

Association of Profilin with Filament-free Regions of Human Leukocyte and Platelet Membranes and Reversible Membrane Binding during Platelet Activation

John H. Hartwig,*[‡] Kristen A. Chambers,* Karen L. Hopcia,* and David J. Kwiatkowski*[§]

*Hematology-Oncology Unit, Department of Medicine, Massachusetts General Hospital, Charlestown, Massachusetts 02129; and [‡]Department of Anatomy and Cellular Biology and [§]Department of Medicine, Harvard Medical School, Boston, Massachusetts 02114

Abstract. Profilin is a conserved, widely distributed actin monomer binding protein found in eukaryotic cells. Mammalian profilin reversibly sequesters actin monomers in a high affinity profilactin complex. In vitro, the complex is dissociated in response to treatment with the polyphosphoinositides, phosphatidylinositol monophosphate, and phosphatidylinositol 4,5-bisphosphate. Here, we demonstrate the ultrastructural

immunolocalization of profilin in human leukocytes and platelets. In both cell types, a significant fraction of profilin is found associated with regions of cell membrane devoid of actin filaments and other discernible structures. After platelet activation, the membrane association of profilin reversibly increases. This study represents the first direct evidence for an interaction between profilin and phospholipids in vivo.

PROFILIN is a 12–15-kD cytoplasmic actin monomer binding protein of eukaryotic cells (Carlsson et al., 1976; Magdolen et al., 1988; Reichstein and Korn, 1979). Mammalian profilin occurs as a single isotype (Ampe et al., 1988; Kwiatkowski and Bruns, 1988) and interacts with actin monomers to sequester them from recruitment into actin filaments. In vitro, the affinity of mammalian spleen profilin for skeletal muscle actin monomer is relatively low, $K_d = 1\text{--}50\ \mu\text{M}$ (Markey et al., 1982; Nishida, 1985), and is higher for nonmuscle actin, $K_d = 10\text{--}400\ \text{nM}$ (Larsson and Lindberg, 1988), whereas profilin and actin can be purified as a high affinity, $K_d = 1\text{--}10\ \text{nM}$, profilactin complex from cells (Blikstad et al., 1980; Carlsson et al., 1976; Harris and Weeds, 1978; Malm, 1984; Markey et al., 1978).

The factor(s) responsible for the high affinity profilactin complex have not been identified (Larsson and Lindberg, 1988). The transient appearance of the high affinity complex before and/or during platelet activation (Lind et al., 1987; Markey et al., 1981) suggests that some mechanism exists inside cells for its rapid formation and dissociation. Lassing and Lindberg (1985) have demonstrated that the high affinity complex can be dissociated by treatment with the polyphosphoinositides, phosphatidylinositol monophosphate (PIP),¹ and phosphatidylinositol 4,5-bisphosphate (PIP₂) in vitro. More recently, human platelet profilin has been shown to bind PIP and PIP₂ reconstituted into vesicles in vitro (Lassing and Lindberg, 1988; Goldschmidt-Clermont, P., J. Baldassare, and T. Pollard, manuscript submitted for publication). These observations raise the question as to whether or

not an interaction between phosphoinositides and profilin occurs in vivo and whether this interaction regulates the transition between the high and low affinity states of the profilactin complex.

PIP and PIP₂ consist of an acidic, phosphorylated inositol side group connected to a glycerol backbone to which two long hydrophobic acyl chains are attached. They are, therefore, likely to reside in cellular membrane compartments, and indeed are widely thought to be localized to the inner leaflet of the plasma membrane (Garrett and Redman, 1975; Mauco et al., 1987). The presence of multiple distinct metabolic pools of PIP and PIP₂ in erythrocytes and platelets (Billah and Lapetina, 1982; King et al., 1987), suggests, moreover, that some PIP and PIP₂ are sequestered in the plasma (and perhaps other intracellular) membrane(s), possibly due to binding to cytoplasmic proteins (Anderson and Marchesi, 1985; Sheetz et al., 1982).

In support of an intracellular interaction between profilin and membrane-bound PIP and PIP₂, here we provide direct morphologic evidence for a close association of a large portion of cellular profilin with membranes in both intact human platelets and leukocytes. In addition, we demonstrate a reversible, transient association between profilin and plasma membrane after the activation of platelets, consistent with previous observations of the formation/dissociation of profilactin complexes during platelet activation.

Materials and Methods

Preparation of Anti-Profilin Antibodies

Human platelets and spleen were obtained as discarded materials from the

1. Abbreviations used in this paper: KRP, Krebs-Ringer phosphate buffer; PIP, phosphatidylinositol monophosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate.

Blood Transfusion Service and Pathology Departments, respectively, of the Massachusetts General Hospital. Profilin was purified from human platelets using a polyproline affinity column as described (Lind et al., 1987), with a 4 M urea rinse and 7 M urea elution step gradient. Antibodies against profilin were raised in rabbits injected every 3–4 wk at multiple sites with 1 ml of an emulsion of 0.25 mg/ml profilin in normal saline, using the RIBI adjuvant system (RIBI Immunochemical Research, Inc., Hamilton, MT). Antibody production was monitored by ELISA assay and the specificity of antibodies validated on immunoblots according to the method of Towbin et al. (1979). Human white blood cell and platelet extracts were prepared by lysis with 1% Triton X-100 in the presence of 2 mM diisopropylfluorophosphate, and were separated by electrophoresis on 15% polyacrylamide slab gels (Laemmli, 1970) before electrophoretic transfer to Immobilon paper (Millipore Continental Water Systems, Bedford, MA).

Anti-profilin IgG was affinity purified. 5 mg of purified human platelet profilin was conjugated to 3 ml CNBr-activated Sepharose beads (Pharmacia Fine Chemicals St. Louis, MO). IgG was precipitated from rabbit serum with 45% saturated ammonium sulfate, the precipitate collected by centrifugation at 10,000 g for 10 min at 4°C, and subsequently dissolved in and dialyzed extensively against PBS, pH 7.4. The dialysate was passed through the profilin-Sepharose column two times, the column was washed with 10 vol PBS, and bound IgG dissociated with 0.05 M glycine-HCl, pH 2.3, followed immediately by neutralization with 0.5 M sodium phosphate buffer, pH 7.7, and dialysis against PBS, pH 7.4.

Preparation of Cells

Platelets. Thin sections were cut from platelets embedded in Lowicryl K4M. The preparation of these blocks has been described by us previously (Hartwig et al., 1989). Briefly, platelets were freshly prepared from anticoagulated blood and used immediately after a 60-min 37°C incubation to insure a resting state. Some cells were fixed with 1% glutaraldehyde and embedded while the rest of the cells were activated by the addition of 1 U of thrombin per milliliter for times of 15, 30, and 300 s. 90% of the cells treated with thrombin for 15 s were activated as judged by the formation of pseudopodia. Gold particles were quantitated only in activated cells displaying pseudopodia or filopodia on their surfaces. Activation was terminated by the immediate fixation of the cells with 1% glutaraldehyde in 10 mM sodium phosphate buffer, pH 7.0.

Human Polymorphonuclear Leukocytes. Normal human blood from JHH and DJK was collected into the anticoagulant acid-citrate-dextrose (8 vol blood/2 vol). The citrated blood was mixed with 0.5 vol of 4% dextran and allowed to sit for 30 min at room temperature. The buffy coat was removed, added to 3 vol of 0.11 M ammonium chloride to lyse contaminating red blood cells, and centrifuged at 280 g for 10 min. The WBC pellet (60–70% polymorphonuclear leukocytes) was resuspended in 0.15 M NaCl, washed by centrifugation and resuspended to 1% (vol/vol) in Krebs-Ringer phosphate buffer (KRP) without added calcium, pH 7.4. Cells used in the electron microscope were fixed for 10 min in 1% glutaraldehyde in 10 mM phosphate buffer, pH 7.4, and embedded in Lowicryl K4M.

Preparation of Cell Sections for the Electron Microscope

Labeling of Sections with Rabbit Anti-Profilin IgG and 8-nm Gold Particles Coated with Goat Anti-Rabbit IgG. Gold particles, 8 nm in diameter, were prepared with tannic acid (Slot and Geuze, 1985) and coated with affinity-purified goat anti-rabbit IgG that had been absorbed with human IgG. Polyclonal rabbit affinity-purified anti-human platelet profilin IgG was used in all immunohistochemical studies.

To label the cell sections with gold, cut sections were placed on the surface of 10- μ l drops of 10 μ g/ml of anti-profilin IgG in PBS containing 1% BSA, pH 8.2, (PBS-BSA) and incubated for 1 h at 25°C. Sections were washed thrice by transferring them to drops of PBS/BSA for 5 min each. They were then transferred to 10- μ l drops of PBS-BSA containing anti-rabbit IgG-coated gold. After the incubation with IgG-gold, the sections were washed twice in PBS, thrice in distilled water, and stained with 2% uranyl acetate for 7 min followed by lead citrate for 1 min. Sections were viewed and photographed in a JEOL 1200-EX electron microscope at an accelerating voltage of 80 kV.

Preparation of Metal Replicas of Membrane Fragments

Adherence and Cleavage of Cells. Drops of a 1% suspension of human polymorphonuclear leukocytes in KRP were placed on glass coverslips 5 mm

in diameter for 3 min. Nonadherent cells were washed off with KRP and the coverslips containing adherent cells were incubated for an additional 5 min at 37°C. The bathing KRP solution was replaced with 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl₂, and 2 μ M phalloidin, pH 6.9 (PHEM buffer of Schliwa et al., 1981) and the exposed membrane surface of cells cleaved from their substrate-adherent basal surface by adhering a polylysine-coated coverslip to their apical surface and removing it. Coverslips containing membrane fragments (unroofed cells) and associated cytoskeletal elements were fixed with 1% glutaraldehyde-PHEM for 10 min, washed in PHEM buffer for 10 min, washed for 2 min in PBS containing 1 mg/ml of sodium borohydride, and then placed in PBS/BSA for 10 min in preparation for gold labeling.

Labeling of Cell Fragments with Rabbit Anti-Profilin IgG and 8-nm Gold Particles Coated with Goat Anti-Rabbit IgG. Coverslips containing cell fragments were covered with 25 μ l of 10 μ g/ml of rabbit anti-profilin IgG in PBS/BSA buffer and incubated for 2 h at 37°C. They were then washed with three changes of PBS/BSA solution and covered with 25 μ l of rabbit anti-goat IgG-coated gold at a 1:10 dilution of the stock and incubated for 1 h at 37°C. Unbound gold was removed using five wash cycles of PBS/BSA followed by three washes of PBS for 5 min each. Gold labeled membranes were then fixed for 10 min at room temperature with 1% glutaraldehyde in 10 mM sodium phosphate buffer, pH 7.0.

Rapid-Freezing and Freeze-Drying. Coverslips containing unroofed cells were washed with three changes of distilled water. They were then placed on the specimen mount of the rapid-freezing apparatus (Med-Vac, Inc, St. Louis, MO) and frozen by slamming them onto a liquid helium-cooled copper block (Hartwig and Shevlin, 1986). Freezing tabs containing the frozen coverslips were transferred to a liquid nitrogen-cooled stage of a Cressington CFE-50 freeze fracture apparatus, the stage temperature raised to -80°C for 45 min, and then rotary coated with platinum at 25° and carbon at 90°. Replicas were separated from the coverslip with 25% hydrofluoric acid, placed in bleach for 30 min, washed into distilled water, and then picked up on the surface of carbon-formvar-coated copper grids. Grids were made hydrophilic before use by glow discharge. Replicas were photographed at $\pm 10^\circ$ of tilt at 100 kV in a JEOL 1200-EX electron microscope.

Quantitation of Gold Label on Cell Sections. The distribution of gold particles was quantitated in different regions of platelets and polymorphonuclear leukocytes. Gold label generally took the form of small aggregates composed of an average of four to eight particles (Table I), although as many as 40 individual particles could be found occasionally in aggregates. The total number of gold particles, the number of gold in aggregates, and the total number of gold aggregates were quantitated. All gold particles and particle aggregates within a defined zone of a cell were counted and the area of the zone determined by tracing its boundaries on the surface of a digitizing pad. Areas, total particles, and particles per micrometer squared of cytoplasm, as well as the number of gold particle aggregates associated with a length of membrane (per micrometer) were determined as previously described (Hartwig et al., 1989; Hartwig and Shevlin, 1986) and are expressed as mean \pm the standard error of the mean per cell. Data means were compared using a two-tailed, unpaired *t* test.

Total gold particles and gold particle aggregates lying in three different intracellular zones of platelets were counted: (a) ≤ 10 nm of the plasma membrane; (b) cytoplasmic, excluding all particles determined to be plasma or intracellular organelle membrane associated; (c) residing on internal cell membranes such as intracellular dense granules, mitochondria, and the platelet canalicular system. Total gold label in each of these compartments was determined and data expressed as percent of total gold label in a cell section. The percent of gold label at the membrane of polymorphonuclear leukocytes was also determined for comparison to membrane labeling in platelets.

Results

Specificity of Rabbit Anti-Human Profilin Antibodies

Fig. 1 shows an immunoblot demonstrating the specificity of affinity-purified rabbit anti-profilin IgG for human profilin. Shown in lanes 1–3 is a Coomassie blue-stained 15% SDS polyacrylamide gel of purified human platelet profilin, and extracts of detergent-solubilized human white blood cells, and human platelets, respectively. Lanes 4–6 show the corresponding immunoblot using the anti-profilin IgG, of an iden-

Table I. Distribution of Anti-Profilin Gold Label in Resting and Thrombin-activated Platelets

Activity state	Location in cell	Total gold/ μm^2	Percent total cellular gold	Gold aggregates/ μm^2	Percent total aggregates	No. gold per aggregate
Resting	PM	244 \pm 17	16.7 \pm 1.6	37.5 \pm 2.1	13.8 \pm 1.3	6.5
	IM	177 \pm 15	16.8 \pm 1.4	43.6 \pm 6.1	22.6 \pm 4.1	4.1
	Cytoplasm	86 \pm 8	66.4 \pm 1.4	15.8 \pm 1.8	63.6 \pm 4.1	5.5
Activated (15 s)	PM	389 \pm 19	24.5 \pm 1.0	62.2 \pm 2.3	26.6 \pm 0.9	6.3
	IM	194 \pm 18	17.7 \pm 1.8	40.0 \pm 2.7	24.8 \pm 2.1	4.9
	Cytoplasm	129 \pm 10	59.3 \pm 3.1	15.9 \pm 1.0	50.0 \pm 2.1	8.1
Activated (30 s)	PM	257 \pm 12	19.8 \pm 1.5	42.7 \pm 1.9	20.4 \pm 1.6	6.1
	IM	169 \pm 12	16.0 \pm 1.1	36.9 \pm 2.7	23.1 \pm 2.7	4.6
	Cytoplasm	117 \pm 9	64.2 \pm 1.7	17.5 \pm 2.0	58.5 \pm 3.6	6.7
Activated (5 min)	PM	262 \pm 17	19.4 \pm 1.2	43.5 \pm 2.5	20.2 \pm 1.2	6.0
	IM	141 \pm 13	15.7 \pm 1.7	28.9 \pm 2.1	21.3 \pm 2.7	4.9
	Cytoplasm	99 \pm 5	64.7 \pm 2.1	14.4 \pm 0.8	58.6 \pm 2.9	6.9

The area (μm^2) for the membrane compartment included gold particles and/or gold aggregates contained in a zone 10 nm from the plasma membrane (plasma membrane length \times 10 nm). Gold labeling is expressed as percent of total label in all cellular compartments. 20 platelets were analyzed to derive these data, which are expressed as mean \pm SEM per platelet. Resting platelets were activated by the addition of 1 U/ml of thrombin. *PM*, peripheral membrane; *IM*, internal membrane structures.

tical set of three lanes. In all cases, only polypeptides with electrophoretic mobilities corresponding to the 15,000-*M*_r profilin polypeptide bound the IgG.

Localization of Profilin in Resting and Activated Platelets: Platelet Profilin Is Associated with Plasma and Internal Cell Membranes

Fig. 2 *a* shows a representative electron micrograph of Lowicryl-embedded resting platelets labeled with affinity-purified rabbit anti-profilin IgG, followed by 8-nm gold particles coated with goat anti-rabbit IgG. The discoid shape of the

gel-filtered platelets indicates that these cells are in a resting state. Anti-profilin gold label, in the form of aggregates containing an average of four to eight and occasionally as many as 40 gold particles (Table I), was observable in several compartments of the cell. Some appeared to reside within cytoplasm separate from any definable organelles. Aggregates of gold were also associated with plasma membrane and intracellular membranes. There was no gold labeling of cells when they were incubated with IgG-coated gold and preimmune rabbit IgG (Fig. 3).

The distribution of profilin label in the sections was quantitated by morphometry (Table I). This analysis focussed on the apportionment of label between plasma membrane, intracellular membranes, and cytoplasm. 16.7 \pm 1.6% of the total anti-profilin gold particles and 13.8 \pm 1.3% (mean \pm SEM) of gold aggregates were associated with the plasma membrane in the resting platelet, whereas 16.8 \pm 1.4 and 22.6 \pm 4.1% of total gold and gold aggregates, respectively, were associated with membrane-bound intracellular granules or organelles. The concentration of gold aggregates per micrometer of plasma membrane was 1.7 \pm 0.1 (n = 17 platelets).

The Distribution of Profilin in Thrombin-activated and Postactivated Cells: The Amount of Profilin Interacting with Membrane Reversibly Increases during Cell Activation

Fig. 2 *b-d* demonstrates the intracellular distribution of profilin in electron micrographs of thin sections of platelets that have been activated with 1 U/ml of thrombin for 15, 30, and 300 s, respectively. Treatment of platelets with this concentration of thrombin resulted in an immediate (15 s) extension of filopodia from the cell margins (Fig. 2 *b*). These extensions were more elaborate 30 s postthrombin treatment, at a time when intracellular granules have become compressed in the cell center (Fig. 2 *c*). After 5 min of incubation with thrombin, however, the cells returned to a more uniform morphology characterized by small, blunt surface protrusions (Fig. 2 *d*). The profilin-membrane association increased markedly after 15 s incubation of the cells with thrombin. Total gold label at the plasma membrane increased to 24.5 \pm 1.0% and gold in aggregates to 26.6 \pm 0.9% at that time,

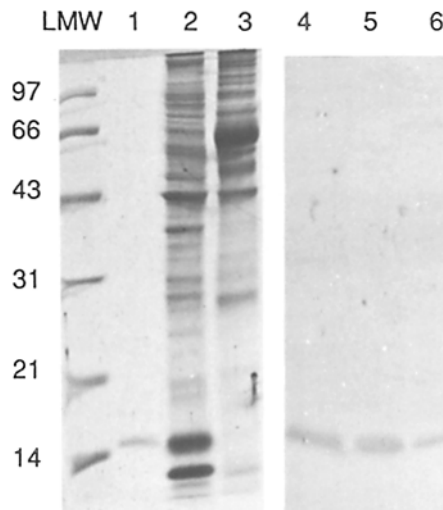


Figure 1. Specificity of anti-profilin IgG by immunoblot. Purified human profilin (lane 1) and detergent-solubilized extracts from human leukocytes (lane 2) and platelets (lane 3) were electrophoresed on a 15% polyacrylamide slab gel in the presence of 0.1% SDS, and stained with Coomassie blue. Molecular mass standards are included in the leftmost lane (*LMW*), with sizes in kilodaltons indicated. Replicate gel lanes, 4-6, respectively, were electrophoretically transferred to Immobilon paper, and incubated with 0.15 $\mu\text{g}/\text{ml}$ of rabbit anti-profilin IgG, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG and 0.01% *o*-dianisidine and H_2O_2 .

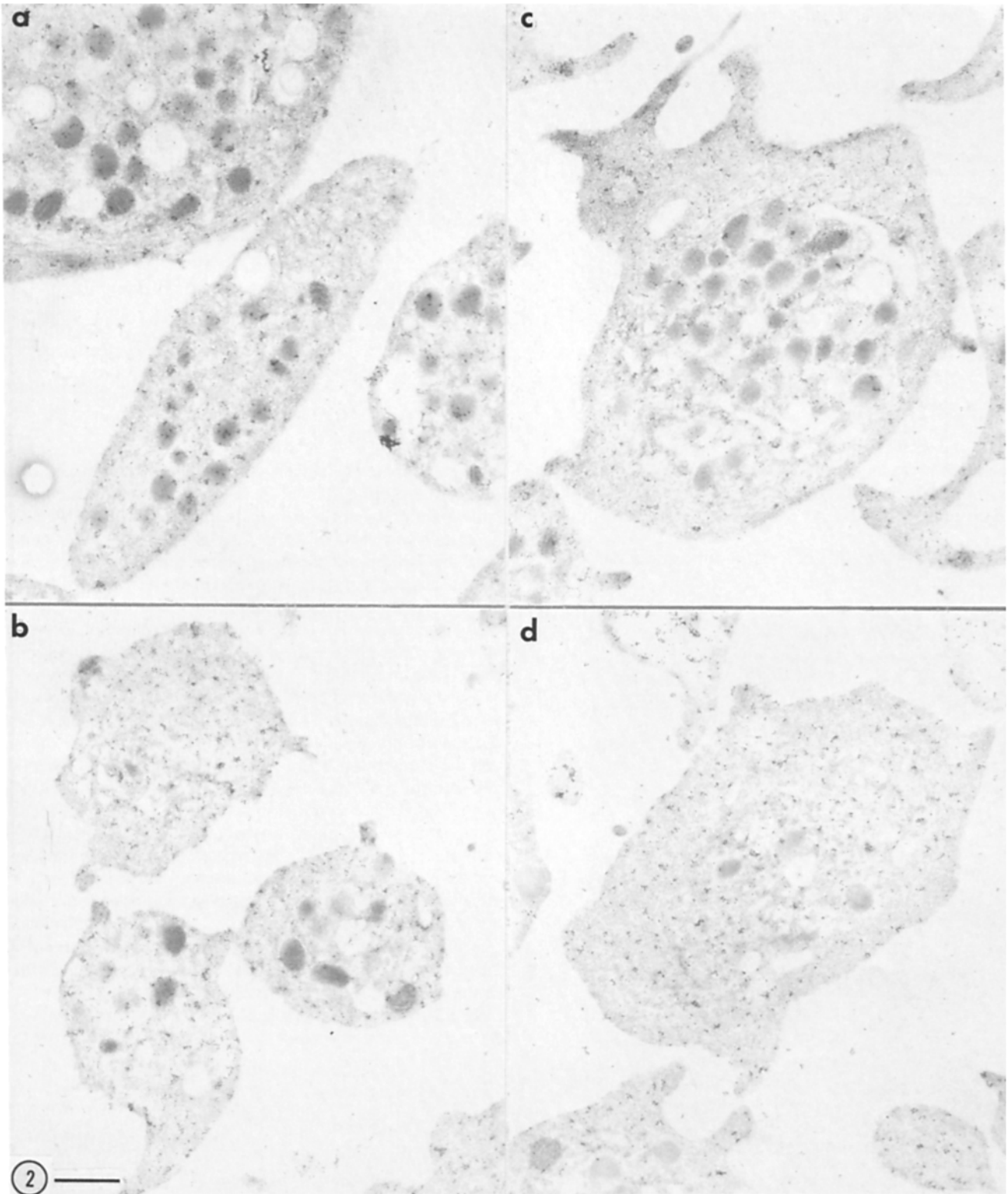


Figure 2. Electron micrographs showing the distribution of anti-profilin gold staining in Lowicryl sections from resting and activated platelets. Platelets, fixed with 1% glutaraldehyde, were embedded in Lowicryl K4M, sectioned, and sequentially treated with rabbit anti-profilin IgG and 8-nm colloidal gold particles coated with goat anti-rabbit IgG. (a) Representative section through a resting platelet. (b) Platelet treated with 1 U/ml of thrombin for 15 s, and then fixed. (c) Platelet treated with thrombin for 30 s, and then fixed. (d) Platelet incubated with thrombin for 5 min. Bar, 0.5 μ m.

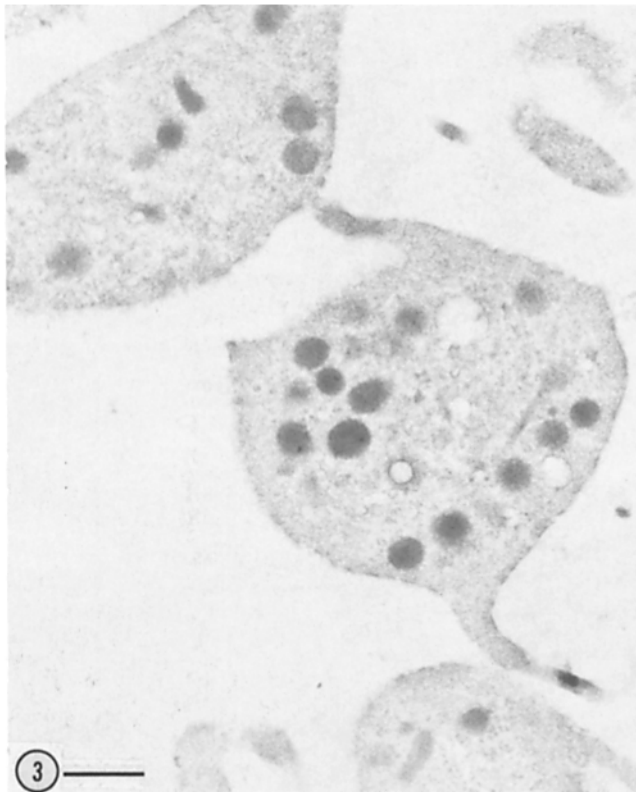


Figure 3. Representative electron micrograph showing the complete lack of gold labeling in platelets treated with thrombin for 15 s, fixed (same as in Fig. 2 b) and incubated with 10 µg/ml of preimmune rabbit IgG and 8-nm colloidal gold particles coated with goat anti-rabbit IgG. Bar, 0.5 µm.

whereas that associated with intracellular granules and the membranes comprising the platelet canalicular system was unchanged (17.7 ± 1.8 and $24.8 \pm 2.1\%$ vs. 16.8 ± 1.4 and $22.6 \pm 4.1\%$ in resting cells, respectively) (Fig. 2 b and Table I). Conversely, anti-profilin gold particle aggregates in cytoplasm diminished with cell activation from $63.6 \pm 4.1\%$ at rest to $50.0 \pm 2.1\%$ after thrombin treatment. Activated platelets had 2.8 ± 0.1 ($n = 24$ platelets) gold aggregates per micrometer membrane 15 s after treatment with thrombin. These data indicate that profilin in the cytoplasm of the resting cell rapidly moved to the plasma membrane in response to activation. After longer treatment with thrombin, the percent association of profilin with the membrane was found to decrease by comparison with 15 s of activation, but was still elevated relative to resting cells (Fig. 2 c and d, Table I). A corresponding return of gold label to cytoplasmic locations was apparent in the 30-s and 5-min postthrombin treated platelets. When preimmune IgG was substituted for the anti-profilin IgG, there was a complete lack of gold labeling bound to the cell sections (Fig. 3).

Distribution of Profilin in Human Polymorphonuclear Leukocytes

The above results indicated that profilin was binding to platelet plasma membranes. We next examined the distribution of profilin in human polymorphonuclear leukocytes. Leukocytes were used both to afford a second cell type for examina-

tion, and because it is possible to mechanically unroof them, permitting a three-dimensional assessment of profilin's membrane interaction in rapidly frozen, freeze-dried cells.

Polymorphonuclear leukocytes labeled densely with the anti-profilin IgG and gold particles. As was found in platelets, anti-profilin label occurred in aggregates composed of many individual gold particles (Fig. 4), and a sizeable portion of the label was associated with cellular membranes, both plasma and intracellular, including nuclear membrane (see Fig. 4 b). 1.9 ± 0.1 (mean \pm SEM, $n = 14$ leukocytes) gold particle aggregates were bound per micrometer of plasma membrane in leukocytes, similar to that found in unactivated platelet plasma membrane (1.7 ± 0.1). We estimate that the plasma membrane-associated label represents $\sim 6.5\%$ of the total anti-profilin staining in these cells.² Little anti-profilin staining was observed in the nucleus of leukocytes.

Anti-Profilin Gold Particles Bind to the Cytoplasmic Side of the Plasma Membrane

To visualize more directly the interaction of profilin with the membrane, unroofed polymorphonuclear leukocytes were incubated with the anti-profilin IgG and anti-rabbit IgG-coated gold conjugate. As shown in Fig. 5, the cytoplasmic side of the adherent cell membrane is exposed by the unroofing procedure and this membrane surface is diffusely decorated with the gold label. Label occurred predominantly as single gold particles, each lying directly on the membrane, although individual particles tended to form loose patches consisting of three to six particles and covering ~ 100 nm² of membrane area. Gold particles (Fig. 5, white arrows) were not associated with the sides or ends of membrane-attached filaments or with regions of membrane decorated with clathrin-coated pits. No labeling of the external side of the plasma membrane was observed. The gold label, therefore, represents binding of anti-rabbit IgG-coated gold conjugate to anti-profilin IgG, which is recognizing either free profilin or profilin-actin complex bound to the plasma membrane.

Discussion

Profilin is an abundant (30–40 µM in platelets) actin monomer sequestering protein which has been found ubiquitously in mammalian cells (Blikstad et al., 1980; Carlsson et al., 1976; Kwiatkowski and Bruns, 1988). Based upon *in vitro* studies of its interaction with PIP₂ in micelles and mixed vesicles (Lassing and Lindberg, 1985, 1988; Goldschmidt-Clermont, P., J. Baldassare, and T. Pollard, manuscript submitted for publication), it has been proposed that intracellular PIP₂ regulates the profilin-actin bond in a physiologically important manner. Here, we provide the first studies of the immunolocalization of profilin in mammalian cells, and are able to demonstrate directly that profilin is found in close association with both the internal leaflet of the plasma membrane and external surfaces of intracellular membranes in human platelets and leukocytes. As profilin is known to bind with high affinity only to actin monomers and phosphoinositides, the observed membrane localization is likely to reflect its direct binding to PIP and PIP₂ inside the cell.

2. This calculation assumes that polymorphonuclear leukocytes have 10 µm diameters.

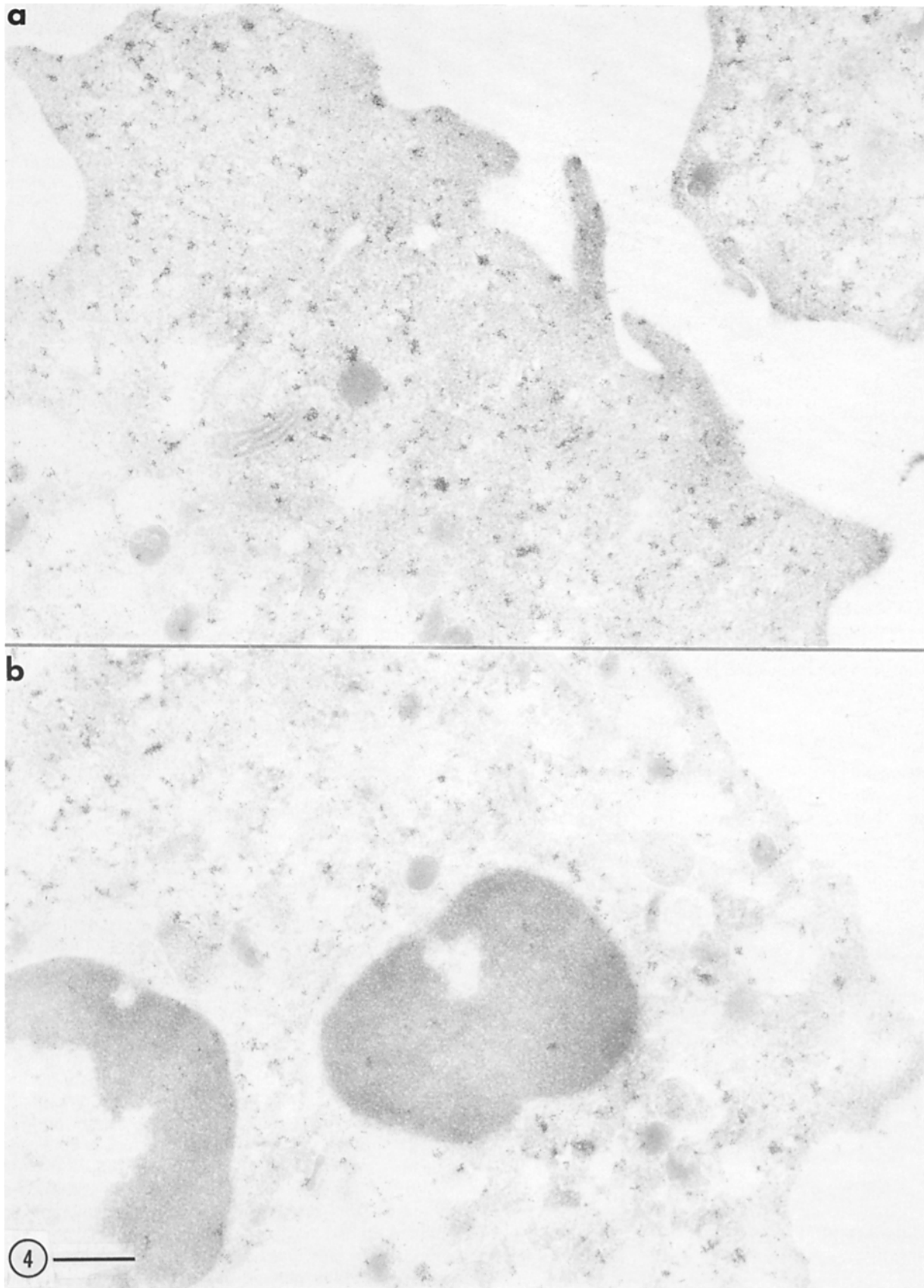


Figure 4. Electron micrographs showing anti-profilin staining in a polymorphonuclear leukocyte. (a) Clusters of anti-profilin label densely stain the peripheral cytoplasm and the plasma membrane at many points. Gold label is also seen on intracellular membranes. (b) Gold labeled the external side of mitochondria, intracellular granules, and the nuclear membrane. Few gold particles are seen in the nucleoplasm. Bar, 0.5 μm .

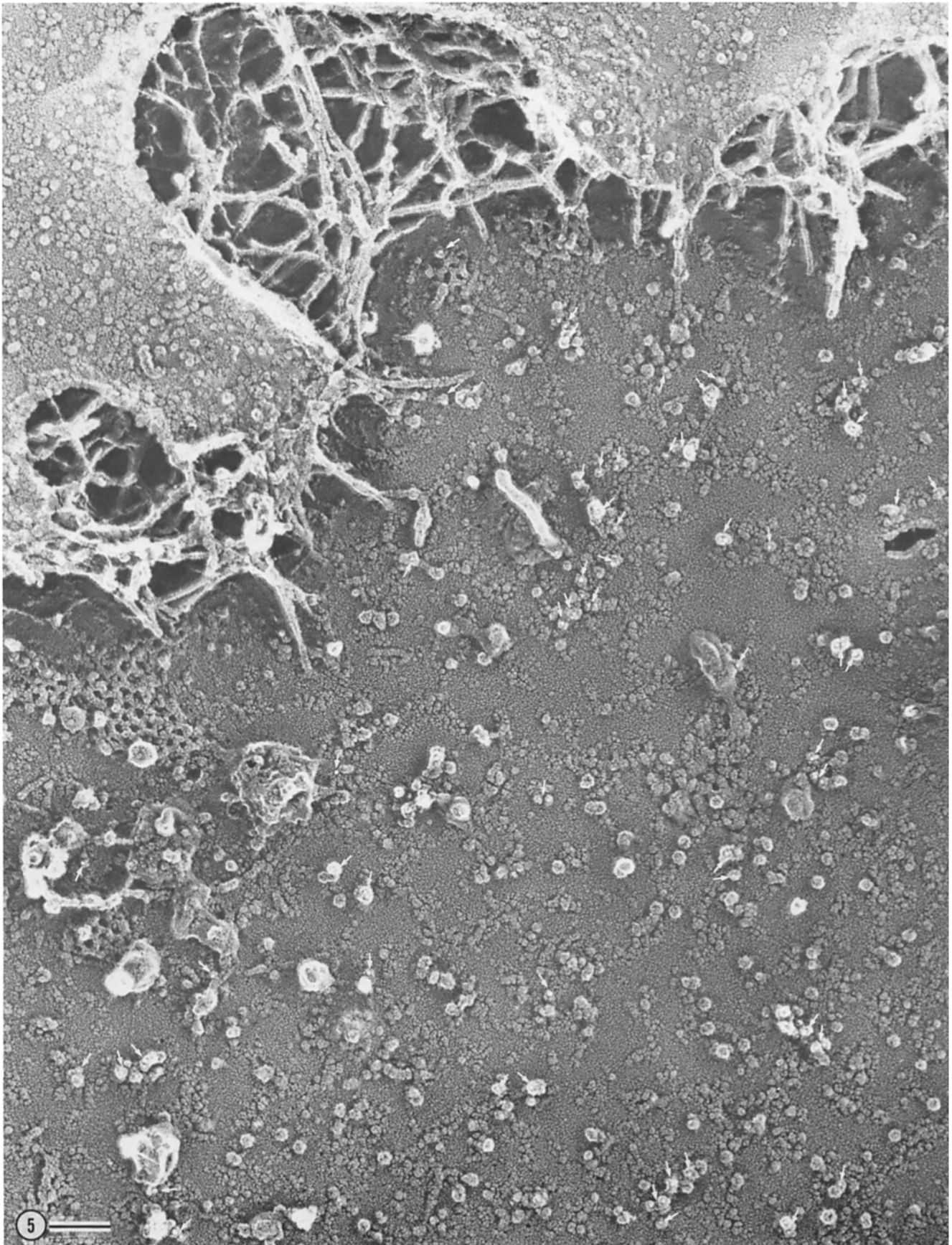


Figure 5. Electron micrograph showing the distribution of anti-profilin gold label on the cytoplasmic side of an unroofed polymorphonuclear leukocyte. Cells, adherent to glass, were unroofed by adhering and removing polylysine-coated coverslips. This representative electron micrograph shows that profilin is found on regions of membrane free of actin filaments and coated pits, and not on the remaining actin filaments adjacent to the membrane at the cell edges. Gold particles are indicated by the white arrows. Bar, 0.1 μm .

Platelet activation with thrombin is well-recognized to cause a marked shape change reaction, accompanied by actin polymerization and alterations in levels of the high affinity profilactin complex. Although the precise time course of events as assessed by different investigators have been somewhat variable (Lind et al., 1987; Markey et al., 1981), all agree that early on (0–10 s) during platelet activation, there are high levels of the profilactin complex as well as unpolymerized actin, while later (30 s–5 min) profilin is found free or in a low affinity complex with actin, and extensive actin polymerization occurs. In our experiments, activation of platelets with thrombin resulted in a rapid and reversible movement of anti-profilin gold label to the plasma membrane (Fig. 3). Maximal movement to the membrane occurred temporally just before the onset of actin polymerization (Lind et al., 1987). These observations are consistent with the hypothesis that the high affinity profilactin complex in platelets must interact with phosphoinositides resident in the plasma membrane to yield free profilin and actin for actin assembly (Lassing and Lindberg, 1985, 1988). The subsequent decreased amount of gold label found at the membrane after longer periods of thrombin treatment, may indicate the transient nature of the profilin phosphoinositide interaction, due either to hydrolysis of PIP₂ to inositol 1,4,5 trisphosphate and diacylglycerol by phospholipase C, or other mechanisms. Indeed, as intracellular Ca²⁺ release, mediated by inositol 1,4,5 trisphosphate, is thought to increase the affinity of phospholipase C for PIP₂ (Manne and Kung, 1987), this event would provide a mechanism for dissociating profilin from the membrane. Because of the coordinated, finely regulated and spatially circumscribed nature of most cell movements, similar morphologic evidence for a transient membrane interaction with profilin will be difficult or impossible to obtain in other cell types. Nonetheless, as a general mechanism, uncoupling of actin from profilin at the membrane, in conjunction with generation of actin nuclei or the uncapping of filament ends, would link receptor stimulation and actin filament growth both temporally and spatially.

These observations also lend further support to the concept that profilin may serve an important physiologic role in the regulation of polyphosphoinositide metabolism. We found that 13.8% and 22.6% of anti-profilin gold label was associated with plasma and internal membranes of resting platelets, respectively. If the profilin concentration in platelets is 30–40 μM (Goldschmidt-Clermont, P., J. Baldassare, and T. Pollard, manuscript submitted for publication) then 11 μM profilin is membrane associated. The binding of profilin to PIP and PIP₂ in the membrane may occur through a single profilin-PIP₂ bond or possibly through interaction with several (up to eight) inositide head groups, as recently proposed (Goldschmidt-Clermont, P., J. Baldassare, and T. Pollard, manuscript submitted for publication). Clustering of acidic phospholipids has been noted in mixed vesicle preparations *in vitro* (Eklund et al., 1988; Haverstick and Glaser, 1987), and may well occur *in vivo*. Thus, profilin could bind 11–88 μM PIP and PIP₂ in platelets, where the PIP and PIP₂ concentrations are estimated to be 100–200 μM (Rittenhouse and Sasson, 1985). Evidence for distinct metabolic pools of phosphoinositides has been obtained in both red blood cells (King et al., 1987) and platelets (Billah and Lapetina, 1982). Short-term labeling experiments in thrombin-stimulated platelets have also suggested that nearly

all the PIP₂ turnover occurs at the plasma membrane (Mauco et al., 1987).

These latter observations are consistent with the possible sequestration of polyphosphoinositides by cellular components. Glycophorin (Anderson and Marchesi, 1985) and gelsolin (Janmey and Stossel, 1989) are other PIP₂ binding proteins that may participate in this process. Our current results provide further evidence for the importance of phospholipid-protein interactions, and suggest that the profilin-phosphoinositide interaction may have wide consequence in control of phosphoinositide metabolism and cell signalling.

Supported by United States Public Health Service grants GM36507, KO8HL01582, and grants from the Council for Tobacco Research, USA and the Edwin S. Webster Foundation.

Received for publication 17 April 1989 and in revised form 2 June 1989.

References

- Ampe, C., F. Markey, U. Lindberg, and J. Vandekerckhove. 1988. The primary structure of human platelet profilin: reinvestigation of the calf spleen profilin sequence. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 228:17–21.
- Anderson, R. A., and V. T. Marchesi. 1985. Regulation of the association of membrane skeletal protein 4.1 with glycophorin by a polyphosphoinositide. *Nature (Lond.)*, 318:295–298.
- Billah, M. M., and E. G. Lapetina. 1982. Evidence for multiple metabolic pools of phosphatidylinositol in stimulated platelets. *J. Biol. Chem.* 257:11856–11859.
- Blikstad, I., I. Sundkvist, and S. Eriksson. 1980. Isolation and characterization of profilactin and profilin from calf thymus and brain. *Eur. J. Biochem.* 105:425–433.
- Carlsson, L., L. E. Nystrom, I. Sundkvist, F. Markey, and U. Lindberg. 1976. Profilin, a low molecular weight protein controlling actin polymerisability. In *Contractile Systems in Non-muscle Tissues*. 39–48. Elsevier/North-Holland, Amsterdam.
- Eklund, K. K., J. Vuorinen, J. Mikkloa, J. A. Virtanen, and P. K. J. Kinnunen. 1988. Ca²⁺-induced lateral phase separation in phosphatidic acid/phosphatidyl choline monolayers as revealed by fluorescence microscopy. *Biochemistry*. 27:3433–3437.
- Garrett, R. J. B., and C. M. Redman. 1975. Localization of enzymes involved in polyphosphoinositide metabolism on the cytoplasmic surface of the human erythrocyte membrane. *Biochim. Biophys. Acta.* 382:58–64.
- Harris, H. E., and A. G. Weeds. 1978. Platelet actin: sub-cellular distribution and association with profilin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 90:84–88.
- Hartwig, J., and P. Shevlin. 1986. The architecture of actin filaments and the ultrastructural location of actin-binding protein in the periphery of lung macrophages. *J. Cell Biol.* 103:1007–1020.
- Hartwig, J., K. Chambers, and T. Stossel. 1989. Association of gelsolin with actin filaments and cell membranes of macrophages and platelets. *J. Cell Biol.* 109:467–479.
- Haverstick, D. M., and M. Glaser. 1987. Visualization of Ca²⁺-induced phospholipid domains. *Proc. Natl. Acad. Sci. USA.* 84:4475–4479.
- Janmey, P., and T. Stossel. 1989. Gelsolin-polyphosphoinositide interaction. Full expression of gelsolin-inhibiting function by polyphosphoinositides in vesicular form and inactivation by dilution, aggregation, or masking of the inositol head group. *J. Biol. Chem.* 264:4825–4831.
- King, C. E., L. R. Stephens, P. T. Hawkins, G. R. Guy, and R. H. Michell. 1987. Multiple metabolic pools of phosphoinositides and phosphatidate in human erythrocytes incubated in a medium that permits rapid transmembrane exchange of phosphate. *Biochem. J.* 244:209–217.
- Kwiatkowski, D. J., and G. A. P. Bruns. 1988. Human profilin. Molecular cloning, sequence comparison, and chromosomal analysis. *J. Biol. Chem.* 263:5910–5915.
- Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680–685.
- Larsson, H., and U. Lindberg. 1988. The effect of divalent cations on the interaction between calf spleen profilin and different actins. *Biochim. Biophys. Acta.* 953:95–105.
- Lassing, I., and U. Lindberg. 1985. Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. *Nature (Lond.)*. 318:472–474.
- Lassing, I., and U. Lindberg. 1988. Specificity of the interaction between phosphatidylinositol 4,5-bisphosphate and the profilin:actin complex. *J. Cell Biochem.* 37:255–267.
- Lind, S. E., P. A. Janmey, C. Chaponnier, T. J. Herbert, and T. P. Stossel. 1987. Reversible binding of actin to gelsolin and profilin in human platelet extracts. *J. Cell Biol.* 105:833–842.
- Magdolen, V., U. Oechsner, G. Muller, and W. Bandlow. 1988. The intron-containing gene for yeast profilin (PFY) encodes a vital function. *Mol. Cell*

- Biol.* 8:5108-5112.
- Malm, B. 1984. Chemical modification of Cys-374 of actin interferes with the formation of the profilactin complex. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 173:399-402.
- Manne, V., and H. Kung. 1987. Characterization of phosphoinositide-specific phospholipase C from human platelets. *Biochem. J.* 243:763-771.
- Markey, F., U. Lindberg, and L. Eriksson. 1978. Human platelets contain profilin, a potential regulator of actin polymerizability. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 88:75-79.
- Markey, F., T. Persson, and U. Lindberg. 1981. Characterization of platelet extracts before and after stimulation with respect to the possible role of profilactin as microfilament precursor. *Cell.* 23:145-153.
- Markey, F., H. Larsson, K. Weber, and U. Lindberg. 1982. Nucleation of actin polymerization from profilactin: opposite effects of different nuclei. *Biochim. Biophys. Acta.* 704:43-51.
- Mauco, G., P. Dajans, H. Chap, and L. Douste-Blazy. 1987. Subcellular localization of inositol lipids in blood platelets as deduced from the use of labelled precursors. *Biochem. J.* 244:757-761.
- Nishida, E. 1985. Opposite effects of cofilin and profilin from porcine brain on rate of exchange of actin-bound adenosine 5'-triphosphate. *Biochemistry.* 24:1160-1164.
- Reichstein, E., and E. D. Korn. 1979. Acanthamoeba profilin: a protein of low molecular weight from *Acanthamoeba Castellani* that inhibits actin nucleation. *J. Biol. Chem.* 254:6174-6179.
- Rittenhouse, S., and J. Sasson. 1985. Mass changes in myoinositol trisphosphate in human platelets stimulated by thrombin. *J. Biol. Chem.* 260:8657-8660.
- Sheetz, M. P., P. Febroriello, and D. E. Koppel. 1982. Triphosphoinositide increases glycoprotein lateral mobility in erythrocyte membranes. *Nature (Lond.)* 296:91-93.
- Slot, J., and H. Geuze. 1985. A new method of preparing gold probes for multiple-labeling cytochemistry. *Eur. J. Cell Biol.* 38:87-93.
- Towbin, J., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.