Journal of Cellular Biochemistry 75:675–685 (1999)

Interaction of the Low-Molecular-Weight GTP-Binding Protein rap2 With the Platelet Cytoskeleton Is Mediated by Direct Binding to the Actin Filaments

Mauro Torti,[1]* **Alessandra Bertoni,**[1] **Ilaria Canobbio,**[1] **Fabiola Sinigaglia,**[2] **Eduardo G. Lapetina,**[3] **and Cesare Balduini**[1]

[1]Department of Biochemistry, University of Pavia, 27100 Pavia, Italy
[2]Institute of Biological Chemistry, University of Genoa, 16132 Genoa, Italy
[3]Molecular Cardiovascular Research Center, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

Abstract The interaction of the low-molecular-weight GTP-binding protein rap2 with the cytoskeleton from thrombin-aggregated platelets was investigated by inducing depolymerization of the actin filaments, followed by in vitro-promoted repolymerization. We found that the association of rap2 with the cytoskeleton was spontaneously restored after one cycle of actin depolymerization and repolymerization. Exogenous rap2, but not unrelated proteins, added to depolymerized actin and solubilized actin-binding proteins, was also specifically incorporated into the in vitro reconstituted cytoskeleton. The incorporation of exogenous rap2 was also observed when the cytoskeleton from resting or thrombin-activated platelets was subjected to actin depolymerization-repolymerization. Moreover, such interaction occurred equally well when exogenous rap2 was loaded with either GDP or GTPγS. We also found that polyhistidine-tagged rap2 immobilized on Ni^{2+}-Sepharose and loaded with either GDP or GTPγS, could specifically bind to cytoskeletal actin. Moreover, when purified monomeric actin was induced to polymerize in vitro in the presence of rap2, the small G-protein specifically associated with the actin filaments. Finally, rap2 loaded with either GDP or GTPγS was able to bind to purified F-actin immobilized on a plastic surface. These results demonstrate that rap2 interacts with the platelet cytoskeleton by direct binding to the actin filaments and that this interaction is not regulated by the activation state of the protein. J. Cell. Biochem. 75:675–685, 1999.

Key words: platelet activation; cytoskeleton; actin polymerization; small GTPases; GDP; GTPγS

rap proteins are low-molecular-weight GTP-binding proteins structurally related to the proto-oncogene product $p21^{ras}$ [Torti and Lapetina, 1994]. Four members of the rap family of proteins have been identified: rap1A, rap1B, rap2A, and rap2B. Human platelets express significant levels of both rap1B (95% identical to rap1A) and rap2B (90% identical to rap2A) [Torti and Lapetina, 1994]. The role of these small GTPases in platelet function is still poorly understood. Rap1 has been originally identified as a transformation suppressor of K-ras [Kitayama et al., 1989]. In fact, the GTP-bound form of rap1 can interact with several targets of $p21^{ras}$, including p120GAP, Raf-1, and ralGDS, without inducing their activation [Frech et al., 1990; Zhang et al., 1993b; Spaagaren et al., 1994]. Based on the ability to interact with these effectors, it has been demonstrated that rap1 is rapidly activated upon platelet stimulation with physiological agonists like thrombin [Torti and Lapetina, 1992; Franke et al., 1997]. By contrast, rap2 does not interact with any known target protein for $p21^{ras}$ and does not antagonize the transforming activity of oncogenic $p21^{ras}$. Moreover, agonist-induced activation of rap2 in platelets, as well as in other cells, has not as yet been reported. Recently, a new protein, called RPIP8 has been identified as potential target for rap2, as it specifically and selectively interacts with the GTP-bound form of rap2 [Janoueix-Lerosey et al., 1998].

Grant sponsor: Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST, Progetti di Ricerca di Interesse Nazionale); Grant sponsor: Consiglio Nazionale delle Ricerche (CNR, Target Project Biotechnology).

*Correspondence to: Mauro Torti, Department of Biochemistry, University of Pavia, via Bassi 21, 27100 Pavia, Italy. E-mail: mtorti@unipv.it

Received 3 December 1998; Accepted 26 May 1999

We have demonstrated that rap2 interacts with the intracellular cytoskeleton upon platelet stimulation with thrombin [Torti et al., 1993]. Translocation of rap2 to the cytoskeleton requires platelet aggregation and is regulated by the fibrinogen receptor, glycoprotein IIb-IIIa [Torti et al., 1994]. In resting platelets, the cytoskeleton is mainly composed of actin filaments [Fox, 1993]. Upon stimulation with extracellular agonists, the amount of polymerized actin increases and other proteins, including myosin and α-actinin, are incorporated in the reorganized cytoskeleton [Fox, 1993]. A further increase in actin polymerization is observed when stimulated platelets undergo aggregation. Moreover, in aggregated platelets, the glycoprotein IIb-IIIa and PECAM-1, and signal transduction molecules, like phospholipase Cγ1, phosphatidylinositol 3-kinase, $pp60^{src}$, $pp72^{syk}$, $pp125^{FAK}$, the small GTPases rap1, rap2, rho, and CDC42, are recruited into the cytoskeleton [Wheeler et al., 1984; Fischer et al., 1990; Grondin et al., 1991; Newman et al., 1992; Zhang et al., 1993a; Torti et al., 1993; Tohyama et al., 1994; Guinebault et al., 1995; Dash et al., 1995]. Therefore, the platelet cytoskeleton is not only responsible for the morphological changes of activated platelets, but also represents a network where different signaling molecules are interacting.

In this work we tried to identify the cytoskeletal component that is able to interact specifically with rap2; we have also analyzed the role of the nucleotide bound to rap2 in this interaction. We found that rap2 directly binds to actin filaments and that this interaction is independent of the functional state of the protein.

MATERIALS AND METHODS

Materials

Thrombin, GDP, GTPγS, N-hydroxysuccinimidobiotin, and polyclonal anti-actin antiserum were purchased from Sigma Chemical Co. (St. Louis, MO). Chelating Sepharose Fast Flow, and purified bovine serum albumin (BSA), ovalbumin, and chymotrypsinogen were from Pharmacia Biotech. Chromozym TH was from Boehringer Mannheim. The polyclonal antiserum against rap2 proteins was obtained as previously described [Winegar et al., 1991]. Bacteria expressing polyhistidine-tagged rap2A were a gift from Dr. J. De Gunzburg (INSERM U248, Istitute Curie, Paris, France). Purified monomeric actin was provided by Professor E. Grazi (Department of Biochemistry and Molecular Biology, University of Ferrara, Italy). Polyhistidine-tagged $SdrCB_{1,2}$ protein, corresponding to a sequence of the *Staphylococcus aureus* fibrinogen receptor was a gift from Dr. Magnus Hook (Department of Biochemistry and Biophysics, Texas A&M University, Houston, Texas) through Dr. L. Visai (Department of Biochemistry, University of Pavia, Italy). Triton X-100 was from ICN. Nitrocellulose membranes were obtained from Costar. Microtiter plates were from Labsystem. Peroxidase-conjugated avidin was from Dako. Peroxidase-conjugated secondary antibodies and molecular-weight markers were obtained from Bio-Rad. Enhanced chemiluminescence substrate and Reflection film was from DuPont-NEN. All others reagents were of analytical grade.

rap2 Purification, Loading, and Biotinylation

Polyhistidine-tagged rap2 expressed in *Escherichia coli* (strain BL21) was purified in the GDP-bound form upon lysis by sonication in the presence of 1 mM GDP by affinity chromatography on Ni^{2+}-Sepharose performed according to the manufacturer's instructions. Recombinant rap2B was purified as described [Molina y Vedia et al., 1990]. Loading with GTPγS was performed by incubating aliquots of the purified protein with 500-fold excess (mol/mol) GTPγS at 30°C for 30 min in 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% dithiothreitol, 1 mM EDTA. The reaction was stopped by adding 5 mM $MgCl_2$. To evaluate loading efficiency, samples were extensively dialyzed, and bound nucleotides were released from the protein by heating at 95°C for 3 min and then analyzed by reverse-phase high-performance liquid chromatography RP-HPLC as previously described [Ramaschi et al., 1996]. To label purified proteins with biotin, aliquots of purified rap2 loaded with either GDP or GTPγS, as well as GDP-loaded rap2B, purified BSA, ovalbumin, chymotrypsinogen, and polyhistidine-tagged $SdrCB_{1,2}$ protein, were incubated with 20% (w/w) N-hydroxysuccinimidobiotin in 0.1 M $Na_2B_4O_7$, pH 9.5, at room temperature for 2 h, and then dialyzed overnight at 4°C with 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 0.5% dithiothreitol. Final protein concentration was determined by densitometric scanning of Coomassie blue-stained gels, using a calibration curve obtained with known amounts of BSA. Protein biotinylation was verified by dot-blot,

followed by staining with peroxidase-conjugated avidin.

Platelet Preparation and Cytoskeleton Extraction

Platelet concentrates were obtained from the local blood bank (Servizio di Immunoematologia e Trasfusione, IRCCS Policlinico S. Matteo, Pavia, Italy). Platelets were centrifuged at 1,200*g* for 20 min at room temperature and resuspended in a buffer containing 135 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 2 mM $MgCl_2$, 0.2 mM EGTA, 5.5 mM glucose, 0.35% BSA, pH 6.5. Cells were washed with the same buffer without EGTA and finally resuspended in a buffer containing 135 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, pH 7.35, at the final concentration of 4×10^9 platelets/ml. Samples, containing 40×10^9 platelets (10 ml), were equilibrated at 37°C for 5 min and then stimulated with 0.6 U/ml thrombin for 5 min with constant stirring to permit maximal aggregation. To prepare samples of thrombin-activated, but not aggregated platelets, stirring was omitted. Samples of resting platelets were treated with buffer, instead of thrombin. Samples were then lysed with 1% Triton X-100, and cytoskeleton was extracted as previously described [Torti et al., 1993]. Cytoskeleton was either solubilized with SDS-sample buffer (25 mM Tris, 192 mM Glycine, pH 8.3, 4% SDS, 1% dithiothreitol, 20% glycerol, 0.02% bromophenol blue, 2% β-mercaptoethanol) for direct electrophoretic analysis or stored at −20°C until use.

Preparation of the Reconstituted Cytoskeleton

Preparation of the reconstituted cytoskeleton was performed essentially as previously described [Payrastre et al., 1991]. Briefly, cytoskeleton from resting, activated, or aggregated platelets was solubilized with 5 ml of KI-buffer (100 mM PIPES, pH 7.4, 100 mM KCl, 0.6 M KI, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 100 μM Na_3VO_4) for 50 min at 4°C under gentle shaking, and centrifuged at 37,000*g* for 30 min at 4°C. KI-soluble and insoluble materials were collected and, in some experiments, directly dissociated with SDS-sample buffer. To induce actin repolymerization, samples containing 1 ml of KI-soluble material were dialyzed overnight with 1 L of dialysis buffer (10 mM PIPES, pH 6.8, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM PMSF, 100 μM Na_3VO_4). In some experiments, 2 μg of exogenous proteins were added to the KI-soluble material before dialysis. Repolymerized actin and actin-binding proteins (reconstituted cytoskeleton, RCSK) were then recovered by centrifugation at 13,000*g* for 10 min at 4°C and washed three times with dialysis buffer. The pellets were finally dissociated with 0.2 ml of 4% SDS, and protein concentration was determined by the bicinchoninic acid assay. To the remaining of the samples, an equal volume of a mixture containing 1% dithiothreitol, 20% glycerol, 0.02% bromophenol blue, and 2% β-mercaptoethanol was added before electrophoresis analysis.

Cytoskeleton Binding Assay

Triton X-100-insoluble material from thrombin-aggregated platelets was extracted with 5 ml of extraction buffer (10 mM HEPES, 0.5 M NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 5 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin, 1 mM Na_3VO_4, 1% Triton X-100, 0.3% sodium deoxycholate) for 50 min at 4°C under gentle shaking and then centrifuged at 37,000*g* for 30 min at 4°C. Supernatant was collected and 5 mM $MgCl_2$ was added. A total of 20 μg of Ni^{2+}-Sepharose-bound polyhistidine-tagged rap2–GDP or polyhistidine-tagged rap2–GTPγS or an equal volume of resin without bound proteins was mixed with 1 ml of cytoskeleton extract and incubated at 4°C for 2 h under shaking. Beads were then recovered by brief centrifugation, washed three times with 1 ml of extraction buffer containing 2% Triton X-100, and finally resuspended in SDS-sample buffer.

Actin Co-sedimentation Assay

In this assay, 1 or 2 μg of biotinylated proteins was incubated at room temperature with 50 μg of G-actin in 1 ml of buffer containing 2 mM Tris-HCl, pH 7.4, 0.2 mM $CaCl_2$, 0.2 mM ATP, 2 mM β-mercaptoethanol, 0.02% NaN_3 for 10 min. Actin polymerization was induced by adding 75 mM KCl and 2 mM $MgCl_2$ for 1 h at room temperature. Samples were then brought up to 1% Triton X-100 and vigorously vortexed for 10 min to disrupt unspecific binding. Samples were centifuged at 50,000*g* for 1 h at 25°C. Pellets of polymerized actin were washed once with the same buffer containing 1% Triton

X-100 and finally dissolved in 50 ml of SDS-sample buffer.

Solid-Phase Binding Assay

Purified monomeric G-actin (50 µl, 0.1 mg/ml) was induced to polymerize by addition of 75 mM KCl and 2 mM $MgCl_2$ for 1 h at room temperature. F-actin was then transferred to wells of a microtiter plate and coating was performed by overnight incubation at 4°C. Plates were washed three times with a solution containing 0.15 M NaCl and 0.1% Tween 20 to remove unbound proteins. Additional binding sites were saturated for 2 h at room temperature with 6% BSA in 10 mM phosphate-buffered saline (PBS). Biotinylated proteins (100 ng) in 50 µl of 6% BSA were then added, and incubation was performed for 2 h at room temperature. Unbound proteins were washed out, and peroxidase-conjugated avidin (1:15,000 dilution in 50 µl of 10 mM PBS containing 6% BSA) was added and incubated for 1 h at room temperature. Bound proteins were detected by a colorimetric reaction using o-phenylenediamine dihydrochloride as substrate. Control samples were prepared by omitting either coating with F-actin or incubation with biotinylated proteins.

Electrophoresis and Immunoblotting

Electrophoresis was performed on 15% and 12% linear or 5–15%, 5–20%, and 10–20% gradient acrylamide gels. Proteins were either stained with Coomassie blue or trasferred to nitrocellulose. Nitrocellulose membranes were blocked overnight at 4°C with 6% BSA in TBS (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl) and incubated with anti-rap2 antiserum (1:1,000 dilution) for 2 h at room temperature. Membranes were washed extensively with 50 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 1 mg/ml polyethylene glycol (PEG) 20,000, 0.1% BSA, and 0.05% Tween 20, and incubated with peroxidase-conjugated secondary antibody (1:20,000 dilution) for 45 min. In some experiments, nitrocellulose membranes were blocked and then incubated directly with peroxidase-conjugated avidin (1: 30,000 dilution) for 45 min. Upon extensive washing, reactive proteins were visualized with a chemiluminescence reaction.

RESULTS

Interaction of rap2 With the Triton X-100-Insoluble Material and With the Reconstituted Cytoskeleton From Thrombin-Aggregated Platelets

The cytoskeleton from resting and thrombin-aggregated platelets was isolated as Triton X-100-insoluble material and subsequently treated with potassium iodine (KI), which causes depolymerization of the actin filaments. The KI-insoluble and KI-soluble fractions were separated and a reconstituted cytoskeleton (RCSK) was obtained by dialysis of the KI-soluble material to induce spontaneous actin repolymerization. All the prepared fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. As shown in Figure 1A, the stimulation of platelets with thrombin led to the incorporation of several proteins into

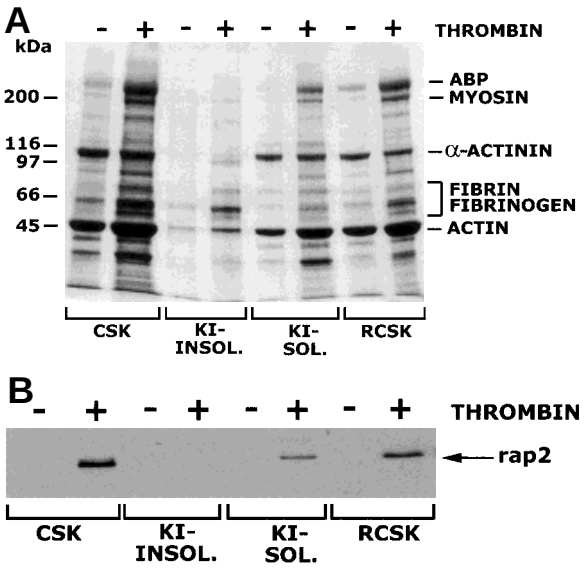


Fig. 1. Fractionation of the platelet cytoskeleton and redistribution of rap2. Cytoskeleton (CSK) from resting and thrombin-aggregated platelets was prepared as Triton X-100-insoluble material and solubilized with 0.6 M KI-containing buffer. KI-insoluble (KI-insol.) and KI-soluble (KI-sol.) fractions were collected. A reconstituted cytoskeleton (RCSK) was obtained by dialysis of the KI-soluble fraction to allow actin repolymerization. **A:** Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 5–15% acrylamide gradient gel and stained with Coomassie brilliant blue. **B:** Proteins were separated by SDS-PAGE on a 10–20% acrylamide gradient gel, transferred to nitrocellulose, and probed with the anti-rap2 antiserum. The amount of cytoskeletal proteins applied to each electrophoretic lane was obtained from 0.15×10^9 platelets for the CSK and from 0.3×10^9 platelets for the other fractions.

the cytoskeleton, including actin-binding protein, myosin, α-actinin, fibrin or fibrinogen, and actin. Upon treatment with KI, many of these proteins were recovered in the soluble fraction, whereas the KI-insoluble material was composed mainly of fibrinogen and fibrin and a small amount of undepolymerized actin. This finding suggests that fibrin and fibrinogen, capable of forming an insoluble network, are probably co-precipitated with the cytoskeleton without being associated with the actin-based structures. The RCSK obtained by spontaneous actin repolymerization exhibited a composition very similar to that of the Triton X-100-insoluble fraction, and all the main contractile proteins were observed.

To investigate the distribution of cytoskeletal-associated rap2 during the depolymerization-repolymerization cycle, samples of Triton X-100-insoluble material, KI-insoluble and soluble fractions, and RCSK were analyzed by immunoblotting using a rap2-specific antiserum. Figure 1B shows that rap2 interacts with the cytoskeleton upon thrombin stimulation, and that totally redistributes to the KI-soluble material upon actin depolymerization. Moreover, rap2 was also able to reassociate to the reconstituted cytoskeleton when actin repolymerization was promoted in vitro by dialysis.

Interaction of Exogenous rap2 With the Reconstituted Cytoskeleton Is Independent of the Bound Nucleotide

We then investigated whether exogenous rap2 added to the KI-soluble fraction could be specifically incorporated into the RCSK. In this study, 2 μg of purified rap2 labeled with biotin, or 2 μg of unrelated biotinylated proteins used as control (BSA, ovalbumin, and chymotrypsinogen), was added to samples of KI-soluble material before the induction of actin repolymerization by overnight dialysis. Chymotrypsinogen was selected as control protein because its molecular mass is similar to that of recombinant rap2. Although chymotrypsinogen could liberate the active protease, which could alter the assay conditions, we never observed the generation of the active enzyme during all our experiments, as evaluated by spettrophotometric determination using the synthetic substrate Chromozym TH (data not shown). Despite the larger molecular mass, BSA and ovalbumin possess a net electric charge similar to rap2 (pI 5.8, 5.1, and 4.7 for BSA, ovalbumin, and rap2, respectively). Aliquots of the reconstituted cytoskeleton, along with aliquots of the biotinylated proteins added to the samples, were separated by SDS-PAGE on a 10–20% acrylamide gradient gel, transferred to nitrocellulose and probed with peroxidase-conjugated avidin. Only biotinylated rap2 was incorporated into the RCSK (Fig. 2A). By densitometric analysis of several experiments, we calculated that about 20–30% of the exogenous purified rap2 associated with the reconstituted cytoskeleton. To rule out the possibility that incorporation of exogenous rap2 into the RCSK could be mediated by unspecific binding through the polyhistidine tag of the protein, we performed control experiments using both recombinant rap2B with no polyhistidine tag purified from *E. coli* in the GDP-bound form by conventional chromatographic methods [Molina y Vedia et al., 1990], and an unrelated protein ($SdrCB_{1,2}$) corresponding to a domain of a bacterial fibrinogen receptor, purified as a polyhistidine-tagged protein [Josefsson et al., 1998]. This protein possesses a molecular mass of about 30 kDa and an electric charge similar to that of rap2 (pI 4.45). Figure 2B shows that rap2B, but not polyhistidine-tagged $SdrCB_{1,2}$ protein, was incorporated into the RCSK. These results indicate that the polyhistidine tag is not responsible for the interaction of rap2 with the RCSK and that, not only rap2A, but also rap2B can associate with the reconstituted cytoskeleton. Therefore, this experimental approach can be used to further investigate the mechanisms of rap2 interaction with the cytoskeleton.

Upon platelet stimulation with thrombin, some proteins, such as myosin or α-actinin, rapidly associate with the actin filaments, whereas others, including several signaling molecules, interact with the cytoskeleton exclusively during platelet aggregation [Fox, 1993]. To identify the cytoskeletal proteins able to interact with rap2, KI-soluble fractions were prepared from the Triton X-100-insoluble material isolated from resting, thrombin-activated, and thrombin-aggregated platelets, and incubated with exogenous rap2 before induction of actin repolymerization. To analyze the role of the nucleotide bound to rap2 in the association with the RCSK, purified rap2 proteins loaded with either GDP or GTPγS were used. Figure 3 shows that both rap2–GDP and rap2–GTPγS

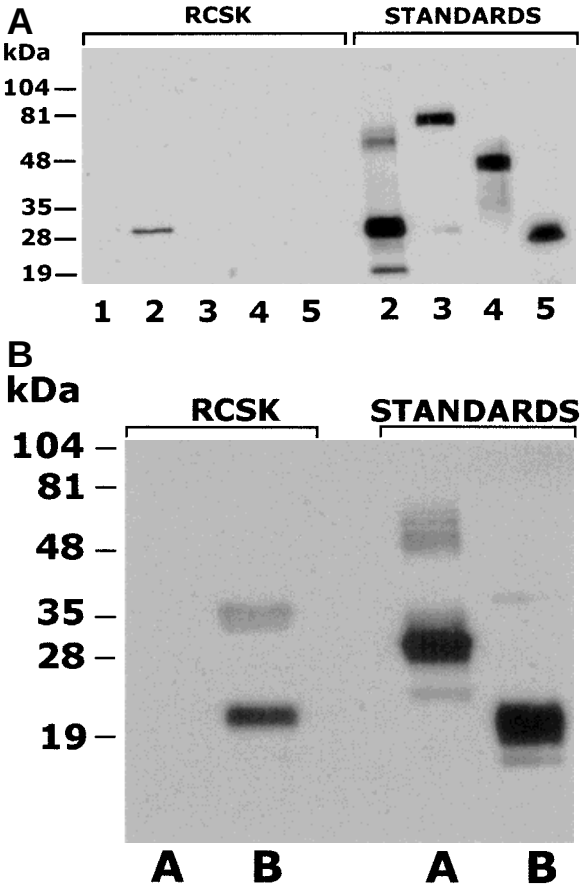


Fig. 2. Exogenous rap2 specifically associates to the reconstituted cytoskeleton. **A:** Samples of KI-soluble material (1 ml) obtained from the cytoskeleton of thrombin-aggregated platelets were incubated with buffer **(lane 1)** or with 2 μg of purified biotinylated rap2 **(lane 2)**, bovine serum albumin (BSA) **(lane 3)**, ovalbumin **(lane 4)**, and chymotrypsinogen **(lane 5)**. Actin polymerization was induced by overnight dialysis, and the reconstituted cytoskeleton (RCSK) was recovered by centrifugation. A total of 20 μg of RCSK proteins from each sample (typically 5–10 μl) was loaded on a 10–20% acrylamide gradient gel together with 50 ng of the biotinylated proteins used (standards): rap2 **(lane 2)**, BSA **(lane 3)**, ovalbumin **(lane 4)**, and chymotrypsinogen **(lane 5)**. Upon transfer to nitrocellulose, biotinylated proteins were detected by staining with peroxidase-conjugated avidin. The band at about 60 kDa in the rap2 standard represents a dimeric form of the purified protein. **B:** The reconstituted cytoskeleton was prepared in the presence of 2 μg of polyhistidine-tagged $SdrCB_{1,2}$ **(A)**, or purified rap2B with no polyhistidine tag **(B)**. A total of 20 μg of RCSK proteins and 50 ng of the biotinylated proteins used (standards) was separated on a 10–20% acrylamide gradient gel, transferred to nitrocellulose and stained with peroxidase-conjugated avidin.

interact with the reconstituted cytoskeleton, indicating that this interaction was independent of the bound nucleotide. Moreover, exogenous rap2–GDP and rap2–GTPγS were able to associate equally well to the RCSK obtained from the cytoskeleton of both resting, thrombin-activated, and thrombin-aggregated platelets. This finding indicates that although rap2 translocates to the cytoskeleton only during aggregation in intact platelets, the cytoskeleton of resting platelets already includes the molecular components that are able to bind rap2.

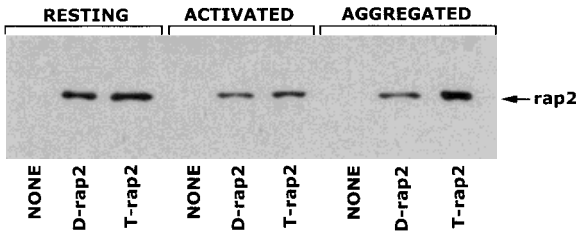


Fig. 3. Role of the guanine nucleotide in the association of purified rap2 with the reconstituted cytoskeleton. RCSK was obtained by dialysis of KI-soluble material prepared from the cytoskeleton of resting, thrombin-activated, and thrombin-aggregated platelets. Before dialysis, 2 μg of rap2 loaded with GDP (D-rap2), 2 μg of rap2 loaded with GTPγS (T-rap2) or buffer (none) were added to the KI-soluble fraction. RCSK proteins from each samples (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10–20% acrylamide gradient gel, transferred to nitrocellulose, and probed with the anti-rap2 antiserum.

Rap2 Binds Directly to the Actin Filaments

On the basis of the results obtained, we investigated the ability of rap2 to bind directly to the actin filaments. Cytoskeleton from thrombin-aggregated platelets was isolated as Triton X-100-insoluble material and extracted with 0.5 M NaCl and 0.3% sodium-deoxycholate. The supernatant (cytoskeleton extract) was mixed with 20 μg of polyhistidine-tagged rap2–GDP or rap2–GTPγS bound to Ni^{2+}-Sepharose beads, or with an equal amount of resin with no bound proteins. The resin beads were recovered by centrifugation, and the presence of actin was analyzed by immunoblotting, using a specific antiserum. As shown in Figure 4A, actin from the cytoskeleton extract was able to bind equally well to immobilized rap2–GDP and rap2–GTPγS, but not to the Ni^{2+}-Sepharose resin alone. Moreover, no immunoreactive proteins were detected when immobilized rap2 was incubated with buffer, ruling out the possibility of cross-reactivity with contaminant proteins (Fig. 4A). To investigate whether any other cytoskeletal protein could specifically bind rap2, identical samples were analyzed by SDS-PAGE and Coomassie blue staining. Figure 4B shows that many bands, in addition to those corresponding to rap2, were evident. However, many of them

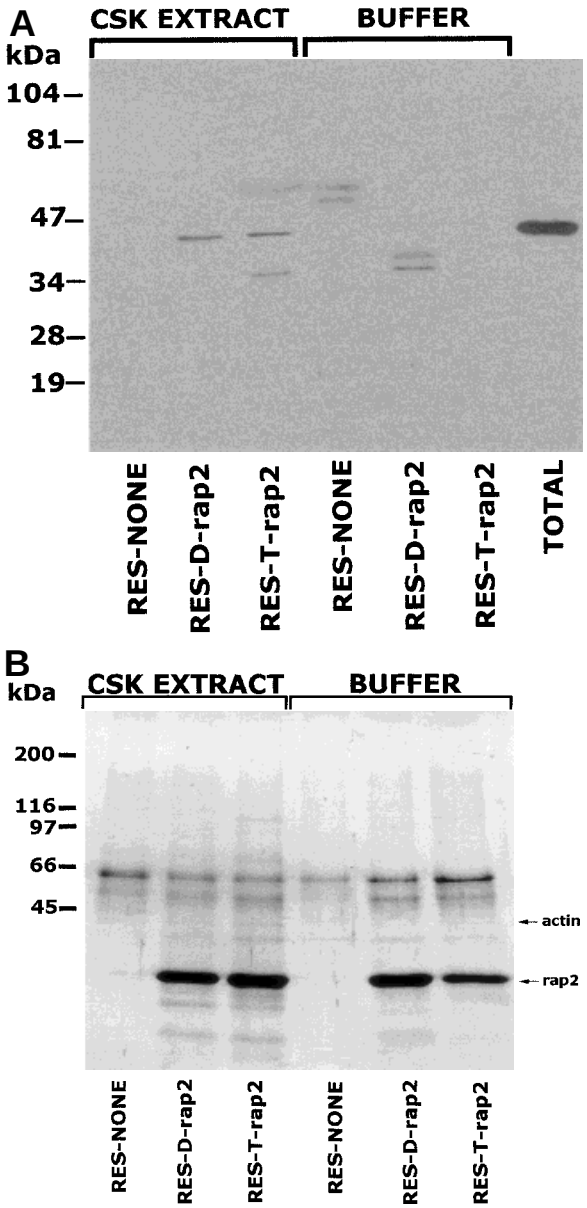
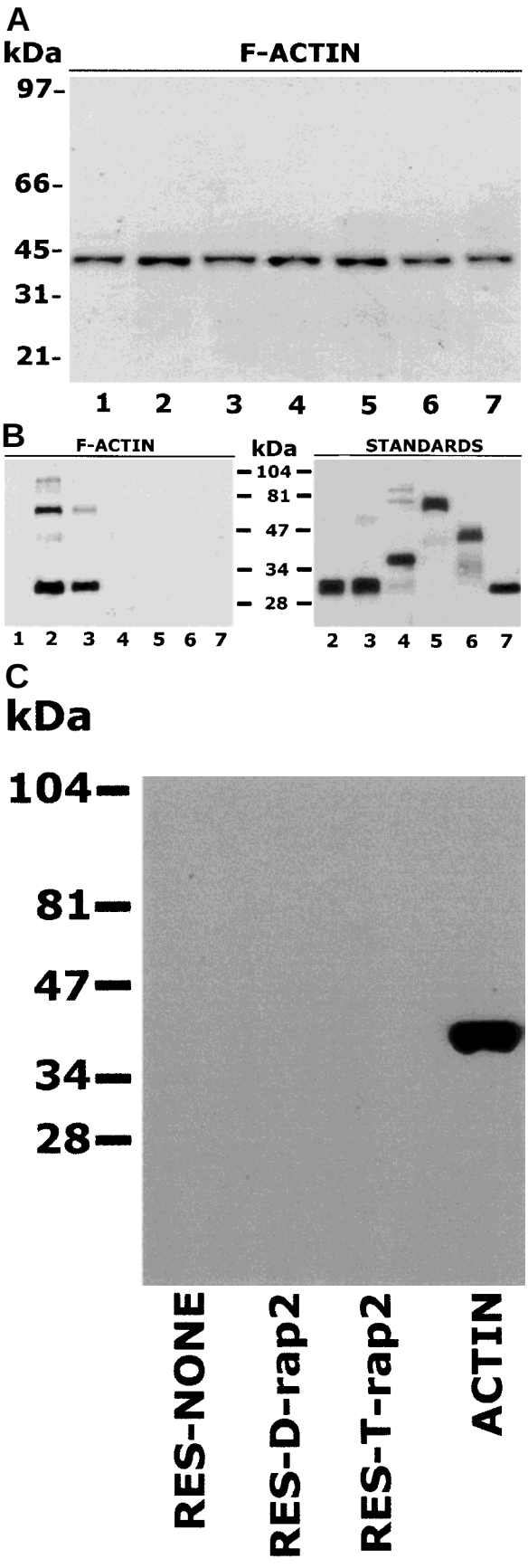


Fig. 4. Rap2 binds to cytoskeletal actin. Cytoskeleton from thrombin-aggregated platelets was extracted with 0.5 M NaCl and 0.3% sodium-deoxycholate. The cytoskeleton extract (CSK extract) was mixed with Ni^{2+}-Sepharose (Res-none) or with Ni^{2+}-Sepharose bound to polyhistidine-tagged rap2 loaded with either GDP (Res-D-rap2) or GTPγS (Res-T-rap2). In parallel samples, the cytoskeletal extract was substituted with an equal volume of buffer (buffer). **A:** Proteins bound to immobilized rap2, as well as an aliquot of the total CSK extract (total), were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10–20% acrylamide gradient gel, transferred to nitrocellulose, and probed with anti-actin antiserum. **B:** Proteins bound to immobilized rap2 were separated by SDS-PAGE on a 5–15% acrylamide gradient gel, and stained with Coomassie blue.

probably represented contaminants or degradation products of the rap2 preparations, since they were also present in the samples lacking the cytoskeleton extract. Only a faint band of about 45 kDa, probably corresponding to actin, was evident when GDP- or GTPγS -loaded rap2 was incubated with the cytoskeleton extract, but not with buffer.

To verify the direct binding of rap2 with actin, 1 μg of rap2, loaded with both GDP and GTPγS and labeled with biotin, or 2 μg of the biotinylated control proteins polyhistidine-tagged $SdrCB_{1,2}$, BSA, ovalbumin, and chymotrypsinogen was mixed with 50 μg of monomeric purified actin (G-actin), and polymerization was induced. Polymerized actin was recovered by centrifugation and analyzed by SDS-PAGE, followed by Coomassie blue staining or Western blotting. Staining with Coomassie blue demonstrated that the same amount of actin was polymerized in the different samples (Fig. 5A). By contrast, staining with peroxidase-conjugated avidin to detect the presence of biotinylated proteins, showed that rap2–GDP and rap2–GTPγS, but not the control proteins, co-sedimented with purified polymerized actin (Fig. 5B). To investigate whether rap2 was also able to bind to monomeric actin, 50 μg of purified actin in monomeric form was mixed with 1 μg of polyhistidine-tagged rap2 loaded with either GDP or GTPγS, without inducing polymerization. Because monomeric actin cannot be recovered by centrifugation, polyhistidine-tagged rap2 bound to Ni^{2+}-Sepharose were used. As a control, a sample containing monomeric purified actin was incubated with the same amount of resin with no bound proteins. Upon recovery of the resin-bound proteins by brief centrifugation, the presence of actin was analyzed by immunoblotting using a specific antiserum. Neither GDP- nor GTPγS-loaded rap2 was able to bind monomeric actin (Fig. 5C).

Finally, binding of rap2 to actin filaments was tested by a solid-phase binding assay. Wells of a microtiter plate were coated with filamentous actin and then incubated with biotinylated rap2–GDP, rap2–GTPγS, BSA, ovalbumin, or chymotrypsinogen. Binding of biotinylated proteins to immobilized actin was tested using peroxidase-conjugated avidin. As shown in Figure 6, only a significant binding of rap2–GDP and rap2–GTPγS to immobilized F-actin was observed. Therefore, we conclude that rap2 di-



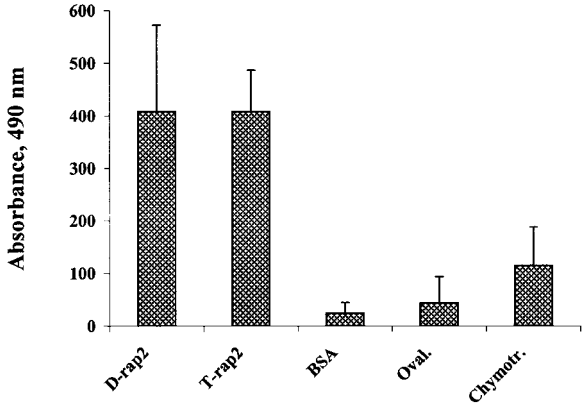


Fig. 6. Solid-phase binding assay. Wells of a microtiter plate were coated with 5 μg F-actin and incubated with 100 ng of biotinylated rap2–GDP (D-rap2), rap2–GTPγS (T-rap2), BSA, ovalbumin (oval.), or chymotrypsinogen (chymotr.). Binding of biotinylated proteins to immobilized F-actin was measured by a peroxidase-conjugated avin-based reaction. Results are means ± SD of three separated experiments.

rectly and specifically interacts with polymerized actin.

DISCUSSION

In this work, we have demonstrated that the small GTP-binding protein rap2 directly binds to the actin filaments of platelet cytoskeleton. By treating the cytoskeleton extracted from thrombin-aggregated platelets with potassium iodine, which is known to cause actin depolymerization and solubilization of the actin-binding proteins, we observed the presence of rap2 in the soluble material. Moreover, by extensive

Fig. 5. Actin co-sedimentation assay. Purified actin (50 μg) was induced to polymerize in the absence of biotinylated proteins **(lane 1)**, or in the presence of 1 μg of biotinylated rap2–GDP **(lane 2)**, rap2–GTPγS **(lane 3)**, or 2 μg of polyhistidine-tagged $SdrCB_{1,2}$ **(lane 4)**, bovine serum albumin (BSA) **(lane 5)**, ovalbumin **(lane 6)**, and chymotrypsinogen **(lane 7)**. F-actin was recoverd by high-speed centrifugation. **A:.** F-actin samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% acrylamide gradient gel and Coomassie blue staining. **B:** F-actin samples were separated by SDS-PAGE on a 12% acrylamide gel, followed by Western blotting and staining with peroxidase-conjugated avidin. On the same gel, 50 ng of the biotinylated rap2 proteins and 100 ng of all the other proteins used (standards) were also loaded (right). **C:** Purified monomeric actin was incubated with Ni^{2+}-Sepharose (Res-none) or with Ni^{2+}-Sepharose bound to polyhistidine-tagged rap2 loaded with either GDP (Res-D-rap2) or GTPγS (Res-T-rap2). In these samples, actin polymerization was not induced. Proteins bound by immobilized rap2, together with 50 ng of purified actin (actin), were loaded on a 10–20% acrylamide gradient gel, transferred to nitrocellulose and probed with the anti-actin antiserum.

dialysis of the KI-soluble fraction, monomeric actin underwent spontaneous polymerization and associated with other solubilized cytoskeletal proteins to form a reconstituted cytoskeleton similar to the Triton X-100-insoluble material. We found that rap2, released from the Triton X-100-insoluble material upon treatment with KI, spontaneously reassociated with the F-actin enriched fraction. Moreover, exogenous purified rap2, but not unrelated proteins, could specifically be incorporated into the actin-filaments network. The results presented herein have been obtained using recombinant rap2A purified as a polyhistidine-tagged protein. However, identical results were also obtained using rap2B purified from recombinant *E. coli* as previously described [Molina y Vedia et al., 1990]. Moreover, an unrelated protein containing a similar polyhistidine tag, molecular mass, and electric net charge as the rap2 proteins used in this work was unable to interact with the reconstituted cytoskeleton. Therefore, interaction of rap2 with the network of actin filaments was not mediated by unspecific binding through the polyhistidine tag, and occurred when both rap2A and rap2B, which are about 90% homologous, were analyzed. The other unrelated proteins used to demonstrate the specificity of the interaction of rap2 with the reconstituted cytoskeleton have little in common with rap2 itself. Therefore, they may not represent as the most appropiated controls, and other small GTPases similar to rap2 may appear more suitable. However, because several low-molecular-weight GTP-binding proteins, including members of the ras, rap, and rho families, are known to interact with the cytoskeleton in platelets and in nucleated cells [Fischer et al., 1990; Hall, 1992; Zhang et al., 1993a; Dash et al., 1995; Tapon and Hall, 1997], they cannot be used as negative controls in our study. On the other hand, the unrelated proteins used in this work display features, including molecular mass, electric charge, and polyhistidine tag, which resemble those of rap2. Therefore, they are useful to rule out the possibility of an association of rap2 attributable to protein trapping or to unspecific electrostatic interaction.

Our findings demonstrate that rap2 interaction with the cytoskeleton is a specific and reversible process that persists as long as actin is maintained in a polymerized form. This is in agreement with our previous work demonstrating that cytochalasin D, which blocks actin polymerization without affecting platelet aggregation, prevents rap2 translocation to the cytoskeleton induced by thrombin [Torti et al., 1994]. The incorporation of rap2 into the actin-based cytoskeleton could be mediated by either direct binding to the actin filaments or binding to some different actin-associated proteins. A direct interaction with actin has been reported for some signaling molecules such as the adaptor protein SHC and phospholipase Cγ1 [Thomas et al., 1995; Pei et al., 1996]. By contrast, the phosphatidylinositol 3-kinase has been found to interact with the actin-based cytoskeleton indirectly by binding to cytoskeletal-associated $pp125^{FAK}$ [Guinebault et al., 1995].

The fact that rap2 is an actin-binding protein is supported by several pieces of evidence: (1) exogenous rap2 is incorporated into the reconstituted cytoskeleton prepared even from resting platelets, in which other signaling molecules are not present and actin is the main component; (2) rap2 immobilized on Sepharose beads is able to interact specifically with the cytoskeletal actin; (3) when purified monomeric actin is induced to polymerize in the presence of rap2, this protein is incorporated into the actin filaments; and (4) rap2 binds specifically to preformed actin filaments immobilized on a plastic surface. These findings suggest that rap2 interacts exclusively with the polymerized, but not with the monomeric form of actin. This was directly confirmed by the evidence that incubation of Sepharose-bound rap2 with purified monomeric actin did not result in any detectable interaction.

It was surprising to find that the interaction of rap2 with actin filaments is independent of the bound nucleotide. In fact, rap2 loaded with either GDP or GTPγS was found to bind equally well to actin in all the experimental approaches that we have used. Therefore, actin cannot be considered an effector for rap2. Moreover, our results indicate that cytoskeletal associated rap2 can potentially undergo nucleotide exchange and activation without being released from the actin filament network. Consequently, cytoskeletal bound rap2 can still bind and regulate unknown downstream effectors. In this regard, binding to the actin filaments may represent a means of correctly locating rap2 in a suitable environment to allow its interaction with other signaling molecules. Alternatively, it is also possible that, in intact cells, the GTP-bound form of rap2 interacts with the cytoskel-

eton and then undergoes inactivation through the stimulated GTPase activity without dissociation from the actin filaments.

Actin filaments represent the main component of the cytoskeleton in both resting, activated and aggregated platelets [Fox, 1993]. Thus, potential binding sites for rap2 are always present in the cytoskeleton of platelets independently of the functional state of these cells. In fact, we have demonstrated that exogenous rap2 can actually interact with the reconstituted cytoskeleton prepared not only from aggregated, but also from activated and even resting platelets, while, in intact cells, endogenous rap2 can be detected only in the cytoskeleton from aggregated platelets. However, it is unlikely that the association of rap2 with the cytoskeleton is exclusively regulated by the amount of polymerized actin. In fact, it is known that the amount of polymerized actin, which represents about 30–40% of the total actin in resting platelets, increases to 60–70% upon stimulation with thrombin [Fox, 1993], and the cytoskeletal rap2 is strongly detectable in the latter case, but totally undetectable in the former one [Torti et al., 1993, 1994]. Moreover, it is known that thrombin-induced actin polymerization also occurs in thrombasthenic platelets, without concomitant translocation of rap2 to the cytoskeleton [Torti et al., 1994]. Therefore, it seems more likely that, although potential binding sites are already present in the cytoskeleton of resting platelets, interaction with rap2 is prevented, and occurs only as consequence of specific signals generated during platelet aggregation. Based on a previous report [Torti et al., 1994], we can consider a major role played by the fibrinogen receptor glycoprotein IIb-IIIa in the generation of the signals promoting rap2 translocation to the cytoskeleton. In fact, glycoprotein IIb-IIIa mediates platelet aggregation and translocates to the cytoskeleton with kinetics similar to that of rap2 and in stoichiometric amount. The experimental model described in this work can be used for future investigation to better understand the mechanisms promoting rap2 interaction with the actin filaments in intact platelets during aggregation.

ACKNOWLEDGMENTS

We thank Dr. J. De Gunzburg (INSERM U248, Istitute Curie, Paris, France) for the bacteria expressing polyhistidine-tagged rap2A, Prof. E. Grazi (Department of Biochemistry and Molecular Biology, University of Ferrara, Italy) for providing purified actin, Dr. Magnus Hook (Department of Biochemistry and Biophysics, Texas A&M University, Houston, Texas), and Dr. L. Visai (Department of Biochemistry, University of Pavia) for recombinant polyhistidine-tagged $SdrCB_{1,2}$, and Marco Bellaviti (Department of Biochemistry, University of Pavia, Italy) for technical assistance in the preparation of the figures.

REFERENCES

Dash D, Aepfelbacher M, Siess W. 1995. Integrin α_{IIb}-β_3-mediated translocation of CDC42Hs to the cytoskeleton in stimulated human platelets. J Biol Chem 270:17321–17326.

Fischer TH, Gatling MN, Lacal JC, White GC II. 1990. Rap1B, a cAMP-dependent protein kinase substrate, associates with the platelet cytoskeleton. J Biol Chem 265: 19405–19408.

Fox JEB. 1993. The platelet cytoskeleton. Thromb Haemost 70:884–893.

Franke B, Akkerman JN, Bos JL. 1997. Rapid Ca^{2+}-mediated activation of rap1 in human platelets. EMBO J 16:252–259.

Frech M, John J, Pizon V, Chardin P, Tavitian A, Clark R, McCormick F, Wittinghofer A. 1990. Inhibition of GTPase activating protein stimulation of ras-p21 GTPase by the Krev-1 gene product. Science 249:169–171.

Grondin P, Plantavid M, Sultan C, Breton M, Mauco G, Chap H. 1991. Interaction of $pp60^{c\text{-}src}$, phospholipase C, inositol-lipid, and diacylglycerol kinases with the cytoskeletons of thrombin-stimulated platelets. J Biol Chem 266: 15705–15709.

Guinebault C, Payrastre B, Racaud-Sultan C, Mazarguil H, Breton M, Mauco G, Plantavid M, Chap H. 1995. Integrin-dependent translocation of phosphoinositide 3-kinase to the cytoskeleton of thrombin-activated platelets involves specific interaction of p85α with actin filaments and focal adhesion kinase. J Cell Biol 129:831–842.

Hall A. 1992. Ras-related GTPases and the cytoskeleton. Mol Biol Cell 3:475–479.

Janoueix-Lerosey I, Pasheva E, De Tand M, Tavitian A, De Gunzburg J. 1998. Identification of a specific effector of the small GTP-binding protein rap2. Eur J Biochem 252:290–298.

Josefsson E, McCrea KW, Ni Eidhin D, O'Connell D, Cox J, Hook M, Foster TJ. 1998. Three new members of the serine-aspartate repeat protein multigene family of *Staphylococcus aureus*. Microbiology 144:3387–3395.

Kitayama H, Sugimoto Y, Matsuzaki T, Ikawa Y, Noda M. 1989. A ras related gene with transformation suppressor activity. Cell 56:77–84.

Molina y Vedia L, Ohmstede CA, Lapetina EG. 1990. Properties of the exchange rate of guanine nucleotides to the novel rap2B protein. Biochem Biophys Res Commun 171:319–324.

Newman PJ, Hillery CA, Albrecht R, Parise LV, Berndt MC, Mazurov AV, Dunlop LC, Zhang J, Rittenhouse SE. 1992. Activation-dependent changes in human platelet PECAM-1: phosphorylation, cytoskeletal association, and surface membrane redistribution. J Cell Biol 119:239–246.

Payrastre B, van Bergen en Henegouwen PMP, Breton M, den Hartigh JC, Plantavid M, Verkleij AJ, Boonstra J. 1991. Phosphoinositide kinase, diacylglycerol kinase, and phospholipase C activities associated to the cytoskeleton: effect of epidermal growth factor. J Cell Biol 115:121–128.

Pei Z, Yang L, Williamson JR. 1996. Phospholipase C-γ1 binds to actin-cytoskeleton via its C-terminal SH2 domain in vitro. Biochem Biophys Res Commun 228:802–806.

Ramaschi G, Torti M, Tolnai Festetics E, Sinigaglia F, Malavasi M, Balduini C. 1996. Expression of cyclic ADP-ribose-synthetizing CD38 molecule on human platelet membrane Blood 87:2308–2313.

Spaargaren M, Bischoff JR. 1994. Identification of the guanine nucleotide dissociation stimulator for Ral as a putative effector molecule of R-ras, H-ras, K-ras, and Rap. Proc Natl Acad Sci USA 91:12609–12613.

Tapon N, Hall A. 1997. Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton. Curr Opin Cell Biol 9:86–92.

Thomas D, Patterson SD, Bradshaw RA. 1995. Src homologous and collagen (SHC) protein binds to F-actin and translocates to the cytoskeleton upon nerve growth factor stimulation in PC12 cells. J Biol Chem 270:28924–28931.

Tohyama Y, Yanagi S, Sada K, Yamamura H. 1994. Translocation of p72syk to the cytoskeleton in thrombin-stimulated platelets. J Biol Chem 269:32796–32799.

Torti M, Lapetina EG. 1992. Role of rap1B and $p21^{ras}$ GTPase activating protein in the regulation of phospholipase C-γ1 in human platelets. Proc Natl Acad Sci USA 89:7796–7800.

Torti M, Lapetina EG. 1994. Structure and function of rap proteins in human platelets. Thromb Haemost 71:533–543.

Torti M, Ramaschi G, Sinigaglia F, Lapetina EG, Balduini C.. 1993. Association of the low-molecular-weight GTP-binding protein rap2B with the cytoskeleton during platelet aggregation. Proc Natl Acad Sci USA 90:7553–7557.

Torti M, Ramaschi G, Sinigaglia F, Lapetina EG, Balduini C.. 1994. Glycoprotein IIb-IIIa and the translocation of rap2B to the platelet cytoskeleton. Proc Natl Acad Sci USA 91:4239–4243.

Wheeler ME, Cox AC, Carroll RC. 1984. Retention of the glycoprotein IIb-IIIa complex in the isolated platelet cytoskeleton. Effects of separable assembly of platelet pseudopodal and contractile cytoskeletons. J Clin Invest 74: 1080–1089.

Winegar DA, Molina y Vedia L, Lapetina EG. 1991. Isoprenylation of rap2 proteins in platelets and human erythroleukemia cells. J Biol Chem 266:4381–4386.

Zhang J, King WG, Dillon S, Hall A, Feig L, Rittenhouse SE. 1993a. Activation of platelet phosphatidylinositide 3-kinase requires the small GTP-binding protein Rho. J Biol Chem 268:22251–22254.

Zhang X, Settleman J, Kyriakis JM, Takeuchi-Suzuki E, Elledge SJ, Marshall MS, Bruder JT, Rapp UF, Avruch J. 1993b. Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature 364:308–313.