Growth Factor Receptor–Bound Protein 2 Contributes to (Hem)Immunoreceptor Tyrosine-Based Activation Motif–Mediated Signaling in Platelets

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Rationale: Platelets are anuclear cell fragments derived from bone marrow megakaryocytes (MKs) that safeguard vascular integrity but may also cause pathological vessel occlusion. One major pathway of platelet activation is triggered by 2 receptors that signal through an (hem)immunoreceptor tyrosine-based activation motif (ITAM), the activating collagen receptor glycoprotein (GP) VI and the C-type lectin-like receptor 2 (CLEC-2). Growth factor receptor–bound protein 2 (Grb2) is a ubiquitously expressed adapter molecule involved in signaling processes of numerous receptors in different cell types, but its function in platelets and MKs is unknown.

Objective: We tested the hypothesis that Grb2 is a crucial adapter protein in (hem)immunoreceptor tyrosine-based activation motif signaling in platelets.

Methods and Results: Here, we show that genetic ablation of Grb2 in MKs and platelets did not interfere with MK differentiation or platelet production. However, Grb2-deficiency severely impaired glycoprotein VI–mediated platelet activation because of defective stabilization of the linker of activated T-cell (LAT) signalosome and activation of downstream signaling proteins that resulted in reduced adhesion, aggregation, and coagulant activity on collagen in vitro. Similarly, CLEC-2–mediated signaling was impaired in Grb2-deficient platelets, whereas the cells responded normally to stimulation of G protein–coupled receptors. In vivo, this selective (hem) immunoreceptor tyrosine-based activation motif signaling defect resulted in prolonged bleeding times but affected arterial thrombus formation only after concomitant treatment with acetylsalicylic acid, indicating that defective glycoprotein VI signaling in the absence of Grb2 can be compensated through thromboxane A2–induced G protein–coupled receptor signaling pathways.

Conclusions: These results reveal an important contribution of Grb2 in (hem)immunoreceptor tyrosine-based activation motif signaling in platelets in hemostasis and thrombosis by stabilizing the LAT signalosome. (Circ Res. 2014;114:444-453.)

Key Words: blood platelets ■ hemostasis ■ mice ■ signal transduction ■ thrombosis

At sites of vascular injury, platelets adhere and aggregate on the exposed subendothelial extracellular matrix and thereby form a plug that seals the wound. This process is crucial for normal hemostasis, but in diseased vessels it may lead to pathological thrombus formation and infarction of vital organs.1 Among the multiple macromolecular constituents of the extracellular matrix, collagens are the most thrombogenic because they directly induce powerful cellular activation. This activation is mediated by glycoprotein VI (GPVI), a receptor of the Ig superfamily, which is closely related to the Fcα receptor (FcαR) and the natural killer cell receptors.2 GPVI is noncovalently associated with an FcRγ-chain homodimer, which contains a classical immunoreceptor tyrosine-based activation motif (ITAM) and initiates a signaling pathway similar to that of immune receptors, such as the T cell receptor (TCR), the B cell receptor (BCR), or the FcγRs. Ligand-induced
cross-linking of GPVI triggers tyrosine phosphorylation of the FcRγ-chain on its ITAMs by the Src kinases Fyn and Lyn. This induces the recruitment and subsequent activation of the tandem Src homology (SH) 2 domain–containing tyrosine kinase Syk, which in turn initiates a downstream signaling cascade involving the adapters linker of activated T cells (LAT) and SH2 domain–containing leukocyte protein of 76 kDa (SLP-76) and culminates in the activation of different effector molecules, including phospholipase C (PLC) γ2 and phosphoinositide-3 kinase. GPVI has emerged as a promising target for antithrombotic therapy because its absence or functional inhibition provides protection from pathological thrombus formation and stroke in mice without causing major bleeding complications.

A similar but not identical signaling pathway is used by the more recently discovered activated platelet surface glycoprotein, C-type lectin-like receptor 2 (CLEC-2), which initiates signaling on tyrosine phosphorylation of only a single YXXL motif (hemITAM) in its cytoplasmic tail. Compared with GPVI, CLEC-2 might also become a target for antithrombotic agents because CLEC-2 deficiency has only a minor effect on hemostasis but prevents formation of stable vessel occluding thrombi. Importantly, however, CLEC-2 is also required for the separation of the lymphatic from the blood vasculature, most likely because of its interaction with its only known physiological ligand, podoplanin. Although several proteins and their functions within the (hem)ITAM signaling pathway have been elucidated, this complex machinery is still not fully understood.

Growth factor receptor–bound protein 2 (Grb2) is an adapter protein comprising a central SH2 domain flanked by 2 SH3 domains. It has been identified as a major mediator in Ras-mitogen-activated protein kinase (MAPK) activation, which is induced by many growth factor receptors because of its association with son of sevenless homolog (SOS1), a GDP–GTP exchange factor for Ras. Grb2 is ubiquitously expressed and has essential functions during embryonic development and malignant transformation. On TCR stimulation, Grb2 associates with LAT or the CD3 complex via SH2 and collagen homology domain–containing protein (Shc). Analysis of mice with a T cell-specific Grb2-deficiency revealed that the adapter plays a pivotal role in early T cell development and that it amplifies TCR signaling at the proximal end of the tyrosine phosphorylation cascade. In addition, Houtman et al have demonstrated a critical role for LAT in amplifying T cell signaling through the generation of an LAT signalosome in association with SOS1. Recently, 2 independent studies on mice with a B cell-specific Grb2-deficiency identified, contrary to T cells, enhanced BCR-induced Ca2+ signaling in the absence of Grb2, whereas conflicting results on the role of Grb2 in MAPK activation in these cells were reported. Grb2 is also expressed in platelets and MKs, and based on in vitro studies, it has been proposed that the Grb2/SOS1/mitogen-activated protein kinase/ERK kinase pathway is of central importance for MK differentiation. Furthermore, the LAT signalosome is key to (hem)ITAM-mediated platelet activation. In platelets, Grb2 is part of the LAT signalosome and becomes tyrosine phosphorylated after GPVI-mediated platelet activation, indicating a possible role of the adapter in GPVI signaling. Interestingly, in human platelets Grb2 associates strongly with LAT after GPVI stimulation, whereas the Grb2-related adapter protein downstream of Shc (Gads) shows only a weak association, and studies in Gads-deficient mice demonstrate only a minor role of Gads in supporting GPVI signaling. In addition, an increased interaction of Grb2 with the adapter protein downstream of tyrosine kinase (Dok) 3 on activation of integrin αIIbβ3 has been reported, implicating a possible role in integrin outside-in signaling. However, because of the lack of an appropriate animal model, the exact role of Grb2 in platelet production and function has remained elusive.

Here, we show that an MK/platelet-specific Grb2-deficiency has no effect on MK differentiation and platelet production but severely affects (hem)ITAM signaling in platelets because of the defective stability of the LAT signalosome.

Methods

Detailed methods are provided in the online-only Data Supplement.

Results

Megakaryocyte-Specific Grb2-Deficiency Has No Effect on Platelet Production

To study the role of Grb2 in platelet physiology in vivo, we crossed mice carrying 2loxP sites flanking exon 2 of the Grb2 gene (Grb2fl/fl) with mice carrying Cre recombinase under the control of the platelet/MK-specific platelet factor 4 promoter. Western blot analysis confirmed the absence of Grb2 in platelets from Grb2fl/fl,PF4-Cre+–/– mice (hereon referred to as Grb2–/– mice), whereas the expression of the protein in splenocytes was unaltered (Online Figure IA). Grb2–/– mice were born in Mendelian ratios, appeared healthy, and did not show any signs of spontaneous bleeding or blood-lymph vessel separation defects. Platelet count and size, as well as surface expression levels of prominent glycoprotein receptors, were unaltered compared with the wild-type control (Online Figure IB and IC, Online Table 1), indicating that megakaryopoiesis and platelet formation can occur independent of Grb2. This

Nonstandard Abbreviations and Acronyms

ASA: acetylsalicylic acid
CLEC-2: C-type lectin-like receptor 2
CRP: collagen-related peptide
ERK1/2: extracellular signal–regulated protein kinase 1/2
FcR: Fc receptor
GPVI: glycoprotein VI
Grb2: growth factor receptor–bound protein 2
ITAM: immunoreceptor tyrosine-based activation motif
LAT: linker of activated T cell
MAPK: mitogen-activated protein kinase
PLC: phospholipase C
SH: Src homology
SLP: Src homology domain–containing leukocyte protein
SOS1: son of sevenless homolog
TxA2: thromboxane A2

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was corroborated by normal MK numbers in the bone marrow of the mutant animals (Grb2+/+; 7.8±0.3 MK/visual field, Grb2−/−; 8.8±0.7 MK/visual field; Online Figure ID).

**Grb2−/− Platelets Show Diminished Responses to GPVI and CLEC-2 Stimulation But Normal Integrin Outside-In Signaling**

To investigate the consequences of Grb2-deficiency on platelet function, we performed ex vivo aggregation studies. Grb2−/− platelets aggregated normally in response to the G protein–coupled receptor agonists thrombin, ADP, and the thromboxane A2 (TxA2) analog U46619 (Figure 1A). In contrast, the mutant platelets displayed a marked aggregation defect in response to intermediate and low doses of the GPVI agonists collagen and collagen-related peptide (CRP). These defects could, however, be overcome by high concentrations of these agonists in which Grb2-deficient platelets showed a slightly delayed aggregation onset but a full aggregation response (Figure 1B). Similar observations were made with the snake venom toxin rhodocytin that induces aggregation via the (hem)ITAM receptor CLEC-2 (Figure 1B). This selective (hem)ITAM receptor signaling defect was confirmed by flow cytometric analysis of integrin αIIbβ3 activation and of degranulation-dependent P-selectin surface exposure (Figure 1C). These results demonstrated that Grb2 is required for (hem)ITAM receptor–induced platelet activation, whereas it is dispensable for platelet activation downstream of G protein–coupled receptors.

**Defective GPVI-Induced Ca2+ Mobilization in Grb2−/− Platelets**

Agonist-induced platelet activation leads to an increase in cytosolic calcium concentrations ([Ca2+]i) through release of ligand-occupied integrin αIIbβ3 mediates outside-in signaling, leading to cytoskeletal reorganization and platelet spreading. Grb2 has been proposed to play a role in this process, as well as in the formation of actin-rich protrusions. To test this directly, Grb2−/− and wild-type platelets were allowed to spread on a fibrinogen-coated surface in the presence of low concentrations of thrombin (Figure 2A). Grb2−/− and wild-type platelets formed filopodia and lamellipodia with similar kinetics, and after 30 minutes, the number of fully spread platelets was comparable between the 2 groups. We confirmed these findings by performing the spreading experiments also in the presence of apyrase and indomethacin to inhibit stimulation by released secondary mediators in the absence (Online Figure IIA) or presence of thrombin (Online Figure IIB). Integrin αIIbβ3 outside-in signaling also regulates clot retraction. Therefore, we induced clot formation in platelet-rich plasma by the addition of a high dose of thrombin (5 U/mL) and 20 mmol/L CaCl2 and monitored retraction over time. No differences between wild-type and Grb2−/− platelets were observed (Figure 2B), demonstrating that the adapter is not required for this integrin αIIbβ3-controlled process.

Figure 1. Impaired aggregation responses, αIIbβ3 activation, and granule release in Grb2−/− platelets in response to glycoprotein VI and C-type lectin-like receptor 2 stimulation. Washed platelets from Grb2+/+ (black line) and Grb2−/− (gray line) mice were activated with the indicated concentrations of (A) ADP, U46619, and thrombin and (B) collagen, collagen-related peptide (CRP), or rhodocytin, and light transmission was recorded on a Fibrintimer 4-channel aggregometer. ADP measurements were performed in platelet-rich plasma. Representative aggregation traces of ≥3 individual experiments are depicted. C, Flow cytometric analysis of integrin αIIbβ3 activation (top) and degranulation-dependent P-selectin exposure (bottom) in response to the indicated agonists in wild-type and Grb2−/− platelets. Results are mean fluorescence intensities (MFI)±SD of 4 mice per group and representative of 4 individual experiments (*P<0.05, **P<0.01, ***P<0.001). CVX indicates convulxin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; and RC, rhodocytin.
Ca\textsuperscript{2+} from intracellular stores and Ca\textsuperscript{2+} entry across plasma membrane Ca\textsuperscript{2+} channels.\textsuperscript{28} To test whether the observed GPVI signaling defect in Grb2\textsuperscript{−/−} platelets was based on impaired Ca\textsuperscript{2+} signaling, we studied agonist-induced changes in [Ca\textsuperscript{2+}]\textsubscript{i} fluorimetrically. Grb2\textsuperscript{−/−} platelets displayed markedly reduced Ca\textsuperscript{2+} store release and entry in response to CRP but showed normal responses to thrombin (Figure 3A and 3B). Notably, passive store depletion with thapsigargin did not lead to any differences in the kinetics of store release or store-operated Ca\textsuperscript{2+} entry between wild-type and Grb2\textsuperscript{−/−} platelets (Figure 3C and 3D).

**Defective Adhesion, Aggregate Formation, and Coagulant Activity of Grb2\textsuperscript{−/−} Platelets on Collagen Under Flow**

Thrombus formation at the site of vascular injury requires stable shear-resistant platelet adhesion on the extracellular matrix and auto- and paracrine platelet activation by locally released secondary mediators.\textsuperscript{3} To address the effect of Grb2-deficiency on these processes, platelet adhesion to collagen was studied in an ex vivo whole blood perfusion system. When blood was perfused over immobilized collagen at a shear rate of 1000 s\textsuperscript{−1}, wild-type platelets rapidly adhered to the collagen surface and recruited additional platelets from the blood stream, resulting in the formation of stable 3-dimensional thrombi (Figure 4A, left). In sharp contrast, adhesion of Grb2\textsuperscript{−/−} platelets was markedly impaired, and aggregate formation was virtually abrogated. As a result, both the surface area covered by platelets and thrombus volume at the end of the perfusion period were reduced by \(\approx 94\%\) for Grb2\textsuperscript{−/−} platelets compared with the wild type.
Impaired Hemostasis and Partially Defective Arterial Thrombus Formation in Grb2<sup>−/−</sup> Mice

To study the effect of Grb2-deficiency on hemostasis, tail bleeding times were determined (Figure 5A). Grb2<sup>−/−</sup> mice exhibited prolonged (mean bleeding time: Grb2<sup>−/−</sup>: 197±107 seconds versus Grb2<sup>+/+</sup>: 381±237 seconds; \( P<0.0005 \)) and highly variable bleeding times, suggesting thrombus instability as recently also described for mice lacking the adapter LAT.\(^{[0,31]}\)

To examine to which extent the in vitro observed defects of Grb2<sup>−/−</sup> platelets influenced thrombotic events in vivo, we studied occlusive thrombus formation in a model where the abdominal aorta is mechanically injured, and blood flow is monitored with an ultrasonic perivascular Doppler flowmeter. Unexpectedly, wild-type and mutant mice formed occlusive thrombi with similar kinetics (mean occlusion time: Grb2<sup>−/−</sup>: 303±101 seconds versus Grb2<sup>+/+</sup>: 351±98 seconds; Figure 5B), whereas the vessels of GPVI-deficient mice did not occlude.\(^{32}\)

We have previously shown that defective GPVI signaling can be compensated through TxA<sub>2</sub>-induced G protein–coupled receptor signaling pathways.\(^{33}\) Therefore, we inhibited TxA<sub>2</sub> synthesis in control and Grb2<sup>−/−</sup> mice by administration of a low dose of acetylsalicylic acid (ASA) intravenously (1 mg/kg) and assessed thrombus formation in the injured aorta after 15 minutes. Strikingly, although ASA treatment had no significant effect on thrombus formation in control mice (14 of 15 occluded, mean occlusion time: 296±84 seconds), a
strong effect was seen in the mutant mice where 7 of 17 vessels did not occlude \((P<0.05)\), and occlusion times for the remaining vessels were increased compared with the wild-type \((419\pm99 \text{ seconds}; P<0.005)\). Similar effects were observed in a model of FeCl$_3$-induced injury of mesenteric arterioles where vessel occlusion times of Grb2-deficient mice were prolonged only in the presence of ASA compared with ASA-treated wild-type mice \((17.89\pm4.13 \text{ versus } 25.73\pm5.20 \text{ minutes}; P<0.005; \text{ Online Figure III})\), confirming that the GPVI signaling defect in these animals is at least, in part, compensated by TxA$_2$-dependent activation pathways in vivo. In contrast, ASA treatment prolonged tail bleeding times of wild-type and \(Grb2^{-/-}\) mice to a similar extent (Figure 5A).

### Grb2 Stabilizes the LAT Signalsome to Mediate (hem)ITAM Signaling

To investigate the molecular basis of Grb2 in transmitting (hem)ITAM-mediated signaling, Grb2 was immunoprecipitated from resting and GPVI-, as well as CLEC-2–, stimulated wild-type platelets, and samples were analyzed for protein tyrosine phosphorylation (Figure 6A). In line with our previous study,\(^{22}\) LAT immunoprecipitated with Grb2 in (hem) ITAM-stimulated platelets, demonstrating that Grb2 forms part of the LAT signalsome (Figure 6A). Significantly, analysis of protein tyrosine phosphorylation of key components of the (hem)ITAM signaling cascade by Western blot analysis revealed that in \(Grb2^{-/-}\) platelets phosphorylation of proteins located upstream of the LAT signalsome, most importantly the FcR \(\gamma\)-chain, and CLEC-2, respectively, and the tyrosine kinase Syk is maintained (Figure 6B and 6C; Online Figure IV). In contrast, phosphorylation of LAT at Y195, which is bound by Grb2 and contributes to PLC\(\gamma\)2 activation, and Y136, the high-affinity binding site for PLC\(\gamma\)2, is strongly reduced in Grb2-deficient compared with wild-type platelets. This resulted in reduced phosphorylation of proteins that lie downstream of the LAT signalsome, including not only key effector proteins such as PLC\(\gamma\)2, but also the adapter SLP-76 and the guanine nucleotide exchange factor Vav3 (Figure 6B and 6C; Online Figure IV). These results position Grb2 as a central adapter molecule of the LAT signalsome in platelets, stabilizing the LAT complex by direct association, thereby enabling downstream signaling and platelet activation.

Furthermore, we analyzed Ras-MAPK signaling because Grb2 has initially been identified as a major mediator of this signaling pathway induced by numerous receptors,\(^4\) but the exact function of MAPK signaling in platelets is still not fully understood.\(^{35}\) Interestingly, phosphorylation of extracellular signal–regulated protein kinase 1/2 (ERK1/2) in response to convulxin was severely reduced in \(Grb2^{-/-}\) platelets compared with wild-type platelets, whereas phosphorylation of p38 was unaltered (Figure 7A). To reveal how Grb2 regulates ERK1/2 phosphorylation, we analyzed MAPK signaling in LAT-deficient platelets, demonstrating a comparable reduction in ERK1/2 phosphorylation after GPVI stimulation (Figure 7B), indicating that Grb2-dependent recruitment of SOS1 to the LAT signalsome seems to be required for full ERK1/2 activation.\(^{35}\) Alternatively, loss of ERK1/2 phosphorylation might be a result of impaired activation by protein kinase C, which lies downstream of PLC\(\gamma\)2 and is critical for the activation of MAPK signaling in platelets.\(^{36}\)

### Discussion

In this study, we have shown that Grb2-deficient platelets display severely impaired (hem)ITAM signaling but unaltered G protein–coupled receptor function and integrin outside-in signaling. This highly specific defect resulted in impaired hemostasis and partially defective arterial thrombus formation, indicating that Grb2-dependent signaling in platelets may contribute to the pathogenesis of acute ischemic disease states.

Grb2 is widely expressed throughout the body and associates with a large variety of receptors and signaling pathways in different cell types.\(^9\) Its physiological importance is best highlighted by the early embryonic lethality of \(Grb2^{-/-}\) mice because of defective differentiation of endodermal cells and formation of the epiblast.\(^{11}\) Grb2 is also strongly expressed in the hematopoietic system, and a previous study suggested a role of the Grb2/SOS1/mitogen-activated protein kinase/ ERK kinase complex in thrombopoietin receptor (c-Mpl) signaling during MK maturation.\(^{37}\) We found, however, normal platelet counts and bone marrow MK numbers in mice with an MK-specific Grb2-deficiency, demonstrating that Grb2 is dispensable for megakaryopoiesis and platelet production in vivo. This might be explained by a compensatory alternative pathway downstream of c-Mpl involving the small GTPase Rap1,\(^{38}\) but further studies will be required to address this in detail.

Grb2 has been implicated in antigen receptor signaling in lymphocytes, with recent reports showing prominent roles of the adapter during B cell maturation by negatively regulating proximal BCR signaling,\(^{16,17}\) and a crucial function in T cell selection by amplifying TCR signaling.\(^{14,38}\) Similar to the BCR and TCR complexes, the GPVI/FcR \(\gamma\)-chain complex and CLEC-2 in platelets use a (hem)ITAM signaling module to transduce extracellular signals, thereby mediating platelet activation, aggregation, and thrombus formation. Previous studies in human platelets demonstrated that Grb2 binds to the membrane-bound adapter molecule LAT\(^{20}\) and that it is phosphorylated after GPVI stimulation,\(^{21}\) indicating a role of Grb2 in ITAM signaling in platelets. However, given the opposing effects of Grb2-deficiency in B and T cells, it was not clear what the exact function of the adapter in platelets would be. We found markedly impaired integrin \(\alpha I b \beta 3\) activation, degranulation, and aggregation of \(Grb2^{-/-}\) platelets in response to GPVI or CLEC-2 stimulation, which phenocopies the defects seen in LAT-deficient mice,\(^{22}\) although to a somewhat lesser extent (Figure 1). Therefore, like in T cells, Grb2 acts as a positive regulator of (hem)ITAM receptor–induced cellular activation.

Compared with immune receptor stimulation, agonist-induced platelet activation requires an increase in \([\text{Ca}^{2+}])\), that is mainly triggered by PLC-mediated inositol 1,4,5-triphosphate production, resulting in \([\text{Ca}^{2+}])\) store release and subsequent \([\text{Ca}^{2+}])\) entry.\(^{28}\) The tyrosine phosphorylation cascade leading to PLC\(\gamma\)2 activation shows striking similarities among the GPVI, the BCR, and the TCR, involving phosphorylation of accessory receptor chains, activity of Src...
and Syk family kinases, and involvement of various adapter proteins, such as SLP-65/SLP-76 and LAT.13 Our data clearly show that Grb2 function in platelets resembles its function in TCR signaling in thymocytes, in that in both cell types deletion of Grb2 results in impaired Ca²⁺ signaling (Figure 3),14 in contrast to B cells in which Ca²⁺ influx is enhanced in the absence of the adapter.15,17 However, although in thymocytes Grb2-deficiency resulted in defects at the proximal end and at the level of the LAT signalosome of the TCR signaling cascade,14,15 in platelets Grb2 seems to only act downstream of platelet GPVI at the level of the LAT signalosome. Our data demonstrate that Grb2 interacts with LAT after (hem)ITAM stimulation and that in the absence of Grb2 tyrosine phosphorylation of LAT at Y136 and Y195 (corresponding to LAT Y132 and Y191 in humans, respectively) is reduced (Figures 6 and 7). Grb2 strongly associates via its SH2 domains with 3 phosphotyrosines of LAT (Y171, Y191, Y226),39,40 whereas PLCγ preferentially binds at Y132.39,41 Previous studies in T-cell lines have shown that mutations of the Gads/Grb2-binding residues of LAT (Y171, Y191, Y226) result in reduced binding of PLCγ1 to LAT and downstream signaling events, including tyrosine phosphorylation of the phospholipase.39,41,42 Therefore, a concept of cooperative binding among LAT-associated proteins that stabilize the signaling complex has been proposed.15,34 In line with this, Grb2-deficient platelets display reduced tyrosine phosphorylation of the key downstream signaling molecules PLCγ2, SLP-76, and Vav3 after GPVI and CLEC-2 stimulation, whereas tyrosine phosphorylation of the key downstream signaling molecules PLCγ2, SLP-76, and Vav3 after GPVI and CLEC-2 stimulation, whereas tyrosine phosphorylation of PLCγ2 and SLP-76 was also reduced (Figure 6C). These findings support the notion that the LAT signalosome is a dynamic and cooperative signaling complex that serves as an adapter and regulator of downstream signaling events.

Figure 6. Defective immunoreceptor tyrosine-based activation motif–induced signal transduction in Grb2−/− platelets. A, Washed platelets (5×10⁸/mL) were stimulated with 10 µg/mL collagen-related peptide (CRP) or 300 nmol/L rhodocytin (RC) for 90 seconds and subsequently lysed with nonidet P-40 (NP-40) detergent. Growth factor receptor–bound protein 2 (Grb2) was immunoprecipitated, and proteins were separated by reducing SDS-PAGE (10%) and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was probed with an anti-pTyr mAb (4G10) and reprobed with an anti–linker of activated T-cell (LAT) and anti-Grb2 antibody. B, Washed platelets (7×10⁸/mL) from Grb2+/+ and Grb2−/− mice were stimulated with 0.5 µg/mL convulxin (CVX) under stirring conditions at 37°C. Aliquots were taken at the indicated time points and subsequently lysed with NP-40 detergent. Proteins were separated by reducing SDS-PAGE (10%), blotted on a PVDF membrane, and stained using the indicated phospho-specific antibodies. Staining of the respective nonphosphorylated proteins or actin served as loading controls. The result shown is representative of 3 individual experiments. C, Washed platelets (5×10⁸/mL) were stimulated with 0.5 µg/mL CVX for the indicated time points and subsequently lysed with NP-40 detergent. Syk, LAT, SLP-76, Vav1, Vav3, and PLCγ2 were immunoprecipitated, and proteins were separated by reducing SDS-PAGE (10%) and transferred to a PVDF membrane. The membrane was probed with an anti-pTyr mAb (4G10) and reprobed with Syk, LAT, SLP-76, Vav1, Vav3, and PLCγ2 antibodies.
Figure 7. Defective extracellular signal–regulated protein kinase 1/2 (ERK1/2) signaling in Grb2–/– and Lat–/– platelets. Determination of mitogen-activated protein kinase (MAPK) phosphorylation. Washed platelets (5×10^8/mL) from Grb2+/+ and Grb2–/– mice (A) or Lat+/+ and Lat–/– mice (B) were stimulated with 0.5 μg/mL convulxin (CVX) under stirring conditions at 37°C. Aliquots were taken at the indicated time points and subsequently lysed with NP-40 detergent. Proteins were separated by reducing SDS-PAGE (10%), blotted on a PVDF membrane, and stained using the indicated phospho-specific antibodies. Staining of the respective nonphosphorylated proteins, GAPDH or tubulin served as loading controls. The result shown is representative of 3 individual experiments.

phosphorylation of the FcR γ-chain, CLEC-2, and Syk is maintained (Figure 6; Online Figure IV). Taken together, our present observations emphasize that Grb2 is an important adapter protein of the LAT signalosome, which stabilizes this signaling complex by its direct association after (hem)ITAM-induced stimulation, thereby enabling downstream signaling and platelet activation.

The residual phosphorylation of LAT and PLCγ2 in the absence of Grb2 implicates that other proteins may partially compensate its loss. One possible candidate is the adapter protein Gads, a second member of the Grb2 family, which has been shown to play a supportive but not essential role in GPVI- and CLEC-2–mediated platelet activation.25 Based on our data, we speculate that Grb2 is the major adapter protein in platelets stabilizing the LAT signalosome, thereby activating PLCγ2, whereas Gads stabilizes SLP-76 binding to support platelet activation in response to weak (hem)ITAM stimuli. This might also explain the less pronounced hem(ITAM) signaling defect in Grb2+/– mice compared with Lat+/– mice.

Besides LAT, further interaction partners of Grb2 are known (http://plateletweb.bioapps. biozentrum.uni-wuerzburg.de/plateletweb.php).43 Thus, it is feasible that Grb2 also exerts functions beyond LAT-dependent signaling. Subtle effects on other signaling pathways may have been masked by the pronounced hem(ITAM) signaling defect that arose from the deletion of the protein. With regard to human platelets, it is conceivable that Grb2 may contribute to the signaling pathway of the ITAM-bearing platelet Fc receptor, FcγRIIa.44,45 However, FcγRIIa is not expressed in murine platelets, demonstrating a principal limitation of mouse studies. Certainly, further investigations involving mouse models with distinct mutations of Grb2 are necessary to explicitly address this point.

The GPVI signaling defect in Grb2–/– platelets translates into severely impaired platelet adhesion, aggregate formation, and procoagulant activity on collagen under flow in vitro. A similar phenotype has been reported in mouse lines lacking LAT21 and Rac1,11 indicating a prominent role for Grb2 in thrombus formation by linking signaling molecules within the LAT signalosome. However, we found unaltered platelet spreading on fibrinogen and normal clot retraction in the absence of Grb2 (Figure 2), excluding a major function of the adapter in integrin outside-in signaling. This stands in contrast to a previous report suggesting a role of Grb2 in this process in human platelets because of its induced association with the adapter proteins Dok-1 and Dok-323 and might be explained by the fact that Dok-3 is not expressed in mouse platelets.

Platelets express 3 different members of MAPK family: p38, c-Jun N-terminal kinase, and ERK1/2.35 In our studies, we found that Grb2 is a positive regulator of ERK1/2 signaling and that p38 was not affected by the loss of Grb2 (we did not see activation of c-Jun N-terminal kinase in wild-type platelets). Furthermore, our data demonstrate that Grb2 mediates ERK1/2 activation in an LAT-dependent manner (Figure 7). This can be explained by recruitment of SOS1 to the LAT–Grb2 complex,15 which in turn activates the Ras-ERK1/2 signaling pathway. The presence of SOS1 in platelets has been described.46 ERK1/2, however, is also regulated downstream of protein kinase C,36 suggesting that this alone is not sufficient to activate the MAPK pathway, leading us to speculate that this delay is as a result of assembly of the LAT–Grb2–SOS1 signaling complex. Regardless of the mechanism, the exact function of MAPKs in platelets is controversially discussed, possibly because of off-target effects of MAPK inhibitors, but increasing evidence suggests a role in promoting thrombus formation.35 Interestingly, Mazharian et al.47 have previously shown that treatment of platelets with an ERK1/2 inhibitor resulted in decreased platelet adhesion to collagen under high shear flow. Based on this observation, it seems possible that the impaired adhesion and thrombus formation of Grb2–/– platelets may, in part, be a consequence of impaired ERK1/2 signaling.

GPVI-deficient mice are protected from pathological thrombus formation but display only mildly prolonged bleeding times.32 In contrast, although LAT deficiency is also protective in experimental thrombosis models, it additionally causes a significant hemostatic defect.30 We found that Grb2–/– mice,
similar to Lat⁺ mice, display highly variable and overall prolonged bleeding times, indicating that Grb2 has an important functional role downstream of GPVI and CLEC-2 in normal hemostasis. Surprisingly, however, we found that Grb2-deficiency alone was not protective in models of arterial thrombosis, suggesting that other signaling pathways can fully compensate the partial loss of hem(IIATAM) signaling in this setting. We have previously shown that defective GPVI signaling can be compensated by TxA2-mediated activation of integrin α2β1 via Gq/G13-induced signaling pathways, thereby enabling platelets to arrest on collagen and to reinforce activation through outside-in signals.49 In this report with line, we found that ASA treatment (1 mg/kg) markedly reduced or delayed occlusive thrombus formation in Grb2⁻/⁻ mice, whereas it had no significant effect in wild-type mice. These results indicate that the residual hem(IIATAM) signaling capacity in Grb2⁻/⁻ platelets is sufficient to trigger arterial thrombus formation by TxA2-mediated reinforcement of platelet activation, whereas hemostasis is affected in the absence of Grb2, independent of ASA treatment.

In summary, our data demonstrate that Grb2 is dispensable for platelet formation but plays an important role in platelet activation in hemostasis and thrombosis by coordinating and stabilizing the formation of the LAT signalosome after GPVI/CLEC-2 receptor stimulation.

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Disclosures

None.

References

Grb2 stabilizes the linker of activated T cell (LAT) signalosome, a protein complex required for signal propagation after (hem)ITAM stimulation.

Novelty and Significance

Stimulation of the major platelet-activating collagen receptor GPVI at sites of vascular injury induces a signaling pathway that culminates in platelet activation required for subsequent platelet aggregation and thrombus development. The formation of a signaling complex called LAT signalosome is central for proper signal transduction of (hem)ITAM-bearing receptors. This signalosome comprises several different adapter and effector proteins, including Grb2, but its exact function in platelets was entirely speculative. By using a genetic mouse model with a megakaryocyte/platelet-specific deletion of Grb2, we demonstrate that Grb2 contributes to the stabilization of this signaling complex. Consequently, platelet activation induced by GPVI and CLEC-2, a related (hem)ITAM receptor expressed in platelets, is strongly impaired by the loss Grb2, resulting in reduced adhesion and aggregation on collagen under flow in vitro. In contrast, the signaling pathways of G protein–coupled receptors that do not use the LAT signalosome were unaffected. In vivo, the impaired (hem)ITAM signaling causes prolonged bleeding times of mice, whereas thrombus formation is only affected under conditions of impaired G protein–coupled receptor signaling (eg, aspirin treatment). These results reveal an important role for the adapter protein Grb2 in propagation of GPVI- and CLEC-2–induced signals, which could be relevant during treatment with certain medications.
Growth Factor Receptor–Bound Protein 2 Contributes to (Hem)Immunoreceptor Tyrosine-Based Activation Motif–Mediated Signaling in Platelets
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SUPPLEMENTAL MATERIAL

DETAILED METHODS

Generation of mice with \textit{Grb2}\textsuperscript{−}\textsuperscript{−} megakaryocytes and platelets

To generate mice lacking Grb2 specifically in MKs and platelets, mice containing the Grb2 gene flanked by loxP sites (\textit{Grb2}\textsuperscript{fl/fl})\textsuperscript{1} were crossed with mice carrying the platelet factor 4 (PF4)–cre transgene (\textit{PF4-cre}\textsuperscript{+/-})\textsuperscript{2}. In the resulting \textit{Grb2}\textsuperscript{fl/fl,PF4-Cre+/-} mice, absence of Grb2 protein was confirmed by Western blot analysis of platelet lysates. Littermates (\textit{Grb2}\textsuperscript{fl/fl,PF4-Cre-/-}) served as controls. Mice were on a C57/Bl-6 background (F5). LAT-deficient animals have been described previously.\textsuperscript{3} Animal studies were approved by the district government of Lower Franconia (Bezirksregierung Unterfranken).

Chemical and reagents

Collagen (Kollagenreagent Horm; Nycomed), ADP, high-molecular-weight heparin, human fibrinogen, apyrase type III (all from Sigma-Aldrich), \(\alpha\)-thrombin (Roche Diagnostics), acetylsalicylic acid (ASA, aspirin i.v., Bayer), U46619 (Alexis Biochemicals), thapsigargin (Molecular probes, Invitrogen), ECL solution (PerkinElmer) were purchased, collagen-related peptide (CRP) was generated as described.\textsuperscript{4}

The anesthetic drugs medetomidine (Pfizer), midazolam (Roche Pharma AG), and fentanyl (Janssen-Cilag GmbH) and the antagonists atipamezol (Pfizer), flumazenil, and naloxon (both from Delta Select GmbH) were used according to the regulation of the local authorities. Anti-Grb2, anti-Vav3, anti-SLP-76 antibody (Millipore), anti-PLC\(_\gamma\)2, anti-Vav1 antibody (Santa Cruz Biotechnology), anti-actin, anti-\(\alpha\)-tubulin antibody (both from Sigma-Aldrich), anti-phospho-PLC\(_\gamma\)2, anti-phospho-LAT (Y191), anti-ERK1/2, anti-phospho-ERK1/2, anti-JNK, anti-phospho-JNK, anti-p38, anti-phospho-p38, anti-Syk, anti-phospho-Syk, anti-GAPDH, anti-rabbit IgG-HRP (all from Cell signaling), anti-phospho-LAT (Y132) (abcam), anti-mouse IgG-HRP (DAKO) were purchased from the respective companies. Anti-Btk antibody was a
kind gift from Dr. Tomlinson, anti-CLEC2 antibody was a kind gift from Dr. Mourão-sá. The antibody against the activated form of integrin αIIbβ3 (JON/A-PE) was from Emfret Analytics. All other antibodies were generated and modified in our laboratory as previously described.5

**Platelet preparation**

Mice were bled under isoflurane anesthesia from the retro-orbital plexus. Blood was collected in a tube containing 20 U/ml heparin, and platelet-rich plasma (prp) was obtained by two cycles of centrifugation at 300 g for 6 minutes at room temperature (RT). For preparation of washed platelets, prp was washed twice at 800 g for 5 minutes at RT and the pellet was resuspended in Tyrodes-HEPES in the presence of prostacyclin (0.1 μg/ml) and apyrase (0.02 U/ml). Platelets were then resuspended in Tyrodes-HEPES buffer containing 2 mmol/L CaCl₂ and 0.02 U/ml apyrase.

**Platelet spreading on fibrinogen**

Cover slips were coated with 100 μg/mL human fibrinogen and blocked with PBS 1% BSA. After rinsing with Tyrode-HEPES buffer, washed platelets (100 μL with 0.03x10⁶ platelets/μL) were either unstimulated or activated with 0.01 U/mL thrombin in the presence or absence of second wave inhibitors apyrase (2 U/mL) and indomethacin (1.4 μM). The cover slips were rinsed again and platelets were visualized with a Zeiss Axiovert 200 inverted microscope (100x/1.4 oil objective). Digital images were recorded using a CoolSNAP-EZ camera (Visitron) and analyzed off-line using Metavue software (Molecular Devises).

**Clot retraction**

Clot retraction studies were performed at 37°C in an aggregometer tube containing diluted prp (3×10⁵ platelets/μL), thrombin (5 U/ml), and CaCl₂ (20 mmol/L). Clot retraction was recorded with a digital camera over a time span of 4.5 hours after activation.
Intracellular Ca\(^{2+}\) measurements

Washed platelets were suspended in Tyrode’s buffer without Ca\(^{2+}\), and loaded with fura-2/AM (5 μmol/L) in the presence of Pluronic F-127 (0.2 μg/ml; Molecular Probes, Invitrogen) for 30 minutes at 37°C. After labeling, platelets were washed once and resuspended in Tyrode–HEPES buffer containing either no or 1 mmol/L Ca\(^{2+}\). Stirred platelets were activated with agonists or treated with thapsigargin (5 μM), and fluorescence was measured with a PerkinElmer LS 55 fluorimeter. Excitation was alternated between 340 and 380 nm, and emission was measured at 509 nm. Each measurement was calibrated using Triton X-100 and EGTA.

Aggregometry

Light transmission was measured on a Fibrintimer 4 channel aggregometer (APACT Laborgeräte und Analysensysteme) using prp (for ADP) or washed platelets (for all other agonists - 160 μL with 1.56x10\(^5\) platelets/μL). Measurements in washed platelets were performed in the presence of 70 μg/ml fibrinogen, except for thrombin.

Flow cytometry

Washed blood was activated with agonists at the indicated concentrations, stained with fluorophore-conjugated monoclonal antibodies at saturating concentrations for 10 minutes at 37°C and analyzed on a FACSCalibur (BD Biosciences). For determination of phosphatidylserine exposure, washed platelets (0.05 x 10\(^6\)/μL) were stimulated with the indicated agonists in the presence of Annexin-V-Dylight 488 for 15 minutes at 37°C. The reaction was stopped by addition of 500 μl buffer and samples were directly analyzed a FACSCalibur (BD Biosciences).
Western blot analysis

Washed platelets or splenocytes were lysed in lysis buffer (300 mM NaCl, 20 mM TRIS, 2 mM EGTA, 2 mM EDTA, 2 mM Na$_3$VO$_4$, 10 mM NaF, pH 7.5, supplemented with protease inhibitor cocktail (Sigma-Aldrich)) containing 1 % Igepal CA-630 for 20 minutes at 4 °C, centrifuged at 14,000 rpm for 10 minutes and the supernatants were collected. 4 x Laemmli sample buffer was added, and the samples boiled at 95 °C for 5 min. Next, samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and blotted onto a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked for 1 hour with 5 % BSA in TBS-T at RT and then incubated with the primary antibody at 4 °C over night. The membrane was washed 3 x 10 minutes in TBS-T, before being incubated with the secondary, HRP-labeled antibody. After extensive washing, the protein was visualized by ECL.

Protein phosphorylation studies and immunoprecipitations

Washed platelets (5-7x10$^5$/µL) from wild-type and Grb2$^{-/-}$ mice were stimulated in suspension with 0.5 µg/mL convulxin or 2 µg/mL rhodocytin under constant stirring conditions at 37 °C in the presence of apyrase (2 U/ml) and indomethacin (10 µM). Stimulation was stopped by addition of 1:1 ice cold lysis buffer containing 2 % Igepal CA-630. Lysates were centrifuged and supernatants were subjected to Western Blotting, as described above, or used for immunoprecipitations.

For immunoprecipitations the platelet lysates were precleared with protein A-sepharose in TBS-T for 1 hour at 4°C. The respective antibody was added to the resultant supernatant and rotated at 4°C overnight. The proteins were immunoprecipitated by addition of protein A- or protein G-sepharose beads. The sepharose pellet was washed sequentially in lysis buffer and TBS-T before the addition of Laemmli sample buffer. Precipitated proteins were separated by SDS-PAGE and subjected to Western blotting as described above.
Adhesion under flow conditions

Heparinized whole blood was perfused over collagen-coated cover slips as described\(^6\). Before perfusion, anticoagulated blood was incubated with Dylight-488–conjugated anti-GPIX derivative (0.2 µg/mL) at 37°C for 5 minutes. Aggregate formation was visualized with a Zeiss Axiovert 200 inverted microscope (40x/0.60 objective). Phase-contrast and fluorescence pictures were recorded with a CoolSNAP-EZ camera, and analyzed off-line using Metavue software.

Bleeding time

Mice were anesthetized and a 1 mm segment of the tail tip was removed with a scalpel. Tail bleeding was monitored by gently absorbing blood with filter paper at 20-second intervals without making contact with the wound site. When no blood was observed on the paper, bleeding was determined to have ceased. Experiments were stopped after 20 minutes.

Mechanical injury of the abdominal aorta

The abdominal cavity of anesthetized mice was opened to expose the abdominal aorta. An ultrasonic flowprobe (0.5PSB699, Transonic Systems, New York, USA) was placed around the vessel and thrombus formation was induced by a single firm compression with a forceps upstream of the flowprobe. Blood flow was monitored for 30 minutes. When indicated, ASA (1 mg/kg bodyweight) was administered to the animals prior to the experiment.

Staining of histological sections

5 µm sections of paraffin-embedded samples were stained with hematoxylin and eosin and analyzed using a Leica DHI 4000B inverse microscope equipped with a Leica digital camera.
Intravital microscopy of thrombus formation in FeCl$_3$-injured mesenteric arterioles

Intravital microscopy was performed as described previously$^7$. In brief, injury of mesenteric arterioles was induced by topical application of 20% FeCl$_3$. Adhesion and aggregation of fluorescently labeled platelets in arterioles was monitored for 40 minutes or until complete occlusion occurred (blood flow stopped for > 2 minutes).

Data Analysis

Results from at least 3 experiments per group are presented as mean ± SD. Differences between two groups were assessed by Welch’s test. The Fischer’s exact test was applied to assess variance in occurrence of occlusion. P-values <0.05 were considered statistically significant.
Online Table I. Levels of platelet glycoproteins in Grb2<sup>+/+</sup> and Grb2<sup>-/-</sup> mice. Expression of glycoproteins on the platelet surface was determined by flow cytometry. Diluted whole blood from the indicated mice was incubated with FITC-labeled antibodies at saturating conditions for 15 minutes at RT, and platelets were analyzed directly. Data are expressed as mean fluorescence intensity ± SD (n=4) and are representative of 3 individual experiments.
Online Figure I. Specific deletion of Grb2 in platelets. (A) Analysis of Grb2 expression in Grb2+/+ , Grb2+/− and Grb2−/− platelets and splenocytes by Western Blot. Expression of GPIIIa and actin were used as loading control. (B) Peripheral platelet counts and (C) platelet volume of wild-type and Grb2−/− mice measured with a blood cell counter are depicted. Results are mean ± SD of 7 mice per group. (D) Determination of MK numbers per visual field (294 x 221 μm) in hematoxylin and eosin stained BM sections. Values are mean ± SD (n ≥ 3).
Online Figure II. Normal integrin outside-in signaling in $Grb2^{-/-}$ platelets. Washed platelets of $Grb2^{+/+}$ and $Grb2^{-/-}$ mice were allowed to spread on fibrinogen (100 μg/ml) for 30 minutes in the presence of high dose apyrase (2 U/mL) and indomethacin (1.4 μM) either without stimulation (A) or after simultaneous stimulation with 0.01 U/ml thrombin (B). Representative differential interference contrast (DIC) images of 2 individual experiments (left) and statistical evaluation of the percentage of spread platelets at different spreading stages (right). 1: roundish, 2: only filopodia, 3: filopodia and lamellipodia, 4: fully spread.
Online Figure III. Thrombosis model of FeCl$_3$-induced injury of mesenteric arterioles.

Small mesenteric arterioles were injured by topical application of FeCl$_3$, and thrombus formation of fluorescently labeled platelets was monitored using intravital microscopy. Time to occlusion in the presence or absence of ASA is depicted. Each symbol represents one arteriole. ** $P < 0.01$. 
**Online Figure IV.** Defective hemITAM-induced signal transduction in Grb2\(^{-/-}\) platelets.

(A) Washed platelets (7 x 10\(^5\)µL) from Grb2\(^{+/+}\) and Grb2\(^{-/-}\) mice were stimulated with 2 μg/ml rhodocytin (RC) under stirring conditions at 37 °C. Aliquots were taken at the indicated time points and subsequently lysed with NP-40 detergent. Proteins were separated by reducing SDS-PAGE (10%), blotted on a PVDF membrane, and stained using the indicated phospho-specific antibodies. Staining of the respective non-phosphorylated proteins or actin served as loading controls. The result shown is representative for three individual experiments. (B) Washed platelets (5 x 10\(^5\)µL) were stimulated with 2 μg/ml RC for the indicated time points and subsequently lysed with NP-40 detergent. Syk, LAT, SLP-76, Vav1, Vav3, and PLC\(\gamma\)2, were immunoprecipitated and proteins were separated by reducing SDS-PAGE (10%) and transferred to a PVDF membrane. The membrane was probed with an anti-pTyr mAb (4G10), and reprobed with Syk, LAT, SLP-76, Vav1, Vav3, and PLC\(\gamma\)2 antibodies.
SUPPLEMENTAL REFERENCES


