Vav family proteins are guanine nucleotide exchange factors for the Rho/Rac family of small GTP-binding proteins. In addition, they have domains that mediate protein-protein interactions, including one Src homology 2 (SH2) and two Src homology 3 (SH3) domains. Vav1, Vav2, and Vav3 play a crucial role in the regulation of phospholipase C\(\gamma\) (PLC\(\gamma\)) isoforms by immuno-tyrosine-based activation motif (ITAM)-coupled receptors, including the T- and B-cell antigen receptors. We have reported in platelets, however, that Vav1 and Vav2 are not required for activation of PLC\(\gamma 2\) in response to stimulation of the ITAM-coupled collagen receptor glycoprotein VI (GPVI). Here we report that Vav3 is tyrosine-phosphorylated upon activation of GPVI but that Vav3-deficient platelets also exhibit a normal response upon activation of the ITAM receptor. In sharp contrast, platelets deficient in both Vav1 and Vav3 show a marked inhibition of aggregation and spreading upon activation of GPVI, which is associated with a reduction in tyrosine phosphorylation of PLC\(\gamma 2\). The phenotype of Vav1/2 triple-deficient platelets is similar to that of Vav1/3 double-deficient cells. These results demonstrate that Vav3 and Vav2 play a crucial role in the activation of PLC\(\gamma 2\) by GPVI. This is the first time that absolute redundancy between two protein isoforms has been observed with respect to the regulation of PLC\(\gamma 2\) in platelets.

Collagen is the most thrombogenic component of the subendothelial matrix, inducing powerful platelet activation through the GPVI\(^1\)-FcR\(\gamma\)-chain receptor complex. GPVI signals through sequential activation of Src and Syk family tyrosine kinases (1, 2). The Src kinases Fyn and Lyn stimulate tyrosine phosphorylation of two conserved tyrosines in the FcR\(\gamma\)-chain immuno-tyrosine-based activation motif (ITAM) (2–4). This leads to engagement of Syk via its two SH2 domains and its subsequent activation. Syk orchestrates a downstream signaling cascade that is regulated through the interaction of several adapter proteins, including LAT, Gads, and SLP-76, and leads to activation of effector enzymes, including phosphatidylinositol (PI) 3-kinases, Tec kinases, and phospholipase C\(\gamma\) (PLC\(\gamma\)). The functional role of many of the proteins in this cascade, including GPV1 (5), FcR\(\gamma\) chain (1), Syk (1), LAT (6), Gads (7), SLP-76 (8), Btk (9), Tec (9), and PLC\(\gamma 2\) (10, 11), has been shown by the impairment or abolition of response in platelets from genetically deficient mice. In sharp contrast, platelets from mice deficient in Vav1 and Vav2 show minimal functional impairment in responses to collagen or GPVI-specific agonists such as convulxin and CRP (12), despite their role in signaling by other ITAM receptors, including the B-cell and T-cell antigen receptors.

The Vav family of GTP exchange factors consists of three members (13–16), which share a common structural arrangement. The amino terminus contains a calponin homology domain and an acidic region, which contains regulatory tyrosine phosphorylation sites. This is followed by Dbl homology, pleckstrin homology, and zinc finger domains, which form the GDP/GTP exchange factor region of Vav family proteins. The COOH-terminal portion contains a short proline-rich region and an SH3-SH2-SH3 region. Vav2 and Vav3 have broad expression profiles, whereas Vav1 is specifically expressed in hematopoietic cells (14–16).

The guanine nucleotide exchange activity of Vav proteins is specific for the Rho family of small G proteins. Vav1 has been shown to selectively activate Rac1, Rac2, RhoG, and to a lesser extent, RhoA. Vav2 and Vav3 activate RhoA and RhoG but show less activity toward Rac1 (15, 17). The GTP/GDP exchange activity of all three Vav family proteins is modulated through phosphorylation on tyrosine by Src and Syk family kinases. Interestingly, however, tyrosine phosphorylation of Vav1 by GPVI in platelets is not associated with activation of Rac demonstrating that additional factors are also required for activation of the Rho family G protein (12). Vav proteins are prominent tyrosine kinase substrates downstream of ITAM receptors, including the T-cell and B-cell antigen receptors (15, 18, 19). Vav family proteins interact with several of the proteins in the ITAM-dependent signaling cascades, including Syk and Zap70, SLP-76 and Blnk, Grb2, Nck, and the p85 regulatory subunit of PI 3-kinase.

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\(^e\) The abbreviations used are: GPVI, glycoprotein VI; ITAM, immunotyrosine-based activation motif; PI, phosphatidylinositol; PLC\(\gamma\), phospholipase C\(\gamma\); CRP, collagen-related peptide; SH2 and SH3, Src homology 2 and 3, respectively; PRP, platelet-rich plasma; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetraakis(acetoxyethyl) ester; PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; PBS, phosphate-buffered saline.

\(1\) The abbreviations used are: GPVI, glycoprotein VI; ITAM, immunotyrosine-based activation motif; PI, phosphatidylinositol; PLC\(\gamma\), phospholipase C\(\gamma\); CRP, collagen-related peptide; SH2 and SH3, Src homology 2 and 3, respectively; PRP, platelet-rich plasma; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetraakis(acetoxyethyl) ester; PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; PBS, phosphate-buffered saline.
Insights into the specific roles of members of the Vav family in hematopoietic cells have come from studying mice engineered to lack individual or combinations of these proteins. T-cell proliferation is severely retarded in Vav1-deficient mice. Vav1−/− T-cells and thymocytes are also defective in their ability to phosphorylate PLCγ1 and mobilize Ca2+ in response to T-cell receptor cross-linking. Significantly, the proliferation ability to phosphorylate PLCγ is impaired in these cells. Vav1−/−/H11002 Vav2−/−/H11001 and Vav3−/−/H11000 mice are reported to lack individual or combinations of these proteins. Some of this work has previously been presented in a recent paper (27). Compound knock-out mice were generated by targeted disruption of the Vav2 gene (Vav2H11002) and Vav3 gene (Vav3H11003). The striking phenotype observed in the absence of Vav1 or Vav2 in T- and B-cells contrasts with the minimal phenotype observed in GPVI-activated platelets (12). Potentially, this can be explained by the presence of Vav3, which is also expressed in platelets and undergoes tyrosine phosphorylation upon engagement of the fibrinogen receptor, αIbβ2 (28). Alternatively, the lack of a role for Vav3 in platelet aggregation may be explained by the presence of Vav1 and Vav2 in platelets. The platelet phenotypes of Vav1−/−/H11002 Vav2−/−/H11001 and Vav3−/−/H11000 mice are reported to lack individual or combinations of these proteins.

MATERIALS AND METHODS

Antibodies and Reagents—Anti-phosphotyrosine monoclonal antibody 4G10 and anti-Lat polyclonal antibody were kindly supplied by Dr. Mike Tomlinson (DNAX, Palo Alto, CA). The anti-mouse SLP-76 polyclonal antibody was purchased from Upstate Biotechnology (Ccs Biology Ltd., Bucks, UK). Anti-human Vav3 antibodies were raised in rabbits as previously described (31). The anti-PLCγ2 and anti-Syk polyclonal antibodies were kindly supplied by Dr. Mike Tomlinson (DNAX, Palo Alto, CA). The anti-mouse SLP-76 polyclonal antibody was a kind gift from Dr. Gary Koretzky (University of Pennsylvania, Philadelphia, PA). Rhodamine phallolidin was from Molecular Probes (Leiden, The Netherlands). The Bio-Rad DC protein assay kit was from Bio-Rad Laboratories (Hemel Hempstead, UK). PD0173952 was a gift from Pfizer Global Research and Development (Ann Arbor, MI). All other reagents were purchased from Sigma (Poole, UK) or obtained from previously described sources (1).

Animals—The generation of mice disrupted in the vav1 gene (Vav1−/−) is described in Turner et al. (32). The generation of mice disrupted in the vav2 gene (Vav2−/−) is described in Doody et al. (24). The generation of mice disrupted in the vav3 gene (Vav3−/−) is described in Fujikawa et al. (27). Compound knock-out mice were generated by appropriate crossing of the individual knock-out 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 Human Platelets—Blood was taken by forearm venepuncture from healthy, drug-free volunteers on the day of the experiment into 1:10 (v:v) sterile sodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation at 200 × g for 20 min. Platelets were isolated from PRP by centrifugation at 1000 × g for 10 min in the presence of 0.1 μg/ml prostacyclin. The platelet pellet was resuspended in modified Tyrodes-HEPES buffer (134 mM NaCl, 0.34 mM NaH2PO4, 2.9 mM KCl, 12 mM NaHCO3, 20 mM HEPES, 5 mM glucose, 1 mM MgCl2, pH 7.3). The platelets were centrifuged at 1000 × g for 10 min in the presence of 0.1 μg/ml prostacyclin and 1.9 (v:v) acid citrate dextrose (120 mM sodium citrate, 110 mM glucose, 80 mM citric acid) and resuspended at a concentration of 5 × 107/ml in Tyrodes-HEPES buffer.

Preparation of Mouse Platelets—Blood was taken from a terminally coagulated limb of the mouse by cardiac puncture on the day of the experiment. The striking phenotype observed in the absence of Vav1 or Vav2 in T- and B-cells contrasts with the minimal phenotype observed in GPVI-activated platelets (12). Potentially, this can be explained by the presence of Vav3, which is also expressed in platelets and undergoes tyrosine phosphorylation upon engagement of the fibrinogen receptor, αIbβ2 (28). Alternatively, the lack of a role for Vav3 in platelet aggregation may be explained by the presence of Vav1 and Vav2 in platelets. The platelet phenotypes of Vav1−/−/H11002 Vav2−/−/H11001 and Vav3−/−/H11000 mice are reported to lack individual or combinations of these proteins.

Platelet Stimulation and Aggregation—Mouse platelets were used at a concentration of 2 × 109/ml. For all protein studies, lotrafiban (10 μM), indomethacin (10 μM), and apyrase (2 units/ml) were included in the resuspension buffer unless otherwise stated. Stimulation of platelets was performed in a PAP-4 aggregometer (Bio/Data Corp., Horsham, PA) with continuous stirring at 1200 rpm at 37 °C for the times shown. Aggregation of platelets was monitored by measuring changes in light transmission in the absence of lotrafiban, indomethacin, and apyrase. Platelets were preincubated with PPI (20 μM), PP2 (20 μM), PD0173952 (25 μM), LY294002 (20 μM), BAPTA-AM (40 μM), and Ro 318220 (10 μM) for 10 min prior to stimulation.

Immunoprecipitation and Immunoblotting—Platelets were lysed with an equal volume of 2× lysis buffer (2% Nonidet P-40, 300 mM NaCl, 20 mM Tris, 10 mM EDTA, 2 mM Na3VO4, 200 mM 4-aminophenylbenzenesulfon fluoride hydrochloride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 μg/ml pepstatin A, pH 7.4). Insoluble cell debris was removed by centrifugation for 5 min at 13,000 × g, 4 °C, and cell lysates were precleared using protein A-Sepharose. Platelet lysates were incubated with the indicated primary antibodies, and the resulting protein complexes and immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed as described previously (12) with detection by enhanced chemiluminescence (ECL, Amersham Biosciences, Bucks, UK). Murine thymocytes and splenocytes in phosphate-buffered saline (PBS) were lysed with an equal volume of 2× lysis buffer and insoluble cell debris removed by centrifugation at 13,000 × g, 4 °C. Protein concentration of each sample was measured using the Bio-Rad DC protein assay using the manufacturer's instructions.

Static Adhesion Spreading Assay—Glass microscope slides were coated with 100 μg/ml Horm collagen solution overnight at 4 °C followed by washing with PBS. Slides were then blocked using 1% heat-denatured bovine serum albumin in PBS for 1 h, followed by washing in PBS. Mouse platelets, suspended in Tyrodes-HEPES at a concentration of 1 × 107 platelets/ml, were transferred to the slides fixed with 3.7% paraformaldehyde at 37 °C for 45 min in a humid atmosphere. Excess platelets were removed and the slides adhered to the slides fixed with 3.7% paraformaldehyde for 10 min at room temperature. The coverslips were washed in PBS, mounted using Immuno Fluore Mounting Medium (ICN Biomedicals, Aurora, CA) and viewed under differential interference contrast microscopy under a 63× oil immersion lens and Slidebook software (Intelligent Imaging Innovations).

Adhesion under Flow—Whole mouse blood was isolated in sodium heparin (10 IU/ml) as described previously (33). Blood was perfused through glass microslides, with inner diameter 1 × 0.1 mm (Camlab, Cambridge, UK), which had been coated with 30 or 100 μg/ml Horm collagen solution overnight at 4 °C before blocking with 2% bovine serum albumin in PBS at room temperature for 1 h. A shear rate of 800 s−1, with a corresponding flow rate of 0.08 ml/min, was generated by a syringe pump (Harvard Apparatus, Southnacht, MA). After 2 min perfusion with whole blood, modified phosphate-free Tyrodes buffer was perfused for 8 min through the microslides at the same shear rate. Platelet thrombi that formed on the surface of the collagen were visualized with an inverted stage video microscope system (DM IRB, Leica, UK). Subsequently, adherent platelets were lysed in 1× ice-cold Nonidet P-40 lysis buffer. Proteins were separated and blotted as above.

RESULTS

Vav3 Is Tyrosine-Phosphorylated in Response to Activation of GPVI—To investigate a potential role for Vav3 down-
stream of GPVI, we analyzed Vav3 tyrosine phosphorylation in platelets stimulated with the GPVI-specific agonist, CRP, or collagen. Aggregation and inside-out signals from the integrin \(\alpha_{IIb}\beta_3\) were blocked using the antagonist lotrafiban and positive feedback signals arising from ADP and thromboxanes were inhibited using apyrase and indomethacin, respectively. CRP stimulated robust tyrosine phosphorylation of Vav3 within 20 s, which was sustained for at least 300 s (Fig. 1a). Tyrosine phosphorylation of Vav3 was maximal between 3 and 10 \(\mu\)g/ml CRP (Fig. 1b). Collagen also stimulates tyrosine phosphorylation of Vav3, although the response is weaker than that induced by CRP, which is consistent with its more powerful action (Fig. 1c). Vav3 is tyrosine-phosphorylated in murine platelets in response to CRP and collagen, with the former again giving a more robust response (Fig. 1d). These results highlight a potential role for Vav3 in the GPVI signaling cascade.

**Vav3 Phosphorylation Is Dependent on Src, Syk, and Btk**

Tyrosine Kinases—The regulation of tyrosine phosphorylation of Vav3 was investigated using a variety of inhibitors and genetically modified murine platelets. Tyrosine phosphorylation of Vav3 by CRP was abolished in the presence of the Src family kinase inhibitors, PP1 and PP2, and the structurally unrelated Src kinase inhibitor PD0173952 confirming that Vav3 lies downstream of the GPVI signaling cascade (Fig. 1e). These results emphasize that Vav3 is regulated downstream of GPVI but do not directly identify the tyrosine kinase(s) that mediates phosphorylation.

**Impaired Activation of Vav1/Vav3 Double-deficient Platelets by GPVI Agonists**—Vav3-deficient mice were used to monitor the functional role of the protein in platelet activation by GPVI. Aggregation of Vav3-deficient platelets in response to stimulation by CRP (Fig. 2a) and collagen (data not shown) was not significantly different from that in controls. Similarly, the ability of platelets to spread and form lamellipodia on a surface of immobilized collagen was not significantly altered in the absence of Vav3 (Fig. 2b). Tyrosine phosphorylation of Syk, LAT, SLP-76, and PLC\(\gamma\)2 in response to CRP was also not altered in Vav3-deficient platelets (Fig. 2c).

In view of the redundancy between Vav family members in B-cells, and also the marked tyrosine phosphorylation of Vav1 that has been reported in both human and murine platelets, we investigated responses in the absence of both Vav1 and Vav3. Aggregation of Vav1/Vav3-deficient murine platelets in response to CRP or collagen was strongly reduced (Fig. 2d), although at maximal concentrations of CRP a small shape change response and weak aggregation could be seen (Fig. 2e). In comparison, aggregation to a submaximal concentration of the G protein-coupled receptor agonist thrombin was not altered (Fig. 2f). Spreading of Vav1/3-deficient platelets on collagen was also largely abolished, with very few cells exhibiting signs of activation such as formation of filopodia (Fig. 2g).

**Flow of Platelet Aggregation**

To investigate the role of Vav family proteins in a more physiological setting, we assayed the ability of Vav1/Vav3-deficient platelets to adhere to a collagen matrix under flow conditions at a flow rate that is typically found in small arteries. Whole blood from Vav1/Vav3-deficient or wild type mice was flowed through a collagen-coated capillary tube at a shear force of 800 s\(^{-1}\) for 2 min. The capillary tube was then washed and analyzed by phase-contrast light microscopy. The capillary...
in the regulation of PLC

These results demonstrate a redundant role of Vav1 and Vav3. SLP-76 and LAT in Vav1/Vav3-deficient platelets (Fig. 3). In addition, tyrosine phosphorylation of PLC, was not altered (Fig. 3). Both Vav1 and Vav3 are essential for phosphorylation (data not shown) to collagen and CRP that is seen in the combined absence of Vav1/Vav3. The Vav1/Vav2/Vav3 triple-deficient cells exhibit residual shape change and aggregation in response to a high concentration of CRP (Fig. 4a). This is accompanied by a residual degree of tyrosine phosphorylation of PLC-γ2 in response to CRP. Syk phosphorylation was not altered (Fig. 4b). Thrombin-induced aggregation of Vav1/Vav2/Vav3 triple-deficient platelets was not significantly altered relative to controls (Fig. 4a). These results demonstrate that Vav2 is unable to compensate for the loss of Vav1 and Vav3 in platelets and indicate that it does not play a functional role in the GPVI signaling cascade. Furthermore, they also show that GPVI is able to mediate weak tyrosine phosphorylation of PLC-γ2 in the absence of Vav family proteins.

Expression of Vav Proteins Is Not Affected by the Absence of Other Vav Isoforms—To investigate whether the redundancy between Vav1 and Vav3 was due to up-regulation of expression of the other isoforms in the family, lysates of Vav1-, Vav3-, and Vav1/3-deficient platelets were Western blotted for other Vav isoforms (Fig. 5a). The level of expression of Vav1 in platelets deficient in Vav3 was similar to that in wild type platelets (Fig. 5a, left panels). A similar picture is seen for expression of Vav3 in the presence or absence of Vav1 (Fig. 5a, middle panels). Expression of Vav2 is not affected by deficiency of Vav1 and Vav3 (Fig. 5a, right panels). In all cases, the level of expression of actin was measured as a marker of platelet protein and was found to be similar under all conditions. These results demonstrate that the redundancy observed between Vav1 and Vav3 is real rather than being a consequence of compensatory up-regulation in the knock-out models.

One potential explanation for the apparent absence of a
functional role of Vav2 in platelets is that it is expressed at a much lower level than the other two isoforms. To address this, we compared the level of expression of all three Vav isoforms in murine platelets to that in murine splenocytes and thymocytes, which are rich in B-cells (70%) and T-cells (95%), respectively. It is relevant in this context that a role for Vav2 in B-cells (24, 25) and T-cells (27) has been reported. The same amount of total protein from platelets, splenocytes, and thymocytes was separated by SDS-PAGE and Western blotted for Vav1, Vav2, and Vav3. The level of expression of Vav1 and Vav3 is similar in murine platelets, splenocytes, and thymocytes, consistent with the functional role of these isoforms in platelets, B-cells and T-cells (Fig. 5b). In comparison, the level of expression of Vav2 is significantly lower in murine platelets than in spleno-
Fig. 4. Vav1/Vav2/Vav3-deficient platelets are identical to Vav1/Vav3-deficient platelets. a, aggregation of platelets from wild type (WT) or Vav1/ Vav2/Vav3-deficient mice was measured as described in the legend to Fig. 2. O.D., optical density. b, proteins were immuno- precipitated (IP) from CRP-stimulated lysates of platelets from wild type (WT) or Vav1/Vav2/Vav3-deficient mice as described in the legend to Fig. 3. The upper panels were Western blotted for phosphotyrosine and the lower panels for the precipitated protein. Results are representative of three to five experiments. IB, immunoblotted.

Fig. 5. Expression levels of Vav proteins in platelets. a, basal platelet whole cell lysates from wild type (WT), Vav1-, Vav3-, and Vav1/Vav3-deficient platelets were separated by SDS-PAGE and Western blotted for Vav1, Vav3, or Vav2 (top panels) and subsequently blotted for actin as a loading control (bottom panels). b, 30 μg of total protein from wild type murine platelets, splenocytes, and thymocytes were separated and Western blotted for Vav1, Vav2, or Vav3. Results are representative of three to five experiments. IB, immunoblotted; V1, Vav1; V2, Vav2; V1/3, Vav1/Vav3.

cytes and thymocytes. These data are consistent with the functional role of Vav2 in B-cells and T-cells and the lack of a role for the isoform in platelets.

DISCUSSION

Tyrosine phosphorylation of Vav family proteins is necessary for guanine nucleotide exchange factor activity and forms docking sites for interactions with other signaling proteins via SH2 domains. Here we show that Vav3 is tyrosine-phosphorylated following stimulation of the ITAM-coupled collagen receptor GPVI in platelets. To our surprise, however, activation of Vav3-deficient platelets by GPVI is indistinguishable from that of wild type cells. Vav1 has previously been reported to be tyrosine-phosphorylated by GPVI, and Vav1-deficient platelets exhibit minimal impairment in activation by GPVI (12). Significantly, in this study we show that platelets deficient in both Vav1 and Vav3 exhibit a marked reduction in functional responses to GPVI, which is associated with a reduced tyrosine phosphorylation of PLCγ2. A similar level of impairment in response is seen in platelets deficient in Vav1, Vav2 and Vav3, providing evidence against a role for Vav2 in the GPVI signaling cascade. This is consistent with the observation that Vav2 expression is very low in platelets relative to that in B-cells where a functional role has been reported. Furthermore, Vav2 does not undergo tyrosine phosphorylation upon engagement of GPVI (12) even in the absence of Vav1 and Vav3 (data not shown). Vav1 and Vav3, but not Vav2, therefore play redundant roles in medi-
Vav1 and Vav3 in Platelets

Vav1 and Vav3 play a critical role in the regulation of PLCγ activity at the level of substrate supply. One such model is that Vav activates phosphatidylinositol-4-phosphate 5-kinase via Rac1 in B-cells, thereby increasing levels of the PLCγ substrate, phosphatidylinositol 4,5-bisphosphate (35). However, Rac1 is not activated by GPVI in platelets, whereas removal of both proteins caused a dramatic reduction in ITAM-mediated tyrosine phosphorylation and cell activation. This study further underscores the similarity in signaling by GPVI and the B-cell and T-cell antigen receptors and emphasizes that Vav family proteins play a universal role in the regulation of PLCγ isoforms by ITAM receptors.

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