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β-Dystroglycan modulates the interplay between actin and microtubules in human-adhered platelets

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Summary

To maintain the continuity of an injured blood vessel, platelets change shape, secrete granule contents, adhere, aggregate, and retract in a haemostatic plug. Ordered arrays of microtubules, microfilaments, and associated proteins are responsible for these platelet responses. In full-spread platelets, microfilament bundles in association with other cytoskeleton proteins are anchored in focal contacts. Recent studies in migrating cells suggest that co-ordination and direct physical interaction of microtubules and actin network modulate adhesion development. In platelets, we have proposed a feasible association between these two cytoskeletal systems, as well as the participation of the dystrophin-associated protein complex, as part of the focal adhesion complex. The present study analysed the participation of microtubules and actin during the platelet adhesion process. Confocal microscopy, fluorescence resonance transfer energy and immunoprecipitation assays were used to provide evidence of a cross-talk between these two cytoskeletal systems. Interestingly, β-dystroglycan was found to act as an interplay protein between actin and microtubules and an additional communication between these two cytoskeleton networks was maintained through proteins of focal adhesion complex. Altogether our data are indicative of a dynamic co-participation of actin filaments and microtubules in modulating focal contacts to achieve platelet function.

Keywords: actin, dystroglycan, focal adhesions, microtubules, platelets.

Platelets respond to a continuity interruption of blood vessels, changing shape, secreting granule contents, adhering to the endothelium, and aggregating to form a haemostatic clot. Filopodia, lamellipodia, stress-like fibres, and contractile ring are four structures formed in full-spread platelets that represent the major actin-based components of all cells and, with a different complement of actin-binding proteins, mediate all aspects of platelet function (Karlsson *et al*, 1984; Bearer, 1995).

The molecular complexity of cell-matrix adhesion occurs at specialized sites where integrins directly bind extracellular matrix molecules, transmitting a signal back into the cytoplasm and coupling them to the actin cytoskeleton (Phillips *et al*, 2001). This linkage is mediated by a sub-membrane interconnecting plaque that consists of structural proteins, such as vinculin, paxillin, talin and α-actinin, and signalling molecules including tyrosine kinases, such as focal adhesion kinase (FAK) and serine/threonine kinases, and various adapter proteins (Goldmann, 2002; Shattil & Newman, 2004). The most common forms of integrin-mediated cell-matrix adhesions are focal adhesions, which are associated with actin stress fibre termini. Studies that involve dynamic remodelling of the actin cytoskeleton as well as the microtubule network have discovered reciprocal cross-talk between these important cytoskeletal systems and their dynamic link to the substrate in focal adhesions.

This interaction may be decisive in determining whether a cell is stationary or migratory. In stationary cells, microtubule tips extend to the cell edge around the entire periphery, whereas the advancing lamellipodium of migrating cells is occasionally penetrated by microtubules (Waterman-Storer & Salmon, 1999). Changes in the microtubule network by means of differential localization and delivery of actin cytoskeletal effectors may control cell migration by influencing focal

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contact formation, stability and remodelling (Jones *et al*, 2005). Microtubule disruption led to RhoA activation, which resulted in stress fibre formation, increased focal contact size, and enhanced paxillin and FAK phosphorylation (Bershadsky *et al*, 1996; Enomoto, 1996).

Dystroglycan is a heterodimeric glycoprotein that is expressed as a single polypeptide, and then post-translationally cleaved into α- and β-subunits. β-dystroglycan (β-Dg) is a 43 kDa single transmembrane protein that binds α-Dg at its N-terminus, and it has a cytoplasmic tail that contains binding sites for numerous proteins, such as dystrophin and utrophin, and several signalling molecules. β-Dg modulates signal transduction, acting as a scaffold for the extracellular-signal-related kinase – mitogen-activated protein (ERK-MAP) kinase cascade. It has also been shown that β-Dg binds and bundles actin filaments directly (Chen *et al*, 2003), sequesters proteins in separate cellular locations to regulate their adhesion-dependent activation (Spence *et al*, 2004), and modulates actin reorganization via Cdc42 (Batchelor *et al*, 2007).

In previous studies, we suggested a feasible association between actin filaments and microtubules in adhered platelets treated with cytochalasin D and colchicine (Cerecedo *et al*, 2002). Additionally, we determined the presence of β-Dg as part of the dystrophin-associated protein complex (DAPC) (Cerecedo *et al*, 2005), as well as the participation of the DAPC focal adhesion complex of adhered platelets (Cerecedo *et al*, 2006).

The present study investigated the role of β-Dg as an interplay protein between actin filament and microtubule systems in adhered platelets using cytoskeleton-disrupting drugs. In addition, we examined the feasible participation of microtubules in targeting contact sites in adhered platelets. Our findings using confocal microscopy, fluorescence resonance energy transfer (FRET) analysis, and immunoprecipitation (IP) assays strongly suggested that microtubules might modulate adhesion contacts, and that β-Dg was part of a microtubule complex that interfaces with the actin cytoskeleton.

Materials and methods

All reagents were purchased from Sigma, Chemical Co. (St Louis, MO, USA) unless otherwise indicated.

Antibodies

Monoclonal antibodies are referred to as mAb, while polyclonal antibodies are pAb. β-Dg (JAF) pAb and MAP2 pAb were a generous gift from Drs D. Mornet and A. Rendón, and were previously characterized (Jancsik *et al*, 1996; Echenne *et al*, 1997) respectively. Integrin β-1 pAb Cat. no. sc-8978; actin pAb Cat. no. sc-1615; α-actinin pAb Cat. no. 7454; vinculin pAb Cat. no. sc-7649; talin pAb Cat. no. 7534; FAK pAb Cat. no. sc-557; Glutathione-S-transferase (GST) mAb Cat. no. sc-138; α-tubulin mAb Cat. no. sc-5286 and β-tubulin pAb Cat no. sc-9110 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Platelet suspension

Platelets were obtained by venepuncture following written consent from healthy donors who had not received any drug treatment 10 d prior to the study. Platelet-rich plasma was obtained from total blood by centrifugation at 100 ***g*** for 20 min at room temperature, and was subsequently mixed with an equal volume of citrate anticoagulant and centrifuged at 400 ***g*** for 10 min. The platelets were washed twice with Hank's balanced saline solution (HBSS) without calcium (137 mmol/l NaCl, 5·3 mmol/l KCl, 1 mmol/l $MgCl_2$, 0·28 mmol/l $Na_2HPO_4{\cdot}12H_2O$. 0·87 mmol/l NaH_2PO_4, 0·44 mmol/l KH_2PO_4, 4·1 mmol/l $NaHCO_3$, 5·5 mmol/l glucose). Platelets were kept in this solution for 60 min at 37°C to reach a resting condition. All incubations and processing were carried out at room temperature.

Preparation of cytoskeleton inhibitors

Cytochalasin D solution (20 μmol/l) was prepared in HBSS from 9·8 mmol/l concentrated solution. Colchicine (Research Organics Inc., Cleveland, OH, USA) was directly dissolved in HBSS.

Induced disruption of actin and microtubule organization

Resting platelet suspension was incubated with an identical volume of the drugs to obtain final concentrations of 10 μmol/l cytochalasin D or 10 mmol/l colchicine (Ikeda *et al*, 2000). Platelets were incubated for 60 min and later allowed to adhere to glass slides for 20 min in presence of the drugs, then fixed and processed for immunofluorescence assays.

Platelet adhesion and fixation

Platelets were set on glass coverslips for 20 min; non-adhered cells were removed by washing with HBSS, fixed, and permeabilized with a mixture of 2% p-formaldehyde, 0·05% glutaraladehyde and 0·04% Nonidet P-40 (NP40) in PHEM solution (100 mmol/l PIPES, 5·25 mmol/l HEPES, 10 mmol/l EGTA, 20 mmol/l $MgCl_2$).

Immunofluorescence staining

Platelets were first incubated with the specific primary antibodies diluted in phosphate-buffered saline 0·1% bovine serum albumin and incubated for 2 h. Cells were washed with PHEM solution and incubated for 1 h with secondary anti-goat, or anti-rabbit/anti-mouse, antibody conjugated to Alexa-Fluor-488 or Alexa-Fluor-568 (Molecular Probes, Eugene, OR, USA) respectively and then washed several times with PHEM and mounted in Vectashield (Vector Laboratories Inc.,

Burlingame, CA, USA). Slides were observed using a Leica confocal instrument model TCS-SP5 Mo, the lasers were configured to 20% (17% outside) for Argon and 45% for He/Ne 543, and the images were taken at 63× zoom 3× at 512 × 512 pixels with an HCX PL APO 63X/1·40–0·60 DIL CS oil immersion. Optical sections (z) were performed at 118 nm with 1 Airy unit. Negative controls included cells incubated with an irrelevant polyclonal antibody, and slides were only exposed to secondary antibodies conjugated to the fluorochromes.

FRET analysis

The FRET analysis was performed confocally in Acceptor Photo Bleaching mode. We used fluorescein isothiocyanate (FITC)- and tetramethylrhodamine isothiocyanate (TRITC)-like donor and acceptor accomplished fluorochromes. The principal line was argon at 20% (33% outside line). Bleaching areas were established by regions of interest and were burned 25 times with paining of 10% at 12 bits. Terminal FRET efficiency for Leica instruments ranged from 0 (0%) to 1 (100%) as established by multicolour indicative bar.

Glutathione-S-transferase binding assays

Human *DAG1* cDNA was removed from pGFP-β-Dg vector by EcoRI digestion and cloned in-frame to the 3′-end of GST in bacterial expression vector pGex-4T1 (Amersham Biosciences Co., Piscataway, NJ, USA) to generate *GSTA1-DAG1* fusion gene. To express and characterize GST and GST-β-Dg fusion proteins, an aliquot (300 ml) of *Escherichia coli* cell culture (strain JM109), expressing either GST or GST-β-Dg, was centrifuged at 3800 ***g*** for 10 min, resuspended in 2 ml of NETN buffer [100 mmol/l (w/v) NaCl, 20 mmol/l (w/v) Tris–HCl (pH 7·5), 1 mmol/l (w/v) EDTA, 0·5% (v/v) NP40, 1 mmol/l (w/v) phenylmethylsulphonyl fluoride, 0·5% (w/v) low-fat dried milk, 1× complete protease inhibitor cocktail], and subjected to sonication. Then, 300 μl of packed glutathione-Sepharose 4B beads (Amersham Biosciences Co.) was added to bacterial lysate and the mixture was incubated overnight at 4°C while rotating. Beads recovered by centrifugation at 1000 ***g*** for 5 min were washed five times with 1 ml ice-cold NETN buffer and resuspended in 300 μl NETN buffer. GST and GST-β-Dg proteins, bound to glutathione-Sepharose, were eluted by adding an equal volume of 2× sample buffer and heating at 95°C, and subsequently analysed by Coomassie brilliant blue staining and immunoblotting. To perform pull-down assays, a similar amount of GST or GST-β-Dg fusion protein immobilized onto 20 μl of glutathione-Sepharose beads was incubated for 2 h at 4°C on a rotator with 1 mg of resting whole platelet extract prepared in radioimmunoprecipitation assay buffer containing 1% (v/v) Triton X-100 and 0·1% (w/v) sodium dodecylsulphate (SDS). Beads were recovered by centrifugation at 6000 rpm for 5 min and washed five times with 1 ml ice-cold NETN buffer. Finally, platelet endogenous proteins bound to glutathione-Sepharose were eluted by adding an equal volume of 2× sample buffer and heating at 95°C, then analysed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Western blotting

Lysates from adhered platelets obtained in SDS and β-mercaptoethanol were boiled for 5 min, subjected to 10% SDS-PAGE, and transferred to nitrocellulose membranes, using a semi-dry system (Thermo Electron Co., Milford, MA, USA). Membranes were incubated with appropriate primary antibodies, and then with horseradish peroxidase-conjugated secondary antibodies, visualized using an enhanced chemoluminescence Western blotting analysis system (Santa Cruz Biotechnlogy Inc.), and documented using X-omat film (Kodak, Rochester, NY, USA). Negative controls comprised transferred strips incubated solely with horseradish peroxidase-conjugated secondary antibodies.

Immunoprecipitation assays

Adhered platelets were lysed for 15 min at 4°C with an equal volume of 2× lysis buffer (2 mmol/l EGTA, 100 mmol/l HEPES, 150 mmol/l NaCl, 2% NP40, pH 7·4) containing a protease inhibitor cocktail. Lysates were incubated for 2 h at 4°C with the immunoprecipitating antibodies and subsequently incubated overnight with Rec Protein G-Sepharose (Santa Cruz Biotechnology Inc.). Immunoprecipitates were separated by centrifugation and washed with NP40-free lysis buffer, then resuspended in 2× sample buffer (125 mmol/l Tris–HCl, 4% SDS, 20% glycerol, 0·01 mg/ml β-mercaptoethanol and bromophenol blue, pH 6·8) and boiled for 5 min. Immunoprecipitated proteins and supernatants were analysed by Western blotting.

Results

Actin and microtubule systems co-operate during the platelet adhesion process

Pharmacological studies showed that actin filament disruption could have effects on microtubule filament organization during platelet adhesion to glass (Cerecedo *et al*, 2002). However, to gain insight into the potential interaction of tubulin and actin filaments during the platelet adhesion process, confocal microscopy was used to investigate the cellular distribution of actin filaments and microtubules from suspended platelets incubated with cytochalasin D and colchicine (inhibitors of actin filaments and microtubules respectively) that had been allowed to adhere to glass. Co-localization was evaluated by FRET and compared with control preparations without drugs. Actin filament immunostaining of control platelets (Fig 1) was observed with radial distribution toward the plasma membrane edge (arrowhead),

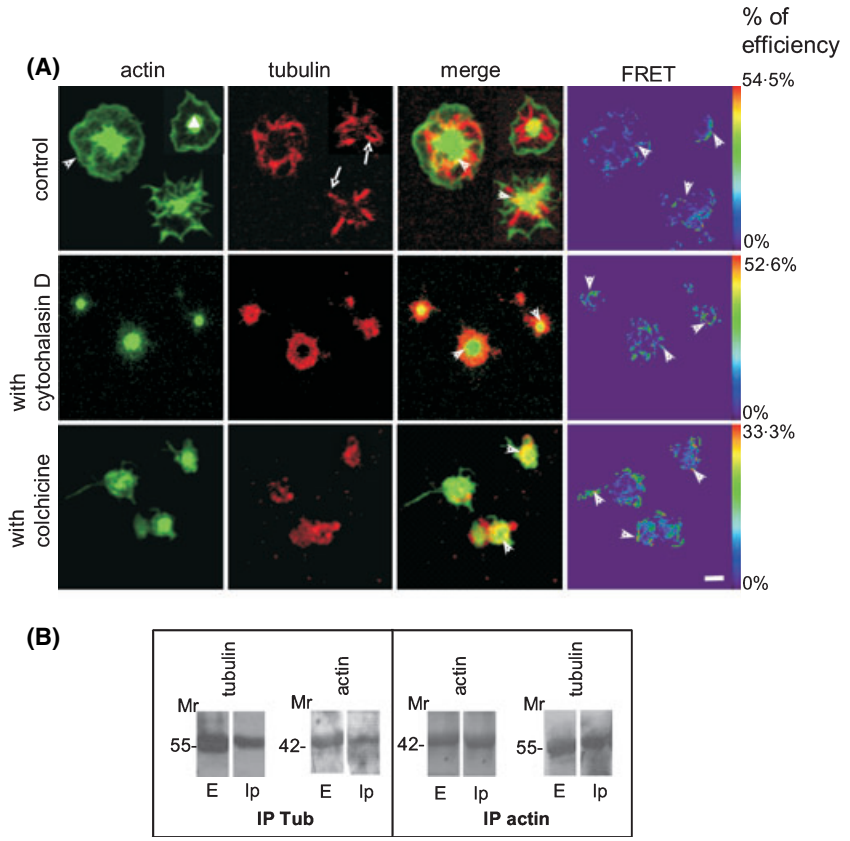

Fig 1. Association of actin filaments and tubulin in human-adhered platelets treated with cytoskeleton disrupters. Panel A, Resting platelets were incubated with cytochalasin D and colchicine for 60 min, adhered on glass, and analysed by confocal microscopy after processing for double-labelling, using fluorescein isothiocyanate (FITC)-phalloidin to detect actin filaments and α-tubulin antibody, followed by a secondary tetramethyl rhodamine isocyanate (TRITC) antibody. The respective merged images as well as the results of fluorescence resonance energy transfer (FRET) analysis are shown. Plasma membrane patches (arrowhead); contractile ring (arrow), granulomere (triangle). Scale bar = 2 μm. Panel B, Resting platelets were adhered on glass and processed for immunoprecipitation assays using anti-actin and anti-α-tubulin antibodies (IP). Proteins from total extracts (E) and immunoprecipitates (Ip) were analysed by immunoblot utilizing antibodies against actin and tubulin. Actin and tubulin were detected with bands of 42 and 55 kDa respectively.

in filopodia, at the granulomere (triangle) and around it, forming the contractile ring.

The circumferential microtubule bundle was disrupted into fragments as described previously (White *et al*, 2001). It is important to note that in full-spread platelets, tubulin polymerized in parallel rays around the granulomere from cytoplasm to plasma membrane (arrows). Merging the respective images showed co-localization at the contractile ring and its quantification by FRET revealed a ratio of 54·5%, which was observed by means of red spots and with less intense green zones corresponding to granulomere (arrowheads). Cytochalasin D treatment did not allow full platelet spreading, and actin filaments were observed as central aggregates at the granulomere and increased tubulin polymerization around plasma membrane with diffuse distribution within cytoplasm with a granular pattern. Apparent co-localization of both proteins was discretely evident at zones where actin filaments and microtubules coalesced (arrowheads). FRET analysis suggested a feasible association, evidenced by red spots at plasma membrane and surrounding the granulomere (arrowheads), with a ratio corresponding to 52·6%. Actin filaments in colchicine-treated adhered platelets showed a scarce distribution at filopodia, the majority of this remaining as a central aggregate, while depolymerized tubulin aggregates were located within the cytoplasm and beneath the plasma membrane. The co-localization rate was lower, at 33·3%; nonetheless, this was observed at plasma membrane and filopodia (arrowheads). These results suggested that actin filaments promote microtubule organization and distribution and that, simultaneously, microtubules might regulate the zone of actin assembly.

To establish the feasible association between actin and microtubule filaments during platelet adhesion, we performed IP assays using anti-tubulin and anti-actin antibodies in adhered platelets. Our results confirmed a close association between these two proteins (Fig 1, panel B), observed by the presence of bands for actin (42 kDa) and tubulin (55 kDa) determinations.

β-Dystroglycan is an actin- and tubulin-binding protein and is distributed to the granulomere membrane during the adhesion process

Recently, we described the presence of β-Dg as a component of the DAPC in actin-based structures located at the plasma membrane of lamellipodia (arrowhead), granulomere (triangle) and contractile ring (arrow) during the adhesion process, co-localizing with actin filaments at these structures, as shown in Fig 2. A FRET rate of 79·2% confirmed its association, showing red spots at the cytoplasmic membrane, filopodia, and granulomere zone (arrowheads).

Cytochalasin D- and colchicine-treated adhered platelets showed diminution of co-localization as evidenced by their respective FRET rates of 55·6 and 57·7%. Thick clumps of β-Dg were observed in cytochalasin D-treated platelets membranes, which were less apparent and more dispersed at colchicine-treated platelets plasma membranes (Fig 2). As in both cases in which adhesion was inhibited, the granulomere zone was not evident, and β-Dg and actin filaments co-localization was observed only at cytoplasmic membrane (arrowheads).

Electron microscopy observations White (1969) and Steiner (1983) revealed connections between membrane structures and microtubules. Therefore, to confirm the spatial correlation between the dynamic microtubule coil and β-Dg, double-labelling immunofluorescence assays were performed using antibodies directed against β-Dg and α-tubulin in cytochalasin D- and colchicine-treated adhered platelets.

Because confocal images corresponding to β-Dg and tubulin distribution were described previously, they are not mentioned further. Co-localization of both proteins was evident at the plasma membrane of filopodial platelets (arrowhead) and contractile ring (arrow) of full-spread control platelets (Fig 2B). Cytochalasin D-treated platelets showed co-distribution of β-Dg and tubulin at the plasma membrane (Fig 2B, arrowheads), while in platelets with depolymerized microtubules (treated with colchicine), the feasible association of tubulin and β-Dg was limited to the granulomere zone (arrowheads), although an incipient lamellipodium was present. FRET rates indicated feasible interaction of β-Dg and microtubules regardless of the drug used (cytochalasin D or colchicine) in relation to control, with values of 76·7, 68·0, and 71·4% respectively.

According to our confocal microscope images and FRET analysis, the association of β-Dg with actin filaments and microtubules was evident. To support these results, we performed IP assays using antibodies against β-Dg, actin and α-tubulin (Fig 2C). Co-immunoprecipitated proteins were analysed by immunoblots utilizing the same antibodies, including one directed against microtubule-associated protein 2 (MAP2).

β-Dystroglycan co-precipitated with actin, tubulin and MAP2, evidenced by the presence of bands of 42 kDa, 55 kDa and 210 kDa respectively; actin pulled-down β-Dg (43 kDa), but not MAP2 (210 kDa). The complementary reaction from tubulin co-precipitates did not detect β-Dg. The presence of β-Dg in extracts and pellets from the corresponding IP (as control) was included (Fig 2C).

β-Dystroglycan stabilizes microtubules at platelet contact sites

When platelets spread on glass surfaces, actin filaments and associated proteins span the cytoplasm and are anchored in ventral plasma membrane in focal contacts (Hagmann, 1993), in which integrin β-1 links adhesive interactions to changes in actin cytoarchitecture (Hynes, 2002). Recently, we suggested scaffolding and signalling roles of DAPCs (Cerecedo *et al*, 2006). To dissect the participation of some proteins making up the DAPC, we evaluated the feasible association of integrin β-1, β-Dg, and tubulin during glass adhesion. Confocal analysis of double immunofluorescence labelling integrin β-1, β-Dg and tubulin was performed in adhered platelets (Fig 3A). Integrin β-1 was distributed at the cytoplasmic plasma membrane (arrowheads), filopodia and contractile ring (arrow), similar to that described for β-Dg and tubulin. Merging of integrin β-1 and tubulin showed an association as discrete patches located at the open canalicular system membrane (arrowheads), and its FRET rate was 75·0%, indicating a high association between these. Integrin β-1 and β-Dg were co-distributed at the contractile ring (arrow), plasma membrane and granulomere zone (arrowheads); FRET analysis highlighted this co-localization (arrowheads), with a rate of 87·2%.

To confirm the association of integrin β-1 with tubulin and β-Dg, IP assays were performed using antibodies raised against integrin β-1, β-Dg and α-tubulin. Integrin β-1 co-precipitated with tubulin, but not with β-Dg, while the complementary IP performed with β-Dg and tubulin pulled-down integrin β-1. These results strongly support a specific relationship of tubulin and β-Dg with integrin β-1-mediating platelet adhesion and spreading, targeting focal adhesions.

Microtubule dynamics induces focal adhesion

Integrin clustering and subsequent recruitment of actin filaments to the cytoplasmic domain of integrins is achieved via a complex of interacting cytoskeletal proteins including talin, vinculin, α-actinin and filamin (Critchley *et al*, 1999). To determine the feasible distribution of cytoskeleton proteins involved in focal adhesion organization, we performed confocal analysis of adhered platelets double-labelled with antibodies against β-Dg (Fig 4A) or α-tubulin (Fig 4B) and proteins of the focal adhesion complex (α-actinin, vinculin and talin).

α-Actinin was distributed as a patched pattern at the plasma membrane (arrowhead) and surrounding contractile ring (arrow) and talin was homogenously distributed at plasma membrane as discrete patches (arrowheads) and at the contractile ring (arrow), while vinculin was observed with a punctuate pattern in the submembrane area (arrowheads).

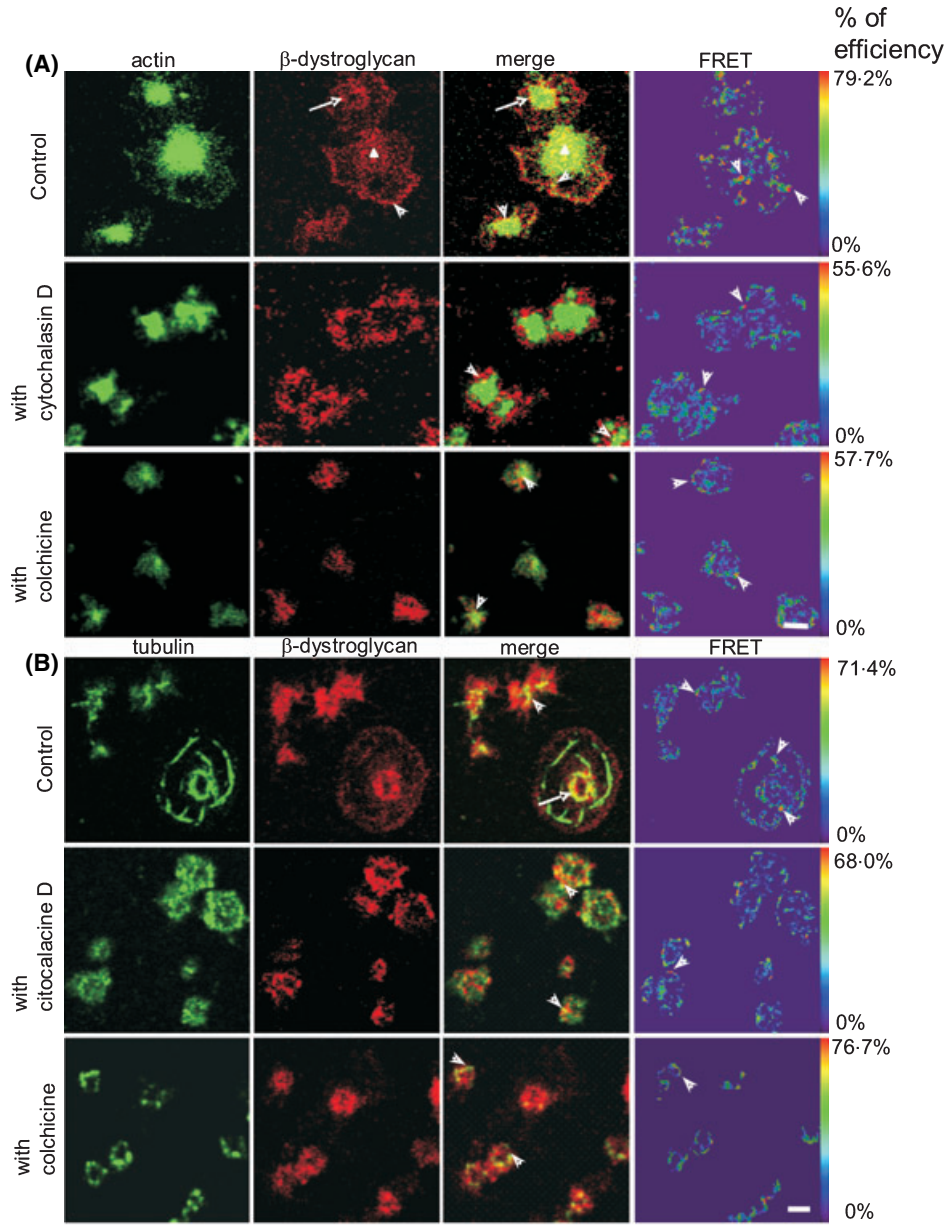

Fig 2. Association of β-dystroglycan (β-Dg) with actin filaments and microtubules in human-adhered platelets treated with cytoskeleton disrupters. Panel A, Resting platelets were incubated with cytochalasin D and colchicine for 60 min, adhered on glass, and analysed by confocal microscopy after processing for double-labeling, using FITC-phalloidin to detect actin filaments and JAF antibody to detect β-Dg, followed by a secondary tetramethyl rhodamine isocyanate (TRITC) antibody. Merges and fluorescence resonance energy transfer (FRET) analysis of the respective pair of images are shown. Plasma membrane patches (arrowhead); contractile ring (arrow), granulomere (triangle). Scale bar = 2 μm. Panel B, Resting platelets were treated with cytoskeleton drugs and prepared for confocal analysis to determine the topographic distribution of tubulin revealed with FITC secondary antibody and β-Dg revealed with TRITC secondary antibody. Panel C, Resting platelets were adhered on glass and processed for immunoprecipitation assays using anti-β-Dg, anti-actin, and anti-α-tubulin antibodies (IP). Proteins from total extracts (E) and immunoprecipitates (Ip) were analysed by immunoblot employing antibodies against β-Dg, actin, tubulin, and MAP2, detecting bands of 43, 42, 55, and 210 kDa respectively.

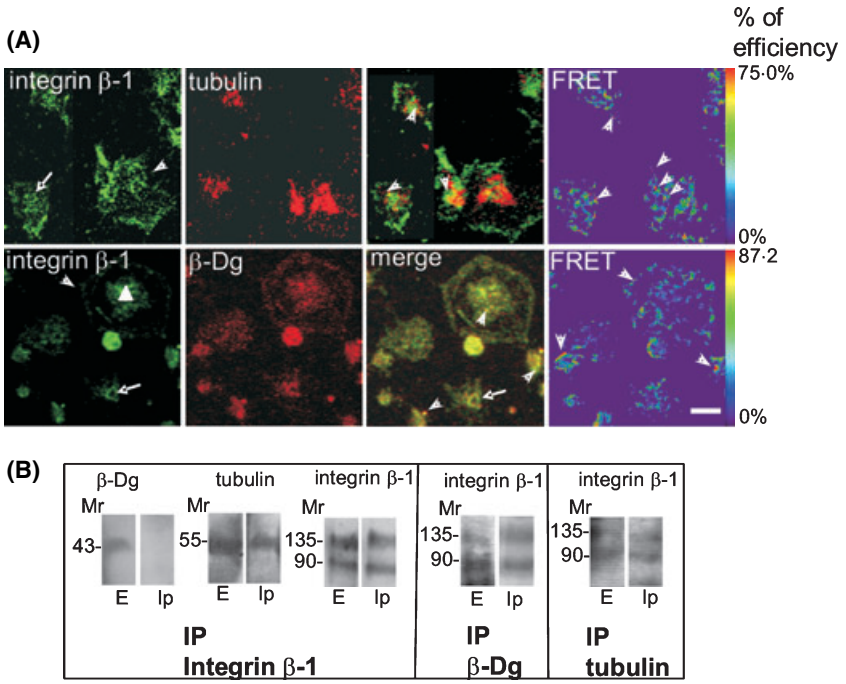

Fig 3. Co-distribution of tubulin and β-dystroglycan (β-Dg) with integrin β-1 in human adhered platelets. Panel A, Confocal microscopy and fluorescence resonance energy transfer (FRET) analysis of glass-adhered platelets after double-labelling with integrin β-1 antibody directed against integrin β-1 subunit revealed with fluorescein isothiocyanate (FITC)-secondary antibody and β-Dg and α-tubulin antibodies revealed with tetramethyl rhodamine isocyanate (TRITC)-secondary antibody. Plasma membrane patches (arrowhead); contractile ring (arrow), granulomere (triangle). Scale bar = 2 μm. Panel B, Immunoprecipitations were performed from human adhered platelets with anti-integrin β-1, anti-β-Dg, and anti-tubulin (IP). Proteins from total extracts (E) and immunoprecipitates (Ip) were analysed by immunoblot using antibodies against integrin β-1 subunit (double band of 135 and 90 kDa), β-Dg (43 kDa) and tubulin (55 kDa).

Co-localization of focal adhesion complex proteins (α-actinin, talin and vinculin) and β-Dg was present mainly at the plasma membrane (arrowheads), contractile ring (arrow) and granulomere (triangle) (Fig 4A). Red spots observed in FRET analysis clearly showed this co-distribution. For α-actinin, interaction with β-Dg was observed at the cytoplasmic membrane (arrowheads) and around granulomere (dashed circle), with an efficiency of 83·0%. A FRET value of 74·0% corresponded to the image of β-Dg with talin, which exhibited discrete interaction at plasma membrane in contrast with the image corresponding to β-Dg and vinculin (75·0%), in which interactions indicated by red spots and yellowish-green zones were located mainly at cytoplasmic membrane (Fig 4A).

Merging of α-actinin, talin and, vinculin with tubulin (Fig 4B) demonstrated an important co-localization label, which was observed embedded within these proteins at the contractile ring (arrow) and plasma membrane patches (arrowheads). It is important to note that vinculin was found on the microtubules located at contractile ring (arrows) or in the cytoplasm radiating from this. The FRET rate efficiency observed between tubulin and talin at plasma membrane corresponded to 79·0%, while FRET analysis of tubulin with α-actinin and vinculin demonstrated a closer interaction along microtubules, with FRET efficiency rates of 88·0 and 94·0% respectively (Fig 4B). High-interaction FRET rates as well as co-distribution between tubulin and the adhesion complex proteins strongly suggested an important role of these proteins during focal contact assembly.

To confirm the participation of microtubules during adhesion assembly, IP assays were performed using extracts from glass-adhered platelets and tubulin and β-Dg antibodies (indicated as IP in Fig 4C). Co-immunoprecipitated proteins

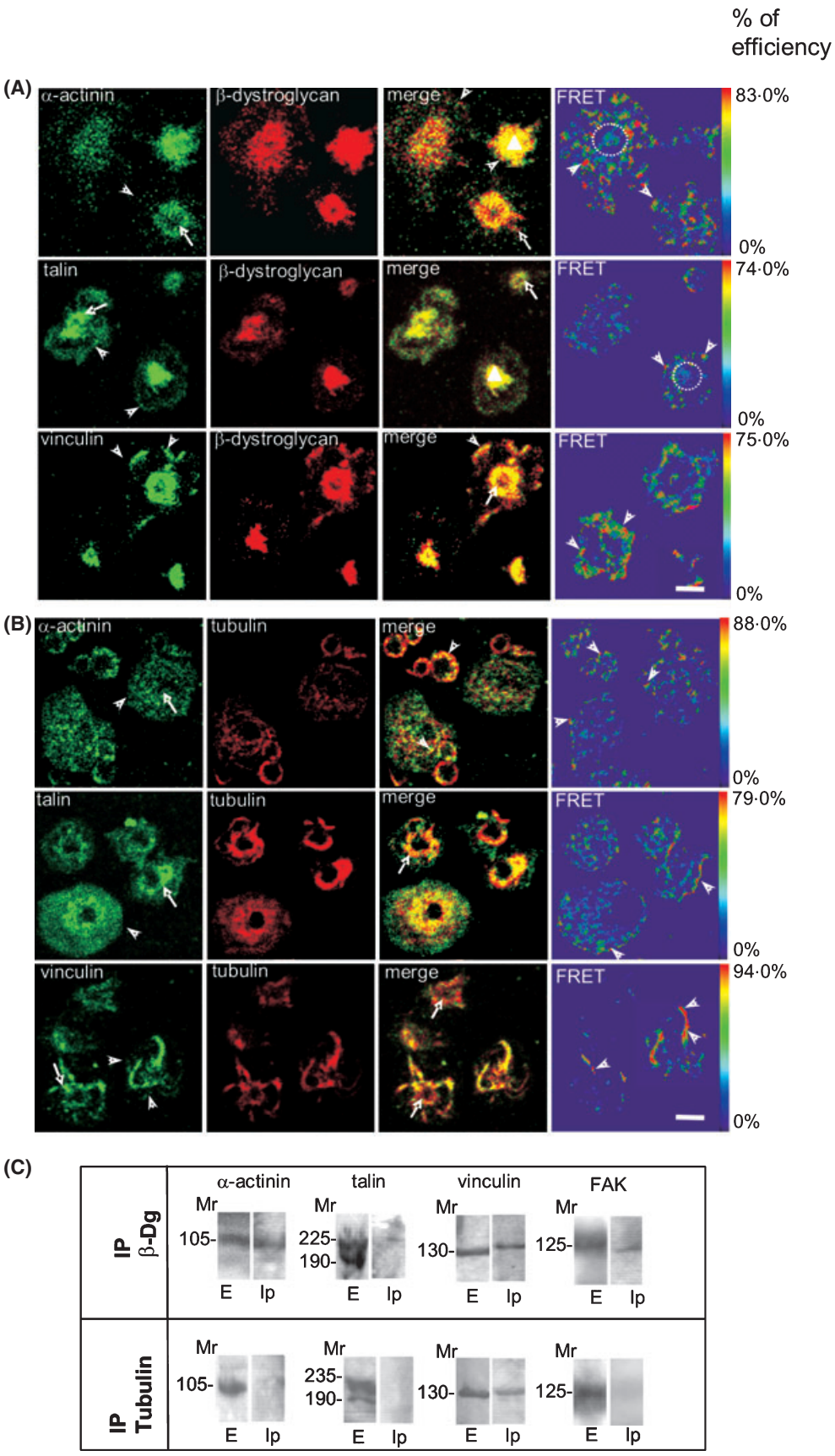

Fig 4. Recruitment of microtubules at focal contacts and participation of β-dystroglycan (β-Dg). Panel A, Glass-adhered platelets were processed for double immunofluorescence assays for observation by confocal microscopy and analysis by fluorescence resonance energy transfer (FRET). Antibodies against focal adhesion complex (α-actinin, talin and vinculin) revealed with fluorescein isothiocyanate (FITC)-secondary antibody and β-Dg revealed with tetramethyl rhodamine isocyanate (TRITC)-secondary antibody. Plasma membrane patches (arrowhead); contractile ring (arrow), contractile ring (dashed circle) in FRET. Scale bar = 2 μm. Panel B, Glass-adhered platelets were processed for double immunofluorescence assays for observation by confocal microscopy and analysis by FRET. Antibodies against focal adhesion complex (α-actinin, talin and vinculin) revealed with FITC-secondary antibody and α-tubulin evidenced with TRITC-secondary antibody. Plasma membrane patches (arrowhead); contractile ring (arrow). Scale bar = 2 μm. Panel C, Adhered platelet extracts were immunoprecipitated using antibodies against β-Dg and α-tubulin (IP). Proteins from total extracts (E) and immunoprecipitates (Ip) were analysed by immunoblot utilizing antibodies against focal adhesion proteins. Bands corresponding to 105 kDa, double band of 235 and 190 kDA, 130 kDa, and 125 kDa were identified for α-actinin, talin, vinculin and FAK respectively.

and total extracts were analysed by immunoblots that employed antibodies against α-actinin, talin, vinculin, and FAK (Fig 4C). β-Dg immunoprecipitated with α-actinin (105 kDa), vinculin (130 kDa), and FAK (125 kDa), whereas tubulin pulled down only with vinculin (130 kDa). These results suggested that β-Dg might anchor microtubules to plasma membrane, while tubulin might be modulate focal adhesion formation through its association with vinculin.

In vitro binding of β-dystroglycan to components of the focal adhesion complex

To corroborate the interaction of β-Dg with proteins involved in the focal adhesion components of the adhesion protein complex by GST pull-down assays, a pGex-4T1-derived vector expressing the GST-β-Dg protein fusion was generated. GST and GST-β-dg protein fusions were isolated by incubation of JM109 bacterial lysates with glutathione-Sepharose beads. Next, GST and GST-β-Dg proteins were characterized by immunoblotting. As expected, the protein band corresponding to GST-β-Dg (66 kDa) was revealed using both anti-GST and anti-β-Dg antibodies, while the GST protein band (28 kDa) was observed only with the anti-GST antibody (Fig 5A). GST or GST-β-Dg fusion protein previously immobilized on glutathione-Sepharose beads was incubated with control-adhered platelet extracts, and GST-bound proteins were eluted and analysed by SDS-PAGE and immunoblotting with antibodies against actin, tubulin, MAP2, integrin-β1, α-actinin, talin, vinculin and FAK. Evaluation of the known interaction of β-Dg was performed using anti-actin antibody, representing the positive control. Figure 5B shows the interaction of GST-β-Dg fusion protein with tubulin and MAP2, but not to GST alone. In contrast, no perceptible binding to GST-β-Dg was observed for talin, integrin-β1, FAK, α-actinin and vinculin.

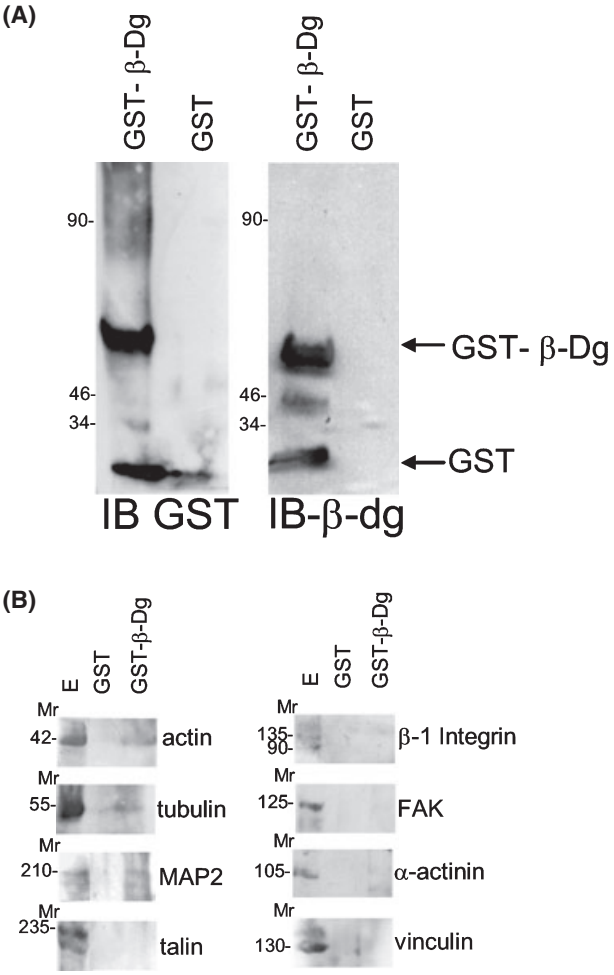

Fig 5. *In-vitro* interactions of β-dystroglycan (β-Dg). Glutathione-S-transferase (GST) and GST-β-Dg proteins isolated from JM109 lysates with glutathione-Sepharose beads were resolved by sodium dodecyl-sulphate polyacrylamide gel electrophoresis and analysed by immunoblot. Panel A, Western blot of GST and GST-β-Dg extracts using antibodies against GST and β-Dg (arrows on the right) denote migration of GST and GST-β-Dg. Panel B, Evaluation of pull-down assays was performed utilizing GST and GST-β-Dg fusion protein immobilized on glutathione-Sepharose beads and incubated with platelet extracts. Pulled-down proteins were analysed by immunoblot assays employing antibodies against actin (as positive control), tubulin, MAP2, talin, integrin β-1, FAK, α-actinin and vinculin. IB, immunoblotting.

Discussion

The results of this study have provided evidence concerning (i) the interaction of microtubules with the cortical actin network; (ii) the existence of interplay between actin filaments and microtubules with β-Dg as a binding molecule; and (iii) the contribution of the two systems (actin filaments and microtubules) in focal adhesion assembly. As the aim of the present

study was to determine the feasible association between proteins, we undertook FRET analysis, which provides a powerful technique for detecting interactions between proteins if the average distance between these is <10 nm (Costes *et al*, 2004). Advantages of this co-localization approach over other dynamic measurements are discussed elsewhere (Gordon *et al*, 1998).

Immunofluorescence studies, FRET analysis, and IP in adhered platelets demonstrated the interaction of microtubules with the cortical actin network in located structures, which may represent an important aspect of cross-talk between these cytoskeletal elements and, as such, be decisive in determining platelet functions. Additionally, the use of compounds depolymerizing actin filaments and microtubules (cytochalasin D and colchicine) aided in determining their interdependence. Cytochalasin D sharply perturbed the zone of actin assembly, such as filopodia, lamellipodia, stress fibres and contractile ring. Treatment of platelets with a microtubule disrupting agent also inhibited Rac-dependent actin-based structures such as lamellipodia protrusion, avoiding spreading, as has been observed in other systems (Bershadsky *et al*, 1991; Waterman-Storer *et al*, 1999).

It is generally believed that leading edge protrusion is driven by actin polymerization (Theriot & Mitchison, 1991; Condeelis, 1993; Brunig *et al*, 2002). However, we observed microtubules physically interacting with actin filaments at the leading edge of the lamellipodium; this might suggest that linking of actin filaments to the relatively rigid extracellular matrix to stable, cell body-anchored microtubules might push the leading edge membrane against the microtubule-linked actin meshwork, as has been proposed elsewhere (Wang *et al*, 2001; Kaverina *et al*, 2002). In addition, specific microtubule co-localization observed at plasma membranes corresponding to cytoplasm and the open canalicular system was in accordance with the findings of Steiner (1983), who suggested that tubulin could be a membrane connection to the cytoskeleton.

In contrast to migrating cells, microtubule tips of stationery cells extend to the cell edge around the entire periphery (Waterman-Storer & Salmon, 1997; Ballestrem *et al*, 2000), platelet tubulin as well as β-Dg coincided with radial distribution extending to the cell edge around the perimeter. In *in-vivo* and *in-vitro* IP assays, co-precipitation of β-Dg with tubulin was indicative of a feasible interaction between these proteins. The fact that complementary IP assays performed with α-tubulin antibody failed to pull-down β-Dg could be due to β-Dg phosphorylation state at specific sites reduces tubulin affinity. In addition, we showed, by complementary IP, the interaction between β-Dg and actin, which is in concordance with other reports (Chen *et al*, 2003). Furthermore, the *in-vivo* and *in-vitro* association observed between β-Dg and MAP2 constitutes another interaction between microtubules and actin filaments, as has been established in other systems (Kim *et al*, 1979; Sattilaro, 1986; Selden & Pollard, 1986). Previous studies have identified motifs within Dp71 (Howard *et al*, 1998) and utrophin (Winder *et al*, 1995; Rybakova *et al*, 2006) that allow their interaction with actin; therefore, these proteins could also serve as bridges for connecting actin and tubulin. Nevertheless, experiments in adhered platelets showed lower association between tubulin and Dp71 and/or utrophins (data not shown) than that obtained with tubulin and β-Dg. Hence, our results strongly suggest that β-Dg is the protein that performs an interplay role with actin-based structures and tubulin at the plasma membrane during the adhesion process.

Integrin ligation by matrix proteins leads to focal adhesion protein assembly (Hynes, 2002), which – in concert with Dg – is required for complete embryonic basement membrane assembly (Henry *et al*, 2001; Li *et al*, 2003). Our data (Fig 3) are indicative of the participation of tubulin and integrin β-1 during stress fibre assembly, although integrin β-1 did not pull-down β-Dg. We think that this inconsistency could be due to indirect interaction of β-Dg with integrin β-1 via actin or vinculin, as we have previously determined (Cerecedo *et al*, 2006).

Our *in-vivo* results suggested intimate interactions at β-Dg adhesion sites with α-actinin, vinculin and FAK, while no association was determined in *in-vitro* assays. It is important to consider that experimental conditions between these two assay types are different, and it is plausible that the complex formed with β-Dg was assembled during cytoskeleton remodelling during the adhesion process. Alternatively, it is possible that phosphorylation of β-Dg, which is not present in the bacterial system, is necessary for interaction with adhesion molecules.

It has been suggested that microtubules confer polarity on the actin cytoskeleton and that the microtubule tip complex guides microtubules into adhesion foci (Kaverina *et al*, 1999; Krylyshkina *et al*, 2003). Based on our observations that microtubules coincided with vinculin-positive contact sites along cytoplasm, we can speculate that the microtubule system and associated proteins stabilize or improve the growth of substrate adhesions or catalyse signalling reactions at adhesion foci by conducting complexes to their vicinity, thus providing another possible link between the microtubule and actin systems.

In platelets, a microtubule-organizing centre (MTOC) in which microtubules are embedded, nucleated and organized has not yet been identified. In different cellular models, Cdc42 is involved in MTOC re-orientation (Stowers *et al*, 1995; Etienne-Manneville & Hall, 2001; Palazzo *et al*, 2001; Tzima *et al*, 2003), and because β-Dg can target local Cdc42 activation (Batchelor *et al*, 2007) it could be conceivable that, in platelets, the MTOC position is determined by actin cytoskeleton interaction via β-Dg with the microtubule network in association with focal adhesions, as has been observed in fibroblasts (Kaverina *et al*, 1998).

Further studies must be performed to elucidate the multiple binding sites that β-Dg appears to possess, as well as the organization of microdomains, which might include membrane proteins (β-Dg and integrin β-1) because lipid rafts play an important role in platelet signalling (Gousset *et al*, 2002; Reineri *et al*, 2007). We conclude that the multiple interrelationships

explored herein suggested that β-Dg directs and anchors microtubule re-location to membrane systems to modulate focal contact assembly during the adhesion process.

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