Distinct but critical roles for integrin $\alpha_{I\beta}3$ in platelet lamellipodia formation on fibrinogen, collagen-related peptide and thrombin

Kelly Thornber¹, Owen J. T. McCarty²,³, Steve P. Watson² and Catherine J. Pears¹

1 Department of Biochemistry, University of Oxford, UK
2 Centre for Cardiovascular Sciences, Institute of Biomedical Research, University of Birmingham, UK
3 Department of Biomedical Engineering, Oregon Health & Science University, Portland, OR, USA

Platelets play an essential role in the formation of a haemostatic plug at the site of vascular injury. This process requires adhesion of the platelet to the exposed subendothelial matrix, followed by powerful intracellular signalling events that lead to platelet–platelet interactions and thrombus formation. One critical feature in this scheme is the dramatic alteration in platelet morphology in response to activation. Thus, the resting, discoid platelet undergoes a series of morphological changes that include rounding, generation of filopodia and lamellipodia, and formation of actin stress fibres [1]. These events serve to stabilize the thrombus, thereby enabling it to withstand the high shear forces found in arteries and arterioles.

Stable adhesion of platelets to the subendothelial matrix is dependent upon sustained activation of integrins [2]. Integrins are glycoprotein heterodimers

1. Integrins are the major receptor type known to facilitate cell adhesion and lamellipodia formation on extracellular matrix proteins. However, collagen-related peptide and thrombin have recently been shown to mediate platelet lamellipodia formation when presented as immobilized surfaces. The aims of this study were to establish if there exists a role for the platelet integrin $\alpha_{I\beta}3$ in this response; and if so, whether signalling from the integrin is required for lamellipodia formation on these surfaces. Real-time analysis was used to compare platelet morphological changes on surfaces of fibrinogen, collagen-related peptide or thrombin in the presence of various pharmacological inhibitors and platelets from ‘knockout’ mice. We demonstrate that collagen-related peptide and thrombin stimulate distinct patterns of platelet lamellipodia formation and elevation of intracellular $\text{Ca}^{2+}$ compared to that induced by the integrin $\alpha_{I\beta}3$ ligand, fibrinogen. Nevertheless, lamellipodia formation on collagen-related peptide and thrombin is dependent upon engagement of $\alpha_{I\beta}3$, consistent with release of $\alpha_{I\beta}3$ ligand(s) from platelet granules. However, the requirement for signalling by the integrin on fibrinogen can be bypassed by the addition of thrombin to the solution. These observations reveal a critical role for $\alpha_{I\beta}3$ in forming lamellipodia on collagen-related peptide and thrombin which is dependent on its ability to function as an adhesive receptor but not necessarily on its ability to signal. These results suggest that integrins may play an important role in lamellipodia formation triggered by nonintegrin ligands in platelets and possibly in other cell types.

Abbreviations

CRP, collagen-related peptide.
composed of α-subunits and β-subunits that exist in an inactive or low-affinity conformation in nonactivated cells. Intracellular signals within the platelet (known as ‘inside-out’ signalling) promote a conformational change in the extracellular domain, leading to an increase in affinity, thereby promoting integrin–ligand interactions [2]. In turn, clustering of integrins generates a series of intracellular signals (‘outside-in’ signalling) that serve to reinforce platelet activation [3].

The major platelet integrin αIIbβ3 is a receptor for fibrinogen, von Willebrand factor, vitronectin, CD40 ligand and fibronectin. Integrin αIIbβ3 plays a vital role in supporting platelet adhesion to the extracellular matrix and promoting platelet–platelet interaction (aggregation). In addition, integrin αIIbβ3 generates outside-in signals that mediate platelet activation. It is now established that engagement of αIIbβ3 activates Src family kinases, leading to activation of Syk [4], SLP-76 [5], Vav1/3 [4] and phospholipase Cγ2 (PLCγ2) [6], and thereby to activation of several second messenger pathways, including protein kinase C [7] and Ca2+ [8]. These signalling events promote actin assembly, leading to formation of filopodia, lamellipodia and stress fibres [9].

In addition to integrin ligands, formation of filopodia and lamellipodia has been described on a monolayer of collagen-related peptide (CRP), which selectively activates the immunoglobulin receptor glycoprotein VI (GPVI) [10,11], and on thrombin that has been immobilized by fibrin. In platelets, thrombin binds to and signals via GPIbα and the G protein-coupled protease-activated receptor (PAR) receptors (PAR 1 and 4) [12,13]. Immobilized thrombin that has become trapped by fibrin is able to promote platelet adhesion and aggregate formation at intermediate rates of flow, leading to the speculation that it may function as an adhesive ligand in vivo [13]. In epithelial cells, it has been suggested that immobilized thrombin can bind to integrins through an RGD site, raising the possibility that it may bind directly to integrins [14].

The present study was undertaken to investigate the mechanism by which CRP and thrombin are able to support platelet lamellipodia formation in comparison to that induced by fibrinogen. It was of particular interest to discern whether thrombin and CRP stimulate lamellipodia formation directly, or whether they require αIIbβ3. The results demonstrate a critical role for αIIbβ3 in promoting platelet lamellipodia formation on CRP, thrombin and on fibrinogen, but that outside-in signalling by the integrin is not required for lamellipodia formation on fibrinogen in the presence of thrombin. These results further emphasize the importance of integrin engagement in lamellipodia formation but demonstrate that signalling by αIIbβ3 is not essential for this response.

**Results**

**Morphological changes of human platelets on fibrinogen, CRP and thrombin**

The three ligands fibrinogen, CRP and thrombin support platelet adhesion and lamellipodia formation when presented as a monolayer, even though they bind to distinct classes of surface receptor. This raises the question of the molecular basis of adhesion to these ligands and whether they induce distinct patterns of change in morphology. To address this, real-time imaging of platelets adhering to each surface was undertaken using a coating of ligands that induces maximal platelet adhesion. These experiments were performed in the presence of concentrations of apyrase and indomethacin shown to fully block the effect of the feedback agonists ADP and thromboxane A2, respectively, in order to directly monitor the ability of each ligand to support adhesion and lamellipodia formation.

Consistent with previous reports, our results demonstrate that platelets exposed to immobilized fibrinogen go through sequential formation of filopodia and lamellipodia over a period of 30 min (Fig. 1 and supplementary Video S1). Both structures were stable and did not retract once formed, although discrete movements could still be seen around the periphery of the cell. Fluorescent labelling of the actin cytoskeleton revealed that stress fibres were formed within platelets that had undergone full lamellipodia formation (Fig. 1B). In contrast, a distinct pattern of platelet morphological changes was observed on the GPVI-specific agonist CRP (Fig. 1A and supplementary Video S2). Limited small filopodia were seen, with wave-like lamellipodia appearing before filopodia formation was complete, in contrast to the spherically synchronized growth on fibrinogen. Full lamellipodia formation was reached within 5–9 min, three times more rapidly than on fibrinogen (Fig. 1C). Strikingly, even after platelets had reached 90% of their final surface area, the lamellipodia were very dynamic (supplementary Video S2). Limited small filopodia were seen, with wave-like lamellipodia appearing before filopodia formation was complete, in contrast to the spherically synchronized growth on fibrinogen. Full lamellipodia formation was reached within 5–9 min, three times more rapidly than on fibrinogen (Fig. 1C). Strikingly, even after platelets had reached 90% of their final surface area, the lamellipodia were very dynamic (supplementary Video S2), even though they were accompanied by formation of stress fibres (Fig. 1B). A similar pattern of rapid yet unstable lamellipodia formation and stress fibre formation was observed for platelets adhering to immobilized thrombin (Fig. 1A,B and supplementary Video S3). Significantly, lamellipodia formation on thrombin was not altered in the presence of the fibrin polymerization inhibitor Gly-Pro-Arg-Pro (GPRP) (data
not shown), demonstrating that this response was not dependent upon fibrin formation.

These results demonstrate that a distinct pattern of morphological change is induced by platelet adhesion to CRP and thrombin, compared to that seen on fibrinogen.

**Different Ca^{2+} mobilization patterns in platelets exposed to fibrinogen, CRP and thrombin**

Experiments were undertaken to investigate whether the distinct pattern of lamellipodia formation on the three ligands is associated with differences in the
The above observations demonstrate a critical role for $\alpha_{\text{IIb}}\beta_3$ in lamellipodia formation on CRP and thrombin. This could be due to a role of $\alpha_{\text{IIb}}\beta_3$ in supporting adhesive events or in generating intracellular signals that drive formation of lamellipodia. It is difficult to distinguish between these two possibilities in the case of CRP, because of the similarity in the signalling pathways used by GPVI and integrin $\alpha_{\text{IIb}}\beta_3$, both of which are mediated by sequential activation of Src and Syk family kinases and subsequent activation of PLC$\gamma$2 [6]. In contrast, thrombin signals through a G protein-dependent pathway and induces a full repertoire of platelet responses in the presence of the Src family kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2). Experiments were therefore designed to investigate the effect of PP2 on the ability of thrombin to promote lamellipodia formation during platelet adhesion.

As previously reported, the Src kinase inhibitor PP2 blocked formation of lamellipodia on fibrinogen [17] (Fig. 4), with average platelet surface area being significantly reduced from $26.3 \pm 0.9$ to $17.6 \pm 0.7 \mu m^2$. 

**Thrombin can bypass $\alpha_{\text{IIb}}\beta_3$ outside-in signalling in mediating lamellipodia formation**

The above observations demonstrate a critical role for $\alpha_{\text{IIb}}\beta_3$ in lamellipodia formation on CRP and thrombin. This could be due to a role of $\alpha_{\text{IIb}}\beta_3$ in supporting adhesive events or in generating intracellular signals that drive formation of lamellipodia. It is difficult to distinguish between these two possibilities in the case of CRP, because of the similarity in the signalling pathways used by GPVI and integrin $\alpha_{\text{IIb}}\beta_3$, both of which are mediated by sequential activation of Src and Syk family kinases and subsequent activation of PLC$\gamma$2 [6]. In contrast, thrombin signals through a G protein-dependent pathway and induces a full repertoire of platelet responses in the presence of the Src family kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2). Experiments were therefore designed to investigate the effect of PP2 on the ability of thrombin to promote lamellipodia formation during platelet adhesion.

As previously reported, the Src kinase inhibitor PP2 blocked formation of lamellipodia on fibrinogen [17] (Fig. 4), with average platelet surface area being significantly reduced from $26.3 \pm 0.9$ to $17.6 \pm 0.7 \mu m^2$. 

**$\alpha_{\text{IIb}}\beta_3$ is required for platelet lamellipodia formation on fibrinogen, CRP and thrombin**

Although the above results demonstrate marked differences between the three ligands in the pattern of Ca$^{2+}$ mobilization, which presumably reflect the differing signalling strengths of their receptors, it is possible that adhesion and lamellipodia formation are mediated through integrin activation, in view of the critical role of integrins in mediating adhesion and cell spreading. To address this, experiments were designed to compare the role of the major platelet integrin $\alpha_{\text{IIb}}\beta_3$, in supporting spreading on the three surfaces, using the $\alpha_{\text{IIb}}\beta_3$ antagonist lotrafiban [15,16].

As expected, lotrafiban abrogated platelet adhesion to fibrinogen (Fig. 2B), but, importantly, dramatically reduced lamellipodia formation on CRP and thrombin surfaces, although it also increased the level of adhesion (Table 1). These findings demonstrate that despite the distinct morphological changes on CRP and thrombin in comparison to those on fibrinogen, the generation of lamellipodia is dependent upon engagement of $\alpha_{\text{IIb}}\beta_3$ in all cases. This is most likely explained by release of fibrinogen, von Willebrand factor and other $\alpha_{\text{IIb}}\beta_3$ ligands from platelet $\alpha$-granules. Consistent with this, secretion of platelet $\alpha$-granules in platelets that had adhered to CRP and thrombin was confirmed by immunofluorescence staining for the $\alpha$-granule marker P-selectin, on the surface of adhered platelets (Fig. 3).

These results demonstrate that formation of lamellipodia on CRP, thrombin and fibrinogen is dependent on engagement of integrin $\alpha_{\text{IIb}}\beta_3$ as a consequence of release of $\alpha_{\text{IIb}}\beta_3$ ligands from platelet $\alpha$-granules.

**Platelet lamellipodia formation via $\alpha_{\text{IIb}}\beta_3$**

The above observations demonstrate a critical role for $\alpha_{\text{IIb}}\beta_3$ in lamellipodia formation on CRP and thrombin. This could be due to a role of $\alpha_{\text{IIb}}\beta_3$ in supporting adhesive events or in generating intracellular signals that drive formation of lamellipodia. It is difficult to distinguish between these two possibilities in the case of CRP, because of the similarity in the signalling pathways used by GPVI and integrin $\alpha_{\text{IIb}}\beta_3$, both of which are mediated by sequential activation of Src and Syk family kinases and subsequent activation of PLC$\gamma$2 [6]. In contrast, thrombin signals through a G protein-dependent pathway and induces a full repertoire of platelet responses in the presence of the Src family kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2). Experiments were therefore designed to investigate the effect of PP2 on the ability of thrombin to promote lamellipodia formation during platelet adhesion.

As previously reported, the Src kinase inhibitor PP2 blocked formation of lamellipodia on fibrinogen [17] (Fig. 4), with average platelet surface area being significantly reduced from $26.3 \pm 0.9$ to $17.6 \pm 0.7 \mu m^2$. 

**$\alpha_{\text{IIb}}\beta_3$ is required for platelet lamellipodia formation on fibrinogen, CRP and thrombin**

Although the above results demonstrate marked differences between the three ligands in the pattern of Ca$^{2+}$ mobilization, which presumably reflect the differing signalling strengths of their receptors, it is possible that adhesion and lamellipodia formation are mediated through integrin activation, in view of the critical role of integrins in mediating adhesion and cell spreading. To address this, experiments were designed to compare the role of the major platelet integrin $\alpha_{\text{IIb}}\beta_3$, in supporting spreading on the three surfaces, using the $\alpha_{\text{IIb}}\beta_3$ antagonist lotrafiban [15,16].

As expected, lotrafiban abrogated platelet adhesion to fibrinogen (Fig. 2B), but, importantly, dramatically reduced lamellipodia formation on CRP and thrombin surfaces, although it also increased the level of adhesion (Table 1). These findings demonstrate that despite the distinct morphological changes on CRP and thrombin in comparison to those on fibrinogen, the generation of lamellipodia is dependent upon engagement of $\alpha_{\text{IIb}}\beta_3$ in all cases. This is most likely explained by release of fibrinogen, von Willebrand factor and other $\alpha_{\text{IIb}}\beta_3$ ligands from platelet $\alpha$-granules. Consistent with this, secretion of platelet $\alpha$-granules in platelets that had adhered to CRP and thrombin was confirmed by immunofluorescence staining for the $\alpha$-granule marker P-selectin, on the surface of adhered platelets (Fig. 3).

These results demonstrate that formation of lamellipodia on CRP, thrombin and fibrinogen is dependent on engagement of integrin $\alpha_{\text{IIb}}\beta_3$ as a consequence of release of $\alpha_{\text{IIb}}\beta_3$ ligands from platelet $\alpha$-granules.

**Thrombin can bypass $\alpha_{\text{IIb}}\beta_3$ outside-in signalling in mediating lamellipodia formation**

The above observations demonstrate a critical role for $\alpha_{\text{IIb}}\beta_3$ in lamellipodia formation on CRP and thrombin. This could be due to a role of $\alpha_{\text{IIb}}\beta_3$ in supporting adhesive events or in generating intracellular signals that drive formation of lamellipodia. It is difficult to distinguish between these two possibilities in the case of CRP, because of the similarity in the signalling pathways used by GPVI and integrin $\alpha_{\text{IIb}}\beta_3$, both of which are mediated by sequential activation of Src and Syk family kinases and subsequent activation of PLC$\gamma$2 [6]. In contrast, thrombin signals through a G protein-dependent pathway and induces a full repertoire of platelet responses in the presence of the Src family kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2). Experiments were therefore designed to investigate the effect of PP2 on the ability of thrombin to promote lamellipodia formation during platelet adhesion.

As previously reported, the Src kinase inhibitor PP2 blocked formation of lamellipodia on fibrinogen [17] (Fig. 4), with average platelet surface area being significantly reduced from $26.3 \pm 0.9$ to $17.6 \pm 0.7 \mu m^2$. 

**$\alpha_{\text{IIb}}\beta_3$ is required for platelet lamellipodia formation on fibrinogen, CRP and thrombin**

Although the above results demonstrate marked differences between the three ligands in the pattern of Ca$^{2+}$ mobilization, which presumably reflect the differing signalling strengths of their receptors, it is possible that adhesion and lamellipodia formation are mediated through integrin activation, in view of the critical role of integrins in mediating adhesion and cell spreading. To address this, experiments were designed to compare the role of the major platelet integrin $\alpha_{\text{IIb}}\beta_3$, in supporting spreading on the three surfaces, using the $\alpha_{\text{IIb}}\beta_3$ antagonist lotrafiban [15,16].

As expected, lotrafiban abrogated platelet adhesion to fibrinogen (Fig. 2B), but, importantly, dramatically reduced lamellipodia formation on CRP and thrombin surfaces, although it also increased the level of adhesion (Table 1). These findings demonstrate that despite the distinct morphological changes on CRP and thrombin in comparison to those on fibrinogen, the generation of lamellipodia is dependent upon engagement of $\alpha_{\text{IIb}}\beta_3$ in all cases. This is most likely explained by release of fibrinogen, von Willebrand factor and other $\alpha_{\text{IIb}}\beta_3$ ligands from platelet $\alpha$-granules. Consistent with this, secretion of platelet $\alpha$-granules in platelets that had adhered to CRP and thrombin was confirmed by immunofluorescence staining for the $\alpha$-granule marker P-selectin, on the surface of adhered platelets (Fig. 3).

These results demonstrate that formation of lamellipodia on CRP, thrombin and fibrinogen is dependent on engagement of integrin $\alpha_{\text{IIb}}\beta_3$ as a consequence of release of $\alpha_{\text{IIb}}\beta_3$ ligands from platelet $\alpha$-granules.
Fig. 2. Different Ca\textsuperscript{2+} signalling patterns on each surface. Washed human platelets were exposed to surfaces of fibrinogen (FG), collagen-related peptide (CRP) or thrombin (THR). (A) Platelets were loaded with Oregon Green-BAPTA 1-AM calcium dye and exposed to each surface for 10 min. The fluorescent Ca\textsuperscript{2+} fluctuations of three single platelets on each surface are shown in the presence (+) or absence (−) of BAPTA-AM (10 μM, added 10 min prior to surface exposure). Representative traces from three individual platelets are shown. (B) Platelets were exposed to each surface for 45 min in the absence or presence of BAPTA-AM (10 μM, added 10 min prior to surface exposure) or the α\textsubscript{IIb}β\textsubscript{3} antagonist lotrafiban (10 μM, 10 min). Images shown are representative of at least three independent experiments. Adhesion and surface area data from these experiments are shown in Table 1.
In contrast, adhesion was not significantly altered. Importantly, the inhibitory effect of PP2 on lamellipodia formation could be completely overcome by addition of thrombin (Fig. 4), which induced a larger increase in surface area to that induced by fibrinogen (37.5 ± 1.5 μm²), most likely reflecting the increased signalling strength of the platelet thrombin receptors PAR1 and PAR4. A similar effect was seen with the PAR1-specific peptide, thrombin receptor activating peptide [TRAP (data not shown)].

Table 1. Human platelet data. See Fig. 2 for experimental details. Values are reported as follows: adherent platelets = mean ± SEM of three experiments; platelet surface area = mean ± SEM of at least 100 cells. On a control surface of BSA, adhesion and surface area data are 1.5 ± 0.1 x 10² mm⁻² and 7.3 ± 0.2 μm², respectively.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Platelet surface area (μm²)</th>
<th>Adherent platelets/mm² (x 10⁴)</th>
<th>Adherent platelets/mm² (x 10⁴)</th>
<th>Platelet surface area (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>57.6 ± 0.9</td>
<td>26.3 ± 0.9</td>
<td>65.7 ± 0.9</td>
<td>35.0 ± 1.1</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>57.2 ± 0.6</td>
<td>17.7 ± 0.6*</td>
<td>50.5 ± 0.6</td>
<td>22.3 ± 1.2*</td>
</tr>
<tr>
<td>Lotrafiban</td>
<td>3.3 ± 0.4a</td>
<td>9.3 ± 0.4*</td>
<td>95.1 ± 1.3*</td>
<td>16.7 ± 0.5*</td>
</tr>
</tbody>
</table>

*P < 0.05 with respect to untreated samples for each surface.

Fig. 3. P-Selectin exposure on collagen-related peptide (CRP) and thrombin. Washed human platelets were exposed to surfaces of CRP or thrombin (THR) for 45 min before fixing and staining with fluorescein isothiocyanate (FITC)-conjugated anti-P-selectin serum. Cells were imaged using differential interference contrast (DIC) or fluorescence (P-selectin). Images shown are representative of at least three independent experiments.

Fig. 4. Thrombin can overcome Src kinase inhibition of lamellipodia on fibrinogen. Washed human platelets were exposed to a surface of fibrinogen for 45 min in the absence or presence of the Src kinase inhibitor PP2 (20 μM, added 5 min prior to surface exposure) and/or thrombin (1 U·mL⁻¹, 1 min) in suspension (+ THR). Images shown are representative of at least three independent experiments.
These results demonstrate that thrombin is able to stimulate formation of lamellipodia on fibrinogen in the absence of signalling downstream of the integrin. Under these circumstances, therefore, the integrin is functioning solely as an adhesive receptor.

Role of $\alpha_{IIb}\beta_3$ and PLC$\gamma_2$ in adhesion and lamellipodia formation of murine platelets

Experiments were undertaken in mouse platelets to confirm the role of integrin $\alpha_{IIb}\beta_3$ in mediating lamellipodia formation on fibrinogen, CRP and thrombin, and to establish the importance of PLC$\gamma_2$ activation in this response. These experiments served to extend the observations made in human to mice platelets and also to provide an alternative line of evidence to support the results obtained above through the use of platelets deficient in the $\alpha_{IIb}$ integrin subunit and PLC$\gamma_2$.

As previously documented [9,18,19], and in contrast to human platelets, mouse platelets generate only filopodia and limited lamellipodia on fibrinogen in the presence of apyrase and indomethacin (Fig. 4A). In contrast, robust lamellipodia formation in mouse platelets is observed on CRP or thrombin surfaces. Adhesion and lamellipodia formation on fibrinogen were abrogated in the presence of the $\alpha_{IIb}\beta_3$ antagonist lotrafiban (Fig. 5A, Table 2) and in mice lacking the $\alpha_{IIb}$ gene (Fig. 5A, Table 2). Similarly, adhesion of mouse platelets to CRP was fully blocked in the presence of lotrafiban or in the absence of $\alpha_{IIb}$ (Fig. 5A, Table 2), demonstrating that adhesion on CRP is critically dependent on functional $\alpha_{IIb}\beta_3$, presumably as a consequence of secretion of fibrinogen and other $\alpha_{IIb}\beta_3$ ligands. In contrast, $\alpha_{IIb}\beta_3$-blocked and $\alpha_{IIb}^{-/-}$ mouse platelets retained the ability to adhere to immobilized thrombin, although lamellipodia formation was eliminated (Fig. 5A, Table 2).

The role of Src kinases and Ca$^{2+}$ mobilization in lamellipodia formation in mouse platelets on fibrinogen and CRP was investigated using platelets deficient in the major isoform of PLC$\gamma$ in platelets, PLC$\gamma_2$ [19,20]. Blockade of Src kinases with PP2 (data not shown) or the absence of PLC$\gamma_2$ led to inhibition of lamellipodia formation on fibrinogen, but had no effect on adhesion (Fig. 5B, Table 2), in agreement with previous observations [18,19]. The addition of thrombin in suspension could overcome this inhibitory effect, leading to extensive lamellipodia formation (Fig. 5, Table 2). In contrast, the absence of PLC$\gamma_2$ abrogated adhesion of mouse platelets to CRP (Fig. 5B), although adhesion and lamellipodia formation could be restored by addition of thrombin (Fig. 5, Table 2).

These experiments extend the observations on human platelets to mouse platelets, namely that integrin $\alpha_{IIb}\beta_3$ is necessary for platelet lamellipodia formation on CRP, thrombin and fibrinogen, but that thrombin is able to mediate lamellipodia formation in the absence of outside-in signalling from the integrin.

The roles of $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ in platelet lamellipodia formation on collagen

Experiments were designed to investigate whether the critical role of $\alpha_{IIb}\beta_3$ in mediating lamellipodia formation in human and mouse platelets on fibrinogen, CRP and thrombin also extends to a further platelet ligand, collagen, which binds directly to a second platelet integrin, $\alpha_2\beta_1$ as well as GPVI. This question was addressed by monitoring lamellipodia formation on collagen in human platelets in the presence of the $\alpha_{IIb}\beta_3$ antagonist lotrafiban and in mouse platelets deficient in the integrin subunit $\alpha_{IIb}$.

Human and mouse platelets generate filopodia and lamellipodia on fibrillar collagen (Fig. 6), and this is reduced by approximately 55% in human platelets in the presence of lotrafiban (28.7 ± 1.1 to 17.8 ± 0.5 μm$^2$, compared to 26.3 ± 0.9 to 9.3 ± 0.4 μm$^2$ on fibrinogen) and in $\alpha_{IIb}$-deficient mouse platelets (from 18.4 ± 0.6 to 11.3 ± 0.4 μm$^2$ in wild-type and $\alpha_{IIb}^{-/-}$-deficient platelets, respectively, compared with 11.1 ± 0.3 to 5.5 ± 0.5 μm$^2$ on fibrinogen). The degree of platelet adhesion, however, was not significantly altered by loss of $\alpha_{IIb}\beta_3$ function in either human or mouse platelets (not shown), presumably because it is mediated through integrin $\alpha_2\beta_1$.

These results demonstrate that $\alpha_{IIb}\beta_3$ contributes to lamellipodia formation on an integrin-binding ligand, namely collagen, but that, in its absence, limited lamellipodia formation is mediated by integrin $\alpha_2\beta_1$.

Discussion

It is well established that integrins play a critical role in lamellipodia formation in a wide variety of cell types, although it is unclear whether lamellipodia formation can also be induced by engagement of nonintegrin receptors. Nevertheless, it is well established that other cell surface receptors may facilitate cell adhesion and actin remodelling through integrin activation and stimulation of actin polymerization. An example of this is Syndecan-1, which has been shown to mediate lamellipodia formation in Raji lymphoblastoid cells independently of integrins [21], although other syndecans are thought to operate through interaction with neighbouring integrins [22]. More recently, two studies
have described lamellipodia formation in platelets upon adhesion to thrombin and the synthetic collagen CRP [10,12]. The present study investigated the molecular basis of lamellipodia formation in platelets on these two surfaces, alongside studies on fibrinogen, which mediates lamellipodia formation through integrin αIIbβ3. The results demonstrate a critical role for integrin αIIbβ3 in mediating lamellipodia formation on
all three surfaces, although each surface induces a distinct pattern of formation of filopodia and lamellipodia. A distinct pattern of lamellipodia formation on CRP was also reported by the Hartwig group [10].

The critical role of αIIbβ3 in mediating lamellipodia formation on CRP and thrombin is likely to be mediated by release of αIIbβ3 ligands from platelet α-granules, which become immobilized on the surface, enabling them to support lamellipodia formation. Moreover, secreted fibrinogen has been shown to be prebound to the platelet surface, which would therefore put it in the right place to support lamellipodia formation [23]. Thrombin may also directly support adhesion through an RDG motif that becomes exposed upon immobilization [14], although it must also bind to other receptors, as it cannot generate lamellipodia in the absence of functional αIIbβ3. Interestingly, blockade of αIIbβ3 caused an increase in platelet adhesion to CRP and thrombin. This can be explained by the reduction in lamellipodia formation and therefore the corresponding increase in available matrix area, and by the observations of Patel et al. that adhered platelets cause the lateral movement of depositing platelets away from themselves and on to the matrix below [24].

The above discussion indicates that the difference in the pattern of lamellipodia between fibrinogen, thrombin and CRP is likely to be due to their different signal

**Table 2.** Murine platelet data. See Fig. 5 for experimental details. Values are reported as follows: adherent platelets = mean ± SEM of three experiments; platelet surface area = mean ± SEM of at least 100 cells. On a control surface of BSA, adhesion and surface area data are 0.9 ± 0.1 x 10² mm⁻² and 4.6 ± 0.6 μm², respectively. THR, thrombin added in suspension; WT, wild-type; LOT, lotrafiban.

<table>
<thead>
<tr>
<th>Genotype/treatment</th>
<th>Fibrinogen</th>
<th>CRP</th>
<th>Thrombin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adherent platelets/ Platelet surface</td>
<td>Adherent platelets/ Platelet surface</td>
<td>Adherent platelets/ Platelet surface</td>
</tr>
<tr>
<td></td>
<td>mm² (x 10⁴) area (μm²)</td>
<td>mm² (x 10⁴) area (μm²)</td>
<td>mm² (x 10⁴) area (μm²)</td>
</tr>
<tr>
<td>WT/None</td>
<td>63.5 ± 0.9 11.1 ± 0.3</td>
<td>52.8 ± 0.9 17.9 ± 0.4</td>
<td>30.3 ± 0.3 15.7 ± 0.5</td>
</tr>
<tr>
<td>WT/LOT</td>
<td>1.1 ± 0.1ᵃ 5.5 ± 0.5ᵃ</td>
<td>5.7 ± 0.4ᵃ 6.5 ± 0.3ᵃ</td>
<td>15.4 ± 0.3ᵃ 5.5 ± 0.4ᵃ</td>
</tr>
<tr>
<td>αIIb⁻/⁻/None</td>
<td>2.4 ± 0.1ᵃ 4.4 ± 0.2ᵃ</td>
<td>4.3 ± 0.3ᵃ 3.7 ± 0.3ᵃ</td>
<td>22.6 ± 0.9ᵃ 5.5 ± 0.3ᵃ</td>
</tr>
<tr>
<td>PLCγ2⁻/⁻/None</td>
<td>62.0 ± 0.5 10.0 ± 0.4ᵃ</td>
<td>6.1 ± 0.3ᵃ 9.0 ± 0.5ᵃ</td>
<td></td>
</tr>
<tr>
<td>PLCγ2⁻/⁻/THR</td>
<td>51.7 ± 0.3ᵇ 18.1 ± 0.5ᵇ</td>
<td>36.8 ± 0.7ᵇ 16.6 ± 0.7ᵇᵇ</td>
<td></td>
</tr>
</tbody>
</table>

ᵃP < 0.05 with respect to untreated wild-type samples for each surface. ᵄP < 0.05 for samples treated with thrombin in suspension compared to the equivalent sample without thrombin.

**Fig. 6.** Lamellipodia formation on collagen in the absence of αIIbβ3. Human washed platelets in the absence or presence of the αIIbβ3 antagonist lotrafiban (10 μM, added 10 min prior to surface exposure), and murine platelets deficient in αIIb (αIIb⁻/⁻) or wild-type (WT) littermate controls were exposed to immobilized collagen for 45 min. Images shown are representative of at least three independent experiments.
strengths rather than the nature of the ligand mediating lamellipodia formation. As shown in the present study, immobilized CRP and thrombin induced a much greater increase in intracellular Ca\(^{2+}\) relative to fibrinogen, and this presumably contributes to the generation of wave-like structures that are absent on fibrinogen.

The ability of integrin \(\alpha_{IIb}\beta_3\) to activate Src kinase-dependent signalling cascades that lead to lamellipodia formation is widely recognized [3]. This study shows, however, that under certain conditions, the ability of the integrin to activate Src kinases is not essential for lamellipodia formation, as these structures can be induced by thrombin or the PAR1-specific agonist TRAP. Nevertheless, there is evidence that outside-in signalling by \(\alpha_{IIb}\beta_3\) is important in supporting thrombus formation in vivo. For example, increased rebleeding is seen following removal of a small portion of the tail in mice with a knock-in mutation of the integrin \(\beta_3\) subunit in which the two conserved tyrosine residues have been replaced by phenylalanine residues, thereby impairing \(\alpha_{IIb}\beta_3\) outside-in signalling [25]. Similarly, thrombus formation in a heat injury model is reduced in mice deficient in the protein tyrosine phosphatase PTP1b, which plays a critical role in \(\alpha_{IIb}\beta_3\)-mediated outside-in signalling [26]. Alternatively, impaired thrombus formation in these studies could be a consequence of reduced clot retraction, which also depends on outside-in signalling.

In summary, the present study has shown that immobilized fibrinogen, CRP and thrombin stimulate distinct patterns of morphological change and Ca\(^{2+}\) signalling during platelet adhesion. However, despite these differences, lamellipodia formation on all surfaces is critically dependent upon integrin \(\alpha_{IIb}\beta_3\), as a consequence of release of \(\alpha_{IIb}\beta_3\) ligands from platelet α-granules. The distinct pattern of lamellipodia may therefore be explained by the differing levels of elevation of intracellular Ca\(^{2+}\) and other intracellular signals. The present study also shows that outside-in signalling from integrin \(\alpha_{IIb}\beta_3\) is not required for lamellipodia formation on fibrinogen in the presence of thrombin. Thus, under these conditions, the essential role of integrin \(\alpha_{IIb}\beta_3\) in supporting lamellipodia formation can be attributed to its ability to function as an adhesive receptor.

**Experimental procedures**

**Reagents**

Fibrinogen depleted of plasminogen, von Willebrand factor and fibronectin were obtained from Kordia Laboratory Supplies, Leiden, NL. Oregon Green bis-(o-aminophenoxy)ethane-N,N',N",N"-tetraacetic acid (BAPTA 1-AM) and rhodamine–phalloidin were purchased from Molecular Probes (Cambridge Bioscience, Cambridge, UK), and fluorescein isothiocyanate-conjugated anti-P-selectin serum was obtained from BD Pharmingen (Erembodegem, Belgium). All other reagents were obtained as described in McCarty et al. [16].

**Preparation of washed platelets**

Human studies were carried out with ethical approval from the Central Oxford Research Committee (Ref: C00:203) and with the understanding and written consent of each subject. Platelets were prepared as previously described [9], and resuspended at \(2 \times 10^7\) ml\(^{-1}\) in modified Heps/Tyrodes buffer (129 mm NaCl, 0.34 mm Na\(_2\)HPO\(_4\), 2.9 mm KCl, 12 mm NaHCO\(_3\), 20 mm Hepes, 5 mm glucose, 1 mm MgCl\(_2\); pH 7.3) containing 0.1 µg·mL\(^{-1}\) prostacyclin. In selected experiments, platelet suspensions were treated with 10 µM lotrafiban, 10 µM BAPTA-AM and 20 µM PP2 for 10 min, or with 1 U·mL\(^{-1}\) thrombin for 1 min, before use. Concentrations of inhibitors were used that are maximally effective. These concentrations were identified in previous studies from the Watson group and others [27–30]. The generation of mice disrupted in the genes encoding \(\alpha_{IIb}\) or PLC\(_{\gamma}2\) was as described [24,31]. Wild-type littermates were used as controls. Washed murine platelets were isolated and resuspended in modified Heps/Tyrodes buffer as previously described [9]. All experiments were performed in the presence of 2.5 U·mL\(^{-1}\) apyrase and 10 µM indomethacin, and in the absence of exogenously added Ca\(^{2+}\). Animals were bred, and blood was removed under an approved Home Office Project licence.

**Adhesion assays**

 Coverslips were incubated with a suspension of fibrinogen (100 µg·mL\(^{-1}\)), CRP (1 µg·mL\(^{-1}\)), thrombin (1 U·mL\(^{-1}\)) or collagen (100 µg·mL\(^{-1}\)) for 1 h at room temperature before washing with phosphate buffered saline and blocking with denatured BSA (5 mg·mL\(^{-1}\)) for 1 h. Platelets were exposed to coverslips for 45 min, before fixing, staining where necessary, and mounting as described in McCarty et al. [9]. Adherent platelets were imaged using Köhler illuminated Nomarski differential interference contrast optics with a Zeiss (Carl Zeiss Ltd., Welwyn Garden City, UK) 63× oil immersion 1.40 NA plan-apochromat lens on a Zeiss Axiosvert ZOOM microscope (Zeiss). Time-lapse events were captured by a Hamamatsu Orca 285 cooled digital camera (Cairn Research, Faversham, UK) using SLIDEBOOK 4.0 (Intelligent Imaging Innovations, Inc., Denver, CO). To compute the surface area of platelets, time-lapse images were manually outlined and quantitated by determining the
number of pixels within each outline using a Java plugin for the IMAGEJ software package (NIH, Bethesda, MD, USA). Imaging a graticule under the same conditions allowed the conversion of pixel size to micrometres. Adhesion data in each experiment were obtained by counting the number of platelets on five random images of each coverslip, with each image encompassing an area of 15 400 μm². For immunofluorescence studies, coverslips were blocked in 0.1% BSA following fixation and stained for 1 h at room temperature before washing, mounting and imaging as described above.

**Single-platelet Ca²⁺ measurement**

Washed human platelets (2 × 10⁸ mL⁻¹) were incubated with the Ca²⁺-sensitive dye Oregon Green BAPTA 1-AM (15 μM) for 1 h at 30 °C. Platelets were subsequently washed, resuspended at 2 × 10⁸ mL⁻¹ and left for a minimum of 30 min before experimentation. Platelets (1 × 10⁴ mL⁻¹) were allowed to sediment onto fibrinogen-, CRP-, or thrombin-coated coverslips over a period of 10 min. Fluorescence changes in single platelets were measured using a Zeiss Axiovert 200M microscope fitted with an Optoscan Monochromator System (Cairn Research). A Hamamatsu Orca 285 camera and SLIDEBOOK software were used for image capture and subsequent analysis.

**Analysis of data**

Experiments were carried out on at least three occasions, and images shown are representative data from one experiment. Unless stated otherwise, 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 < 0.05 were selected to be statistically significant.

**Acknowledgements**

We thank the British Heart Foundation and the Medical Research Foundation for funding this work, and Mark Larson, Simon Calaminus and Andrew Pearce for their help and advice. KT and SPW hold a British Heart Foundation studentship and chair, respectively.

**References**


**Supplementary material**

The following supplementary material is available online:

**Video S1.** Real-time analysis of platelet on fibrinogen.

**Video S2.** Real-time analysis of platelet on collagen-related peptide (CRP).

**Video S3.** Real-time analysis of platelet on thrombin.

This material is available as part of the online article from http://www.blackwell-synergy.com