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# Talin distribution and phosphorylation in thrombin-activated platelets

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## SUMMARY

**We have previously demonstrated that the subcellular distribution of the adhesion plaque protein, talin, changes dramatically in human platelets in response to platelet activation (Beckerle et al., *J. Cell Biol.* 109, 3333-3346, 1989). Talin is uniformly distributed throughout the cytoplasm of resting platelets. However, when platelets are stimulated to become activated and adhesive, a significant amount of the talin population rapidly redistributes to a peripheral, submembranous location. In the present study we have examined talin phosphorylation and proteolytic cleavage as possible mechanisms by which talin's subcellular distribution could be regulated in platelets. We have found that thrombin activation of platelets leads to a fourfold increase in talin phosphorylation. Proteolytic cleavage of talin, however, is not detected in washed platelets activated with thrombin for as long as 30 minutes. Because talin moves to a submembranous location upon platelet activation and has been shown to interact with integrins in vitro, we also investigated whether the major platelet integrin, GPIIb-IIIa, is required for talin redistribution. Using Glanzmann thrombasthenic platelets, which are deficient in GPIIb-IIIa, we found that talin redistribution occurs even in the absence of GPIIb-IIIa. Collectively, our studies suggest that neither proteolytic cleavage of talin nor interactions between talin and GPIIb-IIIa is required for the regulated redistribution of talin in thrombin-activated platelets. Phosphorylation of talin in response to thrombin activation may, however, be one mechanism utilized by platelets to regulate talin distribution and function in human platelets.**

Key words: platelet, talin, phosphorylation, GPIIb-IIIa, Glanzmann thrombasthenia

## INTRODUCTION

Talin is a high molecular mass (225-235 kDa) cytoplasmic protein that has been postulated to be involved in mediating an association between the actin cytoskeleton and the extracellular matrix at sites of cell-substratum contact (Burridge and Connell, 1983). Talin is present in a number of cell types and is generally found at the adhesion plaques of stationary cells and at the leading edge of migrating cells (Geiger et al., 1985; Burridge et al., 1988; Beckerle and Yeh, 1990). In vitro binding studies have suggested that talin can interact with the $\beta_1$ subunit of integrins (Horwitz et al., 1986; Buck and Horwitz, 1987), which are transmembrane glycoproteins that serve as receptors for extracellular matrix molecules such as fibronectin (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Albelda and Buck, 1990). Talin also interacts with the cytoplasmic protein vinculin (Otto, 1983; Burridge and Mangeat, 1984), which, in turn, can associate with the actin cross-linking protein, α-actinin (Belkin and Koteliansky, 1987; Wachsstock et al., 1987). The ability of talin to interact with both cytoskeletal and membrane-associated proteins has led to the suggestion that talin participates in the establishment of transmembrane connections between the actin cytoskeleton and the extracellular environment that are thought to occur at sites of cell-substratum adhesion.

Talin is very abundant in platelets, representing greater than 3% of the total platelet protein (Collier and Wang, 1982; O'Halloran et al., 1985). Interestingly, the subcellular distribution of talin changes dramatically when platelets are activated to become adhesive. In resting, nonadhesive platelets talin exhibits a uniform, cytoplasmic distribution. However, upon platelet activation, talin becomes translocated to a peripheral, submembranous location (Beckerle et al., 1989). Within 1 minute of activation with thrombin, 36% of the cellular talin is located within 40 nm of the plasma membrane and could therefore be membrane-associated. This regulated redistribution of talin to the platelet periphery positions talin at a location where it could participate in the development of adhesive potential upon platelet activation.

How then is the subcellular distribution of talin regulated in platelets? Platelets are anucleate cells with no transcriptional activity and limited translational machinery. Consequently, the regulated redistribution of talin is likely to be mediated by post-translational modifications of proteins upon platelet activation. Talin itself can be post-translationally modified by phosphorylation and proteolytic cleav-

age. For example, talin has been shown to be an in vitro substrate for protein kinase C (Litchfield and Ball, 1986; Beckerle, 1990). Furthermore, treatment of fibroblasts or platelets with tumor-promoting phorbol esters, which results in activation of protein kinase C (Nishizuka, 1984), stimulates the in vivo phosphorylation of talin (Turner et al., 1989; Beckerle, 1990; Litchfield and Ball, 1990). In addition to being phosphorylated, talin can also be proteolytically cleaved by the calcium-dependent protease to generate two fragments of 190-200 kDa and 46 kDa (Fox et al., 1985; O'Halloran et al., 1985; Beckerle et al., 1987). One isoform of the calcium-dependent protease colocalizes with talin in adhesion plaques of cultured cells (Beckerle et al., 1987) and, like talin, has been reported to be redistributed to the plasma membrane upon platelet activation (Wencel-Drake et al., 1991). However, the physiological role of the calcium-dependent protease in platelets has not been fully clarified. Initially, it was reported that proteolytic cleavage of talin occurs in response to platelet aggregation (Fox et al., 1983, 1985); however, recently, investigators have failed to observe a correlation between platelet aggregation and proteolytic cleavage of talin (Elce et al., 1989; Wencel-Drake et al., 1991).

In this paper we have examined talin phosphorylation and proteolytic cleavage in response to platelet activation as possible mechanisms by which talin redistribution is regulated in platelets. We have also studied the role of the major platelet integrin, GPIIb-IIIa, in the translocation of talin to the platelet membrane, since interactions between talin and integrin have been implicated in the attachment of the actin cytoskeleton to the plasma membrane in adhesive cells. Here we report that neither talin cleavage nor GPIIb-IIIa is required for talin redistribution in thrombin-activated platelets. However, we have found that talin is phosphorylated in vivo in response to platelet activation. Taken together these results suggest that activation-dependent phosphorylation of talin may regulate talin's subcellular distribution and function in the adhesive response of platelets.

## MATERIALS AND METHODS

### Platelet isolation

Platelet-rich plasma was prepared from freshly drawn whole blood from aspirin-free donors as described by Fox and Phillips (1982). Patients utilized in the study of Glanzmann thrombasthenic platelets have been well characterized (Bray and Shuman, 1990). Platelet-rich plasma was centrifuged at 560 ***g*** for 20 minutes, and the platelets were subsequently washed twice in a 37°C citrate/glucose/sodium chloride solution (13 mM sodium citrate dihydrate, 30 mM glucose, 120 mM sodium chloride, pH 7.0). Washed platelets were resuspended in HEPES-buffered Tyrode's (138 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, 5.5 mM glucose, 10 mM HEPES, 0.36 mM $Na_2HPO_4$, 1.8 mM $CaCl_2$, 0.49 mM $MgCl_2$) at a concentration of $5\times10^8$ to $1.5\times10^9$ platelets/ml and then incubated at 37°C for 1 hour prior to use in order to guarantee that the cells retained a resting morphology (Nachmias, 1980; Fox et al., 1984). All experiments were performed at physiological temperature.

### Protein purification

Human platelet talin was isolated from outdated platelet-rich plasma by the method of Collier and Wang (1982) as modified by Beckerle et al. (1986). Protein kinase C, isolated from bovine brain (Parker et al., 1984), was generously provided by Dr Peter J. Parker (Ludwig Institute for Cancer Research, London, England). The calcium-dependent protease type II was purified from bovine heart (Croall and DeMartino, 1984) and was the generous gift of Dr Dorothy Croall (University of Maine, Orono, ME).

### Antibody preparation and characterization

Rabbit polyclonal antisera B11 and B5 were raised against human platelet talin and GPIIb-IIIa, respectively, and have been extensively characterized (Beckerle et al., 1989). The anti-phosphotyrosine antibody 54.1 was a generous gift from Dr Benjamin Geiger (Weizmann Institute of Science, Israel). The anti-phosphotyrosine antibody, PY20, was purchased from ICN Biomedicals, Inc., Irvine, CA.

### Talin phosphorylation and immunoprecipitation

In vitro phosphorylation of purified human platelet talin by protein kinase C and cleavage by isolated calcium-dependent protease type II were performed as described previously for chicken smooth muscle talin (Beckerle, 1990).

For in vivo phosphorylation experiments, intact human platelets were labeled with $^{32}P$ on the basis of the methodology of Parise et al. (1990). Briefly, platelets were resuspended after isolation in $Mg^{2+}$- and $PO_4$-free HEPES-buffered Tyrode's ($1.5\times10^9$ platelets/ml). Samples were incubated with 1 mCi $H_3[^{32}P]O_4$ (ICN Biomedicals Inc., Irvine, CA) per ml platelets for 90 minutes at 37°C. The platelets were then washed once in 10 ml $Mg^{2+}$- and $PO_4$-free HEPES-buffered Tyrode's and resuspended at a concentration of $1\times10^9$ platelets/ml in HEPES-buffered Tyrode's containing 0.49 mM $MgCl_2$ and 0.36 mM $Na_2HPO_4$.

Immunoprecipitations were carried out following the procedure described by Burridge and Connell (1983) for chicken embryo fibroblasts. Following platelet activation with thrombin (1 unit/ml; Calbiochem Behring Corp., La Jolla, CA) or phorbol-12-myristate-13-acetate (PMA) (10 μM in DMSO), EGTA (5 mM), hirudin (2 unit/ml) and leupeptin (1 mg/ml) were added to the samples, which were then lysed in 10× Laemmli sample buffer (Laemmli, 1970) containing 100 mM sodium metavanadate and the protease inhibitors phenylmethylsulfonyl fluoride (0.1 mM), benzamidine HCl (0.1 mM), pepstatin A (1 ng/ml), and 1,10-phenanthroline (1 ng/ml) (Sigma). The samples were boiled for 4 minutes and set on ice. The SDS concentration was reduced by raising the sample volume to 10 ml with a solution containing 1% sodium deoxycholate, 1% Triton X-100, 1 mM sodium metavanadate and the protease inhibitors described above. Samples were first precleared with 200 μl of 10% Protein A-agarose beads (Sigma) for 1 hour at 4°C. B11 anti-talin antibody was subsequently added for an overnight incubation at 4°C with constant shaking. Samples were then mixed with Protein A-agarose beads for 4 hours at 4°C with stirring. The immunoprecipitated material was washed 7 times with 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM sodium metavanadate and protease inhibitors, and sedimented after each wash. The final pellets were boiled in Laemmli sample buffer, and the agarose beads were sedimented at 16,000 ***g*** for 1 minute. The supernatants were analyzed on SDS-polyacrylamide gels as described by Laemmli (1970), except that the bisacrylamide concentration was 0.13%. The relative amount of $^{32}P$-labeled talin present in each sample was determined by Phospho Image Analysis (Phospho Imager model 400E (Sunnyvale, CA) with a Molecular Dynamics Image Quant 3.2 software package). Background-subtracted values were normalized with respect to resting samples to account for variation in signals from experiment to experiment.

### Talin cleavage assays

#### Analysis of talin in platelet lysates

Platelets were resuspended to a concentration of $1\times10^9$ platelets/ml in $Ca^{2+}$-free buffer containing 1 mM EDTA and lysed by the addition of an equal volume of buffer containing 1 mM EDTA and 1% Triton X-100. Calcium chloride (2 mM) was then added to platelet lysates to activate the endogenous calcium-dependent protease; control samples were incubated in the presence of EDTA with no added $Ca^{2+}$. Samples were removed at various times following $Ca^{2+}$ addition, solubilized and boiled in Laemmli sample buffer and examined for talin cleavage on Coomassie blue-stained polyacrylamide gels (7.5%) and western immunoblots (performed according to the procedure of Towbin et al. (1979) with the modifications described by Beckerle (1986)). B11 anti-talin antibody, used in the immunoblot analysis, recognizes both intact platelet talin and the 200 kDa cleavage product (Beckerle et al., 1989).

#### Analysis of talin in resting and thrombin-activated platelets

Samples of resting, thrombin-activated (0.1 unit thrombin/ml, not stirred), and aggregated platelets (0.1 unit thrombin/ml, stirred) prepared at a concentration of $5\times10^8$ platelets per ml were solubilized in Laemmli sample buffer. In some experiments EGTA (5 mM) and leupeptin (100 μg) were added immediately prior to solubilization. The samples were boiled and assayed for talin cleavage as described above.

#### Thrombin-mediated cleavage of talin

Thrombin (1 unit/ml) was added to 0.2 mg/ml purified platelet talin. Following incubation at 37°C, samples were solubilized and boiled in Laemmli sample buffer and analyzed for talin cleavage as described above.

### Indirect immunofluorescence

Fixed samples of resting and activated platelets were prepared for indirect immunofluorescence as described previously (Beckerle et al., 1989). PMA-treated platelets were incubated in either 1 μM or 10 μM PMA in DMSO for 5 minutes prior to fixation. In terms of talin's subcellular distribution, no difference was observed in platelets treated with either of these two PMA concentrations. Samples were examined with an MRC-600 laser-scanning confocal microscope (Bio-Rad Microsciences, Cambridge, MA).

## RESULTS

### Phosphorylation of talin in thrombin-activated platelets

In order to investigate whether talin phosphorylation occurs in intact human platelets in response to agonist, we examined the relative levels of phosphorylated talin in resting and thrombin-activated platelets. Talin was immunoprecipitated from $^{32}$P-labeled resting and activated platelets. As shown in Fig. 1, within the first 2 minutes of thrombin stimulation, the amount of phosphorylated talin increases nearly fourfold over the resting level. The level of talin phosphorylation does not change significantly between 2 and 5 minutes of thrombin activation. Protein tyrosine kinases are probably not involved in talin phosphorylation in platelets, since no signal is detected at talin's molecular mass on western immunoblots probed with anti-phosphotyrosine antibodies (Golden and Brugge, 1989; Ferrell and Martin, 1989b; our unpublished observations). Consequently, the

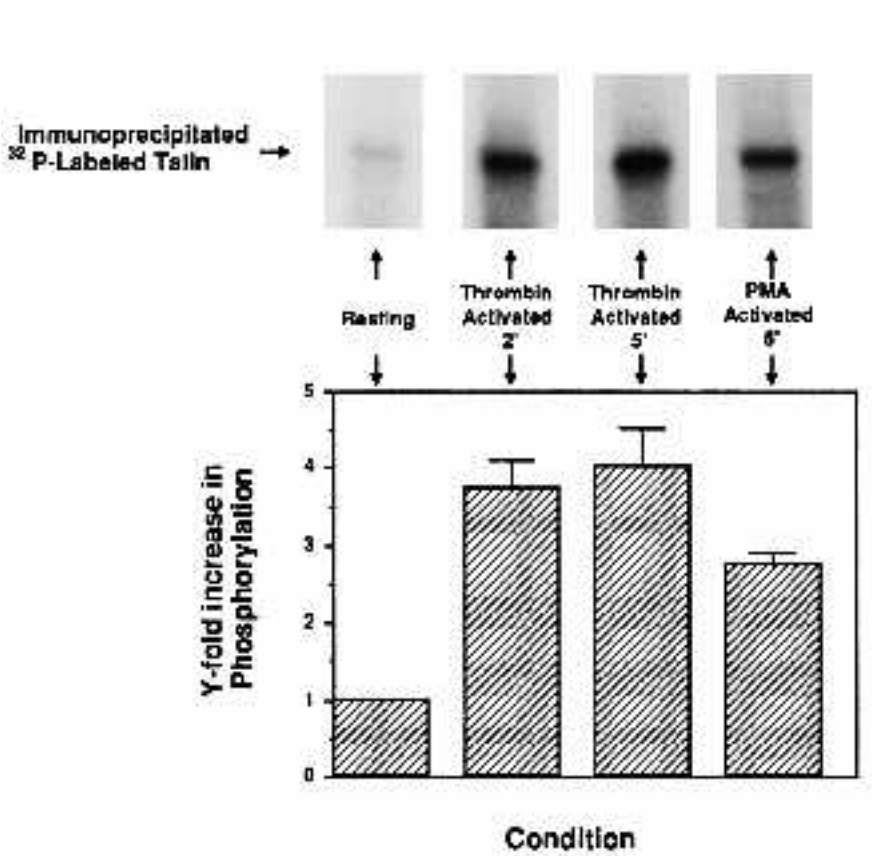


**Fig. 1.** Activation-dependent increase in talin phosphorylation in intact human platelets. $^{32}$P-labeled resting platelets were activated with thrombin (1 unit/ml) or PMA (10 μM) for the given times. Talin was immunoprecipitated and analyzed by autoradiography for $^{32}$P incorporation (A). Quantitative analysis of the relative levels of talin phosphorylation is shown in B. Within the first 2 minutes of thrombin activation there is an almost fourfold increase in the level of talin phosphorylation compared to the resting state. No significant increase in talin phosphorylation occurs between 2 and 5 minutes of thrombin activation. Activation of platelets with PMA, which stimulates protein kinase C activity, results in a two- to threefold increase in talin phosphorylation. The values shown in B were obtained by Phospho Image ananysis and were normalized with respect to resting samples to account for variation in signals from experiment to experiment. Each bar represents the mean ± s.e.m.: Thrombin Activated 2′= 3.73 ± 0.89 ($n$=6); Thrombin Activated 5′= 4.00 ± 1.24 ($n$=6); PMA Activated 5′= 2.74 ± 0.32 ($n$=4).

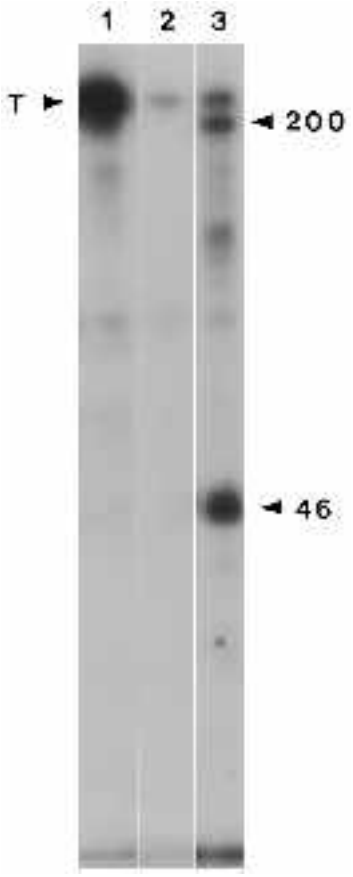


**Fig. 2.** Domain analysis of talin phosphorylated by protein kinase C in vitro. Lane 1, an autoradiograph of talin (T) purified from platelet-rich plasma and phosphorylated in vitro by protein kinase C. Lane 2, because protein kinase C is a phospholipid-dependent kinase, only limited talin phosphorylation is observed in the absence of phosphatidylserine and 1,2-diolein. Lane 3, cleavage of protein kinase C-phosphorylated talin by the calcium-dependent protease type II reveals that both the 200 kDa and 46 kDa domains of talin are phosphorylated. The N-terminal 46 kDa domain of talin is more prominently labeled than is the 200 kDa domain.

talin phosphorylation observed can be attributed to an increased phosphorylation of serine and/or threonine residues. To determine if protein kinase C-mediated phosphorylation of talin could account for the levels of talin phosphorylation we observe in thrombin-activated platelets, $^{32}$P-labeled platelets were treated with PMA for 5 minutes to stimulate directly protein kinase C activity (Nishizuka, 1984). PMA treatment activates platelets and leads to a shape change, secretion and the ability of the cells to aggregate (McNicol et al., 1989; Wheeler-Jones et al., 1989). Exposure of platelets to PMA leads to a two- to threefold increase in talin phosphorylation (Fig. 1B).

Since protein kinase C activation by both thrombin and PMA leads to talin phosphorylation, we have examined the phosphorylation of purified platelet talin by protein kinase C in vitro. As can be seen in Fig. 2, human platelet talin is phosphorylated by protein kinase C in a phospholipid-dependent manner. Cleavage of in vitro phosphorylated talin by the calcium-dependent protease yields the expected 200 kDa and 46 kDa cleavage products (Fig. 2, lane 3). Both the large and small fragments of platelet talin are phosphorylated by protein kinase C; however, the 46 kDa domain of platelet talin is more prominently phosphorylated than the larger domain.

## Calcium-dependent proteolysis of talin does not precede talin redistribution

Proteolysis of talin is another post-translational mechanism that could potentially be utilized by platelets to regulate the

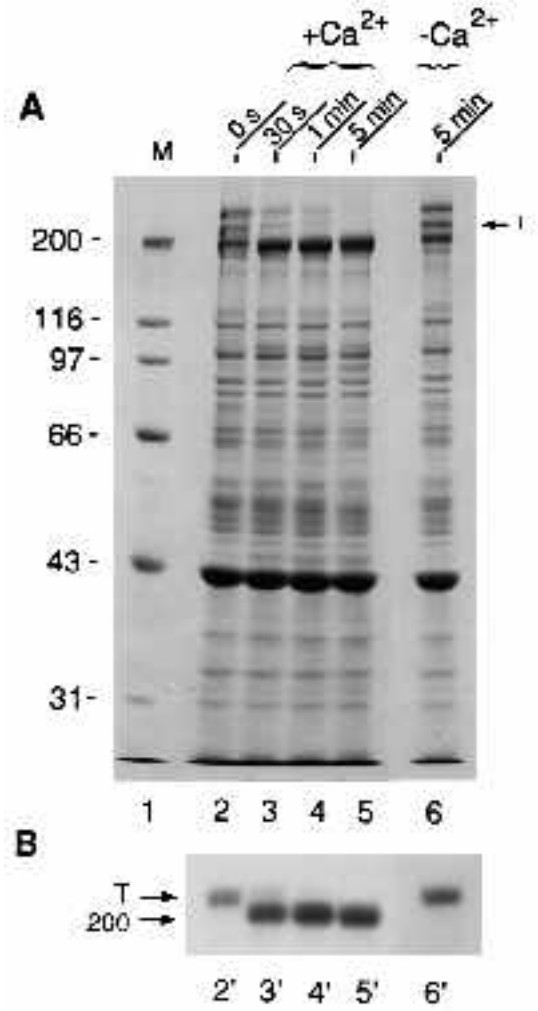


**Fig. 3.** The endogenous calcium-dependent protease cleaves talin in platelet lysates. Detergent lysates of resting platelets were prepared in the presence of EDTA. $Ca^{2+}$ was then added and samples were taken at various time points following $Ca^{2+}$ addition. (A) A Coomassie blue-stained gel of molecular mass (kDa) standards (lane 1), samples prior to $Ca^{2+}$ addition (lane 2) and samples analyzed 30 seconds (lane 3), 1 minute (lane 4) and 5 minutes (lane 5) after $Ca^{2+}$ was added. Control samples were maintained in EDTA-containing buffer ($-Ca^{2+}$) for the 5 minute incubation period (lane 6). The position of talin (T) is marked by an arrow. (B) A corresponding western immunoblot probed with an anti-talin antibody that recognizes intact talin (T) and the 200 kDa cleavage product (200). Endogenous calcium-dependent protease begins to cleave talin within the first 30 seconds of $Ca^{2+}$ addition (lanes 3 and 3′). By 5 minutes most of the talin has been cleaved, as seen by a decrease in intact talin and a corresponding increase in the 200 kDa proteolytic fragment (lanes 5 and 5′). No talin cleavage is detected in control samples (lanes 6 and 6′).

distribution of talin. It has been demonstrated that platelets contain an endogenous calcium-dependent protease (Phillips and Jakabova, 1977). To observe the effect of this protease on talin in vitro, we prepared detergent lysates of resting platelets in the presence of the divalent cation chelator, EDTA, and then added $Ca^{2+}$ to mimic the increase in available calcium ions that occurs upon platelet activation.

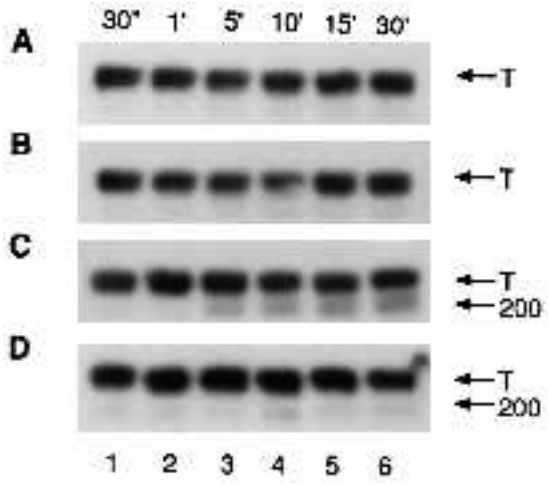


**Fig. 4.** Analysis of talin cleavage in thrombin-activated and aggregated platelets. Resting platelets (A), thrombin-activated, nonaggregated platelets (B) and thrombin-activated, aggregated platelets (C) were solubilized in Laemmli sample buffer at 30 seconds (lane 1) or 1 (lane 2), 5 (lane 3), 10 (lane 4), 15 (lane 5) and 30 (lane 6) minutes after thrombin addition, and examined for talin cleavage by western immunoblot analysis. No significant talin cleavage was detected in either resting (A) or thrombin-activated, nonaggregated platelets (B) even 30 minutes after thrombin addition. Some cleavage of talin (T) was detected in aggregated samples as evidenced by an increase in the 200 kDa cleavage product (200) (C). However, this cleavage could be prohibited to some extent by the addition of leupeptin and EDTA immediately prior to sample solubilization in Laemmli sample buffer (D).

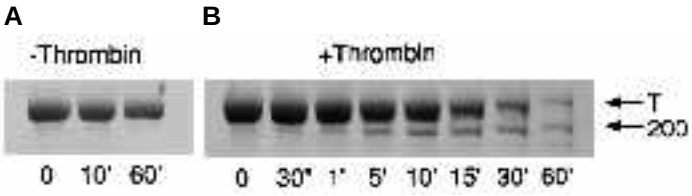


**Fig. 5.** Thrombin cleaves purified platelet talin in vitro. The platelet agonist and protease, thrombin (1 unit/ml), was added to purified platelet talin to determine if talin is cleaved by thrombin. Samples were incubated from 30 seconds to 60 minutes following thrombin addition and examined for talin cleavage on Coomassie blue-stained gels. Talin incubated in the absence of thrombin (A) is not cleaved, even after 60 minutes. As shown in B, the addition of thrombin to talin samples (T) results in the time-dependent cleavage of talin. The 200 kDa cleavage product of talin (200) is itself cleaved by thrombin with time.

As shown in Fig. 3, within 30 seconds of $Ca^{2+}$ addition, the calcium-activated proteases present in the platelet lysate cleave talin (T) (Fig. 3, lane 3). After 5 minutes of $Ca^{2+}$ addition, we can no longer detect intact talin either by Coomassie blue-stained gels (Fig. 3A, lane 5) or by western immunoblot analysis (Fig. 3B, lane 5′). The anti-talin antibody used in this western immunoblot recognizes both intact talin and the 200 kDa cleavage product. Talin cleavage was not detected in platelet lysates that were maintained in the presence of divalent cation chelators without addition of $Ca^{2+}$ (Fig. 3A, lane 6, and Fig. 3B, lane 6′).

To examine more directly the possible role of talin cleavage in the activation-dependent redistribution of talin, we next assayed intact platelets for talin cleavage. Because the concentration of free $Ca^{2+}$ is low in resting platelets, the calcium-dependent protease is inactive in these samples (Fox and Phillips, 1983). Indeed, we did not detect significant talin cleavage in samples of resting platelets (Fig. 4A, lanes 1-6). Thrombin-activated platelets maintained in the absence of stirring to minimize platelet aggregation also had no detectable talin cleavage, even after 30 minutes (Fig. 4B, lane 6). Consistent with results reported previously (Fox and Phillips, 1983; Fox et al., 1985), some talin cleavage was detected in aggregated platelets (Fig. 4C, lanes 3-6). However, this cleavage could be blocked to some extent by the addition of protease inhibitors and EDTA immediately prior to lysis of the sample in Laemmli sample buffer (Fig. 4D); this result shows that postlysis cleavage of talin occurs in aggregated platelet samples in the absence of protease inhibitors. Coupled with our previous observation that talin redistribution occurs within 1 minute of platelet activation in an aggregation-independent manner (Beckerle et al., 1989), the absence of talin cleavage in thrombin-activated, nonaggregated platelets demonstrates that cleavage of talin cannot be required for talin redistribution in platelets.

Because talin cleavage can occur as a postlysis event, we examined the possibility that thrombin, an agonist commonly used to activate platelets, which is itself a protease, could contribute to this cleavage. For these studies thrombin was added to purified platelet talin, and samples were assayed over time for talin cleavage. As shown in Fig. 5, thrombin does cleave talin in vitro, generating a 200 kDa fragment that comigrates with a product formed upon cleavage of talin with the calcium-dependent protease. Collectively, these results suggest that the presence of active thrombin in platelet samples during solubilization could contribute to the proteolysis of platelet talin.

### The activation-dependent redistribution of talin does not require GPIIb-IIIa

We have previously demonstrated that talin is concentrated at the cytoplasmic face of the plasma membrane in activated platelets (Beckerle et al., 1989). Because talin has been shown to bind to integrins (Horwitz et al., 1986; Buck and Horwitz, 1987), we examined whether an interaction between talin and integrin was required to mediate the activation-dependent redistribution of talin to the plasma membrane. The most-abundant integrin on the platelet surface is glycoprotein IIb-IIIa (GPIIb-IIIa), the platelet fibrinogen receptor (Bennett and Vilaire, 1979; Marguerie et al., 1979; Bennett et al., 1983; Parise and Phillips, 1985). To determine whether the platelet integrin, GPIIb-IIIa, is required for the redistribution and localization of talin to the cytoplasmic face of the plasma membrane, we utilized Glanzmann thrombasthenic (GT) platelets, which are deficient in GPIIb-IIIa (Bennett and Vilaire, 1979; George et al., 1984; Nurden et al., 1985; Bray and Shuman, 1990). Glanzmann thrombasthenia is an autosomal recessive disorder. The individuals who provided the thrombasthenic platelets used

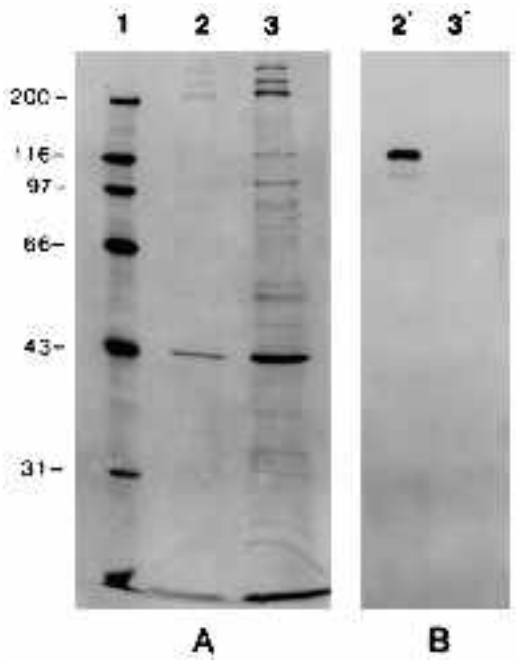


**Fig. 6.** Glanzmann thrombasthenic platelets lack detectable GPIIb-IIIa. Glanzmann thrombasthenic (GT) platelets are unable to aggregate due to a deficiency in GPIIb-IIIa. (A) A Coomassie blue-stained gel of molecular mass standards (lane 1), total platelet protein from normal platelets (lane 2), and total platelet protein from GT platelets (lane 3). (B) GPIIb-IIIa is present in normal platelets, as detected by western immunoblot analysis (lane 2′). No GPIIb-IIIa is detected in GT platelets (lane 3′). The GT lanes were heavily loaded on the gels so that proteins present in small amounts could be detected.

in these studies had inherited two mutant alleles of the gene encoding GPIIIa; these genetic defects have been extensively characterized (Bray and Shuman, 1990). Northern analysis of total platelet RNA obtained from the thrombasthenic platelets revealed a complete loss of GPIIIa mRNA (Bray and Shuman, 1990). We used western immunoblot analysis to confirm that the GT platelets did indeed lack detectable levels of GPIIb-IIIa (Fig. 6). Furthermore, we detected no GPIIb-IIIa in the GT platelets by indirect immunofluorescence (data not shown). Although the gene encoding GPIIb is apparently normal in these individuals, it is believed that GPIIb is itself unstable when not complexed to GPIIIa, and is therefore degraded in the absence of GPIIIa (Bray and Shuman, 1990).

To evaluate the importance of GPIIb-IIIa in the activation-dependent redistribution of talin, we examined the distribution of talin in normal and GT platelets. As can be seen in Fig. 7A and B, in normal platelets the transmembrane protein, GPIIb-IIIa, is detected at the cell periphery in both resting and thrombin-activated platelets by indirect immunofluorescence; each peripheral ring of fluorescence defines the overall shape of a platelet and is consistent with the pattern expected for a membrane-associated protein. As demonstrated previously (Beckerle et al., 1989), talin exhibits a relatively uniform cytoplasmic distribution in normal resting platelets (Fig. 7C). However, within 1 minute of thrombin activation, talin moves to a peripheral, submembranous location (Fig. 7D) and exhibits a staining pattern nearly identical to that of GPIIb-IIIa (see Fig. 7B), suggesting that talin occupies a space very near to the plasma membrane. Interestingly, apparently normal talin redistribution also occurs in GT platelets following thrombin activation (Fig. 7F), despite the fact that these platelets are devoid of detectable GPIIb-IIIa. The distribution of talin in activated GT platelets is virtually indistinguishable from that seen in normal platelets (Fig. 7D,F). In both cases talin moves to a more peripheral location near the plasma membrane. As illustrated in Fig. 7F, GT platelets fail to aggregate, due to the absence of GPIIb-IIIa, which mediates platelet aggregation via fibrinogen binding (Marguerie et al., 1979; Peerschke et al., 1980; Parise and Phillips, 1985). The observation that talin redistribution occurs in GT platelets that are unable to aggregate further supports our earlier finding that platelet aggregation is not required for the activation-dependent translocation of talin (Beckerle et al., 1989).

It has been well documented that in addition to shape change, fibrinogen binding and aggregation, one of the responses of platelets to thrombin treatment is the centralization and fusion of cytoplasmic secretory granules (for review see Zucker and Nachmias, 1985; Kroll and Schafer, 1989). Granule centralization is then followed by release of the contents into the extracellular milieu. Consequently, one important question that arose during our studies was whether the peripheral staining we observed was a reflection of the regulated redistribution of talin or if it was the result of granule centralization and passive exclusion of talin from the center of the platelet following thrombin activation. To address this concern we have identified conditions under which platelet shape change, granule centralization and secretion occur but talin redistribution does not occur (Fig. 8). As noted earlier, PMA treatment of platelets causes stimulation of protein kinase C and results in granule centralization, secretion and platelet aggregation (McNicol et al., 1989; Wheeler-Jones et al., 1989; Litchfield and Ball, 1990; our unpublished observations). However, in contrast to what occurs in thrombin-activated platelets (Fig. 8B), talin's distribution remains diffuse and apparently cytoplasmic in platelets activated with either 1 μM (not shown) or 10 μM PMA for 5 minutes (Fig. 8C). Granule centralization alone is therefore not sufficient to lead to the peripheral distribution of talin observed in thrombin-treated platelets (Fig. 8B). Additionally, this experiment suggests that PMA-induced phosphorylation of talin (Fig. 1) is not sufficient to trigger talin redistribution. We therefore conclude that thrombin stimulation of platelets leads to additional modifications of talin or other proteins that are required for talin redistribution and that granule centralization per se cannot account for the movement of talin to the platelet periphery following thrombin activation.

## DISCUSSION

We previously reported that talin undergoes a dramatic change in its subcellular distribution in response to platelet activation, moving from a diffuse cytoplasmic location to

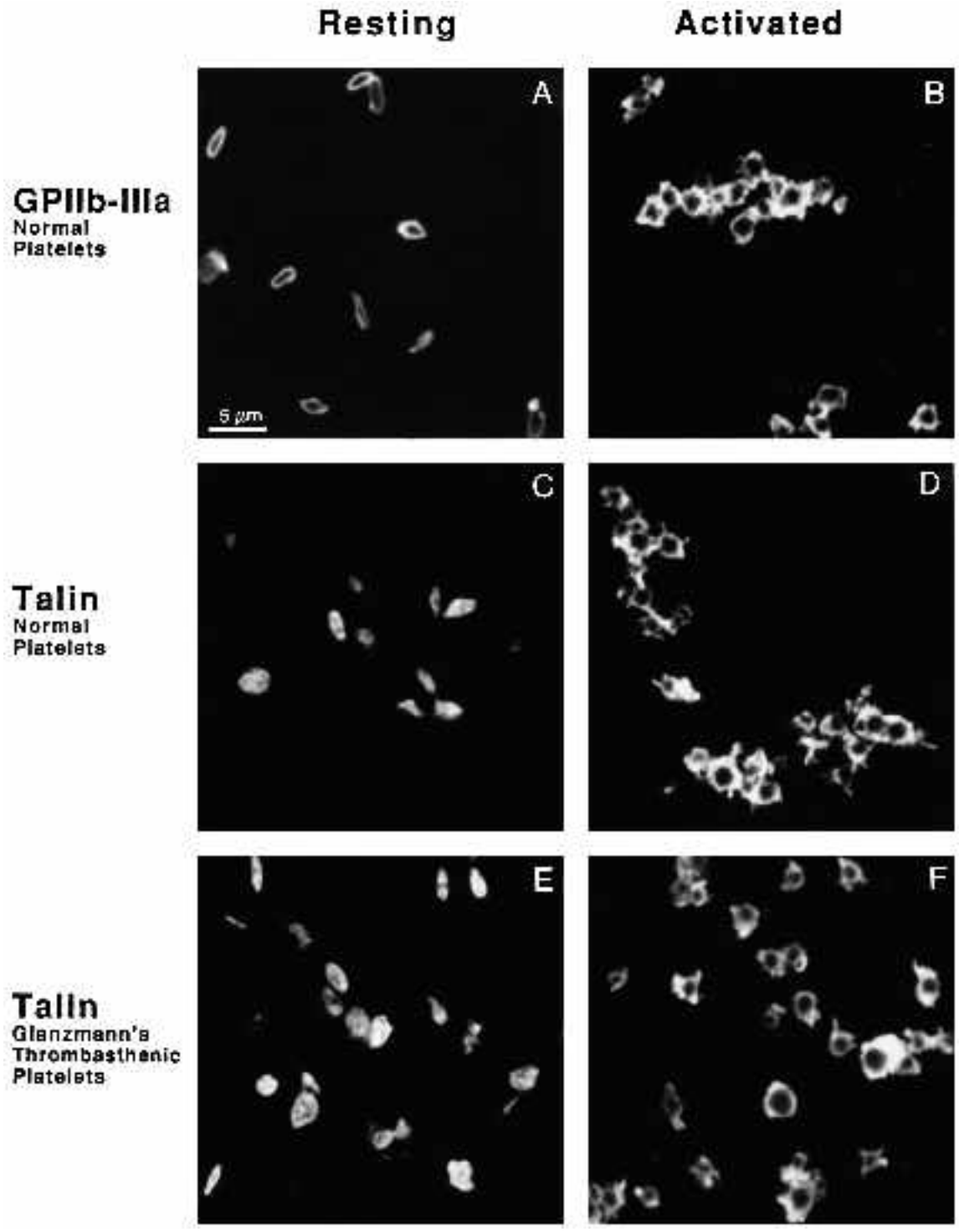


**Fig. 7.** Activation-dependent redistribution of talin occurs in Glanzmann thrombasthenic platelets. Indirect immunofluorescence and confocal microscopy were used to compare the distribution of talin in normal platelets and Glanzmann thrombasthenic platelets, which lack detectable levels of GPIIb-IIIa. GPIIb-IIIa in normal platelets exhibits a peripheral distribution, characteristic of a membrane-associated protein, in both resting (A) and thrombin-activated (B) platelets. In contrast, talin is diffusely distributed in the cytoplasm of both normal (C) and Glanzmann thrombasthenic (E) resting platelets. Upon thrombin activation of normal platelets, talin moves to a peripheral, submembranous location (D). The activation-dependent redistribution of talin also occurs in Glanzmann thrombasthenic platelets (F), even in the absence of GPIIb-IIIa.

a more discrete submembranous distribution (Beckerle et al., 1989). Here we have examined possible mechanisms by which talin's subcellular distribution could be regulated in platelets. Specifically, we have studied talin phosphorylation and proteolytic cleavage in response to platelet activation; these post-translational modifications have been

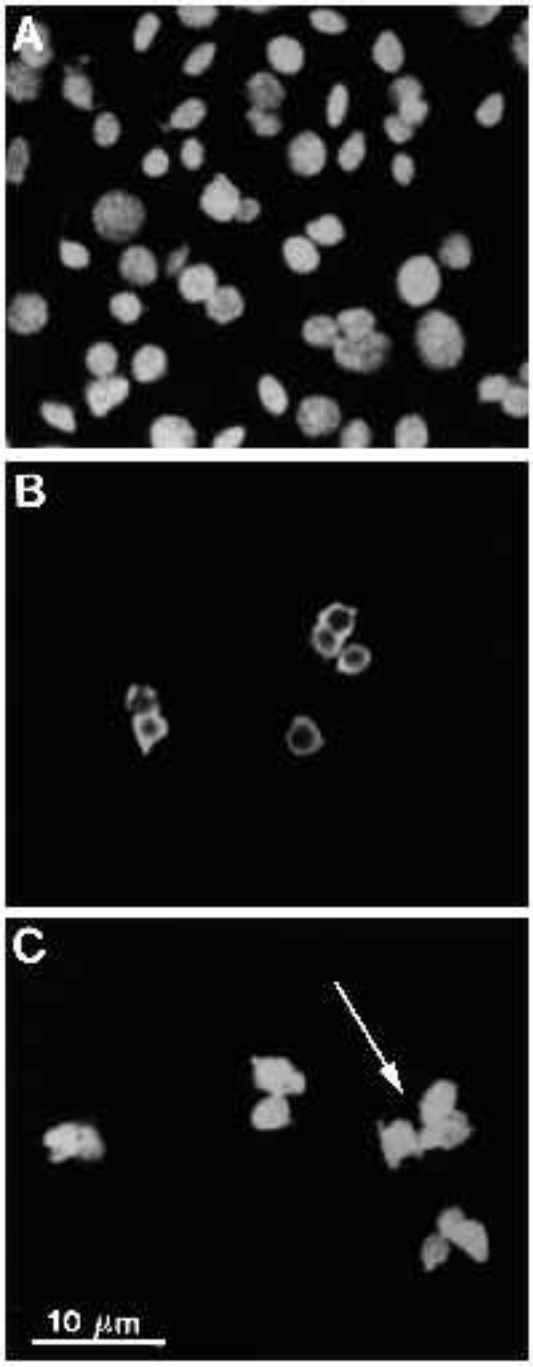


**Fig. 8.** Comparison of talin distribution in thrombin- and PMA-stimulated platelets. Indirect immunofluorescence and confocal microscopy were used to compare the distribution of talin in resting (A), thrombin-activated (B) and PMA-activated (C) platelets. A diffuse, cytoplasmic distribution of talin is seen in resting platelets in the presence of DMSO, the PMA carrier (A). Thrombin activation results in the regulated redistribution of talin to the platelet periphery (B). PMA treatment of platelets, which leads to activation-dependent granule centralization, secretion and aggregation, does not cause talin redistribution to occur (C). The arrow in C points to an aggregate of platelets. Although, in C, platelets were treated with 10 μM PMA, similar results were obtained for platelets treated with 1 μM PMA.

postulated to affect talin function both in platelets and in other cell types. In addition we have used Glanzmann thrombasthenic platelets, which lack detectable levels of the major platelet integrin, GPIIb-IIIa, to determine if GPIIb-IIIa is required for talin redistribution in platelets. Here we report that talin phosphorylation increases approximately fourfold during the first 2 minutes of thrombin activation of platelets. Using western immunoblot analysis we have determined that proteolytic cleavage of talin does not occur in thrombin-activated, nonaggregated platelets under conditions that support talin redistribution. Additionally, the subcellular distribution of talin is not affected by the absence of GPIIb-IIIa in GT platelets. Collectively, our results suggest that neither proteolytic cleavage of talin nor the presence of the integrin GPIIb-IIIa is required for talin redistribution in human platelets. The subcellular distribution of talin may, however, be regulated by the phosphorylation state of talin since thrombin activation of platelets results in both an increase in talin phosphorylation and the translocation of talin to the plasma membrane.

Phosphorylation of proteins is one mechanism by which protein-protein interactions can be regulated in vivo. Thus, the phosphorylation of talin that we observe in response to platelet activation could be involved in the regulation of interactions between talin and other proteins in platelets and, ultimately, could promote the redistribution of talin to the platelet periphery or the anchoring of talin at its new submembranous location. It is also possible that an activation-dependent modification of other platelet molecules is required to generate a binding site for talin near the platelet membrane. The role of talin phosphorylation in activated platelets may be clarified when the stoichiometry of the phosphorylation is determined. Demonstration of a low stoichiometry of phosphorylation of GPIIIa in activated platelets was critical for ruling out the hypothesis that phosphorylation of GPIIIa is required for the activation-dependent development of ligand binding capacity by individual GPIIb-IIIa receptors (Hillery et al., 1991).

Because we detect no phosphotyrosine associated with talin in activated platelets, talin phosphorylation in vivo appears to be mediated by serine/threonine kinases. Talin is a substrate for the serine/threonine kinase, protein kinase C, in vitro (Litchfield and Ball., 1986; Beckerle, 1990) and is phosphorylated in vivo by treatment of platelets with the protein kinase C activator, PMA (Litchfield and Ball, 1990; results shown here). However, protein kinase C-mediated phosphorylation of talin does not appear to be sufficient for the activation-dependent translocation of talin, since talin redistribution does not occur in PMA-treated platelets. Other serine/threonine kinases may therefore be involved in regulating talin's subcellular distribution. It has been shown that thrombin treatment of platelets results in the activation of a number of serine/threonine kinases that are not activated by PMA treatment alone (Ferrell and Martin, 1989a). Phosphorylation of talin by one or more of these kinases, presumably at sites other than those targeted by protein kinase C, could be required for talin redistribution to occur in activated platelets. Indeed we observe qualitatively higher levels of talin phosphorylation in thrombin-treated platelets than in PMA-treated platelets (Fig. 1), suggesting that additional sites may be phosphorylated on talin

by thrombin-stimulated kinases other than protein kinase C.

Although protein kinase C-mediated phosphorylation of talin does not appear to be sufficient to stimulate talin redistribution, it is possible that this modification is important for anchoring talin at its new submembranous location following translocation. We have determined that the phosphorylation of talin by protein kinase C occurs more prominently on the N-terminal 46 kDa region of the protein. This region of talin is related to ezrin and erythrocyte band 4.1, proteins thought to be involved in the attachment of cytoskeletal components to the plasma membrane (Rees et al., 1990). It is therefore intriguing to consider the possibility that phosphorylation of talin's N-terminal domain is involved in mediating interactions between talin and the platelet membrane.

The molecular nature of the linkage between talin and the plasma membrane of activated platelets is not understood. The results reported here for GT platelets show that talin redistribution occurs even in the absence of the plasma membrane-associated glycoprotein complex, GPIIb-IIIa, which is the most-abundant platelet integrin and a protein widely postulated to bind talin in vivo. Three lines of evidence suggest that the activation-dependent redistribution of talin in GT platelets is due to the regulated translocation of talin to the cell periphery and is not caused by exclusion from the platelet center. First, the distribution of talin in activated GT platelets is consistent with that seen in normal platelets in which the subcellular location of talin has been confirmed at the EM level (Beckerle et al., 1989). Second, talin's distribution in thrombin-stimulated normal and thrombasthenic platelets is similar to that of GPIIb-IIIa, a well-characterized membrane-associated protein. Finally, talin redistribution does not occur in PMA-activated platelets, which display granule centralization, secretion and aggregation. Collectively, these results demonstrate that some mechanism other than passive exclusion of talin from the platelet center during granule centralization is involved in mediating the activation-dependent redistribution of talin to the platelet periphery in both normal and GT platelets. Since the regulated redistribution of talin occurs in GT platelets, GPIIb-IIIa must not be absolutely required for the translocation of talin to the platelet periphery. Moreover, the anchoring of talin at this new location is stable during the time course of our experiments. Perhaps other less-abundant integrins or membrane-associated proteins tether talin to the cytoplasmic face of the plasma membrane. Alternatively, activation-dependent modifications of talin, such as phosphorylation, could enable talin to interact directly with the phospholipid bilayer of the plasma membrane, as has been suggested previously (Heise et al., 1991).

Because we failed to detect significant levels of talin cleavage in thrombin-activated, nonaggregated platelets, conditions that support talin redistribution, we conclude that cleavage of talin by the calcium-dependent protease is not required for the talin redistribution that we observe in thrombin-activated platelets. We did detect some talin cleavage in samples of aggregated platelets. However, the addition of protease inhibitors and EDTA immediately prior to lysis of the platelets significantly prohibited this cleavage. As has been suggested by others, the slow or incomplete solubilization of platelet aggregates, which are more resistant to solubilization than single cells, could lead to postlysis cleavage of talin (Wencel-Drake et al., 1991). Our ability to block talin cleavage in aggregated samples by including protease inhibitors immediately prior to sample lysis suggests that the majority of talin cleavage that we observed in samples of aggregated platelets occurs after platelet lysis and is not a consequence of platelet aggregation. Furthermore, the demonstration that the platelet agonist, thrombin, has the capacity to cleave talin in vitro to generate at least one product (200 kDa) that comigrates with a product of calcium-dependent proteolytic cleavage, illustrates that the presence of a 200 kDa proteolytic fragment derived from talin cannot necessarily be attributed to the specific action of the calcium-dependent protease. Our analysis of talin suggests that talin exhibits a protease-sensitive hinge between the 46 and 200 kDa domains (Beckerle et al., 1986).

The detailed physiological function of the calcium-dependent protease in platelets remains to be clarified. In this article we provide evidence demonstrating that proteolytic cleavage of talin is not prerequisite to the activation-dependent redistribution of talin to the platelet membrane. Moreover, calcium-dependent proteolytic cleavage of talin is not required for platelet aggregation. Others have suggested that calcium-dependent proteolysis of proteins associated with the membrane skeleton may be required for the generation of platelet procoagulant activity (Verhallen et al., 1987; Fox et al., 1990, 1991). In addition defects in the activity of the calcium-dependent protease have been correlated with the Montreal Platelet Syndrome, a human disorder in which platelets are abnormally large and aggregate spontaneously (Okita et al., 1989); however, in this case it is not clear if the protease defect is the cause of the disorder.

The role of talin redistribution in the adhesive response of platelets has not been defined. The activation-dependent translocation of some of the cellular talin to a peripheral, submembranous location puts talin in a position where it could participate in the development of adhesive potential in platelets. The results of our present study suggest that talin redistribution is not required for some of the earliest responses of platelets to stimuli such as platelet shape change, secretion or aggregation, since these responses occur in PMA-treated platelets in which talin redistribution is not detected. The localization of talin near the plasma membrane may however be essential for later responses, such as the platelet-mediated retraction of a fibrin clot. The participation of talin in this platelet response would not be surprising, since talin is thought to be involved in the establishment of transmembrane connections between the actin cytoskeleton and the extracellular environment; such an interaction between the platelet cytoskeleton and extracellular fibrin is critical for platelet-mediated retraction of the fibrin clot.

In conclusion, we have demonstrated that talin is post-translationally modified by phosphorylation upon thrombin activation of human platelets. Our results are consistent with the idea that the subcellular distribution of talin is regulated by the phosphorylation state of the protein. Although neither proteolytic cleavage of talin nor the participation of

GPIIb-IIIa is required for the redistribution of talin in thrombin-activated platelets, post-translational modifications of talin may be utilized by these anucleate cells to regulate talin's subcellular location and thus its function in vivo.

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