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Disabled-2 modulates homotypic and heterotypic platelet interactions by binding to sulfatides

John D. Welsh,[1]* John J. Charonko,[2]* Alireza Salmanzadeh,[3,4] Karen E. Drahos,[1,5] Hadi Shafiee,[3,4] Mark A. Stremler,[4] Rafael V. Davalos,[3,4] Daniel G. S. Capelluto,[5] Pavlos P. Vlachos[2] and Carla V. Finkielstein[1]

[1]Integrated Cellular Responses Laboratory, Department of Biological Sciences, [2]Advanced Experimental Thermofluidics Engineering Research Laboratory, Mechanical Engineering Department, [3]Bioelectromechanical Systems Laboratory, Biomedical Engineering Department, [4]Department of Engineering Science and Mechanics, and [5]Protein Signaling Domains Laboratory, Department of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

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Correspondence: Carla V. Finkielstein, Department of Biological Sciences, Virginia Polytechnic Institute and State University, 1981 Kraft Dr., Blacksburg, 24061 VA, USA.
E-mail: finkielc@vt.edu

*These authors contributed equally to this work.

Summary

Disabled-2 (Dab2) inhibits platelet aggregation by competing with fibrinogen for binding to the $\alpha_{IIb}\beta_3$ integrin receptor, an interaction that is modulated by Dab2 binding to sulfatides at the outer leaflet of the platelet plasma membrane. The disaggregatory function of Dab2 has been mapped to its N-terminus phosphotyrosine-binding (N-PTB) domain. Our data show that the surface levels of P-selectin, a platelet transmembrane protein known to bind sulfatides and promote cell-cell interactions, are reduced by Dab2 N-PTB, an event that is reversed in the presence of a mutant form of the protein that is deficient in sulfatide but not in integrin binding. Importantly, Dab2 N-PTB, but not its sulfatide binding-deficient form, was able to prevent sulfatide-induced platelet aggregation when tested under haemodynamic conditions in microfluidic devices at flow rates with shear stress levels corresponding to those found in vein microcirculation. Moreover, the regulatory role of Dab2 N-PTB extends to platelet-leucocyte adhesion and aggregation events, suggesting a multi-target role for Dab2 in haemostasis.

Keywords: Disabled-2, sulfatides, platelets, P-selectin, leucocytes.

The transmembrane glycoprotein P-selectin is an adhesion receptor that, upon platelet activation, localizes at the cell surface where it helps in leucocyte recruitment while exhibiting pro-coagulant activity (Chen & Geng, 2006). The best-characterized ligands for P-selectin are the P-selectin glycoprotein ligand 1 (PSGL-1) and the platelet GPIb complex (Sako *et al*, 1993; Frenette *et al*, 2000), both of which exhibit a number of post-translational modifications needed for receptor binding and signal transduction in which sulfate moieties are consistent components (Pouyani & Seed, 1995; Romo *et al*, 1999). Accordingly, the glycosphingolipid sulfatide (ceramide galactosyl-3′-sulfate), a lipid found at the surface of haemopoietic and certain tumour cells, acts as P-selectin ligand (Heckers & Stoffel, 1972; Aruffo *et al*, 1991; Kushi *et al*, 1996). Evidence has established that (i) P-selectin binds to sulfatides exposed on the cell surface of platelets (Merten & Thiagarajan, 2001), (ii) ligand binding results in further degranulation and increased expression of P-selectin in the platelet membrane, triggering a positive feedback that potentiates aggregation (Merten *et al*, 2005), (iii) the size of the aggregate relates to the level of adhesive P-selectin expressed in the cell surface (Merten & Thiagarajan, 2000), (iv) P-selectin-sulfatide interaction leads to stable aggregates (Merten *et al*, 2005) and (v) sulfatides also enhance the formation of platelet-leucocyte aggregates (Merten *et al*, 2005). An overall conciliatory, sulfatide-centric model of platelet activation and aggregate formation would imply the need for both a competent $\alpha_{IIb}\beta_3$ integrin receptor that would bind to soluble fibrinogen

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bridging adjacent platelets followed, as activation progresses, by P-selectin binding to sulfatides to further stabilize aggregates.

Disabled-2 (Dab2) is an adaptor protein whose role in cellular processes of oncogenesis and normal development spans from endocytosis and growth factor signaling to haematopoietic cell differentiation and cell adhesion (Hasson, 2003; Prunier *et al*, 2004). Among haematopoietic cells, Dab2 is abundantly expressed in α-granules of both megakaryocytic K562 cells (Huang *et al*, 2004) and resting platelets (Huang *et al*, 2006), a localization that is shifted to the cell surface as result of platelet activation (Drahos *et al*, 2009). Dab2 contains an N-terminus phosphotyrosine-binding (N-PTB) domain that partially inhibits platelet adhesion and aggregation by competing with fibrinogen for $\alpha_{IIb}\beta_3$ integrin-receptor binding (Huang *et al*, 2004, 2006; Drahos *et al*, 2009).

More recently, Dab2 has been shown to interact with phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) through the residues Lys53 and Lys90 (Mishra *et al*, 2002) and sulfatides through Lys25, Lys49, Lys51 and Lys53 within the XBBXBX and BXBXBX (B, basic; X, any residue) motifs in N-PTB (Drahos *et al*, 2009). Thus, whereas interaction with PtdIns(4,5)P_2 is speculated to play a role in anchoring Dab2 to the inner platelet membrane, binding to sulfatides sequesters Dab2 at the outer leaflet of activated platelets (Drahos *et al*, 2009). Remarkably, sulfatide binding precludes thrombin, a strong platelet agonist, from cleaving Dab2 within its N-PTB domain and thus, a pool of Dab2 remains anchored to and protected on the cell surface (Drahos *et al*, 2009). Recombinant Dab2 was found to delay and inhibit tissue plasminogen activator-, collagen-, U46619-, and thrombin receptor-activating peptide-stimulated platelet aggregation; however, it was somehow inefficient in preventing thrombin-mediated platelet aggregation (Huang *et al*, 2006). Whereas cleavage of Dab2 by thrombin might lead to loss of its inhibitory activity over platelet aggregation (Huang *et al*, 2006), binding of Dab2 to sulfatides may modulate the progression of platelet aggregation events.

Here, we have investigated the regulatory role of Dab2 binding to sulfatides in platelet activation and in heterotypic association mediated by P-selectin in activated platelets and in aggregate formation under physiological flow conditions. These studies establish a definitive role of Dab2 as a direct modulator of cell aggregation processes.

Methods

DNA cloning, plasmids, and protein expression and purification

Human Dab2 N-PTB (residues 1-241) cDNA was cloned into pGEX6P1 (GE Healthcare, Piscataway, NJ, USA). Site-directed mutagenesis of Dab2 N-PTB was performed using Quick Change (Stratagene, La Jolla, CA, USA) and recombinant proteins were expressed and purified as reported (Drahos *et al*, 2009).

Liposome preparation

Stocks of brain sulfatides, phosphatidylcholine (PC), phosphatidylethanolamine (PE) (Avanti Polar Lipids, Alabaster, AL, USA), and cholesterol (Sigma, St. Louis, MO, USA) were prepared following manufacturer's instructions. Liposomes were obtained in the absence and presence of sulfatides as previously described (Drahos *et al*, 2009). Briefly, sulfatide liposomes were prepared in a weight ratio of 1:1:1:4 of PC:PE:cholesterol:sulfatides. Control liposomes were prepared by adjusting the ratios with PC. Lipid films were generated by lyophilization and hydrated in liposome buffer [20 mmol/l Tris–HCl (pH 6·8), 100 mmol/l NaCl] to a final concentration of 1 mg/ml. Suspensions were incubated at 60°C for 1 h, sonicated, pelleted, and then suspended to a final concentration of 2·5 mg/ml in liposome buffer.

Blood collection and platelet purification

Blood samples were obtained from healthy volunteers by venipuncture according to guidelines set by the Institutional Review Board and ethical approval from the Office of Research Compliance at Virginia Tech. Written informed consent was obtained from all donors. Whole blood was collected into vacutainer ACD solution A blood tubes (Becton, Dickinson and Co, Franklin Lakes, NJ, USA), which were then centrifuged at 200 ***g*** for 20 min to separate a platelet-rich plasma (PRP) fraction from erythrocytes. The PRP fraction was further centrifuged at 1100 ***g*** for 5 min and the pellet was then resuspended in Tyrode's buffer [10 mmol/l HEPES (pH 7·4), 134 mmol/l NaCl, 12 mmol/l $NaHCO_3$, 2·9 mmol/l KCl, 0·34 mmol/l Na_2HPO_4 and 1 mmol/l $MgCl_2$] containing 0·5 μmol/l prostaglandin (PGI_2). Lastly, platelets were washed in Tyrode's albumin buffer without PGI_2 and counted in a haemocytometer.

Flow cytometry

Washed platelets ($2{\cdot}5 \times 10^5$ platelets/μl) were maintained in Tyrode's buffer (unactivated) or treated with ADP (activated; 30 μmol/l). Both unactivated and activated platelets were incubated at 23°C for 6 min with either control liposomes (Lipo-C, 50 μg/ml) or sulfatide-containing liposomes (Lipo-S, 50 μg/ml) along with one of the various N-PTB recombinant proteins [1 μmol/l of Dab2 N-PTB, wild-type; Dab2 N-PTB^{4M}, $Lys^{25}Ala$, $Lys^{49}Ala$, $Lys^{51}Ala$, and $Lys^{53}Ala$; Dab2 N-PTB^{D66E}, $Asp^{66}Glu$; Dab2 N-PTB^{5M}; $Lys^{25}Ala$, $Lys^{49}Ala$, $Lys^{51}Ala$, $Lys^{53}Ala$ and $Asp^{66}Glu$ as previously reported (Drahos *et al*, 2009)]. Platelets were then fixed with 1% formalin in phosphate-buffered saline (PBS) and incubated with either phycoerythrin-labelled anti-CD62P (P-selectin) or allophycocyanin (APC)-labelled CD42b (Biolegend, Franklin Lakes, NJ, USA) antibodies. Antibody-bound cell fractions were quantified using the median fluorescence intensity detected by a FacsAria flow cytometer.

Leucocyte purification and platelet-leucocyte aggregation assay

Whole blood was collected as indicated above and centrifuged at 200 ***g*** to separate the plasma and red blood cell fractions. Plasma and buffy coat layer were carefully removed and diluted 1:1 with PBS. The dilution was then layered onto a Ficoll Plus gradient and spun at 200 ***g*** at room temperature for 20 min. The enriched platelet and leucocyte layer was removed, diluted in PBS, centrifuged, and the pellet resuspended in Tyrode's buffer at a concentration of *c.* 10^8 platelets/ml and *c.* 10^6 leucocytes/ml. Platelet-leucocyte mixtures remained unactivated or were activated using ADP (30 μmol/l). Both unactivated and activated leucocyte/platelet mixtures were incubated at 23°C for 10 min with either control liposomes (Lipo-C, 50 μg/ml) or sulfatide-containing liposomes (Lipo-S, 50 μg/ml) along with one of the various N-PTB recombinant proteins (Dab2 N-PTB, Dab2 N-PTB^{4M}; 1 μmol/l). Reactions were fixed with 1% formalin in PBS and incubated with APC-labelled CD42b and fluorescein isothiocyanate (FITC)-labelled CD45 (Biolegend, San Diego, CA, USA) antibodies. Bound antibodies were quantified using a FacsAria flow cytometer.

Device design

The microfluidic device consisted of a simple straight channel 500 μm wide, 50 μm deep and 3 cm long. The fabrication process was as previously described (Shafiee *et al*, 2009). Briefly, a master stamp was fabricated from a silicon wafer. An AZ 9260 (AZ Electronic Materials, Charlotte, NC, USA) photoresist was spun onto a clean silicon wafer. The wafer was then exposed to UV light for 60 s through a mask. The exposed photoresist was removed using a potassium-based buffered developer AZ400K. Deep Reactive Ion Etching (DRIE) was used to etch the silicon master stamp to a depth of 50 μm. Silicon oxide was grown on the silicon master using thermal oxidation for 4 h at 1000°C and removed using hydrofluoric acid (HF) solvent to reduce surface scalloping. Liquid phase polydimethylsiloxane (PDMS) was prepared by mixing PDMS monomers and the curing agent in a 10:1 ratio (Sylgrad 184; Dow Corning, Midland, MI, USA). Liquid PDMS was poured onto the silicon master, cured for 45 min at 100°C and then removed from the mould. Finally, fluidic connections to the channels were punched with a 15 gauge blunt needle (Howard Electronic Instruments, El Dorado, KS, USA). The PDMS replica was bonded with a clean glass slide after treating with air plasma for 2 min.

Flow was driven using a microsyringe pump (Cole-Parmer, Vernon Hills, IL, USA) with a 1 ml syringe connected to the channel inlet by 30 cm of Cole-Parmer (gauge 20) Teflon tubing. The channel outlet was connected to a waste reservoir by the same Teflon tubing. After priming the system with the sample, the pump was set to 0·05 ml/h (equivalent to an average velocity of 0·55 mm/s in the channel), which is in the range of *in vivo* blood velocity [0·1–1·5 mm/s (Song *et al*, 2009)] and causes a shear rate of 70/s. This flow rate was maintained for 2 min prior to the initiation of all experiments. In all cases, channels were coated with a platelet poor plasma fraction containing native adhesive proteins obtained by centrifugation of the plasma sample at 13 000 ***g*** for 10 min at room temperature. Coating was performed for 2 h at 37°C, 5% CO_2. Platelets flowing through the channel were monitored using an inverted light microscope (DMI 6000B; Leica Microsystems, Buffalo Grove, IL, USA) equipped with a digital camera (DFC420; Leica Microsystems). The quantitative methods developed for platelets and platelet/leucocyte interactions are thoroughly described in the Data S1 section of the Supporting Information.

Results

Dab2 N-PTB modulates surface levels of P-selectin via sulfatide association

To define the role of Dab2 in modulating sulfatide-mediated activation of platelets, we monitored platelet surface P-selectin levels in the presence of Dab2 N-PTB and its integrin (Dab2 N-PTB^{D66E}) and sulfatide (Dab2 N-PTB^{4M}) mutant binding forms (Fig 1A). Surface P-selectin levels were evaluated in activated, washed platelets because previous treatment with ADP is necessary for sulfatides to further activate the cells (Merten *et al*, 2005). Measurements were carried out as described in *Methods*.

As expected, the addition of Lipo-S, but not Lipo-C, to ADP-stimulated platelets resulted in an *c.* 12-fold increase in the median fluorescent signal of the P-selectin marker (Fig 1A, green bar). Remarkably, the addition of Dab2 N-PTB to ADP-stimulated, sulfatide-treated, washed platelets prevented further activation, with levels of P-selectin closely resembling those observed in response to ADP activation (Fig 1A, orange *versus* black bars), an effect that most likely results from Dab2 outcompeting P-selectin for sulfatide binding. To determine the contribution of each pool of Dab2, the $\alpha_{IIb}\beta_3$ integrin receptor-bound or sulfatide-bound pool, to the inhibition of P-selectin surface expression, we employed various mutant forms of Dab2 N-PTB. Interestingly, whereas Dab2 N-PTB^{4M}, a form of Dab2 that is unable to bind sulfatides but remains capable of binding $\alpha_{IIb}\beta_3$ integrin (Drahos *et al*, 2009), was incapable of inhibiting sulfatide-induced expression of surface P-selectin (Fig 1A, cyan bar) Dab2 N-PTB^{D66E}, a form of Dab2 unable to bind to $\alpha_{IIb}\beta_3$ integrin receptor (Huang *et al*, 2006) but still able to associate with sulfatides (Drahos *et al*, 2009), showed surface levels of P-selectin expression comparable to those detected in the presence of the wild-type protein (Fig 1A, grey *versus* orange bars). In this scenario, the pool of Dab2 is shifted towards sulfatide binding (Drahos *et al*, 2009) however the expression of P-selectin resembles the basal levels obtained after Dab2 N-PTB addition, suggesting that $\alpha_{IIb}\beta_3$ integrin does not play a major role in controlling P-selectin expression (Fig 1A, grey *versus* orange bars). Accordingly, Dab2 N-PTB^{5M}, a form of Dab2 N-PTB containing both the 4M

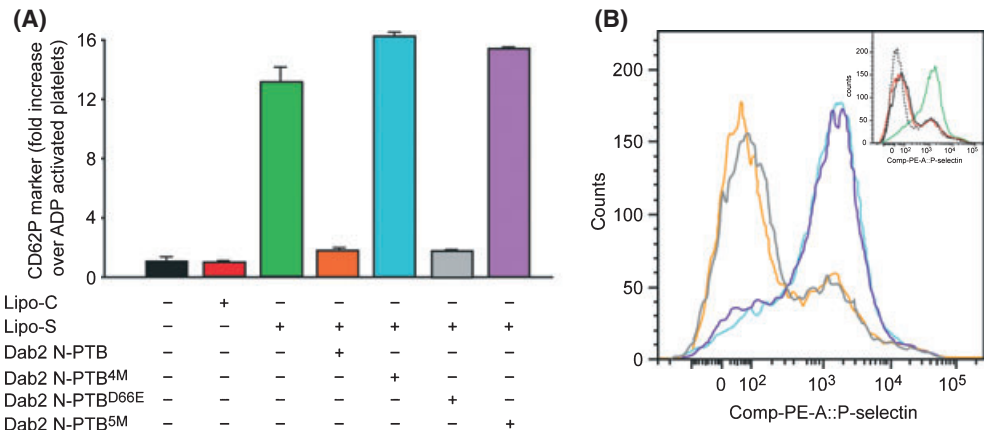

Fig 1. Dab2 N-PTB inhibits sulfatide-induced platelet surface P-selectin expression. (A) ADP-stimulated platelets were incubated with either control (Lipo-C) or sulfatide-loaded (Lipo-S; 50 μg/ml) liposomes in the absence or presence of 1 μmol/l Dab2 N-PTB (orange bar), Dab2 N-PTB^{4M} (cyan bar), Dab2 N-PTB^{D66E} (grey bar), or Dab2 N-PTB^{5M} (purple bar). Samples were incubated for 6 min, fixed, and PE-labelled CD62P antibody added. Median fluorescence intensity for each treatment (n = 3, $P < 0{\cdot}001$) is represented in the graph (mean ± SD). Data is presented as fold increase over ADP-activated platelets (black bar). (B) Representative immunofluorescence histograms show P-selectin expression in unactivated platelets (inset, dashed black), ADP-activated (inset, solid black), and either sulfatide-loaded (50 μg/ml; inset, green) or control liposome (inset, red) in the absence or presence of 1 μmol/l Dab2 N-PTB (orange), Dab2 N-PTB^{4M} (cyan), Dab2 N-PTB^{D66E} (grey), or Dab2 N-PTB^{5M} (purple). Comp: Compensated. In all cases, fluorescence associated with platelets was measured by flow cytometry.

and D66E mutations (Drahos *et al*, 2009), showed no effect on sulfatide-induced activation (Fig 1A, purple bar).

A series of two-dimensional plots showing the distribution of the baseline platelet population in the absence of stimulation, as a result of the sole addition of ADP or in combination with liposomes, is shown in the inset of Fig 1B. In agreement with previous observations, ADP induces a rather modest activation of platelets as measured by P-selectin surface levels and shown by the presence of a peak that represents *c.* 31% of platelets [Fig 1B, inset; dashed *versus* solid black lines and ref. (Merten *et al*, 2005)]. As expected, whereas addition of control liposomes did not significantly impact the fraction of activated platelets, there was a larger population of pre-activated cells that responded to sulfatide treatment raising the value close to 78% (Fig 1B, inset; red *versus* green lines). Further analysis of the responding population of pre-activated platelets to sulfatides showed that *c.* 81% of the platelets remained activated in the presence of Dab2 N-PTB^{4M} and Dab2 N-PTB^{5M} (Fig 1B, cyan *versus* purple lines), and that there was an even distribution of P-selectin on non-responding platelets to Dab2 N-PTB and the integrin-deficient binding form of the protein (Dab2 N-PTB^{D66E}, Fig 1B, orange *versus* grey lines). Interestingly, a roughly 2·5-fold increase in the number of activated platelets resulted in an *c.* 12-fold difference in the level of P-selectin expression, a remarkable increase if looking at the values of P-selectin levels for activated platelets with either ADP or sulfatides alone [this report and (Merten *et al*, 2005)]. Given that the surface expression of P-selectin is expected to augment as result of increasing mobilization of labile pools of secretory granules, our results open the possibility of a second degranulation event triggered by sulfatide binding to P-selectin that is tightly controlled by Dab2 availability.

Dab2 N-PTB inhibits platelet aggregation under shear flow conditions

Initially, we evaluated the role of Dab2 N-PTB in controlling platelet aggregation under conditions that mimic the physiological range of blood flow in veins (Kroll *et al*, 1996). Accordingly, we developed a microfluidic device in which platelets flowed through a channel with a constant velocity and at a shear rate of 70/s. A platelet-poor plasma fraction containing native adhesive proteins was used to coat the channel. This allows the physiological activation of platelets through interactions with the channel's adhesive proteins without the addition of an exogenous agonist (i.e. ADP). Untreated platelets (Fig 2, panel i) and platelets treated with either control (Lipo-C, Fig 2, panel ii) or sulfatide (Lipo-S, Fig 2, panel iii) liposomes were pumped through the channel and monitored by brightfield microscopy. Images were captured within the time frame of action defined for Dab2 and for platelet aggregation (Drahos *et al*, 2009). Untreated and control liposome-treated samples exhibited basal activation and low levels of aggregate formation as a result of adherence to the coated glass surface in the microchannel (Fig 2, panels i and ii). As expected, sulfatide-loaded liposomes promoted the formation of larger aggregates shortly after their addition (Fig 2, panel iii), an event that was reversed by Dab2 N-PTB when sulfatide-treated platelets were pre-incubated in the presence of the protein (Fig 2, panels iv). The direct role of the Dab2 sulfatide binding motifs for modulating aggregation was evidenced by the inability of Dab2 N-PTB^{4M} to prevent this event under similar experimental conditions (Fig 2, panels v). When these haemodynamic studies are analysed in the context of our flow cytometry data (Fig 1), evidence suggests that Dab2 N-PTB significantly reduces the aggregation of

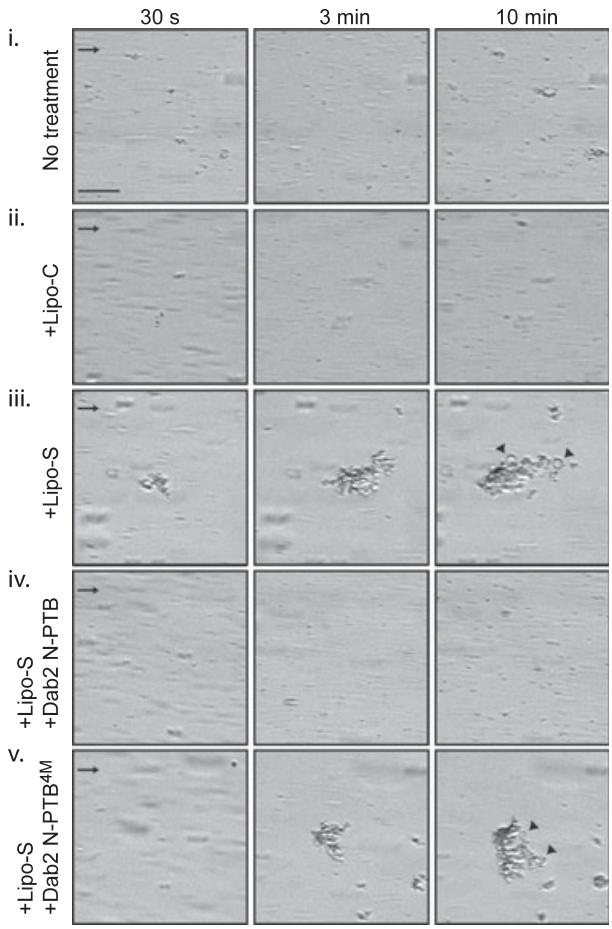

Fig 2. Dab-2 N-PTB prevents sulfatide-mediated dynamic aggregation of platelets. Brightfield images depicting platelets and aggregates within a microchannel taken under physiological flow conditions (70/s). Platelets were incubated in the absence (no treatment, i) or presence of control (Lipo-C, ii) or sulfatide-loaded liposomes (Lipo-S, iii–v) and in the absence (iii) or presence of either of 10 μmol/l Dab2 N-PTB (iv) or Dab2 N-PTB^{4M} (v). Formation of platelet aggregates was monitored over 10 min (Videos S1–S4). Snapshots are single frames from real-time recording at 30 s, 3 and 10 min. Arrowheads indicate sulfatide-loaded liposomes associated to platelet aggregates. Top left arrow indicates the direction of flow. Scale bar represents 45 μm.

circulating platelets most likely by modulating sulfatide availability and, in turn, the surface levels of P-selectin on platelets (Figs 1 and 2). Nevertheless, we cannot completely rule out the contribution of the Dab2 N-PTB sulfatide-binding motifs in modulating the activity and/or expression of other platelet molecules required for adhesion and aggregation.

The presence of Dab2 N-PTB influences the size and number of adhesive platelet aggregates

Perfusion of platelets over a microchannel was performed essentially as described in the previous section, resulting in the adhesion of single platelets to the glass surface that translocates in the direction of flow (Fig 3A, upper panel, black line). In subsequent experiments, platelets were pre-treated with either control (Lipo-C, red line; Video S1) or sulfatide liposomes in the absence (Lipo-S, green line; Video S2) or presence of either Dab2 N-PTB (orange line; Video S3) or Dab2 N-PTB^{4M} (cyan line; Video S4) (Figs 3 and S1) and the number and average size of clusters was determined as described in the Data S1 section of the Supporting Information. To rule out the sole contribution of single protein addition to platelet cluster formation, recombinant proteins [Dab2 N-PTB; magenta line (masked by the *x*-axis) and Dab2 N-PTB^{4M}; blue line] were added to platelet preparations and perfused at low shear rates. As shown in Figs 3 and S1, the effect of the sole addition of the recombinant proteins to untreated samples was negligible; an anticipated result as the few activated platelets were most likely competed off from substrate binding and aggregate formation.

As expected, addition of Lipo-C did not affect the trend of cluster formation over time (Fig 3A, red line) or their mean size, which, in Lipo-C treated samples, remained small (<20 μm^2) and comparable to the untreated sample (black line) throughout the time course analysed. Conversely, samples exposed to sulfatides exhibited a reduced number of total clusters formed over time when compared to Lipo-C that eventually reached a plateau approximately 4 min after perfusion (Fig 3A, red *versus* green lines). Whereas this result seems, at first, to conflict with the proposed role of sulfatides in promoting platelet aggregation, the data suggest that it is not the number but the size of the aggregates that is influenced by sulfatide addition as seen in Fig 3C (upper panel). Analysis of cluster shape and size over a time frame of 10 min showed that the number of new individual platelets interacting with the coated glass surface in the exponential phase (Fig 3B, panel i, Video S2) led to the formation of chain-like structures and other aggregates that arrested transiently, then translocated, and finally incorporated into rolling aggregates that increased in size as they progressed along the surface channel (Fig 3B, panel ii, Video S4). From the standpoint of cluster formation, incubation of Lipo-S-treated platelets with Dab2 N-PTB (Fig 3A, orange line), but not Dab2 N-PTB^{4M} (Fig 3A, cyan line), completely prevented adhesion and aggregation (Videos S1–S4). At low shear rates, platelet integrin interactions with fibrinogen are mostly responsible for platelet adhesion (Savage *et al*, 1996, 1998; Ruggeri, 1997), and thus, the results presented here give a physiologically relevant dimension to our previous observation in which Dab2 N-PTB reduces platelet adhesion (Drahos *et al*, 2009) by competing with the $\alpha_{IIb}\beta_3$ integrin receptor for ligand binding (Huang *et al*, 2006; Drahos *et al*, 2009). Moreover, this haemodynamic system exposes the relevant role of sulfatides in maintaining aggregate stability as evidenced by the partial reduction in cluster size when Lipo-S treated platelets are pre-incubated with Dab2 N-PTB^{4M} (Fig 3C, upper panel).

Our data also expose the presence of sulfatide-independent interactions required for aggregate formation. A snapshot of the time-lapse data presented in Fig 3A,C show quantitative differences among treatments following perfusion and at 500 s (bottom panels). Accordingly, the relevance of the sulfatide

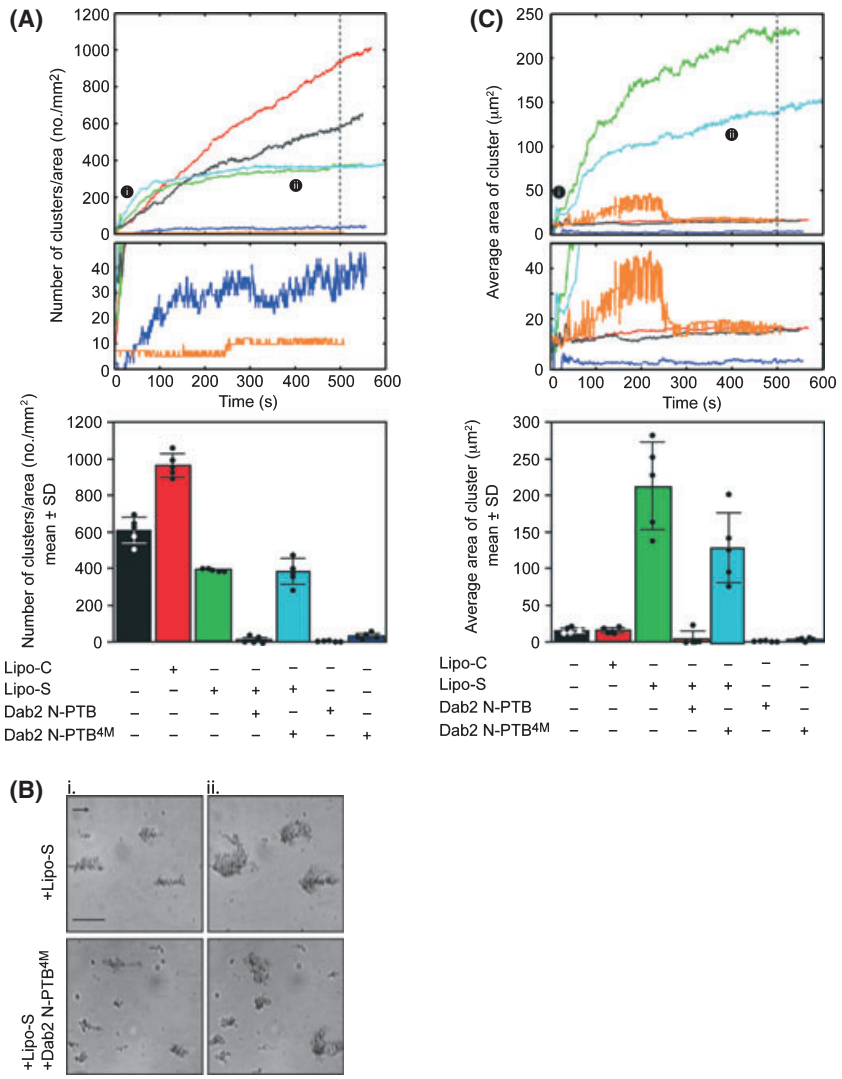

Fig 3. Effect of Dab2 N-PTB in size and number of platelet aggregates formed in a microchannel under low shear rate. (A) Experimental mixtures containing platelets were incubated in the absence (black line) or presence of either control (red line) or sulfatide-loaded liposomes (green line). In other experiments, sulfatide-treated platelets were incubated with either 10 μmol/l Dab2 N-PTB (orange line) or Dab2 N-PTB^{4M} (cyan bar) before data collection as described in the *Methods* section. Other controls include pre-treatment of platelets with either Dab2 N-PTB (magenta, masked by the *x*-axis) or Dab2 N-PTB^{4M} (blue line). Samples were equilibrated for at least 2 min at 70/s before recording started. Formation of platelet aggregates was monitored over 10 min (Videos S1–S4). Top panel. History of the formation of platelet aggregates in the microchannel over time. The lower portion (0–50 clusters/mm^2) of this chart was magnified to better appreciate the effect of Dab2 N-PTB in sulfatide-treated platelets (orange line, Middle panel). Data processing is described in the *Methods* section. Bottom panel. Bar graphs show the average count of platelet aggregates present in five different fields of view on the microchannel surface at 500 s (dashed line in top panel). (B) Snapshots are single frames from real-time recording at 30 s ('i', in top panel) and 400 s ('ii', in top panel) from Videos S2 and S4, respectively. Top left arrow indicates the direction of flow. (C) The average area of clusters was plotted over time (top panel) and the lower portion (0–50 μm^2) magnified for clarity (middle panel) for the experiment presented in A. Bottom panel. Bar graphs show the average area of clusters of all platelet aggregates present in five different sections on the microchannel surface at 500 s (dashed line in top panel). All values are reported as mean ± SD. In each plot, points show individual measurements. Colour code is as described in A throughout the figure.

binding motif for cluster formation and aggregate stability was evidenced by the presence of an equivalent number of clusters in Lipo-S-activated platelets (394 ± 6·7/mm^2) and in Lipo-S-activated-Dab2 N-PTB^{4M}-treated samples (387 ± 70/mm^2) that was significantly larger than those Lipo-S-treated platelets pre-incubated with Dab2 N-PTB instead (9·7 ± 15·8/mm^2). Interestingly, and despite the similarity in cluster number, Lipo-S-activated-Dab2 N-PTB^{4M}-treated aggregates were smaller in size (130·7 ± 48 μm^2) than those formed as result of the sole activation by Lipo-S (231·4 ± 60·7 μm^2). Accordingly, platelet aggregates from Lipo-S-treated samples that had been stable for several minutes exhibited the largest area of cluster (Fig S1A, green line, upper panel) whereas the few aggregates that remained after 4 min of perfusion with either

Lipo-C, (Fig S1A, red line, upper panel) or Lipo-S-activated Dab2 N-PTB-treated (Fig S1A, orange line, upper panel) were significantly reduced in size and cluster area. The relevance of the sulfatide-binding motif in Dab2 N-PTB was more apparent when the effect of Dab2 N-PTB^{4M} was analysed in the context of the largest cluster area data rather than the mean area of a single cluster (Fig S1A *versus* Fig 3C), an observation that directly impacted the total cluster coverage of the channel (Fig S1B). For comparison, a reference time point of 500 s was used to demonstrate these correlations (Figs 3C and S1A,B, bottom panels). Our findings show Dab2 N-PTB can efficiently control platelet aggregation under conditions of low shear stress and that their stability is dependent on membrane sulfatide availability.

Dab2 N-PTB binding to sulfatides controls the extent of heterotypic cell interactions

Platelet-leucocyte interactions play a critical role in haemostasis by contributing to clotting, wound healing, and inflammatory responses (Granger & Kubes, 1994). Major mechanisms controlling heterotypic association involve binding of P-selectin and sulfatides located in the platelet membrane to PSGL-1 and L-selectin on the leucocyte surface, respectively (Bengtsson *et al*, 1996; Merten *et al*, 2005). To determine the role of Dab2 N-PTB in platelet-leucocyte interaction, a mixture of these cells was activated with ADP and incubated with control- or sulfatide-loaded liposomes and heterotypic association monitored on the platelet-leucocyte gated population using flow cytometry.

Consistent with previous observations (Merten *et al*, 2005), we found that sulfatides increased the formation of platelet-leucocyte aggregates threefold [Fig 4A, Lipo-C (red bar) *versus* Lipo-S (green bar)], whereas sulfatide addition to unactivated mixtures of platelets/leucocytes had no significant effect on cell interactions (Merten *et al*, 2005 and data not shown). Interestingly, our data show that addition of Dab2 N-PTB resulted in *c.* 50% inhibition of platelet-leucocyte association ($P < 0{\cdot}016$, orange bar). To investigate whether this effect was mediated by the sulfatide-binding motifs within Dab2 N-PTB, Lipo-S-treated cells were incubated in the presence of Dab2 N-PTB^{4M} and heterotypic association monitored as described above (Fig 4A, cyan bar). Our results show Dab2 N-PTB^{4M} (cyan bar) significantly reversed the inhibitory effect of Dab2 N-PTB (orange bar) over platelet-leucocyte association (Fig 4A; $P < 0{\cdot}03$) to values similar to those obtained as a result of Lipo-S exposure (green bar). These results suggest that Dab2 not only controls platelet-platelet aggregation events but also modulates platelet-leucocyte interactions in a sulfatide-dependent manner.

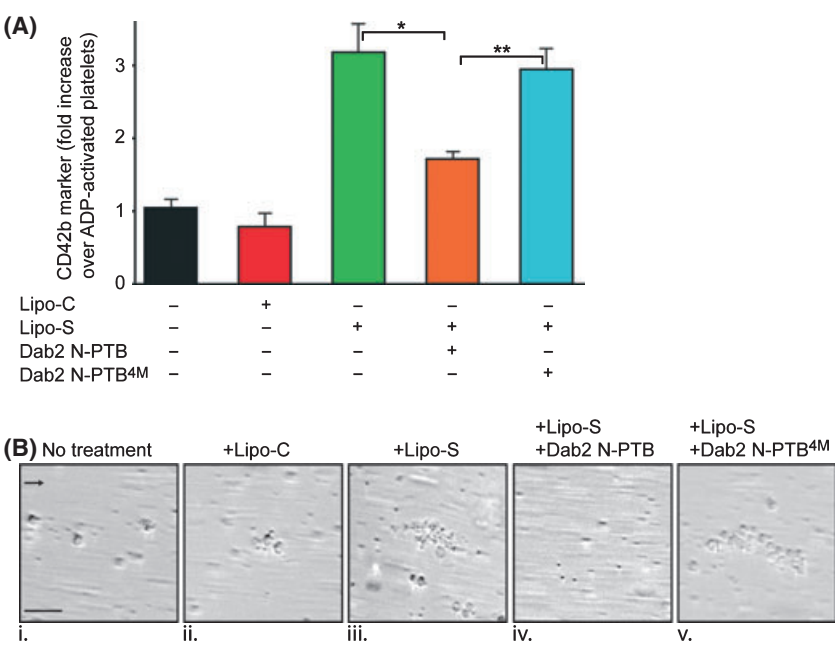

Fig 4. Role of Dab2 N-PTB in heterotypic interactions. (A) Platelet and leucocyte mixtures (10^8 platelets/ml and 10^6 leucocytes/ml) were pre-incubated with Dab2 N-PTB or Dab2 N-PTB^{4M} (1 μmol/l, orange and cyan bars, respectively). Control (Lipo-C, red bar) or sulfatide-loaded liposomes (Lipo-S, green bar) and ADP (30 μmol/l) were added to cell mixtures and incubated for 6 min before fixation and staining with an APC-conjugated CD42b antibody. Median fluorescence intensity for each treatment ($n = 3$, $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}05$) is represented in the graph (mean ± SD). Data is presented as fold increase over ADP-activated platelets (black bar). (B) Brightfield images depicting platelet-leucocyte aggregates in a microchannel under physiological flow conditions (70/s). Platelets were incubated in the absence (no treatment, i) or presence of control (Lipo-C, ii) or sulfatide-loaded liposomes (Lipo-S, iii-v) in the presence of either of 10 μmol/l Dab2 N-PTB (iv), or Dab2 N-PTB^{4M} (v). Formation of aggregates was monitored over 10 min and snapshots are shown for 10 min. Top left arrow indicates the direction of flow. Scale bar represents 45 μm.

Dab2 N-PTB influences platelet-leucocyte interaction under physiological shear flow rates

To directly examine the relationship between heterotypic association and sulfate-mediated Dab2 aggregation under haemodynamic conditions, a mixture of pre-activated platelets and leucocytes were flown through a microchannel under conditions similar to the one described in Fig 2 (Fig 4B). In agreement with our flow cytometry data (Fig 4A), addition of sulfatide-loaded liposomes, but not Lipo-C, to a mixture of platelets and leucocytes resulted in increasing circulating platelet-leucocyte aggregates (Fig 4B, panels ii and iii). The role of Dab2 N-PTB in modulating this interaction was unveiled in a heterotypic preparation pre-incubated with the recombinant protein in the presence of sulfatide-loaded liposomes. In this scenario, aggregation was efficiently prevented (Fig 4B, panel iv). Accordingly, addition of Dab2 N-PTB^{4M} was unable to affect platelet-leucocyte interaction (Fig 4B, panel v). These results point towards a major regulation of heterotypic interaction by Dab2 through its interaction with sulfatides, although alternative modes of regulation are likely to exist and contribute to aggregate stability.

To examine the extent of the disaggregatory effect of Dab2 N-PTB over platelet-leucocyte clusters, we next quantified parameters including the number, area, and coverage of clusters formed under flow conditions. Unlike the case of platelet aggregation, where the number of clusters in untreated or Lipo-C-treated samples accumulated over time and was greater than those observed in samples treated with Lipo-S (Fig 3A), the number of clusters per mm^2 in heterotypic mixtures consistently remained equivalent to control values (Fig 5A, 232·1 ± 40·9/mm^2, black and 252 ± 131·2/mm^2, red bars, $P < 0·05$) unless Lipo-S (345·7 ± 131·2/mm^2, green bar) was added to the samples. The demonstration that Dab2 N-PTB influences cluster formation and size was evident when mean values were under consideration (Fig 5A). Pre-incubation of Lipo-S-treated heterotypic mixtures with Dab2 N-PTB reduced the number of clusters per mm^2 formed from 345·7 ± 131·2/mm^2 to 192·6 ± 87·6/mm^2 (Fig 5A, green *versus* orange bars) whereas no significant difference was observed when Lipo-S-treated samples (345·7 ± 131·2/mm^2, green bar) were compared to Dab2 N-PTB^{4M} (522 ± 86·7/mm^2, cyan bar, $P < 0·05$). Accordingly, the mean value for Dab2 N-PTB-treated samples was comparable to those of Lipo-C (252 ± 131·2/mm^2, red bar) and untreated samples (232·1 ± 40·9/mm^2, black bar) with a $P \leq 0.05$.

The reduction in total cluster coverage was accompanied by a dramatic decrease of the area of the largest cluster considered (Fig 5B,C). Thus, Lipo-S-treated samples show the largest maximum size (626·3 ± 86·3 μm^2, green bar), a significant difference when compared to the values obtained for Lipo-C (119 ± 46·8 μm^2, red bar, $P < 0·05$) and Dab2 N-PTB-treated samples (74·3 ± 33 μm^2, orange bar, $P < 0·05$). Notably, the area of the largest cluster was also reduced as a result of

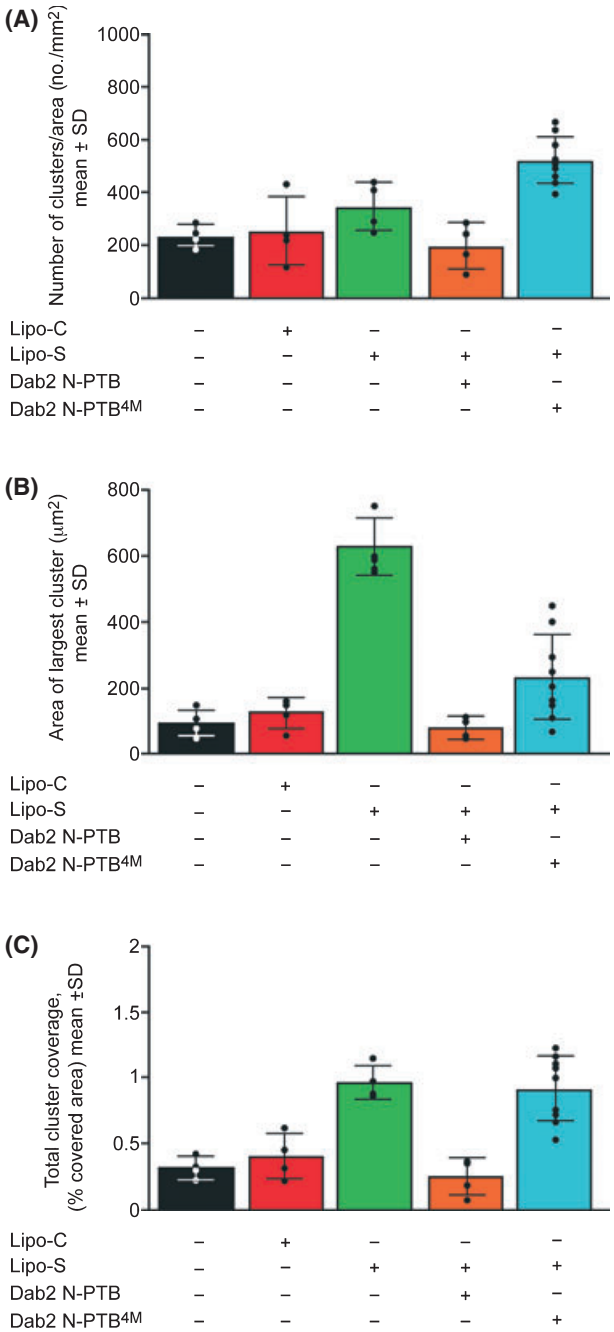

Fig 5. Quantitative analyses on the effect of Dab2 N-PTB for platelet-leucocyte interactions. Platelet-leucocyte mixtures (*c.* 10^8 platelets/ml and *c.* 10^6 leucocytes/ml) were incubated with either control liposomes (Lipo-C, red bar) or sulfatide-containing liposomes (Lipo-S, green bar). In other experiments, sulfatide-treated samples were pre-treated with N-PTB recombinant proteins: Dab2 N-PTB (orange bar) and Dab2 N-PTB^{4M} (cyan bar). Mixtures were flown through the microchannel at a flow of 70/s and data collection was analysed as described in the *Methods* section. Bars indicate platelet/leucocyte aggregates formed at 600 s following injection. All values are reported as mean ± SD. In each plot, points show individual measurements. (A) Number of distinct cell clusters counted in each image. (B) Area of the largest cluster of cells in each image. (C) Percentage of cluster area covered by cells in each field.

Dab2 N-PTB^{4M} addition and when compared with Lipo-S-treated mixtures (626·3 ± 86·3 μm^2, green bar vs. 230·2 ± 130·3 μm^2, cyan bar, $P < 0·05$) than Dab2 N-PTB, mimicking differences observed among treatments in platelet aggregates for the same parameter (Fig S1A). When analysed in the context of the total surface covered among treatments (Fig 5B), the differences between Lipo-S and Dab2 N-PTB^{4M}-treated samples became non-significant (0·96 ± 0·13%, green bar vs. 0·91 ± 0·25%, cyan bar, $P < 0·05$), suggesting that the slightly higher number of clusters observed in Dab2 N-PTB^{4M} compared with Lipo-S sample might compensate for their reduction in size and, therefore coverage (Fig 5B, green *versus* cyan bars). As expected, the mean values for untreated (0·31 ± 0·09%, black bar), Lipo-C (0·40 ± 0·18%, red bar) and Dab2 N-PTB (0·24 ± 0·14%, orange bar) did not differ significantly ($P > 0·95$).

In summary, we demonstrated that the N-terminus region of Dab2 (named N-PTB) is necessary and sufficient to efficiently compete with P-selectin for sulfatide binding, thus, modulating P-selectin surface expression and cell aggregation under conditions that closely resemble the physiology of the microvasculature.

Discussion

The studies presented in this report define a central role for Dab2 in initiating and stabilizing homo- and heterotypic platelet aggregates under haemodynamic conditions that reflect those observed in *in vivo* systems. In this scenario, we show that Dab2 modulates the surface expression of P-selectin, a member of the selectin family of cell adhesion molecules important in a variety of processes including leucocyte rolling on the endothelium, platelet rolling on venules, and platelet-leucocyte adhesion. Aside from its leucocyte and/or endothelial adhesion, P-selectin has proven to contribute to platelet aggregate formation, stabilizing initial $\alpha_{IIb}\beta_3$ integrin-fibrinogen interactions [for review see (Blann *et al*, 2003)].

The direct role of P-selectin in physiological events that relate to platelet aggregation arises from *in vitro* studies that show inhibition of this lectin by monoclonal antibodies achieved 95–100% disaggregation, a value that closely resembles the one obtained after EDTA addition, and that resulted in reduction of size and number of platelet aggregates (Merten & Thiagarajan, 2000). Remarkably, binding of monoclonal antibodies, such as PL-1 to PSGL-1, SZ2 and WM23, to GPIb and anti- $\alpha_{IIb}\beta_3$ had no disaggregated platelets activated with either ADP or thrombin suggesting that, whereas P-selectin is needed for sustaining platelet aggregation, its effect is mediated by receptors other than PSGL-1 (Moore *et al*, 1995; Norman *et al*, 1995; Romo *et al*, 1999).

The current model establishes the formation of large and stable platelet aggregates resulting from binding of protruding P-selectin to nearby sulfatides located on $\alpha_{IIb}\beta_3$-fibrinogen pre-bridged adjacent platelets. Accordingly, platelets do not aggregate in individuals with Glanzmann thrombasthenia, a coagulopathy in which platelets lack expression of $\alpha_{IIb}\beta_3$ but maintain normal levels of P-selectin surface expression (Hawiger, 1995). As expected, sulfatides themselves have a negligible effect on P-selectin expression in unactivated platelets but promote surface expression in platelets pre-activated with ADP [Fig 1 and (Merten *et al*, 2005)]. Interestingly, when these data are analysed in the context of activated cell distribution, our results point toward sulfatide-loaded liposomes promoting a second degranulation event via interaction with P-selectin in pre-activated platelets (Fig 1B, inset, black *versus* green lines). The specificity of this event towards a lipid moiety was established when comparable histograms for ADP-treated platelets alone and in the presence of Lipo-C were obtained (Fig 1B, inset, black *versus* red lines). It seems likely that this second degranulation event increases surface P-selectin levels further, potentiating the formation of stable platelet aggregates. In this context, Dab2 N-PTB acts as a sulfatide antagonist reducing surface levels of platelet P-selectin by *c.* 85% (Fig 1, green *versus* orange bars). In support of this model, lectins other than P-selectin have been linked to the release of secondary, tertiary and secretory granules (Tsang *et al*, 1997; Smolen *et al*, 2000). Of note, we found a rather modest effect of Lipo-S addition on surface levels of $\alpha_{IIb}\beta_3$ [2.5-fold increase, (Drahos *et al*, 2009)] compared to P-selectin (*c.* 12-fold), which we speculate results from the presence of integrin receptor on the cell surface. It is uncertain as to whether Dab2-modulated expression of surface P-selectin favours heterotypic interaction rather than homotypic association and further studies are needed to clarify these relationships.

The direct role of the sulfatide and integrin binding motifs in Dab2 to P-selectin expression has been dissected (Fig 1A). Results show that Dab2 N-PTB, but not the sulfatide-deficient form of Dab2, Dab2 N-PTB^{4M}, is able to prevent P-selectin accumulation (Fig 1A, orange *versus* cyan bars), a result that mimics the effect of the exogenous addition of specific sulfatide antagonists, such as the malaria circumsporozoite protein (MCSP) (Pancake *et al*, 1992) and high-molecular-weight dextran sulfate into platelets (Merten & Thiagarajan, 2001). Moreover, we ruled out the contribution of $\alpha_{IIb}\beta_3$ integrin in promoting P-selectin surface expression by incubating pre-activated platelets with sulfatides in the presence of its integrin-deficient binding form Dab2 N-PTB^{D66E}. This finding is supported by evidence that shows abciximab, a chimaeric monoclonal antibody Fab fragment directed against the platelet $\alpha_{IIb}\beta_3$ receptor, does not influence surface expression of platelet P-selectin (Klinkhardt *et al*, 2002).

Real-time observations of untreated, or PGE_1-treated, platelet interaction with surfaces coated with fibrinogen, fibrin or von Willebrand factor (VWF) were reported at low- (50/s) or high-flow (1500/s, normal arteriolar circulation) rates (Savage *et al*, 1996). Fibrinogen, as well as fibrin, supports the irreversible attachment of platelets that was progressively less efficient when the flow rate increased from 50/s to 1500/s. Although not fully elucidated, this mode of action has been

proposed to depend on the biomechanical properties of the $\alpha_{IIb}\beta_3$-fibrinogen interaction as well as other factors, such as $\alpha_{IIb}\beta_3$ density per unit of area in platelets. On the other hand, VWF favours a slow and continuous movement of platelets on the surface and is particularly efficient in platelet recruitment under very high shear stress conditions leading to irreversible attachment (Savage *et al*, 1996). We showed that Dab2 plays a major role in controlling platelet-platelet interactions under the influence of low shear conditions (Fig 2) by having two complementary roles. First, Dab2 interferes with adhesion by blocking platelet $\alpha_{IIb}\beta_3$ integrin receptor interaction with its fibrin/fibrinogen ligand and then impacts aggregation by preventing sulfatide-P-selectin recognition. Accordingly, we have previously shown that Dab2 N-PTB, but not Dab2 N-PTB^{D66E}, is more efficient than the RGDS (Arg-Gly-Asp-Ser) peptide, a low-affinity fibrinogen receptor antagonist (Basani *et al*, 2001), in reducing platelet adhesion *in vitro* [7% and 13.5%, respectively and (Drahos *et al*, 2009)]. *A priori*, the likelihood of finding clusters under dynamic conditions would be favourable as Dab2 N-PTB only partially prevents adhesion. However, Dab2 N-PTB proved to be an effective modulator of cluster formation when pre-incubated with sulfatide-treated platelets and under dynamic conditions, with *c*. 10 clusters formed over the time course analysed (Figs 3 and S1, orange line and Video S3). In this case, Dab2 N-PTB most likely acts not only by preventing platelets from adhering to the coated surface but also by destabilizing any aggregates. For example, Fig 3C (middle panel, orange line) shows an initial trend of platelet aggregation, albeit for fewer clusters, within the first 300 s of treatment; however, this trend cannot be sustained further. We thus hypothesize that, if partial platelet adhesion occurs due to the limited effectiveness of Dab2 N-PTB action over the integrin receptor, aggregation becomes compromised by effective blocking sulfatides (Figs 3 and S1).

The effect of Dab2 N-PTB^{4M} in aggregate formation under low shear conditions was analysed (Fig 2, panel v and Video S4). In this case, Dab2 N-PTB^{4M} did not completely prevent cluster formation but resulted in reduced size. Although this might result in *a priori* conflict, it is in agreement with our previous observation that Dab2 N-PTB^{4M} inhibited cell adhesion by *c*. 18·5% (Drahos *et al*, 2009) thus, it was expected that a number of platelets would still adhere to some extent to the coated surface of the channel and be able to aggregate. Accordingly, and unlike Dab2 N-PTB, Dab2 N-PTB^{4M} was unable to block sulfatides and, therefore, neither interferes with the process of aggregate stabilization (Figs 2 and S1) nor with P-selectin expression (Fig 1). In support of a direct role of P-selectin in platelet aggregation, anti-P-selectin antibodies G1 and CLB-thromb/6, but not the anti-$\alpha_{IIb}\beta_3$ antibody abciximab, was shown to promote disaggregation of platelets shortly after activation when measured using an aggregometer system (Merten & Thiagarajan, 2000). Overall, our model establishes an interplay between two complementary mechanisms, $\alpha_{IIb}\beta_3$ integrin receptor- and P-selectin-mediated, that are modulated by Dab2 and needed to initiate and sustain platelet aggregation under low shear rates. Nevertheless, we cannot rule out the existence of other, yet unknown, Dab2 targets or additional mechanisms that contribute to these processes.

Direct modulation of heterotypic association by the Dab2 sulfatide binding motifs was apparent for the platelet/leucocyte interaction as monitored by flow cytometry (Fig 4A) and under low shear rates (Fig 4B), a result that also resembles the antagonist effect of MCSP previously observed for platelet-leucocyte association (Merten *et al*, 2005), albeit the endogenous nature of Dab2 compared with MCSP and the rapid control (within 5 min) by Dab2 of the P-selectin/sulfatide association (MCSP needs more than 20 min). Platelet adherence to neutrophils results from P-selectin/PSGL-1 binding at low shear rates, an interaction that is sufficient to mediate further aggregation and does not involve the $\alpha_{IIb}\beta_3$ integrin receptor (Konstantopoulos *et al*, 1998). Accordingly, addition of ZK, a PI_2 analogue that acts on platelets by diminishing the expression of P-selectin, or antibodies, such as EP5C7 (anti-P-selectin) and KPL-1 (anti-PSGL-1), are equally effective in inhibiting platelet/neutrophil adhesion and neutrophil aggregation (Konstantopoulos *et al*, 1998). Moreover, equivalent samples treated with either anti-P-selectin or anti-PSGL-1 antibodies exhibit a decrease in the rate and extent of platelet adhesion as well as a reduction in the number of platelets associated to neutrophils (Konstantopoulos *et al*, 1998). Consistent with our flow cytometry data, quantitative studies under haemodynamic conditions show Lipo-S-treated platelet/leucocyte samples exhibited a significant difference in the total number of clusters formed when compared to Lipo-C (Figs 4 and 5A, red *versus* green bars). Accordingly, pre-treatment of cell mixtures with Dab2 N-PTB, but not Dab2 N-PTB^{4M}, impacted cluster formation by decreasing its total number to basal levels (Fig 5A, cyan *versus* orange bars).

The efficacy by which Dab2 N-PTB acts in preventing heterotypic aggregation is in agreement with its additional role as modulator of platelet adhesion through the $\alpha_{IIb}\beta_3$ integrin receptor at low shear rates (Figs 2, 3, and S1). Indeed, Konstantopoulos *et al* (1998) established that 'platelet capture' is a prerequisite for neutrophil aggregation and Rinder *et al* (1991, 1992) recognized that the dynamic interaction among platelets and leucocytes also correlates with platelet activation. As in the case of platelet-platelet aggregates (Fig 3C), Dab2 N-PTB^{4M} influenced the size of platelet-leucocyte clusters (Fig 5B) supporting the existence of mechanisms that stabilize these aggregates, other than those triggered by Dab2 binding to sulfatides. As expected, the number and area of heterotypic aggregates directly impact surface coverage (Fig 5C).

Taken together, our data places Dab2 as an endogenous platelet modulator of cellular interactions. Because of its unique sulfatide- and integrin-binding capabilities, Dab2 acts as a multi-ligand bridging molecule whose physiological relevance most certainly encompasses inflammatory and thrombotic mechanisms whose deregulation is of pathophysiological significance.

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Authors' contributions

JDW, JJC, MS, RVD, DGSC, PPV and CVF designed the research. JDW performed the experiments in Figs 1, 2, 3B, and 5A. ASD fabricated the microfluidic devices and assisted JDW in performing microchannel perfusion experiments and data collection. JJC and PPV formulated and coded the software for quantitative analyses summarized in Figs 3, 4, and 6. KED, MS, DGSC and RVD contributed with reagents. MS and RVF designed and supervised device fabrication. DGSC and CVF supervise the study and co-wrote the manuscript.

Conflict of interest

The authors declare no competing financial interests in connection to the work described in this paper.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Fig S1. Effect of Dab2 N-PTB in the area covered by platelet aggregates in a microchannel under low shear rate.

Video S1. Shear-induced platelet aggregation *in vitro* in the presence of control liposomes.

Video S2. Shear-induced platelet aggregation *in vitro* in the presence of sulfatide-loaded liposomes.

Video S3. Shear-induced platelet aggregation *in vitro* in the presence of Dab2 N-PTB.

Video S4. Shear-induced platelet aggregation *in vitro* in the presence of Dab2 N-PTB4M.

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