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Electron microscopy of human factor V and factor VIII: Correlation of morphology with domain structure and localization of factor V activation fragments

WALTER E. FOWLER, PHILIP J. FAY, DAVID S. ARVAN, AND VICTOR J. MARDER

Hematology Unit, Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642

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ABSTRACT **Clotting factor V and factor VIII are each represented by the domain structure A1–A2–B–A3–C1–C2 and share 40% sequence homology in the A and C domains. Rotary-shadowed samples of human factor V and factor VIII were examined in the electron microscope. Single-chain factor V molecules exhibited a globular "head" domain 12–14 nm in diameter. In addition, up to 25% of these molecules showed a rod-like "tail" of up to 50 nm. Glycerol-gradient centrifugation of factor V treated with thrombin partially resolved the factor Va heterodimer from a large activation peptide of 150 kDa, as determined by gel electrophoresis. Electron microscopy of factor Va revealed globular molecules with several smaller appendicular structures but lacking the tails seen in factor V. Images of the 150-kDa activation peptide showed rod-like structures, similar in width to the tail of intact factor V and ≈34 nm long. Rotary shadowing was also used to visualize factor VIII that had been fractionated into heterodimers containing heavy chains of distinct sizes. Each factor VIII preparation showed a globular structure ≈14 nm in diameter, but the associated tails were observed much more frequently with factor VIII heterodimers containing the higher-molecular-weight heavy chains. These results, in conjunction with results of studies using other biophysical techniques, suggest a model in which the A and C domains of each cofactor constitute a globular head and the connecting B domain is contained in a two-stranded tail that is released by thrombin cleavage.**

Factor V and factor VIII play key roles in the coagulation cascade, where they function as essential protein cofactors of the prothrombinase and tenase complexes, respectively. These two plasma glycoproteins, which are each synthesized as a single polypeptide chain, are similar in structure and analogous in function (for review, see ref. 1). Both cofactors are represented by the domain structure A1–A2–B–A3–C1–C2 (refs. 2–5; see especially figure 5 of ref. 1). Human factor V is isolated as a single-chain protein (6–8). Factor VIII isolated from human plasma is heterogeneous due to proteolysis within the B domain, appearing as a population of divalent metal ion-linked heterodimers that consist of a variable-sized heavy chain derived from the amino-terminal region (A1–A2 plus various amounts of the B domain) and a light chain derived from the carboxyl-terminal region (A3–C1–C2) (9–11). The A and C domains of factor V and factor VIII showed 40% amino acid identity, whereas the connecting B domains show an insignificant percentage of amino acid identity. Thrombin proteolysis of factor V yields activation fragments of 70 kDa and 150 kDa, which account for the entire B domain; thrombin proteolysis of factor VIII releases multiple small fragments, which account for the remaining B domain. Each activated factor is thus a heterodimer consisting of an amino-terminal-derived heavy chain and a carboxyl-terminal-derived light chain.

Hydrodynamic studies of bovine factor V indicate that the molecule is highly asymmetric with f/f_{min} (i.e., the frictional ratio) of 2.01 (12), while activated factor V (factor Va) is a more globular molecule with f/f_{min} of ≈1.4 (13). This information suggests that much asymmetry observed for intact factor V is from the B domain, which is released during activation. The asymmetry seen for factor VIII heterodimers is also related to the presence of the B domain in that f/f_{min} values of ≈1.7 and 1.3 are obtained for factor VIII containing predominant heavy chains of 155 or 146 kDa and 93 kDa, respectively (9).

Several groups have reported studies of bovine or human factor V structure using EM (14–17). The most comprehensive model of factor V and its activation by thrombin was proposed by Dahlback (17). This model for intact factor V consists of a large central domain ≈14 nm in diameter surrounded by three smaller globular domains. From examination of thrombin-activated factor Va and the largest activation peptide, which is 150 kDa by gel electrophoresis and contains a peptide of 58 kDa by sequence analysis (1), Dahlback proposed that the large central domain of his model represents "fragment 150 kDa." The smaller peripheral domains thus correspond to the heavy chain and light chain of factor Va in this model and were proposed to undergo association after having been cleaved from fragment 150 kDa (17). Mosesson *et al.* (16) conducted similar studies with scanning transmission EM and suggested that intact factor V is pleomorphic, having an irregular oblong shape with dimensions of 10–20 nm and a satellite nodule occasionally associated with the larger structure. These investigators also examined thrombin-activated factor Va and reported no major structural rearrangement with release of the activation peptides (16). Thus, two groups of investigators using similar EM techniques—i.e., negative staining and drying from aqueous solutions—have presented two distinctly different models for the structure of intact factor V and of thrombin-activated factor Va.

We have studied factor V and its activation by thrombin with EM using glycerol spraying/vacuum drying, followed by rotary shadowing. This technique has been valuable for studying large extended molecules (18–20). We have also isolated factor V activation fragment 150 kDa and show that its structure can be related to that of the intact molecule. Similar studies have been done with factor VIII heterodimers containing variable lengths of the B domain. From these data, we propose a molecular model for human factors V and VIII and discuss implications for the function of these molecules in the coagulation cascade.

MATERIALS AND METHODS

Materials. All chemicals were reagent grade or better and were used without further purification. Thrombin was a gift of John Fenton (Albany, NY). Murine monoclonal antibody

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(B-10; ref. 21) to 150-kDa activation peptide of factor V was the gift of Robert Colman (Philadelphia).

Purification of Human Factors V and VIII. Human factor V was a gift from William Kane (Duke University) and was purified from plasma as described (6). Because examination of rotary-shadowed preparations of plasma-derived human factor V initially showed considerable heterogeneity (data not shown), the factor V preparation was fractionated by centrifugation in a glycerol gradient, making appearance of the factor V molecule much more consistent. Human factor VIII was purified from therapeutic concentrates as described (22) and was further fractionated on a Mono Q column (Pharmacia) (9).

Glycerol Gradients. Five-milliliter glycerol gradients, 15–40%, in 0.15 M ammonium formate/2.5 mM calcium chloride/2 mM Hepes, pH 7.2, were centrifuged in an SW 50.1 rotor (Beckman). A set of standards consisting of thyroglobulin, catalase, and bovine serum albumin was included in a separate gradient with every run. The gradients were collected in 0.25-ml fractions. Position of the standards was determined by measuring A_{280} of the gradient fractions. Position of factor V and its thrombin degradation products was determined by gel electrophoresis of a sample from each fraction.

Thrombin Digest of Factor V. Factor V (50 μl of a 1.3 mg/ml stock) in 0.15 M sodium chloride/5 mM calcium chloride/20 mM Tris, pH 7.4, was mixed with thrombin (2 μl of 100 unit/ml stock) and incubated at 37°C for 20 min. Forty microliters of the mixture was diluted to 200 μl with gradient buffer (see above), and this was loaded onto 5-ml glycerol gradient. The gradient was centrifuged at 41,000 rpm for 17 hr at 2°C ($\omega^2T = 1.12$). Gel electrophoresis was done immediately after fractionating the gradient.

Electrophoretic Analysis. Polyacrylamide (5%) slab gels were run according to Laemmli (23) by using a minigel apparatus (Buchler). Current was held constant at 70 mA. Gels were silver stained according to Merril *et al.* (24). Immunoblotting used a dry-transfer process onto Immobilon membranes (Millipore). After being labeled with solutions containing the first antibody, each membrane was incubated in 1:400 dilution of either goat anti-rabbit or goat anti-mouse IgG peroxidase conjugate (Tago) in 0.1% bovine serum albumin/0.05% Tween/phosphate-buffered saline. After 2-hr incubation on the rocker platform at room temperature, the membranes were washed in Tween/phosphate-buffered saline for 10 min three times and then treated with diaminobenzadine in the presence of hydrogen peroxide and nickel chloride. The reaction was stopped with deionized water.

EM. Samples for EM were prepared by using the glycerol spray/vacuum dry method described by Fowler and Erickson (18). Specimens were examined in an electron microscope (model 300, Phillips Electronic Instruments, Mahwah, NJ) at 80 kV accelerating voltage. All micrographs were recorded at a magnification of ×39,000. Measurements in the text are not corrected for the shell of platinum surrounding each molecule.

The micrographs were printed such that the platinum grains appear dark. Final magnification is indicated in the figure legends.

Calculation of Sedimentation Coefficients for Models. Models of single-chain factor V, factor Va, and activation fragment 150 kDa based on electron micrographs were constructed by using multiple contiguous spheres of varying sizes (see Fig. 8). Theoretical sedimentation coefficients for these models were calculated by using the method of Bloomfield *et al.* (25). Application of this calculation to models of proteins based on EM has been described (26, 27).

RESULTS

Factor V. A representative field of gradient-purified human factor V is shown (Fig. 1). Most factor V molecules appear globular with a diameter of 12–14 nm. Up to 25% of the molecules in some fields show a rod-like extension of variable length (up to 50 nm). The ease with which these "tails" could be visualized varied among preparations, suggesting that visualization is near the resolution limit of the preparation techniques. Such distinctive tails were not seen in negative-stained preparations by us (data not shown) or by others (14–17).

Factor Va and Activation Fragment 150 kDa. Gel electrophoresis of the thrombin digest of human factor V (Fig. 2*A*) shows a band corresponding to a large intermediate, two bands corresponding to the heavy and light chains of factor Va, and a band of 150 kDa. The 150-kDa fragment was separated from the other digestion products by glycerol-gradient centrifugation (Fig. 2*B*). The identity of the 150-kDa polypeptide, which is enriched in fractions 18 and 19 (Fig. 2*B*), was confirmed by immunoblotting (Fig. 3) with the monoclonal antibody B-10, which binds to activation fragment 150 kDa [called C1 by Dahlback (17)]. EM of rotary-shadowed preparations from gradient fractions containing a mixture of the large intermediate and factor Va reveals globular molecules with a variety of appendicular structures (see representative fields in Fig. 4). The long tail frequently seen in preparations of the intact molecule is only rarely seen (<5% of molecules) in this thrombin-treated material, and multiple smaller appendages are now visible on the globular portion of the molecule. In contrast, rotary-shadowed preparations of fragment 150 kDa contain rod-like structures ≈34 nm long that are difficult to discern from the background (Fig. 5*A*). Unidirectionally shadowed specimens show similar structures that stand out more clearly from the background (Fig. 5*B*). Gradient fractions containing fragment 150 kDa also rarely showed globular species as large as the globular

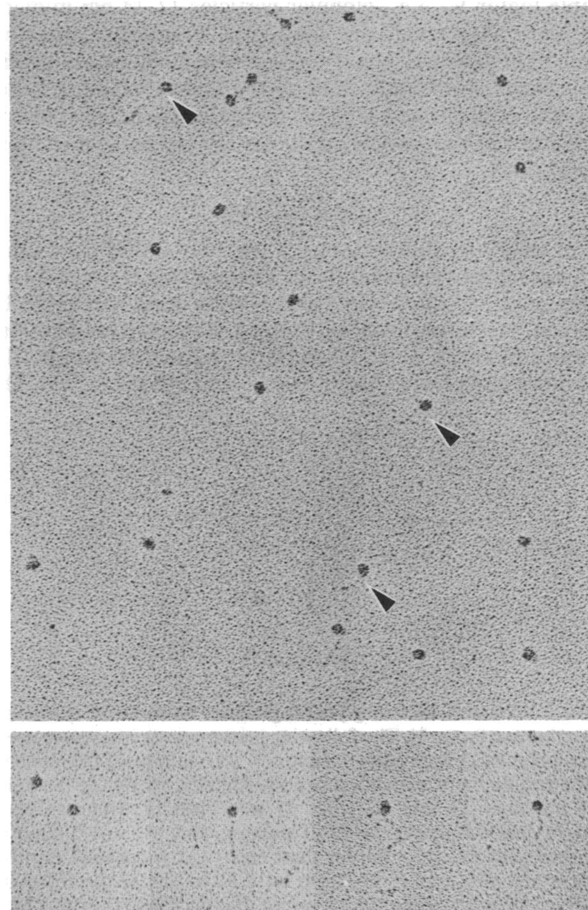

FIG. 1. Electron micrograph of a rotary-shadowed preparation of gradient-purified human factor V. Note the rod-like tails on several molecules (arrowheads). (×109,000.)

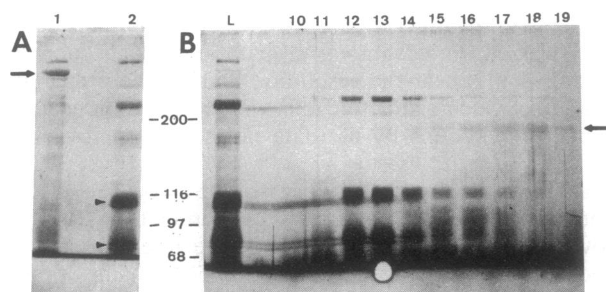

FIG. 2. Gel electrophoretic analysis (silver-stained) of human factor V and the thrombin digest of human factor V. (*A*) Human factor V preparation (lane 1), in which single-chain factor V is the most prominent band (arrow). Thrombin digest of factor V (lane 2) contains a large intermediate, the heavy and light chains of factor Va (arrowheads), and activation fragments. (*B*) Gel electrophoresis of fractions from a glycerol gradient onto which thrombin digest had been loaded (lane L shows the starting material). Gradient fractions are numbered 10–19. Position of fragment 150 kDa is indicated by an arrow at right. $M_r \times 10^{-3}$ markers separate *A* and *B*.

portion of factor V, consistent with the presence of some factor Va in these fractions (Fig. 2*B*).

Factor VIII. Similar studies were done on human factor VIII fractionated on a Mono Q column, which partially separates factor VIII into heterodimers containing heavy chains of various sizes (ref. 9; see also Fig. 6). Representative fields of fractions containing large heavy chains (Fig. 6, fraction 15) and smaller heavy chains (Fig. 6, fraction 18), which were rotary shadowed at the same time, are shown in Fig. 7 *A* and *B*, respectively. The structures strikingly resemble factor V—i.e., globular particles 12–14 nm in diameter often associated with long tails. Fractions containing factor VIII with large heavy chains show molecules with tails much more consistently than do fractions containing factor VIII with smaller heavy chains. Multiple appendages close to the surface of the globular particles are not present in these preparations.

DISCUSSION

EM of rotary-shadowed factor V and factor VIII shows that each molecule consists of a large globular domain ≈12–14 nm in diameter. A single tail up to 50 nm long can often be resolved, extending from the globular domain in preparations of intact factor V (Fig. 1) and preparations of factor VIII containing large heavy chains (i.e., >110 kDa) (Fig. 7*A*). The thin rod-like structure of isolated factor V activation fragment 150 kDa from thrombin-treated factor V (Fig. 5) identifies it as part of the tail domain of the intact molecule; this suggests that the heavy chain present in factor Va, which lacks the tail domain, is largely contained with the light chain in the globular particle. Preparations of factor VIII with small heavy chains (i.e., <110,000 kDa) (Fig. 7*B*) also lack the tail domain but remain activatable by thrombin (9), consistent with localization of the heavy and light chains of factor VIIIa within the globular head region of that molecule. Thus, the B domain, or connecting region of factor V and factor VIII, appears to be a major structural component of the tail domain of both molecules. Examination of factor Va (Fig. 5) shows structures consisting of a globular head region surrounded by multiple peripheral appendages, which are not present in preparations of intact factor V.

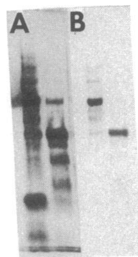

FIG. 3. Immunoblot of glycerol-gradient fractions of thrombin-treated human factor V. The following primary antibodies were used: rabbit polyclonal anti-human factor V (*A*) or mouse monoclonal anti-human factor V activation peptide 150 kDa (B-10) (*B*). Samples from fractions 13 and 18 (Fig. 2) were run in triplicate on a polyacrylamide slab gel. Without staining, this gel was blotted as described and cut into thirds; then each was incubated at room temperature on a rocker platform for 2 hr in one of the following solutions: (*i*) preimmune rabbit serum diluted 1:200, (*ii*) polyclonal anti-human factor V rabbit antiserum diluted 1:200, and (*iii*) ascitic fluid containing mouse monoclonal antibody B-10 diluted 1:200. After incubation, each membrane was processed as described. No staining occurred with preimmune serum (data not shown).

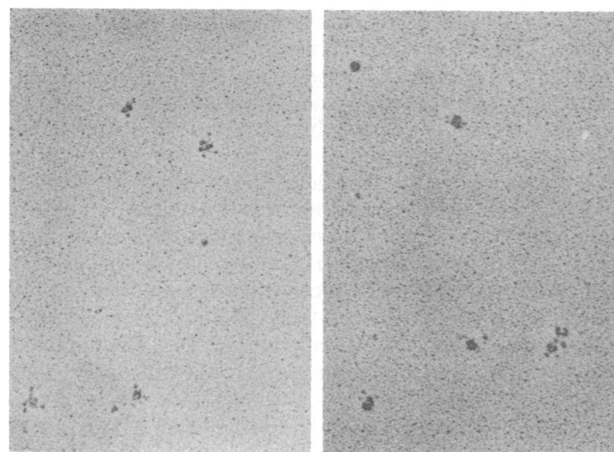

FIG. 4. Electron micrograph of rotary-shadowed preparation of factor Va. This sample was from fraction 13 of the glycerol gradient (see Fig. 2). Note the multiple small peripheral appendages on most globular core domains. (×109,000.)

Previous EM studies of factor V (14–17) have invariably shown globular particles 10–20 nm in diameter, usually with a variable number of smaller globular "satellite nodules." The model of Dahlback for factor V (17), based on EM of negatively stained human and bovine factor V and its thrombin-cleavage products, identified structural features of the intact molecule with distinct thrombin degradation products. Furthermore, that model proposed that the heavy and light chain of factor Va associate after cleavage by thrombin, thus providing a possible mechanism for thrombin activation.

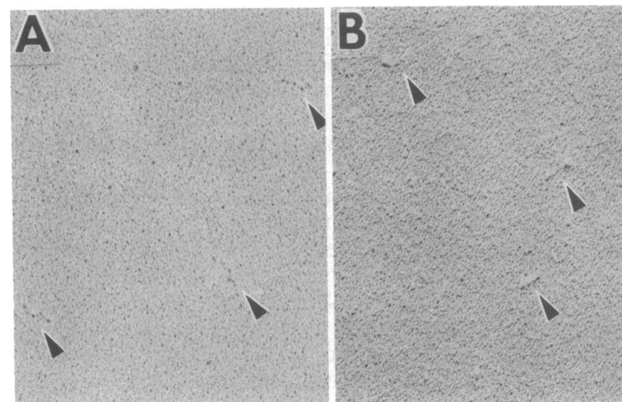

FIG. 5. Electron micrographs of rotary shadowed (*A*) and unidirectionally shadowed (*B*) preparation of the 150-kDa polypeptide. Glycerol-gradient fraction 18 (Fig. 2) was selectively enriched with activation peptide 150 kDa. Rod-like structures ≈34 nm in length are indicated by arrowheads. (×109,000.)

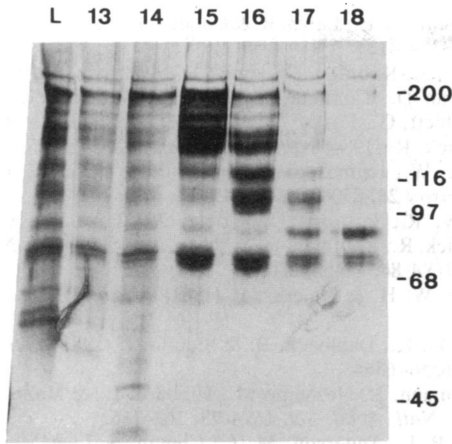

FIG. 6. Polypeptide composition of factor VIII fractions after Mono Q chromatography. SDS/PAGE was done as described. Lane L corresponds to the sample before Mono Q chromatography, and numbered lanes correspond to column fractions. $M_r \times 10^{-3}$ markers are at right.

Mosesson *et al.* (16) examined negatively stained and unstained preparations of bovine factor V in the scanning transmission electron microscope and noted that the central globular particle sometimes exhibited substructure. They also occasionally saw a single satellite nodule but noted no major structural rearrangement of the molecule during thrombin activation (16). Recently Mosesson *et al.* (28, 29) presented images of bovine factor V and porcine factor VIII showing globular particles with tail domains similar to those shown here. From the relative absence of tails in preparations of molecules activated by thrombin, they inferred that the globular head region contained the heavy and light chains of activated factor V.

Our data are inconsistent with the model of Dahlback (17). Using an EM preparation technique that may be less prone to artifact than negative stain but that is more limited in potential resolution (18), we show that fragment 150 kDa is a thin rod and that factor Va consists largely of the remaining globular particle. This fact is consistent with the hydrodynamic studies of Laue *et al.* (13), which suggest "a factor V structure in which the activation peptide resembles a long tail projecting from the more globular" heavy- and light-chain regions at the two ends of the molecule. Models based on the

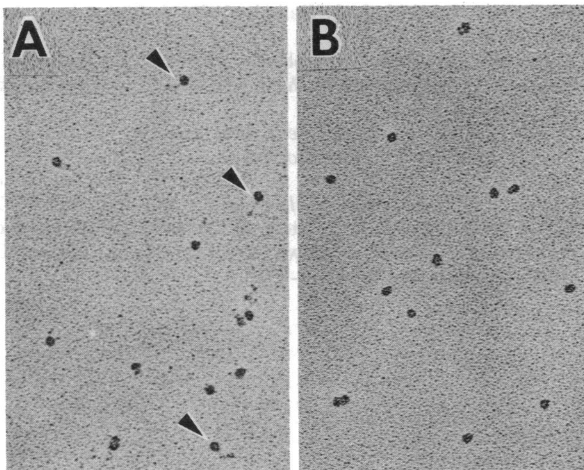

FIG. 7. Electron micrographs showing rotary-shadowed preparations of factor VIII. (*A* and *B*) Factor VIII heterodimers from Mono Q fractions 15 and 18 (Fig. 6), respectively. Arrowheads indicate some molecules with tails, which are predominantly in fraction 15. (×109,000.)

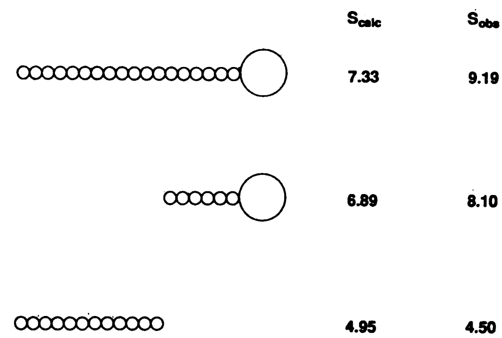

FIG. 8. Models of single-chain factor V (*Top*), factor Va (*Middle*), and activation fragment 150 kDa (*Bottom*) with calculated (s_{calc}) and observed (s_{obs}) sedimentation coefficients for each species. s_{obs} values for single-chain factor V (12) and factor Va (13) were obtained by sedimentation velocity, and s_{obs} for activation fragment 150 kDa was determined by glycerol-gradient centrifugation.

observed structure and dimensions of human factor V and its thrombin-cleavage products (Fig. 8) were used to calculate theoretical sedimentation coefficients with the method of Bloomfield *et al.* (25). The calculated values are compared with those obtained by ultracentrifugation in Fig. 8 and agree reasonably well. The relatively small changes in both the calculated and observed sedimentation coefficients between single-chain factor V and factor Va explain the incomplete resolution of these species by glycerol-gradient centrifugation (Fig. 2).

Molecular models based on current data for human factor V and factor VIII and their cleavage by thrombin are shown in Fig. 9. The amino-terminal heavy chain and the carboxyl-terminal light chain are located close together in each molecule, probably held together by divalent metal ions. This position is consistent with a recent study of human factor VIII by fluorescence energy transfer that indicates a distance of ≈2 nm separating Cys-528 (A2 domain of the heavy chain) and Cys-1858 (A3 domain of the light chain) in the unacti-

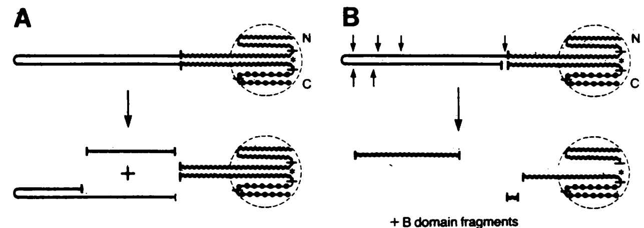

FIG. 9. Schematic models of factor V (*A*) and factor VIII (*B*) relating sequence-derived domain structure to morphologic features. Individual domains are drawn to scale in terms of the length of polypeptide making up each domain (2–5) and are separated by bars. Domains are designated as follows: ⁓, A1, A2, and A3; —, B; and ●—●, C1 and C2. Folding of the polypeptide chain in the globular head structure is for illustrative purposes only, making relative dimensions of the head and tail portions of the molecules close to those observed. *, Divalent cation in globular head. (*A*) Activation of factor V by thrombin results in cleavages within the tail structure, releasing the 70-kDa and 150-kDa activation peptides (30) and generating two free ends. The globular factor Va heterodimer (*Lower*) is composed of a heavy chain (A1–A2 domains) and light chain (A3–C1–C2 domains). (*B*) Factor VIII is heterodimeric with lengths of tail extensions (arrows) proportional to the B-domain content in the heavy chain. Location of the single cleavage invariably present in factor VIII purified from plasma (9–11) is indicated by the discontinuity between B and A3 domains (*Upper*). Thrombin cleaves factor VIII at junctions separating A2–B and A1–A2 domains in the heavy chain and near the amino terminus in the light chain (10). Factor VIIIa (*Lower*) is represented as a dimer composed of a heavy chain-derived subunit (A1 domain) and a light chain (A3–C1–C2 domains) (31).

vated factor VIII molecule (22). The heavy-chain and light-chain portions of the cofactor form the globular domain, while the connecting region of B domain forms the extended rod-like structure.

Factor V exists as a single-chain molecule, and the tail structure can therefore be thought of as double stranded. Although no spectroscopic data are available to assess the secondary peptide structures in the connecting regions of factors V and VIII, the known primary sequence of the connecting region in factor V does not show the heptad repeat characteristic of α-helices. Because the predicted length of the 836-amino acid-connecting region in factor V would be up to 77 nm for a supercoiled α-helix (0.18 nm per amino acid), the observed tail length of 50 nm suggests that the B domain is substantially folded. The connecting regions of both factor V and VIII are not required for procoagulant activity (32, 33), and the rod-like structure of the connecting region explains how this portion of the molecule can be highly susceptible to various proteases without activation or inactivation. The biological function for these tail structures remains to be determined.

Activation of both molecules takes place via cleavages by thrombin in the proximal tail region, releasing fragments (including fragment 150 kDa in factor V) and leaving new "free ends" of the heavy and light chains of the activated factors. The free ends of factor Va (Fig. 9) may be correlated with the appearance of small appendages around the globular head region of factor Va (Fig. 4). Such appendages are not seen in preparations of factor VIII having small heavy chains (Fig. 7*B*), which have not been activated by thrombin. If these structures represent an invariant morphological feature of factor Va (and, by analogy, factor VIIIa), they may be a useful marker for activation.

Thus, although there is no indication at this resolution of a major conformational change in the amino- and carboxyl-terminal regions of the molecule due to thrombin activation, there is evidence for a considerable amount of rearrangement on the surface of the globular head region. We suggest, as have others, that this rearrangement may allow interaction of activated factor V and factor VIII with other components of the coagulation cascade.

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