

# Key role of integrin $\alpha_{\text{IIb}}\beta_3$ signaling to Syk kinase in tissue factor-induced thrombin generation

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**Abstract** The fibrin(ogen) receptor, integrin  $\alpha_{\text{IIb}}\beta_3$ , has a well-established role in platelet spreading, aggregation and clot retraction. How  $\alpha_{\text{IIb}}\beta_3$  contributes to platelet-dependent coagulation is less well resolved. Here, we demonstrate that the potent suppressing effect of clinically used  $\alpha_{\text{IIb}}\beta_3$  blockers on tissue factor-induced thrombin generation is linked to diminished platelet  $\text{Ca}^{2+}$  responses and phosphatidylserine (PS) exposure. The same blockers suppress these responses in platelets stimulated with collagen and thrombin receptor agonists, whereas added fibrinogen potentiates these responses. In platelets spreading on fibrinogen, outside-in  $\alpha_{\text{IIb}}\beta_3$  signaling similarly enhances thrombin-induced  $\text{Ca}^{2+}$  rises and PS exposure. These responses are reduced in  $\alpha_{\text{IIb}}\beta_3$ -deficient platelets from patients with Glanzmann's thrombasthenia. Furthermore, the contribution of  $\alpha_{\text{IIb}}\beta_3$  to tissue factor-induced platelet  $\text{Ca}^{2+}$  rises, PS exposure and thrombin generation in plasma are fully dependent on Syk kinase activity. Tyrosine phosphorylation

analysis confirms a key role of Syk activation, which is largely but not exclusively dependent on  $\alpha_{\text{IIb}}\beta_3$  activation. It is concluded that the majority of tissue factor-induced procoagulant activity of platelets relies on Syk activation and ensuing  $\text{Ca}^{2+}$  signal generation, and furthermore that a considerable part of Syk activation relies on  $\alpha_{\text{IIb}}\beta_3$  signaling. These results hence point to a novel role of Syk in integrin-dependent thrombin generation.

**Keywords** Platelets · Integrin  $\alpha_{\text{IIb}}\beta_3$  · Thrombin generation · Syk kinase · PS exposure

## Introduction

Integrin  $\alpha_{\text{IIb}}\beta_3$  (glycoprotein IIb/IIIa) is among the most abundantly expressed glycoproteins at the platelet surface, which strongly regulates the adhesion and aggregation of platelets. Platelet agonists induce a conformational change in the extracellular part of  $\alpha_{\text{IIb}}\beta_3$  via so-called inside-out signaling events, accumulating in Rap1b activation and talin complex formation, which increases the adhesiveness of the integrin [1–3]. Conversely, binding of  $\alpha_{\text{IIb}}\beta_3$  to its ligands, such as fibrinogen, fibrin and von Willebrand factor, stimulates outside-in signal transduction by a pathway involving activation of Src and Syk protein tyrosine kinases [4–6]. Platelet responses known to rely on  $\alpha_{\text{IIb}}\beta_3$  outside-in signaling include the shedding of microparticles, the formation of lamellipods of fibrinogen-adhered platelets, and the contraction of platelets within a fibrin clot [7–9].

It has been known for a long time that platelets stimulated with strong agonists promote the processes of thrombin generation and blood coagulation [10, 11]. This procoagulant function is triggered by agonist combinations like collagen/thrombin, which evoke prolonged and high rises in

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cytosolic  $[Ca^{2+}]_i$ , and also by stimulating platelets in plasma with tissue factor [12, 13]. The procoagulant activity is caused by exposure of the negatively charged phosphatidylserine (PS), at the membrane surface via a transmembrane protein encoded by TMEM16F [14], which promotes the local assembly of vitamin K-dependent coagulation factors and hence generation of thrombin [15]. Early findings have pointed to a significant role of  $\alpha_{IIb}\beta_3$  in the development of platelet procoagulant activity, in that integrin antagonists were found to suppress tissue factor-induced thrombin generation [12, 16, 17]. Yet, the mechanism by which  $\alpha_{IIb}\beta_3$  blockage interferes with platelet-dependent coagulation has not been resolved. Suggestions are that the integrin is (1) involved in the formation of procoagulant microparticles [17–19], (2) directly binds prothrombin [20], or (3) provides binding sites for factor Va and other coagulation factors [21, 22]. Studies so far are complicated by data that show that distinct  $\alpha_{IIb}\beta_3$  antagonists may differ in their effects on platelet activation and procoagulant activity [19, 23]. Another complicating factor is that part of the role of  $\alpha_{IIb}\beta_3$  may be secondary to that of autocrine ADP, which enhances PS exposure via P2Y<sub>12</sub> receptor stimulation [24–26].

In this paper, we hypothesize that  $\alpha_{IIb}\beta_3$  interaction with its principal ligand, fibrinogen, enhances platelet-dependent thrombin generation via an outside-in signaling mechanism. We demonstrate that signaling via Syk kinase is responsible for the majority of tissue factor-induced thrombin generation of platelets in plasma by stimulating PS exposure.

## Materials and methods

### Materials

Human  $\alpha$ -thrombin was obtained from Enzyme Research Laboratories, recombinant human tissue factor came from Dade Behring, abciximab (reopro) from Centocor; tirofiban (aggrastat) from Merck Sharp & Dohme, and eptifibatid (integrilin) from GlaxoSmithKline. Dimethyl BAPTA (DM-BAPTA), Fura-2 and Fura-Red acetoxymethyl esters were from Molecular Probes, while Syk inhibitor II and IV were from Merck Biosciences. Apyrase, bovine serum albumin (BSA), human and bovine fibrinogen (fraction 1, type III), and non-radioactive protein tyrosine kinase assay kit were all from Sigma. Cangrelor (AR-C69931MX) was kindly provided by The Medicine Company. Ancrod came from NIBSC; fluorescein isothiocyanate (FITC)-labeled annexin A5 from PharmaTarget, and FITC-labeled monoclonal antibody (mAb) against platelet-bound human fibrinogen from WAK Chemie Medical. Rabbit anti-phospho-Syk (Tyr<sup>525/526</sup>) mAb and HRP-linked anti-rabbit IgG were from Cell Signaling Technology, mouse anti-Syk mAb was from

Santa Cruz Biotechnology, rabbit anti- $\alpha$ -tubulin Ab from Abcam, and HRP-linked anti-mouse IgG from GE Healthcare. Pefabloc (Gly-Pro-Arg-Pro-amide, GPRP) was obtained from Kordia Life Sciences. Microbeads coated with human anti-CD31 mAb and MS columns were a kind gift from Miltenyi Biotec. Procoagulant phospholipid vesicles (PS:phosphatidyl choline:phosphatidyl ethanolamine 1:3:1; mol/mol) were prepared as described [27]. Convulxin was purified to homogeneity from the venom of *Crotalus durissus terrificus* [28]. Other materials including fibrinogen were from sources indicated before [27].

### Platelet and plasma preparation

Blood was taken from healthy volunteers and from two patients with Glanzmann's thrombasthenia, with established deficiencies in integrin  $\alpha_{IIb}\beta_3$  [29], after informed consent and in accordance with the Declaration of Helsinki. Approval was received from the local medical ethical committee. Blood was collected into 1/10 volume of 129 mM trisodium citrate. PRP was obtained by centrifuging at 240g for 15 min and platelet-free plasma (PFP) by centrifuging twice at 2,630g for 10 min. Platelet count in PRP was determined with a thrombocounter (Coulter Electronics) and normalized with autologous PFP. Citrate-anticoagulated PFP was partly defibrinated by a 10-min treatment with low ancrod protease (1.3 U/mL). After centrifuging the fibrin clots that were formed, non-turbid plasma was isolated. The remaining fibrinogen content was determined at  $\sim 0.5$  mg/mL according to the conventional Claus method based on turbidimetric measurements [30]. When supplemented with platelets, the ancrod-treated plasma showed normal collagen-induced platelet aggregation responses.

For the preparation of washed platelets, blood was collected into 1/6 volume of acid-citrate glucose solution (ACD, 80 mM trisodium citrate, 52 mM citric acid and 180 mM glucose). Platelets were obtained by centrifugation, washed in the presence of apyrase (0.1 U/mL ADPase), and resuspended in Hepes buffer pH 7.45 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl<sub>2</sub>, 0.1 % glucose and 0.1 % BSA) at a count of  $1.0 \times 10^8$ /mL [27].

For experiments with reconstituted PRP, partly defibrinated plasma was supplemented with washed platelets ( $1.0 \times 10^8$ /mL). Apyrase was not added, because of the presence of autologous exonucleotidase activity in plasma.

### Flow cytometry

Washed, unstirred platelets in Hepes buffer were activated with PAR1 agonist SFLLRN (15  $\mu$ M) or thrombin (10 nM) in combination with convulxin (50 ng/mL). Alternatively,

the washed platelets were resuspended in ancred-treated citrate plasma at  $1.0 \times 10^8$ /mL. The reconstituted PRP was activated with tissue factor (2 pM) and  $\text{CaCl}_2$  (16.6 mM) at 37 °C. After 15 min of activation, PS exposure and integrin activation were determined with FITC-labeled annexin A5 or FITC-labeled mAb against platelet-bound human fibrinogen, respectively, using flow cytometry [13]. For cytosolic  $\text{Ca}^{2+}$  measurements, platelets were loaded with Fura-Red (22  $\mu\text{M}$ ) and pluronic (400  $\mu\text{g}/\text{mL}$ ) in the presence of apyrase (0.1 U/mL ADPase). After a washing step, the loaded platelets were resuspended in ancred-treated citrate plasma, which was triggered by tissue factor (2 pM) and  $\text{CaCl}_2$  (16.6 mM) at 37 °C. Increases in cytosolic  $\text{Ca}^{2+}$ , apparent as decreases in fluorescence, were recorded in time by flow cytometry [26].

### Thrombin generation

Thrombin generation was determined in normalized PRP ( $1.5 \times 10^8$  platelets/mL) or, as a control, in PFP supplemented with phospholipid vesicles (10  $\mu\text{M}$ ). The normalized PRP from control subjects or a Glanzmann patient was activated with tissue factor/ $\text{CaCl}_2$ , and fluorescence accumulation was measured according to the thrombogram method under non-stirred conditions in a Fluoroskan Ascent well-plate reader at 37 °C [12]. Nanomolar thrombin concentrations were obtained by comparison with a human thrombin standard using Thrombinoscope software.

### Spectrofluorometry

Platelets were loaded with Fura-2 when rises in cytosolic  $\text{Ca}^{2+}$  concentration were determined in the absence of plasma [31]. Fura-2-loaded platelets were activated in the presence of 2 mM  $\text{CaCl}_2$  at slow stirring (100 rpm, 37 °C); inhibitors were given before (10 min) or after agonist addition, as indicated. Nanomolar changes in  $\text{Ca}^{2+}$  level were obtained by calibration procedures, described in detail elsewhere [32].

### Fluorescence microscopy

Glass coverslips were coated with 25  $\mu\text{L}$  of fibrinogen solution (1 mg/mL), rinsed twice with saline, and incubated with washed (Fura-2-loaded) platelets in HEPES buffer pH 7.45 ( $1.0 \times 10^8$ /mL) [32]. Where indicated, the coverslips were coated with a low fibrinogen solution (10  $\mu\text{g}/\text{mL}$ ). After 30 min, non-adherent platelets were removed, and the adhered spreading platelets were stimulated with thrombin (10 nM) in the presence of 2 mM  $\text{CaCl}_2$ . Microscopic

phase-contrast and fluorescence images of PS exposure (FITC-labeled annexin A5) were taken using a dual camera imaging system, controlled by Visitech software [33]. Fluorescence ratio images of Fura-2 fluorescence were taken to obtain rises in  $[\text{Ca}^{2+}]_i$ . For calibration, fluorescence values were obtained from  $\text{Ca}^{2+}$ -saturated and  $\text{Ca}^{2+}$ -free lysed platelets containing the fluorescent probe, using the microscopic and camera settings as described [34].

### Platelet isolation from coagulating plasma

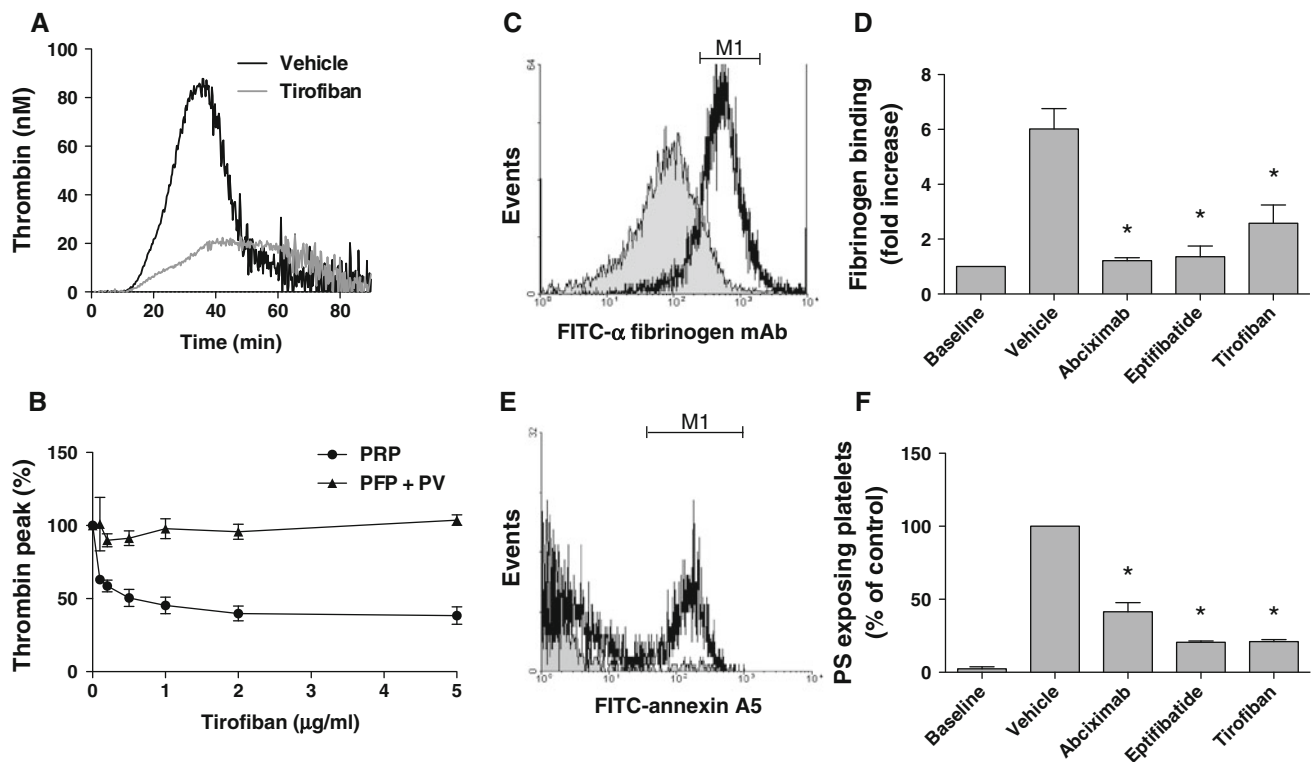
Washed platelets ( $5 \times 10^8$  platelets/mL) were reconstituted in ancred-defibrinated plasma in the presence of GPRP (1 mg/mL) and cangrelor (20  $\mu\text{M}$ ). Samples of reconstituted PRP were preincubated with vehicle, Syk inhibitor II (5  $\mu\text{M}$ ), and tirofiban (10  $\mu\text{g}/\text{mL}$ ), as indicated, and activated with tissue factor (2 pM) and  $\text{CaCl}_2$  (16.6 mM). Initial attempts were made to isolate platelets from the activated PRP by centrifugation or gel filtration, but these were unsuccessful. Hence, a novel method was developed, in which platelets were captured from activated PRP by addition of anti-CD31 mAb-coated magnetic microbeads. After 15 min of activation, these platelets were isolated by passage of the PRP through a separation column, and an immediate rinse to remove all plasma proteins. Isolated platelets in the separation column were immediately lysed by flowing with lysis buffer (600 mM NaCl, 40 mM Tris, 4 mM EGTA, 4 mM EDTA, 4 % nonidet-P40, 10 mM  $\text{Na}_3\text{VO}_4$ , 4 mM PMSF, 20  $\mu\text{g}/\text{mL}$  leupeptin, 20  $\mu\text{g}/\text{mL}$  aprotinin, 5  $\mu\text{g}/\text{mL}$  pepstatin A). Lysates were frozen at  $-80$  °C until use.

### Protein separation and western blotting

Platelet lysates were separated by polyacrylamide gel electrophoresis and subjected to standard western blotting. Blots were stained for phosphorylated Syk with anti-Syk Tyr<sup>525/526</sup> mAb (1:1,000) and secondary HRP-conjugated secondary Ab (1:500). Total Syk was determined by reprobing with anti-Syk mAb (1:1,000) and HRP-conjugated secondary Ab (1:1,000). To control for total platelet proteins, parallel blots were probed for  $\alpha$ -tubulin (1:1,000). Antibody staining was quantified by densitometric analysis [35].

### Statistics

Data are given as mean  $\pm$  SEM. Significance of differences was determined with the Mann–Whitney *U* test or the independent samples *t* test, as appropriate, using the statistical package for social sciences (SPSS 15.0).



**Fig. 1** Blocking of integrin  $\alpha_{IIb}\beta_3$  suppresses PS exposure and thrombin generation in tissue factor-stimulated PRP. **a, b** PRP ( $1.0 \times 10^8$  platelets/mL) or PFP supplemented with phospholipid vesicles (PV, 10  $\mu$ M) was preincubated with vehicle or tirofiban (0.1–5  $\mu$ g/mL) for 20 min. Thrombin generation was stimulated with tissue factor (1 pM) and  $\text{CaCl}_2$ . **a** Representative thrombin generation curves with PRP, **b** dose-dependent inhibition of thrombin generation only in the presence of platelets. Mean  $\pm$  SEM ( $n = 3-7$ ). **c-f** Anecd-treated PRP was preincubated with vehicle, abciximab

(10  $\mu$ g/mL), eptifibatide (10  $\mu$ g/mL) or tirofiban (1  $\mu$ g/mL), and thrombin generation was stimulated as above. After 15 min, platelet activation was evaluated by flow cytometry. **c, d** Platelet fibrin(ogen) binding measured with FITC-labeled anti-fibrinogen mAb. Data are fold increase in fluorescence relative to baseline (prior to activation). **e, f** Platelet PS exposure measured with FITC-annexin A5. Data are fractions of PS-exposing platelets (compared to vehicle control). M1 indicates platelet populations with increased fluorescence. Mean  $\pm$  SEM ( $n = 3-6$ ); \* $p < 0.05$  versus vehicle

## Results

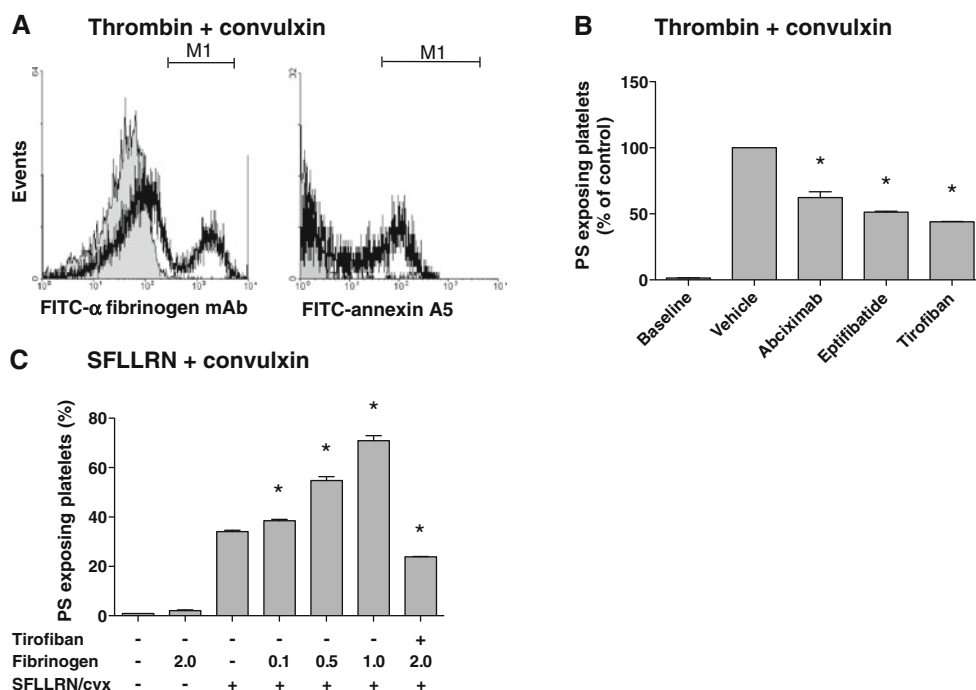
### Roles of integrin $\alpha_{IIb}\beta_3$ in tissue factor-stimulated thrombin generation and platelet activation

Early reports suggest that various integrin  $\alpha_{IIb}\beta_3$  antagonists differently affect thrombin generation in platelet-rich plasma (PRP) [19, 23]. To verify this, we prepared human PRP and determined the effects on tissue factor-induced thrombin generation of three  $\alpha_{IIb}\beta_3$  blockers, all in clinical use: the human/mouse chimeric monoclonal antibody fragment abciximab (blocking integrins  $\alpha_{IIb}\beta_3$  and  $\alpha_v\beta_3$ ), the peptide mimetic eptifibatide, and the non-peptide sulfonamido compound, tirofiban. When added to recalcified PRP, tirofiban dose-dependently suppressed and delayed thrombin generation induced by tissue factor (Fig. 1a, b). A similar reduction in thrombin generation was seen with the other  $\alpha_{IIb}\beta_3$  blockers (Online Resource Fig. 1a, b). Tirofiban, abciximab and eptifibatide reduced the thrombin peak with 60 % at  $\text{IC}_{50}$  values of 0.1, 2 and 1  $\mu$ g/mL,

respectively. In spite of this reduction, a thrombin peak of 20 nM was still reached, which is sufficiently high for maximal thrombin-induced platelet activation. Control experiments indicated that the integrin blockers were similarly effective in suppressing platelet aggregation of PPACK-anticoagulated PRP, stimulated with the PAR1 agonist SFLLRN (not shown). In contrast, none of the  $\alpha_{IIb}\beta_3$  blockers affected thrombin generation in plasma containing phospholipids instead of platelets (Fig. 1b and Online Resource Fig. 1b), thus demonstrating that the blocker effects required the presence of platelets.

In order to study tissue factor-induced platelet activation in plasma, formation of disturbing fibrin clots needed to be prevented. Therefore, human plasma was partly defibrinated with a low concentration (1.3 U/mL) of the snake venom anecrod, which induces fibrin clotting without thrombin generation [36]. After removal of the anecrod clots by centrifugation, the remaining plasma contained a residual concentration of  $\sim 0.5$  mg/mL fibrinogen, which does not form large clots. In this plasma reconstituted with

**Fig. 2** Blocking of integrin  $\alpha_{\text{IIb}}\beta_3$  suppresses PS exposure of convulxin/thrombin-stimulated platelets. Washed platelets containing apyrase were activated with thrombin (10 nM) or SFLLRN (15  $\mu\text{M}$ ) plus convulxin (50 ng/mL), as indicated, in the presence of  $\text{CaCl}_2$  (2 mM) for 15 min. Pretreatment with abciximab (10  $\mu\text{g/mL}$ ), eptifibatide (10  $\mu\text{g/mL}$ ) or tirofiban (1  $\mu\text{g/mL}$ ). **a** Histograms of fibrin(ogen) binding (FITC-anti-fibrinogen mAb) and PS exposure (FITC-annexin A5). **b** Quantitative effect of  $\alpha_{\text{IIb}}\beta_3$  blockage on platelet PS exposure. **c** Effect of added human fibrinogen (0.1–2.0 mg/mL) on PS exposure. Mean  $\pm$  SEM ( $n = 3$ ); \* $p < 0.05$  versus vehicle



platelets,  $\alpha_{\text{IIb}}\beta_3$  blockers suppressed tissue factor-induced thrombin generation with 40–50 % (data not shown). Flow cytometry demonstrated that, after tissue factor stimulation, fibrin(ogen) binding to platelets was antagonized by any of the three  $\alpha_{\text{IIb}}\beta_3$  blockers (Fig. 1c, d). Furthermore, tissue factor induced PS exposure in 35 % of the platelets, as determined by staining with FITC-annexin A5 (Fig. 1e). In the absence of tissue factor, fibrin(ogen) binding and PS exposure were quite low. Strikingly, blockage of  $\alpha_{\text{IIb}}\beta_3$  greatly reduced the tissue factor-induced PS exposure with 60 % (abciximab) or 80 % (eptifibatide, tirofiban) at maximally effective concentrations (Fig. 1f). Flow cytometry furthermore indicated that the integrin blockers suppressed formation of PS-exposing microparticles by >50 %, as reported before [17]. Similar experiments were performed in the presence of apyrase (0.1 U/mL), in which case again abciximab, eptifibatide, and tirofiban reduced the number of PS-exposing platelets by 75–85 %.

A frequently used way of provoking PS exposure is by stimulating washed platelets with thrombin in combination with collagen receptor agonist, convulxin [37]. Considering that these platelets secrete fibrinogen which binds to  $\alpha_{\text{IIb}}\beta_3$  in an autocrine way, we investigated whether in this condition  $\alpha_{\text{IIb}}\beta_3$  blocking may also influence PS exposure. Flow cytometry indicated that the co-stimulation of isolated platelets with thrombin (10 nM) and convulxin (50 ng/mL) resulted in large fractions of platelets binding fibrin(ogen) and exposing PS (Fig. 2a). Dose–response experiments indicated that this concentration of thrombin (10 nM) was maximally effective (not shown), similarly as described before [38]. All integrin blockers caused a

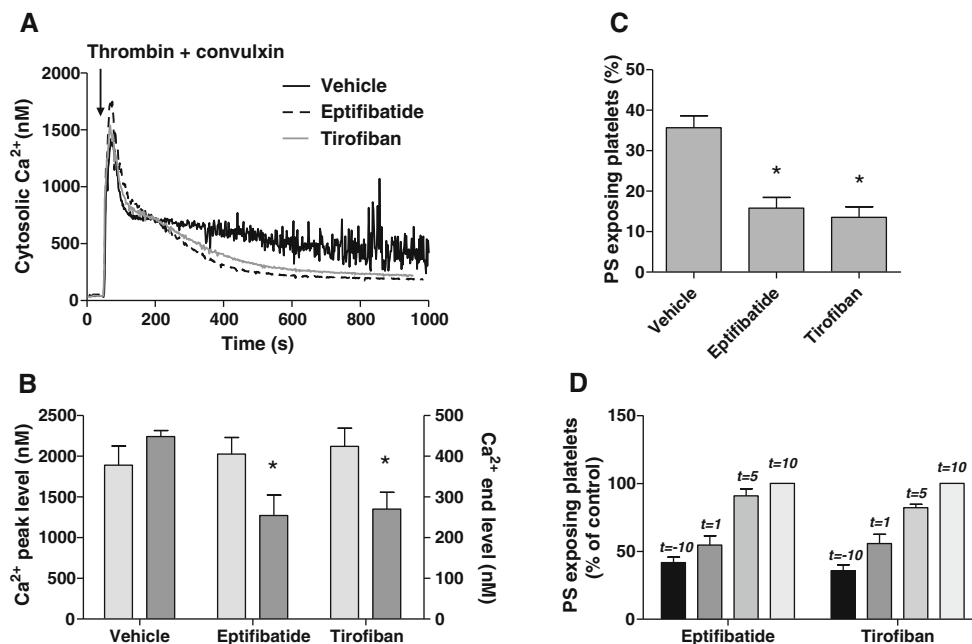
substantial decrease in PS exposure of 35 % (abciximab) or 50 % (eptifibatide, tirofiban) of the control condition (Fig. 2b). This suggested a supportive role of  $\alpha_{\text{IIb}}\beta_3$  in thrombin and collagen receptor-induced PS exposure via interaction with (secreted) fibrinogen.

To further study this under conditions where fibrin clot formation was prevented, the platelets were activated with convulxin plus PAR1 agonist SFLLRN. Addition of exogenous human fibrinogen resulted in a dose-dependent stimulatory effect on PS exposure, increasing the fraction of PS-exposing platelets from 35 up to 70% (Fig. 2c). In contrast, addition of fibrinogen alone, without other agonists, did not stimulate PS exposure. Pretreatment with tirofiban (Fig. 2c) or other integrin blockers (not shown) completely reversed the stimulating effect of fibrinogen. Comparable results were obtained with bovine and human fibrinogen (not shown). Together, these results point to a role of integrin  $\alpha_{\text{IIb}}\beta_3$ , likely via interaction with fibrin (ogen) on platelet PS exposure both in tissue factor-stimulated PRP (resulting in increased thrombin generation), and in washed platelets stimulated with thrombin and collagen receptor agonists.

#### Signaling role of integrin $\alpha_{\text{IIb}}\beta_3$ in $\text{Ca}^{2+}$ and procoagulant platelet responses

Others have suggested that inhibitory effects of  $\alpha_{\text{IIb}}\beta_3$  blockers on platelet PS exposure may occur independently of modulating  $\text{Ca}^{2+}$  responses [22, 39]. Also, in stored platelets, PS exposure can occur independently of elevated  $\text{Ca}^{2+}$  [40]. We therefore re-examined a role of  $\alpha_{\text{IIb}}\beta_3$  in





**Fig. 3** Blocking of  $\alpha_{IIb}\beta_3$  suppresses long-term platelet  $Ca^{2+}$  responses and PS exposure. Fura-2-loaded platelets containing apyrase were preincubated with vehicle (control), eptifibatide (10  $\mu$ g/mL) or tirofiban (1  $\mu$ g/mL). Cells were then activated with thrombin (10 nM) plus convulxin (50 ng/mL) and 1 mM  $CaCl_2$ . **a** Representative platelet  $Ca^{2+}$  responses. **b** Effect of integrin blockers on  $Ca^{2+}$  peak levels (light gray) and 15-min end levels (dark gray). **c** Effect of blockers on

fractions of PS-exposing platelets after 15 min, analyzed by flow cytometry. **d** Effect of addition of eptifibatide or tirofiban at different time points before ( $t = -10$  min) or after ( $t = 1-10$  min) activation. Fractions of PS-exposing platelets after 15 min. Data are relative to control condition without integrin blocker. Mean  $\pm$  SEM ( $n = 3-5$ ); \* $p < 0.05$  versus vehicle

$Ca^{2+}$ -signaling by stimulating washed suspensions of Fura-2-loaded platelets with thrombin and convulxin. While measuring rises in  $Ca^{2+}$ , samples were taken for flow cytometric determination of PS exposure. The agonists caused a potent increase in  $Ca^{2+}$  peak, which was followed by a sustained elevated level (450 nM), persisting during 15 min (Fig. 3a). Pretreatment with eptifibatide or tirofiban did not influence the initial  $Ca^{2+}$  peak, but it markedly reduced the sustained high  $Ca^{2+}$  level to  $56 \pm 10$  or  $60 \pm 8$  % of the control value, respectively (Fig. 3a, b). The reduction in sustained  $Ca^{2+}$  response was accompanied by a proportional decrease in PS exposure from  $36 \pm 3$  to  $16 \pm 3$  or  $14 \pm 3$  %, respectively (Fig. 3c). Reasoning that persistent integrin signaling may prolong these  $Ca^{2+}$  responses and then contribute to PS exposure, we added the integrin blockers at various time points after thrombin/convulxin. Addition of eptifibatide or tirofiban at 1 min after activation still caused a substantial reduction in the fraction of PS-exposing platelets, whereas addition after 5–10 min resulted in progressively less inhibition (Fig. 3d).

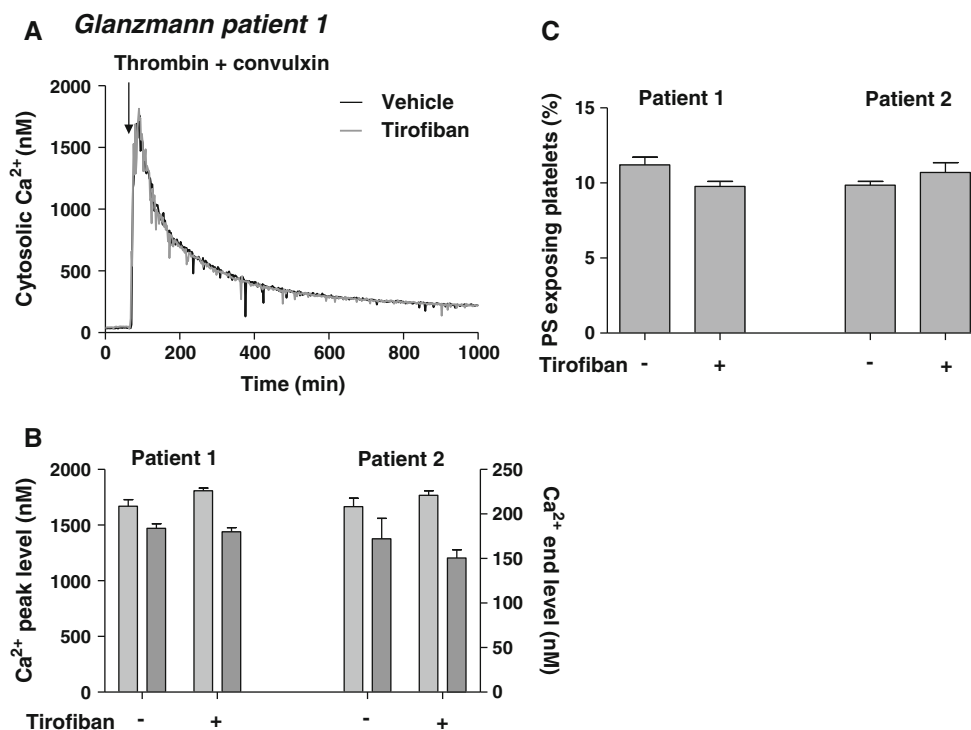
To further confirm the contribution of integrin signaling in PS exposure, platelets were obtained from two Glanzmann patients with complete deficiency in  $\alpha_{IIb}\beta_3$  expression. Loaded with Fura-2, the platelets showed high peak rises in  $Ca^{2+}$  in response to thrombin/convulxin, but

at later time points  $Ca^{2+}$  levels declined to  $\sim 150$  nM (Fig. 4a, b). This corresponded to a low amount of 10–12 % PS-exposing platelets (Fig. 4c). Addition of tirofiban altered neither the late  $Ca^{2+}$  response nor the low exposure of PS. Collectively, these data point to a role of  $\alpha_{IIb}\beta_3$ -mediated signaling in long-term  $Ca^{2+}$  rises induced by thrombin and collagen receptor agonists and thereby in development of platelet procoagulant activity.

Signaling role of integrin  $\alpha_{IIb}\beta_3$  in  $Ca^{2+}$  and procoagulant responses of platelets spreading on fibrinogen

Outside-in signaling by integrin  $\alpha_{IIb}\beta_3$  mediates the spreading of platelets on fibrinogen surfaces [4]. In Fura-2-loaded platelets, we found that spreading was accompanied by irregular, low-amplitude  $Ca^{2+}$  spikes (Fig. 5a). In most of these platelets, thrombin induced a persistently high  $Ca^{2+}$  level and stimulated the spreading process (Fig. 5a, b). To investigate a role of integrin signaling, the platelets were preincubated with tirofiban prior to thrombin stimulation. This retarded the spreading process, and suppressed the  $Ca^{2+}$  response, in a way that the persistent  $Ca^{2+}$  rise changed into a pattern of repetitive  $Ca^{2+}$  spiking. Furthermore, tirofiban reduced the fractions of PS-exposing platelets with thrombin from  $\sim 15$  to only 2.5 %

**Fig. 4** Impaired long-term  $\text{Ca}^{2+}$  responses and PS exposure in activated Glanzmann platelets. Suspensions of Fura-2-loaded platelets containing apyrase from two Glanzmann patients (1, 2) were pretreated with vehicle or tirofiban (1  $\mu\text{g}/\text{mL}$ ). Platelets were then activated with thrombin (10 nM) plus convulxin (50 ng/mL) in the presence of 1 mM  $\text{CaCl}_2$ . **a** Representative traces of  $\text{Ca}^{2+}$  responses. **b** Averaged  $\text{Ca}^{2+}$  peak levels (light gray) and 15-min end levels (dark gray). **c** Fractions of PS-exposing platelets after 15 min, showing no effect of tirofiban. Mean  $\pm$  SEM ( $n = 3$  experiments)



(Fig. 5c). The inhibiting effects of tirofiban were preserved on a surface coated with a low fibrinogen solution of 10  $\mu\text{g}/\text{mL}$  (not shown). Thrombin stimulation of adhered, non-spread platelets (5 min fibrinogen adhesion) similarly resulted in a low fraction of PS-exposing platelets of  $2.5 \pm 0.4\%$ . Control experiments were carried out with DM-BAPTA-loaded platelets, where basal levels of  $\text{Ca}^{2+}$  amounted  $\sim 20$  nM, and thrombin addition resulted in neither  $\text{Ca}^{2+}$  rises nor PS exposure (data not shown).

To further verify a role of  $\alpha_{\text{IIb}}\beta_3$  in thrombin-induced responses of fibrinogen-adhered platelets, similar experiments were performed with the platelets from two Glanzmann patients. While these platelets hardly spread on fibrinogen, they also remained low in PS exposure with only  $\sim 2\%$  annexin A5 binding after thrombin stimulation (Online Resource Fig. 2). These data thus suggest that integrin outside-in signaling during platelet spreading stimulates thrombin-induced procoagulant activity.

#### Contribution of integrin $\alpha_{\text{IIb}}\beta_3$ to platelet procoagulant response via Syk kinase activation

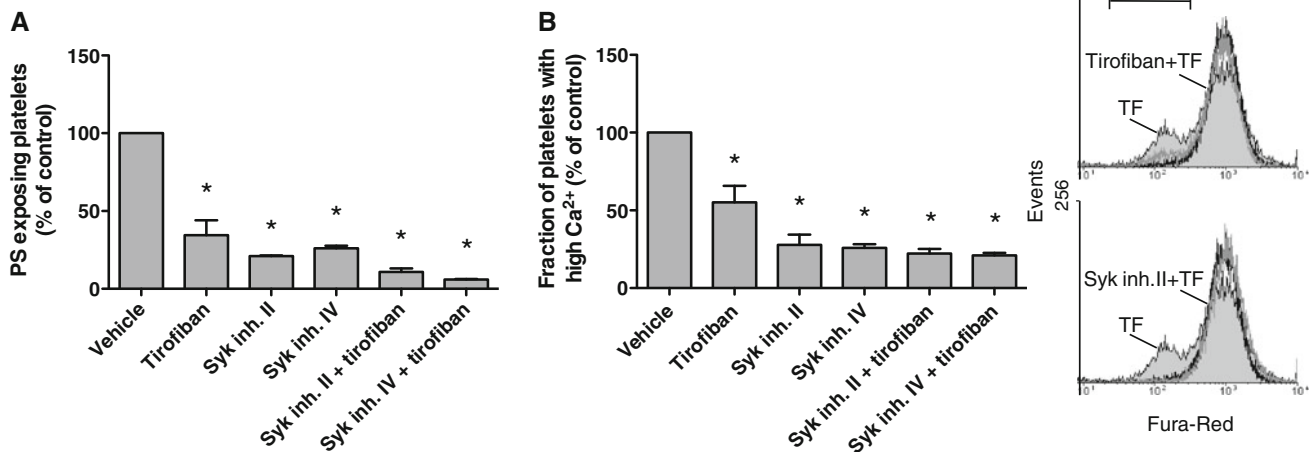
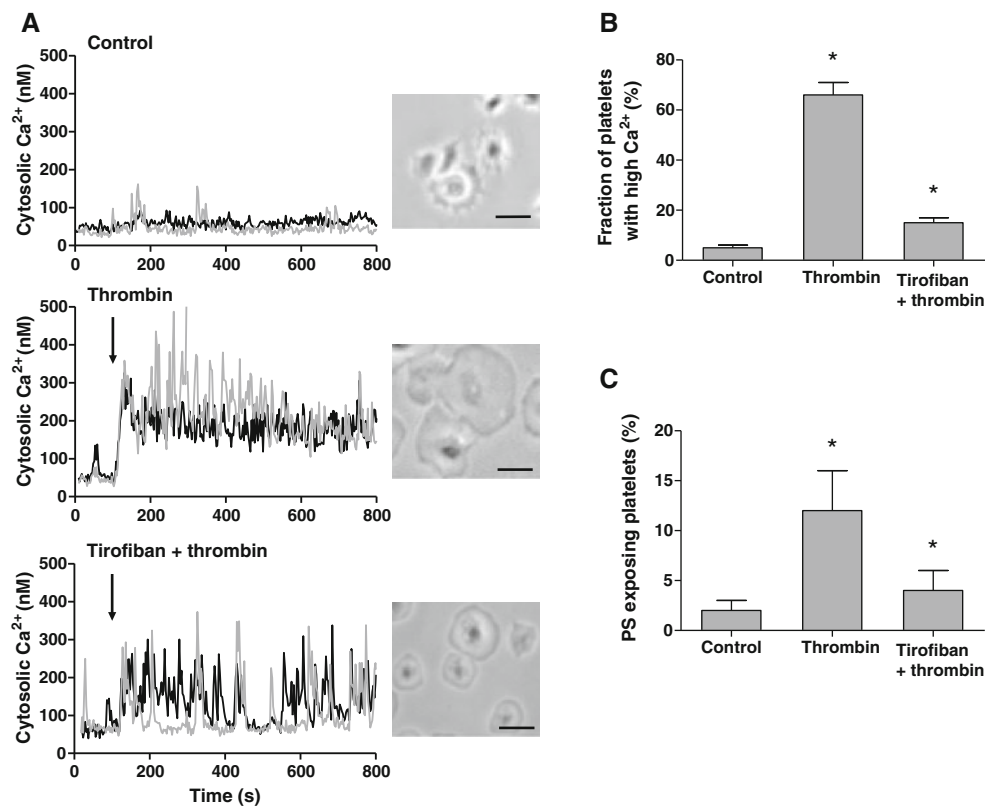
Integrin  $\alpha_{\text{IIb}}\beta_3$ -mediated outside-in signaling triggers inactivation of RhoA and activation of the protein tyrosine kinase Syk, resulting in phospholipase  $\text{C}\gamma 2$  activation [6, 41]. Employing several approaches, we investigated a role of Syk in  $\alpha_{\text{IIb}}\beta_3$ -dependent  $\text{Ca}^{2+}$  rises and procoagulant activity. First, we used the pharmacologic blockers, Syk inhibitor II and IV, which abolished collagen-induced aggregation of platelets in plasma at maximally effective

concentrations of 10  $\mu\text{M}$ . In tissue factor-stimulated PRP, both inhibitors suppressed platelet PS exposure at a similar degree as tirofiban (Fig. 6a). Next, platelets were loaded with the  $\text{Ca}^{2+}$  probe Fura-Red, which allows the monitoring of  $\text{Ca}^{2+}$  rises in the presence of plasma by flow cytometry [42]. Stimulation with tissue factor resulted in a prolonged rise in  $\text{Ca}^{2+}$  in the majority of platelets in plasma (observed as a decrease in Fura-Red fluorescence). This  $\text{Ca}^{2+}$  rise was reduced in the presence of tirofiban and even more so with Syk inhibitor II or IV (Fig. 6b).

Third, we directly examined the activation of Syk in tissue factor-stimulated PRP. It appeared not to be possible to collect these platelets from plasma by centrifugation or gel filtration. Hence, we developed a method to isolate platelets after tissue factor stimulation by using magnetic beads coupled to anti-CD31 mAb. Lysates of the isolated platelets were subjected to gel electrophoresis and western blotting, and probed for phosphorylation of Syk at  $\text{Tyr}^{525/526}$ , which is an essential step in Syk activation [43]. While no Syk phosphorylation was detected in the absence of tissue factor, platelet stimulation with tissue factor stimulation markedly increased the phosphorylation, which event was completely prevented by Syk inhibitor II (Fig. 7a, b). Pretreatment with tirofiban substantially but not completely antagonized  $\text{Tyr}^{525/526}$  phosphorylation, suggesting a prominent role of  $\alpha_{\text{IIb}}\beta_3$ -dependent signaling to Syk in the tissue factor-activated PRP.

Fourth, we directly investigated the effects of Syk inhibitors II and IV on tissue factor-induced thrombin generation. Using citrate-anticoagulated PRP, it appeared

**Fig. 5** Blocking of integrin  $\alpha_{IIb}\beta_3$  reduces  $Ca^{2+}$  responses and PS exposure of spreading platelets. Fura-2-loaded platelets were adhered to coated fibrinogen in the absence (control) or presence of tirofiban (1  $\mu\text{g}/\text{mL}$ ) for 30 min. Rises in  $Ca^{2+}$  in single, adhered platelets in response to thrombin (10 nM) measured by fluorescence ratio imaging. **a** Traces of  $Ca^{2+}$  rises of two representative platelets; also brightfield contrast images after 10 min (*bars* 10  $\mu\text{m}$ ). **b** Fractions of platelets with elevated  $Ca^{2+}$  (>1.2-fold signal) after 10 min. **c** Fractions of platelets binding FITC-annexin A5 after 10 min. Mean  $\pm$  SEM ( $n = 4-6$ ); \* $p < 0.05$  versus control



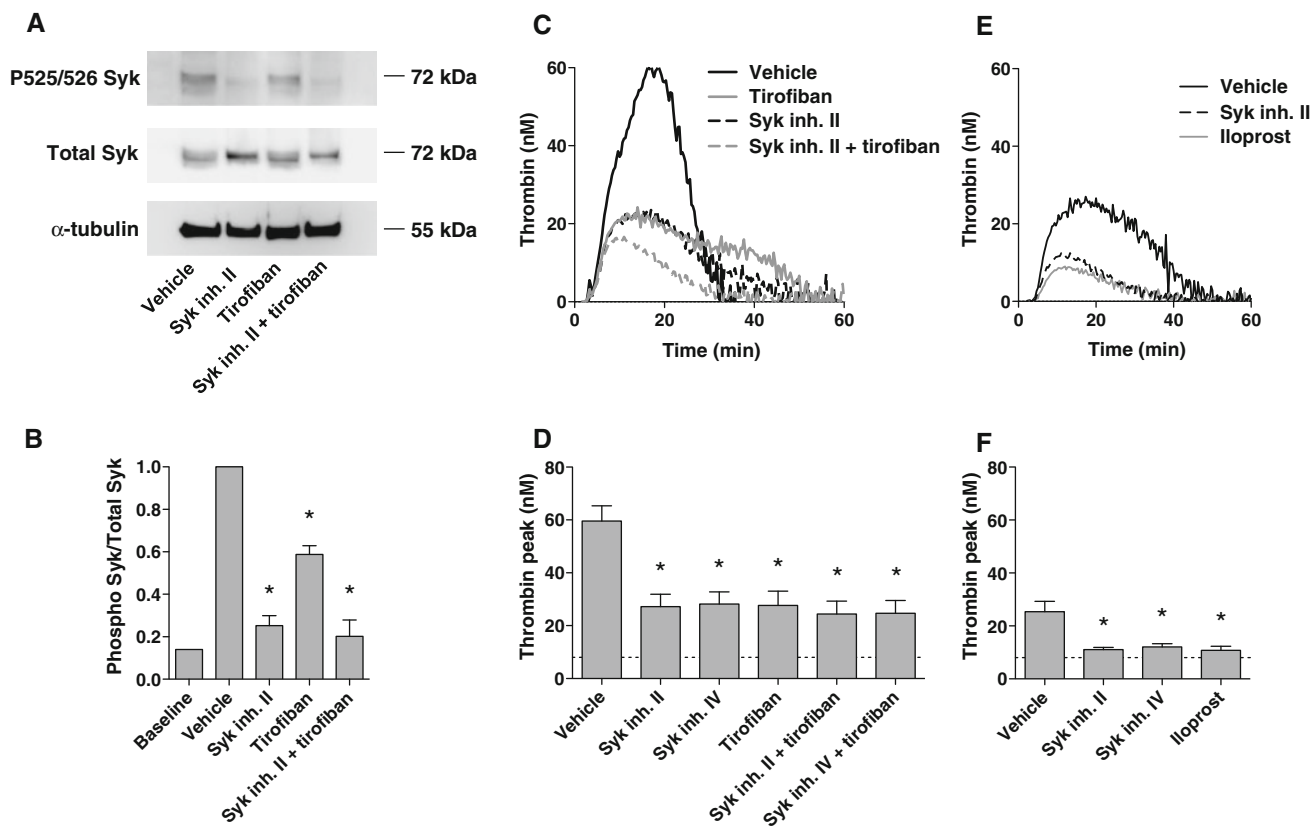
**Fig. 6** Contribution of Syk kinase to platelet activation in tissue factor-stimulated plasma. Fura-Red loaded platelets in anand-treated plasma ( $1.0 \times 10^8/\text{mL}$ ) were preincubated with vehicle, Syk inhibitor II or IV (10  $\mu\text{M}$ ), and/or tirofiban (1  $\mu\text{g}/\text{mL}$ ), as described for Fig. 1. Cangrelor (AR-C, 10  $\mu\text{M}$ ) was present to eliminate  $P2Y_{12}$ -dependent signaling events. PRP was then stimulated with 1 pM tissue factor and  $CaCl_2$  for 15 min. **a** Fractions of PS-exposing platelets determined by

FITC-annexin A5 binding. **b** Fractions of platelets with high  $Ca^{2+}$  as determined by flow cytometry (M1). Flow cytometric histograms of Fura-Red fluorescence. Note decreased Fura-Red fluorescence points to high  $Ca^{2+}$  (M1). Histograms of unstimulated platelets (*black*), and tissue factor-stimulated platelets with vehicle (*light gray*), tirofiban or Syk inhibitor II (*dark gray*). Mean  $\pm$  SEM ( $n = 4$ ); \* $p < 0.05$  versus vehicle

that both compounds were similarly effective as tirofiban in the suppression of thrombin generation, while there was no additional effect of a combination with tirofiban (Fig. 7c, d). As platelet-dependent thrombin generation was still

incompletely blocked, we considered the possibility that the platelets exhibited residual  $\alpha_{IIb}\beta_3$ -dependent signaling during blood collection and PRP preparation. To investigate this, blood was collected on citrate anticoagulant plus





**Fig. 7** Contribution of integrin  $\alpha_{IIb}\beta_3$  signaling and Syk kinase to thrombin generation in tissue factor-stimulated plasma. Reconstituted PRP was preincubated with vehicle, Syk inhibitor II (10  $\mu$ M), iloprost (1  $\mu$ M) and/or tirofiban (1  $\mu$ g/mL), as indicated, and then stimulated with tissue factor (1 pM) and  $\text{CaCl}_2$ . Cangrelor (AR-C, 10  $\mu$ M) was present to eliminate  $\text{P2Y}_{12}$ -dependent signaling events. Control condition (*baseline*) was without tissue factor. **a, b** Effects of tirofiban and Syk inhibitor on  $\text{Tyr}^{525/526}$  phosphorylation of Syk. Platelets were isolated from activated plasma (15 min) using anti-CD31-coupled magnetic beads and examined for protein

phosphorylation. Representative western blots after probing for phospho-Syk ( $\text{Tyr}^{525/526}$ ), and reprobing for total Syk; parallel blots were stained for  $\alpha$ -tubulin. Shown is the ratio of phospho-Syk/total Syk assessed by densitometric analysis ( $n = 6$ ). **c-f** Effects of tirofiban and Syk inhibitor on tissue factor-induced thrombin generation in PRP. Blood was collected on citrate ( $n = 8$ ) (**c, d**); or on citrate plus tirofiban (1  $\mu$ g/mL) ( $n = 3$ ) (**e, f**). Representative thrombin generation curves and thrombin peak heights. Dotted lines in *bar graphs* indicate residual thrombin formed in plasma not containing platelets. Mean  $\pm$  SEM; \* $p < 0.05$  versus vehicle

tirofiban. Indeed, with tirofiban initially present, tissue factor-induced thrombin generation was reduced, while either Syk inhibitor fully reduced the thrombin peak to the level obtained with the strong platelet inhibitor, iloprost (Fig. 7e, f). In fact, this residual, low thrombin generation was also present in plasma devoid of platelets, and could be ascribed to the presence of microparticles. Similar results were obtained with blood collected on Syk inhibitor II (not shown).

Finally, thrombin generation experiments were performed with PRP from a Glanzmann patient. In the patient PRP, tissue factor stimulation evoked limited thrombin generation, which, however, was not influenced by the presence of tirofiban (Online Resource Fig. 3). Similar to control PRP with tirofiban, addition of Syk inhibitor caused an additional decrease in thrombin generation. Taken together, these various sets of data show a substantial role of  $\alpha_{IIb}\beta_3$  and Syk kinase in tissue factor-induced PS

exposure and thrombin generation. Furthermore, they point to the existence of a pathway of thrombin generation that is dependent on Syk, but not on integrin activation.

## Discussion

This paper reveals a new role of integrin  $\alpha_{IIb}\beta_3$ -dependent signaling via Syk kinase in tissue factor-induced platelet procoagulant activity and thrombin generation in plasma. The results point to a pathway where initial traces of thrombin triggered by tissue factor activate platelets to expose procoagulant PS, resulting in a cycle of thrombin generation and platelet activation that is greatly enforced and prolonged by integrin-dependent signaling to Syk activation and  $\text{Ca}^{2+}$  rises. The data furthermore identify a Syk-dependent, but integrin-independent pathway of thrombin generation.

In tissue factor-stimulated PRP, we found that the blockage of  $\alpha_{IIb}\beta_3$  with different antagonists suppresses platelet  $Ca^{2+}$  responses, PS exposure and thrombin generation in a dose-dependent way. However, in washed platelets stimulated via thrombin and collagen receptors, integrin blockage also suppressed long-term  $Ca^{2+}$  rises along with PS exposure, while added fibrinogen enhanced these responses. Furthermore, during platelet spreading on fibrinogen, a process known to rely on  $\alpha_{IIb}\beta_3$  outside-in signaling, blocking of the integrin resulted in  $Ca^{2+}$  signaling and PS exposure in response to maximally effective concentrations of thrombin. Confirmative evidence for a signaling role of  $\alpha_{IIb}\beta_3$  came from the observation that long-term  $Ca^{2+}$  responses and PS exposure were reduced in platelets from two patients with Glanzmann's thrombasthenia, lacking  $\alpha_{IIb}\beta_3$ . Together, these data indicate that, in platelets stimulated with  $Ca^{2+}$  mobilizing agonists,  $\alpha_{IIb}\beta_3$  outside-in signaling prolongs the  $Ca^{2+}$  signal, increases procoagulant activity, and hence supports tissue factor-stimulated thrombin generation on the platelet surface.

Anticoagulant effects of platelet  $\alpha_{IIb}\beta_3$  antagonists have been reported by several authors [12, 16, 17], but the mechanism was not disclosed. Several groups reported that integrin blockers were unable to change platelet  $Ca^{2+}$  responses to collagen and/or thrombin [22, 23, 39]. However, the measurements mostly concerned initial  $Ca^{2+}$  rises, while in our hands only late  $Ca^{2+}$  signals appear to be affected. Interestingly, one study does describe long-term inhibition of collagen/thrombin-induced  $Ca^{2+}$  responses with abciximab but not with other integrin blockers specifically under conditions of stirring [23]. This contrasts with the present findings where appropriate concentrations of different integrin blockers all had similar effects. Another published finding that  $\alpha_{IIb}\beta_3$  blockage reduces shear-dependent  $Ca^{2+}$  responses and microparticle release [19, 44] can be explained by increased fibrinogen secretion of platelets subjected to a high shear rate.

The (patho)physiological relevance of this work comes from recent data that  $\beta_3$ -mutated mice with a deficiency in platelet outside-in signaling and tyrosine phosphorylation are protected from arterial thrombus formation after carotid artery injury with  $FeCl_3$  [45], i.e. a mouse thrombosis model where thrombus formation depends on tissue factor activity and on procoagulant, PS-exposing platelets [13, 46–48].

Platelets from mice deficient in phospholipase  $C\gamma_2$  have shown reduced  $Ca^{2+}$  signals and spreading on immobilized fibrinogen [44, 49]. Based on these and other data a scheme has been proposed of  $\alpha_{IIb}\beta_3$ -induced signaling via Src and Syk kinases to activation of phospholipase  $C\gamma_2$  [6]. This signaling scheme was confirmed by recent proteomic analyses demonstrating the presence of many tyrosine

phosphorylated proteins in human platelets spread on fibrinogen, among which multiple tyrosine kinases [5]. The present finding points to a particular role of Syk phosphorylation and subsequent phospholipase  $C\gamma_2$  activation in  $\alpha_{IIb}\beta_3$ -dependent procoagulant activity and thrombin generation upon triggering with tissue factor. Evidence for this came from the reduction in Syk tyrosine phosphorylation by integrin blockage and by two Syk inhibitors. These experiments were carried out under conditions eliminating a contribution of  $P2Y_{12}$  signaling [24, 50].

Interestingly, in addition to a novel  $\alpha_{IIb}\beta_3$ -dependent role of Syk kinase in platelet procoagulant activity, our data also point to an  $\alpha_{IIb}\beta_3$ -independent role of Syk in this process. We have not yet unraveled the mechanism of  $\alpha_{IIb}\beta_3$ -independent Syk activation, but according to the literature this activation pathway can include  $Fc\gamma RIIa$  [51], glycoprotein Ib–IX–V complex [52, 53], or CLEC2 [54], all of which have been shown to activate Syk. Altogether, our results point to a signaling scheme where fibrin(ogen)-induced integrin activation supports a Syk/phospholipase  $C\gamma_2$  pathway, resulting in prolonged  $Ca^{2+}$  and PS exposure and thrombin generation in plasma.

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