Human platelets contain forms of factor V in disulfide-linkage with multimerin

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Summary
Factor V is an essential cofactor for blood coagulation that circulates in platelets and plasma. Unlike plasma factor V, platelet factor V is stored complexed with the polymeric α-granule protein multimerin. In analyses of human platelet factor V on non-reduced denaturing multimer gels, we identified that approximately 25% was variable in size and migrated larger than single chain factor V, the largest form in plasma. Upon reduction, the unusually large, variably-sized forms of platelet factor V liberated components that comigrated with other forms of platelet factor V, indicating that they contained factor V in interchain disulfide-linkages. With thrombin cleavage, factor Va heavy and light chain domains, but not B-domains, were liberated from the components linked by interchain disulfide bonds, indicating that the single cysteine in the B-domain at position 1085 was the site of disulfide linkage. Since unusually large factor V had a variable size and included forms larger than factor V dimers, the data suggested disulfide-linkage with another platelet protein, possibly multimerin. Immunoprecipitation experiments confirmed that unusually large factor V was associated with multimerin and it remained associated in 0.5 M salt. Moreover, platelets contained a subpopulation of multimerin polymers that resisted dissociation from factor V by denaturing detergent and comigrated with unusually large platelet factor V, before and after thrombin cleavage. The disulfide-linked complexes of multimerin and factor V in platelets, which are cleaved by thrombin to liberate factor Va, could be important for modulating the function of platelet factor V and its delivery onto activated platelets. Factor Va generation and function from unusually large platelet factor V is only speculative at this time.

Keywords
Factor V, multimerin, coagulation, platelets, alpha-granule proteins

Introduction
Factor V is an important coagulation cofactor that accelerates the conversion of prothrombin to thrombin after conversion to factor Va (1, 2). In humans and animals, congenital factor V deficiency results in bleeding and a partial to complete loss of the factor V that circulates in plasma and in platelets (1, 3-5).

The relative contribution of plasma and platelet factor V to hemostasis in humans is uncertain, although platelet factor V can support hemostasis in individuals with plasma factor V deficiency (1, 4, 6-8). Factor V is encoded by a single copy gene (1, 9) and compared to plasma factor V, human platelet factor V has a more heterogeneous reduced size (due to partial activation and proteolysis within the B-domain) and it is also more resistant to
inactivation by activated protein C (10, 11). Moreover, unlike plasma factor V, human platelet factor V is stored complexed to the α-granule protein multimerin, a soluble, disulfide-linked homopolymeric protein that is not normally found in plasma (12, 13). The factor V complexed to multimerin in platelets is functionally active in supporting coagulation, but on thrombin activated platelets, only a small proportion of factor V remains associated with multimerin (12). Multimerin storage appears to influence the point at which factor V forms complexes, but it does not appear to be the major determinant of factor V storage as defective multimerin storage is associated with low levels of circulating multimerin-factor V complexes in plasma, and some individuals with platelet multimerin deficiency have normal platelet factor V storage (14).

In analyses of human platelet proteins on denaturing nonreduced multimerin gels, we identified some unusually large, variably-sized forms of factor V that were larger than single chain plasma factor V. We report that approximately 25% of the factor V in human platelets is composed of unusually large forms that are in covalent linkage with multimerin via the factor V B-domain cysteine, and that these unique forms, like other forms of platelet factor V, remain structurally capable of conversion to factor Va by thrombin.

Materials and methods

Studies were performed with institutional ethics approval and the informed consent of blood donors, who included healthy controls and individuals with congenital factor V deficiency (15), type 3 von Willebrand disease and severe factor XIII deficiency.

Sample preparation

Platelets were isolated from blood anticoagulated with acid citrate dextrose (ACD; v/v: 1/6) using the methods described (16), except phosphate buffered saline-acid citrate dextrose (PBS-ACD, pH 6.1) was used to wash platelets. Lysates were prepared by solubilizing platelets (1 x 10^9/ml) in Tris-saline, pH 7.4, containing 0.5% Triton X-100 and protease inhibitors (sources as described (17), final concentrations: 130 mM NaCl, 30 mM Tris, 4.0 mM pefabloc, 0.3 μM aprotinin, 100 μg/ml soybean trypsin inhibitor, 2.8 μM E64, 1 μM leupeptin, 5-40 mM N-ethyl maleimide [NEM], 1 μM pepstatin, 100 μM phenanthroline, with or without 10 mM EDTA). As a precaution, all lysates and affinity purifed proteins were prepared using reagents containing 5 mM or higher concentrations of NEM as titration analyses using Ellman’s reagent (18) indicated that 0.6 mM NEM was the minimal concentration needed to fully block free-sulphydryls in platelet proteins. Lysates were centrifuged (14,000 g, 15 min.) prior to analyses and affinity purifications. Normal pooled platelet lysate was prepared by mixing equal volume samples from 20 healthy controls.

Releasates of thrombin-stimulated platelets (3 x 10^9/ml in 5 mM Hepes-Tyrode buffer without albumin, pH 7.4, with 2 mM calcium, 1 mM magnesium) were prepared with 1-2 U/ml of human thrombin (Enzyme Research, South Bend, IN; 30 min, 37°C, without stirring; 5 mM NEM added before removing platelets by centrifugation at 2000 g x 10 min, then 14,000 g x 15 min.). Double-centrifuged plasma was prepared from blood collected into 0.105 M (3.2%) buffered sodium citrate. Pooled normal plasma and factor V deficient plasma were purchased from George King Biomedical Inc. (Overland Park, KS). Factor V deficient plasma and factor V deficient platelet lysate (factor V antigen content: 5% of normal pooled lysate) (15) were used as negative controls for Western blots and ELISA.

Antibodies

Primary antisera used included: polyclonal anti-human factor V (sheep antisera: Affinity Biologicals, Hamilton, ON; horse antisera: Haematologic Technologies, Essex, VT), monoclonal anti-human factor V (anti-heavy chain [HC] AHV-5146 from Haematologic Technologies; antibody MK-30 to the 150 kD B-domain fragment [BD], gift from Dr. B. Dählback, Malmö, Sweden), monoclonal (JS-1, RM2) and polyclonal anti-multimerin (16), and polyclonal anti-von Willebrand factor (Dako, Carpinteria, CA). Affinity purified rabbit antibodies to the factor V C2 domain (18) and monoclonal antibody RM2 to recombinant human multimerin, were prepared using standard procedures (19-21). Peroxidase-conjugated donkey or goat secondary antibodies to sheep, rabbit and mouse IgG (minimal cross-reactivity antisera, Jackson ImmunoResearch, BIO/CAN, Mississauga, ON) were used for Western blots and ELISA. Analyses of plasma, factor V deficient samples and affinity purified recombinant human multimerin (22) indicated no crossreactivity of factor V and multimerin antisera used in analyses.

Glycoprotein purification and analyses

Multimerin and factor V were analyzed using ELISA and standards described (14, 15). Two different preparations of affinity purified, recombinant multimerin (confirmed to be >90% pure by silver staining; mean reduced subunit size: 186 kD) were analyzed by ELISA, total protein assays (BCA Protein Assay Kit, Pierce, Rockford, IL) and densitometry of reduced Western blots (with monoclonal and polyclonal antisera), to determine the protein equivalent of 1 U of multimerin antigen, the amount in 10^9 pooled normal platelets (15). These data were used to estimate the molar equivalents of multimerin antigen, where one unit of recombinant multimerin was assumed to be equivalent to one unit of platelet multimerin antigen and the average reduced subunit size of platelet multimerin was taken as 155 kD (16, 23).

Affinity purified factor V was isolated from platelet lysates by incubation with CNBr-activated Sepharose beads conjugated
with sheep anti-human factor V (ShαFV; 50-340 ng factor V/25-50 μl beads), followed by serial washes with PBS/5 mM NEM. For some analyses, affinity captured proteins were incubated (20-60 min., 37°C) with 1 U/ml thrombin, and Western blots of samples separated by reduced SDS-PAGE or two dimensional nonreduced/reduced gels confirmed full cleavage of >95% factor V. Affinity purified platelet multimerin was isolated from lysates using JS-1, or an equal mixture of JS-1 and RM2 conjugated to CNBr-activated Sepharose beads (Pharmacia, Baie d’Urfé, PQ, Canada; 5 ml lysate/ml beads unless otherwise stated). Unbound proteins were removed by serial washes with PBS/5 mM NEM and captured proteins were evaluated before and after washing with 0.5 M NaCl /5 mM NEM. Some analyses of platelet factor V, proportionally enriched in unusually large forms, were done using the factor V that remained bound to affinity captured platelet multimerin in 0.5 M NaCl.

To isolate proteins resistant to dissociation from platelet factor V, double factor V immunoprecipitates were prepared as follows: factor V immunoprecipitates were prepared from 10 ml lysate or 6 ml releasate/ml ShαFV beads. Captured proteins were washed extensively with 0.5 M NaCl/5 mM NEM before elution with 1% SDS. Eluates were renatured in 1% Triton X-100 (final 0.1% SDS) before a second factor V immunoprecipitation and analysis by Western blotting.

Multimerin depleted platelet lysate was prepared by incubating lysate with a 1:1 mixture of JS-1 and RM-2 conjugated Sepharose beads (2 ml lysate/1 ml pooled capture beads; 18 hours, 4°C). Similarly, factor V depleted lysate was prepared using ShαFV conjugated Sepharose beads (2 ml lysate/1 ml beads). Controls included sham depleted samples, prepared using Sepharose beads without conjugated antibody, to correct for sample dilution and nonspecific protein losses.

Multimerin, factor V and von Willebrand factor were analyzed by Western blotting similar to the methods described using appropriate primary and peroxidase-conjugated secondary antibodies and chemiluminescent substrate (14, 23). Proteins were visualized after transfer from 1) single dimension nonreduced and reduced sodium dodecyl sulphate (SDS)-polyacrylamide gels, 2) SDS-multimer gels (containing 1.25% agarose and 1.25% acrylamide) or 3) two dimensional nonreduced/reduced gels (14, 23) using prestained reference markers from Helixx Tech. Inc., Scarborough, ON and Bio-Rad, Mississauga, ON. For Western blots of immunoprecipitates, proteins were eluted using 2% SDS, without boiling or added reducing agents, to prevent coelution of capture IgG. Approximately 20 μg multimerin and 10-35 ng factor V were analyzed per lane on most gels. To compensate for antibody sensitivities, approximately 2.5 and 12 fold more factor V was immunoprecipitated and loaded per lane to visualize HC and LC, compared to BD epitopes, in nonreduced and reduced Western blots. An identical profile of factor V was visualized by BD, HC and polyclonal antibodies in samples loaded directly or after factor V immunoprecipitation. Because of lower sensitivity, samples analyzed with the C2 antibody were tested after immunoprecipitation. Factor V antibodies used for Western blots were verified to recognize nonreduced and reduced factor V and all showed equivalent reactivity for transblotted, nonreduced factor V that was preincubated with or without 5% 2-mercaptoethanol.

To determine the proportions of components visualized in Western blots, data were analyzed using a GS-800 Calibrated Densitometer and Quantity One Analysis Software (Bio-Rad, Hercules, CA) and exposures chosen to visualize components of interest, without maximal saturation of other bands.

Results

Analysis of platelet factor V on nonreduced multimer gels

Western blot analyses of platelet proteins (prepared with NEM to block free sulfhydryl groups) that had been separated on denaturing, SDS-multimer gels unexpectedly revealed forms of factor V that were much larger nonreduced than single chain factor V – the largest form of factor V in plasma (Fig. 1A). The unusually large forms of platelet factor V had a variable nonreduced size and they were recognized by monoclonal and polyclonal human factor V antibodies (Figs. 1-4, and blots probed with horse antisera, not shown). Densitometry analyses confirmed that the unusually large forms of factor V were unique to platelets (Fig. 1B), where they comprised about 25% of the total pool of factor V (data based on shortest gel exposures that revealed 4 distinct bands larger than single chain factor in platelets, Fig. 1B). Factor V deficient platelets contained only traces of unusually large factor V (Fig. 2A, lane def) in contrast to the larger amounts in platelets from healthy controls (n >20 tested; lysates prepared with 5 or 40 mM NEM; Figs. 1-7 and data not shown) and from individuals with severe von Willebrand factor or factor XIII deficiencies (not shown).

In nonreduced Western blots of resting platelet lysates and releasates, separated on SDS-multimer gels, factor V polyclonal (Figs. 2-4), and BD monoclonal antibodies (Figs. 1, 2B, 4) visualized unusually large components in platelet factor V, but they only visualized single chain and smaller proteolyzed forms of factor V in the same samples analyzed after reduction (Fig. 2B, lanes R). These data indicated that platelet factor V was contained in interchain disulfide linkages in the unusually large forms of factor V.

When platelet von Willebrand factor polymers were used as a size reference (multiples of 500 kD dimers), the unusually large components of factor V in resting platelets and thrombin releasates were estimated to include some forms larger than 2000 kD, although most were smaller (Fig. 3). The unusually large components of factor V in thrombin releasates had mobilities and distributions that were different from the unusually large components of factor V in resting platelets.
large components of factor V in resting platelets. Furthermore, their mobilities in lysate and thrombin releasates were different from the major bands recognized by multimerin antibodies, which did not change mobility after exposure to thrombin (Fig. 3). Densitometry analyses of the thrombin releasate lane shown in figure 3 (using a range of exposures that detected unusually large forms and showed more prominent bands with submaximal intensities) confirmed that forms larger than SC factor V comprised about 20-25% of the total factor V/Va, similar to the proportions estimated for platelet lysate (Fig. 1B).

**Figure 1:** Nonreduced SDS-multimer gel analyses of platelet factor V. Arrowheads indicate single chain (SC) factor V. A) Western blots were used to compare BD epitopes on equivalent amounts of factor V (35 ng, analyzed using MK-30, without immunoprecipitation) in normal pooled platelet lysate (lanes Plt; prepared using 5 mM NEM) and plasma (Pla). Epitopes in platelet factor V recognized by HC and C2 antibodies (immunoprecipitates of 350 ng for HC, 1680 ng for LC) are shown for samples run on adjacent lanes. B) Densitometry analyses, illustrating the band profiles and relative optical densities of the lanes probed with the BD antibody, using the lightest exposure that visualized 4 distinct bands larger than SC factor V in platelets. Percentages indicate the proportions of total plasma and platelet factor V that were larger than single chain (SC) factor V.

**Figure 2:** The nonreduced and reduced mobility of platelet factor V. Western blots show the factor V in platelet lysates (Plt), plasma (Pla) and thrombin releasate (rel) (5 mM NEM in all platelet samples), visualized by monoclonal antibodies to the BD domain, or by polyclonal sheep anti-human factor V (poly), after separation on reduced (R) or nonreduced (NR) SDS-multimer gels (arrowheads indicate the position of SC). Panel A shows a long exposure of equal volumes (25 µl lysate/lane) of normal (N) and factor V deficient (def) platelet lysates. Panel B compares 35 ng of platelet and plasma factor V with 25 µl thrombin releasate. Like platelets, thrombin releasates contained unusually large disulfide-linked forms of platelet factor V/Va.

**Epitope mapping analyses of unusually large platelet factor V/Va**

Domain specific factor V antisera (verified to recognize nonreduced and reduced factor V equally well) were used to investigate which domains of factor V were contained in the covalently linked structure of unusually large platelet factor V, before and after thrombin cleavage. The unusually large components of factor V in platelet lysates (Fig. 1) and affinity purified platelet factor V (Figs. 4, 5) were readily evident in blots probed with BD (Figs. 1, 4, 5), LC (Figs. 1, 4, 5) and polyclonal (Figs. 2, 3 and data not shown) antibodies, whereas their detection by HC antibodies was difficult, required long exposures (Figs. 1, 4), and for some analyses were not possible (including the samples evaluated after separation in two-dimensional gels shown in Fig. 5 and data not shown). A much smaller proportion (6%) of the total platelet factor V visualized by HC antibodies was unusually large, compared to the proportion (25%) visualized by both BD and LC antibodies (data from densitometry analyses of samples shown in figures 1 and 4 and additional gels, not shown). Furthermore, the HC antibodies recognized unusually large forms that were spaced further apart than the most abundant unusually large forms recognized by BD and LC antibodies, which had identical mobilities (Figs. 1, 4). These data suggested some components in unusually large platelet factor V were partially proteolyzed and no longer covalently linked to HC domains.

Epitope analyses of affinity purified platelet factor V, before and after exposure to thrombin (Fig. 4) indicated that thrombin
cleavage altered the structure of unusually large platelet factor V, leading to persistent expression of BD, but not LC or HC epitopes (Fig. 4). This suggested that the site of interchain disulfide linkage in unusually large factor V was located within the BD. Two dimensional, nonreduced/reduced analyses (multimer gels followed by SDS-PAGE) of affinity purified platelet factor V (captured using antibodies to factor V, Fig. 5, or multimerin, not shown) and unfractionated platelet lysate (not shown) indicated that partially proteolyzed, 220 kD C-terminal fragments of factor V that expressed BD and LC, but not HC epitopes, were contained in a disulfide linkage in unusually large factor V (Fig. 5).
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On original, prolonged exposures of these two-dimensional gels, the BD antibody (which gave stronger signals than other factor V antibodies) also detected traces of single chain factor V in the unusually large components of platelet factor V. HC epitopes were predominantly contained on components of platelet factor V that were smaller than single chain factor V, before and after reduction (Fig. 5). These data were consistent with the extremely long exposures required to demonstrate any HC components in unusually large factor V in single dimension multimer gels and proportions of proteolysed to single chain factor V platelet detected by HC antibody in platelet lysates analyzed by single dimension, reduced SDS-PAGE (Figs. 1, 4 and data not shown).

Similar two-dimensional, nonreduced-reduced analyses of thrombin-treated affinity purified platelet factor V (Fig. 5) indicated that the 150 kD fragment of the BD was the only part of factor V retained in unusually large platelet factor V after thrombin cleavage, which liberated the heavy and light chain domains that comprise factor Va from unusually large factor V. As the BD of human factor V contains only 1 cysteine (24, 25), we postulated that unusually large platelet factor V was in a disulfide-linkage with another protein, possibly multimerin because of the sizes. Further studies were done to investigate how much platelet multimerin was associated with factor V, and if any was covalently linked to factor V.

The factor V ELISA indicated that there was 2 µg of factor V in 10^9 normal pooled platelets, which (by definition) contained 1 U of multimerin. 1 U of multimerin was determined to be equivalent to 18.5 µg of purified recombinant multimerin (mean of determined values; range: 17.1-19.9 µg; reduced sub-
unit size 186 kD), and to 15.4 µg of platelet multimerin because of its smaller subunit size (155 kD). The conversion of these quantities into molar ratios (assumed molecular masses: 155 kD for multimerin subunits, 330 kD for factor V) suggested that on average, platelets contained approximately 17 multimerin subunits for every factor V molecule. Multimerin undergoes additional, post-translational processing, perhaps catalyzed by platelet/megakaryocyte thiol isomerases. Western blot analyses indicated that multimerin immunodepletion codepleted all detectable unusually large factor V from resting platelet lysates, without removing all of the single chain and smaller, proteolyzed forms of platelet factor V (Fig. 6). Moreover, 0.5 M salt dissociated significant amounts of single chain and smaller proteolyzed platelet factor V from affinity captured multimerin, without dissociating unusually large factor V (Fig. 6). Attempts to purify unusually large factor V by large scale platelet multimerin affinity purifications resulted in successful cocapture of unusually large factor V, but the omission of SDS from the final elution step resulted in extremely low yields of recovered proteins, due to losses from adsorption after elution (not shown). Because affinity purified platelet multimerin, like platelets, contained a large pool of multimerin that was not associated with factor V, other approaches were used to determine if there were forms of multimerin covalently linked to factor V in platelets.

Factor V immunodepletion was used to obtain more data on the proportion of platelet multimerin that was associated with factor V. Factor V immunodepletion removed 97.7 ± 1.6% of the factor V antigen (mean ± 1 S.D., for 6 determinations) and all forms of factor V detectable by Western blotting from pooled platelet lysates (not shown). This removal codepleted 45.4 ± 4.2% of multimerin in contrast to sham immunodepletion, which removed 0 ± 11.5% of multimerin and 13.0 ± 12.2% of factor V (means ± 1 S.D., for 6 determinations). When the factor V/Va in resting platelets and thrombin releasates was separated from noncovalently linked multimerin by double factor V affinity purification (Fig. 7), as outlined in the methods, a subpopulation of multimerin polymers was copurified that had mobilities different from the most abundant multimerin polymers in resting platelets and thrombin releasates. Parallel analyses of multimerin double immunoprecipitates confirmed that the denaturation/renaturation steps were not responsible for the altered multimerin mobility (not shown). Moreover, the multimerin polymers that were isolated by the double factor V immunopurification had altered mobilities after exposure to thrombin and they comigrated with the unusually large factor V/Va visualized by factor V BD antibodies, before and exposure to thrombin (Fig. 7), consistent with covalent linkage to factor V via the BD.

Discussion

Platelets store a number of specialized proteins that are important for supporting hemostasis, including several unusually large homopolymeric proteins that are among the largest known proteins in the body: von Willebrand factor and multimerin (23, 26). In analyses of factor V on nonreducing, denaturing multimer gels, we unexpectedly found that platelets contain a population of unusually large, disulfide-linked forms of factor V. Densitometry analyses suggested that these forms represented approximately 25% of the total factor V stored in platelets. We suspect that the reason that these high molecular weight forms of platelet factor V were not identified in previous studies (11, 27) is due to the following: the much larger proportions of single chain and smaller proteolyzed factor V in platelets; the large pool of factor V that is not covalently bound to multimerin in platelets; the specialized gels needed to resolve unusually large factor V; and the need for special procedures (such as adding denaturing detergents) to recover unusually large factor V in affinity column eluates.

Several lines of evidence from our investigations suggested that the unusually large forms of factor V in human platelets were generated by formation of a disulfide-bridge between the cysteine at position 1085 in the factor V BD and a cysteine residue in multimerin. First, unusually large platelet factor V copurified with and resisted dissociation from multimerin. Second, 150 kD BD fragments were not liberated from unusually large factor V upon thrombin cleavage and the BD in human factor V is known to contain only one cysteine (24, 25). Third, thrombin cleavage of unusually large factor V produced at least 4 distinct disulfide-linked forms containing 150 kD BD fragments, indicating an organization into structures larger than factor V dimers and implying linkage to a protein with a variable nonreduced size. Fourth, even after thrombin cleavage, the unusually large components of platelet factor V comigrated with a subpopulation of multimerin polymers that were resistant to dissociation from factor V when exposed to denaturing detergent. Fifth, the sizes of the subpopulation of multimerin polymers linked to factor V (before and after thrombin cleavage) were compatible with the linkage of variably proteolyzed, single factor V molecules to variably-sized multimerin polymers. In an earlier study, multimerin was illustrated to bind the light chain of factor V and Va via noncovalent interactions (12) and our new data indicate a role for the BD in stabilizing factor V-multimerin complexes. Figure 8 summarizes the types of complexes generated by noncovalent and covalent interactions between factor V and multimerin in platelets and their proposed structures after release and cleavage by thrombin.

The high molecular weight, disulfide-linked forms of factor V in human platelets that are not apparent in plasma suggest factor V undergoes additional, post-translational processing, perhaps catalyzed by platelet/megakaryocyte thiol isomerases
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(28) subsequent to association with multimerin, in order to generate the disulfide-linked forms unique to platelets. Studies of human plasma and platelet factor V have indicated they are similarly effective in supporting prothrombinase assembly once activated by thrombin, and that the pool of platelet factor V bound to multimerin represents a major part of platelet factor V procoagulant activity (11, 12, 27). We observed more heterogeneity in the cleavage products of platelet factor V using domain-specific antisera (Fig. 5) than we had anticipated, and this heterogeneity was less evident in shorter exposures of platelet factor Va analyzed on single dimension gels. It likely reflects variability in sites of factor V proteolysis in platelets. We could not study the functions of covalent factor V-multimerin complexes as denaturing detergent proved necessary to isolate these structurally unique forms from noncovalent multimerin-factor V complexes. Nonetheless, based on their structural ability to generate factor Va, we suspect that the forms of platelet factor V in disulfide linkage with multimerin have procoagulant function once cleaved by thrombin, as outlined in figure 8.

In our current study, we used sensitive quantitative assays rather than the qualitative endpoints used in earlier studies to better estimate the proportions of platelet factor V bound to multimerin (12). We found that a significant proportion, about 43% of the total factor V in platelets, was associated with multimerin. Taking into account the different subunit sizes for recombinant and platelet multimerin, the quantities of multimerin and factor V in platelet lysate (2.0 µg factor V per 15.4 µg multimerin in our current pooled platelet lysate), and the molecular weights of factor V compared to multimerin subunits, we estimated that platelets contain 17-fold more multimerin subunits than factor V molecules. There are some inherent inaccuracies with using densitometry to estimate the proportions of different sized forms. Nonetheless, our estimate that 25% of the total factor V in platelets, and their releasates, was unusually large, further suggests that a small proportion of platelet multimerin, only 1 out of every 68 multimerin subunits, is covalently linked to factor V. This was consistent with the less abundant subpopulation of multimerin found to be covalently linked to factor V. Although the multivalent structure of multimerin (trimers and larger homopolymers) suggests that complex stoichiometries with factor V are possible, the molar ratios suggest that only one molecule of factor V would be present in most disulfide-linked factor V-multimerin complexes in platelets. It also implies that the variable sizes of unusually large factor V originate from their multimerin constituent (Fig. 8).

Our current investigation implicates the C-terminal, thrombin cleavage fragment of the factor V BD as important for multimerin binding, in addition to its role in promoting factor V anticoagulant function (29). It is interesting that the BD cysteine, which is present in human factor V to mediate linkage to multimerin (Fig. 8), is not found in bovine or murine factor V (30, 31) as this suggests that human factor V BD is uniquely capable of forming a disulfide linkage to multimerin. The covalent linkage of the BD of human platelet factor V to multimerin, coupled with the binding of released multimerin to thrombin-activated platelets, may account for the curious observation that thrombin-activated human platelets express the factor V BD on their surface (32). It is unfortunate that antibodies which recognize multimerin in other species are not presently available to determine if covalent multimerin-factor V complexes exist in other species.

**Figure 8:** Proposed model of the structure of unusually large platelet factor V stored in platelet α-granules, before and after secretion and cleavage by thrombin.

The linear structure of factor V (top) indicates the different domains and that the BD is prominent in intact or partially proteolyzed forms in platelet factor V. Multimerin, factor V and factor Va are shown to illustrate proposed covalent (S-S) and noncovalent (dotted lines) linkages, and the calcium bridge between the factor V heavy and light chain before (middle panel) and after thrombin cleavage (lower panel). Multimerin molecules are illustrated as (1+n) subunit trimers and larger polymers of trimers (23). Numbers shown for factor V indicate thrombin cleavage sites, cysteine 1085 and predicted N and C terminal amino acids (2). Factor Va generation and function from unusually large platelet factor V is only speculated at this time. Although not shown, thrombin-cleaved platelet factor Va could be alternatively proteolyzed at 1543, as reported (11).
Human factor V is presently unique in that it is the only participant in the cascade of blood coagulation reactions demonstrated to form variably-sized structures through disulfide-linkage to another protein. As the total pool of factor V bound to multimerin in human platelets has procoagulant function (12), we postulate that like other forms of platelet factor V, the forms covalently linked to multimerin generate factor Vα upon activation by thrombin, as suggested by their structure (Fig. 8). Multimerin could function to hold about half of the platelet pool of factor V in covalent and noncovalent linkages, until granule release occurs and thrombin cleavages liberate factor Vα for prothrombinase assembly on the platelet surface (Fig. 8), akin to the way supporting scaffolds hold pieces of plastic models in a unit until their removal for model assembly is desired. We suspect that the model builder is probably a megakaryocyte. At the moment, there is a lack of functional evidence supporting the role of multimerin-factor Vα complexes in coagulation. A full understanding of the significance of disulfide-linked factor V-multimerin complexes will require detailed dissection of the influences that covalent and noncovalent interactions with multimerin have on factor V functions and its delivery onto activated platelets.

Acknowledgments

We thank Aurelio V. Santos for generating figure 8; Zhihui Song, Alison Cowie, Menaka Pai and Rachel Woram for contributions to preliminary studies; and Dr. Mohan Pai, Dr. J. Drouin, Ms. J. Sek, Mrs. B. Archibald for their help in obtaining patient samples.

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