The association of pp125^{FAK}, pp60^{Src}, CDC42Hs and Rap1B with the cytoskeleton of aggregated platelets is a reversible process regulated by calcium

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Abstract **The integrin $\alpha_{IIb}\beta_3$-mediated redistribution of the tyrosine kinases pp125^{FAK} and pp60^{Src} and the small GTP-binding proteins CDC42Hs and Rap1B from the membrane skeleton to the cytoskeleton was found to be reversible: upon prolonged platelet aggregation (up to 15 min) induced by the thrombin-receptor activating peptide (TRAP) these signalling proteins dissociated from the cytoskeleton and reappeared in the membrane skeleton. Addition of the extracellular Ca^{2+} chelator EGTA and the intracellular Ca^{2+} chelator BAPTA/AM 30 s after TRAP allowed platelet aggregation and the association of pp125^{FAK}, pp60^{Src}, CDC42Hs and Rap1B with the cytoskeleton, but prevented their dissociation from the cytoskeleton. The results indicate that the prolonged elevation of cytosolic Ca^{2+} in stimulated platelets leads to the dissociation of signalling proteins from the cytoskeleton.**

Key words: Platelet aggregation; Membrane skeleton; Cytoskeleton; Protein tyrosine kinase; Small GTP-binding proteins; Thrombin receptor

1. Introduction

Resting platelets have 40–50% of their actin content in the form of filaments in a loose network throughout the cytoplasm as well as in the membrane skeleton (reviewed by Fox in [1]). When platelets are aggregated, there is a rapid increase in actin polymerisation to 70–80% of total platelet actin. This is associated with an extensive reorganisation of filamentous actin into networks at the periphery of the cell and into bundles in filopodia that contribute to the formation of the cytoskeleton. Several regulatory proteins in platelets are known to associate with the actin-rich cytoskeleton in a manner dependent on activation and cross-linking of the integrin $\alpha_{IIb}\beta_3$. These include the integrin $\alpha_{IIb}\beta_3$ itself [2–5], protein tyrosine kinases such as pp60^{Src} [6–12] and pp60$^{c\text{-}Yes}$ [12], the protein tyrosine phosphatase SH-PTP1 [13], small molecular weight GTP-binding proteins like Rho A [14], Rap1B [15,16], Rap2B [17] and CDC42Hs (submitted for publication) and other signalling proteins [6,7]. This complex of specific signalling proteins might regulate integrin–actin interaction in aggregating platelets. Little is known about the regulation of the cytoskeletal association of these proteins. In the present study we demonstrate that the cytoskeletal association of the protein tyrosine kinases pp125^{FAK} and pp60^{Src} and the small GTP-binding proteins CDC42HS and Rap1B is completely reversed upon prolonged platelet aggregation. The regulation of this process by Ca^{2+} was studied.

2. Experimental

2.1. Materials

Monoclonal antibodies against pp125^{FAK} and pp60^{Src} were from Upstate Biotechnology Inc. (Lake Placid, NY). LA 045, a monoclonal antibody recognising rap 1B was obtained from Quality Biotech (Camden, NJ). The horseradish peroxidase conjugated secondary antibodies were from Amersham (Braunschweig, Germany). Apyrase, ADP, EGTA, acetylsalicylic acid, sodium orthovanadate, fetal bovine serum, Triton X-100, leupeptin, pepstatin A, aprotinin, and phenylmethylsulfonyl fluoride were from the Sigma (St. Louis, MO). BAPTA/AM (1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra-(acetoxymethyl)-ester) was from Calbiochem (La Jolla, CA). Tween 20 and the reagents for electrophoresis were obtained from BioRad (Richmond, CA). The chemiluminescence-based Western blot detection system ECL was from the Amersham Corp. (Buckinghamshire, UK). The thrombin-receptor activating peptide, 'TRAP' (SFLLRN) was custom synthesised by Dr. Arnold (Max Planck Institute, Martinsried, Germany).

2.2. Platelet experiments

Washed human platelets were prepared as described previously [18]. Aliquots (1.5 ml) of washed platelet suspension (10^9 platelets/ml) were incubated whilst stirring (1800 rpm) at 37°C in a LABOR aggregometer (Fresenius, Bad Homburg, Germany) for 1 min. $CaCl_2$ (50 μM) was added 15 s before TRAP (10 μM). Aggregation was measured by the change of the light transmission (%). In parallel samples BAPTA/AM (100 μM) and EGTA (5 mM) were added 30 s after TRAP.

2.3. Preparation of platelet cytoskeleton and membrane skeleton

Cytoskeletal fractions were prepared by using a modification of the method described by Phillips et al. [2]. Briefly, platelet suspensions (1.5 ml) were lysed before or at various intervals after agonist addition by adding equal volumes of ice-cold 2 × Triton lysis buffer (pH 7.5) containing 100 mM Tris-HCl, 2% Triton X-100, 10 mM EGTA, 10 mM EDTA, 2 mM sodium orthovanadate, 20 mM leupeptin, 2 mM phenylmethylsulfonyl fluoride, 20 μM pepstatin A and 0.56 trypsin inhibitor unit (t.i.u.)/ml aprotinin. Samples (3 ml) were vortexed for 10 s, left on ice for 30 min and spun at 15,600 × *g* for 15 min at 4°C. The pellets were washed once in 1 × Triton X-100 (1%) lysis buffer. Pellets were resuspended in 300 μl gel electrophoresis sample buffer.

To obtain the membrane skeletal fraction [19], supernatants of detergent extracts were further centrifuged at 100,000 × *g* for 2.5 h at 4°C in a Beckman ultracentrifuge (L5-50) with a 70.1 Ti rotor. The pellets were resuspended in 300 ml gel electrophoresis sample buffer. The Triton-soluble supernatant fractions were mixed at a ratio of 4:1 with 5 × sample buffer.

2.4. Immunoblotting

Platelet proteins were separated by electrophoresis using 12% SDS-polyacrylamide gel. Proteins were electrophoretically transferred to Immobilon-P PVDF membranes (Millipore Corp., Bedford, MA) by using the Nova Blot semidry system (Pharmacia LKB Biotechnology, Bromma, Sweden) and the procedure recommended by the manufac-

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turer. Residual sites on the membranes were blocked by incubating the blots for 1 h at room temperature with 20% (v/v) fetal bovine serum in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.3% Tween 20. The blots were cut horizontally according to the molecular weights of pp125^{FAK}, pp60^{Src}, CDC42Hs and Rap1B, and incubated for 1 h with one of the following primary antibodies: (a) anti-pp125^{FAK} mAb (1:2000), (b) anti-pp60^{Src} mAb (1:6000), (c) anti-CDC42Hs polyclonal antibody (1:200), and (d) LA045 (1:100,000). Following washing, the blots were incubated for 45 min with peroxidase-labeled sheep anti- mouse IgG (1:10,000) or goat anti-rabbit IgG (for CDC42Hs). After 3 washes of the blot, antibody binding was detected using the enhanced chemiluminiscence (ECL) system (Amersham) and bands were quantified by laser densitometry (Ultroscan XL, Pharmacia).

2.5. Cytoskeletal proteins

To analyse the amount of actin in the cytoskeleton and the proteolytic cleavage of actin-binding protein (ABP) and talin, platelet proteins were separated on a 6% SDS-PAGE slab gel, followed by staining of the gel with 0.2% Coomassie brilliant blue, destaining and drying. The amount of actin was quantified by laser densitometry.

3. Results

Platelets were aggregated with TRAP for 15 min. Aggregation was maximal at 5 min and decreased by 34.7 ± 1.3% (mean ± S.D., n = 3) after 15 min of stimulation (see also Fig. 1, tracing A). Cytoskeleton, membrane skeleton and soluble fractions were isolated from platelets aggregated for 0, 2, 5, 10 and 15 min and studied for the presence of pp125^{FAK}, pp60^{Src}, Rap1B and CDC42Hs by immunoblotting.

The immunoblot in Fig. 2 (first five lanes on the left) shows the kinetic of pp125^{FAK} association with the cytoskeleton of aggregated platelets. There was no or very little pp125^{FAK} detectable in the cytoskeleton of resting platelets (0 min). In platelets stimulated for 2 min, 9.5% of total pp125^{FAK} was present in the cytoskeleton. Upon prolonged platelet stimulation (15 min) pp125^{FAK} disappeared from the cytoskeleton, and this was paralleled by partial platelet disaggregation. In the membrane skeleton pp125^{FAK} decreased from 10% to 5.4% of total protein 2 min after stimulation with TRAP and returned to control values 15 min after stimulation (Fig. 3A).

Similar to pp125^{FAK} (Figs. 2 and 3A), two small GTP-binding proteins, Rap1B and CDC42Hs, translocated to the cytoskeleton 2 min after platelet aggregation induced by TRAP and then disappeared from the cytoskeleton upon prolonged aggregation of platelets for 15 min (Fig. 3B and C). In the membrane skeleton, Rap1B and CDC42Hs decreased from 15% and 9% to 10% and 1% of total protein, respectively, 2 min after stimulation with TRAP, and returned to control values 15 min after stimulation. Unlike the proteins mentioned above, pp60^{Src} (10% of total cellular pp60^{Src}) was detected in the cytoskeleton of resting platelets. Upon platelet aggregation cytoskeleton-associated pp60^{Src} increased to 19% 2 min after stimulation, followed by a decline to the control level upon prolonged platelet aggregation for 15 min (Fig. 3D).

In order to further study the regulation of dissociation of these proteins from the cytoskeleton, we next asked whether the level of cytosolic free calcium might determine this process. Platelet activation induced by thrombin receptor activation induces Ca^{2+}-mobilisation from intracellular stores and Ca^{2+}-entry through the plasma membrane, resulting in a very rapid (<1 s) increase of cytosolic Ca^{2+} followed by a slow decrease to a new steady-state Ca^{2+}, the 'Ca^{2+}-plateau' (reviewed in [20]). BAPTA/AM and EGTA chelate intra- and extracellular Ca^{2+}, respectively, and block cytosolic Ca^{2+}-transients in platelets [21]. When added 30 s after TRAP, these substances will not reduce the Ca^{2+}-peak, but the subsequent Ca^{2+}-plateau. Upon

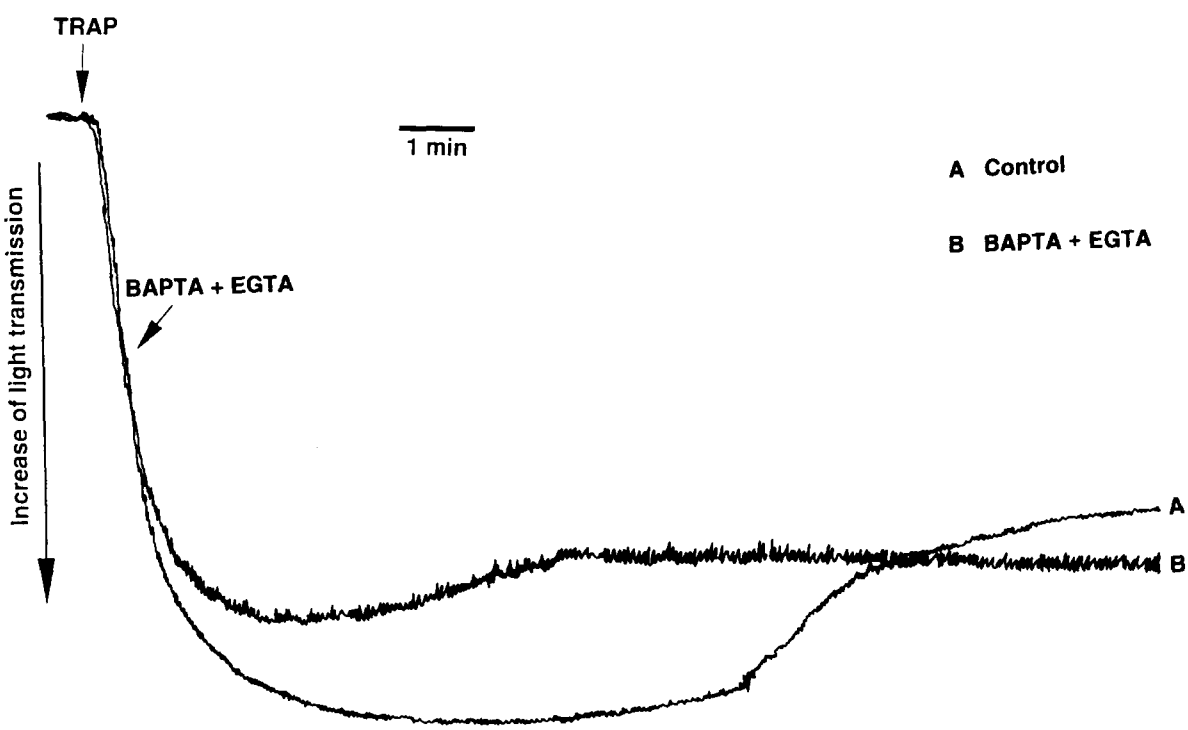

Fig. 1. Platelet aggregation for 15 min induced by TRAP. Effect of BAPTA/AM and EGTA. Platelets were aggregated as described in section 2. BAPTA/AM (100 μM) and EGTA (5 mM) were added (arrow) 30 s after TRAP (tracing B). In control tracing (A) DMSO was added at the same time. The figure is representative of three different experiments.

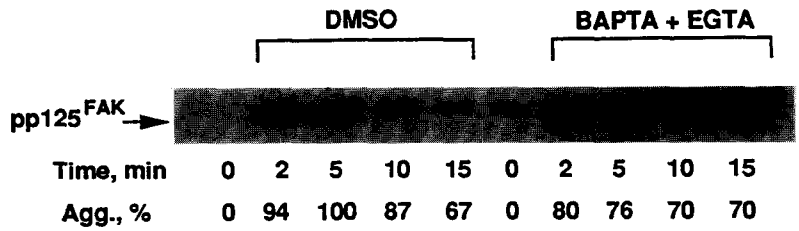

Fig. 2. Cytoskeletal association of pp125^{FAK} in platelets aggregated for 15 min. Effect of BAPTA/AM and EGTA. Platelet aggregation was induced by TRAP (10 μM). DMSO (control), or BAPTA/AM (100 μM) and EGTA (5 mM) were added 30 s after TRAP. The Triton-insoluble cytoskeletal proteins were isolated from the samples at the indicated times, separated by SDS-PAGE and immunoblotted using the anti-pp125^{FAK} antibody. The experiment is representative of four different experiments.

addition of BAPTA/AM (100 μM) and EGTA (5 μM) 30 s after TRAP all proteins studied (pp125^{FAK}, pp60^{Src}, CDC42Hs and Rap1B) still translocated to the cytoskeleton 2 min after platelet stimulation. Under these conditions, maximal platelet aggregation at 5 min was reduced by 18 ± 2% (mean ± S.D.) in comparison to control but it did not further decline (Fig. 1, tracing B). Interestingly, upon continued stimulation for 15 min, the amounts of these proteins in the cytoskeleton did not decrease but remained elevated (Figs. 2 and 3). These results indicate that the sustained elevation of intracellular Ca^{2+} after platelet stimulation might be responsible for the dissociation of pp125^{FAK}, pp60^{Src}, CDC42Hs and Rap1B from the cytoskeleton.

We also measured the amount of actin in the cytoskeleton under these conditions. Cytoskeletal actin increased from 41 ± 7% to 81 ± 9.2% 2 min after aggregation induced by TRAP. Upon prolonged platelet aggregation for 15 min the amount of actin in the cytoskeleton returned to the resting value (40 ± 5.9%) whereas in platelets treated with BAPTA and EGTA the cytoskeletal actin remained elevated (84 ± 8.7%) (mean ± S.D., n = 3).

4. Discussion

In addition to the previously reported aggregation-dependent associaton of pp60^{Src} [6–12], Rap1B [15,16] and CDC42Hs[2] with the cytoskeleton, we found in the present study also an aggregation-dependent translocation of pp125^{FAK} to the platelet cytoskeleton. As the increased binding of CDC42Hs, pp125^{FAK}, pp60^{Src} and Rap1B to the cytoskeleton was paralleled by an increase in cytoskeletal actin, it is conceivable that these proteins associate with actin filaments that are newly formed in aggregating platelets. In previous studies it has been shown that stimulation of actin polymerisation in activated platelets regulates the interaction of signalling proteins with the cytoskeleton. Prevention of increased actin polymerisation in aggregated platelets by cytochalasin D treatment resulted in inhibition of cytoskeletal association of CDC42Hs[2] and pp60^{Src} [10]. Addition of DNase (which depolymerises F-actin) to the Triton-lysate of aggregated platelets also dramatically reduced the amount of cytoskeletal-associated pp60^{Src} [8]. The present study shows to our knowledge for the first time that actin filamentisation and the translocation of protein tyrosine kinases (pp125^{FAK} and pp60^{Src}) and small GTP-binding proteins (CDC42Hs and Rap1B) from the membrane skeleton to the cytoskeleton in aggregated platelets is a completely reversible physiological process.

Chelation of extracellular and cytosolic Ca^{2+} subsequent to platelet aggregation inhibited markedly the cytoskeletal association of Rap1B, but less the association of pp125^{FAK}, pp60^{Src} and CDC42Hs 2 and 5 min after platelet aggregation. In agreement with our results Fischer et al. [16] have reported that Rap1B translocation to the cytoskeleton is dependent on extracellular Ca^{2+} while that of pp60^{Src} is not. Hence, these findings indicate a different regulation for the cytoskeletal association of Rap1B compared to other proteins. The results with chelation of extracellular and cytosolic Ca^{2+} subsequent to TRAP-stimulation reveal that sustained elevation of cytosolic Ca^{2+} is responsible for the decrease of cytoskeletal actin and the dissociation of signalling proteins from the cytoskeleton. Prolonged increase of cytosolic Ca^{2+} might activate calpain I leading to the hydrolysis of cytoskeletal proteins like actin-binding protein (ABP), spectrin, talin (p235) and α-actinin which are involved in the organisation of actin filaments into their three-dimensional structure. However, we did not observe calpain activation in platelets aggregated for 15 min, as judged from proteolysis of ABP and p235 (data not shown). Hence, calpain activation is unlikely to be the cause for the decrease of cytoskeletal actin and dissociation of signalling proteins from the cytoskeleton upon prolonged platelet aggregation. Gelsolin might be another possible candidate responsible for the loss of cytoskeletal actin filaments during prolonged platelet aggregation. Gelsolin binds to filamentous actin in the presence of micromolar Ca^{2+} concentration leading to depolymerisation of actin filaments and disruption of the actin network [22,23]. Notabely, gelsolin activation requires a sustained increase in free Ca^{2+} [24], rather than a rapid and transient increase [25]. Chelation of cytosolic Ca^{2+} by BAPTA/AM and EGTA might result in a diminished actin–gelsolin complex formation and hence unchanged cytoskeletal actin upon prolonged platelet aggregation.

In various studies it has been suggested that the aggregation-induced reorganisation of the actin cytoskeleton may provide a functional platform for the physiological activity of the various signalling proteins that translocate to the cytoskeleton. Our data show that, during prolonged aggregation, the amounts of cytoskeletal actin, pp125^{FAK}, Rap1B, CDC42Hs and pp60^{Src} in the cytoskeleton return to the basal level present in resting platelets. Interestingly, this process was associated with partial platelet disaggregation. These results indicate that sustained elevation of cytosolic Ca^{2+} in stimulated platelets plays a role in the destabilisation of platelet aggregates by diminishing the association of signalling proteins with the actin-rich cytoskeleton. Chelation of Ca^{2+} which preserved the actin-rich cytoskeleton and the cytoskeletal association of signalling proteins led to irreversible platelet aggregates which were stable.

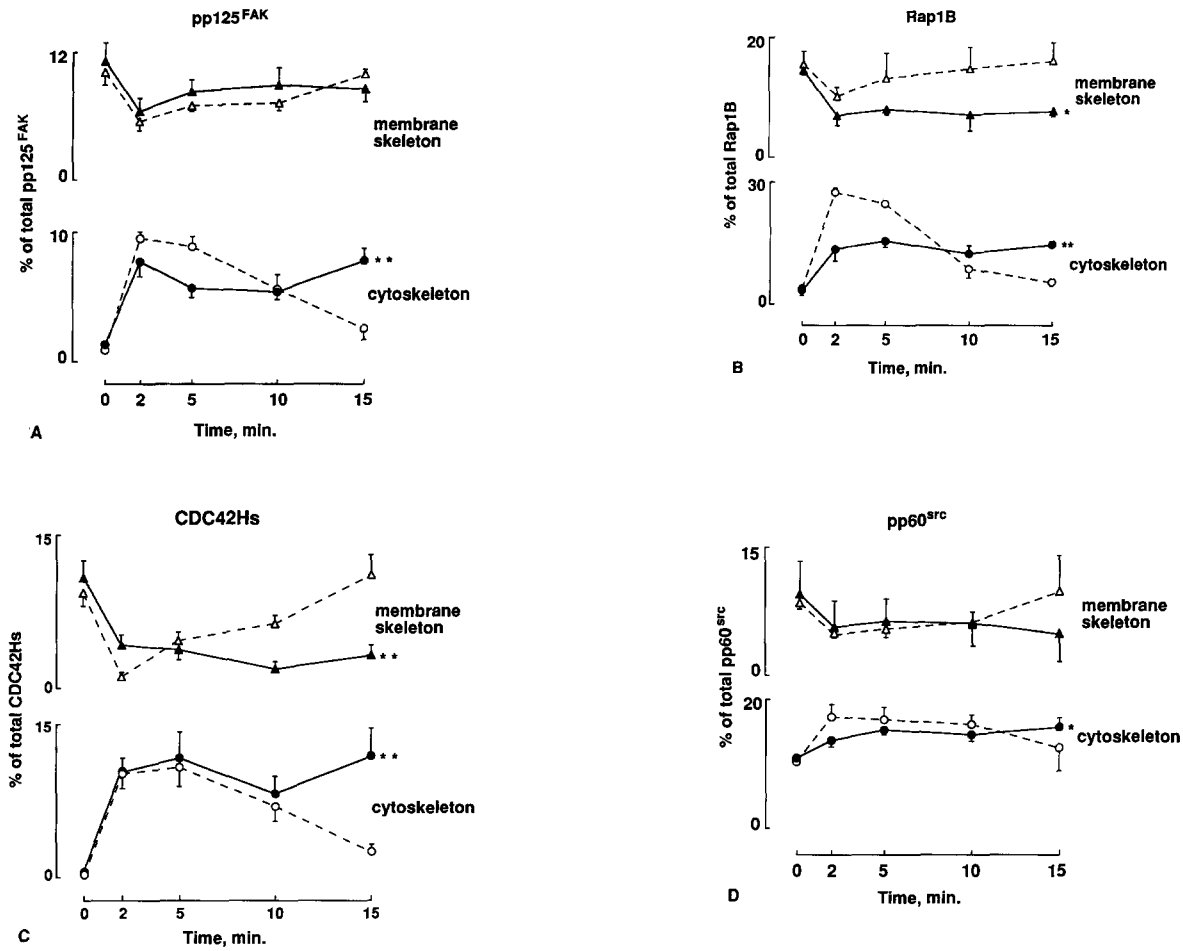

Fig. 3. Distribution of pp125^{FAK} (A), Rap1B (B), CDC42Hs (C) and pp60^{Src} (D) in the cytoskeleton and membrane skeleton during platelet aggregation induced by TRAP. Effect of BAPTA/AM and EGTA. Platelet aggregation was induced by TRAP (10 μM). DMSO (control) or BAPTA/AM (100 μM) and EGTA (5 mM) were added 30 s after TRAP. The cytoskeletal and membrane skeletal fractions were isolated at the indicated time points. Proteins in each fraction were separated by SDS-PAGE and immunoblotted using the respective antibodies. Data are mean ± S.D. of three different experiments. Open symbols-control; closed symbols = BAPTA/AM and EGTA. *$P < 0.05$; **$P < 0.01$ as compared to control at 15 min.

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