The Molecular Adapter SLP-76 Relays Signals from Platelet Integrin αIIbβ3 to the Actin Cytoskeleton*

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Achim Obergfell‡, Barbi A. Judd§§, Miguel A. del Pozo‡, Martin A. Schwartz‡, Gary A. Koretzky§§, and Sanford J. Shattil**

From the Departments of \Vascular Biology and \Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037 and the Graduate Program in Immunology, the \Signal Transduction Program, Leonard and Madlyn Abramson Family Cancer Research Institute, and the \Department of Pathology and Laboratory Medicine, the University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Platelet adhesion to fibrinogen through integrin αIIbβ3 triggers cell rearrangements and cell spreading. Mice deficient in the SLP-76 adapter molecule bleed excessively, and their platelets spread poorly on fibrinogen. Here we used human platelets and a Chinese hamster ovary (CHO) cell expression system to better define the role of SLP-76 in αIIbβ3 signaling. CHO cell adhesion to fibrinogen required αIIbβ3 and stimulated tyrosine phosphorylation of SLP-76. SLP-76 phosphorylation required coexpression of Syk tyrosine kinase and stimulated association of SLP-76 with the adapter, Nck, and with the Rac exchange factor, Vav1. SLP-76 expression increased lamellipodia formation induced by Syk and Vav1 in adherent CHO cells (p < 0.001). Although lamellipodia formation requires Rac, SLP-76 functioned downstream of Rac by potentiating adhesion-dependent activation of PAK kinase (p < 0.001), a Rac effector that associates with Nck. In platelets, adhesion to fibrinogen stimulated the association of SLP-76 with the SLAP-130 adapter and with VASP, a SLAP-130 binding partner implicated in actin reorganization. Furthermore, SLAP-130 colocalized with VASP at the periphery of spread platelets. Thus, SLP-76 functions to relay signals from αIIbβ3 to effectors of cytoskeletal reorganization. Therefore, deficient recruitment of specific adapters and effectors to sites of adhesion may explain the integrin phenotype of SLP-76−/− platelets.

Integrins were originally identified as adhesion receptors for extracellular matrix proteins (ECM), but these αβ transmembrane heterodimers also transmit information into the cell. This process, referred to as “outside-in signaling,” promotes anchorage-dependent cellular responses including motility, growth, differentiation, and survival (1). Most integrin α and β cytoplasmic tails consist of 20–70 amino acid residues and are devoid of enzymatic activity, implying that outside-in signals are transmitted by integrin-associated proteins. Several transmembrane proteins can interact with the extracellular domains of integrins, but attention has focused on proteins that interact with integrin cytoplasmic tails (2, 3). These include α-actinin and talin, cytoskeletal proteins that link integrins to actin filaments (4). Cell attachment to the ECM causes lateral clustering of integrin heterodimers into oligomers and evidence is accumulating that outside-in signaling is triggered, in part, by the co-clustering of integrin-associated adapters, enzymes and substrates into signaling complexes that localize within and modify the actin cytoskeleton (1, 4, 5). This model is directly relevant to integrin αIIbβ3 signaling in blood platelets.

αIIbβ3 is a receptor for fibrinogen and von Willebrand factor that mediates platelet aggregation and spreading on vascular ECM (6). These responses are accompanied by major changes in platelet morphology and in organization of the actin cytoskeleton. For example, platelet attachment to fibrinogen or von Willebrand factor is accompanied by progressive actin polymerization, filopodial and lamellipodial extension, and eventually full spreading (7–9). Recent work has identified at least three sets of signaling responses triggered by ligand binding to αIIbβ3 that potentially mediate these changes. One, detectable within seconds of cell attachment, involves tyrosine phosphorylation and activation of the Syk protein tyrosine kinase (10). The others are detectable only after 30–120 s and involve tyrosine phosphorylation and activation of the FAK tyrosine kinase and tyrosine phosphorylation of the integrin β3 tail (11, 12). In sharp contrast to phosphorylation of FAK and β3, Syk phosphorylation and activation are unaffected by blockade of actin filament barbed ends by cytochalasin D, suggesting that actin polymerization is not required. In fact, two of the most prominent Syk substrates in platelets, Vav1 and SLP-76, may function to promote actin polymerization downstream of αIIbβ3.

Vav1 is an hematopoietic cell-specific guanine nucleotide exchange factor for cdc42 and Rac (13). The GTP-bound forms of cdc42 and Rac promote filopodial and lamellipodial extension, respectively (14). SLP-76, the focus of the present work, is an hematopoietic cell-specific adapter protein that contains an N-terminal SAM domain, several potential tyrosine phosphorylation sites, a central proline-rich region and a C-terminal SH2 domain (Fig. 1). Studies in lymphoid cells indicate that three phosphotyrosines in SLP-76 (Y113, Y128, and Y145) are required for interactions with the SH2 domains of Vav1 and Nck (15, 16). Of potential relevance to αIIbβ3 signaling, Nck is an adapter that can influence cytoskeletal events by recruiting a Rac effector, the PAK1 serine-threonine kinase, to plasma membrane adhesion sites (17). The proline-rich region of SLP-76 interacts with the SH3 domain of Gads, a Grb2-like adapter (18). The SH2 domain of SLP-76 interacts with ty-
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**FIG. 1. Domain structure of SLP-76.** Portions of SLP-76 that interact directly with Vav1, Nck, Gads, and SLAP-130 are indicated. The numbers orient the reader to the approximate location of domains in the linear sequence. Arrows point to potential effectors of Vav1, Nck, and SLP-130 that are evaluated in this study.

rosine-phosphorylated SLP-130 (19, 20), an adapter that can bind in turn through an FP4 polyproline motif to the EVH1 domain of VASP, an actin-binding protein implicated in regulating actin polymerization within lamellipodia and focal adhesions (4, 21, 22). Because all of these SLP-76 binding partners are present in platelets, SLP-76 might be in a pivotal position to transmit αIIbβ3 signals to the cytoskeleton. Consistent with this hypothesis, SLP-76-deficient mice exhibit a bleeding diathesis and defects in platelet function, including reduced spreading on fibrinogen. Retroviral reconstitution of SLP-76−/− bone marrow cells with SLP-76 corrects these platelet defects (23).

Based on these considerations, the present study was carried out to determine more precisely the role of SLP-76 in outside-in αIIbβ3 signaling. Using a CHO cell model system and human platelets, we show here that SLP-76 becomes tyrosine-phosphorylated in fibrinogen-adherent cells in an αIIbβ3 and Syk-dependent manner. Furthermore, SLP-76 enhances lamellipodia formation triggered by an outside-in signaling pathway that involves αIIbβ3, Syk, Vav, and Rac. Whereas SLP-76 does not affect Rac activation, it does enhance adhesion-dependent activation of PAK. Moreover, through its interactions with SLAP-130, SLP-76 may help to localize VASP to αIIbβ3 adhesion sites at the edges of spreading platelets. Thus, SLP-76 functions to relay signals from αIIbβ3 to actin.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Monoclonal antibodies: anti-Syk (4D10) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-phosphotyrosine (4G10 and PY20) from Upstate Biotechnology (Lake Placid, NY) and Transduction Laboratories (Lexington, KY), respectively; anti-VASP from ImmunoGlobe GmbH, Großostheim, Germany; anti-Vav, anti-FLAG M2 and anti-Rac from Upstate Biotechnology; and anti-LIBS6 Fab, an integrin β1 activating antibody, was a gift from Mark Ginsberg (Scripps). Rabbit polyclonal antibody to Nck (5547) was a gift from Michael Grove, PA); fluorescein isothiocyanate-conjugated anti-mouse IgG was from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA); fluorescein isothiocyanate-conjugated anti-mouse IgG from BioSource International Inc. (Camarillo, CA). Rhodamine-phalloidin was from Molecular Probes (Eugene, OR). Purified human fibrinogen from Enzyme Research Laboratories Inc. (South Bend, IN), bovine serum albumin and sodium orthovanadate from Fisher. Protein A and Protein G-Sepharose were from Amersham Pharma Biotech (Piscataway, NJ), glutathione-agarose and leupeptin from Sigma, Pefabloc and aprotinin from Roche Molecular Biochemicals (Indianapolis, IN), and LipofectAMINE from Life Technologies, Inc.

Cell Culture, Plasmids, and Transfections—A5 CHO cells, which stably express αIIbβ3, were maintained in complete Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (26). The following mammalian expression plasmids were used: pEMCV/Syk and pEF/Myc-Vav1 (27); pSAP/CDB-γ (a chimera containing the extracellular and transmembrane domain of human CD8 and the γ subunit of PecR1, Ref. 28); pcDNA3/HA-Rac1 (24); and pEF/FLAG-SLP-76 (wild-type and mutants) (15). Subconfluent cells in 100 mm dishes were transfected with up to 4 μg of plasmid DNA using LipofectAMINE according to the manufacturer’s instructions. When necessary, pcDNA3 was added to maintain equal amounts of DNA in all transfections. Twenty-four hours later, the concentration of fetal calf serum was lowered from 10 to 0.5%, and cells were cultured for an additional 24 h before use in functional assays.

CHO Cell Binding to Fibrinogen—A5 CHO cell transfectants were resuspended using trypsin-EDTA, washed twice with Dulbecco’s modified Eagle’s medium, resuspended to 3 × 106 cells/ml in Dulbecco’s modified Eagle’s medium, and incubated for 45 min with 20 μl cyclohexamide. To test the effects of binding of soluble fibrinogen to αIIbβ3, cells were incubated for an additional 15 min with 250 μg/ml fibrinogen, in the presence or absence of 0.5 mM MnCl2 (to activate integrins) or 10 μM Integrin or 0.5 mM EDTA (to inhibit ligand binding to αIIbβ3) (29). Cells were then washed with phosphate-buffered saline, lysed for 10 min in ice-cold RIPA buffer containing 1 mM sodium vanadate, 0.5 mM leupeptin, 0.5 mg/ml Pefabloc, and 5 μM/ml aprotinin, clarified by centrifugation in a microcentrifuge, and subjected to immunoprecipitation and Western blotting (27, 28). For studies of cells adherent to fibrinogen, bacterial tissue culture plates were precoated with 5 mg/ml bovine serum albumin (BSA) or 100 μg/ml fibrinogen. After blocking for 2 h at room temperature with heat-denatured BSA, 4.5 × 103 cells in 1.5 ml were added to each plate and incubated for 45 min at 37 °C in a CO2 incubator. After 60 min, nonadherent cells from the BSA plates were sedimented at 14,000 rpm for 3 min in a microcentrifuge and lysed immediately in RIPA buffer. Cells adherent to fibrinogen were rinsed twice with phosphate-buffered saline, lysed on the plates directly, and then processed for immunoprecipitation and Western blotting. When coprecipitation of two or more phosphates was being assessed, the cells were lysed in buffer containing 0.5% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 7.4, and inhibitors.

**Immunoprecipitation and Western Blotting—**Equal amounts of each lysate, typically 250–500 μg of protein in 500 μl, were immunoprecipitated with the indicated antibodies, subjected to electrophoresis on 7.5% SDS-polyacrylamide gels, and transferred onto nitrocellulose (27, 28). Membranes were blocked with 5% nonfat dry milk and probed with the indicated antibodies coupled to horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were detected by enhanced chemiluminescence. Blots were scanned in a Hewlett-Packard ScanJet 5300C scanner, and labeled bands were quantified by calibrated densitometry using NIH Image software.

Rac GTPase and PAK kinase Assays—Endogenous PAK was immunoprecipitated from Nonidet P-40 lysates and PAK kinase activity was determined by assay in gel kinase assay with myelin basic protein as substrate (24). Active Rac was monitored using a pull-down assay involving a recombinant PAK of the binding specifically to Rac-GTP (24).

**Conflocal Microscopy—**Twenty-four hours after CHO cell transfection, cells were plated on coverslips coated with 100 μg/ml fibrinogen and incubated in the presence of 0.5% fetal calf serum for an additional 18 h at 37 °C in a CO2 incubator. Adherent cells were then fixed in 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, and stained with primary antibodies to transfected proteins, secondary antibodies conjugated with fluorescein isothiocyanate or Cy5, and rhodamine-phalloidin to stain F-actin. Lamellipodia formation in fibrinogen-adherent cells 3 signals to the cytoskeleton. Consistent with a 3, Syk, Vav, and Rac. Whereas SLP-76 does not affect Rac activation, it does enhance adhesion-dependent activation of PAK. Moreover, through its interactions with SLAP-130, SLP-76 may help to localize VASP to αIIbβ3 adhesion sites at the edges of spreading platelets. Thus, SLP-76 functions to relay signals from αIIbβ3 to actin.
the presence of 2 units/ml apyrase for 30 min (31). Then cells adherent to fibrinogen or suspended over BSA were lysed in Nonidet P-40 buffer, the lysates were immunoprecipitated with antibodies to SLP-76 or SLAP-130, and Western blots were probed with antibodies to phosphotyrosine, SLP-76, SLAP-130, or VASP (25). For confocal microscopy, cells were plated onto fibrinogen-coated coverslips at 10^5/ml for 45 min at 37°C in the presence or absence of 200 nM phorbol myristate acetate (PMA) to enhance spreading. Platelets were fixed, permeabilized, stained with antibodies to SLAP-130 and VASP, and examined by confocal microscopy (8).

RESULTS

\(\alpha_{IIb}\beta_3\) Signals to SLP-76—In platelets, fibrinogen binding to \(\alpha_{IIb}\beta_3\) leads to rapid tyrosine phosphorylation of SLP-76 (23). To study the mechanisms and consequences of integrin signaling to SLP-76, this adapter was transiently expressed in A5 CHO cells, which stably express \(\alpha_{IIb}\beta_3\). To determine whether \(\alpha_{IIb}\beta_3\) signals to SLP-76 in CHO cells as in platelets, A5 cells were first incubated with soluble fibrinogen in the presence of 0.5 mM MnCl₂, an extrinsic activator of integrins (32). Despite successful ectopic expression of SLP-76, no tyrosine phosphorylation of SLP-76 was observed. In contrast, if SLP-76 was cotransfected with Syk, there was now a marked increase in tyrosine phosphorylation of SLP-76 when fibrinogen binding was induced by MnCl₂ (Fig. 2A). This response as well as the tyrosine phosphorylation of Syk itself were inhibited by compounds that block fibrinogen binding to \(\alpha_{IIb}\beta_3\), including the selective \(\alpha_{IIb}\beta_3\) antagonist, Integrillin, and the divalent cation chelator, EDTA. In the experiment shown in Fig. 2A, fibrinogen binding was carried out for 15 min, but similar results were obtained at 30 s, the earliest time point tested. Identical results were obtained if fibrinogen binding was induced by a \(\beta_3\)-specific-activating antibody, anti-LIBS6 Fab, instead of MnCl₂ (not shown). In addition, tyrosine phosphorylation of SLP-76 was observed when cells were plated on immobilized fibrinogen, even in the presence of 10 \(\mu\)M cytochalasin D, an inhibitor of actin polymerization (Fig. 2B). As previously observed (27), cytochalasin D blocked \(\alpha_{IIb}\beta_3\)-dependent tyrosine phosphorylation and activation of FAK but not Syk. These results indicate that SLP-76 is situated downstream of \(\alpha_{IIb}\beta_3\) and Syk in a signaling pathway that becomes activated by fibrinogen binding, independent of actin polymerization.

In antigen-stimulated T cells, SLP-76 phosphorylation is dependent on signals generated through T cell receptor subunits that bear ITAM motifs, and it is abolished by phenylalanine substitution of SLP-76 tyrosines 113, 128, and/or 145 (SLP-76-(3YF), see Ref. 15). To evaluate whether these tyrosine residues are involved in \(\alpha_{IIb}\beta_3\) signaling, SLP-76-(3YF) or M cytochalasin D, an inhibitor of actin polymerization (Fig. 2B). In the presence of 10 \(\mu\)M cytochalasin D (CD) or MnCl₂ control vehicle, after 45 min at 37°C, adherent cells on the fibrinogen plates and nonadherent cells in the BSA plates were lysed in RIPA and immunoprecipitated with an antibody to SLP-76 or FAK. Western blots were probed with antibodies to phosphorytrosine antibodies and reprobed with antibodies to SLP-76 or FAK. A, A5 cells were transfected with wild-type SLP-76 (WT) or SLP-76-(3YF) and then maintained in suspension over BSA for 45 min at 37°C before immunoprecipitation and Western blotting. D, A5 cells were transfected with Syk and either wild-type SLP-76 (WT) or SLP-76-(3YF, Y113F, Y128F, Y145F), referred to as 3YF. F. Then the effect of cell adhesion to fibrinogen on tyrosine phosphorylation of SLP-76 was studied. To compare these results with SLP-76 phosphorylation mediated by an ITAM-dependent mechanism, some cells were cotransfected with Syk, SLP-76, and CDC8 and then maintained in suspension over BSA for 45 min at 37°C before immunoprecipitation and Western blotting. D, A5 cells were transfected with Syk and the indicated SLP-76 deletion mutant, and \(\alpha_{IIb}\beta_3\)-dependent SLP-76 tyrosine phosphorylation was studied. For clarity, SLP-76 and its mutants have been placed next to each other, although on the original gels each migrated as expected according its molecular size. Data shown are representative of three experiments.

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a modest but consistent increase in lamellipodia formation ($p < 0.03$), and the enhancing effect of SLP-76 was maintained in the presence of Vav1 ($p < 0.003$, Fig. 3B).

Because tyrosine-phosphorylated SLP-76 can bind directly to the SH2 domain of Vav1, we wondered if this interaction was necessary for the SLP-76 effect on lamellipodia. In the SLP-76-(3Y3F) mutant, the tyrosines needed for interaction with Vav1 (or Nck) are not present (15, 16). Indeed, unlike the case for wild-type SLP-76, Vav1, and Nck were not observed in SLP-76-(3Y3F) immunoprecipitates from fibrinogen-adherent A5 cells (Fig. 4). Despite this, SLP-76-(3Y3F) was fully able to enhance lamellipodia formation induced by Syk and Vav1 (Fig. 3B). Moreover, a SLP-76 deletion mutant ($\Delta 224-244$) that lacks the polyproline binding site for Gads and a mutant (R448K) that abrogates the SLP-76 SH2 interaction with SLAP-130 (34) were similarly able to enhance lamellipodia formation (Fig. 3B). On the other hand, SLP-76 truncation mutants containing either the N-terminal half (1–266) or the C-terminal half (267–533) of the molecule failed to support lamellipodia formation (Fig. 3B). Taken together, these results indicate that one function of SLP-76 is to enhance lamellipodia formation during $\alpha_{IIb}\beta_3$ signaling. Furthermore, at least when ectopically expressed, the loss of any single known adapter function of SLP-76 does not prevent this effect.

**Downstream Effectors of SLP-76 in $\alpha_{IIb}\beta_3$ Signaling**—Lamellipodial extension involves actin polymerization in localized regions of the cortical cytoskeleton. Because SLP-76 is a multivalent adapter, it has the potential to influence the subcellular localization or function of several proteins that have been implicated in regulating cytoskeletal dynamics. We focused attention on three such proteins, Rac, PAK, and VASP. Rac promotes actin polymerization and lamellipodial extension in many cell types, including platelets and A5 CHO cells (27, 35, 36). In platelets, Rac may be activated to its GTP-bound form by Vav1 (37). Because the Rac exchange activity of Vav1 increases following its tyrosine phosphorylation (13, 38, 39), we examined whether SLP-76 expression influenced tyrosine phosphorylation of Vav1 or levels of Rac-GTP. In A5 cells cotransfected with Syk and Vav1, tyrosine phosphorylation of Vav1 increased 4-fold 30–45 min after cell adhesion to fibrinogen, and cotransfection of SLP-76 had no effect on this response (not shown). Moreover, although levels of Rac-GTP increased 10-fold upon adhesion of Syk/Vav1 transfectants to fibrinogen, SLP-76 did not alter this response (Fig. 5). Thus,
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although SLP-76 enhances a Rac-dependent lamellipodial response triggered by $\alpha_{IIb}\beta_3$, it does not do so by activating Rac. This suggests that SLP-76 functions downstream of Rac and/or independent of it.

Because wild-type SLP-76 interacted with Nck in fibrinogen-adherent A5 cells (Fig. 4), we asked whether SLP-76 expression increased the activity of PKA, a Nck-binding partner and Rac effector (17). In the presence of Syk and Vav1, adhesion of A5 cells to fibrinogen stimulated more than a 3-fold increase in PKA activity ($p < 0.01$). However, in the presence of SLP-76, adhesion-dependent PKA activity increased more than 10-fold ($p < 0.01$) (Fig. 6, A and C). On the other hand, expression of SLP-76-(3Y$\rightarrow$F) had no effect on PKA activity, consistent with a requirement for an interaction between SLP-76 and Nck for this response to take place (Fig. 6, B and C). Thus, one effect of wild-type SLP-76 in $\alpha_{IIb}\beta_3$ signaling is to increase the activity of PKA. However, the results with the SLP-76-(3Y$\rightarrow$F) mutant indicate that this effect is not essential for enhancement of lamellipodia formation, at least when SLP-76-(3Y$\rightarrow$F) is ectopically expressed (Fig. 3B).

VASP is a prominent phosphoprotein in platelets (40), and it may interact directly with SLAP-130 in lymphoid cells (22). Therefore, we asked if there was any relationship between SLP-76, SLAP-130, and VASP during outside-in $\alpha_{IIb}\beta_3$ signaling in platelets. Human platelets were incubated with soluble fibrinogen in the presence or absence of the LIBS-6 Fab $\beta_3$-activating antibody. Then Nonidet P-40 cell lysates were immunoprecipitated with antibodies to SLP-76 or SLAP-130. Western blots of SLP-76 immunoprecipitates revealed a prominent 130 kDa tyrosine-phosphorylated band that comigrated with SLAP-130 (Fig. 7A). Fibrinogen binding to the platelets caused an increase in tyrosine phosphorylation of this band as well as of SLP-76, and this response was inhibited by RO 43-5054, a selective $\alpha_{IIb}\beta_3$ antagonist (30). In both cases, the binding of soluble fibrinogen to the platelets caused little or no increase in the amount of SLAP-130 that coimmunoprecipitated with SLP-76, suggesting that the main effect of $\alpha_{IIb}\beta_3$ ligation under these conditions was on tyrosine phosphorylation of these adapters (Fig. 7, A and B). Thus, SLP-76 and
SLP-130 interact in platelets and their states of tyrosine phosphorylation are modulated by outside-in signaling through αIIbβ3.

For SLP-130 and VASP to play a coordinate role in αIIbβ3-dependent platelet spreading, it would be predicted that they interact with each other and localize within lamellae at the platelet periphery. To assess the subcellular localization of SLP-130 and VASP, platelets were allowed to attach to fibrinogen-coated coverslips for 45 min in the presence or absence of PMA. Adherent cells were then fixed, permeabilized, and stained with antibodies to SLP-130 and VASP, (green and red, respectively, in the merged images). The yellow rim staining in the merged images indicates partial colocalization of these proteins to the periphery of fibrinogen-adherent platelets. Bar = 10 μm. B, washed platelets were maintained in suspension or plated on fibrinogen for 30 min. Then Nonidet P-40 cell lysates were immunoprecipitated with antibody to SLP-130 or SLP-76, and Western blots were probed with antibodies to VASP, SLP-130, and SLP-76. Data shown are representative of three experiments.

DISCUSSION

The goal of this study was to determine how SLP-76 functions during αIIbβ3 signaling to influence cell shape and cytoskeletal organization. The study was motivated by recent observations that normal human and mouse platelets show increased tyrosine phosphorylation of SLP-76 in response to fibrinogen binding to αIIbβ3, suggesting that SLP-76 is involved in outside-in signaling through this integrin. Furthermore, mouse platelets deficient in SLP-76 spread poorly on fibrinogen and display reduced overall tyrosine phosphorylation, and both abnormalities are corrected by in vivo reconstitution of platelets with SLP-76 (23). Here we used a CHO cell model system and human platelets to evaluate the adapter functions of SLP-76 in the context of outside-in αIIbβ3 signaling. The main conclusions are as follows. 1) Ligation of αIIbβ3, whether with soluble or immobilized fibrinogen, induces rapid tyrosine phosphorylation of SLP-76 but only in the presence of Syk. 2) Integrin-dependent SLP-76 phosphorylation involves tyrosines 113, 128, 145 as well as other tyrosine residues in the protein. 3) Phosphorylation of SLP-76 does not require actin polymerization. Rather, SLP-76 expression enhances an actin-driven response, lamellipodia formation, that is triggered by a pathway involving αIIbβ3, Syk, Vav1, and Rac. 4) Whereas SLP-76 interacts with Vav1, enhancement of lamellipodia formation by SLP-76 is not because of increased tyrosine phosphorylation of Vav1 or activation of Rac. Instead, it may be because of interactions of SLP-76 with other proteins, including Nck and SLAP-130. For example, interaction of SLP-76 with Nck may account for the enhanced adhesion-dependent activation of Pak observed in SLP-76 transfectants. Furthermore, interaction of SLP-76 with SLAP-130 may promote localization of SLP-130 and VASP to the periphery of fibrinogen-adherent platelets. Thus, SLP-76 functions to relay signals between αIIbβ3 and effectors that influence the actin cytoskeleton. In addition to defining a key pathway linking αIIbβ3 to changes in F-actin, these results provide a molecular explanation for the integrin phenotype of SLP-76-deficient platelets.

Integrin Signaling to SLP-76—SLP-76 is one of numerous proteins that become tyrosine phosphorylated in response to ligand binding to αIIbβ3. These can be grouped based on their temporal profiles and whether actin polymerization is required (6). For example, Syk and Vav1 become phosphorylated within seconds of fibrinogen binding to αIIbβ3 in platelets or A5 CHO cells, and this response is independent of actin polymerization or cell aggregation (10, 27, 37). In contrast, FAK, Hic-5, SHIP,
and the integrin β3 cytoplasmic tail become phosphorylated later, only after actin polymerization and cell aggregation or spreading have occurred (11, 41–45). The rapid tyrosine phosphorylation of SLP-76 after fibrinogen binding to platelets or A5 CHO cells and its resistance to cytochalasin D places SLP-76 in the group with Syk and Vav1. Indeed, the present study establishes that αι/β3-dependent SLP-76 phosphorylation requires Syk. Moreover, SLP-76 functions with Syk and Vav1 to enhance lamellipodia formation. Thus, SLP-76 is situated downstream of αι/β3 in an early phase of outside-in signaling.

Studies with SLP-76-(3Y→F), which lacks tyrosines 113, 128, and 145, clarify the importance of these residues in integrin signaling and suggest possible differences in SLP-76 function downstream of integrins and immune response receptors. These tyrosines are necessary for the binding of SLP-76 to the SH2 domains of Vav1 and Nck (Refs. 16, 46 and Figs. 1 and 4). In contrast to SLP-76 phosphorylation in response to T cell receptor ligation (46) or to overexpression of an ITAM-bearing CD8/γ chimera in CHO cells, we observed residual tyrosine phosphorylation of SLP-76-(3Y→F) in response to fibrinogen binding to αι/β3 (Figs. 2C and 4). This indicates that one or more additional tyrosines in SLP-76 are targeted by a tyrosine kinase, possibly Syk, during αι/β3 signaling. However, the identity and function of these tyrosines remain to be determined.

In most T cells, a T cell-specific Src family kinase, Lck, and the Syk homologue, ZAP-70, are required for SLP-76 phosphorylation. In platelets and A5 CHO cells, Syk is the relevant ZAP-70 family member, and its optimal activation is dependent on one or more Src family kinases other than Lck (28, 47). Thus, differences in SLP-76 phosphorylation downstream of ITAM receptors and αι/β3 might be caused by, in part, differences in substrate specificities or subcellular localization of related, but not identical, protein tyrosine kinases. However, this cannot explain the differences we observed in SLP-76 phosphorylation downstream of αι/β3 and CD8/γ in CHO cells because both membrane receptors utilized Syk in the same cellular background. Murine platelets express αι/β3 and the ITAM-bearing collagen receptor, GPVI/Fcγ, and both are functionally linked to SLP-76 (47). Because retroviruses can be used to reconstitute SLP-76 in hematopoietic cells of SLP-76−/− mice (23), it should be possible to determine the functional importance of specific SLP-76 tyrosine residues downstream of αι/β3 and GPVI/Fcγ by introducing SLP-76 tyrosine mutants into SLP-76−/− platelets.

Integrin Signaling from SLP-76 to the Actin Cytoskeleton—The spreading defect in fibrinogen-adherent SLP-76−/− mouse platelets (23), and the involvement of SLP-76 in actin cap formation in antigen-activated T cells (16), suggest that one function for SLP-76 in platelets may be to relay αι/β3 signals to the cytoskeleton. This idea is supported by the current findings that SLP-76 phosphorylation occurred rapidly following A5 CHO cell adhesion to fibrinogen, and this response was independent of actin polymerization (Fig. 2B). Furthermore, SLP-76 expression in CHO cells enhanced lamellipodia formation, an event dependent on localized actin polymerization (Fig. 3B). Although caution is warranted in extrapolating results from studies of ectopically expressed SLP-76 in CHO cells to the function of SLP-76 in platelets, these results indicate that SLP-76 has the potential to participate in signaling from αι/β3 to the platelet cytoskeleton, and they provide an explanation for the spreading defect in SLP-76−/− platelets.

How does SLP-76 enhance lamellipodia formation? One possibility we considered is that when tyrosine-phosphorylated SLP-76 binds to Vav1, it may promote either the guanine nucleotide exchange activity of Vav1 or its proximity to effectors, such as Rac. SLP-76 communoprecipitates with Vav1 in activated T cells (16) and in collagen-stimulated platelets (47), implying that it may be close enough to Vav1 to directly influence its function. In addition, Vav1 can activate Rac and promote lamellipodia formation downstream of αι/β3 (Fig. 3B, Ref. 27). Despite this circumstantial evidence, however, we found that expression of SLP-76 in A5 cells affected neither the integrin-dependent tyrosine phosphorylation of Vav1, which regulates Vav1 exchange activity (13, 38, 39), nor the adhesion-dependent activation of Rac (Fig. 5A). Moreover, lamellipodia formation was still enhanced by SLP-76-(3Y→F), despite the fact that it was unable to communoprecipitate with Vav1 (Figs. 3B and 4). Therefore, enhancement of lamellipodia formation by SLP-76 must involve pathways to the cytoskeleton that are downstream of and/or independent of Rac. Studies of PAK and VASP suggest that both possibilities may be correct.

PAK is a Rac effector that has been implicated in regulating actin polymerization, and it is targeted to integrin-based adhesion sites by binding to the central SH3 domain of Nck (17, 48). In this context, adhesion of A5 CHO cells to fibrinogen stimulated the binding of Nck to tyrosine-phosphorylated SLP-76 (Fig. 4). This was associated with an increase in the kinase activity of PAK that was over and above the activity observed in fibrinogen-adherent cells in the absence of SLP-76 (Fig. 6). Thus, one plausible scenario is that fibrinogen binding to αι/β3 triggers activation of Syk followed by tyrosine phosphorylation of SLP-76 and Vav1 in proximity to the integrin. Then whereas Vav1 activates Rac, SLP-76 recruits Nck-PAK to the adhesion site where PAK becomes activated by Rac, resulting in lamellipodia formation. However, this cannot explain how SLP-76-(3Y→F) enhanced lamellipodia formation in A5 CHO cells, because this mutant neither bound to Nck nor increased PAK activation (Figs. 4 and 6, B and C). Interestingly, mutation of other single adapter sites in SLP-76 for Gads or SLAP-130 also failed to prevent lamellipodial enhancement (Fig. 3B). We speculate that when a single adapter region in SLP-76 is mutated, the remaining regions may be able to compensate and promote cytoskeletal changes, particularly if the mutant protein is expressed at high levels, as in the CHO cell system. This interpretation is in keeping with the observation that deletion of larger portions of SLP-76 eliminated its ability to enhance lamellipodia formation (Fig. 3B). It may be useful to determine whether portions of SLP-76 not yet assigned an adapter function, including the SAM domain or phosphotyrosines other than those at positions 113, 128, and 145, are involved in signal relay from αι/β3 to actin.

VASP is an actin-bundling protein that regulates actin dynamics within lamellipodia, possibly by bringing the Arp2/3 actin polymerization machinery and profilin into proximity within integrin-based focal complexes (4, 49–52). VASP has the potential to interact directly with SLAP-130 in lymphocytes (22), where tyrosine-phosphorylated SLAP-130 interacts with VASP in fibrinogen-adherent cells in the absence of SLP-76 (Fig. 6). It may be useful to determine whether portions of SLP-76 not yet assigned an adapter function, including the SAM domain or phosphotyrosines other than those at positions 113, 128, and 145, are involved in signal relay from αι/β3 to actin.
SLP-76-SLAP-130-VASP complex in αIIbβ3 signaling to the platelet cytoskeleton.

These studies, when taken together with recent results obtained with platelets from SLP-76 knockout mice (23), establish that SLP-76 relays signals from αIIbβ3 to act by virtue of the regulated interaction of SLP-76 with multiple downstream effectors that modify the assembly and localization of actin filaments. This model does not require that a given SLP-76 molecule interact simultaneously with all possible binding partners in the relay, because many SLP-76 molecules could be drawn into a large signaling organelle nucleated by αIIbβ3 within focal complexes at sites of platelet adhesion. In this context, antibody-mediated clustering of integrin αIIbβ3 in lymphocytes stimulates tyrosine phosphorylation of SLP-76 and SLAP-130, and overexpression of SLAP-130 enhances lymphocyte migration through fibronectin-coated filters (56). Thus, delineation of the function of SLP-76 in platelet αIIbβ3 signaling may have wider implications for integrin signaling in other hematopoietic cells.

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The Molecular Adapter SLP-76 Relays Signals from Platelet Integrin αIIbβ3 to the Actin Cytoskeleton

Achim Obergfell, Barbi A. Judd, Miguel A. del Pozo, Martin A. Schwartz, Gary A. Koretzky and Sanford J. Shattil

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