Analysis of Fyn function in hemostasis and αIIbβ3-integrin signaling

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Summary

Recent studies have shown that Src-family kinases (SFKs) play an important role in mediating integrin signalling, and the β3 subunit of αIIbβ3 integrin has been shown to interact with multiple SFK members. Here, we analyzed the interactions and functional consequences of Fyn and Src binding to αIIbβ3. Fyn associated with the β3 subunit in resting and thrombin-aggregated platelets, whereas interaction between Src and αIIbβ3 was seen predominantly in resting but not in thrombin-aggregated platelets. We have also observed that Fyn but not Src localized to focal adhesions in CHO cells adherent to fibrinogen through αIIbβ3. On the basis of these differences, we wanted to determine the sequence requirements for the interaction of Fyn and Src within the β3-cytoplasmic domain. Whereas Src association required the C-terminal region of β3, Fyn continued to interact with mutants that could no longer associate with Src and that contained as few as 13 membrane-proximal amino acids of the β3-cytoplasmic tail. Using deletion mutants of β3-cytoplasmic tails expressed as GST-fusion proteins, we narrowed down the Fyn-binding site even further to the amino acid residues 721-725 (IHDRK) of the β3-cytoplasmic domain. On the basis of these observations, we explored whether $Fyn^{-/-}$ mice exhibited any abnormalities in hemostasis and platelet function. We found that $Fyn^{-/-}$ mice significantly differed in their second bleeding times compared with wild-type mice, and platelets from $Fyn^{-/-}$ mice exhibited delayed spreading on fibrinogen-coated surfaces. Using mutant forms of Fyn, it appears that its kinase activity is required for its localization to focal adhesions and to mediate αIIbβ3-dependent cell spreading. Our results suggest that Fyn and Src have distinct requirements for interaction with αIIbβ3; and, consequently, the two SFK can mediate different functional responses.

Supplementary material available online at
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Key words: Integrin, αIIbβ3, Src-family kinases

Introduction

Src-family kinases (SFKs) are structurally related non-receptor tyrosine kinases (Brickell, 1992). Various SFKs have been implicated in signaling pathways that are initiated by multiple transmembrane receptors, including integrins (Appleby et al., 1992; Arias-Salgado et al., 2003; Courter et al., 2005; Klinghoffer et al., 1999). Several SFKs, including Src, Fyn, Lyn and Yes, are readily detected in megakaryocytes and circulating platelets (Melford et al., 1997; Pestina et al., 1997; Stenberg et al., 1998), and multiple lines of evidence suggest that the SFKs are involved in regulating the platelet adhesion, the response key to the role of these cells in thrombosis and hemostasis. For example, platelets treated with pharmacological SFK-inhibitors showed reduced spreading on fibrinogen, a ligand for the major platelet integrin, αIIbβ3; and platelets isolated from the mice lacking several SFKs exhibited poor spreading on fibrinogen (Obergfell et al., 2002). Together, these observations suggest a functionally important relationship between SFKs, αIIbβ3 integrin and the adhesive responses of platelets.

The predominant integrin on the platelet surface is αIIbβ3 (GPIIb-IIIa), and it is also the most prominent membrane protein on these cells. On circulating, non-stimulated platelets, αIIbβ3 exists in a low-affinity conformation and does not bind its plasma ligands such as fibrinogen. Following platelet activation by any of a variety of agonists, αIIbβ3 is rapidly converted to an activated state in which it is able to bind fibrinogen with high affinity (Bennett and Vilaire, 1979; Marguerie et al., 1979; Shattil et al., 1985). This transition is the consequence of inside-out signaling. Ligand binding, in turn, induces a cascade of signaling events collectively termed as outside-in signaling. The cytoplasmic tails of αIIbβ3 must receive and transmit these outside-in signals. Since they do not exhibit any intrinsic enzymatic activity, changes in the proteins associated with these cytoplasmic tails are presumed to be involved in the propagation of outside-in signaling. Many such integrin-associated proteins have been identified, and include cytoskeletal, adaptor and known signaling proteins (reviewed in Buensuceso et al., 2004; Liu et al., 2000; Ma et al., 2007; Phillips et al., 2001). Some of these associated proteins appear to regulate inside-out signaling (Bertoni et al., 2002; Calderwood et al., 1999; Ma et al., 2007; Vinogradova et al., 2002), and a variety of signaling molecules, such as calpain, Rho-family GTPases, tyrosine kinases and lipid kinases, are activated as a consequence of outside-in signaling (Barry et al., 1997; Ferrell, Jr and Martin, 1989; Fox and Phillips, 1983; Fox et al., 1993; Frangioni et al., 1993; Golden et al., 1990; King et al., 1997; Laudanna et al., 1996; Lipfert et al., 1992; Price et al., 1998; Renshaw et al., 1996).

Recent studies have implicated SFKs in the regulation of integrin outside-in signaling (Arias-Salgado et al., 2003; Courter et al., 2005; Obergfell et al., 2002; Wary et al., 1998). Src is the most abundant

SFK is both human and mouse platelets, and Src has been shown to bind constitutively and selectively to the β3-integrins when overexpressed in a Chinese hamster ovary (CHO) cell system (Arias-Salgado et al., 2003). The extreme C-terminal RGT sequence of β3 subunit has been shown to be essential for Src binding (Arias-Salgado et al., 2005), and this same sequence has also been implicated in the binding of Fyn to the β3 subunit (Arias-Salgado et al., 2005). In model cell systems, multiple SFKs had to be knocked out in order to inhibit β3 integrin signaling (Klinghoffer et al., 1999). Thus, it has been presumed that there should be functional redundancy in the ability of integrins to associate with SFKs, and Src should be the predominant SFK in regulating integrin signaling across αIIbβ3 in platelets. Working under the assumption that there is no complete overlap between the various SFKs, we have examined features that might be unique to a particular SFK, Fyn, and its interaction with αIIbβ3. Surprisingly, we found evidence for an interaction of Fyn with αIIbβ3 that is not shared with Src, and that mouse platelets lacking this single SFK exhibit functional defects that are not compensated by other SFKs.

Results

Differential association of Fyn and Src with αIIbβ3

To investigate the association of SFKs with αIIbβ3, we performed co-immunoprecipitation experiments. Lysates from resting or thrombin-treated (aggregated) platelets were immunoprecipitated with either Fyn or Src antibodies, and the immunoprecipitates were analyzed for the presence of αIIbβ3 using β3 subunit antibodies. Consistent with the literature (Arias-Salgado et al., 2003), anti-Src antibody co-precipitated αIIbβ3. Whereas this association was evident in resting platelets, it was much less prominent in thrombin-treated platelets (Fig. 1A). By contrast, anti-Fyn antibody readily immunoprecipitated αIIbβ3 from both resting and thrombin-aggregated platelets (Fig. 1A). Densitometric analysis showed that the β3 subunit that immunoprecipitated with Src from thrombin-aggregated platelets was reduced by 71% compared with that which immunoprecipitated with Src from the resting platelets. However, Fyn-immunoprecipitates from thrombin-aggregated platelets contained only 7% less β3 than that immunoprecipitated from resting platelets (Fig. 1B). The loss of interaction between Src and β3 integrin is probably not because of their translocation to the triton-insoluble fraction. We found that the amounts of Src and β3-integrin in the triton-soluble fraction were comparable in the resting and the thrombin-treated samples (supplementary material Fig. S2). The amounts of Fyn immunoprecipitated from resting and thrombin-treated platelets were also similar (Fig. 1C).

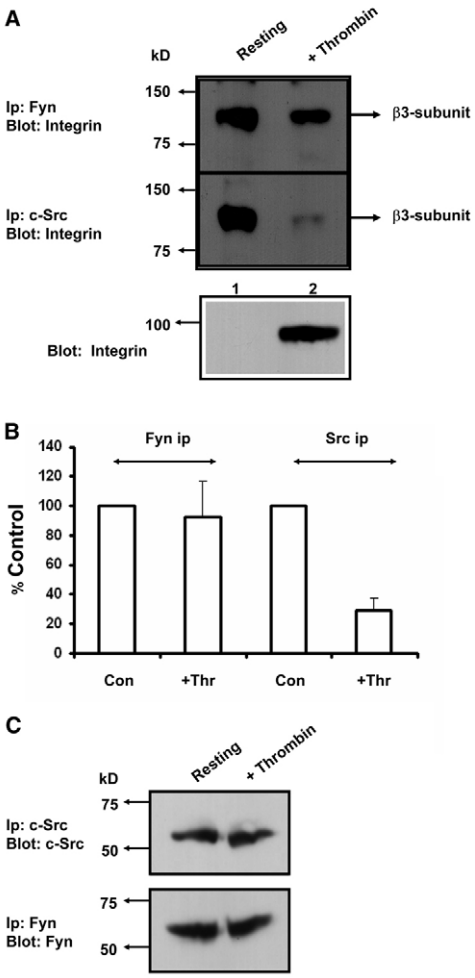

Fig. 1. Co-immunoprecipitation of αIIbβ3 with SFKs from platelets. (A) Equal amounts of total protein in the Triton-X-100-soluble extract of resting platelets or thrombin-treated (aggregated) platelets were subjected to immunoprecipitation using indicated anti-SFK antibodies. The immunoprecipitates were subjected to immunoblot analysis using anti-β3 antibodies. AP3 antibody (lane 2; anti-β3 antibody; positive control) but not control Ig (lane 1; normal mouse serum) immunoprecipitated β3 integrin (bottom panel). (B) Densitometric analysis (n=3) of blots in panel A. (C) Anti-SFK antibodies immunoprecipitated equal amounts of Src and Fyn from resting and thrombin-treated platelets.

Cellular localization of Fyn

To further understand the role of Fyn in αIIbβ3 signaling, we turned to a malleable model system: CHO cells that expressed αIIbβ3. These cells have the advantages that they have been used extensively to analyze αIIbβ3 structure and signaling. Also, unlike platelets, they are transfectable and can express gene products at high levels. To determine the cellular localization of various SFKs, we introduced the cDNAs for Fyn and Src into CHO-αIIbβ3 cells by transfection. Forty-eight hours after transfection, the cells were trypsinized and re-plated onto fibrinogen-coated coverslips and allowed to spread for 24 hours and processed for immunohistochemistry. As shown in Fig. 2, Fyn was primarily localized to the tips of actin-stress fibers in defined structures, the focal adhesions. Endogenous Fyn exhibited a distribution similar to that of transfected Fyn and could be detected in the focal adhesions of the human placental trophoblast cell line 3A-sub-E (supplementary material Fig. S3). Immunohistochemistry experiments showed that the 3A-sub-E cells lacked β1 integrins and primarily attach to immobilized fibrinogen through β3 integrins (supplementary material Fig. S4). Unlike Fyn, Src was primarily distributed in a punctate pattern throughout the cell and was absent from the focal adhesions (Fig. 2). Previous studies in platelets also showed that the majority of Src was present in the cytoplasm (Obergfell et al., 2002). Expression of Src did not alter the formation of stress fibers or focal adhesion (Fig. 2 and supplementary material Fig. S3). Image analyses using the Image Pro-Plus software documented the propensity of Fyn to localize to focal adhesions was 4.7-fold higher compared with the rest of the cell. Similar analyses for Src gave a ratio of 0.55, suggesting that most Src is not present in focal adhesions and may even be selectively excluded from these structures. However, αIIbβ3 was detected in both focal

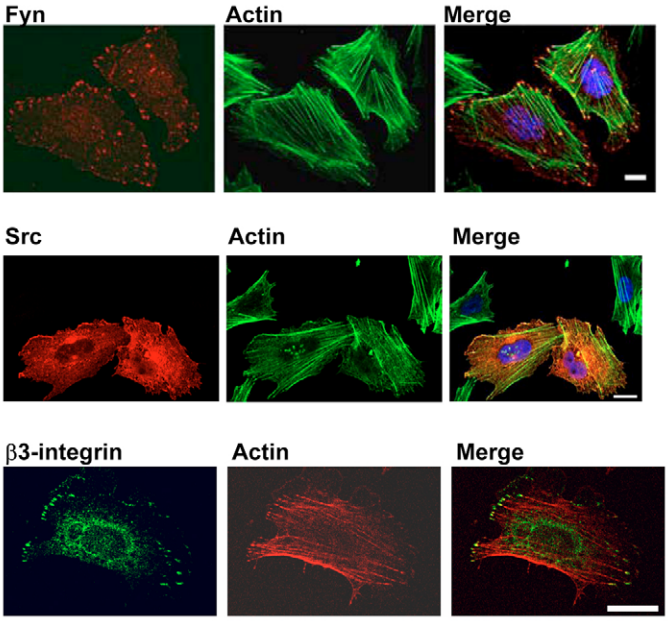

Fig. 2. Images showing the cellular localization of Fyn, Src and αIIbβ3 in CHO cells. CHO cells stably expressing the αIIbβ3 were transfected with the cDNAs encoding either Fyn or Src. 48 hours after transfection, cells were allowed to spread for an additional 24 hours on coverslips coated with fibrinogen. Cells were fixed and subjected to immunohistochemistry using antibodies against Fyn, Src and β3 integrin. Cells were simultaneously stained with fluorescently labeled phalloidin. Nuclei are shown in blue. Scale bars, 20 μm.

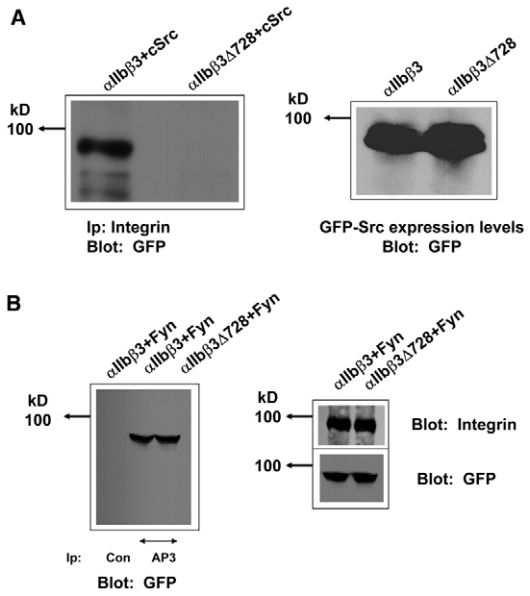

Fig. 3. Fyn associates with both the full-length and the deletion mutant of αIIbβ3. (A,B) CHO cells stably expressing either full-length or truncated β3-subunits, together with the full-length αIIb subunit, were transiently transfected with the cDNAs encoding (A) GFP-Src or (B) GFP-Fyn . 48 hours after transfection, cells were re-plated on fibrinogen-coated plates for 1 hour. Cell extracts were subjected to immunoprecipitation using antibodies against the β3 subunit. The immunoprecipitates were then analyzed for (A) Src or (B) Fyn using anti-GFP antibodies. Expression levels of transfected integrins and GFP fusions are also shown.

adhesions at the tips of actin stress fibers as well as outside focal adhesions in smaller complexes in the peri-nuclear region (Fig. 2). On the basis of these observations, it appears that Fyn colocalizes with αIIbβ3 in focal adhesions, structures formed as a consequence of ligand engagement of the integrin; Src is not associated with these structures. Differences in Src and Fyn distribution was also seen in platelets (Stenberg et al., 1997). Hence, the two SFKs exhibit distinct patterns of colocalization with integrin-containing complexes.

Fyn and Src associate with different regions of αIIbβ3

It has been previously shown that the C-terminal RGT residues (amino acid residues 760-762) of β3 are necessary and sufficient for Src binding (Arias-Salgado et al., 2005). On the basis of the in vitro solid-phase-binding assays, it was shown that the same region was also involved in binding to Fyn (Arias-Salgado et al., 2005). However, on the basis of our observations that Fyn and Src differentially associated with αIIbβ3 in thrombin-treated platelets (Fig. 1) and that they also localized to different regions within the CHO-αIIbβ3 cell (Fig. 2), we explored the possibility that the Fyn- and Src-binding sites within β3 subunit are different. CHO cell lines expressing αIIb in complex with full-length or a truncated form of β3 (truncated at residue 728; β3Δ728) were generated. On the basis of fluorescence-activated cell sorting (FACS)-analysis, expression levels of the two forms of αIIbβ3 were similar (mean fluorescence intensities by FACS obtained by staining with AP3 antibody were 240 for αIIbβ3 and 297 for αIIbβ3Δ728). These cells were transiently transfected with the cDNAs coding for either Src or Fyn as GFP-fusion proteins. The interaction between αIIbβ3 and SFKs were analyzed in co-immunoprecipitation experiments. As reported previously (Arias-Salgado et al., 2003), Src co-immunoprecipitated with αIIbβ3 that contained the full-length but not the truncated form of β3-subunit (Fig. 3A). Expression levels of Src in both the cell lines were comparable (right panel, Fig. 3A). However, Fyn readily co-immunoprecipitated with αIIbβ3 that contained either the full-length or the truncated form of β3 subunit (Fig. 3B). This association between Fyn and truncated β3 was observed in five separate experiments.

Independent verification that Fyn and Src interact differentially with the β3-cytoplasmic domain was obtained using a splice variant of the β3-cytoplasmic tail. Alternative splicing can lead to different forms of the β3-cytoplasmic tail, the β3a and β3c-variants (Kumar et al., 1997; van Kuppevelt et al., 1989), which have a common

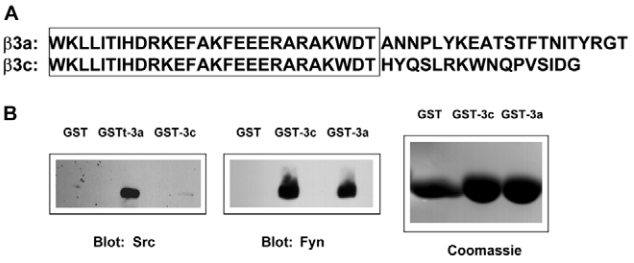

Fig. 4. Differential binding of SFKs with the GST-β3-spliced variants. (A) Amino acid sequences of β3 variants used in this study. Identical residues between the variants are boxed. (B) Various GST-β3 tails were incubated with the platelet lysates. The captured proteins were subjected to SDS-PAGE followed by western blot analyses using indicated antibodies. The protein loading is shown in the Coomassie stain.

Fig. 5. Identification of the Fyn-binding region within the β3-cytoplasmic domain. (A) Amino acid sequences of β3-deletion mutants used in this study. (B) The different GST-β3-deletion mutants were incubated with the platelet lysates. Proteins were subjected to SDS-PAGE followed by western blot analyses using anti-Fyn antibodies. The protein loading is shown in the Coomassie stain.

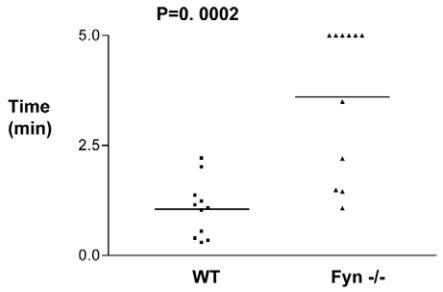

Fig. 6. Fyn$^{-/-}$ mice exhibit prolonged second bleeding times. Tail-bleeding times were measured in Fyn$^{-/-}$ and the corresponding wild-type (WT) mice (n=11) as described in Materials and Methods. Second bleeding was allowed for 5 minutes, at which point the experiment was stopped and tails were pressured to stop bleeding. The mean second bleeding time in Fyn$^{-/-}$ and WT mice were 3.61 minutes and 1.047 minutes, respectively; P=0002.

membrane-proximal sequences (residues 715-741), but differ in their membrane distal regions (residues 742-762; Fig. 4A). We incubated platelet lysates with glutathione S-transferase (GST)–β3-tail variants and analyzed the captured proteins for associated Fyn or Src. Immunoblot analysis showed that both variants efficiently pulled down Fyn from platelet lysates. However, Src efficiently associated with the β3a-variant; and exhibited a poor association with the β3c-variant (Fig. 4B).

To further delineate the Fyn-binding site, we constructed additional deletion mutants of β3-integrin and expressed them as the GST-fusion proteins (Fig. 5A). Platelet lysates were incubated with various deletion mutants and pull-down assays were performed. Consistent with our results (Figs 3, 4), both GST-β3c as well as GST-β3Δ728 brought down Fyn efficiently (Fig. 5B). Deletion of additional two C-terminal residues as in the case of GST-β3Δ726 did not alter the Fyn-binding capacity (Fig. 5B). However, further deletion of an additional five residues – as in GST-β3Δ721 – resulted in the loss of Fyn binding (Fig. 5B), suggesting that residues 721-725 (IHDRK) of β3-integrin are required for Fyn binding. These results suggest that the Fyn and Src interact with different regions within the cytoplasmic domain of β3-subunit. Fyn continues to interact with a β3-cytoplasmic tail that lacks the C-terminal RGT residues.

Characterization of Fyn$^{-/-}$ mice

Although several SFKs have been shown to interact with β3-tails in in-vitro binding studies (Arias-Salgado et al., 2003; Arias-Salgado et al., 2005), on the basis of the results presented in Figs 1-5, which show that Fyn is more prominently associated with αIIbβ3 than Src in aggregated platelets, interacts with an unique region of β3-integrin, and is present in focal adhesions, we examined Fyn$^{-/-}$ mice for defects in platelet-dependent hemostasis. As a first step, we measured the bleeding times in Fyn$^{-/-}$ mice and found that there were no significant differences in the first bleeding times (1.419±0.45 minutes for wild type, 1.00±0.58 minutes for Fyn$^{-/-}$, see Materials and Methods for details). There was also no significant difference in the interval time between the first and second bleeding time (1.917±1.74 minutes for wild type, 0.97±0.69 minutes for Fyn$^{-/-}$). However, we observed significant differences in the second bleeding time (P=0002). Fyn$^{-/-}$ mice exhibited prolonged second bleeding times compared with the corresponding wild-type mice (Fig. 6). The mean second bleeding time in Fyn$^{-/-}$ was measured to be 3.61±0.5153 minutes compared with 1.04±0.1947 minutes in wild-type mice (n=11, Fig. 6). The observed bleeding defect in Fyn$^{-/-}$ mice was not due changes in levels of other SFKs in platelets (supplementary material Fig. S1).

On the basis of these differences, we explored whether platelets from Fyn$^{-/-}$ mice exhibited other abnormalities. We used the JON/A antibody to study the agonist-induced signaling. This antibody reacts selectively with the activated conformer of αIIbβ3 (Bergmeier et al., 2002). We did not observe any significant differences between wild-type and Fyn$^{-/-}$ mice with respect to JON/A binding that was induced by thrombin or two different doses of ADP (Table 1). In addition, platelets from Fyn$^{-/-}$ mice aggregated normally in response to various agonists tested, similar to the platelets from wild-type mice (supplementary material Fig. S5). Typically, 0.1 U thrombin induced ~80% aggregation under stirring conditions in wild-type and Fyn$^{-/-}$ mice (~3×10^7 platelets/ml). Under similar conditions, collagen (5 μg/ml) and ADP (50 μM) induced ~80% and ~50% aggregation, respectively, with no obvious differences between platelets from wild-type and Fyn$^{-/-}$ platelets. Although we could not demonstrate a prominent role of Fyn in αIIbβ3 activation, a role in outside-in signaling was observed. We found that platelets from Fyn$^{-/-}$ mice exhibited delayed spreading on immobilized fibrinogen (Fig. 7A). Whereas 63% of platelets from wild-type mice were fully spread at 30 minutes after plating onto fibrinogen, only 17% of platelets from Fyn$^{-/-}$ mice were fully spread at the same time (Fig. 7B top panel, data from an independent experiment is provided in the bottom panel). However, these differences were not maintained at later time points (95% of the Fyn$^{-/-}$ platelets were spread at 3 hours, data not shown). These observations suggest that

Table 1. JON/A binding to mouse platelets

	% Positives for JON/A binding	
Treatment	WT	Fyn$^{-/-}$
Thrombin (0.1 U)	94.8	96.9
ADP (10 μM)	41.77	47.83
ADP (50 μM)	52.36	64

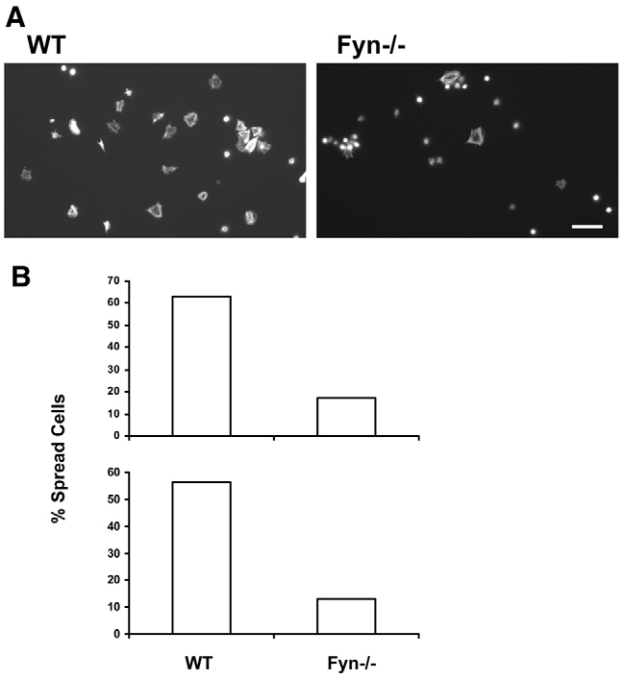

Fig. 7. Spreading of platelets on fibrinogen-coated surface. (A) Washed platelets from wild-type (WT) and Fyn$^{-/-}$ mice were plated onto fibrinogen-coated coverslips. Pooled platelets from three to four mice were used for these studies. Platelet numbers were 10^6 cells per well in six-well plates. Cells were allowed to adhere for 30 minutes, fixed, stained with fluorescently labeled phalloidin and then images were captured. Scale bar, 10 μm. (B) The cells from six different fields (a total of 200-300 cells) were counted to determine the percentage spread cells. Data from an independent experiment are given in the bottom panel.

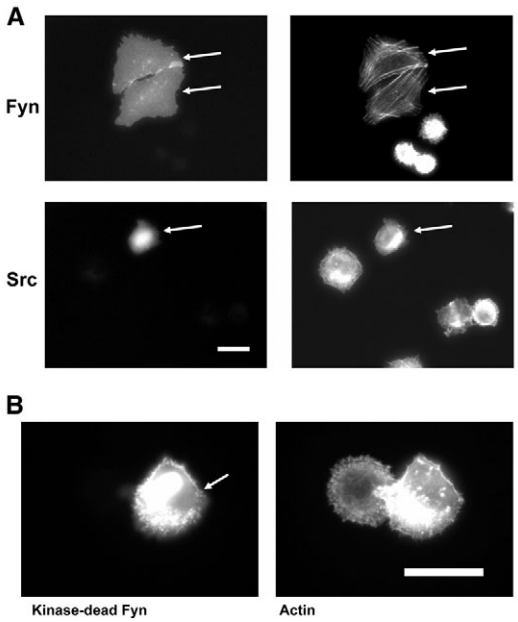

Fig. 8. Transient expression of Fyn restores cell spreading in CHO cells that express the truncated form of β3-integrin. (A) CHO cells expressing the deletion mutant αIIbβ3Δ754 were transfected with the cDNAs encoding either Fyn (top panels) or Src (bottom panels). 48 hours after transfection, cells were allowed to spread for 30 minutes in serum-free medium on coverslips coated with fibrinogen. Cells were fixed and stained with TRITC-labeled phalloidin. Cells transfected with Fyn and Src were identified by the GFP fluorescence (indicated with arrows). (B) Cells were transfected with cDNA encoding a kinase-dead version of Fyn (FynK299M) and then simultaneously stained using anti-Fyn antibodies and phalloidin. Scale bars, 20 μm.

the absence of Fyn results in a modest but clear effect on αIIbβ3-dependent platelet function. Spreading defects in Fyn$^{-/-}$ mice were observed on fibrinogen, an αIIbβ3 ligand, but were not observed on collagen, a ligand for other integrins (supplementary material Fig. S6). The extent of ADP-induced spreading of platelets was similar in wild-type and Fyn$^{-/-}$ mice, even at earlier time points (supplementary material Fig. S7). A preliminary report from Eto et al, also suggested spreading defects in Fyn$^{-/-}$ platelets (Eto et al., 2007).

Distinct role for Fyn in αIIbβ3-mediated signalling

Several independent studies have demonstrated that αIIbβ3-mediated cell spreading requires an intact cytoplasmic β3 subunit containing its two NxxY motifs at residues 744-747 and 756-759. Deletion of or point mutations in these motifs results in a loss of αIIbβ3-mediated cell spreading (O'Toole et al., 1995; Schaffner-Reckinger et al., 1998; Ylanne et al., 1995). Consistent with these previous reports, we found that cells that express a truncated form of integrin (αIIbβ3Δ754) failed to spread (untransfected cells, Fig. 8A). As shown in Fig. 8A, expression of Fyn but not Src rescued cell spreading in CHO cells expressing truncated β3 integrins. This rescue by Fyn was seen in 94% of transfected cells. To determine whether the kinase activity of Fyn is required for integrin-mediated cell spreading, we transfected the CHO-αIIbβ3 cells with cDNA encoding the kinase-dead form of Fyn (Fyn-KD; Fyn K299M). Immunohistochemical analysis showed that Fyn-KD was present throughout the cell, but was absent from the focal adhesions (Fig. 9A), i.e. it displayed a pattern similar to Src but distinct from that of wild-type Fyn. In addition, Fyn-KD failed to rescue the cell spreading in CHO cells expressing truncated forms of β3-integrin (Fig. 8B). This failure to rescue was observed in all cells transfected with Fyn-KD. However, a constitutively active form of Fyn (FynΔ525) was readily incorporated into focal adhesions, similar to wild-type Fyn (Fig. 9B). On the basis of these results, it appears that the kinase activity of Fyn is required for both, its targeting to focal adhesions as well as for mediating integrin signaling.

Discussion

Integrins are bidirectional signaling receptors, whose cytoplasmic tails function as both receivers and transmitters of various signaling pathways (Ginsberg et al., 2005; Hynes, 1992; Hynes, 2002; Liddington and Ginsberg, 2002). The integrin cytoplasmic tails, including those of the αIIb and β3 subunits, are generally short and do not have any known catalytic activity. Searches to define other mechanisms of integrin signaling have led to the identification of multiple cytoskeletal and signaling proteins that directly associate with the cytoplasmic tails of either the α- or β-subunits and influence the integrin-induced signaling (reviewed in Buensuceso et al., 2004; Liu et al., 2000; Phillips et al., 2001). A recent focus of such studies has been the association and functional consequences the non-receptor tyrosine kinases, including specific SFKs in regulating integrin signaling (Arias-Salgado et al., 2003; Arias-Salgado et al., 2005; Obergfell et al., 2002). Several SFKs have been shown to bind to the cytoplasmic domains of integrins, and of these, Src and

Fyn exhibited selective interaction with αIIbβ3 (Arias-Salgado et al., 2003; Obergfell et al., 2002). Src has been shown to associate constitutively with unactivated αIIbβ3, binding to the extreme C-terminal RGT residues of the β3 subunit through its SH3-domain (Arias-Salgado et al., 2005). In vitro assays further suggested that the SH3-domain of Fyn also exhibits RGT-dependent association with β3 (Arias-Salgado et al., 2005). However, the data presented here suggest that two SFK members, Src and Fyn, interact differently with αIIbβ3. Whereas both Fyn and Src exhibited an interaction with αIIbβ3 in resting platelets, only the interaction between Fyn and αIIbβ3 is sustained following platelet aggregation; that of Src, however, was lost (Fig. 1). The diminution of Src association during platelet aggregation might be a consequence of cleavages in the C-terminal region of β3 by calpain that occur in thrombin-aggregated platelets and would delete the Src-binding site (Du et al., 1995) (supplementary material Fig. S2).

The conclusion that Fyn and Src bind to a distinct site has been independently corroborated using variant forms of the β3 subunit expressed in heterologous cells (Fig. 3). A β3 spliced variant and a β3-integrin-deletion mutant lacking C-terminal RGT residues retained the capacity to interact with Fyn whereas this variant failed to bind Src (Fig. 4). Further studies using deletion mutants of the β3 cytoplasmic domain revealed that the residues IHDRK (residues 721-725) are sufficient to support an interaction with Fyn (Fig. 5). Together, these results indicate that Fyn can interact with a distinct set of residues from Src in αIIbβ3. Our results cannot exclude the possibility that the association of Fyn with this small segment of β3 is indirect, i.e. mediated through another molecule that binds to this region of β3 and to Fyn. In either case, Src does not share this binding mechanism. Amino acid residues 721-725 of β3-integrin have also been implicated in binding to other cellular proteins such as skelemin and FAK (Reddy et al., 1998; Schaller et al., 1995; Lyman et al., 1997). Although the interaction between integrin and Fyn is absent in the $Fyn^{-/-}$ mice, it is important to realize that other known interactions between the β3 cytoplasmic domain and various cellular proteins (e.g. the interactions with other SFK members, FAK and/or skelemin) will be present in $Fyn^{-/-}$ mice and might explain the mild nature of the bleeding defect.

From the literature, it is clear that the αIIbβ3 integrin uses multiple signaling intermediates to mediate its biological effects leading to cell spreading and cytoskeletal reorganization. The small GTPase Rac1 is one such signaling intermediate and has been implicated in αIIbβ3-mediated cell spreading. Rac1 is activated is within 30 minutes upon adhesion of CHO-αIIbβ3 cells to immobilized fibrinogen (Berrier et al., 2002). This Rac1 activation was dependent on the two NxxY motifs present within the cytoplasmic tails of β3-integrin (Berrier et al., 2002). Deletion mutants that lacked NxxY motifs failed to activate Rac1 and to spread. Expression of a constitutively active form of Rac1 restored cell spreading in CHO cells expressing the deletion mutants of αIIbβ3 [our unpublished observations and Berrier et al. (Berrier et al., 2002)]. On the basis of these observations, it appears that the full-length integrins with the intact NxxY motifs mediate signals primarily by activating small molecular weight GTPases. We find that the expression of Fyn but not Src restored cell spreading in cells expressing the deletion mutants (Fig. 8), suggesting that integrins can mediate cell spreading by recruiting Fyn, in the absence of NxxY motifs and activation of small GTPases. We believe that these β3-mediated signals through Fyn might constitute an alternative pathway, possibly leading to Rac1 activation or bypassing Rac1 activation altogether to support cell spreading. The spreading defect observed in platelets from $Fyn^{-/-}$ mice suggests that this alternative, Fyn-dependent mechanism is functionally significant.

The above observations led us to analyze the platelet function in $Fyn^{-/-}$ mice. $Fyn^{-/-}$ mice were previously generated by Stein et al. and Appleby et al. (Appleby et al., 1992; Stein et al., 1992). Both groups have reported defective T-cell receptor signaling in $Fyn^{-/-}$ mice. Double and triple knockout mice of Fyn, Src and Yes were also reported (Stein et al., 1994). Most of the Fyn-Src or Yes-Src double mutants die perinatally; a substantial proportion of Fyn-Yes double mutants are viable but undergo degenerative renal changes leading to diffuse segmental glomerulosclerosis. However, abnormalities in hemostasis in the various SFK-knockout mice have not been reported. We specifically noticed prolonged second bleeding times in the $Fyn^{-/-}$ mice (Fig. 6). Abnormalities in second bleeding times have not been noted when tested in a number of different mouse strains (Hoover-Plow, personal communication), but may be analogous to the cyclic reflow observed in thrombosis models conducted in larger animals (Folts, 1991). The prolongation in second but not first bleeding is consistent with the absence of an aggregation defect with $Fyn^{-/-}$ platelets and suggests that defects in platelet spreading impact on clot stability. Knockout mice with defects in platelet spreading and clot stability have been described; the DiYF mice in which the two tyrosine residues in the β3 cytoplasmic tail have been mutated to phenylalanines, precluding their phosphorylation and, thereby, blunting outside-in signaling (Law et al., 1999). The retention of Fyn association with αIIbβ3 in thrombin-aggregated platelets is consistent with an effect on outside-in signaling, which occurs after ligand binding and clustering of the occupied integrin. Since the defect in spreading of $Fyn^{-/-}$ platelets is overcome with time, the functional effects

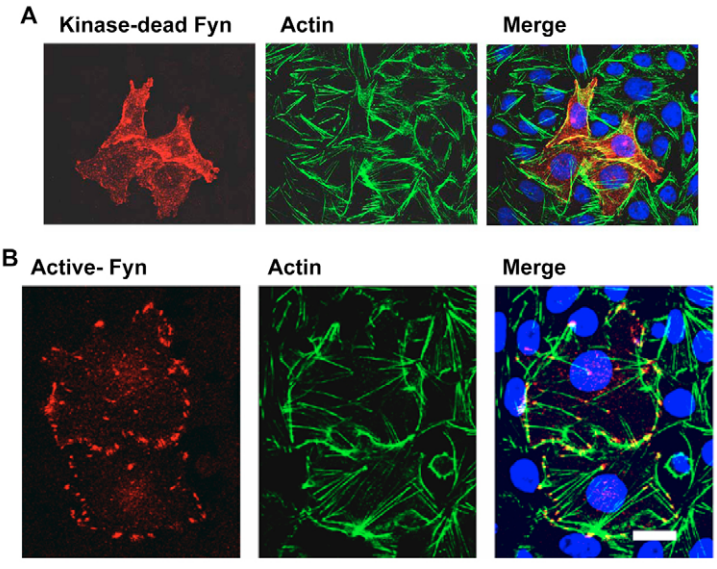

Fig. 9. Transient expression of kinase-dead version of Fyn fails to localize to focal adhesions. (A,B) CHO-αIIbβ3 were transfected with the cDNAs coding for (A) a kinase-dead version of Fyn (FynK299M) or (B) a constitutively active form of Fyn (FynΔ525). Transfected cells were allowed to spread for 24 hours on coverslips coated with fibrinogen. Cells were fixed and stained for Fyn (red), actin (green) and nuclei (blue). Scale bar, 20 μm.

of Fyn might be exerted temporally, fast-tracking but not essential for platelet spreading. This Fyn-dependent pathway might accelerate Rac activation or might bypass it altogether.

It is well established that several other SFKs (Src, Yes and Lyn) also bind to β3 integrin (Arias-Salgado et al., 2003; Arias-Salgado et al., 2005). We have shown that multiple SFKs are expressed in $Fyn^{-/-}$ mice (supplementary material Fig. S1). The fact that $Fyn^{-/-}$ mice exhibit only a mild phenotype might be because the other SFKs are compensating for the loss of Fyn. In addition, several other previously characterized interactions (reviewed in Liu et al., 2000), might be contributing to this mild phenotype. However, it is important to note that $Fyn^{-/-}$ mice exhibit a detectable but milder phenotype despite several compensatory interactions.

In summary, our studies identify multiple and distinct roles for Fyn in integrin signaling: Fyn is readily detected in focal adhesions of spread cells. The interaction between Fyn and αIIbβ3 is retained during platelet activation, whereas that of Src is less stable. Expression of Fyn restored the spreading of CHO cells expressing a deletion mutant of αIIbβ3. These functional activities of Fyn translate into a subtle defect in the hemostatic response of $Fyn^{-/-}$ mice.

Materials and Methods

Reagents

Antibodies against Fyn and Src and Protein-G Sepharose were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other primary antibodies used were purchased from BD Biosciences (San Jose, CA). Western blot reagents were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA). Fluorescently labeled fibrinogen and secondary antibodies were purchased from Invitrogen Corporation (Carlsbad, CA). GST-agarose, ECL reagents and bovine thrombin were from GE-Amersham Biosciences (Piscataway, NJ). Monoclonal antibody against the integrin β3 subunit, AP3, was purchased from ATCC.

Mice and tail-bleeding assays

$Fyn^{-/-}$ mice and corresponding control wild-type mice of the same genetic background were purchased from The Jackson Laboratory. As previously reported (Stein et al., 1992), the $Fyn^{-/-}$ mice exhibited no overt phenotype and appeared healthy. The absence of Fyn in these mice was confirmed by western blotting of the platelet lysates. The expression levels of other SFK members in platelets were comparable to that of wild-type mice (supplementary material Fig. S1). All procedures on the mice were performed under a protocol approved by the IACUC of the Cleveland Clinic. Tail-vein-bleeding times were measured as described previously (Hoover-Plow et al., 2006). Mice (~8 weeks of age) were anesthetized with ketamine-xylazine (80 mg/kg, 5 mg/kg), the tails were pre-warmed for 5 minutes in 10 ml of saline at 37°C in a water bath. The tails were briefly lifted from the saline and a 1-cm tail segment was amputated and immediately returned to the saline. The first bleeding time was measured as the time between the start of bleeding to cessation of bleeding. With the tail remaining in the same saline solution, the interval time was measured from the time between first cessation of bleeding and the start of the second bleeding. The second bleeding time was measured as the time between the start of the second bleeding to cessation of second bleeding.

Platelet analyses

Human and mouse platelets were isolated from the fresh blood drawn into acid citrate dextrose by differential centrifugation (Lam et al., 1989). Isolated platelets were washed once with modified Tyrode's buffer (10 mM HEPES pH 7.5, containing 12 mM Sodium bicarbonate, 150 mM NaCl, 0.1% dextrose and 0.1% BSA) and aggregated with thrombin (2 U/ml). Thrombin treatment was performed in 15-ml conical tubes for 10 minutes at 37°C. The platelets were spun down at 2000 ***g*** for 10 minutes. Spun platelets were disrupted by addition of lysis buffer (final concentration: 50 mM Tris-HCl pH 8.0, containing 150 mM NaCl, 1% Triton X-100 and protease inhibitors). After 20 minutes on ice, insoluble material was removed by centrifugation at 16,000 ***g*** for 10 minutes. Equal amounts of protein (~2 mg total protein) from the lysates of non-stimulated or thrombin-aggregated platelets were incubated with either anti-Fyn or anti-Src antibodies. The immune-complexes were captured onto protein-G Sepharose (Santa Cruz). After washing the beads, the immunoprecipitates were subjected to SDS-PAGE under reducing conditions followed by immunoblotting analysis with β3 subunit antibodies.

For aggregation assays, platelets were isolated by the gel filtration from the pooled blood of three to four mice. Platelets at ~3×10^7/ml were subjected to agonist- induced aggregation (whole-blood aggregometer model 810, Chrono-Log Corporation, Havertown, PA). Thrombin, collagen and ADP were used as agonists.

Platelet adhesion assays were performed as described previously (Obergfell et al., 2002). Pooled platelets from three to four mice were used for each set of adhesion assays. Washed platelets from $Fyn^{-/-}$ and the corresponding wild-type mice were allowed to adhere to fibrinogen coated coverslips (25 μg/ml), in Tyrode buffer, containing 1 mM Ca^{2+} and Mg^{2+}. At different times after adhesion at 37°C (30 minutes to 3 hours), platelets were fixed with 1.48% formaldehyde for 10 minutes, permeabilized by adding PBS containing 0.2% Triton X-100, blocked and stained with fluorescently labeled phalloidin to visualize the actin filaments. Images were captured with a Leica-DMR microscope equipped with Q-imaging system (Retiga Exi Fast digital camera). Quantification was based on counting spread cells versus round cells in four to six different fields.

GST pull-down assays

The cDNAs coding for specific β3-cytoplasmic tails (Fig. 4) were PCR-amplified and cloned into pGEX-GST vectors (GE-Amersham Biosciences). β3-cytoplasmic tail-deletion mutants were generated using the Quick Change mutagenesis kit (Stratagene). Recombinant proteins were expressed as described previously (Reddy et al., 1998). Protein lysates from freshly isolated platelets (~1 mg of total protein) were incubated with ~200 μg β3-tail proteins coupled to GST beads. The incubations were carried out for 2 hours at 4°C. After the incubation, the beads were washed three times in lysis buffer and captured proteins were subjected SDS-PAGE followed by western blot analysis.

Cell transfections

CHO cells (ATCC, CCL61) were routinely grown in F12 nutrient mixture (Invitrogen) containing 10% fetal bovine serum. Our preparation of CHO cells stably expressing wild-type αIIbβ3 integrin has been described previously (Reddy et al., 1998). The cDNAs coding for the deletion mutants of β3 subunits [residues 1-753 (β3Δ754) and 1-727 (β3Δ728)] were obtained from Joan Fox (Cleveland Clinic Foundation, Cleveland, OH). CHO cells were transfected with 5 μg of each of the cDNAs encoding the deletion mutants of the β3-subunit and full-length αIIb-subunit together with 2 μg pCR 3.1 vector (Invitrogen) using LipofectAMINE reagent (Invitrogen). Stable cell lines were selected in the growth medium containing 500 μg/ml G418 (Invitrogen). CHO cells expressing wild-type αIIbβ3 or its mutants were identified in FACS using FITC-labeled αIIbβ3antibody (Roche Applied Science, Indianapolis, IN).

CHO cells expressing the full-length αIIbβ3 and deletion mutants of the integrin were transfected with 5 μg of the cDNAs coding for indicated cDNAs. The cDNA for GFP-Fyn (full length) was provided by Marc Philips (NYU School of Medicine, New York, NY). The cDNA coding for GFP-Src (full length) was obtained from Margaret Frame and Giulio Supert-Furga (EMBL, Heidelberg, Germany). The cDNAs for the kinase-dead version of Fyn (FynK299M) and the Fyn constitutively active version (FynΔ525) were obtained from Todd Holmes [New York University (see Nitabach et al., 2001)]. After 48 hours, cells were processed for further analyses. In some cases, the SFK-transfected cells were enriched by flow cytometry.

For immunocytochemistry, cells transfected with various SFKs were fixed using 1.4% formaldehyde solution for 10 minutes, permeabilized by adding PBS supplemented 0.2% Triton X-100, blocked with PBS containing 10% bovine serum, and stained with a SFK antibody followed by fluorescently labeled secondary antibody. Cells were simultaneously labeled with fluorescently labeled phalloidin to visualize the actin filaments, and images were visualized as described above for platelets and quantified using the Image Pro-Plus software.

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