Published in final edited form as:

J Thromb Haemost. 2012 June ; 10(6): 1120–1132. doi:10.1111/j.1538-7836.2012.04715.x.

Targeted gene inactivation reveals a functional role of calpain-1 in platelet spreading

S. M. KUCHAY^{*,†,1}, A. J. WIESCHHAUS^{*,†,1}, M. MARINKOVIC[‡], I. M. HERMAN[‡], and A. H. CHISHTI^{*,‡}

^{*}Department of Pharmacology, University of Illinois College of Medicine, Chicago, IL

[†]Howard Hughes Medical Institute, New York University School of Medicine, New York, NY

[‡]Department of Molecular Physiology and Pharmacology, Sackler School Programs in Physiology, Pharmacology, and Microbiology, Tufts University School of Medicine, Boston, MA, USA

Summary

Background—Calpains are implicated in a wide range of cellular functions including the maintenance of hemostasis via the regulation of cytoskeletal modifications in platelets.

Objectives—Determine the functional role of calpain isoforms in platelet spreading.

Methods and Results—Platelets from calpain-1^{−/−} mice show enhanced spreading on collagen- and fibrinogen-coated surfaces as revealed by immunofluorescence, differential interference contrast (DIC) and scanning electron microscopy. The treatment of mouse platelets with MDL, a cell permeable inhibitor of calpains 1/2, resulted in increased spreading. The PTP1B-mediated enhanced tyrosine dephosphorylation in calpain-1^{−/−} platelets did not fully account for the enhanced spreading as platelets from the double knockout mice lacking calpain-1 and PTP1B showed only a partial rescue of the spreading phenotype. In non-adherent platelets, proteolysis and GTPase activity of RhoA and Rac1 were indistinguishable between the wild-type (WT) and calpain-1^{−/−} platelets. In contrast, the ECM-adherent calpain-1^{−/−} platelets showed higher Rac1 activity at the beginning of spreading, whereas RhoA was more active at later time points. The ECM-adherent calpain-1^{−/−} platelets showed an elevated level of RhoA protein but not Rac1 and Cdc42. Proteolysis of recombinant RhoA, but not Rac1 and Cdc42, indicates that RhoA is a calpain-1 substrate *in vitro*.

© 2012 International Society on Thrombosis and Haemostasis

Correspondence: Athar Chishti, Department of Molecular Physiology and Pharmacology, Tufts University School of Medicine, 136 Harrison Avenue, Room 701A, Boston, MA 02111, USA., Tel.: +1 312 355 1293; fax: +1 312 355 1297., athar.chishti@tufts.edu. Shafi Kuchay, Howard Hughes Medical Institute, New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA., Tel.: +1 212 263 9267; fax: +1 212 263 5107., shafi.kuchay@nyumc.org.

¹These authors contributed equally to this work.

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:

Figure S1. PTP1B null mouse platelet spreading.

Figure S2. Effect of MDL 28 170 on mouse and human platelet spreading.

Figure S3. Effect of MDL 28 170 on Rho GTPase proteolysis in non-adherent mouse platelets.

Figure S4. Effect of ROCK inhibitor Y27632 on wild type and calpain-1^{−/−} mouse platelet spreading.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Conclusions—Potentiation of the platelet spreading phenotype in calpain-1^{-/-} mice suggests a novel role of calpain-1 in hemostasis, and may explain the normal bleeding time observed in the calpain-1^{-/-} mice.

Keywords

calpain-1; platelets; proteolysis; PTP1B; RhoA; spreading

Introduction

Calpain-1 and calpain-2 are ubiquitously expressed cysteine proteases active in both human and murine platelets. We have previously shown that calpain-1 regulates platelet aggregation and clot retraction pathways through PTP1B by utilizing knockout mouse models [1]. The specific role of calpain-2 in platelet functions remains unclear as its genetic inactivation leads to embryonic lethality in mice [2]. The murine counterparts of human calpains share 88% amino acid sequence similarity suggesting a conserved function across species [3]. In mouse platelets, ~80% of total calpain protease activity is contributed by calpain-1, whereas calpain-2 accounts for the remaining 20% [4]. Previous studies have shown that calpain-1 is activated at micromolar calcium concentrations, whereas calpain-2 requires a millimolar calcium concentration for activation *in vitro*, although the precise calcium requirement for calpain activation *in vivo* remains poorly defined [5,6]. There is considerable evidence indicating that calpains can be regulated by a variety of signals originating from integrins and G-protein coupled receptors (GPCRs) leading to the mobilization of internal calcium and polyphosphoinositides [7–10].

An important aspect of platelet physiology is their ability to spread on extracellular matrix proteins (ECM) such as collagen and fibrinogen, thus sustaining normal hemostasis during wound healing [11]. The remodeling of the actin cytoskeleton during platelet spreading has been previously demonstrated implying a role of GTPases in the regulation of platelet spreading [12]. Similarly, a functional role of calpains in the remodeling of the actin cytoskeleton in nucleated cells has been proposed using small molecule inhibitors [13,14]. In the spreading cells, the role of small G protein family members such as Cdc42, Rac and RhoA has been extensively investigated during the formation of filopodia, lamellipodia and stress fibers [15,16]. Interestingly, calpains are known to modulate Rac and RhoA activation in the nucleated cells where active calpains have been localized at integrin clusters [16,17]. However, a precise function of calpain activity in the formation of focal adhesion complexes induced by Rac and RhoA remains controversial [18–20]. For example, Vav, an activator of Rho GTPases, is a known calpain substrate and has been implicated in platelet spreading [21]. The calpain-mediated cleavage of integrin β_3 was shown to suppress cell spreading in transfected Chinese hamster ovary (CHO) cells by promoting RhoA-mediated cell retraction [22]. In contrast, the amino-terminal cleavage of RhoA by calpains has been shown to generate a dominant negative form of RhoA that can potentially inhibit integrin-dependent cell spreading [17]. Moreover, tyrosine phosphorylation of integrin β_3 at its cytoplasmic tyrosine residue-759 was shown to suppress its susceptibility to calpain cleavage, and this resistance was suggested to enhance cell spreading [23]. These findings reflect the consequences of differential activation of calpain-1 and calpain-2 in both nucleated and enucleated cells.

One possible mechanism by which calpains may regulate platelet spreading is through the GPCR signaling pathway. The engagement of known GPCRs such as the protease-activated receptors (PAR's), thromboxane A₂ receptor (TPR) and ADP receptors (P2Y₁₂ and P2Y₁) leads to calcium mobilization resulting in platelet shape change and spreading [24–26]. Similarly, a functional role of calpains in thrombin-activated human platelet spreading has

been previously investigated using synthetic inhibitors of calpains [27]. In spite of these efforts, a precise role of individual calpain isoforms in the regulation of platelet spreading remains unclear at this stage.

As synthetic inhibitors of calpains often lack specificity [28,29], the availability of viable calpain-1 null mice provided us with an opportunity to investigate differential regulation of Rho GTPases by calpain-1 upon activation of integrin ($\alpha_{IIb}\beta_3$) and collagen ($\alpha_2\beta_1$ and GPVI) receptors. In the present study, we examined the functional consequence of calpain-1 deficiency in platelet spreading, and utilized platelets from the double knockout mice lacking calpain-1 and PTP1B to determine the role of PTP1B proteolysis in platelet spreading. Our findings revealed an enhanced spreading phenotype of calpain-1 null platelets adhering to fibrinogen- and collagen-coated surfaces. An increased PTP1B level in calpain-1 null platelets did not fully account for this phenotype as platelets from the double knockout mice failed to completely rescue the spreading defect. In the ECM-adherent calpain-1 null platelets, the Rac-1 activity is elevated at the beginning of spreading whereas the RhoA activity is enhanced at later time points, thus correlating with the increased level of RhoA protein. In contrast, activation of RhoA, Rac-1 and Cdc-42 was unaltered in the non-adherent calpain-1 null platelets, suggesting that calpains may not target Rho GTPases in non-adherent and resting mouse platelets.

Material and methods

Antibodies and reagents

Anti-Rho antibody (ARH01) from Cytoskeleton Inc., anti-Rac1 antibody from EMD Inc., anti β -actin antibody from Sigma and horseradish peroxidase-conjugated secondary antibodies were purchased from Upstate Biotechnology and Santa Cruz Biotechnology. Anti-Cdc42 antibody was kindly provided by Dr Dolly Mehta of UIC. MDL 28 170 was obtained from Aventis Pharmaceuticals. Bovine thrombin, Apyrase, Prostaglandin E_1 , calpain-1 (C6108), Leupeptin and Aprotinin were purchased from Sigma. Antifade (P36934) and Phalloidin- Alexa-594 were obtained from Molecular Probes (Eugene, OR, USA). Y27632 (688000) was purchased from EMD Biosciences (Billerica, MA, USA).

Isolation of mouse platelets

Mouse platelets were harvested by gel filtration as previously described [1]. Platelet count was adjusted to $0.2\text{--}20 \times 10^8 \text{ mL}^{-1}$, and platelets were allowed to rest at room temperature (RT) for 30 min. Resting platelets were supplemented with 1.0 mM CaCl_2 and 1.0 mM MgCl_2 before measurements.

Scanning electron microscopy

Glass cover slips were coated with fibrinogen ($200 \mu\text{g mL}^{-1}$) in 100 mM NaHCO_3 overnight at 4 °C. Gel-filtered platelets ($2 \times 10^7 \text{ mL}^{-1}$) from wild-type (WT) and calpain-1 null mice were spread for 30 min in a 37 °C incubator with 5% CO_2 . Platelets were fixed with 2% glutaraldehyde and 2% paraformaldehyde in 100 mM Cacodylate buffer (pH 7.2) for 10 min at RT. Dehydration of fixed platelets was carried out by stepwise immersion in ethanol gradient (25–100%), and samples were dried in a critical point dryer. Platelets were sputter-coated with Palladium, and viewed with a JEOL 5600LV scanning electron microscope (Peabody, MA, USA) at the UIC EM facility.

Spreading of mouse and human platelets

Glass cover slips were coated overnight at 4 °C with either fibrinogen ($200 \mu\text{g mL}^{-1}$) in 100 mM NaHCO_3 (pH 8.3) or rat collagen type 1 ($50 \mu\text{g mL}^{-1}$) in phosphate-buffered saline. Gel-filtered platelets ($2 \times 10^7 \text{ mL}^{-1}$) in modified Tyrode's buffer from WT and knockout

mice were spread for 1.5 and 15 min in a 37 °C incubator with 5% CO₂. Human platelets were harvested by gel filtration similar to mouse platelets. In experiments involving MDL28170, platelets were pre-incubated for 20 min with MDL before spreading for 30 min. In experiments involving ROCK inhibitor Y27632, mouse platelets were pre-incubated with 150 nM Y27632 ($K_i = 150$ nM) for 1 h before spreading for 30 min. For experiments with fluorescence and differential interference contrast (DIC) microscopy, platelets were fixed with 3% paraformaldehyde for 20 min, washed and permeabilized with 0.1% Triton X-100 for 30 min. Phalloidin staining was performed for 25 min. After washing, cover slips were dried and mounted on glass slides. Platelet spreading was visualized using a 100 × objective on the Nikon Eclipse TE2000-E microscope. DIC and fluorescence images were acquired with a CCD camera (Photometrics CoolSNAP™; Tucson, AZ, USA) attached to the microscope using METAMORPH® software (Molecular Devices, Sunnyvale, CA, USA). For quantification of platelet surface area, 10 random fields of acquired images were analyzed and statistical analysis was performed using the Student's *t*-test and one-way ANOVA.

Expression of recombinant Rho GTPases

Glutathione-S-transferase (GST) fusion proteins containing human RhoA, Rac1 and Cdc42 were expressed in DH5α *Escherichia coli*, and purified using standard protocols. Expression of recombinant proteins was confirmed with Western blotting using specific antibodies.

Proteolysis of Rho GTPases in platelets

Gel-filtered platelets (2×10^9 cells mL⁻¹) from WT and calpain-1 knockout mice were incubated with 2.0 μM calcium ionophore (A23187) at 37 °C with stirring. In experiments with MDL, platelets were incubated with 300 μM MDL for 30 min at RT prior to activation with A23187. Lysates of resting and aggregated platelets were prepared by the addition of SDS-sample buffer, and proteolysis of GTPases was assessed by Western blotting using specific antibodies. For *in vitro* proteolysis assays, 5 μg of recombinant GTPases was incubated with one unit of purified calpain-1 at 37 °C for up to 30 min. Proteolysis was initiated by the addition of 2 mM CaCl₂ to each reaction and 1.0 mM EDTA was used to chelate calcium as a negative control. Reactions were stopped by the addition of SDS-sample buffer.

Measurement of Rho GTPase activity in platelets

Platelets (2×10^9 cells mL⁻¹) from WT and calpain-1 knockout mice were reconstituted with mouse fibrinogen (20 μg mL⁻¹). Platelets were activated with thrombin (0.5 U mL⁻¹) for 1.0 min, and lysates were prepared in a lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 5% v/v glycerol, 1% Triton X-100, 1.0 mM EGTA, 1.5 mM MgCl₂, 1.0 mM sodium vanadate, 10 μg mL⁻¹ Leupeptin and 10 μg mL⁻¹ Aprotinin. Soluble lysates from resting and activated platelets were incubated with the GST-PBD for 45 min at 4 °C. Protein complex bound to beads was analyzed by Western blotting using an anti-Rac1 antibody. For RhoA activity measurements, soluble lysates were analyzed using the G-LISA kit according to manufacturer's protocol (Cytoskeleton Inc., Denver, CO, USA). For RhoA activity measurements in adherent platelets, the gel-filtered WT and calpain-1^{-/-} platelets were spread on fibrinogen for 5 and 25 min. Protein lysates (0.5 mg mL⁻¹) from adherent spread platelets were prepared and examined for RhoA activity using G-LISA. For measurements of Rac1 and Cdc42 activity, platelets were spread on fibrinogen for 2 and 45 min, and protein lysates (0.5 mg mL⁻¹) from adherent spread platelets were prepared and subjected to GST-PBD pull down assay. Bound protein complex was analyzed by Western blotting using an anti-Rac1 and Cdc42 antibodies.

Measurement of dense granule secretion in platelets

Platelet secretion was measured using the Luciferin/Luciferase reagent (Chrono-lume) to detect ATP release from platelet dense granules. WT and calpain-1^{-/-} gel-filtered platelets (2.5×10^8 platelets mL⁻¹) were incubated for 3 min at 37 °C before the addition of Chrono-Lume reagent. After 2 min of incubation, platelets were stimulated with specific agonists (0.6 µg mL⁻¹ collagen, 1 µM U46619, and 80 µM TRAP4), and platelet secretion response was recorded. All experiments were recorded in real time in a model 700 Chrono-log lumiaggregometer at 37 °C with stirring (8154 g).

Results

Calpain-1 null mouse platelets show enhanced platelet spreading

Previous findings from our laboratory have shown that platelet aggregation and clot retraction are compromised in calpain 1-null mice in spite of a normal tail bleeding phenotype [1,4]. To reconcile these findings, we examined the platelet spreading phenotype on fibrinogen- and collagen-coated surfaces. First, platelet spreading on fibrinogen-coated cover slips at 1.5, 15, and 30 min was visualized by scanning electron microscopy (SEM). The images show a significant increase in the spreading of calpain-1 null platelets (Fig. 1). Using DIC and fluorescence microscopy, enhanced spreading of calpain-1 null platelets was confirmed on both fibrinogen (Fig. 2) and collagen (Fig. 3) coated surfaces at 1.5- and 15-min time points, respectively. Calpain-1 null mice showed no change in the mean platelet volume (MPV) of their resting platelets as compared with WT counterparts (MPV in fL: Males: WT: 8.23 and KO 8.33; Females: WT 8.0 and KO 8.1).

Calpain-1 null mouse platelet spreading is not dependent on secretion

To investigate the dependence of the enhanced platelet spreading phenotype on granule secretion of calpain-1 null platelets adhering to extracellular matrix proteins (fibrinogen/collagen), spreading experiments were carried out in the presence of indomethacin and apyrase to block the action of secretory agonists. As expected, treatment with indomethacin and apyrase blocked WT platelet spreading upon adhesion to fibrinogen for 15 and 45 min (Fig. 4A, top panel). Under similar conditions, the enhanced spreading phenotype in calpain-1 null platelets was not affected by the treatment of either indomethacin or apyrase (Fig. 4A, lower panel). Similar results were obtained when the platelet spreading was carried out on the collagen-coated surface (data not shown). Our published data have indicated a mild dense granule secretion defect in the calpain-1 null mice, which were on mixed genetic background (C57BL/129SvJ) [4]. To revisit this observation, we backcrossed the calpain-1 null mice to 20 generations on the C57BL background, and analyzed their secretion phenotype. Again, the U46619- and TRAP4-induced ATP secretion was only slightly reduced, whereas the collagen-mediated dense granule secretion was not altered in the calpain-1 null platelets (Fig. 4B). Together, these results suggest that the enhanced spreading phenotype in calpain-1 null platelets is not regulated by platelet secretory pathways.

Partial rescue of enhanced platelet spreading in double knockout mice

Our previous findings have demonstrated that calpain-1 mouse platelets exhibit increased tyrosine dephosphorylation of multiple proteins as a result of the upregulation of PTP1B [1]. To further investigate if the increased PTP1B activity in calpain-1 null platelets can account for the enhanced spreading phenotype, platelet spreading was compared between WT, calpain-1 null- and double knockout (DKO) mice lacking both calpain-1 and PTP1B. Platelet spreading was examined on both fibrinogen- (Fig. 2) and collagen (Fig. 3)-coated surfaces. The enhanced platelet spreading phenotype in calpain-1 null mice was only partially rescued in the double knockout mice (Figs 2 and 3, Table 1), suggesting a minor

role of elevated PTP1B in the regulation of the platelet spreading phenotype under these conditions.

PTP1B null mice show reduced platelet spreading

To further examine whether PTP1B alone can modulate the platelet spreading phenotype, we evaluated platelet spreading of PTP1B null mice on the fibrinogen-coated surface. The PTP1B null platelets showed reduced spreading (Fig. S1), consistent with the previously published findings [30]. As the enhanced platelet spreading phenotype in calpain-1 null mice is only partially rescued in the double knockout platelets lacking both calpain-1 and PTP1B (Figs 2 and 3, Table 1), we conclude that the stimulatory effect of calpain-1 deletion on platelet spreading is only in part mediated by the regulation of PTP1B.

Quantification of platelet spreading phenotype

Platelet spreading on the extracellular matrix does not follow a homogenous spreading pattern, and usually shows variations in the spreading phenotype at a given time point. To quantify the spreading phenotype, we performed a detailed comparative analysis of the WT, calpain-1 null and DKO platelets spreading on fibrinogen and collagen surfaces for 1.5 and 15 min to capture both early and late events of cell spreading. As summarized in Table 1, statistical analysis and quantification of platelet surface area ($\mu\text{m}^2 \pm \text{SD}$) showed that the average surface area of WT platelets spreading for 1.5 and 15 min on the fibrinogen surface was 6.9 ± 4.0 and 9.4 ± 5.1 , respectively. Similarly, quantification of calpain-1 null platelets for 1.5 and 15 min indicated the average surface area of 11.4 ± 6.0 and 19.2 ± 9.2 , respectively. In the DKO platelets, the average surface area was estimated as 9.8 ± 3.8 and 14.3 ± 6.1 at 1.5 and 15 min, respectively.

To further investigate the relative extent of platelet spreading at each time point, we compared the percentage of platelets of each genotype that had spread either greater or equal to the average surface area of WT and calpain-1 null platelets. After 1.5 min of spreading, ~34% of WT platelets showed a surface area that was greater than their average surface area, whereas only ~12% of WT platelets spread more than the average surface area of calpain-1 null platelets. In calpain-1 null mice, ~36% of platelets spread greater than their average surface area, whereas ~74% of calpain-1 null platelets showed a surface area that is greater than the average WT platelet surface area. Similarly, in the double knockout mice, ~75% of platelets displayed a surface area that is greater than the average surface area of WT platelets, but only ~30% of platelets showed a surface area that is greater than the average surface area of calpain-1 null platelets. A one-way ANOVA analysis of variance ($P < 0.0001$) and Student's *t*-test indicated significant differences between these groups. A similar analysis was performed on platelets after 15 min of spreading on the fibrinogen surface, and spreading for 1.5 and 15 min on the collagen surface (Table 1).

Pharmacological inhibition of calpains exerts opposite effects on spreading in mouse and human platelets

The treatment of WT mouse platelets with MDL enhanced the platelet spreading phenotype on glass- (Fig. S2A), collagen- (Fig. S2C) and fibrinogen-coated surfaces (Fig. 5). Notably, the mouse platelet spreading phenotype was more pronounced on the ECM-coated surfaces as compared with the glass. These results are consistent with the enhanced platelet spreading phenotype in the calpain-1 null mice. Surprisingly, human platelets treated with MDL under similar conditions showed inhibition of spreading on glass- (Fig. S2B, top), fibrinogen- (Fig. S2B, lower) and collagen-coated surface (Fig. S2D). In addition, pre-incubation of MDL-treated WT platelets (Fig. S2C) and human platelets (Fig. S2D,E) with thrombin also produced opposite phenotypes on both collagen and fibrinogen. Thus, pharmacological inhibition of both calpains with MDL exerts opposite effects on spreading in mouse and

human platelets under these conditions. The precise molecular basis of the differential effects of MDL on platelets is not known at this stage. It is to be noted that pharmacological inhibition of calpains by MDL is predicated on the assumption that this inhibitor shows strict specificity for platelet calpains in two species. A potential caveat of utilizing only the pharmacological approach for calpain inhibition is illustrated by calpeptin, another peptidyl calpain inhibitor similar to MDL, which is known to inhibit SHP-2, a major tyrosine phosphatase, in addition to calpains [28,29].

Substrate specificity of Rho GTPases

Rescue experiments suggested that PTP1B activity does not play a major role in the calpain-1-mediated platelet spreading phenotype, a finding that is distinct from the effects of PTP1B on platelet aggregation and clot retraction pathways [1]. As Rho GTPases are known to play important roles in the regulation of cellular shape change, including cell spreading, we examined the proteolytic sensitivity of Rac1, Cdc42 and RhoA by calpain-1 *in vitro*. The results indicate that Rac1 and Cdc42 are not cleaved by calpain-1 *in vitro* (Fig. 6A, lower two panels). In contrast, RhoA was completely degraded by calpain-1 *in vitro* (Fig. 6A, top panel). As expected, chelation of calcium with EDTA blocked RhoA proteolysis by calpain-1. This observation was confirmed by Western blotting using an anti-GST antibody, which detected the degradation products of RhoA (Fig. 6B) but not of Rac1 (Fig. 6C).

Proteolysis of Rho GTPases in non-adherent platelets

Proteolysis experiments indicated that calpain-1 degraded GST-RhoA, but not GST-Rac1 and GST-Cdc42 under *in vitro* conditions (Fig. 6). To investigate this observation in mouse platelets, the total protein content of RhoA, Rac1 and Cdc42 was compared in non-adherent WT and calpain-1 null platelets under resting and activated conditions. Activation of WT platelets with calcium ionophore (A23187) induced proteolysis of RhoA (Fig. 7A), consistent with published evidence of RhoA cleavage in aggregating platelets [17]. Interestingly, proteolysis of RhoA was similar in the WT and calpain-1 null platelets (Fig. 7A). This observation suggests that in calpain-1 null platelets either calpain-2 or as yet an unknown protease compensates for calpain-1 deficiency resulting in the proteolysis of RhoA. In contrast to the *in vitro* data, proteolysis of both Rac1 and Cdc42 was detected in the mouse platelets activated with calcium ionophore, A23187 (Fig. 7B,C). Again, there was no difference in the proteolysis of Rac1 and Cdc42 in the WT and calpain-1 null platelets under these conditions (Fig. 7B,C). These observations suggest that Rac1 and Cdc42 are degraded by either calpain-2 or as yet an unknown protease in mouse platelets activated by calcium ionophore, A23187. However, MDL did not protect Rho GTPases from the calcium ionophore-induced proteolysis in both WT and calpain-1 null platelets under non-adherent conditions (Fig. S3). These findings suggest that calpains are unlikely to mediate proteolysis of Rho GTPases in non-adherent mouse platelets. These observations are consistent with similar protein content and degradation profile of RhoA, Rac1 and Cdc42 in non-adherent calpain-1 null platelets. Thus, the molecular pathways regulating proteolysis of Rho GTPases in non-adherent platelets remain unknown.

Activation of Rho GTPases in non-adherent platelets

To examine if the activation of Rho GTPases is altered in the calpain-1 null platelets, RhoA activity was measured in the resting and thrombin-activated platelet lysates supplemented with fibrinogen to induce integrin activation. The activity of RhoA was similar in the WT and calpain-1 null platelets upon thrombin activation (Fig. 7D). Next, the activity of Rac1 was measured in the resting and thrombin-activated platelet lysates. Mouse platelets were stimulated for 1.0 min with 0.5 U mL⁻¹ of thrombin, and the GTP-bound Rac1 was recovered by affinity purification using the GST-PBD domain of PAK1. Again, Rac1

activation was similar in the WT and calpain-1 null platelets under these conditions (Fig. 7E). Several attempts to pull down activated Cdc42 were unsuccessful, and therefore the status of Cdc42 activity in non-adherent platelets remains unknown at this stage. Together, these findings suggest that total protein content and activity of RhoA and Rac1 remain unaltered in the calpain-1 null platelets under non-adherent conditions.

Proteolysis and activation of Rho GTPases in ECM-adherent platelets

As proteolysis of Rho GTPases was not altered in the calpain-1 null platelets under non-adherent conditions, we examined the status of Rho GTPases in adherent platelets spreading on the fibrinogen-coated surface. Biochemical analysis at both the early and late stages of platelet spreading showed a significant increase in the total protein content of RhoA in the calpain-1 null platelets (Fig. 8A). In contrast, a similar analysis for Rac1 and Cdc42 in adherent platelets showed no change in their protein content between WT and calpain-1 null platelets (Fig. 8B,C). These findings suggest that calpain-1 specifically regulates RhoA protein content in the adherent mouse platelets spreading on the fibrinogen-coated surface.

It is well recognized that the ‘inside-out’ signaling makes a significant contribution to the fibrinogen-supplemented platelets under non-adherent conditions. Therefore, it is likely that the activity of Rho GTPases in non-adherent platelets is not reflective of their function in platelets spreading on the immobilized ligands where the ‘outside-in’ signaling predominates [7]. To examine this issue further, we measured the activation RhoA, Rac-1 and Cdc42 on immobilized fibrinogen at both the early and late stages of platelet spreading. In spite of the increased level of RhoA protein, the RhoA activity was similar in the WT and calpain-1 null platelets spreading for 5 min (Fig. 8D). In contrast, the activity of RhoA was increased by ~26% in the calpain-1 null platelets spreading for 25 min on the fibrinogen-coated surface, consistent with the increased level of RhoA protein. Interestingly, the activity of Rac1 was enhanced in the calpain-1 null platelets spreading for 2 min, whereas no measurable difference was observed at later time points between WT and calpain-1 null platelets (Fig. 8E). The activity of Cdc42 was not altered in the calpain-1 null platelets under adherent conditions (Fig. 8F). Together, these findings suggest that a positive correlation exists between the enhanced platelet spreading phenotype and increased Rac-1 activity at early and RhoA activation at late stages of spreading in the calpain-1 null platelets.

Finally, we examined the effect of ROCK inhibitor Y27632 to determine if the enhanced spreading phenotype observed in calpain-1 null platelets can be reversed by blocking the downstream signaling pathways of RhoA. As expected, the treatment of WT platelets with 150 nM Y27632 inhibited spreading (Fig. S4, top panel) whereas the inhibitory effect of Y27632 was not as pronounced in the calpain-1 null platelets (Fig. S4, bottom panel). This finding is consistent with the enhanced RhoA activity in the calpain-1 null platelets, which may require a significantly higher concentration of Y27632 to achieve the same level of inhibition of platelet spreading under these conditions. It is noteworthy that our data also show an increase in the abundance of actin-rich nodules in the calpain-1 null platelets (Fig. S4, bottom panel). This observation is consistent with the published data on actin nodule formation [12], and enhanced RhoA activity in the calpain-1 null platelets (Fig. 8D).

Discussion

There is considerable interest in understanding the physiological role of calpains in platelets [1,4,27,31–37]. Previous generation of calpain-1 null mice in our laboratory [4] led to the demonstration that calpain-1 regulates platelet signaling by suppressing tyrosine dephosphorylation of key platelet proteins [1]. PTP1B, a major tyrosine phosphatase, was identified as a physiological target of calpain-1, and upregulation of PTP1B protein in calpain-1 null platelets correlated with alterations in platelet aggregation, clot retraction and

the tyrosine dephosphorylation cascade [1]. Interestingly, in spite of reduced platelet aggregation and clot retraction defects, the tail bleeding times were nearly normal in the calpain-1 null mice [4]. To reconcile these observations, we investigated the role of calpain-1 in platelet spreading using the calpain-1 null mice to better understand the physiological function of calpain-1 in normal platelet spreading.

Our results demonstrate that calpain-1 null mouse platelets exhibit enhanced spreading on glass-, collagen- and a fibrinogen- coated surface as compared with the WT mouse platelets (Figs 1–3 and 5). As the calpain-1 null platelets express an elevated level of PTP1B, the enhanced spreading phenotype we observed in the present study could be explained by prior findings showing reduced platelet spreading in the PTP1B null mice [30]. We also observed reduced platelet spreading in a different model of PTP1B null mice (Fig. S1). This PTP1B null mouse model was originally used to generate the double knockout mice lacking calpain-1 (DKO). However, the enhanced platelet spreading phenotype observed in the calpain-1 null mice was only partially rescued in the DKO mice lacking calpain-1 and PTP1B (Figs 2 and 3, Table 1). These results suggested the existence of PTP1B-independent substrates of calpain-1 that can potentially regulate the platelet spreading phenotype in mice. Moreover, the pharmacological inhibition of platelet secretion also failed to rescue the enhanced spreading phenotype in the calpain-1 null platelets, suggesting this phenotype is regulated by platelet secretion independent pathways (Fig. 4A).

During the course of these studies, an unexpected finding was the observation that MDL exerts differential effects on the spreading of mouse and human platelets. Our results indicate that pharmacological inhibition of calpains with MDL enhanced the spreading of mouse platelets, consistent with the enhanced spreading phenotype observed in the calpain-1 null platelets (Figs 1–3, 5 and S2A,C). Conversely, MDL treatment inhibited the spreading phenotype in human platelets under the same conditions (Fig. S2B,D,E). The MDL-mediated inhibition of spreading in human platelets is consistent with previous evidence demonstrating reduced spreading of human platelets treated with pharmacological inhibitors of calpains [27]. Although the precise reason for these differential effects is not known at this stage, it is noteworthy that human and mouse platelets are known to display marked differences in their spreading pattern on fibrinogen [38]. Differential expression of PAR1 and PAR3 receptors in human and mouse platelets, respectively, may endow differential signaling downstream of the G-protein coupled pathways on platelet spreading [39–41]. Recently, a direct comparison of human and mouse platelet transcriptome by genome-wide RNA-seq analysis showed that human and mouse platelets contain differential expression of a wide variety of RNA transcripts, although most of the orthologs are shared between the two species [42]. In contrast to human platelets, which express robust amount of TIMP1 transcripts, this matrix metalloproteinase inhibitor expression is undetectable in mouse platelets. Without question, such significant differences in protease inhibitor activity could significantly impact murine vs. human platelet adhesion, spreading and/or hemostatic function [42]. Thus, future elucidation of the mechanisms underlying differential effects of MDL in human and mouse platelet spreading would entail proteomics and candidate-based screens of the inhibitor-treated platelets between two species.

Rescue experiments using the DKO mice suggested that the calpain-1-mediated effect on mouse platelet spreading is only partially dependent on the activity of PTP1B (Figs 2 and 3, Table 1). This partial rescue of the platelet spreading phenotype by PTP1B is reminiscent of the partial rescue of thrombosis in the DKO mice [1]. To investigate alternate mechanisms, we examined the status of Rho GTPases previously known to regulate multiple cellular spreading phenotypes [15]. Our results indicate that proteolysis of Rac1, Cdc42 and RhoA is not altered in the non-adherent calpain-1 null platelets activated by calcium ionophore (Fig. 7). In fact, proteolysis of Rho GTPases is still evident in non-adherent mouse platelets when

both calpains are inhibited by MDL (Fig. S3). Moreover, the activity of RhoA and Rac-1 is not altered in the calpain-1 null platelets consistent with the proteolysis status of Rho GTPases in non-adherent and resting platelets (Fig. 7). These observations suggest that calpains do not play a major role in targeting Rho GTPases in non-adherent mouse platelets.

Further biochemical analysis of Rho GTPases in platelets spreading on the fibrinogen surface revealed an elevated level of RhoA but not Rac-1 or Cdc42 in the calpain-1 null platelets (Fig. 8). The activity of Rac-1 was increased during the early phase of cell spreading whereas the activation of RhoA was amplified at the later stage of platelet spreading. The observed increase in total RhoA is consistent with the enhanced RhoA activity in the calpain-1 null platelets. However, the mechanism of calpain-1-mediated regulation of Rac-1 activity during the early phase of platelet spreading is not known at this time. There remains a possibility that activity of a specific Rho GTPase might be altered in the calpain-1 null platelets owing to the modulation of a particular GEF, GAP and/or unknown adaptor proteins. Together, our results are consistent with the notion that calpains can translocate to their sites under non-adherent conditions, but the ECM-adhesion is required for full activation of calpains [7].

Based on findings reported in the present study, and previously published data, we propose a model of calpain-1-mediated regulation of mouse platelet spreading on the ECM surface (Fig. 9). Calpain-activation leads to limited cleavage, release and activation of PTP1B. Activated calpain-1 also causes degradation of RhoA and activated PTP1B. The observed activation of Rac-1 at the beginning of platelet spreading is limited by calpain-1, presumably via a regulator of Rac1. Activation of PTP1B and increased Rac1 activity at the beginning of platelet spreading and subsequent activation of RhoA at the later stage of spreading contributes to the increased spreading phenotype observed in the calpain-1 null platelets. Thus, activation of RhoA and Rac1 plays a key regulatory role in coordinating the spreading of calpain-1 null mouse platelets on extracellular matrix substrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Professor Guy Le Breton of UIC for many helpful comments and suggestions throughout the course of this study. We also thank Dr Brian P. Kennedy of Merck Frosst Canada for sharing the PTP1B null mice. We appreciate Dr Jeff Skaar of NYU School of Medicine for his help with the proof reading of the manuscript. Financial support from the NIH grants HL089517 and HL095050 to AHC is acknowledged.

References

1. Kuchay SM, Kim N, Grunz EA, Fay WP, Chishti AH. Double knockouts reveal that protein tyrosine phosphatase 1B is a physiological target of calpain-1 in platelets. *Mol Cell Biol.* 2007; 27:6038–52. [PubMed: 17576811]
2. Dutt P, Croall DE, Arthur JS, Veyra TD, Williams K, Elce JS, Greer PA. m-Calpain is required for preimplantation embryonic development in mice. *BMC Dev Biol.* 2006; 6:3. [PubMed: 16433929]
3. Goll DE, Thompson VF, Li H, Wei W, Cong J. The calpain system. *Physiol Rev.* 2003; 83:731–801. [PubMed: 12843408]
4. Azam M, Andrabi SS, Sahr KE, Kamath L, Kuliopulos A, Chishti AH. Disruption of the mouse mu-calpain gene reveals an essential role in platelet function. *Mol Cell Biol.* 2001; 21:2213–20. [PubMed: 11238954]
5. Jacquemond V. Indo-1 fluorescence signals elicited by membrane depolarization in enzymatically isolated mouse skeletal muscle fibers. *Biophys J.* 1997; 73:920–8. [PubMed: 9251808]

6. Maravall M, Mainen ZF, Sabatini BL, Svoboda K. Estimating intracellular calcium concentrations and buffering without wavelength ratioing. *Biophys J*. 2000; 78:2655–67. [PubMed: 10777761]
7. Fox JE, Taylor RG, Taffarel M, Boyles JK, Goll DE. Evidence that activation of platelet calpain is induced as a consequence of binding of adhesive ligand to the integrin, glycoprotein IIb–IIIa. *J Cell Biol*. 1993; 120:1501–7. [PubMed: 8449989]
8. Lokuta MA, Nuzzi PA, Huttenlocher A. Calpain regulates neutrophil chemotaxis. *Proc Natl Acad Sci USA*. 2003; 100:4006–11. [PubMed: 12649322]
9. Saido TC, Shibata M, Takenawa T, Murofushi H, Suzuki K. Positive regulation of mu-calpain action by polyphosphoinositides. *J Biol Chem*. 1992; 267:24585–90. [PubMed: 1332961]
10. Du X, Saido TC, Tsubuki S, Indig FE, Williams MJ, Ginsberg MH. Calpain cleavage of the cytoplasmic domain of the integrin beta 3 subunit. *J Biol Chem*. 1995; 270:26146–51. [PubMed: 7592818]
11. Watson SP. Platelet activation by extracellular matrix proteins in haemostasis and thrombosis. *Curr Pharm Des*. 2009; 15:1358–72. [PubMed: 19355974]
12. Calaminus SD, Thomas S, McCarty OJ, Machesky LM, Watson SP. Identification of a novel, actin-rich structure, the actin nodule, in the early stages of platelet spreading. *J Thromb Haemost*. 2008; 6:1944–52. [PubMed: 18761725]
13. Potter DA, Tirnauer JS, Janssen R, Croall DE, Hughes CN, Fiacco KA, Mier JW, Maki M, Herman IM. Calpain regulates actin remodeling during cell spreading. *J Cell Biol*. 1998; 141:647–62. [PubMed: 9566966]
14. Shuster CB, Herman IM. Indirect association of ezrin with F-actin: isoform specificity and calcium sensitivity. *J Cell Biol*. 1995; 128:837–48. [PubMed: 7876308]
15. Fox JE. On the role of calpain and Rho proteins in regulating integrin-induced signaling. *Thromb Haemost*. 1999; 82:385–91. [PubMed: 10605728]
16. Kulkarni S, Saido TC, Suzuki K, Fox JE. Calpain mediates integrin-induced signaling at a point upstream of Rho family members. *J Biol Chem*. 1999; 274:21265–75. [PubMed: 10409684]
17. Kulkarni S, Goll DE, Fox JE. Calpain cleaves RhoA generating a dominant-negative form that inhibits integrin-induced actin filament assembly and cell spreading. *J Biol Chem*. 2002; 277:24435–41. Epub 2002 Apr 18. [PubMed: 11964413]
18. Beckerle MC, Burridge K, DeMartino GN, Croall DE. Colocalization of calcium-dependent protease II and one of its substrates at sites of cell adhesion. *Cell*. 1987; 51:569–77. [PubMed: 2824061]
19. Bialkowska K, Kulkarni S, Du X, Goll DE, Saido TC, Fox JE. Evidence that beta3 integrin-induced Rac activation involves the calpain-dependent formation of integrin clusters that are distinct from the focal complexes and focal adhesions that form as Rac and RhoA become active. *J Cell Biol*. 2000; 151:685–96. [PubMed: 11062268]
20. Huttenlocher A, Palecek SP, Lu Q, Zhang W, Mellgren RL, Lauffenburger DA, Ginsberg MH, Horwitz AF. Regulation of cell migration by the calcium-dependent protease calpain. *J Biol Chem*. 1997; 272:32719–22. [PubMed: 9407041]
21. Miyakawa Y, Oda A, Druker BJ, Ozaki K, Handa M, Ohashi H, Ikeda Y. Thrombopoietin and thrombin induce tyrosine phosphorylation of Vav in human blood platelets. *Blood*. 1997; 89:2789–98. [PubMed: 9108397]
22. Flevaris P, Stojanovic A, Gong H, Chishti A, Welch E, Du X. A molecular switch that controls cell spreading and retraction. *J Cell Biol*. 2007; 179:553–65. [PubMed: 17967945]
23. Xi X, Flevaris P, Stojanovic A, Chishti A, Phillips DR, Lam SC, Du X. Tyrosine phosphorylation of the integrin beta 3 subunit regulates beta 3 cleavage by calpain. *J Biol Chem*. 2006; 281:29426–30. Epub 2006 Aug 25. [PubMed: 16935858]
24. Brace LD, Venton DL, Le Breton GC. Thromboxane A2/prostaglandin H2 mobilizes calcium in human blood platelets. *Am J Physiol*. 1985; 249(1 Pt 2):H1–7. [PubMed: 3925795]
25. Mazharian A, Roger S, Berrou E, Adam F, Kauskot A, Nurden P, Jandrot-Perrus M, Bryckaert M. PAR4 induces full platelet spreading on a fibrinogen matrix: involvement of ERK2 and p38 and Ca²⁺ mobilization. *J Biol Chem*. 2007; 282:2.

26. Soulet C, Hechler B, Gratacap MP, Plantavid M, Offermanns S, Gachet C, Payrastre B. A differential role of the platelet ADP receptors P2Y₁ and P2Y₁₂ in Rac activation. *J Thromb Haemost.* 2005; 3:2296–306. [PubMed: 16194206]
27. Croce K, Flaumenhaft R, Rivers M, Furie B, Furie BC, Herman IM, Potter DA. Inhibition of calpain blocks platelet secretion, aggregation, and spreading. *J Biol Chem.* 1999; 274:36321–7. [PubMed: 10593923]
28. Schoenwaelder SM, Burrige K. Evidence for a calpeptin-sensitive protein-tyrosine phosphatase upstream of the small GTPase Rho. A novel role for the calpain inhibitor calpeptin in the inhibition of protein-tyrosine phosphatases. *J Biol Chem.* 1999; 274:14359–67. [PubMed: 10318859]
29. Schoenwaelder SM, Petch LA, Williamson D, Shen R, Feng GS, Burrige K. The protein tyrosine phosphatase Shp-2 regulates RhoA activity. *Curr Biol.* 2000; 10:1523–6. [PubMed: 11114521]
30. Arias-Salgado EG, Haj F, Dubois C, Moran B, Kasirer-Friede A, Furie BC, Furie B, Neel BG, Shattil SJ. PTP-1B is an essential positive regulator of platelet integrin signaling. *J Cell Biol.* 2005; 170:837–45. [PubMed: 16115959]
31. Brass LF, Shattil SJ. Inhibition of thrombin-induced platelet activation by leupeptin. Implications for the participation of calpain in the initiation of platelet activation. *J Biol Chem.* 1988; 263:5210–6. [PubMed: 2833498]
32. Ezumi Y, Takayama H, Okuma M. Differential regulation of protein-tyrosine phosphatases by integrin alpha IIb beta 3 through cytoskeletal reorganization and tyrosine phosphorylation in human platelets. *J Biol Chem.* 1995; 270:11927–34. [PubMed: 7538126]
33. Fox JE. Cytoskeletal proteins and platelet signaling. *Thromb Haemost.* 2001; 86:198–213. [PubMed: 11487008]
34. Fox JE, Austin CD, Reynolds CC, Steffen PK. Evidence that agonist-induced activation of calpain causes the shedding of procoagulant-containing microvesicles from the membrane of aggregating platelets. *J Biol Chem.* 1991; 266:13289–95. [PubMed: 2071604]
35. Schoenwaelder SM, Yuan Y, Cooray P, Salem HH, Jackson SP. Calpain cleavage of focal adhesion proteins regulates the cytoskeletal attachment of integrin alphaIIb beta3 (platelet glycoprotein IIb/IIIa) and the cellular retraction of fibrin clots. *J Biol Chem.* 1997; 272:1694–702. [PubMed: 8999848]
36. Schoenwaelder SM, Yuan Y, Jackson SP. Calpain regulation of integrin alpha IIb beta 3 signaling in human platelets. *Platelets.* 2000; 11:189–98. [PubMed: 10938897]
37. Cooray P, Yuan Y, Schoenwaelder SM, Mitchell CA, Salem HH, Jackson SP. Focal adhesion kinase (pp125FAK) cleavage and regulation by calpain. *Biochem J.* 1996; 318(Pt 1):41–7. [PubMed: 8761450]
38. McCarty OJ, Larson MK, Auger JM, Kalia N, Atkinson BT, Pearce AC, Ruf S, Henderson RB, Tybulewicz VL, Machesky LM, Watson SP. Rac1 is essential for platelet lamellipodia formation and aggregate stability under flow. *J Biol Chem.* 2005; 280:39474–84. [PubMed: 16195235]
39. Sambrano GR, Weiss EJ, Zheng YW, Huang W, Coughlin SR. Role of thrombin signalling in platelets in haemostasis and thrombosis. *Nature.* 2001; 413:74–8. [PubMed: 11544528]
40. Leger AJ, Covic L, Kuliopulos A. Protease-activated receptors in cardiovascular diseases. *Circulation.* 2006; 114:1070–7. [PubMed: 16952995]
41. Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Invest.* 1999; 103:879–87. [PubMed: 10079109]
42. Rowley JW, Oler AJ, Tolley ND, Hunter BN, Low EN, Nix DA, Yost CC, Zimmerman GA, Weyrich AS. Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. *Blood.* 2011; 118:e101–11. [PubMed: 21596849]

Fig. 1. The effect of calpain-1 deficiency on platelet spreading. Gel-filtered wild-type (WT, top row) and calpain-1 null (calpain-1^{-/-}, bottom row) platelets spread on fibrinogen for 1.5 (lane 1), 15 (lane 2) and 30 (lane 3) minutes. Scanning electron microscopy (SEM) was performed as described in methods. Photographs show representation of two independent experiments. Bar = 2 μm.

Fig. 2. Platelet spreading on fibrinogen. Gel-filtered wild-type (WT), calpain- 1^{-/-}, and double knockout (DKO) platelets spreading on fibrinogen for (A) 1.5 and (B) 15 minutes. F-actin was detected with Phalloidin-conjugate to visualize platelets. Images of platelets were captured by fluorescence microscopy (top) and DIC (bottom). Photographs taken with 100 × objective and Nikon Eclipse TE2000-E microscope are representative of multiple fields from each platelet sample out of three independent experiments.

Fig. 3. Platelet spreading on collagen. Gel-filtered wild-type (WT), calpain- 1^{-/-} and double knockout (DKO) platelets spreading on collagen for (A) 1.5 and (B) 15 minutes. F-actin was detected with Phalloidin-conjugate to visualize platelets. Images of platelets were captured by fluorescence microscopy (top) and DIC (bottom). Photographs taken with 100 × objective and Nikon Eclipse TE2000-E microscope are representative of multiple fields from each platelet sample out of three independent experiments.

Fig. 4.

Enhanced spreading in calpain-1 null platelets is not driven by secretion. (A) Gel-filtered wild-type (WT) (top panels), calpain-1^{-/-} (lower panels) platelets spreading on fibrinogen in the presence of Indomethacin (10 μM) and Apyrase (2 U mL⁻¹) for 15 and 45 minutes. F-actin in paraformaldehyde-fixed platelets was detected by Phalloidin-conjugate. Photographs of platelets, taken with 100 × objective and Nikon Eclipse TE2000-E microscope, are representative of multiple fields from each platelet sample out of three independent experiments. Bar size = 5 μm. (B) ATP secretion from platelet dense granules of WT and Calpain-1^{-/-} gel-filtered platelets (2.5 × 10⁸ mL⁻¹) was measured using the Luciferin/Luciferase reagent (Chrono-lume). Agonists used to induce secretion from platelet dense granules were U48819 (1.0 μM), TRAP4 (80 μM) and collagen (0.6 μg mL⁻¹). AU, arbitrary units

Fig. 5.

The effect of MDL 28170 on mouse platelet spreading. Gel-filtered wild-type platelets were incubated with 0.1% dimethylsulfoxide (DMSO) (control, left panels) and 400 μM MDL in DMSO (right panels), and spread on fibrinogen. Platelets were stained for polymerized actin using Phalloidin. Images are representative of three independent experiments from multiple fields from each platelet sample. Bar graph shows quantification of WT platelet surface area of DMSO and MDL-treated platelets using METAMORPH software. Bar size = 10 μm.

Fig. 6.

Substrate specificity of recombinant Rho GTPases. (A) Immunoblotting was performed by anti-RhoA (top), anti-Rac1 (middle) and anti-Cdc42 (bottom). Lane 1 shows protein amount of each GTPase. Lanes 2–4 show the amount of GTPase left after incubation with calpain-1 (min), and lane 5 shows the GTPase amount after inhibition of calpain-1 with EDTA. (B) Immunoblotting of GST-RhoA by anti-GST antibody. Lane 1 shows GST-RhoA used for proteolysis. Lanes 2–4 show GST-RhoA left after incubation with calpain-1. Degradation products of RhoA are shown in lanes 2 and 3. (C) Immunoblotting of GST-Rac1 using an anti-GST antibody. Lane 1 shows GST-Rac1 used for proteolysis. Lanes 2–4 show GST-Rac1 after incubation with calpain-1.

Fig. 7.

Proteolysis and activation of Rho GTPases in non-adherent platelets. (A) Resting (bottom panel, lanes 1, 2) and calcium ionophore-activated (bottom panel, lanes 3, 4) platelet lysates from the wild-type (WT) (+/+, lanes 1, 3) and calpain-1^{-/-} (-/-, lanes 2, 4) were probed with an anti-RhoA monoclonal antibody. A doublet of RhoA, the lower band represents the N-terminally cleaved RhoA, is seen in the ionophore-activated platelets (lanes 3, 4). Top panel shows the corresponding Ponceau-stained image. (B) Resting (bottom panel, lanes 1, 2) and calcium ionophore-activated (bottom panel, lanes 3, 4) platelet lysates from the WT (+/+, lanes 1, 3) and calpain-1^{-/-} (-/-, lanes 2, 4) were probed with an anti-Rac1 monoclonal antibody. Top panel shows the corresponding Ponceau-stained image. (C) Resting (bottom panel, lanes 1, 2) and calcium ionophore-activated (bottom panel, lanes 3, 4) platelet lysates from the WT (+/+, lanes 1, 3) and calpain-1^{-/-} (-/-, lanes 2, 4) were probed with an anti-Cdc42 antibody. Top panel shows the corresponding Ponceau-stained image. (D) Gel-filtered WT and calpain-1^{-/-} platelets were supplemented with fibrinogen, and activated with thrombin (0.5 U mL⁻¹) for 1.0 min. Soluble lysates from resting and activated platelets were used for RhoA activity. Bar graph shows RhoA activity in the WT and calpain-1^{-/-} platelets under resting and thrombin-activated conditions. Error bars represent standard error of the mean (SEM). Statistical analysis was done with unpaired *t*-test. Data are representative of three independent experiments. (E) Gel-filtered WT and

calpain-1^{-/-} platelets were supplemented with fibrinogen, and activated with 0.5 U mL⁻¹ thrombin for 1.0 min. Soluble lysates from resting and activated platelets were used for Rac1 activity using a GST-PBD pull down assay. Active Rac1 bound to PBD beads in the resting (top panel, lanes 1, 3) and thrombin-activated (top panel, lanes 2, 4) WT (lanes 1, 2) and calpain-1^{-/-} (lanes 2, 4) platelets were probed with an anti-Rac1 antibody. Middle panel shows the total amount of Rac1 in the WT (lanes 1, 2) and calpain-1^{-/-} (lanes 3, 4) platelets used for the GST-PBD pull down assay. Bottom panel shows the amount of β -actin in the platelet lysates from WT (lanes 1, 2) and calpain-1^{-/-} (lanes 3, 4) platelets.

Fig. 8.

Proteolysis and activation of Rho GTPases in extracellular matrix proteins (ECM)-adherent platelets. Gel-filtered wild-type (WT) and calpain-1^{-/-} platelets were spread on fibrinogen for 5 and 30 minutes. Protein lysates (20 μ g) prepared at each time point from the adherent spread platelets were probed with antibodies for (A) RhoA, (B) Rac1, and (C) Cdc42. (D) Gel-filtered WT and calpain-1^{-/-} platelets were spread on fibrinogen for 5 and 25 minutes. Protein lysates (0.5 mg mL⁻¹) prepared at each time point were used for RhoA activity measurements using G-LISA kit. Bar graph shows RhoA activity in the WT and calpain-1^{-/-} platelet lysates. Error bars represent standard error of the mean (SEM). Statistical analysis was done with unpaired *t*-test. Data are representative of three independent experiments. (E, F) Gel-filtered WT and calpain-1^{-/-} platelets were spread for 2 and 45 minutes on fibrinogen. Soluble lysates (0.5 mg mL⁻¹) were used for the GST-PBD pull down assay. Active Rac1 (E) and Cdc42 (F) were probed with specific antibodies. Middle panels show total Rac1 and Cdc42 used for GST-PBD pull down assay. Bottom panel shows the β -actin blot.

Fig. 9.

Regulation of platelet spreading by calpain. Model (A) In the wild-type extracellular matrix proteins (ECM)-adherent platelets, activation of both calpains leads to the release and subsequent proteolysis of PTP1B. RhoA is also a target of calpain-1. Proteolysis of PTP1B and RhoA exerts synergistic effects limiting platelet spreading. Rac-1 activation at the beginning of platelet spreading is limited by calpain-1 presumably by targeting a regulator of Rac1. (B) Genetic inhibition of calpain-1 leads to an increase in PTP1B and RhoA in mouse platelets. Together, PTP1B activation and increased Rac1 activity at the beginning of spreading, and RhoA activation at the later stage of spreading participate in potentiating the platelet spreading phenotype in the calpain-1^{-/-} mice.

Table 1**Quantification of mouse platelet surface area**

A. Platelet spreading on fibrinogen for 1.5 minutes						
Genotype	<i>N</i>	Area $\pm$ SD μm^2	$\overline{f}\%$	6.9 μm^2	$\overline{s}\%$	11.4 μm^2
WT	170	6.9 $\pm$ 4.0	34		12	
Calpain-1 ^{-/-}	168	11.4 $\pm$ 6.0	74		36	
DKO	208	9.8 $\pm$ 3.8	75		30	
B. Platelet spreading on fibrinogen for 15 minutes						
Genotype	<i>N</i>	Area $\pm$ SD μm^2	$\overline{f}\%$	9.4 μm^2	$\overline{s}\%$	19.1 μm^2
WT	301	9.4 $\pm$ 5.1	34		7.51	
Calpain-1 ^{-/-}	322	19.2 $\pm$ 9.2	85		45.9	
DKO	252	14.3 $\pm$ 6.1	69		21.8	
C. Platelet spreading on collagen for 1.5 minutes						
Genotype	<i>N</i>	Area $\pm$ SD μm^2	$\overline{f}\%$	6.3 μm^2	$\overline{s}\%$	12.3 μm^2
WT	130	6.3 $\pm$ 2.4	48.4		2.3	
Calpain-1 ^{-/-}	130	12.3 $\pm$ 4.8	95.3		44.6	
DKO	141	10.6 $\pm$ 3.8	92.1		26.2	
D. Platelet spreading on collagen for 15 minutes						
Genotype	<i>N</i>	Area $\pm$ SD μm^2	$\overline{f}\%$	10.2 μm^2	$\overline{s}\%$	19.4 μm^2
WT	115	10.2 $\pm$ 3.6	43.5		2.6	
Calpain-1 ^{-/-}	120	19.5 $\pm$ 6.8	94.2		45.8	
DKO	249	15.8 $\pm$ 5.3	88.4		20.1	

Platelet spreading was quantified in the WT, Calpain-1^{-/-}, and DKO platelets on the ECM substrates using the METAMORPH software. Mean spread surface area of platelets spreading on fibrinogen for 1.5 minutes (A) and 15 minutes (B), and on collagen for 1.5 minutes (C) and 15 minutes (D), is shown. *N* represents the platelet number in each genotype.

$\overline{f}$ represents the average surface area of WT platelets, and

$\overline{s}$ represents the average surface area of calpain-1^{-/-} platelets. SD represents standard deviation. Statistical analysis was performed using one-way ANOVA and unpaired *t*-test. WT, wild type; DKO, double knockout; SD, standard deviation.