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Haemostatic Role of Intermediate Filaments in Adhered Platelets: Importance of the Membranous System Stability

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ABSTRACT

The role of platelets in coagulation and the haemostatic process was initially suggested two centuries ago, and under appropriate physiological stimuli, these undergo abrupt morphological changes, attaching and spreading on damaged endothelium, preventing bleeding. During the adhesion process, platelet cytoskeleton reorganizes generating compartments in which actin filaments, microtubules, and associated proteins are arranged in characteristic patterns mediating crucial events, such as centralization of their organelles, secretion of granule contents, aggregation with one another to form a haemostatic plug, and retraction of these aggregates. However, the role of Intermediate filaments during the platelet adhesion process has not been explored. J. Cell. Biochem. 114: 2050–2060, 2013.

KEY WORDS: ADHERED PLATELETS; INTERMEDIATE FILAMENTS (IF); DYSTROPHIN-ASSOCIATED PROTEINS (DAPs); PLECTIN, GRANULOMERE

In the present work, we described, by confocal and electron microscopy analysis and Immunoprecipitation assays, the presence of desmin and vimentin and their association with members of the Dystrophin-associated proteins as well as with microfilaments and microtubules through plectin. We have also undertaken a pharmacological approach using Acrylamide and Brefeldin A to evaluate the participation of vimentin and desmin in granule trafficking. Our findings strongly suggest that Microfilaments, Microtubules, and Intermediate filaments modulate platelet membranous system organization.

Platelets are one of the most widely studied elements of blood tissue, including its role as haemostatic initiators, and its receptors and ligands, underlying the biochemical signaling that control all of these processes. In performing the initial arrest of bleeding, platelets become activated and dynamic responses to many stimuli occurring within seconds involve platelet cytoskeleton remodeling, which has been extensively investigated. It is well known that platelets circulate as anucleated discoid cell fragments, containing 40–50% of their actin in a filamentous form [Fox, 1993]. Upon adhesion to a substrate, actin is reorganized as bundles on the filopodia and netlike arrangements are displayed in lamellipodial regions. Microtubules that appear as a concentric marginal bundle below the outer limiting membrane of the resting platelets become either radially arranged or arranged in parallel bundles in the long axis of spread cells, as well as straight fragments radiating from the central zone [White and Sauk, 1984]. In relation to Intermediate filaments [IF], the presence has been revealed of an isotropic network of individual vimentin-like proteins distributed throughout the platelets, forming a peripheral ring with additional fibrillar presence throughout the platelets [Tablin and Taube, 1987].

The IF network is a highly dynamic structure that is continually reorganized in diverse cellular processes including cell division [Magin et al., 2007], cell migration, cell adhesion [Helfand et al., 2011], intracellular transport, and specific arrangements of

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organelles [Gao and Sztul, 2001; Styers et al., 2005]. IF are differentially expressed during embryonic development, in parallel with distinct routes of differentiation, indicating that IF possess distinct tissue-specific functions [Lazarides, 1982; Hesse et al., 2001]. Hence, in epithelial cells, a highly diverse group of keratins is expressed; vimentin is a hallmark of mesenchymal, endothelial, and hematopoietic cells [Dellagi et al., 1983].

Plectin is considered a universal crosslinking element of the cytoskeleton that possesses binding sites for all types of IF subunit proteins. In muscle cells, it is known that IF are linked with plectin 1 through actin and α-dystrobrevin, as well as with β-dystroglycan [Rezniczek et al., 2007; Hijikata et al., 2008]. β-dystroglycan and α-dystrobrevin are members of the oligomeric dystrophin-glycoprotein complex that is also comprised of α-dystroglycan, sarcoglycans, sarcospan, and syntrophins. α-Dystroglycan mediates binding to components of the Extracellular matrix [ECM], while β-dystroglycan is a single pass transmembrane protein that binds to α-dystroglycan [Boffı et al., 2001] and dystrophin [Huang et al., 2000]. All syntrophins function as adaptor proteins involved in anchoring cell-signaling molecules to the plasma membrane [Piluso et al., 2000], while dystrobrevins are expressed in a wide array of tissues with α-dystrobrevins expressed predominantly in skeletal muscle [Sadoulet-Puccio et al., 1996], binding to dystrophin carboxy terminus [Sadoulet-Puccio et al., 1997; Peters et al., 1998].

In platelets, our work team has identified the participation of β-dystroglycan, α-syntrophin and α-dystrobrevins in key haemostatic functions during the adhesion process forming part of actin-based structures [Cerecedo et al., 2005], acting as a membrane scaffold as well as adaptor proteins [Cerecedo et al., 2006b]. In addition, we have demonstrated that α-dystrobrevins in association with microtubules are involved in granule trafficking events [Cerecedo et al., 2010].

Despite advances in the understanding of the structure and function of the microfilament [MF] and microtubule [MT] networks in the biology of the platelet, the feasible haemostatic role of IF in platelets remains elusive. To address this unexplored field, we have undertaken a pharmacological and biochemical approach to identify the association of two members of type-III IF proteins, desmin and vimentin, with MF and MT networks, as well as with DAPSs, identifying their feasible participation in the haemostatic platelet role. Our findings are consistent with the idea that IF in association with actin filaments, MT, and DAPSs, contribute to granule trafficking and participate in maintaining and modulating the function of platelet membranous systems.

MATERIALS AND METHODS

PLATELET PREPARATION

Platelets were obtained by venopuncture from healthy donors who had not received any drug during the 10 days prior to sampling and who gave consent for the procedure to be carried out. Blood was immediately mixed with citrate anticoagulant including dextrose at pH 6.5 at a blood:anticoagulant ratio of 9:1. Platelet-rich plasma was obtained from total blood by centrifugation at 100*g* for 20 min at room temperature [White, 1983].

ANTIBODIES USED

Monoclonal antibodies are referred to as mAb, while polyclonal antibodies are pAb. Actin mAb Catalogue [Cat.] no. sc-8432, P-selectin pAb Cat. no. sc-6941, α-dystrobrevin pAb Cat. no. sc-13812, α-tubulin mAb Cat no. sc-5286, β-tubulin pAb Cat no. sc-9110, Ubiquitous kinesin heavy chain [UKHC] pAb Cat. no. sc-28538, α-syntrophin pAb Cat. no. sc-13757, β-dystroglycan pAb Cat. no. sc-30405, α-dystrobrevin pAb Cat. no. sc-271874, desmin pAb Cat. no. sc-7559, vimentin pAb Cat. no. sc-7557, and plectin mAb Cat. no. sc-33649 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), while α-dystrobrevin pAb (α-DB) and α-syntrophin pAb [P6] were a kind gift of D. Mornet [Rivier et al., 1997].

ELECTRON MICROSCOPY

Spurr resin beds were prepared and polymerized within plastic Petri dishes according to the supplier's instructions (Electron Microscopy Sciences, Fort Washington, PA). Human resting platelets were incubated with 10 µmol/L Dimethyl sulfoxide (DMSO) or with 10 µg/ml brefeldin A [BFA] for 1 h and were settled for 20 min at room temperature on resin beds. Method used to ultrastructure experiments was described in an earlier report [Cerecedo et al., 2006a]. Respective thin sections recovered on copper grids were stained with uranyl acetate and lead citrate and then examined in a Jeol 1400 transmission electron microscope (JEOL, Tokyo, Japan) at 80 keV.

PREPARATION OF CYTOSKELETON NETWORKS FOR STRUCTURAL CHARACTERIZATION

Platelets (1×10^5/ml) were settled on 200 mesh nickel grids covered with formvar film (Polysciences, Warrington, PA) for 20 min. Platelets were rapidly washed with PHEM (10 mM HEPES, 10 mM EGTA, 1 mM $MgCl_2$), 50 mg/ml N-Tosyl-L-phenylalanine chloromethyl ketone [TPCK], 50 mg/ml Na-p-Tosyl-L-lysine chloromethyl ketone [TLCK], and 17.4 mg/ml Phenylmethylsulfonyl fluoride [PMSF] and incubated with 0.1% Triton X-100 in PHEM for 5 min, and then rinsed twice with PHEM [modified from [Patron et al., 2005]. Samples were dehydrated in increasing concentrations of ethanol, critical point dried in a CO_2 atmosphere in a Samdry-780A apparatus (Tousimis Research Co.) and coated by the evaporation of Platinum-carbon [Pt/C] rods at 458 in a BAF 400T freeze fracture system (Balzers, Austria) and then observed in the Transmission electron microscopy [TEM].

PREPARATION OF INHIBITORS

A 5 mmol/L Acrylamide (ACR) solution (2X) (Sigma Chemical Co., St. Louis, MO) was directly dissolved in HBSS and a 10 µg/ml Brefeldin A (BFA) solution (2X) (Invitrogen, Carlsbad, CA), was prepared in HBSS from a 0.5 mg/ml concentrated solution diluted in DMSO.

TREATMENT OF PLATELETS WITH CYTOSKELETON INHIBITORS

Resting platelets (1×10^6/ml) in suspension were incubated with the same volume of the drugs in order to obtain final concentrations of 10 µg/ml of BFA [Styers et al., 2006] and 5 mM ACR [Sager, 1989], for 60 min at room temperature.

EVALUATION OF sP-SELECTIN

Platelets [1×10^6/ml] incubated with cytoskeleton inhibitors were allowed to adhere to glass Petri dishes in a wet camera. After 20 min, the glass Petri dishes with adhered platelets were rinsed with HBSS; supernatants were collected and centrifuged to eliminate non-adhered platelets. Samples were immediately processed for enzyme-linked immunosorbent assay [ELISA] for quantitative detection of soluble human P-selectin (sP-selectin) [Invitrogen, Camarillo, CA, USA] levels according to the manufacturer's instructions [Cerecedo et al., 2010]. Three independent experiments were performed and average Standard errors of the mean [± SEM] results are presented graphically.

IMMUNOFLUORESCENCE ASSAYS

Control platelets and platelets treated with cytoskeleton inhibitors were allowed to adhere to glass cover slips in a wet camera for 20 min, fixed, and permeabilized in PHEM solution. This method has been described in more detail elsewhere [Cerecedo et al., 2005]. Slides were observed using a Leica confocal instrument model TCS-SP5 (Mannheim, Germany), lasers were configured to 20% (17% outside) for Argon and 45% for He/Ne 543, and images were taken at 63X zoom 7X at 512 × 512 pixels with an HCX PL APO 63/1.40–0.60 DIL CS oil immersion. Optical sections [z] were performed at 118 nm with one Airy unit. Negative controls included cells incubated with an irrelevant polyclonal antibody and slides were only exposed to secondary antibodies conjugated to the fluorochromes. Likewise, platelets incubated with 0.1% DMSO was processed for 1 h as the solvent control.

WESTERN BLOTTING

Lysates from adhered platelets obtained in Sodium dodecyl sulfate (SDS) and β-mercaptoethanol were subjected to 10% SDS–Polyacrylamide gel electrophoresis (PAGE), and transferred onto nitrocellulose membranes. Membranes were incubated with appropriate primary antibodies, then with Horseradish peroxidase [HRP]-conjugated secondary antibodies [Cerecedo et al., 2005] and documented using T-mat G/RA film [Kodak, Rochester, NY]. Negative controls comprised transferred strips incubated solely with HRP-conjugated secondary antibodies.

IMMUNOPRECIPITATION ASSAYS

Adhered platelets were lysed for 15 min at 4°C with an equal volume of 2X lysis buffer Lysates were incubated with the immunoprecipitating antibodies and subsequently incubated overnight with Rec Protein G-Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitates [Ip] were separated by centrifugation and washed with NP40-free lysis buffer, then re-suspended in 2X sample buffer and boiled for 5 min. Immunoprecipitated proteins [Ip] and supernatants were analyzed by Western blotting [Cerecedo et al., 2005].

STATISTICAL ANALYSIS

Data obtained from sP-selectin quantification was expressed as mean ± standard deviation [SD]. Statistical analysis was carried out with the GraphPad Prism for Windows version 5 (GraphPad Software, Inc. La Jolla, CA). One-way Analysis of variance [ANOVA] with a multiple comparison test (Tukey test) was utilized for data analysis. Statistical significance was defined as $P < 0.05$.

RESULTS

DESMIN AND VIMENTIN ARE ASSOCIATED WITH ACTIN FILAMENTS IN HUMAN ADHERED PLATELETS

To determine the feasible association between platelet Intermediate filaments [IF] in adhered platelets in relation to actin filaments, we performed double immunofluorescence staining using TRITC-phalloidin to label actin filaments and with antibodies raised against desmin and vimentin identified with the aid of FITC secondary antibodies. Platelets treated with Acrylamide [ACR] 5 mM were also processed.

Confocal analysis showed that both desmin and vimentin apparently have the same distribution, localizing around the plasma membrane at the cytoplasm, but their main location corresponded to the granulomere. Actin filament bundles were observed radiating from the center of the adhered platelet to the front of the plasma membrane, concentrating at the central zone or granulomere (Fig. 1, Panel A control). The merge of the respective images showed certain association in particular at the granulomere, along the actin filament, bundles and to a lesser extent at the plasma membrane (Fig. 1 Panel A, control merge).

Disruption of IF networks in ACR-treated platelets (Fig. 1 Panel A, ACR), exhibited a heterogeneous morphology that ranged from platelets with filopodial extensions to partially extended lamellipodia with high concentration of fragmented IF at the granulomere zone.

To assess the feasible association between IF [desmin and vimentin] and actin filaments, we performed immunoprecipitation assays [IP] from fully adhered platelet extracts using desmin, vimentin, actin, and plectin antibodies. Total extracts (E) and immunoprecipitated extracts (Ip) were resolved by Western blot (Fig. 1 Panel B). Anti-desmin pulled down actin (42 kDa) with a weak band, but plectin did not, while anti-vimentin pulled down actin (42 kDa) and plectin (500 kDa) (Fig. 1 Panel B, IP desmin, IP vimentin). Complementary immunoprecipitation assays performed using actin antibody pulled down vimentin (57 kDa), but desmin (55 kDa) was observed with a very faint band (Fig. 1 Panel B, IP actin). All of these results showed that the association of actin with vimentin is privileged compared to the association between actin and desmin.

MICROTUBULES ARE CLOSELY ASSOCIATED WITH PLATELET INTERMEDIATE FILAMENTS

To know the cellular distribution of Intermediate filaments (IF) in relation to microtubules (MT), we compared immunofluorescence labels of fully adhered control platelets with platelets treated with Acrylamide [ACR] 5 mM. We employed desmin and vimentin antibodies revealed with FITC-labeled secondary antibodies, while microtubules were identified using primary anti-tubulin antibody revealed with a TRITC secondary antibody. Control platelets showed polymerized microtubules around the plasma membrane and at the center of the spread platelet, co-localizing with desmin and vimentin in both zones (Fig. 2 Panel A, control). ACR-treated platelets showed a lesser spread morphology affecting the polymerization and

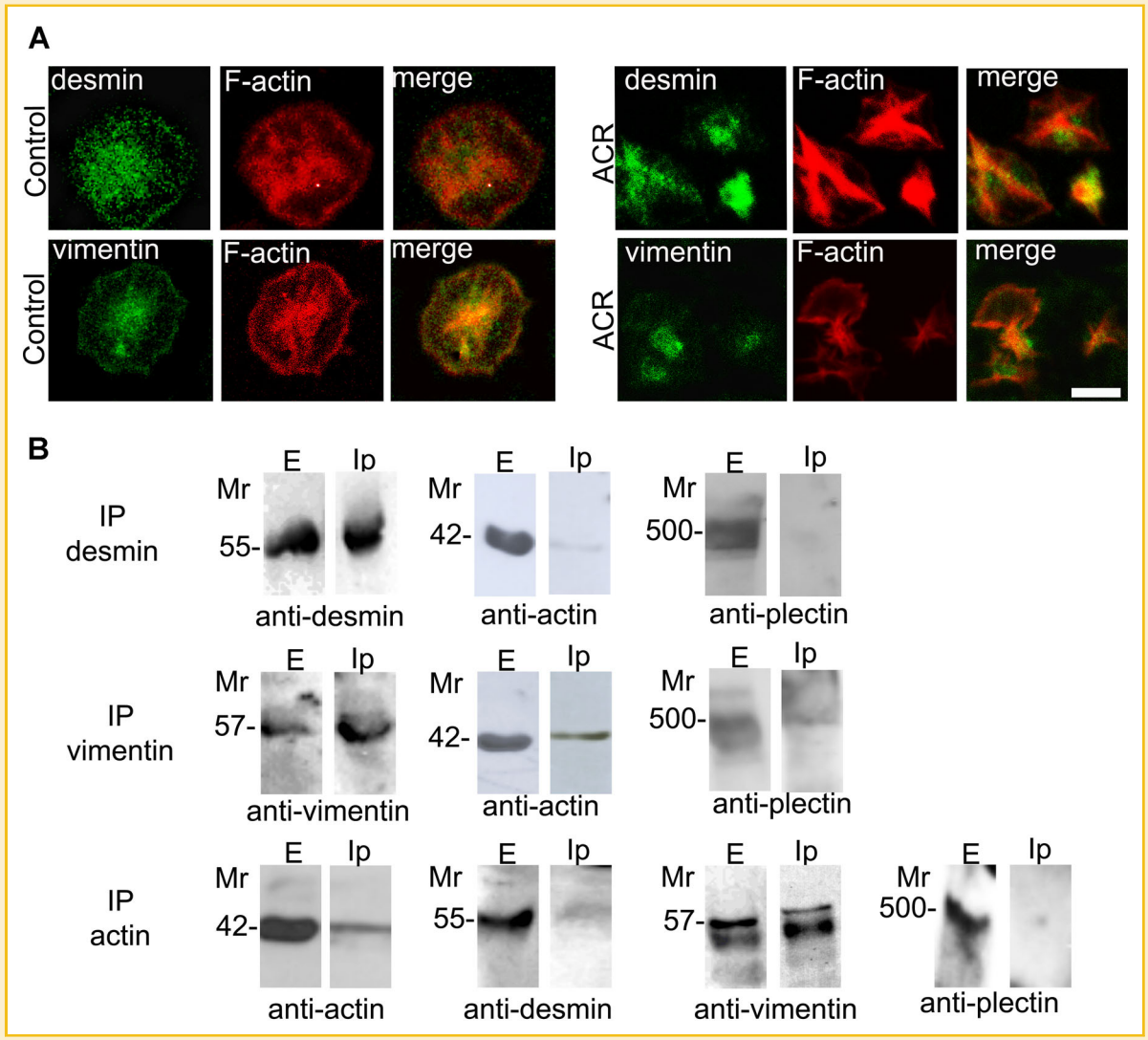

Fig. 1. Desmin and vimentin are associated with actin filaments in human adhered platelets. Panel A. Resting platelets were adhered to glass for 20 min and analyzed by confocal microscopy after processing for double-labeling, using desmin and vimentin antibodies revealed with fluorescein isothiocyanate [FITC] and tetramethyl rhodamine isothiocyanate [TRITC]-phalloidin. The respective merged images are shown. Scale bar = 1.5 μm. Panel A. Resting platelets were incubated with Acrylamide [ACR] for 60 min, adhered to glass, and analyzed by confocal microscopy after processing for double-labeling, using desmin and vimentin antibodies revealed with FITC and TRITC-phalloidin. The respective merged images are depicted. Scale bar = 1.5 μm. Panel B. Resting platelets were adhered to glass and processed for Immunoprecipitation (IP) assays using anti-desmin, anti-vimentin and anti-actin antibodies [IP]. Proteins from total extracts (E) and Immunoprecipitates (Ip) were analyzed by immunoblot utilizing antibodies against desmin, vimentin, actin, and plectin detected with bands of 55, 57, 42, and 500 kDa, respectively.

reorganization of microtubules at the granulomere zone, and to a lesser extent at the plasma membrane co-localizing with IF at these compartments (Fig. 2 Panel A, ACR).

To confirm the feasible association of MT and IF, we carried out immunoprecipitation assays using antibodies raised against desmin and vimentin. Immunoprecipitated proteins (Ip) as well as total extracts (E) were assayed by Western blot (Fig. 2, Panel B). Anti-desmin and anti-vimentin pulled down tubulin (55 kDa), but not the Ubiquitous kinesin heavy chain (UKHC; 100 kDa). Complementary IP showed that anti-tubulin antibody pulled down desmin (55 kDa) but not vimentin. In addition to establishing the feasible connection between MT and IF is due to plectin, we performed Western blot and immunoprecipitation assays using an antibody raised against plectin. Plectin pulled down desmin (55 kDa), vimentin (55 kDa), and tubulin (55 kDa) (Fig. 2, Panel B). Together these results show that plectin is not only present in platelets, but that as in other cellular systems, it is the interplay molecule among actin, tubulin, and IF.

DYSTROPHIN ASSOCIATED PROTEINS BIND DESMIN AND VIMENTIN

To explore the feasible association between IF and DAPs, we performed double immunofluorescence labeling using antibodies against desmin and vimentin, which were revealed with FITC secondary antibodies, while antibodies against the DAPs (β-dystroglycan, α-syntrophin, and α-dystrobrevin) were revealed with TRITC secondary antibodies.

From the images corresponding to desmin and the three different DAPs in control adhered platelets, we could observe that β-dystroglycan (β-dg) is distributed at the plasma membrane, granulomere, and

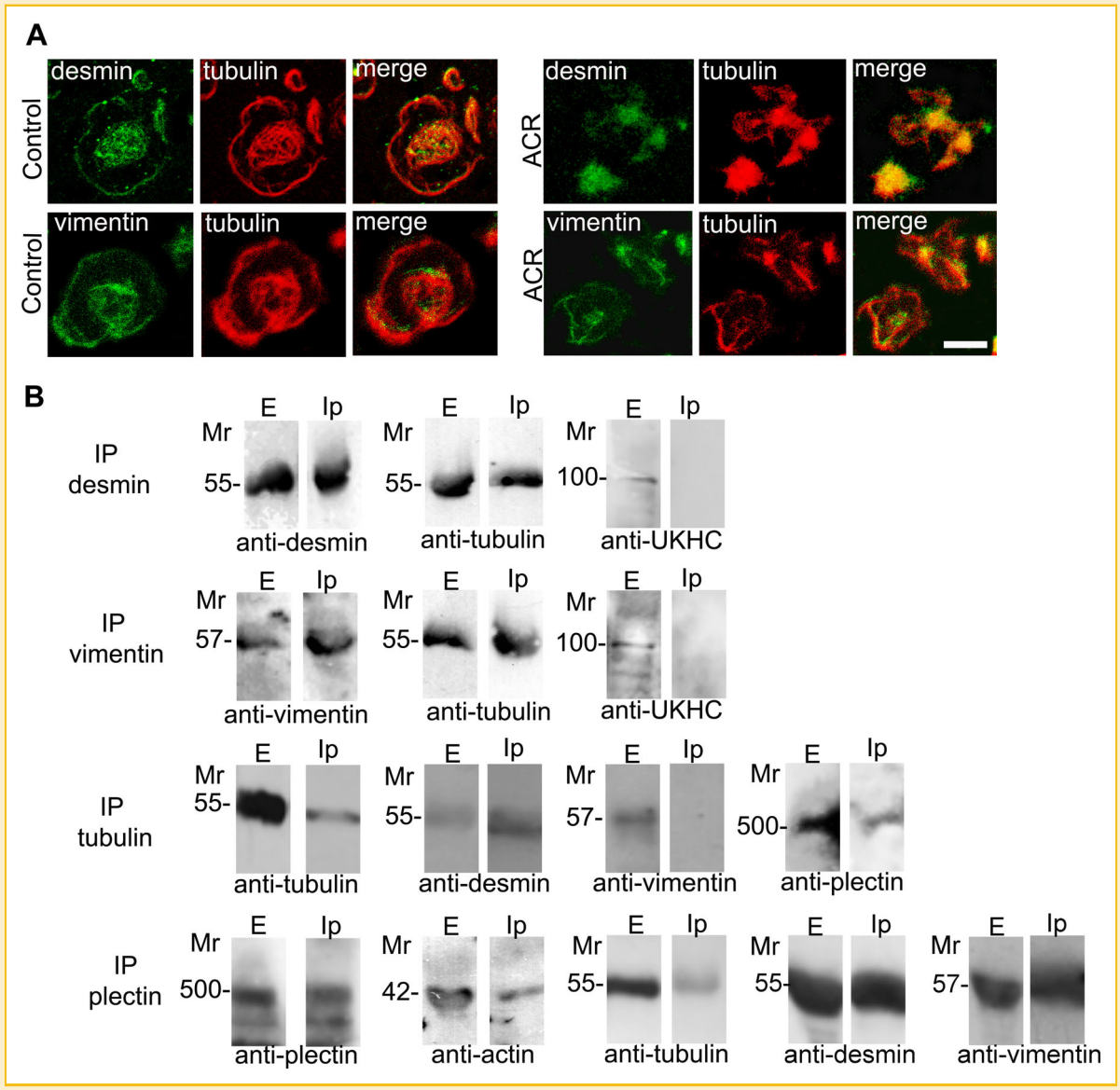

Fig. 2. Microtubules are closely associated with platelet intermediate filaments [IF]. Panel A. Resting platelets were adhered to glass for 20 min and analyzed by confocal microscopy after processing for double-labeling, using desmin and vimentin antibodies revealed with Fluorescein isothiocyanate [FITC] and α-tubulin antibody, identified with a Tetramethyl rhodamine isothiocyanate [TRITC] secondary antibody. The respective merged images are depicted. Panel A. Resting platelets were incubated with Acrylamide [ACR] for 60 min, adhered to glass, and analyzed by confocal microscopy after processing for double-labeling, using desmin and vimentin antibodies revealed with FITC and α-tubulin antibody, identified with a TRITC secondary antibody. The respective merged images are depicted. Scale bar = 1.5 μm. Panel B. Resting platelets were adhered to glass and processed for Immunoprecipitation [IP] assays using anti-desmin, -vimentin, -α-tubulin, and -plectin antibodies (IP). Proteins from total extracts (E) and immunoprecipitates (Ip) were analyzed by immunoblot utilizing antibodies against desmin, vimentin, tubulin, Ubiquitous kinesin heavy chain (UKHC); and actin. Desmin and vimentin were detected with bands of 55- and 57 kDa, respectively; actin and tubulin were detected with bands of 42- and 55 kDa, respectively. UKHC was detected only in the [E] lane with a relative molecular mass of 100 kDa, while plectin showed its presence with bands approximately of 500 kDa.

cytoplasm, co-localizing with desmin mainly at the granulomere zone, while α-syntrophin (α-syn) and α-dystrobrevin (α-db) showed a more granulated pattern around the granulomere and discrete patches at the plasma membrane. In both compartments, they co-localize with desmin (Fig. 3, Panel A, control). Platelet-disrupted IF exhibited a filopodial shape and a granulated pattern of desmin that co-localize with DAPs around the granulomere (Fig. 3 Panel A, ACR).

The pattern distribution observed in the images corresponding to double labeling between vimentin and DAPs in control adhered platelets is very similar to that described for desmin and DAPs (Fig. 3, Panel B, control). Platelets treated with the IF-disrupting agent (Acrylamide, ACR) maintained the localization of IF proteins as aggregates mainly at the granulomere zone of platelets; aggregates of the three DAPs were also observed at the plasma membrane, granulomere, and, scarcely, at the

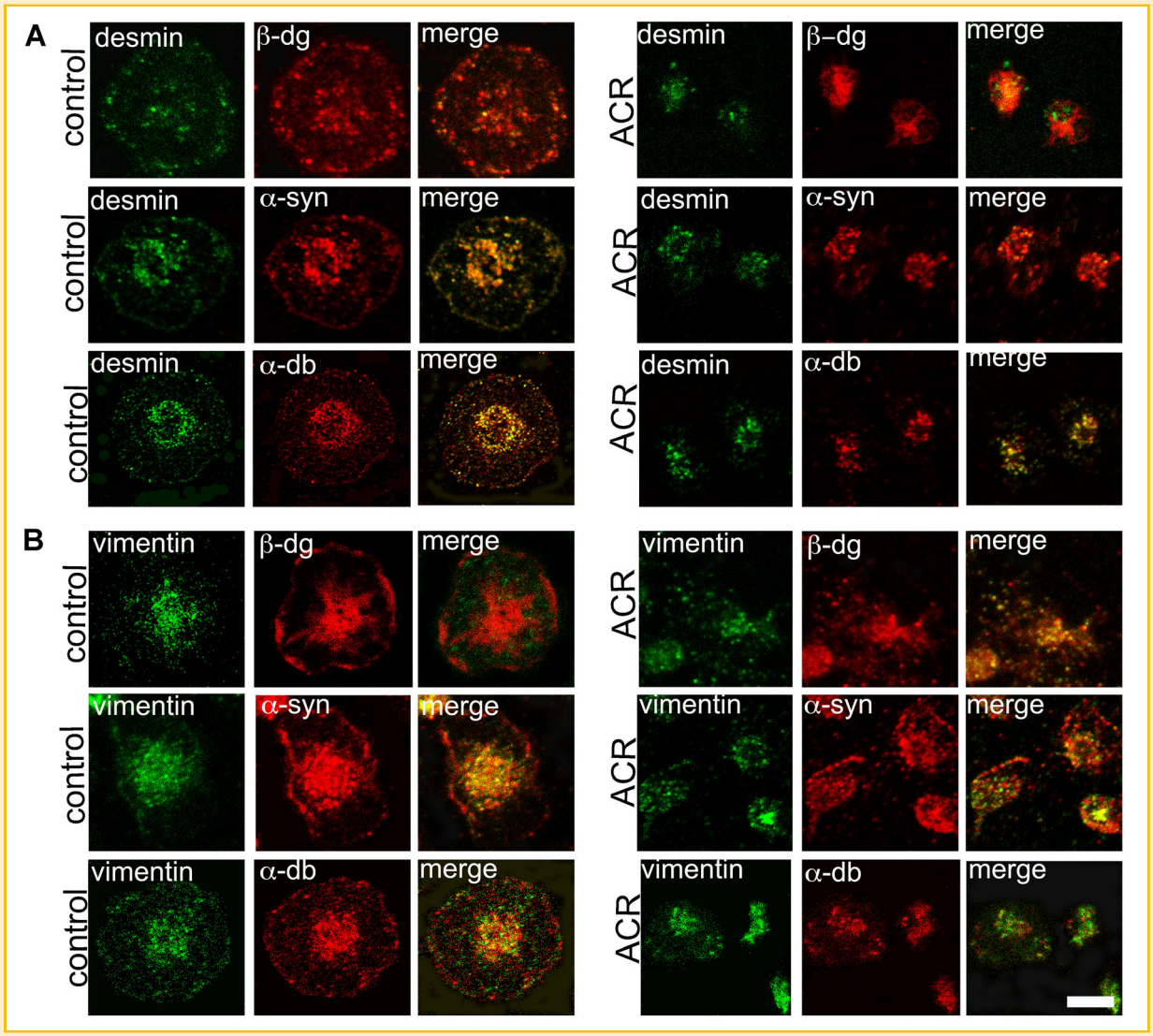

Fig. 3. Dystrophin-associated proteins [DAPs] bind desmin and vimentin. Panel A. Resting platelets were adhered to glass for 20 min and analyzed by confocal microscopy after processing for double-labeling, using desmin antibody revealed with Fluorescein isothiocyanate [FITC] and β-dystroglycan [β-dg], α-syntrophin [α-syn], and α-dystrobrevin [α-db] antibodies, identified with Tetramethyl rhodamine isothiocyanate [TRITC] secondary antibodies. The respective merged images are depicted. Panel A. Resting platelets were incubated with Acrylamide [ACR] for 60 min, adhered to glass, and analyzed by confocal microscopy after processing for double-labeling, using desmin antibody revealed with FITC and β-dystroglycan [β-dg], α-syntrophin [α-syn], and α-dystrobrevin [α-db] antibodies, identified with TRITC secondary antibodies. The respective merged images are depicted. Panel B. Resting platelets were adhered to glass for 20 min and analyzed by confocal microscopy after processing for double-labeling, using vimentin antibody revealed with FITC and β-dystroglycan [β-dg], α-syntrophin [α-syn], and α-dystrobrevin [α-db] antibodies, identified with TRITC secondary antibodies. The respective merged images are depicted. Resting platelets were incubated with Acrylamide [ACR] for 60 min, adhered to glass, and analyzed by confocal microscopy after processing for double-labeling, using desmin antibody revealed with FITC and β-dystroglycan [β-dg], α-syntrophin [α-syn], and α-dystrobrevin [α-db] antibodies, identified with TRITC secondary antibodies. The respective merged images are depicted. Scale bar = 1.5 μm.

cytoplasm of filopodial and partially spread platelets (Fig. 3, Panel B, ACR).

DESMIN AND VIMENTIN ARE CLOSELY ASSOCIATED WITH DYSTROPHIN ASSOCIATED PROTEINS (DAPS)

To confirm the feasible association between IF and DAPs [β-dystroglycan, α-syntrophin, and α-dystrobrevin] observed with immunofluorescence double staining, we processed adhered platelets protein extracts by immunoprecipitation assays using antibodies directed against desmin, vimentin, β-dystroglycan, α-syntrophin, and α-dystrobrevin (Fig. 4, IP). Immunoprecipitates (Ip) and total extracts (E) were resolved by Western blot. Desmin and vimentin antibodies pulled down β-dystroglycan (42 kDa), α-syntrophin (55 kDa), and α-dystrobrevin (55 kDa). β-Dystroglycan immunoprecipitated desmin (55 kDa) and vimentin (57 kDa). α-Syntrophin, pulled down desmin and α-dystrobrevin immunoprecipitated both desmin (55 kDa) and vimentin (57 kDa) (Fig. 5). These results thus show a close association of IF with DAPs in adhered platelets.

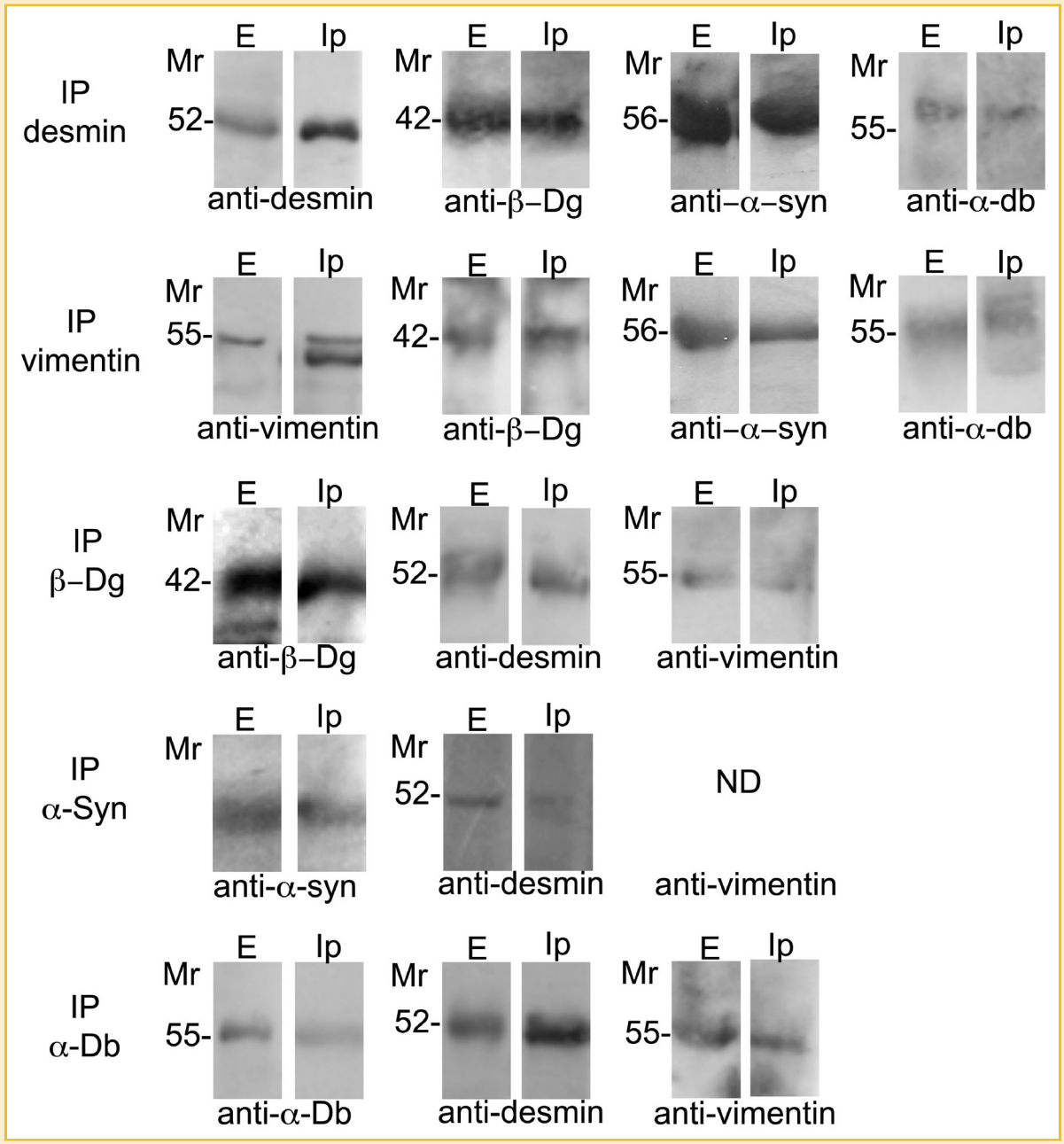

Fig. 4. Desmin and vimentin are closely associated with dystrophin-associated proteins [DAPSs]. Panel A. Resting platelets were adhered to glass and processed for Immunoprecipitation [IP] assays using desmin, vimentin, β-dystroglycan [β-dg], α-syntrophin [α-syn], and α-dystrobrevin (α-db) antibodies (IP). Proteins from total extracts (E) and Immunoprecipitates (Ip) were analyzed by immunoblot utilizing antibodies against desmin, vimentin, β-dystroglycan [β-dg], α-syntrophin [α-syn], and α-dystrobrevin [α-db]. Desmin and vimentin were detected with bands of 55- and 57 kDa, respectively, while DAPs were identified with bands of 42- for β-dystroglycan [β-dg], of 56- for α-syntrophin [α-syn], and of 55 kDa for α-dystrobrevin [α-db]. ND, Not done.

BREFELDIN A DISRUPTS THE ARCHITECTURE OF VIMENTIN AND DESMIN NETWORKS

We focused on Brefeldin A [BFA] to evaluate the association between platelet IF networks and the vesicular transport process. Therefore, we treated platelets with BFA (10 µg/ml) and performed double immunofluorescence labeling using antibodies raised against desmin and vimentin, which were revealed with FITC secondary antibodies, in order to observe by confocal analysis their distribution in relation to actin filaments, tubulin, and P-selectin (a component of α-granules) revealed with TRITC-phalloidin for actin filaments and TRITC secondary antibodies for tubulin and P-selectin.

BFA-treated platelets exhibited abnormal intermediate filament structures. Desmin and vimentin were clearly disrupted and small protein clumps were observed at the center of the platelet. Platelets developed filopodial structures with poor lamellipodia and the presence of the granulomere was apparently absent. In addition, tubulin maintained the marginal ring and in some platelets was reorganized in filopodial structures (Fig. 5). BFA-treated platelets do

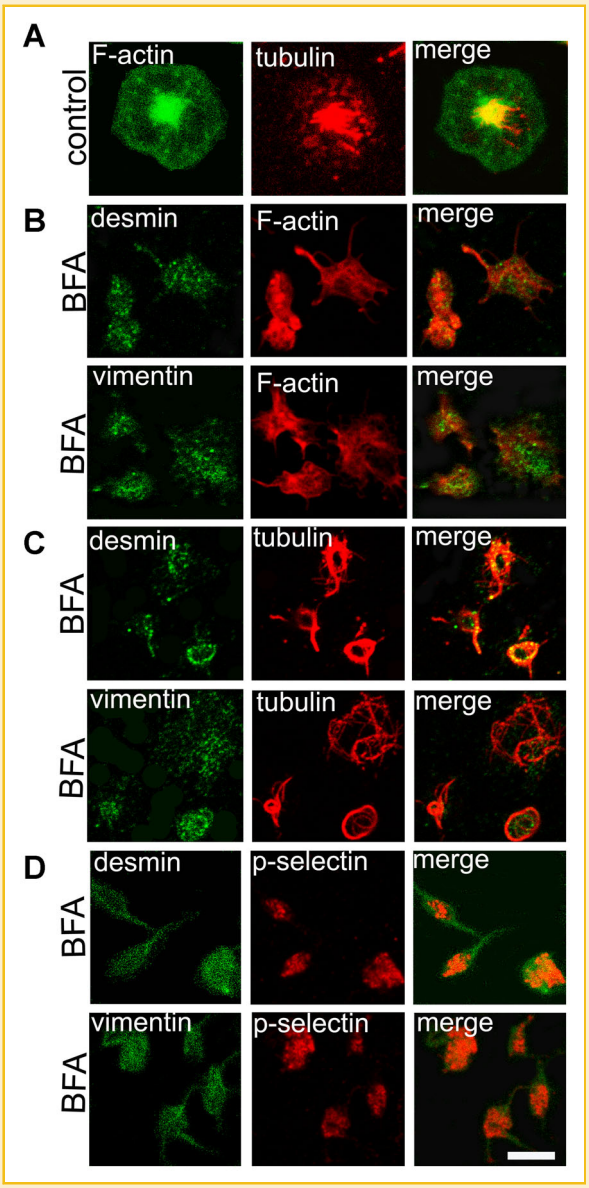

Fig. 5. Brefeldin A (BFA) disrupts the architecture of vimentin and desmin networks. Panel A. Control resting platelets were adhered to glass for 20 min and analyzed by confocal microscopy after processing for double-labeling, using Fluorescein isothiocyanate [FITC]-phalloidin to detect actin filaments and α-tubulin antibody, followed by a secondary Tetramethyl rhodamine isothiocyanate [TRITC] secondary antibody. The respective merged images are depicted. Resting platelets were incubated with BFA for 60 min, adhered to glass, and analyzed by confocal microscopy after processing for double-labeling, using desmin and vimentin antibodies revealed with FITC and TRITC-phalloidin to label F-actin (Panel B), α-tubulin antibody (Panel C), and P-selectin (Panel D) antibody, both identified with a TRITC secondary antibody. The respective merged images are depicted. Scale bar = 1.5 μm.

not spread sufficiently to visualize α-granules dispersion and were observed at the central zone of the platelet (Fig. 5, BFA).

Our results indicated that treatment with BFA, an inhibitor of specific steps of vesicular membrane transport, led to drastic changes in the architecture of vimentin and desmin filaments.

INTERMEDIATE FILAMENTS ORGANIZE THE PLATELET MEMBRANOUS STRUCTURES

To better visualize the perturbations of adaptor-based vesicular membrane transport and/or the integrity of membranous organelles triggered by BFA treatment, we processed control and BFA-treated platelets by electron microscopy under resting and adhered conditions. Electron microscopy images revealed several types of organelles in the cytoplasm, as well as components of the Open canalicular system [OCS] that appear as vacuoles; these canaliculi increase the surface area of the platelet and provide a route for substances extruded during platelet activation.

Resting platelets treated with BFA compared with control platelets conserved their lentiform shape, maintaining their large population of granules randomly dispersed in the cytoplasm, as did the resting platelets but with evident amplification of the OCS (Fig. 6, arrows). BFA-treated adhered platelets showed filopodial shape without developing the Granulomere (G), which is clearly observed in control adhered platelets (Fig. 6, Panel B, DMSO).

To assess further whether modifications in desmin and vimentin cytoskeleton triggered by ACR and BFA were equivalent or whether OCS perturbations observed by electron microscopy exerted some effects on α-granule secretion, 1.5×10^6 platelets/ml were incubated in the presence of ACR and BFA for 1 h before they were adhered. Soluble P-selectin [sP-selectin], obtained from supernatants of adhered platelets, was quantified using an ELISA kit (Fig. 6, Panel C). Control supernatant obtained from platelets without drugs had a mean sP-selectin concentration of 7.3 ng/ml while supernatants of ACR- or BFA-treated platelets exhibited a reduced sP-selectin concentration relative to control supernatants, with mean values of 2.7 and 2.44 ng/ml, respectively. One-way ANOVA analysis and Tukey multiple comparisons showed that sP-selectin levels in supernatants employing ACR and BFA were statistically significant ($P < 0.05$; asterisks in Fig. 6, Panel C). These results strongly suggested that the intermediate filaments network participated in α-granule trafficking of adhered platelets.

MICROFILAMENTS, MICROTUBULES, AND INTERMEDIATE FILAMENTS ARE RESPONSIBLE FOR GRANULOMERE ORGANIZATION IN FULLY ADHERED PLATELETS

To better observe the spatial relationship of the three cytoskeleton elements in adhered platelets, we extracted the cytoskeleton and processed it for electron microscopy. Figure 7, Panel A depicts the different zones that can be identified, which are evidently composed of actin filaments. According to Loftus et al. [1984], these zones comprise the following: the Peripheral web [PW]; the Outer filamentous zone [OFZ]; the Inner filamentous zone [IFZ], and the Granulomere [G]. The enlarged area corresponding to the granulomere showed the confluence of MT, MF, and IF (Fig. 7, Panel A right). In this image, MT, MF, and IF radiating from the center of the granulomere were evident, as well as the presence of residual granules. Because plectin has been considered the most important crosslinker to bind MF, MT, and IF, it could be organizing the three cytoskeleton elements at the granulomere. To test this hypothesis, we performed double immunofluorescence of adhered platelets to

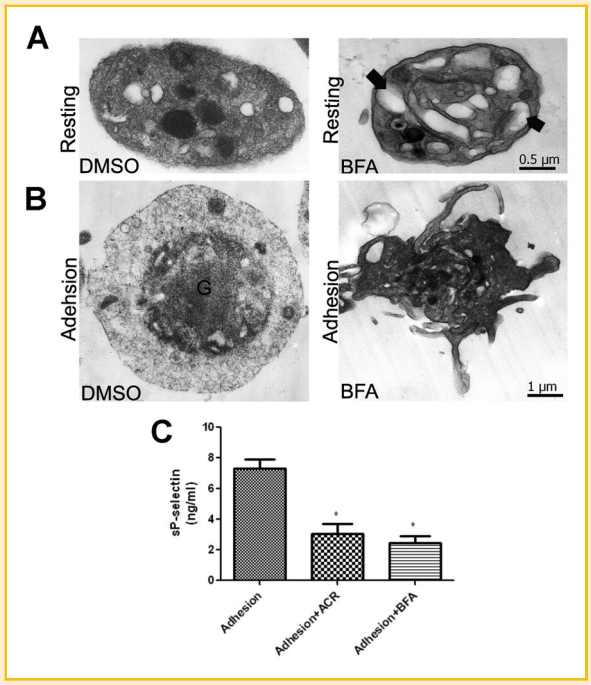

Fig. 6. Intermediate filaments [IF] organize the platelet membranous structures. Panel A. Electron micrographs of resting platelets were incubated with vehicle (DMSO) and Brefeldin A (BFA) for 60 min and processed by transmission electron microscopy. Granules were randomly dispersed in the cytoplasm and the open canalicular system (arrows) was amplified at BFA-treated platelets. Micrographs are representative of triplicates. Scale bar = 0.5 µm. Panel B. Electron micrographs of transverse sections of adhered platelets incubated with DMSO and BFA, settled for 20 min, and processed by transmission electron microscopy. In platelets incubated with DMSO, the granulomere is evident (G), but is absent in platelets treated with BFA. Micrographs are representative of triplicates. Scale bar = 1.0 µm. Panel C. Control adhered platelets incubated with DMSO and adhered platelets treated with Acrylamide (ACR) and Brefeldin A (BFA) were adhered to glass for 20 min. Supernatants were isolated and quantified for sP-selectin by Enzyme-linked immunosorbent assay (ELISA) ($^{*}P < 0.05$; n = three independent experiments). Error bars represent Standard error of the mean ± SEM.

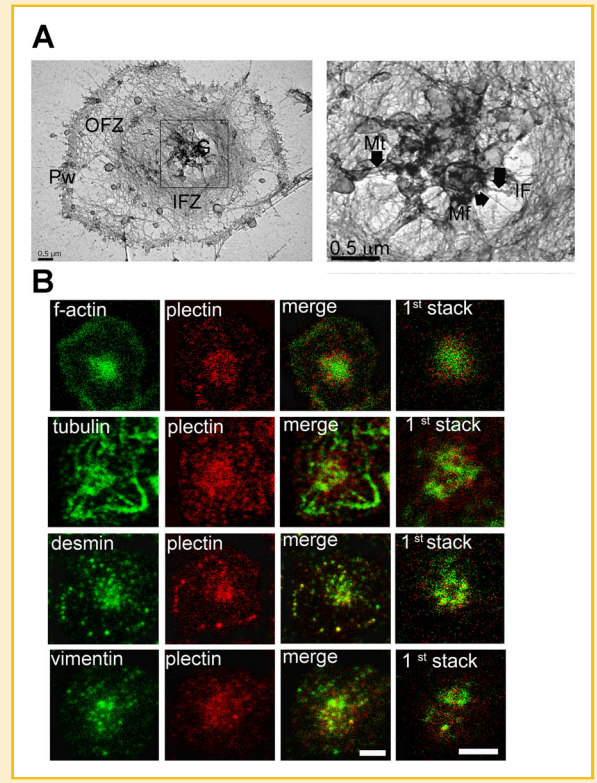

Fig. 7. Microfilaments [MF], microtubules [MT], and intermediate filaments [IF] are responsible for granulomere organization in fully adhered platelets. Panel A. Control adhered platelets were settled for 20 min on nickel grids and processed for cytoskeleton network extraction for structural characterization. Distribution of actin filaments was observed at the plasma membrane or Peripheral web [PW], the Outer filamentous zone [OFZ], the Inner filamentous zone [IFZ], and the Granulomere [G]. The boxed area is enlarged to show the confluence of MT, MF, and IF [arrows]. Panel B. Control resting platelets were adhered to glass for 20 min and analyzed by confocal microscopy after processing for double-labeling, using Fluorescein isothiocyanate [FITC]-phalloidin to detect actin filaments and α-tubulin, desmin, and vimentin labeled with FITC secondary antibodies and plectin detected with a tetramethyl rhodamine isothiocyanate [TRITC] secondary antibody. The respective merged images are depicted. Scale bar = 1.5 µm. Merged images corresponding to first stack of adhered platelets are depicted. Scale bar = 1.5 µm.

observe the cellular distribution of plectin and the feasible association with actin, tubulin, desmin, and vimentin. FITC-labeled phalloidin revealed F-actin, while TRITC secondary antibody-labeled plectin exhibited a patched pattern decorating plasma membrane and cytoplasm, but its presence was abundant at the granulomere, where it co-localizes with F-actin. Tubulin was revealed with a FITC-labeled anti-tubulin antibody and co-localizes with plectin around the granulomere. Equivalent images observed with anti-desmin and -vimentin antibodies revealed with FITC secondary antibodies showed a similar distribution with plectin, especially at the granulomere zone (Fig. 7, Panel B).

Because the granulomere comprises the most important platelet compartment in which the granules centralize, we included an amplification of the first z stack, which corresponded to the plane nearest to the glass where platelets adhere and spread. The granulomere is the first visible structure where the three-cytoskeleton systems interact via plectin.

DISCUSSION

In the present work, we provide evidence of the existence of two members of type-III IF proteins: desmin, and vimentin. Both proteins have shown similar distribution, at specific cellular compartments co-distributed in the following defined cellular compartments: Surrounding the plasma membrane; cytoplasm, and granulomere zone.

Based on Intermediate filaments [IF] properties and according to our results, the main function of desmin and vimentin is maintenance of platelet mechanical integrity by binding to other cytoskeleton components such as microtubules (MT) and microfilaments (MF) at the plasma membrane, highlighting the interaction with which this takes place at special attachment sites such as the granulomere. Therefore, taken together, these three filament systems operate as an

integrated, dynamic network, as has been reported in nucleated cells [Parry and Steinert, 1999; Fuchs and Karakesisoglou, 2001]. It has been described that an association between tubulin and IF could be established through molecular motors [Prahlad et al., 1998], but immunoprecipitation with desmin and vimentin did not pull down the Ubiquitous kinesin heavy chain [UKHC]. However, we have demonstrated the existence of interaction among IF, MT, and MF mediated by linker proteins such as plectin, which is capable of linking three different cytoskeleton systems [Sonnenberg et al., 2007]. A biophysical property of IFs is their elasticity in response to mechanical stretch, becoming more viscoelastic when exposed to an external mechanical force [Schopferer et al., 2009]. Platelets are extremely sensitive to shear stress and enhance the attachment of platelets to the vessel wall and the growth of platelet aggregates on adherent platelets [Turitto and Hall, 1998]. It is feasible that changes in platelets IFs proprieties may inflict changes in responses to mechanosensing and mechanotransduction as has been observed for muscle cells [Kreplak and Bar, 2009]. However, the presence of two elements of class III IF protein which apparently have the same distribution, function and protein interactions should indicate that one can replace the other, explaining the absence of bleeding in patients suffering of desminopathies [Clemen et al., 2013].

According to our results, components of the dystrophin associated proteins DAPs, such as β-dystroglycan [β-dg], α-syntrophin [α-syn], and α-dystrobrevin [α-db] maintain a close relationship with IF and are co-distributed at the granulomere zone; this interconnection is probably established through plectin, as has been shown for muscle cells [Rezniczek et al., 2007; Hijikata et al., 2008]. IF also possess specialized non-mechanical functions that participate in the intracellular distribution of organelles and their functioning [Toivola et al., 2005]. In this respect, we observed that platelets treated with the IF disrupting agent Acrylamide [ACR] generated low values of sP-selectin in relation to control platelets, suggesting that IF participate in α-granules distribution.

The membrane system in platelets comprises the Open canalicular system [OCS] and the Dense tubular system [DTS]. The OCS is a series of conduits of the plasma membrane and it provides a potential route for the release of granule contents to the outside environment [White, 1974], while the DTS is a closed channel network of residual endoplasmic reticulum that functions as a reservoir of calcium [Menashi et al., 1982]. Upon platelet adhesion, the α-granules are centralized and fuse with the OCS, in addition to fusing with each another and with the plasma membrane [Escolar and White, 1991].

We have demonstrated that in fully adhered platelets, perturbation of platelet membrane system using the drug Brefeldin A (BFA) leads to rapid reorganization of the desmin and vimentin cytoskeleton, forming part of long filopodia as previously described in other biological system [Styers et al., 2006]. Additionally, platelets were also impeded from releasing α-granules contents because sP-selectin quantification revealed these to have a low value in relation to control platelets. This value is lower than that obtained with ACR, whose main target is IF disruption. In addition, morphological analysis performed with the electron microscope demonstrates the effect of BFA on the OCS in resting and adhered platelets. Therefore, we assumed that BFA-treated platelets release their granule contents, fusing only with the plasma membrane, emphasizing the role of the OCS as an important route to release granule contents to the outside.

According to our results, we speculate that at the early stages of the adhesion process, platelets tether to the substrate in an integrin-independent manner but through dystroglycans, triggering matrix-driven mechanical signal transduction via plectin, as has been shown in alveolar epithelial cells [Takawira et al., 2011]. These events may occur at the very beginning of the adhesion process before the platelet begins to spread, but as the process continues, MF, MT and IF are reorganized to display filopodia, lamellipodia, and granulomere in order to centralize platelet granules. Simultaneously, the OCS amplifies conducting granule contents to the outer environment, but as the process continues, the OCS is evaginated to increase platelet surface [Escolar et al., 1989]. At this point, granules are directed via MF and MT to the plasma membrane for direct discharge of their contents; at the end of the adhesion process, cytoskeleton organization facilitates platelet contraction.

Overall, these results show that IF play essential roles in regulating both localization and trafficking platelet granules, as well as modulating the maintenance and integrity of membranous compartments, influencing granulomere organization. Because all of the proteins studied in the present work are interlinked, we propose that the central membranous system or OCS, in association with MF, MT, IF, DAPs, and plectin, operates as an integrated structure that contributes to organizing the granulomere and stabilizing cytoskeleton networks.

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