Coactosin-like 1 integrates signaling critical for shear-dependent thrombus formation in mouse platelets

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Coactosin-like 1 integrates signaling critical for shear-dependent thrombus formation in mouse platelets

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SUPPLEMENTAL METHODS

Platelet preparation
Mice were bled under isoflurane anesthesia into heparin (20 U mL\(^{-1}\), Ratiopharm) and blood was centrifuged twice for 6 minutes at 300 g. Platelet-rich plasma (PRP) was supplemented with 2 \(\mu\)L mL\(^{-1}\) apyrase (0.02 U mL\(^{-1}\); A6410, Sigma-Aldrich) and 5 \(\mu\)L mL\(^{-1}\) PGI\(_2\) (0.1 \(\mu\)g mL\(^{-1}\); P6188, Sigma-Aldrich) and platelets were pelleted by centrifugation for 5 minutes at 800 g, washed twice with Tyrode-HEPES buffer (134 mM NaCl, 0.34 mM Na\(_2\)HPO\(_4\), 2.9 mM KCl, 12 mM NaHCO\(_3\), 5 mM HEPES, 5 mM glucose, 0.35% BSA, pH 7.4) containing 2 \(\mu\)L mL\(^{-1}\) apyrase and 5 \(\mu\)L mL\(^{-1}\) PGI\(_2\) and allowed to rest for 30 minutes prior to experiments.

Flow cytometry
50 \(\mu\)L of blood were withdrawn under isoflurane anesthesia, washed twice with Tyrode-HEPES buffer and finally diluted (1:20) in Tyrode-HEPES buffer containing 2 mM Ca\(^{2+}\). Samples (washed blood) were activated with the indicated agonists. Activation of αIIbβ3- (JON/A-PE, Emfret) (1) and P-selectin exposure (WUG.E9-FITC) were determined using fluorophore-conjugated antibodies (15 minutes at 37°C). Analysis was performed on a FACSCalibur (BD Biosciences) (2).

Aggregometry
Light transmission of washed platelets (1.5 \(\times\) 10\(^5\) platelets per \(\mu\)L) supplemented with 100 \(\mu\)g mL\(^{-1}\) fibrinogen (Sigma-Aldrich) was monitored over time using a four-channel aggregometer (APACT, Laborgeräte und Analysensysteme, Hamburg). Platelet aggregation was induced by addition of the indicated agonists.

Platelet adhesion under flow
Mice were bled under isoflurane anesthesia into heparin (20 U mL\(^{-1}\), Ratiopharm). Anticoagulated whole blood was perfused over collagen I-coated cover slips (100 \(\mu\)g mL\(^{-1}\) Horm collagen, Nycomed) at a shear rate of 150, 1 000, 1 700 and 3 000 s\(^{-1}\). Prior to perfusion, platelets were labeled with a Dylight-488-conjugated anti-GPIX derivative (0.2 \(\mu\)g mL\(^{-1}\)) for 5 minutes at 37°C. Phase-contrast and fluorescence images were recorded with a Zeiss Axiovert 200 inverted microscope (40x/0.60 objective) equipped with a CoolSNAP-EZ camera (Visitron) and analyzed off-line using Metavue software.
Coagulation flow chamber

Mice were bled under isoflurane anesthesia into sodium citrate (0.129 M). Collagen-coated and BSA-blocked coverslips were perfused with citrated blood, which was recalcified with Tyrode’s-HEPES buffer supplemented with 63 mM CaCl₂ and 32 mM MgCl₂ by co-infusion in a 1:10 ratio via a Y-shaped dual inlet tube at a shear rate of 1 000 s⁻¹. Prior to perfusion, blood samples were pre-labeled with Alexa Fluor 488-labeled fibrinogen and an Alexa Fluor 647-labeled anti-GPIX derivative for 5 min at 37°C. Blood was perfused for 6 min and afterwards coverslips were washed for another 6 min. At the end of the perfusion time, phase-contrast and fluorescence images were recorded with a Zeiss Axiovert 200 inverted microscope (40x/0.60 objective) equipped with a CoolSNAP-EZ camera (Visitron) and analyzed off-line using Metavue software.

Tail bleeding time

Tail bleeding assays were performed as described (3). Mice were anesthetized and a 2 mm segment of the tail tip was removed using a scalpel. Tail bleeding was monitored by gently absorbing blood on a filter paper at 20 s intervals without making contact with the wound site. Bleeding was determined to have ceased when no blood was observed on the paper. Experiments were stopped after 20 minutes by cauterization. Differences between the mean bleeding time was statistically assessed using the Student’s t-test and differences between occluded and non-occluded vessels by Fisher’s t-test.

Aorta injury model

The abdominal cavity of anesthetized mice was opened to expose the abdominal aorta. An ultrasonic flow probe (0.5PSB699; Transonic Systems, USA) was placed around the abdominal aorta, and thrombus formation was induced by a single firm compression with forceps upstream of the flow probe. Blood flow was monitored for 30 minutes or until vessel occlusion occurred (blood flow stopped for > 5 minutes). Differences between occluded and non-occluded vessels were statistically assessed using the Fisher’s t-test.

F-actin assembly

F-actin polymerization has been described previously (4). Washed platelets were incubated with a DyLight-649-labeled anti-GPIX antibody derivative (20 μg mL⁻¹) and either remained resting or were stimulated with the indicated agonists for 2 min. The cells were fixed with 55% of total volume of 10% paraformaldehyde in PHEM buffer, centrifuged and resuspended in 55 μL of Tyrode’s buffer supplied with Ca²⁺ and 0.1% Triton™ X100. Subsequently, platelets were stained with 10 μM phalloidin-FITC (P5282, Sigma-Aldrich) for 30 min and immediately analyzed on a FACSCalibur.
**Immunostaining of spread platelets**

Spreading and immunostaining of platelets were performed as described previously (5). Briefly, coverslips were coated with fibrinogen (100 μg mL⁻¹; F4883, Sigma-Aldrich) overnight at 4°C. Washed platelets were stimulated with 0.01 U mL⁻¹ thrombin (10602400001, Roche) and allowed to spread. At the indicated time points, platelets were fixed and permeabilized in PHEM buffer supplemented with 4% para-formaldehyde (PFA) and 1% IGEPAL® CA-630 and stained with phalloidin-Atto647N (170 nM, 65906, Fluka) and anti-α-tubulin Alexa F488 (3.33 μg mL⁻¹, 322588 (B-5-1-2), Invitrogen). Samples were mounted with Fluoroshield (F6182, Sigma-Aldrich) and images acquired using a Leica TCS SP5 confocal microscope (Leica Microsystems).

**TxB₂ ELISA**

Washed platelets were either left untreated or stimulated with CRP (5 and 1 μg mL⁻¹) or thrombin (0.01 and 0.001 U mL⁻¹) for 5 min. The reaction was stopped by adding 5 mM EDTA and 1 mM aspirin. Platelets were removed by centrifugation at 14 000 rpm for 5 min and the supernatant was collected. TxB₂ concentrations in the supernatant were measured using the TxB₂ ELISA kit according to the manufacturer’s instructions (DRG, Marburg, Germany).

**Transmission electron microscopy**

To visualize platelet ultrastructure, platelet-rich plasma was prepared and platelets were fixed with 2.5% glutaraldehyde (16210, Electron Microscopy Sciences) in 50 mM cacodylate buffer (pH 7.2; 12201, AppliChem). Platelets were embedded in epon 812 (14900, Electron Microscopy Sciences), ultrathin sections were generated and stained with 2% uranyl acetate (22400, Electron Microscopy Sciences) and lead citrate (17800, Electron Microscopy Sciences). The visualization of the cytoskeleton of resting and spread platelets was performed as previously described (6). Images were acquired on an EM900 electron microscope (Carl Zeiss).

**Immunoblotting**

Denatured or native lysates of resting and activated platelets were separated by SDS-PAGE and blotted onto PVDF membranes. GAPDH (1 µg mL⁻¹, G5262, Sigma-Aldrich), Twf1 (1 µg mL⁻¹), Twf2a (1 µg mL⁻¹), full length (#3311, Cell Signaling) and phosphorylated n-cofilin (#5175, Cell Signaling) were probed with the respective antibodies and detected using horseradish peroxidase-conjugated secondary antibodies (0.33 μg ml⁻¹) and enhanced chemiluminescence solution (JM-K820-500, MoBiTec). Images were recorded using a Multimage® II FC Light Cabinet (Alpha Innotech cooperation) device. Anti-Twf1 and -Twf2 antibodies were a kind gift from Pekka Lappalainen (Helsinki, Finland).
Real-time deformability cytometry
RT-DC measurements of platelets were performed as described by Otto et al. (7). Briefly, whole blood was collected into EDTA-microvettes (Sarstedt). Afterwards, the mechanical properties of several thousands of platelets were calculated from shear-induced deformations inside a 300 µm long constriction of 15 µm x 15 µm cross-section. This microfluidic system is assembled on the commercial AcCellerator system (Zellmechanik Dresden) consisting of an inverted microscope, a pulsed 2 µs brightfield illumination and a syringe pump driving the cell suspension through the constriction. Measurements were performed at flow rates of 6 nL s⁻¹ using heparinized peripheral blood diluted 1:20 in PBS (with 0.6% (w/v) methylcellulose) and a size gate was applied to analyze exclusively platelets, only. For reference a measurement was taken in the reservoir of the microfluidic chip before cells were exposed to mechanical stress. Deformation was calculated from the circularity of each cell:

\[
Deformation = 1 - \text{circularity} = 1 - \frac{2\sqrt{\pi} \times \text{Area}}{\text{Perimeter}},
\]

where the area and perimeter are calculated in real-time using ShapeIn software (Zellmechanik Dresden) for up to 1,000 cells per second from the brightfield images. Data analysis was performed using three experimental replicates where statistical significance was calculated based on linear mixed models and using ShapeOut software (Zellmechanik Dresden) (8). Before starting a measurement and between experimental replicates the entire fluidic system was flushed with 70% ethanol and de-ionized water. Flow was equilibrated for 2 min between samples.

Lipid mediator extraction and analysis
Lipid mediator extraction and analysis of platelets were performed as described by Peng et al. (9). In summary, washed platelets were either left untreated or stimulated with 1 U mL⁻¹ thrombin (Sigma) or 5 µg mL⁻¹ CRP for 5 min at 37°C. After centrifugation for 5 min at 300 g, the pellet and supernatant were separated and shock frozen in liquid nitrogen and lipid extraction was performed as follows: 225 µL of MeOH (4 °C) were added to the platelet cell pellet or supernatant in an Eppendorf polypropylene tube that was placed on ice. After a few seconds of treatment with ultrasonication and vortexing, 10 µL of the internal standard mixture (5 mg L⁻¹ of 12-HETE-d8, 5-HETE-d8, 5-OxoETE-d7 and AA-d8; 0.5 mg L⁻¹ of PGE2-d9 and TXB2-d4; 0.2 mg L⁻¹ of LTB4-d4, LTC4-d5 and LTD4-d5; and 0.25 mg L⁻¹ of DHA-d5), 20 µL of acetic acid (99.99%, 17.5 M) and 750 µL of MTBE (4 °C) were added. The mixture was incubated for 1 h at 4 °C in a thermomixer at 650 rpm. Subsequently, 235 µL (for pellet) or 188 µL (for supernatant) of ultrapure water was added to induce phase separation. The samples were centrifuged at 10 000 g for 10 min at 4 °C, afterwards the upper organic layer
was transferred to another Eppendorf tube and dried under continuous N₂ flow. The dried lipid extract was resuspended in 50 µL of MeOH for further MS analysis. The lower layer of pellet extraction was used to precipitate proteins by adding 1220 µL MeOH after removing the interphase and samples were stored at -80 °C for 3 h in order to perform protein precipitation. Protein pellets were collected after centrifugation at 19 000 g for 30 min at 4 °C and stored in 1 % SDS, 150 mM NaCl, 50 mM Tris (pH 7.8) solution at -80 °C. Protein amount was quantified by BCA (bicinchoninic acid assay, Thermo Fisher Scientific, Rockford, USA).

For the reverse-phase liquid chromatography (LC), an UltiMate 3000-system from Thermo Fischer Scientific (Darmstadt, Germany) was employed. The chromatographic separation was performed on an Ascentis Express C18 main column (150 mm × 2.1 mm, 2.7 µm, Supelco) fitted with a guard cartridge (50 mm × 2.1 mm, 2.7 µm, Supelco). The temperatures of the autosampler and the column oven were set at 10 °C and 30 °C, respectively. Solvent A was ACN/H₂O (3:7, v/v) and 0.1 % formic acid while solvent B was IPA/ACN (1:1, v/v). The separation was carried out at a flow rate of 0.4 mL min⁻¹ with the following 20 min long gradient: initial (0 % B), 0.0-1.0 min (hold 0 % B), 1.0-13.0 min (0-100 % B), 13.0-16.0 min (hold 100 % B), 16.0 min (0 % B), 16.0-20.0 min (hold 0 % B). Solvent A was also used as transfer solution for injection. Samples and standards were injected with a volume of 5 µL and analyzed in triplicates, respectively. The LC was coupled to a QTRAP 6500 (Applied Biosystems, Darmstadt, Germany) which was equipped with an electrospray ion source (Turbo V Ion Source). The following ESI source settings were used for negative mode: curtain gas 10 arbitrary units, temperature 525 °C, ion source gas I 30 arbitrary units, ion source gas II 55 arbitrary units, collision gas medium; ion spray voltage -4500 V, entrance potential -10 V, and exit potential 10 V. In multiple reaction monitoring (MRM), at least two fragments per lipid were added into the transition list. Skyline (64-bit, 4.1.0.11796) was used to visualize results, integrate signals, and quantify all lipids that were detected by MS.

**Data analysis**

The presented results are mean ± standard deviation (S.D.) from at least three independent experiments, if not stated otherwise. Unless stated otherwise, data distribution was analyzed using the Shapiro-Wilk-test and differences between WT and Cotl1⁺⁻ mice were statistically analyzed using Student’s-t- or Wilcoxon-Mann-Whitney-test (SigmaPlot, Systat Software Inc., Version 14.0). Differences between more than two groups were statistically analyzed using ANOVA + post hoc test for multiple comparisons. P-values < 0.05 were considered as statistically significant *P< 0.05; **P< 0.01; ***P< 0.001.
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SUPPLEMENTAL TABLE

Supplemental Table 1. Platelet surface glycoprotein expression in Cotl1-/- platelets.
Diluted whole blood was stained with fluorophore-labeled antibodies and analyzed on a FACSCalibur (Becton Dickinson, Heidelberg). Platelets were gated by FSC/SSC characteristics. Results are given as the MFI ± S.D. of 12 mice per group.

| Glycoprotein | WT       | Cotl1-/- |
|--------------|----------|----------|
| GPIb         | 268±36   | 294±16   |
| GPV          | 213±4    | 222±5    |
| GPIX         | 384±10   | 396±10   |
| CD9          | 668±20   | 661±21   |
| GPVI         | 36±2     | 36±2     |
| CLEC-2       | 110±6    | 113±5    |
| Integrin α2  | 43±4     | 45±3     |
| Integrin β1  | 131±2    | 136±4    |
| Integrin αIIbβ3 | 438±31 | 425±21  |
**Supplemental Figure 1. Cytoplasmic localization of Cotl1.** (A) Immunoblot on platelet lysates proved the complete loss of Cotl1 in mutant platelets. GAPDH served as loading control. (B, C) Localization of Cotl1 in resting and spread platelets was assessed by confocal imaging of resting and spread (15 min) platelets on fibrinogen (100 μg mL⁻¹), which were immunostained for F-actin (red), α-tubulin (green) and α-Cotl1 (cyan). Scale bars, 3 μm. Images were acquired with a TCS SP8 confocal microscope (100x/1.4 oil STED WHITE objective, Leica Microsystems) and are representative of at least 10 individuals per group.
Supplemental Figure 2. Cotl1 is dispensable for actin dynamics in platelets. (A, B) Relative F-actin content of resting and activated platelets was determined by flow cytometry. Values are mean ± S.D. of 4 mice per group. The values shown are the ratio of MFI from activated and resting platelets. (C) Washed platelets were allowed to spread (15 min) on fibrinogen (100 μg mL⁻¹) and phase abundance was determined. (D) Confocal fluorescence
microscopy images of spread platelets (15 and 30 min) on fibrinogen (100 μg mL\(^{-1}\)) immunostained for F-actin (red) and α-tubulin (green). Scale bar, 3 μm. Images were acquired with a TCS SP8 confocal microscope (100x/1.4 oil STED WHITE objective, Leica Microsystems) and are representative of at least 10 individuals per group. (E) Representative images of the platelet cytoskeleton ultrastructure on fibrinogen (15 min). Scale bar, 1 μm.
Supplemental Figure 3. Unaltered activation in Cotl1-deficient platelets under static conditions in vitro. (A, B) Activation of platelet αIbβ3 integrin (JON/A-PE) (A) and degranulation (α-P-selectin-FITC) (B) in Cotl1−/− platelets upon stimulation with the indicated agonists was determined by flow cytometry (n = 12). U46, U46619; CRP, collagen-related peptide; Rhd, rhodocytin.
Supplemental Figure 4. Unaltered platelet aggregation in Cotl1-deficient platelets. (A) Aggregation responses of washed platelets (A) and platelet-rich plasma (PRP) (B) in turbidometric aggregometry after stimulation with the indicated agonists (n = 6). The arrow indicates addition of the agonist.
Supplemental Figure 5. Impaired adhesion and thrombus formation of Cotl1−/− platelets on collagen under flow. Heparinized whole blood of WT and Cotl1−/− mice was perfused over a collagen I-coated surface for 4 min at a shear rate of 1,700 s⁻¹. Representative brightfield images were acquired at the indicated time points during the perfusion period. Values are mean ± S.D. (n = 12). Scale bar, 50 μm.
Supplemental Figure 6. Reduced aggregate formation of Cotl1−/− platelets under flow in the presence of coagulation. Assessment of platelet adhesion (A, B) and aggregate formation (A, C) on Horm collagen (70 μg mL⁻¹) under flow (1000 s⁻¹). Collagen-coated coverslips were perfused with citrated blood, which was recalcified with Tyrode’s-HEPES buffer supplemented with 63 mM CaCl₂ and 32 mM MgCl₂. Before each experiment, blood samples from WT and Cotl1−/− mice were pre-labeled with Alexa Fluor 488-labeled fibrinogen and an Alexa Fluor 647-labeled anti-GPIX antibody. Values are mean ± S.D. (n = 9). Scale bar, 50 μm.
Supplemental Figure 7. The 5-LO pathway. 5-LO catalyzes the two initial steps of LT biosynthesis, (1) the oxygenation of AA to 5-HPETE and (2) the subsequent dehydration into the epoxide LTA₄ (10, 11). Likewise, 5-HPETE can be reduced to 5-HETE, which is metabolized to 5-oxo-ETE. AA is also converted to thromboxanes (TxA/B₂), prostacyclin (PGI₂) and prostaglandines (PGE/F₂) by cyclooxygenases (Cox).
Supplemental Figure 8. Increased thromboxane B₂ production in Cotl1⁻/⁻ platelets. TxB₂ levels were measured in the supernatant of resting and stimulated washed platelets by ELISA. Results are expressed as mean TxB₂ generation [ng mL⁻¹] ± S.D. of 6 mice per group. *P<0.05.
Supplemental Figure 9. LTB₄ treatment does not induce platelet activation under static conditions. (A- C) Assessment of platelet αIIbβ3 integrin (JON/A-PE) activation and degranulation (α-P-selectin-FITC) in WT and Cotl1⁻/⁻ platelets by flow cytometry. Platelets were stimulated with (A) 250-0.025 nM LTB₄ or (B) co-stimulated with 10 µg mL⁻¹ CRP. *P< 0.05; **P< 0.01; ***P< 0.001.
Supplemental Figure 10. LTB₄ treatment does not influence platelet aggregation. (A, B)
Assessment of platelet aggregation in WT and Cotl1⁻/⁻ platelets stimulated with (A) 250-0.025 nM LTB₄ or (B) co-stimulated with 1 µM U466199 or 0.01 µg mL⁻¹ CRP in turbidometric aggregometry.
Supplemental Figure 11. Effect of LTB₄ pre-incubation on WT blood under flow. (A-C)
Assessment of platelet adhesion (A, B) and aggregate formation (A, C) under flow (1700 s⁻¹)
on Horm collagen (70 μg mL⁻¹). WT samples were either left untreated or were pre-incubated
for 5 min with LTB₄ (25- 0.025 nM). Values are mean ± S.D. (n = 12). Scale bar, 50 μm. **P< 0.01. NS, not significant.
Supplemental Figure 12. Defective shear-dependent thrombus formation in Grb2-deficient mice is not rescued by exogenous addition of leukotriene B₄. (A-C) Assessment of platelet adhesion (A, B) and aggregate formation (A, C) of anticoagulated blood from WT and Grb2⁻/⁻ mice on Horm collagen (70 μg mL⁻¹) under flow (1700 s⁻¹). Samples were either left untreated or were pre-incubated for 5 min with LTB₄ [0.25 nM]. Images are representative of at least 12 mice per group. Values are mean ± S.D. Scale bar, 50 μm. *P< 0.05; ***P< 0.001.
Supplemental Figure 13. Defective shear-dependent thrombus formation in RhoA-deficient mice is not rescued by exogenous addition of leukotriene B4. (A-C) Assessment of platelet adhesion (A, B) and aggregate formation (A, C) in whole, anticoagulated blood from WT and RhoA<sup>−/−</sup> mice on Horm collagen (70 μg mL<sup>−1</sup>) under flow (1700 s<sup>−1</sup>). Samples were either left untreated or were pre-incubated for 5 min with LTB<sub>4</sub> [0.25 nM]. Images are representatives of at least 12 mice per group. Values are mean ± S.D. Scale bar, 50 μm. *P<0.05. NS, not significant.