

# Differential Requirement for LAT and SLP-76 in GPVI versus T Cell Receptor Signaling

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## Abstract

Mice deficient in the adaptor Src homology 2 domain-containing leukocyte phosphoprotein of 76 kD (SLP-76) exhibit a bleeding disorder and lack T cells. Linker for activation of T cells (LAT)-deficient mice exhibit a similar T cell phenotype, but show no signs of hemorrhage. Both SLP-76 and LAT are important for optimal platelet activation downstream of the collagen receptor, GPVI. In addition, SLP-76 is involved in signaling mediated by integrin  $\alpha$ Ib $\beta$ 3. Because SLP-76 and LAT function coordinately in T cell signal transduction, yet their roles appear to differ in hemostasis, we investigated in detail the functional consequences of SLP-76 and LAT deficiencies in platelets. Previously we have shown that LAT<sup>-/-</sup> platelets exhibit defective responses to the GPVI-specific agonist, collagen-related peptide (CRP). Consistent with this, we find that surface expression of P-selectin in response to high concentrations of GPVI ligands is reduced in both LAT- and SLP-76-deficient platelets. However, platelets from LAT<sup>-/-</sup> mice, but not SLP-76<sup>-/-</sup> mice, aggregate normally in response to high concentrations of collagen and convulxin. Additionally, unlike SLP-76, LAT is not tyrosine phosphorylated after fibrinogen binding to integrin  $\alpha$ Ib $\beta$ 3, and collagen-stimulated platelets deficient in LAT spread normally on fibrinogen-coated surfaces. Together, these findings indicate that while LAT and SLP-76 are equally required for signaling via the T cell antigen receptor (TCR) and pre-TCR, platelet activation downstream of GPVI and  $\alpha$ Ib $\beta$ 3 shows a much greater dependency on SLP-76 than LAT.

Key words: SLP-76 • LAT • GPVI • integrin  $\alpha$ Ib $\beta$ 3 • T cell receptor

## Introduction

Adaptor proteins lack intrinsic effector functions, but contain distinct modular domains which mediate protein-protein interactions. These molecules thus serve as the scaffolding around which effectors and their substrates are assembled into signaling complexes (1). Two such hematopoietic cell-specific adaptors are the Src homology 2

(SH2)\* domain-containing leukocyte phosphoprotein of 76 kD (SLP-76; reference 2), and linker for activation of T cells (LAT; reference 3). Expression of SLP-76 and LAT is restricted to T cells, NK cells, macrophages, mast cells, and

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\*Abbreviations used in this paper: CRP, collagen-related peptide; Cvx, convulxin; GEM, glycolipid-enriched membrane microdomain; GFP, green fluorescent protein; LAT, linker for activation of T cells; Rag, recombination activating gene; SEM, scanning electron microscopy; SH2, Src homology 2; SLP-76, SH2 domain-containing leukocyte phosphoprotein of 76 kD.

megakaryocyte-lineage cells (2, 4, 5). SLP-76 is composed of three modular domains including an NH<sub>2</sub>-terminal acidic region which contains key tyrosine residues (2), a central proline-rich region and a COOH-terminal SH2 domain. Tyrosine residues within the acidic region of SLP-76 become phosphorylated after TCR ligation (6, 7), and then bind the guanine nucleotide exchange factor, Vav (8–11), the adaptor, Nck (12), and the Tec family kinase, Itk (13–15). In vivo reconstitution studies have demonstrated that the NH<sub>2</sub>-terminal tyrosines are critical for normal T cell development and TCR function (16), however, which of these intermolecular interactions are most critical for these events remains to be determined. The SH2 domain of SLP-76 binds to the SLP-76-associated phosphoprotein of 130 kD, SLAP-130/FYB (17, 18). SLAP-130/FYB is also an adaptor molecule and has been implicated in proliferation and integrin function in T cells (19, 20), and has been shown to colocalize and associate with vasodilator-stimulated phosphoprotein (VASP) in platelets (21, 22). The central proline-rich region of SLP-76 is required for constitutive association with the Grb-2 family member Gads (23–25). After antigen recognition by the TCR, Gads associates with LAT bringing SLP-76 to the membrane and localizing it to glycolipid-enriched membrane microdomains (GEMs) (26, 27), specialized regions within the membrane in which signal transduction complexes are formed (28). GEMs can also be isolated from platelets, where their role is under investigation (29).

The transmembrane adaptor LAT is a 36-kD protein which is resident in GEMs due to palmitoylation of two conserved cysteine residues (26). Within the cytoplasmic domain of LAT are six tyrosine residues which in T cells become phosphorylated after activation of the Syk family member ZAP-70. These phosphorylated tyrosines are critical for T cell activation (30, 31) and mediate protein-protein interactions with multiple proteins including Gads, Grb2, PI3K, and PLC $\gamma$ 1 (3, 23–25). In platelets, LAT is part of a large signaling complex and coprecipitates with SLP-76, PLC $\gamma$ 2, Cbl, Grb2, Gads, and Src kinase-associated phosphoprotein homologue (SKAP-HOM) (32).

Much has been learned about the functions of SLP-76 and LAT through analysis of mice made deficient for either molecule. SLP-76-null mice lack peripheral T cells (33, 34) and have reduced viability associated with perinatal hemorrhage (35). In addition, platelets from SLP-76<sup>-/-</sup> animals fail to phosphorylate PLC $\gamma$ 2 or aggregate in response to collagen (35, 36), and show a defect in spreading downstream of the fibrinogen receptor, integrin  $\alpha$ IIB $\beta$ 3 (37). LAT-deficient mice also lack T cells; however, they are born at expected frequencies and show no signs of bleeding (38). We have shown previously that LAT<sup>-/-</sup> platelets have impaired responses to collagen-related peptide (CRP), including decreased PLC $\gamma$ 2 phosphorylation and granule release (39).

As SLP-76 and LAT inducibly associate via a third adaptor, Gads (23–25), and mice null for each of these proteins exhibit defects in T cell development and activation (33, 34, 38, 40), we next asked if these molecules function co-

ordinately and are each required similarly for T cell versus platelet activation. Using biochemical and genetic approaches, we show that, in contrast to SLP-76, LAT is dispensable for platelet aggregation in response to high concentrations of GPVI collagen receptor agonists and for platelet spreading on the  $\alpha$ IIB $\beta$ 3 ligand, fibrinogen. Furthermore, while the phosphorylated tyrosine residues of SLP-76 are critical for both T cell development and platelet activation, the Gads binding domain is dispensable for platelet spreading on fibrinogen, but important for platelet granule release and normal T cell development.

## Materials and Methods

**Antibodies, Reagents, and Mice.** Anti-P-selectin was from BD PharMingen and anti-PLC $\gamma$ 2 was from Santa Cruz Biotechnology, Inc. Purified human fibrinogen and  $\alpha$ -thrombin were from Enzyme Research Laboratories, and collagen from Chronolog Corp. Purified convulxin (Cvx) from the venom of *Crotalus durissus terrificus* was the gift of Mark Kahn (University of Pennsylvania; reference 41). CRP (GKP\*(GPP)<sub>10</sub>GKP\*G, where P represents hydroxyproline) was cross-linked via lysine residues as described previously (42). LAT-deficient mice (38) were the gift of Larry Samelson (National Institutes of Health, Bethesda, MD). SLP-76-deficient mice have been described previously (31) as have Gads-deficient mice (40). SLP-76<sup>-/-</sup>, LAT<sup>-/-</sup>, and Gads-deficient mice were housed under pathogen-free conditions at the University of Pennsylvania or the Washington University Animal Care Facilities under National Institutes of Health guidelines and approved animal protocols. Recombinant hematopoietic growth factors were from R&D Systems.

**Platelet Isolation.** Whole blood was obtained from mice by cardiac puncture and anticoagulated with 0.1 vol acidic-citrate-dextrose. 4 ml of Pipes-Saline buffer (150 mM NaCl, 20 mM PIPES, pH 6.5) were added and platelet-rich plasma (PRP) was obtained by centrifugation at 200 g for 20 min at 25°C. Washed platelets were then obtained by adding apyrase (1 U/ml final concentration) and prostaglandin E1 (1  $\mu$ M), centrifuging at 600 g for 20 min at 25°C, and resuspending the platelet pellet in a modified Tyrode's buffer (134 mM NaCl, 2.9 mM KCl, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>-12H<sub>2</sub>O, 12 mM NaHCO<sub>3</sub>, 20 mM HEPES, 1 mM MgCl<sub>2</sub>, 5 mM glucose, pH 7.35). Human whole blood was obtained from medication-free volunteers and washed platelets were prepared as described (43).

**Aggregation, Immunoprecipitation, and Western Blotting.** Washed murine platelets were resuspended to a concentration of 4  $\times$  10<sup>8</sup>/ml in a volume of 250  $\mu$ l modified Tyrode's buffer. Aggregation was assayed using a Chronolog aggregometer to measure light transmission. After a 3-min aggregation period, platelets were lysed using 2 $\times$  NP-40 buffer including inhibitors of proteases (50  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 50  $\mu$ g/ml pepstatin A, and 1 mM pefablock) and phosphatases (400  $\mu$ M sodium vanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate). Clarified lysates were subjected to immunoprecipitation with anti-human LAT, immunoprecipitates were probed on Western blots with anti-phosphotyrosine mAbs, 4G10 (Upstate Biotechnology) and PY20 (Transduction Laboratories), and immunoreactive bands were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

For human platelet studies, bacterial Petri dishes were coated overnight at 4°C with 100  $\mu$ g/ml fibrinogen or 5 mg/ml BSA in coating buffer (150 mM NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 50 mM

Na<sub>2</sub>HPO<sub>4</sub>, pH 8.0), followed by blocking in 5 mg/ml BSA for 2 h at room temperature. Platelet adhesion to fibrinogen was performed by adding washed platelets (10<sup>8</sup> platelets/ml) to the dishes or coverslips for 30 min at 37°C. After removing nonadherent platelets from the fibrinogen-coated Petri dishes with three washes of PBS, adherent platelets were lysed for 30 min on ice in 1% RIPA lysis buffer including inhibitors of proteases and phosphatases. Platelets incubated in BSA-coated dishes served as suspension controls and were pelleted for 5 s at 16,000 g before lysis. Clarified lysates were subjected to immunoprecipitation with anti-human SLP-76(44) or anti-human LAT (Upstate Biotechnology), immunoprecipitates were probed on Western blots with anti-phosphotyrosine mAbs.

**Confocal Microscopy and Scanning Electron Microscopy.** Washed platelets were allowed to adhere to fibrinogen-coated coverslips for 45 min at 37°C. Coverslips were then washed and platelets fixed in 1% glutaraldehyde for scanning electron microscopy (SEM) or 3.7% formaldehyde followed by 1% Triton for confocal microscopy. Coverslips for SEM were dehydrated and sputter-coated with gold followed by analysis using an Hitachi S-4000 (University of Iowa Microscopy Facility). Coverslips for confocal microscopy were stained with 4G10 and PY20, Cy5-anti-mouse IgG and rhodamine-phalloidin, and confocal microscopy was performed as described (43).

**Flow Cytometry.** Washed murine platelets (10<sup>6</sup>) were stimulated with 0.5 U/ml human  $\alpha$ -thrombin, 40 or 10 nM Cvx, 40 or 20  $\mu$ g/ml CRP in the presence of 5  $\mu$ g/ml FITC anti-murine P-selectin or biotinylated anti-murine P-selectin along with streptavidin-allophycocyanin (APC), and binding was quantified by flow cytometry.

**Genetic Reconstitution of SLP-76-deficient Bone Marrow.** A 1.6-kb murine SLP-76 cDNA was cloned into the BglII site of the MigR1 vector (45). This BglII site lies directly 5' of an internal ribosome entry site (IRES) which is followed by a cDNA coding for enhanced green fluorescent protein (GFP). Bosc23 cells were transfected with MIGR1 and viral supernatants were collected as described (46). Bone marrow cells were harvested from the tibias and femurs of SLP-76-deficient mice and cultured for 4 d at a density of  $2 \times 10^6$  cells/ml in the presence of 6 ng/ml rmIL-3, 10 ng/ml rmIL-6, and 50 ng/ml rmSCF in DMEM containing 15% fetal calf serum and 5% WEHI-conditioned medium. At 24 and 48 h, cells were spin infected with retroviral supernatants followed by intravenous injection into lethally irradiated 4 to 6-wk-old C57/BL6 or RAG2<sup>-/-</sup> recipients as described (45). Mice were killed and tissues harvested for analysis 6–8 wk after reconstitution.

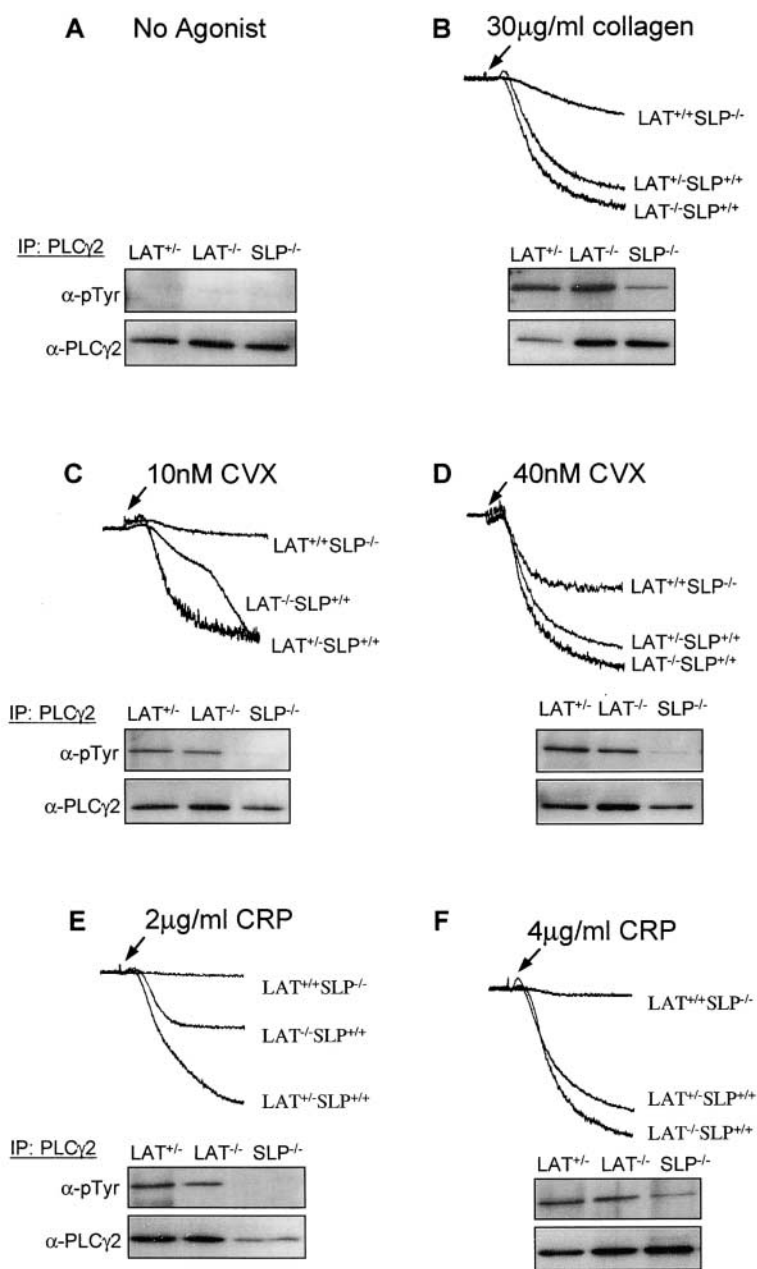
## Results

**LAT<sup>-/-</sup> Platelets Aggregate in Response to High Concentrations of Collagen.** The differential requirement of SLP-76 and LAT for hemostasis led us to further assess the roles of these molecules in platelet function. Previously we have shown that platelets from LAT<sup>-/-</sup> mice exhibit a marked reduction in both surface expression of P-selectin and phosphorylation of PLC $\gamma$ 2 in response to CRP (39). We therefore asked whether LAT-deficient platelets can respond to a more physiologic agonist, collagen. Surprisingly, aggregation in response to 30  $\mu$ g/ml collagen occurs similarly in platelets from LAT<sup>-/-</sup> and LAT<sup>+/-</sup> mice (Fig. 1 B). In comparison, SLP-76-deficient platelets exhibit only

minimal responses to this high concentration of collagen. Because limited amounts of material precluded a direct assessment of PLC $\gamma$ 2 function in response to collagen receptor ligation, we next examined inducible tyrosine phosphorylation of PLC $\gamma$ 2 which has been linked to its enzymatic activation. After a 3-min aggregation period, platelets were lysed and PLC $\gamma$ 2 was immunoprecipitated. Immune complexes were then separated by SDS-PAGE, transferred to nitrocellulose and blotted with anti-phosphotyrosine antibodies. As shown, LAT deficiency results in a modest reduction of PLC $\gamma$ 2 phosphorylation when platelets are treated with 30  $\mu$ g/ml collagen, even though LAT<sup>-/-</sup> platelets aggregate fully (Fig. 1 B). However, in the absence of SLP-76 there is a marked decrease in PLC $\gamma$ 2 tyrosine phosphorylation levels compared with that seen in SLP-76<sup>+/+</sup> platelets.

Because collagen is known to bind multiple surface receptors (47), we investigated whether the response of LAT-deficient platelets to high concentrations of collagen is due to signal transduction events occurring downstream of GPVI or through another collagen receptor. When activated with 10 nM of the potent GPVI-selective agonist, Cvx, there is intermediate aggregation and PLC $\gamma$ 2 phosphorylation in the absence of LAT, but no apparent response by either measure in the absence of SLP-76 (Fig. 1 C). A higher concentration of Cvx (40 nM) induces aggregation of LAT<sup>-/-</sup> platelets similarly to LAT<sup>+/-</sup> platelets (Fig. 1 D). At this dose, SLP-76<sup>-/-</sup> platelets also aggregate, however, to a far lesser degree than LAT<sup>-/-</sup> or LAT<sup>+/-</sup> cells. Similar to results seen with 30  $\mu$ g/ml collagen, the degree of PLC $\gamma$ 2 phosphorylation is slightly reduced in platelets deficient for LAT, while SLP-76-deficient platelets reveal a marked diminution in PLC $\gamma$ 2 phosphorylation (33).

A relatively low concentration (2  $\mu$ g/ml) of CRP induces a moderate aggregation response in LAT<sup>-/-</sup> platelets, but no detectable response in SLP-76<sup>-/-</sup> platelets (Fig. 1 E). This correlates with a modest increase in PLC $\gamma$ 2 phosphorylation in platelets deficient for LAT, and no detectable PLC $\gamma$ 2 phosphorylation in platelets deficient for SLP-76. When stimulated with 4  $\mu$ g/ml CRP, platelets from SLP-76-null animals fail to aggregate or inducibly phosphorylate PLC $\gamma$ 2 while LAT<sup>-/-</sup> platelets aggregate fully and exhibit increases in PLC $\gamma$ 2 phosphorylation (Fig. 1 F). It should be noted that the response to CRP appears to depend heavily on assay conditions, as we have found previously that under other activation conditions, CRP is a poor agonist for PLC $\gamma$ 2 phosphorylation and activation in LAT-deficient platelets compared with wild-type (39). The different results from our two studies are likely attributable to differences in stimulation conditions (in the previous work platelets were not stirred) reinforcing the notion that in the absence of LAT, more potent GPVI engagement is required for platelet activation. Together, these data demonstrate that unlike SLP-76-deficient platelets which manifest severe signaling defects after GPVI engagement, there is only a partial defect in these signaling pathways in LAT-null platelets.

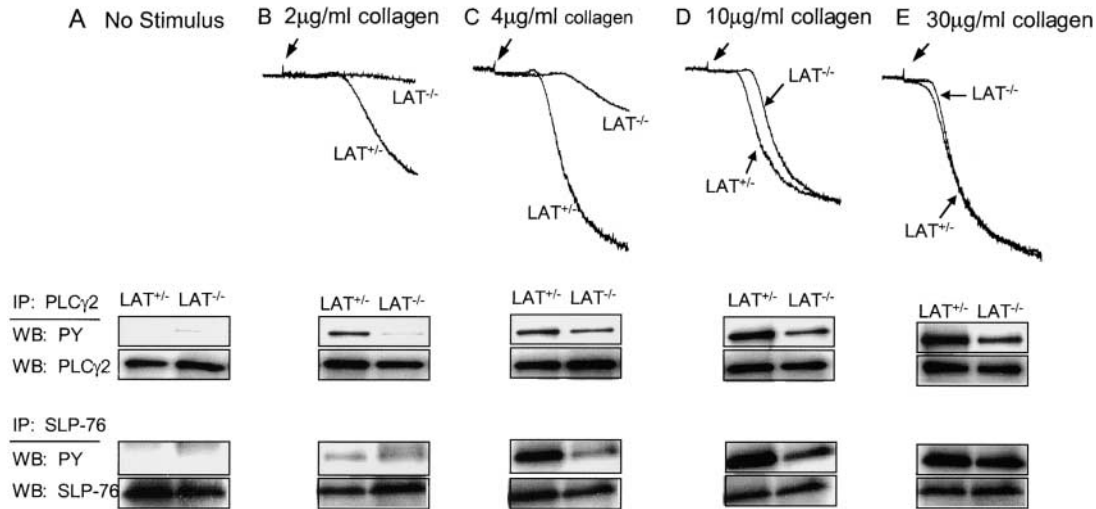


**Figure 1.** LAT<sup>-/-</sup> platelets, but not SLP-76<sup>-/-</sup> platelets, aggregate normally in response to potent GPVI agonists. Platelets isolated from either, LAT<sup>+/-</sup>SLP<sup>+/+</sup>, LAT<sup>-/-</sup>SLP<sup>+/+</sup>, or LAT<sup>+/+</sup>SLP<sup>-/-</sup> mice were (A) left unstimulated or stimulated (arrows) with (B) 30 μg/ml collagen, (C) 10 nM Cvx, (D) 40 nM Cvx, (E) 2 μg/ml CRP, or (F) 4 μg/ml CRP for 3 min with stirring in an aggregometer. Platelets were then lysed and PLCγ2 was immunoprecipitated followed by Western analysis for phosphotyrosine. Blots were stripped and reprobed with anti-murine PLCγ2.

*Dose-dependent Responses of LAT<sup>-/-</sup> Platelets to Collagen.* Because platelets from LAT-deficient mice aggregate in response to 30 μg/ml collagen but do not undergo efficient granule release in response to CRP, we next asked if this was due to the different agonists used or to intrinsic differences in aggregation and release responses to collagen. To address this, the responses of LAT-deficient and normal platelets were compared over a range of collagen concentrations. At the lowest concentration of collagen tested (2 μg/ml), LAT<sup>-/-</sup> platelets fail to aggregate (Fig. 2 B). Concentrations of collagen greater than or equal to 4 μg/ml induce aggregation of LAT<sup>-/-</sup> platelets (Fig. 2 C) and aggregation is nearly normal at 10 μg/ml collagen (Fig. 2 D); a dose which results in no response in SLP-76<sup>-/-</sup> platelets (data not shown). After the 3-min aggregation period,

platelets were lysed and PLCγ2 was immunoprecipitated followed by Western blotting with anti-phosphotyrosine antibodies. Western analysis revealed that at high concentrations of collagen, PLCγ2 is heavily phosphorylated in LAT<sup>-/-</sup> platelets, while at concentrations less than 5 μg/ml phosphorylation of PLCγ2 is significantly diminished. These data are consistent with a strength of signal model in which LAT modulates signal transduction events after collagen binding, but can be bypassed when platelets are activated with high concentrations of agonist.

*P-selectin Upregulation Is Reduced in Platelets from LAT-deficient Mice and Absent in Platelets from SLP-76-deficient Mice.* P-selectin expression at the platelet surface is a marker of platelet α-granule release. Due to the aggregation response of LAT-deficient platelets, we next asked if

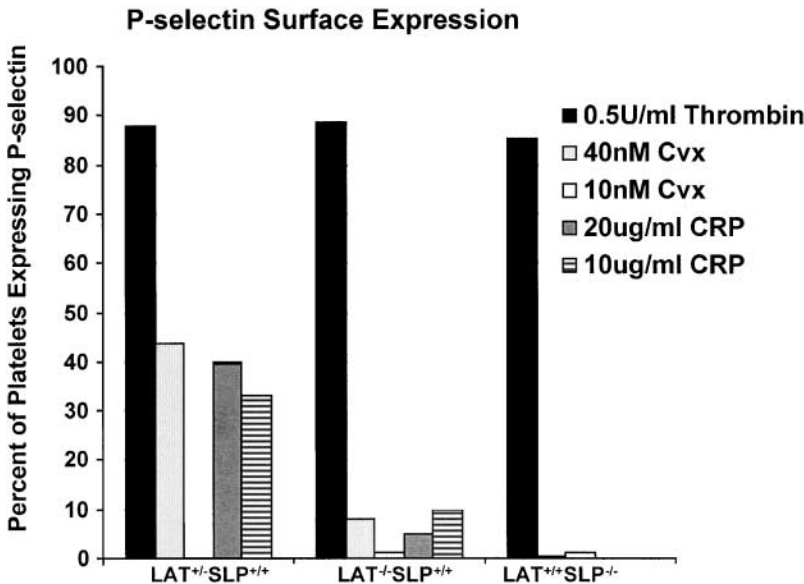


**Figure 2.** LAT is required for optimal platelet activation in response to collagen. Washed murine platelets were analyzed for aggregation responses to either (A) no stimulus (B) 2  $\mu\text{g/ml}$ , (C) 4  $\mu\text{g/ml}$ , (D) 10  $\mu\text{g/ml}$ , or (E) 30  $\mu\text{g/ml}$  collagen (arrows). After stimulation for 3 min,  $\text{LAT}^{+/+}$  and  $\text{LAT}^{-/-}$  platelets were lysed and PLC $\gamma$ 2 and SLP-76 were sequentially immunoprecipitated followed by Western analysis for phosphotyrosine. Blots were then stripped and reprobed for either PLC $\gamma$ 2 or SLP-76.

$\text{LAT}^{-/-}$  platelets undergo granule release normally in response to high concentrations of CRP and Cvx. Platelets from  $\text{LAT}^{+/+}$ ,  $\text{LAT}^{-/-}$ , and  $\text{SLP-76}^{-/-}$  mice express P-selectin in response to the G-protein-coupled receptor agonist, thrombin. In addition, platelets from LAT heterozygous littermates responded normally to Cvx and CRP, with as many as 42% of the platelets expressing P-selectin on their surface in response to 40nM Cvx (Fig. 3). In contrast, only 8% of  $\text{LAT}^{-/-}$  platelets respond to the highest concentration of Cvx, a dose which induces full platelet aggregation. Also as shown, LAT-deficient platelets exhibit defective responses to both 10 and 20  $\mu\text{g/ml}$  CRP.  $\text{SLP-76}^{-/-}$  platelets fail to inducibly express P-selectin in response to both

GPVI agonists. These results confirm that both LAT and SLP-76 are required for optimal granule release. Thus, it is possible that the aggregation defect with low concentrations of GPVI agonists observed in the LAT-deficient mice relates to defective release of positive feedback stimuli such as ADP from platelet granules.

*Differential Requirement for SLP-76 and LAT Downstream of  $\alpha\text{IIb}\beta$ 3 Engagement.* We have shown that in addition to defects in collagen signaling,  $\text{SLP-76}^{-/-}$  platelets exhibit defects in outside-in signaling downstream of integrin  $\alpha\text{IIb}\beta$ 3. Because both SLP-76 and Syk are inducibly phosphorylated after fibrinogen binding to  $\alpha\text{IIb}\beta$ 3 (22, 37, 48), we asked if LAT plays a role in this pathway. Washed hu-



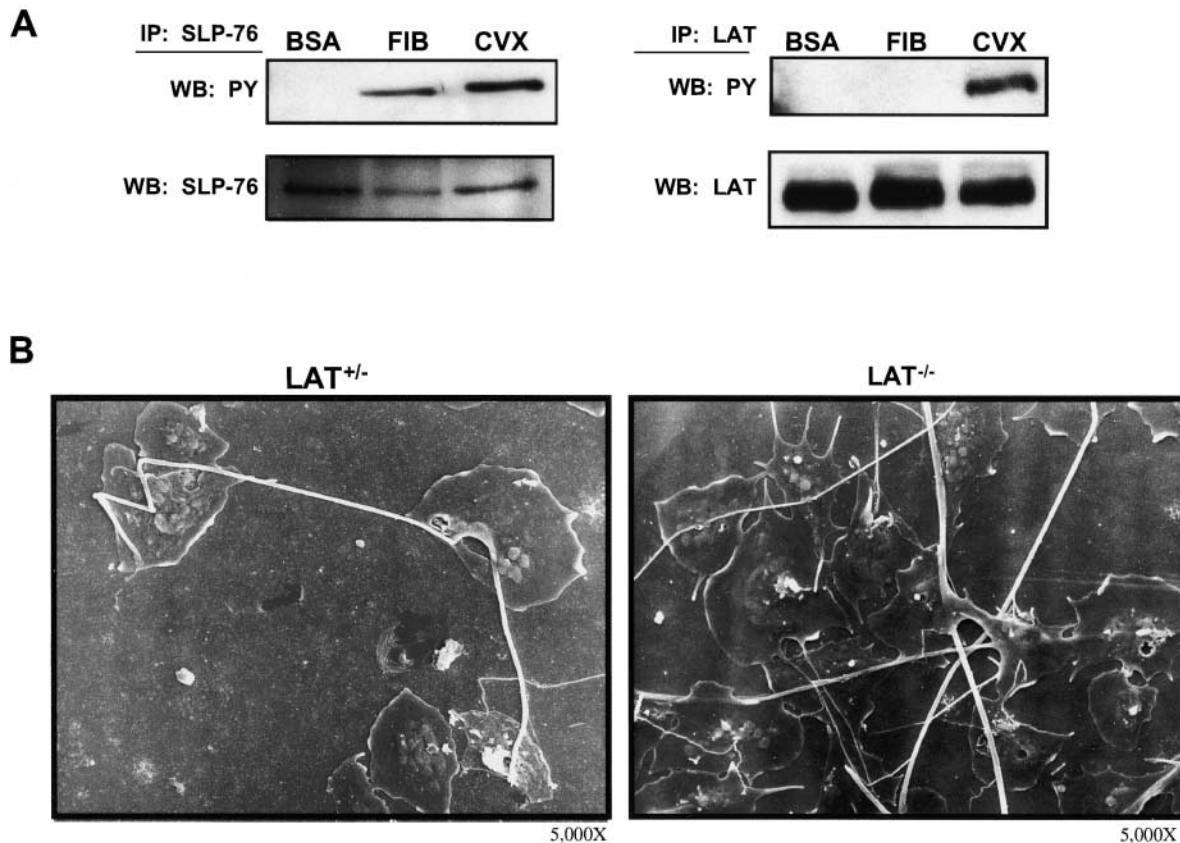
**Figure 3.** P-selectin upregulation is reduced in  $\text{LAT}^{-/-}$  platelets and absent in  $\text{SLP-76}^{-/-}$  platelets. Washed murine platelets were stimulated with 0.5 U/ml thrombin, 40 nM Cvx, 10 nM Cvx, 20  $\mu\text{g/ml}$  CRP, or 10  $\mu\text{g/ml}$  CRP for 10 min at 37°C in the presence of FITC- $\alpha$ -P-selectin followed by fixation and flow cytometric analysis. Results were then plotted as the percent of platelets staining positive for P-selectin.

man platelets were incubated on Petri plates coated with BSA, fibrinogen, or Cvx for 30 min at 37°C. As expected, nonadherent platelets incubated on BSA-coated plates do not exhibit tyrosine phosphorylation of either LAT or SLP-76. As we have shown previously, platelets adherent to fibrinogen manifest SLP-76 phosphorylation (22, 37). Surprisingly, there is no detectable tyrosine phosphorylation of LAT (Fig. 4 A), although both molecules are inducibly phosphorylated when platelets adhere to the GPVI ligand, Cvx. Consistent with these findings, LAT-deficient platelets activated with collagen spread normally on fibrinogen coated coverslips (Fig. 4 B). These data demonstrate that in addition to a LAT-independent signaling pathway downstream of GPVI, outside-in signaling through integrin  $\alpha$ IIb $\beta$ 3 can occur in the absence of this adaptor.

**Retroviral Rescue of SLP-76-deficient Mice.** The formation of a SLP-76/LAT complex has been shown to be critical for integration of signals initiated by the TCR (49). In T cells, SLP-76 binds constitutively to Gads which, in turn inducibly associates with LAT resulting in GEM localization of the signaling complex (27). Recently (16), we have found that transgenic reconstitution of SLP-76-deficient

thymocytes with wild-type SLP-76 rescues T cell development and function, whereas expression of a mutant variant of SLP-76 which cannot be phosphorylated on tyrosine residues fails to rescue these events. Expression of a mutant SLP-76 which cannot bind Gads results in delayed thymocyte development and severe defects in TCR signaling in peripheral T cells underscoring the importance of the SLP-76/Gads interaction for T cell function. However, given our findings that platelet activation appears to differentially require SLP-76 and LAT, we wondered if formation of the SLP-76/Gads/LAT complex is as important for platelet function as it appears to be for T cells.

As the transgenic constructs are not expressed in platelets, we used a retroviral reconstitution system to study the structure/function relationships of SLP-76 in this lineage. The retroviral system makes use of an IRES for expression of the marker gene, GFP, and the gene of interest from the same transcript. Four viral constructs were used to transduce SLP-76-deficient bone marrow: GFP alone (MigR1), full length murine SLP-76 (WT-SLP-76), SLP-76 in which each of the three critical tyrosine residues have been mutated to phenylalanine (Y3F-SLP-76), and SLP-76



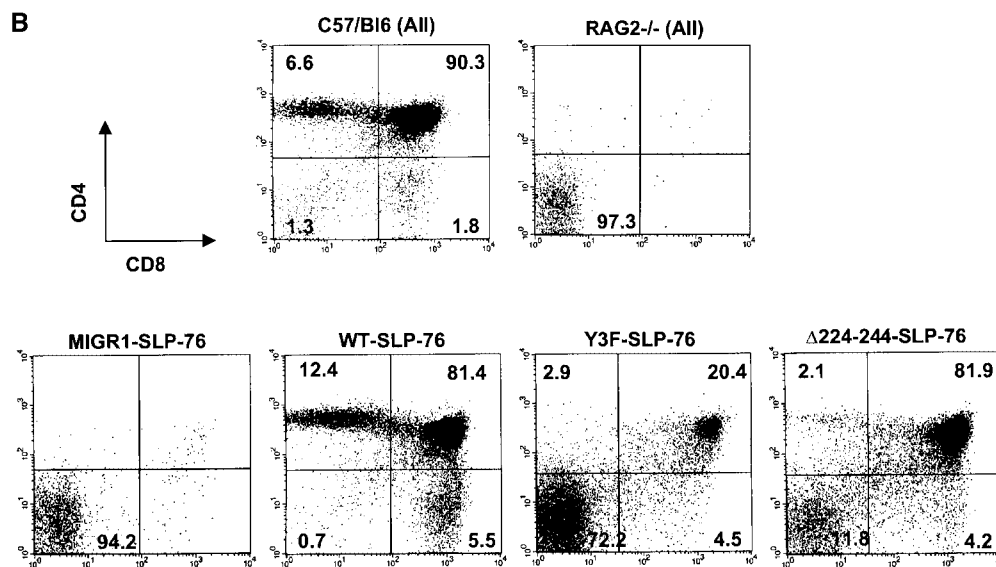
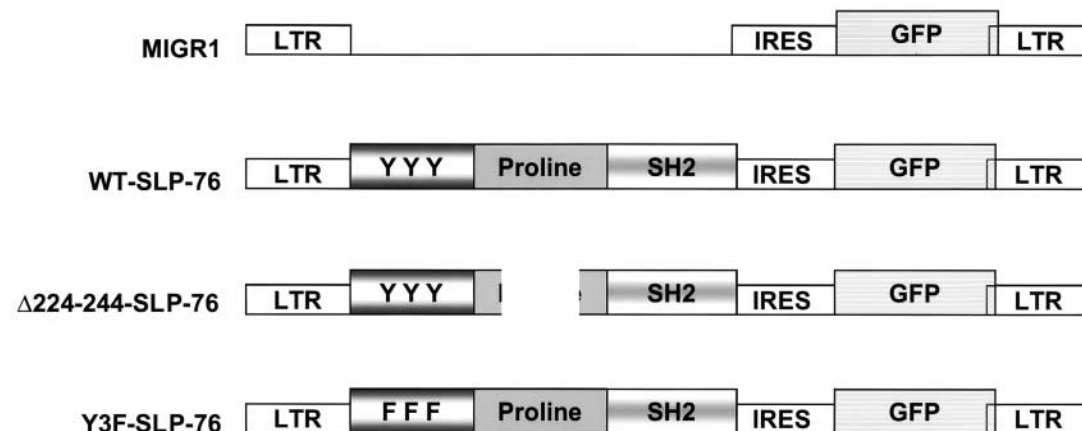
**Figure 4.** LAT<sup>-/-</sup> platelets spread in response to collagen and fibrinogen. (A) Washed human platelets were incubated for 30 min at 37°C on Petri dishes coated with 5 mg/ml BSA, 100  $\mu$ g/ml fibrinogen (FIB), or 1  $\mu$ g/ml Cvx. Nonadherent platelets were isolated by centrifugation (5 s, 16,000 g) and then lysed with RIPA buffer (BSA condition). Fibrinogen or Cvx-adherent platelets were lysed directly on the Petri dishes. Equal amounts of protein from each lysate were subjected to immunoprecipitation (IP) with anti-human SLP-76 or anti-human LAT followed by Western analysis for phosphotyrosine. Blots were reprobbed with anti-human SLP-76 or LAT to demonstrate equal loading. WB, Western blot. (B) Washed LAT<sup>+/-</sup> or LAT<sup>-/-</sup> platelets were incubated on coverslips coated with 100  $\mu$ g/ml fibrinogen. Cells were stimulated with 30  $\mu$ g/ml collagen for 45 min at 37°C followed by fixation and analysis by SEM. Each image is  $\times$ 5,000.

which cannot bind to Gads ( $\Delta 224-244$ -SLP-76) (Fig. 5 A). Lethally irradiated recombination activating gene ( $Rag2^{-/-}$ ) mice were reconstituted with the retrovirally transduced SLP-76 $^{-/-}$  bone marrow and  $\sim 6$  wk later, mice were killed and tissues harvested for study.

As a proof of principle, we first examined thymocyte development in the reconstituted mice (Fig. 5 B) and found that this readout in the transplanted mice is consistent with our findings using the T cell-specific transgenes. As shown, infection of SLP-76-deficient bone marrow with retrovirus encoding GFP alone (MigR1) fails completely to restore thymocyte development, while reconstitution by WT-SLP-76 rescues T cell development with restored thymic cellularity and the appearance of double-positive as well as CD4 and CD8 single-positive cells. The success of thymocyte de-

velopment (as evidenced by the number of single-positive cells) in the wild-type SLP-76 restored mice varies, but is always at least as efficient as seen after transfer of wild-type bone marrow. This is likely due to levels of SLP-76 achieved after retroviral infection, similar to what we have observed previously following rescue with SLP-76 transgenes (16). The data in Fig. 5 B demonstrate also that mutation of the three critical tyrosine residues within the NH<sub>2</sub> terminus of SLP-76 (Y3F-SLP-76) results in a profound decrease in both double- and single-positive cells. Deletion of the Gads binding domain ( $\Delta 224-244$ -SLP-76) results in an intermediate phenotype, also manifesting severe abnormalities in T cell developmental progression. The T cell phenotypes observed with the mutant variants of SLP-76 introduced by retroviral infection are also consistent with what

## A Retroviral Constructs



**Figure 5.** Structure/function analysis of SLP-76 by retroviral reconstitution. (A) Retroviral constructs used for coexpression of SLP-76 and GFP in transduced SLP-76 $^{-/-}$  hematopoietic cells. Virus expressing GFP alone (MigR1), or GFP with WT-SLP-76, SLP-76 which cannot be tyrosine phosphorylated (Y3F-SLP-76), or SLP-76 which cannot bind Gads ( $\Delta 224-244$ -SLP-76) were used to transduce SLP-76-deficient stem cells followed by transfer to lethally irradiated  $Rag2^{-/-}$  hosts. After  $\sim 6$  wk, mice were killed and tissues harvested for analysis. (B) Thymocytes were removed from the reconstituted mice, fixed, and stained for CD4 and CD8 followed by flow cytometric analysis. In the top two panels all thymocytes (All) from a nonmanipulated C57/Bl6 (a normal thymic profile) and  $Rag2^{-/-}$  (a profile from mice deficient for T cells) mouse were analyzed. For retrovirally reconstituted mice, gates were set to distinguish GFP-positive cells (GFP) which were then analyzed for CD4 and CD8 expression. Numbers indicate the percentage of cells in each quadrant.

manipulated C57/Bl6 (a normal thymic profile) and  $Rag2^{-/-}$  (a profile from mice deficient for T cells) mouse were analyzed. For retrovirally reconstituted mice, gates were set to distinguish GFP-positive cells (GFP) which were then analyzed for CD4 and CD8 expression. Numbers indicate the percentage of cells in each quadrant.

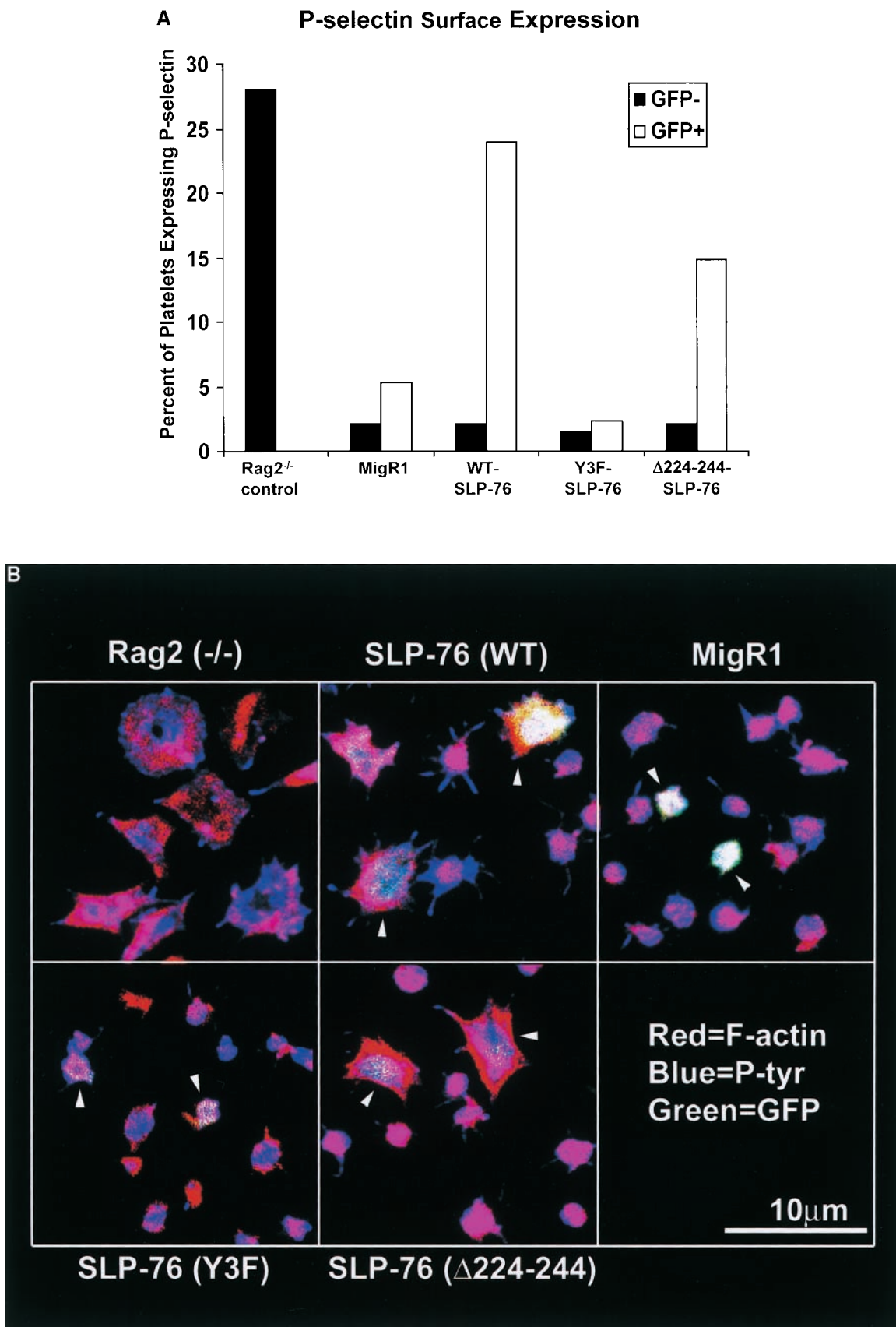


Figure 6 (continues on facing page)



we found using the transgenic approach (16), further validating the use of retroviral reconstitution to examine the structure/function relationships of SLP-76 in various hematopoietic lineages, including platelets.

**The Role of the Gads Binding Region of SLP-76 in Platelet Function.** Platelets harvested from retrovirally reconstituted mice described for Fig. 5 B were stimulated with 40 nM Cvx for 10 min. Cells were then assessed for expression of P-selectin by flow cytometry. Gates were set to distinguish GFP-positive (transduced) from GFP-negative (non-transduced) cells and results were then plotted as the percentage of platelets staining positive for P-selectin (Fig. 6 A). As shown previously, WT-SLP-76 reconstitutes P-selectin release in response to 40 nM Cvx to levels near that of the RAG2<sup>-/-</sup> control platelets while both nontransduced (GFP-negative) cells and cells transduced with GFP alone (MigR1) do not respond to Cvx (32). In contrast to WT-SLP-76, Y3F-SLP-76 consistently fails completely to reconstitute P-selectin expression suggesting that the NH<sub>2</sub>-terminal tyrosine residues of SLP-76 are required for granule release. Platelets expressing the Gads binding mutant of SLP-76 ( $\Delta$ 224–244-SLP-76) exhibit an intermediate, but variable, response to Cvx in comparison to rescue with wild-type SLP-76. On average, cells expressing the Gads binding mutant responded  $\sim$ 60% as well as those infected with WT-SLP-76 ( $n = 6$ ). Interestingly, although wild-type responses are not achieved, platelets expressing the  $\Delta$ 224–244-SLP-76 mutation always demonstrate increased inducible P-selectin expression when compared with LAT<sup>-/-</sup> cells, suggesting an alternative, albeit inefficient, means by which this SLP-76 mutant may be recruited to LAT and GEMs. Alternatively, it is possible that GPVI may signal through SLP-76 and LAT leading to granule release even when the two adaptors do not physically interact.

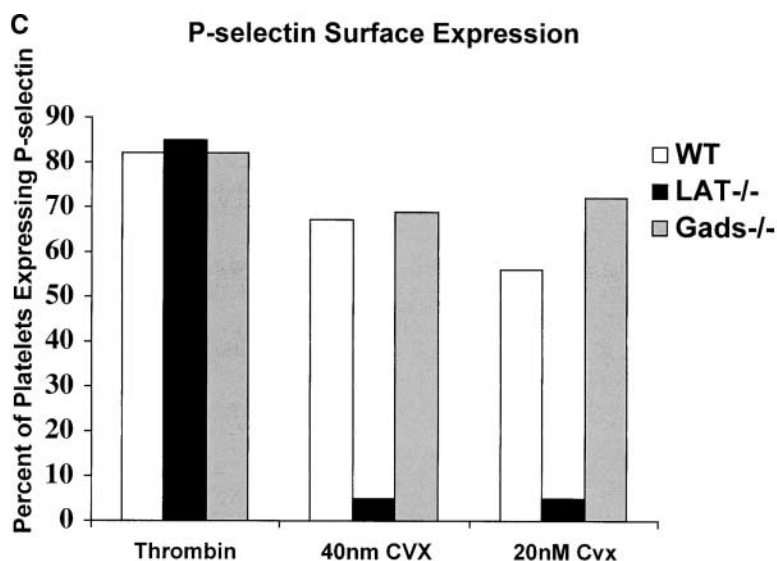
Our findings indicating that the Gads binding domain of SLP-76 is not essential for rescue of Cvx-mediated granule release suggests the possibility that signaling via GPVI may

not require Gads. We tested this hypothesis directly using mice deficient in this adaptor protein. Platelets from Gads-deficient mice, which show significant defects in T cell maturation and function (40), were stimulated with 40 and 20 nM Cvx followed by flow cytometric analysis of P-selectin surface expression. In contrast to LAT-deficient platelets, platelets lacking Gads undergo granule release normally (Fig. 6 C). The absence of a defect in the Gads<sup>-/-</sup> cells may be due to the ability of Grb2 to bind SLP-76, an interaction which has been shown to occur in vitro and is mediated by amino acids 224–244 of SLP-76 (44), and subsequently bring SLP-76 to LAT.

Given that LAT-deficient platelets spread efficiently following collagen and fibrinogen receptor engagement (Fig. 4 B), we predicted that the Gads binding region of SLP-76 would be dispensable for this platelet response. To test this directly, platelets from reconstituted mice were analyzed by confocal microscopy (Fig. 6 B). Platelets deficient for SLP-76 (GFP-negative cells and MigR1-transduced cells) fail to spread under these conditions, while reconstitution of SLP-76 expression (WT-SLP-76) restores platelet spreading. The phosphorylated tyrosine residues of SLP-76 are critical for this response as Y3F-SLP-76 transduced cells fail to spread. Consistent with our finding that LAT-deficient cells can respond to high concentrations of collagen and transduce outside-in signals through  $\alpha$ IIB $\beta$ 3 (Fig. 4), the  $\Delta$ 224–244-SLP-76 mutant restores platelet spreading similarly to WT-SLP-76. This finding indicates that although SLP-76 and its NH<sub>2</sub>-terminal tyrosines are critical for platelet spreading, this aspect of platelet function does not depend on a trimolecular interaction between SLP-76/Gads/LAT (nor solely the interaction between Gads and SLP-76).

## Discussion

The adaptor molecules SLP-76 and LAT have overlapping expression patterns and appear to function coordi-



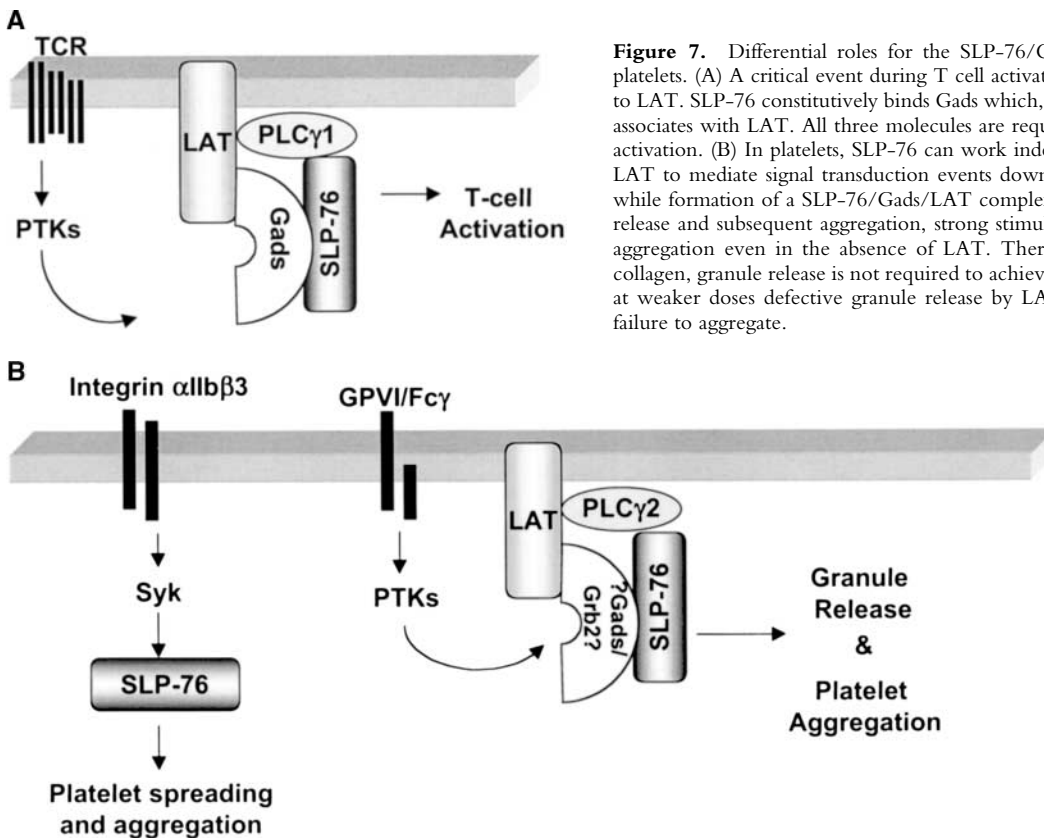
**Figure 6.** Structure/function analysis of SLP-76 in platelets. (A) Retrovirally transduced or nonmanipulated Rag2<sup>-/-</sup> platelets were either left unstimulated, stimulated with thrombin, or Cvx for 10 min in the presence of  $\alpha$ -P-selectin followed by fixation and analysis by flow cytometry with gates set to distinguish GFP-positive cells from GFP-negative cells. Results were then plotted as the percent of platelets staining positive for P-selectin. (B) Washed platelets were incubated on coverslips coated with 100  $\mu$ g/ml fibrinogen in the presence of 30 mg/ml collagen for 45 min. Cells were fixed, permeabilized, and then stained for phosphotyrosine (blue) and F-actin (red). Arrows indicate GFP-positive cells. The bar in the lower right corner corresponds to 10  $\mu$ m. (C) Washed murine platelets from wild-type (WT), LAT<sup>-/-</sup>, or Gads<sup>-/-</sup> were stimulated with 0.5 U/ml thrombin, 40 nM Cvx, or 20 nM Cvx for 10 min at 37°C in the presence of FITC- $\alpha$ -P-selectin followed by fixation and flow cytometric analysis. Results were then plotted as the percent of platelets staining positive for P-selectin.

nately in T cell activation. Both molecules are similarly required for signal transduction after TCR ligation and during T cell development (33, 34, 38). In addition to a similar phenotype in the T cell compartment, mice made deficient for either SLP-76 or LAT manifest mast cell signaling defects (50, 51). Furthermore, in contrast to LAT-deficient mice, SLP-76-null animals have a bleeding diathesis and decreased perinatal survival (34, 35). This contrast in phenotypes argues that SLP-76 is required in pathways in which LAT is not. To further explore this issue, we have now expanded our studies of both LAT-deficient and SLP-76-deficient platelets.

Previously we have shown that platelets from LAT<sup>-/-</sup> mice exhibit reduced P-selectin surface expression, and  $\alpha$ IIb $\beta$ 3 activation in response to CRP (39). In the current report we confirm defective signaling to this agonist and find further that the failure to express normal levels of P-selectin is not overcome at higher concentrations of CRP or after stimulation with the more powerful GPVI agonist, Cvx. Surprisingly, however, we find that LAT-deficient platelets phosphorylate PLC $\gamma$ 2 and aggregate fully in response to high concentrations of collagen, Cvx or CRP. This observation suggests a model in which GPVI-mediated granule release can be uncoupled from platelet aggregation. Our observation that at lower concentrations of collagen, CRP, and Cvx there is an aggregation defect of the LAT-deficient cells reinforces the notion that a complete response to less potent GPVI engagement requires efficient granule release.

Our current studies also indicate that although expressed in both platelets and T cells, mechanisms of action of SLP-76 and LAT do not overlap completely in the two cell types. First, we found that while engagement of the fibrinogen receptor,  $\alpha$ IIb $\beta$ 3, results in tyrosine phosphorylation of SLP-76, there is no similar change detectable in LAT. Consistent with this, when stimulated with collagen and allowed to adhere to a fibrinogen matrix, LAT-deficient cells spread normally whereas SLP-76-deficient platelets fail to spread under these conditions (37). Collectively, our studies comparing SLP-76- and LAT-deficient platelets suggest that the interaction between SLP-76 and LAT (mediated by the adaptor protein, Gads), while essential for TCR signaling, is dispensable for platelet spreading in response to collagen and fibrinogen binding (Fig. 7). We tested this hypothesis further by reconstituting SLP-76 deficient platelets with wild-type or mutant variants of the adaptor.

As shown, retroviral reconstitution of WT-SLP-76 restores T cell development as well as platelet function in response to collagen and fibrinogen. In contrast, reconstitution with a mutant variant of SLP-76 incapable of becoming tyrosine phosphorylated (Y3F-SLP-76) results in a severe block in T cell development, and in granule release and spreading in cells expressing this mutant. These data suggest that some of the protein-protein interactions mediated by SLP-76 are similarly required for T cell and platelet function. We speculate that the binding and recruitment of Vav may play a critical role in this process. Analysis of mice deficient in expression of multiple Vav



**Figure 7.** Differential roles for the SLP-76/Gads/LAT axis in T cells versus platelets. (A) A critical event during T cell activation is the recruitment of SLP-76 to LAT. SLP-76 constitutively binds Gads which, upon T cell activation, inducibly associates with LAT. All three molecules are required for T cell development and activation. (B) In platelets, SLP-76 can work independently of its association with LAT to mediate signal transduction events downstream of  $\alpha$ IIb $\beta$ 3. Furthermore, while formation of a SLP-76/Gads/LAT complex is required for efficient granule release and subsequent aggregation, strong stimulation of GPVI results in platelet aggregation even in the absence of LAT. Therefore, at high concentrations of collagen, granule release is not required to achieve efficient aggregation, however, at weaker doses defective granule release by LAT-deficient platelets results in a failure to aggregate.

isoforms will be required to address the importance of this intermolecular relationship (52).

In contrast to the NH<sub>2</sub>-terminal tyrosines of SLP-76, we found a differential requirement for the Gads binding region of SLP-76 in platelets versus T cells. Thus, mutation of the Gads binding region of SLP-76 ( $\Delta$ 224–244-SLP-76) disrupts T cell development and function, however, platelet spreading in response to collagen and fibrinogen is normal with this mutant. The SLP-76 structure/function analysis is consistent with our observation that signaling via integrin  $\alpha$ IIb $\beta$ 3 involves phosphorylation of SLP-76 but not LAT, suggesting the likelihood that the SLP-76/Gads/LAT axis is not critical for  $\alpha$ IIb $\beta$ 3 signal transduction (Fig. 7). It is likely that the ability of SLP-76 to couple to the PLC $\gamma$  signaling cascade in the absence of association with LAT is due to a direct interaction between the proline rich region of SLP-76 (outside of the Gads binding site) and PLC $\gamma$  (53). Importantly, in our transgenic reconstitution studies we found that, although unable to bind Gads, the  $\Delta$ 224–244-SLP-76 construct still binds to PLC $\gamma$  as well as the other known SLP-76 interacting proteins (16).

Granule release in response to Cvx is rescued with the  $\Delta$ 224–244 mutant of SLP-76, however, as predicted based on the defects seen in the LAT<sup>-/-</sup> platelets, the restoration is not to wild-type levels. These data suggest, therefore, that while in the absence of Gads binding to SLP-76 granule release can occur, maximal granule secretion in response to GPVI agonists requires the Gads binding region of SLP-76, likely to facilitate recruitment of SLP-76 and its partner proteins to signaling complexes within the cell. However, studies of Gads-deficient platelets indicate that this adaptor is not critical for platelet granule release or aggregation in response to several GPVI agonists (Fig. 6 C, and results not shown). One plausible explanation for this is that in the absence of Gads, association of SLP-76 with LAT may occur through other known binders including Grb2 (which associates with the same region of SLP-76 as Gads) or PLC $\gamma$ 2 (which associates with other residues within the SLP-76 proline rich region [53]). Thus, in the absence of the preferred in vitro SLP-76 binder, Gads, Grb2, or another family member may substitute bringing SLP-76 to LAT. However, it should be noted that in Gads-deficient T cells, there is no evidence of increased Grb2 association with SLP-76 (40), suggesting that either platelet interactions differ from those seen in T cells or perhaps another Grb2 family member may substitute in platelets. Additionally, in the case of  $\Delta$ 224–244-SLP-76, which cannot associate with Grb2 family members, LAT and SLP-76 may still form a complex mediated by yet other interacting proteins (e.g. PLC $\gamma$ 2). Further mutational analysis of SLP-76 and its associated molecules will be required to determine if this is the case.

Signal transduction in platelets has been investigated extensively in recent years. Due to difficulties manipulating platelets and the lack of an easily transfectable representative cell line, models of platelet signal transduction have largely been based on known functions of key proteins in other cell types, including lymphocytes. Because GPVI as-

sociates with the ITAM bearing Fc $\gamma$  chain and ligation of this receptor leads to Syk activation, many parallels do exist between this platelet signal transduction pathway and those downstream of both the T and B cell antigen receptors. Our studies of LAT and SLP-76 reinforce the notion that there are many similarities in platelet and T cell activation. It appears, however, that there are also important differences as LAT and SLP-76 seem to be differentially required for platelet activation downstream of GPVI and  $\alpha$ IIb $\beta$ 3. Future studies using additional “knock-out” mice and tissue-specific expression of signaling molecules in megakaryocytes and platelets will further elucidate the molecular mechanisms unique to platelet activation. Finally, although these studies provide further information about platelet signaling in SLP-76- and LAT-deficient mice, it remains unclear how the signaling defects relate to the bleeding diathesis of the SLP-76-null animals. Further studies of these and other genetically altered mice will be required to provide a complete explanation for this failure of hemostasis.

We are grateful to Dr. Mark Kahn for thoughtful discussions and critical reading of this manuscript as well as Jian Shao and the University of Iowa Central Microscopy Center for their expert help and technical assistance.

This work was supported in part by grants from the National Institutes of Health (W.S. Pear, S.J. Shattil, and G.A. Koretzky). S.P. Watson is supported by the Wellcome Trust and the British Heart Foundation. W.S. Pear is a recipient of a Scholar Award from the Leukemia and Lymphoma Society.

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