Supplementary information

Methods

Mouse blood collection, platelet preparation, aggregation and dense granule secretion assays

Mouse blood collection and platelet preparation were performed using standard methods as reported previously. Briefly, mice were sacrificed and blood collected immediately by cardiac puncture into a syringe containing ACD (2.5% Sodium citrate, 2% D-glucose and 1.5% citric acid) [at 1 (ACD): 9 (blood) ratio] for aggregation assays and in 4% citrate [at 1 (citrate): 9 (blood) ratio] for flow cytometry, clot retraction and thrombus formation assays. The blood was centrifuged at 203 g for 8 minutes in a fixed rotor centrifuge at room temperature and platelet-rich plasma (PRP) collected into fresh tubes. After addition of PGI₂ (12.5 ng/ml), the PRP was further centrifuged at 1028 g for 5 minutes. The resultant platelet pellet was resuspended in modified Tyrodes-HEPES buffer to the required density of platelets. The platelets were rested for 30 minutes before aggregation assays performed using collagen (Nycomed, Austria), cross-linked collagen-related peptide (CRP-XL, obtained from Prof R Farndale, University of Cambridge, UK) and thrombin (Sigma Aldrich, UK) by optical aggregometry. Dense granule secretion assays were performed using luciferin-luciferase luminescence substrate (Chrono-log, USA) as described previously.

Immunoblotting, immunoprecipitation and electron microscopy analysis

SDS-PAGE and immunoblotting were performed using standard protocols as described previously. Rabbit anti-EphB2 and rabbit anti-ephrinB1 (Santa Cruz Biotechnology, USA) antibodies were used to detect and immunoprecipitate EphB2 and ephrinB1 in mouse platelets respectively. Rabbit anti-14-3-3ζ (Santa Cruz Biotechnology, USA) was used as control for protein loading on immunoblots. Phospho-specific antibodies against various signalling proteins (PLCγ2 Y1197, integrin β3 Y773, Lyn Y507 and AKT S473) and non-phospho specific antibodies for proteins such as integrin β3 were obtained from Epitomics, USA. Anti-phosphotyrosine antibody 4G10 was obtained from Millipore, UK. The phospho-specific anti-Src Y418 antibody and secondary antibodies (Cy5® goat anti-rabbit IgG and Cy3® goat anti-mouse IgG) for immunoblotting were obtained from Invitrogen, UK. Rap1-GTP assay reagents were obtained from Millipore, UK and performed according to manufacturer’s protocols. Immunoprecipitation of proteins was performed following lysing of platelets with NP40 buffer and incubation with appropriate antibodies at a concentration of 1µg/ml and pureproteome™ protein A magnetic beads for 3-6 hours at 4ºC (Millipore, UK). Transmission electron microscopy analysis was performed as described previously.
**Flow cytometry**

The expression levels of various surface receptors such as GPVI, GPIbα, and integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ were measured on mouse platelets using FITC-conjugated antibodies (Emfret analytics, Germany). Similarly, CRP-XL- or thrombin- or ionomycin-stimulated fibrinogen binding and P-selectin exposure were measured in mouse whole blood using FITC-labelled rabbit anti-human fibrinogen antibodies (Dako UK Ltd) and FITC-conjugated P-selectin antibody (Emfret analytics, Germany) respectively.

Calcium mobilisation in mouse platelets was measured upon CRP-XL stimulation using flow cytometry. Three microliters of mouse whole blood was mixed with 122µl of Fluo-4 NW dye (Invitrogen, UK) and the final volume was made up to 225µl using HEPES buffered saline and incubated for 30 minutes at 37°C, and data acquisition initiated using an Accuri C6 flow cytometry (BD Accuri flow cytometers, USA). After 10 seconds of data collection, CRP-XL (1µg/ml) was injected into the well and data acquisition continued up to 5 minutes. The median fluorescence intensity and the rate of calcium mobilisation were calculated.

**Clot retraction**

Mouse PRP (200µl) was mixed with 5µl of red blood cells and the final volume raised to 1ml with modified Tyrodes-HEPES buffer as described previously. Fibrin clot formation was initiated by adding thrombin (1U/ml). Clot retraction around a glass capillary added prior to clot formation was observed over a period of 2 hours at room temperature. Clots were removed from glass capillary and weighed as a measure of clot retraction at different time points.

**Platelet spreading**

Washed mouse platelets were prepared at a density of $2\times10^7$ cells/ml and 200µl of suspension allowed to spread on fibrinogen (100µg/ml) coated cover-glass for 45 minutes. Unbound platelets were washed away using modified Tyrodes-HEPES buffer and adhered cells fixed using 2% formaldehyde in modified Tyrodes-HEPES buffer. Images were obtained using a Nikon A1-R Confocal microscope using 100X objective. Following optimisation of platelet density to ensure no platelet-platelet contact occurred during adhesion, multiple differential interference contrast images were obtained by Nikon eclipse microscope (TE2000-U) (Nikon instruments, UK) using 100X objective. The number of platelets in different stages of spreading such as initial adhesion, formation of filapodia, formation of lamellapodia and fully spread were analysed using ImageJ (NIH, USA).
Thrombus formation in vitro

DiOC₆ (Sigma Aldrich, UK) labelled mouse citrated blood was perfused over a collagen (400µg/ml) coated Vena8 BioChips (Cellix Ltd, Ireland) at a shear rate of 40 dynes/cm² as reported previously.¹² Z-stack images (wide field) of thrombi were obtained every 30 seconds for up to 10 minutes using a Nikon eclipse (TE2000-U) microscope (Nikon Instruments, UK) using 10X objective. Fluorescence intensity and thrombus volume were calculated by analysing the data using Slidebook5 software (Intelligent Imaging Innovations, USA).

In vivo thrombus formation

In vivo thrombus formation was performed as described previously.¹ Briefly, the mice were anesthetised by intraperitoneal injection of ketamine (125mg/kg), xylazine (12.5mg/kg) and atropine (0.25mg/kg) and maintained with 5mg/kg pentobarbital as required through a jugular vein cannula. The cremaster muscle was exteriorized following removal of connective tissues. Platelets were labelled with Alexa fluor®488 conjugated anti-mouse GPIb antibody (0.2µg/gram of mouse weight) (Emfret Analytics, Germany). The injury on the cremaster arteriole was induced with a Micropoint® Ablation Laser Unit (Andor technology plc). Thrombus formation was observed using an Olympus BX61W1 microscope (Olympus Imaging Ltd). Images were captured prior and after the injury by a Hamamatsu digital camera C9300 (Hamamatsu Photonics UK Ltd) charge-coupled device (CCD) camera and analyzed using Slidebook5 software (Intelligent Imaging Innovations).

Assessment of haemostasis

Mice (7–8 weeks old) were anesthetized using ketamine (80mg/kg) and xylazine (5mg/kg) administered via the intraperitoneal route 20 minutes prior to the experiment and placed on a heated mat at 37°C. 1mm of tail tip was removed using a scalpel blade and the tail tip was placed in sterile saline at 37°C. The time to cessation of bleeding into warmed saline was measured up to 10 minutes.

Statistical analysis

The data obtained from aggregation, fibrinogen binding, granule secretion, spreading and clot retraction assays were analysed using student T-test. Median fluorescence intensity values obtained in fibrinogen binding and granule secretion assays were converted into percentage for comparison. The data obtained from in vitro and in vivo thrombus formation assays were analysed using two-way Anova. The data obtained in calcium mobilisation experiments were analysed by ‘R’ statistical software (http://www.r-project.org/) and GraphPad Prism (version 5.04) from GraphPad Software Inc using a nonparametric (Mann-Whitney) test. Tail bleeding assay data were also analysed using the Mann-Whitney test.
References
