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Distinct and overlapping functional roles of Src family kinases in mouse platelets

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Summary

Background and objectives—Src family kinases (SFKs) play a critical role in initiating and propagating signals in platelets. The aims of this study were to quantitate SFK members present in platelets and to analyze their contribution to platelet regulation using glycoprotein VI (GPVI) and intregrin αIIbβ3, and *in vivo*.

Methods and Results—Mouse platelets express four SFKs, Fgr, Fyn, Lyn and Src, with Lyn expressed at a considerably higher level than the others. Using mutant mouse models, we demonstrate that platelet activation by collagen-related peptide (CRP) is delayed and then potentiated in the absence of Lyn, but only marginally reduced in the absence of Fyn or Fgr, and unaltered in the absence of Src. Compound deletions of Lyn/Src or Fyn/Lyn, but not of Fyn/Src or Fgr/Lyn, exhibit a greater delay in activation relative to Lyn-deficient platelets. Fibrinogen-adherent platelets show reduced spreading in the absence of Src, potentiation in the absence of Lyn, but no change in the absence of Fyn or Fgr. In mice double-deficient in Lyn/Src or Fgr/Lyn, the inhibitory role of Lyn on spreading on fibrinogen is lost. Lyn is the major SFK-mediating platelet aggregation on collagen at arterial shear and its absence leads to a reduction in thrombus size in a laser injury model.

Conclusion—These results demonstrate that SFKs share individual and overlapping roles in regulating platelet activation, with Lyn having a dual role in regulating GPVI signaling and an inhibitory role downstream of αIIbβ3, which requires prior signaling through Src.

Keywords

GPVI; integrin αIIbβ3; Lyn; platelets; Src family kinases

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Introduction

Src-family kinases (SFKs) are a family of eight structurally related tyrosine kinases, namely Lyn, Fyn, Src, Fgr, Blk, Hck, Yes and Lck, characterized by a SH2 and SH3 domain and a tyrosine kinase group. The greatest sequence diversity is found in the myristoylated N-terminal region which is sometimes called a SH4 domain [1,2]. In six of the SFKs, this region contains a cysteine residue which supports palmitoylation and localization to lipid rafts. The two exceptions are Src and Blk which are located outside of these domains [3,4]. SFKs are regulated through phosphorylation on inhibitory and activatory tyrosines [1,5]. Receptor tyrosine phosphatases regulate the activity of SFKs through dephosphorylation of the inhibitory site, with CD148 and PTP1B performing this function in platelets [6–8].

Src-family kinases have a critical role in many cellular processes, including proliferation, differentiation, motility and adhesion [1,5,9]. In platelets, SFKs are critical for initiating activation by the collagen receptor glycoprotein VI (GPVI) and the integrin αIIbβ3 [10–12]. They also contribute to signaling by G protein-coupled and cytokine receptors[13]. In addition, SFKs inhibit platelet activation via phosphorylation of immunoreceptor tyrosine-based inhibition motifs (ITIMs) in several membrane immunoglobulin receptors including PECAM-1 [14,15].

The GPVI–FcRγ-chain receptor complex has a conserved immunoreceptor tyrosine-based activation motif (ITAM), characterized by two YxxLs separated by seven amino acids. Clustering of GPVI leads to phosphorylation of the conserved tyrosines in the immunoreceptor tyrosine-based activation motif (ITAM) by SFKs and binding of Syk via its tandem SH2 domains. Syk then initiates a signaling cascade that consists of adapter and effector proteins, including further roles for SFKs [11,12]. GPVI signaling takes place in lipid rafts which are enriched in receptors and signaling proteins, including SFKs and the membrane adapter LAT, which nucleates a signalosome that provides a docking site for a variety of proteins including PLCγ2 [16–18].

The integrin αIIbβ3 is excluded from membrane rafts but also signals via SFKs and uses many of the same adapter and effector proteins as used by GPVI, with the notable exception of LAT [19]. In human platelets, αIIbβ3 also signals via the low-affinity immune receptor, FcγRIIA [20], but this is absent in the mouse genome and there is as yet no evidence of ITAM involvement in αIIbβ3 signaling in mouse platelets.

A critical question in regard to each of the SFKs is their net overall contribution to the regulation of platelets. Fyn, Lyn and Src are recognized as the major SFKs in human and mouse platelets. Expression of Fgr, Hck, Lck and Yes in platelets has also been reported but not in all studies, whereas the presence of Blk has not been described [21–23].

Lyn and Fyn are constitutively associated with a proline-rich domain (PRD) in the GPVI cytosolic tail via their SH3 domains with Lyn being held in an active conformation [24–26]. This arrangement positions the collagen receptor in a ‘ready-to-go’ state that can be triggered by clustering, with Lyn being the major kinase mediating activation [25]. Deletion of the PRD delays GPVI signaling in both transfected cell lines and in platelets revealing a

second, yet to be characterized, route of SFK activation [25,27]. This delay in response is overcome at later times under static conditions but results in a marked inhibition in activation at arteriolar rates of shear [25,28]. A similar delay in activation by GPVI and inhibition of response under shear is observed in Lyn-deficient platelets [25]. The similar phenotype of the proline-rich mutant of GPVI and the Lyn-deficient platelets suggests a causative association. In contrast, platelet adhesion on collagen under flow is not altered in the absence of Fyn, even although a partial reduction in aggregation to collagen-related peptide (CRP) has been reported [25,28]. Mice double deficient in Fyn and Lyn have a greater defect response to CRP, demonstrating that the two SFKs work in synergy to regulate platelet activation [28].

Fyn and Src have been shown to associate with distinct regions of the β3-cytoplasmic tail [8,10,29]. Further, platelets deficient in Fyn or Lyn exhibit a mild defect or marked potentiation in spreading on fibrinogen, respectively [29,30]. On the other hand, spreading on fibrinogen is not altered in the absence of Fgr, Hck and Lyn but is blocked by the additional absence of Src revealing a critical role in αIIbβ3 signaling [31]. Together, these results reveal that several SFKs participate in integrin-mediated spreading, with individual SFKs performing distinct roles.

A full understanding of the role of each of the SFKs in platelet activation is essential for the understanding of thrombo-inflammatory processes and platelet regulation. In the present study, we have measured the levels of SFKs in mouse platelets and investigated their roles downstream of the collage receptor GPVI and the integrin αIIbβ3 through the use of mouse models deficient in one or more SFKs.

Methods

Materials, mutant mice [32-34], preparation of mouse platelets and aggregation [35], flow cytometry, western blotting and immunoprecipitation studies [34], quantitation of SFKs in mouse platelets, lipid raft isolation [36], static adhesion and spreading studies [37,38], whole blood flow studies on immobilized collagen [37,39], the laser-induced thrombus formation model [7,40] and statistical analysis are described in Data S1.

Results

Expression and localization of SFKs in mouse platelets

There are several reports of expression of Fyn, Lyn and Src in mouse platelets supported by specific antibodies, gene expression arrays on platelets and megakaryocytes, and functional studies using mutant mice platelets [25,28,29,31,34,41]. On the other hand, there are contrasting reports of expression of Fgr, Hck, Lck and Yes, and no reports of expression of Blk [21,23,42].

We re-investigated the presence of Blk, Fgr, Hck, Lck and Yes in mouse platelets by western blotting of platelets and control tissues that are known to express SFKs from wild-type (WT) and mutant mouse models. A band corresponding to Fgr was present in the spleen and platelets from WT mice but, as expected, not from Fgr-deficient mice (Fig. 1A).

Robust expression of Hck and Yes was observed in the spleen and lung, respectively, and was abolished in mice deficient in the corresponding SFKs (Fig. 1A). In contrast, we did not observe a specific band corresponding to Hck or Yes by comparison of control and Hck- and Yes-deficient platelets, respectively, even after long exposure times (Fig. 1A). Robust expression of Blk and Lck was also observed in the spleen but not in mouse platelets (Fig. 1A). Thus, four of the eight SFK members, Fgr, Fyn, Lyn and Src, were detected in mouse platelets by western blotting (Fig. 1A and S1).

A quantitative western blotting approach, illustrated in Fig. 1B for Fyn, was used to compare the levels of the four SFKs. Lyn is the most abundant with an estimated intracellular concentration of 72 ± 3.2 μmol L^{-1}. The levels of Fyn, Src and Fgr were considerably lower at 5.6 ± 0.7, 6.0 ± 2.4 and 1.2 ± 0.04 μmol L^{-1}, respectively (Fig. 1B). We also used a pan phospho-specific antibody to the highly conserved activation site of all SFKs (anti-SFK pY418) to investigate expression (Fig. S1). The pan phospho-specific antibody detected three bands in mouse platelets, with the lower two co-migrating with Lyn. These two bands were absent in Lyn-deficient mouse platelets (Fig. S1). The upper band migrates with Fgr, Fyn and Src and is reduced by 40%–50% in mice deficient in Fyn or Src, but is unaltered in mice deficient in Fgr (Fig. S1). This is consistent with the similar level of expression of Fyn and Src and the much lower level of expression of Fgr (with the caveat that the stoichiometry of phosphorylation is not known).

Lipid rafts are cholesterol-rich, plasma microdomains enriched in a variety of signlling proteins. We investigated the distribution of SFKs in lipid rafts in platelets using Brij-58 and sucrose gradient ultracentrifugation. Samples were western blotted for the different SFKs and for the membrane adapter LAT to identify the raft fraction [19] and analyzed by densitometry. Similar levels of Fgr, Fyn and Lyn co-localized with LAT in the raft fraction in resting and CRP-stimulated platelets, whereas Src was absent from this fraction (Fig. 1C). Fgr, Fyn and Lyn were also present in the non-raft fraction where Src is exclusively present. Densitometry analysis shows that there is no significant difference in expression and localization of Fgr, Fyn, Lyn and Src in resting and CRP-stimulated platelets (not shown). Thus, Fgr, Fyn and Lyn are capable of localizing to lipid rafts in platelets whereas Src is excluded from these domains.

Impact of deficiency of single SFKs on platelet activation by GPVI and integrin αIIbβ3

The functional role of Fyn, Lyn and to a limited extent Src has been previously investigated in platelets using mice deficient in the individual SFKs and in the case of Fyn and Lyn, mice deficient in both kinases [25,28,29,31,34,41]. Spreading of platelets on fibrinogen from mice deficient in Fgr, Hck and Lyn or Fgr, Hck, Lyn and Src has also been reported [31].

Mice deficient in Fgr, Fyn, Lyn or Src were born at Mendelian frequency and were viable, although Src-deficient mice tended to be smaller than their littermates most probably as a result of under nourishment as they had to be fed on a liquid diet as previously reported [43]. When measured at 8 weeks of age, the platelet count and size, and the corresponding parameters of other blood cells, was similar in the four SFK-deficient mice to their littermate controls (not shown). The platelet count was reduced by approximately 25% in Lyn-deficient mice of 12 weeks of age as previously reported [44] and thus experiments were

conducted prior to this time. The level of expression of the glycoproteins GPVI, α_2- and αIIb-integrin subunits and GPIbα were similar in platelets from the four SFK-deficient mice and controls, and deletion of one SFK had no significant effect on the level of other SFKs (not shown and [28,34]).

We and others have previously reported that aggregation and dense granule secretion of platelets by the GPVI-specific ligands, CRP or convulxin, is delayed and then potentiated in the absence of Lyn and partially reduced in the absence of Fyn [25,28]. These observations have been confirmed in the present study (the effect was less marked than previously observed, possibly because of batch variation in CRP) and extended to mice deficient in Fgr or Src (Fig. 2A). Aggregation and secretion of dense- and α-granules induced by low concentrations of CRP were inhibited in the absence of Fgr to a similar extent to those observed in the absence of Fyn (Fig. 2A). In contrast, responses to low concentrations of CRP were not altered in the absence of Src (Fig. 2A and not shown). Thus, Fgr and Fyn play minor roles in mediating activation by GPVI with Lyn being the major SFK initiating platelet activation by the collagen receptor as shown by the delay in onset of response.

Spreading of different single SFK-deficient platelets on fibrinogen was monitored over 45 min. During this time course, platelets continuously bind and spread on the matrix protein such that the final picture is a net summary of capture of platelets at various stages of spreading as determined by the time of contact with the surface. Monitoring of spreading in this way therefore provides a kinetic capture of spreading from various times up to 45 min. Spreading but not adhesion of platelets on fibrinogen is abolished in the presence of the SFK inhibitor PP2 but is not altered in the presence of its inactive analogue PP3 (Fig. 2B and not shown). The degree of spreading is reduced by over 70% in Src-deficient platelets, with adhesion again being unaffected (Fig. 2B). In comparison, there was no difference in spreading of Fgr- or Fyn-deficient platelets on fibrinogen (Fig. 2B), whereas Lyn-deficient platelets show an approximate doubling in surface area but no change in adhesion (Fig. 2B). These observations establish Src as the major SFK mediating spreading on fibrinogen and demonstrate that Lyn has a net inhibitory role. The greater inhibitory effect of PP2 relative to the reduction in the Src-deficient platelets suggests that one or more of the other SFKs contributes to spreading.

Platelet aggregation in single SFK-deficient mice under flow conditions

Platelet aggregation on collagen under flow conditions involves signaling through a combination of platelet surface receptors including GPVI, integrin αIIbβ3, ADP and thromboxane receptors, all which signal through SFKs to varying extents. The net contribution of SFKs to aggregation of platelets on fibrillar collagen was monitored at a shear rate of 1000 s^{-1} as, under these conditions, adhesion and aggregation are critically dependent on signaling through GPVI [39]. Blood from Fgr, Fyn or Src-deficient mice formed robust aggregates on collagen fibers that were similar in size and surface area to each other and to those of litter-matched controls (Fig. 2C). In contrast, blood from Lyn-deficient mice formed only small aggregates along the length of the collagen fibers resulting in a significant decrease in surface area (Fig. 2C). However, this effect was less than that observed in the presence of Dasatinib, a SFK inhibitor which (unlike PP2) is bioavailable in

blood [8,29,45], suggesting that one or more other SFKs mediates this response in association with Lyn. Interestingly, there was no significant change in blood loss in mice deficient in Lyn or any of the other SFKs relative to littermate controls as measured by a tail bleeding assay (not shown). In contrast, tail bleeding was increased from 2.83 ± 1.2 mg in non-treated mice to 69.8 ± 21.9 mg in mice treated with the pan-SFK inhibitor Dasatinib, in agreement with previous reports [8,29,45].

However, a role for Lyn in supporting thrombus formation *in vivo* was observed in a laser-induced vessel injury model. The overall time course of thrombus formation in the Lyn-deficient platelets was similar to that in littermate controls but the magnitude was significantly reduced (Fig. 2D), which may reflect a role for Lyn in platelet activation by several receptors which contribute to thrombus growth including GPIb and PAR-4 [46,47]. It is unclear whether the observed role for Lyn in the laser injury model relative to the tail bleeding assay is as a result of a fundamental difference in these two assays or if the former is simply a more sensitive assay given that the time course of thrombus formation was the same to that of control platelets with only height being affected.

Impact of double deficiency of SFK on platelet activation by GPVI

The above studies confirm Lyn and Src as the major SFKs mediating platelet activation by the collagen receptor GPVI and the integrin αIIbβ3, respectively, but suggest that additional SFKs also play a role for both receptors in view of the more pronounced inhibitory effect of the pan SFK inhibitors such as Dasatinib and PP2. To investigate this, we generated various combinations of mice deficient in two SFKs, namely mice deficient in Fyn/Lyn ($fyn^{-/-}lyn^{-/-}$), Fgr/Lyn ($fgr^{-/-}lyn^{-/-}$) and Lyn/Src ($lyn^{-/-}src^{-/-}$). In addition, we generated mice deficient in Fyn/Src ($fyn^{-/-}src^{-/-}$) as these two SFKs are constitutively associated with the β3-integrin tail [8,29]. Mice deficient in Lyn/Src and Fyn/Src and their matched controls were generated as radiation chimeras by transplantation of fetal liver cells because of the high level of lethality during gestation as previously reported [48]. Bone marrow depletion was confirmed by western blotting of radiation chimeric platelets with appropriate antibodies as previously described [34].

The platelet counts of the $fyn^{-/-}lyn^{-/-}$ and $fyn^{-/-}src^{-/-}$ mice were not significantly different to their respective controls, whereas the platelet counts of the $lyn^{-/-}src^{-/-}$ and $fgr^{-/-}lyn^{-/-}$ mice were reduced to approximately 40% and 70% of littermate controls at 8 weeks, respectively (Table 1). A significant increase in the level of monocytes was also observed in these two mice models (not shown). Platelet size was increased by approximately 15% ($P < 0.01$) in the $fyn^{-/-}lyn^{-/-}$ and the $fgr^{-/-}lyn^{-/-}$ mice but was not altered in the two other double-deficient mice (Table 1). The level of expression of GPVI, α_2- and αIIb-integrin subunits and GPIbα were similar in all double-deficient platelets compared with WT platelets. GPIbα was reduced by approximately 15% in platelets from $fgr^{-/-}lyn^{-/-}$ and $lyn^{-/-}src^{-/-}$ mice ($P < 0.05$) (Table 1). The level of the remaining SFKs, assessed by western blot, was similar in all double-deficient and WT platelets (not shown and [34]).

An increased delay in aggregation and dense-granule secretion to CRP was observed in $fyn^{-/-}lyn^{-/-}$ and $lyn^{-/-}src^{-/-}$ platelets relative to $lyn^{-/-}$ platelets and, for a low

concentration of the GPVI-specific peptide, the magnitude of aggregation and secretion also dramatically reduced. The defect in response was considerably greater in $lyn^{-/-}src^{-/-}$ platelets than in $fyn^{-/-}lyn^{-/-}$ platelets (Fig. 3A,B). A slight delay in aggregation and dense-granule secretion to CRP was also seen in $fyn^{-/-}src^{-/-}$ platelets but this was less than that observed in the absence of Lyn (Fig. 3A,B). Interestingly, $fgr^{-/-}lyn^{-/-}$ platelets show a reduced delay in aggregation and dense-granule secretion in comparison to WT or $lyn^{-/-}$ platelets, which could reflect a weak inhibitory role for Fgr in signaling by GPVI (Fig. 3A,B). These results demonstrate that, in the absence of Lyn, Src and Fyn play compensatory roles in supporting aggregation and secretion by GPVI, with Src having the more significant role. In the presence of Lyn, Fyn and Src have only a minor role in GPVI signaling.

The molecular basis of the difference in response to CRP in the double SFK-deficient platelets was investigated by measurement of tyrosine phosphorylation in whole cell lysates and of key signaling proteins in the GPVI cascade, namely FcR γ-chain, Syk and PLCγ2. A direct comparison of the phosphorylation results is complicated by the different time course of response in the various double SFK-deficient models. In consideration of this, we have focused on the pattern of tyrosine phosphorylation and on the level of phosphorylation of key signaling proteins relative to each other. A representative set of blots from platelets that have been stimulated by CRP (3 μg mL^{-1}) for 3 min is shown in Fig. 4. CRP stimulates a similar pattern of increase in tyrosine phosphorylation in whole cell lysates in mice deficient in Lyn/Fyn, Lyn/Src and Fyn/Src-deficient platelets, but with quantitative differences in individual bands. There is a significant reduction in tyrosine phosphorylation of the FcR γ-chain in all three double-deficient platelets relative to WT mice, consistent with a critical role of SFKs in mediating phosphorylation of the FcR γ-chain ITAM. On the other hand, a significant reduction in phosphorylation of Syk and PLCγ2 was only observed in platelets deficient in Fyn/Src and Lyn/Src, whereas potentiation of phosphorylation of PLCγ2 was observed in Fyn/Lyn double-deficient platelets relative to controls, consistent with a negative feedback role of Lyn that is more pronounced in the Fyn/Lyn relative to Lyn/Src-deficient platelets. Thus, these results demonstrate qualitatively distinct roles of SFKs and emphasize that the interpretation of protein phosphorylation measurements needs to be considered in the context of the time course and pattern of response. It is noteworthy that potentiation of PLCγ2 phosphorylation in Fyn/Lyn-deficient platelets was not seen in our previous study [28] using a lower concentration of CRP (1 μg mL^{-1}). This could reflect the relative contribution of the opposing actions of Lyn in GPVI signaling at the two different concentrations of the synthetic collagen peptide, although it is likely that other factors have also contributed to this difference including the use of lotrafiban rather than EGTA and the more mild nature of the defect in both the Fyn- and Lyn-deficient platelets relative to that seen in our previous study [28] as mentioned above.

Impact of double deficiency of SFK on platelet activation by GPVI and integrin αIIbβ3

Investigation of adhesion/spreading on fibrinogen was complicated by a significant increase in spreading of WT radiation chimeric platelets relative to those from non-irradiated mice ($P < 0.05$). To account for this, spreading was normalized by comparison of the WT radiation chimeric platelet response (= 100%) to that in the presence of the SFK inhibitor PP2 (= 0%),

which is assumed to represent complete inhibition. The surface area of platelets on fibrinogen from Lyn/Src- and Fyn/Src-deficient mice was reduced by over 70% and 50%, respectively, relative to litter-matched controls (Fig. 5A), whereas Fyn/Lyn-deficient platelets exhibited a significant increase in spreading of 135% which is similar to that seen in Lyn-deficient platelets (Figs 2B and 5B). The surface area of platelets from mice deficient in Fgr/Lyn was not significantly different to controls (Fig. 5B), although there was a reduction in the number of platelets with filopodia (41.3 ± 1.9% vs. 73.4 ± 3.4%; $P < 0.05$). These results highlight the differential role of Src and Lyn in the regulation of the integrin αIIbβ3 outside-in signaling, with Lyn having an inhibitory role and Src a positive role. In platelets double deficient in Lyn/Src or Fgr/Lyn, spreading is abolished or similar to that of control platelets, respectively, suggesting that the potentiation induced in the absence of Lyn is mediated downstream of Src and Fgr (Fig. 5A,B).

Platelet aggregation in SFK double-deficient mice under flow conditions

The increase in spreading in the radiation chimeric platelets relative to controls did not result in a significant change in adhesion and aggregation of platelets on collagen at 1000 s^{-1} (not shown). On the other hand, a significant reduction in surface coverage relative to controls was observed in the absence of Fyn/Lyn or Lyn/Src, which was similar in magnitude to that in Lyn-deficient platelets but less than that seen in the presence of Dasatinib (Figs 2C and 6). In addition, there was also a significant reduction in aggregate size in all three sets of platelets (Figs 2C and 6), with the net size of *lyn*$^{-/-}$*src*$^{-/-}$ platelets being less than that of *lyn*$^{-/-}$ platelets at 300 ± 30 and 390 ± 15 μm^2, respectively, although this is also is influenced by the decrease in platelet count in the double-deficient platelets. The surface coverage of *fyn*$^{-/-}$*src*$^{-/-}$ platelets on collagen at 1000 s^{-1} was similar to that in control platelets, although the size of the aggregates was also significantly reduced (Fig. 6). A reduction in aggregation was also observed in Fgr/Lyn-deficient platelets but again this is likely to also reflect the reduction in platelet count (not shown). There was no significant increase in blood loss using a tail bleeding assays in the Fyn/Lyn or Fyn/Src double-deficient mice relative to their matched controls (not shown).

Discussion

The present study demonstrates that mouse platelets express four SFKs, Fgr, Fyn, Lyn and Src, and establishes their roles in regulating platelet activation after engagement of the collagen receptor GPVI and the integrin αIIbβ3. Lyn is the major SFK regulating GPVI signaling and its absence causes a delay in activation and a marked reduction in aggregation on collagen under shear and in thrombus formation after laser injury *in vivo*. Mice deficient in Lyn/Src and, to a lesser extent, mice deficient in Lyn/Fyn have a further delay in activation suggesting that all three SFKs participate in GPVI signaling. In addition, Lyn has a powerful inhibitory role which results in a delayed potentiation of aggregation to low concentrations of GPVI agonists and increased spreading on the αIIbβ3-ligand, fibrinogen, which signals mainly via Src. Fgr plays a minor role in signaling by both receptors, although it abrogates the inhibitory effect of Lyn on fibrinogen in accordance with a previous report on Fgr, Hck and Lyn-deficient platelets [31] (Hck is undetectable in mouse platelets).

Overall, these results demonstrate that SFKs have distinct and overlapping roles in regulating platelet activation by GPVI and integrin αIIbβ3.

Lyn is the most highly expressed of the SFKs and is present at over 10 times the level of Fyn and Src, and over 50 times that of Fgr. Lyn is localized to membrane rafts and is the major SFK mediating activation by GPVI. Src, on the other hand, is the major SFK mediating signaling by integrin αIIbβ3, with both Src and the integrin being localized outside of lipid rafts. However, the role of SFKs in platelet activation cannot be explained solely on the basis of their localization to rafts. For example, a significant proportion of Lyn is present in the non-raft fraction and yet functions as the major inhibitory SFK downstream of both GPVI and integrin αIIbβ3, presumably through phosphorylation of platelet ITIM receptors, including PECAM-1 and G6b. This class of receptor recruit the SH2 domain-containing tyrosine phosphatases, SHP-1 and SHP-2, and the inositol phosphatase, SHIP1, to mediate inhibition [15,49,50]. Similarly, mice deficient in Lyn and Src show an enhanced delay in activation relative to Lyn-deficient platelets even although Src is localized to the non-raft fraction.

Src family kinases are also known to participate in signaling by G protein-coupled receptors and it is therefore important to consider to what extent the results could reflect this role, bearing in mind that ADP and TxA_2 are critical positive feedback agonists in platelet activation, and that thrombin also supports platelet activation *in vivo*. The general consensus is that SFKs play a minor role in aggregation and secretion to low concentrations of G_i and G_q protein-coupled receptor agonists [46,47,51–57]. Consistent with this, platelet activation in the individual and double-deficient SFK mice was unaltered to thrombin (Fig. S2), TxA_2 mimetic, U46619 and ADP (not shown). Spreading of mouse platelets on fibrinogen under the conditions used is independent of ADP and TxA_2 [58] and so results cannot reflect a role downstream of either of these receptors. Nevertheless, it is possible that observations at high shear on a collagen surface or *in vivo* could reflect a role for SFKs in signaling by G protein-coupled receptors, as well as by other receptors including GPIb [46,47].

The observation that the SFK inhibitors, PP2 and Dasatinib, have a more powerful inhibitory effect than loss of any of the four SFKs against GPVI and integrin αIIbβ3 signaling strongly suggests that one or more SFKs work in combination with Lyn and Src to mediate platelet activation by GPVI and integrin αIIbβ3. In the case of GPVI, the additional role is played by Src and to a lesser extent by Fyn, and in the case of αIIbβ3, this role is played by Lyn and to a lesser extent by Fyn. Fgr also appears to play a minor role in signaling by GPVI and integrin αIIbβ3. The redundancy between SFKs is further illustrated by the greater level of inhibition induced by the pan-SFK inhibitor Dasatinib on aggregation under flow conditions relative to the single and double SFK-deficient platelets and by the observation that Dasatinib causes a significant increase in tail bleeding times whereas these are not altered in the various SFK-mutant mouse models.

The conclusion that mice platelets express only four SFKs needs to be considered in light of previous studies which have reported expression of the SFKs, Hck, Lck and Yes in mouse platelets. We have been unable to detect any expression of these three kinases in spite of demonstration of the efficacy and specificity of each antibody for different members through

western blotting of deficient control tissues of different SFK members. Explanations for the contrasting results in the literature include contamination with other cell types (notably in the genomic studies) and limitations in the specificity of available antibodies given the close sequence homology of SFKs. While the sensitivity limits for detection by western blotting is antibody dependent, the strong signals observed in control tissues and the detection range of western blotting strongly suggest that none of these kinases are likely to be expressed in platelets at a significant level. This is further underscored by the observation that an antibody against the phosphorylated conserved activatory site in SFKs detects three bands which appear to correspond to Fgr, Fyn, Lyn and Src as described above. The lack of expression of Blk, Lck, Hck and Yes is corroborated by a SAGE library from mouse megakaryocyte generated within our laboratory [49]. We found no tags for Blk, Lck, Hck or Yes, consistent with a lack of expression of these kinases within mouse platelets. In contrast to this, Lyn, Fyn and Src all have multiple SAGE tags. A recent expression database published by Rowley *et al*. [59] also demonstrates high expression of Lyn, Src and Fyn, low expression of Fgr and very little to no expression of Yes or Lck. In addition, Yes-deficient platelets show normal platelet aggregation in response to CRP stimulation and normal spreading on fibrinogen, which correlate with no or little expression of Yes in mouse platelets (not shown).

This study has investigated the roles of Fgr, Fyn, Lyn and Src in hemostasis and thrombosis in mouse platelets using both single-deficient mice and several combinations of double mutants. We show for the first time that Lyn plays a dominant role in regulating aggregation on collagen at arteriolar shear rates, as blood from Lyn-deficient mice forms only small aggregates along the length of the collagen fibers. This is presumably the consequence of the delay in adhesion that occurs in the absence of Lyn, as similar results are seen in the presence of αIIbβ3-blockade in platelets expressing a proline-depleted form of GPVI which is unable to bind to Lyn [see Introduction; 25]. Lyn-deficient mice have defective thrombus formation as shown using a laser injury model, although presumably sufficient thrombus is formed to prevent an increase in bleeding at least in a tail bleeding assay.

One of the striking observations in this study was the marked reduction in platelet counts in mice deficient in Fgr/Lyn and Lyn/Src that was evident by 8 weeks after birth. Lyn-deficient mice have been shown to develop a weak myeloproliferative disease (MPD) which begins after 8 weeks and is associated with increased myeloid progenitors and a reduced number of mature B cells [44,60]. These effects are believed to be due to altered ITIM phosphorylation and loss of membrane recruitment of the inositol 5-phosphatase, SHIP1 [44]. The onset of the MPD is associated with a mild reduction in platelet count in the Lyn-deficient platelets with age and may be mediated at the level of the hematopoietic stem cell. The combined defect of loss of Lyn and several other SFKs also induces profound effects on the development and function of hematopoietic lineages. These include a marked increase in neutrophil and dendritic cell activation in mice deficient in Fgr/Hck [61], altered hematopoietic stem cells, differentiation, marked fibrosis and invasion of inflammatory cells in the lung which results in death within the first 2 months of life in Lyn/Hck-deficient mice [13]. The defect in platelet count in the Fgr/Lyn and Lyn/Src mice observed in this study is

therefore likely to be as a result of altered HSC function and possibly to an early onset of a MPD.

In conclusion, this study demonstrates that although Lyn and Src are the major SFKs mediating platelet activation by the collagen receptor GPVI and the integrin αIIbβ3, Fyn and Fgr also have synergistic and complementary roles in regulating platelet function, and that each of the four SFKs plays a unique role in supporting platelet activation. Significantly, the present study also demonstrates that loss of any of the four SFKs does not give rise to excessive bleeding. Given the central role of SFKs in many biological processes, these observations have important implications for the development of inhibitors that target individual SFKs, in particular Lyn. However, the individual SFKs are unlikely to be targets for the development of anti-thrombotics because of their multiple roles in other tissues, notably on the immune system, unless irreversible inhibitors can be developed to selectively target platelets.

Supplementary Material

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Acknowledgments

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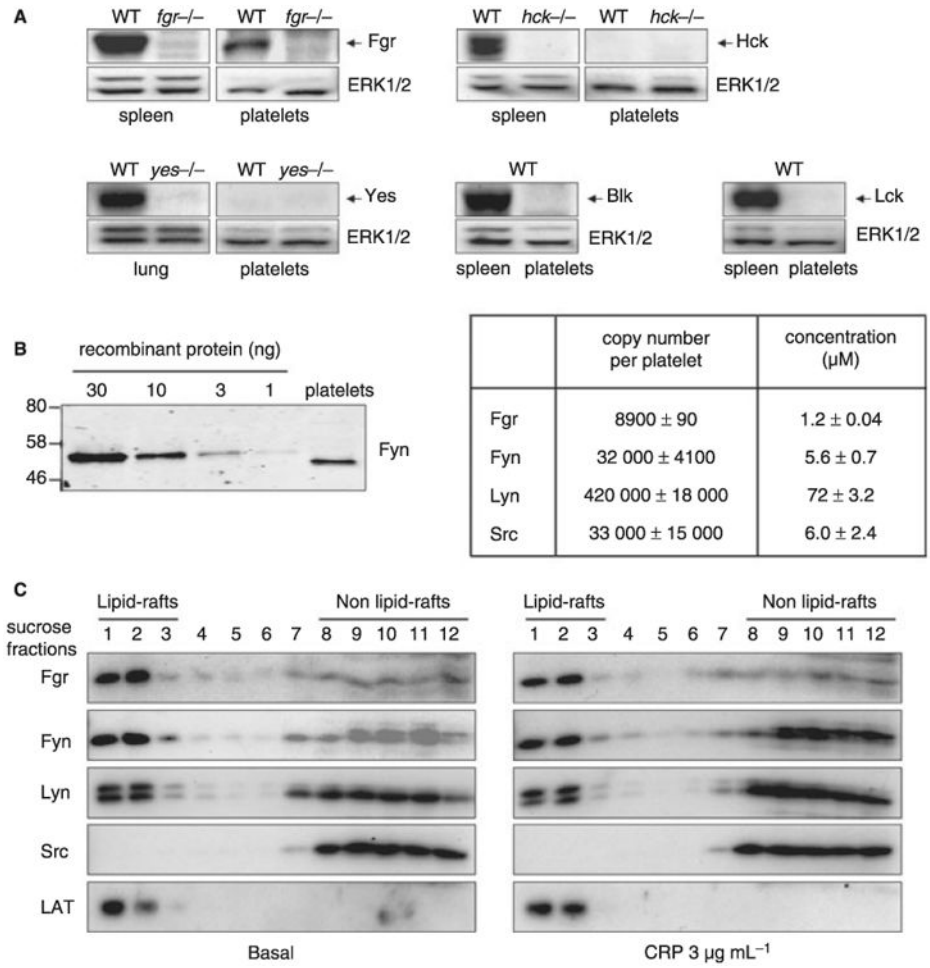
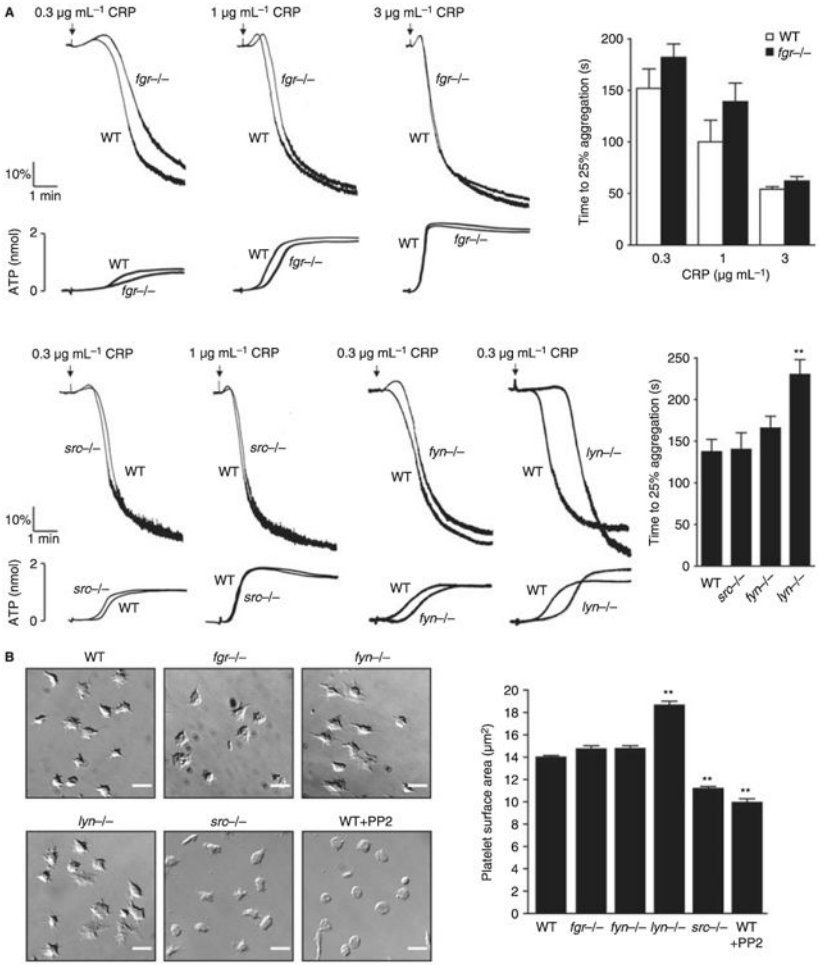


	copy number per platelet	concentration (µM)
Fgr	8900 ± 90	1.2 ± 0.04
Fyn	32 000 ± 4100	5.6 ± 0.7
Lyn	420 000 ± 18 000	72 ± 3.2
Src	33 000 ± 15 000	6.0 ± 2.4

Fig. 1.

Expression and localization of Lyn, Fyn, Src and Fgr in mouse platelets. (A) Whole cell lysates were prepared from spleen, lung or washed platelets of control and different members of Src family kinase (Yes, Hck and Fgr)-deficient mouse as described in the Methods. Expression of the different Src family kinase members (Yes, Hck, Fgr, Blk and Lck) was detected by western blot using specific antibodies. Data are representative of at least three independents experiments. (B) The protein level of Fgr, Fyn, Lyn and Src were quantitated by densitometry in mouse platelets as described in the Methods. Representative western blot is shown in the left panel. (C) Resting and collagen-related peptide (CRP)-stimulated platelets were lysed in 1% Brij 58 lysis buffer and lipid raft were isolated by sucrose gradient ultracentrifugation. The lipid rafts fractions were detected using an anti-LAT and localization of Fgr, Fyn, Lyn and Src was detected using specific antibodies. Data are representative of two independents experiments.



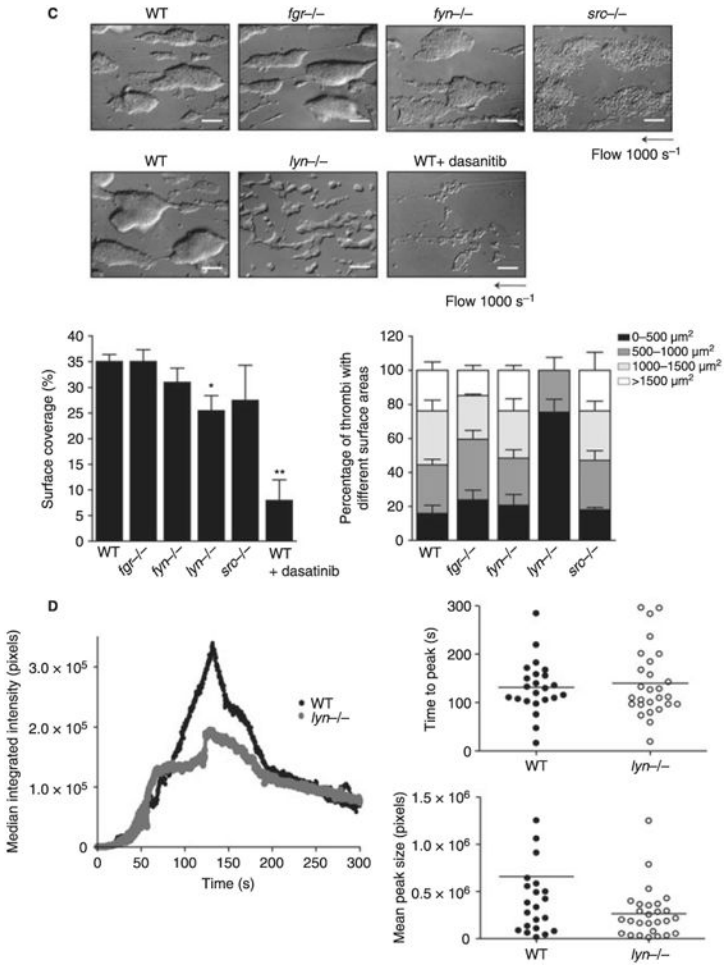


Fig. 2.

Impact of deficiency of Src family kinases (SFK) in platelet responses. (A) Washed platelets (2×10^8 mL^{-1}) prepared from *fyn*$^{-/-}$, *lyn*$^{-/-}$, *src*$^{-/-}$ or *fgr*$^{-/-}$ mice and their respective litter-matched controls were stimulated with different doses of collagen-related peptide (CRP) (0.3, 1 and 3 µg mL^{-1}). Platelet aggregation was measured as a change in light transmission and ATP secretion was measured as luciferin/luciferase-mediated luminescence, using a lumi-aggregometer. Representative images are shown (n = 3–6 mice per condition). The time to 25% of aggregation was calculated and data are shown as mean ± standard error of the mean (SEM). One-way ANOVA test, $^{**}P < 0.01$ vs. wild type (WT). (B) Washed platelets (2×10^7 mL^{-1}) from *fgr*$^{-/-}$ *fyn*$^{-/-}$, *lyn*$^{-/-}$ or *src*$^{-/-}$ and their respective litter-matched controls or WT platelets treated for 10 min with 10 µmol L^{-1} PP2 were placed on a fibrinogen-coated surface for 45 min. Platelets were fixed and images captured using differential interference contrast (DIC) microscopy (scale bar: 5 µm). Representative DIC images of platelets captured from four independent experiments for each genotype are shown. The surface area of platelets per condition were measured using ImageJ software (mean ± standard error of the mean [SEM]; n = 150–300 individual platelets per mice; four mice per genotype). Oneway ANOVA test, $^{*}P < 0.05$ vs. WT. (C) Anticoagulated blood from *lyn*$^{-/-}$, *fyn*$^{-/-}$, *src*$^{-/-}$ or *fgr*$^{-/-}$ and their respective litter-matched controls or WT platelets

treated for 10 min with 3 μmol L^{-1} Dasatinib was perfused through collagen-coated capillary tubes at 1000 s^{-1} for 4 min. DIC images of fixed platelets on collagen fibers after being flowed through collagen-coated capillary tubes at 1000 s^{-1} for 4 min (scale bar: 20 μm) are shown. Representative images of thrombus formed from at least three independent experiments of each genotype are shown. Area covered by platelet thrombi and the percentage of thrombi with different surface were measured in each of at least three different experiments and mean ± SEM is shown. One-way ANOVA test, *$P < 0.05$, **$P < 0.01$ vs. WT. (D) Platelets from WT or Lyn-deficient mice were fluorescently labeled *ex vivo* with rat anti–mouse αIIb primary antibody and Alex488-conjugated secondary antibody before being reintroduced into recipient mice. Arterioles in cremaster muscles of recipients were subsequently injured by laser, and the accumulation of platelets into the thrombi was assessed. Curves represent the median integrated thrombus fluorescence intensity in arbitrary units (pixels) for 23–25 thrombi induced in five mice of each genotype. Dot plot represents time to peak (seconds) and mean peak size (pixels) from between 23 and 27 thrombi from five mice.

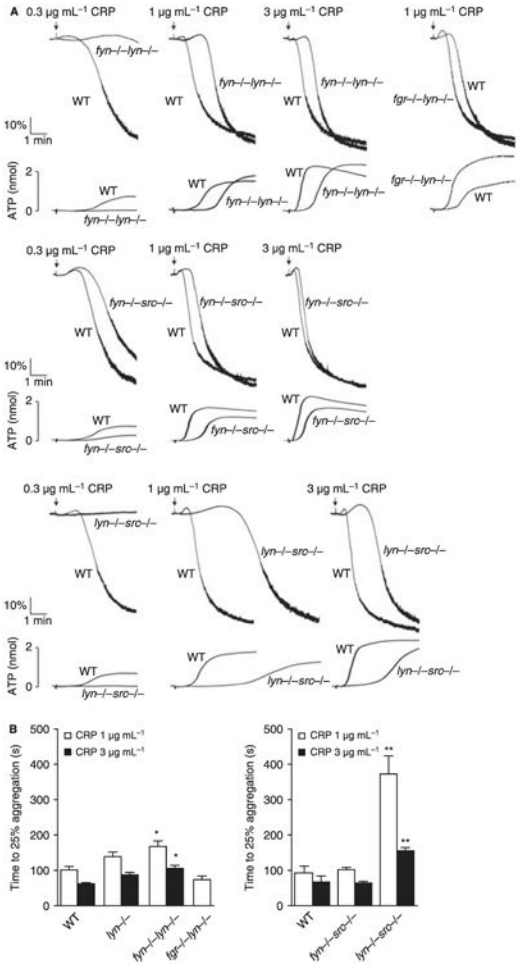


Fig. 3.

Impact of double deficiency of Src family kinases (SFK) in glycoprotein VI (GPVI)-induced platelet aggregation. (A) Washed platelets (2×10^8 mL^{-1}) prepared from $fyn^{-/-}lyn^{-/-}$ (top), $fgr^{-/-}lyn^{-/-}$ (top), $fyn^{-/-}src^{-/-}$ (middle) or $lyn^{-/-}src^{-/-}$ (bottom) mice and their respective litter-matched control were stimulated with different doses of CRP (0.3, 1 and 3 µg mL^{-1}). Platelet aggregation was measured as a change in light transmission and ATP secretion was measured as luciferin/luciferase-mediated luminescence, using a lumi-aggregometer. Representative images are shown (n = 4 mice per condition). (B) Time to 25% of aggregation was calculated and data are shown as mean ± SEM. One-way ANOVA test, *P < 0.05, **P < 0.01 vs. wild type (WT).

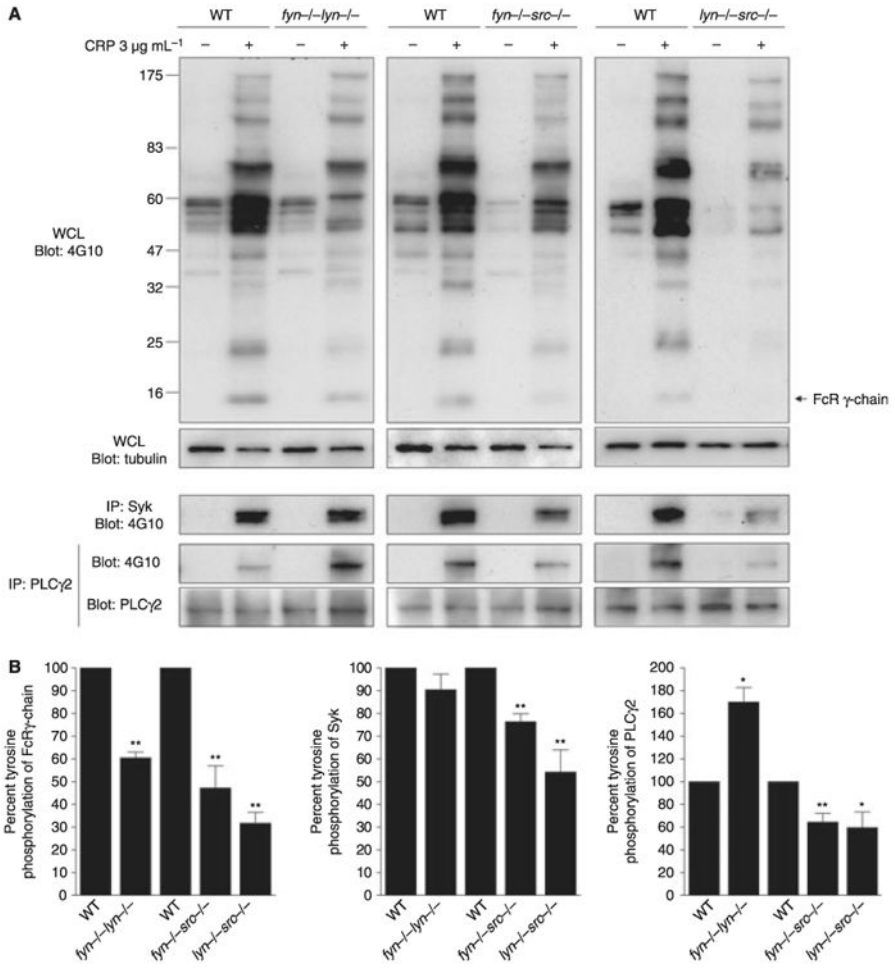


Fig. 4.

Impact of double deficiency of Src family kinases (SFK) in protein tyrosine phosphorylation in response to glycoprotein VI (GPVI) stimulation. Washed platelets (2×10^8 mL^{-1}) from *fyn*$^{-/-}$*src*$^{-/-}$, *lyn*$^{-/-}$*src*$^{-/-}$ or from *fyn*$^{-/-}$*lyn*$^{-/-}$ mice in comparison to their respective litter-matched controls were activated for 3 min with collagen-related peptide (CRP) (3 µg mL^{-1}) in the presence of lotrafiban (10 µmol L^{-1}) and lysed. Whole platelet lysates were immunobloted with anti-phosphotyrosine antibody. The FcRγ-chain was observed on long exposure of whole cell lysates. Syk and PLCγ2 were immunoprecipitated from whole platelet lysates, and immunoprecipitates were immunoblotted with an anti-phosphotyrosine antibody and Syk or PLCγ2 antibodies (A) Representative blots of three independent experiments are shown. (B) Data represent mean ± standard error of the mean (SEM). One-way ANOVA test, **P* < 0.05, ***P* < 0.01 vs. respective controls.

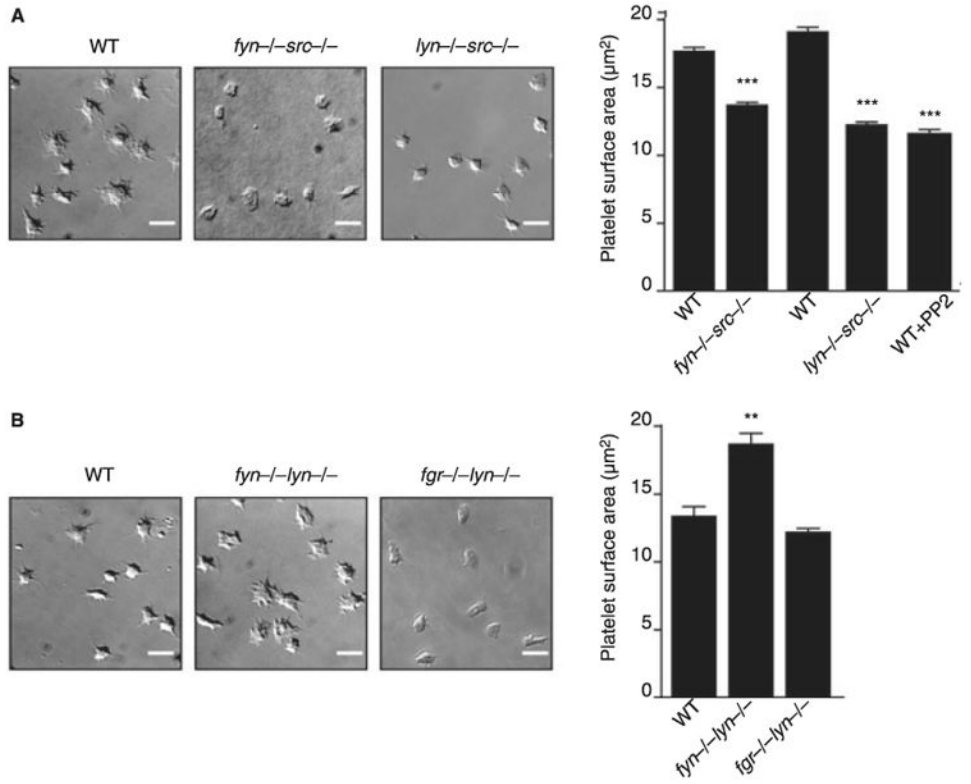


Fig. 5.

Impact of double deficiency of Src family kinases (SFK) in $\alpha_{IIb}\beta_3$ outside-in signaling. Washed platelets (2×10^7 mL^{-1}) from $fyn^{-/-}src^{-/-}$ or $lyn^{-/-}src^{-/-}$ radiation chimeric mice (A) or from $fyn^{-/-}lyn^{-/-}$ or $fgr^{-/-}lyn^{-/-}$ mice (B) in comparison to their respective litter-matched controls or wild-type (WT) platelets treated for 10 min with 20 µmol L^{-1} PP2 were placed on a fibrinogen-coated surface for 45 min and then fixed. Images captured by differential interference contrast (DIC) microscopy (scale bar: 5 µm). Representative DIC images of platelets captured from four independent experiments for each genotype are shown. The surface area of platelets per condition were measured using ImageJ software (mean ± standard error of the mean [SEM]; n = 150–250 individual platelets per mice; four mice per genotyping). One-way ANOVA test, $^{**}P < 0.01$, $^{***}P < 0.005$ vs. respective controls.

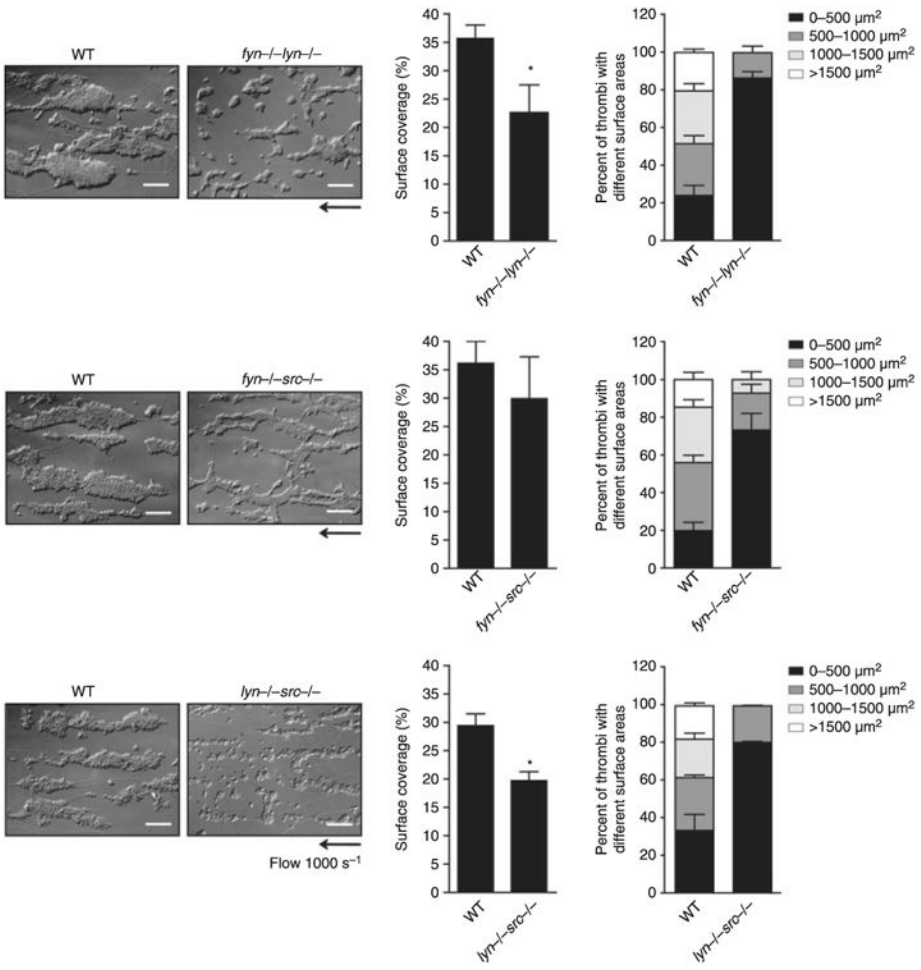


Fig. 6.

Impact of double deficiency of Src family kinases (SFK) in thrombus formation on collagen fibers under flow conditions. Anticoagulated blood from $lyn^{-/-}fyn^{-/-}$, $fyn^{-/-}src^{-/-}$ or $lyn^{-/-}src^{-/-}$ and their respective litter-matched controls was perfused through collagen-coated capillary tubes at 1000 s^{-1} for 4 min. Differential interference contrast (DIC) images of fixed platelets on collagen fibers after being flowed through collagen-coated capillary tubes at 1000 s^{-1} for 4 min (scale bar: 20 µm) are representative pictures of thrombus formed from at least three independent experiments of each genotype. Area covered by platelet thrombi and the percentage of thrombi with a different surface were measured in each of at least three different experiments and mean ± standard error of the mean (SEM) is shown. Student's *t*-test, $*P < 0.05$ vs. wild-type (WT) control.

Table 1

Platelet count, volume and surface glycoprotein expression in control and deficient platelets

	Control	*fyn*$^{-/-}$*lyn*$^{-/-}$	*fgr*$^{-/-}$*lyn*$^{-/-}$	Chimeric control	*fyn*$^{-/-}$*src*$^{-/-}$	*lyn*$^{-/-}$*src*$^{-/-}$
PC, 10^3 mm^{-3}	366 ± 42	823 ± 47	267 ± 49**	744 ± 52	813 ± 32	481 ± 49**
MPV, µm^3	5.5 ± 0.1	6.2 ± 0.2**	6.3 ± 0.2**	5.4 ± 0.1	5.2 ± 0.1	5.7 ± 0.1
GPIb	50.4 ± 1.0	50.8 ± 4.9	37.9 ± 3.5*	41.2 ± 2.5	44.5 ± 3.3	36.2 ± 1.9*
GPIIb	89.5 ± 12.8	108.9 ± 15.6	104 ± 23	103.5 ± 17.0	118.3 ± 12.3	89.6 ± 7.9
GPVI	16.7 ± 1.0	22.2 ± 1.6	15.9 ± 0.5	19.9 ± 2.4	18.5 ± 2.5	16.7 ± 0.4
α_2	3.6 ± 0.5	3.3 ± 0.4	3.7 ± 1.0	3.4 ± 0.3	3.6 ± 0.1	3.1 ± 0.4

Whole blood platelet count and mean platelet volume (MPV) for control and deficient mice platelets were analyzed with an automated blood analyzer (ABX Pentra 60). Results are expressed as means ± standard error of the mean (SEM) for at least six mice per group. Expression of glycoproteins on the platelet surface was determined by flow cytometry using specific fluorescein isothiocyanate-labeled antibodies. Results are expressed as median fluorescence intensity ± SEM for at least four mice per group. One-way ANOVA.

* $P < 0.05$,

** $P < 0.01$ vs. respective control.

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