Detergent-Resistant Cytoskeleton of the Surface-Activated Platelet Differs From the Suspension-Activated Platelet Cytoskeleton

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This study contrasts the protein composition of the detergent-resistant cytoskeleton of platelets fully spread on glass with the cytoskeletal composition of resting platelets and platelets aggregated in suspension with thrombin. Complete Triton X-100-insoluble cytoskeletons were isolated from spread, resting, and suspension-activated platelets in the presence of protease inhibitors, solubilized in sodium dodecyl sulfate/EDTA and analyzed on reduced, one-dimensional polyacrylamide gels. The protein composition of the cytoskeletons differed both qualitatively and quantitatively. Most notable were more extensive incorporation of total protein, talin, and vinculin into the cytoskeleton of spread platelets than the cytoskeleton of suspension-activated platelets. Varying the concentration and time of exposure to thrombin during suspension activation did not mimic the cytoskeletal changes of surface activation. Scanning electron microscopy, measurement of lipid phosphorus content, and varying the duration of Triton extraction did not show incomplete solubilization or nonspecific trapping of constituents in the spread platelet cytoskeleton. Proteolysis of talin was minimal in suspension-activated platelets and in platelets spread for 50 minutes. The differences in the detergent-resistant cytoskeletons of surface- and suspension-activated platelets indicate significant divergence in the physiologies of platelet spreading on surfaces and platelet activation in suspension.

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Materials and Methods

Platelet Preparation

Blood was obtained from healthy adult human donors following procedures in accordance with the University of Minnesota Committee on the Use of Human Subjects in Research. Blood was collected by venipuncture into citrate-citric acid dextrose (CCD) anticoagulant (93 mmol/L sodium citrate, 70 mmol/L citric acid, and 140 mmol/L dextrose) pH 6.5, in a ratio of nine parts blood to one part anticoagulant. Platelet-rich plasma (PRP) was separated from whole blood by centrifugation at 100g for 15 minutes at room temperature. PRP was diluted 1:1 with CCD, and platelets were separated by centrifugation at 220g for 20 minutes at room temperature. The supernatant was removed and the platelet pellet resuspended in nine parts Tris Balanced Salt Solution to one part CCD supplemented with adenosine 10 mmol/L, theophylline 6 mmol/L, pH 7.0. The resuspended platelets were centrifuged at 220g for 15 minutes and the wash step was repeated. The platelets were finally suspended in Tyrode's buffer (138 mmol/L NaCl, 2.9 mmol/L KCl, 12 mmol/L NaHCO3, 0.36 mmol/L Na2PO4, 5.5 mmol/L glucose, 1.8 mmol/L CaCl2, 0.4 mmol/L MgCl2, pH 7.4) and incubated for at least 30 minutes at 37°C before activation studies.

Suspension and surface platelet activation. Platelets were activated in suspension with thrombin (Parke Davis, Elkgrove, IL). Washed platelets were adjusted to a cell concentration of 1 x 10^9 cells/mL, and aggregated by exposure to thrombin (0.2, 1.0 U/mL) for 30 seconds and 20 minutes at room temperature in a stirred lumaggregometer. Aggregation was confirmed by change in light transmission and secretion by the luciferase reaction.

Platelets were surface-activated on 10-cm diameter Pyrex culture dishes (Corning Glass, Corning, NY). A dilute suspension of platelets (1 x 10^6 cells/mL) was interacted with the plates for 20 to 50 minutes at room temperature and nonadherent platelets poured off. The plates were washed twice with Tyrode's buffer, and the surfaces were examined by video light microscopy to assure completeness of platelet spreading and to count the number of adherent platelets. The surfaces were visualized with a Diaphot inverted microscope equipped with Hoffman differential interference optics and 40× and 100× achromatic objectives (Nikon, Garden City, NY). Microscope images were projected onto a video camera using a 2× video relay lens and images optimized using a sync stripper (Model 302-2, Colorado Video, Boulder) and video processor (Model 604, Colorado Video). Images were directed to a computer with an image processor in which images were digitized and analyzed using Java Image Analysis Software (Jandel Scientific, Corte Madera, CA).

Detergent-resistant cytoskeletons. Resting and activated platelets were extracted in 1% Triton X-100 (LKB Instruments, Gaithersburg, MD). In Tris-Cl 50 mmol/L, pH 7.4, containing protease inhibitors (ethylene glycol bis[B-aminoethyl ether]-N,N,N',N'-tetraacetic acid (EGTA) 5 mmol/L, leupeptin 2 x 10^-5 mol/L, benzamidine 50 mmol/L, and phenylmethylsulfonyl fluoride 1 mmol/L). Scanning electron microscopy, measurement of lipid phosphorus content, and varying the duration of Triton extraction did not show incomplete solubilization or nonspecific trapping of constituents in the spread platelet cytoskeleton. Proteolysis of talin was minimal in suspension-activated platelets and in platelets spread for 50 minutes. The differences in the detergent-resistant cytoskeletons of surface- and suspension-activated platelets indicate significant divergence in the physiologies of platelet spreading on surfaces and platelet activation in suspension.

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mmol/L) for 20 minutes at room temperature. Because of concern over previous reports of incomplete solubilization of platelet membranes in thrombin aggregates, thrombin-aggregated platelets were gently disrupted 10 minutes into the Triton extraction by drawing the suspension through a pasteur pipette twice. Surface-activated platelets were solubilized for 1, 10, or 30 minutes at room temperature.

Triton-insoluble protein (putative cytoskeletons) of surface-activated platelets remained attached to the glass surface. The Triton extraction buffer was decanted, surfaces washed once in Tris HCl 100 mmol/L containing protease inhibitors and the adherent material removed with 2% sodium dodecyl sulfate (SDS) containing 0.25 mmol/L EDTA. Protein was visualized by light microscopy on the Triton-extracted plates before SDS/EDTA solubilization after fixation in 1% glutaraldehyde in cacoodylate buffer and staining with Coomassie Brilliant Blue. No material was visualized after SDS/EDTA solubilization. Protein collected from the plates was concentrated fivefold with Cenricon-10 filters (Amicon, Beverly, MA), 10,000 molecular weight exclusion limit. Each experiment typically required four to six glass (10-cm diameter) plates to obtain sufficient protein for analysis.

Triton-insoluble material (cytoskeletons) of resting and thrombin-aggregated platelets were isolated from the Triton-extracted preparations by centrifugation at 100,000g for 3 hours at 4°C in a Beckman Ultracentrifuge Model L-50 (Beckman Instruments, Irvine, CA), using an SW40-TI rotor. The supernatant was decanted and the sedimented material solubilized in 2% SDS, 0.25 mmol/L EDTA. Resuspension of the thrombin-aggregated cytoskeleton required vigorous vortexing and brief sonication for 10 seconds.

Triton X-100 supernatants from Triton-extracted spread platelets were also subjected to ultracentrifugation at 100,000g for 3 hours to determine if any Triton-insoluble material remained in the supernatant. In other experiments, the total protein in the supernatants was precipitated in 5 volumes of cold acetone for 10 minutes at −20°C and collected by centrifugation at 13,000g for 10 minutes at 4°C. The acetone was poured off and the sample dried under nitrogen before solubilization in SDS/EDTA. The larger volumes of the Triton supernatants precluded concentration of the protein by filtration.

Lipid phosphorus. Lipid was extracted from washed platelets and Triton-insoluble material of activated platelets by modification of a method previously described. Washed platelets and thrombin-aggregated cytoskeletons were extracted twice in equal volumes of chloroform:methanol (2:1, vol/vol). Triton-insoluble surface-adherent material was extracted twice in a 10-fold excess of chloroform:methanol because of the volume requirements of the plates. The chloroform:methanol phases were collected after gravity sedimentation, pooled, placed in acid-washed tubes, and dried under nitrogen. The samples were digested in perchloric acid and phosphorus determined by the method of Chen et al.

The Triton-insoluble thrombin cytoskeletons isolated by ultracentrifugation were difficult to resuspend in aqueous solution. Experiments were performed that showed solubilization of platelets in 2% SDS, 0.25 mmol/L EDTA did not interfere with the measurement of lipid phosphorus in the samples. Washed platelets in Tyrode's buffer yielded virtually identical lipid phosphorus content to platelets solubilized in 2% SDS, 0.25 mmol/L EDTA (13.9 ± 2.1 versus 14.3 ± 1.9 μg phosphorus/10⁶ platelets). The lipid phosphorus content of thrombin-aggregated cytoskeletons in this report is based on SDS/EDTA-solubilized preparations.

Electron microscopy. Washed platelets were allowed to interact with disk-shaped coverslips for 20 minutes at room temperature in a moist chamber. Unattached platelets were removed by washing with Hanks' Balanced Salt Solution and the buffer was replaced by 1% glutaraldehyde in cacoodylate buffer containing 1% Triton X-100. Simultaneous fixation and detergent extraction were continued at room temperature for 30 minutes. After washing, the coverslips were critical point dried and prepared for study in the scanning electron microscope by shadowing with carbon and platinum. The coverslips were cemented to special stubs and examined in a Cambridge S4-10 scanning electron microscope.

Gel electrophoresis. The protein concentration of SDS/EDTA-solubilized proteins was determined by Micro-Lowry (Sigma, St Louis, MO) and samples were prepared for gel electrophoresis using the method described by Laemmli. Mercaptoethanol-reduced samples were separated on one-dimensional, 6% to 12% continuous gradient polyacrylamide gels without stacking gel using a Mini-Slab Gel Apparatus (Idea Scientific, Minneapolis, MN). The usual protein load was 5 μg protein per lane. Gels were electrophoresed by the method of Laemmli with a modification in the running buffer by addition of 0.1 mol/L sodium acetate. Proteins were stained with colloidal Coomassie G-250 and percentage of protein determined with a Shimadzu CS-9000 Scanning Densitometer (Kyoto, Japan).

Western blot procedure. Proteins were transferred to nitrocellulose sheets as described by Towbin et al using a Genie Transfer Apparatus (Idea Scientific) and 6-A battery charger. Nonspecific sites were blocked with Blotto (5% Carnation nonfat dry milk, 0.01% Sigma Antifoam A emulsion) for 1 to 24 hours. Identification and location of proteins in the gel were confirmed with the sequential use of a primary antibody, biotinylated secondary antibody, and streptavidin/peroxidase system. A polyclonal IgG rabbit antibody against human talin was provided by Dr Keith Burridge (Chapel Hill, NC). Biotinylated secondary antibodies and streptavidin/peroxidase were obtained from Amersharm (Arlington Heights, IL).

Data analysis. Statistical significance, means, and SD were derived using an unpaired t-test.

RESULTS

Washed platelets became tightly adherent and fully spread into "fried egg" forms after 20 minutes of contact with the glass surface (Fig 1). Washed platelets activated in suspension by thrombin (0.2 and 1.0 U/mL) for 30 seconds or 20 minutes underwent shape change, aggregation, and secretion in the lumiauggregometer before detergent extraction.

Scanning electron microscopy confirmed the persistence of platelet cytoskeletons after simultaneous fixation and detergent extraction of cells spread on glass (Fig 2). Membranes were completely removed by the procedure, leaving filamentous elements of the cytoplasm and the circumferential microtubule. Filaments often seemed to radiate from specialized, detergent-resistant structures resembling adhesion plaques.

The total amount of protein remaining adherent to the surface after platelet spreading and Triton extraction was significantly greater than the protein content of the thrombin-aggregated Triton-insoluble residue. Spreading for 20 minutes incorporated 41% ± 3% of total platelet protein into a Triton-insoluble structure, whereas thrombin, 0.2 U/mL, for 30 seconds only incorporated 31% ± 4% of total platelet protein (n = 5, P < .05). Both forms of activation resulted in greater protein content than Triton-insoluble residues sedimented under resting conditions (22% ± 3% of total platelet protein). High-speed centrifugation of the
supernatant from the Triton-extracted spread platelets did not yield enough protein for analysis, indicating little protein in the supernatant assembled into a cytoskeleton. The Triton-resistant material remaining adherent to the surface after platelet spreading contained the entire formed platelet cytoskeleton. Quantitation of the protein content of the Triton supernatant by acetone precipitation showed 63% (n = 2) of the total platelet protein in the supernatant. This is the amount of protein expected by subtraction from measurements of adherent protein, indicating removal of most of the adherent protein by SDS/EDTA treatment.

Coomassie-stained polyacrylamide gels of the detergent-resistant cytoskeletons of resting, thrombin-activated, and spread platelets showed qualitative and quantitative differences. Electrophoresis of the cytoskeletons applied at equal protein load showed several qualitative differences, most notably, a significant increase in the amount of talin and vinculin in the spread platelet cytoskeleton in comparison with the thrombin-activated cytoskeleton (Fig 3, left). The spread cytoskeletons contained somewhat less \( \alpha \) actinin and proteins migrating at positions A and B on the gel than the thrombin cytoskeletons, and contained a novel band between \( \alpha \) actinin and talin not present in whole platelets. Candidate proteins migrating at positions A and B on the gel would include the \( \beta \) and \( \gamma \) chains of fibrinogen and the two isoforms of tubulin.\(^9,10\) Identification and quantitation of these bands will require immunoblotting and immunoprecipitation studies and are not the subject of this report. Analysis of the detergent-resistant material isolated from an equal number of platelets more clearly illustrates the quantitative discrepancies between the cytoskeletons (Fig 3, right).

Laser densitometric analysis confirmed significant differences between the content of individual proteins comprising the detergent-resistant cytoskeletons of resting, thrombin, and spread platelets (Fig 4). Spreading tended to incorporate more of each cytoskeletal protein into a detergent-resistant structure than thrombin activation with the exception of \( \alpha \) actinin and the aforementioned proteins at positions A and B. The talin and vinculin contents of spread platelet cytoskeletons were significantly greater than found in thrombin-aggregated cytoskeletons.

Platelets were exposed to a higher concentration of thrombin and for a longer time interval to determine if the differences observed between suspension- and surface-activated platelets were caused by unusual features of thrombin dose response rather than the types of stimulus. The detergent-resistant cytoskeletons resulting from platelet stimulation with thrombin (0.2 and 1.0 U/mL) for 30 seconds or 20 minutes showed few qualitative differences...
when evaluated at constant protein load (Fig 5). No differences were found in the total amount of protein incorporated into the detergent-resistant cytoskeletons at the low and high doses of thrombin (data not shown). Exposure of platelets to either concentration of thrombin for 20 minutes resulted in a loss of one third of the total detergent-resistant protein assembled at the shorter time interval. The studies indicate the differences between

surface and suspension activation are not caused by peculiarities of the dose response of thrombin activation.

The lipid phosphorus contents of thrombin-aggregated and spread platelet cytoskeletons were measured to evaluate the completeness of Triton solubilization of platelet membranes, and address the question of nonspecific trapping of platelet components in the cytoskeletal preparations. Cytoskeletons of platelets spread for 20 minutes and extracted in Triton X-100 for only 1 minute contained only $6\pm 3\%$ of total platelet lipid phosphorus, yet $39\pm 4\%$ of total platelet protein ($n = 3$). Cytoskeletons from platelets activated with thrombin for 30 seconds and extracted in Triton for 20 minutes contained $13\pm 2\%$ of total platelet lipid phosphorus, and $31\pm 3\%$ of total platelet protein ($n = 3$). The results of the experiments suggest that the greater protein content of spread platelet cytoskeletons is unlikely to be caused by artifactual trapping of constituents.

Immunoblot with an antitalin antibody confirmed the P235 band in spread cytoskeletons as talin (Fig 6). The major proteolytic fragment of talin is a 200-Kd peptide retaining reactivity with the antitalin antibody used in this study. Talin proteolysis was minimal during platelet spreading monitored for up to 50 minutes when platelets were directly solubilized in SDS/EDTA (Fig 6, left). Talin
proteolysis was also minimal 30 seconds after thrombin-induced platelet aggregation (Fig 6, left).

Incorporation of talin into the spread cytoskeleton was not diminished by prolonged solubilization in Triton X-100. Talin remained associated with the spread cytoskeleton even after 30 minutes of exposure to Triton (Fig 6, right). Proteolysis was not as well controlled in the Triton-extracted cytoskeletons as in the whole platelets. Platelets spread for 20 minutes and then solubilized in Triton for 1, 10, or 30 minutes (Fig 6, right) underwent more proteolysis than platelets directly solubilized in SDS/EDTA after spreading for the same time periods of 20, 30, and 50 minutes (Fig 6, left). The studies indicate the talin incorporation of the spread platelet cytoskeleton is slightly underestimated by densitometric analysis.

**DISCUSSION**

The most notable differences between the detergent-resistant cytoskeletons of thrombin and surface-activated platelets were the greater protein content and significant incorporation of talin and vinculin in the cytoskeleton of the spread platelet in comparison to the cytoskeletons of resting or suspension-activated platelets. Varying the concentration of thrombin or time of exposure to thrombin did not support a simple restriction of all the platelet filaments and stress fibers to one or the other. One might speculate that the 40% of talin resistant to Triton extraction in spread platelets may be related to incorporation of the protein into the detergent-resistant adhesion plaque structures.

The increased protein, talin, and vinculin content of spread platelet cytoskeletons were not caused by artifacts of incomplete solubilization of platelet membranes and nonspecific trapping of cytoskeletal components. The issue of nonspecific trapping of protein has been raised in thrombin-aggregated cytoskeletal agglomerations but seems less likely in cytoskeletons prepared from a monolayer of surface-adherent cells. Examination of Triton-extracted spread platelet cytoskeletons by scanning electron microscopy showed complete removal of membranes allowing visualization of filamentous elements. Furthermore, spread platelet cytoskeletons contained less lipid phosphorus than suspension-activated cytoskeletons despite greater protein content.
Platelet spreading was not associated with significant proteolysis of talin. Talin, like glycoprotein Ib and actin-binding protein, is a sensitive substrate for calcium-activated neutral protease (calpain). Proteolysis of these sites of attachment of the membrane to the cytoskeleton were originally hypothesized as integral steps in platelet activation, although recent studies have cast doubt on the significance and extent of calpain-induced proteolysis in platelet activation and aggregation in suspension. Through our study and a recent report by Nachmias and Golla also indicate little role for proteolysis in platelet surface activation and spreading.

REFERENCES

34. Wencel-Drake JD, Okita JR, Amnis DS, Kunicki TJ: Activation of calpain I and hydrolysis of calpain substrates (actin-binding protein, glycoprotein Ib, and talin) are not a function of thrombin-


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