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Coordinate interactions of Csk, Src, and Syk kinases with αIIbβ3 initiate integrin signaling to the cytoskeleton

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Integrins regulate cell adhesion and motility through tyrosine kinases, but initiation of this process is poorly understood. We find here that Src associates constitutively with integrin αIIbβ3 in platelets. Platelet adhesion to fibrinogen caused a rapid increase in αIIbβ3-associated Src activity, and active Src localized to filopodia and cell edges. Csk, which negatively regulates Src by phosphorylating Tyr-529, was also constitutively associated with αIIbβ3. However, fibrinogen binding caused Csk to dissociate from αIIbβ3, concomitant with dephosphorylation of Src Tyr-529 and phosphorylation of Src activation loop Tyr-418. In contrast to the behavior of Src and Csk, Syk was associated with αIIbβ3 only after fibrinogen binding. Platelets multiply deficient in Src, Hck, Fgr, and Lyn, or normal platelets treated with Src kinase inhibitors failed to spread on fibrinogen. Inhibition of Src kinases blocked Syk activation and inhibited phosphorylation of Syk substrates (Vav1, Vav3, SLP-76) implicated in cytoskeletal regulation. Syk-deficient platelets exhibited Src activation upon adhesion to fibrinogen, but no spreading or phosphorylation of Vav1, Vav3, and SLP-76. These studies establish that platelet spreading on fibrinogen requires sequential activation of Src and Syk in proximity to αIIbβ3, thus providing a paradigm for initiation of integrin signaling to the actin cytoskeleton.

Introduction

Outside-in signaling refers to the process whereby ligation of an integrin adhesion receptor by a cognate ligand stimulates inward signals that affect a variety of cellular responses, including cytoskeletal reorganization, proliferation, differentiation, and apoptosis (Aplin et al., 1998; Giancotti and Ruoslahti, 1999; Geiger et al., 2001; Schwartz, 2001). Tyrosine phosphorylation of proteins is a major outside-in signaling event, implying that there are key relationships between integrins and specific protein tyrosine kinases and phosphatases. In this regard, Src family kinases have been shown to play a significant role in several phases of outside-in signaling in many cell types (Kaplan et al., 1995; Lowell et al., 1996; Schlaepfer and Hunter, 1997; Klinghoffer et al., 1999; Suen et al., 1999; Feng et al., 2001; Suzuki-Inoue et al., 2001; Timpson et al., 2001). A prominent example is in adherent fibroblasts, where Src forms a complex with tyrosine-phosphorylated focal adhesion kinase (FAK),* and where downstream effectors of this signaling complex regulate cell growth, migration, and focal adhesion turnover (Guan, 1997; Polte and Hanks, 1997; Schlaepfer et al., 1997; Schaller et al., 1999). Since formation of the Src–FAK complex occurs at actin-rich adhesion sites that assemble over minutes to hours, it is likely to be preceded by integrin-dependent signals that are required for actin polymerization and reorganization. However, the process by which integrins initiate outside-in signaling is poorly understood.

Blood platelets are frequently used to study outside-in signaling because they represent a physiologically important cell, and the platelet-specific integrin, αIIbβ3, is required for hemostasis. Outside-in signaling in platelets is triggered when fibrinogen or von Willebrand factor binds to αIIbβ3, and it is dependent on close functional, if not physical, relationships between αIIbβ3 and the signaling machinery of the cell (Shattil et al., 1998). Signals transduced by αIIbβ3 regulate platelet filopodial extension, spreading, aggregation, and granule secretion (Shattil et al., 1998; Phillips et al., 2001). Since these responses involve polymerization

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*Abbreviation used in this paper: FAK, focal adhesion kinase.

Key words: integrin; signaling; Src; Syk; tyrosine kinase

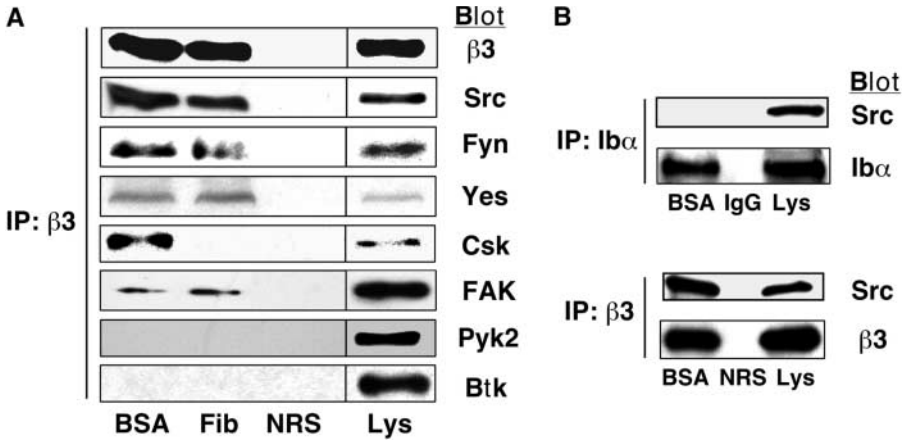

Figure 1. **Association of tyrosine kinases with αIIbβ3.** (A) Washed human platelets were plated on fibrinogen (Fib) for 45 min or maintained in suspension in a BSA-coated dish (BSA). Cells were then lysed in NP-40 detergent, the detergent-soluble fraction was immunoprecipitated with an antibody to β3 or normal rabbit serum (NRS) as a control, and immunoprecipitates were probed on Western blots with antibodies to Src and other tyrosine kinases, as indicated. (B) Platelets in suspension (BSA) were lysed in NP-40, and the presence of Src in β3 and GP Ibα immunoprecipitates was compared. Results are representative of five separate experiments.

and rearrangements of actin filaments (Fox, 1993; Hartwig et al., 1999), αIIbβ3 signals presumably regulate actin dynamics.

Specific nonreceptor tyrosine kinases have been implicated in αIIbβ3 outside-in signaling (Jackson et al., 1996; Shattil et al., 1998; Phillips et al., 2001). For example, Syk becomes activated within seconds of fibrinogen binding to platelets, whereas FAK becomes activated later during platelet aggregation and spreading (Lipfert et al., 1992; Clark et al., 1994). Syk activation does not require actin polymerization since it is unaffected by inhibitors such as cytochalasin D, whereas FAK activation requires actin polymerization (Lipfert et al., 1992; Clark et al., 1994). Thus, activation of Syk, but not FAK, may be a key early event in outside-in signaling. This idea is supported by several recent observations. First, platelet adhesion to fibrinogen stimulates a direct interaction of Syk with the cytoplasmic tail of β3, correlating with Syk activation (Woodside et al., 2001). Second, Syk heterologously expressed in CHO cells is activated in response to clustering of αIIbβ3 complexes (Hato et al., 1998). Third, adhesion of platelets or αIIbβ3-CHO cells to fibrinogen induces Syk-dependent tyrosine phosphorylation of proteins implicated in cytoskeletal regulation, including Vav1 and SLP-76 (Cichowski et al., 1996; Judd et al., 2000; Obergfell et al., 2001). The biological relevance of these interactions for outside-in signaling in platelets is suggested by studies of mice deficient in SLP-76, which exhibit a bleeding diathesis and, among other platelet abnormalities, defective spreading on fibrinogen (Clements et al., 1999; Judd et al., 2000).

In addition to Syk and FAK, platelets contain several Src family members (Src, Fyn, Fgr, Hck, Lyn, Yes), with Src itself being the most abundant (Golden et al., 1986; Huang et al., 1991; Stenberg et al., 1997). Studies using chemical cross-linking and immunoprecipitation techniques have suggested that one or more Src kinases may be associated with αIIbβ3, either before or after thrombin-induced platelet activation (Dorahy et al., 1995; Kralisz and Cierniewski, 1998). Furthermore, Src becomes activated and partitions to the detergent-insoluble actin cytoskeleton in thrombin-aggregated platelets, suggesting that it is regulated by fibrinogen binding to αIIbβ3 (Horvath et al., 1992; Clark and Brugge, 1993; Fox et al., 1993).

Based on these considerations, we used biochemical and genetic approaches here to determine how outside-in signaling is initiated in platelets. The results establish that Src is constitutively associated with αIIbβ3 and that platelet adhesion to fibrinogen selectively activates this pool of Src, possibly by inducing the dissociation of an Src-regulatory kinase, Csk, from the αIIbβ3 complex. Furthermore, Src kinases are required for αIIbβ3-dependent activation of Syk, and both Src and Syk are required to initiate cytoskeletal events responsible for platelet spreading on fibrinogen.

Results

Interactions between αIIbβ3 and Src in platelets

To begin to explore relationships between αIIbβ3 and Src in outside-in signaling, we asked whether Src is associated with αIIbβ3, either before or after platelet adhesion to fibrinogen, a response dependent on αIIbβ3. Human platelets were incubated for 45 min over a BSA matrix, to which they do not adhere, or a fibrinogen matrix, to which they adhere and gradually spread. Then platelets were solubilized in a buffer containing NP-40 detergent, the αIIbβ3 complex was immunoprecipitated with a polyclonal antibody to β3, and immunoprecipitates were probed on Western blots with an antibody to Src. As shown in Fig. 1 A, Src was detected in β3 immunoprecipitates whether or not the platelets had become adherent to fibrinogen. The same results were obtained if A2A9, an αIIbβ3 complex–dependent antibody, was used to immunoprecipitate the integrin (unpublished data). A comparison of the Src that did and did not quantitatively coprecipitate with αIIbβ3 indicated that ~3% of the Src solubilized from platelets was associated with αIIbβ3. Fyn and Yes, the only other Src family members tested, were also associated with αIIbβ3 (Fig. 1 A). Note in Fig. 1 A that the amounts of β3 and Src were slightly less in β3 immunoprecipitates from adherent platelets compared with nonadherent ones. This was attributed to a minor redistribution of these proteins to the detergent-insoluble actin cytoskeleton because it was prevented by preincubation of platelets with 10 μM cytochalasin D to inhibit actin polymerization (see Fig. 2 A).

Two other nonreceptor protein tyrosine kinases, Pyk2 and Btk, were not present in β3 immunoprecipitates, and FAK was present to a relatively minor degree despite being well represented in the detergent-soluble lysate (Fig. 1 A). The specificity of the Src association with αIIbβ3 was supported

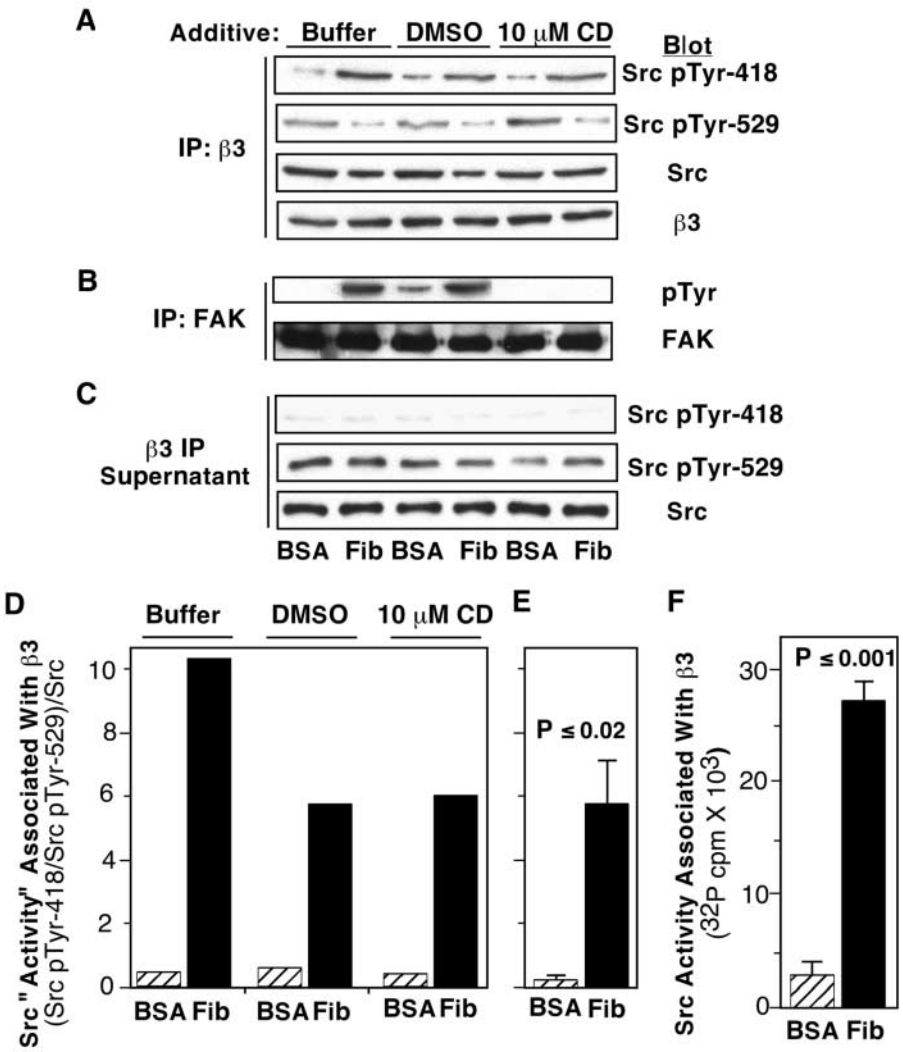

Figure 2. **Effect of platelet adhesion to fibrinogen on Src activation.** As described in the legend to Fig. 1, lysates from fibrinogen-adherent and -nonadherent platelets were immunoprecipitated with an antibody to β3 (A) or FAK (B), and immunoprecipitates were probed on Western blots as indicated. (C) An analysis of the pool of Src that did not coimmunoprecipitate with αIIbβ3 after two sequential immunoprecipitations with the anti-β3 antibody. In all panels, platelets were pretreated for 10 min with buffer, cytochalasin D (CD), or diluent (DMSO) before plating. (D) The raw data from the single experiment in A as Src "activity" associated with β3, expressed as the normalized ratio of Src pTyr-418/pTyr-529. (E) The means ± SEM of this ratio for five experiments. (F) The effects of platelet adhesion on Src activity in β3 immunoprecipitates, measured by an in vitro kinase assay as described in Materials and methods. Results represent means ± SEM of four experiments.

further by the observation that Src could not be detected in immunoprecipitates of GP Ibα, another prominent platelet adhesion receptor (Fig. 1 B). Csk, an Src-like tyrosine kinase that has been implicated in the negative regulation of Src family members (Okada et al., 1991; Latour and Veillette, 2001), was found to be associated with αIIbβ3 in suspended platelets but not adherent ones (Fig. 1 A). These results reveal a specific and constitutive interaction between Src and αIIbβ3 that is unaffected by platelet adhesion to fibrinogen, and an interaction between Csk and αIIbβ3 that is disrupted by adhesion.

To determine whether platelet adhesion to fibrinogen affects the activation state of Src, Western blots of αIIbβ3 immunoprecipitates were probed with phospho-specific antibodies to Src tyrosine residues 418 and 529. Autophosphorylation of Tyr-418 within the kinase activation loop is a marker of Src activation. On the other hand, phosphorylation of Tyr-529 (the target residue of Csk) promotes intramolecular interactions of the Src COOH terminus with the SH2 domain, effectively inhibiting kinase activity (Xu et al., 1999; Young et al., 2001). Thus, phosphorylation at Tyr-529 is a marker of Src suppression. Platelet adhesion to fibrinogen for 45 min caused a simultaneous increase in the phosphorylation of Src Tyr-418 and a decrease in the phosphorylation Tyr-529 (Fig. 2 A). This effect was observed within 15 min, the earliest time point at which sufficient numbers of adherent platelets could be analyzed (unpublished data). Src activation in fibrinogen-adherent platelets was unaffected by 10 μM cytochalasin D, implying that it did not require actin polymerization (Fig. 2 A). Adhesion-dependent tyrosine phosphorylation of FAK was blocked by cytochalasin D, as reported previously (Fig. 2 B) (Lipfert et al., 1992). In contrast to these results for integrin-associated Src, the bulk of the Src in platelets that did not coprecipitate with αIIbβ3 failed to become activated during platelet adhesion to fibrinogen (Fig. 2 C).

To analyze these results quantitatively, Src "activity" was expressed arbitrarily as the ratio of the phospho-specific Tyr-418 and Tyr-529 immunoblot signals after normalization for the amount of Src in the β3 immunoprecipitates (Fig. 2, D and E). Platelet adhesion to fibrinogen caused a mean 35-fold increase in Src activity as assessed by this method ($P < 0.02$). This effect was confirmed by direct measurements of Src kinase activity in β3 immunoprecipitates ($P < 0.001$) (Fig. 2 F).

In contrast to platelet adhesion to immobilized fibrinogen, binding of soluble fibrinogen to platelets requires prior activation of αIIbβ3. Therefore, to investigate whether soluble fibrinogen binding is sufficient to activate Src, platelets were in-

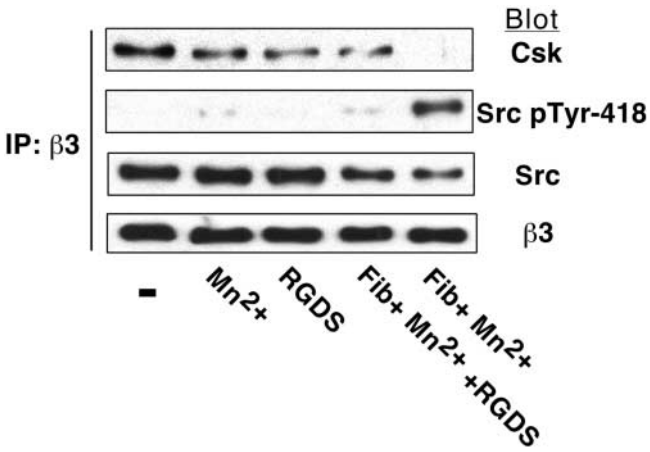

Figure 3. **Effect of soluble fibrinogen binding to platelets on αIIbβ3-associated Csk and Src.** Platelets were incubated as indicated in the presence or absence of 250 μg/ml fibrinogen, 0.5 mM $MnCl_2$, and 2 mM RGDS for 20 min. Then the presence of Csk and Src in β3 immunoprecipitates was analyzed as described in the legends to Figs. 1 and 2. Results are representative of two experiments.

cubated in the presence of 250 μg/ml fibrinogen, and 0.5 mM $MnCl_2$ was added to directly activate αIIbβ3 (Bazzoni and Hemler, 1998). Fibrinogen binding caused both the dissociation of Csk from the αIIbβ3 complex and the increased phosphorylation of Src Tyr-418 (Fig. 3, Fib + Mn^{2+} lane). These responses were observed as early as 1 min, were stable for at least 20 min, and were blocked by 2 mM RGDS, which inhibits fibrinogen binding to αIIbβ3. Interestingly, $MnCl_2$ or RGDS each induced a small amount Csk dissociation and Src Tyr-418 phosphorylation, suggesting that both integrin activation and ligation contribute to Src activation (Fig. 3). Together with the data for adherent platelets, these results establish that fibrinogen binding to αIIbβ3 causes dissociation of Csk from the αIIbβ3 complex at the same time that the integrin-associated pool of Src becomes activated. These responses require neither actin polymerization nor tyrosine phosphorylation of FAK.

The results presented so far are consistent with the idea that Src activation may occur in localized regions of the platelet where αIIbβ3 first comes in contact with fibrinogen. Therefore, the distribution of activated Src in fibrinogen-adherent platelets was determined by confocal microscopy using the anti–pTyr-418 antibody as a marker. Although this antibody may also react with the corresponding activation loop phosphotyrosine of other Src family members, it reacted on Western blots of platelet lysates with an apparent single band at ~60 kD, consistent with Src (unpublished data). Anti–pTyr-418 antibody staining was confined to the filopodia and edges of spreading platelets and to a central region corresponding to the granulomere. In contrast, the distribution of "total" Src determined with antibody 327 was more diffuse (Fig. 4). Thus, Src activation takes place in association with αIIbβ3 and at the periphery of spreading platelets.

Role of Src family kinases in outside-in signaling through αIIbβ3

To determine whether an Src kinase is required for αIIbβ3-dependent cytoskeletal changes, platelets were preincubated

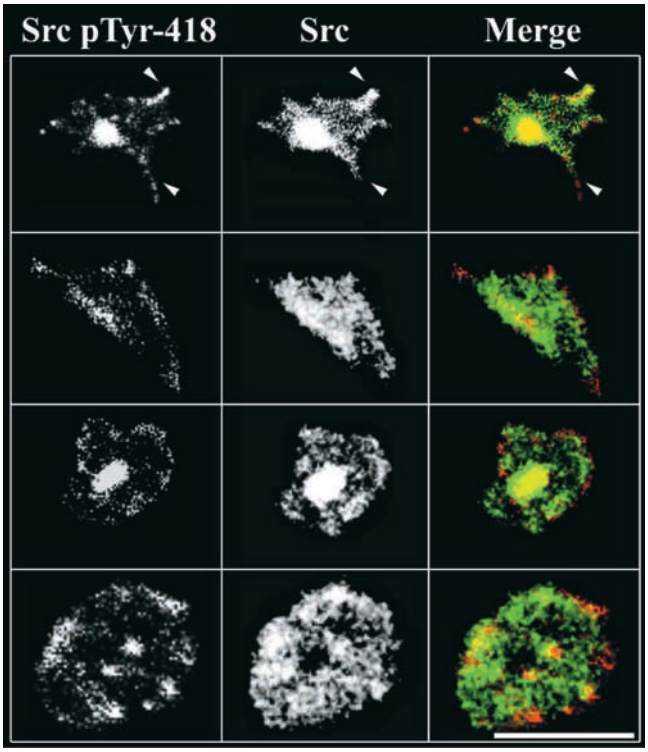

Figure 4. **Distribution of activated Src and total Src in fibrinogen-adherent platelets.** Cells were plated on fibrinogen-coated coverslips for 45 min and prepared for confocal microscopy as described in Materials and methods. Images represent four platelets in various stages of spreading. In the merged images, activated Src (Src pTyr-418) is red and total Src is green. Arrowheads point to some of the filopodia that stained heavily for activated Src. The results are from a single experiment representative of three so performed. Bar, 10 μm.

for 30 min with PP2, an inhibitor of Src family kinases (Hanke et al., 1996), and then plated on fibrinogen. PP3, an inactive analogue of PP2, was used as a control. 5 μM PP2 abolished adhesion-dependent tyrosine phosphorylation of Src Tyr-418 in β3 immunoprecipitates, but not phosphorylation of Tyr-529 or the association of Src with αIIbβ3 (Fig. 5). Control platelets adherent to fibrinogen underwent morphological changes ranging from filopodia protrusion to complete spreading, with F-actin and tyrosine-phosphorylated proteins evident at the cell periphery. In contrast, platelets treated with 5 μM PP2 generally showed fewer and smaller filopodia and spread very poorly (Fig. 6 A). The differences in spreading between PP2-treated and control platelets were significant as assessed by computerized image analysis of cell surface areas ($P < 0.001$). Although not shown, results identical to those with PP2 were obtained with 2 μM SU6656, a pharmacologically distinct, selective inhibitor of Src family kinases (Blake et al., 2000). Furthermore, the spreading defect of platelets treated with PP2 or SU6656 could be overcome if the cells were stimulated during adhesion with a combination of agonists to G protein–coupled receptors (1 mM PAR-4 receptor–activating peptide, 50 μM ADP, and 50 μM epinephrine).

To address the role of Src kinases without using chemical inhibitors, mouse bone marrow chimeras deficient in Src family members were generated. Murine platelets are smaller

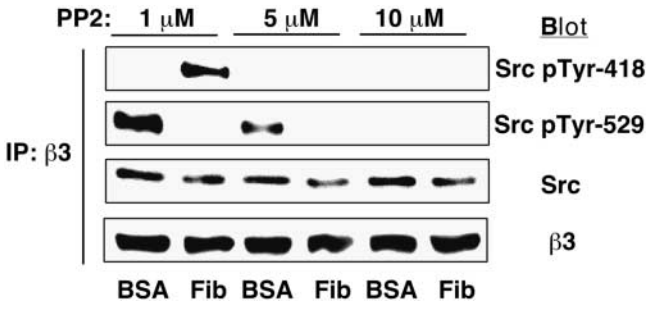

Figure 5. **Effect of PP2 on Src activity and Src association with αIIbβ3.** Platelets were preincubated for 30 min with PP2, plated on fibrinogen or suspended over BSA for 45 min, and Src in αIIbβ3 immunoprecipitates was analyzed as described in the legend to Fig. 1. Results are representative of two experiments.

than their human counterparts and spread less well on fibrinogen (Judd et al., 2000). Nonetheless, adherent wild-type platelets exhibited filopodial extensions, variable degrees of spreading, and tyrosine-phosphorylated proteins. The same was true for platelets lacking three Src kinases (Fgr, Hck, and Lyn). In sharp contrast, platelets deficient in Src as well as Fgr, Hck, and Lyn spread poorly on fibrinogen and exhibited minimal tyrosine phosphorylation (Fig. 6 B). Analysis of cell surface areas confirmed that the spreading defect of these latter platelets was significant ($P < 0.0001$). Thus, Src and possibly other Src family members are involved in αIIbβ3-dependent platelet spreading.

To identify potential downstream effectors of αIIbβ3 and Src, the effect of PP2 or SU6656 on adhesion-dependent tyrosine phosphorylation of several platelet proteins was examined. The results with PP2 are shown in Fig. 7 and are identical to those obtained with SU6656. PP2, but not PP3, inhibited adhesion-dependent tyrosine phosphorylation of Syk (Fig. 7 A), tyrosine phosphorylation of putative Syk substrates Vav1, Vav3, and SLP-76, and tyrosine phosphorylation of SLAP-130, an adaptor that binds to SLP-76 (Fig. 7 B) (Judd et al., 2000; Obergfell et al., 2001). On the other hand, PP2 had no effect on the adhesion-dependent association of Syk with αIIbβ3 (Fig. 7 C). Thus, one or more Src family kinases appear to be required for αIIbβ3-dependent activation of Syk and for tyrosine phosphorylation of Syk substrates implicated in cytoskeletal regulation (Judd et al., 2000; Obergfell et al., 2001).

Role of Syk in outside-in signaling through αIIbβ3

Platelets genetically deficient in Syk were evaluated to establish if Syk was required for either Src activation or for cytoskeletal changes that promote platelet spreading. The perinatal lethality of $syk^{-/-}$ mice was overcome by generation of $syk^{-/-}$ bone marrow chimeras. Compared with wild-type platelets, $syk^{-/-}$ platelets displayed fewer and less prominent filopodia, reduced tyrosine phosphorylation, and less spreading and ($P < 0.001$) (Fig. 8). However, when $syk^{-/-}$ platelets were stimulated during the adhesion process with a combination of PAR-4 receptor–activating peptide, ADP, and epinephrine, they now spread fully and exhibited tyrosine phosphorylation at cell edges (Fig. 8). As expected, $syk^{-/-}$ platelets spread poorly on fibrinogen when stimulated with collagen, which activates platelets in part through an Syk-coupled receptor, GP VI/FcR γ (Watson and Gibbins, 1998). Thus, Syk is required for platelet spreading mediated by outside-in signaling through αIIbβ3. However, spreading can occur in a manner independent of Syk if platelets are costimulated with agonists to G protein–coupled receptors.

To identify platelet proteins whose tyrosine phosphorylation is dependent on Syk, $syk^{-/-}$ platelets were subjected to immunoprecipitation and Western blot analysis. Compared with wild-type platelets, $syk^{-/-}$ platelets exhibited virtually

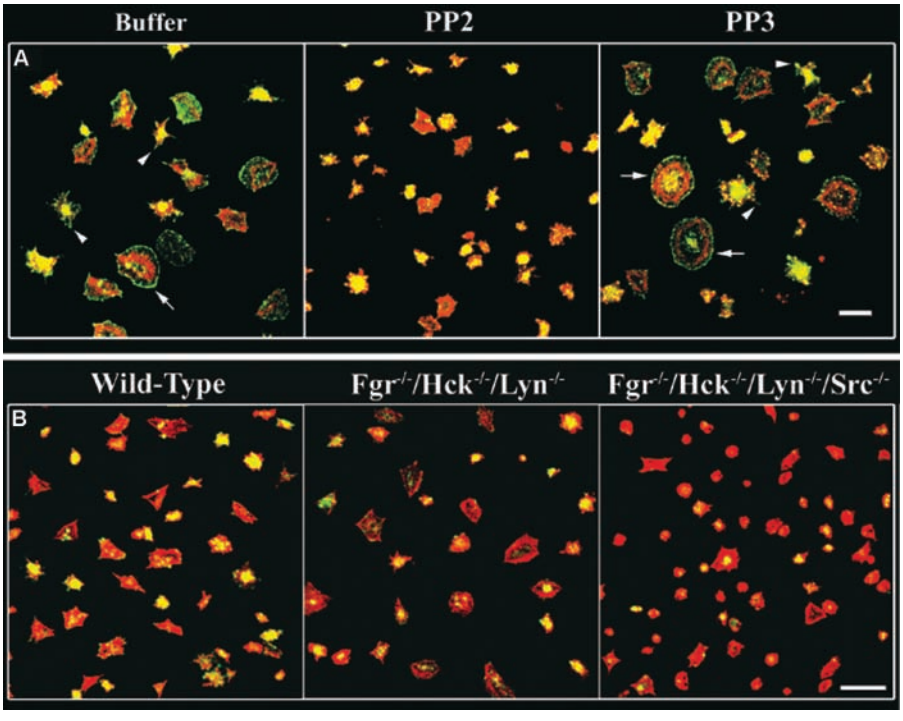

Figure 6. **Role of Src family kinases in platelet spreading on fibrinogen.** (A) Human platelets were preincubated for 30 min with buffer, 5 μM PP2, or 5 μM PP3. After adhesion to fibrinogen for 45 min, cells were fixed, permeabilized, stained for F-actin (red) and phosphotyrosine (green), and analyzed by confocal microscopy. Arrowheads orient the reader to filopodia and arrows to the peripheral rims of some of the spreading platelets. Results are representative of three experiments. (B) Platelets from wild-type or chimeric mice were plated on fibrinogen for 45 min, stained as above, and analyzed by confocal microscopy. Note that murine platelets are smaller than human platelets. Bars, 10 μm.

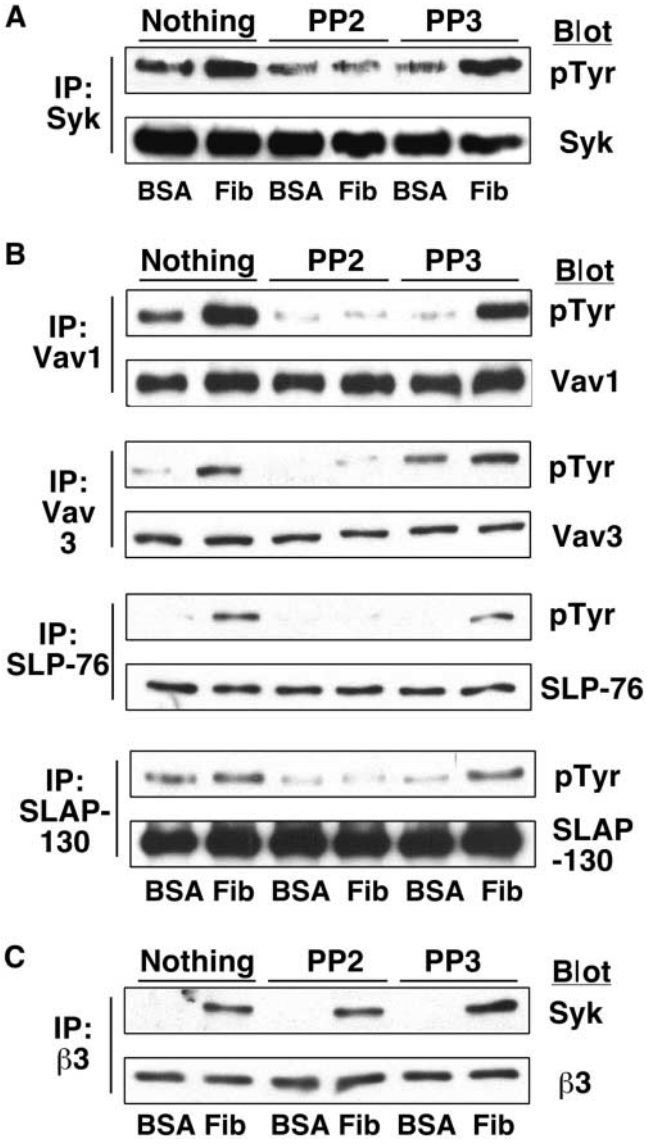

Figure 7. **Effect of inhibition of Src kinases on Syk and its substrates.** Platelets were processed as described in the legends to Figs. 1 and 5. (A) The effect of 5 μM PP2 on tyrosine phosphorylation of Syk. (B) The effect on tyrosine phosphorylation of Vav1, Vav3 and SLP-76. (C) The effect on adhesion-dependent association of Syk with αIIbβ3. Results are representative of two experiments.

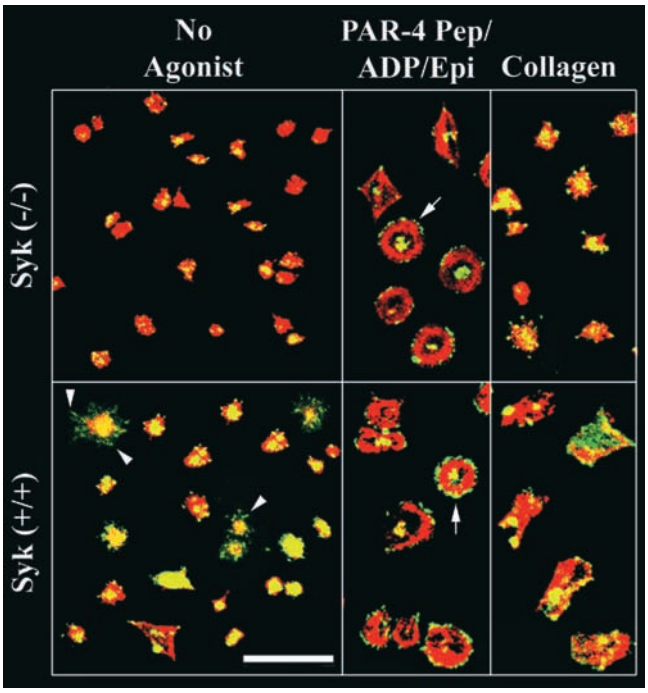

Figure 8. **Platelet spreading on fibrinogen is defective in syk$^{-/-}$ murine platelets.** Platelets were obtained from syk$^{-/-}$ and wild-type mice and plated on fibrinogen for 45 min with no agonist; with a combination of PAR-4 receptor–activating peptide (1 mM), ADP (50 μM), and epinephrine (50 μM); or with collagen (10 μg/ml). Cells were then fixed, permeabilized and stained for F-actin (red) and phosphotyrosine (green), and analyzed by confocal microscopy. Arrowheads point to filopodia and arrows to the peripheral rims of some of the spreading platelets. Results are representative of three experiments. Bar, 10 μm.

no adhesion-dependent tyrosine phosphorylation of Vav1, Vav3, or SLP-76, and reduced tyrosine phosphorylation of SLAP-130 (Fig. 9). In contrast, both the association of Src with αIIbβ3 and the adhesion-dependent activation of Src were normal in *syk*$^{-/-}$ platelets (Fig. 10). Altogether, these results indicate that Syk is downstream of Src but upstream of Vav1, Vav3, and SLP-76 in an αIIbβ3 signaling pathway that regulates the platelet actin cytoskeleton.

Discussion

Outside-in signaling through integrins mediates numerous anchorage-dependent cellular responses (Aplin et al., 1998; Giancotti and Ruoslahti, 1999; Schwartz, 2001). Components of the actin cytoskeleton are particularly important targets of integrin signals because they are often concentrated within integrin-based adhesion structures known as focal complexes and focal adhesions, and they participate in the regulation of cell shape and gene expression (Schoenwaelder and Burridge, 1999; Geiger et al., 2001). In adherent cells, an increase in tyrosine phosphorylation of cytoskeletal and signaling proteins is one of the most prominent events within actin-rich filopodia, lamellipodia, and focal adhesions (Hall, 1998; Geiger et al., 2001; Schwartz, 2001). In fibroblasts, the cell types most commonly studied in this regard, Src and FAK, have been identified as a prominent signaling complex that functions downstream of integrins to regulate cell migration and focal adhesion turnover (Guan, 1997; Polte and Hanks, 1997; Schlaepfer et al., 1997; Schaller et al., 1999). In hematopoietic cells, including platelets, Syk has been identified as a third type of tyrosine kinase that is activated by integrins (Clark et al., 1994; Lin et al., 1995). In fact, platelet adhesion to fibrinogen stimulates both the direct association of Syk with the cytoplasmic tail of β3 and Syk activation (Woodside et al., 2001). Unlike activation of FAK, Syk activation in adherent platelets precedes actin polymerization, and Syk substrates, including Vav1 and SLP-76, have been implicated in cytoskeletal regulation (Miranti et al., 1998; Judd et al., 2000; Obergfell et al., 2001). Therefore, Syk may participate in the initiation of αIIbβ3-dependent actin polymerization and platelet spread-

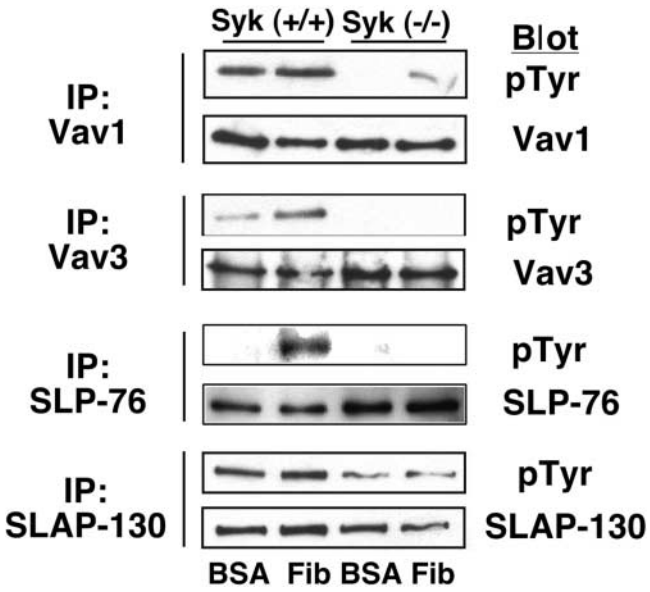

Figure 9. **Effect of Syk deficiency on adhesion-dependent tyrosine phosphorylation in platelets.** After plating on BSA or fibrinogen for 45 min, wild-type and syk$^{-/-}$ platelets were subjected to immunoprecipitation and Western blotting as indicated. Results are representative of three experiments.

ing. Src is also activated during platelet aggregation (Clark and Brugge, 1993), but its precise role in outside-in signaling in platelets has not been established.

Human- and gene-targeted mouse platelets were used here to define the relationships between αIIbβ3, Src, and Syk in outside-in signaling. The major new findings are: (a) Src and its regulatory kinase, Csk, are constitutively associated with αIIbβ3 in resting platelets. (b) Upon soluble fibrinogen binding to αIIbβ3 or platelet adhesion to immobilized fibrinogen, Csk dissociates from αIIbβ3 and Src becomes activated, independent of actin polymerization. Activated Src localizes to the periphery of spreading platelets, including filopodia. (c) The activity of Src is required for αIIbβ3-dependent tyrosine phosphorylation of Syk and for platelet spreading on fibrinogen. (d) Syk is also required for platelet spreading but not for Src activation. Thus, outside-in signaling in platelets is initiated by the sequential activation of Src and Syk in proximity to αIIbβ3, providing a molecular basis for signal generation from αIIbβ3 to the actin cytoskeleton.

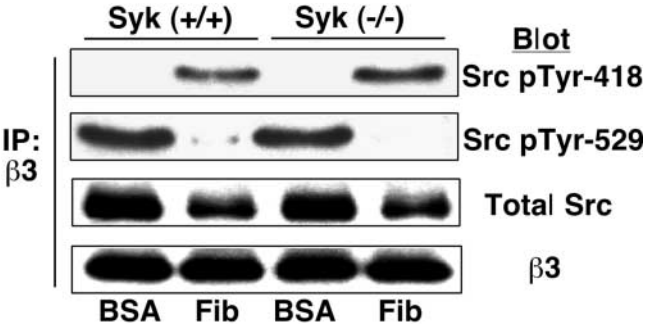

Figure 10. **Effect of Syk deficiency on adhesion-dependent activation of Src.** Wild-type and syk$^{-/-}$ platelets were plated on fibrinogen or suspended over BSA for 45 min, and β3 immunoprecipitates were analyzed on Western blots as indicated. Results are representative of three experiments.

Immunoprecipitation analysis of NP-40 detergent extracts revealed that Src and the related kinases, Fyn and Yes, were associated with αIIbβ3, both before and after platelet adhesion to fibrinogen. These interactions are specific because no association was observed between αIIbβ3 and two other nonreceptor tyrosine kinases, Pyk2 and Btk. Furthermore, Src did not coprecipitate with GP Ibα, another abundant platelet membrane adhesion receptor (Fig. 1). These results are consistent with a brief report that Src coprecipitates with αIIbβ3 from Triton X-100 lysates of resting platelets (Kralisz and Cierniewski, 1998). In another study, Dorahy et al. (1995) detected association of Src (and Lyn) with αIIbβ3, but only in the presence of a cell-permeable chemical cross-linker which simultaneously activated the platelets. Our ability to detect Src, Fyn, and Yes in αIIbβ3 immunoprecipitates without cross-linkers reflects differences in the conditions used for platelet preparation and analysis. Although we did not analyze αIIbβ3 immunoprecipitates for all Src family members expressed in platelets, these results indicate that Src associates with and is regulated by αIIbβ3, and this functional relationship may extend to some other Src family members.

These studies do not resolve whether the association of Src with αIIbβ3 is direct or indirect. Several proteins are capable of binding directly to the cytoplasmic tails of αIIb or β3 in vitro, and one of these might serve to link Src to αIIbβ3 in platelets (Shattil et al., 1998; Liu et al., 2000; Phillips et al., 2001). FAK and Syk warrant discussion in this regard. FAK can bind to peptides derived from integrin β tails (Schaller et al., 1995), and we found it to be associated with αIIbβ3 in platelet lysates (Fig. 1). Furthermore, when FAK becomes auto-phosphorylated at Tyr-397 in adherent fibroblasts, it forms a bimolecular complex with Src (Guan, 1997; Polte and Hanks, 1997; Schaller et al., 1999; Schaller, 2001). However, a FAK–Src complex cannot mediate the interaction we observed between αIIbβ3 and Src in platelets because, in contrast to FAK activation, the αIIbβ3/Src association was neither adhesion-dependent nor inhibited by cytochalasin D (Fig. 2 B). Syk is also unlikely to serve as a necessary link between Src and αIIbβ3 because it coprecipitated with the integrin only after platelet adhesion (Fig. 1), even in Syk-null platelets (Fig. 10) (Woodside et al., 2001). The precise mode of interaction between αIIbβ3 and Src remains to be determined.

How does fibrinogen binding to αIIbβ3 lead to activation of Src? Src is stabilized in an inactive conformation by intramolecular interactions of the SH2 domain with pTyr-529 and the SH3 domain with a polyproline helix in the SH2 kinase linker region (Xu et al., 1999; Young et al., 2001). Tyr-529 is likely maintained in the phosphorylated state by Csk (Okada et al., 1991; Latour and Veillette, 2001), a kinase that was associated with αIIbβ3 in nonadherent platelets. Fibrinogen interaction with platelets resulted in the dissociation of Csk (but not Src) from αIIbβ3 (Figs. 1 and 3), suggesting that Src may become activated at integrin adhesion sites following its physical separation from Csk. This idea is consistent with

the localization of activated Src to filopodia and edges of fibrinogen-adherent platelets (Fig. 4), and with the observation that only the integrin-associated pool of Src became activated in such platelets (Fig. 2). In T lymphocytes, the proximity of Csk to Src kinases is influenced by specific transmembrane proteins, such as PAG/Cbp, which are enriched in lipid rafts and bind Csk when tyrosine-phosphorylated (Brdicka et al., 2000; Kawabuchi et al., 2000). PAG/Cbp is also present in platelets (Watson et al., 2001), but additional studies will be required to determine if it is involved in regulating the association of Csk with αIIbβ3. Fibrinogen binding to platelets might also activate Src by influencing the localization or activity of a protein tyrosine phosphatase that can dephosphorylate Src Tyr-529. Three such phosphatases have been implicated in integrin signaling in other cell types, including receptor-like protein-tyrosine phosphatase-α, PTP-1B, and SHP-2 (Oh et al., 1999; Su et al., 1999; Cheng et al., 2001). In theory, Src might also be subject to regulation in platelets by proteins within nascent adhesion sites that engage the Src SH2 or SH3 domains (Xu et al., 1999; Young et al., 2001).

The results with PP2 and SU6656 strongly suggest that αIIbβ3-dependent tyrosine phosphorylation of Syk and platelet spreading on fibrinogen are controlled by an Src kinase. The defect in spreading of murine platelets lacking Src, Fgr, Hck, and Lyn confirms this assessment, and the normal spreading of platelets lacking Fgr, Hck, and Lyn suggests that Src itself plays a dominant role. However, the current studies do not exclude the involvement of other Src family members in specific phases of outside-in signaling.

PP2 and SU6656 blocked adhesion-dependent tyrosine phosphorylation of the Rac exchange factors, Vav1 and Vav3, and the molecular adaptor, SLP-76, all of which have been implicated in cytoskeletal regulation downstream of integrins in hematopoietic cells (Fig. 7) (Cichowski et al., 1996; Judd et al., 2000; Moores et al., 2000). Since these proteins are direct substrates of Syk, the results imply that Src kinases lie upstream of Syk in an αIIbβ3 pathway, possibly directly upstream. Although not studied, other substrates of Src kinases and Syk, such as cortactin and tubulin, might also couple αIIbβ3 to cytoskeletal events (Gallet et al., 1999; Faruki et al., 2000). Unlike Syk activation by immune response receptors that contain ITAM domains, αIIbβ3 activation of Syk is ITAM-independent (Gao et al., 1997; Turner et al., 2000; Woodside et al., 2001). Based on the current results, we speculate that fibrinogen binding to platelets induces clustering of αIIbβ3 complexes, leading to activation of Src kinases, interaction of Syk with the cytoplasmic tail of β3, and activation of Syk by the Src kinases. Activation of additional Syk molecules might then proceed by autophosphorylation in trans.

Syk$^{-/-}$ mouse platelets have a very subtle defect in agonist-induced fibrinogen binding (Law et al., 1999), and a profound defect in aggregation induced by collagen (Watson and Gibbins, 1998). These abnormalities cannot explain the spreading defect we observed in fibrinogen-adherent *syk*$^{-/-}$ platelets (Fig. 8) because no agonists were added in this experiment, and agonists are not required for platelet attachment to immobilized fibrinogen via αIIbβ3 (Savage et al., 1992; Law et al., 1999). By the same token, neither Src nor Syk were required for agonist enhancement of platelet spreading on fibrinogen (Fig. 8). Like platelets, Syk-deficient neutrophils exhibit defective integrin-dependent responses, such as the respiratory burst (unpublished data).

Although integrins and Src kinases are ubiquitous, Syk was thought until recently to be confined to hematopoietic cells. However, Syk is more widely distributed and may regulate the anchorage-dependent growth of epithelial and endothelial cells (Coopman et al., 2000; Inatome et al., 2001; Tsujimura et al., 2001; Yamada et al., 2001). Moreover, Syk can interact with integrin β1 and β2 tails as well as β3 (Woodside, D., and M. Ginsberg, personal communication), and our preliminary studies indicate that Src is associated with β1 integrins in platelets. Consequently, some of the functions attributable to Syk in hematopoietic and nonhematopoietic cells may require coordinated interactions between integrins, Src, and Syk. Thus, the paradigm delineated here for initiation of outside-in αIIbβ3 signaling in platelets may be relevant to integrins in a variety of biological contexts.

Materials and methods

Reagents

Anti-Csk antibody was from Transduction Laboratories; anti-Src pTyr-418 and anti–Src-pTyr-529 from BioSource International, Inc. Anti-Src antibody 327 recognizes the SH3 domain of c-Src (Lipsich et al., 1983) (unpublished data). Antibody 2-7 to Yes was generated by immunization of mice with the noncatalytic domain of Yes purified from *E. coli* extracts (Sudol and Hanafusa, 1986) (unpublished data). Antibodies to Fgr, Hck, Lyn, Syk, and Vav1 were from Santa Cruz Biotechnology, Inc., and antibodies to FAK, phosphotyrosine, Fyn, Btk, and Pyk2 were from Upstate Biotechnology. Antibody A2A9 is specific for αIIbβ3 and antibody #8053 is specific the β3 subunit (a gift from Mark Ginsberg, La Jolla, CA, The Scripps Research Institute, La Jolla, CA) (Bennett et al., 1983; Woodside et al., 2001). Antibodies AP-1 and #3584 to GPIbα were from Tom Kunicki and Zaverio Ruggeri (The Scripps Research Institute), respectively (Okita et al., 1985; Vicente et al., 1990). Antibodies to SLP-76 and SLAP-130 were from Gary Koretzky (University of Pennsylvania, Philadelphia, PA) (Motto et al., 1996). Antiserum #2206 to Vav3 was produced by rabbit immunization with a bacterially expressed GST-Vav3 fusion protein containing Vav3 amino acid residues 563–847. It did not cross-react with endogenous or recombinant Vav1 or Vav2 by Western blotting. HRP-conjugated secondary antibodies were from Bio-Rad Laboratories. Texas red– and FITC-conjugated anti–mouse and anti–rabbit IgG were from Jackson ImmunoResearch Laboratories, Inc. Rhodamine-phalloidin was from Molecular Probes; purified human fibrinogen was from Enzyme Research Laboratories, Inc.; collagen, leupeptin, and cytochalasin D were from Sigma-Aldrich, and sodium orthovanadate and sodium fluoride were from Fisher Scientific. Protein A Sepharose was from Amersham Pharmacia Biotech; Pefabloc and aprotinin were from Roche Molecular Biochemicals; PP2 and PP3 were from Calbiochem; and SU6656 was from SUGEN.

Bone marrow chimeras

src$^{+/-}$ mice (Soriano et al., 1991) were bred to the *hck*$^{-/-}$/*fgr*$^{-/-}$/*lyn*$^{-/-}$ background (Meng and Lowell, 1997) and mated to obtain *hck*$^{-/-}$/*fgr*$^{-/-}$/*lyn*$^{-/-}$/*src*$^{-/-}$ mutant embryos. *hck*$^{-/-}$/*fgr*$^{-/-}$/*lyn*$^{-/-}$/*src*$^{+/+}$ embryos were generated from the same pregnancy. *syk*$^{+/-}$ mice (Turner et al., 1995) were mated to obtain Syk$^{-/-}$ embryos. Fetuses were genotyped by PCR. Bone marrow chimeras were generated by injecting mutant or wild-type littermate fetal liver cells into lethally irradiated C57BL/6 recipients. Repopulation of the hematopoietic compartment by transplanted cells was confirmed by absence of the relevant tyrosine kinase(s) in platelets, as determined by Western blotting, and by absence of peripheral B-cells in the *syk*$^{-/-}$ chimeras, as determined by flow cytometry. Platelets from chimeras were studied 6–8 wk after transplantation. In some experiments, *syk*$^{+/-}$ rather than *syk*$^{+/+}$ chimeras were used as controls, with no differences in results from these two sources.

Interactions of platelets with fibrinogen

Washed human platelets were obtained from fresh, anticoagulated whole blood and resuspended to 3×10^8 cells/ml in a platelet incubation buffer

(Leng et al., 1998). To test the effects of soluble fibrinogen binding to αIIbβ3, platelets were incubated at room temperature for 15 min with 250 μg/ml fibrinogen in the presence or absence of 0.5 mM $MnCl_2$ (to activate integrins) (Bazzoni and Hemler, 1998) and 2 mM RGDS (to block fibrinogen binding). Platelets were then sedimented at 180 g for 5 min, washed once with phosphate-buffered saline, and solubilized for 10 min on ice in a buffer containing 0.5% NP-40, 50 mM NaCl, 50 mM Tris, pH 7.4 and inhibitors (1 mM sodium vanadate, 0.5 mM sodium fluoride, 0.5 mM leupeptin, 0.25 mg/ml Pefabloc, 100 μg/ml aprotinin). Lysates were routinely clarified by sedimentation at 10,000 rpm in a 4° microcentrifuge, and subjected to immunoprecipitation and Western blotting. Identical results were obtained if lysates were clarified at 100,000 *g* for 30 min. For studies of platelet adhesion to fibrinogen, 100 mm bacterial tissue culture plates were precoated with 5 mg/ml BSA or 100 μg/ml fibrinogen (Haimovich et al., 1993). After blocking with heat-denatured BSA, 4.5×10^8 platelets in 1.5 ml were added and incubated for the indicated periods of time at 37°C in a CO_2 incubator. Nonadherent cells from the BSA plates were sedimented at 10,000 rpm for 3 s in a microcentrifuge and lysed immediately. Platelets adherent to fibrinogen were gently rinsed twice in phosphate-buffered saline, lysed on the plates directly and processed for immunoprecipitation and Western blotting.

For studies of mouse platelets, anticoagulated mouse blood was obtained by cardiac puncture (Judd et al., 2000). Platelet preparation and experimental procedures were similar to those with human platelets.

Immunoprecipitation and Western blotting

500 μl aliquots of each platelet lysate containing equal amounts of protein (ranging from 500–750 μg between experiments) were immunoprecipitated with the indicated antibodies and protein A. Samples were electrophoresed in 7.5% SDS-polyacrylamide gels, transferred onto nitrocellulose and subjected to Western blotting (Gao et al., 1997; Miranti et al., 1998). Immunoreactive bands were detected by enhanced chemiluminescence with reaction times ranging from 5 s to 5 min. Blots were scanned in a Hewlett-Packard ScanJet 5300C scanner, and labeled bands were quantified by calibrated densitometry using NIH Image software.

Src Kinase Assay

αIIbβ3 was immunoprecipitated from platelet lysates with antibody #8053, and the protein A beads were washed twice. Beads were resuspended in kinase buffer and Src kinase activity was measured with a peptide substrate (KVEKIGEGTYGVVYK) according to the manufacturer's instructions (Src Assay Kit, Upstate Biotechnology Inc.)

Confocal microscopy

Washed human or mouse platelets were plated on fibrinogen-coated coverslips for 45 min at room temperature. Adherent cells were fixed in 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, and stained with primary and FITC- or Texas red–conjugated secondary antibodies as indicated. Rhodamine-phalloidin was used to stain F-actin. Single images were acquired with a Leica fluorescence microscope equipped with a laser scanning confocal system (MRC 1024; Bio-Rad Laboratories). Images were processed in Adobe Photoshop. Surface areas of at least 100 platelets per sample were measured using Image-Pro Plus Software (Media Cybernetics, Inc.). Statistical analyses were performed using Student's *t* test.

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