Identification of a novel, actin-rich structure, the actin nodule, in the early stages of platelet spreading

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Summary. Background: During platelet spreading, the actin cytoskeleton undergoes marked changes, forming filopodia, lamellipodia and stress fibres. In the present study, we report the identification of a novel actin-rich structure, termed an actin nodule, which appears prior to lamellipodia and stress fibre formation. Methods: Platelet spreading was monitored using human platelets and mouse GFP-actin platelets using real-time and end-point DIC, and fluorescent and electron microscopy (EM). Results: We identified a small, novel actin structure, the actin nodule, in the early stages of adhesion and spreading, which we hypothesize to be a precursor of lamellipodia and stress fibres. Nodule formation shows an inverse correlation to Rho kinase and myosin-II activity, is independent of PI3-kinase, but dependent on Src kinase activity. Actin nodules contain multiple proteins, including Arp2/3, Fyn, Rac, and β1- and β3-integrins, but not Src. EM analysis revealed that actin filaments extend in all directions from the nodules. Actin nodules are present on multiple matrices, including fibrinogen, laminin and VWF + botrocetin. Conclusion: This work identifies a novel platelet actin structure, which we propose is a precursor to both lamellipodia and stress fibres and acts to drive platelet spreading.

Keywords: actin cytoskeleton, Arp2/3, platelet spreading, Rho kinase, Src kinases.

Introduction

Upon vascular damage and exposure of subendothelial matrix proteins, platelets assemble actin-rich structures such as filopodia, lamellipodia and stress fibres that serve to support thrombus formation and stability [1–4]. The actin cytoskeleton is also capable of forming many other actin structures, including focal adhesions [5], focal complexes [6], invadopodia [7], podosomes [8], endocytic actin patches [9], and dorsal ruffles [10]. Focal adhesions have been described in platelets that have been allowed to spread on fibrinogen, but have only been partially characterized and their functional role is unknown [11]. Podosomes have been identified in megakaryocytes [12], but not in platelets, and to our knowledge there are no reports of the presence of dorsal ruffles, invadopodia, or endocytic- or granule-associated actin structures in platelets.

In the present study, we have used fixed platelets stained with the actin-binding toxin FITC-phalloidin and mouse platelets expressing a transgene encoding GFP-actin to monitor actin polymerization during spreading on various matrix proteins. For the first time, we have visualized real-time actin dynamics in platelets and have found punctate areas of actin staining, which we have termed the actin nodules. Actin nodules are first seen at the onset of lamellipodia formation, but are absent in platelets with stress fibres. They can be labeled with antibodies to several key actin regulatory proteins, including the Arp2/3 complex and cortactin. We propose that actin nodules are formed during the early stages in actin polymerization and that they play an important role in driving formation of later actin structures, including lamellipodia and stress fibres.

Methods

Reagents

Y27632, blebbistatin, latrunculin A and Ly294002 were obtained from Merck Biosciences (Nottingham, UK). PD0173952 was a gift from Pfizer Global Research and Development (Ann Arbor, MI, USA). Polyethyleneglycol (MW 40 kDa) was obtained from Universal Biologicals (Cambridge, UK). Antibodies to Arp2/3, cortactin, phosphotyrosine, Src, WASP and Rae were obtained from Upstate Technology (Chandlers Ford, UK). P-selectin and CD63 were obtained from Pharmingen (Oxford, UK). Csk and αIIbβ3 were obtained from Santa Cruz (Santa Cruz, CA, USA). BL90, the Fyn antibody, was a gift from Mike Tomlinson (Birming-
ham, UK). Antibodies to talin and vinculin and other materials were from previously described sources [13,14] or Sigma (Poole, UK).

**Platelet preparation**

Human venous blood was drawn by venipuncture and prepared as previously described [13]. Mouse platelets were prepared as previously reported [13]. GFP mice were obtained from Professor A. Matus, Friedrich Miescher Institute, Basel, Switzerland.

**Platelet morphology studies**

Coverslips were coated in the presence of 100 μg mL⁻¹ fibrinogen, 10 μg mL⁻¹ VWF + 2 μg mL⁻¹ botrocetin, 10 μg mL⁻¹ VWF + 1 mg mL⁻¹ ristocetin, or 50 μg mL⁻¹ laminin as previously described [13]. All experiments were performed in the presence of apyrase (2 unit mL⁻¹) and indomethacin (10 μM). Platelets (2 × 10⁷ mL⁻¹) were incubated for 2 min with either DMSO (0.1%), Y27632 (50 μM), blebbistatin (100 μM), PD0173952 (20 μM), Ly294022 (25 μM) or latrunculin A (3 μM) as appropriate, prior to spreading on the specific matrix for 45 min at 37 °C. In a separate set of experiments, platelets (2 × 10⁷ mL⁻¹) were spread for 22.5 min before addition of DMSO (0.1%), PD0173952 (20 μM), Ly294022 (25 μM) or latrunculin A (3 μM), and spreading continued for 22.5 min. For both sets of experiments, platelets were fixed after 45 min with paraformaldehyde (3.7%). Differential interference contrast (DIC) analysis was performed using Köhler illuminated Nomarski DIC optics after mounting with Hydromount (Fischer, Dublin, Ireland).

For immunohistochemistry, platelets were fixed as above and lysed with Triton X-100 (0.2%). Platelets were washed with PBS before incubation with FITC-phalloidin (2 μM) or rhodamine phalloidin (2 μM) and the appropriate antibody for 60 min in the dark. Platelets were washed with PBS, before addition of the appropriate secondary antibody, and incubated for 60 min. Platelets were mounted with Hydromount, and imaged using both fluorescent imaging with a Zeiss 63× oil immersion 1.40 NA plan-apochromat lens on a Zeiss Axiovert 200 m microscope or DM IRE2 confocal microscope (Leica, Milton Keynes, UK), and DIC microscopy as above.

For 3D reconstruction platelets were fixed, stained and imaged with a Nikon 100× oil immersion 1.40 NA plan-apochromat lens on a Nikon Eclipse TE2000-E microscope (Nikon, Surrey, UK). Image analysis was completed using Openlab 4.0.3 (Improvision, Coventry, UK). In other experiments platelets were fixed, stained with a FITC-conjugated GPIb antibody, before lysis with Triton X-100 (0.2%) for 3 min, prior to staining with rhodamine phalloidin (2 μM) for 60 min in the dark. Image analysis was completed using Slidebook 4.0 (Intelligent imaging Innovations Inc., Denver, CO, USA).

**Real-time platelet morphological studies**

Platelets (2 × 10⁷ mL⁻¹) were incubated with apyrase (2 unit mL⁻¹) and indomethacin (10 μM) for 2 min before being allowed to spread on fibrinogen-coated coverslips at 37 °C for 30 min. In some experiments, platelets were allowed to spread for 15 min before addition of either PBS or 0.03 units mL⁻¹ thrombin, and spreading continued for another 15 min. Platelet spreading was monitored using either DIC or fluorescent microscopy as above.

**Electron microscopy**

Platelets (2 × 10⁷ mL⁻¹) were allowed to spread on fibrinogen-coated coverslips in the presence of apyrase (2 unit mL⁻¹), indomethacin (10 μM) and either DMSO (0.1%), Y27632 (50 μM) or blebbistatin (100 μM) for 45 min at 37 °C. Platelets were then prepared as previously described [15]. Images were processed using a Philips XL-30 FEG Environmental scanning EM (Oxford Instruments, High Wycombe, UK).

**Analysis of results**

Where applicable, results are shown as mean ± SEM. Statistical significance of differences between means was determined by Student’s t-test or one-tailed ANOVA. If means were shown to be significantly different with an ANOVA, multiple comparisons were performed using a Tukey test. Probability values of P < 0.05 were selected to be statistically significant. In some cases, a frequency distribution of the data was obtained from a combination of three separate experiments.

**Results**

**Monitoring actin polymerization during platelet spreading**

Spreading of human platelets on fibrinogen was monitored with real-time DIC microscopy (Supporting Information video S1) and in fixed platelets at specific times using both DIC and fluorescent microscopy (Fig. 1A,B). After initial contact with fibrinogen, actin-rich filopodia were observed to extend from the platelet body. This was followed by lamellipodia formation, leading to the characteristic shape of a fully spread platelet. The membrane of the fully spread platelet underwent continuous movement, reflecting peripheral ruffle formation (Supporting Information video S1). Filopodia and limited lamellipodia formation can be seen in the majority of platelets after 10 min, with a minority having undergone full spreading and stress fibre formation (Fig. 1A,Bi). In contrast, by 45 min, the majority of the platelets have fully spread, while stress fibres were observed in nearly 60% of platelets (Fig. 1C). An intermediate phenotype was seen at 20 min (Fig. 1Bi). The sequential formation of filopodia, lamellipodia and stress fibres is consistent with previous observations in other cells [16].

Notably, during platelet spreading, we observed a punctuate pattern of staining in the FITC-phalloidin images, which
co-localized with nodule-like structures in the DIC images (Fig. 1Bii). We have termed the individual components that make up the punctate appearance, actin nodules, because they are readily stained with phalloidin. Actin nodules were observed in the majority of platelets on fibrinogen at 10 min, with each platelet containing, on average, 4.9 ± 0.7 nodules, with a range of 1–13 nodules per platelet (data not shown). At 45 min, the number of platelets containing actin nodules had decreased to <40%, although the mean number of nodules per platelet (from the population that contained nodules) was not significantly changed at 5.16 ± 0.6, with a range of 1–25 nodules per platelet (data not shown). It was notable that actin nodules were absent in the majority of platelets with stress fibres.

Using real-time DIC recordings (Supporting Information video S1), the punctate structures that correspond to actin nodules were observed to be highly dynamic structures that move continuously throughout lamellipodia formation, but which disappear following stress fibre formation. Due to the small size and compact nature of non-activated platelets, we were not able to establish whether actin nodules were present prior to onset of spreading.

Fig. 1. Identification of actin nodules in platelets spread on fibrinogen. Human platelets (2 × 10⁷ mL⁻¹) were allowed to spread on fibrinogen-coated coverslips in the presence of apyrase (2 unit mL⁻¹) and indomethacin (10 μM) for 10 min, 20 min or 45 min. Platelets were fixed with paraformaldehyde (3.7%) and (A) imaged using DIC microscopy or (B & ii) lysed with Triton X-100 (0.2%), and stained with FITC-phalloidin (2 μM). Images were taken using fluorescent and DIC microscopy and are representative of more than three experiments. White arrows indicate presence of actin nodules, both within fluorescent and DIC images. (C) Platelets with stress fibres, actin nodules or both actin nodules and stress fibres were analyzed over three distinct fields: a minimum of 100 platelets was counted per experiment.

Actin nodules are generated in mouse platelets

Studies were designed to investigate actin nodule formation in mouse platelets, and to characterize their formation using mice expressing a transgene for GFP-actin. In contrast to human platelets, mouse platelets do not form extensive lamellipodia and stress fibres on fibrinogen unless stimulated by an agonist such as thrombin [17]. Nevertheless, fluorescent imaging of FITC-phalloidin-stained cells revealed the presence of punctate actin-rich structures in mouse platelets that have spread on fibrinogen (Fig. 2A), consistent with actin nodule formation being independent of full spreading and stress fibre formation. When imaged after 45 min, our data show that over 80% of mouse platelets spread on fibrinogen contained actin nodules, with a mean of 4.7 ± 0.6 actin nodules per platelet over a range of 1–16 actin nodules per platelet (Fig. 2A & data not shown). Stimulation with an intermediate concentration of thrombin (0.1 units mL⁻¹) induces extensive spreading and stress fibre formation in mouse platelets such that, at 45 min, 92.3 ± 1.8% of platelets contain stress fibres and only...
6.3 ± 1.7% contain one or more actin nodules, with little cross-over in the two populations (Fig. 2B).

To further investigate the formation of actin structures, we used mouse platelets expressing a transgene for GFP-actin [18] to enable real-time recording of platelet actin dynamics. These studies revealed the appearance of nodule-like structures in the early stage of spreading on fibrinogen and demonstrate that these undergo continuous dynamic movement (Fig. 3, and Supporting Information video S2). Strikingly, the addition of thrombin (0.03 units mL\(^{-1}\)) at 15 min led to the disappearance of actin nodules, and both enrichment of actin labeling at the cell periphery and stress fibre formation (Fig. 3 and Supporting Information video S3). Staining of GFP-actin platelets that have spread on fibrinogen with rhodamine phalloidin demonstrated colocalization of GFP-actin and F-actin staining, confirming that these two methods detect the same structure (Supporting Information Fig. S1). Thus, these results confirm that the punctuate structures labeled with FITC-phalloidin correspond to a dynamic actin structure that is generated early during platelet spreading.

**Actin nodules are correlated with low RhoA and ROCK activity**

The above data suggest a reciprocal relationship between the presence of actin nodules and stress fibres. In order to characterize the role of RhoA and ROCK activity in nodule formation, we utilized the ROCK inhibitor, Y27632, and the myosin-II inhibitor, blebbistatin, to inhibit stress fibre formation [1]. Our data demonstrate that Y27632 and blebbistatin inhibit stress fibre formation in more than 95% of human platelets and cause an approximate doubling of the number of platelets with actin nodules (Fig. 4A,B). However, neither inhibitor had an effect on adhesion or lamellipodia formation (data not shown). These data suggest that ROCK and myosin-II activity are not required for (and appear antagonistic to) actin nodule formation.

**Actin nodule formation requires actin polymerization and Src kinase activity**

To identify the mechanism of actin nodule formation, we characterized nodule formation in the presence of the actin polymerization inhibitor latrunculin A, the Src kinase inhibitor PD0173952, and the PI3-kinase inhibitor Ly294002. Pharmacological inhibition of Src kinase, PI3-kinase and actin polymerization has been previously reported to disrupt platelet spreading [17,19–21]. Our data demonstrate that actin nodules were absent in platelets treated with PD0173952 or latrunculin.
A, but were abundant in platelets treated with Ly294002 (data not shown). The absence of nodules may reflect either inhibition of their formation or an increased rate of breakdown. To address this, platelets were allowed to spread on fibrinogen for 22.5 min before addition of the above inhibitors. Platelets were then allowed to spread for a further 22.5 min. Our data show that addition of PD0173952, Ly294002 or latrunculin A at 22.5 min abrogated stress fibres by 45 min. However, only PD017952 and Ly294002 induced a partial loss of lamellipodia (Fig. 5A). Interestingly, treatment of platelets with Ly294002 increased the number of platelets with actin nodules while, in contrast, PD017952 or latrunculin A dramatically reduced the number of platelets containing actin nodules (Fig. 5B). These data suggest that actin nodules are dynamic in nature, and that their formation and stability require continuous actin polymerization downstream of Src kinases, but are independent of PI3-kinase.

Actin nodules extend through the platelet

The actin nodules were further characterized through analysis of z stacks of spread platelets stained with phalloidin to ascertain whether nodules were localized to basal or apical portions of the platelet, or whether they are pancellular. Deconvolution and 3D restoration of the images clearly demonstrate that the majority (~90%) of nodules appear to extend throughout the platelet, from the basal to the apical surface. Only a minority of the actin nodules were associated with either the upper or lower surface alone (Supporting Information Fig. S2 and Supporting Information video S4). To identify the position of the plasma membrane, z stacks of platelets labeled with GPIb prior to lysis and phalloidin staining were taken. These sections confirmed the pancellular distribution of the nodules (Supporting Information video S5). Because spread platelets are very thin, assigning localization of the nodules more precisely is limited by the resolution of the light microscope. However, taken together, Supporting Information Fig. S2 and Supporting Information videos S4 and S5 provide strong evidence that the nodules are pancellular.
Electron microscopy techniques were utilized to further characterize the actin nodules. Stress fibres were readily identified in platelets spread on fibrinogen in the presence of DMSO (Supporting Information Fig. S3A). The stress fibres are revealed as bundles of parallel actin fibres that circle around a central core. In contrast, thick rings of actin fibres that correspond to stress fibres were absent in electron micrographs of platelets spread on fibrinogen treated with Y27632 or blebbistatin (Supporting Information Fig. S3B and data not shown). Instead, actin filaments could be seen in all directions within the spread platelet, with the majority originating or finishing in raised, circular nodule-like structures that correspond to actin nodules (see white arrows in Supporting Information Fig. S3C). Thus, these studies confirm the presence of actin nodules in platelets treated with Y27632 or blebbistatin and suggest that they function as terminals for organization of actin filaments.

**Actin nodules co-localize with actin regulatory proteins**

The presence of actin binding and regulatory proteins within actin nodules was investigated using immunohistochemistry. These studies reveal the presence of Rac, Fyn, the Arp2/3 complex, cortactin, talin, β1- and β3-integrin subunits within the actin nodules (Fig. 6 and data not shown). Proteins that are not associated with actin nodules include ROCK1, Myosin-II.

![Image](https://example.com/fig6)

**Fig. 6.** Proteins associated with actin nodules present on fibrinogen. Platelets ($2 \times 10^7$ mL$^{-1}$) were allowed to spread on fibrinogen-coated coverslips in the presence of apyrase (2 unit mL$^{-1}$) and indomethacin (10 μM) for 45 min. Platelets were fixed with paraformaldehyde (3.7%), lysed with Triton X-100 (0.2%), and stained with (i–iii) FITC-phalloidin (2 μM) or (iv) rhodamine-phalloidin (2 μM) and an antibody to the designated protein. Platelets were analyzed by confocal microscopy. Images are representative of three experiments.
and Src, which have been shown to associate with other actin structures in other cells, including podosomes (Fig. 6 and Supporting Information Fig. S4). In addition, the α- or dense granules markers, P-selectin and CD63 respectively, do not colocalize with the actin nodules, confirming that they do not represent a secretory granule. The observation that actin nodules are rich in proteins required for actin polymerization, cell signaling and adhesion implies an important role in actin regulation and adhesion.

**Actin nodules are present on multiple matrices.**

Due to the observation that actin nodules contain integrins we investigated whether actin nodules were formed on other extracellular matrices. A set of experiments were designed to determine if actin nodules were present in platelets spread on VWF in the presence of botrocetin, a snake C-type lectin, which binds to the A1 domain of VWF, or ristocetin, a bacterial glycopeptide that binds to the proline rich region of the A1 domain in VWF. VWF/botrocetin and VWF/ristocetin signal differentially, with the former signaling via the GPIb-IX-V complex, and the latter via GPIb-IX-V and integrin αIbβ3 [4]. VWF/botrocetin generates weak signals, resulting in partial lamellipodia and limited stress fibre formation, whilst VWF/ristocetin generates full spreading and robust stress fibre formation. Strikingly, actin nodules were readily seen in platelets spread on VWF/botrocetin, but were absent in platelets spread on VWF/ristocetin (Fig. 7), consistent with the reciprocal relationship between actin nodule formation and stress fibre formation. Actin nodules were also observed in platelets that had spread on laminin, which induces spreading through the combination of α6β1 and GPVI [22], although they were absent in platelets with stress fibres (Fig. 7). Thus, these results demonstrate that actin nodules are also formed on other matrix proteins, including VWF/botrocetin and laminin, but that they disappear upon stress fibre formation.

**Discussion**

The present study identifies the formation of a novel actin structure, the actin nodule, within platelets spread on multiple matrix proteins. The nodules are independent of filopodia formation, but are present at the very early stage of lamellipodia formation and undergo continuous movement throughout the generation of full lamellipodia, although they disappear upon stress fibre formation. The generation of actin nodules is dependent on continuous Src kinase activity and actin polymerization, consistent with their dynamic nature. We propose that actin nodules represent an early actin structure that precedes and possibly contributes to stress fibre formation.

The extent to which actin nodules are similar to actin structures identified in other cells should be considered. There are multiple actin-rich structures that have been reported in other cell types, including podosomes, focal adhesions, focal complexes, invadopodia, peripheral and dorsal ruffles, and endocytic vesicles. However, only podosomes and focal adhesions are close in structure to the actin nodule. Podosomes are F-actin enriched, circular structures containing the Arp2/3 complex, WASP, Src, β1 and β3 integrins, and are heavily tyrosine phosphorylated [8]. Their formation is dependent on WASP and PKC and they are typically present as a rosette structure within spreading cells [8]. Moreover, they have been described in megakaryocytes that have spread on collagen [12]. Actin nodules, however, are not present as a rosette structure and are much fewer in number than podosomes described in other cell types. The strongest argument against the actin nodules being a variant of a podosome is their small size and the absence of an enriched core of actin and outer ring, which is typical of podosomes [8]. The actin nodules are also unlikely to represent focal adhesions, as these are formed at the tips of stress fibres [7] and actin nodules disappear on stress fibre formation. Further, actin nodules are present in Rac1−/− mouse platelets [13], whereas focal adhesions are critically dependent on Rac activity, at least in fibroblasts [23].

Thus, it would appear that the actin nodules do not correspond to previously described actin structures and that they are unique both in composition of proteins and in structure. Further, our results suggest that actin nodules are not required for filopodia formation, as actin nodules are absent in platelets treated with the Src kinase inhibitor, PD0173952, whereas filopodia are still present. However, the presence of actin nodules at the beginning of lamellipodia formation and absence in platelets with stress fibres raises the possibility they may play a critical role in the formation of these two structures.
Alternatively, the availability of actin monomers at the outset of spreading may facilitate their formation, while their disappearance may be due to consumption of actin monomers within actin structures. The present study has shown that the actin nodule is enriched in many actin cytoskeletal regulatory proteins, such as the Arp2/3 complex, Rac1, talin and cortactin, and they require continuous signaling through one or more Src family kinases. Moreover, the early time course of their formation, the presence of Rac1, cortactin and the Arp2/3 complex, and their presence in Rac1−/− mice, provide evidence for a role in mediating lamellipodia formation. Certainly, the presence of these, and other important proteins, including the β1 and β3 integrins, indicates an important role for the nodules in platelet spreading. The observation from electron microscopy that the actin nodules appear to act as termini for actin fibres makes them well placed to play a role in lamellipodia and stress fibre formation, perhaps by helping to organize the actin with the cell. Further investigation of the role of the actin nodules necessitates investigating the effect of their disruption on platelet spreading and function. Actin nodules have been observed in previous studies in platelets, although there was no specific comment on their presence [4,13,21,24–26]. In conclusion, this study demonstrates the formation of a novel actin structure, which is illustrated to form prior to both lamellipodia and stress fibre formation. It requires both actin polymerization and Src kinase activity, whilst being negatively correlated to ROCK and myosin-II activity, and stress fibre formation. Actin nodules are present on multiple matrices and have all the proteins required for a major role within actin dynamics.

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Disclosure of Conflict of Interests
The authors state that they have no conflict of interest.

Supporting information
Additional Supporting Information may be found in the online version of this article:

Fig. S1. Identification that condensation spots within GFP-actin mice colocalize with actin nodules. Platelets (2 × 10^7 mL⁻¹) were allowed to spread on fibrinogen in the presence of apyrase (2 unit mL⁻¹) and indomethacin (10 μM) for 45 min. Platelets were fixed, stained with rhodamine phalloidin (2 μM) and imaged using confocal microscopy. Actin spots present within the GFP-actin platelets colocalize with F-actin spots stained by rhodamine phalloidin. The results are representative of three experiments.

Fig. S2. 3D representation of a spread platelet demonstrating that the majority of actin nodules are pancellular. Platelets (2 × 10^7 mL⁻¹) were allowed to spread on fibrinogen in the presence of apyrase (2 unit mL⁻¹) and indomethacin (10 μM) for 45 min. Platelets were fixed and stained with rhodamine-phalloidin (2 μM). A z stack of optical sections, with a separation of 0.1 μm, was collected. Images were deconvolved using Openlab software, before 3D restoration. The image demonstrates that actin nodules, viewed in the z orientation, extend from the basal to the apical regions of the cell.

Fig. S3. Scanning electron microscopy of spread platelets. Platelets (2 × 10^7 mL⁻¹) were allowed to spread on fibrinogen-coated coverslips in the presence of apyrase (2 unit mL⁻¹), indomethacin (10 μM) and either DMSO (0.1%), Y27632 (50 μM) or blebbistatin (100 μM) for 45 min. Platelets were fixed, dried with graded alcohols, and critical point dried. Samples were analyzed using scanning electron microscopy. (A) Stress fibre formation in a single, control platelet. (B) White arrows identify formation of actin nodules in a single platelet that has been allowed to spread in the presence of blebbistatin (100 μM). (C) An enlargement of part B demonstrating that actin filaments radiate out in all directions from an actin nodule. Images are representative of two experiments.

Fig. S4. ROCK1 and myosin-II do not colocalize to actin nodules. Platelets (2 × 10^7 mL⁻¹) were allowed to spread on fibrinogen in the presence of apyrase (2 unit mL⁻¹) and indomethacin (10 μM) for 45 min. Platelets were fixed and stained with antibodies to (A) ROCK1 and (B) myosin light chain. Actin was stained with rhodamine phalloidin (2 μM). Images are representative of three experiments.

Video Clip S1. Real-time platelet spreading on fibrinogen. Platelets (2 × 10^7 mL⁻¹) were allowed to spread on fibrinogen in the presence of apyrase (2 unit mL⁻¹) and indomethacin (10 μM) for 30 min. Platelet spreading was monitored using real-time fluorescent microscopy, with an image taken every 5 s. Platelets initially form filopodia and short lamellipodia sheets before undergoing full spreading. At the periphery of the platelet, peripheral ruffle formation can be identified. The video is representative of three experiments.

Video Clip S2. Real-time spreading of GFP-actin platelets on fibrinogen. Platelets (2 × 10^7 mL⁻¹) were allowed to spread on fibrinogen in the presence of apyrase (2 unit mL⁻¹) and indomethacin (10 μM) for 30 min. Platelet spreading was monitored using real-time fluorescent microscopy, with an image taken every 5 s. Platelets initially form filopodia and short lamellipodia sheets before undergoing full spreading. At the periphery of the platelet, peripheral ruffle formation can be identified. The video is representative of three experiments.

Video Clip S3. Real time spreading of GFP-actin platelets on fibrinogen with addition of thrombin at 15 min. Platelets (2 × 10^7 mL⁻¹) were allowed to spread on fibrinogen, in the
presence of apyrase (2 unit mL^{-1}) and indomethacin (10 μm) for 15 min. At this time, thrombin (0.03 unit mL^{-1}) was added and spreading allowed to continue for a further 15 min. Time 0 is the point at which the platelet enters the field of view. Platelet spreading was monitored using real-time fluorescent microscopy, with an image taken every 5 s. Platelets initially form filopodia and actin nodules. The actin nodules can be seen to move continually throughout the recording. The video is representative of three experiments.

**Video Clip S4.** 3D representation of a spread platelet demonstrating that the majority of actin nodules are pancellular. Platelets were treated as described for Supporting Information Fig. S2. The video demonstrates the pancellular distribution of the actin nodule as it extends across the platelet, from basal to apical regions.

**Video Clip S5.** Z stack of a spread platelet demonstrating that the majority of actin nodules are pancellular. Platelets (2 × 10^3 mL^{-1}) were allowed to spread on fibrinogen, in the presence of apyrase (2 unit mL^{-1}) and indomethacin (10 μm) for 45 min. Platelets were fixed and stained with FITC-conjugated GPIb antibody, before lysis with Triton X-100 for 45 min. Platelets were stained with rhodamine phalloidin conjugated GPIb antibody, before lysis with Triton X-100 for 15 min. At this time, thrombin (0.03 unit mL^{-1}) was collected. Images were deconvolved using Slidebook 4.0 before 3D restoration. The video shows actin (top left), GPIb (top right) and the merged image (bottom left) (actin, red; GPIb, green). The video demonstrates that actin nodules extend across the platelet, from the basal membrane (0 μm) to the apical membrane (2 μm).

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