

Vav family proteins are required for optimal regulation of PLC γ 2 by integrin α IIB β 3

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Vav proteins belong to the family of guanine-nucleotide-exchange factors for the Rho/Rac family of small G-proteins. In addition, they serve as important adapter proteins for the activation of PLC γ (phospholipase C γ) isoforms by ITAM (immunoreceptor tyrosine-based activation motif) receptors, including the platelet collagen receptor GPVI (glycoprotein VI). Vav proteins are also regulated downstream of integrins, including the major platelet integrin α IIB β 3, which has recently been shown to regulate PLC γ 2. In the present study, we have investigated the role of Vav family proteins in filopodia and lamellipodia formation on fibrinogen using platelets deficient in Vav1 and Vav3. Wild-type mouse platelets undergo a limited degree of spreading on fibrinogen, characterized by the formation of numerous filopodia and limited lamellipodia structures. Platelets deficient in Vav1 and Vav3 exhibit

reduced filopodia and lamellipodia formation during spreading on fibrinogen. This is accompanied by reduced α IIB β 3-mediated PLC γ 2 tyrosine phosphorylation and reduced Ca²⁺ mobilization. In contrast, the G-protein agonist thrombin stimulates full spreading of control and Vav1/3-deficient platelets. Consistent with this, stimulation of F-actin (filamentous actin) formation and Rac activation by thrombin is not altered in Vav-deficient cells. These results demonstrate that Vav1 and Vav3 are required for optimal spreading and regulation of PLC γ 2 by integrin α IIB β 3, but that their requirement is by-passed upon G-protein receptor activation.

Key words: fibrinogen, integrin α IIB β 3, phospholipase C γ 2 (PLC γ 2), platelet spreading, signalling, Vav.

INTRODUCTION

Platelets are small anucleate cells that circulate in a quiescent state in the vasculature. Following vascular damage, platelets are recruited to the site of injury and undergo explosive activation to stem the loss of blood from the wound. Following tethering, platelets are activated by prolonged close proximity of the platelet immunoglobulin receptor GPVI (glycoprotein VI) with subendothelial collagen (reviewed in [1]). GPVI stimulates an increase in the affinity of the major platelet integrin α IIB β 3 for its ligands, vWF (von Willebrand factor) and fibrinogen. Outside-in signalling by the integrin contributes to spreading over the subendothelial matrix and recruitment of additional platelets which become cross-linked by fibrinogen, thereby generating a vascular plug [2]. Finally, the clot is retracted and stabilized by an active process dependent on α IIB β 3 and the cytoskeleton [3].

The Vav family of GEFs (guanine-nucleotide-exchange factors) consists of three members [4–7] which share a common structural arrangement. The N-terminus contains a calponin homology domain and an acidic region, which contains regulatory tyrosine phosphorylation sites. This is followed by Dbl homology, PH (pleckstrin homology) and zinc-finger domains, which form the guanine-nucleotide-exchange region of Vav family proteins. The C-terminal portion contains a short proline-rich region and an SH3-SH2-SH3 (SH is Src homology) region. Vav2 and Vav3 are

widely expressed, whereas Vav1 is specifically expressed in haemopoietic cells [5–7]. The guanine-nucleotide-exchange activity of Vav proteins is specific for the Rho family of small G-proteins. The individual Vav proteins have specificity towards different Rho family G-proteins. For example, Vav1 selectively activates Rac1, Rac2 and RhoG, whereas Vav2 and Vav3 show less activity towards Rac1 [6,8]. The guanine-nucleotide-exchange activity of all three Vav proteins is modulated through tyrosine phosphorylation of regulatory tyrosine residues by Src and Syk family kinases [9].

Vav proteins are prominent tyrosine kinase substrates downstream of ITAM (immunoreceptor tyrosine-based activation motif) receptors, including the platelet collagen receptor GPVI, and the T- and B-cell antigen receptors [6,9–12]. The importance of Vav in signalling by ITAM receptors has been demonstrated by studying mice that lack individual or combinations of Vav family proteins [13–21]. In all cases, Vav proteins have been shown to play a critical role in the regulation of PLC γ (phospholipase C γ) by ITAM receptors. Vav1^{-/-} T-cells have significantly reduced TCR (T-cell receptor) signalling, with a more pronounced phenotype observed in Vav1/3^{-/-} and Vav1/2/3^{-/-} cells. B-cells deficient in Vav1 have a mild reduction in B-cell receptor signalling, whereas Vav1/2^{-/-} B-cells have a severe phenotype that is even more pronounced in Vav1/2/3^{-/-} cells [17,18,20]. These observations therefore demonstrate a limited redundancy between the three members of the Vav family.

Abbreviations used: AEBSEF, 4-(2-aminoethyl)benzenesulfonyl fluoride; BAPTA-1/AM, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester); CHO, Chinese-hamster ovary; DIC, differential interference contrast; F-actin, filamentous actin; Gads, Grb2-related adaptor downstream of Shc; GEF, guanine-nucleotide-exchange factor; GPVI, glycoprotein VI; GST, glutathione S-transferase; ITAM, immunoreceptor tyrosine-based activation motif; LAT, linker for activation of T-cells; PAK, p21-activated kinase; PI3K, phosphoinositide 3-kinase; PLC γ , phospholipase C γ ; SH, Src homology; SLP-76, SH2 domain-containing leucocyte protein of 76 kDa; TCR, T-cell receptor.

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Vav1 and Vav3 are expressed at significant levels in platelets, whereas there is only residual expression of Vav2 [10,11,22]. In striking contrast with B- and T-cells, however, Vav1 and Vav3 are completely redundant in platelets [11]. Platelets deficient in Vav1 or Vav3 respond normally to GPVI stimulation, whereas cells deficient in both Vav isoforms display a severe block in activation through the collagen receptor [10,11]. Vav2 is not phosphorylated in platelets, and cells deficient in Vav1, Vav2 and Vav3 exhibit a similar defect in response to GPVI agonists as platelets deficient in Vav1 and Vav3 [10,11].

Importantly, Vav family proteins have been demonstrated to play a role in ITAM signalling independent of GEF activity and Rac activation, most notably through regulation of PLC γ isoforms downstream of the antigen receptors [12,21,23–26]. This action has been attributed to the adapter function of Vav isoforms in the formation and stabilization of signalling complexes [21,23,27–29]. There appear to be at least two pathways through which Vav regulates PLC γ downstream of ITAM receptors, namely via regulation of Tec family kinases and through assembly of a LAT–Gads–SLP-76–PLC γ (where LAT is linker for activation of T-cells, Gads is Grb2-related adaptor downstream of Shc, and SLP-76 is SH2 domain-containing leucocyte protein of 76 kDa) signalosome [12,21,23–26,30]. The regulation of Tec family kinases is dependent on PI3K (phosphoinositide 3-kinase) and has been proposed to be regulated through Rac1 [30–32]. On the other hand, the role of Vav in the assembly of the LAT–Gads–SLP-76–PLC γ signalosome is independent of PI3K and, presumably, Rac activation. In addition, Rac has been shown to directly activate PLC γ isoforms *in vitro* independently of PI3K [33]. Therefore Vav is able to control PLC γ activity via at least two pathways that differ in their dependence on PI3K and Rac, although the relative contribution of these pathways is unclear.

Vav family proteins have been shown to be tyrosine-phosphorylated downstream of β 1, β 2 and β 3 integrins in a number of haemopoietic cells, including platelets and in CHO (Chinese-hamster ovary) cells transfected with α IIB β 3 [22,34–38]. Furthermore, Vav proteins have been shown to be critical for outside-in signalling by β 2 integrins in neutrophils and to contribute to their stable adhesion and spreading [39]. Vav proteins have also been shown to bind to tyrosine-phosphorylated β 3 in a K562 cell line model of α v β 3 function [40].

In platelets, the integrin α IIB β 3 activates a signalling pathway that uses many of the same proteins as ITAM receptors (reviewed in [41]) including Src kinases [22,42], Syk [22,43], SLP-76 [44] and PLC γ 2 [45–47]. Platelets deficient in these proteins do not spread on fibrinogen, demonstrating the critical importance of outside-in signalling by the integrin for the spreading response. Vav1 and Vav3 undergo tyrosine-phosphorylation downstream of α IIB β 3 when platelets spread on fibrinogen [22] and when platelets undergo aggregation in suspension [37]. In addition, Vav proteins interact with several of the membrane-proximal signalling proteins in the α IIB β 3 signalling cascade, including Syk [48] and SLP-76 [49–51].

These observations raise the possibility that Vav family proteins may play a role in the regulation of PLC γ 2 by integrin α IIB β 3 in platelets. This hypothesis has been investigated in the present study using platelets from mice deficient in Vav1 and Vav3 to assess the role of Vav family proteins in α IIB β 3 outside-in signalling. We show that Vav1/3^{-/-} platelets have reduced spreading on fibrinogen compared with wild-type platelets, which is associated with defective PLC γ 2 phosphorylation and Ca²⁺ mobilization. Pre-treatment of platelets with thrombin overcomes the spreading defect in Vav1/3^{-/-} platelets. These results demonstrate that Vav family proteins are required for normal regulation of PLC γ 2 by α IIB β 3 and for optimal platelet spreading on fibrinogen.

MATERIALS AND METHODS

Antibodies and reagents

Anti-phosphotyrosine monoclonal antibody 4G10 and anti-Rac monoclonal antibody were purchased from Upstate Biotechnology (through TCS Biologicals). The anti-PLC γ 2 and anti-Syk polyclonal antibodies were kindly supplied by Dr Mike Tomlinson (DNAX, Palo Alto, CA, U.S.A.). The FITC-conjugated rat anti-(mouse α IIB) (MwReg30), anti-(mouse β 3) (Luc.A5) and anti-(mouse α IIB β 3) (Leo.D2) antibodies and rat IgG were purchased from Emfret Analytics. The cDNA for GST (glutathione S-transferase)–PAK (p21-activated kinase) CRIB domain was a gift from Dr Doreen Cantrell (School of Life Sciences, Dundee University, Dundee, U.K.). FITC–phalloidin and Oregon Green BAPTA-1/AM [bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester)] were from Molecular Probes. All other reagents were purchased from Sigma or obtained from sources described previously [43,52].

Animals

The generation of mice disrupted in the *vav1* gene (Vav1^{-/-}) is described by Turner et al. [53]. The generation of mice disrupted in the *vav2* gene (Vav2^{-/-}) is described by Doody et al. [17]. The generation of mice disrupted in the *vav3* gene (Vav3^{-/-}) is described by Fujikawa et al. [20]. Compound knockout mice were generated by appropriate crossing of the individual knockout genotypes. Mutant and control mice were age- and background-matched. All animals were maintained using housing and husbandry in accordance with local and national legal regulations.

Preparation of mouse platelets

Blood was taken from a terminally CO₂-narcosed mouse by cardiac puncture on the day of the experiment into 1:10 (v/v) ACD (85 mM sodium citrate, 110 mM glucose, 71 mM citric acid). Blood was diluted 1:6 (v/v) in Tyrode's Hepes buffer (134 mM NaCl, 0.34 mM Na₂HPO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM Hepes, 5 mM glucose and 1 mM MgCl₂, pH 7.3) and centrifuged at 200 *g* for 6 min to obtain platelet-rich plasma. Platelet-rich plasma was centrifuged in the presence of 0.1 μ g/ml prostacyclin at 1000 *g* for 6 min, and the platelet pellet was resuspended in Tyrode's Hepes buffer.

Flow cytometry staining

Washed platelets (1 \times 10⁷/ml) were stained with the indicated FITC-conjugated antibodies for 15 min at room temperature (22 °C). Staining was quenched with 400 μ l of Tyrode's Hepes buffer, and samples were analysed using a FACScalibur flow cytometer and CellQuest software (Becton Dickinson).

Static adhesion spreading assay

Glass coverslips were coated with 100 μ g/ml fibrinogen solution overnight at 4 °C followed by washing with PBS. Slides were then blocked using 0.5% heat-denatured BSA in PBS for 1 h at room temperature, followed by washing in PBS. Mouse platelets, resuspended at a concentration of 3 \times 10⁷/ml in Tyrode's Hepes containing 2 units/ml apyrase and 10 μ M indomethacin, were transferred to the coverslips and incubated at 37 °C for 45 min in a humid atmosphere. Excess platelets were removed, and the adherent platelets were fixed with 3.7% (w/v) paraformaldehyde for 10 min at room temperature. The coverslips were washed in PBS, mounted using HydroMount mounting medium (National Diagnostics) and viewed under DIC (differential interference

contrast) microscopy under a 63 \times oil-immersion lens and Slidebook software (Intelligent Imaging Innovations). Surface areas were calculated using a Java plugin for ImageJ software.

Immunoprecipitation and immunoblotting

Platelets at 2×10^8 /ml were incubated over fibrinogen-coated dishes for 45 min at 37°C in the presence of 2 units/ml apyrase and 10 μ M indomethacin. Non-adherent basal platelets were removed and lysed in an equal volume of $2 \times$ lysis buffer {2% Nonidet P40, 300 mM NaCl, 20 mM Tris/HCl, 2 mM EDTA, 2 mM EGTA, 2 mM Na₃VO₄, 200 μ g/ml AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride] hydrochloride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 1 μ g/ml pepstatin A, pH 7.4}. Adherent platelets were lysed in a final volume of 1 ml of $1 \times$ lysis buffer. Insoluble cell debris was removed by centrifugation at 13000 *g* for 5 min at 4°C, and cell lysates were pre-cleared using Protein A–Sepharose. Platelet lysates were incubated with the indicated primary antibodies, and resulting protein complexes and immunoprecipitates were resolved by SDS/PAGE (10% polyacrylamide) and transferred on to PVDF membranes. Immunoblotting was performed as described previously [11] with detection by ECL[®] (enhanced chemiluminescence) (Amersham Biosciences).

Rac activation assay

Rac activity was measured as described previously [10]. The CRIB domain of PAK1 (amino acids 67–150) was expressed as a GST-fusion protein and bound to glutathione–Sepharose beads. Platelet stimulations were stopped with an equal volume of $2 \times$ Rac assay lysis buffer [2% (v/v) Nonidet P-40, 1% (w/v) *N*-octylglucoside, 300 mM NaCl, 20 mM Tris/HCl, 2 mM EDTA, 2 mM EGTA, 20 mM MgCl₂, 2 mM Na₃VO₄, 200 μ g/ml AEBSF hydrochloride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 1 μ g/ml pepstatin A, pH 7.4]. Insoluble material was removed by centrifugation at 13000 *g* for 5 min at 4°C, and freshly prepared GST–PAK1 was added to the samples and incubated for 1 h at 4°C. The beads were then washed with $1 \times$ Rac assay lysis buffer, and the bound protein was taken up into Laemmli buffer. The resulting samples were separated by SDS/PAGE (12% polyacrylamide) and transferred on to PVDF membranes for immunoblotting as described above.

F-actin (filamentous actin) assay

F-actin was measured using a modification of the method of Machesky and Hall [54]. Briefly, platelets at a concentration of 2×10^8 /ml were fixed with an equal volume of 3.7% (w/v) formaldehyde containing FITC–phalloidin (20 mM KH₂PO₄, 10 mM Pipes, 5 mM EGTA, 2 mM MgCl₂, 0.1% Triton X-100, 3.7% formaldehyde and 2 μ M FITC–phalloidin) and rotated for 1 h at room temperature. Platelets were then pelleted for 2 min in a microcentrifuge and washed in 0.1% saponin, 20 mM KH₂PO₄, 10 mM Pipes, 5 mM EGTA and 2 mM MgCl₂. Platelets were rotated in methanol for 1 h to extract the FITC–phalloidin. FITC–phalloidin binding was measured by measuring the emission at 520 nm using an excitation wavelength of 488 nm on a spectrofluorimeter.

Ca²⁺-mobilization assay

Platelets were incubated with 15 μ M Oregon Green BAPTA-1/AM in DMSO containing 1.2 mg/ml pluronic acid for 90 min in the dark at room temperature. Excess dye was removed by washing the platelets in Tyrode's Hepes buffer in the presence

of 0.1 μ g/ml prostacyclin. Platelets were allowed to settle on to fibrinogen-coated coverslips in the presence of 2 units/ml apyrase and 10 μ M indomethacin and were imaged in real time using the 63 \times oil-immersion lens on a Zeiss Axiovert 200M microscope using Slidebook software (Intelligent Imaging Innovations).

Analysis of data

Experiments were performed on at least three occasions and are shown as representative data. Where appropriate, data are shown \pm S.E.M. Where statistical significance is indicated, data have been subjected to Student's *t* test. For comparison of surface areas of platelets in Table 1(a), where multiple comparisons are required, Tukey's test has been used. *P* < 0.01 was selected to represent statistical significance.

RESULTS

Vav1/3^{-/-} platelets exhibit reduced spreading on fibrinogen

To investigate whether Vav family proteins are involved in outside-in signalling by α IIB β 3, platelets from wild-type and Vav1/3^{-/-} mice were incubated on fibrinogen-coated coverslips for 45 min and imaged by DIC microscopy. These experiments were carried out in the presence of apyrase and indomethacin to block the effects of the secreted G-protein-coupled secondary agonists, ADP and thromboxane A₂. Under these conditions, wild-type mouse platelets undergo partial spreading, with formation of filopodia and limited lamellipodia-like structures (Figure 1A). This limited spreading response has previously been shown to be independent of activation of Rac, as shown using Rac1/Rac2^{-/-} platelets [55], but dependent on PLC γ 2 [45]. Vav1/3^{-/-} platelets adhere with the same efficiency as wild-type platelets (Figure 1B). However, in contrast with wild-type platelets, Vav1/3^{-/-} platelets do not form lamellipodia and have fewer filopodia than wild-type cells (Figure 1A). The number of filopodia formed by wild-type and Vav1/3^{-/-} platelets were counted and plotted as a histogram. Vav1/3^{-/-} platelets exhibit significantly fewer filopodia than wild-type cells (Figure 1C). The surface area of each set of platelets was measured and plotted as a frequency distribution. The distribution of surface area of Vav1/3^{-/-} platelets is shifted slightly to the left relative to wild-type platelets (Figure 1D), thereby demonstrating a small, but significant, reduction in surface area as shown in Table 1. Platelets deficient in Vav1 or Vav3 alone exhibited an intermediate phenotype, with a slightly lower average surface area than wild-type platelets (Table 1A). The surface area of Vav1/3^{-/-} platelets was reduced significantly relative to the single knockouts, demonstrating a degree of redundancy in the role of these proteins in platelet spreading on fibrinogen (Table 1a). Consistent with a residual expression of Vav2 in platelets, the defect in spreading in Vav1/2/3^{-/-} platelets was not significantly different to that in Vav1/3^{-/-} platelets (Table 1a).

A potential explanation for the decreased spreading of Vav1/3^{-/-} platelets on fibrinogen could be due to reduced surface expression of the fibrinogen receptor α IIB β 3 in these cells. To investigate this, we have compared the levels of the integrin on wild-type and Vav1/3^{-/-} platelets by staining with antibodies against each of the subunits of the integrin and to the integrin complex. Expression of α IIB, β 3 and α IIB β 3 is identical between wild-type and Vav1/3^{-/-} platelets (Figure 1E). These results demonstrate that the defective spreading of Vav1/3^{-/-} platelets on fibrinogen is not due to a lower expression level of the receptor.

Activation of PLC γ 2 by α IIB β 3 is impaired in Vav1/3^{-/-} platelets

Platelet spreading on fibrinogen has previously been shown to be critically dependent on Ca²⁺, PKC and PLC γ 2 [45,56]. Since

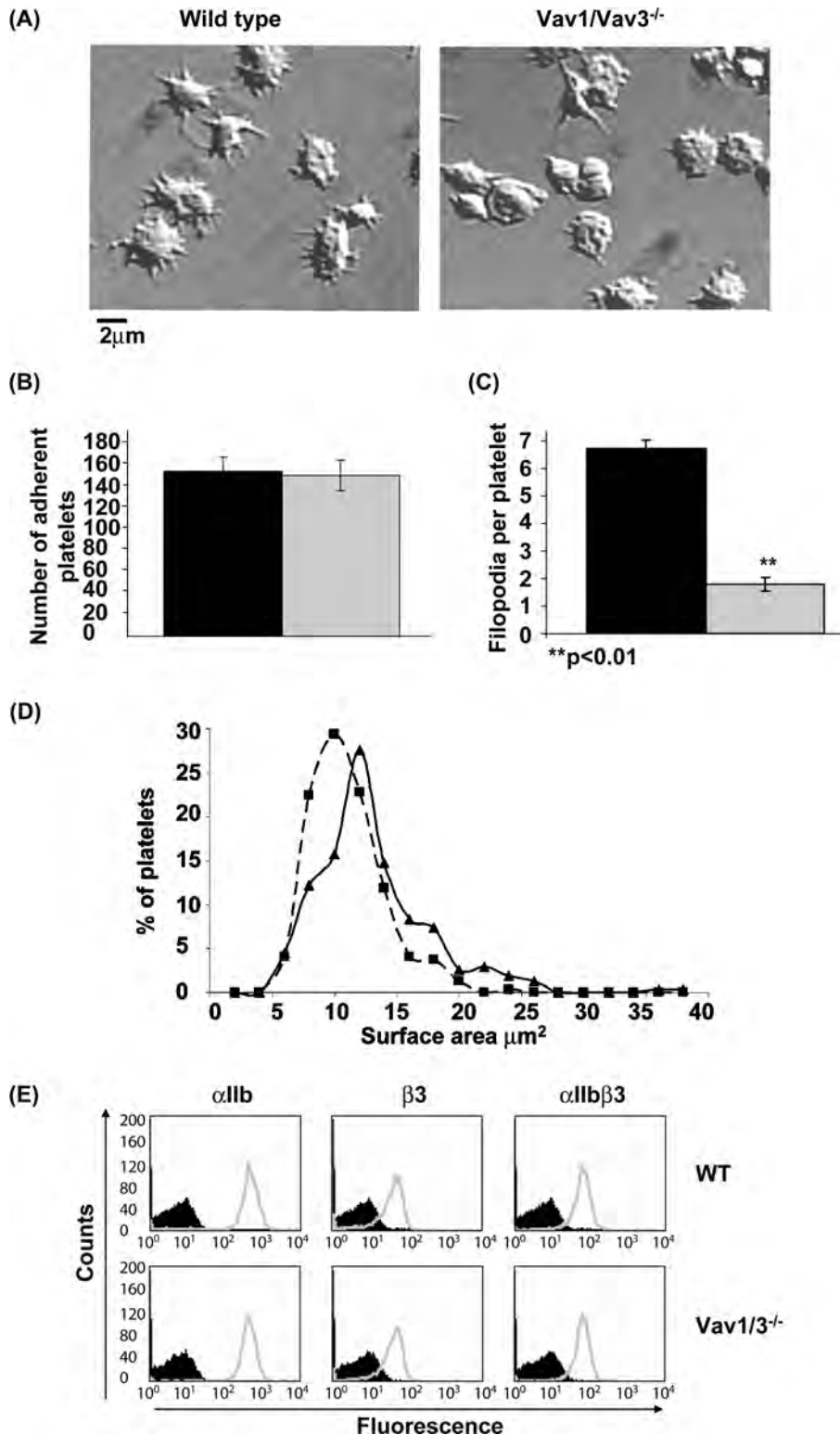


Figure 1 Vav1/3^{-/-} platelets exhibit reduced spreading on fibrinogen

(A) Washed platelets from wild-type (left-hand panel) or Vav1/3^{-/-} (right-hand panel) mice were incubated over fibrinogen-coated coverslips in the presence of 2 units/ml apyrase and 10 μM indomethacin for 45 min at 37 °C and subsequently imaged by DIC microscopy. A representative field of view is shown. (B) Mean \pm S.E.M. number of adherent wild-type (black bar) and Vav1/3^{-/-} (grey bar) platelets per 13500 μm^2 . (C) The mean \pm S.E.M. number of filopodia per platelet were counted in 50 wild-type (black bar) and Vav1/3^{-/-} (grey bar) platelets chosen at random. (D) The surface area of 301 wild-type (solid line) and 319 Vav1/3^{-/-} (broken line) platelets on fibrinogen was calculated by imaging slides as above with a graticule standard and using ImageJ software to measure surface area. Surface areas are plotted as a frequency distribution. (E) Washed platelets from wild-type (WT) or Vav1/3^{-/-} mice were stained with FITC-conjugated rat IgG (black) or rat anti-mouse αIIb , $\beta 3$ or $\alpha\text{IIb}\beta 3$ (grey). Results are representative of three experiments.

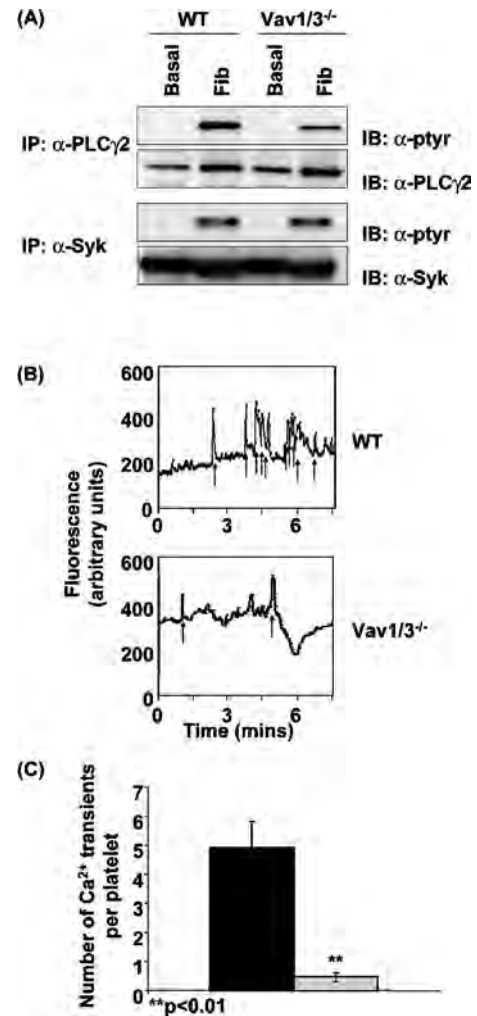
Table 1 Comparison of surface areas

(a) Washed platelets from the genotypes of mice indicated were incubated over fibrinogen-coated coverslips in the presence of 2 units/ml apyrase and 10 μ M indomethacin for 45 min at 37°C. Slides were imaged by DIC microscopy and the surface area of the platelets was calculated with a graticule standard and using ImageJ software. The surface areas of Vav1^{-/-}, Vav3^{-/-}, Vav1/3^{-/-} and Vav1/2/3^{-/-} cells were compared with wild-type surface areas by Tukey's test; **P* < 0.01 relative to wild-type. Vav1^{-/-}, Vav3^{-/-} and Vav1/2/3^{-/-} surface areas were compared with that of Vav1/3^{-/-} using Tukey's test; †*P* < 0.01 relative to Vav1/3^{-/-}. (b) Platelets were stimulated with 1 unit/ml thrombin before adding to coverslips and imaging as above. Surface areas were compared using Student's *t* test and were not significantly different (*P* < 0.01).

(a)	
Genotype	Mean surface area (μm^2)
Wild-type	12.6 ± 0.22 (<i>n</i> = 301)
Vav1 ^{-/-}	10.2 ± 0.16*† (<i>n</i> = 299)
Vav3 ^{-/-}	11.3 ± 0.16*† (<i>n</i> = 289)
Vav1/3 ^{-/-}	9.3 ± 0.13* (<i>n</i> = 319)
Vav1/2/3 ^{-/-}	9.44 ± 0.13* (<i>n</i> = 330)
(b)	
Genotype and treatment	Mean surface area (μm^2)
Wild-type + thrombin	19.3 ± 0.41 (<i>n</i> = 300)
Vav1/3 ^{-/-} + thrombin	17.9 ± 0.41 (<i>n</i> = 300)

Vav family proteins have been shown to be critical for regulation of PLC γ 2 by ITAM receptors in platelets and in other cells, we investigated whether a defect in PLC γ 2 regulation by the integrin could underlie the reduced spreading on fibrinogen that is observed in the combined absence of Vav1 and Vav3. Wild-type and Vav1/3^{-/-} platelets were incubated over a fibrinogen-coated surface for 45 min, before removal and lysis of basal (non-adherent) and stimulated (adherent) platelets. Following lysis and immunoprecipitation, PLC γ 2 phosphorylation was measured by Western blotting using the anti-phosphotyrosine antibody 4G10. Wild-type platelets exhibit a robust increase in tyrosine phosphorylation of PLC γ 2 when spread on fibrinogen, which is reduced by approx. 50% in Vav1/3^{-/-} platelets (Figure 2A). In contrast, tyrosine phosphorylation of the upstream kinase Syk is not significantly different in wild-type and Vav1/3^{-/-} platelets (Figure 2A). These results suggest that the same amount of signal is transmitted by the receptor into the cell but that the efficiency of transmitting this signal to PLC γ 2 is reduced in the absence of Vav1/3.

In order to investigate whether the defect in platelet spreading is due to the defect in signalling of α IIB β 3 to PLC γ 2, we assessed the ability of Vav1/3^{-/-} platelets to mobilize Ca²⁺ during spreading on fibrinogen. Platelets from wild-type and Vav1/3^{-/-} mice were labelled with the Ca²⁺ reporter dye Oregon Green BAPTA-1/AM. This dye is a highly sensitive Ca²⁺ reporter dye that can be used to detect fluctuations in intracellular Ca²⁺ levels without chelating sufficient Ca²⁺ to block Ca²⁺-dependent functional responses ([57,58], and results not shown). Labelled platelets were incubated over fibrinogen-coated coverslips, and spreading was monitored by fluorescence microscopy. Wild-type platelets exhibit a series of oscillations in fluorescence, indicative of transient elevation of cytosolic Ca²⁺, as shown in Figure 2(B) and Supplementary Video S1(A) (see <http://www.BiochemJ.org/bj/401/bj4010753add.htm>). In contrast, Vav1/3^{-/-} platelets exhibit significantly less Ca²⁺ transients over a 7 min recording period as shown by the single-cell records (Figure 2B, and Supplementary Video S1B at <http://www.BiochemJ.org/bj/401/bj4010753add.htm>) and pooled data (Figure 2C). These results

**Figure 2 Activation of PLC γ 2 by α IIB β 3 is impaired in Vav1/3^{-/-} platelets**

(A) Washed wild-type (WT) and Vav1/3^{-/-} platelets were incubated over fibrinogen-coated Petri dishes in the presence of 2 units/ml apyrase and 10 μ M indomethacin for 45 min at 37°C. Basal (non-adherent) and fibrinogen-stimulated (adherent; Fib) cells were lysed in Nonidet P40 lysis buffer, and PLC γ 2 and Syk were immunoprecipitated (IP). Immunoprecipitates were separated by SDS/PAGE and were Western-blotted (IB) with anti-phosphotyrosine monoclonal antibody 4G10 (α -ptyr). Membranes were subsequently stripped and reprobed with anti-PLC γ 2 (α -PLC γ 2) and anti-Syk (α -Syk) antibodies respectively. (B) Washed wild-type (WT) and Vav1/3^{-/-} platelets were labelled with the Ca²⁺ reporter dye Oregon Green BAPTA-1/AM and incubated over fibrinogen-coated coverslips in the presence of 2 units/ml apyrase and 10 μ M indomethacin and imaged in real time using the FITC channel on a fluorescence microscope. The fluorescence intensity of a representative platelet is shown. Spikes in fluorescence indicative of transient cytoplasmic Ca²⁺ elevation are indicated by arrows. (C) The number of Ca²⁺ transients in 30 platelets chosen at random from wild-type (black bar) and Vav1/3^{-/-} (grey bar) mice were counted. Results are means \pm S.E.M. and are representative of three experiments.

demonstrate that Vav1 and Vav3 are required for α IIB β 3-mediated Ca²⁺ mobilization, regulated downstream of PLC γ 2.

Vav1 and Vav3 do not regulate Rac in fibrinogen- or thrombin-stimulated platelets

In addition to their role in regulating PLC γ 2 downstream of ITAM receptors, Vav family proteins also function as GEFs for Rho family G-proteins. The defect in spreading that is observed in the absence of Vav1 and Vav3 is unlikely to be due to blockade of Rho activity, since platelets are not thought to form stress fibres under these conditions. Furthermore, we have been unable to detect

activation of Rho in platelets that have adhered to fibrinogen. However, we have also been unable to detect activation of Cdc42 during adhesion of human or murine platelets to fibrinogen (results not shown). This may reflect that the small G-protein is not activated by the integrin or that it is activated at a level below that required for detection. We have reported previously that spreading of mouse platelets on fibrinogen is independent of activation of Rac, as shown using *Rac1/2^{-/-}* mice [55]. Furthermore, we were unable to detect activation of *Rac1/2* in human and mouse platelets using GST-PAK, which selectively binds to the GTP-bound form of the protein, to precipitate active Rac [55]. In the present study, we have been able to detect weak activation of Rac in murine platelets that have spread on fibrinogen (Figure 3A). In order to investigate whether Rac activation by the integrin is regulated by *Vav1/3*, Rac activation in *Vav1/3^{-/-}* platelets was assayed. α IIB β 3-induced Rac activation is normal in *Vav1/3^{-/-}* platelets (Figure 3A). These results demonstrate that *Vav1/3* does not lie upstream of Rac in α IIB β 3 signalling.

In contrast with signalling by the integrin, thrombin induces robust activation of Rac. In order to investigate whether *Vav1/3* lies upstream of Rac in thrombin-stimulated platelets, active Rac was precipitated from thrombin-stimulated platelets as above. Activation of Rac by thrombin is not altered in the absence of *Vav1* and *Vav3* (Figure 3B). Furthermore, thrombin induced F-actin polymerization is normal in *Vav1/3^{-/-}* platelets (Figure 3C). These results demonstrate that *Vav1/3* does not lie upstream of Rac or actin assembly in thrombin-stimulated platelets. Importantly this is consistent with the observation that thrombin induces full spreading and formation of extensive lamellipodia in both wild-type and *Vav1/Vav3^{-/-}* platelets (Figure 3D and Table 1), as demonstrated by the indistinguishable frequency distribution curves of platelet surface area (Figure 3E).

DISCUSSION

In the present study, we have investigated the role of *Vav* family proteins in α IIB β 3-mediated platelet activation and spreading on fibrinogen. Platelets deficient in the major *Vav* family proteins, *Vav1* and *Vav3*, have reduced spreading on fibrinogen, in association with reduced tyrosine phosphorylation of PLC γ 2 and reduced elevation of intracellular Ca^{2+} . These results therefore demonstrate a critical role for *Vav1* and *Vav3* in the regulation of PLC γ 2 by α IIB β 3, as has previously been shown to be the case for regulation of PLC γ 2 by the ITAM-coupled collagen receptor, GPVI [11]. The defect in spreading in the *Vav1/3^{-/-}* platelets is bypassed in the presence of thrombin, a G-protein-coupled receptor agonist, which stimulates a powerful activation of PLC β isoforms in platelets. Thus the role of *Vav1* and *Vav3* in mediating spreading on fibrinogen is specifically linked to α IIB β 3-mediated outside-in signalling.

Vav family proteins have been implicated in integrin-dependent responses in a variety of cells [34,39,40]. *Vav*-deficient neutrophils display defective β 2-integrin-mediated spreading and phagocytosis [39], a K562 model of α v β 3-mediated cell adhesion demonstrates recruitment of *Vav1* to the phosphorylated β 3 subunit in fibronectin-adherent cells [40] and CHO cells overexpressing α IIB β 3, Syk, SLP-76 and *Vav1* form extensive lamellipodia on fibrinogen [38]. In the present study, we demonstrated that *Vav1* and *Vav3* are required for normal platelet spreading on fibrinogen, as a consequence of a reduction in α IIB β 3-mediated activation of PLC γ 2 and Ca^{2+} mobilization. Since β 2 integrins in neutrophils also exhibit PLC γ 2-dependent Ca^{2+} mobilization [59], we suspect that *Vav* proteins are likely to be involved in PLC γ 2 regulation by β 2 integrins in neutrophils

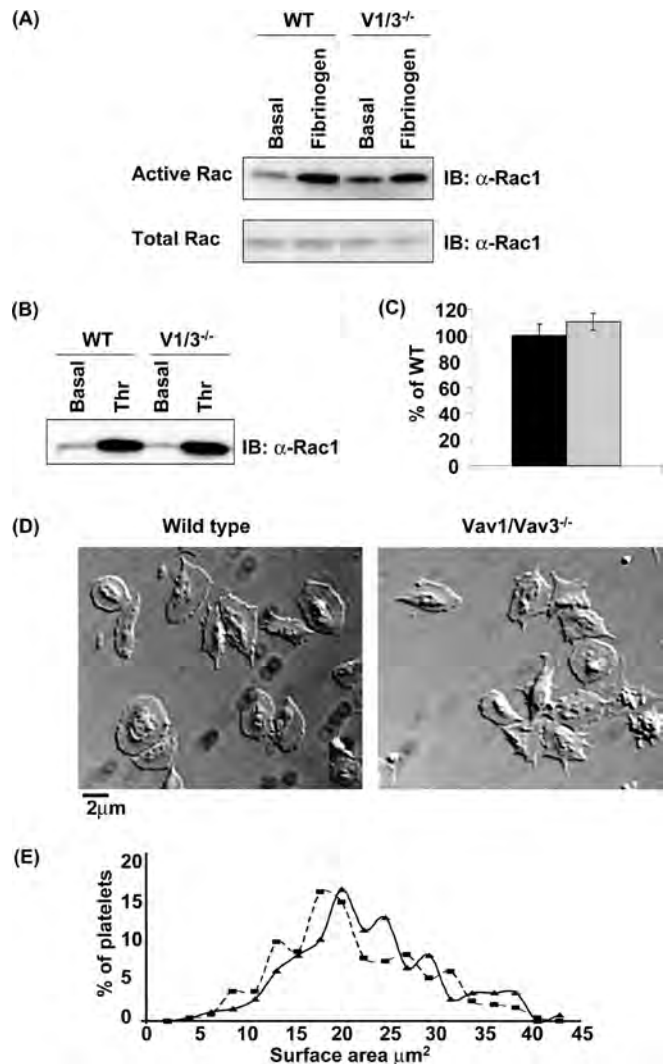


Figure 3 *Vav1* and *Vav3* do not regulate Rac in fibrinogen- or thrombin-stimulated platelets

(A) Washed wild-type (WT) and *Vav1/3^{-/-}* (*V1/3^{-/-}*) platelets were incubated over fibrinogen-coated Petri dishes in the presence of 2 units/ml apyrase and 10 μ M indomethacin for 45 min at 37 °C. Basal (non-adherent) and fibrinogen-stimulated (adherent) cells were lysed in Rac assay lysis buffer. An aliquot of lysate was taken into Laemmli buffer for total Rac analysis by Western blotting (IB) with anti-Rac1 (α -Rac1). Remaining lysates were incubated with GST-PAK for 1 h, and active Rac was precipitated. Precipitates were separated by SDS/PAGE and were Western-blotted for Rac. (B) Washed wild-type (WT) and *Vav1/3^{-/-}* (*V1/3^{-/-}*) platelets in suspension in the presence of 2 units/ml apyrase and 10 μ M indomethacin were stimulated with 1 unit/ml thrombin for 60 s and lysed in Rac assay lysis buffer, and active Rac was precipitated and Western-blotted as above. (C) Platelets were stimulated with 1 unit/ml thrombin for 60 s, fixed and F-actin-stained with FITC-phalloidin. Specifically bound FITC-phalloidin was eluted and quantified in a spectrofluorimeter. The amount of F-actin (black bar) and *Vav1/3^{-/-}* (grey bar) is expressed as the mean \pm S.E.M. percentage of thrombin induced F-actin formation in wild-type (WT) cells for triplicate samples. (D) Washed platelets from wild-type (left-hand panel) or *Vav1/3^{-/-}* (right-hand panel) mice were stimulated with 1 unit/ml thrombin and immediately incubated over fibrinogen-coated coverslips for 45 min at 37 °C in the presence of 2 units/ml apyrase and 10 μ M indomethacin and were subsequently imaged by DIC microscopy. A representative field of view is shown. (E) The surface area of 300 wild-type (solid line) and 300 *Vav1/3^{-/-}* (broken line) platelets on fibrinogen was calculated by imaging slides as above with a graticule standard and using ImageJ software to measure surface area. Surface areas are plotted as a frequency distribution. Results are representative of three experiments.

and that this contributes to the defective functional responses in these cells [39]. Interestingly, platelet responses to the G-protein-coupled receptor agonist thrombin are not affected by

deficiency of Vav1 and Vav3. Similarly, neutrophil responses to G-protein-coupled chemoattractants are normal in Vav-deficient cells, suggesting that Vav is also dispensable for GPCR signalling in these cells [39].

It is now recognized that integrins use many of the same signalling proteins as ITAM receptors [41]. In platelets, for example, critical roles for Src [22,42] and Syk [22] tyrosine kinases, the adapter protein SLP-76 [44] and PLC γ 2 [45–47] have been reported downstream of both GPVI and α IIB β 3. On the basis of the data in the present study, a further similarity in the signalling pathways activated by these two classes of receptors is the requirement of Vav proteins for efficient regulation of PLC γ 2 [10,11]. It is noteworthy that Vav proteins appear to be essential for optimal regulation of PLC γ isoforms by all ITAMs. It will therefore be of interest to know whether Vav proteins are required downstream of all integrins for the regulation of PLC γ . Interestingly, we have also recently demonstrated a role for Vav1 and Vav3 in the activation of PLC γ 2 by the C-type lectin family receptor, CLEC-2, in platelets which has a single rather than dual YXXL motif in its cytosolic tail [60]. There are, however, significant differences in signalling by these three classes of receptor in platelets. For example, GPVI signalling takes place in lipid rafts and is critically dependent on the transmembrane adaptor protein, LAT [61,62], whereas signalling by α IIB β 3 is independent of these specialized membrane domains and LAT [45,62]. In comparison, signalling by CLEC-2 is partially dependent on the adapters LAT and SLP-76, whereas the latter is essential for responses to GPVI.

Cell spreading involves extensive cytoskeletal re-modifications, which can be regulated by Rho family small G-proteins. The small G-proteins Cdc42, Rac and Rho are implicated in the formation of filopodia, lamellipodia and stress fibres respectively [63–65]. In the present study, we have assayed spreading mediated directly by the integrin, by performing all studies in the presence of apyrase and indomethacin. Under these conditions, mouse platelets undergo a limited spreading response that is dependent on PLC γ 2 [45], but independent of Rac [55]. Consistent with this, we have been able to detect only weak activation of Rac by α IIB β 3 in platelets that have undergone spreading on fibrinogen, and this activation is not reduced in Vav1/3^{-/-} platelets. These results demonstrate that the defective regulation of PLC γ 2 by α IIB β 3 in platelets is not due to defective regulation of Rac. Interestingly, we have been unable to detect activation of Cdc42 (results not shown) during spreading on fibrinogen, despite robust formation of filopodia. This may represent a low level of activation of the small G-protein that is below the level of detection, or may represent Cdc42-independent formation of filopodia, as has been reported in other cells [66,67]. We cannot therefore rule out the possibility that the defect in spreading on fibrinogen in the absence of Vav1 and Vav3 is, at least partially, due to a direct inhibition of Cdc42 activation or a related G-protein, although it should be noted that a role of Vav family GEFs in the regulation of Cdc42 is controversial [6,8,68–71]. It is also extremely unlikely that the spreading defect of Vav1/3^{-/-} platelets is due to impairment of activation of Rho as there is no evidence of stress fibre formation in platelets spread under these conditions. Under physiological conditions, the limited spreading response initiated by α IIB β 3 is reinforced by G-protein-coupled receptor agonists, such as ADP and thrombin, leading to Rac activation and Rac-dependent full spreading [10,55]. In the present study, we have shown that Vav proteins are not required for G-protein-coupled receptor signalling leading to Rac activation or actin polymerization, suggesting that this process would occur normally in Vav-deficient mice.

It is well established that Vav family proteins have GEF-independent functions thought to be due to their roles as adaptor

proteins and stabilizing signalling complexes [21,23,27,28]. Indeed, Vav family proteins have been shown to be required for normal formation of the LAT–Gads–SLP-76–PLC γ 2 signalosome downstream of the TCR [23]. It is likely that this function of Vav proteins is responsible for their critical role in the regulation of PLC γ isoforms by ITAM receptors, including GPVI. Demonstration of inhibition of PLC γ 2 downstream of α IIB β 3 in the present study provides strong evidence that this mechanism also applies to the regulation of PLC γ isoforms downstream of integrin receptors.

In summary, the data presented here demonstrate an important role for Vav family proteins in signalling by the platelet integrin α IIB β 3 and for normal regulation of PLC γ 2, intracellular Ca²⁺ mobilization and platelet spreading on fibrinogen. These results add to previous studies that demonstrate a pivotal role for Vav family proteins in the regulation of PLC γ 2 by distinct classes of surface glycoproteins, including integrins, ITAM and lectin receptors.

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