

# Activation-dependent Redistribution of the Adhesion Plaque Protein, Talin, in Intact Human Platelets

Mary C. Beckerle, Diane E. Miller, Maria E. Bertagnolli, and Sarah J. Locke

Department of Biology, University of Utah, Salt Lake City, Utah 84112

**Abstract.** Talin is a high molecular weight protein localized at adhesion plaques in fibroblasts. It binds vinculin and integrin and appears to participate in generating a transmembrane connection between the extracellular matrix and the cytoskeleton. We have recently shown that talin is an abundant protein in platelets, cells highly specialized for regulated adhesion. Although talin constitutes >3% of the total protein in intact human platelets, its location within the cells had not been defined. In the work reported here, we have investigated the distribution of talin in resting and acti-

vated human platelets by immunofluorescence and immunoelectron microscopy. We have found that talin undergoes an activation-dependent change in its subcellular location. In resting platelets, which are nonadhesive, talin is uniformly distributed throughout the cytoplasm. In contrast, in thrombin- and glass-activated, substratum-adherent platelets, talin is concentrated at the cytoplasmic face of the plasma membrane. This dramatic, regulated redistribution of talin raises the possibility that talin plays a role in the controlled development of platelet adhesion.

**P**LATELETS are specialized cells that exhibit highly regulated adhesive properties. Resting platelets in the circulation are nonadhesive, biconcave disk-shaped cells. When such platelets encounter a discontinuity in a blood vessel wall, they become activated; they polymerize extensive arrays of actin filaments, undergo a dramatic change in cell shape, secrete the contents of intracellular granules into the medium, and become adhesive. The activated platelets bind fibrinogen, the precursor of the major extracellular matrix component of a blood clot, and aggregate with each other (Marguerie et al., 1980). Finally, the platelets carry out an actomyosin-dependent retraction of the clot which serves to consolidate the fibrin strands into a dense meshwork that restricts the flow of blood beyond the vessel wall.

For effective clot retraction to occur, the platelets must establish a transmembrane connection between surface receptor-ligand complexes and the force-generating contractile elements of the cytoskeleton. Recent studies have clarified to some extent the mechanism by which this transmembrane linkage between fibrin and actin is developed. Platelet adhesion to fibrin and its precursor, fibrinogen, is mediated by the transmembrane glycoprotein complex, gpIIb-IIIa (Bennett et al., 1982; Nachman and Leung, 1982; Phillips et al., 1980), a member of the integrin family of cell adhesion molecules (Pytela et al., 1986; Hynes, 1987; Ruoslahti and Pierschbacher, 1987). When platelets are activated with a physiological stimulus such as thrombin, gpIIb-IIIa acquires the ability to bind fibrinogen (Hawiger et al., 1980) and becomes stably associated with the actin-rich cytoskeleton (Phillips et al., 1980). A small percentage of the gpIIb-IIIa

appears to associate directly with actin filaments (Painter et al., 1985). In addition, there may be indirect associations between actin and gpIIb-IIIa that are mediated by other cellular proteins.

Indirect connections between actin and elements of the extracellular matrix are believed to occur at sites of substratum adhesion in fibroblasts. Fibroblasts in culture adhere to extracellular matrix components such as fibronectin at specialized regions of the plasma membrane called adhesion plaques. At these sites a structurally and functionally significant transmembrane connection between the extracellular matrix and the cytoskeleton is established (Ali and Hynes, 1977; Ali et al., 1977; Singer, 1979). A fibronectin receptor that, like gpIIb-IIIa, is a member of the integrin family of extracellular matrix receptors is localized at adhesion plaques (Damsky et al., 1985). This receptor does not appear to interact directly with actin, but can associate with at least two other adhesion plaque components, a 225-kD polypeptide called talin (Burrige and Connell, 1983; Horwitz et al., 1986; Buck and Horwitz, 1987) and a recently discovered 100-kD protein called fibulin (Argaves et al., 1989). Talin can also bind vinculin (Otto, 1983; Burrige and Mangeat, 1984), a 130-kD protein that is widely found at sites of cell-cell and cell-substratum interaction (Geiger, 1979; Geiger et al., 1985). Bundles of actin filaments terminate at the adhesion plaques, where the fibronectin receptor, talin, fibulin, and vinculin are concentrated; however, the detailed molecular mechanism by which they associate with these regions has yet to be defined.

Although the complex interactions that result in a transmembrane connection between the extracellular matrix and

the cytoskeleton are not fully understood, talin has emerged as a potentially pivotal component in this chain since it can interact with proteins in both the membrane and cytoplasmic domains of the adhesion plaque. Interestingly, we have recently found that talin is a very abundant protein in platelets, cells for which adhesion is a primary function (O'Halloran et al., 1985; Beckerle et al., 1986). In human platelets, talin is a 235,000-D protein (P235) that represents >3% of the total platelet protein (Collier and Wang, 1982). The fact that talin is significantly enriched in platelets relative to other cell types suggests that it is important for performance of some differentiated function in the platelet.

Because of the abundance of talin in platelets, cells highly specialized for regulated adhesion, and because of our knowledge of talin's role at sites of cell-substratum adhesion in fibroblasts, we have investigated the distribution of talin in resting (nonadhesive) and activated (adhesion-competent) platelets. In this paper we show that talin undergoes a dramatic redistribution in response to platelet activation, moving from a diffuse, cytoplasmic distribution to a more discrete, submembranous location. Our results demonstrate that the subcellular distribution of talin is regulated in living platelets and is responsive to the physiological state of the platelet.

## Materials and Methods

### Preparation and Characterization of Antibodies

Three independently raised rabbit polyclonal antisera were used for indirect immunofluorescence. A2 serum was raised against avian smooth muscle talin and has been shown previously to cross react with human platelet talin (Beckerle et al., 1986). B11 serum was raised against human platelet talin. N681 serum was raised against human platelet talin and prepared in collaboration with Keith Burrige and Theresa O'Halloran at the University of North Carolina at Chapel Hill (Chapel Hill, NC). These three unfractionated antisera, as well as the affinity-purified antibody described below, gave indistinguishable results in indirect immunofluorescence and specifically recognized talin in immunoblot experiments.

The N681 serum was affinity purified against isolated human platelet talin (O'Halloran et al., 1985) coupled to activated CH-Sepharose-4B (Pharmacia Fine Chemicals, Piscataway, NJ). Affinity-purified antibody was stored in small aliquots at  $-20^{\circ}\text{C}$  in PBS with added BSA (5 mg/ml) as carrier. The specificity of the affinity-purified antibody was determined by Western immunoblot analysis performed essentially as described previously (Beckerle et al., 1986), except for that  $^{125}\text{I}$ -protein A (ICN Radiochemicals, Irvine, CA) was used to visualize the position of the primary antibody on the nitrocellulose strip. Affinity-purified antibody was used in all immunoelectron microscopy studies described here.

A rabbit polyclonal antiserum (B5) was raised against the platelet glycoprotein complex, gpIIb-IIIa. gpIIb-IIIa was purified from outdated human platelet-rich plasma by a previously published procedure (Fitzgerald et al., 1985). Western immunoblot analysis demonstrated that the B5 antiserum recognized both gpIIb and gpIIIa but exhibited a stronger reaction against gpIIb.

### Isolation of Platelets

Platelet-rich plasma drawn from healthy donors in the previous 24-h period was obtained from IHC Blood Services (Salt Lake City, UT). Platelets were isolated by gel filtration at  $22-24^{\circ}\text{C}$  through Sepharose-2B (Pharmacia Fine Chemicals) equilibrated with Hepes-buffered Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 0.5 mM  $\text{MgCl}_2$ , 3.3 mM  $\text{Na}_2\text{HPO}_4$ , 5.5 mM glucose, 3.8 mM Hepes, pH 7.4, 0.35% BSA) (Timmons and Hawiger, 1978). In some cases, we omitted the BSA from the column buffer without a detectable effect on the morphology of resting platelets. Fractions rich in platelets were pooled, and the platelets were incubated at  $37^{\circ}\text{C}$  (Nachmias, 1980; Fox et al., 1984) for 30-60 min. The platelet suspension was then recalcified to yield a final calcium concentration of 2 mM, and the platelet

concentration was adjusted to  $1-5 \times 10^8$  cells/ml. To activate the platelets, thrombin (Calbiochem-Behring Corp., La Jolla, CA) was added at a final concentration of 0.1 NIH U/ml. Resting and thrombin-activated platelet samples were either agitated gently on a rocking platform (Lab-Quake; Labindustries, Inc., Berkeley, CA) or allowed to sit undisturbed until fixation. In some experiments, platelet-rich plasma was incubated with  $10 \mu\text{g/ml}$  prostaglandin  $\text{E}_1$  ( $\text{PGE}_1$ )<sup>1</sup> for 30 min at  $22-24^{\circ}\text{C}$  before gel filtration to insure that the platelets would remain resting during isolation.

For the preparation of platelets to be spread on glass coverslips, suspensions of resting platelets were centrifuged, and the cells were resuspended in Hanks' balanced salt solution. The platelets were allowed to spread on  $22 \times 22$ -mm glass coverslips for 30 min at room temperature. The coverslips were then rinsed with Hanks' balanced salt solution and fixed for immunoelectron microscopy as described below.

### Indirect Immunofluorescence

Samples of resting or thrombin-activated cells were dispensed into 3 vol of 3.7% formaldehyde in PBS and fixed for a minimum of 10 min. Parallel platelet samples were mixed with Laemmli SDS-PAGE sample buffer (1:1) (Laemmli, 1970), immediately boiled for 3 min, and then frozen in liquid  $\text{N}_2$  to minimize sample proteolysis. These samples were analyzed by SDS-PAGE and Western immunoblot to determine whether talin had been cleaved by the calcium-dependent protease.

For indirect immunofluorescence, the fixed platelets were allowed to settle for 30 min at room temperature onto coverslips that had been previously coated with Alcian blue (Sigma Chemical Co., St. Louis, MO). Once the cells had attached to the coverslips, indirect immunofluorescence was performed as described previously (Beckerle, 1986), except it was not necessary to permeabilize the cells stained with antibodies against gpIIb-IIIa. Antisera were used at dilutions of 1:500 (B11, A2, and N681) or 1:2,000 (B5). Preimmune sera were used at the same concentrations as the corresponding postimmune sera. Indirect immunofluorescence experiments were performed a minimum of three times using unrelated platelet samples with the same results.

Photomicrographs were taken on a fluorescence microscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY) equipped with a  $100\times$  Plan-Neofluor objective. Photomicrographs of cells stained with preimmune serum were taken with exposure times greater than or equal to the optimal exposure times used for postimmune serum-stained cells. Similarly, prints of preimmune serum-stained cells were made with abbreviated paper exposure times to maximize our ability to detect faint signals.

### Preparation of Cells for Immunoelectron Microscopy

Resting (plus or minus  $\text{PGE}_1$ ) and thrombin-activated platelets to be used for immunogold labeling were fixed in suspension with filtered 2% paraformaldehyde (prepared fresh), 0.1% glutaraldehyde in Dulbecco's PBS, pH 7.4, for 90 min at room temperature. The fixed cells were then sedimented in a microfuge (Eppendorf; Brinkmann Instruments Co., Westbury, NY) (10 min at 10,000 rpm), and the pellets were washed three times for 10 min each time in PBS. The samples were incubated with NaBorohydride (0.5 mg/ml in PBS) for 30 min to reduce free aldehydes and were then washed in PBS (three times for 10 min each time). The platelets were then dehydrated in ethanol (graded series) and embedded in LR White as above. Thin sections were cut on an ultramicrotome (Ultratome II; LKB Instruments Inc., Bromma, Sweden) and collected on 200-mesh thin-bar grids (Ted Pella, Inc., Redding, CA).

Platelets that had been spread on glass coverslips were fixed in situ and embedded as above. The glass coverslips were separated from the polymerized resin by immersion in liquid  $\text{N}_2$  before thin sectioning.

### Immunogold Labeling

Grids containing thin sections were incubated for 3 h at  $37^{\circ}\text{C}$  with  $30 \mu\text{l}$  affinity-purified anti-talin antibody (7.5  $\mu\text{g/ml}$ ) in BSA blocking buffer (2.5 mg/ml BSA [fraction V], 0.05% Tween-20, 0.2% gelatin in TBS). Incubation with primary antibody was continued overnight at  $4^{\circ}\text{C}$  in TBS. The grids were then washed and incubated with protein A conjugated to 10-nm gold (a generous gift of Dr. Laszlo Kömüves, Baylor College of Medicine, Houston, TX) for 1 h at  $37^{\circ}\text{C}$ . Finally, the grids were washed and stained for 10-60 s with 2% uranyl acetate. Samples were viewed under an electron

1. Abbreviation used in this paper:  $\text{PGE}_1$ , prostaglandin  $\text{E}_1$ .

microscope (201; Philips Electronic Instruments Inc., Mahwah, NJ) at 80 kV. Micrographs of random fields were taken at a magnification of 10,000 $\times$ .

### Quantitative Analysis of Immunogold Labeling

The distribution of immunogold label was determined for cells in four treatment categories: (a) resting, treated with PGE<sub>1</sub>; (b) resting, not treated with PGE<sub>1</sub>; (c) thrombin activated; and (d) glass activated. All gold particles present in ten cell sections were counted for each treatment. Individual micrographs were selected at random and were projected onto a digitizing pad (GTCO Corporation, Columbia, MD) at a final magnification of 100,000 $\times$ . Up to three cells per negative were chosen for analysis based on whether or not they met the following criteria: (a) cell diameter between 1 and 2  $\mu$ m; (b) well-defined cell boundary with entire cell visible in the field; and (c) no filopodial extensions associated with resting cells.

Each cell's perimeter was defined by tracing the cell boundary onto a sheet of paper, and the total cross-sectional membrane length in micrometers was determined. The shortest distance between each gold particle and the cell boundary (plasma membrane) was traced using the digitizer pen, and the distance was computed. The data derived from each cell were grouped into five categories that classified the gold particles according to their distance from the plasma membrane as follows: (a) gold particles falling within 0–40 nm of the plasma membrane (for the purposes of this analysis, these particles will be referred to as membrane associated); (b) gold particles falling within 40–80 nm of the plasma membrane (these particles fall within subplasmalemmal cortical cytoplasm); (c) gold particles falling within 80–160 nm from the cell boundary; (d) gold particles falling within 160–320 nm from the plasma membrane; and (e) gold particles in the central cytoplasm,  $\geq$ 320 nm from the plasma membrane. Some gold particles were associated with internal membranes in both resting and activated platelets; however, the fixation and embedding procedure required for immunogold labeling made it difficult in most cases to determine unequivocally whether a particular membrane cross section resulted from an invagination of the plasma membrane or a bonafide intracellular vesicle. We therefore measured the shortest distance from a gold particle to the well-defined plasma membrane at the cell boundary.

All statistical analyses were performed on a computer (IBM Instruments, Inc., Danbury, CT) using the statistical analysis system (SAS Institute Inc., 1985). The number of gold particles in each of the distance categories was expressed as a percentage of the total gold counted per cell. The gold particle distributions for the various treatments were compared in a pair-wise fashion using a Chi square test. To determine whether the percentage of membrane-associated gold (i.e., gold in the 0–40-nm category) varied significantly as a function of treatment, a one-way analysis of variance was performed, and the mean values were compared by *t* test using Tukey's honestly significant difference correction for multiple comparisons. We have also determined the number of membrane-associated gold particles per micrometer of plasma membrane. These data were analyzed as above except that the values for gold particles per micrometer were square root transformed to meet the assumptions of the analysis of variance model. Probability (*p*) values  $>0.05$  were not considered to be statistically significant.

### Results

Resting platelets isolated from platelet-rich plasma by gel filtration are nonadhesive and exhibit a characteristic disk-like shape (Fig. 1 *a*). Few, if any, filopodia extend from the surface of the cells. When platelets are activated in suspension with a physiological agonist such as thrombin, they exhibit a dramatic change in cell shape (Fig. 1 *b*). Numerous long, thin filopodia that are beneath the resolution of the light microscope extend from the spheroidal cell bodies. Platelets can also be activated by exposure to glass, which provides a substratum to which they adhere. Glass-activated platelets extend filopodia that spread out to form extremely thin lamellopodia, ultimately giving rise to the flattened, pancake-like cell morphology seen in Fig. 1 *c*. All of the images presented in Fig. 1 are at the same final magnification to illustrate the dramatic transformations in overall cell shape un-

dergone by the platelets as they become activated and adhere to a two-dimensional substratum.

### Distribution of Talin and gpIIb-IIIa in Resting and Thrombin-activated Platelets

We have compared the distributions of the abundant, high molecular weight protein, talin, with the transmembrane glycoprotein complex, gpIIb-IIIa, in resting and activated platelets by indirect immunofluorescence. Resting platelets exhibit a cytoplasmic distribution of talin as detected by indirect immunofluorescence (Fig. 2 *a*). The cytoplasmic staining is not completely homogeneous, however, it is not as punctate as one would expect if specific intracellular organelles were stained (Ginsberg et al., 1980). Upon activation of the platelets by exposure to the physiological agonist, thrombin (0.1 NIH U/ml), the intracellular distribution of talin changes dramatically. As can be seen in Fig. 2 *b*, indirect immunofluorescent localization of talin in thrombin-activated cells reveals that talin has become concentrated at the periphery of the cells, giving rise to a halo-like fluorescence image. We have obtained this staining pattern using three independently raised polyclonal antisera directed against talin, as well as an affinity-purified anti-talin antibody. We have confirmed that the halo of fluorescence results from a peripheral subcellular distribution of talin by optical sectioning of

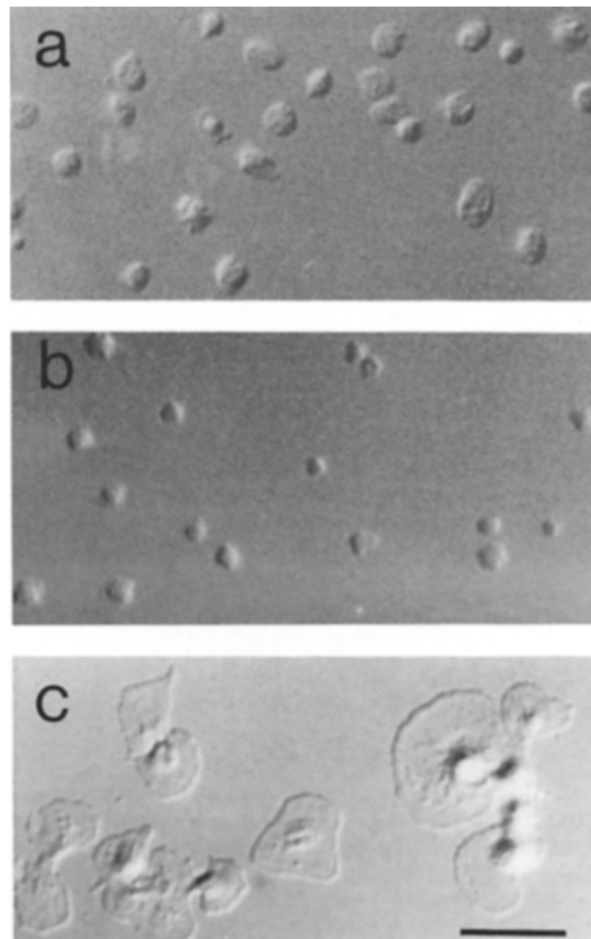
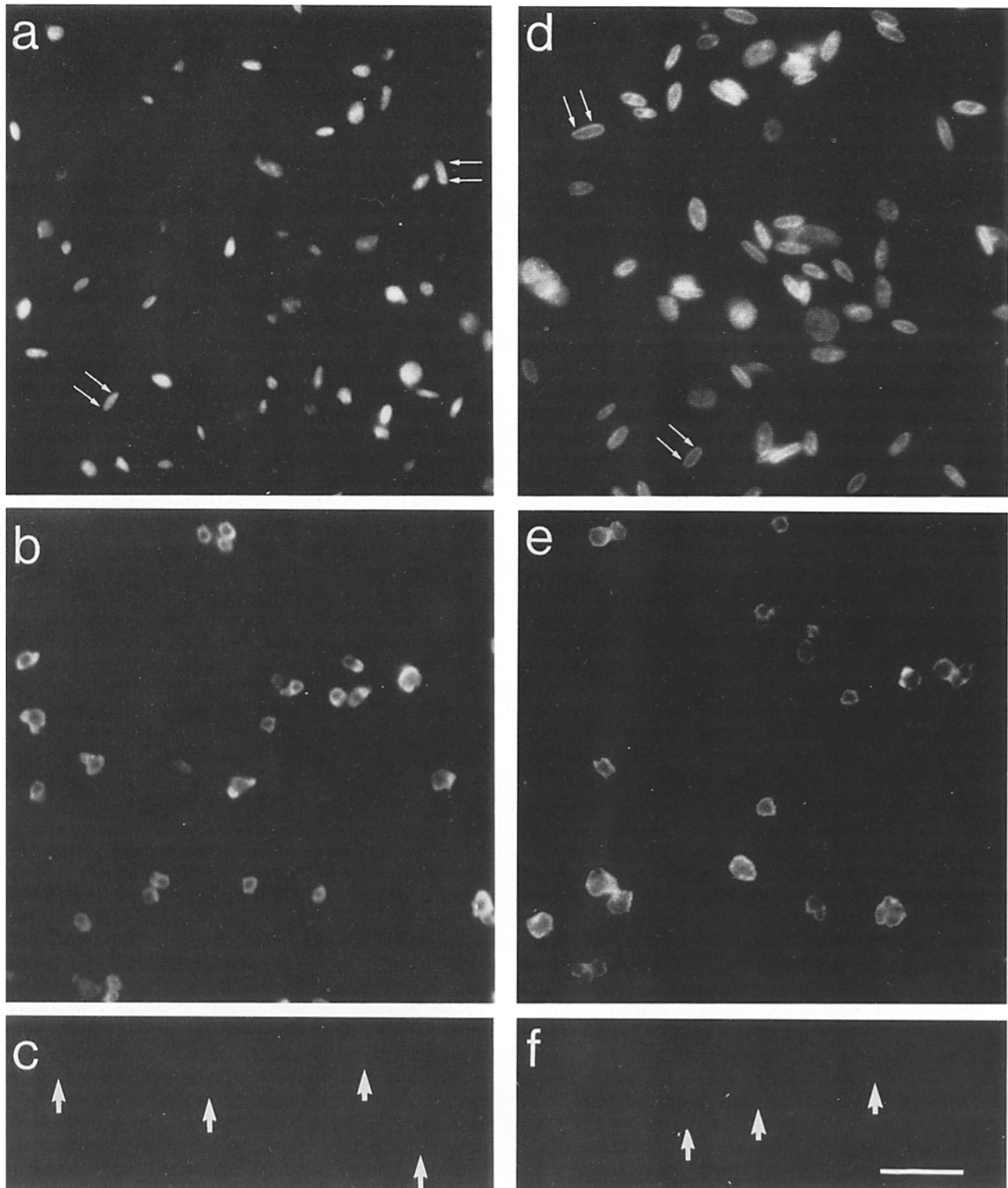


Figure 1. Morphology of platelets. Resting (*a*), thrombin-activated (*b*), and substratum-adherent (*c*) platelets visualized by Nomarski differential interference contrast microscopy. Bar, 10  $\mu$ m.

Talin

gpIIb-IIIa



**Figure 2.** Comparison of the distribution of talin and gpIIb-IIIa in resting and thrombin-activated platelets by indirect immunofluorescence. (a-c) Distribution of talin. (a) Resting platelets stained with anti-talin antibody. Talin appears to be uniformly distributed throughout the cytoplasm. (b) In platelets that had been exposed to 0.1 NIH U/ml of thrombin for 1 min, talin has redistributed to the cell periphery giving rise to rings of fluorescence. This platelet sample was not stirred, and no macroscopic aggregates were observed during the incubation period. (c) Preimmune serum does not label activated platelets. The positions of the cells are indicated by the large arrows. (d-e) Distribution of gpIIb-IIIa. (d) The membranous distribution of gpIIb-IIIa is apparent in resting cells that are lying on their sides (paired arrows). (e) Peripheral rings of fluorescence, indicating the subcellular location of gpIIb-IIIa, are visible in activated platelets. (f) Negligible staining is obtained with preimmune serum (large arrows). Bar, 10  $\mu$ m.

labeled platelets using a laser scanning confocal microscope (data not shown). The platelet sample shown in Fig. 2 *b* was exposed to thrombin for 1 min at 22–24°C in the absence of stirring before being fixed for indirect immunofluorescence. The fact that the redistribution of talin in response to thrombin occurs in the absence of stirring indicates that platelet aggregation is not necessary to stimulate the change in talin's subcellular location. The thrombin-triggered redistribution of talin to the cell periphery does not appear to be a transient phenomenon since talin exhibits this subcellular localization in platelets exposed to thrombin for up to 30 min (data not shown). Indirect immunofluorescent labeling of platelets with preimmune serum results in extremely faint staining of activated (Fig. 2 *c*) or resting platelets.

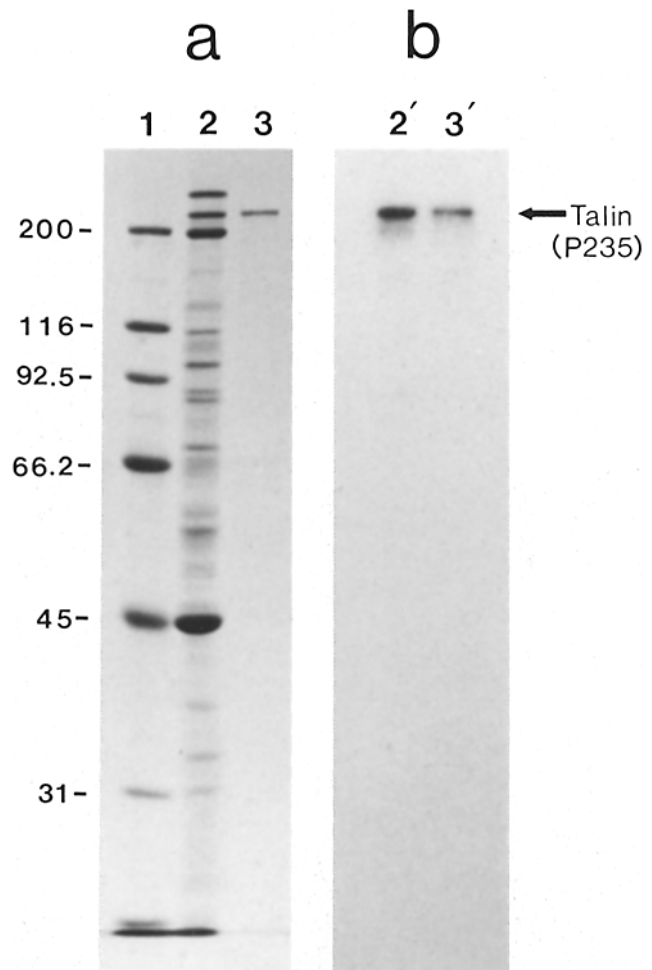
To determine whether the talin distribution noted in resting platelets could result if talin were membrane associated in unactivated cells, we have compared talin's distribution in resting and activated platelets with the distribution of the well-characterized platelet membrane glycoprotein complex, gpIIb–IIIa. In contrast with the staining pattern obtained with anti-talin antiserum, when antibodies directed against gpIIb–IIIa are used, both resting (Fig. 2 *d*) and activated (Fig. 2 *e*) platelets exhibit a narrow peripheral band of fluorescence that defines the boundaries of the cells. In resting platelets this peripheral staining is primarily evident when the cells are attached to the coverslip via a side edge (Fig. 2 *d*, *paired arrows*). (Compare with the uniform distribution of talin in cells in this orientation; Fig. 2 *a*, *paired arrows*.) The peripheral staining pattern we have observed with antibody against gpIIb–IIIa is evident in both detergent-permeabilized and unpermeabilized cells. Preimmune serum again resulted in very low background labeling of the cells (Fig. 2 *f*).

These observations suggest that talin and gpIIb–IIIa exhibit distinct subcellular locations in resting platelets. gpIIb–IIIa is a transmembrane glycoprotein that has been shown to be associated with the plasma membrane as well as certain classes of internal membranes in resting cells (Wencel-Drake et al., 1986). Talin, on the other hand, does not exhibit a discrete membrane-localized distribution in unactivated platelets. Both talin and gpIIb–IIIa are concentrated at the cell periphery in thrombin-activated cells.

#### Characterization of the Subcellular Distribution of Talin in Platelets by Immunoelectron Microscopy

We have analyzed the distribution of talin in platelets at higher resolution in the electron microscope by immunogold labeling. For these studies, affinity-purified antibodies against talin were used. The specificity of the affinity-purified anti-talin antibody was determined by Western immunoblot analysis (Fig. 3). The antibody recognizes purified platelet talin (Fig. 3, lane 3') as well as a protein corresponding to platelet talin in samples of total human platelet proteins (Fig. 3, lane 2'). Some faint lower molecular weight immunoreactive bands can be detected in the sample of total platelet protein (Fig. 3, lane 2'); these represent proteolytic fragments of talin present in this sample of platelet proteins.

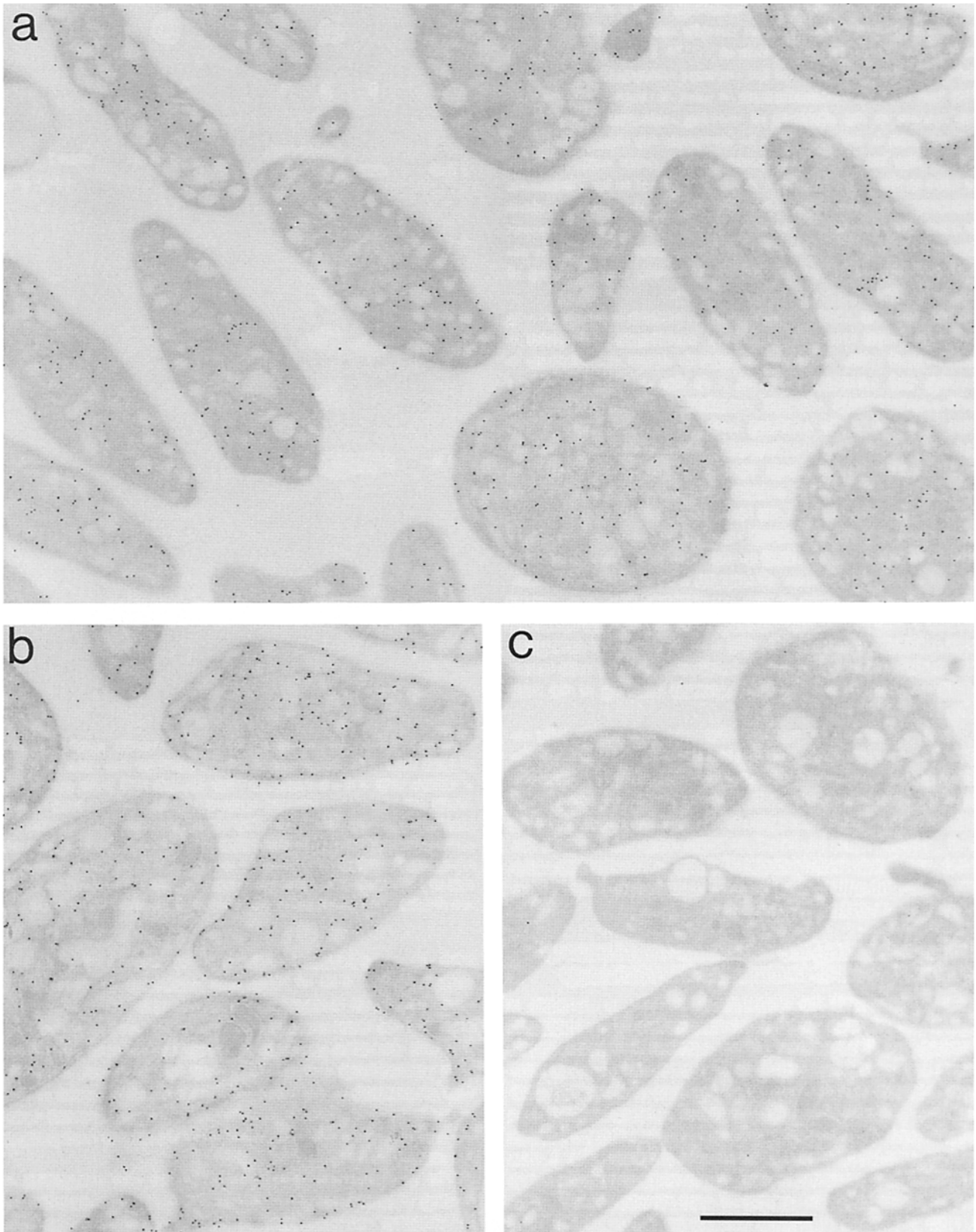
For immunoelectron microscopic localization of talin in resting platelets, the cells were fixed in suspension, pelleted, dehydrated, embedded in LR White, and thin sectioned. Sections were labeled with affinity-purified anti-talin antibody followed by gold-conjugated protein A. The distribution of



**Figure 3.** Characterization of antibody directed against human platelet talin. The specificity of affinity-purified anti-talin antibody is shown here. *a* is a Coomassie blue-stained 10% SDS-polyacrylamide gel: (lane 1) molecular mass standards; (lane 2) total human platelet proteins; (lane 3) purified human platelet talin. *b* shows the corresponding Western immunoblot. The affinity-purified antibody recognizes purified talin (lane 3') as well as a comigrating polypeptide of 235,000 D in the platelet extract. A 200-kD proteolytic product of talin is also detected in this sample of platelet extract (lane 2').

talin in resting platelets isolated in the absence (Fig. 4 *a*) or presence (Fig. 4 *b*) of PGE<sub>1</sub> was examined. In both cases, talin was found throughout the cytoplasm. Anti-talin antibody did not label intracellular organelles such as mitochondria and secretory granules. Although some gold associated with intracellular membranes could be identified, a significant proportion of the label was not located adjacent to any identifiable subcellular organelle. Very low background staining was observed when sections were incubated with protein A-gold alone (Fig. 4 *c*).

The gold particle distributions in the two categories of resting platelets (plus or minus PGE<sub>1</sub>) were analyzed by quantitative morphometry as described in Materials and Methods. Fig. 5 shows the distribution of immunogold-labeled talin in the two categories of resting cells as a function of distance from the plasma membrane. There was no statistically significant difference between the distributions of gold particles in resting platelets whether or not they were first treated with PGE<sub>1</sub>. In both categories of resting plate-



**Figure 4.** Distribution of talin in resting platelets. Immunogold labeling of talin in thin sections of resting human platelets isolated in the absence (a) or presence (b) of PGE<sub>1</sub>. Talin appears to be uniformly distributed throughout the cytoplasm of resting cells. (c) Control. Thin sections of resting platelets were labeled with protein A-gold alone. Very little nonspecific labeling is observed. Bar, 1  $\mu$ m.

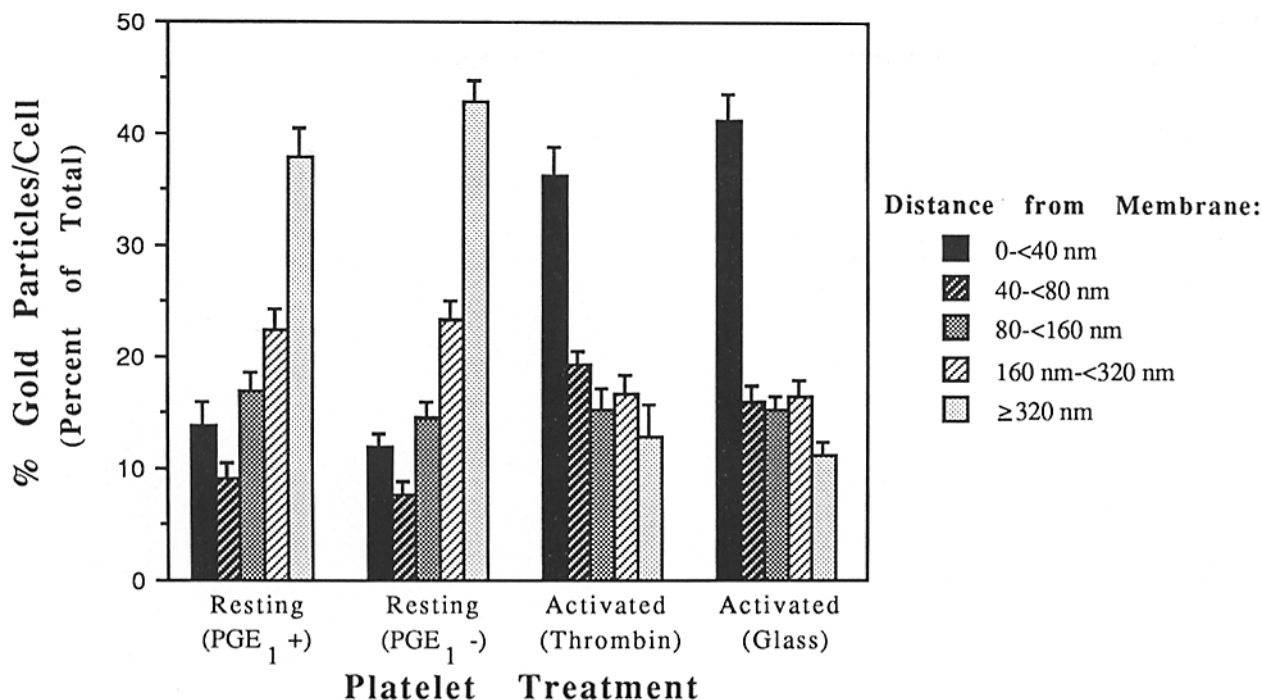


Figure 5. Analysis of the distribution of talin in resting and activated platelets. The histograms show the percentage of gold label per cell found at various distances from the plasma membrane in resting (both plus and minus PGE<sub>1</sub>), thrombin-activated, and glass-activated platelets. Data are expressed as mean  $\pm$  SEM.

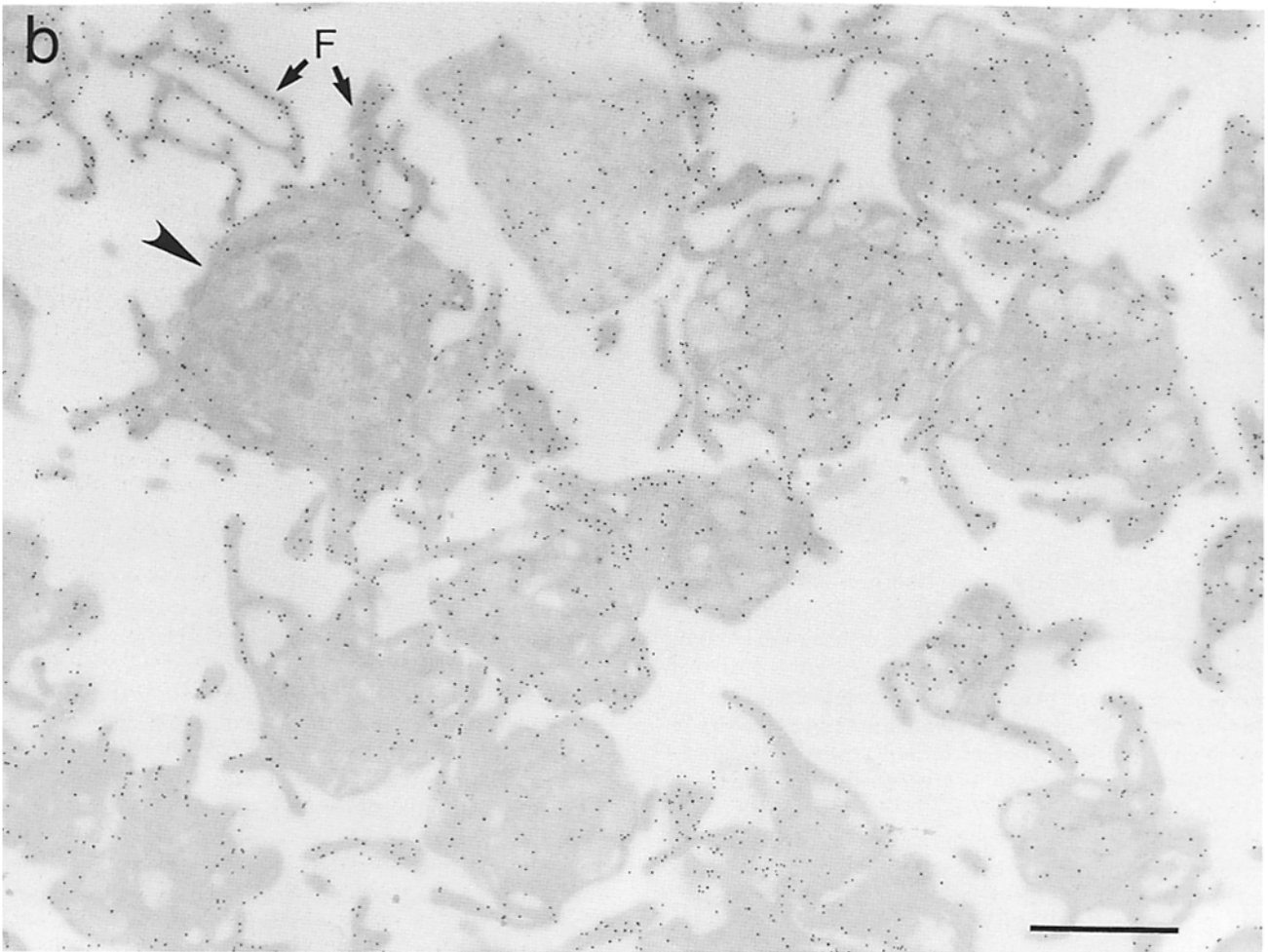
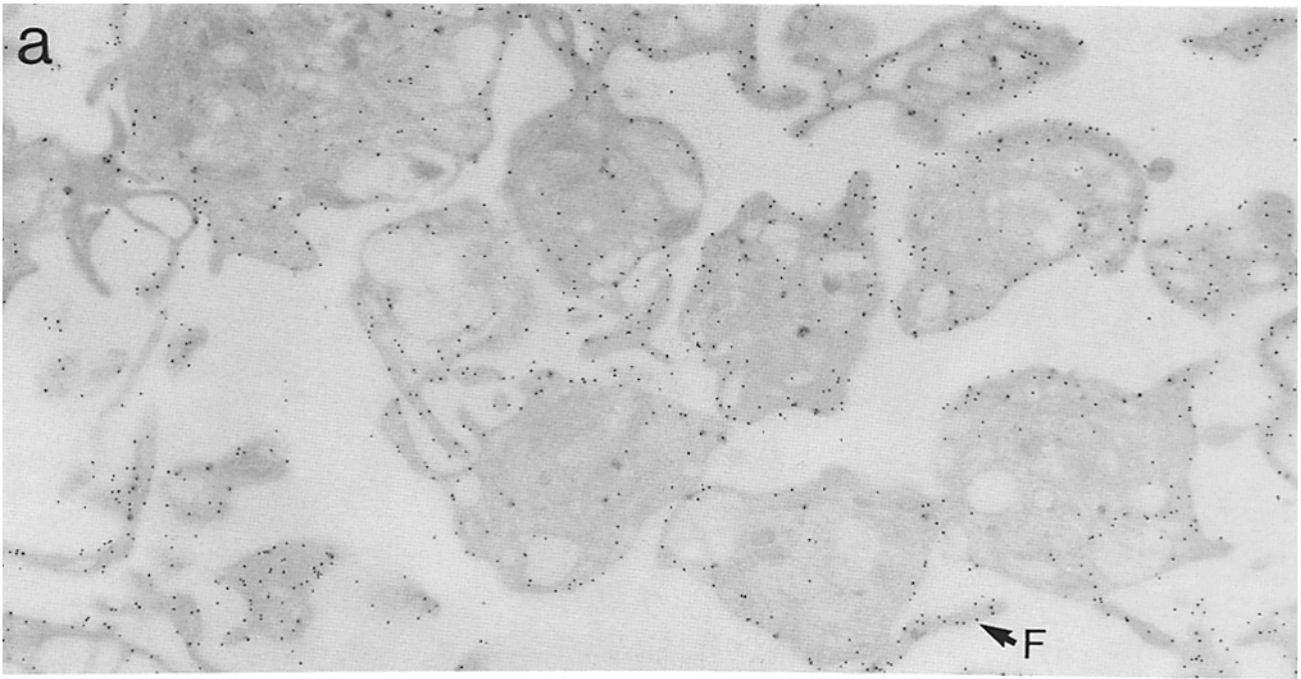
lets, the majority of the gold particles were found >80 nm away from the plasma membrane. In the remainder of this paper, only the data from non-PGE<sub>1</sub>-treated resting cells were used for comparison with the activated cells since the activated cells were not pretreated with PGE<sub>1</sub>.

We have examined the distribution of talin by immunoelectron microscopy in two populations of activated platelets, cells activated with thrombin and cells activated by contact with glass. The distribution of talin in platelets activated by exposure to thrombin (0.1 NIH U/ml) for 1 min and visualized by immunoelectron microscopy is shown in Fig. 6. There are large numbers of filopodia that are extensively labeled with anti-talin antibody. (These were excluded from the quantitative analysis because of their small cross-sectional diameters.) The distribution of immunogold-labeled talin in thrombin-activated platelets as a function of the distance of the gold from the plasma membrane is illustrated in Fig. 5 and is significantly different from the distribution of talin in resting cells ( $p < 0.0001$ ). Most notably, there is a dramatic increase in the percentage of the total cellular gold particles located within 40 nm of the membrane. Since talin is a 60-nm flexible rod at physiological ionic strength (Molony et al., 1987), talin located within 40 nm of the membrane could be directly associated with some membrane-bound component.

To study the distribution of talin in a population of activated, substratum-attached cells, we examined the distribution of talin in platelets that were allowed to spread on glass coverslips. These cells offer the additional advantage that, unlike thrombin-activated cells, they do not exhibit a dense array of centralized granules that could, in theory, exclude talin nonspecifically from the central cytoplasm. Furthermore, we examined well-spread cells that no longer exhibited numerous thin filopodial extensions. When the platelets are

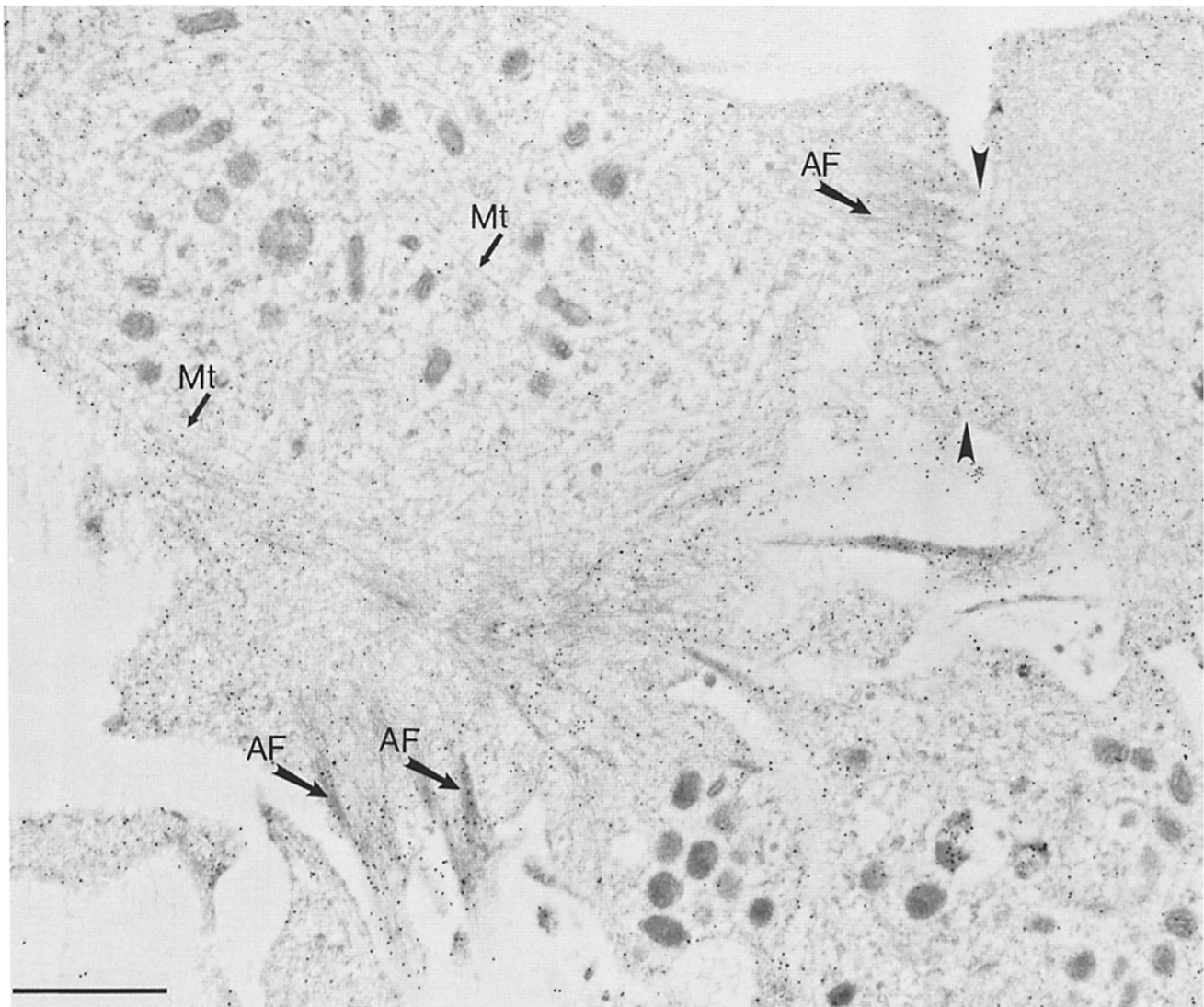
sectioned parallel to the substratum, it is possible to obtain en face sections through the spread platelets at a level very close to the glass substratum (Fig. 7). In such sections, one can see the bundles of actin filaments impinging upon the plasma membrane of the cell in a manner very similar to what occurs with fibroblast stress fibers. Accumulations of talin were typically observed in association with actin filament bundles near the plasma membrane. By analogy with the fibroblast, the termini of the actin filament bundles would be expected to occur at regions of close membrane-substratum contact. In addition, talin was found associated with the plasma membrane in areas where no contact with the glass coverslip had occurred; this is apparent when deeper sections through the substratum-attached platelets are cut and labeled with anti-talin antibody (Figs. 8 and 9). Under the conditions in which these cells were activated, it is likely that extracellular ligands, such as fibrinogen, were associated with the platelet plasma membranes (Stenberg et al., 1984). Quantitative morphometric analysis (Fig. 5) confirms that the distribution of talin in the glass-activated cells is similar to that in thrombin-activated cells and significantly different from that in resting cells ( $p < 0.0001$ ).

We have compared the extent of anti-talin antibody labeling at the plasma membrane (0-40-nm category) in resting with activated platelets (Table I). We have analyzed the gold particle distribution data in two ways. First, we have determined the percentage of gold particles per cell that are found in close association with the plasma membrane; this normalizes for any differences in the absolute amount of gold counted per cell. We have compared the percentages of membrane-associated gold particles in resting, thrombin-activated, and glass-activated cells. In resting platelets, an average of 11.8% of the total gold per cell is found within 40 nm of the plasma membrane, whereas, in thrombin- or glass-activated cells,



**Figure 6.** (*a* and *b*) Distribution of talin in thrombin-activated platelets. Platelets were activated with 0.1 NIH U/ml thrombin for 1 min. Many filopodia (*F*) extending from the activated cells are densely labeled with anti-talin antibody. In some cells (*arrowhead*), it is apparent by visual inspection that talin is localized in the region underlying the plasma membrane. Bar, 1  $\mu$ m.





**Figure 7.** En face section through a platelet allowed to spread on a glass coverslip. Microtubules (*Mt*) and bundles of actin filaments (*AF*) can be seen. In this substratum-adjacent section, immunogold-labeled talin is concentrated at the cell periphery, particularly in regions that are rich in actin filament bundles. The termini of actin filament bundles occur at regions of cell-cell contact (area between large arrowheads) and, by analogy with other systems, cell-substratum contact. Immunogold labeling of talin is dense in these regions. Bar, 1  $\mu\text{m}$ .

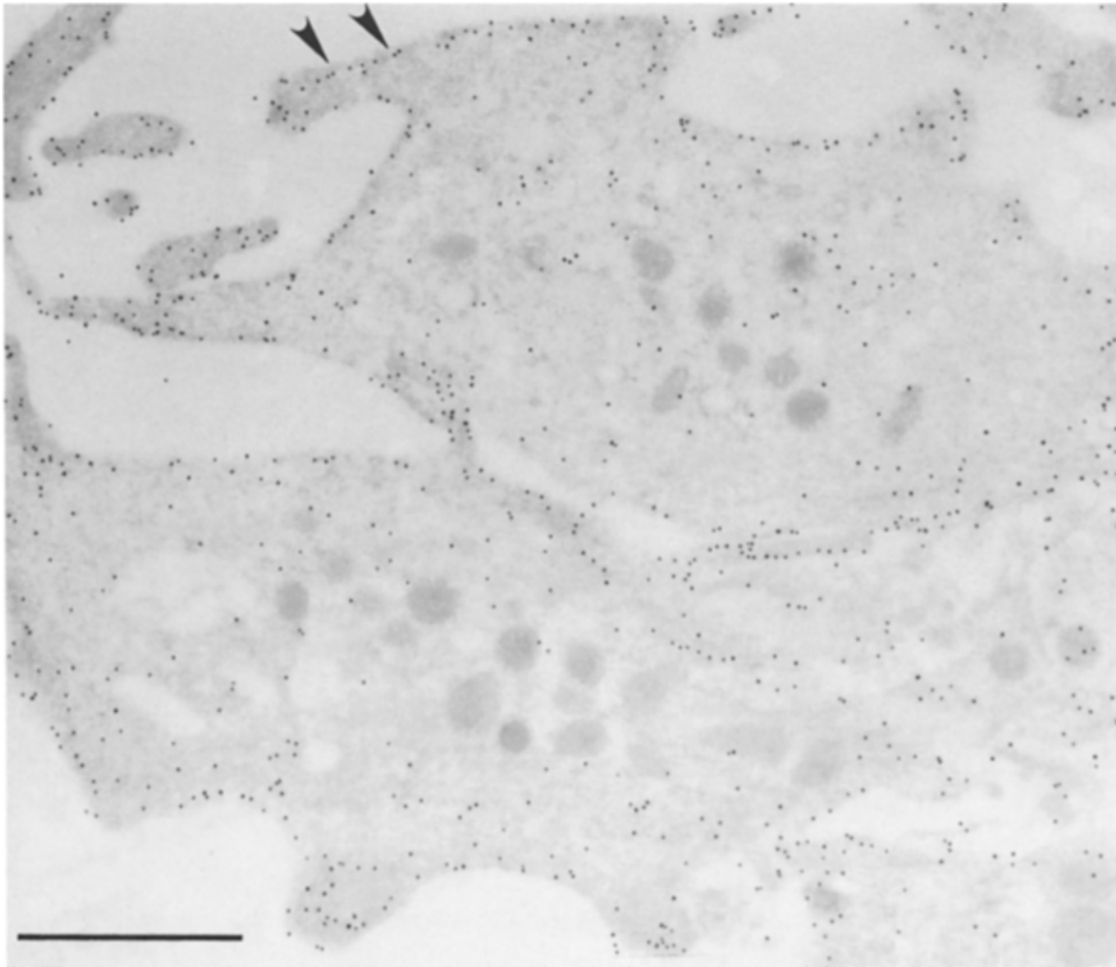
the percentage of gold in this region increases significantly to 36.1 ( $p < 0.0001$ ) and 41.1% ( $p < 0.0001$ ), respectively.

In addition, we have measured the density of membrane-associated label in resting and thrombin-activated cells by determining the number of gold particles per micron of plasma membrane; this normalizes for variation in cell surface area. Statistical analysis of these data reveals that the density of gold particles near the plasma membrane increases significantly when the cells are activated by thrombin ( $p < 0.001$ ), whereas the overall particle density per square micrometer remains constant (data not shown). The number of gold particles at the plasma membrane increased from 1.9 gold particles/ $\mu\text{m}$  in resting cells to 4.1 gold particles/ $\mu\text{m}$  in thrombin-activated cells.

#### ***Calcium-dependent Proteolysis of Talin Does Not Precede its Redistribution***

Since calcium-dependent proteolysis of talin has been shown to occur in platelets in response to thrombin, we have inves-

tigated the possibility that cleavage of talin is occurring before talin redistribution. The calcium-dependent protease cleaves talin at a single site to generate two proteolytic products of 200 and 46 kD (Fox et al., 1985; O'Halloran et al., 1985). Our anti-talin antibodies recognize both the intact and 200-kD cleavage product of talin (Beckerle et al., 1986). By Western immunoblot analysis of platelet proteins prepared from resting and thrombin-activated platelets, we have detected no proteolytic cleavage of talin within 1 min of thrombin stimulation (Fig. 10), although significant talin redistribution had occurred by that time. We have noted the appearance of the 200-kD calcium-dependent proteolytic cleavage product of talin after longer periods of thrombin stimulation in stirred platelet samples. Our observations are consistent with the results of Fox and co-workers (1985) who reported that talin cleavage by the calcium-dependent protease is dependent on the platelet aggregation response. We have shown here that the redistribution of talin to the plasma membrane in activated platelets is independent of platelet aggregation.



**Figure 8.** Talin distribution in substratum-adherent platelets. This section is farther away from the substratum than is shown in Fig. 7. Consequently, bundles of actin filaments are not prominent. Talin is concentrated near the plasma membrane (*arrowheads*). Little label is found in the central cytoplasm. Bar, 1  $\mu\text{m}$ .

## Discussion

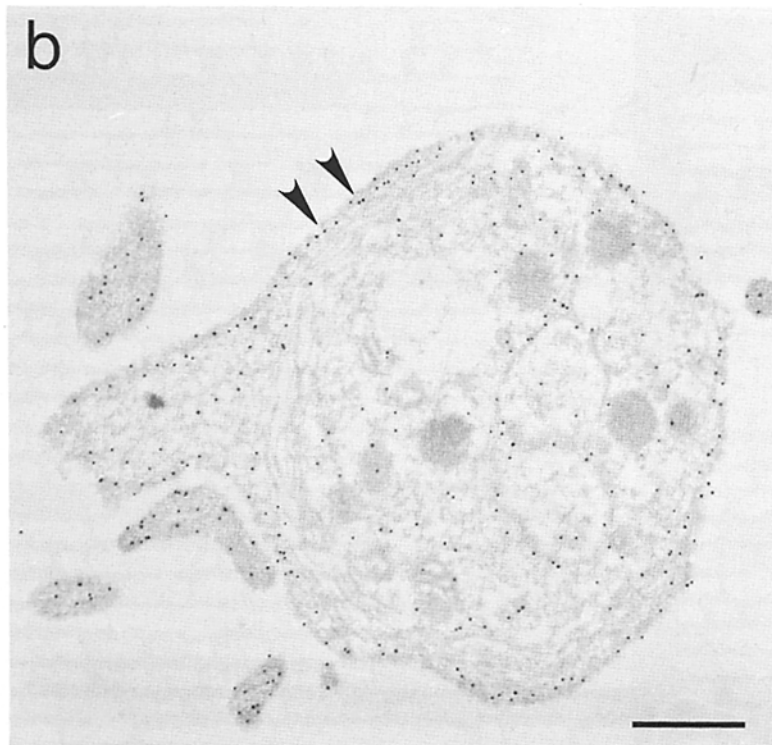
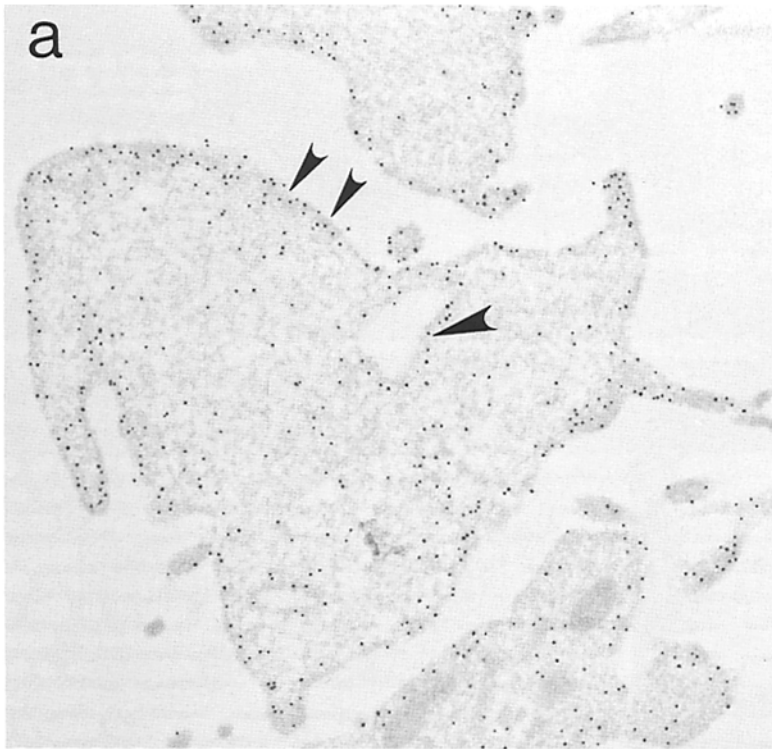
We have previously demonstrated that talin, a component of adhesion plaques in fibroblasts, is a major platelet protein (O'Halloran et al., 1985). In this paper we report that talin undergoes a dramatic change in subcellular distribution in response to platelet activation. In resting, discoid human platelets, the majority of talin is found in the cytoplasm. In contrast, in thrombin- or glass-activated platelets talin becomes concentrated at the cell periphery underlying the plasma membrane.

Although many of the gold particles in resting platelets appear to be located in the cytoplasmic matrix rather than in association with specific intracellular organelles or membrane-bound vesicles, we cannot completely exclude the possibility that some population of talin in resting cells is associated specifically with intracellular membranes or elements of the surface-connected canalicular system. However, if talin were extensively localized on a subset of intracellular membranes, we expect that we would have detected that localization in our immunoelectron microscopy studies. Others have shown, for example, that gpIIb-IIIa is associated with both the plasma membrane as well as certain classes of intra-

cellular vesicles using immunoelectron microscopic methods (Wencel-Drake et al., 1986). Furthermore, the distribution of talin we have observed in resting platelets is not unique to human cells. We have also observed a general cytoplasmic distribution of talin in resting, avian thrombocytes of immunoelectron microscopy (Drenckhahn et al., 1988).

The platelet response to activating agents is complex. Upon exposure to thrombin, for example, platelets reorganize their cytoskeletons, undergo a dramatic change in cell shape, secrete the contents of intracellular granules, bind extracellular ligands such as fibrinogen, and aggregate with each other upon contact (Zucker and Nachmias, 1985). We have reported here that the subcellular distribution of talin changes in response to platelet activation. We have shown that platelet aggregation is not a prerequisite to talin redistribution, but we do not yet know which of the other stages in the platelet response most closely correlates with the redistribution of talin.

Some previously reported experimental observations provide biochemical support for the idea that talin is indeed more closely associated with the platelet plasma membrane in activated cells. When membrane-permeable cross-linking agents were used to identify sets of interacting proteins in in-



**Figure 9.** Talin is localized at the cell periphery in activated, adherent platelets. (*a* and *b*) Tangential, immunogold-labeled sections through platelets adhering to a glass coverslip. In these activated platelets, talin is found predominantly at the cell periphery (*paired arrowheads*). A cross section through a cell surface invagination is marked by a single arrowhead. Bar, 0.5  $\mu\text{m}$ .

tact resting and activated platelets, an abundant 230-kD protein (presumably talin) was incorporated into a high molecular weight complex with a number of other proteins including gpIIb-IIIa exclusively in thrombin-activated platelets (Davies and Palek, 1982).

The biochemical compositions of Triton X-100-insoluble "cytoskeletons" prepared from resting and activated platelets

have also been compared by a number of investigators in an effort to define activation-dependent changes in the composition of the platelet cytoskeleton. Somewhat surprisingly, talin is not found in stable association with Triton X-100-insoluble material isolated from either resting or activated platelets even though the transmembrane glycoprotein complex, gpIIb-IIIa, is present in the detergent-insoluble residue

Table I. Analysis of Talin Associated with the Plasma Membrane in Resting and Activated Platelets\*

Platelet treatment	Gold particles counted	Membrane-associated gold particles per cell (percent of total) <sup>‡</sup> §	Number of gold particles per micrometer of membrane <sup>‡</sup> §**
Resting (without PGE <sub>1</sub> )	1,147 (n = 10 cells)	11.8 ± 1.4 (a)	1.9 ± 0.3 (a)
Activated (thrombin)	760 (n = 10 cells)	36.1 ± 2.8 (b)	4.1 ± 0.4 (b)
Activated (glass)	1,335 (n = 10 cells)	41.1 ± 2.6 (b)	ND <sup>‡‡</sup>

\* All gold particles falling within 40 nm of the plasma membrane were considered to be membrane associated.

‡ Each value represents the mean ± SEM. Values within columns with different letters have significantly different means.

§ Mean values are based on untransformed data.

||  $p < 0.0001$ .

\*\*  $p < 0.001$ .

‡‡ These data were not included in the statistical analysis since the immunolabeling of the glass-activated cells was performed on different days than were resting and thrombin-activated cell labeling experiments.

(operationally defined as cytoskeletons) isolated from aggregated platelets (for example see Phillips et al., 1980; Jennings et al., 1981; Rogman et al., 1982). We have observed that talin is easily extracted from the adhesion plaques of cultured cells by a variety of nonionic detergents. Since talin's association with focal contacts is detergent labile, it is possible that interactions between talin and the platelet membrane and cytoskeleton are undetectable in the presence of Triton X-100.

In chicken embryo fibroblasts, talin and the transmembrane heterodimer, integrin, are colocalized at sites of substratum adhesion (Damsky et al., 1985) and the two proteins

have been shown to interact with each other in vitro by an equilibrium gel filtration binding assay (Horwitz et al., 1986). The association between talin and integrin is mediated at least in part by the cytoplasmic domain of the integrin  $\beta_1$  chain (Buck and Horwitz, 1987). The cytoplasmic domain of chicken  $\beta_1$  integrin is closely related to that of platelet gpIIIa, also an integrin  $\beta$  chain (Tamkun et al., 1986; Fitzgerald et al., 1987). The structural conservation of the protein domain involved in talin binding raises the possibility that talin binding will be a conserved function of integrin  $\beta$  chain cytoplasmic domains. In fact, recent preliminary results suggest that human platelet talin interacts directly with the platelet integrin, and gpIIb-IIIa interacts via its  $\beta$  chain, gpIIIa (Isenberg, W. M., J. E. B. Fox, and D. E. Phillips, unpublished observations).

Other members of the integrin family of transmembrane glycoprotein complexes are also found on platelets (Giancotti et al., 1987; Piotrowicz et al., 1988; Hemler et al., 1988). Because these proteins are structurally and immunologically related to the chicken  $\beta_1$  integrin that binds talin (Piotrowicz et al., 1988; Takada et al., 1987; Marcantonio and Hynes, 1988), they emerge as candidates for linking talin to the plasma membrane. More work obviously needs to be done to define the biochemical basis for talin's localized distribution in activated platelets. Although integrins may be involved, other proteins such as platelet vinculin (Rosenfeld et al., 1985) or some as yet unanticipated protein may also be playing significant roles in determining the subcellular distribution of talin.

The observations reported in this paper raise the question of how talin's subcellular distribution is regulated in vivo. What molecular event(s) associated with platelet activation induces the movement of talin from its apparently diffuse location in the cytoplasm of resting cells to the more discrete subplasmalemmal position it occupies in activated cells? Talin function in platelets has been proposed to be regulated to some extent by calcium-dependent proteolysis. When platelets are activated by exposure to a physiological stimulus such as thrombin, specific proteolysis of talin occurs (Fox et al., 1985). We have found that the activation-dependent redistribution of talin precedes talin cleavage. The onset of calcium-dependent proteolysis of talin occurs later and corresponds to the period of platelet aggregation and clot retraction; consequently, it seems more likely that cleavage of talin is somehow required for execution of these platelet functions (Fox et al., 1985). Interestingly, the calcium-dependent protease has, as shown here for talin, been reported to exhibit

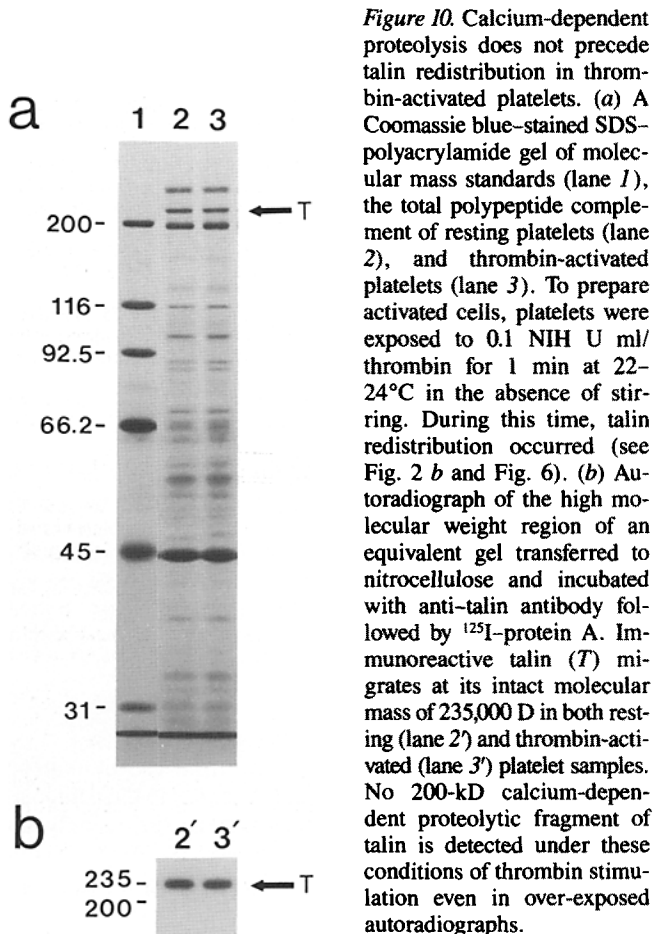


Figure 10. Calcium-dependent proteolysis does not precede talin redistribution in thrombin-activated platelets. (a) A Coomassie blue-stained SDS-polyacrylamide gel of molecular mass standards (lane 1), the total polypeptide complement of resting platelets (lane 2), and thrombin-activated platelets (lane 3). To prepare activated cells, platelets were exposed to 0.1 NIH U/ml thrombin for 1 min at 22–24°C in the absence of stirring. During this time, talin redistribution occurred (see Fig. 2 b and Fig. 6). (b) Autoradiograph of the high molecular weight region of an equivalent gel transferred to nitrocellulose and incubated with anti-talin antibody followed by <sup>125</sup>I-protein A. Immunoreactive talin (T) migrates at its intact molecular mass of 235,000 D in both resting (lane 2') and thrombin-activated (lane 3') platelet samples. No 200-kD calcium-dependent proteolytic fragment of talin is detected under these conditions of thrombin stimulation even in over-exposed autoradiographs.

an activation-dependent redistribution to the plasma membrane in human platelets (Okita, J. R., J. D. Wencel-Drake, and T. J. Kunicki, unpublished observations). Although the specific role of talin proteolysis in the platelet has not yet been determined, it is interesting to note that the calcium-dependent protease is colocalized with talin at sites of substratum adhesion in fibroblasts and epithelial cells (Beckerle et al., 1987). The codistribution of these two proteins in a variety of cell types raises the possibility that regulation of talin by the calcium-dependent protease may have broad relevance for control of talin function at sites of cell-substratum adhesion.

Some recent experiments have raised the interesting possibility that protein kinase C-mediated phosphorylation events may participate in regulating talin's distribution within cells. Burn and co-workers (1988) have examined talin's ability to associate with antibody-induced integrin caps on lymphocytes *in vivo*. They found that talin accumulates at the region of the integrin cap only if the lymphocytes had been exposed to the tumor promoting phorbol ester, phorbol 12-myristate 13-acetate, before cap generation. The major known effect of phorbol 12-myristate 13-acetate on cells is that it stimulates protein kinase C activity. Therefore, a likely interpretation of these results is that talin's ability to be redistributed to the cell periphery underlying integrin caps is regulated, either directly or indirectly, by protein kinase C-mediated phosphorylation events. Since talin has been shown to be a substrate for protein kinase C *in vitro* (Litchfield and Ball, 1986), it will be interesting to examine whether talin phosphorylation by protein kinase C affects its subcellular distribution or its affinity for membrane-associated ligands such as integrin. Additional experiments are clearly required to define the mechanism by which talin's subcellular distribution is regulated.

In summary, we have found that talin, a major protein in platelets and a component of adhesion plaques in cultured cells, undergoes an activation-dependent redistribution in intact human platelets. In resting platelets, talin is uniformly distributed throughout the cytoplasm. However, upon platelet activation and adhesion, talin becomes concentrated at the cell surface underlying the plasma membrane. Because the adhesive properties of intact platelets can be readily manipulated *in vitro*, these cells provide an extremely useful model system for defining the mechanism(s) by which talin distribution and function is regulated in living cells.

We are particularly grateful to Laszlo Kömüves for providing us with an excellent immunogold reagent. In addition, we appreciate the valuable technical advice of Ed King, Detlev Drenckhahn, Jim Tidball, David Williams, and Marjorie Zucker and the skilled typing of Maurine Vaughan. We are also grateful to Clyde Lulhum who, during a demonstration of the Bio-Rad Laboratories confocal microscope, performed the optical sectioning of fluorescently labeled platelets. We also thank our colleagues in the Department of Biology, in particular Dave Gard, for help with the digitizer and statistical analysis, as well as critical reading of this manuscript.

This work was supported by grants from the National Science Foundation (DCB 8602131), the American Heart Association (86 677), and, most recently, the National Institutes of Health (HL 41553) to M. C. Beckerle. M. C. Beckerle is an Established Investigator of the American Heart Association.

Received for publication 16 January 1989 and in revised form 14 August 1989.

## References

- Ali, I. U., and R. O. Hynes. 1977. Effects of cytochalasin B and colchicine on attachment of a major surface protein of fibroblasts. *Biochim. Biophys. Acta.* 471:16-24.
- Ali, I. U., V. Mautner, R. Lanza, and R. O. Hynes. 1977. Restoration of normal morphology, adhesion, and cytoskeleton in transformed cells by addition of a transformation-sensitive surface protein. *Cell.* 11:115-126.
- Argraves, W. S., K. Dickerson, W. H. Burgess, and E. Ruoslahti. 1989. Fibulin, a novel protein that interacts with the fibronectin receptor  $\beta$  subunit cytoplasmic domain. *Cell.* 58:623-629.
- Beckerle, M. C. 1986. Identification of a new protein localized at sites of cell-substrate adhesion. *J. Cell Biol.* 103:1679-1687.
- Beckerle, M. C., T. O'Halloran, and K. Burridge. 1986. Demonstration of a relationship between talin and P235, a major substrate of the calcium-dependent protease in human platelets. *J. Cell. Biochem.* 30:259-270.
- Beckerle, M. C., K. Burridge, G. N. DeMartino, and D. E. Croall. 1987. Colocalization of calcium-dependent protease II and one of its substrates at sites of cell adhesion. *Cell.* 51:569-577.
- Bennett, J. S., G. Vilaire, and D. B. Cines. 1982. Identification of the fibrinogen receptor on human platelets by photoaffinity labeling. *J. Biol. Chem.* 257:8049-8054.
- Buck, C. A., and A. F. Horwitz. 1987. Integrin, a transmembrane glycoprotein complex mediating cell-substratum adhesion. *J. Cell. Sci. Suppl.* 8:231-250.
- Burn, P., A. Kupfer, and S. J. Singer. 1988. Dynamic membrane-cytoskeletal interactions: specific association of integrin and talin arises *in vivo* after phorbol ester treatment of peripheral blood lymphocytes. *Proc. Natl. Acad. Sci. USA.* 85:497-501.
- Burridge, K., and L. Connell. 1983. A new protein of adhesion plaques and ruffling membranes. *J. Cell Biol.* 97:359-367.
- Burridge, K., and P. Mangeat. 1984. An interaction between vinculin and talin. *Nature (Lond.).* 308:744-745.
- Collier, N. C., and K. Wang. 1982. Purification and properties of human platelet P235. *J. Biol. Chem.* 257:6937-6943.
- Damsky, C. H., K. A. Knudsen, D. Bradley, C. A. Buck, and A. F. Horwitz. 1985. Distribution of cell-substratum attachment (CSAT) antigen on myogenic and fibroblastic cells in culture. *J. Cell Biol.* 100:1528-1539.
- Davies, G. E., and J. Palek. 1982. Platelet protein organization: analysis by treatment with membrane-permeable cross-linking reagents. *Blood.* 59:502-513.
- Drenckhahn, D., M. C. Beckerle, K. Burridge, and J. Otto. 1988. Identification and subcellular location of talin in various cell types and tissues by means of vinculin overlay, immunoblotting and immunocytochemistry. *Eur. J. Cell Biol.* 46:513-522.
- Fitzgerald, L. A., B. Leung, and D. R. Phillips. 1985. A method for purifying the platelet membrane glycoprotein IIb-IIIa complex. *Anal. Biochem.* 151:169-177.
- Fitzgerald, L. A., B. Steiner, S. C. Rall, S.-S. Lo, and D. R. Phillips. 1987. Protein sequence of endothelial glycoprotein IIIa derived from a cDNA clone: identity with platelet glycoprotein IIIa and similarity to integrin. *J. Biol. Chem.* 262:3936-3939.
- Fox, J. E. B., J. K. Boyles, C. C. Reynolds, and D. R. Phillips. 1984. Actin filament content and organization in unstimulated platelets. *J. Cell Biol.* 98:1985-1991.
- Fox, J. E. B., D. E. Goll, C. C. Reynolds, and D. R. Phillips. 1985. Identification of two proteins (actin-binding protein and P235) that are hydrolyzed by endogenous  $Ca^{2+}$ -dependent protease during platelet aggregation. *J. Biol. Chem.* 260:1066-1066.
- Geiger, B. 1979. A 130K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. *Cell.* 18:193-205.
- Geiger, B., T. Volk, and T. Volberg. 1985. Molecular heterogeneity of adhesion junctions. *J. Cell Biol.* 101:1523-1531.
- Giancotti, F. G., L. R. Languino, A. Zanetti, G. Peri, G. Tarone, and E. Dejana. 1987. Platelets express a membrane protein complex immunologically related to the fibroblast fibronectin receptor and distinct from gpIIb-IIIa. *Blood.* 69:1535-1538.
- Ginsberg, M. H., L. Taylor, and R. G. Painter. 1980. The mechanism of thrombin-induced platelet factor 4 secretion. *Blood.* 55:661-668.
- Hawiger, J., S. Parkinson, and S. Timmons. 1980. Prostacyclin inhibits mobilization of fibrinogen binding sites on human ADP- and thrombin-treated platelets. *Nature (Lond.).* 283:195-197.
- Hemler, M. E., C. Crouse, Y. Takada, and A. Sonnenberg. 1988. Multiple very late antigen (VLA) heterodimers on platelets: evidence for distinct VLA-2, VLA-5 (fibronectin receptor) and VLA-6 structures. *J. Biol. Chem.* 263:7660-7665.
- Horwitz, A., K. Duggan, C. Buck, M. C. Beckerle, and K. Burridge. 1986. Interactions of plasma membrane fibronectin receptor with talin—a transmembrane linkage. *Nature (Lond.).* 320:531-533.
- Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48:549-554.
- Jennings, L. K., J. E. B. Fox, H. H. Edwards, and D. R. Phillips. 1981.

- Changes in the cytoskeletal structure of human platelets following thrombin activation. *J. Biol. Chem.* 256:6927-6932.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Litchfield, D. W., and E. H. Ball. 1986. Phosphorylation of the cytoskeletal protein talin by protein kinase C. *Biochem. Biophys. Res. Commun.* 134:1276-1283.
- Marcantonio, E., and R. D. Hynes. 1988. Antibodies to a conserved cytoplasmic domain of the integrin  $\beta_1$  subunit react with proteins in vertebrates, invertebrates, and fungi. *J. Cell. Biol.* 106:1765-1772.
- Marguerie, G. A., T. S. Edgington, and E. F. Plow. 1980. Interaction of fibrinogen with its receptor is part of a multistep reaction in ADP-induced platelet concentration. *J. Biol. Chem.* 255:154-161.
- Molony, L., D. McCaslin, J. Abernethy, B. Paschal, and K. Burridge. 1987. Properties of talin from chicken gizzard smooth muscle. *J. Biol. Chem.* 262:7790-7795.
- Nachman, R. L., and L. L. K. Leung. 1982. Complex formation of platelet membrane glycoproteins IIb and IIIa with fibrinogen. *J. Clin. Invest.* 69:263-269.
- Nachmias, V. T. 1980. Cytoskeleton of human platelets at rest and after spreading. *J. Cell Biol.* 86:795-802.
- O'Halloran, T., M. C. Beckerle, and K. Burridge. 1985. Identification of talin as a major cytoplasmic protein implicated in platelet activation. *Nature (Lond.)* 317:449-451.
- Otto, J. J. 1983. Detection of vinculin-binding proteins with an  $^{125}\text{I}$ -vinculin gel overlay technique. *J. Cell Biol.* 97:1283-1287.
- Painter, R. G., K. N. Prodouze, and W. Gaarde. 1985. Isolation of a subpopulation of glycoprotein IIb-IIIa from platelet membranes that is bound to membrane actin. *J. Cell Biol.* 100:652-657.
- Phillips, D. R., L. K. Jennings, and H. H. Edwards. 1980. Identification of membrane proteins mediating the interaction of human platelets. *J. Cell Biol.* 86:77-86.
- Piotrowicz, R. S., R. P. Orzechowski, D. J. Nugent, K. Y. Yamada, and T. J. Kunicki. 1988. Glycoprotein Ic-IIa functions as an activation-independent fibronectin receptor on human platelets. *J. Cell Biol.* 106:1359-1364.
- Pytela, R., M. D. Pierschbacher, M. H. Ginsberg, E. F. Plow, and E. Ruoslahti. 1986. Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp-specific adhesion receptors. *Science (Wash. DC)* 231:1559-1562.
- Rosenfeld, G. C., D. C. Hou, J. Dingus, I. Meza, and J. J. Bryan. 1985. Isolation and partial purification of human platelet vinculin. *J. Cell Biol.* 100:669-676.
- Rotman, A., J. Heldman, and S. Linder. 1982. Association of membrane and cytoplasmic proteins with the cytoskeleton in blood platelets. *Biochemistry* 21:1713-1719.
- Ruoslahti, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. *Science (Wash. DC)* 238:491-497.
- SAS Institute Inc. 1985. SAS User's Guide: Statistics Version 5 Edition. SAS Institute Inc., Cary, NC. 956 pp.
- Singer, I. I. 1979. The fibronexus: a transmembrane association of fibronectin-containing fibers and bundles of 5 nm microfilaments in hamster and human fibroblasts. *Cell* 16:675-685.
- Stenberg, P. E., M. A. Shuman, S. P. Levine, and D. F. Bainton. 1984. Redistribution of alpha-granules and their contents in thrombin-stimulated platelets. *J. Cell Biol.* 98:748-760.
- Takada, Y., J. L. Strominger, and M. E. Hemler. 1987. The very late antigen family of heterodimers is part of a super family of molecules involved in adhesion and embryogenesis. *Proc. Natl. Acad. Sci. USA* 84:3239-3243.
- Tamkun, J. W., D. W. DeSimone, D. Fonda, R. S. Patel, C. Buck, A. F. Horwitz, and R. O. Hynes. 1986. Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. *Cell* 46:271-282.
- Timmons, S., and J. Hawiger. 1978. Separation of platelets from plasma proteins including factor VIII<sub>WF</sub> by a combined albumin gradient gel filtration method using Hepes buffer. *Thromb. Res.* 12:297-306.
- Wencel-Drake, J. D., E. F. Plow, T. J. Kunicki, V. L. Woods, D. M. Keller, and M. H. Ginsberg. 1986. Localization of internal pools of membrane glycoproteins involved in platelet adhesive responses. *Am. J. Pathol.* 124:324-334.
- Zucker, M. B., and V. T. Nachmias. 1985. Platelet activation. *Arteriosclerosis* 5:2-18.