Cellular Regulation of Platelet Glycoprotein VI: *in vivo* and *in vitro* studies in mice

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Tamer Rabie
aus
Kairo

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Mitglieder der Promotionskommission:

Vorsitzender:	Prof. Dr. U. Scheer
1. Gutachter:	Prof. Dr. B. Nieswandt
2. Gutachter:	Prof. Dr. G. Krohne

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Summary

Platelet interaction with the subendothelium is essential to limit blood loss after tissue injury. However, upon rupture of atherosclerotic plaques, this interaction may result in blood vessel occlusion leading to life threatening diseases such as myocardial infarction or stroke. Among the subendothelial matrix proteins, collagen is considered to be the most thrombogenic component as it directly activates platelets. Platelets interact with collagen, either indirectly through glycoprotein (GP) Ib-V-IX receptor complex, or directly through the major collagen receptor on the platelet surface, GPVI. The work presented here focused on studying the cellular regulation of GPVI. In addition, a possible role for GPVI in thrombus formation induced by atherosclerotic plaque material was investigated and it was found that GPVI plays an important role in this process. Using a recently published mitochondrial injury model, it was found that GPVI contains a cleavage site for a platelet-expressed metalloproteinase. Further studies showed that platelet activation by CRP, or thrombin induced down-regulation of GPIbα, but not GPVI. In parallel, cellular regulation of GPV was studied and it was found that GPV is cleaved *in vitro* by the metalloproteinase ADAM17. In previous studies it was shown that injection of mice with the anti-GPVI mAb, JAQ1, induces GPVI down-regulation, which is associated with a strong, but transient, thrombocytopenia. Using new anti-GPVI mAbs, which bind different epitopes on the receptor, it is shown in this study that GPVI down-regulation occurs in an epitope-independent manner. Further experiments showed that antibody treatment induces a transient, but significant increase in bleeding time. Using different genetically modified mice, it is shown that, upon antibody injection, GPVI is both, shed from the platelet surface and internalized into the platelet. Signaling through the immunoreceptor tyrosine-based activation motif (ITAM) of the FcRγ chain is essential for both processes, while LAT and PLCγ2 are essential for the shedding process only. Antibody-induced increase in bleeding time and thrombocytopenia were absent in LAT deficient mice, showing that it is possible to uncouple the associated side effects from the down-regulation process. As antibody-induced GPVI internalization still occurs in LAT and PLCγ2 deficient mice, this suggests a novel signaling pathway downstream of GPVI that has not been described so far.

Zusammenfassung

Plättchen Interaktion mit dem Subendothel ist für die Blutstillung essentiell. Dies kann jedoch nach dem Aufbrechen atherosklerotischer Plaques zu lebensbedrohlicher Erkrankungen wie Infarkt oder Schlaganfall führen. Kollagen, welches die Plättchen dirket aktiviert, ist der thrombogenste Bestandteil der Extrazellularmatrix (EZM). Die Bindung zwischen Plättchen und Kollagen wird sowohl indirekt durch den Glykoprotein (GP) Ib-V-IX Rezeptorkomplex, als auch direkt durch den Kollagenrezeptor GPVI, auf der Plättchenoberfläche vermittelt. In der vorliegenden Arbeit wurde die zelluläre Regulation von GPVI untersucht. Des Weiteren wurde die Rolle von GPVI in durch atheroklerotisches Plaquematerial induzierter Thrombusbildung studiert. Hierbei wurde festgestellt, dass GPVI eine wichtige Funktion in diesem Prozess spielt. Mittels eines jüngst publizierten mitochondrialen Verletzungsmodels, konnte gezeigt werden, dass GPVI eine Erkennungsstelle für eine in den Plättchen exprimierte Metalloproteinase besitzt. Mehrere Versuche haben gezeigt, dass Plättchenaktivierung durch CRP, und Thrombin zur Runterregulierung von GPIbα aber nicht von GPVI führt. Parallellaufende Untersuchungen zeigten, dass GPV durch die Metalloproteinase ADAM17 *in vitro* abgespalten wird. Vorherige Studien ergaben, dass die *in vivo* Behandlung von Mäusen mit dem anti-GPVI Antikörper, JAQ1, zur Runterregulierung des Rezeptors führt. Dieses ist mit einer starken, transienten Thrombozytopenie assoziiert. Mittels neu generierte anti-GPVI Antikörper (JAQ2, 3), die unterschiedliche Bindungsstellen auf GPVI erkennen, konnte demonstriert werden, dass die Antikörper vermittele GPVI Runterregulierung Epitop unabhängig ist. Weitere Untersuchungen ergaben, dass Anitkörperinjektion eine transiente Erhöhung der Blutungszeit verursacht. Mittels genetisch modifizierter Mäuse konnte dargestellt werden, dass die Antikörpergabe GPVI sowohl von der Plättchenoberfläche abgespalten, als auch internalisiert wird. Während die Signaltransduktion durch das ITAM Motif der FcRγ Kette essentiell für beide Prozesse ist, sind LAT und PLCγ2 nur für das Abspalten wichtig. Antikörper induzierte Erhöhung der Blutungszeit und Thrombozytopenie sind abwesend in LAT-defizienten Mäuse, was zeigt, dass möglicherweise die GPVI Runterregulierung von den assoziierten Nebenwirkungen zu trennen ist. Da die GPVI Runterregulierung in LAT und –PLCγ2 defizienten Mäusen weiterhin stattfindet, zeigt dies einen neuen GPVI Signalweg, der bisher noch nicht beschrieben wurde.

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A. Introduction

Platelets are anucleated cells that originate from megakaryocytes in the bone marrow. They circulate in the blood, surveying the integrity of the blood vessels. Vessel wall injury triggers sudden platelet activation and platelet plug formation, followed by coagulant activity and the formation of fibrin-containing thrombi that occlude the site of injury. These events are crucial to limit blood loss at sites of tissue trauma but may also block diseased vessels, leading to ischemia and infarction of vital organs.

A.1 Platelet morphology and structure

Blood platelets are formed from megakaryoctes in the bone marrow. The process of megakaryocyte maturation involves nuclear duplication without cell division, leading to the formation of large cells. It is not clear how megakaryocyte fragmentation into individual platelets takes place. However, it is likely to be due to shear forces in the circulating blood, mainly in the pulmonary circulation [1]. In humans, the physiological platelet count in peripheral blood lies between 200,000 and 400,000 per µL blood. However, in the mouse, the platelet count is about 1 $x10^6$ per µL. Platelets are the smallest corpuscular components of circulating blood and have a diameter of 2-4 µm [2]. In contrast to other mammalian cells, platelets do not have a cell nucleus and thus exhibit no or a very limited ability for *de novo* synthesis of proteins. The physiological lifetime of platelets in the peripheral blood stream is about 10 days. In the non-activated state, platelets have a typical discoid shape with an average surface area of 8μm^2. Activation of platelets by soluble agonists such as ADP or thrombin or by adhesion to the subendothelium leads to a shape change with formation of pseudopods that represent protrusions of the plasma membrane. In this way the surface area increases to as much as 13 μm^2 (Figure A.1).

Platelets contain several organelles which are dispersed in the cytoplasm, including mitochondria, glycogen stores, and three different forms of storage organelles: dense bodies (or dense granules), α-granules, and lysosomes. The dense granules contain a series of low molecular weight compounds (ADP, ATP, Ca^{2+}, serotonin, etc.) that promote aggregation processes. The α-granules contain a number of proteins that influence adhesion and aggregation (fibrinogen, fibronectin, P-selectin, von Willebrand factor, etc.). The lysosomes contain hydrolytic enzymes (collagenase, elastase, etc.) and are similar to the lysosomes of other cells.

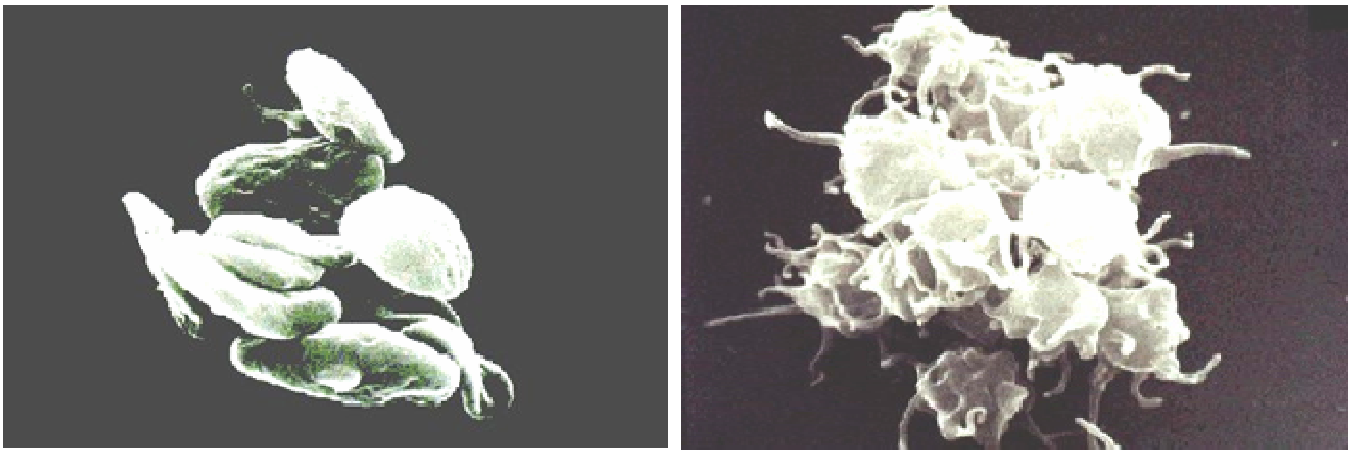

Figure A.1. Scanning electron micrograph of resting (left) and activated (right) platelets.
From: www.akh-wien.ac.at/biomed-research/htx/platweb1.htm

A.2 Thrombus formation

Platelet thrombus formation is a complex process that can be separated into two steps: platelet adhesion to the extracellular matrix (ECM), and platelet cohesion/aggregation. At least four platelet receptors, GPIIb/IIIa, GPIa/IIa, GPIb-V-IX, and GPVI play a role in these processes [3-5]. To form a stable bond with components of the ECM e.g. collagen, platelets need to initially slow down and attach to the ECM, a process that is termed *tethering*. The current model of platelet thrombus formation on the collagen surface suggests that this initial step is mediated by the interaction of the von Willebrand factor (vWF) receptor complex, GPIb-V-IX, with the exposed ECM on the injured subendothelium. Subsequent to this, platelet activation takes place. This is mediated by the interaction of GPVI with collagen on the exposed subendothelium. This leads to the activation of integrins, GPIa/IIa (also known as $\alpha 2\beta 1$) and GPIIb/IIIa (also known as $\alpha IIb\beta 3$), leading to firm platelet adhesion and thrombus growth [6] (Figure A.2). The most important receptors involved in platelet tethering/adhesion and platelet activation will be next discussed with special emphasis on GPVI due to its importance for this study.

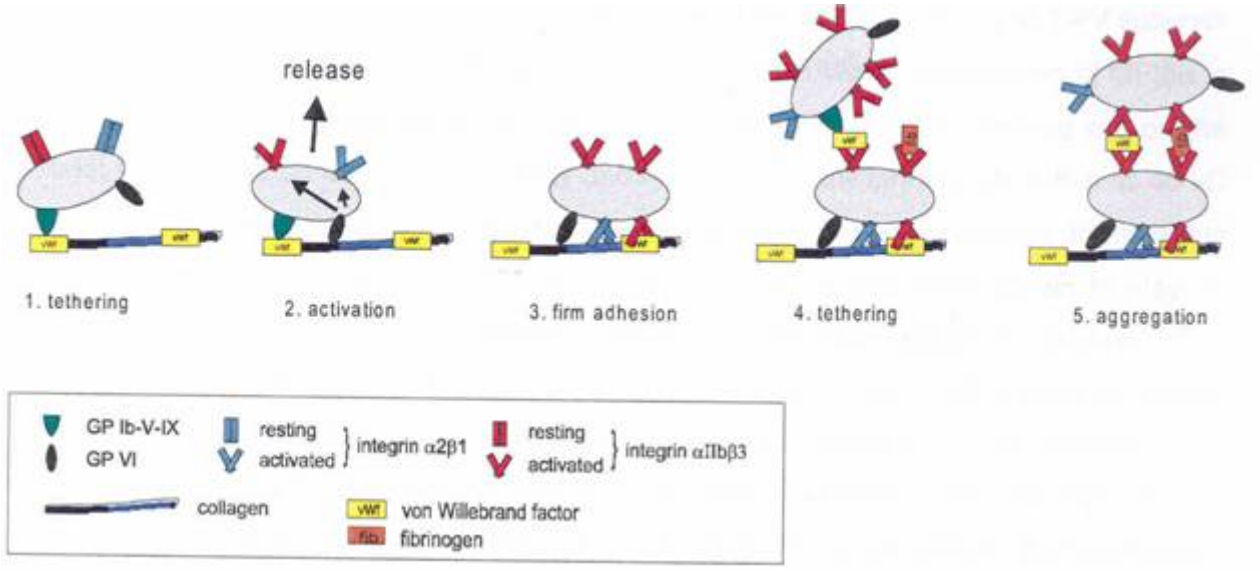

Figure A.2. Current model of platelet thrombus formation on a collagen surface

A.2.1 *Adhesion receptors*

A.2.1.1 *GPIb-V-IX receptor complex*

The membrane GPIb-V-IX complex plays a central role in platelet adhesion and aggregation at sites of vascular injury as it mediates the initial platelet tethering to the damaged vessel wall under conditions of elevated shear by interacting with collagen-bound von Willebrand factor (vWF) [3]. Although the receptor complex is considered as an indirect collagen receptor, recent evidence suggests that the GPV subunit of the receptor complex binds directly to collagen and participates in platelet adhesion and aggregation [7]. The receptor complex consists of four distinct polypeptides, GPIbα (M_r =143,000), Ibβ (M_r =22,000), V (M_r =83,000), and IX (M_r =20,000), in 2:2:1:2 stoichiometry with approximately 12.500 copies per platelet (Figure A.3). Each of the four subunits of the receptor complex has the orientation of a typical type I transmembrane protein, with the carboxy terminus in the cytoplasm and the amino terminus towards the exterior. They are homologous to each other and belong to the leucine-rich motif (LRM) family of proteins/receptors [8]. The receptor complex is associated with the regulatory cytoplasmic protein calmodulin which is involved in the free Ca^{2+} uptake upon platelet activation [9], but its exact role for GPIb-V-XI function is unclear. While the role of GPIbβ, GPIX, and GPV subunits of the receptor complex is still open to speculation, it is well known that GPIbα is a thrombin receptor on the platelet surface [10]. The importance of the GPIb-V-XI complex is emphasized by the

study of the Bernard-Soulier syndrome, an inherited bleeding disorder where the complex is congenitally missing or dysfunctional due to mutations in the genes encoding GPIb or GPIX [8]. Correspondingly, targeted deletion of the *GPIbα* [11] or *GPIbβ [12]* genes in mice reproduces the Bernard-Soulier phenotype, as characterized by thrombocytopenia, giant platelets and massively prolonged bleeding times. Cellular regulation of this receptor complex has been of major interest for many investigators in the last years. It is well known that thrombin directly cleaves GPV, releasing a 69 kDa soluble fragment (GPV f1) of unknown function. In addition, elastase and calpain are capable of cleaving GPV as well releasing 75 kDa and 82 kDa fragments, respectively, but the significance of these observations is unresolved. In addition, very recently, it was shown that the GPIbα subunit of the receptor complex is down-regulated by the metalloproteinase ADAM17 (see A.3.1), belonging to the "a disintegrin and metalloproteinase" (ADAM) family of metalloproteinases [13].

A.2.1.2 *Integrins*

The integrins are a major class of adhesive and signaling molecules on the surface of platelets. They consist of non-covalently associated heterodimers of α and β subunits and are generally involved in linking adhesive molecules to the cytoskeleton. Integrins usually exist in different affinity states; when the platelets are not activated they are in a low affinity state, but upon platelet activation they turn to the high affinity state where they can bind their ligands [14;15].

A.2.1.2.1 *β3 family*

αIIbβ3. αIIbβ3 (also know as GPIIb/IIIa) is the only integrin expressed uniquely on platelets. αIIbβ3 is the major platelet integrin (and receptor) with 50.000-80.000 copies per platelet [16-19], and serves as a receptor for fibrinogen. Patients where αIIbβ3 integrin is either missing or dysfunctional (Glanzmann's Thrombastenia) show a severe aggregation defect and suffer from an increased bleeding tendency.

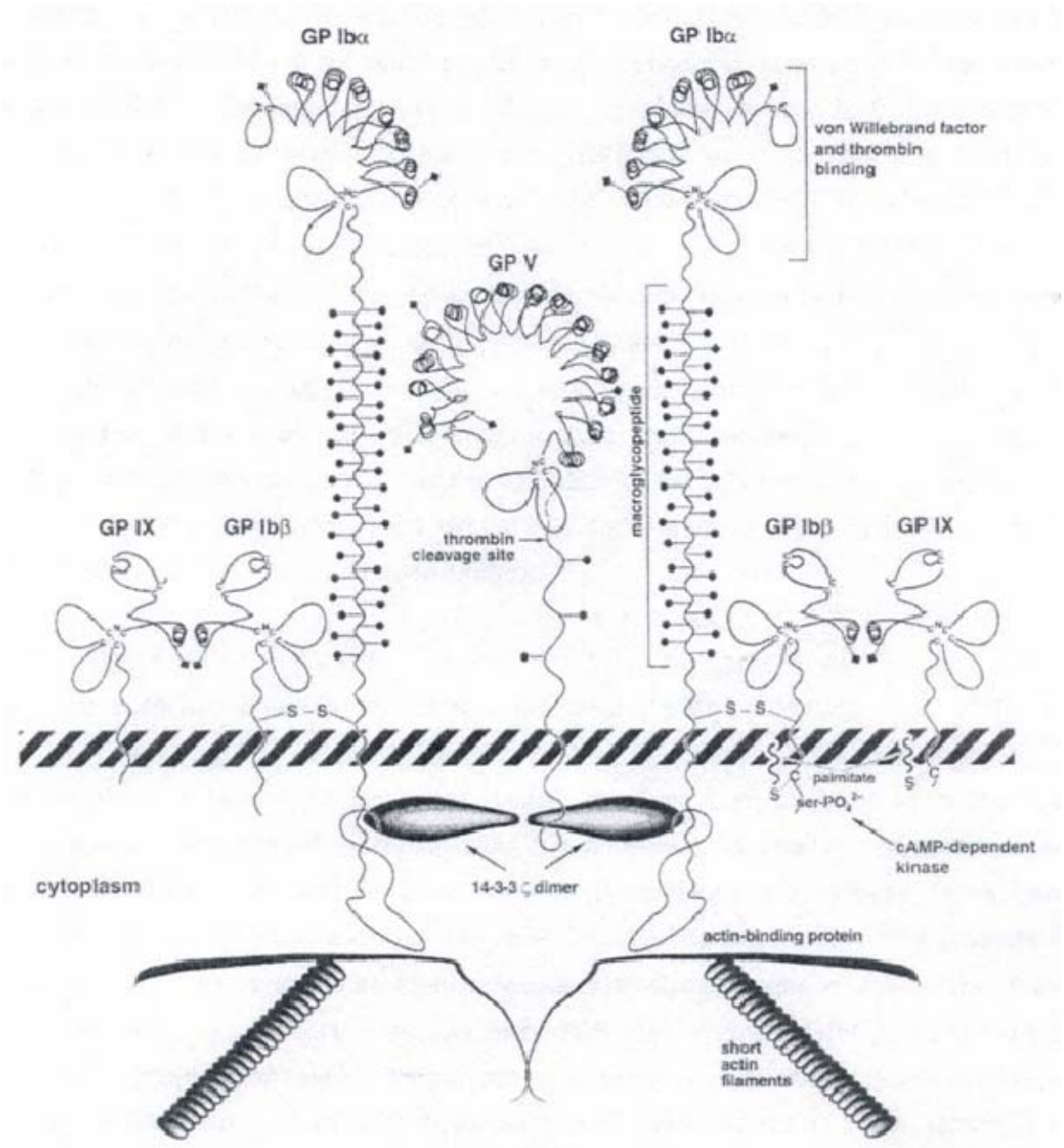

Figure A.3. Schematic drawing of the GPIb-V-IX complex [20]

In addition to the adhesive functions of αIIbβ3, this integrin serves as a bidirectional mediator for biochemical and mechanical information flow across the platelet membrane [21]. Similar to humans, in mouse platelets αIIbβ3 integrin plays an essential role in platelet adhesion and aggregation [22].

αvβ3. Integrin αvβ3 is present on platelets in rather small amounts (several hundred copies per platelet) and serves as a receptor for vitronectin. Its significance for platelet function is unclear.

A.2.1.2.2 *β1 family*

- **α2β1**. α2β1 (also known as GPIa/IIa) is the second most important integrin on platelets and serves as a direct collagen receptor [23]. There are 2000-4000 copies per platelet.
- **α5β1.** α5β1 serves as a fibronectin receptor on platelets that supports platelet adhesion at injury sites [24].
- **α6β1.** α6β1 is a laminin receptor on platelets [25].

Receptor	Ligand	Mol. Weight	Expression	CD name
GPIIb/IIIa (αIIbβ3)	fibrinogen, vWf, fibronectin, vitronectin	125α-22β/95	Mg	CD 41/61
GPIa/IIa (α2β1)	collagen	153/130	broad expression	CD 49b/29
GPIc/IIa (α5β1)	fibronectin	160/130	broad expression	CD 49e/29
GPIc'/IIa (α6β1)	laminin	160/130	broad expression	CD 49f/29
αvβ3	vitronectin thrombospondin	195/95	Mg, EC	CD 51/61
GPIb/IX	vWf, thrombin, P-selectin	143α-22β/22	Mg	CD 42a-c
GPV	collagen	89	Mg	CD 42d
GPIV	thrombospondin	88	Mg, Ma, B	CD 36
P-selectin	PSGL-1	140	Mg, EC	CD 62P

Table A.1. Major adhesive receptors on the platelet surface. Abbreviations: B= B-cell; EC= endothelial cell; Ma= macrophage; Mg= megakaryocyte; PSGL-1= P-selectin glycoprotein ligand-1

A.2.2 *Activation receptors*

A.2.2.1 *G-protein-coupled receptors*

While collagen acts on platelets via GPVI (discussed below) and α2β1 integrin, many other platelet agonists (e.g. thrombin, ADP, thromboxane A_2, epinephrine) activate platelets through G-protein coupled receptors. Prominent members of this family are protease activated receptor (PAR)-1, (PAR)-3, and (PAR)-4 (thrombin receptors [26]), $P2Y_1$ and $P2Y_{12}$ (ADP receptors [27;28]), and TPα (thromboxane A_2 receptor [29]). Irrespective of the agonist, intracellular signaling events lead to the activation of

second messenger-generating enzymes like adenylyl cyclase, phospholipase C and phospholipase A_2, leading finally to the activation of integrins like αIIbβ3, which is a prerequisite for firm platelet adhesion and aggregation.

Table A.2. Major G protein-coupled receptors on the platelet surface

Agonist	**Receptor**	**G protein**
thrombin	PAR-1/3	$G\alpha_{i,}$ $G\alpha_q$, $G\alpha_{12/13}$
	PAR-4	$G\alpha_i$, $G\alpha_q$, $G\alpha_{12/13}$
thromboxane A_2	TP	$G\alpha_q$, $G\alpha_{12/13}$
ADP	$P2Y_1$ $P2Y_{12}$	$G\alpha_q$ $G\alpha_i$
epinephrine	α_{2A}	$G\alpha_i$
serotonin	$5\text{-}HT_{2A}$	$G\alpha_q$

A.2.3 *The activating collagen receptor GPVI*

GPVI was first identified as a 60 to 65 kDa glycoprotein by 2-D gel electrophoresis more than 20 years ago [30]. The first indication that GPVI may be an important platelet receptor for collagen, however, came from studies on a patient who was presented to the clinic with an autoimmune thrombocytopenia caused by autoantibodies to a 65 kDa protein that was present in healthy individuals but absent in the patient [31]. Gel electrophoresis (2-D) demonstrated that the antiserum identified GPVI. Platelets from this patient were unresponsive to collagen, whereas activation by other stimuli was normal. A $F(ab)_2$ preparation of the IgG fraction from this patient was found to strongly activate platelets from healthy individuals, whereas monovalent F(ab) fragments inhibited collagen-induced activation. A few additional patients with low levels of GPVI have been described [32-35]. In most cases, the patients display a mild bleeding phenotype and their platelets exhibit defective aggregation to collagen. These early studies provided the first evidence for a key role of GPVI in platelet-collagen interaction. The current model of platelet interaction with the subendothelium places GPVI in a central position in this process, as, in this model, GPVI is the activating receptor, without which no integrin activation takes place (Figure A.2); [6]. Recently, mice deficient in GPVI were generated and

described [36]. Similar to GPVI deficient patients, these mice do not have a major bleeding phenotype. However, platelets from these mice failed to aggregate in response to collagen. Moreover, no thrombus growth was observed when platelets lacking GPVI were allowed to flow over collagen-coated coverslips under high shear conditions.

A.2.3.1 GPVI structure

GPVI belongs to the immunoglobulin (Ig) superfamily [37;38] and non-covalently binds to the FcRγ chain, which acts as the signaling subunit of the receptor complex [39] [40]. GPVI expression is dependent on the expression of the FcR γ chain, as FcR γ chain deficient platelets do not express GPVI [41]. GPVI is composed of 319 amino acid residues and a signal sequence of 20 amino acids. The cytoplasmic region of GPVI contains 51 amino acids that show no apparent homology with other receptors [42]. Mouse GPVI has 319 amino acids and shares 64.4% and 67.3% identity with human GPVI on protein and nucleotide levels, respectively [38]. Murine GPVI has an intracellular tail of 27 amino acids that lacks the 24 amino acids that lie C-terminal to the proline-rich region of the human GPVI [38].

A.2.3.2 GPVI ligands, physiological and synthetic

So far, collagen is the main physiological ligand for GPVI. Very recently, the collagen-binding site on human GPVI was reported where lysine at position 59 was identified as crucial for GPVI-collagen interaction [43]. However, several synthetic tools (synthetic collagens, snake venoms and antibodies) have been developed in order to study GPVI function. Collagen contains a GPO repeat (G= glycine; P= proline; O= hydroxyproline). This sequence makes up approximately 10% of collagens type I and III. The GPO repeat sequence forms monomeric chains, which, upon cross-linking, results in fibrillar collagen, the predominant structure in the ECM. The most commonly used collagen preparation for platelet studies (Horm collagen) consists of a mixture of collagen I and III. For certain experiments, a digested form of collagen (soluble collagen) is also used. Another experimental tool used for platelet studies is the collagen related peptide (CRP). This peptide consists of a repeat of GPO motifs. Upon cross-linking of CRP, it becomes a strong and specific agonist for GPVI [32;39;44].

Several snake venom peptides that mediate their actions through GPVI are also used as powerful tools to study the function of this receptor. The multimeric snake toxin convulxin, the venom of the tropical rattlesnake *Crotalus terrificus*, is one example.
In the year 2000, the first anti-GPVI mAb, JAQ1, was generated which proved to be a powerful tool to study the function and regulation of GPVI *in vitro* and *in vivo* [41;45;46].

A.2.3.3 *JAQ1*
JAQ1 is the first rat monoclonal antibody detecting mouse GPVI. JAQ1 binds to the major collagen binding site on GPVI as it blocks platelet aggregation induced by collagen or CRP [41]. While collagen-induced platelet aggregation in presence of JAQ1 was restored by increasing the collagen concentration, CRP-induced platelet aggregation was abolished even at high CRP concentrations, suggesting the existence of two distinct epitopes within collagen for activation of mouse platelets and that CRP binds to the major collagen binding site on GPVI [46]. On its own, JAQ1 does not induce platelet aggregation, which only occurs upon subsequent cross-linking with anti-rat IgG [41]. However, incubation of platelets with JAQ1 induces a weak and subliminal level of protein tyrosine phosphorylation in a profile similar to that observed upon platelet activation with the GPVI-specific agonist, CRP [46].
JAQ1 has the unique property that upon its injection into mice it induces irreversible GPVI down-regulation. This down-regulation lasts for a period of more than two weeks, during which mice are protected against thromboembolism, providing a promising therapeutic intervention in cardiovascular diseases. However, this treatment was accompanied by a rapid, but transient, drop in the platelet count, limiting the therapeutic usefulness of this treatment [45].

A.2.3.4 GPVI signaling
As mentioned above, GPVI non-covalently binds to the FcR γ chain, which acts as the signaling subunit of the receptor complex [39;40]. Upon ligand binding, tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) of the FcRγ chain by the Src family kinases is induced [47;48]. The phosphorylated ITAM then provides a binding site for the Syk family kinases Syk and ZAP-70, which are then phosphorylated by Src family kinases. Activated ZAP-70 then phosphorylates the linker for activation of T-cell (LAT) [49], which finally leads to the activation of

phospholipase C γ2 (PLCγ2) [50]. Platelets from LAT deficient mice show a reduced aggregation response to collagen. However, this was overcome by increasing the collagen concentration, suggesting the existence of another pathway downstream of GPVI that is independent of LAT [51]. Consistent with this, PLCγ2 phosphorylation in response to CRP activation was shown to be significantly reduced, but not abolished in platelets from LAT deficient mice [52]. In contrast to LAT, PLCγ2 seems to be essential for signaling downstream of GPVI, as platelets from PLCγ2 deficient mice failed to aggregate in response to collagen [50].

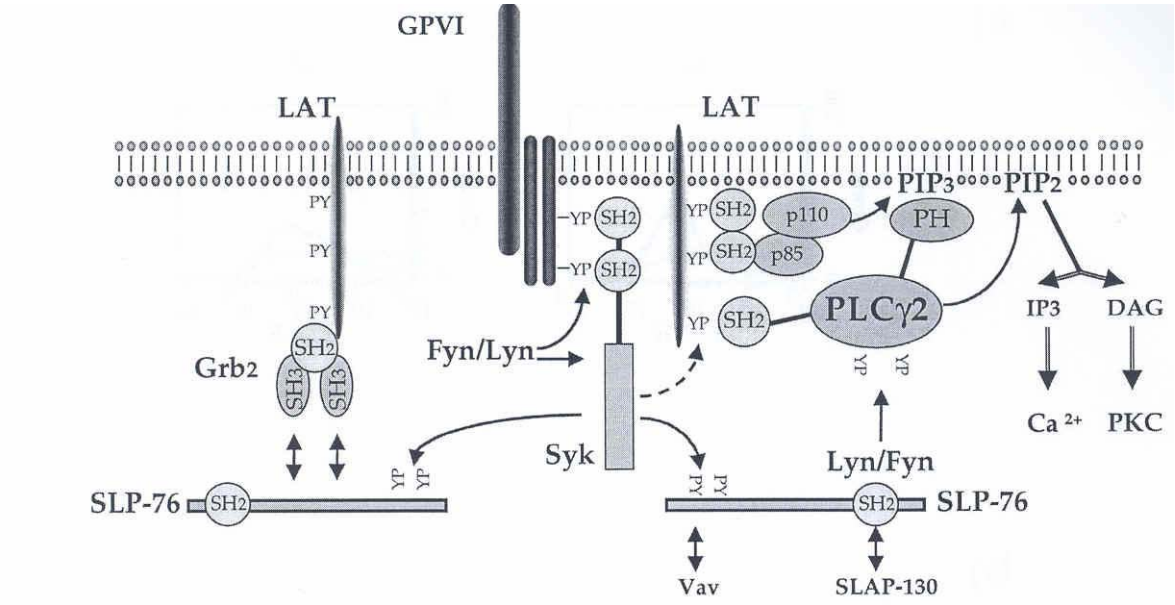

Figure A.4. Signaling pathways downstream of GPVI [52]

A.2.3.5 GPVI regulation

In spite of the crucial role of GPVI in platelet collagen interactions, very little is known about its cellular regulation. As mentioned above, injection of mice with the anti-GPVI antibody, JAQ1, induces the depletion of the receptor from the surface of circulating platelets [45]. The mechanism of this down-regulation was, however, unclear. Although evidence was provided that GPVI is internalized into the platelets upon antibody injection [45], it remained unclear whether GPVI is cleaved from the platelet surface as well. *In vitro*, JAQ1 did not induce GPVI down-regulation, showing that additional *in vivo* signals are required for this process.

A.3 Metalloproteinase-disintegrins (ADAMs)

The role of protein ectodomain shedding is emerging as an important regulatory mechanism of surface receptors. Metalloproteinase-disintegrins are a family of transmembrane glycoproteins that play roles in cell-cell interaction and in the processing of the ectodomains of proteins such as tumor necrosis factor α (TNFα). They are zinc-dependent and characterized by a conserved domain structure, consisting of an N-terminal signal sequence followed by a prodomain, metalloproteinase and disintegrin domains, a cysteine-rich region, usually containing an EGF repeat, and finally a transmembrane domain and a cytoplasmic tail [53-55] [56]. Thus, family members are referred to as ADAMs (for a disintegrin and metalloproteinase domain). Metalloproteinase-disintegrins have been implicated in diverse processes, including protein-ectodomain processing or shedding of cytokines, cytokine receptors, adhesion proteins and other extracellular protein domains. Platelets express ADAM10 and ADAM17, but their role in platelet function is still elusive. In addition to ADAMs, platelets express another family of metalloproteinases, namely, matrix metalloproteinases (MMPs). In human platelets, activation of MMPs-1, -2, -3, and -9 and their translocation to the surface membrane have been demonstrated in response to various agonists (reviewed in [57]). Further studies have shown that these enzymes may prime platelets for agonist-induced platelet aggregation (MMP-1,-2) or counteract the aggregation response (MMP-9).

A.3.1 ADAM17

ADAM17 is one of the best characterized members of the ADAM family of metalloproteinases. It was identified as the major enzyme cleaving TNFα and hence was also termed TNFα converting enzyme (TACE), as T cells that have a targeted disruption in the Zn^{2+} binding domain of TACE (TACE $^{\Delta Zn}$) show a severe defect of TNFα release [58]. TACE $^{\Delta Zn/\Delta Zn}$ mice die between embryonic day 17.5 and the first day of birth. The severe phenotype was unexpected in light of the predicted role of TACE in TNFα shedding, because animals that lack either TNFα or the TNF receptors p55 TNFR and p75 TNFR are viable and appear normal [59-61]. In addition, it was also shown that L-selectin is a substrate for TACE, which indicates that TACE has a more general role than initially anticipated [62].

A.4 Platelets and atherosclerosis

The pathogenesis of atherosclerosis is multifactorial, but the main aspect considered of great relevance is the deposition of lipids that are then metabolized abnormally and oxidized in the vascular wall, leading to inflammation. Under certain conditions, rupture of an atherosclerotic plaque occurs, exposing thrombogenic material to the flowing blood, a process which can lead to acute myocardial infarction or ischemic stroke. Although the role of tissue factor in plaques in stimulating the blood coagulation has been recognized [63-65], it is unknown, whether plaques can cause thrombus formation through direct activation of platelets.

A.5 Antiplatelet therapy

Although the platelet was initially viewed only as a bystander in hemostasis, it is now clear that it is a key mediator of thrombosis as well as of inflammation. Therefore, many efforts have been undertaken to develop antiplatelet agents for the prevention and / or treatment of cardiovascular diseases.

A.5.1 *Aspirin*

Aspirin is the prototype of antiplatelet agents. It acts by irreversibly inhibiting the cyclooxygenase 1 (COX1) enzyme and thereby blocking the synthesis of TxA_2 [66]. Although there is a lot of clinical data supporting the efficacy of aspirin, many limitations have also been reported. Therefore, efforts have been dedicated to develop better antiplatelet agents.

A.5.2 *αIIbβ3 integrin blockers*

The intravenous αIIbβ3 blocker abciximab, the Fab fragment of a humanized murine monoclonal antibody, was the first in this class of agents. There is substantial evidence supporting the utility of abciximab in patients undergoing percutaneous coronary interventions (PCI), as it confers a significant long-term mortality benefit in these patients [67;68].

A.5.3 *Thienopyridines*

Clopidogrel, and its predecessor ticlopidine, are thienopyridines that act via ADP receptor antagonism. These agents are irreversible platelet inhibitors, active for the

life of the platelet. Both compounds are specific for the $P2Y_{12}$ receptor, which is associated with amplification of platelet activation / aggregation and secretion.

A.6 Aim of the study

The aim of this study was to investigate the mechanism of GPVI regulation. As GPVI down-regulation could be induced *in vivo* by antibody treatment, it was essential to understand the mechanisms underlying this process, as this is crucial for the development of anti-GPVI based therapeutics. As the reported antibody-induced thrombocytopenia represents a limitation for anti-GPVI treatment, it was essential to test whether it is possible to uncouple thrombocytopenia from the down-regulation process.

B. Materials and Methods

B.1 Materials

B.1.1 *Chemicals*

ADP	Sigma (Deisenhofen, Germany)
adrenaline	Sigma (Deisenhofen, Germany)
apyrase (grade III)	Sigma (Deisenhofen, Germany)
bovine serum albumin (BSA)	Pierce (Rockford, IL, USA)
carbonyl cyanide m-chlorophenylhydrazone (CCCP)	Sigma (Deisenhofen, Germany)
enhanced chemoluminiscence (ECL) detection substrate	MoBiTec (Göttingen, Germany)
EZ-Link sulfo-NHS-LC-biotin	Pierce (Rockford, IL,USA)
fetal calf serum (FCS)	PAN (Aidenbach, Germany)
fibrilar type I collagen (Horm)	Nycomed (Munich, Germany)
fluorescein-isothiocyanate (FITC)	Molecular Probes (Oregon, USA)
GM6001	Calbiochem (Bad Soden, Germany)
GW280264X	GlaxoSmithKline (Stevenage, UK)
HAT stock (50x)	Roche Diagnostics (Mannheim, Germany)
high molecular weight heparin	Sigma (Deisenhofen, Germany)
hirudin	Aventis (Frankfurt, Germany)
horseradish peroxidase (HRP)-labeling kit	Zymed (Berlin, Germany)
human fibrinogen	Sigma (Deisenhofen, Germany)
immobilized papain	Pierce (Rockford, IL, USA)
immobilized pepsin	Pierce (Rockford, IL, USA)
Nonidet P-40 (NP-40)	Roche Diagnostics (Mannheim)
PD-10 column	Pharmacia (Uppsala, Sweden)
penicillin/ streptomycin	PAN (Aidenbach, Germany)
phorbol 12-myristate 13-acetate (PMA)	Sigma (Deisenhofen, Germany)
polyethylene glycol 1500 (PEG 1500)	Roche Diagnostics (Mannheim)
prostacyclin	Calbiochem (Bad Soden, Germany)
protein G sepharose	Pharmacia (Uppsala, Sweden)
recombinant human (rh) ADAM17	R&D Systems (Minneapolis, USA)
R-phycoerythrin (PE)	EUROPA (Cambridge, UK)

RPMI media	PAN (Aidenbach, Germany)
TNFα protease inhibitor-2 (TAPI-2)	Calbiochem (Bad Soden, Germany)
thrombin	Roche Diagnostics (Mannheim)
3,3,5,5-tetramethylbenzidine (TMB)	EUROPA (Cambridge, UK)
U46619	Alexis Biochemicals (San Diego, USA)
W13	Calbiochem (Bad Soden, Germany)

Collagen related peptide (CRP) was kindly provided by S.P Watson (University of Birmingham, UK). Convulxin was obtained from the venom of the tropical rattlesnake *Crotalus durissus terrificus* and was kindly provided by M. Leduc and C. Bon (Institute Pasteur, Paris, France).
All other chemicals were obtained from Sigma (Deisenhofen, Germny) or Roth (Karlsruhe, Germany).

B.1.2 *Monoclonal antibodies (mAbs)*
All mAbs used were generated and modified in our laboratory.

antibody	**isotype**	**antigen**	**described in**
JAQ1	IgG2a	GPVI	[45]
DOM1	IgG1	GPV	[69]
DOM2	IgG2a	GPV	[69]
JON/A	IgG2b	GPIIb/IIIa	[70]
JON1	IgG2b	GPIIb/IIIa	[69]
EDL1	IgG2a	GPIIb/IIIa	[69]
ULF1	IgG2a	CD9	[69]
p0p4	IgG2b	GPIbα	[69]
p0p6	IgG2b	GPIX	[69]
21H4	IgG2b	α2 integrin	Unpublished
BRU1	IgG1	P-selectin	Unpublished

B.1.3 *Polyclonal antibodies (pAbs)/ secondary reagents*
Rabbit anti-rat IgG (-FITC, -HRP) and streptavidin-HRP were purchased from DAKO (Hamburg, Germany).

B.1.4 *Animals*

Specific-pathogen-free mice (NMRI, C57Bl/6) and rats (Wistar) 6 to 10 weeks of age were obtained from Charles River, Sulzfeld, Germany. C57Bl/6 mice deficient in the FcRγ-chain were obtained from Taconics (Germantown, NY, USA), deficient in PLCγ2 were obtained from F. Lanza (INSERM, Strasbourg, France), deficient in LAT were obtained from S.P. Watson (University of Birmingham, UK), and FcRγ–YF mice were obtained from T. Saito (Chiba University, Chiba, Japan).

B.1.5 *Cell lines*

The mouse myeloma cell line *Ag14* was kindly provided by D. Männel (Regensburg, Germany)

B.2 Methods

B.2.1 *Buffers and Media*

All buffers were prepared and diluted using aqua $_{bidest}$

- Phosphate-buffered saline (PBS), pH 7.14

NaCl	137 mM (0.9 %)
KCl	2.7 mM
KH_2PO_4	1.5 mM
$Na_2HPO_4x2H_2O$	8 mM

- PBS/EDTA

PBS	
EDTA	5 mM

- Tris-buffered saline (TBS), pH 7.3

NaCl	137 mM (0.9%)
Tris/HCl	20 mM

- Storage buffer, pH 7.0

Tris	20 mM

NaCl	0.9 %
BSA	0.5 %
NaN_3	0.09 %

- Tyrode's buffer, pH 7.3

NaCl	137 mM (0.9 %)
KCl	2.7 mM
$NaHCO_3$	12 mM
NaH_2PO_4	0.43 mM
Glucose	0.1 %
Hepes	5 mM
BSA	0.35 %
$CaCl_2$	1 mM
$MgCl_2$	1 mM

- Acid-citrate-dextrose (ACD) buffer, pH 4.5

Trisodium citrate dehydrate	85 mM
Citric acid anhydrous	65 mM
Glucose anhydrous	110 mM

- IP buffer

Tris/HCl (pH 8.0)	15 mM
NaCl	155 mM
EDTA	1 mM
NaN_3	0.005 %

- SDS sample buffer, 2X

β-mercaptoethanol (for red. conditions)	10 %
Tris buffer (1.25 M), pH 6.8	10 %
Glycerin	20 %
SDS	4 %
Bromophenolblue	0.02 %

- Laemmli buffer

Tris	40 mM
Glycin	0.95 M
SDS	0.5 %

- Blotting buffer A

Tris, pH 10.4	0.3 M
Methanol	20 %

- Blotting buffer B

Tris, pH 10.4	25 mM
Methanol	20 %

- Blotting buffer C

ε-amino-n-caproic acid	4 mM
Methanol	20 %

- Digestion buffer (for generation of Fab fragments), pH 7.0

$NaH_2PO_4.H_2O$	20 mM
NaCl	10 mM
Cystein HCl	20 mM

- Digestion buffer (for generation of $F(ab)_2$ fragments), pH 4.5

Sodium acetate	20 mM

- Washing buffer

PBS	
Tween 20	0.1 %

- Coating buffer, pH 9.0

$NaHCO_3$	50 mM

- Biotinylation buffer, pH 9.0

$NaHCO_3$	50 mM
NaCl	0.9 %

- Coupling buffer, pH 9.0

$NaHCO_3$	160 mM
Na_2CO3	80 mM

- DNA digestion buffer

Tris-HCl (pH 8.0)	50 mM
EDTA	100 mM
NaCl	100mM
SDS	1 %
Proteinase K (10 mg/mL)	35 µL

- Pen/strep-solution

Penicillin	10,000 U/mL
Streptomycin	10 mg/mL
in 0.9 % NaCl	

- RPMI standard media

RPMI 1640	470 mL
FCS	25 mL
Pen/Strep	5 mL

- HAT media

RPMI 1640	435 mL
Pen/Strep	5 mL
FCS	50 mL
HAT stock (50x)	10 ml

- Coomassie Stain

Acetic acid	10 %
Methanol	40 %

Brilliant blue in H_2O	1 g

- Destaining solution

Acetic acid in H_2O	10 %

B.2.2 *Modification of antibodies*

B.2.2.1 *F(ab) /F (ab)$_2$ fragments*

Antibody (whole IgG; 3 mg) was dialyzed overnight against digestion buffer at 4°C. Antibody was then concentrated in a concentrator tube (VivaSpin®, Exclusion size 10 kDa, VivaScience, Hannover) by centrifugation at 4000 rpm until volume of 0.5-1.0 mL was reached (approximately 10 min). To generate F(ab) fragments, dialyzed IgG was mixed with digestion buffer and immobilized papain (washed 2x with digestion buffer) in the ratio of 1:1:1 and left to incubate at 37°C for 4-6 h under shaking conditions. To test the efficacy of the digestion, samples (20 µL) were taken at different time points (0, 1, 2, 4, and 6), mixed with 20 µL non-reducing sample buffer and separated by SDS-PAGE (15 %). To visualize the protein bands, the gel was then coomassie-stained (overnight), then destained (incubated with destaining solution overnight). If the digestion was not complete, antibody was left to incubate for further 2-4 h. Antibody was next dialyzed overnight against PBS at 4°C. To remove remaining Fc parts, F(ab) fragments were incubated overnight with 70 µL protein-G-sepharose. Samples (20 µL) were taken before and after the clearing process, mixed with 20 µL non-reducing sample buffer and were separated by SDS-PAGE (12 %). Finally, 1 % BSA was added to the F(ab) fragments. To generate F (ab)$_2$ fragments, dialyzed IgG was mixed with digestion buffer and immobilized pepsin (washed 2x with digestion buffer) in the ratio of 1:1:1 and left to incubate at 37°C for 48 h under shaking conditions. Samples (20 µL) were taken at 0, 6, 24, and 48 h, mixed with 20 µL non-reducing sample buffer and separated by SDS-PAGE (15 %). Same procedure as above was then performed.

B.2.2.2 *FITC labeling*

Affinity purified antibodies were FITC-labeled to a fluorescein/protein ratio of approximately 3:1. Antibody (4 mg) was dialyzed against coupling buffer overnight at

4°C. FITC was dissolved in anhydrous DMSO to a final concentration of 1 mg/mL. 50 µL of this solution was added to the antibody and left to incubate at RT for 8 h. The reaction was then stopped by addition of 100 µL of 1 M NH_4Cl. FITC-labeled antibody was separated from the unreacted FITC by gel filtration on a PD-10 column.

B.2.2.3 *Biotinylation*

Antibody (3 mg) was dialyzed against biotinylation buffer overnight at 4°C. After that EZ-link sulfo-NHS-LC-biotin (3 mg/mL in biotinylation buffer) was added to a final concentration of 300 µg/mL for 20 min at RT with rotation. Reaction was stopped by addition of 100 µL of 1 M NH_4Cl and the antibody was finally dialyzed against TBS for 5 h at RT. At the end, NaN_3 was added to a final concentration of 0.01%.

To check the efficiency of the biotinylation, washed platelets were incubated with the biotinylated antibody (20 µg/mL) for 10 min at RT, then centrifuged (2800 rpm, 5 min) to remove unbound antibody, and subsequently incubated with FITC-labeled streptavidin (1.5 µg/mL; 10 min, RT). Reaction was stopped by addition of 500 µL PBS, and samples were analyzed immediately by flow cytometric analysis.

B.2.3 *Production of monoclonal antibodies*

B.2.3.1 *Immunization*

Female Wistar rats, 6-8 weeks of age, were immunized either with 0.5 x 10^9 washed mouse platelets or purified antigens obtained by immunoprecipitation from 0.5 x 10^9 washed mouse platelets. Rats were immunized repeatedly (5 x in intervals of 17 days) intraperitoneally and intramuscularly with the immunogens.

B.2.3.2 *Generation of hybridomas*

The rat spleen was removed under aseptic conditions and filtered through a Nitex filter to obtain a single cell suspension. Spleen cells were washed twice in RPMI/pen-strep medium (160 *g*, 5 min, RT), mixed with mouse myeloma cells (Ag14, 10^8 cells / fusion) and washed once with RPMI/pen-strep. Supernatant was removed carefully and 1 mL of polyethylene glycol 1500 was added dropwise (over a time period of 2 min). This was followed by slow addition of 10 mL RPMI/pen-strep medium (over a period of 5 min). Cells were then seeded into 96-well plates and fed with HAT

medium until screening. Positive hybridomas were left to grow in RPMI/ pen-strep/ 10 % fetal calf serum.

B.2.3.3 *Screening of hybridomas*

Hybridomas producing mAbs directed against platelet receptors were identified by flow cytometry. A 1:1 mixture of resting and thrombin-activated platelets (10^6) was incubated with 100µL of the hybridoma supernatant for 20 min, RT. To prepare this mixture, washed platelets (10^6; prepared as mentioned under B.2.6) were divided into two parts. One part was left untreated and the other was activated by thrombin (0.2 U/mL; 5 min, 37°C), followed by the addition of hirudin to stop thrombin function. Samples were then washed once with 1 mL PBS (2800 *rpm*, 10 min) and stained with FITC-conjugated rabbit anti-rat Ig for 15 min at RT. Samples were finally analyzed on a FACScalibur (Becton Dickinson, Heidelberg, Germany).

B.2.3.4 *Determination of isotype subclass*

96-well ELISA plates (BD Falcon, Heidelberg, Germany) were coated with rabbit anti-rat IgG/M (H+L) antibodies (1:1000 in coating buffer) for 2 h at 37°C or overnight at 4°C. After blocking, serial dilutions of purified rat anti-mouse platelet mAbs were added in duplicate wells (1 h, 37°C). Plates were washed and subsequently incubated for 1 h with AP-conjugated isotype specific antibodies (BD Pharmingen; 1:1000, 37°C). After several washing steps, Sigma104 substrate was added to each well and absorbance at 405 nm was recorded on Anthos Reader 2010 (Anthos Labsysteme, Krefeld, Germany).

B2.4 *Genomic DNA isolation from mouse tails*

0.5 cm tail was dissolved in 700 µL DNA digestion buffer by overnight incubation at 56°C under shaking conditions. Samples were then vortexed for 5 min, followed by the addition of 250 µL of saturated NaCl (6 M) solution. Samples were then centrifuged at 14000 rpm for 10 min. 750µL supernatant was taken and 500µL isopropanol was added and samples were shaken for 2 min. After centrifugation at 14000 rpm for 10 min, the DNA pellet was washed twice with ice cold 70% ethanol. The DNA pellet was left to dry and finally resuspended in 50µL H_2O.

B2.5 *Genotyping protocols*

- Primers (PLC γ2 -/- mice)

P1: TTCACCGCATCCTCCTTTGAGTCC

P2: GCCTCTGCACAGCACACATATGG

P3 (Neo): CAAGGTGAGATGACAGGAGATCC

PCR buffer (10x)	5µL
$MgCl_2$ (10x)	5µL
dNTPs	2µL
P1 (10 pmol)	2µL
P2 (10 pmol)	2µL
P3 (10 pmol)	2µL
DNA	100ng
Taq polymerase	0.5 µL
H_2O	ad 50µL

- PCR program (PLC γ2 -/- mice)

1. 95°C 3 min
2. 95°C 45 s
3. 62°C 45 s
4. 72°C 1 min
5. repeat step 2.-4. 30x
6. 72°C 7 min
7. 4°C hold

- Primers (LAT -/- mice)

5PII AGC ACC TTT CCA GAG CCA ACA

3PIII TCA TCC AGT TCC GCA AAG CTT

Neo 5'-2 GCA TCG CCT TCT ATC GCC TTC

Two PCR reactions are needed; one to detect the wild-type band, and the other to detect the knock-out band.

PCR buffer (10x)	5µL
$MgCl_2$ (10x)	5µL
dNTPs	2µL
5PII (10 pmol)	2µL
3PIII (or Neo) (10 pmol)	2µL
DNA	100ng
Taq polymerase	0.5 µL
H_2O	ad 50µL

- PCR program (LAT -/- mice)

1. 95°C 5 min
2. 95°C 1 min
3. 60°C 1 min
4. 72°C 2 min
5. repeat step 2.-4. 34x
6. 72°C 5 min
7. 4°C hold

B.2.6 *Platelet preparation and washing*

Mice were bled under ether anesthesia from the retroorbital plexus. Blood was collected into a tube containg ACD buffer or 20 U/mL heparin in TBS, pH 7.3 (both 300 µL). Blood was centrifuged at 1800 rpm for 5 min. Supernatant was taken and centrifuged at 800 rpm for 6 min at RT to obtain platelet rich plasma (prp). To wash platelets, prp was centrifuged at 2800 rpm for 5 min in the presence of prostacyclin (PGI_2) (0.1 µg/mL) and the pellet was resuspended in Tyrode's buffer containing PGI_2 (0.1 µg/mL) and apyrase (0.02 U/mL) and left to incubate at 37 °C for 5 min. After a second centrifugation step, platelets were resuspended in the same buffer and incubated at 37°C for 5 min. Platelets were finally centrifuged as above, resuspended in Tyrode's buffer containing apyrase (0.02 U/mL) (500µL) and left to incubate for at least 30 min at 37 °C before analysis.

B.2.7 *Platelet counting*

For determination of platelet counts, blood (20 µL) was obtained from the retroorbital plexus of anesthetized mice using siliconized microcapillaries and immediately diluted 1:100 in Unopette kits (Becton Dickinson, Heidelberg, Germany). The diluted blood sample was allowed to settle for 20 minutes in an Improved Neubauer haemocytometer (Superior, Bad Mergentheim, Germany), and platelets were counted under a phase contrast microscope at x400 magnification.

B.2.8 *Preparation and washing of human platelets*

Blood from aspirin free healthy volunteers was collected in TBS-EDTA (5 mM) containing syringes. To obtain prp, blood was centrifuged at 200 *g* for 20 min and supernatant (prp) was collected carefully. To wash platelets, PGI_2 (0.1 µg/mL) was added to prp which was then centrifuged at 500 *g* for 20 min and platelet pellet resuspended in PGI_2-containing Tyrode's buffer (0.1 µg/mL). The centrifugation step was repeated once and platelets were finally suspended in Tyrode´s buffer and adjusted to 2 $x10^8$ platelets/mL.

B.2.9 *Platelet surface biotinylation*

To biotinylate platelet surface molecules, washed platelets were suspended in biotinylation buffer at a concentration of 2 $x10^9$ platelets/mL. EZ-link sulfo-NHS-LC-biotin (3 mg/mL in biotinylation buffer) was then added at a final concentration of 25 µg/mL and left to incubate for 10 min with rotation. Reaction was stopped by addition of 100 µL of 1M NH_4Cl solution and platelets were washed twice in PBS/EDTA. To check the efficacy of the biotinylation, a sample (1:20 dil. in PBS; 50 µL) was incubated with FITC-labeled streptavidin (1.5 µg/mL; 10 min, RT), reaction was stopped by the addition of 500µL PBS, and samples were analyzed immediately by flow cytometry.

B.2.10 *Immunoprecipitation*

For immunoprecipitation, biotinylated platelets (1 $x10^9$) were solubilized in 1 mL of IP buffer containing NP-40 (1 %) by incubation at RT for 10 min, followed by centrifugation at 14000 rpm for 10 min. Supernatants were then incubated with 10 µg of the required antibody (supernatant from 1 $x10^9$ platelets is sufficient for 3 different

antibodies) for 30 min at 4°C with rotation, followed by addition of 25 µL G-sepharose (washed 3x in IP buffer). Samples were left to incubate overnight at 4°C with rotation. Samples were then washed once with IP buffer containing 1 % NP-40 and twice with IP buffer (14000 rpm, 1 min).

B.2.11 *Immunoblotting*

For Western blot analysis, platelets were washed 3x in PBS/EDTA and finally solubilized in 150 µL IP buffer containing 1% NP-40. Samples were separated by 12 % SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. To prevent non-specific antibody binding, membrane was incubated in 10 % fat-free milk (dissolved in washing buffer) for 1 h at RT. After that, membrane was incubated with the required antibody (5 µg/mL) for 1 h at RT. For washing, the membrane was incubated 3x with washing buffer for 10 min at RT. After the washing steps, HRP-labeled secondary reagent was added and left to incubate for 1 h at RT. After several washing steps, proteins were visualized by ECL.

B.2.12 *In vitro analysis of platelet function*

B.2.12.1 *Flow cytometry*

Platelets (1 x 10^6) were activated with the indicated agonists or reagents (10 min, RT), stained for 15 min with saturating amounts of fluorophore-conjugated antibodies, reaction was stopped by addition of 500 µL PBS, and sample was immediately analyzed on a FACScalibur. For a two-color staining, the following settings were used:

Detectors/Amps:

Parameter	Detector	Voltage
P1	FSC	E01
P2	SSC	380
P3	Fl1	650
P4	Fl2	580
P5	Fl3	150

Threshold:

Value	Parameter
253	FSC-H
52	SSC-H
52	Fl1-H
52	Fl2-H
52	Fl3-H

Compensation

Fl1	2.4 % of Fl2
Fl2	7.0 % of Fl1
Fl2	0 % of Fl3
Fl3	0 % of Fl2

B.2.12.2 *Aggregometry*

To determine platelet aggregation, light transmission was measured using washed platelets adjusted to a platelet concentration of 3 x 10^8 platelets/mL with Tyrode's buffer. Alternatively, heparinized prp was used. Agonists or reagents were added as 100-fold concentrates and light transmission was recorded over 14 min on an Apact 4-channel optical aggregation system. Before starting the measurements, Tyrode's buffer (for washed platelets) or plasma (for prp) was set as 100% aggregation and washed platelet suspension (for washed platelets) or prp (for prp) was set as 0% aggregation.

B.2.12.3 *Mitochondrial damage*

Washed platelets resuspended at a concentration of ~1.5 x 10^9 platelets/mL in Tyrode's buffer containing PGI_2 (0.1 µg/mL) were treated for 1 h at 37°C with 100 µM CCCP (dissolved in H_2O) in the presence or absence of the broad spectrum metalloproteinase inhibitor GM6001 (100 µM ; dissolved in DMSO). At the end of the incubation period, platelets were washed once (centrifuged at 2800 *rpm* for 5 min) and resuspended in Tyrode's buffer.

B.2.12.4 *GPVI shedding ELISA assay*

Washed platelets were pre-incubated with biotinylated JAQ1 (10 µg/mL) for 15 min at RT. After centrifugation (2800 *rpm*, 5 min), platelets were resuspended in Tyrode's buffer containing PGI_2 (0.1 µg/mL) and, to induce GPVI shedding, the cells were then treated with CCCP (100 µM) in presence or absence of GM6001 (100µM) for 1 h at 37 °C. Platelets were then centrifuged (2800 *rpm*, 5 min) and the supernatants were incubated on JAQ3-coated (10 µg/mL) ELISA plates for 1 h at 37 ° C. After extensive washing, plates were incubated with HRP-labeled streptavidin for 45 min at 37°C and developed using 3,3,5,5-tetramethylbenzidine (TMB). The reaction was stopped by addition of 2 N H_2SO_4 and absorbance at 450 nm was recorded on a Multiskan MCC/340 (Labsystems, Germany).

B.2.12.5 *Adhesion under flow conditions*

Blood (1 vol) was collected into 0.5 vol of Hepes buffer, pH 7.4, containing 20 U/mL heparin and Ca^{2+} (1 mM). Coverslips (24 x 60 mm) were coated with fibrillar (Horm) collagen (0.25 mg/mL, Nycomed, Munich, Germany), or human plaque material, and blocked for 1 h with 1% bovine serum albumin. Perfusion studies were performed as follows. Transparent flow chambers with a slit depth of 50 µm, equipped with the coated coverslips, were connected to a syringe filled with the anticoagulated blood. Perfusion was performed using a pulse-free pump under high shear stress equivalent to a wall shear rate of 1000 s^{-1} (4 min). Thereafter, chambers were rinsed by a 10 min perfusion with Hepes buffer at the same shear stress and phase-contrast images were recorded from at least five different microscope fields (40x objectives). Analysis was performed using MetaVue ® software.

B.2.13 *In vivo experiments*

Ether anaesthetized mice were injected intravenously with biotinylated JAQ1 (20µg; in 200 µL sterile PBS) and blood (50 µL) was collected at different time points. Blood was centrifuged at 2800 *rpm* for 5 min, plasma collected and incubated on JAQ3-coated ELISA plates. ELISA was performed as described in B.2.12.4.

B.2.14 *Bleeding time experiments*

Mice were anesthetized by intraperitoneal injection of ketamine/xylazine (ketamine 100 mg/kg, Parke-Davis; xylazine 5 mg/kg, Bayer AG), and a 3-mm segment of the

tail tip was cut off with a scalpel. Tail bleeding was monitored by gently absorbing the bead of blood with a filter paper without contacting the wound site. When no blood was observed on the paper after 15-second intervals, bleeding was determined to have ceased. The experiment was stopped after 20 minutes.

B.2.15 *Data analysis*

The results shown are mean ± S.D. Statistical analysis were performed using Student's *t* test with $P < 0.05$ taken as the level of significance.

C. Results

C.1 Human atherosclerotic plaques stimulate thrombus formation by activating GPVI

In collaboration with the group of Prof. Dr. Siess, Institute for Prevention of Cardiovascular Diseases, University of Munich, a study was carried out with the aim of identifying the major thrombogenic constituent(s) of atherosclerotic plaques. For this, atherosclerotic plaques from 60 patients with carotid stenosis were isolated and the material was tested in platelet aggregation studies and under flow conditions using whole blood. Thereby, collagen was identified as a major thrombogenic component of the atherosclerotic plaque as it induced platelet aggregation under stirring conditions in a similar manner as collagen. Moreover, atherosclerotic plaque induced thrombus formation of anti-coagulated whole blood under flow conditions. It was previously shown that GPVI plays a key role in arterial thrombus growth in a mouse model *in vivo* [71]. To test the relevance of this finding in humans, a possible role of GPVI in plaque-induced platelet activation was tested. .

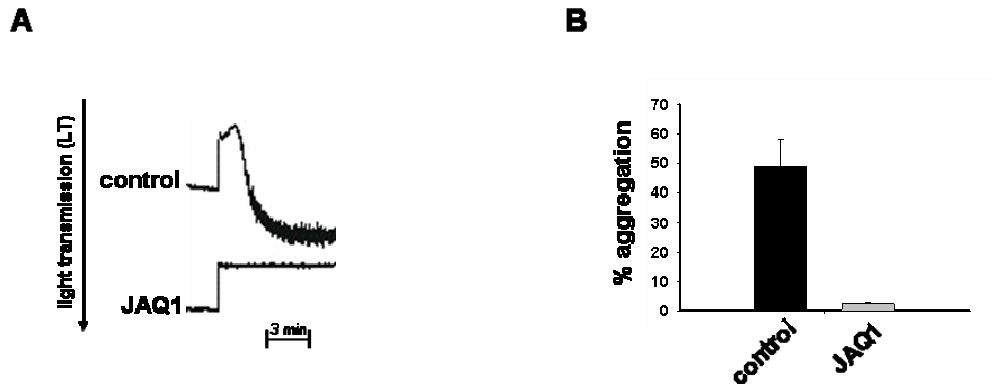

Fig.C.1 Platelet aggregation induced by plaque material is abolished in the presence of JAQ1. Heparinized prp was incubated with plaque material in the presence or absence of JAQ1 (20µg/ml) and aggregation was recorded. Results are representative (**A**) or given as mean ± S.D of six individual experiments (**B**).

C.1.1 *Platelet activation by human plaque material*

To test the hypothesis that plaque material can directly activate platelets, platelet rich plasma (prp) was incubated with the plaque material from different patients under stirring conditions and light transmission was recorded. Surprisingly, plaque material from most of the patients (56 out of 60) induced strong and irreversible platelet aggregation (Fig C.1).

C.1.2 *Plaque-induced platelet aggregation is abolished in the presence of JAQ1*

To test a possible role of GPVI in the platelet aggregation induced by atherosclerotic plaque material, prp was incubated with the plaque material in the presence or absence of JAQ1 (20µg/mL) under stirring conditions and light transmission was measured. Plaque-induced platelet aggregation was abolished in the presence of JAQ1 demonstrating that, under these conditions, GPVI is essential for this process (Fig C.1).

C.1.3 *GPVI is essential for plaque-induced thrombus formation of anti-coagulated blood*

To test the involvement of GPVI in plaque-induced platelet activation under more physiological conditions, anti-coagulated mouse blood was allowed to flow over cover slips coated with the atherosclerotic plaque material under high shear conditions in the presence or absence of JAQ1 (20µg/mL). Surprisingly, no thrombus was formed when blood was pre-incubated with JAQ1. To further confirm the role of GPVI in this process, anti-coagulated blood from JAQ1-treated, and thus GPVI-depleted mice [45] or mice deficient in the FcR γ-chain, which also lack GPVI, was used. Indeed, no thrombus formation was observed in both cases, demonstrating that GPVI is essential in plaque-induced platelet activation (Fig C.2).

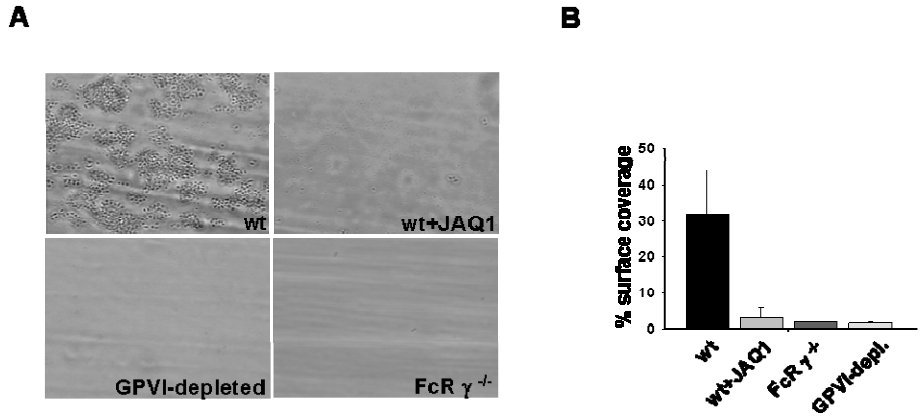

Fig. C.2 Plaque-induced platelet adhesion and aggregate formation is abolished in the absence of GPVI.

Heparinized whole blood from wild type mice ± JAQ1 (100µg/ml), GPVI–depleted mice, or from FcR γ -/- mice was allowed to flow over coverslips coated with plaque material under conditions of high shear rates (1000 s-1). Results shown are representative (**A**) or given as mean ± S.D of six individual experiments (**B**).

C.2 GPVI down-regulation in murine platelets through metalloproteinase-dependent shedding

In contrast to GPVI, the cellular regulation of GPIb has been intensively studied and it is known that the receptor can be down-regulated in response to agonist-induced platelet activation by internalization of the GPIb-IX complex and/or proteolysis of GPIbα [72-75]. On mouse platelets, these two pathways can be accurately discriminated using a two color flow cytometric assay utilizing antibodies against the N-terminal 45 kDa domain of GPIbα and GPIX [13;72]. This system was used to quantitiate GPIbα shedding / internalization in response to various platelet agonists. Partly, the following experiments were done in collaboration with W. Bergmeier, Harvard Medical School, Boston.

C.2.1 *Platelet activation induced GPIbα, but not GPVI down-regulation*

To test the effect of platelet activation on GPVI expression levels, platelets were activated with thrombin (0.1 U/mL) or CRP (5 µg/mL) and GPVI level on the platelet surface was measured. As a control, expression level of GPIbα was measured in parallel. Platelet stimulation with thrombin or CRP resulted in significant, but not complete shedding of GPIbα (Fig.C.3A, left panel). To test a possible involvement of metalloproteinases in this process, platelets were activated in the presence or absence of the broad spectrum metalloproteinase inhibitor GM6001 (100µM). Consistent with previous results [13], in all cases agonist-induced shedding of GPIbα, but not platelet activation (not shown), was inhibited in the presence of GM6001. The ~20% reduction in GPIbα surface expression observed in the presence of GM6001 was due to internalization of the GPIb-IX complex as shown by a similar reduction of GPIX levels (Fig.C.3A, left panel; white bars). This demonstrates that cellular stimulation through different signaling pathways activates platelet metalloproteinases that are essential for proteolysis of GPIbα. Very unexpectedly, however, under the same experimental conditions GPVI levels either remained unchanged (CRP) or even increased (thrombin) (Fig. C.3A, right panel). Immunoprecipitation experiments confirmed the presence of cleaved GPIbα, but not GPVI, in the supernatant of thrombin- or CRP-activated platelets (Fig. C.3B). Further studies demonstrated that platelet activation with other agonists like ADP, the stable thromboxane A2 analog U46619, or adrenaline even at very high concentrations failed to induce GPVI down-

regulation (data not shown). Together, these findings show that stimulation of the GPVI/FcRγ pathway or G-protein-mediated signaling is not sufficient to trigger GPVI down-regulation.

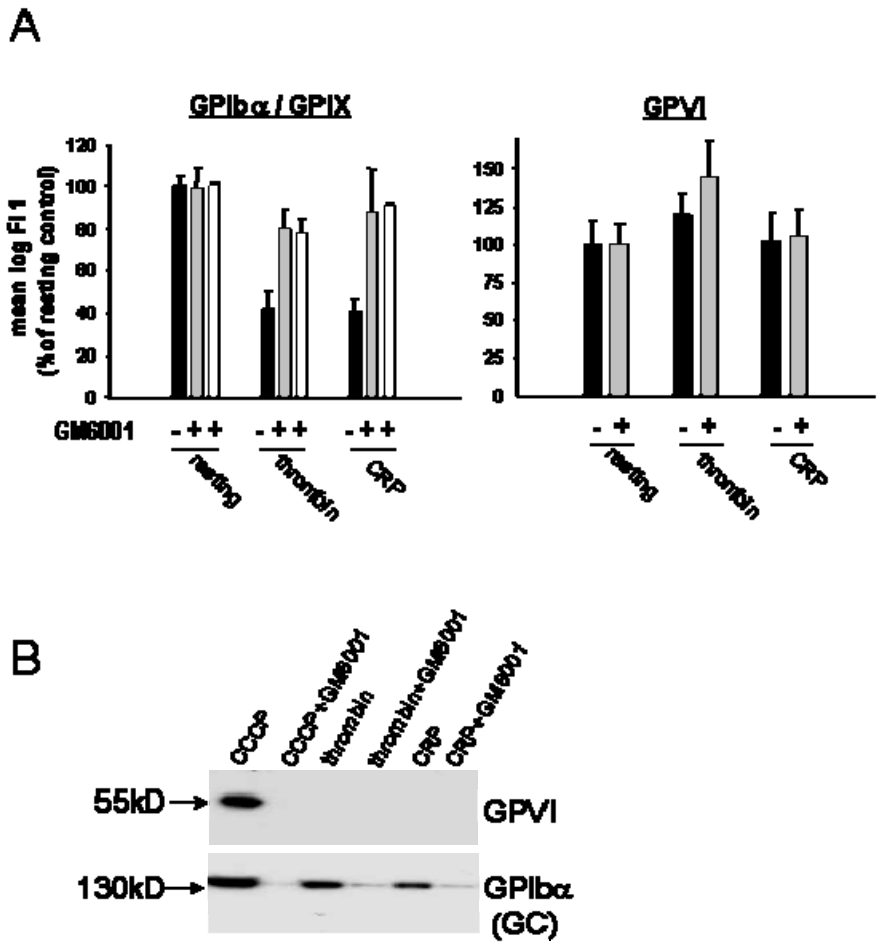

Fig. C.3 Agonist-induced platelet activation leads to metalloproteinase-dependent shedding of GPIbα, but not GPVI. (A) Washed platelets were left untreated or stimulated with CRP (5µg/ml) or thrombin (0.1 U/ml) for 10 min in the absence or presence of GM6001. Samples were stained with fluorophore-conjugated antibodies against GPIbα (black and gray bars, left panel), GPIX (white bars, left panel), or GPVI (right panel) for 15 min and analyzed directly. The data are the mean ± S.D. of six experiments and are presented as % of mean log fluorescence determined at t=0. **(B)** Biotinylated platelets were left untreated or stimulated with CRP (5 µg/ml) or thrombin (0.3 U/ml) in the presence or absence of GM6001. Subsequently, GPVI or GPIbα were precipitated from the supernatant of the cells. Precipitates were separated by SDS-PAGE under reducing conditions, blotted onto a PVDF membrane and visualized using streptavidin-HRP and ECL. The data shown are representative of 3 identical experiments.

C.2.2 *Mitochondrial damage markedly reduces GPVI activity in platelets*

Recently, an *in vitro* model was described, where induction of mitochondrial damage by carbonyl cyanide m-chlorophenylhydrazone (CCCP), induced the activation of metalloproteinases leading to profound shedding of GPIbα [13]. CCCP is a lipid-soluble amphiphatic molecule that uncouples mitochondrial oxidative phosphorylation

thereby generating pores in the inner mitochondrial membrane [76]. In addition to the effect on GPIbα expression level, mitochondrial damage induced various changes in platelets that are similar to those observed during *in vitro* storage. These changes include the rearrangement of the cytoskeleton, low levels of phosphatidyl serine (PS) exposure and P-selectin expression. This experimental system was also used in the current study to test whether GPVI is cleaved by a metalloproteinase *in vitro*. First, the effect of mitochondrial damage on platelet function was further characterized. For this, washed platelets were treated with CCCP (100 µM) for 1 h and their reactivity towards various agonists was tested. As previously reported, CCCP-treated platelets almost normally aggregated in response to thrombin (Fig. C.4A, left panel) or the stable thromboxane A2 analog U46619 (not shown), suggesting that G-protein coupled signaling pathways are largely preserved in those platelets. In contrast, the aggregation response to collagen was markedly delayed and reduced (Fig. C.4A, middle panel), suggesting an impaired function of the GPVI-dependent/mediated signaling pathway. This was confirmed when CCCP-treated platelets were stimulated with the GPVI-specific agonist CRP (5 µg/mL). While control platelets showed robust aggregation in response to CRP, CCCP-treated platelets were virtually unresponsive to this agonist (Fig. C.4A, right panel). Consistent with these observations, flow cytometric studies demonstrated that activation of integrin αIIbβ3 and degranulation (as measured by surface-expression of P-selectin) was markedly impaired in >90% of the CCCP-treated platelets activated with CRP, whereas only a minor inhibitory effect was observed upon stimulation with thrombin (Fig. C.4B). These findings demonstrated that the induction of mitochondrial damage with CCCP selectively affects GPVI-dependent activation pathways in platelets, whereas G-protein-mediated signaling was largely preserved. It is important to note, however, that CCCP is a cytotoxic compound that induces various alterations in platelets finally leading to cell death. At the time point chosen, 5-10% of the CCCP-treated platelets show signs of apoptosis [13], a fact that may explain why a small subpopulation of the CCCP-treated platelets was unresponsive towards thrombin.

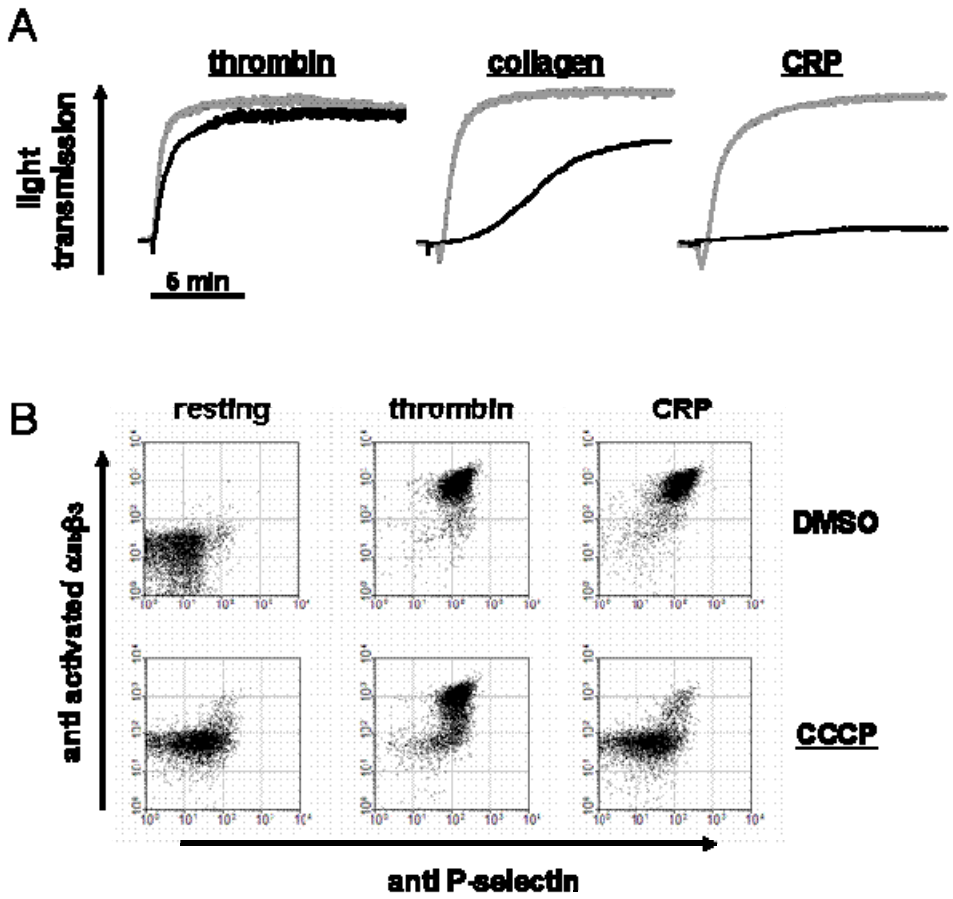

Fig. C.4 Mitochondrial damage impairs GPVI-mediated signaling. **(A)** Washed platelets were treated with DMSO (gray line) or CCCP (black line) for 60 min and platelet responses were tested in standard aggregometry by adding 0.5 U/mL thrombin, 5 μg/mL collagen, or 5 μg/mL CRP. The bar indicates 5 min along the x axis. Results are representative of 3 separate experiments. **(B)** Washed platelets were treated for 60 min with DMSO or CCCP and then activated for 10 min with 0.5 U/mL thrombin or 5 μg/mL CRP. Samples were stained for activated αIIbβ3 and P-selectin and immediately analyzed on a FACScalibur. Results are representative of 5 separate experiments.

C.2.3 *CCCP induces clearing of GPVI from the platelet membrane*

To test whether the reduced GPVI activity in CCCP-treated platelets was based on impaired GPVI-dependent signaling or, alternatively, down-regulation of the receptor, GPVI levels on CCCP-treated platelets were determined by flow cytometry. Strikingly, GPVI was undetectable on platelets treated with CCCP for 1 h suggesting that the receptor had been cleared from the surface of these cells. Whereas GPIbα levels were also reduced by more than 95%, surface levels of other receptors, including β1- and β3-integrins, CD9, and GPIX were unchanged or slightly increased, probably due to translocation of these glycoproteins from internal pools to the surface membrane (Fig. C.5A). Time course experiments further showed that CCCP-induced down-regulation of GPIbα and GPVI occurred with slightly different kinetics (Fig. C.5B).

To test a possible involvement of platelet metalloproteinases in GPVI down-regulation, washed platelets were incubated with CCCP in the presence or absence of the broad spectrum metalloproteinase inhibitor GM6001. As shown in figure C.5B and C, GM6001 blocked the down-regulation of both GPIbα and GPVI. Thus, GPVI can be efficiently cleared from the platelet surface by a metalloproteinase-dependent mechanism.

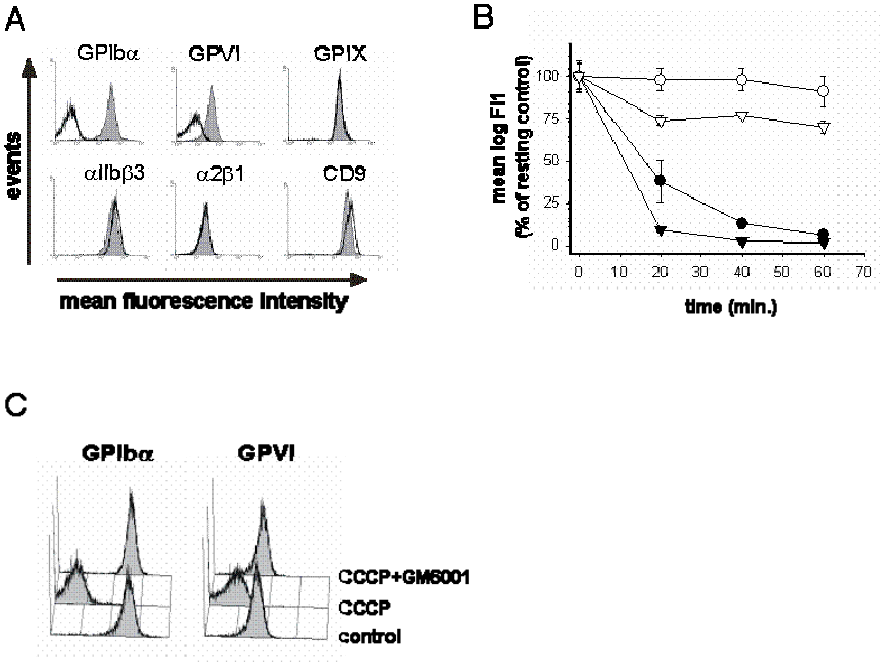

Fig. C.5 GPVI is cleared from the platelet surface by metalloproteinase-dependent mechanisms. Flow cytometric analysis of glycoprotein expression. **(A)** Washed platelets were incubated in the absence (shaded area) or presence of CCCP (black line) for 1 h, stained with the indicated fluorophore-labeled antibodies for 15 min, and analyzed directly. **(B)** Time course of GPIbα (triangles) and GPVI (circles) clearing from platelets treated with CCCP in the absence (filled symbols) or presence (open symbols) of GM6001. The data are the mean ± S.D of six experiments and are presented as % of mean log fluorescence determined at t=0. **(C)** Washed platelets were incubated with CCCP for 1 h in the presence or absence of the broad range metalloproteinase inhibitor GM6001, stained with the indicated fluorophore-labeled antibodies for 15 min, and analyzed directly. (**A**, **C**) The data shown are representative of 6 individual experiments.

C.2.4 *GPVI down-regulation is mediated by metalloproteinase-dependent shedding of the receptor*

To test whether CCCP-induced removal of GPVI from the cell surface is based on shedding or internalization of the receptor, the presence of GPVI in supernatants and in lysates of CCCP-treated platelets was tested. For this, platelets were surface-biotinylated and treated with CCCP for 1 h in the presence or absence of GM6001. Subsequently, immunoprecipitation of GPVI (JAQ1), GPIbα (p0p4), or, as a control,

GPIIIa (EDL1) from platelet lysates or supernatants was performed. As shown in figure C.6A (upper panel), JAQ1 precipitated a band of approximately 55 kDa, corresponding to the extracellular domain of GPVI, from the supernatant of CCCP-treated, but not control platelets or platelets treated with CCCP in the presence of GM6001. Consistently, the level of intact GPVI (62 kDa) present in the lysate of CCCP-treated platelets was markedly reduced as compared to the controls. Identical results were obtained when JAQ2 or JAQ3 were used for immunoprecipitation (not shown). Similarly, CCCP-induced release of the soluble extracellular 130 kDa fragment of GPIbα (GC) was inhibited by GM6001 (Fig C.6A, middle panel). In contrast, GPIIIa was found in comparable amounts in all platelet lysates but not in any supernatant (Fig C.6A, lower panel). In parallel, the same experiment was performed with FcRγ chain-deficient platelets, which are known to lack GPVI [41]. As shown in figure C.6B, JAQ1 did not precipitate any protein from lysates or supernatants of those platelets, whereas CCCP-induced shedding of GPIbα normally occurred and was inhibited by GM6001, suggesting that the GPVI/FcRγ complex is not required for the activation of metalloproteinases in this system.

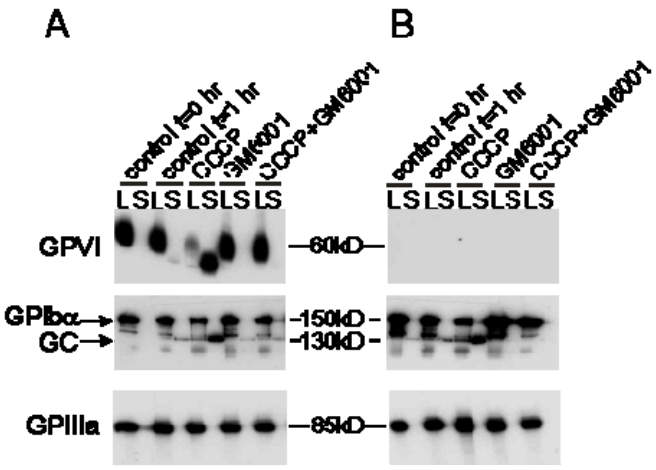

Fig. C.6 CCCP induces metalloproteinase-dependent shedding of GPVI. Biotinylated platelets from wild-type **(A)** or FcRγ-chain -/- **(B)** mice were treated with CCCP in the presence or absence of GM6001 for 1 h. Subsequently, GPVI (JAQ1), GPIbα (p0p4), or GPIIIa (EDL1) were precipitated from the supernatant (S) or the lysate (L) of the cells. Precipitates were separated by SDS-PAGE under reducing conditions, blotted onto a PVDF membrane and visualized using streptavidin-HRP and ECL. The data shown are representative of 6 identical experiments.

Together, these results demonstrated for the first time that the extracellular domain of GPVI can be efficiently shed from the platelet surface by a platelet-derived protease.

The profound inhibition of GPVI cleavage by GM6001 reveals an essential role of metalloproteinases in this process, but it is unclear whether they directly or indirectly act on GPVI. The above experiments demonstrated that inhibition of metalloproteinases prevents CCCP-induced shedding of GPVI, but it remained to be seen whether this restores the activity of the receptor. To address this question, platelets were treated with CCCP in the absence or presence of GM6001 and then stimulated with CRP. Inhibition of metalloproteinase activity during mitochondrial injury restored platelet aggregation (Fig. C.7A) as well as integrin activation and degranulation (Fig. C.7B) in response to CRP. These observations indicate that the impairment of GPVI-mediated responses in CCCP-treated platelets is mediated by a metalloproteinase-dependent pathway.

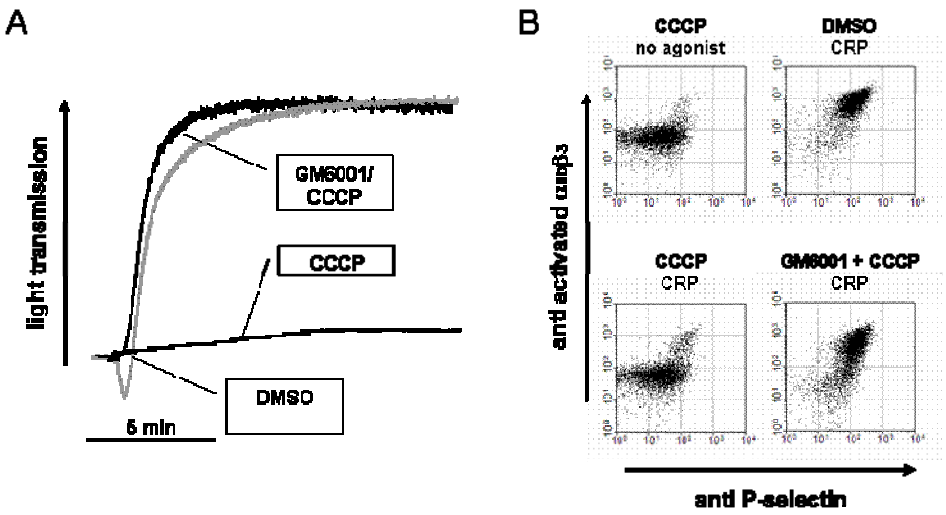

Fig. C.7 Inhibition of metalloproteinases during mitochondrial damage restores GPVI activity. (A) Washed platelets were treated for 60 min with CCCP in the absence or presence of GM6001, and platelet responses were tested in standard aggregometry by adding 5 μg/mL CRP. The bar indicates 5 min along the x axis. Results are representative of 3 separate experiments. **(B)** DMSO-, CCCP-, or GM6001/CCCP-treated platelets (60 min) were activated for 10 min with 5 μg/mL CRP. Samples were stained for activated αIIbβ3 and P-selectin and immediately analyzed on a FACScalibur. Results are representative of 5 separate experiments.

C.3 Evidence for a role of ADAM17 (TACE) in the regulation of GPV

As mentioned above, platelet activation induces GPIbα down-regulation, which is dependent on metalloproteinases. As GPV is a subunit of the GPIb-V-IX receptor complex, cellular regular of GPV was investigated in parallel.

C.3.1 *GPV down-regulation in human platelets is metalloproteinase-dependent*

To study GPV regulation on the platelet surface, human platelets were stimulated with PMA (100 ng/mL), ADP (5 µM) or collagen-related peptide (CRP, 2.5 µg/mL) and surface levels of GPV were determined by flow cytometry. As a positive control, platelets were incubated with thrombin (0.1 U/mL) which directly cleaves the receptor. GPV was down-regulated in response to PMA to a similar extent as in the thrombin control, whereas a weaker effect was observed in response to CRP. In contrast, ADP, which is only a weak platelet agonist, did not induce significant down-regulation of GPV (Fig.C.8A).

To investigate whether the decrease in GPV surface expression is accompanied by the down-regulation of the entire GPIb-V-IX complex from the cell surface, surface levels of GPIbβ were determined, as this subunit is not specifically cleaved upon platelet activation. GPIbβ surface levels were only slightly decreased or unchanged in PMA- or CRP-stimulated platelets, respectively, indicating that down-regulation of GPV was rather the effect of shedding than internalization (Fig.C.8B).

PMA- or CRP-induced shedding of GPV occurred independently of thrombin activity as it was unaltered in the presence of the thrombin inhibitor, hirudin (not shown). In contrast, GM6001 markedly blocked GPV shedding in response to PMA and CRP, but not thrombin (Fig.C.8C).

C.3.2 *Metalloproteinase- and thrombin–mediated GPV cleavage lead to the generation of different soluble GPV variants*

Next, GPV regulation in murine platelets was studied. In these cells, PMA induced virtually complete down-regulation of the receptor whereas the effect of CRP was weaker and ADP did not alter GPV surface levels (Fig. C.9A). GPIX surface levels remained virtually unchanged under all conditions, confirming that GPV shedding is not accompanied by down-regulation of the entire GPIb-V-IX complex from the cell surface (Fig. C.9A). Like in human platelets, GM6001 almost completely inhibited GPV shedding in response to PMA or CRP, but not thrombin (Fig. C.9B).

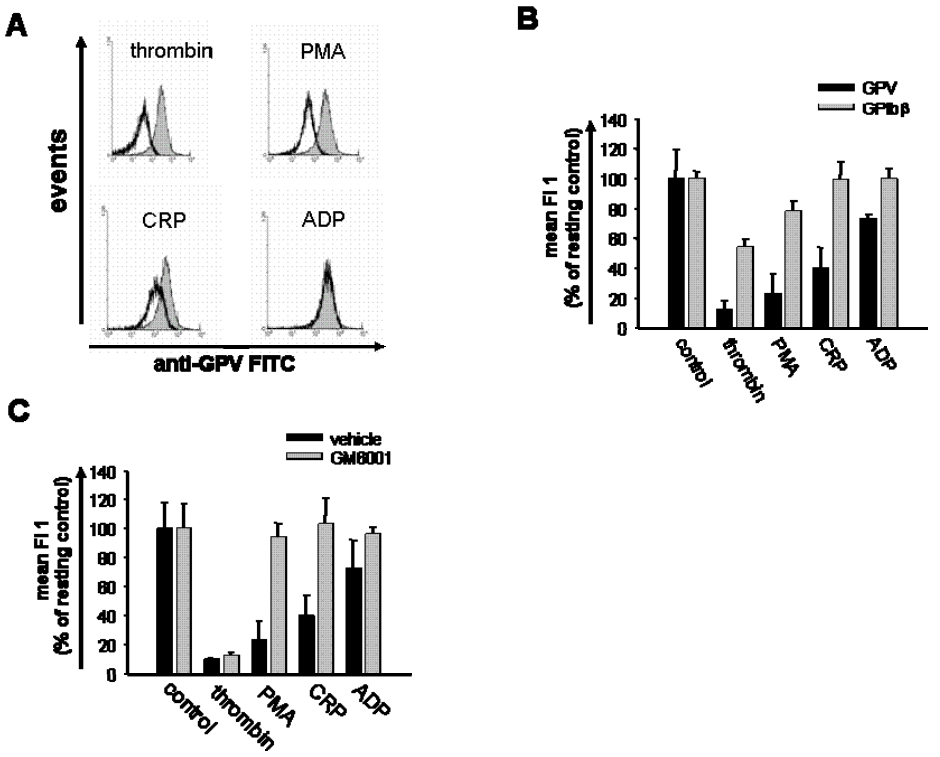

Fig. C.8 GPV down-regulation in human platelets is metalloproteinase-dependent Washed human platelets were incubated for 15 min at 37°C in the absence (shaded area) or presence (black line) of the indicated agonists, stained with FITC-labeled anti-GPV antibody (**A**) or anti-GPIbβ antibody (**B**), in absence or presence of GM6001 (100µM) (**C**) and analyzed immediately on a FACScalibur. (**A**) Data shown are representative of 3 identical experiments. (**B, C**) Data shown are the mean ± S.D of six separate experiments and are presented as % of mean fluorescence determined at t=0.

Immunoprecipitation experiments demonstrated that the metalloproteinase-generated fragment of GPV has a MW of ~82 kDa and thereby differs significantly from GPV f1 (~69 kDa) released by thrombin (Fig. C.9C). As a control, immunoprecipitations were also performed with an anti-GPIIIa antibody which did not yield a band under any condition (Fig. C.9C). These results demonstrated that platelet activation by thrombin, PMA and CRP induces limited proteolysis of GPV via different mechanisms. PMA or CRP-stimulated cleavage involves a platelet-derived metalloproteinase and occurs in close vicinity to the transmembrane domain

C.3.3 *Inhibition of ADAM17 blocks GPV shedding from the platelet surface*

As ADAM17 is known to be potently induced by PKC activators, such as PMA, a possible role of ADAM17 in this process was tested. Therefore, first, platelets were tested for the expression of ADAM 17 by Western blot analysis. As shown in Fig. C.10A, ADAM 17 (~130 kDa) was specifically detectable in whole cell lysates of both

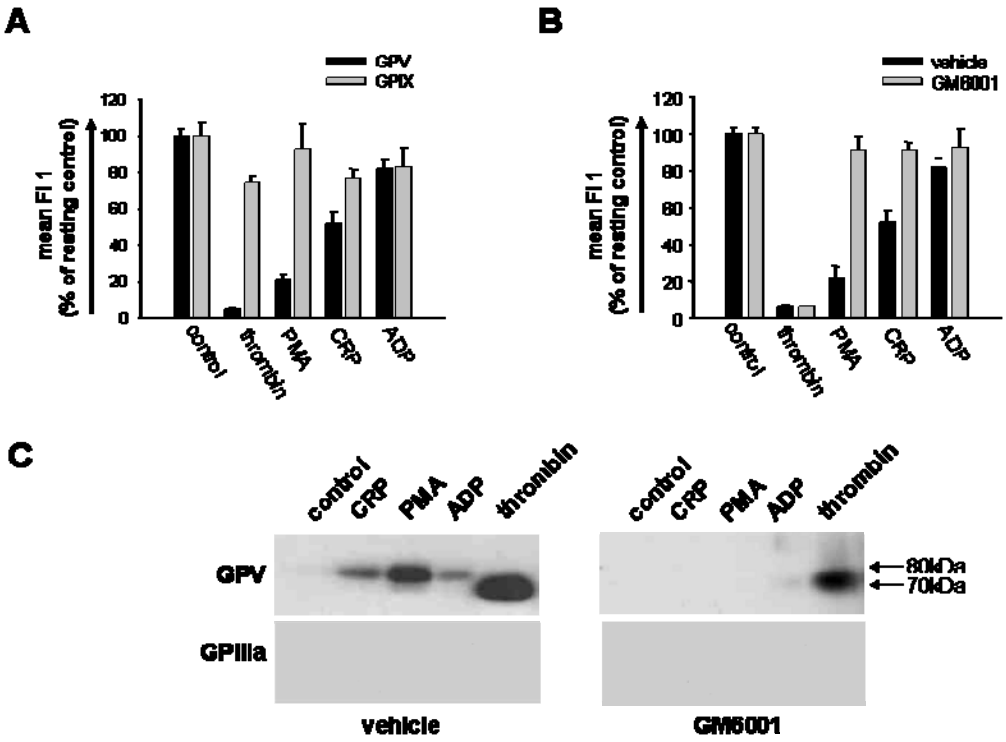

Fig. C.9 Metalloproteinase- and thrombin–mediated GPV cleavage lead to the generation of different soluble GPV variants. Washed mouse platelets were incubated with the indicated agonists for 15 min at 37°C, stained with FITC-labeled anti-GPV or anti-GPIX antibody (**A**) in the presence or absence of GM6001 (**B**), and analyzed immediately on a FACScalibur. The data shown are the mean ± S.D of six separate experiments and are presented as % of mean fluorescence determined at t=0. (**C**) Surface-biotinylated mouse platelets were incubated with the indicated agonists for 15 min at 37°C in the absence or presence of GM6001 (100µM). Subsequently, GPV and GPIIIa were immunoprecipitated from the supernatants of the cells. Immunoprecipitates were separated by SDS-PAGE under reducing conditions, blotted onto a PVDF membrane and visualized using streptavidin-HRP and ECL. The data shown are representative of 3 identical experiments.

human and mouse platelets. Next, the effect of the two potent ADAM17 inhibitors, GW 280264X (GlaxoSmithKline) [77;78] and TAPI-2 (Immunex/Amgen) [79;80] on GPV down-regulation was tested. Both compounds inhibited PMA- and CRP–induced GPV shedding in human and mouse platelets (Fig. C.10B), suggesting that ADAM17 mediates ectodomain shedding of GPV. To test this directly, platelets were incubated with different concentrations of recombinant human ADAM17 ectodomain (rhADAM17) and GPV levels were determined by flow cytometry. Indeed, rhADAM17 dose-dependently down-regulated GPV (Fig. C.10C), but not GPIX or GPIIb/IIIa (not shown) from the platelet surface and this effect was abrogated in the presence of GM6001.

C.3.4 *GPV shedding is abolished in ADAM17$^{\Delta Zn/\Delta Zn}$ mice*

To confirm the role of ADAM17 in this process, platelets from mice lacking functional ADAM17 (ADAM17$^{\Delta Zn/\Delta Zn}$) were used. Since ADAM17$^{\Delta Zn/\Delta Zn}$ mice die perinatally [62], blood was collected from mutant and control mice immediately after birth. Blood was washed once in Tyrode's buffer, activation with PMA or CRP was then performed and surface levels of GPV was determined by flow cytometry. As shown in figure C.11, no GPV shedding was observed in these mice, demonstrating that ADAM17 is the major sheddase that cleaves GPV.

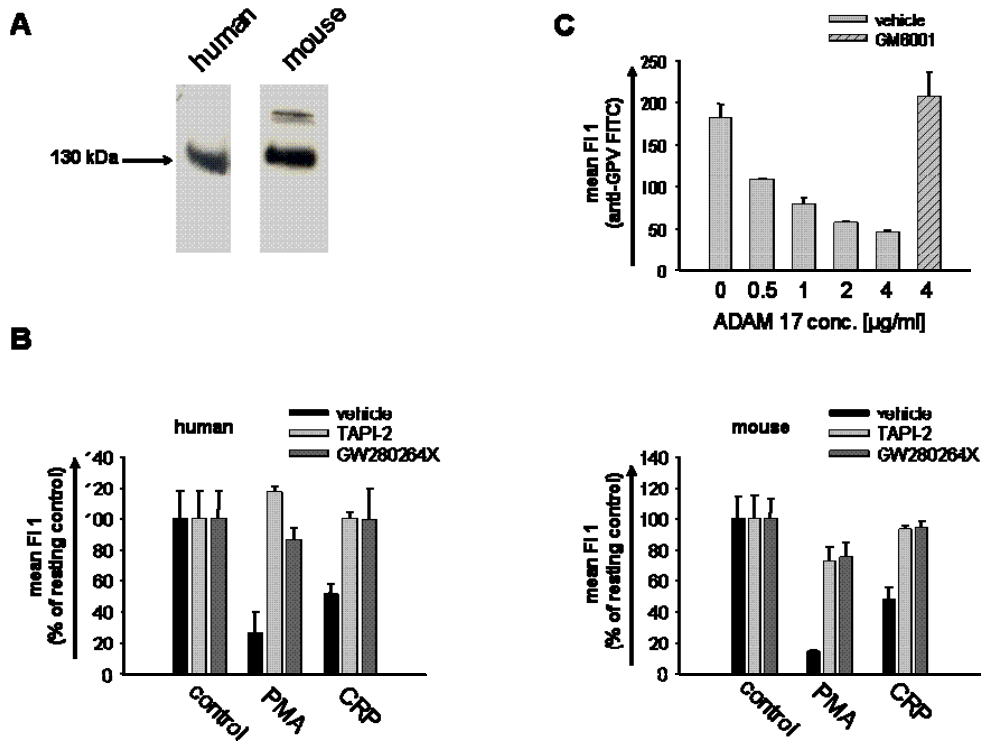

Fig. C.10 Inhibition of ADAM17 blocks GPV shedding from the platelet surface (A) Whole platelet proteins were separated by SDS-PAGE under reducing conditions and immunoblotted with anti-ADAM17 antibodies. **(B)** Human (left) and mouse (right) platelets were activated in presence or absence of TAPI-2 (100µM) or GW280264X (10µM), stained with FITC-labeled anti-GPV antibody and analyzed immediately on a FACScalibur. Data shown are the mean ± S.D of six separate experiments and are presented as % of mean fluorescence determined at t=0. (**C**) Washed mouse platelets were incubated with the indicated concentrations of recombinant human ADAM17 for 1 h at 37°C, stained with FITC-labeled anti-GPV antibody and analyzed immediately on a FACScalibur. Where indicated, the experiment was performed in the presence of GM6001 (100µM). Data shown are the mean ± S.D of three separate experiments.

C.3.5 *Inhibition of calmodulin induces metalloproteinase-dependent shedding of GPV in mouse and human platelets*

Besides GPV, other receptors such as L-selectin on leukocytes [81] are known to be associated intracellularly with calmodulin and to undergo rapid ectodomain shedding

upon treatment with calmodulin inhibitors such as W13 [82;83]. During the course of this study, this was further confirmed by Gardiner *et al*, reporting that a similar mechanism exists for GPVI in human platelets [84]. In order to examine the implication of calmodulin in ADAM17-dependent GPV shedding, platelets were incubated with vehicle or the

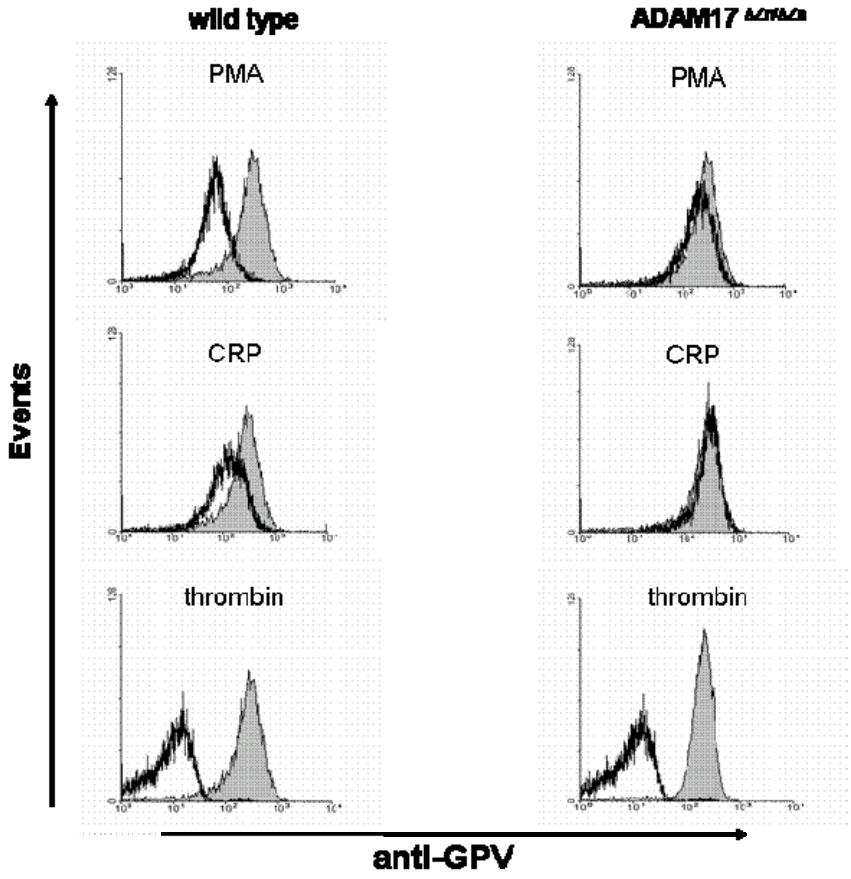

Fig. C.11 GPV shedding is abolished in ADAM17$^{\Delta Zn/\Delta Zn}$ mice. Platelets from wild-type or ADAM17 $^{\Delta Zn/\Delta Zn}$ mice were incubated in absence (shaded area) or presence (black line) of the indicated agonists for 15 min. at 37°C, stained with FITC-conjugated anti-GPV antibody and analyzed immediately on a FACScalibur. The results shown are representative of 4 independent experiments.

calmodulin inhibitor W13 (200 µM) and GPV surface levels were determined at different time points. As shown in figure C.12A, GPV was rapidly down-regulated in the presence, but not in the absence of W13 in both mouse and human platelets. Similar results were obtained with a second calmodulin inhibitor, W7 (not shown). As with CRP- or PMA-stimulated platelets, W13-induced GPV shedding was inhibited in the presence of GM6001, GW 280264X, or TAPI-2 (Fig. C.12A and not shown) and was absent in platelets from ADAM $^{\Delta Zn/\Delta Zn}$ mice (Fig. C.12B). Moreover, immunoprecipitation experiments showed the accumulation of the ~82 kDa variant of soluble GPV in the supernatant of W13-treated platelets (Fig. C.12C). Together,

these results indicate that calmodulin is a negative regulator of ADAM17-mediated GPV cleavage.

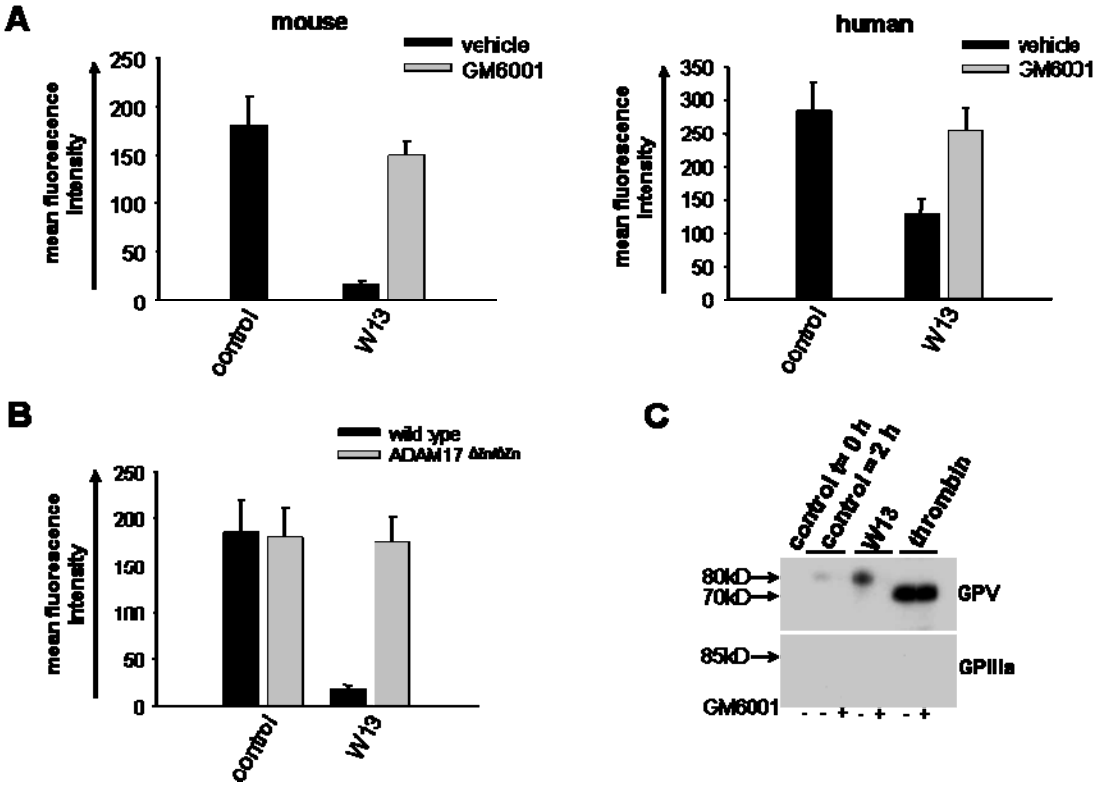

Fig. C.12 Inhibition of calmodulin induces metalloproteinase-dependent shedding of GPV in mouse and human platelets (A) Washed human and mouse platelets were incubated, in presence or absence of GM6001, with W13 (200µM) for 2 h at 37°C, stained with FITC-labeled anti-GPV antibody and analyzed immediately on a FACScalibur. The data shown are the mean ± S.D of six separate experiments. **(B)** Washed platelets from wild type or ADAM17 $^{\Delta Zn/\Delta Zn}$ mice were incubated with W13 (200µM) for 2 h at 37°C, stained with FITC-labeled anti-GPV antibody and analyzed immediately on a FACScalibur. The data shown are the mean ± S.D of 4 separate experiments. **(C)** Surface-biotinylated mouse platelets were incubated with W13 (200µM) for 2 h at 37°C in presence or absence of GM6001 (100µM). Subsequently, GPV and GPIIIa were immunoprecipitated from the supernatants of the cells. Immunoprecipitates were separated by SDS-PAGE under reducing conditions, blotted onto a PVDF membrane and visualized using streptavidin-HRP and ECL. The data shown are representative of 3 identical experiments.

C.3.6 *GPV shedding occurs independently of GPIIb/IIIa outside-in signaling*

Besides GPV, a number of other platelet membrane glycoproteins can undergo ectodomain shedding on the surface of activated platelets, including P-selectin, CD40-L, and GPVI and it appears that different regulatory mechanisms and proteases may be involved in these processes. Shedding of CD40-L has been reported to be largely dependent on activated GPIIb/IIIa, suggesting that outside-in signaling through the integrin regulates the proteolytic process [85;86]. In contrast, GPVI shedding occurs completely independent of GPIIb/IIIa signaling [84]. To test the involvement of GPIIb/IIIa outside-in signaling in ADAM17-mediated GPV

shedding, mouse platelets were stimulated with PMA, CRP, or thrombin under stirring conditions in the presence or absence of a blocking antibody against GPIIb/IIIa (JON/A, 50µg/mL, [70]). Cleaved GPV was quantitated in the supernatant with a newly established ELISA system. While JON/A blocked platelet aggregation (Fig. C.13A), it had no significant effect on GPV shedding in response to PMA, thrombin (Fig. C.13B), or CRP (not shown), demonstrating that this process occurs independently of outside-in signaling through GPIIb/IIIa.

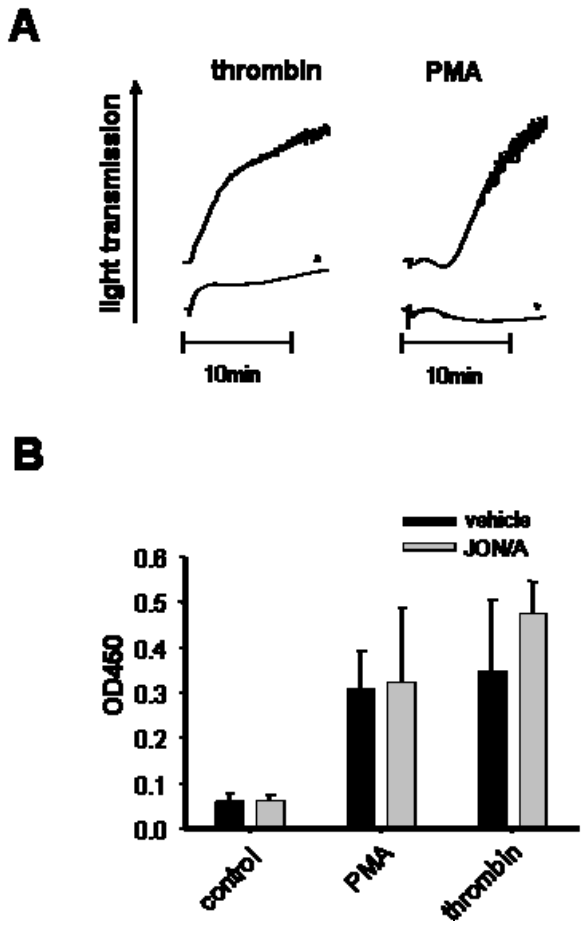

Fig. C.13 GPV shedding occurs independently of GPIIb/IIIa outside-in signaling. Washed mouse platelets (thrombin) or heparinized prp (PMA) were incubated with the indicated activators in absence or presence (*) of JON/A (50µg/ml) under stirring conditions and **(A)** light transmission was recorded or **(B)** GPV levels in the supernatants were determined by ELISA as described in Materials and Methods. Results shown are the mean ± S.D of six individual experiments.

C.4 Targeting of the collagen binding site on glycoprotein VI is not essential for *in vivo* depletion of the receptor

Until the beginning of the study there was only one anti-mouse GPVI antibody available, JAQ1. JAQ1 binds to the major collagen site on the platelet surface and, upon injection into mice, induces GPVI down-regulation *in vivo* [45]. An open question at that time was whether GPVI down-regulation is dependent on the binding

site of the antibody. To answer this question, new anti-GPVI antibodies were generated. Together with V. Schulte, Rudolf Virchow Center, Würzburg, the new antibodies were characterized *in vitro* and *in vivo*.

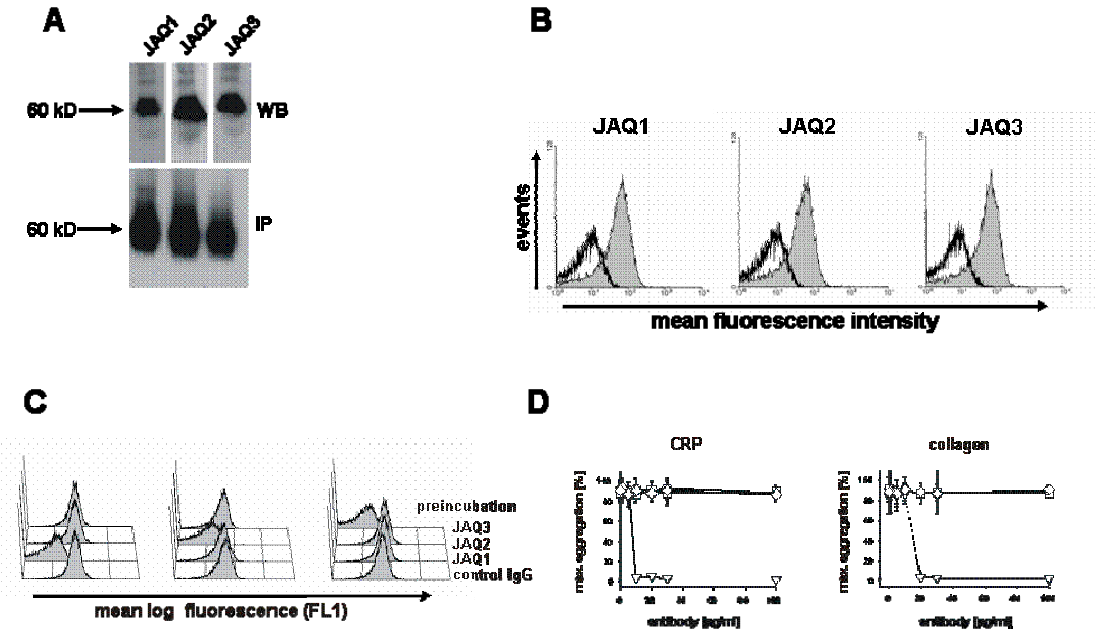

Fig. C.14 JAQ1, JAQ2 and JAQ3 bind to different epitopes on mouse GPVI. (**A**) Whole platelet proteins were separated by SDS-PAGE under nonreducing conditions and immunoblotted with the indicated antibodies. Bound mAb was detected by HRP-labeled rabbit anti-rat Ig and ECL (*WB*). Surface biotinylated platelets were lysed and immunoprecipitation was performed with the indicated antibodies. Precipitated proteins were detected with streptavidin-HRP and ECL (*IP*). (**B**) Washed platelets from wild-type (shaded area) or FcRγ chain -/- (black line) mice were incubated with the indicated antibodies (5 µg/ml) for 10 min, washed and incubated with FITC-labeled anti-rat IgG for 10 min. and samples were analyzed immediately on a FACScalibur. (**C**) Platelets were preincubated with the indicated antibodies (20 µg/ml, 30 min), washed and binding of FITC-labeled JAQ1, JAQ2, or JAQ3 was determined (**D**) Heparinized prp was incubated with different concentrations of IgG2a (circles), JAQ1 (triangles), JAQ2 (squares), or JAQ3 (diamonds) for 5 min before the addition of CRP (0.25 mg/mL, left) or collagen (5 µg/mL, right) and aggregation was recorded. Results are expressed as mean ± S.D. of 6 mice per group.

C.4.1 *The new anti-GPVI antibodies bind to different epitopes*

Two new anti-GPVI antibodies, JAQ2 and JAQ3; (both rat IgG2a) were generated. Both antibodies precipitated a single chain protein of an apparent molecular weight of approximately 60 kDa (Fig. C.14A, IP). The identity of the precipitated protein with GPVI was verified by immunoblotting with JAQ1 (not shown). JAQ2 and JAQ3 also recognized GPVI in Western blot analysis under nonreducing conditions (Fig. C.14A, WB). Moreover, JAQ2 and JAQ3 did not bind to platelets from FcRγ-chain-deficient mice which are known to lack GPVI [41] (Fig C.14B). Flow cytometric preincubation studies demonstrated that JAQ1, JAQ2, and JAQ3 do not block each others binding

indicating that they recognize different epitopes on GPVI (Fig. C.14C). Consistent with this, JAQ2 and JAQ3 (up to 100 µg/mL) did not inhibit CRP-induced platelet aggregation, whereas this was completely blocked by JAQ1 (Fig. C.14D). Similarly, collagen-induced aggregation was largely inhibited by JAQ1 in a dose-dependent manner, whereas it was not affected by JAQ2 or JAQ3 (Fig. C.14D). Together, these results demonstrated that JAQ2 and JAQ3 bind to epitopes on mouse GPVI that are different from the collagen binding site on the receptor.

C.4.2 *JAQ2 and JAQ3 induce GPVI down-regulation in vivo*

To test the effects of JAQ2 and JAQ3 on the *in vivo* expression of GPVI, mice received 100 µg of the respective antibodies and platelets were tested on day 5 for the presence of GPVI in Western blot analysis. As shown in figure C.15A, GPVI was undetectable in platelets from mice treated with JAQ1, JAQ2, or JAQ3 whereas normal amounts of GPIIIa were found in all platelets. These results demonstrate that the antibody-induced depletion of GPVI from circulating platelets *in vivo* is not dependent on the exact binding epitope recognized by the antibody.

C.4.3 *F (ab) fragments of JAQ2 and JAQ3 induce GPVI down-regulation in vivo*

To address the question whether the Fc part or the dimeric form of anti-GPVI antibodies plays a role in depletion of the receptor, monovalent F(ab) fragments of JAQ1, JAQ2, and JAQ3 were produced, injected into mice (100µg) and their effect on the *in vivo* expression of GPVI was tested. F (ab) fragments of JAQ2 and JAQ3 induced the depletion of GPVI. However, there was a more rapid increase of GPVI levels in platelets after F (ab) injection as compared to intact IgG, as very low levels of GPVI were detected in platelet lysate on day 3 in Western blot analysis (Fig. C.15B), strongly suggesting that *in vivo* stability and avidity of anti-GPVI agents may have a major influence on their potential to induce long-term antithrombotic protection. These findings show that neither the Fc part nor the dimeric form of anti-GPVI antibodies is responsible for the depletion of the receptor. It is important to note that JAQ1-3 did not induce GPVI down-regulation *in vitro* (Fig. C.15C), suggesting the involvement of other signals that are present *in vivo* only.

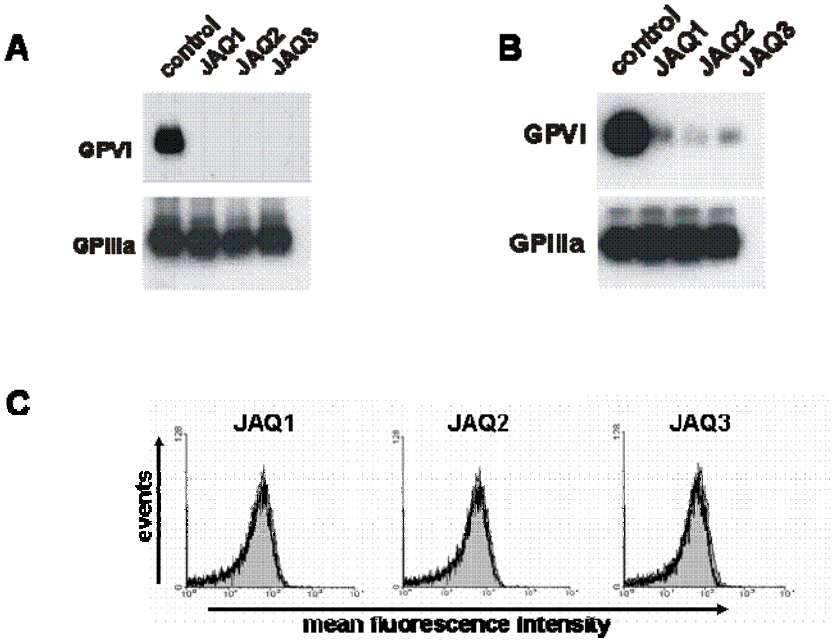

Fig. C.15 JAQ1, JAQ2 and JAQ3 induce GPVI down-regulation *in vivo*, but not *in vitro*. (A) Mice were injected (i.p) with 100µg of indicated antibodies. On day 5 after antibody injection, whole platelet proteins were separated on SDS-PAGE under nonreducing conditions and immunoblotted with HRP-labeled JAQ1 (anti-GPVI) or EDL1 (anti-GPIIIa) followed by ECL. (**B**) Mice were injected (i.p) with 100µg of F (ab) fragments of the indicated antibodies. On day 3 after antibody injection, whole platelet proteins were separated on SDS-PAGE under nonreducing conditions and immunoblotted with HRP-labeled JAQ1 (anti-GPVI) or EDL1 (anti-GPIIIa) followed by ECL. (**C**) Washed platelets were incubated with indicated antibodies for 10 min, washed and incubated with FITC-labeled anti-rat IgG for 10 min. Samples were analyzed immediately on a FACScalibur.

C.5 Diverging signaling events control the pathway of GPVI down-regulation *in vivo*

As mentioned above, injection of anti-GPVI antibodies (JAQ1, JAQ2, and JAQ3) induces the depletion of the receptor from circulating platelets. The major question that was still open at that time is how JAQ1-3 induce GPVI down-regulation. Although there was evidence that GPVI is internalized upon antibody treatment [45], it was unclear whether receptor cleavage also takes place. Based on the studies described in C.2, it was known that GPVI contains a cleavage site for a metalloproteinase(s). Therefore, it was intriguing to speculate that, besides internalization, GPVI cleavage takes place *in vivo* after antibody injection. In order to test this, an assay system that specifically detects soluble GPVI was established. As the available anti-GPVI antibodies (JAQ1-3) detect different epitopes on GPVI, an ELISA assay was the method of choice. For this, biotinylated JAQ1 was used in combination with JAQ3. To validate this ELISA system, washed platelets from wild-type or FcR γ-chain deficient mice were preincubated with JAQ1-biotin for 10 min. After centrifugation and

resuspension in tyrode's buffer (see Materials and Methods) platelets were incubated, in the presence or absence of GM6001 (100µM), with CCCP (100µM) for 1 h. Supernatants were then transferred onto an ELISA plate coated with JAQ3 (20µg/mL) and incubated for 1 h (at 37°C). Plates were incubated with HRP-labeled streptavidin for 1 h and were then developed. As shown in figure C.16, soluble GPVI could be detected in the supernatant of platelets from wild type, but not FcR γ-chain deficient mice, showing that the soluble GPVI/JAQ1 complex could be specifically detected using this ELISA system.

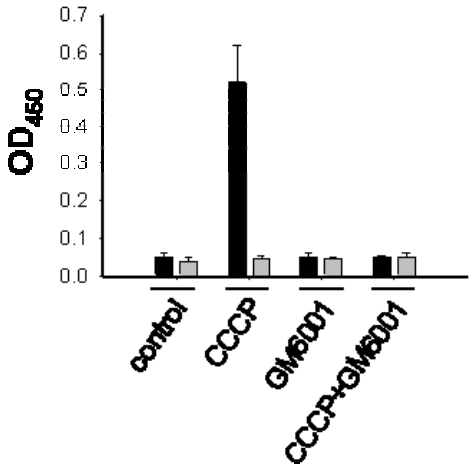

Fig. C.16 Detection of soluble GPVI in an ELISA system. Washed platelets from wild-type (black bars) or FcRγ chain -/- (gray bars) mice were preincubated with biotinylated JAQ1 (10µg/ml) for 15 min and ELISA was performed as described in Materials and Methods.

C.5.1 *JAQ1 induces GPVI shedding in vivo*

To study the mechanisms underlying antibody-induced GPVI depletion *in vivo*, wild-type and FcRγ chain deficient mice were injected with biotinylated JAQ1 (20 µg; i.v.) and platelets were analyzed at various time points after antibody administration. Consistent with previous results [45], JAQ1 injection, in wild-type mice, induced strong and transient thrombocytopenia with a maximum drop in platelet count of 80% reached at 30 min time point and a return to almost normal counts after 48 h post antibody injection (Fig. C.17A). In addition, JAQ1, measured with FITC-labeled streptavidin, was undetectable on the surface of circulating platelets showing that GPVI is depleted from the platelet surface (Fig. C.17B). Moreover, GPVI and biotinylated JAQ1 were detected in platelet lysate only at early time points after antibody injection, but not at later time points (Fig. C.17C).This demonstrates that GPVI, at least in part, is internalized into the platelets and is subsequently degraded.

Surprisingly, however, as soon as 30 min after antibody injection, high levels of JAQ1/GPVI complex were detected in the plasma of antibody-treated mice. Over time, plasma JAQ1/GPVI levels decreased reaching control levels 24 h after injection. Importantly, JAQ1/GPVI was undetectable in plasma of FcRγ-chain-deficient mice at any time point after JAQ1 injection confirming the specificity of the ELISA system (Fig. C.17D).

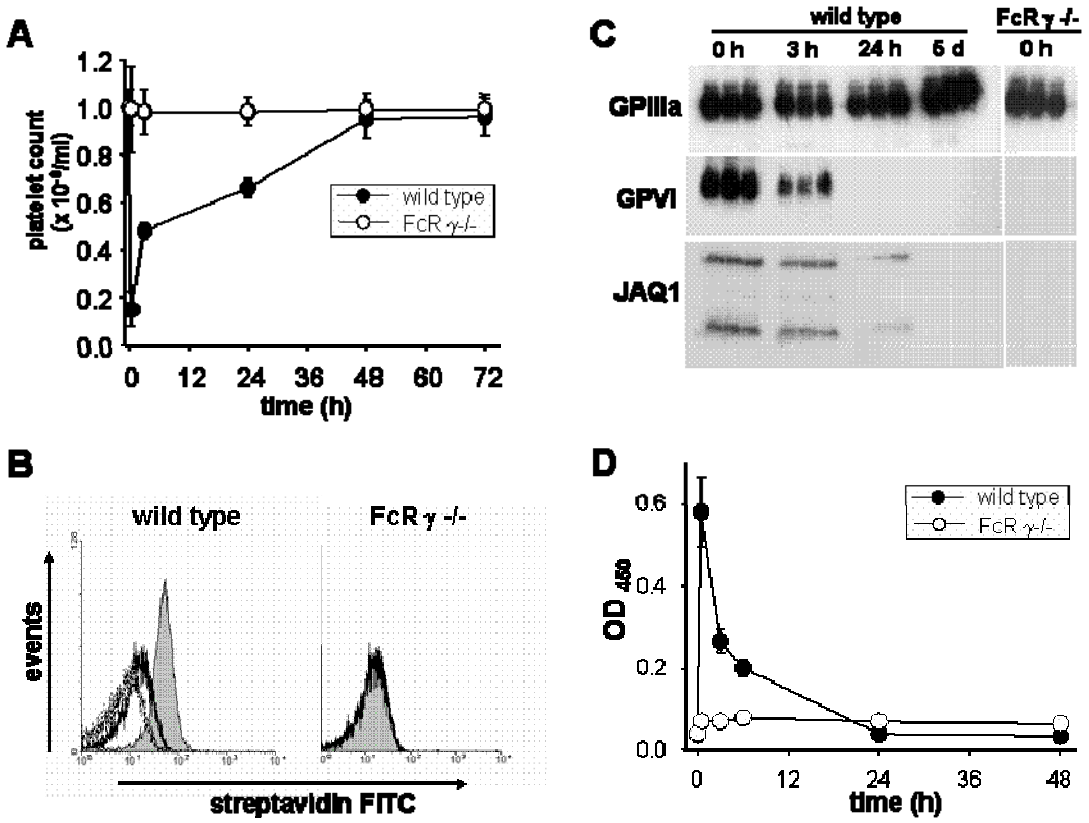

Fig. C.17 JAQ1 induces GPVI shedding *in vivo*. Wild-type or FcRγ chain -/- mice were injected with biotinylated JAQ1 (20µg; i.v). (**A**) Blood was withdrawn at the indicated time points and platelet count was determined. (**B**) Platelets from untreated mice (shaded area), 30 min (black line), or 24 h (gray line) after antibody injection were incubated with FITC-labeled streptavidin for 10 min and samples were analyzed directly. (**C**) Blood was withdrawn at the indicated time points after antibody injection, platelets were prepared and lysed. Platelet proteins were separated on SDS-PAGE and immunoblotted with HRP-labeled JAQ1 (anti-GPVI), or- EDL (anti-GPIIIa), or- streptavidin (anti-JAQ1) followed by ECL. (**B**, **C**) Results shown are representative of 4 different experiments. (**D**) Blood was withdrawn at the indicated time points. Plasma was collected and transferred onto JAQ3-coated plates and ELISA was performed as described. (**A, D**) Results shown are the mean S.D. of 4 separate experiments.

To test the physiological relevance of antibody treatment, bleeding time experiments were performed. As shown in figure C.18, antibody-treated mice showed significantly increased bleeding time on day one after antibody injection. However, the bleeding time decreased gradually and reached normal values on day 5 after antibody injection. These data demonstrate that binding of JAQ1 to GPVI induces both, rapid

proteolytic cleavage of the receptor from circulating platelets *in vivo*, and its internalization into the platelets, which is associated with a transient thrombocytopenia and increased bleeding time.

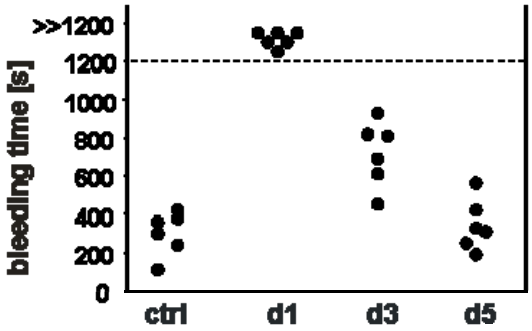

Fig. C.18 Increased bleeding time after antibody-treatment .
Wild-type mice were injected with biotinylated JAQ1 (20µg; i.v) and tail bleeding time was determined at the indicated time points after antibody injection. Experiment was stopped manually after 20 min. Each symbol represents 1 individual.

C.5.2 *Signaling downstream of GPVI is essential for antibody-induced down-regulation of the receptor*

To investigate the role of GPVI signaling in antibody-induced GPVI down-regulation (shedding/internalization), mice with defective GPVI-mediated signaling pathways were used. In these mice the FcRγ-chain was replaced by a point-mutated γ-chain, where tyrosine at position 65 and 76 in the ITAM motif was replaced by phenylalanine (FcRγ-YF) [87]. These mice express virtually normal levels of GPVI on their platelets, but do not respond to stimulation with CRP (Fig. C.19).

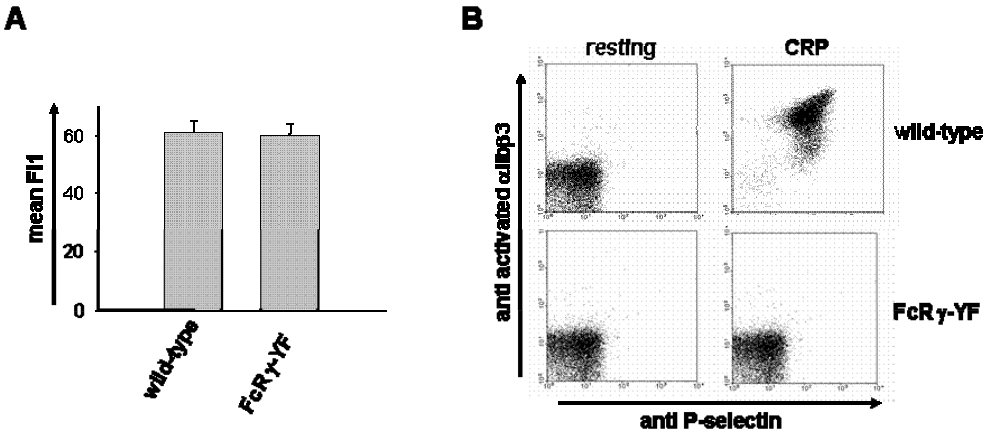

Fig. C.19 FcRγ–YF mice express normal GPVI levels, but do not respond to CRP. (**A**) Washed platelets from wild-type or FcRγ–YF mice were incubated with FITC-labeled JAQ1 for 10 min and samples were analyzed immediately on a FACScalibur. Results shown are the mean ± S.D of 4 separate experiments. (**B**) Platelets from wild-type or FcRγ-YF mice were stimulated with CRP (5 µg/mL) and analyzed for P-selectin expression and GPIIb/IIIa activation by flow cytometry. The results shown are representative of 3 independent experiments.

Wild-type and FcRγ-YF mice were injected with JAQ1-biotin (20µg; i.v) and were analyzed as mentioned above. Surprisingly, and in contrast to wild type mice, JAQ1-treated FcRγ-YF mice did not show any thrombocytopenia (Fig. C.20A), demonstrating the essential role of ITAM-mediated (GPVI signaling) in this process. Moreover, JAQ1, measured with FITC-labeled streptavidin, was detected on the surface of circulating platelets in antibody-treated FcRγ-YF mice, showing that GPVI is not down-regulated in these animals (Fig. C.20B). Consistent with this, no JAQ1/GPVI complex was detected in the plasma of antibody-treated FcRγ-YF mice, while maximal levels of this complex were detected in plasma of wild-type mice (Fig. C.20C).

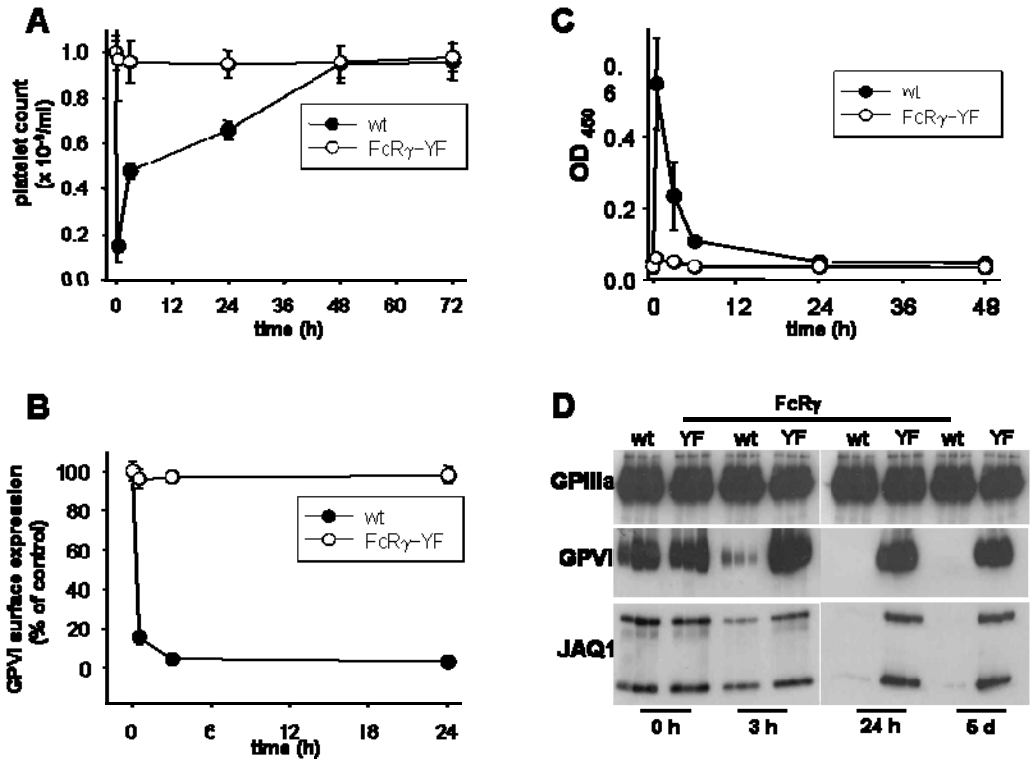

Fig. C.20 Signaling downstream of GPVI is essential for antibody-induced down-regulation of the receptor. Wild-type or FcRγ-YF mice were injected with biotinylated JAQ1 (20µg; i.v). (**A**) Blood was withdrawn at the indicated time points and platelet count was determined. (**B**) Blood was withdrawn at the indicated time points, platelets were incubated with FITC-labeled streptavidin for 10 min and samples were analyzed immediately on a FACScalibur. Results shown are the mean ± S.D of 4 individual experiments and expressed as % of untreated control mice. (**C**) Blood was withdrawn at the indicated time points. Plasma was collected and transferred onto JAQ3-coated plates and ELISA was performed. (**A**, **C**) Results shown are the mean ± S.D of 4 individual experiments. (**D**) Blood was withdrawn at the indicated time points after antibody injection, platelets prepared and lysed. Platelet proteins were separated on SDS-PAGE and immunoblotted with HRP-labeled JAQ1 (anti-GPVI), or-EDL (anti-GPIIIa), or- streptavidin (anti-JAQ1) followed by ECL. Results shown are representative of 4 different experiments.

In addition, both JAQ1 and GPVI were detected in the platelet lysate of antibody-treated FcRγ-YF mice while in wild-type mice they were detected at early time points only (Fig. C.20D). These results show that signaling downstream of GPVI is essential for antibody-induced GPVI down-regulation and thrombocytopenia.

C.5.3 *LAT-deficient mice show no thrombocytopenia and no GPVI shedding upon antibody treatment*

To dissect the mechanisms underlying antibody-induced GPVI down-regulation, mice deficient in the adaptor molecule LAT were used. Wild-type and LAT deficient mice were injected with JAQ1-biotin (20µg; i.v) and treated as above. Similar to FcRγ-YF mice, JAQ1 treatment did not induce thrombocytopenia in LAT deficient mice (Fig.C.21A)

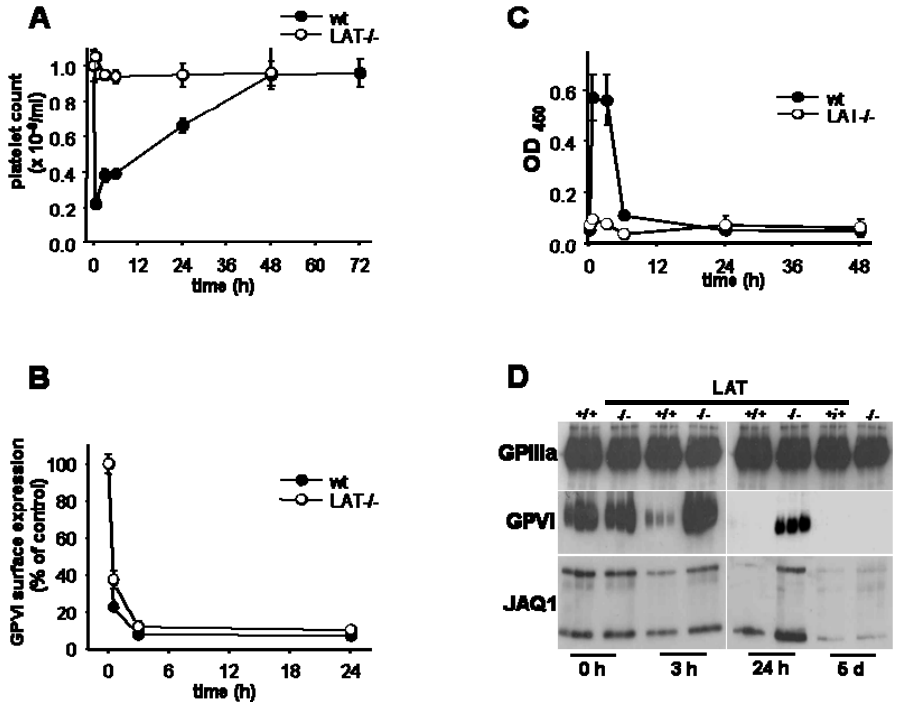

Fig. C.21 LAT-deficient mice show no thrombocytopenia and no GPVI shedding upon antibody treatment. Wild-type or LAT -/- mice were injected with biotinylated JAQ1 (20µg; i.v). (**A**) Blood was withdrawn at the indicated time points and platelet count was determined. (**B**) Blood was withdrawn at the indicated time points, platelets were incubated with FITC-labeled streptavidin for 10 min and samples were analyzed immediately on a FACScalibur. Results shown are the mean ± S.D of 4 individual experiments and expressed as percent of untreated control mice. (**C**) Blood was withdrawn at the indicated time points. Plasma was collected and transferred onto JAQ3-coated plates and ELISA was performed. (**A**, **C**) Results shown are the mean ± S.D of 4 individual experiments. (**D**) Blood was withdrawn at the indicated time points after antibody injection, platelets prepared and lysed. Platelet proteins were separated on SDS-PAGE and immunoblotted with HRP-labeled JAQ1 (anti-GPVI), or- EDL (anti-GPIIIa), or- streptavidin (anti-JAQ1) followed by ECL. Results shown are representative of 4 different experiments.

Surprisingly, JAQ1, measured with FITC-labeled streptavidin, was undetectable on the surface of circulating platelets, indicating that GPVI was down-regulated from the platelet surface (Fig C.21B). However, JAQ1/GPVI complex was not detected by ELISA in plasma of these mice, demonstrating that no proteolytic cleavage takes place in these mice (Fig. C.21C). To test whether GPVI is internalized upon JAQ1 treatment, the presence of GPVI and/or JAQ1 in the platelet lysate of antibody-treated LAT -/- mice was tested. Indeed, both GPVI and JAQ1 were detected in platelet lysate of antibody-treated mice at early, but not later time points after antibody injection (Fig. C.21D), demonstrating that LAT is essential for the shedding process, but not for the internalization process. To further confirm the absence of GPVI, platelets from antibody-treated (5 d after antibody injection) wild-type or LAT -/- mice were incubated with the strong GPVI agonist convulxin under stirring conditions and light transmission was recorded. As expected, no platelet aggregation could be induced in platelets from antibody-treated wild-type and LAT -/- mice, while platelets from these mice aggregated normally in response to ADP (Fig C.22). To test the physiological relevance of GPVI depletion in LAT -/- mice, bleeding time experiments were performed. In contrast to wild-type mice, antibody-treated LAT -/- mice did not show increased bleeding times on day one after antibody injection (Fig. C.23). Taken together, the results show that LAT is essential for antibody-induced thrombocytopenia. Therefore, LAT -/- mice are protected from the associated increase in bleeding time. Moreover, these results suggest the existence of a signaling pathway downstream of GPVI which bypasses LAT.

C.5.4 *GPVI signals in a LAT- and PLCγ2-independent manner*

Initial studies using platelets from PLCγ2 deficient mice suggested PLCγ2 to be indispensable for the signaling cascade downstream of GPVI as collagen-induced platelet aggregation was nearly abolished in platelets from these mice [50]. However, a residual aggregation response was still observed but not discussed. In a subsequent study, PLCγ1 was suggested to be responsible for this residual response, still confirming that PLCγ isoforms are crucial for the GPVI signaling cascade [88;89]. To test this hypothesis and to further investigate the mechanism of JAQ1-induced

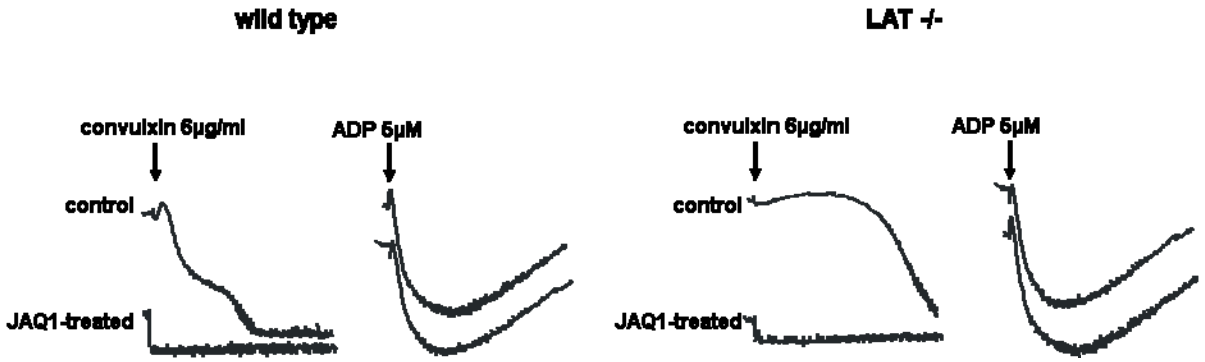

Fig. C.22 Antibody-treated LAT -/- mice do not respond to convulxin.
Wild-type or LAT -/- mice were injected with biotinylated JAQ1 (20µg; i.v). Blood was withdrawn at day 5 after antibody injection, prp was prepared and incubated with convulxin (6 µg/ml) or ADP (5 µM) under stirring conditions. Results shown are representative of 3 different experiments.

GPVI down-regulation, mice deficient in PLCγ2 were used. Wild-type and PLCγ2 deficient mice were treated as mentioned above. As expected, and similar to LAT deficient mice, JAQ1-treated PLCγ2 deficient mice did not show any thrombocytopenia (Fig. C.24A). Very unexpectedly, JAQ1 was undetectable on the surface of circulating platelets indicating the depletion of surface GPVI (Fig. C.24B). However, JAQ1/GPVI complex was not detected in plasma of PLCγ2 deficient mice, demonstrating that no ectodomain shedding takes place in these mice (Fig. C.24C).

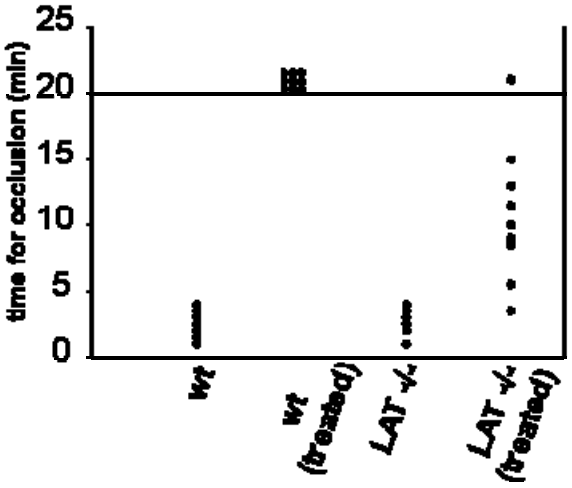

Fig. C.23 JAQ1 treatment did not induce increased bleeding time in LAT -/- mice.
Wild-type or LAT -/- mice were injected with biotinylated JAQ1 (20µg; i.v). Tail bleeding time (wt, n= 7; JAQ1-treated wt, n= 9; LAT -/-, n=6; JAQ1-treated LAT -/-, n=9) was performed. Experiment was stopped manually after 20 min. Each symbol represents 1 individual.

Similar to LAT -/- mice, both GPVI and JAQ1 were detected in platelet lysate of antibody-treated mice at early, but not later time points after antibody injection (Fig

C.24D), demonstrating that GPVI was internalized in these mice. These results suggest for the first time that there is a signaling pathway downstream of GPVI which bypasses not only LAT, but also PLCγ2. In addition, this shows that, similar to LAT, PLCγ2 is essential for the shedding process, but not for the internalization of the GPVI/FcRγ chain complex.

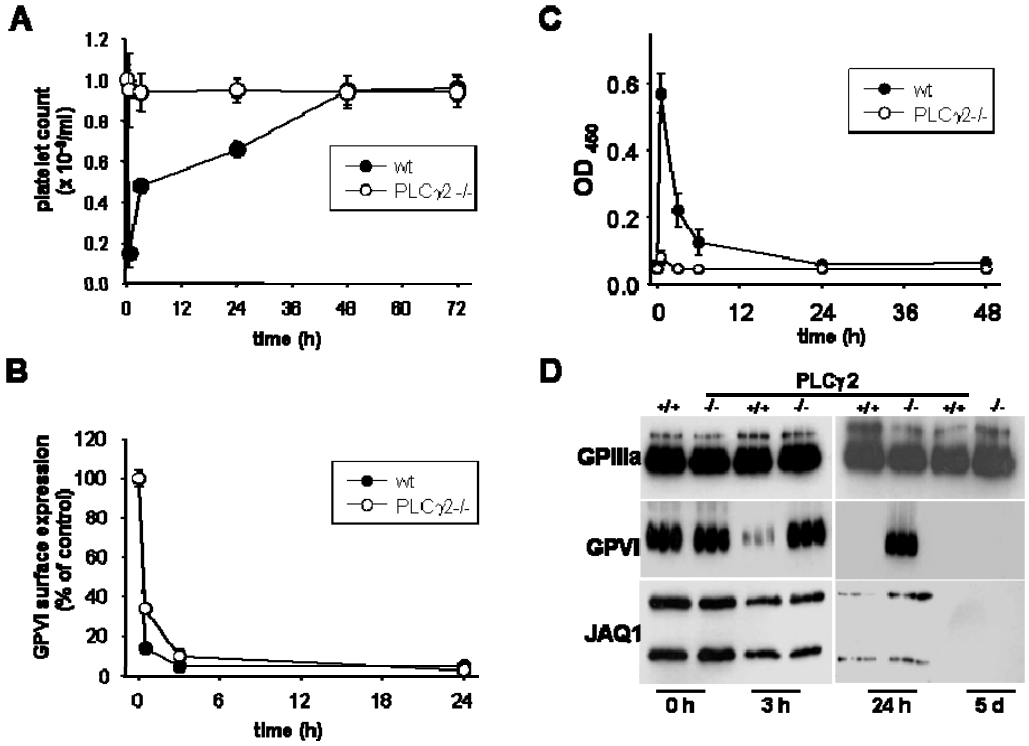

Fig. 24 GPVI signals in a LAT- and PLCγ2-independent manner. Wild-type or PLCγ2 -/- mice were injected with biotinylated JAQ1 (20µg; i.v). (**A**) Blood was withdrawn at the indicated time points and platelet count was determined. (**B**) Blood was withdrawn at the indicated time points, platelets were incubated with FITC-labeled streptavidin for 10 min and samples were analyzed immediately on a FACScalibur. Results shown are the mean ± S.D of 4 individual experiments and expressed as % of untreated control mice. (**C**) Blood was withdrawn at the indicated time points. Plasma was collected and transferred onto JAQ3-coated plates and ELISA was performed. (**A**, **C**) Results shown are the mean ± S.D of 4 individual experiments. (**D**) Blood was withdrawn at the indicated time points after antibody injection, platelets prepared and lysed. Platelet proteins were separated on SDS-PAGE and immunoblotted with HRP-labeled JAQ1 (anti-GPVI), or- EDL (anti-GPIIIa), or- streptavidin (anti-JAQ1) followed by ECL. Results shown are representative of 4 different experiments.

D. Discussion

Over the last years, efforts have been made to develop safe and effective antiplatelet agents. The main aim of antiplatelet agents has been the prevention / treatment of (arterial) thrombosis. Arterial thrombi are predominantly composed of platelets and are formed under conditions of elevated shear at site of (atherosclerotic) vascular injury and disturbed blood flow. Aspirin, the prototype of antiplatelet agents, has been in clinical use since almost half a century. It acts by irreversibly acetylating the active site of cyclooxygenase-1 (COX-1), the enzyme responsible for thromboxane A_2 (TXA_2) production [66]. TXA_2 is released by platelets and stimulates specific surface receptors, which activate PLC, release intracellular calcium, and ultimately release more TXA_2, a positive feedback loop, enhancing aggregation and recruitment of platelets to the primary plug [90]. However, aspirin treatment has been associated with strong gastrointestinal side effects [91]. Moreover, and importantly, aspirin is a weak antithrombotic agent that was very useful in the prevention, but not treatment of acute vascular events that lead to myocardial infarction [92].

Clopidogrel, another antiplatelet agent currently used clinically in patients who are sensitive or resistant to aspirin, selectively and irreversibly binds the ADP receptor $P2Y_{12}$ [93]. Similar to TXA_2, ADP is released from activated platelets, binds to specific receptors, providing a positive feedback loop, enhancing platelet aggregation. Similar to aspirin, clodipgrel has been a successful prophylactic antithrombotic agent [94;95].

Interruption of platelet aggregation, which is the final step of platelet activation, by agents that block αIIbβ3 integrin such as abciximab proved to have a significant mortality benefit in patients suffering from acute coronary diseases. However, in acute phases, abciximab was reported to cause a strong bleeding problem, limiting its usefulness as a prophylactic agent [68].

Therefore, clinically used antiplatelet agents could be classified as agents that inhibit platelet activation (aspirin, clopidogrel), or agents that block the final step of thrombus formation (e.g. abciximab).

As mentioned in A.2, the current model of platelet thrombus formation on a collagen surface suggests that the initial step of platelet interaction with the injured subendothelium is mediated by the interaction of the vWf receptor complex, GPIb-V-IX with the exposed ECM on the injured subendothelium. Subsequent to this, platelet activation takes place which is mediated by the interaction of GPVI with collagen on

the exposed subendothelium [6]. The interruption of this initial step of thrombus formation could represent a promising approach in the treatment of cardiovascular diseases. So far, there are no clinically used agents that interfere with GPIb-vWf binding. However, several animal studies have been performed using different agents that block this interaction. It should be noted that these studies evaluated the safety of such a treatment, but not its efficacy in the treatment of cardiovascular diseases. Moreover, these studies gave conflicting results concerning the effect on the primary hemostasis. Antibodies directed against vWf induced a marked increase in bleeding time in baboon [96], guinea pig [97], but not in hamster [98]. On the other hand, antibodies against GPIbα induced a marked increase in bleeding time in baboons [99] and in mice (Nieswandt B, unpublished observations). Taken together, it seems that such treatment will be associated with increased bleeding, although a direct correlation between bleeding time and bleeding risk has not yet been established [100].

GPVI, which plays an essential role in the initial step of thrombus formation, may represent a more promising target for treatment of cardiovascular diseases. Clinical evidence supporting the safety of an anti-GPVI treatment came from patients who lack the receptor, but have only a mild bleeding problem [32;33;35;101;102]. Although antibody-induced GPVI depletion induces an increase in bleeding time, this effect is only transient (Fig. C.18). Moreover, GPVI deficient mice display no major bleeding phenotype [36]. However, as mentioned above, a direct correlation between bleeding time and bleeding risk has not yet been established [100]. Taken together, it seems that the absence of GPVI does not represent a major clinical risk, making GPVI a good target for prophylactic agents.

Evidence supporting the idea that anti-GPVI agents could have an antithrombotic effect came from an *in vivo* study by Massberg and coworkers. In that study, injury of the carotid artery was induced and thrombus formation on the injured vessel wall was monitored using intravital fluorescence microscopy. In mice depleted of GPVI, no thrombus formation took place, while occlusive thrombi were formed in wild-type mice. The authors concluded that GPVI is essential for the process of initial attachment of platelets to the injured subendothelium and thus in thrombus formation [71]. Moreover, as discussed below, GPVI plays an essential role in thrombus formation induced by human atherosclerotic plaque material. Taken together, these

experimental data suggest that GPVI might be a suitable / promising target for therapeutic agents.

D.1 *Human atherosclerotic plaques stimulate thrombus formation by activating platelet glycoprotein VI*

Abrupt rupture of atherosclerotic plaques frequently leads to lethal ischemic cardiovascular or cerebrovascular events such as myocardial infarction or stroke. Therefore, it is essential to understand the mechanisms underlying the process of atherogenesis, the nature of the atherosclerotic plaques and the interaction between atherosclerotic plaques and blood vessels / platelets. The generally held view suggests tissue factor (TF) in the plaque material as the main triggering factor in the process of thrombus formation after plaque rupture [64;65;103;104].This implies that, upon plaque rupture, thrombin is generated by the coagulation system, which then activates platelets. In this scenario, inhibition of coagulation would be sufficient to prevent thrombus growth after plaque rupture. In the current study it was shown that plaque material induces thrombus formation in anticoagulated blood under flow conditions demonstrating that TF is not essential for this process. It has to be noted that the atherosclerotic plaque material is heterogeneous, containing various adhesive molecules. The aim of this study was to evaluate the role of collagen, as a component of the atherosclerotic plaque material, in the thrombogenicity of ruptured plaques. Collagen was found to be an essential thrombogenic component of that material. Several lines of evidence supported that finding; under static conditions, using human platelets, adhesion to the plaque material was blocked in the presence of antibodies against the collagen receptors GPVI and $\alpha 2\beta 1$ integrin. Furthermore, in aggregometry assays, when human prp was incubated with the plaque material, aggregation was induced, which was abolished in presence of antibodies against GPVI, but not GPIbα. As the used anti-GPIbα antibody blocks binding of GPIb to its ligand, vWf [105], this result indicates that vWf present on the surface of the plaque material is not responsible for the observed aggregation response. The role of GPIbα has been appreciated in the initial tethering of platelets to the vessel wall *in vivo* allowing GPVI to bind collagen and activate platelets [6]. According to that model, the role of GPIbα is to facilitate the access of GPVI to collagen and not to activate the platelets. Therefore, a role for GPIb-vWf binding in aggregometry would not be

expected. In contrast, incubation of prp with RGDS, which blocks αIIbβ3-fibrinogen binding, abolished platelet aggregation, but not shape change. This is to be expected as this binding is thought to be crucial for platelet aggregation [22], regardless of the stimulus. The presence of shape change in this case indicates that platelet activation is still possible, showing that the thrombogenic component on the surface of the plaque material induces platelet activation. Taken together, the above results provide evidence that collagen is the main thrombogenic component in the human atherosclerotic plaque material. This was further supported by studies that more resemble physiological conditions. When heparinized human blood was allowed to flow over cover slips coated with the plaque material, thrombus formation was induced, which was inhibited in the presence of a blocking α2 integrin antibody and was abolished in the presence of an anti-GPVI antibody. This is consistent with the current model of platelet adhesion to the subendothelium where GPVI is the central receptor in this process [6]. Significantly, when these experiments were performed using mouse blood, similar results were obtained. Incubation of mouse prp with the plaque material induced aggregation, which was abolished in the presence of JAQ1. Consistent with this, when prp from GPVI-depleted mice or mice deficient of FcRγ-chain, which also lack GPVI [41], was used, no aggregation took place (Fig. C.1). Consistent results were obtained in flow chamber experiments; when heparinized blood from wild-type mice was allowed to flow over cover slips coated with the plaque material, thrombi were formed. This was abolished when blood was incubated with JAQ1, when depletion of GPVI was induced, or when blood from FcRγ-chain deficient mice was used (Fig. C.2). These results demonstrate that GPVI plays an important role in thrombus formation induced by the human atherosclerotic plaque material.

D.2 *GPVI down-regulation in murine platelets through metalloproteinase-dependent shedding*

Although the therapeutic potential of GPVI as a drug target has been now appreciated, very little is known about its cellular regulation. In order to develop anti-GPVI agents, a thorough understanding of its cellular regulation is essential. Among the mechanisms of cellular regulation of membrane receptors, protein ectodomain shedding is emerging as an important post-translational mechanism for regulating the function of membrane-anchored proteins.

Shedding involves the proteolytic processing of membrane-tethered proteins leading to the release of their extracellular- or ectodomain [106-109]. About 2-4% of the proteins on the cell surface are released by metalloproteinases in response to phorbol ester stimulation [110]. These molecules comprise a variety of structurally and functionally distinct proteins, including epidermal growth factor ligands such as TGFα [106], tumor necrosis factor (TNF) family members, and other cytokines, receptors such as p55 and p75 TNF receptors and the interleukin-6 receptor. Ectodomain shedding seems to be essential for processes such as signaling via epidermal growth factor receptor ligands [62;111-115], and limiting TNFR-mediated inflammatory reactions [116]. Even if the functional consequences of ectodomain release remain to be evaluated for most shed proteins, it seems likely that ectodomain shedding will affect the function of the majority of its substrate proteins.

The results presented here (C.2) provide evidence that GPVI is a novel substrate for metalloproteinases. The cleavage site for this platelet-derived protease is located on the extracellular domain of GPVI. Cleavage occurs near the surface membrane and leads to the release of a ~55 kDa fragment of the receptor. Utilizing the broad range metalloproteinase inhibitor GM6001, a key role for endogenous metalloproteinase(s) in the proteolysis of GPVI was identified.

To induce metalloproteinase activity, a previously reported model of mitochondrial injury was used [13]. In this model, uncoupling of the oxidative phosphorylation by the lipid-soluble amphiphatic molecule CCCP was shown to induce GPIbα shedding by a metalloproteinase-dependent mechanism in both mouse and human platelets. Moreover, this study demonstrated that mitochondrial injury induces alterations in platelets that are similar to those observed in human and mouse platelets aged *in vitro*, including shape change and disruption of membrane asymmetry [13;117]. It is therefore feasible to speculate that metalloproteinase-dependent shedding of GPVI occurs in mouse and human platelets under more physiological conditions, thereby having a strong effect on the hemostatic function of these cells. Although it was difficult to adequately address this question in the current study, there is support for this hypothesis by a recent report showing that both the collagen response and GPVI function in canine platelets decline progressively with age *in vivo* [118]. However, platelet activation by the GPVI-specific agonist CRP (Fig. C.3) did not result in the proteolysis of the receptor. Furthermore, no significant down-regulation of GPVI was detectable on platelets activated through various agonists of G-protein-coupled

receptors such as thrombin (Fig. C.3), ADP, U46619, or adrenaline (not shown). Interestingly, subsequent to this study, Gardiner and coworkers showed that activation of human platelets by collagen, CRP, or the specific GPVI agonist convulxin induced GPVI down-regulation [84], suggesting that different mechanisms of GPVI down-regulation exist for mouse and human platelets. As mentioned before, the intracellular tail of human GPVI contains 24 amino acids more than the mouse counterpart [38]. This could have an impact on cytoplasmic binding partners of GPVI, which might explain the difference between the two species. In agreement with the current study, thrombin activation did not induce GPVI down-regulation, suggesting that, in human platelets, signaling downstream of GPVI, but not through G-protein coupled receptors is essential for the observed GPVI down-regulation. This is supported by the finding that inhibiting GPVI signaling, by inhibiting Src and Syk kinases, which lie downstream of GPVI, inhibited agonist-induced GPVI down-regulation [84]. Preliminary experiments showed that inhibiting the same signaling molecules did not influence CCCP-induced GPVI down-regulation (not shown), suggesting that signaling downstream of GPVI is not involved in this process which is consistent with the fact that CRP did not induce GPVI down-regulation in mouse platelets (Fig. C.3). Another discrepancy between the study of Gardiner and coworkers and the current study is the effect of calmodulin inhibition on GPVI expression level. It was previously shown that calmodulin binds to the intracellular domain of human [119], and mouse [120] GPVI and that platelet activation with collagen, CRP [119], or convulxin [120] disrupts this binding. Gardiner and coworkers found that disrupting the binding of calmodulin to GPVI by incubation of platelets with a calmodulin inhibitor, induces GPVI down-regulation in human platelets. However, preliminary experiments performed during the course of this study using mouse and human platelets did not support this finding (not shown). This discrepancy is difficult to interpret. Very recently, Stephens and coworkers demonstrated that activation of human platelets with collagen and convulxin induces GPVI down-regulation [121], supporting the results of Gardiner and coworkers discussed above. However, in contrast to the latter results and to the results shown in the current study (Fig. C.3), Stephens and coworkers show that platelet activation by thrombin induces down-regulation of GPVI. Again, this discrepancy is difficult to explain. Stephens and coworkers also found that, *in vitro*, incubation of human platelets with an anti-GPVI antibody induces GPVI down-regulation. This result is in contrast to the results

presented in the current study showing that *in vitro* incubation of mouse platelets with JAQ1 did not induce GPVI down-regulation (Fig. C15C). However, the antibody used by Stephens and coworkers induces platelets activation [122], while JAQ1-3 used in this study do not. As activation of human platelets via GPVI signaling induces GPVI down-regulation [84;121], and such activation does not induce GPVI down-regulation in mouse platelets (Fig. C3), this could explain the discrepancy between the two studies.

Similar to the regulation of human and mouse GPVI, different mechanisms seem to govern the regulation of GPIbα and GPVI as platelet activation induced GPIbα, but not GPVI down-regulation (Fig. C.3). Further studies will be required to determine the identity of the metalloproteinase(s) and to elucidate whether metalloproteinase activity is directly or indirectly responsible for the cleavage of the receptor. It was reported recently that GPIbα is cleaved *in vitro* and *in vivo* by ADAM17 [123]. As different mechanisms exist for the regulation of GPIbα and GPVI, it is unlikely that ADAM17 is the responsible metalloproteinase for GPVI cleavage, but this needs to be investigated in future studies. Similar to the identity of the responsible metalloproteinase, the mechanism of its activation remains elusive. It appears likely, however, that metalloproteinase activity is an indirect consequence of mitochondrial injury, possibly through mechanisms similar to those induced during apoptosis [13] [124] but this needs to be addressed in future experimentation. A tight regulation of the surface expression of GPVI may be important in different physiological and pathological situations, as GPVI-mediated activation of human [125;126] and mouse platelets [127] showed a strong dependency on the receptor-density on the cell surface.

As mentioned above, infusion of anti GPVI-mAbs induces GPVI-deficiency in mice [45] ; C.3. The data presented here gave the first indication that proteolysis may be the responsible mechanism for antibody-induced depletion of GPVI in humans and mice (discussed in D.4).

D.3 *Targeting of the collagen binding site on glycoprotein VI is not essential for in vivo depletion of the receptor*

In the above mentioned study it was shown that GPVI contains a cleavage site for an endogenous metalloproteinase. It was then essential to study the *in vivo* relevance of

this finding. In a previous study it was reported that injection of mice with the anti-GPVI antibody, JAQ1, induces down-regulation of the receptor [45]. JAQ1 binds to the major ligand binding site on mouse GPVI and largely blocks its function [46]. As mentioned in A.2.3, Sugiyama and coworkers reported a patient that lacks GPVI. In plasma of that patient anti-GPVI antibodies were isolated that were found to block collagen-induced platelet activation [31]. Therefore, it was tempting to speculate that that patient suffered from an acquired antibody-induced GPVI deficiency and that targeting of the collagen binding site on GPVI is essential for this process. To test this hypothesis, new anti-GPVI antibodies were generated. All three antibodies (JAQ1-3) bind to different epitopes as they did not block each others binding in flow cytometric preincubation studies. This was an essential prerequisite for this study. As JAQ2, 3 do not bind to the same epitope on GPVI as JAQ1, it was expected that they do not inhibit CRP- or collagen-induced platelet aggregation. Indeed, this was observed in this study (Fig. C14D). Similar to JAQ1, JAQ2, 3 induced the down-regulation of GPVI *in vivo* when injected into mice (Fig. C15A). This effect demonstrated that anti-GPVI agents that do not directly activate the receptor can induce its down-regulation. A significant clinical relevance of this finding came from a patient lacking GPVI who developed anti-GPVI antibodies that did not block collagen–induced platelet activation (P. Newman, personal communications), showing that similar mechanisms exist in humans. The finding that F(ab) fragments of anti-GPVI antibodies also induce GPVI down-regulation shows that neither receptor dimerization, nor the Fc part of the antibody is essential for the down-regulation process. This is particularly important in clinical settings as F(ab) fragments are less immunogenic than the intact IgG. Immunogenicity of antibodies used clinically is a major limitation as they induce an immunresponse which results in their clearance from the circulation. Using F(ab) fragments of such antibodies could overcome this problem, though. In case of anti-GPVI antibodies, however, it seems that their affinity / avidity is essential for the efficacy of the down-regulation process as the duration of GPVI depletion induced by the F(ab) fragments of JAQ1-3 was significantly shorter than that induced by the intact IgG (Fig. C15B). These findings showed for the first time that it is possible to down-regulate a receptor irreversibly by antibodies or, possibly, small molecules regardless of their binding site on the receptor.

D.4 *Diverging signaling events control the pathway of GPVI down-regulation in vivo*

The above discussed experiments showed that antibody-induced GPVI down-regulation *in vivo* is not depending on the exact binding site on the receptor. However, the mechanism underlying this process was unclear. From previous results it was known that JAQ1 injection induces GPVI internalization followed by intracellular degradation [45]. However, as mentioned under C.2, GPVI contains a cleavage site for an endogenous metalloproteinase. In the light of these results it was intriguing to speculate that antibody treatment induces GPVI shedding as well as internalization. Indeed, this was found to be the case (Fig. C.17). Until these experiments had been performed it was not described that a soluble form of GPVI exists. However, very recently, soluble GPVI was found to be present in the plasma of healthy individuals [35], showing that GPVI shedding takes place in humans under physiological conditions as well. The *in vivo* triggering factors for this shedding are still elusive. The mitochondrial injury model described above, which was used to show that GPVI shedding takes place *in vitro* (C.2), could resemble *in vivo* conditions where apoptosis-like processes take place, as CCCP treatment was shown to induce apoptosis in the human promyelocytic cell line HL-60 [128]. Therefore, it could be argued that GPVI cleavage is a consequence of platelet aging. However, this hypothesis does not explain the physiological significance of the shedding process. Alternatively, GPVI shedding could be a marker for platelet aging, triggering platelet removal from the circulation. This is supported by a work by Bergmeier and coworkers showing that inhibition of GPIbα cleavage *in vitro* improves the posttransfusion platelet survival suggesting that GPIbα cleavage induces platelet clearance *in vivo* [13]. This suggests that receptor cleavage in general may act as a signal for platelet clearance. Similar to the conditions governing the cleavage process, the role of the cleaved, soluble GPVI under physiological / pathological conditions is unclear. It could be speculated that soluble GPVI plays a protective role against thrombosis, by competitively binding collagen present on the exposed subendothelium. However, this seems to be unlikely as it was shown by Miura and coworkers that only the dimeric form of GPVI, but not the monomeric form is able to bind to fibrous collagen [129]. The hypothesis that soluble GPVI has no antithrombotic effect was further supported by a very recent study by Grüner and coworker. In that study the authors expressed the soluble dimeric human GPVI, comprising the extracellular domain of the receptor fused to the human

immunoglobulin Fc domain (GPVI-Fc), and tested its antithrombotic potential. Using intravital fluorescence microscopy and ultrasonic flow measurements they observed that GPVI-Fc had no effect on platelet adhesion and thrombus formation at sites of injured arterial wall. They found similar results with a fusion protein comprising the extracellular domain of mouse GPVI and human IgG-Fc. The authors then concluded that even the dimeric form of soluble GPVI has no antithrombotic effect [130]. In contrast to that study, Massberg and coworker found in a similar experimental approach that the dimeric form of GPVI has an antithrombotic effect [131]. This discrepancy may be related to slightly different experimental conditions.

GPVI belongs to the immunoglobulin superfamily and signals in a way similar to immunoglobulin receptors (e.g. B-cell receptor or T-cell receptor). According to this, phosphorylation of the ITAM motif of the receptor-associated γ chain is central to the signaling cascade. This is followed by the recruitment and activation of the tyrosine kinase Syk. The signaling cascade culminates in the activation of phosphatidylinositol 3-kinase (PI3-K) and, finally, PLCγ2. Between ITAM phosphorylation and PLCγ2 activation lie several signaling and adaptor molecules, which are either important or indispensable for the signaling process, including, Syk, SLP-76, and LAT. The absence of Syk leads to a complete loss of phosphorylation of SLP-76, LAT and PLCγ2, whereas deletion of SLP-76 leads only to a loss of phosphorylation of PLCγ2 [52;132-134]. In LAT-deficient mice, certain level of phosphorylation of both SLP-76 and PLCγ2 in CRP-activated platelets remains, suggesting a LAT-independent activation pathway for PLCγ2 [52]. In the current study, subsequent to the observation that JAQ1 induces GPVI cleavage *in vivo* (Fig. C.17), a mouse genetically engineered to have a point mutation in the ITAM motif of the FcRγ-chain (FcRγ-YF) and thus impaired GPVI signaling, was used to test the role of signaling downstream of GPVI in JAQ1-induced GPVI down-regulation (shedding / internalization). In these mice, GPVI levels on the surface of platelets remained unaltered upon JAQ1 injection, demonstrating that signaling via the ITAM motif of the FcRγ-chain is essential for the down-regulation process. In addition, no thrombocytopenia was observed after antibody injection (Fig. C.20). A possible role for the adaptor molecule LAT was then investigated by using LAT deficient mice. LAT was found to be essential for JAQ1-induced thrombocytopenia, GPVI shedding, but

not GPVI internalization (Fig. C.21). Moreover, deficiency of LAT protected the mice from JAQ1-induced transient increase in tail bleeding time, showing that it is possible to uncouple the down-regulation process from the associated side effects (Fig. C.23). A role for PLC γ2 in JAQ1-induced GPVI down-regulation was finally tested and, similar to LAT, PLC γ2 was found to be essential for antibody-induced thrombocytopenia, GPVI shedding, but not GPVI internalization (Fig. C.24). Consistent with the above model, the results presented here (Fig C.20) show the essential role of the ITAM motif of the FcRγ chain in the signaling process downstream of GPVI, as no GPVI down-regulation took place in FcRγ-YF mice. According to the current model, PLCγ2 lies downstream of LAT in the signaling cascade downstream of GPVI [50]. However, it seems that there is a LAT-independent pathway downstream of GPVI leading to PLCγ2 activation, as it was reported that weak PLCγ2 phosphorylation takes place in platelets from LAT deficient mice in response to CRP activation [52]. A subsequent study [46] and the results shown in Fig. C.21 further support the idea that there is a signaling pathway downstream of GPVI which bypasses LAT. Very surprisingly, however, the fact that GPVI down-regulation took place in PLCγ2 -/- mice (Fig. C.24) shows that GPVI signaling in these mice is, at least in part, still functional. Therefore, a novel pathway for signaling downstream of GPVI is suggested which bypasses PLCγ2. This is the first time where such a pathway is described. However, signaling molecules involved in this pathway are still unknown. Preliminary experiments showed that Rac-1 might lie in that pathway. Rac-1 belongs to the family of the small GTPases. During the course of this study, experiments using platelets from Rac-1 deficient mice were used. These platelets were found to have a severe defect in GPVI-induced platelet activation as CRP failed to induce aggregation of these platelets (A. Strehl, Würzburg, unpublished observations) showing that Rac-1 lies in the signaling pathway downstream of GPVI. This is consistent with a previous study showing that, in human plartelets, Rac-1 plays a role in collagen induced platelet activation [135]. However, CRP-induced tyrosine phosphorylation profile in platelets from wild-type mice was similar to that observed in platelets from Rac-1 deficient mice, showing that Rac-1 does not lie in the classical GPVI signaling pathway. It is unlikely, however, that PLCγ1 is involved in this pathway as no GPVI shedding took place in platelets from PLCγ2 deficient mice, meaning that PLCγ2 function is not compensated.

Although GPVI is a promising antithrombotic target, the reported strong drop in platelet count at the early phase of treatment and the associated increase in bleeding time represent a limitation for anti-GPVI based therapeutics. Therefore, the finding that the process of GPVI down-regulation could be uncoupled from the associated thrombocytopenia and increase in bleeding time is very important. Although the mechanism of this transient thrombocytopenia is unclear, it could be argued that antibody treatment induces weak platelet activation which leads to their transient sequestration from the circulation. The absence of thrombocytopenia in all genetically modified mice tested, which all have a defect in GPVI signaling cascade supports this hypothesis. However, this finding was somehow surprising as it was thought that opsonizing platelets with antibodies generally induces their removal from the circulation [136]. While impairment of the “classical” GPVI signaling pathway abolishes only the shedding process, GPVI internalization takes place normally and with comparable kinetics to control. The physiological relevance of this finding is manifested by the fact that antibody-treated LAT -/- mice are protected from the increased bleeding time observed in antibody-treated wild-type mice on day one after antibody injection (Fig. C.23).

In summary, the above results show that GPVI signals through two different pathways: the “classical” pathway, where LAT and PLCγ 2 are involved; this pathway is required for antibody-induced thrombocytopenia, for metalloproteinase activation and GPVI shedding, and another “novel” pathway, which bypasses LAT and PLCγ2 and is involved in antibody-induced GPVI internalization. While it is still unclear how the responsible metalloproteinase(s) is activated, it appears that Ca^{2+} mobilization is essential for this process as LAT and PLCγ2 are required for the shedding process. Further experiments are essential to understand the mechanisms of the internalization/shedding as well as to identify the responsible metalloproteinase(s).

D.5 *Evidence for a Role of ADAM17 (TACE) in the Regulation of Platelet Glycoprotein V*

ADAM 17 is a well-known sheddase that cleaves a variety of receptors, including L-selectin, VCAM-1 [62;137;138] and, as shown very recently, platelet GPIbα [123]. Similar to the regulation of these molecules, the release of GPV from the platelet surface can be stimulated by the phorbol ester PMA through activation of ADAM17, a

pathway that appears to be conserved across multiple cell types. This is also supported by the observation that calmodulin plays a central role in the regulation of the shedding of GPV and L-selectin. Calmodulin is known to bind to the endodomain of both receptors, and prevention of such interaction by calmodulin inhibitors stimulates ectodomain shedding (Fig. C.12 and [139;140]). Besides its interaction with receptors, calmodulin also acts as a cellular intermediate of multiple Ca^{2+} actions and to interfere with the PKC pathway [107;141]. Although PKC itself is a potent activator of ADAM17, PKC-independent pathways of ADAM17 activation have been reported [139;142]. Similar to the mechanism of TACE activation, its substrate specificity is unclear as the sequences cleaved by the enzyme in different proteins are highly variable, suggesting that it recognizes more complex motifs and/or sequences distal to the cleavage site. It remains to be elucidated how ADAM17 becomes activated and what exactly determines the substrate specificity of the enzyme [143].

In conclusion, the results in this study show that the endogenous metalloproteinase ADAM17 is the major sheddase for GPV on the platelet surface. The shedding process can be regulated via calmodulin inhibition, and is independent of outside-in signaling through the GPIIb/IIIa complex. The physiological relevance of this shedding process needs to be further elucidated regarding both, the impact of GPV shedding on the platelet itself and the biological properties of the GPV fragment released from the membrane.

D.6 *Concluding remarks*

The work presented here shows studies on the regulation of the major collagen receptor on the platelet surface *in vitro* and *in vivo*. For this, different genetically modified animals were used for *in vivo* studies. Moreover, an assay system for measuring soluble GPVI in plasma was developed. The major findings are:

- GPVI plays an important role in thrombus formation induced by human atherosclerotic plaque material.
- GPVI contains a cleavage site for an endogenous metalloproteinase.
- GPVI shedding takes place *in vivo*.

- Two distinct mechanisms govern the regulation of GPVI *in vivo*; ectodomain shedding and receptor internalization followed by intracellular degradation.
- A novel signaling pathway downstream of GPVI is described, where both LAT and PLCγ2 are not required.
- It is possible to uncouple GPVI down-regulation from the associated thrombocytopenia and the increase in bleeding time.
- GPV is cleaved *in vitro* in an ADAM17-dependent manner.

D.6.1 *Future work*

Future experiments are required to identify the responsible metalloproteinase as well as the mechanisms of both, shedding and internalization. One of the candidate metalloproteinases is ADAM17. Therefore, experiments using ADAM17 $^{\Delta Zn/\Delta Zn}$ mice will be needed. As these mice die between E17.5 and birth, it is planned to create chimeric mice, where wild-type mice will be lethally irradiated to destroy their bone marrow, which will then be replaced by fetal liver cells derived from ADAM17 $^{\Delta Zn/\Delta Zn}$ mouse embryos (E17.5). This will give rise to mice that carry the mutation on their hematopoietic cells only.

As platelets from Rac-1 deficient mice have a defect in GPVI signaling, it is tempting to speculate that Rac-1 lies in the LAT- and PLC γ2 –independent GPVI signaling pathway. To test this hypothesis, Rac-1 deficient mice will be injected with biotinylated JAQ1 and GPVI levels on the platelet surface and in the plasma will be measured. If GPVI down-regulation and shedding took place, it would be interesting to test whether GPVI internalization also occurs. To better asses this and to prevent antibody-induced GPVI shedding, Rac-1 -/- mice are currently being crossed with LAT -/- mice to obtain Rac-1 / LAT double knock out mice. These mice will be a useful tool to study signaling down-stream of GPVI.

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Appendix

Abbreviations

ADAM	a disintegrin and metalloproteinase protein	IP	immunoprecipitation
ADP	adenosine diphosphate	ITAM	immunoreceptor tyrosine-based activation motif
BSA	bovine serum albumin	LAT	linker for activation for T cell
CCCP	Carbonyl cyanide m-chloro phenylhydrazon	mAb	monoclonal antibody
CRP	collagen related peptide	MMP	matrix metalloproteinase
cvx	convulxin	NP-40	Nonidet P-40
DMSO	dimethylsulfoxide	PAGE	polyacrylamide gel electrophorasis
ECL	enhanced chemiluminiscence	PBS	phosphate buffered saline
ECM	extracellular matrix	PLCγ2	phospholipase γ2
EDTA	ethylenediaminetetraacetic acid	PMA	phorbol 12-myristate acetate
ELISA	enzyme linked immunosorbent assay	prp	platelet rich plasma
FcR	Fc receptor	RT	room temperature
FCS	fetal calf serum	SDS	sodium dodecyl sulfate
FITC	fluorescein isothiocyanate	TACE	TNFα converting enzyme
FSC/SSC	forward scatter / side scatter	TBS	Tris buffered saline
GP	glycoprotein	TMB	3,3,5,5-tetramethyl benzidine
GPO	glycine-proline-hydroxyproline	TNFα	tumor necrosis factor alpha
HRP	horseradish peroxidase	TxA_2	thromboxane A_2
Ig	immunoglobulin	vWf	von Willebrand factor

Curriculum vitae

NAME	Tamer Rabie
Date of Birth	15/03/1975
Nationality	Egyptian

School education

1981-1993	primary, secondary and high school, Cairo
1993	Egyptian high school diploma

University education

1993-1998	Faculty of Pharmacy, University of Cairo
1998	BSc in Pharmacy and Pharmaceutical Sciences

Postgraduate education

1999-2000	MSc in Molecular Genetics, University of Leicester, England
2000-2002	PhD student at GBF, University of Braunschweig,
2002-now	PhD student at Rudolf-Virchow Center, Univesity of Würzburg

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Publications

Articles

Schulte V, **Rabie T**, Prostredna M, Aktas B, Gruner S, Nieswandt B. “Targeting of the collagen-binding site on glycoprotein VI is not essential for the *in vivo* depletion of the receptor” *Blood* 2003;101(10):3948-52

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Rabie T, Schulte V, Lanza F, Watson S, Saito T, Nieswandt B “Diverging Signaling Events Control The Pathway Of GPVI Down-Regulation *in vivo*” *manuscript in preparation*

Oral presentations

Rabie T, Schulte V, Lanza F, Watson S, Saito T, Nieswandt B “Diverging Signaling Events Control The Pathway Of GPVI Down-Regulation *in vivo*” The International Society on Thrombosis and haemostasis XXth Congress, August 2005, Sydney, Australia

Bergmeier W*, **Rabie T***, Strehl A, Piffath CL, Prostredna M, Wagner DD, Nieswandt B. **"**GPVI down-regulation in murine platelets through metalloproteinase-dependent shedding." XVIIIth European Platelet Meeting, October 2003, Giessen, Germany

Poster

Rabie T, Schulte V, Lanza F, Watson S, Saito T, Nieswandt B "Diverging Signaling Events Control The Pathway Of GPVI Down-Regulation *in vivo*" XIXth European Platelet Meeting, October 2004, Bad Brückenau, Germany

Erklärung gemäß §4 Abs. 3 der Promotionsordnung:

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Arbeit selbständig angefertigt und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe.

Diese Dissertation hat weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen.

Ich habe bisher außer den mit dem Zulassungsbesuch urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Tamer Rabie Würzburg, im Mai 2005