THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 52, Issue of December 24, pp. 53955–53962, 2004

Printed in U.S.A.

Vav1 and Vav3 Have Critical but Redundant Roles in Mediating Platelet Activation by Collagen*

Received for publication, September 9, 2004
Published, JBC Papers in Press, September 27, 2004, DOI 10.1074/jbc.M410355200

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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 Cγ (PLCγ) 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 PLCγ2 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 PLCγ2. The phenotype of Vav1/2/3 triple-deficient platelets is similar to that of Vav1/3 double-deficient cells. These results demonstrate that Vav3 and Vav1 play crucial but redundant roles in the activation of PLCγ2 by GPVI. This is the first time that absolute redundancy between two protein isoforms has been observed with respect to the regulation of PLCγ2 in platelets.

Collagen is the most thrombogenic component of the subendothelial matrix, inducing powerful platelet activation through the GPVI[1]-FcRγ-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 FcRγ-chain immunotyrosine-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 Cγ (PLCγ). The functional role of many of the proteins in this cascade, including GPVI (5), FcRγ chain (1), Syk (1), LAT (6), Gads (7), SLP-76 (8), Btk (9), Tec (9), and PLCγ2 (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 activation 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.

* This work was supported in part by the Wellcome Trust, the British Heart Foundation, and the Biotechnology and Biological Sciences Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡‡ Holds a Medical Research Council Senior Fellowship.

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[1] The abbreviations used are: GPVI, glycoprotein VI; ITAM, immunotyrosine-based activation motif; PI, phosphatidylinositol; PLCγ, phospholipase Cγ; 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 tetrakis(acetoxymethyl 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 hematopoeitic 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 Ca^{2+} in response to T-cell receptor cross-linking. Significantly, the proliferation defects can be partially rescued through restoration of the Ca^{2+} flux with ionophore (20). Cytoskeletal remodeling, formation of cap structures, and T-cell receptor clustering are also defective in Vav1-deficient T-cells (21, 22). The loss of Vav1 from B-cells causes mild defects in B-cell development and signaling via the B-cell receptor (23). However, Vav2 is also required for these processes and a stronger phenotype is seen in Vav1/Vav2-deficient B-cells, demonstrating a degree of redundancy in this system. $Vav1^{-/-}Vav2^{-/-}$ mice display a block in B-cell development and a severe impairment of phosphorylation of PLCγ2 and Ca^{2+} mobilization following antigen receptor triggering (24, 25). Vav3 is also expressed in B- and T-cells and has recently been shown to play a role in regulating PLCγ phosphorylation downstream of the B- and T-cell receptors (26, 27). It is presently unclear whether the role of Vav family proteins downstream of ITAM receptors is a consequence of their GDP/GTP exchange factor activity or through their function as adapter proteins.

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, $\alpha_{IIb}\beta_3$ (28). Alternatively, the lack of a role for Vav1 and Vav2 in platelets may represent a novel mechanism of ITAM coupling to PLCγ. In the present study, we demonstrate that Vav3 is tyrosine phosphorylated downstream of GPVI in platelets but that, as with Vav1, platelets deficient in Vav3 are activated normally by GPVI. Strikingly, however, platelets double-deficient in Vav1 and Vav3 show a marked impairment in functional responses, which is associated with loss of phosphorylation of PLCγ2. A similar phenotype is seen in platelets deficient in all three Vav proteins. Some of this work has previously been presented in abstract form (29).

MATERIALS AND METHODS

Antibodies and Reagents—Anti-phosphotyrosine monoclonal antibody 4G10 and anti-Lat polyclonal antibody were purchased from Upstate Biotechnology (TCS Biologicals Ltd., Bucks, UK). Anti-human Vav3 antibodies were raised in rabbits as described previously (30). Anti-mouse Vav3 antibodies were raised in rabbits against a peptide of amino acids 556–590 of murine Vav3. Anti-Vav2 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 phalloidin was from Molecular Probes (Leiden, The Netherlands). The RC DC protein assay kit was from Bio-Rad (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 Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, 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 5×10^8/ml in Tyrodes-HEPES buffer.

Preparation of Mouse Platelets—Blood was taken from a terminally CO_2-narcosed mouse by cardiac puncture on the day of the experiment into 1:10 (v:v) acid citrate dextrose. Blood was diluted 1:6 (v:v) in Tyrodes-HEPES and centrifuged at 200 × *g* to obtain PRP. PRP was centrifuged in the presence of 0.1 μg/ml prostacyclin at 1000 × *g*. The platelet pellet was resuspended at a concentration of 5×10^8/ml in Tyrodes-HEPES.

Platelet Stimulation and Aggregation—Mouse platelets were used at a concentration of 2×10^8/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 PP1 (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 Na_3VO_4, 200 μg/ml 4-(2-aminoethyl)benzenesulfonyl 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. The protein concentration of each sample was measured using the Bio-Rad RC 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 3×10^7 platelets/ml, were transferred to the slides and incubated at 37 °C for 45 min in a humid atmosphere. Excess platelets were removed and the platelets 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 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, Southnatick, 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-

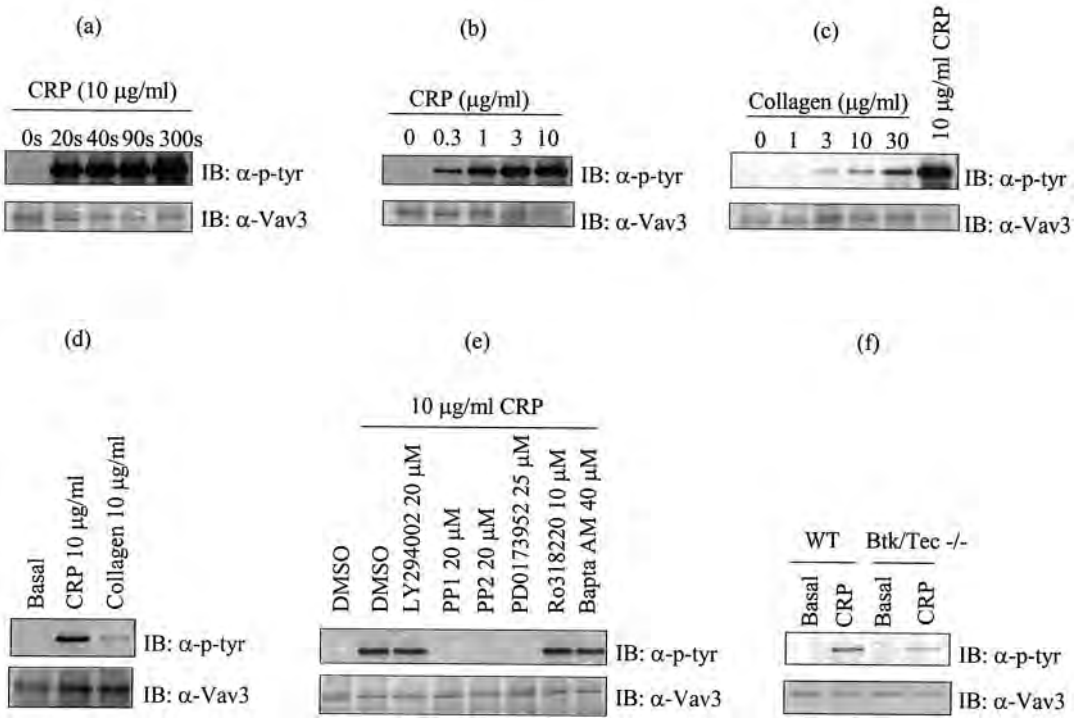

FIG. 1. **Vav3 is tyrosine-phosphorylated by GPVI.** Vav3 was immunoprecipitated from lysates of human (*a*, *b*, *c*, and *e*) or mouse (*d* and *f*) platelets. Protein complexes were separated by SDS-PAGE and sequentially Western blotted for phosphotyrosine (*α-p-tyr*, *top panels*) and Vav3 (*α-Vav3*, *bottom panels*). *e*, human platelets were preincubated with the inhibitors for 10 min. *DMSO*, dimethyl sulfoxide. Results are representative of three to five experiments. *IB*, immunoblotted; *WT*, wild type.

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. 1*a*). Tyrosine phosphorylation of Vav3 was maximal between 3 and 10 μg/ml CRP (Fig. 1*b*). 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. 1*c*). Vav3 is tyrosine-phosphorylated in murine platelets in response to CRP and collagen, with the former again giving a more robust response (Fig. 1*d*). These results highlight a potential role for Vav3 in the GPVI signaling cascade.

Vav3 Phosphorylation Is Dependent on Src, Syk, and Btk/Tec 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. 1*e*), although it may not necessarily be regulated directly by Src kinases. Vav3 tyrosine phosphorylation was also abolished in response to CRP in murine platelets deficient in the tyrosine kinase Syk (data not shown) and reduced in platelets deficient in the Tec family kinases, Btk, and Tec (Fig. 1*f*). In contrast, tyrosine phosphorylation of Vav3 was not altered in the presence of the protein kinase C inhibitor Ro 318220 (10 μM), the intracellular calcium chelator, BAPTA-AM (40 μM), or the PI 3-kinase inhibitor, LY294002 (20 μM) (Fig. 1*e*). These results show that CRP-induced tyrosine phosphorylation of Vav3 is completely dependent on Src family kinases and Syk and is partially dependent on Tec family kinases. 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. 2*a*) 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. 2*b*). Tyrosine phosphorylation of Syk, LAT, SLP-76, and PLCγ2 in response to CRP was also not altered in Vav3-deficient platelets (Fig. 2*c*).

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. 3*a*), although at maximal concentrations of CRP a small shape change response and weak aggregation could be seen (Fig. 3*a*). In comparison, aggregation to a submaximal concentration of the G protein-coupled receptor agonist thrombin was not altered (Fig. 3*a*). 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. 3*b*).

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 arterioles. 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

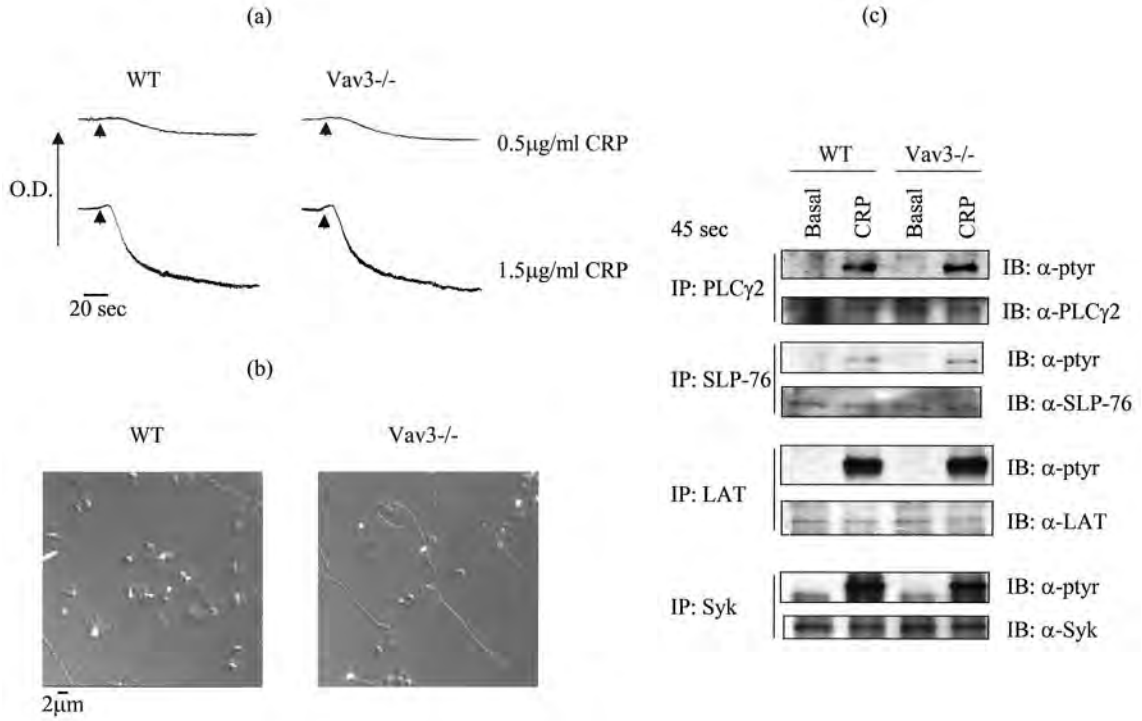

FIG. 2. **Vav3−/− platelets are activated normally by GPVI.** *a*, platelets from wild type (*WT*) or Vav3-deficient mice were stimulated with the indicated concentration of agonist in a Born aggregometer with continuous stirring in the absence of lotrafiban, apyrase, or indomethacin. The reduction in optical density (*O.D.*), indicative of platelet aggregation, was plotted against time. Addition of agonist is indicated by an *arrowhead*. *b*, platelets from wild type (*WT*) and Vav3-deficient mice were incubated on collagen-coated coverslips. After 45 min adherent platelets were fixed and imaged by differential interference contrast microscopy. *c*, PLCγ2, SLP-76, LAT, and Syk were immunoprecipitated (*IP*) from CRP-stimulated lysates of platelets from wild type (*WT*) and Vav3-deficient mice, separated by SDS-PAGE, and sequentially Western blotted for phosphotyrosine (*α-p-tyr*, *top panel*) and Vav3 (*α-Vav3*, *bottom panel*). Results are representative of three to five experiments. *IB*, immunoblotted.

tubes were subsequently flushed with lysis buffer and the resulting lysates blotted for actin as an indication of total platelet protein. Robust thrombus formation was observed in wild type platelets, whereas this was restricted to adhesion of single platelets in the absence of Vav1/Vav3 (Fig. 3*c(i)*). This reduction in platelet recruitment was confirmed by the reduction in the level of actin (Fig. 3*c(ii)*).

To investigate the molecular basis of the defect in platelet activation in the absence of Vav family proteins, we measured tyrosine phosphorylation of PLCγ2, the major PLCγ isoform in platelets, in platelet suspensions stimulated by CRP. Tyrosine phosphorylation of PLCγ2 was reduced in Vav1/3-deficient platelets but was not abolished (Fig. 3*d*). This is in contrast to the result observed in the absence of either Vav isoform alone, where phsophorylation is not altered. In contrast, tyrosine phosphorylation of Syk, which lies upstream of phosphorylation of Vav1 and Vav3, was not altered (Fig. 3*d*). In addition, there was a slight reduction in tyrosine phosphorylation of SLP-76 and LAT in Vav1/Vav3-deficient platelets (Fig. 3*d*). These results demonstrate a redundant role of Vav1 and Vav3 in the regulation of PLCγ2 and platelet activation by GPVI. This role is downstream of the tyrosine kinase Syk and may be mediated in part through regulation of tyrosine phosphorylation of LAT and SLP-76. These results are consistent with a role for Vav1 and Vav3 in the assembly of the PLCγ2 signalosome in platelets.

Platelets from Vav1/2/3-deficient Mice Are Identical to Platelets from Vav1/3-deficient Mice—To investigate whether the residual response observed in the absence of Vav1 and Vav3 was due to the presence of Vav2, we compared activation of Vav1/Vav3 double-deficient platelets with Vav1/Vav2/Vav3 triple-deficient cells. The triple-deficient platelets displayed the same decrease in aggregation (Fig. 4*a*) and spreading (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. 4*a*). This is accompanied by a residual degree of tyrosine phosphorylation of PLCγ2 in response to CRP. Syk phosphorylation was not altered (Fig. 4*b*). Thrombin-induced aggregation of Vav1/Vav2/Vav3 triple-deficient platelets was not significantly altered relative to controls (Fig. 4*a*). 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 PLCγ2 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 within the family, lysates of Vav1-, Vav3-, and Vav1/3-deficient platelets were Western blotted for other Vav isoforms (Fig. 5*a*). The level of expression of Vav1 in platelets deficient in Vav3 was similar to that in wild type platelets (Fig. 5*a*, *left panels*). A similar picture is seen for expression of Vav3 in the presence or absence of Vav1 (Fig. 5*a*, *middle panels*). Expression of Vav2 is not affected by deficiency of Vav1 and Vav3 (Fig. 5*a*, *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

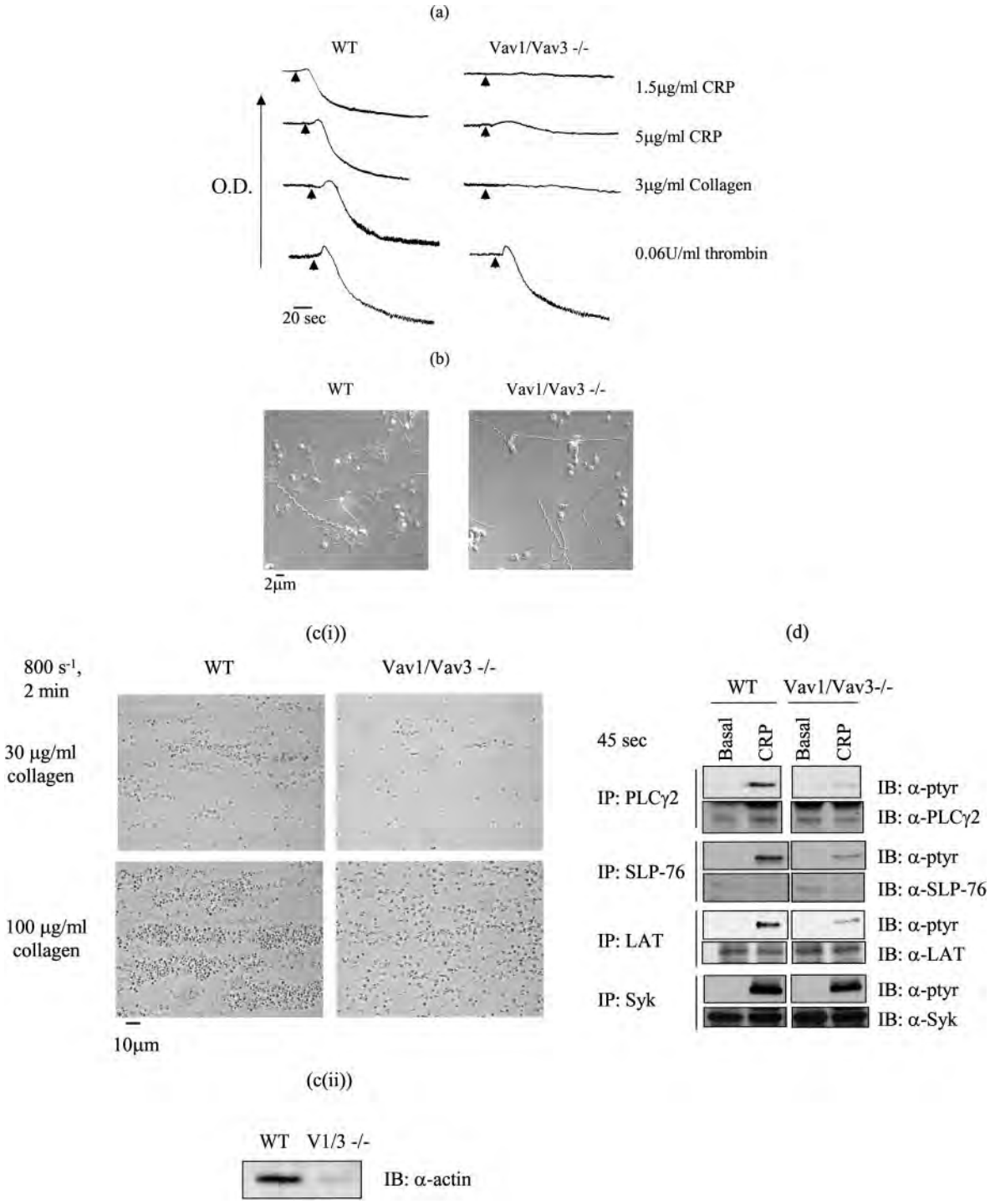

FIG. 3. **Vav1/Vav3 double-deficient platelets exhibit impaired activation by GPVI.** *a*, aggregation of platelets from wild type (*WT*) and Vav1/Vav3-deficient mice was measured as described in the legend to Fig. 2. *b*, spreading of platelets from wild type (*WT*) and Vav1/Vav3-deficient mice was measured as described in the legend to Fig. 2. *c(i)*, whole blood from wild type (*WT*) or Vav1/Vav3-deficient mice was flowed through capillary tubes coated in the presence of the indicated concentration of collagen. Blood was flowed for 2 min at a shear force of 800 s^{-1}. Thrombi were imaged using phase contrast light microscopy. *c(ii)*, capillary tubes of experiments using 100 μg/ml collagen were eluted in lysis buffer, and whole cell lysates were Western blotted for actin (*α-actin*). *WT*, wild type; *IB*, immunoblotted. *d*, the indicated proteins were immunoprecipitated (*IP*) from CRP-stimulated lysates of platelets from wild type (*WT*) or Vav1/Vav3-deficient mice, separated by SDS-PAGE, and sequentially Western blotted for phosphotyrosine (*α-p-tyr*, *upper panels*) and the immunoprecipitated proteins (*lower panels*). Results are representative of three to five experiments. *IB*, immunoblotted.

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. 5*b*). In comparison, the level of expression of Vav2 is significantly lower in murine platelets than in spleno-

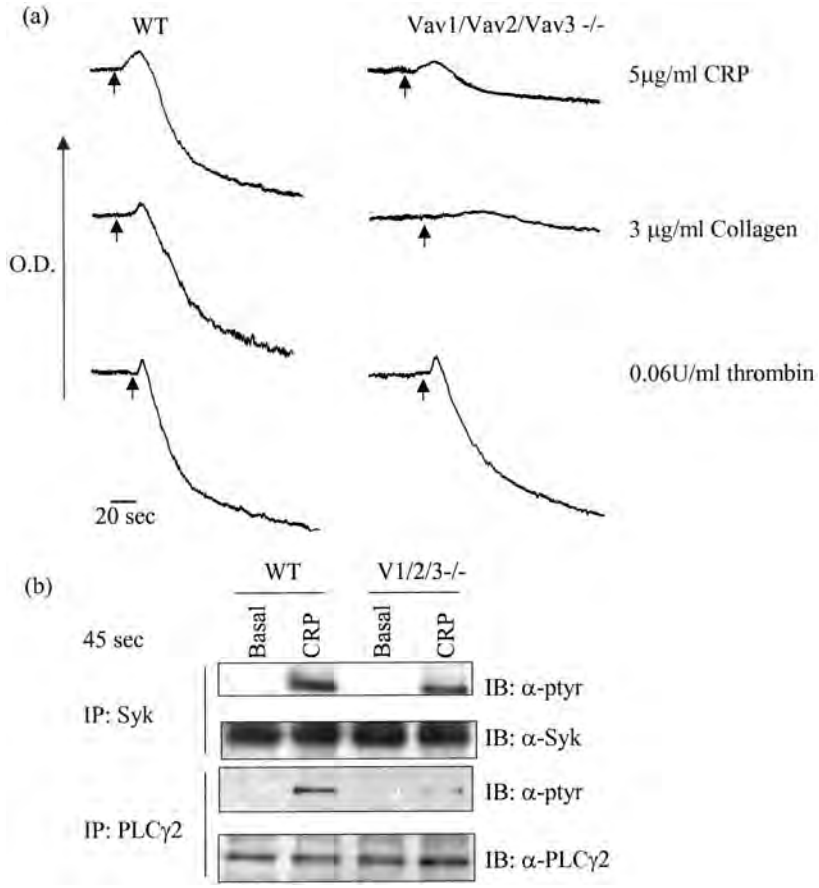

FIG. 4. **Vav1/Vav2/Vav3-deficient platelets are identical to Vav1/Vav3-deficient platelets.** *a*, aggregation of platelets from wild type (*WT*) or Vav1/Vav2/Vav3-deficient mice was measured as described in the legend to Fig. 2. *O.D.*, optical density. *b*, proteins were immunoprecipitated (*IP*) from CRP-stimulated lysates of platelets from wild type (*WT*) or Vav1/Vav2/Vav3-deficient mice as described in the legend to Fig. 1. 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.

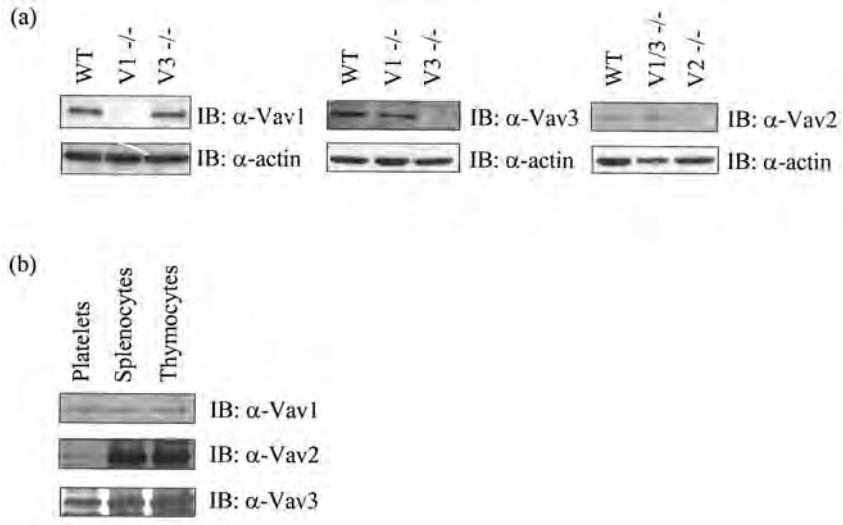

FIG. 5. **Expression levels of Vav proteins in platelets.** *a*, basal platelet whole cell lysates from wild type (*WT*), Vav1-, Vav3-, and Vav1/Vav3-deficient platelets were separated by SDS-PAGE and Western blotted for Vav1, Vav3, or Vav2 (*top panels*) and subsequently blotted for actin as a loading 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-

ating platelet activation by GPVI. GPVI, like other ITAM-coupled receptors such as B- and T-cell antigen receptors, requires Vav family proteins to efficiently activate PLCγ.

The residual aggregation and phosphorylation of PLCγ2 that was seen in the absence of Vav family proteins in platelets demonstrates that GPVI is able to signal to a minimal extent in the absence of the GDP/GTP family of exchange factors. A similar observation was made in Vav1/Vav2/Vav3 triple-deficient B-cells, which exhibit a weak Ca^{2+} flux in response to B-cell receptor cross-linking (27). Thus, while Vav family proteins play a critical role in regulation of PLCγ isoforms, it appears that their function can be by-passed to a very limited degree. This observation resembles the case for many of the other proteins that are recruited to the GPVI-driven PLCγ2 signalosome in platelets. For example, a residual degree of platelet activation in response to GPVI is seen in platelets deficient in the Tec kinases, Btk and Tec (9), in the absence of the adapters SLP-76 or LAT (6, 8) or in the presence of the PI 3-kinase inhibitors wortmannin or LY294002 (34). Thus, it appears that the GPVI signaling pathway requires a large number of proteins for optimal regulation of PLCγ2 but that a limited degree of signaling can take place in their absence. Thus it seems as if very few proteins are absolutely essential for the regulation of PLCγ2 but that the majority are required for optimal signaling through the cascade.

The mechanism through which Vav proteins regulate PLCγ isoforms is unclear. Vav proteins have been proposed to control 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 (12). In addition, Vav proteins have been shown to directly bind the p85α catalytic subunit of PI 3-kinase (36). It is noteworthy, however, that inhibition of PLCγ1 phosphorylation is greater in Vav1$-/-$ thymocytes than in the presence of PI 3-kinase inhibition demonstrating that its role is not simply to maintain the formation of 3-phosphorylated lipids (37). The observation that the inducible association of PLCγ1 with the SLP-76·Gads complex is defective in Vav1$-/-$ thymocytes (37), coupled with the observation of reduced tyrosine phosphorylation of LAT in Vav1$-/-$ thymocytes (38), suggest that the role of Vav proteins may be to stabilize the PLCγ signalosome by serving as adapters. This is consistent with the slightly reduced phosphorylation of LAT and SLP-76 observed in the present study.

One observation of general interest is the apparent absence of a defect in the separation of the vascular and lymphatic systems in the double or triple deficient Vav mice, which is seen in mice-deficient in Syk, SLP-76, and PLCγ2 (39). This underscores the fact that impairment in platelet activation by GPVI (or by other ITAM receptors) is not responsible for this defect. In this context, it is of interest that Syk, SLP-76, and PLCγ2 also participate in signaling by integrins and that Vav1 and Vav3 are also tyrosine-hosphorylated downstream of GPIIb-IIIa in platelets (28, 40). We previously reported a small defect in late-stage aggregation in Vav1-deficient platelets, which we attributed to a decrease in outside-in signaling through the integrin (12). This defect was not seen in the absence of Vav3 or in the combined absence of Vav1 and Vav3 in this study, a result that may be due to the different background of the Vav3 and Vav1/Vav3 knock-out mice.

In summary, we have shown that Vav1 and Vav3 play important and redundant roles in the regulation of PLCγ2 by GPVI in platelets. This redundancy is unique as it is complete; unlike Vav deficiencies in other cells, removal of either of the proteins has no discernable effect on signaling by GPVI, 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.

Acknowledgments—We gratefully acknowledge Helen Reynolds and the Small Animal Barrier Unit staff for animal care. We thank Dr. Owen McCarty for help with the imaging studies.

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Mechanisms of Signal Transduction:

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J. Biol. Chem. 2004, 279:53955-53962.
doi: 10.1074/jbc.M410355200 originally published online September 27, 2004