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Platelet integrins exhibit anisotropic mechanosensing and harness piconewton forces to mediate platelet aggregation

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Platelet aggregation at the site of vascular injury is essential in clotting. During this process, platelets are bridged by soluble fibrinogen that binds surface integrin receptors. One mystery in the mechanism of platelet aggregation pertains to how resting platelets ignore soluble fibrinogen, the third most abundant protein in the bloodstream, and yet avidly bind immobile fibrinogen on the surface of other platelets at the primary injury site. We speculate that platelet integrins are mechanosensors that test their ligands across the platelet–platelet synapse. To investigate this model, we interrogate human platelets using approaches that include the supported lipid bilayer platform as well as DNA tension sensor technologies. Experiments suggest that platelet integrins require lateral forces to mediate platelet–platelet interactions. Mechanically labile ligands dampen platelet activation, and the onset of piconewton integrin tension coincides with calcium flux. Activated platelets display immobilized fibrinogen on their surface, thus mediating further recruitment of resting platelets. The distribution of integrin tension was shown to be spatially regulated through two myosin-signaling pathways, myosin light chain kinase and Rho-associated kinase. Finally, we discovered that the termination of integrin tension is coupled with the exposure of phosphatidylserine. Our work reveals the highest spatial and temporal resolution maps of platelet integrin mechanics and its role in platelet aggregation, suggesting that platelets are physical substrates for one another that establish mechanical feedback loops of activation. The results are reminiscent of mechanical regulation of the T-cell receptor, E-cadherin, and Notch pathways, suggesting a common feature for signaling at cell junctions.

Platelet-to-platelet aggregation is vital for hemostasis (Fig. 1A). The major adhesion molecule involved in platelet aggregation at low shear conditions is the \( \alpha_{\text{IIb}} \beta_{3} \)-integrin receptor present at high density both in the plasma membrane and in \( \alpha \)-granules (1). Despite its abundance, this integrin is maintained in an inactive state and ignores soluble ligands, such as fibrinogen and fibronectin within the bloodstream. However, upon activation, platelets at the primary injury site undergo inside–out triggering of their integrins and rapidly bind soluble fibrinogen (2). Receptor-bound fibrinogen then functions to recruit quiescent platelets from the bloodstream (3). While recruitment of platelets is accelerated and amplified in the presence of agonists such as adenosine diphosphate (ADP) and thrombin, surface-anchored fibrinogen is sufficient to trigger outside–in integrin activation, which leads to platelet activation, platelet-to-platelet cohesion, and initiation of clot formation (4).

Soluble fibrinogen is the third most abundant protein in blood plasma (2–4 mg/mL), and thus platelets must discriminate between soluble and membrane-bound fibrinogen with high fidelity. Previous work showed that fibrinogen displayed a lower binding affinity to platelet integrins compared with monomeric fibrin (5). In addition to this biochemical contribution, we here postulate that platelet integrins mechanically test their ligands to distinguish between soluble and immobilized states, thereby mediating the process of platelet cohesion. This hypothesis is supported by recent work showing that platelets are more activated when exposed to fibrinogen immobilized onto stiffer polymer scaffolds (4). Still, there is little understanding of how the mechanics of integrin ligands regulate the initiation of platelet activation. Elucidating how chemo-mechanical coupling aids in the recognition of immobilized ligands is central to understanding platelet aggregation.

Following initial recruitment and adhesion of a resting platelet from the bloodstream, it rapidly undergoes a well-defined signaling cascade (Ca\textsuperscript{2+} influx, degranulation, phosphatidylserine exposure, etc.) that leads to the application of contractile mechanical forces, creating a plug and sealing the injury site. Accordingly, a platelet must deliver precise contractile forces that are coordinated in space and time. For example, polymer pillar force sensor experiments showed that microclots containing tens of platelets apply a collective force of \( \sim 80 \) nN after exposure to immobilized ligand (6). Our previously reported atomic force microscopy (AFM)-based mechanical measurements revealed a contractile force of up to \( \sim 70 \) nN per platelet (7). Traction force microscopy (TFM) measurements suggested a cumulative force of \( \sim 34 \) nN per activated platelet (8). Taken together, these studies clearly confirm that platelets exert nanonewton (nN)


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Significance

During initial growth of a clot, it is necessary for platelets to aggregate, which is mediated by binding fibrinogen molecules that bridge platelets through their integrin receptors. How platelet integrin receptors rapidly bind fibrinogen that is attached to the surface of another platelet but yet ignore soluble fibrinogen remains a mystery. This is difficult to understand since fibrinogen is the third most abundant protein in blood plasma. Here we show that differentiation between soluble and immobilized platelet ligands is mediated by mechanical forces. We demonstrate that the platelet integrins apply specific piconewton forces to test their ligands within the platelet junction. Our results provide insights into how clotting functions.
Thrombin activation and fibrinogen polymerization into fibrin, this last observation suggests that cross-linking of platelets by fibrin is rapidly accelerated as platelets terminate their contractile phase of activation.

**Results**

**Platelet Integrin Activation Requires Tangential Forces.** Soluble fibrinogen molecules bind to the \(\alpha_{IIb}\beta_3\) integrins on the surface of activated platelets at the primary injury site (3). Surface-bound fibrinogen molecules then, in turn, provide adhesion sites for recruiting inactive platelets from the blood stream, resulting in platelet aggregation and the generation of a growing thrombus (Fig. 1A). Knowing that platelets discriminate between soluble fibrinogen and immobilized ligands, we first asked how the lateral mobility of integrin ligands influences their potency for platelet triggering. The rationale was that membrane-anchored ligands confined to a 2D plane are amenable to clustering and oligomerization, which may enhance integrin activation.

To address this question, we mimicked the platelet–platelet junction by engaging human platelets to SLBs decorated with integrin ligands. The SLB is composed of phospholipids that are laterally mobile and modified with a variety of proteins, thus obscuring nascent, weak, or disorganized adhesion forces. Moreover, TFM resolves forces with \(\sim 1\mu\text{m}\) spatial resolution, which is particularly problematic for studying platelet mechanotransduction because the size of platelets is \(\sim 2\) to \(\sim 3\mu\text{m}\). Although AFM is sensitive to pN forces, its application is limited because the technique is serial, which prevents mapping of forces across the cell surface. Therefore, current approaches are unable to image initial mechanical signaling events during platelet activation and subsequent aggregation. Mapping platelet forces with pN resolution and submicron spatial resolution is paramount to understanding the biophysical mechanisms of clot retraction and how mechanical stability of the clot is established, both of which are associated with pathological states when dysregulated (9, 10).

In this report, we recapitulated the platelet–platelet junction by immobilizing integrin ligands onto a supported lipid bilayer (SLB) with controlled mobility. These experiments show that platelets failed to activate on laterally fluid but axially fixed ligands, suggesting that platelet integrins display selective activation in response to tangential tension over forces that are in an orientation perpendicular to the substrate plane. Thus, ligand lateral mobility tunes ligand potency. In agreement with this result, we show that immobilizing integrin ligands using labile anchors dampens activation as measured by cell spreading and immunostaining. We found that, when a platelet is activated, it rapidly sequesters soluble fibrinogen from solution and transports the ligand into a central zone at the initial site of adhesive platelet–substrate contact. The centrally transported fibrinogen exhibits limited lateral mobility, thus placing it in an active mechanical state that should accelerate the recruitment of additional platelets. Next, we generate the first pN maps of integrin tension during platelet activation using our recently developed molecular tension probes. Our results demonstrate that integrin tension is associated with the earliest steps of platelet activation and coincides with \(Ca^{2+}\) flux. In addition, we demonstrate that the distribution of integrin tension is spatially regulated through two myosin-signaling pathways, myosin light chain kinase, and Rho-associated kinase. Finally, we discovered that the termination of platelet forces is highly coupled with the exposure of plasma membrane phosphatidylserine (PS). Since PS initiates
glass substrates on which the ligand was immobilized. We used platelet adhesion density and spreading areas as a proxy for platelet activation in accordance with the literature (4, 17).

When human platelets were incubated on the fluid SLBs for 20 min, there was minimal cell adhesion and no detectable platelet spreading, as measured using reflection interference contrast microscopy (RICM) (Fig. 1B–D and Movie S2). Limiting long-range ligand mobility similarly resulted in no detectable platelet activation (Fig. 1B–D). In contrast, when ligands at equivalent or lower density were immobilized on a fixed substrate, there was a drastic increase in platelet adhesion and spreading (Fig. 1B–D). Pretreating platelets with ADP, an agonist that drives platelet aggregation, led to platelet spreading on the SLB surfaces, which is consistent with inside-out activation of integrins (SI Appendix, Fig. S5). Given that these surfaces present chemically identical ligands that differ only in their physical mobility, the data demonstrate that platelet integrins are mechanosensors. Moreover, SLBs offer little resistance to shear forces but strongly resist normal forces (18). Therefore, lateral forces are necessary for platelet integrin activation. Notably, our results are consistent with the conclusions of Springer and colleagues (19) stating that lateral forces are the most physiologically relevant mechanism for activating integrins. This conclusion was based on modeling showing that lateral forces applied to the integrin–ligand complex stabilize its high-affinity active state. This is in contrast to adding shear force (shearing) to stabilize the low-affinity closed integrin conformation (4, 19, 20). Our results also agree with previous studies of nucleated cells showing that lateral resistance is necessary for the maturation of integrin adhesions (21, 22).

To further confirm the role of mechanics in platelet activation, cells were incubated on substrates coated with the recently reported DNA tension gauge tether (TGT) (23–25) to physically cap integrin αIIbβ3 tension (Fig. 1E and F). The TGT is a DNA duplex designed to rupture at forces exceeding its “tension tolerance” (Ttol), defined as the force required to denature the duplex when a constant force is applied for less than 2 s. One DNA strand was modified with the cRGD peptide and a fluorescent reporter, while the complement was fixed onto the substrate. In this way, TGTs maintain the chemical recognition between the integrin and its ligand; however, the TGT sets the upper limit of forces experienced during mechanosensation. We challenged platelets with TGT constructs of Ttol values of 12 and 56 pN, which correspond to identical DNA sequences but differ in orientation, dissociating in either unzipping or shearing modes. Indeed, the weaker 12-pN TGT displayed more dissociation (twofold difference) compared with the 56-pN TGT probes (Fig. 1G and SI Appendix, Fig. S6). Platelet activation was quantified using PAC-1, an antibody that binds to the activated integrins αIIbβ3 (26). Platelets that adhered to the 56-pN (shearing) TGT displayed higher PAC-1 intensity compared with platelets spreading onto the 12-pN (unzipping) TGT (Fig. 1H). Capping the receptor force at 12 pN leads to dampening of platelet activation, likely by hampering the lifetime of the mechanical tension and also by limiting the peak tension. Thus, human platelet integrins mechanically test their integrin ligands using forces in the range of ∼12–56 pN.

Finely Tuned pN Forces Are Transmitted Through Individual Integrins in a Dynamic Spatiotemporal Pattern During Platelet Activation.

The process of clot formation requires mechanical contraction of platelets following their aggregation (Fig. 2A). The magnitude and spatial/temporal distribution of integrin forces during platelet activation is unknown, and thus we used DNA-based molecular tension fluorescence microscopy (MTFM) to directly map integrin mechanics upon ligand binding (Fig. 2B) (15, 25, 27). In brief, MTFM probes consist of a DNA hairpin labeled with a Cy3B-BHQ2 fluorophore-quencher pair. One end of the probe was immobilized onto a glass slide, while the other terminus was coupled to the cRGD ligand. The DNA hairpin unfolds and generates a 34-± 2-fold increase in fluorescence when the applied force exceeds the F1/2 (defined as the force at which 50% of hairpins unfold at equilibrium). By varying the guanine-cytosine (GC) content and stem-loop structure of the hairpins, F1/2 can be tuned from 4.7 to 19.3 pN to visualize different magnitudes of integrin tension (Fig. 2C) (15, 28).

The cRGD probes with F1/2 of 4.7 pN were first used to map tension dynamics spanning platelet activation from initial landing to spreading (Fig. 2D and Movie S3). Within minutes of cell seeding, the platelets engaged the surface as shown by RICM. Simultaneously, a punctate 4.7-pN tension signal was colocalized with RICM. Shortly thereafter, filopodia appeared in RICM, and was accompanied by tension radiating from the center to the edge of the filopodia. Once the filopodia became mature (∼4 min after initial contact), the platelet lamellipodia filled in the gaps between the filopodial projections and developed tension at the front of the growing lamellipodium. The spreading resulted in a ring of tension that at the platelet periphery. The lamellipodia, along with the tension signal, continued growing until platelets fully spread to a diameter of ∼10 μm within 10 min of the initial contact. In contrast to the dynamic lamellipodial tension signal, the central region maintained more static tension.

When the tension signal was integrated, the total intensity increased and then reached a plateau, which was maintained for 20–30 min (Fig. 2E and SI Appendix, Fig. S7) (7, 8). Radial distribution analysis of tension at steady state (n = 15 platelets) revealed two local peaks that were consistent with the lamellipodial ring pattern and a high-intensity central region that is consistent with the conclusions of Springer and colleagues (19) showing that lateral forces applied to the integrin–ligand complex stabilize its high-affinity active state. The lamellipodial tension signal accounted for ∼40% of the total, while the remaining signal was primarily localized at the central zone (Fig. 2E and F). Subsequently, platelets displayed an abrupt and complete loss of tension, accompanied by a reduction in RICM contrast (Fig. 2E). Taken together, MTFM provides integrin tension maps with spatial and temporal resolution unachievable by conventional TFM and micropipette arrays and reveals that platelets generate a distinct mechanical architecture and spatiotemporal pattern during activation compared with that reported in cell-free systems such as fibroblasts, epithelial cells, and smooth muscle cells (29–31).

To better define the magnitude of integrin tension during platelet activation, we challenged platelets with probes of F1/2 = 13.1 and 19.3 pN (15, 28). Although platelets displayed similar levels of adhesion and spreading on these two probes, the mean tension signal per platelet was 60–70% weaker (Fig. 2G). This shows that a significant fraction of integrins transduce a force of less than 13.1 pN during platelet activation (Fig. 2G). Nonetheless, integrin forces are heterogeneous, and while the signal at 4.7 pN probes was diffuse and less quantitative because fibrinogen dimers can cross-link the biotinylated probe (21), the signal at the lamellipodial edge was diminished for the 13.1 and 19.3 pN probes, the central signal was maintained (Fig. 2H). Further image analysis showed that the ratio of the lamellipodal edge signal normalized to the total tension decreased from 0.32 ± 0.05 for the 4.7 pN probes to 0.15 ± 0.06 and 0.04 ± 0.02 for the 13.1 and 19.3 pN tension probes, respectively (SI Appendix, Fig. S8). These results show that a subset of integrins at the center of the platelet contact experience forces >19.3 pN.

To further test the mechanosensor model of platelet adhesion, we simultaneously measured integrin tension and calcium flux, a proxy for platelet activation (21, 27 and SI Appendix, Fig. S11 and S12 and Movie S5). We hypothesized that, if platelets mechanically test integrin ligands with pN forces as a mechanism of discrimination between soluble and immobile ligand, then we would expect to observe tension preceding early markers of platelet activation. Representative time-lapse images and
quantitative analysis of a single platelet show that the initiation of integrin tension and calcium flux are synchronized within the temporal resolution of our experiments. Importantly, these data further support the mechanosensor model.

Following initial platelet aggregation, inside-out signaling leads to integrin binding of soluble fibrinogen, which is required for cross-linking of adjacent platelets. To examine the relationship between the pool of soluble and immobile integrin ligands, we exposed activated platelets to soluble alexa-488-labeled fibrinogen (10 μg/mL) and performed two-color time-lapse total internal reflection fluorescence microscopy (TIRFM) to map integrin tension and fibrinogen localization (SI Appendix, Fig. S13A and Movie S6). Soluble fibrinogen rapidly bound to the cell surface forming puncta that translocated along the basal face of the cell to the center of the cell–substrate junction. We tracked 45 fibrinogen clusters from n = 9 platelets and plotted their velocities over time. Cluster velocity decreased as fibrinogen moved toward the center of the platelet (SI Appendix, Fig. S13B and Movie S7). There was no significant change in integrin tension upon addition of soluble fibrinogen, suggesting minimal mechanical cross talk between these different pools of integrins. Finally, FRAP analysis showed that platelet-bound fibrinogen was immobile (SI Appendix, Fig. S13C), suggesting a mechanism in which soluble fibrinogen is bound and mechanically primed to recruit platelets from the bloodstream.

Spatial Distribution of Platelet Tension Is Regulated by Two Myosin-Signaling Pathways. Akin to nucleated cells, platelet forces are derived from myosin activity regulated by myosin light chain kinase (MLCK) and Rho-associated protein kinase (ROCK) (32). To clarify the role of these two kinases in platelet tension generation, we used inhibitors to interfere with myosin activity and measured integrin tension. Platelets were pretreated with 0.1% DMSO (vehicle control), MLCK inhibitor (10 μM ML-7), and ROCK inhibitor (30 μM Y27632) for 1 h and then incubated on the 4.7-pN cRGD probe surfaces. After 20 min, platelets treated with Y27632 displayed diminished tension signal but stably adhered and spread onto substrates (Fig. 3 A and B). Platelets treated with ML-7 adhered to substrates but failed to spread and generate tension signal. Importantly, platelets treated with Y27632 lacked the strong central tension signal typical of untreated cells. Indeed, the ratio of lamellipodial edge signal over the total tension signal increased from 0.32 ± 0.06 to 0.44 ± 0.06 (Fig. 3C, n = 20). Radial intensity profiling of 25 platelets confirmed this result, showing a peak corresponding to the lamellipodial edge without the peak at the center of platelets (Fig. 3D).

To investigate the role of these two kinases following initial adhesion, we treated platelets with inhibitors after seeding the cells onto the surface for 20 min. ROCK inhibition led to a gradual decrease in the total tension signal as well as attenuation of the central tension zone in a period of 10 min (Fig. 3E). In contrast, MLCK inhibition dramatically reduced tension within ~1 min, eradicating signal within the lamellipodial region but sparing a small amount of central platelet-generated tension (Fig. 3F and Movie S8). Together, these results suggest that RhoA and MLCK kinase-signaling pathways have distinct roles in the spatial regulation of myosin activities and integrin forces. RhoA primarily drives the high-magnitude integrin forces at the center of the platelets, while MLCK is necessary for spreading, lamellipodia extension, and filopodial protrusion. Inhibiting MLCK abolishes remodeling of the cytoskeleton at the platelet periphery, which is critical for platelet spreading and formation of stable adhesions.

Correlation of PS Exposure with Platelet Tension. PS is a negatively charged lipid typically localized to the inner leaflet of the platelet membrane (33). PS exposure to the outer leaflet promotes platelet procoagulant activity and is also associated with the proteolysis of the cytoskeleton (Fig. 4A) (34, 35). However, the relationship between PS exposure and platelet tension remains
and Cessation of platelet tension coincides with αIIbβ3 cells were analyzed for each group. The horizontal = receptor (32) and αSI Appendix
0.001). (D) Plot of mean tension intensity for inhibitor-
treated platelets. The data were collected from at least three different
substrates, where n > 20 cells were analyzed for each group. The horizontal
lines indicate the mean and SD (**p < 0.001). (C) Histogram of ratios of the
lamellipodial edge tension to the whole-platelet tension. The data were
analyzed from platelets provided by a single donor and added to four
substrates. Twenty platelets were analyzed to produce the histogram. Solid
line is a Gaussian fit of the data used to determine the mean value and the
corresponding error. (D) Tension signal radial intensity profiling of 25 platelets
(two donors) averaged from four substrates. (E and F) The representative time-
lapse RICM and tension images of platelets treated with ROCK and MLCK in-
hibitors, respectively. (Scale bar: 2 μm.)

Fig. 3. Platelet integrin tension is modulated differentially by the MLCK
and ROCK pathways. (A) RICM and integrin tension images of ROCK or MLCK
inhibitor-pretreated platelets engaged to cRGD-tension probes with F1/2 =
4.7 pN. (Scale bar: 3 μm.) (B) Plot of mean tension intensity for inhibitor-
treated platelets. The data were collected from at least three different
substrates, where n > 20 cells were analyzed for each group. The horizontal
lines indicate the mean and SD (**p < 0.001). (C) Histogram of ratios of the
lamellipodial edge tension to the whole-platelet tension. The data were
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lapse RICM and tension images of platelets treated with ROCK and MLCK in-
hibitors, respectively. (Scale bar: 2 μm.)

unclear. We performed time-lapse experiments measuring PS ex-
posure and integrin tension using confocal microscopy. Platelets
were incubated with annexin V-Alexa 488, which binds PS, and then
allowed to engage the 4.7-pN tension probe surface. Interestingly, we
found that the abrupt loss of tension signal 30–50 min after initial
platelet adhesion (Fig. 2D) coincided with PS exposure (Fig. 4B and
Movie S9). Analysis of the temporal delay (Δt) between the loss of
tension and PS exposure showed a mean value of 14.7 s for n =
23 platelets (Fig. 4 C and D and SI Appendix, Fig. S14). This
proves that proteolysis of cytoskeleton and the resulting integrin
tension cessation is upstream of PS exposure. Three-dimensional
reconstruction of platelet morphology during this process showed
that as integrin tension decreased and PS level mounted, cells
rounded up. This result agrees with the reduction in RICM contrast
(Fig. 2D and SI Appendix, Figs. S14 and S15). Therefore, PS ex-
posure follows cytoskeletal reorganization and loss of integrin tension.

Discussion
By controlling ligand mobility and ligand mechanical resistance, we
showed that chemically identical ligands display differential
levels of potency in activating platelets. This shows that outside–
intrinsic platelet activation requires mechanical tension. The
platelet response is pseudobinary, and only laterally immobile
ligands trigger cell activation, suggesting that αIIbβ3 activation
is an anisotropic mechanosensor (36). Our observation of force-
dependent activation of platelet integrins qualitatively agrees with
cadherin and integrin activation on fluid membranes in nucleated
cells (21, 37). For example, Yu et al. (21) showed that focal adhesion
maturation is dependent on lateral resistive forces within a fluid
membrane. However, there are distinct differences in platelet ad-
hesion and spreading compared with nucleated cells. At the time-
scale of our image acquisitions, we did not detect platelet adhesion
and spreading on fluid and partially fluid membranes, suggesting
strong suppression of integrin activation on laterally fluid ligands.
In contrast, nucleated cells, such as fibroblasts and epithelial cells, use
integrins to adhere to mobile ligands presented on lipid membranes,
forming activated integrin clusters (21). Likewise, cadherins can be
triggered on fluid membranes in a kinetic nucleation process (21, 37).
Our results support a concept where platelets perform a me-
chanical checkpoint of their integrin ligands to determine
whether to activate and adhere. It is likely that the mechanism
mediating this observation is driven by mechanical stabilization
of αIIbβ3-integrin in the open conformation that enhances ligand
affinity (1). This type of response is well documented for αβ1,
and likely to be the case for αIIbβ3 (38). Normal forces are in-
sufficient to activate platelets, thereby suggesting that platelet
integrins require tangential forces to mediate platelet activation.
Such a model has been proposed for the αIIbβ3 receptor (32) and
for the T-cell receptor (39). The result of this mechanical testing
is that low (lateral) mechanical resistance results in bond disso-
ciation, while high resistance forces stabilize integrins in their
active state, triggering activation.

Importantly, our mechanical checkpoint model may explain how
platelets discriminate between soluble fibrinogen and platelet-
bound fibrinogen. The mechanical resistance of fibrinogen is likely
due to two parameters. First, soluble fibrinogen becomes immobile
once bound to integrins on the surface of activated platelets. Sec-
ond, fibrinogen rapidly polymerizes forming fibrin networks that
exist at significant mechanical stiffness (40). Both of these mecha-
nisms likely contribute to enhancing the fidelity of mechanical
testing of fibrinogen to avoid unintended platelet activation.
Platelet forces accumulate in two zones: a central region with
greater tension and a ring of peripheral signal along the lamel-
lipodial edge. This tension signal pattern resembles αIIbβ3-
integrin receptor distribution in platelets spread on a fibrinogen-
coated surface (41) and is distinct from the tension distribution in
nucleated cells that lack a central region of tension. We also
found that platelets produced a greater tension signal on cRGD
peptide compared with AGD peptide, both of which are widely
used in integrin αIIbβ3 studies. This difference in mechanical
response may be due to the weaker affinity of the AGD ligand
compared with the cRGD peptide. MLCK contributed to the
integrin tension at the cell edge, similar to its function in
nucleated cells. Interestingly, ROCK inhibition in nucleated cells leads
to the loss of stable focal adhesions and central stress fibers (42);
in contrast, ROCK regulates the central contractile forces in

Fig. 4. Cesation of platelet tension coincides with PS exposure. (A) Schematic of PS exposure on plate-
lets within a clot. (B) Time-lapse images of platelet tension and PS exposure on surface presenting 4.7-pN
tension probes. Annexin V-Alexa 488 was used to stain for PS. Regions of interest (ROIs) I and II high-
light two platelets during tension termination and PS exposure. The third platelet in the middle did not
generate a detectable PS signal. (Scale bar: 2 μm.) (C) A plot of tension and PS intensity for ROI I in B. The
term “Δt” is defined as the time difference between the time points of tension cessation and PS exposure.
(D) The histogram of Δt indicates that tension disappearance is concurrent with PS exposure; n = 23 cells from three experiments.
platelets but did not abolish tension signal. Note that the central zone of tension was not reported in previous TFM measurements (8). This is likely due to the low spatial resolution of TFM and its inability to read out forces normal to the substrate.

Whereas biological agonists such as thrombin, ADP, and epinephrine are known to activate platelet aggregation, our data show that the mechanical microenvironment plays a role in this process as well. Specifically, the data suggest that fibrinogen anchoring results in a positive feedback loop of platelet activation, which may further drive platelet aggregation contributing to the presence of groups of highly activated platelets within a nascent clot.

Using DNA-based probes, we show that the magnitude of tension generated by platelet integrins ranges from 4.7 to 19.3 μN with a subpopulation that exceeds 19.3 pN. Previous papers reporting on the contractile forces applied by single platelets indicate forces of 29 nN (AFM) and 34 nN (TFM) (7, 8). By estimating the number of hairpins unfolded by a single platelet, we calculate that the force per cell is between ~6 and ~24 nN, which is consistent with prior reports. Note that this is a lower limit of the total tension since values below F1/2 are not detected and forces exceeding F1/2 are underestimated.

We also determined the temporal relationship between PS exposure, a prerequisite for platelet-dependent thrombin generation, and mechanical signaling. PS presentation rapidly follows the termination of platelet contraction, suggesting that PS-exposing cells do not actively contribute to the clot retraction process. Therefore, the release of tension by PS-positive platelets may relax the strained clot. As fibrinolysis can be retarded in the strained clot, our results suggest that PS-positive platelets not only play a biochemical role in clot fibrinolysis, but also mechanically contribute to fibrinolysis.

Materials and Methods

The whole-blood samples were obtained from healthy blood donors, and consent was obtained according to the protocol (H15258) approved by the Institutional Review Board at Georgia Tech. Whole blood was drawn into collection tubes with acid citrate dextrose (ACD). Platelet-rich plasma (PRP) was collected after centrifugation under 150 × g for 15 min. Additional ACD was added into PRP (10% of the PRP volume), which was then centrifuged at 900 × g for another 5 min. Platelet-poor plasma was then removed, and the platelet pellet was resuspended in Tyrode’s buffer containing 0.1% BSA.

More detailed information about the materials and methods used in this study is provided in **SI Appendix, SI Materials and Methods**.

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