that complete the primary structure. The A1 and A2 domains are highly homologous, both to one another within and across species, and to corresponding domains in the heavy chain of coagulation factor VIII, the A3 domains in the light chains of FVa and factor VIII(a), and the A1, A2, and A3 domains of ceruloplasmin (8–10,44). The A1 domain spans amino acid residues 1–303 in the heavy chains of both species and share 84.5% and 94.7% sequence homology and similarity, respectively. This is followed by thirteen amino acids in both species (residues 304–316) that differ only at positions 308, 311, and 314 (L->P, I->L, and E->D, human -> bovine). This is followed by the A2 domain. The A2 domain of the bovine heavy chain (residues 317–660) is four residues longer than the A2 domain of the human heavy chain by virtue of the lack of an arginine residue corresponding to position 439 in the human sequence, and the replacement of threonine residue corresponding to position 560 in the human sequence with the six residues NFTLPA at positions 559–564 in the bovine sequence. This latter insert has no counterpart in the human sequence. The A2 domains of the two species share 88.7% and 97.1% sequence homology and similarity, respectively. The A2 domain of the heavy chains of both species is followed by 53 amino acids, spanning residues 661–713 in the bovine heavy chain and residues 657–709 in the human heavy chain. In contrast to the high degree of similarity exhibited by the other regions of sequence of the two species, these latter 53 residues display species differences at 21 of the 53 positions when the sequences are aligned by discounting the glycine at position 667 of the bovine sequence and discounting the glutamate at position 691 of the human sequence.

The functional differences observed in the current work can be attributed to one or more of the differences in primary structure between the two species of FVa. If one assumes that regions of substantial differences in sequence account for these functional differences, likely candidates comprise the inserts and deletions in the A2 domains and the regions spanning the 53 residues at the COOH-termini of the heavy chains. Recent studies have suggested that the pentapeptide sequence DYDYQ in the heavy chain of human FVa at positions 695–699 may be involved in the interaction of prothrombin with FVa in prothrombinase assembled on PCPS vesicles (27). A similar pentapeptide sequence DSDYQ is present in the heavy chain of bovine FVa at positions 699–703. These sequences of residues start 34 residues from the COOH-terminal end of the A2 domains in both species. These sequences are

followed at their COOH-termini by ten amino acids, the last of which is the COOH-terminal residue of the heavy chain. In the human heavy chain, the sequence is NRLAAALGIR, whereas in the bovine heavy chain it is DELALILGLR. These are similar or identical except for the first two amino acids, which are NR and DE in human and bovine heavy chains, respectively. These two residues profoundly affect the calculated pI values of the two sequences. In human FVa, the pI value of the ten amino acids is 12.5, whereas in bovine FVa, it is 4.26. With bovine FVa, the net negative charge of these ten residues would augment the negative charge of the DSDYQ pentapeptide and perhaps specifically facilitate the interaction with Pre-1 required for cleavage at Arg320. Conversely, with human FVa, the net positive charge of these ten residues would partially negate the negative charge of the DYDYQ pentapeptide, and thereby would not facilitate the interaction with Pre-1. This is supported by the observations that the initial rates of Pre-2 accumulation, which reflects cleavage at Arg271, were nearly identical between the two species, whereas cleavage at Arg320 was 8.5-fold faster with bovine FVa.

Toso and Camire recently published studies of prothrombin and Pre-1 activation by prothrombinase containing recombinant human FVa variants truncated from the COOH-terminus of the heavy chain (32). They found that the truncation of as many as 51 residues had no effect on prothrombin activation. In contrast, truncation of the ten COOH-terminal residues increased the K_m for Pre-1 activation by 7-fold or more, with little effect on kcat. Their results imply that the ten amino acids of the COOH-terminus of the human FVa heavy chain influence the K_m of Pre-1 activation, but not the kcat. Our results suggest that replacement of the ten amino acids of human FVa heavy chain with those of the bovine heavy chain do not affect K_m, but increase kcat 6.4-fold. Both studies suggest that the identity of the last 51 residues of the heavy chain may influence the kinetics of Pre-1 activation.

Since both species of FVa share a common physiologic property (enhanced activation of prothrombin) but differ substantially in a functional variant of that property (enhanced activation of the prothrombin derivative, Pre-1), the two properties together should be very useful in efforts to correlate the structure of FVa with its function and to consequently further characterize the mechanism by which prothrombin is activated by the prothrombinase complex.

Acknowledgments

The abbreviations used are

DYDYQ	hirudin-like pentapeptide sequence found at position 695–699 of human factor Va heavy chain
DSDYQ	pentapeptide sequence found at position 699–703 of bovine factor Va heavy chain
PCPS	phosphatidyl-L-choline (75%), phosphatidyl-L-serine (25%)
DAPA	dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide
FVa	factor Va
FXa	factor Xa
Pre-1	prethrombin-1

Pre-2   prethrombin-2

PPAck   D-Phe-Pro-Arg chloromethyl ketone

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| 1. What is known on this topic	2. What this paper adds
• The heavy chain of factor Va is necessary for efficient activation of prothrombin but the molecular basis for this is not known. • Several groups have shown independently that the truncation of the heavy chain from the C-terminus by at least 51 residues substantially reduces the efficiency of prothrombin activation. • One group has concluded that the hirudin-like sequence DYDYQ in the C-terminus of the heavy chain is important for prothrombin activation and it determines the pathway of prothrombin activation. Another group, however, concluded that this sequence is irrelevant to prothrombin activation. They did find, however, that mutants lacking the ten residues C-terminal to the DYDYQ sequence are deficient in prethrombin-1 activation. • Prethrombin-1 activation kinetics are much slower than prothrombin activation kinetics with human prothrombinase because of the lack of membrane binding. • The precise role of the heavy chain of factor Va in prothrombin activation is yet to be determined.	• Substantial difference exists between human and bovine factor Va in the activation of prethrombin-1 by prothrombinase. • Differences in kinetics can be attributed to the differences in sequences of the C- terminus of heavy chain, where the human and bovine differ substantially in their respective pI values, despite high sequence homology between the conserved domains of the heavy chain. • The difference in prethrombin-1 activation is specifically due to retardation of Arg320 cleavage of prethrombin-1 with human Va, but not with bovine Va. • These studies suggest that the residues C- terminal to the DYDYQ sequence influence cleavage at Arg320 in prethrombin-1 and may do so in prothrombin.
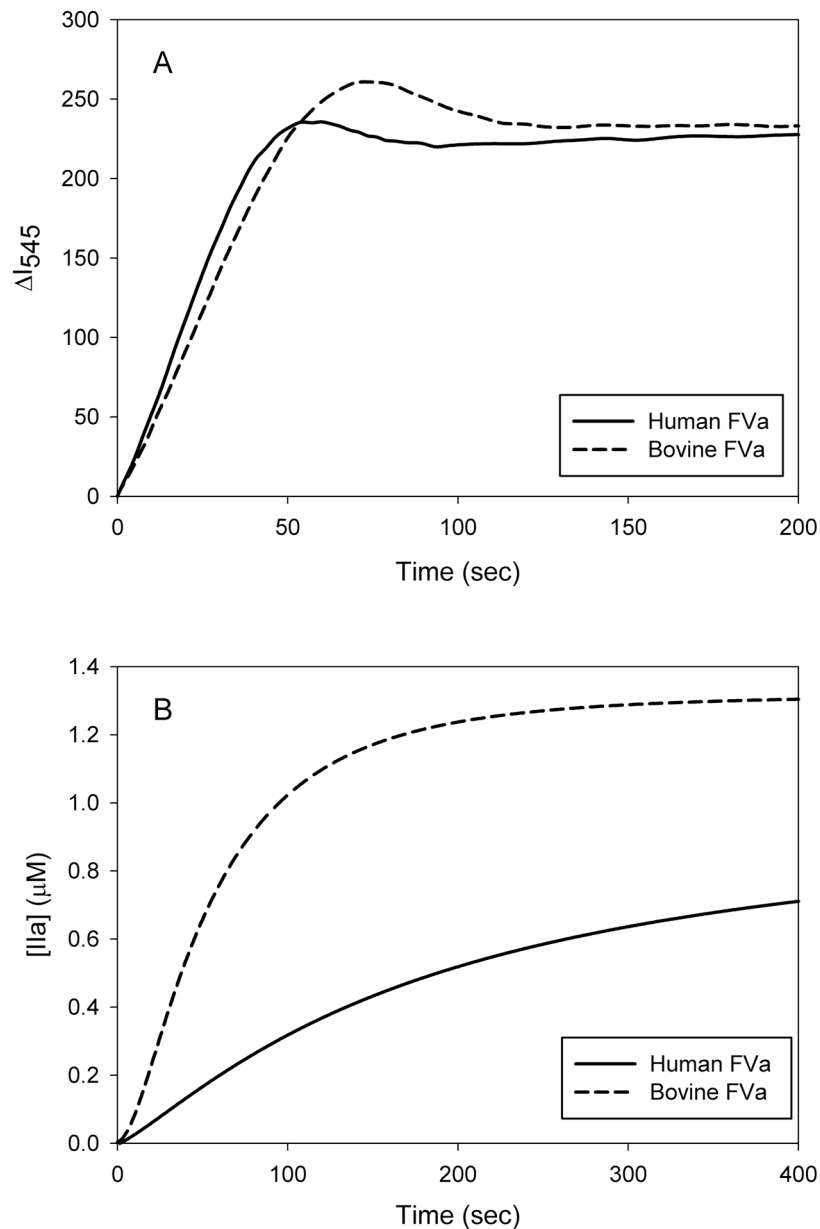
Figure 1. Time course of the prothrombinase-catalyzed activation of prothrombin and prethrombin-1 in the presence of human or bovine factor Va

(A) Human prothrombin (1.0μM) was activated in 0.02M Tris-HCl, 0.15M NaCl, 2.0mM CaCl$_2$, 20μM PCPS, 3.0μM DAPA, and 1.0nM FXa at 22°C. Thrombin formation was monitored by the fluorescence intensity of the DAPA-Thrombin (and meizothrombin) complex (λex=335nm, λem=545nm). Since a meizothrombin transient was present during prothrombin activation the values of the vertical axis are expressed in arbitrary units. (B) Human Pre-1 (1.4μM) was activated in 0.02M Tris-HCl, 0.15M NaCl, 2.0mM CaCl$_2$, 20μM PCPS, 3.0μM DAPA, and 2.5nM factor Xa at 25°C. Thrombin formation was monitored by the fluorescence intensity (λex=280nm, λem=545nm) and quantified. Both prothrombin and Pre-1 were converted to thrombin in the presence of human or bovine FVa at a concentration of 10nM.
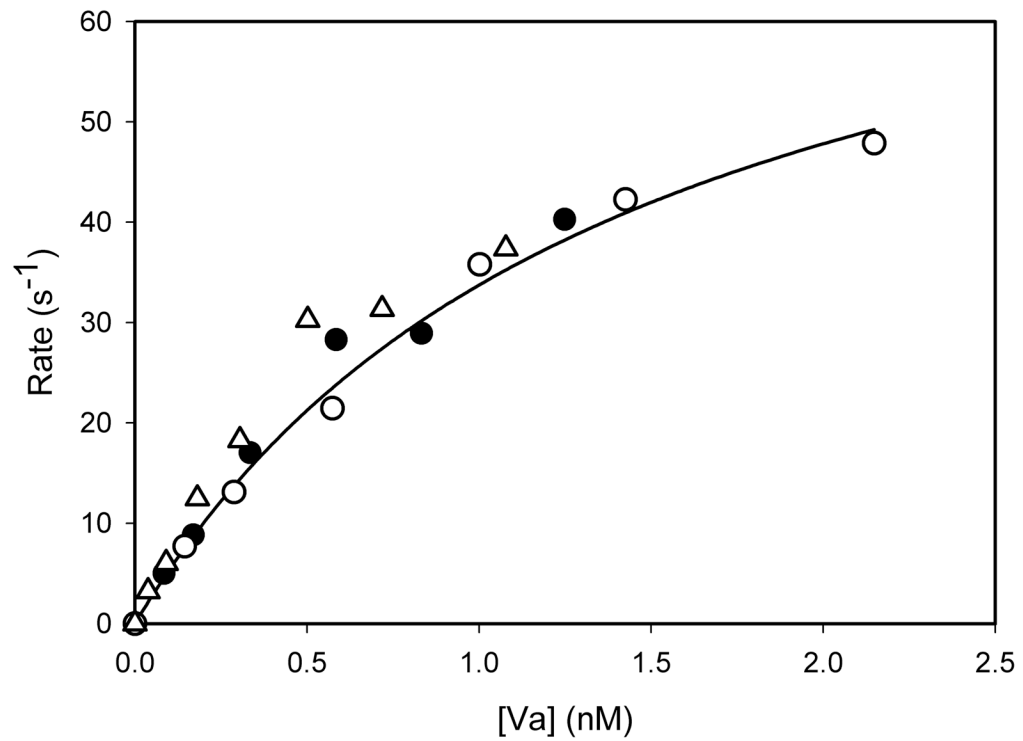
Figure 2. The dependence of initial rates of prothrombin activation on the concentration of various preparations of factor Va

The initial rates of activation of human prothrombin (1.0μM) were measured at 22°C by DAPA fluorescence in the presence of human FXa (1.0nM), PCPS vesicles (20μM phospholipid), $CaCl_2$ (2.0mM), DAPA (3.0μM) and the indicated concentrations of human FVa (closed circles), bovine FVa (open circles), or a preparation of human FVa reassembled from its isolated heavy and light chains (open triangles). Rates are expressed as mol thrombin/sec/mol FXa.
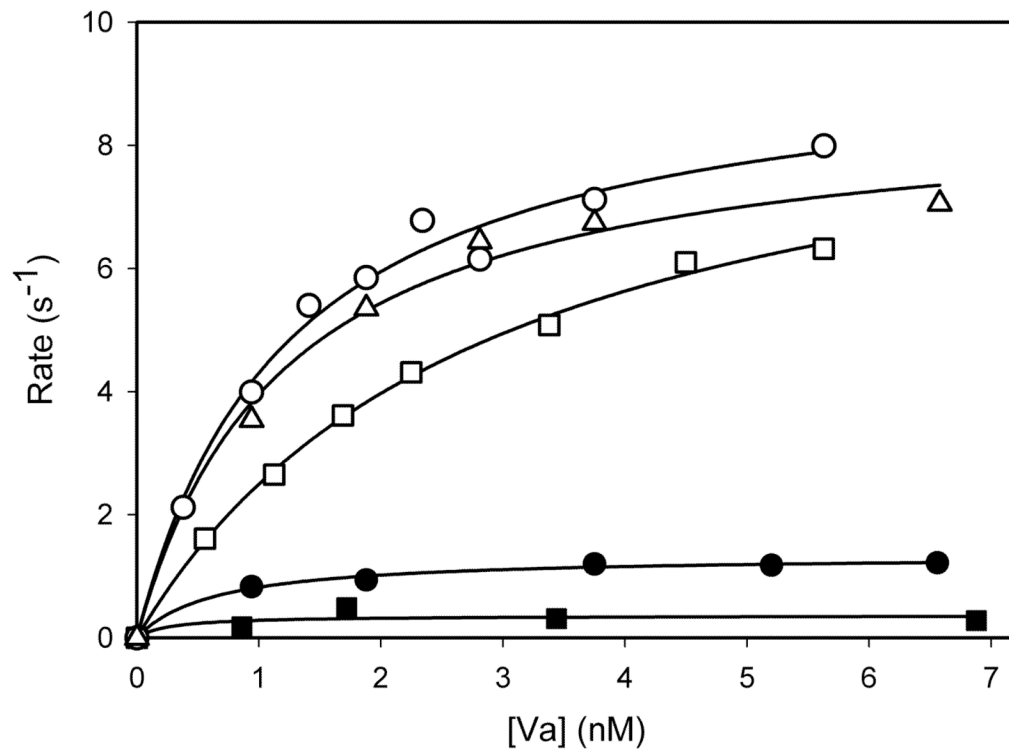
Figure 3. The dependence of initial rates of prethrombin-1 activation on the concentration of various preparations of factor Va

The initial rates of activation of human Pre-1 (1.0μM) were determined under conditions such as those described in the legend of Figure 2. The indicated data were obtained with preparations of FVa that included bovine FVa (open circles); bovine FVa reassembled from its heavy and light chains (open triangles); FVa reassembled from the bovine heavy chain and the human light chain (open squares); human FVa(closed circles); and FVa reassembled from the human heavy chain and the bovine light chain (closed squares).
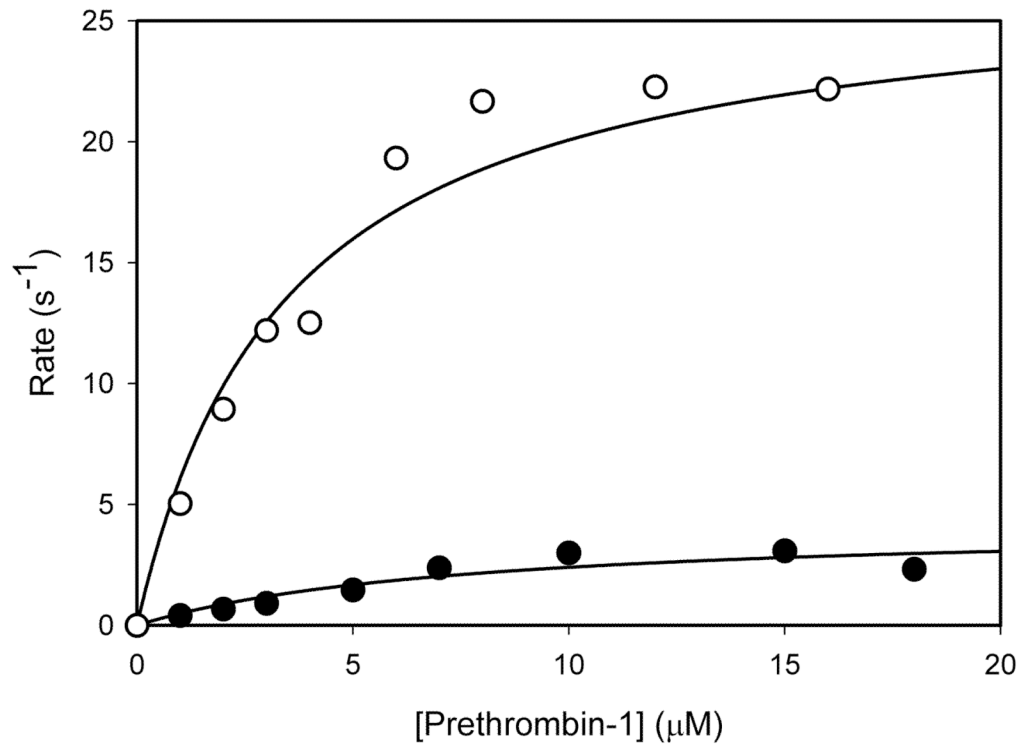
Figure 4. The substrate concentration dependence of prethrombin-1 activation in the presence of human (closed circles) or bovine (open circles) factor Va
Initial rates of activation of human Pre-1 (indicated as mol thrombin/sec/mol FXa) were measured at 22°C by DAPA fluorescence at initial concentrations of prethrombin-1 indicated by the units of the horizontal axis. The data were fit by non linear regression analysis to the equation v=v(max)[Pre-1]/(K_m+[Pre-1]) to yield the kcat and K_m values.
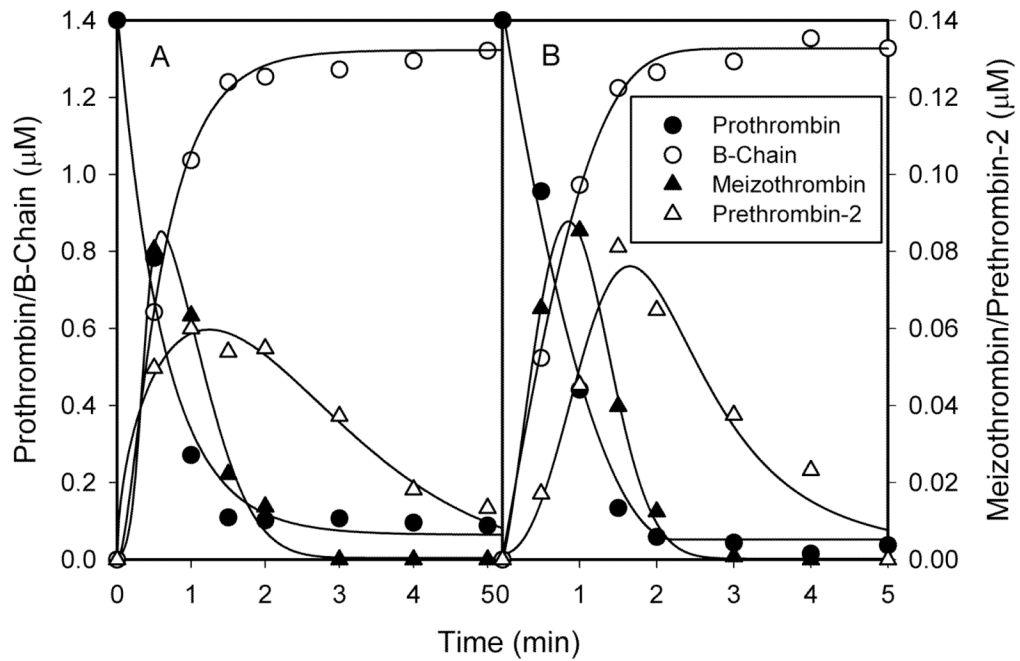
Figure 5. The time courses of human prothrombin, meizothrombin, prethrombin-2 and the B-chain during prothrombin activation by prothrombinase in the presence of A) human or B) bovine factor Va

Prothrombin (1.4μM) was incubated with either human or bovine FVa (10nM), $CaCl_2$ (2.0mM), PCPS vesicles (20μM) and DAPA (3.0μM) in 0.02M HEPES, 0.15M NaCl, pH 7.4 and0.01% (v/v) Tween-80, and its activation was initiated by the addition of FXa (1.0nM). Samples were withdrawn at various times and subjected to 10–14.5% SDS-PAGE. The gels were fixed in 50% methanol, 20% ethanol, and 6% trichloroacetic acid for at least 2 hours and were stained with Coomassie Blue and destained. The time courses were determined by quantitative densitometry.
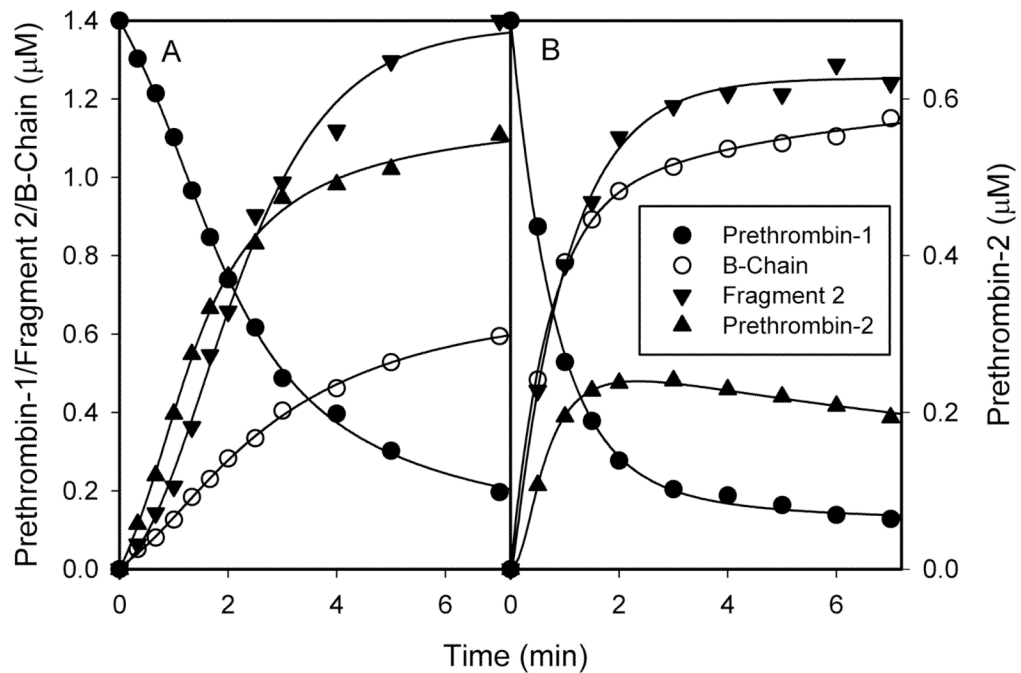
Figure 6. The time courses of human prethrombin-1, prethrombin-2, fragment 2 and the B-chain during prethrombin-1 activation by prothrombinase in the presence of A) human or B) bovine factor Va

Pre-1 (1.4μM) was incubated with either human or bovine FVa (10nM), CaCl$_2$ (2.0mM), PCPS vesicles (20μM) and DAPA (3.0μM) in 0.02M HEPES, 0.15M NaCl, pH 7.4 and 0.01% (v/v) Tween-80, and its activation was initiated by the addition of FXa (2.5nM). Samples were withdrawn at various times, and were analyzed as in Figure 5.

```
Human    1   aqlrqfyvaa qgiswsyrpe ptnsslnlsv tsfkkivyre yepyfkkekp    50
Bovine   1   aklrqfyvaa qsirwnyrpe sthlsskpfe tsfkkivyre yeayfqkekp    50

        51   qstisgllgp tlyaevgdii kvhfknkadk plsihpqgir ysklsegasy   100
        51   qsrtsgllgp tlyaevgdim kvhfknkahk plsihaqgik yskfsegasy   100

       101   ldhtfpaekm ddavapgrey tyewsiseds gpthddppcl thiyyshenl   150
       101   sdhtlpmekm ddavapgqey tyewiisehs gpthddppcl thiyysyvnl   150

       151   iedfnsglig pllickkgtl teggtqktfd kqivllfavf deskswsqss   200
       151   vedfnsglig pllickkgtl tedgtqkmfe kqhvlmfavf deskswnqts   200

       201   slmytvngyv ngtmpditvc ahdhiswhll gmssgpelfs ihfngqvleq   250
       201   slmytvngyv ngtmpditvc ahdhiswhli gmssgpelfs ihfngqvleq   250

       251   nhhkvsaitl vsatsttanm tvgpegkwii ssltpkhlqa gmqayidikn   300
       251   nhhkisaitl vsatsttanm tvspegrwti asliprhfqa gmqayidikn   300

       301   cpkktrnlkk itreqrrhmk rweyfiaaee viwdyapvip anmdkkyrsq   350
       301   cakktrnpkk ltrdqrrhik rweyfiaaee viwdyapiip anmdkkyrsl   350

       351   hldnfsnqig khykkvmytq yedesftkht vnpnmkedgi lgpiiraqvr   400
       351   hldnfsnrig khykkvvykq yqddsftkrl edpssegdgi lgpiiraqvr   400

       401   dtlkivfknm asrpysiyph gvtfspyede vnssftsgrn ntmiravqpg   450
       401   dtlkivfknm asrsysiyph gvtfspydne vnssstsg-s ntmiravrpg   449

       451   etytykwnil efdeptenda qcltrpyysd vdimrdiasg liglllicks   500
       450   etytykwnil esdeptenda qcltrpyysn vditrdlasg liglllicks   499

       501   rsldrrgiqr aadieqqavf avfdenkswy ledninkfce npdevkrddp   550
       500   rsldrrgiqr aadieqqavf avfdenkswy iedniykfce npekvkrddp   549

       551   kfyesnimst ------ingy vpesittlgf cfddtvqwhf csvgtqneil   594
       550   kfyesnims- nftlpaingy vpesipilgf cfddtvqwhf csvgtqndil   598

       595   tihftghsfi ygkrhedtlt lfpmrgesvt vtmdnvgtwm ltsmnssprs   644
       599   tihftghsfi ygkrhedtlt lfpmqgesvt vtmdnvgtwm lttmnsnprs   648

       645   kklrlkfrdv kcipdddeds yeifeppest vmatrkmhdr lepedeesda   694
       649   kklrlrfrda kcirndddds yeiiyepsgs tamttkkihd sseiedenda   698

       695   dydyqnrlaa algir   709
       699   dsdyqdelal ilglr   713
```

A1 Domain

A2 Domain

Figure 7. Comparison of amino acid sequence between human and bovine factor Va heavy chain
The heavy chain comprises of the A1 domain that spans residues 1 to 303, followed by a thirteen residue connecting region, followed by the A2 domain that spans residues 317 to 656 in human and residues 317 to 660 in bovine, and finally the carboxy-terminal end region that spans residues 657 to 709 in human and residues 661 to 713 in bovine. The hirudin-like peptide D(Y/S)DYQ (695–699 in human and 699–703 in bovine) is located in this carboxy-terminal region. Human Factor V sequence pubmed accession #AAB59532; Bovine Factor V sequence pubmed accession #AAA30512.

Table 1

Maximum rates and factor Va concentrations required for half-maximal rates of human prethrombin-1 activation with various forms of factor Va included in the prothrombinase complex

Data such as those indicated in Figure 4 were fit by non-linear regression analysis to the equation rate = rate(max)· [Va]/($K_{1/2}$+[Va]). k_{max} is rate(max)/[factor Xa]. Values of k_{max} and $K_{1/2}$ are indicated ± S.E.M. The various preparations of factor Va are: hFVa, human FVa; bFVa, bovine FVa; hH + hL and bH + bL, human and bovine FVa, respectively, reassembled from their isolated chains; hH + bL, FVa reassembled from the human heavy and bovine light chains; and bH + hL, FVa reassembled from the bovine heavy and human light chains.

Substrate	Factor Va	$K_{1/2}$ (nM)	k_{max} (s^{-1})
Prothrombin	hFVa	0.9±0.4	63±15
Prothrombin	hH+hL	0.7±0.1	62±7
Prothrombin	bFVa	0.7±0.1	51±2
Prothrombin	bH+bL	0.9±0.3	63±7
Prothrombin	hH+bL	0.5±0.1	67±6
Prethrombin-1	bFVa	0.9±0.2	8.6±0.5
Prethrombin-1	bH+hL	2.9±0.2	9.7±0.4
Prethrombin-1	hFVa	0.5±0.1	1.2±0.1
Prethrombin-1	hH+bL	0.7±0.3	0.3±0.1

Table 2

The initial rates of prethrombin-1 consumption and intermediate/product accumulation measured by SDS-PAGE analysis (Fig. 6).

	Rate (s^{-1})	
	Human factor Va	**Bovine factor Va**
Prethrombin-1	2.32	8.81
B-chain	0.99	8.61
Fragment-2	1.71	7.09
Prethrombin-2	1.50	1.84
[a] Arg271 Cleavage	1.50	1.84
[b] Arg320 Cleavage	0.82	6.97

[a]As measured by the initial rate of Pre-2 accumulation.

[b]Difference between the initial rate of Pre-1 consumption and Pre-2 accumulation.