CLP36 Is a Negative Regulator of Glycoprotein VI Signaling in Platelets

Shuchi Gupta,* Attila Braun,* Martina Morowski, Thomas Premsler, Markus Bender, Zoltan Nagy, Albert Sickmann, Heike M. Hermanns, Michael Bösl, Bernhard Nieswandt

Rationale: At sites of vascular injury, exposed subendothelial collagens not only trigger sudden platelet adhesion and aggregation, thereby initiating normal hemostasis, but also can lead to acute ischemic diseases, such as myocardial infarction or stroke. The glycoprotein (GP) VI/Fc receptor γ-chain complex is a central regulator of these processes because it mediates platelet activation on collagens through a series of tyrosine phosphorylation events downstream of the Fc receptor γ-chain–associated immunoreceptor tyrosine-based activation motif. GPVI signaling has to be tightly regulated to prevent uncontrolled intravascular platelet activation, but the underlying mechanisms are not fully understood.

Objective: We studied the role of PDZ and LIM domain family member CLP36 in platelet physiology in vitro and in vivo.

Methods and Results: We report that CLP36 acts as a major inhibitor of GPVI immunoreceptor tyrosine-based activation motif signaling in platelets. Platelets from mice either expressing a low amount of a truncated form of CLP36 lacking the LIM domain (Clp36ΔLIM) or lacking the whole protein (Clp36−/−) displayed profound hyperactivation in response to GPVI agonists, whereas other signaling pathways were unaffected. This was associated with hyperphosphorylation of signaling proteins and enhanced Ca2+ mobilization, granule secretion, and integrin activation downstream of GPVI. The lack of functional CLP36 translated into accelerated thrombus formation and enhanced procoagulant activity, assembling a prothrombotic phenotype in vivo.

Conclusions: These data reveal an inhibitory function of CLP36 in GPVI immunoreceptor tyrosine-based activation motif signaling and establish it as a key regulator of arterial thrombosis. (Circ Res. 2012;111:1410-1420.)

Key Words: calcium ■ CLP36 ■ degranulation ■ glycoprotein VI ■ platelets ■ thrombosis

Platelet activation, adhesion, and aggregation on the subendothelial collagen matrix are important processes in normal hemostasis, but under pathological conditions abnormal platelet activation can result in thrombosis and ischemia.1–5 Glycoprotein (GP) VI is an immunoglobulin-like collagen receptor expressed exclusively in platelets and megakaryocytes that is essential for activation of the cells on the matrix protein.3 GPVI has been proposed as a promising antithrombotic target because antibody-induced or genetic deficiency of the receptor protects mice from arterial thrombosis and ischemic stroke while only moderately affecting normal hemostasis.4,9 GPVI is noncovalently associated with the Fc receptor γ (FcRγ) chain, the immunoreceptor tyrosine-based activation motif (ITAM), bearing signal-transducing subunit of the receptor complex.10 On collagen binding, signaling through GPVI involves tyrosine phosphorylation of the FcRγ chain by the Src-family kinases Fyn and Lyn.11,12 ITAM phosphorylation provides a docking site for the recruitment of the kinase Syk to the GPVI/FcRγ complex, thereby triggering a series of tyrosine phosphorylation events. This results in the recruitment of adaptor proteins and finally culminates in the activation of effector enzymes and Ca2+ mobilization from intracellular stores, followed by Ca2+ influx through store-operated Ca2+ channels on the plasma membrane. Stromal interaction molecule 1 (STIM1) has been identified as the central regulator of this so-called store-operated Ca2+ entry (SOCE). Both gain-of-function13 and loss-of-function mutations in STIM1 result in defective GPVI signaling, and evidence exists that this defect is not only based on altered Ca2+ homeostasis.13

Original received January 11, 2012; revision received August 29, 2012; accepted September 5, 2012. In July 2012, the average time from submission to first decision for all original research papers submitted to Circulation Research was 11.2 days.

From the Vascular Medicine, University Hospital (S.G., A.B., M.M., M.B., B.N.), Rudolf Virchow Center, DFG Research Center for Experimental Biomedicine (S.G., A.B., M.M., B.N.), and Cardiovascular Physiology, Institute of Physiology (T.P.), University of Würzburg, Würzburg, Germany; and Leibniz-Institut für Analytische Wissenschaften-ISAS-e.V., Dortmund, Germany (A.S.).

*These authors contributed equally to this work.

The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.112.264754/-/DC1.

Correspondence to Bernhard Niesswandt or Attila Braun, Vascular Medicine, Rudolf Virchow Center, University of Würzburg, Josef-Schneider-St 2, D-15, 97080 Würzburg, Germany. E-mail bernhard.niesswandt@virchow.uni-wuerzburg.de or attila.braun@virchow.uni-wuerzburg.de

© 2012 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.112.264754
Enzymatic activity of Src family kinases and the recruitment of adaptor proteins to the GPVI/FcRγ-chain complex have to be tightly regulated to limit thrombus growth and to prevent inappropriate intravascular platelet activation, but the underlying mechanisms are only partially understood. It has been shown that lack of the immunoreceptor tyrosine-based inhibitory motif–bearing receptors, such as platelet endothelial cell adhesion molecule-1,17 carcinoembryonic antigen cell adhesion molecule-1,18 or the T-cell ubiquitin ligand-2 phosphatase TULA-2,17 results in a selective platelet hyperreactivity to GPVI agonists, which is based on hyperphosphorylation of Src-family kinases and phospholipase C (PLC) γ2 and leads to a prothrombotic phenotype in vivo.

The PDZ and LIM domain (PDLIM) protein family comprises 7 members (PDLIM1–7) sharing similar domain structures and biological functions in mammalian cells.19 They are composed of 1 or 2 PDZ domains at the N-terminus, a short linker region, and 1 or 3 LIM domains at the C-terminus.20–22 The PDLIM protein family contains muscle-specific and ubiquitously expressed proteins that associate with actin cytoskeleton proteins (β-tropomyosin, α-actinin)23,24 and different kinases (Src, Clik, protein kinase C).25–27 Muscle-specific PDLIM proteins are associated with Z-disc structures via α-actinin, and their loss leads to cardiac and skeletal myopathies in mice.28,29 In Drosophila cell lines, loss of PDLIM function disrupts integrin-mediat -ed cell adhesion, thereby inducing abnormal cell shape and spreading in vitro, confirming similar phenotypes observed in mammalian cells.30 CLP36 (Elfph, PDLM1) is an adapt -or protein that contains 1 PDZ domain at the N-terminus, a short linker region, and 1 LIM domain at the C-terminus. The knockdown of CLP36 in a trophoblast-derived choriocar -cinoma cell line (BeWo) led to impaired formation of stress fibers and focal adhesions, resulting in the loss of cell morphology.31 In human platelets, the PDZ domain of CLP36 has been shown to build a bridge between stress fibers and α-actinin-1 and to link plasma membrane Ca2+ ATPase 4b to the actin cytoskeleton.32,33 However, as a result of the lack of genetic models, the in vivo function of CLP36 has not been studied to date.

Here, we show that CLP36 is the only PDLIM family member expressed in platelets. To study its function in platelet physiology, we generated 2 mutant mouse lines: Clp36ΔLIM expressing low amounts of a truncated chimeric form of CLP36 and Clp36ΔLIM mice lacking the entire protein. Although platelets from both mouse lines showed unaltered actin turnover, the lack of functional CLP36 resulted in marked hyperactivity of the GPVI-ITAM signaling pathway and a prothrombotic phenotype.

**Results**

**Generation of Clp36ΔLIM Mice**

The partially overlapping expression profiles of PDLIMs in different mouse tissues and the similar domain structure of actinin-associated LIM protein, reversion-induced LIM protein, and CLP3622 indicated that the biological function of these proteins may be redundant in vivo. To assess the expression of members of the PDLIM family in platelets, reverse-transcriptase polymerase chain reaction was performed. Interestingly, we found only CLP36 mRNA but no other PDLIM mRNAs were expressed in platelets (Figure 1A). The unique expression of CLP36 in platelets suggested that disruption of CLP36 function cannot be compensated by other PDLIM proteins. Immunofluorescence confocal microscopy of wild-type (Wt) platelets spread on fibrinogen showed that CLP36 is highly expressed with dotted appearance throughout the platelet cytoskeleton, except for the central granule body (Figure 1B).

To study the physiological function of CLP36 in vivo, we generated mice in which the Clp36 gene was disrupted by insertion of an intronic geomycin (Geo) gene-trap cassette into intron 5 located upstream of exons encoding the LIM domain (Online Figure IA). Insertion of the Geo cassette resulted in expression of a chimeric CLP36ΔLIM–Geo fusion protein. Immunoblotting with an antibody recognizing the PDZ domain (ab64971) of CLP36 detected the CLP36ΔLIM–Geo protein at the expected size of ≈130 kDa, but at very low levels in comparison with the Wt protein (Online Figure IB). The absence of the C-terminal LIM domain was verified using another antibody (ab17022; Online Figure IB). The mice homozygously expressing this low amount of the CLP36ΔLIM–Geo protein are referred to as Clp36ΔLIM mice. Clp36ΔLIM mice were born following the Mendelian distribution and developed normally. Histological analysis of different hematopoietic organs of Clp36ΔLIM mice revealed no obvious hematological diseases or other alterations until the age of 12 months (data not shown). Transmission electron microscopy and determination of the mean platelet volume of resting platelets revealed a slightly increased size of the mutant platelets compared with Wt platelets (Figure 1C). However, blood platelet counts and surface expression of major surface receptors, such as GPIb-V-IX, GPVI, and β1 and β3 integrins, were normal, except for a slight elevation of CD9 expression (Online Table I). The life span of Clp36ΔLIM platelets was also similar to that of Wt platelets (Figure 1D). Together, these results demonstrated that the LIM domain of CLP36 is dispensable for megakaryopoiesis and platelet production. To study the role of CLP36 in actin rearrangements, Wt and Clp36ΔLIM platelets were allowed to spread on fibrinogen-coated coverslips in the presence of thrombin (0.01 U/mL). Wt and Clp36ΔLIM platelets spread with similar kinetics, and after 20 minutes the number of fully spread platelets was comparable between the 2 groups (Online Figure IC). In addition, F-actin assembly (Online Figure ID) was similar between Wt and mutant platelets, and their cytoskeleton (actin and tubulin) appeared indistinguishable during activation and spreading (Online Figure IIA).
Figure 1. PDZ and LIM domain (PDLIM) family member CLP36 is expressed in murine platelets. A, mRNA expression profile of PDLIM family members in wild-type (Wt) and Clp36^{ΔLIM} platelets. mRNA from Wt thymus was used as a positive control. Actin was used as loading control. B, Washed Wt platelets were allowed to spread on fibrinogen (200 µg/mL) after stimulation with 0.01 U/mL thrombin for 20 minutes and then were stained with phalloidin Atto647N and CLP36-PDZ antibody to detect CLP36 protein. Representative confocal microscopy images are shown. Scale bar, 3 μm. C, Representative transmission electron microscopy images of resting Wt and Clp36^{ΔLIM} platelets. Scale bar, 1 μm. Platelet width was analyzed with electron microscopy images, and the mean platelet volume (MPV) of Wt and Clp36^{ΔLIM} mice measured with a blood cell counter is depicted. Data are presented as mean±SD of 20 mice per group. D, The platelet life span was determined by percentage of fluorescently labeled platelets in Wt (black) and Clp36^{ΔLIM} (gray) mice during a 5-day period after intravenous injection of a Dylight-488 anti-glycoprotein IX immunoglobulin derivative (0.5 mg/kg; n=5).

Enhanced GPVI Signaling in Clp36^{ΔLIM} Platelets

To assess the function of CLP36 in platelet activation, flow cytometric analysis of integrin αIIbβ3 activation using the JON/A-PE antibody and P-selectin surface exposure as a marker of α-granule release was performed with Wt and Clp36^{ΔLIM} platelets. In this experimental setting, highly diluted platelet suspensions were used, which largely excluded the accumulation of released secondary mediators.

Figure 2. Enhanced glycoprotein VI (GPVI)-induced integrin activation and degranulation in Clp36^{ΔLIM} platelets. A, Flow cytometric analysis of integrin αIIbβ3 activation (binding of JON/A-PE) and degranulation-dependent P-selectin exposure in wild-type (Wt; black bar) and Clp36^{ΔLIM} (gray bar) platelets in response to the indicated G protein-coupled receptor agonists and rhodocytin (B) in response to GPVI agonists. Results are given as mean fluorescence intensities (MFIs)±SD of 5 mice per group and are representative of 4 individual experiments. *P<0.05, **P<0.01, and ***P<0.001. U46 indicates U46619; CRP, collagen-related peptide; CVX, convulxin; RC, rhodocytin; FITC, fluorescein isothiocyanate.
Activation of Clp36<sup>ΔLIM</sup> platelets was normal in response to the G-protein–coupled receptor agonists ADP, thrombin, and the stable thromboxane A2 analog U46619 (Figure 2A). In contrast, the response of Clp36<sup>ΔLIM</sup> platelets to GPVI agonists (collagen-related peptide [CRP], convulxin) was markedly increased, and this effect was most evident at low agonist concentrations (Figure 2B). Of note, no significant differences on agonist-induced activation were observed between Wt mice and mice heterozygous for the mutation (Clp36<sup>ΔLIM</sup>; Online Figure IIB). To study the functional consequences of increased αIIβ3 integrin activation and degranulation in Clp36<sup>ΔLIM</sup> platelets, aggregation responses to different agonists were assessed. Clp36<sup>ΔLIM</sup> platelets aggregated normally in response to G-protein–coupled receptor agonists (thrombin, ADP, U46619) at all tested concentrations (Figure 3A, left). In contrast, on stimulation with GPVI agonists (CRP, collagen, convulxin), a markedly enhanced aggregation response was observed in Clp36<sup>ΔLIM</sup> platelets. This effect was most detectable at threshold concentrations of these agonists that did not induce aggregation of Wt platelets (Figure 3A, right). In contrast, at higher concentrations of GPVI agonists, no significant difference in aggregation was detected between Wt and mutant platelets. Notably, Clp36<sup>ΔLIM</sup> platelets did not aggregate spontaneously or on stimulation with epinephrine (data not shown), indicating that they were not in a preactivated state per se. The enhanced GPVI-induced aggregation response also was associated with enhanced dense granule secretion as shown by faster and increased ATP release in response to low concentrations of collagen or CRP. In contrast, no differences in ATP release between Wt and Clp36<sup>ΔLIM</sup> platelets were detectable in response to thrombin (data not shown) or at high concentrations of GPVI agonists (Figure 3B).

**Figure 3.** Enhanced glycoprotein VI–induced aggregation and ATP release in Clp36<sup>ΔLIM</sup> platelets. A, Washed platelets from wild-type (Wt; black line) or Clp36<sup>ΔLIM</sup> (gray line) mice were stimulated with the indicated agonists, and light transmission was recorded on a Fibrintimer 4-channel aggregometer. ADP measurements were performed in platelet-rich plasma (PRP; n=4). B, Washed Wt (black line) or Clp36<sup>ΔLIM</sup> (gray line; 240 µL with 0.3 x 10<sup>6</sup> platelets/µL) platelets were incubated with luciferase-luciferin reagent followed by agonist addition. ATP release and aggregation were measured simultaneously on a Lumi-aggregometer. The concentration of released ATP is given. Mean percentage of maximal ATP release±SD of Wt (black bar) or Clp36<sup>ΔLIM</sup> (gray bar) platelets. **P<0.01, ***P<0.001. CRP indicates collagen-related peptide; CVX, convulxin.

**Increased GPVI-Induced Tyrosine Phosphorylation, Inositol-1,4,5-Trisphosphate Production, and Ca<sup>2+</sup> Mobilization in Clp36<sup>ΔLIM</sup> Platelets**

Agonist-induced platelet activation and aggregation require an increase in [Ca<sup>2+</sup>], that occurs through release of Ca<sup>2+</sup> from intracellular stores, followed by Ca<sup>2+</sup> entry through the plasma membrane Ca<sup>2+</sup> channels. To test whether the observed GPVI-ITAM–induced activation response of Clp36<sup>ΔLIM</sup> platelets was based on altered Ca<sup>2+</sup> signaling, we studied agonist-induced changes in [Ca<sup>2+</sup>], fluorometrically. Store release in the absence of extracellular Ca<sup>2+</sup> in response to CRP was significantly elevated in Clp36<sup>ΔLIM</sup> platelets compared with Wt controls (CRP 1 µg/mL; Wt: 30±12 nmol/L vs Clp36<sup>ΔLIM</sup>: 60±10 nmol/L; P<0.05; Figure 4A and Online Figure IIC). As a result, the subsequent Ca<sup>2+</sup> influx also was markedly increased in the presence of extracellular Ca<sup>2+</sup> in Clp36<sup>ΔLIM</sup> platelets (CRP 1 µg/mL; Wt: 180±20 nmol/L vs Clp36<sup>ΔLIM</sup>: 380±35 nmol/L; P<0.01; Figure 4B and Online Figure IIC, bottom). In contrast, no differences were observed in Ca<sup>2+</sup> store release or Ca<sup>2+</sup> influx in response to thrombin or ADP (Figure 4A and 4B).
To further assess the mechanism underlying the hyperactivity of Clp36ΔLIM platelets to GPVI stimulation, changes in tyrosine phosphorylation patterns were analyzed. At low concentrations of CRP (0.1 µg/mL), Wt platelets displayed only a small increase in tyrosine phosphorylation, whereas a marked increase in tyrosine phosphorylation of numerous platelet proteins, including those comigrating with PLCγ2, Fyn, Lyn, and the FcRγ chain, was observed in Clp36ΔLIM platelets (Figure 5A). At high concentrations of CRP (5 µg/mL), the increases in tyrosine phosphorylation were similar in Wt and mutant platelets (data not shown).

To confirm that the enhanced phosphorylation of signaling molecules in Clp36ΔLIM platelets resulted in an increased PLCγ2 activity, we measured the amount of inositol-1,4,5-trisphosphate indirectly using an inositol monophosphate ELISA.35 Although inositol monophosphate production in response to thrombin was similar in Wt and Clp36ΔLIM platelets (1551±307 nmol/L [Wt] vs 1742±186 nmol/L (Clp36ΔLIM); P=0.42), the response to CRP at both tested concentrations was markedly elevated in Clp36ΔLIM platelets compared with Wt, suggesting enhanced activity of PLCγ2 in the mutant platelets (427±45 nmol/L [Wt] vs 842±197 nmol/L (Clp36ΔLIM); P<0.001 for CRP 1 µg/mL and Wt; 311±99 nmol/L vs Clp36ΔLIM: 1049±493 nmol/L; P<0.01 for CRP 0.1 µg/mL; Figure 5B and Online Figure 2D). Taken together, these results suggested that CLP36 acts as a negative regulator of GPVI signaling.

Enhanced Aggregate Formation and Procoagulant Activity of Clp36ΔLIM Platelets on Collagen Under Flow
To study the consequences of enhanced GPVI signaling in Clp36ΔLIM platelets for thrombus formation under flow, anticoagulated whole blood was perfused over collagen at different shear rates. At high shear rates (1700 s⁻¹), no significant differences in surface coverage were observed between the 2 groups, but increased thrombus volumes were found for Clp36ΔLIM platelets (Online Figure IIIA). In contrast, at intermediate shear rates (1000 s⁻¹), Clp36ΔLIM platelets displayed both increased surface coverage and thrombus volumes (Figure 6A) compared with Wt controls (31.4±4% [Wt] vs 46.5±5% (Clp36ΔLIM); P<0.001). Interestingly, Clp36ΔLIM and Wt platelets
adhered to a similar extent when whole blood was perfused over fibrinogen-coated (60 µg/mL) coverslips at an intermediate shear rate of 1000 s⁻¹ (Online Figure IIIB).

Activated αIIbβ3 integrin and increased Ca²⁺ mobilization have been implicated in the coagulant activity of platelets.36,37 To determine the role of CLP36 in this process, anticoagulated whole blood from Wt or Clp36ΔLIM mice was perfused over a collagen-coated surface at a shear rate of 1700 s⁻¹. Exposure of procoagulant phosphatidylserine was determined using Annexin V-Dylight-488 staining. The number of phosphatidylserine-positive platelets was significantly increased in Clp36ΔLIM blood samples compared with Wt controls (Figure 6B). Thus, the enhanced GPVI-mediated activation responses resulted in an increased procoagulant activity of Clp36ΔLIM platelets.

Accelerated Arterial Thrombus Formation but Normal Bleeding Times in Clp36ΔLIM Mice

To assess the in vivo consequences of enhanced GPVI signaling in Clp36ΔLIM platelets, thrombus growth was studied in a model of arterial thrombosis in which the abdominal aorta was mechanically injured and blood flow was monitored by an ultrasonic perivascular Doppler flowmeter. In this model, thrombus formation was triggered predominantly by collagen and thus occurred in a GPVI-ITAM-PLCγ2–dependent manner.38 To rule out the possibility that deficiency of the LIM domain in cells of the vessel wall might influence thrombus formation and hemostatic function, mutation of the LIM domain was restricted to the hematopoietic system by transferring bone marrow cells from Clp36ΔLIM donor mice into lethally irradiated Wt recipient mice and vice versa. Although

--

**Figure 6.** Increased thrombus formation and procoagulant activity of Clp36ΔLIM platelets. A, Heparinized whole blood from wild-type (Wt; black) or Clp36ΔLIM (gray) mice was perfused over immobilized collagen (0.2 mg/mL) at a shear rate of 1000 s⁻¹. Representative phase contrast images (top) and fluorescent images (anti-glycoprotein IX-DyLight-488; bottom) are shown. B, Clp36ΔLIM platelets display enhanced procoagulant activity. Whole blood was perfused over a collagen-coated (0.2 mg/mL) surface at a shear rate of 1700 s⁻¹ for 4 minutes. Adherent platelets were stained with Annexin V Dylight-488 (0.25 µg/mL). Representative phase contrast (top) and fluorescence images (bottom) are shown (scale bar, 50 µm). C, The abdominal aorta of Wt and Clp36ΔLIM bone marrow chimeric mice was injured by tight compression with a forceps, and blood flow was monitored for 30 minutes with an ultrasonic flow probe. The time to stable vessel occlusion is shown. Each symbol represents 1 individual. D, Normal hemostasis in Clp36ΔLIM bone marrow chimeric mice. Tail bleeding times of Wt and Clp36ΔLIM bone marrow chimeric mice was measured in saline at 37°C. Each symbol represents 1 individual. The white dots represent Wt mice, and the black dots and gray dots represent Wt and Clp36ΔLIM bone marrow chimeric mice, respectively. n.s indicates nonsignificant; BMC, bone marrow chimeras.
irreversible occlusion of the aorta occurred with similar kinetics in Wt controls and Clp36Δ− bone marrow chimeras (BMC) with mean time to occlusion for Wt control of 260±59 seconds and for Clp36Δ− BMC of 350±131 seconds (P=0.07), occlusion times were markedly reduced in Clp36Δ+/+ BMC (132±56 s; P<0.001), reflecting faster occlusive thrombus formation (Figure 6C).

Tail bleeding times were measured to determine the hemo-
static function of Clp36Δ+/+ platelets. No significant hemostat-
ic defect was observed in Wt and Clp36Δ+/+ mice or in BMC
mice transplanted with Clp36Δ− or Clp36Δ−/+ cells (mean
bleeding time: 47±22 s [Clp36Δ+/+] vs 57±54 s [Clp36Δ+/+]; P=0.48; Figure 6D).

Enhanced GPVI Signaling in Clp36Δ−/+ Mice

The expression of chimeric proteins even at extremely low
levels might lead to aberrant signaling. To exclude the possi-
bility that low amount of the CLP36Δ+/+β-GEO protein
could cause the observed hyperreactive GPVI/ITAM signaling
in Clp36Δ+/+ platelets, we generated Clp36Δ−/+ mice (Online
Figure 1A, bottom). Clp36Δ−/+ mice had normal platelet char-
acteristics with platelet size, counts, and surface glycopro-
tein expression profile similar to Wt platelets (Online Table
II). Strikingly, Clp36Δ−/+ platelets, very similar to Clp36Δ+/+
platelets, displayed a pronounced hyperreactivity to GPVI
agonists, as revealed by flow cytometric analysis of αIbb3
integrin activation and P-selectin exposure (Figure 7A) as
well as aggregometry (Figure 7B), and again this effect
was most evident at low agonist concentrations. In line
with the data obtained with Clp36Δ+/+ platelets, Clp36Δ−/+-
platelets displayed unaltered activation responses to the
G-protein–coupled receptor agonists ADP, thrombin, and
U46619 (Figure 7A and 7B). Furthermore, when Clp36Δ−/+-
platelets were perfused over collagen at an intermediate
shear rate (1000 s−1), they showed increased surface cover-
age (38.4±4.1% [Wt] vs 60.5±9.8% [Clp36Δ−/+]; P<0.001;
Figure 7C) and thrombus volumes at the end of the perfu-
sion phase. Similar to Clp36Δ+/+ platelets, Clp36Δ−/+ platelets
also adhered and spread normally on fibrinogen (Online Figure
IIIC), and their actin cytoskeleton also was unaltered (Online
Figure IIID). Importantly, the results obtained for Clp36Δ−/+
platelets were similar to those obtained with Clp36Δ+/+ plate-
lets, further suggesting that the Clp36Δ+/+ mutation results
in a knockout-like phenotype, at least in platelets. Together,
these results confirmed that CLP36 is degraded in a calpain-
dependent manner during platelet activation.

CLP36 Interacts With Components of the
GPVI Signalosome and Is Degraded During
Platelet Activation

Based on the marked hyperreactivity of Clp36 mutant plate-
lets to GPVI agonists, we speculated that CLP36 might inter-
act with components of the GPVI signalosome. To test this
directly, we performed coimmunoprecipitation experiments.
We were unable to detect a direct interaction between CLP36
and GPVI in the absence of chemical crosslinkers (data not
shown). However, CLP36 could be coimmunoprecipitated with
GPVI when platelets have been treated beforehand with the
reducible crosslinker dimethyl 3,3′-dithiobispropionimidate
2 HCl (Figure 8A). We also detected an interaction between
CLP36 and linker of activated T cells (LAT) in Wt platelets
on chemical crosslinking. Apart from the weak interaction
of CLP36 with GPVI and LAT, we identified strong binding of
CLP36 to the calcium sensor protein STIM1 by mass spectro-
metric analysis of the platelet STIM1 complex (Online Figure
IVA and Online Table III). This is an interesting observation
because both the gain-of-function or loss-of-function muta-
tions of Stim1 in platelets result not only in a defect in SOCE
but also in a selective defect in the GPVI-ITAM signaling
pathway13,14 that seems to be at least partially independent of
its SOCE-regulating function.13

Platelet activation is controlled by various mechanisms,
including proteolytic degradation of intracellular proteins.
Calpain, the major calcium-dependent protease in platelets,
undergoes activation on increase in [Ca2+]. In vitro experi-
ments in mouse muscle cells with calpain-3 knockout mice
revealed that CLP36 was cleaved directly by the protease.19
To study this process in platelets, we analyzed degradation
of CLP36 on stimulation with the GPVI agonist convulxin and
the Ca2+ ionophore ionomycin in the absence or presence of
the calpain inhibitor calpeptin. Although ionomycin induced
the virtually complete loss of intact CLP36 within 10 minutes,
a more delayed effect was seen with convulxin, which could
be inhibited in the presence of calpeptin (Figure 8B). These
results demonstrated that CLP36 is degraded in a calpain-
dependent manner during platelet activation.

Discussion

In this study, we identified the PDLIM family protein CLP36
as a major regulator of GPVI-induced platelet activation.
We demonstrate that the loss of CLP36 function results in
markedly enhanced platelet activation in response to collagen
in vitro and a prothrombotic phenotype in vivo. This find-
ing was unexpected because PDLIM family members have,
sor far, not been associated with ITAM signaling in platelets
or other cell types. In contrast, most of PDLIMs associate
with stress fibers and with the focal adhesion complex via
α-actinin.21,24,41 Stress fibers and focal adhesions were lost
in knockout or knockdown cell lines of PDLIMs, and this
induced altered cell morphology and spreading.80 Therefore,
PDLIMs were proposed to play a critical role in stress fiber
formation41 and integrin-mediated focal adhesion.30,31 Our
reverse-transcriptase polymerase chain reaction studies re-
vealed that CLP36 is the only PDLIM family member ex-
pressed in platelets (Figure 1A), making these cells a good
model to study its function.

The targeting strategy used to generate Clp36Δ+/+ mice did
not ablate CLP36 expression but resulted in the expression of
a truncated CLP36 protein fused with β-GEO protein at very
low levels. Clp36Δ+/+ platelets displayed normal spreading
and stress fiber formation on fibrinogen (Online Figure IC
and ID). Interestingly, the subcellular localization of the
CLP36Δ+/+ protein in spread platelets was similar to the Wt
protein in control platelets (Online Figure IVB). This might
be attributed to the intact N-terminal PDZ domain of the
chimeric CLP36 protein, which still could associate with the
actin cytoskeleton. The interaction of CLP36 with α-actinin,
however, was not essential for actin turnover in platelets because Clp36−/− platelets were able to form filopodia and lamellipodia, and, finally, were able to spread with similar kinetics as Wt platelets (Online Figure IIIC and IIID). Together, these data demonstrate that CLP36 is dispensable for actin rearrangements in platelets.

The enhanced integrin αIIbβ3 activation and granule secretion of Clp36ΔLIM and Clp36−/− platelets (Figures 2 and 7) in response to GPVI agonists were associated with an increased PLCγ2 activity (Figure 5) caused by abnormal function of downstream signaling molecules of GPVI that are not required for G-protein–coupled receptor signaling. In both mutant mouse lines, this defect resulted in enhanced aggregation and an increased ability of the platelets to form aggregates on collagen under flow conditions (Figures 6A and 7C). This strongly suggests that Clp36ΔLIM platelets display a CLP36 knockout-like phenotype and that the low amounts of CLP36ΔLIM–β-GEO protein have no detectable effect on GPVI-ITAM signaling or actin rearrangements. Surface expression of GPVI in Clp36ΔLIM and Clp36−/− platelets was unaltered compared with Wt controls (Online Tables I and II), thus excluding the possibility of enhanced signaling as a result of higher receptor numbers on the platelet surface.

Several proteins have been identified by knockout approaches to negatively regulate GPVI-ITAM-PLCγ2 signaling, including platelet endothelial cell adhesion molecule-1,15 TULA-2,17 and carcinoembryonic antigen cell adhesion molecule-1.16 Mice lacking these proteins, similar to Clp36ΔLIM and Clp36−/− mice, display platelet hyperreactivity toward GPVI agonists in vitro and a prothrombotic phenotype in vivo, demonstrating the (patho)physiological importance of the negative feedback loop that controls GPVI signaling in platelets. Previous studies have shown that PDLIMs act as adapters between kinases and the actin cytoskeleton.23–27 This is based on the observation that the PDZ domain of PDLIMs associates, in some cases, with the actin cytoskeleton via α-actinin or β-tropomyosin and, in some cases, with different kinases via their LIM domains. The exact mechanism how CLP36 is associated with the negative feedback loop of GPVI could not be completely elucidated here, but our results indicate a complex mechanism that involves weak interactions of CLP36 with components of the GPVI signalosome (Figure 8A). Studies by Bertipaglia et al39 revealed calpain-mediated cleavage of CLP36 in muscle cells. Interestingly, we also observed the loss of intact CLP36 on GPVI-induced platelet activation, which was blocked by the calpain inhibitor calpeptin (Figure 8B). Together, these results support a model in which CLP36 might serve as a brake to prevent unwanted platelet activation through GPVI in the intact vascular system. After an initial stimulus that triggers increases in [Ca2+]i, calpain is activated and degrades CLP36, thereby releasing the brake of GPVI signaling and thus allowing full platelet activation.

Figure 7. Enhanced glycoprotein VI signaling in Clp36−/− platelets. A, Flow cytometric analysis of integrin αllb3 activation (JON/A-PE) and P-selectin exposure in Clp36−/− platelets in response to the indicated agonists. Results are given as mean fluorescence intensities (MFI) ±SD of 5 mice per group and are representative of 4 individual experiments. **P<0.01, ***P<0.001. B, Washed platelets from Wt (black line) or Clp36−/− (gray line) were stimulated with the indicated agonists, and light transmission was recorded on an aggregometer (n=4). C, Heparinized Wt (black) or Clp36−/− (gray) whole blood was perfused over immobilized collagen (0.2 mg/mL) at a shear rate of 1000 s−1. Representative phase contrast images (top) and fluorescent images (anti-GPIX-DyLight-488; bottom). Mean surface coverage±SD% (n=10 per group). ***P<0.001. Thr indicates thrombin; CRP, collagen-related peptide; RC, rhodocytin; FITC, fluorescein isothiocyanate.
peractivity of GPVI signaling as in the GPVI-ITAM pathway independently of its role in Ca\textsuperscript{2+} indicating that lack or aberrant localization of STIM1 affects both mutations result in defective GPVI-mediated signaling, \(^{(Stim1Sax)}\) signalosome with STIM1 and CLP36.

To dissect the molecular interplay of the GPVI signalosome in resting platelets, but further studies will be required to dissect the molecular interplay of the GPVI signalosome with STIM1 and CLP36. The function of CLP36 identified here as an inhibitor of GPVI-ITAM signaling is highly relevant in vivo because lack of functional CLP36 in the hematopoietic compartment resulted in accelerated occlusive thrombus formation in BMC mice in an arterial injury model. In contrast, tail bleeding times were unaltered in the mutant animals as well as in the chimeras, suggesting that the negative regulation of GPVI signaling may be particularly important to prevent intravascular occlusive thrombus formation. Based on this assumption, one may speculate that altered expression or activity of CLP36 in platelets might have an impact on the risk of acute ischemic diseases, such as myocardial infarction.

Taken together, we have shown that loss of the CLP36 LIM domain in platelets leads to increased PLC\textgamma\textsubscript{2} activity downstream of GPVI and thereby accelerates Ca\textsuperscript{2+} store depletion and Ca\textsuperscript{2+} influx, which in turn induces faster \(\alpha\)-granule and dense granule secretion and integrin activation. These findings establish CLP36 as an important regulator of platelet activation and might provide a basis for the development of novel strategies to control intravascular platelet activation.

**Acknowledgments**

The authors thank Sylvia Hengst for providing excellent technical assistance.

**Sources of Funding**

This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 688 and grant Ni556/10–1 to B. Nieswandt and A. Braun), the Rudolf Virchow Center, and the Ministerium für Innovation, Wissenschaft und Forschung des Landes Nordrhein-Westfalen. S. Gupta was supported by a grant of the German Excellence Initiative to the Graduate School of Life Sciences, University of Würzburg.
Disclosures

None.

References

What Is Known?

- Glycoprotein VI (GPVI) is the major collagen receptor on the surface of platelets and is activated on vascular injury to enforce platelet adhesion and thrombus formation.
- GPVI signaling is tightly regulated in both resting and activated platelets to allow hemostasis.
- Unbalanced GPVI activation may increase the risk of thrombotic disease, such as myocardial infarction or stroke.

What New Information Does This Article Contribute?

- CLP36 is identified as a crucial negative regulator of GPVI-mediated signaling.
- CLP36 is associated with the GPVI signalosome in resting platelets, and its inhibitory effect is overcome by calpain-dependent degradation during platelet activation.
- Loss of functional CLP36 results in accelerated thrombus formation in vivo.

Novelty and Significance

Increasing experimental evidence suggests the existence of a negative feedback mechanism that controls GPVI signaling in platelets. This regulation seems to be crucial to prevent uncontrolled platelet activation in the intact vasculature and to limit thrombus growth at sites of injury. We show that the PDZ and LIM domain–containing adaptor protein, CLP36, acts as an important inhibitor of GPVI signaling. Platelets from mice lacking functional CLP36 displayed a marked hyperreactivity to GPVI agonists in vitro, and the animals showed a prothrombotic phenotype in vivo. The study concludes that modulating CLP36-dependent inhibition of GPVI signaling might represent a novel therapeutic strategy to treat or prevent thrombotic disorders.
CLP36 Is a Negative Regulator of Glycoprotein VI Signaling in Platelets
Shuchi Gupta, Attila Braun, Martina Morowski, Thomas Premsler, Markus Bender, Zoltan Nagy, Albert Sickmann, Heike M. Hermanns, Michael Bösl and Bernhard Nieswandt

Circ Res. 2012;111:1410-1420; originally published online September 5, 2012;
doi: 10.1161/CIRCRESAHA.112.264754

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/111/11/1410

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2012/09/05/CIRCRESAHA.112.264754.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Detailed Methods

Mice

Animal studies were approved by the local authorities (Bezirksregierung Unterfranken). Clp36\textsuperscript{ΔLIM} chimeric mice were generated by microinjection of an embryonic stem (ES) cells (ID: XC 262, BayGenomics, USA) into C57Bl/6 blastocysts. After germ line transmission, heterozygous and homozygous knockin animals were genotyped by polymerase chain reaction (PCR) using mouse tail DNA and Western blotting. Clp36 chimeric mice were generated by microinjection of embryonic stem (ES) cell clone IST12013D3 (TIGM) into C57Bl/6 blastocysts. After germ line transmission, heterozygous and knockout animals were genotyped by PCR using mouse tail DNA and Western blotting detecting different domains of CLP36.

Generation of bone marrow chimeras

Five to six week old C57Bl/6 female mice were lethally irradiated with a single dose of 10 Gy and bone marrow cells from Wt or Clp36\textsuperscript{ΔLIM} mice were injected intravenously into the irradiated mice (4 x 10\textsuperscript{6} cells/mouse). All recipient animals were given water containing 2 g/L neomycin sulfate for 2 weeks after transplantation. The mice were tested by Western blotting to confirm the genotype.

Primer sequences for RT PCR

Platelet mRNA was isolated using Trizol reagent (Invitrogen) and cDNA was synthesized using Superscript reverse transcriptase (Invitrogen) as described by the manufacturer. PCR was performed using primer sequences as depicted here.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Size bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP/F-RT</td>
<td>5’-TAA AGC AGC GTC CTA CCA GT-3’</td>
<td>340</td>
</tr>
<tr>
<td>ALP/R-RT</td>
<td>5’-TGA GGG GGC ACT GAA GCT GT-3’</td>
<td></td>
</tr>
<tr>
<td>RIL/F-RT</td>
<td>5’-TAA AGG CTG CCA CGA TCA TC-3’</td>
<td>360</td>
</tr>
<tr>
<td>Gene</td>
<td>Primer 5′-3′ Sequences</td>
<td>Product Length</td>
</tr>
<tr>
<td>------</td>
<td>------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Ril/R-RT</td>
<td>5′-CAG GTC TAC CCT GCA GTC CC-3′</td>
<td>360 bp</td>
</tr>
<tr>
<td>CLP36/F-RT</td>
<td>5′-CAA GGG CTG CGC AGA CAA CA-3′</td>
<td></td>
</tr>
<tr>
<td>CLP36/R-RT</td>
<td>5′-CTT GTC AAT GAT AAG GCT GC-3′</td>
<td></td>
</tr>
<tr>
<td>Mystique/F-RT</td>
<td>5′-CCG ACA GAG CGC CTC ACC CC-3′</td>
<td>440 bp</td>
</tr>
<tr>
<td>Mystique/R-RT</td>
<td>5′-TTC CTC CAG ATC CAA ACT GC-3′</td>
<td></td>
</tr>
<tr>
<td>Enh1/F-RT</td>
<td>5′-CAC TTC ATC ACA TGC TTC CC-3′</td>
<td>250 bp</td>
</tr>
<tr>
<td>Enh1/R-RT</td>
<td>5′-AAC TCC GTG TTG CGC TCC AC-3′</td>
<td></td>
</tr>
<tr>
<td>Enigma/F-RT</td>
<td>5′-ATA TTG ACG GTG AGA ACG CG-3′</td>
<td>280 bp</td>
</tr>
<tr>
<td>Enigma/R-RT</td>
<td>5′-GCA ACT GTC CAT TCT GCC GC-3′</td>
<td></td>
</tr>
<tr>
<td>Zasp/F-RT</td>
<td>5′-AGA GGC TCT GCG AAG GTC AA-3′</td>
<td>280 bp</td>
</tr>
<tr>
<td>Zasp/R-RT</td>
<td>5′-CTC GCT GTA GCT GGT ATG GG-3′</td>
<td></td>
</tr>
</tbody>
</table>

**Chemicals and antibodies**

ADP (Sigma-Aldrich, Deisenhofen, Germany), U46619 (Alexis Biochemicals, San Diego, CA), thrombin, Nonidet P-40 (Roche Diagnostics, Mannheim, Germany), DTBP and Triethanolamine (Thermoscientific, IL, USA), Calpeptin (Calbiochem, USA), Ionomycin (Molecular probes, Invitrogen), Collagen (Kollagenreagent Horm; Nycomed, Munich, Germany), Collagen-related peptide (CRP) was generated as described., thapsigargin (Molecular probes, Invitrogen) ECL solution (PerkinElmer LAS, Boston, USA). Anesthetic drugs: medetomidine (Pfizer, Karlsruhe, Germany), midozolam (Roche Pharma, Grenzach-Wyhlen, Germany), and fentanyl (Janssen-Cilag, Neuss, Germany); and antagonists: atipamezol (Pfizer, Karlsruhe, Germany), flumazenil, and naloxon (Delta Select, Dreieich, Germany) were used according to the regulations of the local authorities. Anti-CLP36 antibodies (ab64971 and ab17022) were purchased from Abcam (Abcam, Cambridge, UK), Anti-LAT antibody was from Cell Signaling. Rabbit anti-α-actinin antibody (H-300) was from Santa Cruz Biotechnology. JON/A antibody directed against activated integrin αIIbβ3 and anti-GPVI antibody, JAQ1, were from Emfret Analytics (Eibelstadt, Germany). All other antibodies were generated and modified in our laboratory as described previously.¹
Platelet preparation and aggregometry

Platelet-rich plasma and washed platelets were prepared as recently described.² To determine platelet aggregation, changes in light transmission was measured using platelet rich plasma (PRP) or washed platelets (200 μL with 0.5 x 10⁶ platelets/μL) on a Fibrintimer 4 channel aggregometer (APACT Laborgeräte und Analysensysteme, Hamburg, Germany). Aggregation of washed platelets was induced by addition of thrombin, CRP, collagen, U46619 or CVX. ADP induced aggregation studies were performed with PRP.

Platelet spreading and immunofluorescence

Platelet spreading was analyzed as described previously.² For confocal fluorescence microscopy, spread platelets on fibrinogen were stained with an anti-CLP36 antibody (ab64971, Abcam, Cambridge, UK) for 2 h followed by washing with PBS and incubation for 1 hour with an Alexa 488-labeled anti-rabbit Ig antibody (Invitrogen) and phalloidin-Atto647N (AttoTec GmbH (Siegen, Germany)) and visualized using a Leica SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). To visualize the chimeric protein, 27% of the argon laser power was used in Clp36ΔLIM platelets. To image the CLP36 protein in Wt platelets, 8% of the argon laser power was used. To visualize tubulin cytoskeleton, staining with anti-α-tubulin-Alexa 488 (catalog-322588, Invitrogen, Germany) was performed. For analysis with STED (stimulated emission depletion) microscopy, spread platelets on fibrinogen were stained with phalloidin-Atto 647N and visualised using a Leica SP5 microscope. Images were further processed using Image J software (National Institute of Health, USA).

Electron microscopy

For transmission electron microscopy, platelets were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and embedded in epon. Thin sections were stained with uranyl
acetate and lead citrate and examined under a CM120 transmission electron microscope (Zeiss, Oberkochen, Germany).

**Determination of platelet life span**

Mice were intravenously injected with Dylight-488 conjugated anti-GPIX Ig derivative. 50 µL of blood was collected 1 hour (day 0) after injection and each day thereafter for 5 days and the percentage of fluorescently labeled platelets was determined by flow cytometry.

**Bleeding time**

Mice were anesthetized and a 1 mm segment of the tail tip was removed with a scalpel and immersed in saline at 37 °C. The bleeding time was defined as the time at which all visible signs of bleeding from the incision had stopped. The experiment was stopped after 20 min.

**Aorta occlusion model**

The abdominal cavity of anesthetized mice was opened by a longitudinal incision and the abdominal aorta was exposed. An ultrasonic flow probe was placed around the vessel and thrombus formation was induced by a single firm compression with a forceps. Blood flow was monitored until complete occlusion of the vessel or experiments were stopped manually after an observation period of 30 minutes.

**Flow cytometry and western blot analysis**

Flow cytometric measurements were performed as described. For western blot analysis, blotted platelet lysates were probed with an anti-CLP36 antibody recognizing different domains of the protein. Proteins were visualized by enhanced chemiluminescence (ECL). GPIIIa levels were used as loading controls. The blot images were further processed using Image J software (National Institute of Health, USA).
Measurement of ATP release

ATP secretion was measured using CHRONO-LUME reagent according to the manufacturer’s protocol on a Chronolog aggregometer (Chrono-Log Corp. Philadelphia, PA, USA).³ Luciferase (5 μl) was added directly to the platelets (0.5 X 10⁶ platelets/μL) under constant stirring and indicated concentrations of various agonists were added to study ATP release. The luminescence intensity was measured at a setting of ×0.01.

Measurement of inositol 1 phosphate (IP₁)

Washed platelets were adjusted to the final concentration of 0.6 x 10⁶/μL in a modified phosphate-free Tyrode-HEPES buffer containing 50 mM LiCl₂ and 2 mM Ca²⁺. Indomethacin (10 μM) and apyrase (2 U/mL) were also added. Platelets were activated with the indicated agonists for 5 minutes at 37 °C with constant shaking at 450 rpm. After stimulation, platelets were lysed in the buffer supplied with the kit. 50 µL of lysed platelets were used for the IP₁ ELISA assay according to the manufacturer’s protocol (Cisbio, France).

Intracellular calcium measurements

Platelet intracellular Ca²⁺ measurements were performed as described.⁴ Platelets isolated from blood were washed and suspended in Tyrode’s buffer without Ca²⁺. The washed platelets were loaded with fura-2/AM (5 μM) in the presence of Pluronic F-127 (0.2 μg/mL; Molecular Probes) for 30 minutes at 37 °C. After 30 minutes platelets were washed and resuspended in Tyrode’s buffer containing no or 1 mM Ca²⁺. Stirred platelets were activated with different agonists, and fluorescence was measured with a PerkinElmer LS 55 fluorimeter (Waltham, MA). Excitation was alternated between 340 and 380 nm, and emission was measured at 509 nm. Each measurement was calibrated using Triton X-100 and EDTA.
Whole cell tyrosine phosphorylation

Washed platelets from Wt and Clp36<sup>ΔLIM</sup> mice were stimulated in suspension with indicated CRP concentrations under constant stirring conditions at 37 °C for different times. Membranes were blocked for 2 hours in 5% BSA in PBS and then incubated with primary anti-phosphotyrosine antibody 4G10 (Upstate, CA, USA) for 12 h at 4 °C. Membranes were then washed extensively with PBS-T and were subsequently incubated with secondary anti-mouse horseradish peroxidase-conjugated antibody in PBS-T. The membranes were developed using an enhanced chemiluminescence detection system.

Immunoprecipitation

For co-immunoprecipitation of proteins, washed platelets (100x10<sup>6</sup> platelets) were prepared and incubated with 3 mM of DTBP in 0.1M triethanolamine-HCl for 30 min. The crosslinking reaction was stopped by addition of equal volume of IP lysis buffer containing protease inhibitors and Brij 97 added to 1% f.c. The protein lysates were incubated on ice for 1 hour followed by incubation with 2 µg/mL of the anti-CLP36 antibody (ab64971) or anti-GPVI antibody for 2 hours at 4 °C. Precipitation was carried out for 1 hour at 4 °C by addition of 25 µL protein G-Sepharose to the above incubated lysates. Samples were incubated at 37 °C for 30 min under reducing conditions in 4x NuPage sample buffer (Invitrogen) and then incubated for 5 min at 70 °C. The samples were separated on 4-12% NuPage Bis-Tris gradient gels followed by transfer onto a polyvinylidene difluoride (PVDF) membrane. To prevent non-specific antibody binding, membranes were blocked in blocking buffer for 2 hours at RT. Membranes were incubated with the required antibodies o/n with gentle shaking at 4 °C. Afterwards, membranes were washed three times with washing buffer for 15 min at RT. Next, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 hour at RT. The membranes were developed using an enhanced chemiluminescence detection system.
Calpain mediated degradation of CLP36

Washed platelets were pre-incubated with or without 5µg/mL calpeptin in the presence 2 mM Ca^{2+} for 5 minutes at 37 °C. The platelets were then treated with convulxin (1µg/ml) or 10µM ionomycin followed by incubation for different time intervals. Western blots were performed using anti-CLP36 antibody (ab17022, Abcam) to detect CLP36.

Actin polymerization

F actin assembly measurements were performed as described.\(^6\)

Clot retraction assay

Clot retraction studies were performed at 37 °C in an aggregometer tube containing diluted PRP (500 µL, 3 x 10^8 mL^{-1} platelets), thrombin (3 U mL^{-1}), and CaCl\(_2\) (20 mM). Clot retraction was recorded with a digital camera over time span of 4 hours after activation.

Adhesion under flow conditions and determination of phosphatidylserine-exposing platelets after perfusion

Perfusion was performed at room temperature using a pulse-free pump at shear rate of 1000 s^{-1} during perfusion, microscopic phase-contrast images were recorded in real time. For determination of phosphatidylserine (PS) exposure, adhesion experiments were performed as previously described.\(^6\) To prevent coagulation, chamber and tubing were prewashed extensively with HEPES buffer supplemented with heparin (5 U/mL). The blood was perfused through the flow chamber with a 1 mL syringe and a pulse-free pump at a shear rate of 1700 s^{-1} for 4 minutes. The flow chamber was perfused with HEPES buffer containing heparin (5 U/mL), 1 mM CaCl\(_2\) and 250 ng/mL of Annexin-V-Dylight-488 for 4 min followed by washing with HEPES buffer for 2 minutes to remove unbound Annexin-V-Dylight-488. Phase contrast and fluorescent images were recorded from at least 5 different visual fields (40x/0.75 NA objective; Carl Zeiss, Heidelberg, Germany). Image analysis was performed
off-line using Metamorph software (Visitron, Munich, Germany). Thrombus formation was expressed as the mean percentage of total area covered by thrombi, and as the mean integrated fluorescence intensity per square millimeter. For flow adhesion assay over fibrinogen, 60 μg of fibrinogen was coated overnight at 4 °C in a humid chamber. The coverslips were blocked for 1 hour and the whole blood was perfused at a shear rate of 1000s⁻¹ for 4 minutes and analysis was performed as described above.

**MS analysis of the STIM1 interactome**

1D-SDS-PAGE of IP samples, gel silver staining, tryptic digestion of differential protein spots and sample preparation for subsequent MS analysis was performed as published previously.⁷ Tryptic peptides were separated on an Ultimate nano-HPLC system (Dionex, Idstein, Germany) as reported⁸ and directly eluted into the online ESI ion source of a QStar Elite mass analyzer (Applied Biosystems, Darmstadt, Germany). Using an ionization voltage of 2.2 kV, full 0.5-sec MS survey scans from 380 to 1,500 m/z were acquired and the three most intense peaks were subjected to CID MS/MS. Mass spectra were recorded via the Analyst QS 2.0 operating software, including the mascot.dll plug-in 1.6b7 for conversion of LC-MS raw data into Mascot generic format. Applying the Mascot™ search algorithm for MS/MS spectra (Mascot Daemon 2.2.02 software platform and Mascot server version 2.2.04)⁹, the generated data were searched against the UniProtKB/ Swiss-Prot database (www.uniprot.org, 11/2009, murine subset), with Mascot parameters being set as published.⁸ All MS/MS spectra with a minimum Mascot score of 34 (P-value of 0.05) were taken into consideration for further interpretation and were additionally validated manually.

**Data Analysis**

Results from at least 3 experiments per group are presented as mean (±) SD. Differences between groups of wild-type and Clp36ΔLIM mice were assessed by the Welch $t$ test. $P$-values <0.05 were considered statistically significant.
References for Supplemental Methods


Online Figure I. (A) Targeting strategy for the generation of Clp36ΔLIM and Clp36−/− mice. Intronic Geo gene-trap cassette is denoted as Lac Z. (B) upper panel: Detection of Wt and the chimeric CLP36ΔLIM-β-GEO fusion protein in Wt and Clp36ΔLIM platelets using different anti-CLP36 antibodies. Lower panel: analysis of CLP36 expression in Wt and Clp36−/− platelets by Western blot. (C) Unaltered spreading in Clp36ΔLIM platelets. Representative differential interference contrast (DIC) images of 4 individual experiments from the indicated
time points (right) and statistical evaluation of the percentage of spread platelets at different spreading stages (left). 1: no filopodia, 2: only filopodia, 3: filopodia and lamellipodia, 4: full spreading. Scale bar represents 5 µm. (D) Quantification of F-actin assembly in Clp36ΔLIM platelets. After activation of washed platelets with thrombin (1 U/mL), CRP (10µg/mL) or CRP (1 µg/mL) for 2 minutes at 37°C, platelets were fixed, permeabilized, stained with phalloidin-FITC and analyzed by flow cytometry. The mean fluorescence intensity (MFI) of resting control platelets was set to 1. MFI of resting and activated platelets was measured. The ratio of polymerized actin in activated versus resting platelets was determined. ***, P < 0.001. The black bars and gray bars represent Wt and Clp36ΔLIM platelets, respectively.
Online Figure II. (A) Analysis of filamentous actin (red) and tubulin (green) structure in spread (20 minutes) Clp36ΔLIM and Wt platelets by confocal microscopy. Scale bar represents 3 μm. (B) Flow cytometric analysis of integrin αIIβ3 activation (JON/A-PE) and P-selectin exposure in Clp36+/ΔLIM platelets in response to the indicated agonists. Results are given as mean fluorescence intensities (MFI) ± SD of 5 mice per group and are representative of 3 individual experiments. Abbreviations: Thr, thrombin; CRP, collagen related peptide; CVX, convulxin; RC, rhodocytin. **P<0.01, ***P<0.001. (C) Fura-2–loaded Wt (black line) or
Clp36\textsuperscript{ΔLIM} (gray line) platelets were stimulated with 0.1 and 0.02 μg/mL CRP in the presence of 0.5 mM EGTA (C upper panel) or in the presence of 1 mM Ca\textsuperscript{2+} (C lower panel) and changes in [Ca\textsuperscript{2+}]\textsubscript{i} were monitored fluorimetrically. Representative measurements and maximal increase in [Ca\textsuperscript{2+}]\textsubscript{i} compared with baseline levels before stimulus (Δ[Ca\textsuperscript{2+}]\textsubscript{i}) ± SD (n = 5 mice per group) are shown. (D) IP\textsubscript{1} production upon platelet activation using low doses of CRP (0.1 and 0.01μg/mL). Platelets were lysed and IP\textsubscript{1} ELISA assays were performed. Results are given as the mean IP\textsubscript{1} concentration (nM) ± SD (n=4 per group). The black bars and gray bars represent Wt and Clp36\textsuperscript{ΔLIM} platelets, respectively.

Online Figure III. (A) Normal surface coverage, but increased thrombus volume of Clp36\textsuperscript{ΔLIM} platelets on collagen at a shear rate of 1700 s\textsuperscript{-1}: Heparinized whole blood from Wt and
Clp36ΔLIM was perfused over immobilized collagen (0.2 mg/mL) at a shear rate of 1700 s⁻¹ (4 min) followed by 1 min perfusion with Tyrode HEPES buffer at the same shear rate. Mean surface coverage and relative thrombus volume ± SD (n=10 per group). *P < 0.05. (B) Heparinized whole blood from Wt and Clp36ΔLIM was perfused over fibrinogen coated coverslips (60 μg/mL) at a shear rate of 1000 s⁻¹. Representative phase contrast images are shown. Mean surface coverage ± SD (n=6 per group). (C) Different spreading stages of Wt and Clp36⁻/⁻ platelets were analyzed and compared. 1: no filopodia, 2: only filopodia, 3: filopodia and lamellipodia, 4: full spreading. Scale bar represents 5 μm. Representative DIC images of 4 individual experiments. (D) Visualization of actin cytoskeleton in Wt and Clp36⁻/⁻ platelets using STED microscopy. Fully spread platelets on fibrinogen coated surface after 0.01 U/mL thrombin activation were fixed and stained with Phalloidin-Atto647N. Scale bar represents 3 μm.
**Online Figure IV.** (A) 1D-SDS-PAGE and subsequent silver staining of STIM1-specific IP samples of native *Stim1*−/− and *Wt* platelet lysates. In each case a loading volume of 20 μL was applied and separated on 10% Bis-Tris gels. Arrows indicate differential protein bands which were subsequently excised and analyzed by protein mass spectrometry. (B) Washed platelets of *Wt* and *Clp36ΔLIM* mice were allowed to spread on fibrinogen (200 μg/mL) after stimulation with 0.01 U/mL thrombin. Platelets were allowed to spread for 10 or 20 minutes and then stained with phalloidin Atto647N and CLP36-PDZ antibody to detect *Wt* (left panel)
and Clp36ΔLIM (right panel) protein. To visualize the chimeric protein, 27% of the argon laser power was used in Clp36ΔLIM platelets. To image the CLP36 protein in Wt platelets, 8% of the argon laser power was used. Representative confocal microscopy images are shown. Scale bar: 3 μm. (C) Clot retraction of PRP from Wt, Clp36ΔLIM and Clp36−/− mice upon activation with 3 U/mL thrombin in presence of 20 mM CaCl₂ at the indicated time points. Representative images of 3 different experiments are depicted (D) Basal [Ca²⁺], calcium store release and store operated calcium entry (SOCE) was measured fluorimetrically after incubating platelets with thapsigargin (5 μM) for 5 minutes followed by the addition of CaCl₂ (1 mM).
Supplementary Tables

Online Table I. Platelet glycoprotein expression in Wt and Clp36ΔLIM mice. Expression levels of prominent glycoproteins were determined by flow cytometry. Diluted whole blood from the indicated mice was incubated with FITC-labeled antibodies at saturating concentrations for 15 min at RT and platelets were analyzed directly. Results are expressed as mean fluorescence intensity ± SD (n=5) and are representative of 4 individual experiments. Platelet count and platelet size was determined using a Sysmex automated cell analyzer. *P<0.05.

<table>
<thead>
<tr>
<th></th>
<th>Wt</th>
<th>Clp36ΔLIM</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPIb</td>
<td>291±5.6</td>
<td>285±10.0</td>
</tr>
<tr>
<td>GPV</td>
<td>271±2.6</td>
<td>280±14.3</td>
</tr>
<tr>
<td>GPIX</td>
<td>440±5.3</td>
<td>460±25.0</td>
</tr>
<tr>
<td>GPVI</td>
<td>21±1.25</td>
<td>21±2.6</td>
</tr>
<tr>
<td>CD9</td>
<td>1585±31.0</td>
<td>1657±35.0</td>
</tr>
<tr>
<td>GPIibilla</td>
<td>509±38.0</td>
<td>506±29.0</td>
</tr>
<tr>
<td>Integrin α</td>
<td>59±3.8</td>
<td>57±3.3</td>
</tr>
<tr>
<td>Integrin β1</td>
<td>167±3.8</td>
<td>163±18.0</td>
</tr>
<tr>
<td>Clec2</td>
<td>136±13.0</td>
<td>138±7.0</td>
</tr>
<tr>
<td>Platelets (nL⁻¹)</td>
<td>128±29.0</td>
<td>112±11.21</td>
</tr>
<tr>
<td>MPV(μm)</td>
<td>5.2±0.1542</td>
<td>5.4±0.1857</td>
</tr>
</tbody>
</table>

Online Table II. Platelet glycoprotein expression in Wt and Clp36⁻/⁻ mice. Expression levels of prominent glycoproteins were determined by flow cytometry. Diluted whole blood from the indicated mice was incubated with FITC-labeled antibodies at saturating concentrations for 15 min at RT and platelets were analyzed directly. Results are expressed
18

as mean fluorescence intensity ± SD (n=5) and are representative of 4 individual experiments. Platelet count and platelet size was determined using a Sysmex automated cell analyzer. *P<0.05

Online Table III. Individual proteins identified in ten differential protein bands of STIM1-specific IP samples of Wt mouse platelet lysates. Summary of QStar Elite nano-LC-ESI-MS/MS data. Hits are listed according to corresponding protein bands and with decreasing Mascot scores. MW: molecular weight [Da]; PB: protein band in Suppl. Fig IVA.