Ultrastructural Localization of Coagulation Factor V in Human Platelets

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The distribution and transport in platelets of human coagulation Factor V was investigated by immunofluorescent and immunoelectron microscopy. In resting intact platelets, little surface staining was observed by immunofluorescence. In permeable resting cells, punctate staining similar to that reported for fibrinogen (Fbg), thrombospondin (TSP), fibronectin (Fn), von Willebrand factor (VWF), B-thromboglobulin (BTG), and platelet Factor 4 (PF4) was observed.1,2 Double label immunofluorescent staining for Fbg and Factor V demonstrated colocalization, suggesting their presence in the same intracellular structure. Thrombin stimulation induced the appearance of larger (~0.5 μm) immunofluorescent masses of these proteins which exactly colocalized. Thus, at the light level, Factor V and Fbg are localized in the same structure in resting and thrombin-stimulated cells. On the ultrastructural level, an alpha granule localization for Fbg has previously been established.3,4 We have extended our immunofluorescent observations regarding the localization of Factor V in human platelets by use of transmission electron microscopy of antibody-stained ultrathin frozen sections. In resting cells, staining of virtually all alpha granules was observed for Factor V. In contrast, consistent staining was absent from other organelles including plasma membranes, mitochondria, and vacuolar structures which may represent the open canalicular system. These data thus establish at the ultrastructural level an alpha granule localization of human coagulation Factor V.

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MATERIALS AND METHODS

Chemicals. The following were purchased from Sigma Chemicals (St. Louis, Mo): poly-L-lysine, bovine serum albumin, and cadocyclic acid. Paraformaldehyde and EM grade glutaraldehyde were obtained from Polysciences (Warrington, Pa). Sepharose 2B and dextran T-10 were obtained from Pharmacia (Piscataway, NJ). Triton X-100 was purchased from J.T. Baker Chemicals (Phillipsburg, NJ). Phosphotungstic acid was purchased from Ted Pella, Inc. (Tustin, Calif).

Platelet preparation. Platelet-rich plasma was prepared from acid citrate dextrose anticoagulated whole blood obtained from healthy aspirin-free volunteers as described.14 Washed human platelets were subsequently prepared by centrifugation and gel-filtration on Sepharose 2B as described.15

Purified proteins. Purified human alpha-thrombin was the generous gift of Dr. John Fenton, New York State Department of Health, Albany, NY. This material was diluted to 100 u/mL in modified Tyrode's buffer, stored in aliquots at -70 °C, and thawed once prior to use. Purified human fibrinogen was prepared as previously described.16 Human coagulation Factor V was prepared according to the method of Dahlback17 with the following modification: the molarity of the NaH4Cl in the buffer used to dissolve the polyelectylyene glycol precipitate was lowered to 0.05 mol/L from 0.1 mol/L, to ensure binding to the DEAE-sepharose. The purified material produced a single Coomassie blue staining band of apparent Mr ~ 330,000, under reducing conditions in 5% to 10% SDS-polyacrylamide gels. Purified Factor V (0.5 mg/mL in 50% glycerol, 25 mmol/L Tris-HCl, pH 7.5, 0.05 mol/L NaH4Cl, 5 mmol/L benzamidine, and 5 mmol/L CaCl2) was labelled with 125I by the lactoperoxidase method of Thorell et al.18 The labelled Factor V was isolated by gel filtration on a 0.9 x 55 cm column packed with...
Plasma, purified Factor V, or a platelet extract. Rabbit antiserum tested against plasma in crossed immunoelectrophoresis. Additional-
were obtained by sectioning at \(-54^\circ\text{C}\). Sections were transferred to acid washed glass slides and stained as described for whole cells.

**Immunolabelling and negative staining.** Rabbit antihuman Factor V or preimmune rabbit serum were used as primary antibodies in an indirect immunolabelling procedure. Affinity purified and biotinylated goat anti-rabbit IgG (Hyclo Laboratories, Logan Ut) was used as the second antibody. Antibody-antigen interactions were visualized by means of a colloidal gold-avidin conjugate.

Five nanometer colloidal gold-avidin was prepared as previously described.\(^6\) Prior to immunolabelling, the thawed ultrathin frozen sections were conditioned with 2% gelatin and 0.1% bovine serum albumin. Following completion of immunolabelling, the sections were negatively stained with a mixture of 0.2% phosphotungstic acid and 1% dextran. Sections were viewed on a Hitachi 12-A transmission electron microscope with an accelerating voltage of 75 KV, and were photographed with Kodak electron microscope film No. 4489.

**RESULTS**

**Localization of Factor V in resting and thrombin-stimulated platelets.** Intact resting platelets demonstrated minimal surface staining. When cells were made permeable with Triton X-100, however, punctate immunofluorescent staining for Factor V and Fbg was observed. Antibody specificity was documented in double-label immunofluorescent cross-blocking studies. In these studies, mixtures of rabbit antihuman Factor V antibody and rhodaminated goat F(ab')\(_2\) antihuman Fbg were absorbed overnight at 4\(^\circ\text{C}\) with either a 1.4-fold molar excess of purified Fbg, or an equimolar concentration of purified Factor V or buffer. Permeable platelets were subsequently stained with these absorbed antibodies and counterstained with fluoresceinated goat F(ab')\(_2\) antirabbit IgG. As shown in Fig 2A and B, antibodies absorbed with buffer alone demonstrated punctate intracellular staining for Factor V which exactly colocalizes with Fbg. However, when platelets were stained using the absorbed antibodies, fluorescence was blocked only when an antibody was absorbed with its parent antigen, confirming specificity of staining (Fig 2C and F). Additionally, control testing with preimmune rabbit or goat IgG failed to stain either intact or permeable cells (data not shown).

To determine the localization of Factor V antigen immediately following thrombin stimulation, cells fixed after one minute’s stimulation at 24\(^\circ\text{C}\) were double stained for Factor V and Fbg. Thrombin stimulation resulted in the time-dependent translocation of Fbg and Factor V into larger immunofluorescent masses similar to those that have been reported for TSP, Fn, VWF, BTG, and PF4 (Fig 2G and H).\(^1,2\) These masses were defined as smooth fluorescent structures, \(\geq 0.5\) microns in diameter, and were not seen in

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**Fig 2.** Colocalization of Factor V and Fbg in resting and thrombin-stimulated platelets. Resting, permeable platelets were stained with a mixture of rabbit antihuman Factor V and rhodaminated goat F(ab')\(_2\) antihuman Fbg and counterstained with fluoresceinated goat F(ab')\(_2\) antirabbit IgG. Antibodies absorbed with buffer alone (A and B) demonstrated punctate intracellular staining for Factor V which exactly colocalized with Fbg. Incubation of the antibody mixture prior to staining with either purified Factor V (C and D) or Fbg (E and F) only blocked fluorescence when an antibody was absorbed with its parent antigen. When platelets were fixed one minute following thrombin stimulation, we noted the appearance of large, peripheral immunofluorescent masses which are defined as smooth fluorescent structures \(\geq 0.5\) \(\mu\text{m}\) in diameter and which double stained for Factor V and Fbg (G and H, arrowheads). Original magnification, \(\times 1,400\); Current magnification, \(\times 1,050\).

**Fig 3.** Reduced immunofluorescent staining for Factor V and Fbg in Gray platelets. Frozen thick sections of normal and Gray platelets were double stained for Factor V and Fbg as described in Materials and Methods. The left panels illustrate Factor V staining and the right illustrate Fbg staining of the same field of platelets. Normal cells are depicted in the upper panels and gray platelets are shown below. Original magnification, \(\times 1,400\); Current magnification, \(\times 770\).
Fig 4. Indirect immunolabeling of Factor V in cryostat sections of human platelets. Ultrathin frozen sections were incubated with rabbit antihuman Factor V as the primary antibody. Biotinylated goat antirabbit IgG was employed as the second antibody. Antibody-antigen interactions were visualized by means of a 5 nm colloidal gold-avidin conjugate. Panel A, a magnification of the whole cell shown in panel C, demonstrates heavy labeling over structures identified as alpha granules (g) with minimal labeling of mitochondria (m), cristae (cr), or the platelet cytosolic matrix (c). Panel B represents a corresponding illustration, with intact membranes designated by solid lines and presumptive membranes, by dashed lines. (original magnification (A), 107,500 X; bar 0.1 μm; current magnification, 80,625 X; (B) original magnification, 107,500 X; current magnification, 80,625 X; (C) original magnificationm 28,000 X, bar 0.5 μm; current magnification, 21,000 X).
resting cells. The above data suggested that Factor V and Fbg are stored in the same intracellular organelle and are processed to the cell surface via common structural intermediates.

As an alpha granule localization for Fbg has been established, it seemed likely that platelet Factor V would also be stored in alpha granules. To examine this possibility frozen thick sections of gray platelets, which are selectively deficient in alpha granules, were double stained for Factor V and Fbg. As seen in Fig 3, immunofluorescent labeling of gray platelets revealed markedly decreased staining for both Factor V and Fbg (lower panels) when compared to normal platelets (upper panels).

**Ultrastructural localization of Factor V in frozen thin sections.** To investigate the ultrastructural localization of Factor V by transmission electron microscopy, we prepared negatively stained, ultrathin frozen sections of resting platelets. Platelet morphology in negatively stained cryosections has been described previously. Alpha granules are identified on the basis of their size, 0.1 to 0.4 μ, roughly spherical shape, single limiting electron lucent membrane of 100 ± 21 angstroms in thickness, and the occasional appearance of a darkly staining eccentric nucleoid. Other identifiable subcellular organelles include mitochondria with characteristic double membranes and cristae.

Indirect immunolabelling of frozen sections with an anti-Factor V antibody demonstrated heavy labelling of structures identified by the above criteria as alpha granules (Fig 4A). The labelling pattern was neither restricted to the nucleoid nor to the electron lucent periphery, but rather appeared inhomogeneous. In contrast, label was absent from plasma membrane and mitochondria. Only background levels of staining were associated with the platelet cytoplasmic matrix. In contrast, control sections, stained with preimmune rabbit serum, demonstrated only occasional random colloidal gold particles (not shown).

**DISCUSSION**

In the present study we have investigated the intracellular localization of human coagulation Factor V and its initial redistribution in response to thrombin. Utilizing immunofluorescence techniques we have found that Factor V and Fbg colocalize in the interior of resting platelets. On the ultrastructural level, Factor V was localized within structures morphologically identified as alpha granules by means of indirect immunolabeling of ultrathin frozen sections. Following thrombin stimulation, Factor V is consolidated into larger structural intermediates which double stain for Fbg.

It has long been recognized that Factor V is an essential cofactor in the assembly of the prothrombinase complex. Activated Factor V (Factor Va) functions as the platelet-bound receptor for activated Factor X (Factor Xa). While the platelet receptor for Va does not require platelet release for its expression, platelet Factor V contributes to functional prothrombinase complex assembly. In this regard, subcellular fractionation studies have suggested an alpha granule localization for Factor V. However, in recent studies Tracy et al have suggested that normal Factor V levels are present in gray platelets (which are selectively deficient in alpha granules). While these results may represent partially activated platelets with surface-bound Factor V or reflect heterogeneity among gray platelet patients (as the patient we examined demonstrated markedly reduced Factor V antigen as judged by immunofluorescence), the present studies and the subcellular fractionation studies of Chesney et al localize the bulk of Factor V antigen in the resting cell to alpha granules. Additionally, following thrombin stimulation, Factor V was shown to become consolidated into larger structural intermediates that double stain for Fbg and have been suggested to represent compound granules. Thus, intraplatelet Factor V is apparently stored and processed to the cell surface by a mechanism common to a growing number of alpha granule proteins. Whether the apparent functional differences between platelet and plasma Factor V are due to structural differences, as has been shown for von Willebrand factor, another alpha granule protein, or simply reflect a local concentration effect brought on by a fusing compound granule, remains to be evaluated.

**REFERENCES**


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