Coordinated Regulation of Platelet Actin Filament Barbed Ends by Gelsolin and Capping Protein

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Abstract. Exposure of cryptic actin filament fast growing ends (barbed ends) initiates actin polymerization in stimulated human and mouse platelets. Gelsolin amplifies platelet actin assembly by severing F-actin and increasing the number of barbed ends. Actin filaments in stimulated platelets from transgenic gelsolin-null mice elongate their actin without severing. F-actin barbed end capping activity persists in human platelet extracts, depleted of gelsolin, and the heterodimeric capping protein (CP) accounts for this residual activity. 35% of the ~5 μM CP is associated with the insoluble actin cytoskeleton of the resting platelet. Since resting platelets have an F-actin barbed end concentration of ~0.5 μM, sufficient CP is bound to cap these ends. CP is released from OG-permeabilized platelets by treatment with phosphatidylinositol 4,5-bisphosphate or through activation of the thrombin receptor. However, the fraction of CP bound to the actin cytoskeleton of thrombin-stimulated mouse and human platelets increases rapidly to ~60% within 30 s. In resting platelets from transgenic mice lacking gelsolin, which have 33% more F-actin than gelsolin-positive cells, there is a corresponding increase in the amount of CP associated with the resting cytoskeleton but no change with stimulation. These findings demonstrate an interaction between the two major F-actin barbed end capping proteins of the platelet: gelsolin-dependent severing produces barbed ends that are capped by CP. Phosphatidylinositol 4,5-bisphosphate release of gelsolin and CP from platelet cytoskeleton provides a mechanism for mediating barbed end exposure. After actin assembly, CP reassociates with the new actin cytoskeleton.

Actin assembly is required for the shape changes that occur when platelets are activated (9, 14) to perform essential hemostatic tasks. The cytoplasmic concentration of actin in platelets is ~500 μM, 40% of which is polymerized as filaments (F-actin) at rest (34). Exposure of platelets to agonists such as thrombin, initiates a signal transduction cascade that doubles the F-actin content (6). Actin assembly is preceded by a rapid severing of cytoplasmic actin filaments (17). Severing amplifies the number of barbed ends 10-fold in permeabilized cells (17). As a result, platelets transform from resting discoid forms to activated forms having long thin filopods containing bundles of F-actin and flat lamellae which contain an orthogonal array of short filaments (17).

Barbed end actin filament capping proteins regulate the burst of actin polymerization in platelets. Actin filament barbed ends in the resting platelet are inaccessible for actin polymerization, however, 1 min after thrombin receptor ligation up to 500 barbed ends support actin polymerization (19). Platelet shape change and actin assembly are blocked by addition of cytochalasins (9, 14, 17), specific inhibitors of monomer addition at the barbed end. Furthermore, the use of cytochalasin demonstrates that the burst of actin assembly in response to thrombin receptor ligation can be attributed solely to polymerization at the barbed end (17). Free barbed ends result from a signal transduction cascade from the thrombin receptor that includes activation of the small GTPase Rac which is upstream of polyphosphoinositide (ppI)[1]-dependent "uncapping" of barbed ends (19). This places the proteins that generate free barbed ends and regulate the availability of barbed ends for polymerization at a critical step in cytoskeletal assembly and reorganization in platelets.

We demonstrate here that platelets have capping protein (CP), the mammalian homologue of an *Acanthamoeba* protein (23) and nonmuscle homologue of chicken capZ (8), at a concentration that exceeds the number of barbed ends in the resting platelet by a factor of 10. CP represents the bulk of capping activity in cell extracts depleted of gelsolin by affinity chromatography. CP is found in both F-actin associated and soluble compartments, and redistributes upon platelet activation from the soluble

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1. *Abbreviations used in this paper*: CP, capping protein; OG, n-octyl β-D glucopyranoside; ppI, polyphosphoinositide; PRP, platelet-rich plasma; TRAF, thrombin receptor activating fragment.

compartment to the F-actin associated compartment. The translocation of CP to the actin cytoskeleton depends on gelsolin. However, CP in permeabilized human platelets, that respond to the thrombin receptor activating fragment (TRAF) by exposing barbed filament ends (19), is released upon treatment with TRAF. Release of gelsolin and CP from permeabilized platelets is also achieved by addition of phosphatidylinositol (4,5)-bisphosphate ($PI_{4,5}P_2$). The results suggest in platelets gelsolin and capping protein together control actin filament barbed end exposure.

Materials and Methods

Reagents

All reagents are purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. All antibodies were produced in the laboratory of John A. Cooper (Department of Cell Biology and Physiology, Washington University Medical School, St. Louis, MO).

Isolation of Platelets and Platelet Fractions

Platelets are isolated from blood by differential centrifugation and chromatography as previously described (17). Briefly, blood is drawn into1/10th volume Aster-Jandl anticoagulant and platelet-rich plasma (PRP) is obtained by centrifugation at 110 *g* for 10 min. Platelets are separated from the serum by chromatography on a Sepharose 2B column equilibrated and run in 145 mM NaCl, 10 mM Hepes, 10 mM glucose, 0.2 mM Na_2HPO_4, 5 mM KCl, pH 7.4 (platelet buffer) with or without 0.3% BSA. Platelets are then allowed to rest for 30 min in a 37°C water bath before use.

For large scale protein preparations, platelets are isolated from blood bank platelet packs that are no more than 5-d old. The platelets are sedimented by centrifugation at 800 *g* for 15 min and the cell pellet resuspended in 1/8th of the original volume in platelet buffer. The platelets are again pelleted by centrifugation at 800 *g* for 15 min and resuspended in the same volume of platelet buffer containing in addition 10 mM EGTA, protease inhibitors (42 nM leupeptin, 10 mM benzamidine, and 0.123 mM aprotinin), and 1% n-octyl β-D-glucopyranoside (OG). The cytoskeletal and soluble fraction are segregated by ultracentrifugation at 100,000 *g* for 1 h, all fractions are frozen in liquid nitrogen and stored at −80°C.

Activation of Platelets

Resting platelets are activated with thrombin, 1 U/ml, or the TRAF, 25 μM. At the desired times, the platelets are permeabilized by adding 1/10 vol of 10× PHEM (0.6 M Pipes, 0.25 M Hepes, 0.1 M EGTA, 20 mM $MgCl_2$, pH 6.9), 1% Triton X-100, protease inhibitors (as above), 10 μM phallacidin and 20 U/ml Hirudin (thrombin-treated cells only). Cytoskeletal and soluble fractions are separated as above. Samples for SDS-PAGE are solubilized with 60 μM Tris-HCl, 2% sodium dodecyl sulfate, 10% glycerol, 10 μM DTT (SDS sample buffer) overnight at 37°C.

In these experiments, 1 μM phallacidin is used to prevent F-actin depolymerization after cell permeabilization, to avoid release of capping protein due to actin filament depolymerization. The depolymerization of F-actin upon platelet extraction, and its prevention by addition of phallacidin, have been reported previously (17). Since phallatoxins might also induce actin polymerization, we used FACS® analysis with 1 μM fluorescein tagged phalloidin, in place of phallacidin, to confirm that the concentration of phallatoxin used in the studies presented here has no stimulatory effect on F-actin content for a period of up to at least 30 min after platelet extraction.

Fractionation of Soluble Proteins from Resting Platelets

The supernatant obtained from OG permeabilized platelets is depleted of gelsolin by using affinity chromatography on an anti-gelsolin monoclonal IgG (C4)-CL-4B sepharose resin. The nonadherent material is collected and reapplied to ensure removal of gelsolin. Gelsolin removal is confirmed by SDS-PAGE and immunoblotting. The nonadherent fraction is diluted with platelet buffer without NaCl or BSA to give a final NaCl concentration of 50 mM, and applied to a Pharmacia Mono Q column equilibrated in platelet buffer with 50 mM NaCl and no BSA. Adherent protein is eluted in the same buffer using a linear 50 to 500 mM NaCl gradient. Fractions having capping activity are pooled and concentrated by force filtration in a Centricon 10 filter (Amicon Corp., Beverly, MA), and applied to a Superose 12 column equilibrated in 10 mM Tris, 100 mM KCl, 2 mM $MgCl_2$, pH 7.4 (buffer B).

Analysis of $PI_{4,5}P_2$ effect on actin-associated proteins uses column purified human platelets at 2×10^8/ml extracted for 30 s in 0.4% OG with 1/10 volume 10× PHEM (here the concentration of $MgCl_2$ is 2 mM instead of 20 mM for all other experiments), 0.1 μM phallacidin and protease inhibitors as above and centrifuged for 1 min at 10,000 *g*. A portion of the supernatant is retained for immunoblotting. From this point the sample is kept on ice when possible. The pellet is washed twice by resuspending in 1× PHEM with 0.1 μM phallacidin and protease inhibitors and centrifuged again. Samples of the supernatants are recovered with each wash for immunoblotting. After the final wash the pellet is resuspended in 1× PHEM with 0.1 μM phallacidin and protease inhibitors in 1/4 the volume prior to extraction. To this is added either buffer (control) or $PI_{4,5}P_2$ (final concentration = 100 μM) and incubated for 2 min and then centrifuged for 1 min at 10,000 *g*. The supernatant is recovered and the pellet is resuspended in an equivalent volume.

Viscometric and Fluorometric Assays for Actin Filament Barbed End Capping Activity

Viscosity Assay. The effect of gelsolin-depleted platelet supernatants on actin's viscosity is determined in a falling ball apparatus. Samples for viscosity assays are diluted into buffer B containing 0.5 mM ATP, 0.5 mM 2-mercaptoethanol, and 1 μM G-actin. The sample is drawn into a 100 μl capillary micropipet, allowed to stand at room temperature for 1 h, and then the time for a stainless steel ball bearing (outer diam 0.7 mm; The Microball Co., Peterborough, NH) to fall a fixed distance through the solution is recorded. Fall times are converted to centipoises (cp) using glycerol as a standard.

Depolymerization Assay. Pyrene-labeled F-actin, at a concentration of 10 μM, is diluted to 80 nM in buffer B (270 μl) and the column fraction being tested (30-μl each). The rate of fluorescence decrease is monitored in a Perkin-Elmer model LS-50 fluorimeter using 366 and 386 nm as the excitation and emission wavelengths, respectively. The fluorescence data is normalized so that time 0 = 100. Since there are sometimes artificial changes in fluorescence in the first 40 s of the assay (see Fig. 4 *a*), the slope of the fluorescence decay is calculated by linear regression of the data points from 40–180 s.

Electron Microscopy

Immunogold labeling for CP is done as described previously (17). Platelets, isolated by column chromatography, are centrifuged onto poly-L-lysine coated 5 mm coverslips in a 96-well microtiter plate at 1,450 *g* for 5 min. Adherent cells are mechanically unroofed (18) by compressing and removing a second poly-L-lysine coated coverslip onto the platelet coated coverslip in 1× PHEM with 5 μM phallacidin and protease inhibitors. This procedure exposes the underlying cytoskeleton and its attachments to the cytoplasmic surface of the plasma membrane. CP in these specimens is visualized by immunogold labeling with 8 nM particles. Alternatively, cytoskeletons of coverslip adherent platelets are prepared by permeabilizing the cells in PHEM buffer containing 0.75% Triton X-100, 2 μM phallacidin, and protease inhibitors. Rabbit anti–chicken capZ β subunit, affinity purified using a fusion protein containing an NH_2-terminal fragment that is identical in both β1 and β2 subunits, at a concentration of 10 μg/ml is applied in 1× PHEM, pH 7.8, containing 0.1 μM phallacidin, and protease inhibitors for 1 h, and then washed three times in the same buffer without antibody. Colloidal gold, 8 nm, coated with goat anti–rabbit IgG is applied to the sample in 1× PHEM with 1 μM phallacidin and protease inhibitors for 1 h. Unbound gold is removed by washing three times in 1× PHEM with 1 μM phallacidin and protease inhibitors. Samples are fixed with 1% glutaraldehyde with 0.1 μM phallacidin, washed into distilled water, rapidly frozen, freeze-dried, and coated with 1.4 nm of tungsten–tantalum with rotation and 2.5 nm of carbon without rotation.

Polyacrylamide Gel Electrophoresis and Immunoblot Analysis

SDS-PAGE is performed in a modified Laemmli system (28). Samples are run on either 12% acrylamide or linear gradient gels of 5–15%. Gels are stained in 0.2% Coomassie brilliant blue R250 in 20% methanol and 5%

acetic acid. Immunoblots are obtained by electrophoretic transfer of protein from polyacrylamide gels using a tank transfer apparatus in 25 mM Tris, 195 mM glycine, and 20% methanol onto Immobilon P membrane (PVDF, 0.45 μm; Millipore Corp., Bedford, MA). Primary and secondary antibodies are incubated with the membrane in PBS containing 0.05% Tween-20 and 5% Carnation nonfat dry milk. Chemiluminescence detection is done using Renaissance (DuPont/NEN, Boston, MA) as described by the manufacturer. Densitometric and quantitative analysis of immunoblots used a Molecular Dynamics densitometer. Two-dimensional gel electrophoresis is done as previously described (38). The concentration of CP in platelets is determined on quantitative immunoblots using a monoclonal antibody specific for the chicken CP β2 subunit. This antibody is used since platelets have only the β2 isoform. The region of the β2 isoform recognized by the antibody is highly conserved (38) and contains the actin binding site (22). The protein assay is standardized using a CP fusion protein including glutathione S transferase and the 27 carboxy-terminal amino acids of chicken CP β2 subunit. The immunoblot shows a linear response from 0.5 to 10 ng of fusion protein standard and to increasing amounts of platelet lysate (Fig. 1 *b*).

Antibodies used are as follows: Fig. 1 *a*, goat anti–chicken CP polyclonal previously described (39). Fig. 1 *b*, mouse anti-β2 subunit monoclonal antibody affinity purified using the antigen a fusion protein containing the 27 COOH-terminal amino acids of chicken capping protein β2 subunit. Fig. 1 *c*, rabbit anti–β1 and –β2 subunit polyclonal antibody and anti-α monoclonal antibody, which recognizes all known α isoforms, affinity purified.

Results

Human and Mouse Platelets Contain a Homologue of Chicken CP

Immunoblot analysis is used to analyze platelet samples for heterodimeric CP. A polyclonal antibody that recognizes both the α (36 kD) and β (32 kD) subunits of chicken skeletal muscle CP (Fig. 1 *a*, lane *6*) reacts strongly with a polypeptide with the same electrophoretic mobility as the chicken CP α subunit and weakly with a band corresponding to chicken CP β subunit in lysates of whole human platelets (Fig. 1 *a*, lane *1*).

Immunoblots of two-dimensional gel electrophoresis demonstrate that platelets have predominantly the β2 isoform of CP. Using antibodies that distinguish β2 from β1 isoform we estimate that at least 95% of the β isoform is β2 (data not shown). The β1 isoform, predominant in muscle, runs at a more acidic pI than the α isoforms whereas the β2 isoform runs with a more basic pI than the α subunits (38). An additional, minor, α subunit is sometimes seen with a more acidic pI. Whether this is an α1 or α2 isoform remains to be determined. An additional β2 isoform, β2′, is also seen in platelets and has been reported in chicken brain (38) where it is suggested that this isoform may arise by posttranslational modification. Cytoskeletal and soluble fractions from resting platelets show identical isoform composition (Fig. 1 *c*).

The concentration of capping protein in human platelets is 5.6 μM based on quantitative analysis of one-dimensional immunoblots (Fig. 1 *b*). Since the actin concentration in platelets is ~0.5 mM (34), the ratio of capping protein to total actin is ~1:90.

Removal of Gelsolin from Soluble Platelet Extracts Reveals CP-related Actin Filament Capping Activity

Previous studies demonstrated that gelsolin is a major barbed end capping protein in platelets (17, 26, 27, 30, 31). Therefore, to determine the amount of capping activity attributable to CP, a partial purification and characterization of capping protein activity in the 1% OG lysate soluble protein phase of human platelets first depleted of gelsolin by affinity adsorption was pursued. The polypeptide composition of unbound platelet proteins after passage through the gelsolin affinity matrix is unchanged (Fig. 2, compare lanes *2* and *4*) except for the removal of the gelsolin polypeptide (band of 88 kD, Fig. 2). This procedure removes most of the gelsolin from the platelet extract and reapplication of the unbound fraction to the anti-gelsolin IgG affinity column removes all gelsolin based on Coomassie blue staining and detection by immunoblot (Fig. 2). We also validated that gelsolin is removed in pyrene–actin assembly and disassembly assays. After removal of gelsolin from the platelet supernatant, calcium-dependent nucleation, severing or capping activity is no longer detected (data not shown). Removal of gelsolin did, however, facilitate the detection of calcium-independent barbed end capping activities.

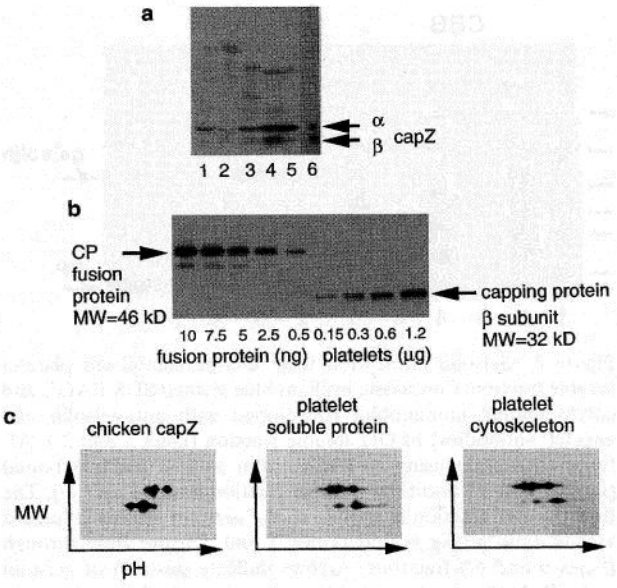


Figure 1. Human platelets contain actin capping protein, a homologue of chicken muscle capZ. (*a*) Western blot of platelet fractions using a polyclonal antibody raised against the α and β subunits of chicken muscle capZ. Lane *1*, whole platelets; lane *2*, 40% ammonium sulfate pellet of platelet soluble fraction after octylglucoside extraction; lane *3*, 55% ammonium sulfate pellet of 40% ammonium sulfate supernatant; lane *4*, 70% ammonium sulfate pellet of 55% ammonium sulfate supernatant; lane *5*, pooled fractions 24–26 from Superose 12 column; lane *6*, chicken capZ standard. Arrows indicate α and β subunits of capZ. (*b*) Quantitation of capping protein in platelets. Western blot with serial dilutions of CP fusion protein (*left*) and serial dilutions of platelet total protein (*right*). The primary antibody used is a monoclonal antibody that is specific of the β2 subunit of capping protein. Densitometric analysis of CP in platelets and fusion protein detected by western blot are linear over the concentration used. (*c*) Two-dimensional gel electrophoresis of purified chicken capZ, for reference, platelet soluble proteins and cytoskeletal fractions after permeabilization with Triton X-100 and ultracentrifugation. The primary antibodies used recognize all α and β isoforms.

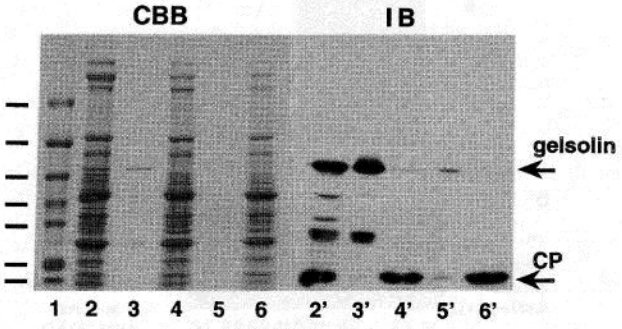


Figure 2. Gelsolin adsorption from OG-permeabilized platelet soluble fraction. Coomassie brilliant blue stained SDS-PAGE and corresponding immunoblot (developed with anti-gelsolin and anti-CP antibodies) of OG soluble fraction (lanes *2* and *2′*). Affinity adsorption using an anti-gelsolin column yields a bound (lanes *3* and *3′*) and flow through fraction (lanes *4* and *4′*). The flow through fraction is applied to the gelsolin affinity column a second time giving bound (lanes *5* and *5′*) and flow through (lanes *6* and *6′*) fractions. Arrows indicate position of gelsolin and CP. Molecular weight standards are marked and shown in lane *1*. From top: 195, 112, 84, 63, 35, and 32.5 kD.

Characterization of calcium-insensitive capping activity in the gelsolin-depleted extract is accomplished by sequential chromatographic separations. The gelsolin depleted supernatant is first applied to an anion exchange column and bound protein eluted using a linear NaCl gradient. The unbound fraction from this column did not alter the viscosity of F-actin. Viscometric analysis of adherent, salt eluted fractions demonstrates calcium-independent activities that reduce viscosity of F-actin solutions. This activity is broadly distributed over the column although the eluted fractions vary in their polypeptide composition (data not shown). However, fractions eluting between 320 and 360 mM NaCl consistently decrease the viscosity of F-actin. Fractions with this activity are complex having >20 Coomassie blue stained bands ranging from 20–30 kD up to the prominent high molecular weight polypeptides of the platelet: actin binding protein-280, spectrin, talin and myosin.

Fractions eluted from the ion exchange column with high activity are pooled and further fractionated by gel filtration chromatography. This procedure segregates the high molecular weight proteins from smaller proteins (20–70-kD range) and only the latter (fractions 24–26), lower the viscosity of F-actin solutions (Fig. 3 *a*). Fractions with activity are composed of six to eight prominent polypeptides, with a major polypeptide of 55 kD and two minor polypeptides of 30–36 kD which are the CP subunits (detected by immunoblot Fig. 1 *a*, lane *5*). Based on relative molecular weight standards, CP elutes from this gel filtration column as a globular protein in the 60–80-kD range. Fractions 24–26 consistently lower the viscosity of actin in the absence or presence of calcium ion (Fig. 3 *a*).

Eluted fractions which diminish the viscosity of actin gels also slow the rate of actin filament depolymerization when pyrene-labeled F-actin is diluted below its critical concentration. In the absence of added material, dilution of F-actin to this concentration results in depolymeriza-

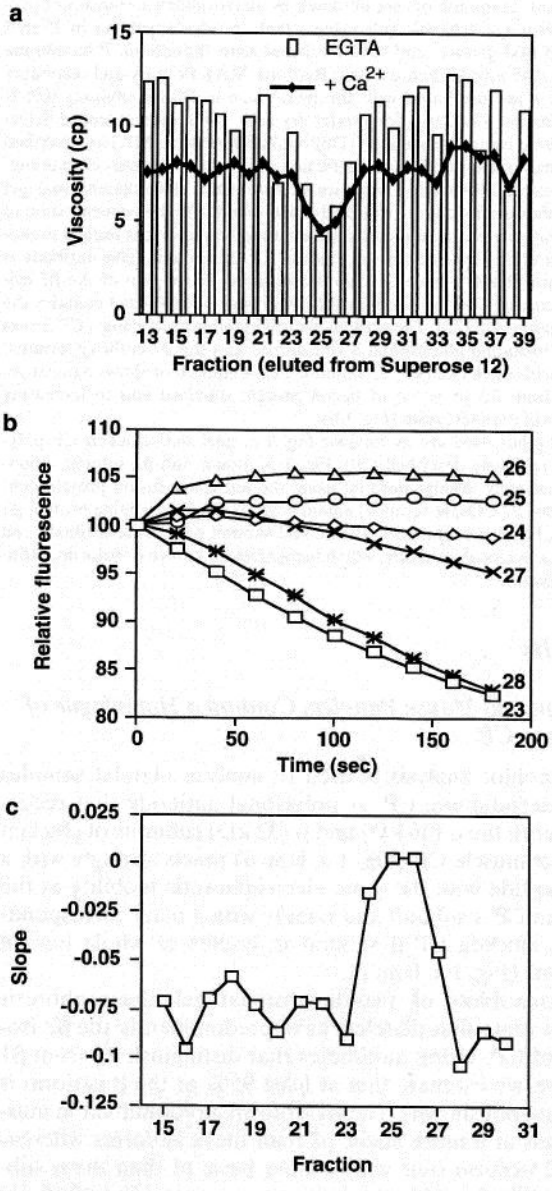


Figure 3. Activity in the partially purified sample, obtained after gel filtration, is a calcium-independent actin filament barbed end capping protein. (*a*) Low shear falling ball assay of the entire included volume from the Superose 12 column. The assay is done either in 1 mM EGTA, open bars or in the presence of 0.2 mM $CaCl_2$, line graph. The reduction in viscosity of all actin samples in Ca^{2+} may be due to Ca^{2+} effects on actin polymerization. (*b*) Analysis of pyrene-labeled F-actin depolymerization in the presence of fractions eluted from the Superose 12 column. The fluorescence measurements at time 0 are normalized to equal 100. Measurements are shown over the first 180 s immediately following dilution of F-actin into buffer containing 30 μl of fraction 23, 24, 25, 26, 27, or 28. (*c*) Linear regression analysis from 40 to 180 s after start of depolymerization assay is used to determine the change in fluorescence over time (*slope*) for fractions 15–30. Under these conditions depolymerization should occur at both ends of the actin filament, with the barbed end depolymerizing 10 times faster than the pointed end.

tion, as measured by the decreasing fluorescence of pyrene–actin. Fractions 24–26 slow the rate of F-actin depolymerization, with fractions 25 and 26 inhibiting depolymerization under these conditions. The fluorescence measurements taken at 20-s intervals over the first 2 min following dilution of the F-actin in the presence of fractions 23–28 is shown in Fig. 3 *b*. Since there are some abnormalities in the fluorescence readings in the first 40 s, linear regression analysis to determine the slope of the fluorescence decay employed data from 40–180 s (Fig. 3 *c*). The slope of the fluorescence decay is directly proportional to the rate of actin depolymerization. The same observations are obtained when depolymerization is monitored in the presence of calcium ion (data not shown). These results suggest these fractions contain calcium-insensitive actin filament capping proteins.

All fractions with calcium-insensitive barbed end capping activity contain platelet capping protein detected in immunoblots with chicken CP antibody. Fig. 1 shows that the fractionation scheme used above enriches for human CP (Fig. 1 *a*, lane *5*). An alternative method to enrich for CP uses the gelsolin-depleted platelet extract which is treated by a series of ammonium sulfate precipitations, conditions which mimic the purification of capZ from chicken skeletal muscle (7), (Fig. 1 *a*, lanes *2–4*). Using this method, the CP is found in the 50 and 70% ammonium sulfate pellets. This distribution is the same as reported for chicken skeletal muscle capZ.

Platelet Activation Increases the Fraction of Capping Protein Bound to the Cytoskeleton

The platelet actin filament–based cytoskeleton can be separated from soluble cytoplasmic components by ultracentrifugation after detergent permeabilization of the cells (13). A Coomassie-stained gel and corresponding immunoblot, for one experiment, show the cytoskeletal pellets and soluble fractions from resting and thrombin activated platelets (Fig. 4 *a*). The relative amount of CP associated with the resting and activated cytoskeleton can be determined by densitometric quantitation of immunoblots. The resting human platelet has 35.2 ± 4% (from five experiments) of its total CP associated with cytoskeletal actin filaments (Fig. 4 *b*). When platelets are stimulated with thrombin, CP is translocated into the cytoskeleton. Fig. 4 *b* shows that by 20 s after the addition of thrombin the amount of cytoskeletal CP increases by 1.7 times to 59.0 ± 7.4% (from five experiments). Maximal incorporation of CP into the cytoskeleton, therefore, occurs at the time of actin filament assembly and maximal exposure of barbed ends (19). Uncapping of CP-capped actin filament barbed ends is not detected during platelet activation, only association of CP is observed suggesting that barbed ends formed by some other mechanism are capped by CP to slow actin polymerization.

Capping Protein Is Associated with Actin Filament Ends in Platelets

Anti-CP immunogold bound to the actin cytoskeleton demonstrates the localization of CP in glass activated platelets (Fig. 5). The distribution of 8 nm anti-CP immunogold at the margin of a platelet cytoskeleton prepared

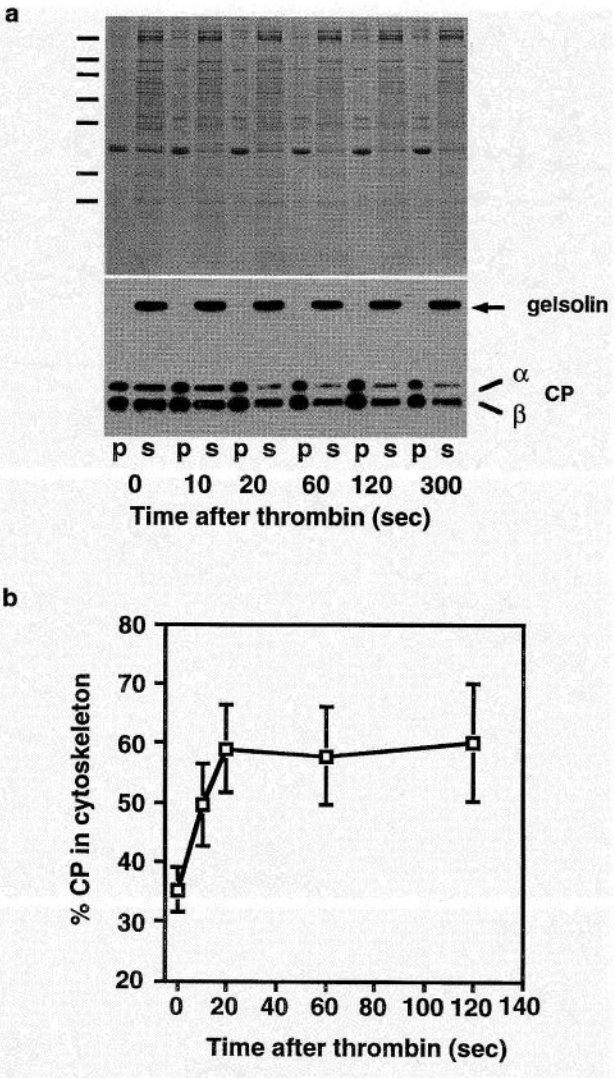
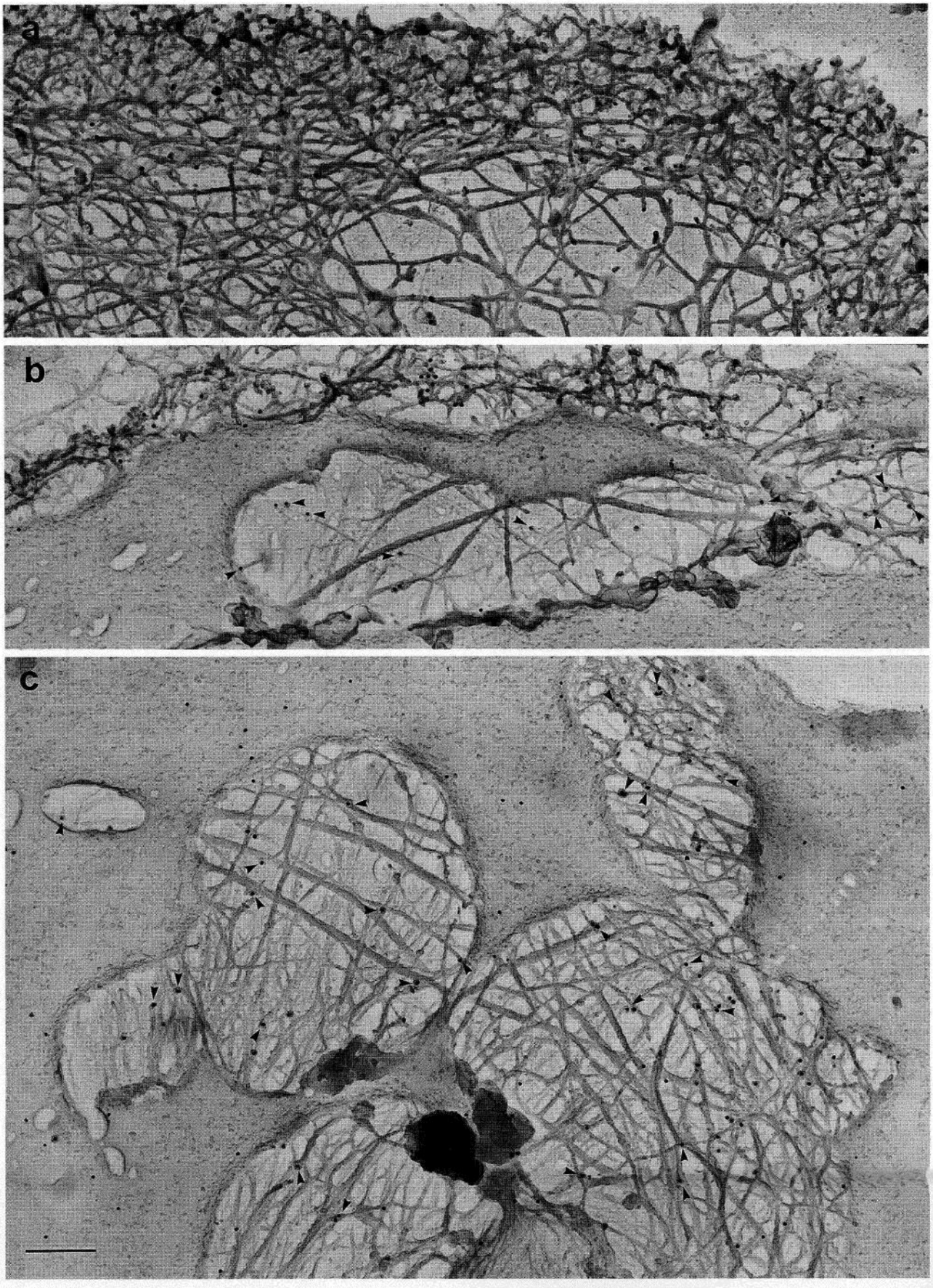


Figure 4. Analysis of capping protein in resting and activated platelet soluble and cytoskeletal fractions. (*a*) Coomassie blue–stained gel (5–20% acrylamide, *top*) and immunoblot analysis (15% acrylamide, using antibodies that recognize gelsolin, the α subunit and the β subunit of CP) bottom. 100,000 *g* pellets (*p*) and supernatants (*s*) are shown for resting platelets (time 0) and platelets activated for the indicated times with 1 U/ml thrombin. Marks to right of Coomassie-stained gel are molecular weight standards from top: 195, 112, 84, 63, 52.5, 35, and 32.5 kD. (*b*) The capping protein β subunit, using the monoclonal antibody 3F2, is quantitated using densitometric analysis in both soluble and cytoskeletal fractions and the percentage of cytoskeletal calculated in resting and activated platelet fractions. Error bars are standard deviation based on results from five experiments.

using Triton X-100 is displayed in Fig. 5 *a*. Fig. 5, *b* and *c*, demonstrate the distribution of anti-CP immunogold in unroofed platelets. Fig. 5 *b* shows the edge, while Fig. 5 *c* shows the center, of representative unroofed platelets. Gold particles are most abundant in the orthogonal networks of short filaments at the edge of the cytoskeleton. The density of filaments in this region makes it difficult to determine if the immunogold is on filament ends. How-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ever, immunogold found in the interior of cytoskeletons or unroofed cells is bound selectively at filament ends (Fig. 5, *arrowheads*). Gold is not associated with microtubules and is rarely found on the cytoplasmic side of the plasma membrane in the absence of an associated actin filament. Controls using only secondary gold-conjugated antibody show no background labeling (data not shown).

Mouse Platelets That Lack Gelsolin Have Increased Amounts of Capping Protein Bound to the Cytoskeleton of Resting Cells

The increase in binding of CP to the activated platelet cytoskeleton suggests that as the F-actin content of activated cells increases more binding occurs. To investigate this further, we analyzed CP binding to the cytoskeleton in platelets isolated from transgenic mice lacking gelsolin. Relative to normal mouse and human platelets, resting platelets from these animals have more of their actin incorporated in filaments. Specifically, gelsolin containing platelets have 40% of their actin polymerized into filaments compared to 53%, a 33% increase, in gelsolin null platelets. The transgenic, gelsolin null platelets, also respond to agonist by exposing 75% fewer barbed ends (43).

The amount of capping protein bound to cytoskeletons of normal resting mouse platelets, 33 ± 7% (from 6 experiments), is similar to that found in human platelets (compare Fig. 4 and Fig. 6). Normal mouse platelets, as in human platelets, also have elevated capping protein, maximally ~50%, in the cytoskeletal fraction after thrombin stimulation (Fig. 6). Fig. 6 shows that mouse platelets from transgenic animals lacking gelsolin have significantly higher ($P < 0.002$) resting levels of capping protein bound to their cytoskeleton (Fig. 6). The amount of CP bound, 49 ± 3.9% (from 6 experiments), is nearly that seen maximally in activated human or normal mouse platelets. However, gelsolin null platelets fail to change their capping protein content in the cytoskeletal fraction when stimulated with thrombin (Fig. 6), despite their ability to generate barbed ends, albeit fewer compared to normal platelets, and to polymerize actin (43).

pH Influences the Association of Gelsolin and CP with the Cytoskeleton of Resting and Activated Platelets

The pH of the extraction buffer changes the solubility of both gelsolin and CP, indicating that proper buffer during extraction is of critical importance (Fig 7). Resting platelets extracted in PHEM buffer at pH 6.0 have a surprisingly high amount of cytoskeletal-associated gelsolin, and a corresponding reduction in the amount of CP, associated with the cytoskeleton. Platelets activated with 1 U/ml thrombin for 60 s and then permeabilized at pH 6.0 show a reduction of gelsolin and an increase in the amount of CP associated with the cytoskeleton, although the amount of

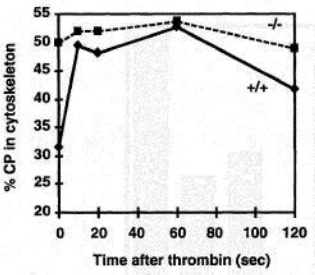

Figure 6. CP association with the cytoskeleton in platelets from normal mice and from transgenic mice lacking gelsolin. Effect of thrombin (1 U/ml) activation on capping protein cytoskeletal association in normal (+/+) and gelsolin deficient (−/−) platelets.

CP bound is less than that found in cells permeabilized at neutral pH. Gelsolin is not found in the cytoskeleton of unstimulated cells nor in cells 1 min after thrombin activation when the cells are extracted at pH 6.9. Small amounts of gelsolin (<10%) are found in the cytoskeleton transiently between 10 s and 1 min after thrombin activation when extracted at neutral pH (data not shown).

$PI_{4,5}P_2$ Treatment Releases Gelsolin and CP But Not ABP-280 from OG-extracted Platelets

Previous studies implicate $PI_{4,5}P_2$ in the regulation of actin assembly by inhibiting gelsolin's (24) and CP's (21) association with actin filament barbed ends. We investigated the effect of $PI_{4,5}P_2$ on the association of endogenous gelsolin, CP and ABP-280, an actin filament cross-linking protein, with the cytoskeleton of resting OG-permeabilized platelets. Treatment of platelets with 0.4% OG permeabilizes them, leaving 100–200 nm holes while maintaining the signal transduction cascade from thrombin receptor to actin filament barbed ends (19). Resting platelets extracted in 0.4% OG release a portion of ABP-280, gelsolin and CP into the soluble fraction (Fig. 8 *a*, lane *1*), all three of which are cleared from the soluble fraction with subsequent washings (Fig. 8 *a*, lanes *2* and *3*). After washing, the cytoskeletal pellet contains 11% of the CP, 7% of the gelsolin, and 70% of the ABP in the starting material. Addition of 100 μM $PI_{4,5}P_2$ micelles to the washed pellet effects the release of both gelsolin and CP to the soluble fraction (Fig 8 *a*, lane *6*) but not ABP-280 (Fig. 8 *a*, lane *6*) compared to buffer treated control (Fig. 8 *a*, lane 5). Although gelsolin and CP are always released by $PI_{4,5}P_2$ treatment the amount released is variable. This variability is likely a result of differences in the degree of OG permeabilization and the micellisation of $PI_{4,5}P_2$. The percent gelsolin and CP released by 100 μM $PI_{4,5}P_2$ from OG permeabilized and washed platelets are 8.6 ± 10% ranging from 1 to 25% and 8.3 ± 6.2% ranging from 2.5 to 19%, respectively (based on results from seven experiments, average ± SD). Phosphatidylserine added to washed, OG extracted platelets caused no detectable enhancement of gelsolin or capping protein release compared to buffer controls (data not shown). The CP released by $PI_{4,5}P_2$ re-

Figure 5. Ultrastructural localization of capping protein in activated platelets. (*a*) Triton X-100 permeabilized platelet cytoskeleton showing both the dense cortical network, upper region of panel, and the underlying cytoskeleton, lower region. Gold particles are black dots being most abundant in the cortical cytoskeleton. (*b*) Unroofed platelet where the membrane at the edge of the cell has been removed. Gold particles are labeled with arrowheads. (*c*) Unroofed platelet with the interior cytoskeleton exposed. Labeling is sparse compared to the dense cortical network where the density of actin filament ends is greatest. Since the network here is more open the specificity for actin filaments and ends of filaments can be seen. Gold particles are labeled with arrowheads. Bar, 200 nm.

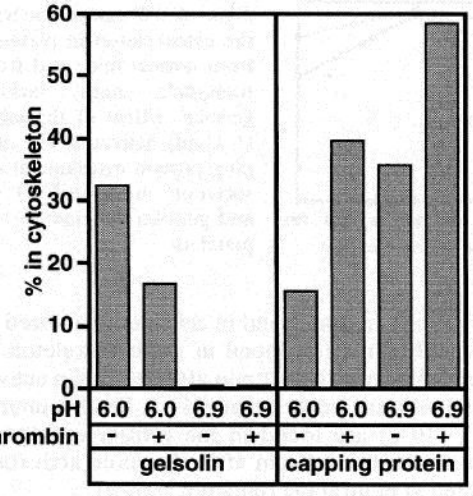

Figure 7. Gelsolin and CP association with the platelet cytoskeleton are sensitive to pH. Lowering the pH of extraction increases gelsolin association and depresses CP association with the cytoskeleton. The percentage CP and gelsolin associated with the cytoskeleton of resting or thrombin activated platelets extracted at pH 6.0 or 6.9 solutions are determined by densitometric analysis of western blots using the monoclonal antibody specific for the CP β subunit, 3F2.

mains soluble following ultracentrifugation (Fig. 8 *b*, compare lanes *1–3* and *1′–3′*) suggesting that released CP is not bound to F-actin. After ultracentrifugation most CP remains in the soluble fraction compared to control where the bulk of CP is found in the high speed pellet. Densitometric analysis of the immunoblot shown in Fig. 8 *b* indicates that in the buffer control only 13% of the CP β subunit is recovered in the high speed supernatant, whereas, in samples treated with 25 μM $PI_{(4,5)}P_2$ and 50 μM $PI_{(4,5)}P_2$, 59 and 83% are recovered, respectively.

CP Is Released from OG-permeabilized Platelets upon Thrombin Receptor Activation

Since movement of CP to the soluble fraction could not be observed in intact activated platelets, we reasoned uncapping to be masked as soluble CP concurrently capped newly accessible barbed ends. Addition of the TRAF to permeabilized platelets triggers a release of CP to the soluble fraction (Fig 8 *b*, lane *4*). Of the CP released, 60% is soluble following ultracentrifugation (Fig 8 *b*, lane *4′*). Fig. 8 *d* shows soluble fractions following ultracentrifugation of samples treated with buffer, increasing amounts of $PI_{4,5}P_2$, phosphatidylserine, or TRAF. TRAF and $PI_{4,5}P_2$ cause a dose dependent release of CP into the high speed supernatant but not phosphatidylserine. Quantitative analysis of Fig. 8 *c* is shown in Fig. 8 *d*.

Discussion

Greater than 98% of the actin filament barbed ends in resting platelets are capped (17). After treatment with agonists such as thrombin (17, 19), cold (42), or phorbol 12-myristate 13-acetate (PMA) (Hartwig, J.H., et al., manuscript submitted for publication) 20–25% of the 2,000 actin

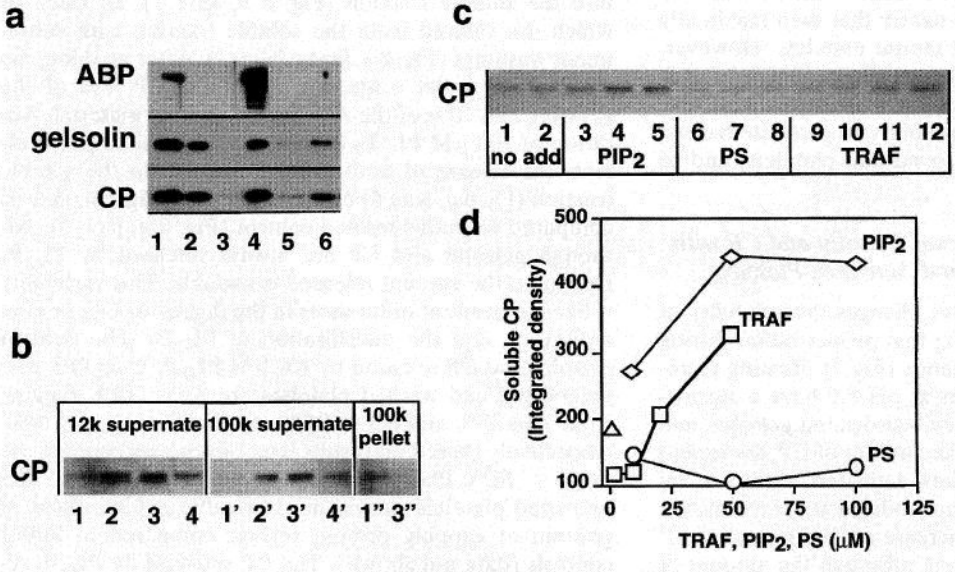

Figure 8. $PI_{4,5}P_2$ releases gelsolin and CP but not ABP-280 while activation of the thrombin receptor releases CP from OG-extracted and washed platelets. (*a*) Immunoblot analysis of platelet fractions after extraction with OG developed using anti-ABP-280 (*ABP*), anti-gelsolin, and anti-CP IgG's. Protein remaining soluble (12,000 *g* supernatant) following OG permeabilization is shown in lane *1*. Soluble material remaining following two successive buffer washes of the OG insoluble pellet are shown in lanes *2* and *3*. Lanes *4* and *5* are the pellet and supernatant fractions of subsequent to buffer treated, washed platelets (*control*), and lane *6* is the supernatant of OG-permeabilized washed platelets treated with 100 μM $PI_{4,5}P_2$. Based on sample volume of starting material the ratio of sample loaded in (*a*) are, 1 (lanes *1–3*):4 (lane *4*):20 (lane *5* and *6*). (*b*) Immunoblot of CP in low speed supernatants (12k *g* for 1 min, lanes *1–4*), high speed supernatants (100k *g* for 60 min, lanes *1′–4′*), and high speed pellets (lanes *1″* and *3″*) of OG-permeabilized washed platelets treated with $PI(_{4,5})P_2$ or TRAF. Lanes *1*, *1′*, and *1″* are buffer treated controls. Lanes *2* and *2′* are treated with 25 μM $PI(_{4,5})P_2$; lanes *3*, *3′*, and *3″* are treated with 50 μM $PI(_{4,5})P_2$; lanes *4* and *4′* are treated with 25 μM TRAF. All lanes are loaded equivalently based on volume of starting material. (*c*) Immunoblot of CP in high speed supernatants (100k *g* for 60 min) from OG-permeabilized platelets. Lanes *1* and *2* are buffer treated controls; lanes *3–5* are treated with 10, 50, and 100 μM $PI(_{4,5})P_2$, respectively; lanes *6–8* are treated with 10, 50, and 100 μM phosphatidylserine, respectively; lanes *9–12* are treated with 2, 10, 20, and 50 μM TRAF, respectively. (*d*) Quantitative analysis of the CP bands shown in *c*. Triangle indicates CP in buffer control.

filament barbed ends become accessible for monomer addition and net polymerization of actin (17, 19). Barbed end capping proteins, therefore, both stabilize the resting cell by preventing filament elongation and disassembly, and in response to agonist, allow net assembly by dissociating from these ends. Although dissociation of barbed end capping proteins best explains the results, the de novo formation of actin nuclei (32) may also account for a small portion of the newly formed barbed ends in agonist activated platelets and other cells (reviewed in 3, 4).

We have found that human and mouse platelets contain abundant amounts (5.6 μM, a concentration equivalent to that of gelsolin) of heterodimeric actin filament capping protein. This actin filament barbed end capping protein is homologous to the capping protein first described in *Acanthamoeba* (23) and extensively studied in chicken muscle and yeast (1, 2, 8, 10). In muscle, CP is found primarily at the Z lines (8) where it is thought to cap and help anchor actin filaments (37). Genetically altered yeast lacking CP have aberrant actin filament organization (1). In *Dictyostelium*, capping protein participates in barbed end exposure when cells are treated with the chemoattractant, cAMP (12, 12a, 15, 16, 20, 36). Antibodies specific for chicken CP crossreactive to bands at 32 and 36 kD, which comigrate with chicken CP subunits, in platelets (Fig. 1 *a*). Two-dimensional gel electrophoresis demonstrates that an additional α subunit may exist, the β subunit is predominantly the β2 isoform, and the subunit composition is identical in both cytoskeletal and soluble fractions.

Finding CP in human and mouse platelets is not surprising, since human and murine cDNA and gene characterization have been previously reported (5, 38). However, defining its role in actin polymerization is important for understanding proteins that influence this essential event. Therefore, to assess CP's contribution to the total capping activity in platelets, we characterized the residual capping activity contained in platelet lysates after removal of endogenous gelsolin by affinity adsorption. Fractionation of barbed end capping activity in gelsolin-depleted platelet extracts by sequential ion exchange and gel filtration chromatography revealed calcium-independent actin filament capping activity eluting only with fractions containing CP as determined by viscometry, F-actin depolymerization assays and immunoblotting. CP is an abundant capping protein in neutrophils (11) where it represents the bulk of capping activity in cell lysates after immunoadsorption of gelsolin, consistent with our findings in platelets.

Resting platelets have 0.2 mM of their actin assembled into F-actin. Since each filament contains ~400 monomers on average (17), the barbed end concentration is ~0.5 μM. More than sufficient CP (~35% of total CP, or 2.0 μM) is associated with the resting cytoskeleton, enough to cap all its barbed actin filament ends. Why there is more CP associated with the cytoskeleton than barbed ends is not clear. Potential reasons for this discrepancy are: (*a*) our calculations may overestimate the amount of CP. The CP concentration in platelets is reported by Nachmias (33) to be 2.2 μM, the discrepancy with our results may be due to the use of different antibodies; (*b*) The calculation of actin filament number may be an underestimation; and (*c*) CP may associate with other components of the cytoskeleton in addition to barbed ends. However, even with these uncertainties, it is clear that >95% of actin filament barbed ends in resting platelets are capped and CP is the major barbed end capping protein associated with both the resting and activated cytoskeleton. Immunolocalization demonstrates CP is associated with the actin cytoskeleton. Although high efficiency immunogold labeling is not achieved, electron microscopy demonstrates capping protein to be specifically associated with cortical actin where filament ends are most abundant in activated platelets and, when ends could be identified, at only one end of filaments.

After activation of normal human or mouse cells with thrombin, a portion of the previously soluble CP translocates into the cytoskeleton. However, in mouse platelets lacking gelsolin, activation neither increases nor decreases CP association with the actin cytoskeleton, although more capping protein is associated with the actin filament rich cytoskeleton of these resting cells. Either the filament ends in gelsolin-negative platelets become free by dissociation of unknown capping proteins, de novo nucleation or no net change in CP–cytoskeletal interaction is observed because uncapping and reassociation of CP with barbed ends occur rapidly. The increased proportion of CP associated with the resting cytoskeleton of gelsolin-deficient cells is best explained by the fact that these cells have 33% more polymerized actin than normal gelsolin containing platelets (43). Although we do not know if this increase in F-actin results in longer filaments or more filaments, the increased binding of CP suggests the latter. The failure to bind more CP to the actin cytoskeleton when gelsolin null cells activate suggests that severing by gelsolin creates most of the ends to which CP binds. How the gelsolin null mice are able to expose barbed ends and polymerize actin without detectable release of CP is not yet clear.

The lack of CP release from cytoskeletons of thrombin-activated platelets was unexpected. However, this finding does not exclude CP dissociation as a mechanism to generate free barbed ends for actin monomer addition. There are several possibilities why we fail to measure release of CP in these cells but observe CP uncapping in OG-permeabilized cells. First, platelet gelsolin is activated within seconds of thrombin treatment. We observed, consistent with previously published results (31), a rapidly reversible association of gelsolin with the cytoskeleton, and that only a fraction (5–10%) of the total gelsolin is associated with the activated cytoskeleton at any time. Since the gelsolin concentration is ~5 μM, and the bulk of gelsolin is inactive in the resting cell, the activation of gelsolin severing activity can increase the filament number by 10-fold from 2,000 filaments in resting platelets to as many as 20,000 following platelet activation by thrombin. However, biochemical assays detect ~500 new barbed ends at any given time up to 1 min after thrombin. Therefore, binding of CP to gelsolin generated barbed ends is likely to far exceed the number of ends exposed by CP-dependent uncapping. Second, dissociation and reassociation of CP to actin filament ends may be so rapid that we cannot detect dissociation, particularly since CP's affinity for barbed ends is 0.1–0.6 nM. If actin polymerization is driven in platelets by as little as a 10 μM monomer supply, and then filaments 0.5 μm in length, as in the activated cell, would require only seconds to form (35).

The OG permeabilization of platelets leaves behind plasma membrane and the thrombin signaling system re-

mains responsive to TRAF (19), a receptor activating peptide. TRAF treatment of OG-permeabilized cells exposes actin filament barbed ends and releases CP into the soluble fraction, showing release of CP to cause barbed end exposure. We further exploited the OG-permeabilized platelet system to identify the capping protein(s) targeted by $PI_{4,5}P_2$. A portion of the gelsolin and CP remains associated with the extracted platelets and can be released by $PI_{4,5}P_2$. The amount of gelsolin remaining in the OG-extracted platelets is surprisingly high compared to that seen after Triton X-100 suggesting that a portion of the gelsolin seen in the OG-permeabilized platelets associates with membrane rather than barbed ends. This does not seem to be the case for CP since its recovery in OG-permeabilized, washed platelets is 73% less than after Triton X-100. This could result from the dissociation of CP from filament ends or loss of CP attached to the barbed ends of small actin filament during the low speed centrifugation following OG permeabilization and buffer washes. Another possibility is that ppI metabolism remains partially active after OG permeabilization causing release of some CP. The release of only ~10% of the CP present in the washed platelets with $PI_{44,5}P_2$ is probably due to steric hindrances influencing $PI_{4,5}P_2$ micelle access to CP capped barbed ends. Similarly, the small amount of CP released in these OG-permeabilized cells upon TRAF treatment is likely to result from loss of signal transducing elements due to the extensive washing of the OG-permeabilized cells.

CP has been identified recently in platelets and its interaction with cytoskeletal actin characterized (33). Our findings contrast strikingly with the results of Nachmias et al. (33) where they observe CP release from the activated platelet cytoskeleton during the period between 5 and 15 s following activation with thrombin. We have failed repeatedly to observe a transition of CP from the cytoskeleton to the soluble fraction in intact platelets activated with thrombin or TRAF, despite uncovering a transition of CP to the soluble fraction in OG-permeabilized platelets treated with either $PI_{4,5}P_2$ or TRAF. Our preparations differ from that of Nachmias et al. in that we include phallacidin in the permeabilization buffer to prevent depolymerization of small actin filaments. The amount of CP release may also be affected by the pH of the buffer upon platelet permeabilization. Other discrepancies between our results and those of Nachmias et al. (33) are in the two-dimensional gel analysis. We find little β1 isoform in platelets and no difference in segregation of CP isotypes into the soluble or cytoskeletal fractions.

Our findings demonstrate that CP has an important role in maintaining the resting form of platelets by binding available barbed ends. A stabilization role can be extended to the activated platelet where association of CP to elongated actin filaments, could prevent their depolymerization when the actin monomer pool size decreases. However, since the amount of CP in the cytoskeleton of activated gelsolin null platelets does not change, it is possible that other proteins, in addition to CP, participate in barbed end exposure.

ppI's are emerging as important regulators of actin filament barbed end capping proteins. The synthesis of polyphosphoinositides by the activation of ppI kinases is an early event following thrombin activation of platelets (29). Thrombin receptor activation leads to the production ppI's phosphorylated in the D3 and D4 position. Both a rise in $PI_{3,4}P_2$ (40) and a rapid but transient rise in $PI_{3,4,5}P_2$ (41) have been observed in activated platelets, although, the inhibition of ppI phosphorylation in the D3 position does not affect actin assembly in thrombin activated platelets (25). The amount of ppI's phosphorylated in the D4 position (PI_4P and $PI_{4,5}P_2$) increase rapidly after thrombin stimulation, concurrent with barbed end exposure and actin polymerization (19). The ability of ppI's to uncap filament ends in a permeabilized platelet system has been measured by detecting barbed end exposure and characterized to be in a signal cascade downstream from the GTPase Rac (19). Furthermore, a peptide, derived from the gelsolin sequence, which binds $PI_{4,5}P_2$, inhibits thrombin receptor-dependent exposure of barbed ends (19). Taken together, these results and the observation of ppI dependent release of capping proteins from OG-permeabilized platelets presented here, suggest a model for actin polymerization in thrombin stimulated platelets where exposure of the barbed end, to allow actin polymerization, is mediated by ppI interaction with capping proteins.

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