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Integrin-dependent interaction of lipid rafts with the actin cytoskeleton in activated human platelets

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Summary

Dynamic connections between actin filaments and the plasma membrane are crucial for the regulation of blood platelet functions. Protein complexes associated with αIIbβ3 integrin-based cytoskeleton structures are known to play a role in these processes. However, mechanisms involving lateral organizations of the plasma membrane remain to be investigated. Here, we demonstrate that a large fraction of platelet lipid rafts specifically associates with the actin cytoskeleton upon activation. This association was inhibited by antagonists of fibrinogen-αIIbβ3 binding and did not occur in type I Glanzman's thrombasthenic platelets. The raft-cytoskeleton interaction is a reversible process correlating with the intensity and stability of platelet aggregation. Although only a minor fraction of αIIbβ3 was recovered in rafts upon activation, this integrin specifically upregulated the level of PtdIns(4,5)*P*$_2$ in membrane microdomains and induced the recruitment of several actin-modulating proteins known to directly or indirectly interact with this lipid. Controlled disruption of rafts did not affect αIIbβ3-mediated platelet aggregation in response to high concentrations of thrombin but significantly inhibited fibrin clot retraction. We propose that rafts participate in the organization of membrane-cytoskeleton interactions where αIIbβ3-mediated tension forces apply during the late phase of platelet activation.

Key words: Platelets, Lipid rafts, Actin, Cytoskeleton, Integrins, Phosphoinositides

Introduction

The interactions between the plasma membrane and the cytoskeleton are essential for a wide variety of cellular processes such as endocytosis, cell morphology, cell adhesion, formation of cell-cell contacts and cell motility. These interactions are regulated by signaling complexes formed by the assembly of transmembrane and cytosolic proteins and lipids (Engqvist-Goldstein and Drubin, 2003; Fais and Malorni, 2003; Sechi and Wehland, 2000). These complexes are thought to participate in the control of the elongation of actin filaments leading to the formation of membrane protrusions, or in the contraction of the actomyosin network leading to membrane retraction. Blood platelets represent an attractive model to investigate these mechanisms as the actin cytoskeleton and its interactions with the plasma membrane are critical for their physiological functions (Hartwig et al., 1999). Indeed, platelets must respond rapidly to a vessel injury by a series of coordinated events including adhesion, shape change, spreading, aggregation and clot retraction. αIIbβ3 is the most prominent platelet integrin, and, upon activation by 'inside-out' signaling, is capable of binding to several adhesive proteins, including fibrinogen, to support platelet aggregation. During aggregation, αIIbβ3 is also involved in transmitting an 'outside-in' signal leading to the formation of a network of signaling and structural proteins strongly interacting with the newly remodeled actin cytoskeleton (Hartwig et al., 1999). These protein complexes were previously co-isolated with the cytoskeleton as a Triton X-100 insoluble pellet obtained at low speed centrifugation (15,000 ***g***) from aggregated platelets (Fox, 1993). The characterization of this cellular fraction led to the identification of several key signaling molecules recruited to αIIbβ3-based cytoskeletal structures, where they are thought to play an essential role in stabilization of platelet aggregation (Hartwig et al., 1999).

At the sites of interaction with the cytoskeleton, the plasma membrane is subjected to important mechanical forces, especially during platelet fibrin clot retraction, a post-aggregation event involved in the maintenance of hemostasis. During retraction, integrins take part in the formation of a transmembrane linkage between proteins from the extracellular matrix and from the actomyosin filament network, allowing the transmission of contractile forces. It is currently hypothesized that the areas of membrane interacting with the cytoskeleton have a specific lipid composition adapted to these forces. In this context, cholesterol and sphingolipid enriched membrane microdomains, known as detergent resistant membranes (DRMs) or lipid rafts, are of major interest. Their lipid composition confers a restricted fluidity state called a liquid ordered phase to these membrane microdomains (Brown and London, 2000). Although the existence of rafts has been

controversial in the past few years (Munro, 2003), recent data place them at the forefront of cell biology and biomembrane research (del Pozo et al., 2004; Sharma et al., 2004). Interactions between rafts and cytoskeleton were proposed in various cell types (Brdickova et al., 2001; Harder and Simons, 1999; Laux et al., 2000; Moran and Miceli, 1998; Rozelle et al., 2000; Villalba et al., 2001). The actomyosin cytoskeleton seems to be involved in the lateral mobility of these microdomains (Seveau et al., 2001) and the stabilization of raft platforms requires cytoskeleton rearrangements in their vicinity (Villalba et al., 2001). However, the molecular mechanisms involved in raft-cytoskeleton interactions are still poorly documented. In platelets, lipid rafts were isolated on the basis of their insolubility in the non-ionic detergent Triton X-100 at 4°C and their low density (Bodin et al., 2001; Bodin et al., 2003a; Dorahy et al., 1996). Strong evidence support their role in the early phases of signal transduction induced by FcγRIIa (Bodin et al., 2003b), the collagen receptor GpVI (Wonerow et al., 2002) and the von Willebrand factor receptor GpIb-IX-V complex (Baglia et al., 2003; Shrimpton et al., 2002). However, the importance of rafts in post-integrin activation events as well as their potential interactions with the cytoskeleton remain unknown.

Interestingly, several years ago, a detergent-resistant pool of lipids was described in the cytoskeleton isolated from aggregated platelets (Hinchliffe et al., 1996; Livne et al., 1988; Schick et al., 1983; Tuszynski et al., 1984; Zucker and Masiello, 1983). The origin and the role of these ‘membrane fragments’ are still unclear. Detergent resistance is considered as a valuable means to assess protein association with the cytoskeleton, but little is known about the relevance of a lipid-rich domain co-precipitating with the actin filaments upon cell activation. In this context, we decided to investigate in detail the interactions between the actin cytoskeleton and membrane domains and their functional roles during the late phases of platelet activation.

We demonstrated that a large fraction of DRMs specifically associated with the actin cytoskeleton upon platelet activation in an αIIbβ3-dependent manner. Although only a minor fraction of αIIbβ3 was recovered in lipid rafts, this integrin induced a specific increase of phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] in rafts and the recruitment of several actin cytoskeleton regulatory proteins known to interact with this lipid. Moreover, we found that rafts are not mandatory for αIIbβ3-mediated platelet aggregation upon high concentration of thrombin but we provide evidence for their role in platelet-mediated fibrin clot retraction. Thus, αIIbβ3-mediated local modifications of cholesterol-rich membrane microdomains play an important role in the organization of the membrane-cytoskeleton linkage, through which tension forces may apply during the late phase of platelet activation.

Materials and Methods

Reagents

SFLLRNP (TRAP) and RGDS peptides, wortmannin, Triton X-100, Methyl-β cyclodextrin (MβCD), atroxin (*Bothrops atrox* venom), phosphatidylinositol 4-monophosphate [PtdIns(4)*P*] and phosphatidylserine (PS) were from Sigma. SR-121566 was a gift from P. Savi (Sanofi-Synthelabo, Toulouse, France). The human α-thrombin was from Enzyme Research Laboratories. [γ-^{32}P]ATP (3000 Ci/mmol) was from Dupont NEN. [^{32}P]orthophosphate and enhanced chemiluminescence (ECL) immunoblotting reagents were from Amersham Pharmacia Biotech. Rabbit polyclonal anti-FAK (C-20), anti-Rac (C-14), anti-Cdc42 (P-1), anti-VASP (H-90), anti-β3 (H-96) and anti-β1 (M-106) integrin antibodies were from Santa-Cruz Biotechnology and used at 1:1000. Rabbit polyclonal anti-actin (MAB1501) antibody was from Chemicon and used at 1:1000. Monoclonal anti-CD36 (Cat No. 0765) antibody was from Immunotech and used at 1:1000. Rabbit polyclonal anti-Arp3 and anti-moesin antibodies were kindly provided by P. Cossart (Institut Pasteur, Paris, France) and P. Mangeat (Université Montpellier II, France), respectively. Rabbit polyclonal anti-LAT antibody was generated in our laboratory and used at 1:1000 in western blot experiments (Ragab et al., 2003). All other reagents were purchased from Sigma (St Quentin-Fallavier, France) unless otherwise indicated.

Preparation and stimulation of platelets

Human platelets were isolated from concentrates obtained from the local blood bank (Etablissement Français du Sang, Pyrénées-Mediterranée). Platelets were washed twice in a washing buffer [140 mM NaCl, 5 mM KCl, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 10 mM HEPES pH 6.5, 5 mM glucose, 0.2% BSA (w/v)] and resuspended at 1.5×10^9 cells/ml in a stimulation buffer (washing buffer plus 1 mM $CaCl_2$, pH 7.4). For inositol lipid analysis, platelets were labeled with 0.5 mCi/ml [^{32}P]orthophosphate in a phosphate-free washing buffer (60 minutes, 37°C), washed and resuspended in the stimulation buffer (1.5×10^9 cells/ml). Platelet aggregation was monitored by a turbidimetric method as described previously (Gratacap et al., 1998). When indicated, wortmannin, RGDS and SR-121566 were used at 50 nM, 500 μM and 400 μM, respectively. For studies with platelets from three Glanzmann type I thrombasthenic patients, blood was anticoagulated with 124 mM tri-sodium citrate, 130 mM citric acid, 110 mM glucose at one volume for nine volumes of blood and centrifuged for 15 minutes at 190 ***g*** to obtain the platelet-rich plasma (PRP). Platelets were then washed as described above.

Clot retraction experiments

Clot retraction studies were performed as described (Schoenwaelder et al., 1997). Blood was drawn from healthy control donors, anticoagulated as described above and the platelet-rich plasma (PRP) was obtained as described above. Platelets were then washed as previously indicated and resuspended (3×10^8 cells/ml) without heparin in 2 ml of their autologous platelet-poor plasma containing 2 mM $MgCl_2$ and 2 mM EGTA. They were activated with thrombin (0.5 IU/ml) and atroxin (0.1 μg/ml) or thrombin alone (2 IU/ml) under gentle shaking for 20 seconds at 37°C and the reaction mixtures were left unstirred for 2 hours at 37°C. The extent of clot retraction was quantified by measuring the residual volume of serum after removal of the fibrin clot and expressed as percentage of total reaction volume.

Isolation of the cytoskeleton fraction

Platelets (2×10^9 cells/ml) were lysed by addition of 0.5 ml of a 3× ice-cold cytoskeleton (CSK) lysis buffer [1.5% Triton X-100 (v/v), 3 mM PMSF, 3 mM Na_3VO_4, 6 μg/ml each of leupeptin and aprotinin, 60 mM EGTA and 300 mM Tris, pH 7.4] incubated for 10 minutes and centrifuged (15,000 ***g***, 15 minutes, 4°C) to obtain the Triton X-100 insoluble low-speed pellet. This fraction was washed with 1× CSK lysis buffer and then with CSK lysis buffer without Triton X-100 to obtain the cytoskeleton.

Raft isolation procedures

Platelets (1.5×10^9 in 750 μl) were lysed by addition of 250 μl ice-cold 4× buffer [2% Triton X-100 (v/v), 150 mM NaCl, 100 mM Mes, pH

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6.5, 3 mM PMSF, 3 mM Na_3VO_4, 8 μg/ml each of leupeptin and aprotinin] and incubated for 5 minutes. Procedures were performed at 4°C as described (Bodin et al., 2001). The 1.5 ml lysate was adjusted to 1.37 M (40%) sucrose by addition of 1 ml of 2.74 M sucrose prepared in Mes-buffered saline (MBS) (150 mM NaCl, 25 mM Mes, pH 6.5, 1 mM PMSF, 1 mM Na_3VO_4). A step sucrose gradient was made by successive additions of 1 M (30%), 0.8 M (25%), 0.6 M (20%), 0.5 M (15%), 0.3 M (10%), 0.15 M (5%) sucrose solution (1.33 ml each) on the top of the 1.37 M (40%) homogenate. The gradients were ultracentrifuged (200,000 ***g***, 4°C, 16 hours) and eight fractions of equal volume were harvested from the top. Rafts were recovered mainly in fractions 2-4. When rafts were isolated from the cytoskeleton fraction, the Triton X-100 insoluble low speed pellet was first incubated with 1 ml of 0.6 M KI (30 minutes, 4°C) in order to depolymerize actin filaments (Payrastre et al., 1991). Then, 1 ml of the 2.74 M sucrose solution was added, and rafts were isolated as described above. For analysis of the protein content in rafts, the gradient fractions 2 to 4 were pooled. Proteins were precipitated with trichloroacetic acid and resuspended in Laemmli buffer. Western blot experiments were performed using the relevant antibodies, peroxidase-conjugated secondary antibodies and the ECL system. When indicated, quantifications were performed using NIH Image J software.

Cholesterol depletion experiments

Controlled cholesterol depletion treatments were performed as previously described (Bodin et al., 2001). Platelets (1×10^9 cells/ml) were incubated in the washing buffer containing 5 mM methyl-β-cyclodextrin MβCD) (10 minutes), washed and resuspended in the stimulation buffer. The cholesterol levels before and after depletion were quantified by gas chromatography (Bodin et al., 2001).

Major phospholipid and cholesterol quantification

Lipids were extracted from the cytoskeleton fraction or the isolated rafts following the acidified Bligh and Dyer method (Bligh and Dyer, 1959). Sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylethanolamine (PE) were separated by TLC using $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (75/45/12/6, v/v), identified by iodine vapor staining, in reference to lipid standards and scraped off. The quantification was performed according to phosphorus content as previously described (Bodin et al., 2001). Cholesterol quantification was performed by gas chromatography.

Phosphoinositide analysis

[^{32}P]-labeled lipids were extracted from whole platelets or isolated rafts following the acidified Bligh and Dyer method (Bligh and Dyer, 1959) and resolved by TLC using $CHCl_3/CH_3COCH_3/CH_3COOH/H_2O$ 80/30/26/24/14 (v/v). Spots corresponding to [^{32}P]PtdInsP_2 were visualized with a PhosphorImager 445 SI (Molecular Dynamics) scraped off, deacylated by 20% methylamine and analyzed by high performance liquid chromatography (HPLC) on a Whatman Partisphere 5 SAX column (Whatman International Ltd., UK) as described (Gratacap et al., 1998).

PtdIns(4)*P* 5-kinase in vitro assay

Rafts isolated from platelets (1.5×10^9 cells/gradient) were diluted three times in MBS, pelleted by ultracentrifugation (100,000 ***g***, 1.5 hours, 4°C, Rotor 60 Ti, Beckmann), resuspended in Tris-HCl (50 mM, pH 7.4) and incubated at 37°C for 20 minutes with PtdIns(4)*P*/PS sonicated vesicles (20 μg/40 μg) and 2× kinase buffer (100 mM Tris HCl, 3 mM DTT, 200 mM NaCl, 20 mM $MgCl_2$, 1 mM EDTA, 100 μM ATP and 30 μCi [γ-^{32}P]ATP) with gentle shaking. The reaction was stopped by addition of $CHCl_3/CH_3OH$ (v/v) and lipids were immediately extracted. The [^{32}P]PtdIns(4,5)P_2 produced was quantified as described above.

Results

A large fraction of lipid rafts co-isolates with the actin cytoskeleton upon human platelet activation

Small amounts of lipids were detected in the cytoskeleton isolated from resting platelets. Conversely, significant amounts of cholesterol and sphingomyelin (SM) were found in the cytoskeleton isolated from platelets stimulated by thrombin under stirring conditions (Fig. 1A). Phosphatidylcholine (PC) and, to a lesser extent, phosphatidylserine (PS) and phosphatidylethanolamine (PE) were also detected in this fraction. This remarkable lipid composition was qualitatively

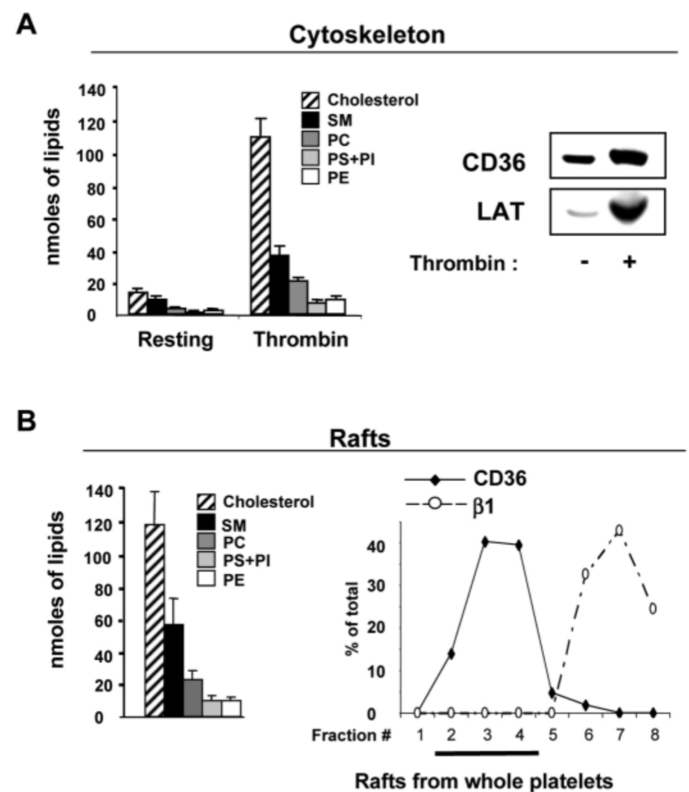

Fig. 1. Similarities in the lipid composition of rafts and actin cytoskeleton isolated from activated platelets. (A) Cytoskeleton was isolated from platelets (3×10^9 cells) stimulated or not with 1 IU/ml thrombin for 3 minutes. The major phospholipid and cholesterol composition of isolated cytoskeleton was analyzed as described in the Methods. Results are expressed as the mean±s.d. of three different experiments. Proteins from the isolated cytoskeleton were separated by 7.5% SDS-PAGE and the relative amounts of the raft protein markers CD36 and LAT were analyzed by western blotting. Results shown in the right panel are representative of two independent experiments. (B) Rafts were isolated from 3×10^9 resting platelets as previously described (Bodin et al., 2003b) and their lipid composition was analyzed. Results are expressed as the mean±s.d. of four different experiments. To illustrate the validity of the raft isolation procedure, the level (% of control) of CD36, a raft marker and β1 integrin, a transmembrane non-raft protein, in each fraction of the sucrose gradient is represented after quantification of western blots by densitometric analysis (NIH Image). Results shown are the means of two independent experiments.

and quantitatively strikingly similar to the composition of lipid rafts (Fig. 1B, left panel) isolated by the conventional method (flotation in a sucrose gradient as illustrated in Fig. 1B, right panel, by the distribution of CD36, a raft marker protein and the β1 integrin subunit a non-raft transmembrane protein) from the same number of platelets. The distribution of lipids in the cytoskeleton and rafts (rich in cholesterol and SM, poor in PS and PE) was clearly different from that of whole platelets (see Bodin et al., 2001; Bodin et al., 2003b). CD36 and LAT, two raft marker proteins (Bodin et al., 2001; Brown and Rose, 1992; Simons and Toomre, 2000), were weakly or not detected in the actin cytoskeleton isolated from resting platelets. However, they were clearly present in this fraction upon stimulation (Fig. 1A, right panel) with a concomitant decrease in the 'Triton-X100 soluble fraction' (not shown). Based on these results, we tried to isolate rafts by flotation from the cytoskeleton of activated platelets. For this purpose, we designed a protocol (Fig. 2A) allowing the tightly packed actin cytoskeleton to disassemble using potassium iodine (KI) treatment (Payrastre et al., 1991) before ultracentrifugation in a sucrose density gradient (Bodin et al., 2001; Brown and Rose, 1992; Simons and Toomre, 2000). Under these conditions, a light refractive band characteristic of lipid rafts was observed in the low-density fractions of the gradient (10-20% sucrose density) (Fig. 2A). In agreement, the raft markers, LAT and CD36, were strongly enriched in the low-density fractions of the gradient whereas more than 85% of actin was recovered at the bottom of the gradient (Fig. 2B). Of the phospholipids associated with the cytoskeleton of activated platelets, 90±5% were found in the raft fraction whereas 10±5% were recovered at the bottom of the gradient (not shown). It is noteworthy that the lipid composition of rafts isolated from cytoskeleton treated with KI was similar to the lipid composition of crude cytoskeleton (compare Fig. 1A and Fig. 2C) and of rafts from whole platelets, indicating that KI treatment did not modify their composition. These data demonstrate that lipid rafts co-isolated with the cytoskeleton of activated platelets and represented the large majority of the lipids associated with the cytoskeleton.

In resting platelets, most of the rafts were recovered in the 'Triton-X100 soluble fraction' (Fig. 2C) corresponding to the supernatant as defined in Fig. 2A. This observation indicates that rafts do not associate with the cytoskeleton in resting platelets and that they do not sediment in the presence of 1% Triton-X100 by low speed centrifugation (15,000 ***g***, 30 minutes, 4°C). Furthermore, after high-speed centrifugation of the 'Triton X-100 soluble fraction' (100,000 ***g***, 60 minutes, 4°C), a procedure classically used to sediment the membrane skeleton (Fox, 1993), rafts were not pelleted, as judged by the absence of cholesterol and SM in the pellet (not shown). After thrombin stimulation, a large fraction of lipid rafts associated to the platelet cytoskeleton with a concomitant decrease of their presence in the supernatant (Fig. 2C).

To rule out any non-specific trapping of lipid rafts into the newly polymerized actin filaments of activated platelets, we added radioactively labeled rafts isolated from [^{32}P]-labeled platelets stimulated with thrombin, to the lysate of unlabeled thrombin-activated platelets. All raft-resident phospholipid classes were labeled with [^{32}P] and no significant exchange between

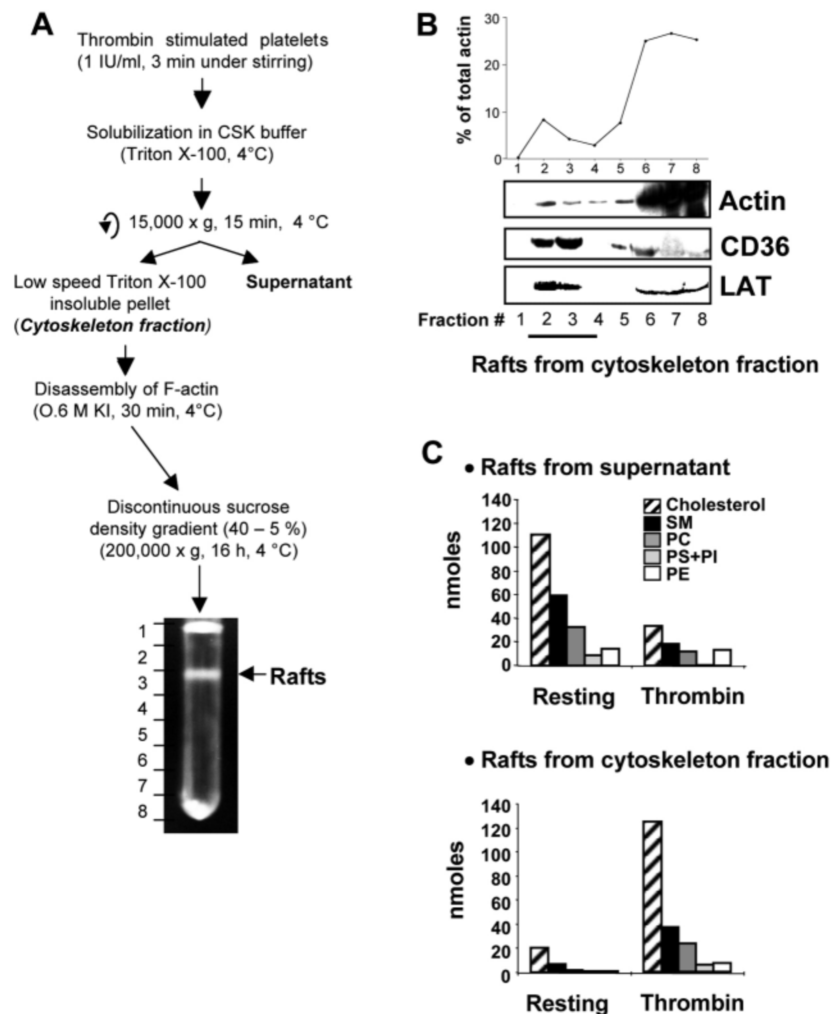

Fig. 2. Lipid rafts can be isolated from the actin cytoskeleton of activated platelets. (A) Schematic representation of the isolation procedure of lipid rafts from the cytoskeleton of thrombin-activated platelets. A representative picture of the gradient obtained after centrifugation is shown.
(B) Distribution of the raft marker proteins actin, CD36, LAT and in the eight fractions of the sucrose gradient containing the depolymerized actin cytoskeleton isolated from 3×10^9 activated platelets (as described in A). Results are representative of two independent experiments. In the top panel, the distribution of actin was determined by densitometry analysis of western blots (10-15% of F-actin associated to lipid rafts). (C) Rafts were isolated from both the cytoskeleton fraction pre-treated with KI (0.6 M) and the supernatant (as described in A) prepared from resting or thrombin-activated platelets (3×10^9 cells). The lipid composition of rafts (pooled fractions 2-4) was analyzed qualitatively and quantitatively. Results are expressed as nmoles of lipids and are the mean of two independent experiments with very similar results. Similar results were obtained in the presence or absence of 0.6 M of KI in the supernatant.

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exogenous radio-labeled lipids and endogenous ones could be observed as illustrated by the stability of the lipid composition of labeled rafts for at least 15 minutes at 4°C under these conditions (not shown). After a 10-minute incubation at 4°C, this mix was subjected to the classical cytoskeleton isolation procedure (as defined in Fig. 2A). The large majority (72 and 74%, n=2) of exogenous [^{32}P]-labeled rafts remained in the supernatant. Altogether, these data strongly suggest the existence of a specific and regulated interaction of rafts with the cytoskeleton during platelet activation.

The association of lipid rafts with the actin cytoskeleton requires αIIbβ3 engagement

The engagement of αIIbβ3 integrins induced through the binding of fibrinogen is a key event for platelet aggregation. In human platelets, thrombin is a potent agonist that activates protease activated receptors (PARs) 1 and 4 and interacts with GpIb. To show that activation of PAR1 is sufficient to induce raft-cytoskeleton interaction, we stimulated platelets with the TRAP peptide. As expected, RGDS, the antagonist for fibrinogen binding to αIIbβ3, abolished platelet aggregation induced by stimulation of PAR1 with TRAP (Fig. 3A, left panel) whereas actin cytoskeleton reorganization still occurred to a large extent (Fig. 3A, right panel). Interestingly, the amounts of cholesterol and SM associated with the actin cytoskeleton, reflecting the presence of lipid rafts, were dramatically reduced upon RGDS treatment (Fig. 3B). A similar result was obtained with the compound SR-121566 (Fig. 3B), another competitive antagonist for fibrinogen binding to αIIbβ3 that prevents platelet aggregation (Savi et al., 2001).

This αIIbβ3-mediated raft-cytoskeleton interaction was further analyzed by isolating DRMs from whole platelets and measuring the amount of bound F-actin. Trace amounts of actin were observed in rafts from resting platelets, but a dramatic increase in actin was found in rafts upon TRAP stimulation (Fig. 3C). This association correlated with a shift of rafts to a higher density in sucrose gradients (Fig. 3C). Inhibition of fibrinogen binding to αIIbβ3 by pretreatment with RGDS or SR-121566 inhibited both the increase of actin level in rafts and the shift of rafts in the density gradient (Fig. 3C). Similar results were obtained using another raft isolation procedure (Drevot et al., 2002) performed at 37°C in the presence of Brij 98 as a detergent (not shown) indicating that this association was not due to a lipid phase transition occurring at 4°C.

To prove the role of αIIbβ3 in the association of rafts with the cytoskeleton, we used platelets from type I Glanzmann thrombastenia patients, a disease characterized by the absence of αIIbβ3 (Nair et al., 2002). As expected, the αIIbβ3-deficient platelets were unable to aggregate in response to the potent agonist thrombin (not shown) and partially reorganized their actin cytoskeleton (Fig. 4A). The amounts of CD36, LAT and cholesterol (Fig. 4A,B) did not increase significantly in the cytoskeleton of activated Glanzmann thrombastenic platelets indicating a lack of raft association. The lipid composition analysis was limited to cholesterol because of the sample size.

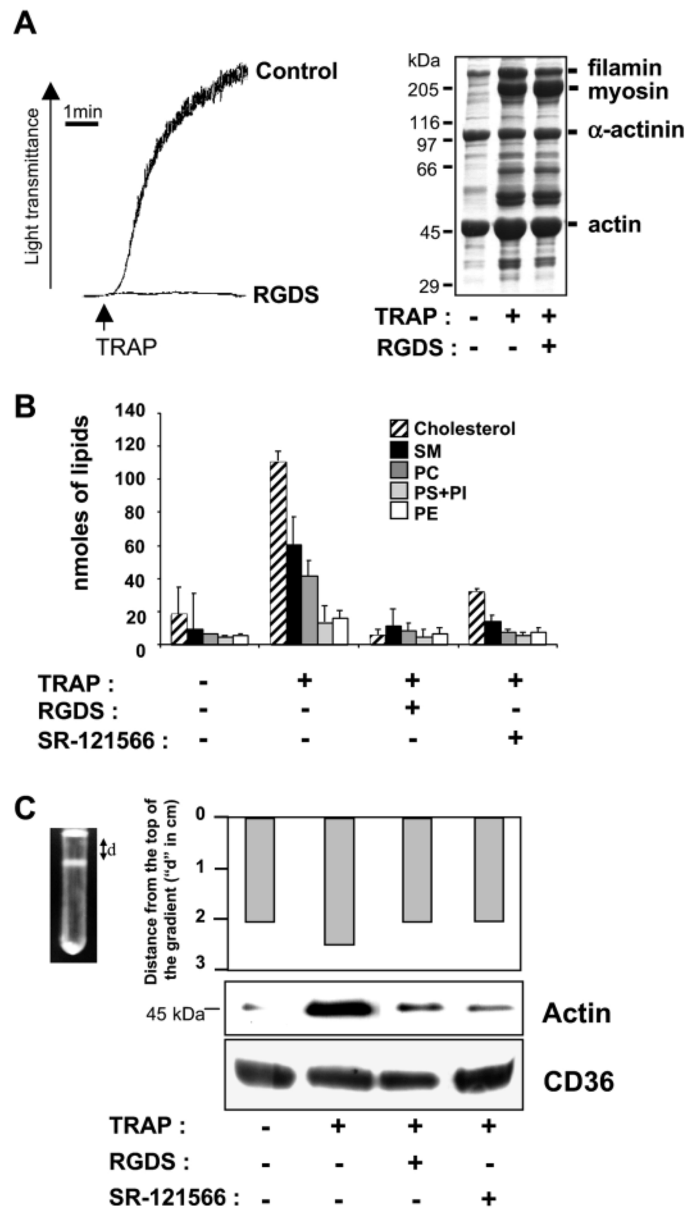

Fig. 3. The association of lipid rafts with the actin cytoskeleton is dependent on αIIbβ3 integrin engagement. Platelets pretreated or not with 500 μM RGDS for 1 minute (A,B,C) or 400 μM SR-121566 for 10 minutes (B,C) were stimulated at the concentration of 2×10^9 cells/ml, with 5 μM TRAP for 3 minutes and their actin cytoskeleton was isolated. (A) The effect of RGDS treatment on platelet aggregation was assessed using a chrono-Log dual-channel aggregometer with stirring at 900 rpm (left panel). Proteins from the isolated actin cytoskeleton were separated by SDS-PAGE (7.5%) and stained with Coomassie Blue (right panel). (B) The quantification of the major phospholipids and cholesterol associated with the actin cytoskeleton isolated from 3×10^9 platelets was performed as described in Fig. 1A. Results shown are the mean±s.d. of four independent experiments. (C) Lipid rafts were isolated from whole platelets (2×10^9 cells) pretreated or not with RGDS or SR-121566 and stimulated or not with 5 μM TRAP for 3 minutes and their position in the sucrose density gradient is shown. The distance '*d*', indicated by the gray bars, represents the height in cm from the top of gradient where the light refractive band corresponding to the raft fraction was observed. The relative amount of actin present in rafts (pooled fractions 2-4) was determined by western blotting using an anti-actin antibody. As a loading control, the amount of CD36 was also analyzed by western blotting. Data shown are representative of three independent experiments.

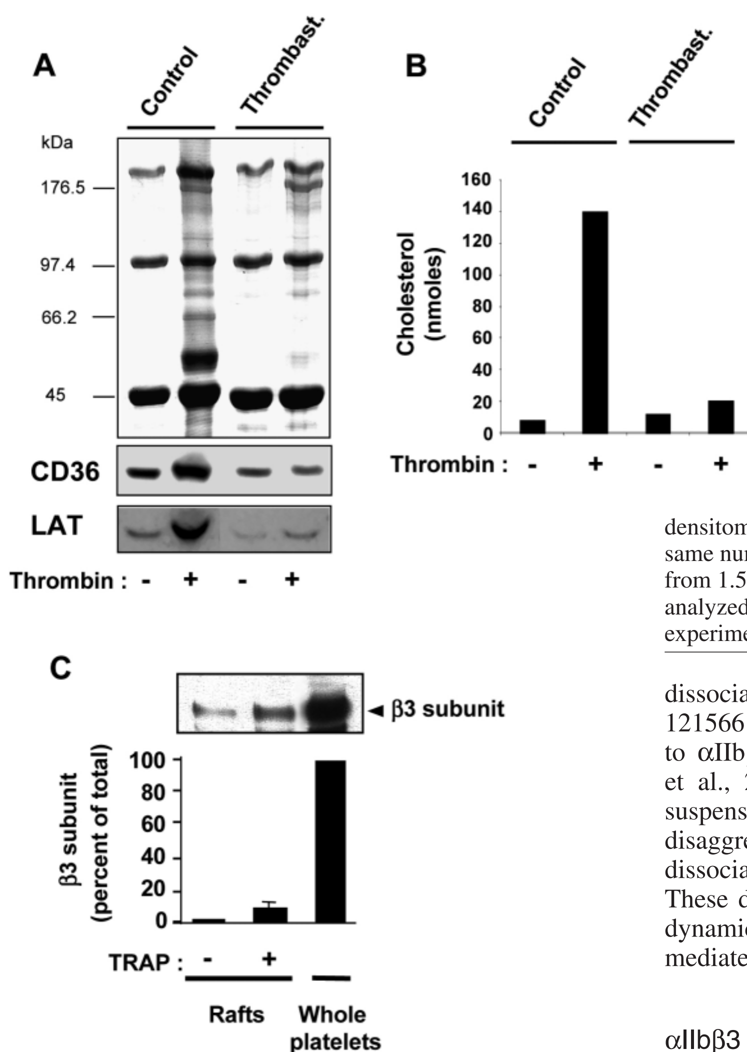

Fig. 4. Lipid rafts do not associate with the actin cytoskeleton of platelets from patients with Glanzmann thrombasthenia. Platelets from healthy volunteer or from patients with type I Glanzmann thrombasthenia were stimulated or not with 1 IU/ml thrombin for 3 minutes in an aggregometer with stirring at 900 rpm (2×10^9 cells/ml). (A) Proteins from the isolated cytoskeleton were separated by 7.5% SDS-PAGE followed by Coomassie Blue staining (upper panel) or by western blotting (bottom panel) to determine the relative amount of the raft markers CD36 and LAT using specific antibodies. (B) The amount of cholesterol in cytoskeleton isolated from 3×10^9 platelets was determined as described in Fig. 1. Results shown are representative of three independent experiments. (C) Rafts were isolated from resting or 5 μM TRAP-stimulated control platelets and the relative amount of β3 subunits in rafts was quantified by western blotting and densitometric analysis and compared to the total amount of β3 in the same number of platelets. In the western blot shown, rafts isolated from 1.5×10^9 platelets and a homogenate from 4×10^8 platelets were analyzed. Results shown are mean±s.d. of three independent experiments.

The amount of SM could, however, be measured in a sample from one patient and was also very low (3 nM) in the cytoskeleton (from 3×10^9 cells) upon activation. This critical role of αIIbβ3 in raft-cytoskeleton association prompted us to check for the presence of integrin in these microdomains. In agreement with a recent study (Wonerow et al., 2002), only a minor pool (10±5%, n=3) of β3 integrin subunit was recovered in lipid rafts upon TRAP stimulation of normal platelets (Fig. 4C).

The raft-cytoskeleton association is reversible

As indicated by the quantification of cholesterol and SM in the cytoskeleton, the extent of raft-cytoskeleton association followed the aggregation amplitude and remained stable during an irreversible aggregation (see Fig. 5A,B). We then investigated this association during a pharmacologically induced reversible platelet aggregation. Inhibition of PI 3-kinase by addition of wortmannin shortly (2 minutes) after TRAP stimulation is known to induce a rapid reversion of platelet aggregation without significantly affecting the actin polymerization (Kovacsovics et al., 1995; Trumel et al., 1999). Under these conditions (Fig. 5B), we observed a rapid dissociation of lipid rafts from the actin cytoskeleton. The SR-121566 compound is known to displace the fibrinogen bound to αIIbβ3 and to induce the reversion of aggregation (Savi et al., 2001). When SR-121566 was added to the platelet suspension 1.5 minutes after TRAP stimulation, the platelet disaggregation (Fig. 5A) was again accompanied by a dissociation of rafts from the actin cytoskeleton (Fig. 5B). These data indicate that the raft-cytoskeleton interaction is a dynamic and reversible process requiring a stable αIIbβ3-mediated platelet aggregation.

αIIbβ3 upregulates the PtdIns(4,5)P_2 level specifically in rafts and the concomitant recruitment of actin regulatory proteins known to directly or indirectly interact with this lipid

PtdIns(4,5)P_2 plays important roles in regulating actin assembly as well as interactions between membranes and cytoskeleton (Raucher et al., 2000; Sechi and Wehland, 2000). This lipid interacts with and regulates the activity of many actin modulating proteins such as the ERMs (ezrin, radixin, moesin) proteins acting as a link between transmembrane proteins an actin filaments, and the nucleation promoting factors (NPFs) of the WASP family proteins (Wiskott Aldrich Syndrome proteins) that activate the Arp2/3 complex (Actin-related protein 2/3 complex) (Higgs and Pollard, 2001). Interestingly, a pool of PtdIns(4,5)P_2 has been previously described in rafts (Bodin et al., 2001; Pike and Casey, 1996) and the modulation of its level has been proposed to participate in actin accumulation in the vicinity of these microdomains (Laux et al., 2000). Therefore, we checked whether the amount of PtdIns(4,5)P_2 in rafts was modulated by an αIIbβ3 engagement-dependent process. As shown in Fig. 6A, 3 minutes after TRAP stimulation, the [^{32}P]-PtdIns(4,5)P_2 level significantly increased in rafts (3.0±0.8 fold) and was sustained up to 6.5 minutes of stimulation (2.9±0.8 fold). The amount of other phospholipids (SM, PC, PS and PE) in rafts did not change upon stimulation (not shown) indicating a selective

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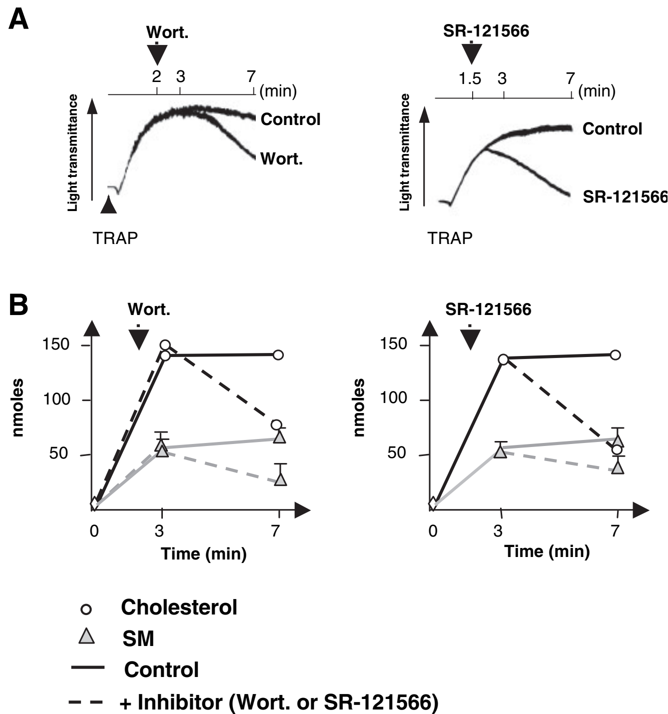

Fig. 5. The association of lipid rafts with the actin cytoskeleton is reversible. Platelets (3×10^9 cells) were stimulated by TRAP (5 μM) as in Fig. 4A. In order to reverse platelet aggregation, 50 nM wortmannin (A and B, left panels) or 400 μM SR-121566 (A and B, right panel) was added 2 and 1.5 minutes after addition of the agonist, respectively. (A) Platelet aggregation profiles show disaggregation after the addition of wortmannin (left panel) or SR-121566 (right panel). (B) The actin cytoskeletons were isolated at 0, 3 or 7 minutes and their cholesterol (O) and SM (Δ) content was determined as in Fig. 1A. The drop in cholesterol and SM concomitant with platelet disaggregation induced either by wortmannin or by SR-121566 (dotted lines) illustrates a transient association of rafts with the cytoskeleton under these conditions. Results shown are mean±s.d. of two (for cholesterol) or three (for SM) independent experiments.

effect of αIIbβ3. In whole platelets, the amount of [^{32}P]PtdIns(4,5)P_2 did not change significantly upon TRAP stimulation indicating that the increase was specific for rafts (Fig. 6A). Furthermore, when platelets were pretreated with SR-121566 or RGDS (Fig. 6A), the increase in [^{32}P]PtdIns(4,5)P_2 in rafts was inhibited indicating its requirement for αIIbβ3 engagement. Accordingly, the PtdIns(4)P 5-kinase activity measured in isolated rafts increased fivefold upon TRAP stimulation in an αIIbβ3 engagement-dependent manner (Fig. 6B, upper panel). This is consistent with the αIIbβ3-dependent recruitment in raft of a fraction (10% of total) of the small GTPase Rac (Fig. 6B, lower panel). Indeed, Rac is known to associate and activate PtdIns$P$ 5-kinase Iα (Chatah and Abrams, 2001) leading to the production of a pool of PtdIns(4,5)P_2 involved in actin assembly in platelets (Tolias et al., 2000).

Several proteins involved in the re-organization of the actin cytoskeleton and known to be regulated directly or indirectly by PtdIns(4,5)P_2 associated with rafts in an αIIbβ3-dependent manner (Fig. 6C). This was the case for the only ERM protein expressed in platelets, moesin (14% of total), which is involved in cytoskeleton rearrangements, lamellipodia and filopodia formation (Shcherbina et al., 1999) (Fig. 6C). Cdc42 (11% of total), a small GTPase of the Rho family highly expressed in platelets (Azim et al., 2000) was also recruited to rafts in an αIIbβ3-dependent manner (Fig. 6B) whereas RhoA, also abundantly expressed in platelets, did not (not shown). VASP (vasodilator-stimulated phosphoprotein) (18% of the total) and the Arp3 subunit of the Arp2/3 complex (11% of the total) known to promote actin nucleation and branching (Higgs and Pollard, 2001) (Fig. 6C) were recruited to the microdomains in the same way. However, we were unable to detect the presence of WASP suggesting that the recruitment of the Arp2/3 complex was mediated by another NPF such as N-WASP or Scar/WAVE. Furthermore, under 50% cholesterol depletion by MβCD, the increase in PtdIns(4,5)P_2 in the disorganized rafts did not occur upon TRAP stimulation and the association of these microdomains with the cytoskeleton was largely abolished (not shown), whereas actin polymerization was only partly affected (12% inhibition). Altogether, these results point to PdIns(4,5)P_2 as a key component of the αIIbβ3-mediated cytoskeleton-rafts interaction in platelets.

The integrity of lipid rafts is required for fibrin clot retraction

Irreversible platelet aggregation and fibrin clot retraction are two post-integrin engagement events that require firm membrane-cytoskeleton interactions. To investigate the role of lipid rafts in these processes, we performed a controlled cholesterol depletion (50±2% depletion of total cholesterol) using MβCD in order to disorganize these microdomains (Bodin et al., 2001; Bodin et al., 2003b; Pizzo et al., 2002). Disruption of rafts was illustrated by the sharp decrease in CD36 and LAT levels in the light density fractions of the sucrose gradients (Fig. 7A). As previously published (Bodin et al., 2001; Bodin et al., 2003b), cholesterol depletion affected early platelet responses induced by stimulation with low (0.2 IU/ml) concentrations of thrombin (Fig. 7A) or TRAP (5-40 μM) (not shown). At high thrombin concentrations (⩾0.5 IU/ml) the intensity and the stability of the aggregation response was not affected by raft disorganization indicating that the microdomains are not mandatory for αIIbβ3-mediated platelet aggregation.

We then examined the cellular retraction of fibrin clots in a washed platelet assay system. Platelet stimulation by thrombin (0.5 IU/ml) in the presence of atroxin, an N-terminal fibrinopeptide A cleaving snake venom enzyme (Meh et al., 1995), or by thrombin alone at high concentration (2 IU/ml) resulted in retraction of fibrin clots (Fig. 7B). This clot retraction was strongly inhibited by cholesterol depletion and raft disruption. One way of evaluating the extent of clot retraction is to measure the residual volume of serum after removal of the clot. Disorganization of lipid rafts led to a significant reduction of clot retraction (30±5% when platelets were stimulated by 0.5 IU/ml thrombin in the presence of atroxin and 39±12% when they were stimulated with thrombin alone; $P<0.01$, $n=3$). This result suggests that lipid rafts are involved in the organization of a functional membrane scaffold involved in the efficiency of actomyosin contraction.

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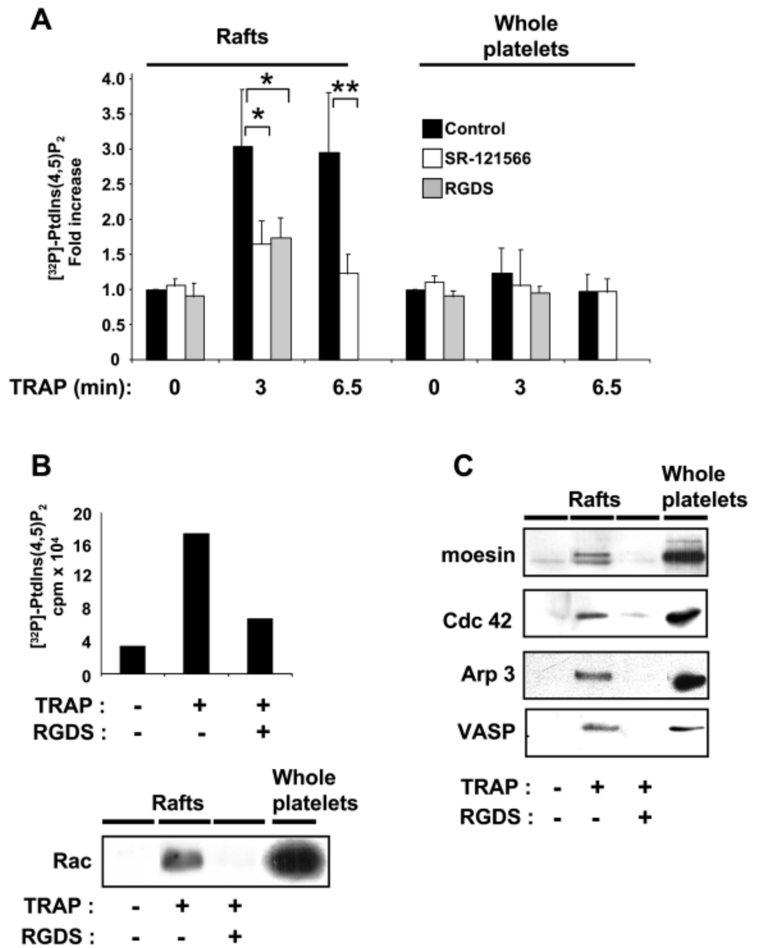

Fig. 6. αIIbβ3 integrin-dependent upregulation of PtdIns(4,5)P_2 in rafts and concomitant recruitment of cytoskeleton regulatory proteins. (A) [^{32}P]-labeled platelets (1.5×10^9 cells) preincubated or not with 400 μM SR-121566 for 10 minutes (white bars) or with 500 μM RGDS for 1 minute (gray bars) were stimulated with 10 μM TRAP for the indicated time. Lipids were extracted from isolated rafts or from whole cells and the amount of [^{32}P]PtdIns(4,5)P_2 was quantified by HPLC. ([^{32}P]PtdIns(4,5)P_2 concentration in rafts and platelets after RGDS treatment and 6.5-minute TRAP stimulation have not been determined.) Results are the mean±s.d. of four independent experiments and are expressed as fold increase compared to the level of [^{32}P]PtdIns(4,5)P_2 under resting conditions in the absence of RGDS or SR-121566 treatment (corresponding to 7.9×10^4 cpm of [^{32}P]PtdIns(4,5)P_2 in rafts isolated from 1.5×10^9 platelets and 1.1×10^6 cpm in whole cells). *$P<0.05$; **$P<0.01$. (B) Rafts were isolated from 3×10^9 platelets pretreated or not with 500 μM RGDS for 1 minute and stimulated or not with 10 μM TRAP for 3 minutes. Isolated rafts were submitted to an in vitro PtdIns(4)P kinase assay as described in Materials and methods. The level of [^{32}P]PtdIns(4,5)P_2 produced was quantified by HPLC. Results are expressed as cpm of [^{32}P]PtdIns(4,5)P_2 produced and are the means of two independent experiments with similar results (upper panel). The amount of Rac associated with rafts (isolated from 1.5×10^9 cells) or present in 4×10^8 platelets was estimated by western blotting and densitometry analysis (lower panel). (C) Rafts were isolated from platelets pre-incubated or not with 500 μM RGDS for 1 minute and stimulated or not with 5 μM TRAP for 3 minutes. Proteins from rafts (isolated from 1.5×10^9 cells) and from whole platelets lysate (from 4×10^8 cells) were analyzed by western blotting. Results are representative of two to three independent experiments. CD36 was used as a loading control. The amount of this raft marker was constant under the different conditions tested as shown in Fig. 3C.

Discussion

Several years ago, a Triton X-100 insoluble pool of lipids was shown to co-isolate with the actin cytoskeleton from aggregated platelets (Hinchliffe et al., 1996; Livne et al., 1988; Schick et al., 1983; Tuszynski et al., 1984; Zucker and Masiello, 1983). The origin of these lipids and the relevance of this observation remained unclear until now. Here, we show that the lipid composition of the cytoskeleton isolated from activated platelets was qualitatively and quantitatively identical to the composition of lipid rafts isolated from whole platelets. We developed a method to separate the pool of lipids from the tightly packed actin filament system in order to isolate it by flotation in a sucrose density gradient. Characterization of this fraction clearly indicated that the membrane fragments co-isolating with the cytoskeleton of activated platelets are lipid rafts.

We found that in resting platelets, rafts did not sediment by high-speed centrifugation in the presence of 1% Triton X-100 excluding an eventual association with the membrane skeleton (Fox, 1993). Upon platelet stimulation, the interaction of rafts with the cytoskeleton paralleled the aggregation process, was delayed compared to the rapid and massive actin assembly induced by thrombin or TRAP (S.B. and B.P., personal observation) and did not result from non-specific trapping in newly polymerized actin filaments. The raft-cytoskeleton interaction was also observed when the DRMs were directly isolated from thrombin-stimulated platelets by the conventional method. Under these conditions, a significant amount of F-actin was found associated with the microdomains and correlated with a slight increase in raft density. Interestingly, competitors of fibrinogen binding to αIIbβ3 integrins strongly inhibited the raft-cytoskeleton interaction under conditions that preserved a large part of the agonist-mediated cytoskeleton reorganization. The critical role of αIIbβ3 in this process was confirmed using Glanzman's thrombastenic platelets lacking this integrin (Nair et al., 2002). The extent of cytoskeleton-raft interaction paralleled the platelet aggregation intensity and was stable during an irreversible aggregation. Under conditions where platelet aggregation was reversed by addition of either SR-121566, a potent fibrinogen binding antagonist (Savi et al., 2001), or PI 3-kinase inhibitors (Trumel et al., 1999) after TRAP stimulation, the raft-cytoskeleton association became reversible. Thus, both the initiation and the stability of this interaction require sustained αIIbβ3 engagement and a stable aggregation.

The existence of raft-cytoskeleton interactions were previously shown in nucleated cells (Brdickova et al., 2001; Harder and Simons, 1999; Laux et al., 2000; Moran and Miceli, 1998; Rozelle et al., 2000; Villalba et al., 2001). In lymphocytes, a T-cell receptor (TCR)-CD44 co-stimulation participates in the Rac-dependent actin-mediated clustering of

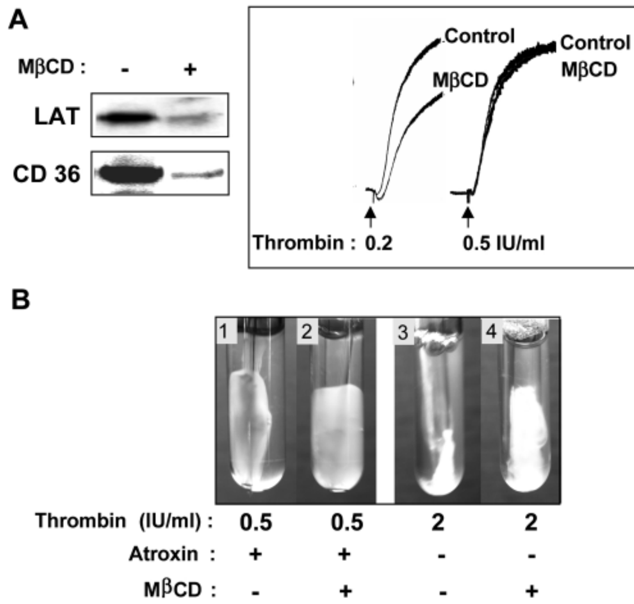

Fig. 7. The integrity of lipid rafts is required for efficient platelet fibrin clot retraction. (A) Control showing raft disruption by cholesterol depletion (left panel). The level of LAT and CD36 in raft (pooled fractions 2-4 of the sucrose gradient) isolated from platelet pre-treated or not with 5 mM MβCD for 10 minutes (leading to 50±2% depletion of total cholesterol and 56±4% depletion of cholesterol in rafts) was analyzed by western blotting. The effect of raft disruption on platelet aggregation induced by low (0.2 IU/μl) or high (0.5 IU/μl) thrombin concentration is shown in the right panel. (B) Effect of raft disruption on fibrin clot retraction. Platelets treated or not with 5 mM MβCD for 10 minutes, were resuspended at 3×10^8/ml in 2 ml autologous plasma prior to treatment with either 0.5 IU/ml thrombin and 0.1 μg/ml atroxin or 2 IU/ml thrombin alone at 37°C. For the study with 0.5 IU/ml thrombin, atroxin was added to induce clot formation. After 2 hours, the extent of clot retraction was observed.

lipid rafts (Foger et al., 2001). The F-actin dependent spatial reorganization at the immune synapse was shown to involve the Vav1/Rac pathway (Villalba et al., 2001). It was recently demonstrated in fibroblasts that Rac1 targeting to lipid rafts is controlled by integrin signals, and local regulation of rafts by integrins as a cause of the recruitment of signaling molecules involved in anchorage-dependent growth was proposed (del Pozo et al., 2004). Interestingly, upon TRAP stimulation, we measured a significant αIIbβ3-dependent increase in PtdIns(4)*P* 5-kinase activity in platelet rafts and a concomitant production of PtdIns(4,5)P_2 specifically in these microdomains. This is consistent with a previous study reporting an increase in PtdIns(4,5)P_2 in the cytoskeleton isolated from thrombin-stimulated platelets (Hinchliffe et al., 1996). Moreover, a PtdIns*P* 5-kinase Iα activated by Rac was shown to translocate to membranes upon thrombin PAR1 receptor stimulation (Chatah and Abrams, 2001) and to produce a pool of PtdIns(4,5)P_2 involved in actin assembly (Tolias et al., 2000). Thus, the integrin-dependent recruitment of Rac to lipid rafts observed in activated platelets and in other cell types (del Pozo et al., 2004) may be essential for the regulation of this localized production of PtdIns(4,5)P_2. PtdIns(4,5)P_2 raft-modulating proteins (Laux et al., 2000) such as the myristoylated alanine-rich C-kinase substrate MARCKS which is present in platelets (Elzagallaai et al., 2000), may also play a role in the recruitment or in the stabilization of these PtdIns(4,5)P_2 pools. The regulation of PtdIns(4,5)P_2 in membrane domains is particularly important for dynamic processes linked to the organization of the cytoskeleton. This phosphoinositide has been shown to induce the actin-based movement of raft-enriched intracellular vesicles (Rozelle et al., 2000). In neuronal cells, GAP43-like proteins, a group of PtdIns(4,5)P_2 raft-modulating proteins, accumulate at the inner leaflet of rafts where they concentrate PtdIns(4,5)P_2 and then promote F-actin assembly (Laux et al., 2000). We show here that, concomitant with the increase of PtdIns(4,5)P_2 in the microdomains, several proteins known to interact directly or indirectly with this phosphoinositide, and which are involved in actin cytoskeleton organization, relocated to lipid rafts in an αIIbβ3-dependent manner. For example, the association of Arp3 with rafts upon platelet aggregation suggests the presence of the actin branching and nucleating Arp2/3 complex which is known to be activated by WASP and N-WASP, which are themselves stimulated by PtdIns(4,5)P_2 and Cdc42 (Higgs and Pollard, 2001). We also detected recruitment of the ERM protein moesin whose activity is modulated by an interaction with PtdIns(4,5)P_2 (Barret et al., 2000) and that acts as a crosslinker between actin filaments and the plasma membrane. Consequently, moesin participates notably in cell-cell adhesion processes (Mangeat et al., 1999) and such a role in platelets has recently been suggested via interaction with PECAM-1 (Gamulescu et al., 2003), a protein that we detected in rafts (not shown). The major role for αIIbβ3 in regulating the recruitment of actin cytoskeleton modulating proteins to rafts, resulting in a strong interaction with the actin network of activated platelets, contrasts with the low level of αIIbβ3 found in these microdomains. Using different methods, Wonerow and colleagues found very low levels of activated αIIbβ3 in DRMs and showed that early tyrosine phosphorylation events downstream of αIIbβ3 engagement do not require lipid raft integrity (Wonerow et al., 2002). A recent report (Heijnen et al., 2003) described cholesterol-rich membrane domains concentrated at the tips of filopodia and at the leading edge of spreading platelets interacting with a fibrinogen coated surface. Interestingly, this αIIbβ3-dependent spreading on fibrinogen (Litjens et al., 2003) required raft integrity (Heijnen et al., 2003). Thus, despite its apparent low affinity for rafts, αIIbβ3 can, under certain conditions, regulate raft-dependent events including a local increase in PtdIns(4,5)P_2, which may then play an important role in anchoring the microdomains to the actin filament system. αIIbβ3 could localize in the vicinity of the microdomains (Wonerow et al., 2002), possibly via an association with raft-resident proteins such as tetraspanins (Berditchevski, 2001) while having a higher affinity for the actin cytoskeleton than for rafts. These data also raise the question of the communication between molecules located outside and inside rafts. This area of research remains to be investigated and may implicate the lateral mobility of particular lipids and membrane proteins in and out of raft and non-raft membrane areas.

What is the role of raft-cytoskeleton interactions in post-integrin engagement events? The present study demonstrates that disruption of raft integrity by controlled cholesterol depletion does not prevent platelet aggregation induced by high

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thrombin concentrations. Thus, αIIbβ3 inside-out and outside-in signaling can function, at least in part, independently of lipid rafts as previously proposed (Wonerow et al., 2002). However, we found that fibrin clot retraction, a post-aggregation event requiring αIIbβ3 engagement, was strongly affected by raft disruption. The interaction of the actin cytoskeleton with membrane domains with a restricted fluidity state may be well adapted to develop αIIbβ3-mediated tension forces during the late phase of platelet activation. Although the molecular mechanisms leading to clot retraction are still poorly characterized, this final step of hemostasis is thought to be important for the mechanical stability of thrombi under conditions of high shear stress and may provide less surface area for fibrinolytic proteins.

In conclusion, our study demonstrates for the first time the existence of a strong and specific αIIbβ3-dependent interaction of lipid rafts with the actin cytoskeleton in activated human platelets. In addition to the previously described raft-cytoskeleton interaction driven by the immune receptors and their partners, we show the existence of a novel mechanism involving integrins. We propose a model where αIIbβ3, although mainly located outside the rafts, controls the level of PtdIns(4,5)P_2 in the microdomains and the local rearrangement of the actin cytoskeleton. Although rafts are not mandatory for αIIbβ3-mediated platelet aggregation, they appear to play an important role in clot retraction. Thus, the raft-cytoskeleton interaction may participate in the organization of αIIbβ3-mediated tension forces required for this post-aggregation event. Whether the raft-cytoskeleton interaction also plays a role in other cellular processes such as adhesion, cell-cell contact or cell migration involving different integrins represents an important area for future investigation.

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