Evaluation of the role of platelet integrins in fibronectin-dependent spreading and adhesion

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Summary. Background: Recent studies have shown that platelet adhesion and subsequent aggregation can occur in vivo in the absence of the two principal platelets adhesive ligands, von Willebrand factor and fibronectin. These results highlight a possible role for fibronectin in supporting thrombus formation. Objective and methods: To evaluate the platelet integrins and subsequent activation pathways associated with fibronectin-dependent platelet adhesion utilizing both human and murine platelets. Results: Platelets can adhere to fibronectin via the integrin αIIbβ3, leading to formation of lamellipodia. This is mediated through an interaction with the tenth type III domain in fibronectin. Spreading on fibronectin promotes αIIbβ3-mediated Ca²⁺ mobilization and tyrosine phosphorylation of focal adhesion kinase and phospholipase C γ². In contrast, studies with blocking antibodies and αIb mice demonstrate that αIβI and αIβ3 support adhesion and promote formation of filopodia but not lamellipodia or tyrosine phosphorylation of these proteins. Further, neither αIβI nor αIβ3 is able to induce formation of lamellipodia in the presence of platelets agonists, such as collagen-related-peptide (CRP). Conclusions: These observations demonstrate that integrins αIβI and αIβ3 support platelet adhesion and the generation of filopodia but that, in contrast to the integrin αIIbβ3, are unable to promote formation of lamellipodia.

Keywords: αIβI, αIIbβ3, fibronectin, platelet.

Introduction

Platelet adhesion to the subendothelium of an injured vessel wall and subsequent aggregation are critical events in hemostasis and thrombosis. The interaction of platelets with adhesive proteins both in the extracellular matrix (ECM) and plasma provides signals that affect their morphology, activation and binding. Following initial platelet-collagen adhesion, a variety of receptor–ligand interactions synergistically promote homotypic platelet aggregation leading to thrombus formation [1–3]. A critical event in this process is the activation of platelet integrins, which increases their affinity for their respective adhesive ligands. The key integrin in thrombus formation is αIIbβ3, which is a receptor for a number of adhesive proteins including fibrinogen, von Willebrand factor (VWF) and fibronectin [2,4]. Engagement of αIIbβ3 promotes platelet–platelet interaction [5] and generates ‘outside-in’ signals that reinforce activation [6,7]. Until recently, VWF and fibrinogen were thought to be the principal ligands in vivo that engage αIIbβ3. However, Ni et al. have shown that mice lacking both of these adhesive ligands retain the ability to develop thrombi at sites of vascular injury in vivo, highlighting a role for alternative adhesive proteins in promoting thrombus formation [8]. In this context, it is pertinent to consider a role for fibronectin, a recognized ligand for αIIbβ3 that is present in high levels in plasma and which has been shown to promote thrombus growth and stability in injured arterioles [9,10].

Fibronectin is present in plasma, the subendothelium of the vessel wall, and is stored in low amounts in platelet α-granules [11]. This soluble dimeric protein is a mosaic of three different types of homologous repeating modules that have been designated type I, type II and type III. The Arg-Gly-Asp (RGD) sequence in FNIII domain 10 (10FIII) is crucial for interactions with its integrin receptors, namely αIIbβ3, αIβ3 and αIβI. In addition, other sequences outside of this RGD region are sometimes required for full adhesive activity [12,13]. This is exemplified by a set of residues in the FNIII domain 9 (9FIII)
which have been shown to contribute to high affinity binding to \( \alpha_{\text{IIb}}\beta_3 \) integrin in baby hamster kidney (BHK) cells [14]. This region has been termed the synergy site.

The initial binding of an integrin to an adhesive protein, such as fibronectin, stimulates recruitment of signaling proteins to the vicinity of the integrin cytoplasmic tails [15]. This initial phase of outside-in signaling contributes to further platelet activation. This is illustrated by \( \alpha_{\text{IIb}}\beta_3 \) signaling mediated via Src and Syk tyrosine kinases leading to activation of a number of downstream pathways including focal adhesion kinase (FAK), PI 3-kinase and phospholipase C \( \gamma_2 \) (PLC\( \gamma_2 \)), which leads to an increase in intracellular \( \text{Ca}^{2+} \); together these events are crucial for platelet spreading [16–19]. The contribution made by other platelet integrins which are expressed in lower levels, such as the \( \alpha_{\text{IIb}}\beta_1 \) and \( \alpha_c\beta_3 \) integrins, to activation has yet to be defined.

This study focuses on characterizing the potential role of the platelet integrin receptors for fibronectin, \( \alpha_{\text{IIb}}\beta_3, \alpha_c\beta_3 \), and \( \alpha_{\text{IIb}}\beta_1 \), in mediating adhesion and activation. We demonstrate that platelet \( \alpha_{\text{IIb}}\beta_1 \) and \( \alpha_c\beta_3 \) integrins are capable of supporting platelet adhesion, but that lamellipodia formation and intracellular \( \text{Ca}^{2+} \) mobilization requires \( \alpha_{\text{IIb}}\beta_3 \). Furthermore, we demonstrate that platelet adhesion and spreading is critically dependent upon the RGD-sequence containing 10FIII module of fibronectin, although the 9FIII region is required to produce equivalents levels of signaling to that induced by full-length fibronectin. These results demonstrate that the integrins \( \alpha_{\text{IIb}}\beta_3, \alpha_c\beta_3 \), and \( \alpha_{\text{IIb}}\beta_1 \) are able to support platelet adhesion but that only the first of these is able to mediate formation of lamellipodia and mobilization of \( \text{Ca}^{2+} \).

**Experimental procedures**

**Reagents**

The blocking anti-\( \alpha_5 \) (JBS5) monoclonal antibody (mAb) was purchased from Serotec (Oxford, UK). The blocking anti-\( \beta_1 \) (P4C10) mAb was purchased from Chemicon (Temecula, CA, USA). Polyclonal rabbit anti-FAK (C-903) antibody (pAb) was from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Anti-PLC\( \gamma_2 \) pAb was a gift from Dr Tomlinson (DNAX, Palo Alto, CA, USA). The \( \alpha_c\beta_3/\alpha_{\text{IIb}}\beta_3 \) antagonists lotrafiban and abiciximab were supplied by GlaxoSmithKline (King of Prussia, PA, USA) and Eli Lilly and Company, Ltd (Basingstoke, UK), respectively. AR-C67085 was a gift from AstraZeneca R & D Charnwood (Loughborough, UK). BAPTA-AM, A3P5P, apyrase, indomethacin, thrombin and ADP were from Sigma (Poole, UK). D-phenyl-alanyl-1-prolyl-1 arginine chloromethyl ketone (PPACK) was purchased from Calbiochem (La Jolla, CA, USA). Fibrinogen depleted of fibrinogen, recombinant fibrinogen proteins (200 \( \mu \text{g mL}^{-1} \)) or spectroscopy (data not shown). Oregon Green bis-(o-aminophenoxy)ethane-N,N,N,N′-tetra-acetic acid (BAPTA 1)-AM was purchased from Molecular Probes (Cambridge Bioscience, Cambridge, UK). CRP was synthesized by Tana Laboratories (Houston, TX, USA). All other reagents were from Sigma (Poole, UK) or previously named sources [16,18,20].

**Preparation of human platelets**

Human venous blood was drawn by venipuncture from healthy volunteers into either sodium citrate and acid/citrate/dextrose (ACD) or 50 \( \mu \text{M} \) PPACK anticoagulant as previously described [16,21]. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 160 \( \text{g} \) for 20 min. Platelet-poor plasma (PPP) was obtained by further centrifugation of the blood at 1100 \( \text{g} \) for 10 min. The final platelet count in the PRP was adjusted to the desired levels with platelet-poor plasma.

In selected experiments, platelets were isolated from PRP by centrifugation at 1100 \( \text{g} \) for 10 min in the presence of prostacyclin (0.1 \( \mu \text{g mL}^{-1} \)). The pellet was resuspended in modified HEPES-Tyrodes buffer (in mM: 129 NaCl, 0.34 Na2HPO4, 2.9 KCl, 12 NaHCO3, 20 HEPES, 5 glucose, 1 MgCl2; pH 7.3) containing 0.1 \( \mu \text{g mL}^{-1} \) prostacyclin. The platelets were washed once via centrifugation (1100 \( \text{g} \) for 10 min) and resuspended at the desired concentration with HEPES-Tyrodes buffer, containing physiological levels of fibrinogen (3 mg mL\(^{-1}\)), fibrinectin (300 \( \mu \text{g mL}^{-1} \)) and VWF (10 \( \mu \text{g mL}^{-1} \)) in selected experiments. In separate experiments, platelet suspensions were treated with 0.2 \( \mu \text{g mL}^{-1} \) CRP, 10 \( \mu \text{M} \) ADP, 1 and 0.06 U mL\(^{-1} \) thrombin, 10 \( \mu \text{M} \) BAPTA-AM, 5 mM Mn\(^{2+} \), 10 \( \mu \text{M} \) lotrafiban (LOT), 20 \( \mu \text{g mL}^{-1} \) JBS5 and P4C10, 1 U mL\(^{-1} \) apyrase or 1 mM A3P5P and 1 \( \mu \text{M} \) AR-C67085, or 10 \( \mu \text{M} \) indomethacin for 10–30 min before use in the assays. All experiments were performed in the absence of exogenously added Ca\(^{2+} \).

**Preparation of murine platelets**

The generation of mice disrupted in the \( \alpha_{\text{IIb}} \) gene (\( \alpha_{\text{IIb}}^{-/-} \)) is described in Emambokus et al. [22]. Mice deficient in PLC\( \gamma_2 \) were generated as described [23]. Wild type littermates were used as controls. Murine blood was drawn from CO2 terminally anesthetized mice by cardiac puncture and taken into 100 \( \mu \text{L} \) of acid-citrate-dextrose. PRP was obtained by centrifugation at 300 \( \text{g} \) for 6 min PPP was then obtained by further centrifugation of the blood at 1000 \( \text{g} \) for 6 min and used to adjust the platelet concentration of PRP. In selected experiments, washed platelets were obtained via centrifugation of PRP at 1000 \( \text{g} \) in the presence of prostacyclin (0.1 \( \mu \text{g mL}^{-1} \)) for 6 min. The pellet was resuspended in modified HEPES-Tyrodes buffer to the desired platelet level.

**Adhesion assays**

Cover slips were incubated with a suspension of fibrinogen, fibrinectin, recombinant fibrinectin proteins (200 \( \mu \text{g mL}^{-1} \)) or
denatured BSA (5 mg mL\(^{-1}\)) overnight at 4 °C. Surfaces were washed twice with PBS and blocked with denatured BSA (5 mg mL\(^{-1}\)) for 1 h at room temperature (RT). Surfaces were then subsequently washed twice with PBS before use in spreading assays. Platelets failed to bind or become activated to surfaces coated with denatured BSA.

For the spreading experiments, washed murine platelets (2 \times 10^5 \text{ mL}^{-1}) and washed human platelets (2 \times 10^5 \text{ mL}^{-1}) were incubated on coated coverslips at 37 °C for 45 min in a humid environment. After removal of unbound platelets by washing with PBS, adhered platelets were fixed, permeabilized, and stained by TRITC-conjugated phalloidin, as described previously [24]. Platelets were viewed on an inverted fluorescent microscope (Carl Zeiss MicroImaging, Inc., Herts, UK), and digital imaging was performed using Openlab software for Macintosh.

Platelet spreading (6 \times 10^7 \text{ mL}^{-1}) was imaged in real time using Köhler illuminated Nomarski differential interference contrast optics with a Zeiss 63\times oil immersion 1.40 NA axioplan lens on a Zeiss Axiovert 100 Microscope. Time-lapse events were captured by a QICAM Mono 10-bit camera (QImaging, Burnaby, BC, Canada) using Openlab software for Macintosh. To compute the surface area of spreading platelets, time-lapse images were manually outlined and quantitated by determining the number of pixels within each outline using a Java plugin for the Image J software package. Imaging a graticule under the same conditions allowed the conversion of pixels size to microns.

**Single platelet Ca\(^{2+}\) measurement**

Washed human platelets at 4 \times 10^8 \text{ mL}^{-1} were loaded with the Ca\(^{2+}\) sensitive dye Oregon Green BAPTA 1-AM (15 \text{ µM}) for 1 h at 30 °C. Loaded platelets were subsequently washed, resuspended at 2 \times 10^5 \text{ mL}^{-1} and left for a minimum of 30 min before experimentation. Platelets (1 \times 10^7 \text{ mL}^{-1}) were allowed to sediment onto either a fibronectin- or BSA-coated coverslip over a period of 30 min. Fluorescence changes in single platelets were measured using a Nikon TE200 microscope, fitted with a Zeiss 75 W xenon source and an epifluorescence attachment with excitation and emission filter wheels containing a green filter set as previously described by Harper et al. [25]. A Hamamatsu Orca 1 CCD camera and AQM Orca 2001 software was used for image capture and subsequent analysis (Kinetic Imaging, Wirral, UK).

**Phosphorylation studies**

For protein precipitation studies, six well plates were incubated overnight with an equal molar concentration (1 \text{ µM}) solution of either fibronectin or purified fibronectin domains. Following the aforementioned washing and BSA blocking procedure, 500 \text{ µL} washed platelets (4 \times 10^8 \text{ mL}^{-1}) were incubated on six-well plates at 37 °C for 45 min in a humid environment. Unbound platelets were removed by two washes with PBS followed by aspiration, and adhered cells were solubilized with an equal volume of ice-cold immunoprecipitation buffer (2% v/v NP-40, 20 mM Tris, 300 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM NaF, VO\(_4\), 10 µg mL\(^{-1}\) leupeptin, 10 µg mL\(^{-1}\) aprotonin, 1 µg mL\(^{-1}\) pepstatin A, pH 7.3). A sample of the suspension over BSA was taken and used as a control. After removing debris and preclearing, selected supernatants were incubated with 6 µL anti-FAK pAb or 2 µL anti-PLC\(\gamma\)2 pAb and 20 µL protein A-Sepharose at 4 °C overnight. Subsequent immunoprecipitation and Western blotting were carried out as previously described [16,18,24].

**Analysis of data**

Experiments were carried out on at least three occasions, and images shown are representative data from one experiment. Where applicable, results are shown as mean ± SEM. Statistical significance of differences between means was determined by one-way ANOVA. If means were shown to be significantly different, multiple comparisons by pairs were performed by the Tukey test. Probability values of \( P < 0.01 \) were selected to be statistically significant.

**Results**

**Spreading of human platelets on fibronectin-coated surfaces**

To determine the molecular basis of platelet binding to fibronectin, we gently pipetted washed human platelets over surface-immobilized proteins and subsequently measured adhesion and spreading through labeling of the actin cytoskeleton using rhodamine-labeled phalloidin. We initially confirmed that very few platelets adhere to a BSA-coated surface or undergo spreading (Fig. 1A). In contrast, platelets bind and spread on immobilized fibronectin in the absence of external stimulation (Fig. 1B). The majority of adhered platelets had spread fully after 45 min, and both filopodia and lamellipodia structures could be seen. Adhesion and spreading on fibronectin was not significantly altered in the presence of the cyclooxygenase inhibitor indomethacin (10 \text{ µM}) and the P2Y\(_1\) and P2Y\(_{12}\) ADP receptor antagonists A3P5P (1 mM) and AR-C67085 (1 \text{ µM}), suggesting that these changes are directly mediated through engagement of fibronectin receptors (Fig. 1C). External stimulation with ADP (10 \text{ µM}) slightly enhanced platelet spreading and led to most of the platelets forming stress fibres (Fig. 1D).

Experiments were designed to elucidate the molecular constituents mediating platelet adhesion to fibronectin utilizing truncated portions of fibronectin containing either the single 10FIII domain or the 9–10FIII domain pair. Previous work has shown that the RGD sequence in 10FIII is critical for integrin engagement, and that the synergy site in 9FIII domain potentiates this interaction [26]. Our results show that the 9–10FIII sequence, as well as the 10FIII sequence alone, support spreading to a similar extent induced by native fibronectin (Fig. 1E–F).
Identification of platelet integrins mediating adhesion to fibronectin

We examined the role of platelet membrane integrins in mediating adhesion to fibronectin using blocking antibodies and receptor antagonists. The integrin αIIbβ3 is the most abundant receptor on the platelet surface, with 60,000–80,000 copies per activated cell. αIIbβ3 has previously been shown to mediate platelet adhesion to fibrinogen-, VWF- and fibronectin-coated surfaces [2,4]. Blockade of αIIbβ3 with the antagonist lotrafiban, which also blocks the minor platelet integrin αvβ3 [27], has no effect on the extent of adhesion to fibronectin (Table 1) but eliminates platelet formation of lamellipodia but not filopodia (Fig. 1G). Furthermore, prior stimulation with either the secondary mediator ADP, the GPVI-specific agonist CRP or the G-protein-coupled receptor agonist thrombin failed to potentiate αIIbβ3-blocked platelet spreading (Table 1). This αIIbβ3-independent adhesion of platelets to fibronectin was abrogated in the presence of antibodies against the α5β1 integrin in combination with lotrafiban (Fig. 1I). In contrast, α5β1-blockade alone had no effect on the adhesion or degree of spreading in the absence of an αIIbβ3 antagonist (Table 1). Platelet adhesive interactions were unaltered by the presence of an IgG control mAb (data not shown). Taken together, our data demonstrates that formation of lamellipodia on fibronectin is eliminated in the presence of αIIbβ3/αvβ3 blockade, and that while the α5β1 integrin can support platelet-fibronectin binding, it is insufficient to mediate platelet spreading.

We further examined platelet adhesion and spreading using Normarski differential interference contrast microscopy. This technology provides a clear resolution between lamellipodia and filopodia and also enables monitoring of adhesion and spreading in real time. Initial studies confirmed that platelets adhere extensively to fibronectin but not to BSA and demonstrated that lamellipodia but not filopodia formation is inhibited in the presence of the αIIbβ3 antagonist lotrafiban but that additional blockade of α5β1 is required to block adhesion (Fig. 2A-D). The time course of spreading was monitored by time-lapse video microscopy as illustrated in Fig. 2(E–F). Platelet binding to fibronectin is associated with a characteristic series of shape changes (Fig. 2E), as previously described for fibrinogen-coated surfaces [28]. Platelet rounding is followed by formation of filopodia and then lamellipodia resulting in an increase in size after 600 s from 11.6 ± 0.7 μm² to 43.7 ± 1.7 μm² (Fig. 2G). The generation of stable filopodia and lamellipodia is drastically reduced by the presence of the αIIbβ3 antagonist lotrafiban, although dynamic extension and retraction of filopodia is observed as exemplified by the arrowhead at 245 s and 455 s, respectively, in Fig. 2. A quantitative assessment of the platelet surface area both in the absence and presence of lotrafiban over 20 min is shown in Fig. 2(G). The results demonstrate a uniform rate of spreading of platelets on fibronectin, which is complete within 600 s.

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contrast, a smaller increase in surface area is seen in the presence of lotrafiban, which reaches a plateau in 200 s.

Role of Ca$^{2+}$ mobilization and PLC$\gamma_2$ on platelet spreading on fibronectin

To further assess the potential ability of the $\alpha_{IIb}\beta_3$ integrin to transduce outside-in signaling, we examined the elevation of Ca$^{2+}$ in single platelets that had bound to immobilized fibronectin in the absence and presence of the $\alpha_{IIb}\beta_3$ antagonist lotrafiban. Intracellular Ca$^{2+}$ was measured in washed platelets loaded with the Ca$^{2+}$ reporter dye Oregon Green BAPTA 1-AM utilizing dynamic video imaging. The majority of Oregon Green-loaded platelets moved continually on a BSA-coated surface, while the minority that settled did not exhibit an increase in Ca$^{2+}$ (Fig. 3A). More specifically, once the platelet arrived within the region of interest (as denoted by an arrowhead in Fig. 3), a basal Ca$^{2+}$ level was observed with little fluctuation, as previously shown by Atkinson et al. [29]. In contrast, platelets readily formed stable adhesions on fibronectin and, after a variable lag phase of usually greater than 200 s,
exhibited an increase in Ca\(^{2+}\) consisting of a series of oscillatory changes (Fig. 3B). This increase in Ca\(^{2+}\) was abolished in the presence of lotrafiban (Fig. 3C), demonstrating that while platelet activation and spreading via \(\alpha_{IIb}\beta_3\) elicits an increase in intracellular Ca\(^{2+}\), platelet \(\alpha_5\beta_1\)-mediated binding and dynamic synthesis of filopodia is independent of Ca\(^{2+}\) mobilization. Consistent with these findings, treatment of washed platelets with the intracellular Ca\(^{2+}\) chelating agent BAPTA-AM abrogated platelet lamellipodia formation but did not block extension and retraction of filopodia in the presence or absence of lotrafiban (data not shown).

In an attempt to further corroborate the notion that calcium mobilization is not required for filopodia formation, we examined adhesion of PLC\(\gamma_2\)-null murine platelets to fibronectin, bearing in mind that regulation of cytosolic calcium flux by fibrinogen has been shown to be dependent upon activation of this phospholipase [30]. Wild type mouse platelets extend filopodia and form partial lamellipodia on fibronectin in contrast to the full spreading response observed in human platelets (Fig. 4A). A similar difference between spreading of murine and human platelet on fibrinogen has previously been reported [17]. In comparison, the majority of PLC\(\gamma_2\)-null platelets produce filopodia but fail to form lamellipodia structures (Fig. 4B). This is illustrated by the reduction in platelet surface area between wild type and PLC\(\gamma_2\)-null platelets (Table 2). Furthermore, filopodia formation was still observed for PLC\(\gamma_2\)-null platelets in the presence of the \(\alpha_{IIb}\beta_3\) antagonist lotrafiban (data not shown). It is noteworthy that treatment of PLC\(\gamma_2\)-null platelets with thrombin led to lamellipodia formation (as evidenced by an increase in surface area; Table 2), consistent with activation of phospholipase C-\(\beta\) (PLC-\(\beta\)) by the G-protein coupled receptor agonist [31].

**\(\alpha_{IIb}\)-deficient mouse platelets adhere but do not spread on fibronectin**

To further validate the aforementioned findings and determine the role of \(\alpha_5\beta_3\) in these events, we monitored fibronectin-dependent binding of platelets from mice with a mutation that disrupts the GPIIb gene, resulting in the absence of the \(\alpha_{IIb}\beta_3\)

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**Fig. 3.** Ca\(^{2+}\) elevation induced by a fibronectin surface is abolished by blockade of \(\alpha_{IIb}\beta_3\) integrins. Washed human platelets loaded with the Ca\(^{2+}\)-sensitive dye Oregon Green BAPTA 1-AM were imaged as they made contact with either a BSA- or fibronectin-coated slide. The Ca\(^{2+}\) traces are all from the same experiment and were analyzed using AQM Orca 2001 software. The graph shows the platelets arrive at the region of interest as denoted by an arrowhead and a basal level of Ca\(^{2+}\), then subsequent Ca\(^{2+}\) fluctuations are shown over a 20-min period of observation. The scale is in arbitrary units derived from the intensity of fluorescence emission. Traces shown are representative of 3 parallel experiments.

**Fig. 4.** PLC\(\gamma_2\) is required to promote platelet lamellipodia but not filopodia formation during adhesion to immobilized fibronectin. Purified (A) wild type murine platelets and (B) PLC\(\gamma_2\)-null murine platelets were exposed to a fibronectin-coated surface and observed in real time using Nomarski differential interference contrast microscopy. A representative time course of a single platelet spreading is shown. Bar = 10 \(\mu\)m.
Table 2 Effects of external stimulation on murine platelet surface area following adhesion to fibronectin

<table>
<thead>
<tr>
<th>Mouse genotype</th>
<th>Treatment</th>
<th>Platelet surface area (μm²)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>–</td>
<td>15.1 ± 0.6</td>
</tr>
<tr>
<td>WT</td>
<td>LOT</td>
<td>6.0 ± 0.3</td>
</tr>
<tr>
<td>WT</td>
<td>ADP</td>
<td>19.0 ± 0.6</td>
</tr>
<tr>
<td>WT</td>
<td>CRP</td>
<td>20.1 ± 0.9</td>
</tr>
<tr>
<td>WT</td>
<td>thrombin</td>
<td>20.2 ± 0.6</td>
</tr>
<tr>
<td>PLC2/–/–</td>
<td>–</td>
<td>9.2 ± 0.6</td>
</tr>
<tr>
<td>PLC2/–/–</td>
<td>thrombin</td>
<td>19.6 ± 0.5</td>
</tr>
<tr>
<td>αIIb/–/–</td>
<td>–</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>αIIb/–/–</td>
<td>ADP</td>
<td>5.7 ± 0.3</td>
</tr>
<tr>
<td>αIIb/–/–</td>
<td>CRP</td>
<td>5.8 ± 0.4</td>
</tr>
<tr>
<td>αIIb/–/–</td>
<td>thrombin</td>
<td>5.9 ± 0.5</td>
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Wild type, PLC2/–/– and αIIb/–/– murine platelets were layered onto a fibronectin-coated slide for 45 min. In selected experiments, platelets were treated with the αIIb/β3/αIIb, antagonist lotrafiban (10 μM LOT), ADP (10 μM), CRP (0.2 μg mL⁻¹) or thrombin (1 U mL⁻¹) before being layered onto the slide. Values are reported as follows: platelet surface area = mean ± SEM of 75 cells.

complex [22]. Purified wild type murine platelets adhere and spread on surface-immobilized intact fibronectin (Fig. 5A). These changes were not altered in the presence of indomethacin and the A3P5P and AR-C67085 antagonists, confirming that they are independent of the major secondary mediators (data not shown). As with human platelets, ADP further enhanced spreading of murine platelets and resulted in most of the platelets forming stress fibres (Fig. 5B). In contrast, platelet spreading was absent in αIIb/– murine platelets (Fig. 5C), although these platelets retain the ability to bind to the fibronectin surface. Furthermore, stimulation with ADP, CRP or thrombin failed to induce formation of lamellipodia or stress fibers in the absence of the αIIb/β3 complex (Fig. 5D and Table 2). Together, these results demonstrate that platelet spreading is dependent upon the presence of the αIIb/β3 complex. However, in the absence or presence of external stimulation, platelets retain the ability to adhere to fibronectin in an αIIb/β3-independent manner, presumably through α3β1 and α5β1 integrins.

Further studies were undertaken to validate the ability of α3β1 to support adhesion. For these studies, we used fibrinogen, which binds αIIb/β3 and α5β1. In accordance with previous work, Fig. 6 (A) demonstrates that purified wild type murine platelets adhere to and spread on surface-immobilized fibrinogen [17]. The absence of the αIIb/β3 complex in the αIIb–/– murine platelets results in complete abrogation of platelet adhesion to fibrinogen (Fig. 6B). However, adhesion but not spreading on fibrinogen in the αIIb–/– platelets upon cellular stimulation with Mn²⁺, which acts through the metal ion-dependent adhesion site (MIDAS) of the insertion I-like domain and promotes integrin activation in the absence of calcium (Fig. 6C) [32]. A smaller increase, relative to Mn²⁺ stimulation, in the number of cells undergoing adhesion of 59.1 ± 5.9% was seen in the presence of the weak agonist ADP. Importantly, lotrafiban (30 μM), which acts simultaneously as an αIIb/β3 and α5β1 antagonist, eliminates binding of Mn²⁺ stimulated αIIb–/– murine platelets to the fibrinogen surface (Fig. 6D). These results demonstrate that the presence of the activated form of the α5β1 integrin is sufficient to support platelet binding to fibrinogen, but that it is incapable of forming lamellipodia in the absence of the αIIb/β3 complex.

Fig. 5. Murine platelets lacking the αIIb/β3 complex fail to spread on fibronectin. Purified wild type murine platelets in the (A) absence or (B) presence of ADP (10 μM) and αIIb/– murine platelets in the (C) absence or (D) presence of ADP (10 μM) were layered on a fibronectin-coated slide for 45 min at 37°C. Slides were then stained with rhodamine-phalloidin and imaged by fluorescence microscopy. One experiment representative of 3 is depicted. Bar = 10 μm.

Fig. 6. Exogenous stimulation of the platelet integrin α5β1 supports binding but not spreading to fibrinogen in the absence of αIIb/β3. Purified (A) wild type murine platelets or (B) αIIb/– murine platelets were layered on a fibrinogen-coated slide for 45 min at 37°C. αIIb/– murine platelets were treated with 5 mM Mn²⁺ in the (C) absence or (D) presence of αIIbβ3 antagonist lotrafiban (30 μM). Slides were then stained with rhodamine-phalloidin and imaged by fluorescence microscopy. One experiment representative of 3 is depicted. Bar = 10 μm.
Regulation of FAK and PLCγ2 activity in platelets on fibronectin

It is well established that platelet spreading on fibrinogen mediates αIIbβ3-dependent tyrosine phosphorylation and that this is enhanced by actin polymerization [16,17]. Experiments were designed to determine whether the interaction of fibronectin with αIIbβ3 and α5β1 induces a similar set of signaling events. For these studies, purified platelets were allowed to attach and spread on fibronectin or recombinant proteins consisting of either the single 10FIII domain or the 9–10FIII domain pair. The actions of ADP and thromboxanes were eliminated in these experiments through the incorporation of specific inhibitors. Platelets suspended over BSA-coated dishes were used as a control. Phosphorylation was assessed in whole cell lysates and following immunoprecipitation of specific proteins by Western blotting with antiphosphotyrosine antibodies. Our data shows that platelet spreading on fibronectin was associated with an increase in tyrosine phosphorylation. Furthermore, 10FIII stimulates a similar pattern of tyrosine phosphorylation, which is further increased when this fragment is complexed with 9FIII (Fig. 7A). A similar set of observations was made for tyrosine phosphorylation of FAK and PLCγ2. Immunoprecipitation of these two proteins and Western blotting for tyrosine phosphorylation demonstrated that, while FAK and PLCγ2 are tyrosine phosphorylated by 10FIII and 9–10FIII (Fig. 7B–C), there is a decrease in magnitude of tyrosine phosphorylation in 10FIII samples of 58 ± 4% and 79 ± 7%, respectively, as assessed via densitometry. Tyrosine phosphorylation of FAK and PLCγ2 was abrogated in the presence of a concentration of lotrafiban (10 μM) that causes complete blockade of the αIIbβ3 complex but does not prevent binding to α5β1 (Fig. 7D). Taken together, our data indicate that αIIbβ3 binding to fibronectin promotes tyrosine phosphorylation independent of α5β1 integrin involvement.

Spreading of platelets on fibronectin in plasma

This study, in common with many others in the literature, has utilized washed platelets in order to examine the effects of proteins on their own and to facilitate measurement of phosphorylation. Physiologically however, adhesion and spreading occur in the presence of plasma proteins, which have the potential to influence these processes. In order to investigate this, we layered platelet-rich plasma over surface-immobilized fibronectin and compared the results to those obtained with washed platelets. In contrast to studies using washed platelets, there was minimal adhesion to fibronectin or BSA in plasma, and the very few platelets that did adhere failed to undergo spreading (Fig. 8A–B). Washed platelets resuspended in platelet-poor plasma also failed to attach or spread on the fibronectin surface demonstrating that the difference in the two conditions was not due to the method of platelet isolation (data not shown). Stimulation of platelet-rich-plasma with the GPVI-specific agonist collagen-related peptide (CRP, 0.2 μg mL⁻¹) resulted in a dramatic increase in both the number of bound platelets and the degree of platelet spreading (Fig. 8C). These platelets exhibited a time-dependent increase in platelet surface area similar to that observed for washed platelets (data not shown).

Fig. 7. Fibronectin induced tyrosine phosphorylation of platelets. Human washed platelets were placed in dishes coated with BSA or fibronectin (FN), 9–10FIII or 10FIII in the presence of apyrase and indomethacin and absence or presence of lotrafiban (10 μM LOT) for 45 min at 37°C. Dishes coated with fibronectin were washed twice to remove non-adherent cells. Platelets adherent to the immobilized proteins or in suspension over BSA were lysed in ice-cold immunoprecipitation buffer and used directly for SDS-PAGE or subjected to immunoprecipitation for FAK or PLCγ2 and immunoblotted for tyrosine phosphorylated proteins. Samples were adjusted prior to immunoprecipitations so that there were similar amounts of protein in each group. Results are representative of 3–5 experiments.

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Exogenously Added Plasma Proteins

Fig. 8. Platelets in plasma proteins require external stimulation to spread on fibronectin. Human platelets in PRP were layered onto a (A) BSA- or a fibronectin-coated slide for 45 min at 37°C in the (B) absence or (C) presence of the GPVI-specific agonist CRP (0.2 μg mL⁻¹). In selected experiments, PRP was pretreated with the (D) αIIbβ3 antagonist lotrafiban (10 μM). Slides were then stained with rhodamine-phalloidin and imaged by fluorescence microscopy. In separate experiments, washed platelets were resuspended in buffer containing fibrinogen (3 mg mL⁻¹) and imaged using Nomarski differential interference contrast microscopy. One experiment representative of 3 is depicted. Bar = 10 μm.

Similar results were observed using sodium citrate/ACD, PPACK, a selective thrombin inhibitor, or heparin (not shown) as anticoagulant (Table 3 and not shown). These results are in accordance with reports indicating that the affinity of integrins for fibronectin is up-regulated upon stimulation [33].

CRP-induced platelet spreading on fibronectin was solely dependent upon the αIIbβ3 complex, as evidenced by the absence of spreading in the presence of the αIIbβ3 blocker lotrafiban (10 μM; Fig. 8D). However, CRP-stimulated platelets retained their ability to bind to the fibronectin surface in the presence of an αIIbβ3/α5β1 antagonist, consistent with a role for α5β1 in mediating adhesion.

In an attempt to delineate the potential inhibitory role of soluble adhesive proteins present in plasma, which might account for the absence of adhesion in plasma by virtue of their ability to bind to platelet integrins in an active conformation (see Discussion), we resuspended human washed platelets in buffer containing physiological concentrations of the three major components of plasma: fibrinogen (3 mg mL⁻¹), fibronectin (300 μg mL⁻¹) and VWF (10 μg mL⁻¹). Similar to studies using PRP, there was minimal adhesion of washed platelets to immobilized fibronectin in the presence of exogenously added plasma proteins (Fig. 8E). However, an increase in both the number of bound platelets and the degree of platelet spreading was observed upon stimulation with CRP (Fig. 8F). Platelet adhesive interactions were unaltered by the presence of similar levels of a non-adhesive protein (3.3 mg mL⁻¹ BSA; data not shown).

**Discussion**

In this study, we demonstrate that engagement of αIIbβ3 by fibronectin is sufficient to mediate platelet adhesion and subsequent development of stable filopodia and lamellipodia. This result is in accordance with the paradigm that ligand binding to αIIbβ3 triggers an ‘outside-in’ signaling cascade that supports platelet spreading [6,16,17]. Additionally, our data confirm the involvement of a second platelet surface receptor, α5β1, in mediating platelet-fibronectin adhesion [9]. In both cases, adhesion to immobilized fibronectin via αIIbβ3 or α5β1 was achieved in the absence of external stimulation of cellular activation and in the presence of inhibitors of the major platelet feedback agonists, ADP and thromboxane A2, suggesting that it is mediated through binding to the low affinity conformations of the two integrins or to a small population of ‘activated’ integrin. We favor the latter of these two explanations in view of the observation that plasma concentrations of the αIIbβ3 ligands, fibrinogen, VWF and fibronectin, block adhesion of washed platelets to immobilized ligands. These observations therefore demonstrate that fibronectin engagement of integrin αIIbβ3 in resting platelets leads to outside-in signaling independent of external stimuli [16,30] and that adhesion is reinforced through binding to α5β1 [9,34].

Our data show that in the absence of the αIIbβ3 complex, α5β1 and α5β3 are unable to support formation of lamellipodia. It is important to consider whether this reflects the inability of

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Anticoagulant</th>
<th>Treatment</th>
<th>Adherent platelets per mm² (×10⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>NaCit/ACD</td>
<td>–</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td>FN</td>
<td>NaCit/ACD</td>
<td>–</td>
<td>0.8 ± 1.3</td>
</tr>
<tr>
<td>FN</td>
<td>NaCit/ACD</td>
<td>CRP</td>
<td>46.6 ± 5.4</td>
</tr>
<tr>
<td>BSA</td>
<td>PPACK</td>
<td>–</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>FN</td>
<td>PPACK</td>
<td>–</td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td>FN</td>
<td>PPACK</td>
<td>CRP</td>
<td>55.6 ± 3.5</td>
</tr>
</tbody>
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Platelets (3 × 10⁷ mL⁻¹) in PRP anticoagulated with either sodium citrate and acid/citrate/dextrose (NaCit/ACD) or PPACK were layered on a BSA- or fibronectin-coated slide for 45 min in the absence or presence of CRP (0.2 μg mL⁻¹). The number of adherent platelets was recorded for five fields of view (7550 μm²). Values are mean ± SEM of three experiments.
ζβ1 and αβ3 to initiate a signaling response or the low level of expression of the integrin giving rise to a weak intracellular signal. Upon contact with the fibronectin surface, platelets begin to extend filopodia. However, in the presence of an αIIBβ3 antagonist, these protrusions are unsuccessful in their attempt to anchor to the surface and are subsequently retracted, as demonstrated in Fig. 2(F). This dynamic extension/retraction process demonstrates that αIIBβ3-blocked platelets retain their ability to undergo cytoskeletal rearrangements, suggesting a limited degree of intracellular signaling. However, this is insufficient to generate lamellipodia, robust protein tyrosine phosphorylation or a rise in intracellular Ca2+.

The accepted model for integrin mediated signaling is based on integrin clustering and conformational changes leading to an increase in avidity/affinity for the ligand. In turn, ligand engagement initiates a complex network of signaling and structural cytoskeletal proteins leads to outside-in signaling, marked by an increase in tyrosine phosphorylation. In platelets, this process has been thoroughly documented for αIIBβ3 engagement by fibrinogen [2,6]. Our data demonstrate that formation of lamellipodia on fibronectin, or its active domains 10FIII or 9–10FIII, is associated with signaling events that have been attributed to αIIBβ3, including tyrosine phosphorylation of FAK and PLCγ2. Further, inhibitor studies confirmed that they are mediated through an αIIBβ3-dependent, but αβ3-independent pathway. The absence of tyrosine phosphorylation of PLCγ2 by αβ3, combined with the observation that PLCγ2αIIBβ3−/− platelets retain the ability to form filopodia clearly demonstrates that the dynamic process of platelet filopodia formation is independent of activation of this phospholipase. This conclusion is corroborated by the lack of an increase in intracellular Ca2+ in the absence of αIIBβ3 engagement.

The data further support the notion that the information needed for platelets to adhere to fibronectin resides in the 120-kDa central cell-binding domain, which contains 9–10FIII [35]. Interestingly, while platelet spreading was unaltered by the presence of 9FIII, incorporation of this fragment with 10FIII yielded an increase in tyrosine phosphorylation as compared to 10FIII alone. This suggests that tyrosine phosphorylation is not rate-determining in mediating the extent of spreading and adhesion on fibronectin. The differences between 9FIII and 10FIII is thought to arise from differences in affinity of the ligands for the receptor, whereby 10FIII binds to the receptor with low affinity compared to 9–10FIII which binds to the receptor with high affinity [26,36]. Although this phenomenon might not have a direct effect on the pattern or degree of platelet spreading under static conditions, an increase in receptor affinity may have increased relevance in the physiological setting whereby platelet adhesion occurs in the presence of shear forces [37]. Along these lines, it has been suggested that enhanced platelet affinity for fibronectin is requisite for the formation of stable thrombi in vivo [10].

Under physiological conditions, platelet–ligand interactions occur in the presence of soluble plasma proteins. However, many of the studies in the literature have utilized washed platelets. Our data demonstrate that both PRP and washed platelets resuspended in purified plasma proteins fail to spread on fibronectin in the absence of external stimulation, suggesting that the presence of soluble proteins in the plasma has the ability to prevent platelets from binding and spreading. This is likely to be a consequence of the high concentrations of integrin-ligands present in the plasma [38], which effectively out compete integrin engagement of surface-immobilized proteins [39]. Support for this is provided by the observation that addition of soluble fibronectin, fibrinogen and VWF completely block adhesion of washed platelets on fibronectin. Importantly, modulation of the affinity conformation of integrins via GPVI-specific leads to adhesion and spreading of platelets in plasma, presumably through integrin activation and possibly an increase in the level of platelet surface glycoproteins which are present in intracellular granules [40,41]. Significantly, the difference between plasma and washed conditions is unlikely to be due to integrin activation during the preparation of washed platelets since adhesion was seen in the presence of ADP and thromboxane inhibitors or upon resuspension of washed platelets in purified plasma. We therefore propose that a small proportion of the integrins are in an active conformation, but are dynamically bound to soluble fibrinogen and fibronectin in plasma, thereby preventing interaction with immobilized ligand. The role of plasma proteins in influencing platelet–ligand interactions may provide a degree of clarification for differences in the adhesive properties of washed platelets that has been reported in the literature [16,24,30,33].

In summary, this study demonstrates that while αβ3, αβ3 and αIIBβ3 are able to support adhesion of washed platelets to fibronectin, only αIIBβ3 is able to mediate formation of lamellipodia. This may reflect a difference in outside-in signaling from the integrin and/or the level of expression. The study also shows that αIIBβ3-dependent adhesion in plasma requires prior stimulation, demonstrating that plasma provides an anti-adhesive medium, which prevents adhesion in the absence of stimulating agonists such as GPVI.

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