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Platelet Thrombosthenin: Subcellular Localization and Function *

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Summary. Thrombosthenin, an immunologically distinct contractile protein was isolated in relatively pure form from human platelets. The protein, which was of high molecular weight appeared to be composed of multiple polypeptide subunits, probably polymeric in nature.

Thrombosthenin had magnesium-dependent ATPase activity, releasing an average of 3 μg phosphorus per mg protein in 30 min. After the addition of ATP, there was a reversible alteration in viscosity with calculated ATP sensitivity ranging from 64 to 90%. These biochemical properties of thrombosthenin resemble those of smooth muscle.

Specific antisera to thrombosthenin significantly inhibited the ATPase activity of the protein. Clot retraction of recalcified platelet-rich plasma and clot retraction of clotted fibrinogen-platelet mixtures were also inhibited by the antisera. The findings suggest that thrombosthenin is an important component of the clot retraction system.

Thrombosthenin was extracted from isolated platelet granule and membrane fractions. The contractile protein derived from the membrane compartment was more active as an ATPase and appeared to be more homogeneous on immunologic analysis.

Introduction

Certain biochemical and physiological properties of blood platelets suggest that they possess contractile activity similar in some respects to that found in muscle tissue. Clot retraction requires the presence of platelets, and during the formation of a hemostatic plug, platelet aggregates appear to actively contract as part of the phenomenon known as viscous metamorphosis (1). Actomyosin-like ATPase activity is present in platelets (2) and glycolysis is a major pathway of energy metabolism (3). An actomyosin-like protein, thrombosthenin, was first extracted from human platelets by Bettex-Galland and Lüscher (2). A similar protein in pig platelets was extracted and partially characterized by Grette (4). Of further interest is the description of intracellular contractile proteins in flagellated protozoans, mitochondria, sarcoma cells (5), and chick embryo fibroblast cells (6). This report describes the isolation and further characterization of human platelet thrombosthenin. Furthermore, its distribution in specific subcellular compartments will be defined.

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Methods

Thrombosthenin preparation. The general procedure utilized to separate thrombosthenin was based mainly on the technique as described by Grette (4) and is patterned after methods employed for the extraction of actomyosin from muscle tissue (7). The starting ma-

terials were platelet concentrates[1] prepared from 10–20 units of freshly collected whole blood, using acid-citrate-dextrose solution as the anticoagulant. Usually the concentrates were processed to the point of butanol extraction on the day of collection. Rarely they were stored at 4° C overnight before processing. Platelets were separated by means of the "oil bottle" centrifugation technique (8), washed three times in Alsever's solution (9), and washed three times more in 0.9% NaCl containing 0.1 volume of 3.14% sodium citrate dihydrate at 4° C. 2 ml of cold 0.6 M KCl in 0.015 M Tris buffer (pH 7.5) was added to each gram (wet weight) of platelets, followed by 25 μl of *n*-butanol for each milliliter of final solution. Lysis of the cells occurred upon the addition of butanol (4). The extraction proceeded for 24 hr at 4° C, and after being warmed to room temperature the material was centrifuged at 12,100 *g* for 15 min at 22° C. The supernatant was cooled to 4° C and 2 volumes of cold 0.002 M $MgSO_4$ were added. The pH was adjusted to 6.5 with 0.1% acetic acid, and the mixture was kept at 4° C for 1–3 hr, after which it was centrifuged at 8,700 *g* for 10 min at 4° C. The contractile protein precipitated in the form of a gel and was redissolved in 2 volumes of 0.6 M KCl in 0.015 M Tris. It was then reprecipitated five times over the following 24–36 hr. Final solubilization was carried out in the KCl-Tris solution. In some instances the thrombosthenin preparation was further purified by column chromatography on polyacrylamide gel. Bio-Gel P-300,[2] suspended in KCl-Tris solution, was poured into a column 12 inches long and 1 inch in diameter. The column was then charged with 50–75 mg of thrombosthenin solution and the bulk of the protein appearing in the void volume was concentrated by ultrafiltration and reprecipitated once with $MgSO_4$.

Viscosity experiments. Solutions of thrombosthenin in KCl-Tris buffer were used for relative viscosity determinations. They were carried out in a Cannon-Manning Semi-Micro Viscometer.[3] Specific viscosity values were computed according to the formula: $Z_n = 2.3 \log \eta_{rel}/$ concentration (grams/liter) (2). Sensitivity to ATP was calculated according to the method of Portzehl (10). The concentrations of membrane thrombosthenin solutions were 0.45% in 0.6 M KCl, 0.015 M Tris (pH 7.5). Granule thrombosthenin was studied in 0.3% solutions under the same conditions. The times obtained in each viscosity experiment represent the average of triplicate determinations which were then used to calculate relative viscosity.

Superprecipitation studies. In a low magnesium environment and in the presence of ATP, muscle proteins flocculate and contract to small volume. This phenomenon is known as superprecipitation (2). Superprecipitation experiments on whole platelet thrombosthenin were carried out in the presence and absence of 10^{-3} M ATP. Magnesium sulfate (6×10^{-3} M) was always present in the test system. The protein concentrations were adjusted to 0.2%, ionic strength 0.08, and the pH was 6.3. In the studies involving thrombosthenin derived from the platelet membranes and granules, EDTA (4×10^{-3} M) was added. The membrane thrombosthenin solutions contained 0.18% protein in a final volume of 1 ml of 0.12 M KCl containing $MgSO_4$ and EDTA. The solutions of granule thrombosthenin contained 0.15% protein in a final volume of 1 ml of 0.12 M KCl, with the same concentrations of Mg^{++}, ATP, and EDTA.

ATPase experiments. 1 mg of reprecipitated thrombosthenin in a final volume of 1 ml was incubated at 22° C with 5×10^{-4} M ATP. The medium also included KCl 5×10^{-2} M; $MgSO_4$ 1×10^{-4} M; and borate buffer 0.2 M (pH 7.0) (4). After a 30 min incubation period, the reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid (TCA) and the mixture allowed to stand for 30 min at room temperature. The tubes were then centrifuged at 3,000 *g* for 15 min and inorganic phosphorus in the supernatant fraction was determined by the method of Marsh (11). Tissue blanks, ATP blanks, and reagent blanks were included in all determinations. Further ATPase experiments were carried out in the presence of the following: (*a*) mersalyl[4] in a final concentration of 2.5×10^{-4} M; (*b*) ouabain 1×10^{-3} M; (*c*) rabbit antithrombosthenin globulin, 1 mg/ml borate buffer; and (*d*) rabbit antifibrinogen globulin, 1 mg/ml borate buffer. Finally, another group of experiments was performed in the absence of $MgSO_4$. Mersalyl was prepared in borate buffer (0.2 M, pH 7.0). Mersalyl acid was converted to the sodium salt by dissolving initially in 0.2 M borax (calculated as sodium borate) with heating at 50° C. The pH was adjusted to 7.0 with 0.2 M boric acid and the solution brought to volume with borate buffer. Ouabain was dissolved directly in borate buffer with heating at 37° C.

Immunologic experiments. For the preparation of antisera, thrombosthenin extracts were reprecipitated six times. A single intravenous injection of 1–2 mg of the thrombosthenin in KCl-Tris buffer was given to two rabbits at four weekly intervals. The animals were then "boosted" with subcutaneous and intramuscular injections of 2–4 mg of protein in complete Freund's adjuvant at biweekly intervals for 8–12 wk. After inactivation at 56° C for 30 min, the sera were stored at −20° C until used. Specimens from individual blood collections were not pooled.

Antisera to isolated human platelet granules, membranes, and the soluble supernatant fractions were produced in individual rabbits by repeated subcutaneous and intramuscular injections of washed subcellular fractions (see below) in Freund's adjuvant. Antihuman platelet serum was prepared as previously described (8). Antisera to human albumin, human IgG, whole human serum, and human fibrinogen were obtained commercially.[5]

[1] Generously supplied by the New York Blood Center, New York, N. Y.

[2] Bio-Rad Laboratories, Richmond, Calif.

[3] No. 150, 1 ml capacity at 22° C, Cannon Instrument Co., State College, Pa.

[4] Winthrop Laboratories, Special Chemical Department, New York, N. Y.

[5] Lloyd Bros., Inc., Cincinnati, Ohio.

Antisera to actomyosin derived from human striated and smooth muscle were kindly provided by Dr. Carl Becker.[6] Antithrombosthenin and antifibrinogen globulin were prepared by precipitation with equal volumes of 30% sodium sulfate at room temperature, followed by washing with 15% sodium sulfate. Before use in ATPase inhibition studies, the globulins were dialyzed in borate buffer.

For studies involving fluoresceination, the antithrombosthenin globulin was prepared from antithrombosthenin serum which was previously adsorbed with 10 mg of lyophilized human fibrinogen[7] and 5 mg of lyophilized normal plasma per ml of antiserum. The antiserum was incubated for 1 hr at 37° C and overnight at 4° C and then cleared by centrifugation. The adsorbed antiserum did not react in immunodiffusion with purified fibrinogen at antigen concentrations ranging from 1 to 10 mg/ml. The adsorbed antithrombosthenin globulin was then dialyzed into a 1 : 10 dilution of bicarbonate buffer (0.05 M, pH 9) in saline. Fluorescein isothiocyanate[8] (0.05 mg/mg protein) was dissolved slowly with stirring for 18 hr at 4° C. After dialysis into phosphate buffer (0.01 M, pH 7.2), the dialyzate was passed through a Sephadex G-25[9] column in phosphate buffer and ultrafiltered to the original volume of the serum. Washed human platelets were prepared for examination by application to a glass slide with a Pasteur pipette and fixation in acetone for 10 min. Bone marrow spicules were prepared in a similar fashion. 2 drops of fluoresceinated antithrombosthenin globulin (diluted 1 : 4) were added to the slide which was then incubated at room temperature for 30 min. The slides were washed in phosphate buffer and mounted in phosphate-buffered glycerol. The specimens were examined in a Leitz Ortholux fluorescent microscope containing a mercury vapor lamp (CS 150), a BG 0.12 excitor filter, a dark field condensor, and a Leitz barrier filter.[10]

Immunoelectrophoresis and immunodiffusion studies were carried out by methods previously described (12, 13). Agar was prepared in the presence of 0.6 M KCl.

Clot retraction was evaluated by a modification of the technique of Shulman (14). Studies on clot retraction inhibition were carried out as follows: tubes containing 0.9 ml of platelet-rich plasma and 0.1 ml of the various antisera were recalcified with 0.1 ml of 0.5 M $CaCl_2$ and incubated at 37° C for 1 hr. Clot retraction inhibition was also studied in a plasma-free system by incubating 0.5 ml of washed platelets in Tris-buffered saline (15) with 0.1 ml of purified fibrinogen (2 *g*/100 ml) and 0.2 ml of glucose (200 mg/100 ml). 0.1 ml of antiserum was added and the system was clotted with 0.1 ml of thrombin[11] (10 units in 0.025 M calcium chloride).

Starch urea gel electrophoresis in glycine buffer was carried out by the method of Cohen (16). Peptide mapping of thrombosthenin purified by column chromatography was performed as previously described (17). When pronase[12] was used for digestion, 2 units of enzyme were added for every 10 mg of protein. The incubation was carried out in 0.2 M sodium bicarbonate (pH 8.0) for 24 hr at 37° C.

Studies on subcellular platelet fractions. The technique for preparing platelet homogenates and their subsequent separation on a continuous sucrose density gradient has been described in detail (18). Two subcellular "compartments" and the soluble fraction ("cell sap") were studied. Fractions to be used for ATPase experiments were washed in borate buffer (0.2 M, pH 7.0), and those from which thrombosthenin was extracted were washed in citrate-saline solution (4). The granules were washed by ultracentrifugation at 36,220 *g* for 20 min. The membranes were washed at 198,400 *g* for 60 min. Prior to ATPase studies, the washed pellets were resuspended in borate buffer and stored overnight in ice or frozen at −85° C. Washed pellets for thrombosthenin extraction were stored at − 85° C. The starting material for these extractions was 1.5 − 2.7 g (wet weight) of granules and 0.6–1.4 g (wet weight) of membranes. This was the equivalent of fractions derived from platelets isolated from 5–10 units of whole blood. The extraction procedure was essentially the same as for whole platelet thrombosthenin except for modifications necessitated by the quantity and nature of the material. For each gram (wet weight) of granules, 2 ml of KCl-Tris buffer was added. For comparable membrane material, 3.3 ml of KCl-Tris buffer was added. After the addition of $MgSO_4$, the mixture was kept at 4° C overnight and then centrifuged at 12,100 *g* for 15 min at 4° C. The resulting precipitate was redissolved in 1–2 ml of KCl-Tris solution and used for ATPase assay. For some viscosity and superprecipitation studies, a single reprecipitation step was carried out. When whole fractions were assayed for ATPase activity, about 1 mg of protein was used. Slightly less was employed when the extracted thrombosthenin was similarly assayed. The ATP concentration in the assay mixture was 1×10^{-3} M. After the reaction was stopped with cold TCA, the tubes were centrifuged at 10,000 rpm for 15 min at 4° C. Inorganic phosphate in the clear supernate was determined by the method of Dryer, Tammes, and Routh (19). Tissue blanks, ATP blanks, and reagent blanks were included in all assays.

Protein determinations were carried out by the Folin method (20). Human striated muscle actomyosin was prepared by the method of Levy and Fleisher (7).

Results

Whole platelet thrombosthenin

Unless otherwise stated, the following results were obtained on thrombosthenin that was sixfold reprecipitated.

[6] Department of Pathology, Cornell University Medical College, New York, N. Y.

[7] Mann Research Biochemicals, New York, N. Y.

[8] Dajac Laboratories, The Borden Chemical Co., Philadelphia, Pa.

[9] Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

[10] Leitz, Inc., New York, N. Y.

[11] Bovine thrombin, Parke, Davis & Co., Detroit, Mich.

[12] Calbiochem., Los Angeles, Calif.

Viscosity. A 0.3% solution of soluble thrombosthenin in KCl–Tris buffer had a relative viscosity of 1.8. This value was not appreciably affected by the addition of 2% (by volume) of 0.6 M KCl. A typical experiment is shown in Figure 1. Addition of ATP (final concentration 10^{-3} M) resulted in a marked drop in relative viscosity which returned to the original value after 20 min. The calculated sensitivity to ATP was 74%, and the specific viscosity was 0.13. The results of three additional, separate extraction experiments are shown in Table I. The calculated ATP sensitivity characterizes the presumed degree of dissociation of the macromolecular subunits of thrombosthenin in the presence of ATP. The values observed are comparable to those obtained with smooth muscle (5).

Superprecipitation. Solutions of thrombosthenin showed marked superprecipitation in the presence of ATP. The superprecipitation response varied from batch to batch, but in general maximal effects were observed 15 min after exposure to ATP. These experiments were performed on three separate occasions with three separate batches of extracted protein. The results were similar on all occasions.

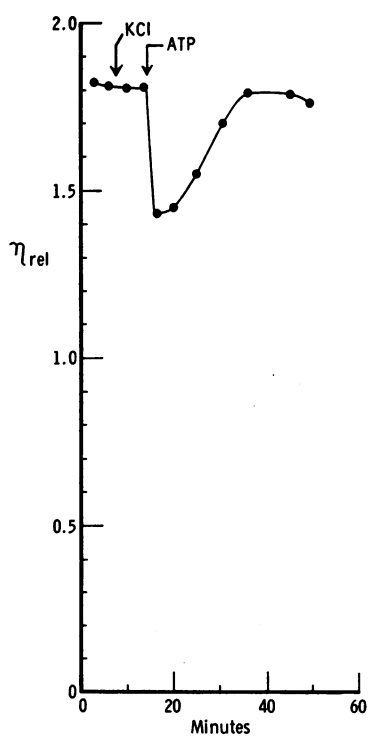

FIG. 1. REVERSIBLE CHANGES IN VISCOSITY OF THROMBOSTHENIN AFTER THE ADDITION OF ATP.

TABLE I

*Viscosity changes of whole platelet thrombosthenin**

Experiment	η_{rel}	η_{rel} ATP	η_{rel} 30 min after ATP	ATP sensitivity
1	1.9	1.4	1.8	90
2	1.9	1.45	1.7	73
3	1.8	1.43	1.7	64

* 0.3% solutions of thrombosthenin in KCl–Tris buffer. 10^{-3} ATP (2% by volume) added. Each experiment was performed in triplicate.

ATPase studies. In four separate control experiments the thrombosthenin preparations hydrolyzed ATP, releasing inorganic phosphate at an average rate of 3 μg of phosphorus per mg of protein in 30 min. Various conditions under which the ATPase activity of extracted thrombosthenin was inhibited were then studied (Table II). Deletion of magnesium resulted in a significant but incomplete loss of ATPase activity. Virtually complete inhibition was noted with mersalyl and slight inhibition was found with ouabain. Significant loss of ATPase activity occurred when antithrombosthenin globulin was added to the assay system. Antifibrinogen globulin had no effect on the enzymatic activity of thrombosthenin.

Immunologic studies. A preparation of reprecipitated thrombosthenin, further purified by chromatography, was subjected to immunoelectrophoresis using a potent rabbit antithrombosthenin serum. As shown in Figure 2, a single precipitin

TABLE II

Effect of inhibitory conditions on ATP hydrolysis by thrombosthenin

Preparation	Per cent of control value*
Control‡	100 (3μ g P/mg protein per 30 min)
Control without magnesium (2)§	30
Control with mersalyl 2.5×10^{-4} M (2)	10
Control with ouabain 1×10^{-3} M (2)	80
Control with antithrombosthenin (2)	17
Control with antifibrinogen (1)	94

* Average of indicated number of experiments.
‡ 1 mg of thrombosthenin in 1 ml of borate buffer, pH 7.0, 0.2 M, containing KCl, 5×10^{-2} M; $MgSO_4$, 1×10^{-4} M; ATP, 5×10^{-4} M. (Repeated three times.)
§ Number of experiments.

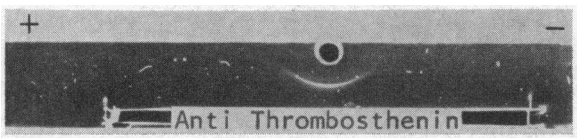

FIG. 2. IMMUNOELECTROPHORESIS OF CHROMATOGRAPHED THROMBOSTHENIN IN 0.6 M KCl AGAR.

line in the beta globulin region was observed. The procedure was repeated using the thrombosthenin preparation against antifibrinogen, anti–whole human serum, anti-albumin, and anti–gamma globulin. In no instance was reactivity observed. However, when less purified, unchromatographed samples of thrombosthenin were used as antigen, the antiserum to thrombosthenin precipitated two antigens, one of which appeared to be identical with human fibrinogen. The antithrombosthenin in the unadsorbed state thus appeared to have antifibrinogen activity; however in no case did antifibrinogen react with purified preparations of thrombosthenin. Antisera to striated and smooth muscle actomyosin of human origin failed to react with thrombosthenin. Similarly, antithrombosthenin failed to react with a preparation of striated muscle actomyosin.

A preparation of washed human platelets was exposed to fluoresceinated antithrombosthenin that had been previously adsorbed with human fibrinogen and normal human plasma. A diffuse, "speckled" type of staining was observed (Figure 3). In contrast, erythrocytes and buffy coat preparations were not stained with the antiserum. Intense fluorescence was noted in megakaryocytes in two normal bone marrow preparations.

Clot retraction experiments. The effect of thrombosthenin antiserum on clot retraction of recalcified platelet-rich plasma was examined. A typical experiment is shown in Figure 4, in which it is seen that antisera to human platelets and thrombosthenin completely inhibited clot retraction. This phenomenon was abolished by prior adsorption of the antisera with thrombosthenin. If the antisera were adsorbed with normal human plasma or purified human fibrinogen, clot retraction inhibition was still observed. No inhibition of clot retraction was observed when anti-albumin, anti–gamma globulin, anti–whole serum, or antifibrinogen were used. This series of experiments was repeated on four occasions with two separate batches of antisera. Identical results were obtained. Finally, antisera to actomyosin derived from smooth and striated muscle were completely ineffective as inhibitors of clot retraction. In order to determine whether plasma proteins in this system competed with platelets for antiserum binding, thereby masking the potential inhibitory effect of antiserum, we repeated these experiments in a purified fibrinogen (plasma-free) system. No inhibition of clot retraction was observed when anti–gamma globulin or anti-albumin were added to the fibrinogen-platelet mixtures. Antisera which did not inhibit clot retraction remained

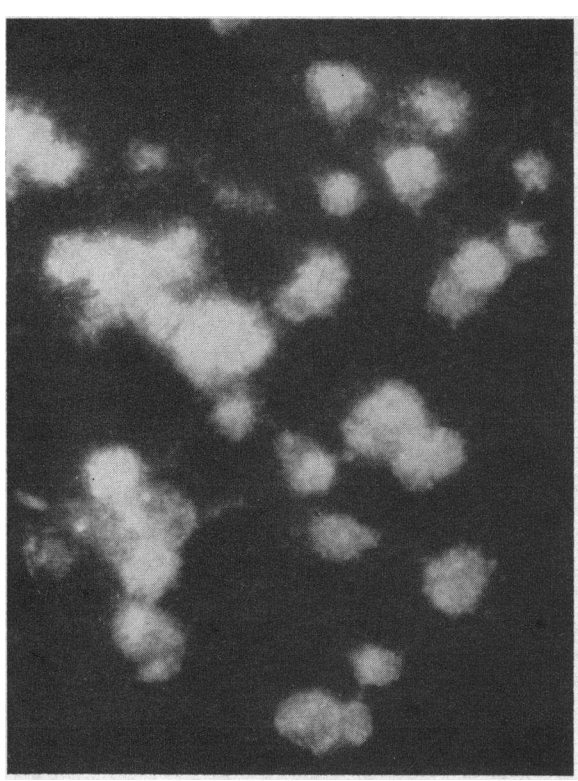

FIG. 3. FLUORESCEIN STAINING OF WASHED HUMAN PLATELETS WITH FLUORESCEINATED ANTITHROMBOSTHENIN.

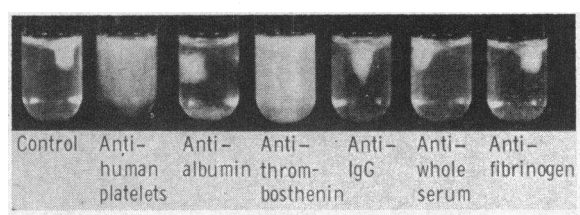

FIG. 4. CLOT RETRACTION INHIBITION STUDIES USING CITRATED PLATLET-RICH PLASMA AND VARIOUS ANTISERA. Calcium was added after 5 min incubation at 37° C.

ineffective after dilution with buffered saline in ranges from 1 : 4 to 1 : 64.

Structural studies. A chromatographically purified thrombosthenin extract was subjected to starch gel electrophoresis in urea glycine. Two major bands and three to four less discrete minor components were observed. Peptide map "fingerprints" of pronase-digested thrombosthenin revealed only 12–13 clear spots. When an extract of striated muscle actomyosin was similarly treated, over 40 different spots were observed. A peptide map of trypsin-digested thrombosthenin showed only 8 spots.

Thrombosthenin associated with subcellular compartments

The distribution of thrombosthenin among the three main subcellular platelet fractions was as follows. The contractile protein was found in the membranes and granules, but none was present in the soluble (cell sap) compartment. Evidence for these conclusions is presented in the following series of experiments.

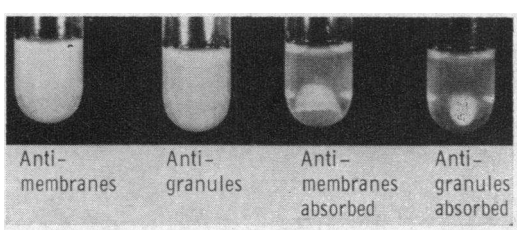

FIG. 5. CLOT RETRACTION INHIBITION USING CITRATED PLATLET-RICH PLASMA WITH ANTISERA. The adsorbed antisera were exposed to 5 mg chromatographed thrombosthenin for 1 hr at 37° C.

Clot retraction studies. Antiserum to platelet membranes and granules completely inhibited clot retraction of recalcified platelet-rich plasma. If the antiserum was adsorbed with a thrombosthenin extract, this property was abolished. Adsorption with lyophilized human plasma or purified human fibrinogen did not remove this activity. A typical experiment is shown in Figure 5. Identical results were obtained in three separate experiments. In contrast, antiserum to the soluble fraction present at the top of the sucrose density gradient after ultracentrifugation had no effect on clot retraction.

TABLE III

ATPase activity of platelet subcellular fractions and thrombosthenin extracted from the granules and membranes

Preparation	No. of experiments	μg P/mg protein per 30 min
Granules	3	1.03 ± 0.14
Membranes	3	1.16 ± 0.14
Soluble layer	3	1.34 ± 0.42
Granule thrombosthenin	2	2.78 ± 0.51
Membrane thrombosthenin	2	4.57 ± 0.91

Thrombosthenin extraction from subcellular fractions. It was possible to extract thrombosthenin from isolated platelet granules and membranes, as described in the methodology section. An extensive effort was made to extract thrombosthenin from the soluble (cell sap) fraction derived from six ultracentrifugal runs. The pooled fractions were concentrated to a small volume in a rotary evaporator and ultrafiltered. They were then dialyzed against Tris buffer to remove the sucrose, and finally against KCl–Tris buffer. The addition of $MgSO_4$ and subsequent adjustment to appropriate pH left no precipitate after standing overnight at 4° C and centrifugation (12,000 *g* for 15 min). It was concluded that thrombosthenin was not present in the soluble subcellular platelet compartment.

ATPase activity of subcellular platelet particles. In these experiments, the granules, membranes, and soluble layer were tested first. This was followed by studies on thrombosthenin isolated from the granules and membranes. ATPase activity was present in all three subcellular compartments and in the thrombosthein extracted from the gran-

TABLE IV

Effect of inhibitory conditions on ATP hydrolysis by platelet fractions and extracted thrombosthenin

Preparation	Per cent of control values		
	Mersalyl (2.5×10^{-4} M)	Ouabain (1×10^{-3} M)*	No Mg^{++}
Granules	48	73	40
Membranes	45	87	61
Soluble	100	100	100
Granule thrombosthenin	53	97	—
Membrane thrombosthenin	57	100	—

* 1×10^{-5} M ouabain was used for the thrombosthenin experiments.

ules and membranes (Table III). Attempts were then made to distinguish this ATPase activity from that of the sodium-dependent, potassium-stimulated "pump" ATPase. Ouabain produced partial inhibition of the ATPase activity in the granule and membrane fractions, suggesting the presence of "pump" ATPase activity in these particles. However, ouabain had no inhibitory effect on the ATPase activity of the thrombosthenin extracted from the subcellular particles. Mersalyl inhibited the ATPase activity of the subcellular particles and the thrombosthenin extracted from them. None of the inhibitory conditions influenced the ATPase activity of the soluble compartment. The results are summarized in Table IV.

Superprecipitation and viscosity. The experiments were performed with thrombosthenin extracted from isolated membranes and granules. Solutions of membrane and granule thrombosthenin showed clear-cut superprecipitation properties in the presence of ATP in two separate batches of extracted material.

Soluble thrombosthenin was extracted from platelet membranes in two separate experiments. The relative viscosity of these preparations was 1.6 and 1.7, respectively. In the presence of ATP (10^{-3} M) there was a moderate fall in relative viscosity (Table V). In 30 min the relative viscosity returned to the approximate starting values. ATP sensitivity of the membrane thrombosthenin solutions was 48 and 45% respectively. Soluble thrombosthenin extracted from platelet granules showed similar but less striking changes in viscosity following the addition of ATP.

Immunologic studies. Antithrombosthenin, adsorbed with lyophilized human plasma and human fibrinogen, reacted with thrombosthenin preparations from granules, membranes, and whole platelets (Figure 6). The reaction against the soluble subcellular compartment was negligible.

TABLE V

Viscosity changes of thrombosthenin prepared from platelet subcellular compartments

Preparation	Experiment	η_{rel}	η_{rel} ATP	η_{rel} 30 min after ATP	ATP sensitivity
Membranes	1	1.6	1.4	1.54	48
	2	1.7	1.44	1.6	45
Granules	1	1.3	1.19	1.28	50
	2	1.3	1.16	1.25	76

Membrane protein concentrations were 0.45%. Granule protein concentrations were 0.3%. 10^{-3} M ATP (2% by volume) was added. Each experiment was performed in triplicate.

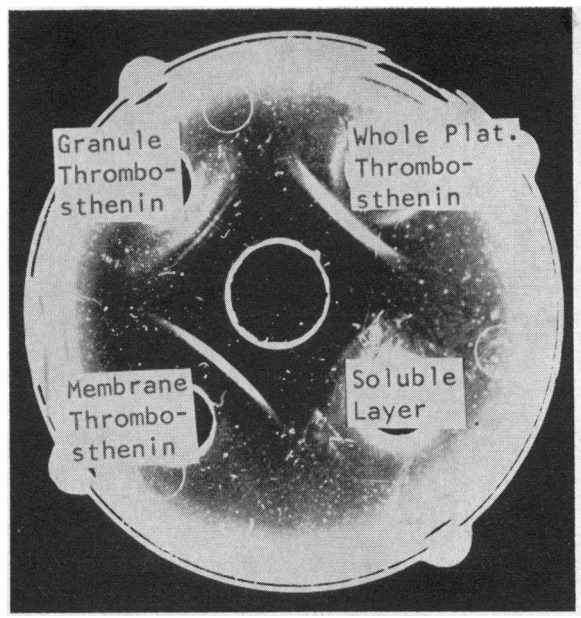

FIG. 6. IMMUNODIFFUSION ANALYSIS IN 0.6 M KCl AGAR OF THROMBOSTHENIN PREPARATIONS EXTRACTED FROM WHOLE PLATELETS, GRANULES, AND MEMBRANES. Soluble platelet protein derived from the top layer of the subcellular gradient was dialyzed into 0.6 M KCl and ultrafiltered. The protein concentrations in the antigens were equal. The middle well contained antithrombosthenin adsorbed with fibrinogen and plasma.

Discussion

There is little doubt that proteins associated with the plasma membrane of the platelet, as well as those located intracellularly, are of great functional significance. In these studies, the contractile protein thrombosthenin was first isolated from whole platelets and subsequently from platelet granules and membranes. A variety of experiments on its *in vitro* properties were then carried out.

The results corroborate previous reports that thrombosthenin has physicochemical properties similar to those of contractile protein of muscle tissue. The protein was soluble only at relatively high ionic strengths. In the presence of ATP it showed the phenomenon of superprecipitation and underwent reversible alterations in viscosity. The

ATP sensitivity of whole platelet thrombosthenin preparations was similar to that reported for actomyosin derived from smooth muscle. In addition, purified thrombosthenin showed magnesium-dependent ATPase activity which was slightly inhibited by ouabain and almost totally inhibited by mersalyl, a mercurial compound which is a specific inhibitor of contractile proteins of the actomyosin group (5). The finding that ouabain exerted a mild inhibitory effect on the ATPase activity of whole platelet thrombosthenin may have reflected contamination of the extracted protein with other ATPase moieties or could have been due to the relatively high concentration of ouabain used in the experiments (10^{-3} M). Ouabain at a concentration which completely inhibited "pump" ATPase activity of the subcellular fractions (10^{-5} M) had no inhibitory effect on thrombosthenin from these particles. This suggested that the Na-dependent, K-stimulated ATPase activity of platelets was distinct from that associated with thrombosthenin. These experiments were carried out on one batch of extracted subcellular thrombosthenin since qualitative rather than precise quantitative effects were sought, as a means of distinguishing between the types of ATPase activity present. Experiments on the "pump" ATPase of platelets will be reported elsewhere. The physical properties of platelet thrombosthenin closely resembled those of contractile protein extracted from rat sarcoma cells (5).

The immunologic studies indicated that thrombosthenin could not be detected in normal human plasma or serum and was a protein apparently unique to platelets. Furthermore, there was no cross-reactivity with actomyosin derived from human smooth and striated muscle. In general, the thrombosthenin extracts which were not further purified chromatographically contained varying amounts of platelet fibrinogen and possibly other contaminants. Therefore, the thrombosthenin antisera were adsorbed with fibrinogen and plasma before use. When washed normal human platelets were exposed to previously adsorbed fluoresceinated antithrombosthenin, a diffuse "speckled" type of staining was observed. There did not appear to be a preferential binding of the fluorescein label to the plasma membrane. Similar intense staining was noted in the megakaryocytes of normal bone marrow. The fluoresceinated antiserum did not stain normal leukocytes or erythrocytes, suggesting that it was specific for platelets.

Anti–whole platelet and antithrombosthenin sera completely inhibited clot retraction of recalcified platelet-rich plasma. This inhibitory activity could be completely abolished by adsorption of the antisera with a thrombosthenin extract. Other antisera, particularly antifibrinogen, did not significantly affect the ability of platelets to support clot retraction. Prior adsorption of the thrombosthenin antiserum with either fibrinogen or human plasma did not remove its inhibitory properties. The fact that anti–whole platelet sera inhibited clot retraction, which could be abolished by prior adsorption with extracted thrombosthenin, suggested that this property of anti–whole platelet sera was probably related to a population of thrombosthenin antibodies.

Antisera to the soluble subcellular "compartment" of platelets had no effect on clot retraction. This was taken as further evidence of the specificity of the clot retraction inhibition reaction. The soluble (cell sap) subcellular component did not contain thrombosthenin, but was previously found to contain practically the entire complex of soluble platelet protein (21). It is possible that the antigen-antibody complex involving thrombosthenin in or on the platelet membrane caused nonspecific damage, thereby destroying other undefined components of the clot retraction system. However, if this were true, one might expect a deleterious effect on clot retraction from other nonspecific immune reactions. No such effects were observed in experiments with anti-albumin, anti-IgG, anti–whole serum and antifibrinogen. Anti-IgG and anti-albumin were also ineffective in a plasma-free system, which is significant since albumin and gamma globulin are identifiable in platelets even after extensive washing (22). Therefore, not all antigen-antibody reactions involving platelet components impair clot retraction. This favors the specificity of the thrombosthenin-antithrombosthenin reaction. Perhaps immune drug reactions which affect clot retraction may be deleterious to the steric and functional integrity of thrombosthenin.

Since disrupted platelets do not support clot retraction, it would appear that thrombosthenin must retain a specific steric relationship in the cell in order to participate in the retraction process.

Platelet fibrinogen is another necessary component for clot retraction (23) and a recent report by Morse, Jackson, and Conley indicated that enzymatic splitting of fibrinopeptide B from fibrinogen by thrombin is a necessary prerequisite for clot retraction (24). Clot retraction probably represents the culmination of a complex series of physiological events, requiring at least a source of energy in an intact platelet, proteolytic digestion of platelet fibrinogen, and the presence of the contractile protein thrombosthenin. The capacity of thrombosthenin to hydrolyze ATP may be an important part of its function in mediating clot retraction. The observation that antithrombosthenin significantly blocks the ATPase activity of thrombosthenin suggests that an enzymatic site(s) of the molecule may be necessary for its activity. However, the antiserum probably also contains antibodies to nonenzymatic portions of the molecule. Chambers, Salzman, and Neri have proposed that the ATPase activity of thrombosthenin on the outer platelet membrane may be related to the action of ADP in aggregating platelets (25).

Our structural studies on purified thrombosthenin suggest that the molecule may be composed of subunit polypeptide chains, possibly of polymeric nature as illustrated by multiple bands in starch urea gel electrophoresis. The molecular weight was greater than 300,000, as evidenced by exclusion of the active material by a P-300 Bio-Gel column. It was of interest that only eight peptides were identifiable after tryptic digestion of the purified protein. Pronase digestion revealed 12–13 spots on fingerprinting, which was in marked contrast to over 40 spots when striated muscle actomyosin was similarly treated. The small number of peptides in the presence of a large molecular weight suggests the presence of repeating units.

Studies on the subcellular platelet fractions indicated that thrombosthenin was associated with platelet granules and membranes. Anti-granule and anti-membrane sera, previously adsorbed with fibrinogen and human plasma, inhibited clot retraction. Furthermore, it was possible to directly extract KCl-soluble, magnesium-precipitable protein from isolated granules and membranes. This material showed potent ATPase activity partially inhibited by mersalyl and not inhibited by ouabain. In similar fashion, the isolated granules and membranes themselves actively hydrolyzed ATP. That these fractions contained other ATPase activities such as the cation "pump" was evident by partial inhibition of enzyme activity by ouabain. The specific activity of the extracted subcellular thrombosthenin was greater than that associated with the whole particle fractions (Table III). It appeared that thrombosthenin derived from the platelet membrane was more active than that extracted from the granules. These materials reacted strongly with the adsorbed antithrombosthenin serum. The granule thrombosthenin showed two to three precipitin lines with the antiserum, while whole platelet and membrane thrombosthenin showed single precipitin arcs in agar. Both the granule and membrane thrombosthenin underwent superprecipitation in the presence of ATP and ATP caused reversible changes in their relative viscosity (Table V). However, these changes were less marked than those observed with whole platelet thrombosthenin, possibly due to the intricate series of manipulations involved in processing the material as well as to its inherent lability (2). In general, the properties of thrombosthenin extracted from the membranes more closely resembled those of whole platelet thrombosthenin than did the granule preparation. The reasons for these differences are not clear, but it is possible that hydrolytic enzymes known to be present in platelet granules (18) may have altered some of the properties of thrombosthenin during the separation procedure. Finally, the granule thrombosthenin may have contained more contaminants than the membrane thrombosthenin.

The specificity of the subcellular distribution of thrombosthenin may still require further examination. For example, thrombosthenin made available after platelet homogenization might denature, precipitate, and then nonspecifically sediment in the sucrose gradient (22). However, the absence of extractable thrombosthenin in the soluble (cell sap) layer of the sucrose gradient and its relatively intact physicochemical and immunological character in the subcellular fractions suggest that the protein may be a normal constituent of platelet membranes. The significance of the differences between granule and membrane thrombosthenin noted in the viscosity and immunochemical studies remains to be determined. It is of interest that a contractile protein has recently been isolated from the membrane of liver cells (26).

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