CLP36 is a Negative Regulator of GPVI Signaling in Platelets

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ABSTRACT

Rationale: At sites of vascular injury exposed subendothelial collagens trigger sudden platelet adhesion and aggregation thereby initiating normal hemostasis but can also lead to acute ischemic diseases such as myocardial infarction or stroke. The glycoprotein (GP)VI/Fc receptor γ(FcRγ)-chain complex is a central regulator of these processes as it mediates platelet activation on collagens through a series of tyrosine phosphorylation events downstream of the FcRγ-chain-associated immunoreceptor tyrosine-based activation motif (ITAM). 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 (PDLIM) 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-ITAM 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 pro-coagulant activity assembling a pro-thrombotic phenotype in vivo.

Conclusion: These data reveal an inhibitory function of CLP36 in GPVI-ITAM signaling and establish it as a key regulator of arterial thrombosis.

Keywords: CLP36, GPVI, degranulation, calcium, thrombosis, platelets

Non-standard Abbreviations:
- ITAM: immunoreceptor tyrosine-based activation motif
- PDLIM: PDZ and LIM domain
- SFKs: Src-family kinases
- SOCE: store operated calcium entry
- STIM1: stromal interaction molecule 1

INTRODUCTION

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. Glycoprotein VI (GPVI) is an Ig-like collagen receptor expressed exclusively in platelets and megakaryocytes that is essential for activation of the cells on the matrix protein. GPVI has been proposed as a promising antithrombotic target as antibody-induced or genetic deficiency of the receptor protects mice from arterial thrombosis and ischemic stroke while only moderately affecting normal hemostasis. GPVI is non-covalently associated with the FcRγ chain, the immunoreceptor tyrosine-based activation motif (ITAM) bearing signal-transducing subunit of the receptor complex. Upon collagen binding, signaling through GPVI involves tyrosine phosphorylation of the FcRγ-chain by the Src-family kinases (SFKs) Fyn and Lyn. 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 Ca^{2+} mobilization from intracellular stores followed by Ca^{2+} influx through store operated Ca^{2+} (SOC) channels on the plasma membrane. Stromal interaction molecule 1 (STIM1) has been identified as the central regulator of this so-called store operated Ca^{2+} entry (SOCE). Both gain-of-function^{13} and loss-of-function mutations in STIM1^{14} result in defective GPVI signaling and evidence exists that this defect is not only based on altered Ca^{2+} homeostasis^{13}.

Enzymatic activity of SFKs 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 (ITIM)-bearing receptors, such as PECAM1,^{15} CEACAM1,^{16} or the phosphatase TULA-2^{17} results in a selective platelet hyperreactivity to GPVI agonists which is based on hyperphosphorylation of SFKs and phospholipase C (PLC) γ2 and leads to a prothrombotic phenotype in vivo.

The PDLIM protein family comprises seven members (PDLIM1-7) sharing similar domain structures^{18} and biological functions in mammalian cells.^{19} They are composed of one or two PDZ domains at the N-terminus, a short linker region and one or three LIM domains at the C-terminus.^{20-22} The PDLIM protein family contains muscle specific and ubiquitously expressed proteins that associate with actin cytoskeletal proteins (β-tropomyosin, α-actinin)\(^{23,24}\) and different kinases (Src, Clik, PKC).^{25-27} Muscle specific PDLIM proteins are associated with Z-disc structures via α-actinin and their loss leads to cardio- and skeletal myopathies in mice.\(^{28,29}\) In Drosophila cell lines, loss of PDLIM function disrupts integrin mediated cell adhesion, thereby inducing abnormal cell shape and spreading in vitro, confirming similar phenotypes observed in mammalian cells.\(^{30}\) CLP36 (Elfin, PDLIM1) is an adaptor protein that contains one PDZ domain at the N-terminus, a short linker region and one LIM domain at the C-terminus. The knockdown of CLP36 in a trophoblast-derived choriocarcinoma 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 Ca^{2+} ATPase 4b (PMCA4b) to the actin cytoskeleton.\(^{24,32}\) However, due to 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 two mutant mouse lines: Clp36\(^{-\Delta \text{LIM}}\) expressing low amounts of a truncated chimeric form of CLP36 and Clp36\(^{-/-}\) mice lacking the entire protein. While 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.

**METHODS**

A detailed description of materials and methods is provided in supplemental methods.
RESULTS

**Generation of Clp36ΔLIM mice.**

The partially overlapping expression profiles of PDLIMs in different mouse tissues and the similar domain structure of ALP, RIL and CLP36 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, RT-PCR was performed. Interestingly, we found only CLP36 mRNA but no other PDLIM mRNAs to be expressed in platelets (Fig. 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 *Wt* platelets spread on fibrinogen showed that CLP36 is highly expressed with dotted appearance throughout the platelet cytoskeleton except for the central granule body (Fig. 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 Geo gene-trap cassette into intron 5 located upstream of exons encoding the LIM domain (Online Fig. IA). Insertion of the Geo cassette resulted in expression of a chimeric CLP36ΔLIM-β-GEO fusion protein. Immunoblotting with an antibody recognizing the PDZ domain (ab64971) of CLP36 detected the CLP36ΔLIM-β-GEO protein at the expected size of approximately 130 kDa, but at very low levels in comparison to the *Wt* protein (Online Fig. IB). The absence of the C-terminal LIM domain was verified using another antibody (ab17022) (Online Fig. IB). The mice homozygously expressing this low amount of the CLP36ΔLIM-β-GEO protein are further 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 (TEM) and determination of the mean platelet volume of resting platelets revealed a slightly increased size of the mutant platelets compared to *Wt* platelets (Fig. 1C). However, blood platelet counts and surface expression of major surface receptors like GPIb-V-IX, GPVI, 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 (Fig. 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 also comparable between the two groups (Online Fig. IC). Additionally, F-actin assembly (Online Fig. ID) was similar between *Wt* and mutant platelets and their cytoskeleton (actin and tubulin) appeared indistinguishable during activation and spreading (Online. Fig. IIA)

**Enhanced GPVI signaling in Clp36ΔLIM 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 *Clp36ΔLIM* platelets. In this experimental setting, highly diluted platelet suspensions are used which largely exclude the accumulation of released secondary mediators. Activation of *Clp36ΔLIM* platelets was normal in response to the G protein-coupled receptor (GPCR) agonists ADP, thrombin and the stable thromboxane A2 (TxA2) analog U46619 (Fig. 2A). In contrast, the response of *Clp36ΔLIM* platelets to GPVI agonists (CRP: collagen related peptide, convulxin) was markedly increased and this effect was most evident at low agonist concentrations (Fig. 2B). Of note, no significant differences upon agonist induced activation were observed between *Wt* mice and mice heterozygous for the mutation (*Clp36+/ΔLIM*) (Online Fig. IIB). To study the functional consequences of
increased αIIbβ3 integrin activation and degranulation in Clp36\textsuperscript{ΔLIM} platelets, aggregation responses to different agonists were assessed. Clp36\textsuperscript{ΔLIM} platelets aggregated normally in response to GPCR agonists (thrombin, ADP, U46619) at all tested concentrations (Fig. 3A, left panel). In contrast, upon stimulation with GPVI agonists (CRP, collagen, convulxin: CVX), a markedly enhanced aggregation response was observed in Clp36\textsuperscript{ΔLIM} platelets. This effect was best detectable at threshold concentrations of these agonists that did not induce aggregation of Wt platelets (Fig. 3A, right panel). In contrast, at higher concentrations of GPVI agonists, no significant difference in aggregation was detected between Wt and mutant platelets. Notably, Clp36\textsuperscript{ΔLIM} platelets did not aggregate spontaneously or upon stimulation with epinephrine (data not shown), indicating that they were not per se in a pre-activated state. The enhanced GPVI-induced aggregation response was also 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\textsuperscript{ΔLIM} platelets were detectable in response to thrombin (data not shown) or at high concentrations of GPVI agonists (Fig. 3B).

Increased GPVI-induced tyrosine phosphorylation, IP\textsubscript{3} production and Ca\textsuperscript{2+} mobilization in Clp36\textsuperscript{ΔLIM} platelets.

Agonist induced platelet activation and aggregation requires an increase in the intracellular Ca\textsuperscript{2+} concentration [Ca\textsuperscript{2+}]; that occurs through release of Ca\textsuperscript{2+} from intracellular stores followed by the Ca\textsuperscript{2+} entry through the plasma membrane Ca\textsuperscript{2+} channels.\textsuperscript{34} To test whether the observed GPVI-ITAM-induced activation response of Clp36\textsuperscript{ΔLIM} platelets was based on altered Ca\textsuperscript{2+} signaling, we studied agonist-induced changes in [Ca\textsuperscript{2+}] using fluorimetrically. Store release in the absence of extracellular Ca\textsuperscript{2+} in response to CRP was significantly elevated in Clp36\textsuperscript{ΔLIM} platelets compared to Wt controls (CRP 1 µg/mL: Wt: 30±12 nM vs. Clp36\textsuperscript{ΔLIM}: 60±10 nM; P<0.05) (Fig. 4A and Online Fig. IIC). As a result, the subsequent Ca\textsuperscript{2+} influx was also marked increased in the presence of extracellular Ca\textsuperscript{2+} in Clp36\textsuperscript{ΔLIM} platelets (CRP 1 µg/mL: Wt: 180±20 nM vs. Clp36\textsuperscript{ΔLIM}: 380±35 nM, P<0.01) (Fig. 4B and Online Fig. IIC, lower panel). In contrast, no differences were observed in Ca\textsuperscript{2+} store release or Ca\textsuperscript{2+} influx in response to thrombin or ADP (Fig. 4A, B).

To further assess the mechanism underlying the hyperreactivity of Clp36\textsuperscript{ΔLIM} platelets to GPVI stimulation, changes in tyrosine phosphorylation patterns were analysed. 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 co-migrating with PLC\textgamma{}2, Fyn, Lyn and the FcR-chain was observed in Clp36\textsuperscript{ΔLIM} platelets (Fig. 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 Clp36\textsuperscript{ΔLIM} platelets resulted in an increased PLC\textgamma{}2 activity, we measured the amount of inositol-1,4,5-trisphosphate (IP\textsubscript{3}) indirectly using an IP\textsubscript{3} ELISA.\textsuperscript{35} While IP\textsubscript{3} production in response to thrombin was similar in Wt and Clp36\textsuperscript{ΔLIM} platelets (Wt: 1551±307 nM vs. Clp36\textsuperscript{ΔLIM}: 1742±186 nM, P=0.42), the response to CRP at both tested concentrations was markedly elevated in Clp36\textsuperscript{ΔLIM} platelets compared to Wt, suggesting enhanced activity of PLC\textgamma{}2 in the mutant platelets (Wt: 427±45 nM vs. Clp36\textsuperscript{ΔLIM}: 842±197 nM, P<0.001 for CRP 1 µg/mL and Wt: 311±99 nM vs. Clp36\textsuperscript{ΔLIM}: 1049±493 nM, P<0.01 for CRP 0.1 µg/mL; Fig. 5B and Online Fig. 2D). Taken together, these results suggested that CLP36 acts as a negative regulator of GPVI signaling.

Enhanced aggregate formation and pro-coagulant activity of Clp36\textsuperscript{ΔLIM} platelets on collagen under flow.

To study the consequences of enhanced GPVI signaling in Clp36\textsuperscript{ΔLIM} platelets for thrombus formation under flow, anti-coagulated 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 two groups, but increased thrombus volumes were found for Clp36ΔLIM platelets (Online Fig. IIIA). In contrast, at intermediate shear rates (1000 s⁻¹), Clp36ΔLIM platelets displayed both increased surface coverage and thrombus volumes (Fig. 6A) when compared to Wt controls (Wt: 31.4 ± 4 % vs. Clp36ΔLIM: 46.5 ± 5 %, P<0.001). Interestingly, Clp36ΔLIM and Wt platelets adhered to a similar extent when whole blood was perfused over fibrinogen (60 µg/mL) coated coverslips at intermediate shear of 1000 s⁻¹ (Online Fig 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, anti-coagulated whole blood from Wt or Clp36ΔLIM mice was perfused over a collagen coated surface at the shear rate of 1700 s⁻¹. Exposure of procoagulant phosphatidylserine (PS) was determined using Annexin-V-Dylight-488 staining. The number of PS positive platelets was significantly increased in Clp36ΔLIM blood samples compared to Wt controls (Fig. 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 where the abdominal aorta is mechanically injured and blood flow is monitored by an ultrasonic perivascular Doppler flowmeter. In this model, thrombus formation is triggered predominantly by collagen and thus occurs in 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. While irreversible occlusion of the aorta occurred with similar kinetics in Wt controls and Clp36ΔLIM bone marrow chimeras (BMC) with mean time to occlusion for Wt control: 260 ± 59 s and for Clp36ΔLIM BMC: 350 ± 131 s (P=0.07), occlusion times were markedly reduced in Clp36ΔLIM BMC (132 ± 56 s; P<0.001), reflecting faster occlusive thrombus formation (Fig. 6C).

Tail bleeding times were measured to determine hemostatic function of Clp36ΔLIM platelets. No significant hemostatic defect was observed in Wt and Clp36ΔLIM mice or in bone marrow chimeric mice transplanted with Clp36ΔLIM or Clp36ΔLIM cells (mean bleeding time: 47 ± 22 s (Clp36ΔLIM) vs. 57 ± 54 s (Clp36ΔLIM) P=0.48;Fig. 6D).

**Enhanced GPVI signaling in Clp36ΔLIM mice.**

The expression of chimeric proteins even at extremely low levels might lead to aberrant signaling. To exclude the possibility that low amount of the CLP36ΔLIM-β-GEO protein caused the observed hyperreactive GPVI/ITAM signaling in Clp36ΔLIM platelets, we generated Clp36ΔLIM mice (Online Fig. IA, lower panel). Clp36ΔLIM mice had normal platelet characteristics with platelet size, counts and surface glycoprotein expression profile similar to Wt platelets (Online Table II). Strikingly, Clp36ΔLIM platelets, very similar to Clp36ΔLIM platelets, displayed a pronounced hyperreactivity to GPVI agonists as revealed by flow cytometric analysis of αIIbβ3 integrin activation and P-selectin exposure (Fig. 7A) as well as aggregometry (Fig. 7B) and again this effect was most evident at low agonist concentrations. In line with the data obtained with Clp36ΔLIM platelets, Clp36ΔLIM platelets displayed unaltered activation responses to the GPCR agonists ADP, thrombin and U46619 (Fig. 7A,B). Furthermore, when Clp36ΔLIM platelets were perfused over collagen at an intermediate shear rate (1000 s⁻¹) they showed increased surface coverage (Wt: 38.4± 4.1 %, vs. Clp36ΔLIM: 60.5± 9.8 %, P<0.001; Fig. 7C) and thrombus volumes at the end of the perfusion phase. Similar to Clp36ΔLIM platelets, Clp36ΔLIM platelets also adhered and spread normally on fibrinogen (Online Fig IIIC) and their actin cytoskeleton was also unaltered (Online Fig IIID). Importantly, the results obtained for Clp36ΔLIM platelets were similar to those obtained with Clp36ΔLIM.
platelets, further suggesting that the Clp36ΔLIM mutation results in a knockout-like phenotype, at least in platelets. Together, these results confirmed that CLP36 acts as a negative regulator of GPVI signaling also under flow.

CLP36 interacts with components of the GPVI signalosome and is degraded during platelet activation.

Based on the marked hyperreactivity of CLP36 mutant platelets to GPVI agonists we speculated that CLP36 might interact with components of the GPVI signalosome. To test this directly, we performed co-immunoprecipitation 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 co-immunoprecipitated with GPVI when platelets have been treated beforehand with the reducible crosslinker DTBP (Fig. 8A). We also detected an interaction between CLP36 and Linker of activated T cells (LAT) in Wt platelets upon 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 spectrometric analysis of the platelet STIM1 complex (Online Fig. IVA and Online Table III). This is an interesting observation as both the gain-of-function or loss-of-function mutations of Stim1 in platelets result not only in a defect in SOCE but also in a selective defect in the GPVI-ITAM signaling pathway that appears to be at least partially independent of its SOCE-regulating function.

Platelet activation is controlled by various mechanisms, including proteolytic degradation of intracellular proteins. Calpain, the major calcium dependent protease in platelets, undergoes activation upon increase in [Ca^{2+}]. In vitro experiments in mouse muscle cells with calpain-3 knockout mice revealed that CLP36 was cleaved directly by the protease. To study this process in platelets, we analyzed degradation of CLP36 upon stimulation with the GPVI agonist convulxin and the Ca^{2+} ionophore ionomycin. While ionomycin induced the virtually complete loss of intact CLP36 within 10 min, a more delayed effect was seen with convulxin which could be inhibited in the presence of the calpeptin (Fig. 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 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 finding was unexpected as PDLIM family members have so 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. Stress fibers and focal adhesions were lost in knockout or knockdown cell lines of PDLIMs and this induced altered cell morphology and spreading. Therefore, PDLIMs were proposed to play a critical role in stress fiber formation and integrin mediated focal adhesion. Our RT-PCR studies revealed that CLP36 is the only PDLIM family member expressed in platelets (Fig 1A), making these cells a good model to study its function.

The targeting strategy used to generate Clp36ΔLIM 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. Clp36ΔLIM platelets displayed normal spreading and stress fiber formation on fibrinogen (Online Fig IC, D). Interestingly, the subcellular localization of the CLP36ΔLIM protein in spread platelets was similar to the Wt protein in control platelets (Online Fig. IVB). This might be attributed to the intact N-terminal

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PDZ domain of the chimeric CLP36 protein, which could still associate to the actin cytoskeleton. The interaction of CLP36 with α-actinin, however, was not essential for actin turnover in platelets as Clp36ΔLIM platelets were able to form filopodia, lamellipodia and finally spread with similar kinetics as Wt platelets (Online Fig. IIIC, D). Together, these data demonstrate that CLP36 is dispensable for actin rearrangements in platelets.

The enhanced integrin αIIbβ3 activation and granule secretion of Clp36ΔLIM and Clp36Δ/- platelets (Fig. 2 and Fig. 7) in response to GPVI agonists was associated with an increased PLCγ2 activity (Fig. 5) caused by abnormal function of downstream signaling molecules of GPVI that are not required for GPCR 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 (Fig. 6A and Fig. 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 Clp36Δ/- platelets was unaltered compared to Wt controls (Online Table I. and II.), thus excluding the possibility of enhanced signaling due to higher receptor numbers on the platelet surface.

Several proteins have been identified by knockout approaches to negatively regulate GPVI-ITAM-PLCγ2 signaling, including PECAM,15 TULA-2,17 and CEACAM1.16 Mice lacking these proteins, similar to Clp36ΔLIM and Clp36Δ/- mice, display platelet hyperreactivity towards 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, on one hand, to the actin cytoskeleton via α-actinin or β-tropomyosin and on the other hand, to different kinases via their LIM domains. The exact mechanism, how CLP36 is associated to 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 (Fig. 8A). Studies by Bertipaglia et al. revealed calpain mediated cleavage of CLP36 in muscle cells.39 Interestingly, we also observed the loss of intact CLP36 upon GPVI induced platelet activation which was blocked by the calpain inhibitor calpeptin (Fig. 8B). Together, these results support a model where CLP36 might serve as a "brake" to prevent unwanted platelet activation through GPVI in the intact vascular system. Following an initial stimulus that triggers rises in [Ca2+], calpain is activated and degrades CLP36, thereby releasing the brake of GPVI signaling and thus allowing full platelet activation.

However, CLP36 may additionally contribute to the control of GPVI-ITAM signaling by other mechanisms. We identified CLP36 along with α-actinin and β-tropomyosin isoforms as interaction partners of the calcium sensor STIM1 during the mass spectrometric analysis of STIM1-Co-IP samples (Online Table III). STIM1 deficiency virtually abolishes SOCE in platelets14 while a gain-of-function mutation of STIM1 (Stim1Sax) leads to enhanced SOCE.13 Interestingly, however, both mutations result in defective GPVI-mediated signaling indicating that lack or aberrant localization of STIM1 affects the GPVI-ITAM pathway independently of its role in Ca2+ signaling. Based on this assumption, we speculate that CLP36 might negatively regulate the association of STIM1 to 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.

Our studies with Clp36Δ/- platelets revealed a similar hyperactivity of GPVI signaling as in Clp36ΔLIM platelets, indicating that this effect is not based on an unphysiological activity of the chimeric CLP36 protein (Fig. 7 and data not shown). Earlier, CLP36 was proposed to be involved in the regulation
of Ca^{2+} homeostasis in human platelets.\textsuperscript{32} It was shown that the PDZ domain of CLP36 interacts with plasma membrane Ca^{2+}-ATPase (PMCA4b) and the authors speculated that this association might regulate late events in platelet activation such as clot retraction and stability. However, we found no impairment of clot retraction in Clp36\textsuperscript{ALIM} or Clp36\textsuperscript{ΔLIM} platelets (Online Fig. IVC), demonstrating that CLP36 is dispensable for this process. The increased IP\textsubscript{3} production observed in Clp36\textsuperscript{ALIM} platelets upon GPVI activation (Fig. 5B) led to a faster and enhanced Ca^{2+} release (Fig. 4A) from the intracellular stores which subsequently increased SOCE.\textsuperscript{42} Notably, passive store depletion with thapsigargin (TG) did not lead to any differences in the kinetics of store release or SOCE between \textit{Wt} and Clp36\textsuperscript{ALIM} platelets (Online Fig. IVD). Therefore, we conclude that CLP36 has no direct effect on the regulation of Ca^{2+} store depletion or on the assembly and activation of the SOC complex. Apart from SOCE, platelets express ligand-gated channels like transient receptor potential cation channel (TRPC) and P2X\textsubscript{1}.\textsuperscript{34} TRPC6 has been identified as the major diacylglycerol (DAG) induced receptor operated calcium (ROC) channel in these cells.\textsuperscript{43,44} Enhanced phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) hydrolysis by PLC\textgreek{y} in platelets lacking functional CPL36 may lead to increased cytoplasmic DAG levels thereby potentially accelerating TRPC6 mediated Ca^{2+} influx.

The here identified function of CLP36 as an inhibitor of GPVI-ITAM signaling is highly relevant \textit{in vivo} as lack of functional CLP36 in the hematopoietic compartment resulted in accelerated occlusive thrombus formation in bone marrow chimeric 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\textgreek{y} activity downstream of GPVI and thereby accelerates Ca^{2+} store release and Ca^{2+} influx, which in turn induces faster \alpha 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.

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DISCLOSURE
None.
REFERENCES


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FIGURE LEGENDS

**Figure 1.** PDLIM family member CLP36 is expressed in murine platelets. (A) mRNA expression profile of PDLIM family members in Wt and Clp36ΔLIM 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. Platelets were allowed to spread for 20 minutes and were then stained with phalloidin Atto647N and CLP36-PDZ antibody to detect CLP36 protein. Representative confocal microscopy images are shown. Scale bar: 3 μm. (C) Representative TEM images of resting Wt and Clp36ΔLIM platelets. Scale bar represents 1 μm. Platelet width was analyzed with EM images and the mean platelet volume (MPV) of Wt and Clp36ΔLIM mice measured with a blood cell counter are 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 Clp36ΔLIM (gray) mice over a 5 day period after i.v injection of a Dylight-488 anti-GPIX Ig derivative (0.5 mg/kg), (n=5).

**Figure 2.** Enhanced GPVI-induced integrin activation and degranulation in Clp36ΔLIM platelets. (A) Flow cytometric analysis of integrin αIIbβ3 activation (binding of JON/A-PE) and degranulation-dependent P-selectin exposure in Wt (black bar) and Clp36ΔLIM (gray bar) platelets in response to the indicated GPCR agonists and rhodocytin, (B) in response to GPVI agonists. Results are given as mean fluorescence intensities (MFI) ± SD of 5 mice per group and are representative of 4 individual experiments. Abbreviations: U46: U46619; CRP: collagen related peptide; CVX: convulxin; RC: rhodocytin. *P<0.05, **P<0.01, ***P<0.001

**Figure 3.** Enhanced GPVI-induced aggregation and ATP release in Clp36ΔLIM platelets. (A) Washed platelets from Wt (black line) or Clp36ΔLIM (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 Clp36ΔLIM (gray line) (240 μl with 0.3x10^6 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 % of maximal ATP release ± SD of Wt (black bar) or Clp36ΔLIM (gray bar) platelets. **P<0.01, ***P<0.001. Abbreviations: CRP: collagen related peptide; CVX: convulxin.

**Figure 4.** Enhanced GPVI-induced Ca^{2+} mobilization in Clp36ΔLIM platelets. (A) Fura-2–loaded Wt (black) or Clp36ΔLIM (gray) platelets were stimulated with 0.1 U/mL thrombin, 10 μM ADP or 1 μg/mL CRP in the presence of 0.5 mM EGTA or (B) in the presence of 1 mM Ca^{2+} and changes in [Ca^{2+}] were monitored fluorimetrically. Representative measurements and maximal increase in [Ca^{2+}] compared with baseline levels before stimulus (Δ[Ca^{2+}]) ± SD (n = 5 mice per group).

**Figure 5.** Enhanced GPVI-induced tyrosine phosphorylation in Clp36ΔLIM platelets. (A) Determination of whole cell tyrosine phosphorylation. Washed platelets from Wt and Clp36ΔLIM mice were stimulated with 0.1 μg/mL of CRP under stirring conditions at 37°C. 50 μL aliquots were taken at the indicated time points. Samples were probed with the anti-phosphotyrosine antibody 4G10. Expression of GPIIIa served as loading control. (n=3). (B) Quantification of produced IP₁ upon platelet activation. Washed platelets from Wt (black) and Clp36ΔLIM (gray) mice were stimulated with the indicated agonists for 5 min at 37°C (450 rpm). Platelets were lysed and IP₁, a specific metabolite of IP₃, was quantified using an ELISA assay. Results are given as the mean IP₁ concentration (nM) ± SD (n=4 per group).

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Figure 6. Increased thrombus formation and pro-coagulant activity of Clp36<sup>ΔLIM</sup> platelets. (A) Heparinized whole blood from Wt (black) or Clp36<sup>ΔLIM</sup> (gray) mice was perfused over immobilized collagen (0.2 mg/mL) at a shear rate of 1000 s<sup>-1</sup>. Representative phase contrast images (upper panel) and fluorescent images (anti-GPIX-DyLight-488; lower panel) are shown. Lower panel: mean surface coverage and relative thrombus volume ± SD (n=10 per group). (Bar: 50 µm) (B) Clp36<sup>ΔLIM</sup> platelets display enhanced pro-coagulant activity. Whole blood was perfused over a collagen-coated (0.2 mg/mL) surface at a shear rate of 1700 s<sup>-1</sup> for 4 min. Adherent platelets were stained with Annexin-V-Dylight-488 (0.25 µg/mL). Representative phase contrast (upper panel) and fluorescence images (lower panel) are shown (Bar: 50 µm). Mean percentage of Annexin-V positive platelets ± SD (n≥4) for the indicated shear rates. ***P < 0.001. (C) The abdominal aorta of Wt and Clp36<sup>ΔLIM</sup> bone marrow chimeric mice was injured by tight compression with a forceps and blood flow was monitored for 30 min with an ultrasonic flow probe. The time to stable vessel occlusion is shown. Each symbol represents one individual. (D) Normal hemostasis in Clp36<sup>ΔLIM</sup> bone marrow chimeric mice. Tail bleeding times of Wt and Clp36<sup>ΔLIM</sup> bone marrow chimeric mice was measured in saline at 37°C. Each symbol represents one individual. The white dots represent Wt mice, the black dots and gray dots represent Wt and Clp36<sup>ΔLIM</sup> bone marrow chimeric mice, respectively.

Figure 7. Enhanced GPVI signaling in Clp36<sup>-/-</sup> platelets. (A) Flow cytometric analysis of integrin αIIbβ3 activation (JON/A-PE) and P-selectin exposure in Clp36<sup>-/-</sup> 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. Abbreviations: Thr: thrombin; CRP: collagen related peptide; RC: rhodocytin. **P<0.01, ***P<0.001. (B) Washed platelets from Wt (black line) or Clp36<sup>-/-</sup> (gray line) were stimulated with the indicated agonists and light transmission was recorded on an aggregometer (n=4). (C) Heparinized Wt (black) or Clp36<sup>-/-</sup> (gray) whole blood was perfused over immobilized collagen (0.2 mg/mL) at a shear rate of 1000 s<sup>-1</sup>. Representative phase contrast images (upper panel) and fluorescent images (anti-GPIX-DyLight-488; lower panel). Mean surface coverage ± SD % (n=10 per group). ***P < 0.001.

Figure 8. CLP36 interacts with the GPVI signalosome and is degraded during platelet activation. (A) Co-immunoprecipitation of CLP36 and GPVI in Wt, Clp36<sup>-/-</sup> and Gp6<sup>-/-</sup> platelets followed by Western blot analysis. (B) Degradation of CLP36 upon activation with convulxin (CVX) or ionomycin in presence and absence of the calpain inhibitor calpeptin. Coomassie staining of blots served as loading control.
Novelty and Significance

What Is Known?

- Glycoprotein VI (GPVI) is the major collagen receptor on the surface of platelets and is activated upon 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.

Increasing experimental evidence suggests the existence of a negative feedback mechanism that controls GPVI signaling in platelets. This regulation appears 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.
A

MFI (JON/A-PE)

- 0.1
- 0.01
- 0.001
- 10
- 10/3
- 1.2

Thr (U/mL) ADP (µM) ADP + RC (µg/mL) U46

B

MFI (JON/A-PE)

- 5
- 1
- 0.1
- 0.1
- 0.05

CRP (µg/mL) CVX

MFI (anti-P-selectin FITC)

- 0.1
- 0.01
- 0.001
- 10
- 10/3
- 1.2

- 5
- 1
- 0.1
- 0.1
- 0.05

CRP (µg/mL) CVX
A

Thrombin 0.1 U/mL

[Ca$^{2+}$]$_i$ (nM)

100 200 300 (s)

ADP 10 μM

[Ca$^{2+}$]$_i$ (nM)

100 200 300 (s)

CRP 1 μg/mL

[Ca$^{2+}$]$_i$ (nM)

100 200 300 (s)

Δ[Ca$^{2+}$]$_i$ (nM)

Thr ADP CRP

+ 0.5 mM EGTA

B

Thrombin 0.1 U/mL

[Ca$^{2+}$]$_i$ (nM)

100 200 300 (s)

ADP 10 μM

[Ca$^{2+}$]$_i$ (nM)

100 200 300 (s)

CRP 1 μg/mL

[Ca$^{2+}$]$_i$ (nM)

100 200 300 (s)

Δ[Ca$^{2+}$]$_i$ (nM)

Thr ADP CRP

+ 1 mM Ca$^{2+}$