

Glycoprotein VI but not $\alpha 2\beta 1$ integrin is essential for platelet interaction with collagen

Bernhard Nieswandt¹, Cord Brakebusch², Wolfgang Bergmeier, Valerie Schulte, Daniel Bouvard², Rabée Mokhtari-Nejad, Theo Lindhout³, Johan W.M. Heemskerk³, Hubert Zirngibl and Reinhard Fässler^{1,2}

Department of Molecular Oncology, General Surgery, Witten/Herdecke University, 42117 Wuppertal, Germany, ²Department of Experimental Pathology, Lund University, 221 85 Lund, Sweden and ³Department of Biochemistry, Maastricht University, Maastricht, The Netherlands

¹Corresponding authors

e-mail: nieswand@klinikum-wuppertal.de or reinhard.fassler@pat.lu.se

C.Brakebusch and W.Bergmeier contributed equally to this work

Platelet adhesion on and activation by components of the extracellular matrix are crucial to arrest post-traumatic bleeding, but can also harm tissue by occluding diseased vessels. Integrin $\alpha 2\beta 1$ is thought to be essential for platelet adhesion to subendothelial collagens, facilitating subsequent interactions with the activating platelet collagen receptor, glycoprotein VI (GPVI). Here we show that Cre/loxP-mediated loss of $\beta 1$ integrin on platelets has no significant effect on the bleeding time in mice. Aggregation of $\beta 1$ -null platelets to native fibrillar collagen is delayed, but not reduced, whereas aggregation to enzymatically digested soluble collagen is abolished. Furthermore, $\beta 1$ -null platelets adhere to fibrillar, but not soluble collagen under static as well as low (150 s^{-1}) and high (1000 s^{-1}) shear flow conditions, probably through binding of $\alpha \text{IIb}\beta 3$ to von Willebrand factor. On the other hand, we show that platelets lacking GPVI can not activate integrins and consequently fail to adhere to and aggregate on fibrillar as well as soluble collagen. These data show that GPVI plays the central role in platelet–collagen interactions by activating different adhesive receptors, including $\alpha 2\beta 1$ integrin, which strengthens adhesion without being essential.

Keywords: collagen/Cre/loxP/GPVI/ $\alpha 2\beta 1$ integrin/platelets

Introduction

Damage to the integrity of the vessel wall results in exposure of the subendothelial extracellular matrix (ECM), which triggers adhesion and aggregation of platelets (Weiss, 1975). The consequence of this process is the formation of a thrombus, which prevents blood loss at sites of injury or leads to occlusion and irreversible tissue damage or infarction in diseased vessels. Integrins play a central role in adhesion and aggregation of platelets (Phillips *et al.*, 1991; Shattil *et al.*, 1998). Integrins are heterodimeric transmembrane receptors composed of an α

and a β subunit. Resting platelets express their integrins in a low affinity state. After activation, mediated by other platelet receptors, integrins shift to a high affinity state and bind their ligands efficiently (Phillips *et al.*, 1991; Shattil *et al.*, 1998).

The ECM of the vessel walls is rich in collagens, which play essential roles in thrombus formation by providing a substrate for platelet adhesion and by activating platelets (Baumgartner, 1977). Besides GPIb-V-IX and $\alpha \text{IIb}\beta 3$ integrin, which interact indirectly with collagen via von Willebrand factor (vWF) (Savage *et al.*, 1998), a large number of collagen receptors have been identified on platelets, including most importantly $\alpha 2\beta 1$ integrin (Santoro, 1986) and glycoprotein VI (GPVI) (Moroi *et al.*, 1989). A current model of platelet adhesion to collagen suggests that the GPIb–vWF interaction mediates initial tethering of platelets at high shear, followed by $\alpha 2\beta 1$ integrin-mediated firm adhesion, which halts platelet translocation and allows collagen interactions with GPVI, finally resulting in platelet activation and thrombus growth (Sixma *et al.*, 1997; Barnes *et al.*, 1998; Santoro, 1999).

The first indication that $\alpha 2\beta 1$ integrin may be an essential platelet collagen receptor came from patients with excessive post-traumatic bleeding and menorrhagia. The platelets of these patients expressed markedly reduced levels of $\alpha 2$ integrin and displayed profound defects in collagen-induced adhesion/aggregation (Nieuwenhuis *et al.*, 1985; Kehrel *et al.*, 1988). Subsequent *in vitro* studies, however, gave rise to conflicting data on the role of $\alpha 2\beta 1$ in platelet–collagen interactions. Some studies showed that inhibition of $\alpha 2\beta 1$ markedly reduced or abolished adhesion and aggregate formation in stasis and flow (Saelman *et al.*, 1994; Kamiguti *et al.*, 1997; Verkleij *et al.*, 1998), while others reported only minor effects of such treatment on adhesion to collagen (Morton *et al.*, 1994; Savage *et al.*, 1999; Siljander and Lassila, 1999) and collagen-induced aggregation (Coller *et al.*, 1989; Hers *et al.*, 2000), suggesting the involvement of other receptors. These conflicting results may at least partly be explained by the use of different collagen preparations. *In vivo*, collagen molecules are secreted by cells, processed and finally assembled into long, insoluble fibrils. However, collagens can be solubilized, e.g. by pepsin treatment, and such soluble collagen preparations are frequently used for *in vitro* analysis of platelets. Experimental evidence suggests that the interaction of platelets with soluble collagen is exclusively mediated by $\alpha 2\beta 1$ integrin (Nakamura *et al.*, 1998).

Another collagen receptor on platelets is GPVI. GPVI belongs to the immunoglobulin superfamily (Clemetson *et al.*, 1999; Jandrot-Perrus *et al.*, 2000) and is non-covalently associated with the Fc γ chain, which serves as the signal-transducing part of the receptor in human as well as mouse platelets (Gibbins *et al.*, 1997; Tsuji *et al.*,

1997; Nieswandt *et al.*, 2000a). GPVI-deficient patients suffer from a mild bleeding diathesis and their platelets respond poorly to collagen (Moroi *et al.*, 1989; Arai *et al.*, 1995) or the collagen-related peptide (CRP) (Kehrel *et al.*, 1998), which contains a glycine–proline–hydroxyproline repeat motif and mimics many of the signaling actions of collagen (Asselin *et al.*, 1997). Platelets from FcR γ chain-deficient mice (Takai *et al.*, 1994) lack GPVI (Nieswandt *et al.*, 2000a) and fail to respond to collagen or CRP (Poole *et al.*, 1997). We recently generated the monoclonal antibody (mAb) JAQ1 (Nieswandt *et al.*, 2000a) and showed that it blocks the CRP binding site on mouse GPVI, and inhibits platelet aggregation induced by low, but not high, concentrations of collagen, suggesting that collagen can activate platelets by a second mechanism (Schulte *et al.*, 2001). *In vivo* application of JAQ1 induces virtually complete internalization and degradation of GPVI on mouse platelets. Such GPVI-depleted mice have significantly prolonged bleeding times and their platelets fail to aggregate in response to low and high concentrations of collagen, suggesting that GPVI is essential for both activation pathways induced by collagen (Nieswandt *et al.*, 2001).

To study the role of $\alpha 2\beta 1$ and GPVI in platelet adhesion to collagen and to understand how $\alpha 2\beta 1$ becomes activated *in vivo*, we generated mice with $\beta 1$ -null platelets using the cre/loxP system. We show that mice with $\beta 1$ -null platelets display no increased bleeding tendency. Furthermore, we demonstrate that $\alpha 2\beta 1$ is dispensable for platelet adhesion, activation, and thrombus growth on fibrillar collagen under static and flow conditions, whereas these processes are abolished in the absence of GPVI.

Results

Mice with $\beta 1$ -null platelets have normal platelet counts and bleeding time

To generate mice lacking $\beta 1$ integrin expression on platelets, mice carrying a $\beta 1$ integrin gene flanked by loxP sites (Potocnik *et al.*, 2000) were crossed with mice expressing the cre recombinase under the control of the inducible Mx promoter (Kühn *et al.*, 1995). Deletion of the $\beta 1$ gene from the mice carrying Mx-cre was induced in 4- or 5-week-old animals by three intraperitoneal (i.p.) injections of polyinosinic–polycytidylic acid (pI–pC) at 2-day intervals. Mx-cre-mediated deletion of the floxed $\beta 1$ gene in various tissues 1 week after the first pI–pC injection was quantified by Southern blot analysis of genomic DNA by comparing the band intensities between the floxed and null alleles. Mx-cre-mediated deletion was almost complete in liver and bone marrow, and ~70% complete in spleen, 40% in kidney, 40% in heart, and very little in tail and brain (data not shown).

By flow cytometry, the level of $\beta 1$ integrin expression was undetectable on circulating platelets 2 weeks after the last pI–pC injection in all mice used in this study. Expression of the $\alpha 2$, $\alpha 5$ and $\alpha 6$ subunits was also lost, while $\beta 3$ as well as other major platelet receptors, including GPVI, CD9 and all subunits of the GPIb–V–IX complex, were unchanged (Table I). Western analyses of platelet lysates confirmed the loss of the $\beta 1$ integrin and normal expression of GPVI (Figure 1A). Integrins $\alpha 1$, $\alpha 4$,

Table I. Surface expression of different glycoproteins on control and $\beta 1$ -null platelets

	Control	$\beta 1$ -null
GPIIa ($\beta 1$)	128.3 \pm 13.2	7.3 \pm 2.1
GPIa ($\alpha 2$)	39.1 \pm 9.5	8.4 \pm 3.3
GPIc ($\alpha 5$)	27.2 \pm 5.1	6.6 \pm 1.4
GPIc' ($\alpha 6$)	87.3 \pm 10.2	5.9 \pm 1.7
GPVI	43.5 \pm 10.7	45.2 \pm 12.9
GPIIb/IIIa	347.8 \pm 17.5	359.5 \pm 21.4
GPIIIa	169.7 \pm 10.2	176.1 \pm 16.2
GPIb–IX	295.8 \pm 21.2	287.7 \pm 26.1
GPV	143.4 \pm 15.9	149.5 \pm 14.3
CD9	545.9 \pm 41.1	537.7 \pm 51.8

The surface expression of the indicated glycoproteins was detected by flow cytometry on control and $\beta 1$ -null platelets. Platelets were gated by forward scatter/side scatter (FSC/SSC) characteristics. Results are expressed as mean log fluorescence \pm SD for six mice per group.

$\alpha 7$, $\beta 4$ and $\beta 7$ were undetectable on normal or $\beta 1$ -null platelets (not shown).

Integrins play important roles in blood cell differentiation and proliferation (Fässler *et al.*, 1996). To test platelet production in mutant mice, blood was isolated and platelets were counted. No significant differences were found between control and mutant mice (Figure 1B). Bleeding time was determined next, since several reports showed increased bleeding in patients with reduced expression of $\alpha 2\beta 1$ integrin on platelets (Nieuwenhuis *et al.*, 1985; Kehrel *et al.*, 1988). To restrict the Mx-cre-induced deletion of the $\beta 1$ integrin gene to the hematopoietic system, bone marrow derived from mice carrying floxed $\beta 1$ gene and the Mx-cre transgene was transferred into irradiated normal recipient mice. Four weeks after transfer, the $\beta 1$ integrin gene was deleted by pI–pC injections as described above and the absence of the protein on circulating platelets was confirmed by flow cytometry (data not shown). Surprisingly, no significant differences were found in the bleeding time between control and $\beta 1$ -null bone marrow chimeric mice (Figure 1C). These results show that $\beta 1$ -null platelets develop normally and display no major defects *in vivo*.

Collagen-induced aggregation of $\beta 1$ -null platelets is delayed, but not reduced

$\alpha 2\beta 1$ integrin was reported to play a major role during collagen-induced platelet aggregation *in vitro* (Nieuwenhuis *et al.*, 1985; Kehrel *et al.*, 1988). To test this directly, we induced aggregation of normal and $\beta 1$ -null platelets using fibrillar type I collagen. While no difference was observed in the dose–response characteristics and in the maximum aggregation of control and mutant platelets, $\beta 1$ -null platelets displayed a significant delay in the onset of aggregation that became more evident when lower concentrations of collagen were used (Figure 2A and B). This finding was due to the lack of $\alpha 2\beta 1$ but not other $\beta 1$ integrins, since a blocking mAb against the $\alpha 2$ integrin subunit revealed a similar delayed onset of platelet aggregation (not shown).

Collagen-induced platelet aggregation leads to tyrosine phosphorylation of several intracellular signaling molecules (Watson and Gibbins, 1998). To determine the phosphorylation pattern, lysates from normal and $\beta 1$ -null platelets were analyzed by western blotting. Although the

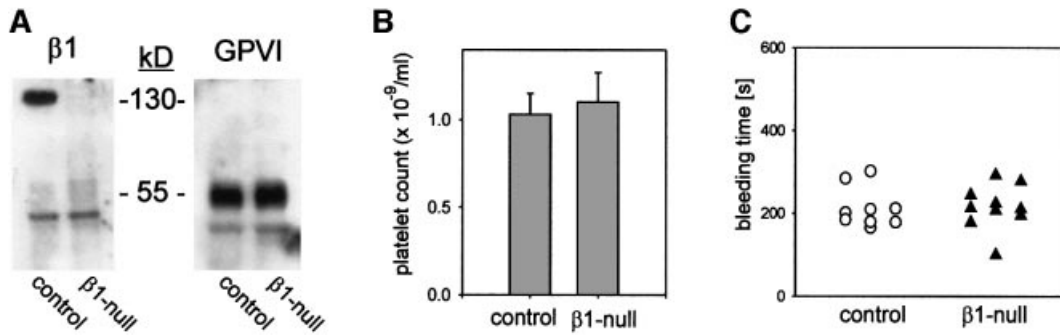


Fig. 1. Characterization of mice with $\beta 1$ -null platelets. (A) Western blot analysis of $\beta 1$ integrin and GPVI in control and $\beta 1$ -null platelets. (B) Platelet counts are expressed as the mean count \pm SD for groups of six mice. (C) Tail bleeding times were determined in groups of nine control and 10 $\beta 1$ -null mice. Each point represents one individual.

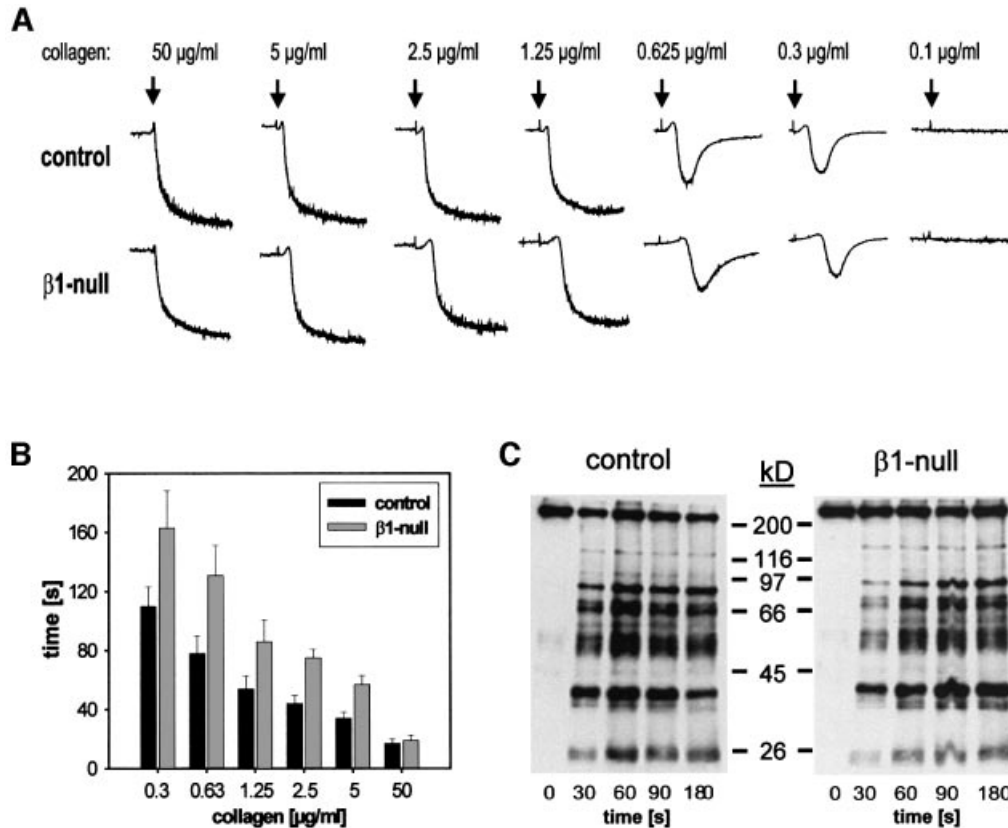


Fig. 2. Aggregation of $\beta 1$ -null platelets in response to fibrillar collagen. (A) Heparinized platelet-rich plasma (prp) from control and $\beta 1$ -null mice was stimulated with the indicated concentrations of fibrillar collagen, and light transmission was recorded in a standard aggregometer. (B) The delay in platelet aggregation is expressed as time (s) between addition of collagen and maximum shape change. Results are given as mean \pm SD ($n = 6$). (C) Washed platelets were stimulated with fibrillar collagen (5 $\mu\text{g/ml}$) and samples were lysed at the indicated time points. Protein tyrosine phosphorylation was detected by immunoblotting.

phosphorylation pattern was comparable between normal and $\beta 1$ -null platelets, the onset of phosphorylation was significantly delayed in the absence of $\beta 1$ integrin (Figure 2C).

In addition to collagen, agonists such as CRP, snake venom toxins, thrombin and ADP can induce platelet aggregation. Therefore, these agonists were used to test whether the loss of $\beta 1$ integrin was associated with a general delay or other defects in platelet aggregation. The GPVI-specific agonists CRP and convulxin, as well as thrombin and ADP, induced aggregation of normal and $\beta 1$ -null platelets to the same extent (Figure 3) and with the same kinetics (not shown).

Altogether, these data indicate that the signaling machinery leading to platelet activation is not altered in $\beta 1$ -null platelets, that GPVI-mediated platelet activation is not affected by the absence of $\beta 1$ integrins and that $\alpha 2\beta 1$ integrin facilitates fibrillar collagen-induced platelet activation, but is not essential.

GPVI mediates platelet activation by two different pathways, only one of which involves $\alpha 2\beta 1$

We recently reported the generation of the mAb JAQ1 against mouse GPVI (Nieswandt *et al.*, 2000a). JAQ1 can completely block platelet aggregation in response to low concentrations of CRP and collagen. The inhibitory effect

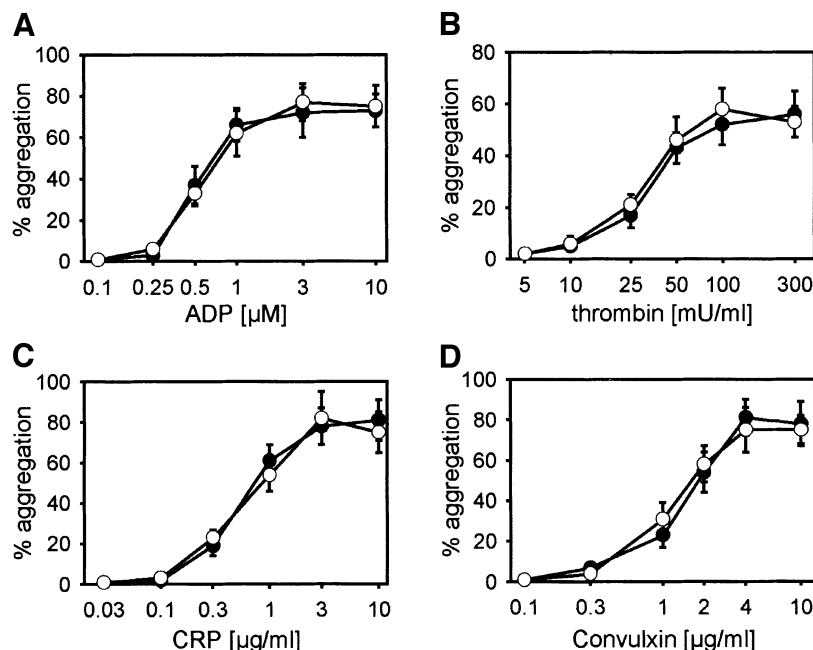


Fig. 3. Normal aggregation of $\beta 1$ -null platelets in response to various agonists. Platelets from control (filled circles) and $\beta 1$ -null (open circles) mice were stimulated with different concentrations of ADP (A), thrombin (B), CRP (C) and convulxin (D). Results are given as percent aggregation and are expressed as mean \pm SD for groups of six mice.

of JAQ1 can be overcome with high doses (greater than $\sim 7 \mu\text{g/ml}$) of collagen, but not CRP. These findings suggested that collagen can activate platelets by a mechanism(s) that is independent of the CRP binding site on GPVI (Schulte *et al.*, 2001). To test whether $\alpha 2\beta 1$ integrin is involved in this activation process, normal and $\beta 1$ -null platelets were incubated with $20 \mu\text{g/ml}$ JAQ1 Fab fragments and subsequently stimulated with low ($5 \mu\text{g/ml}$) or high ($50 \mu\text{g/ml}$) concentrations of fibrillar collagen. While low concentrations of collagen failed to induce aggregation of normal or $\beta 1$ -null platelets in the presence of JAQ1, high collagen concentrations induced aggregation of normal but not $\beta 1$ -null platelets (Figure 4A), suggesting that an $\alpha 2\beta 1$ -dependent pathway mediates this activation. The presence of such a pathway was confirmed by the finding that aggregation was also abolished when normal platelets were pre-incubated with JAQ1 and a blocking antibody against $\alpha 2$ integrin (not shown). On the other hand, GPVI-depleted and FcR γ chain-deficient platelets failed to aggregate in response to high concentrations of fibrillar collagen, confirming earlier reports (Poole *et al.*, 1997; Nieswandt *et al.*, 2001) and demonstrating that GPVI is strictly required for the $\alpha 2\beta 1$ -dependent pathway.

Cells secrete monomeric procollagen, which is converted into collagen by the proteolytic removal of the N- and C-propeptides and assembled into cross-striated fibrils that occur in the extracellular matrix of connective tissue (Kadler *et al.*, 1996). Soluble collagen is normally not found in tissues, but is frequently used for *in vitro* analysis of platelets (Morton *et al.*, 1994; Savage *et al.*, 1999; Siljander and Lassila, 1999). It has been reported that platelet activation by soluble collagen is greatly dependent on $\alpha 2\beta 1$ integrin (Siljander and Lassila, 1999). Indeed, soluble collagen induced irreversible aggregation

of normal, but not of $\beta 1$ -null platelets (Figure 4B). The aggregation response, however, was also abolished with normal platelets pre-treated with JAQ1 as well as with GPVI-depleted and the FcR γ chain-deficient platelets, demonstrating a critical role of GPVI in this activation process. These results were further confirmed by tyrosine phosphorylation studies showing that phosphorylation induced by soluble collagen I was abolished in $\beta 1$ -null (Figure 4C) as well as GPVI-depleted and FcR γ chain-deficient platelets (not shown).

In summary, these data show that GPVI is the central collagen receptor for platelet activation, which only requires cooperation with $\alpha 2\beta 1$ when signaling through the CRP binding site is insufficient or blocked.

Adhesion to fibrillar collagen is GPVI-, but not $\alpha 2\beta 1$ -dependent

A current model suggests that, *in vivo*, platelets adhere firmly to collagen through $\alpha 2\beta 1$ integrin, which allows subsequent collagen-GPVI interactions leading to platelet activation (Sixma *et al.*, 1997; Barnes *et al.*, 1998; Santoro, 1999). To test this model, normal and $\beta 1$ -null platelets were isolated and allowed to adhere to fibrillar collagen under static conditions. Integrin $\alpha 2\beta 1$ binding depends on the presence of $\text{Mg}^{2+}/\text{Ca}^{2+}$ (Onley *et al.*, 2000) and therefore the adhesion assays were performed in the presence or absence of these divalent cations. Both normal and $\beta 1$ -null platelets adhered with and without $\text{Mg}^{2+}/\text{Ca}^{2+}$ to fibrillar collagen in a time-dependent manner (Figure 5A). When the CRP binding site on GPVI was blocked with JAQ1 Fab fragments ($20 \mu\text{g/ml}$), normal platelets adhered to fibrillar collagen in the presence, but not in the absence of $\text{Mg}^{2+}/\text{Ca}^{2+}$, suggesting that the attachment is mediated by $\alpha 2\beta 1$ integrin. This was confirmed by the abrogated adhesion of $\beta 1$ -null platelets

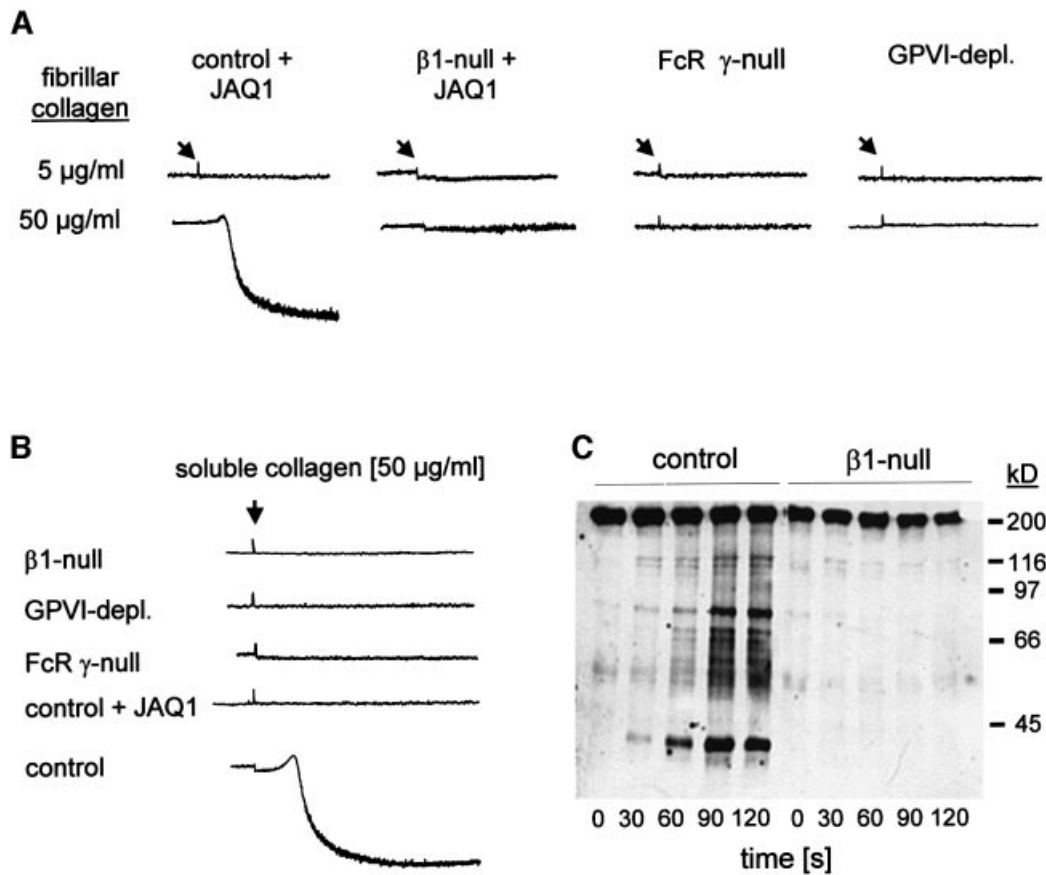


Fig. 4. GPVI mediates two activation pathways, one of which involves $\alpha 2\beta 1$. (A) Heparinized prp from control and $\beta 1$ -null mice was pre-incubated with 20 $\mu\text{g/ml}$ JAQ1 Fab fragments and then stimulated with fibrillar collagen (5 or 50 $\mu\text{g/ml}$). As a control, prp from GPVI-depleted and FcR γ chain-deficient mice was stimulated with collagen in the absence of JAQ1. (B) Prp from the indicated mice was stimulated with soluble collagen (50 $\mu\text{g/ml}$). Where indicated, the prp was pre-treated with JAQ1 Fab fragments (20 $\mu\text{g/ml}$) for 5 min. (C) Washed platelets from control and $\beta 1$ -null mice were stimulated with soluble collagen (50 $\mu\text{g/ml}$) and samples were lysed at different time points. Protein tyrosine phosphorylation was detected by immunoblotting.

in the presence of JAQ1. We have recently demonstrated that GPVI-depleted platelets display markedly delayed and reduced, but not abolished, adhesion to fibrillar collagen under static conditions, which is mediated by $\alpha 2\beta 1$ integrin (Nieswandt *et al.*, 2001). This low level of adhesion, however, is based on residual GPVI activity as it is abrogated in the presence of JAQ1 (Figure 5A). The strict requirement for GPVI was confirmed by the finding that FcR γ chain-deficient platelets were unable to adhere to fibrillar collagen in the absence and presence of $\text{Mg}^{2+}/\text{Ca}^{2+}$.

To test whether this defect was based on impaired integrin activation, adhesion was analyzed in the presence of Mn^{2+} cations, which are known to activate $\beta 1$ and $\beta 3$ integrins directly (Humphries, 2000). As shown in Figure 6A, FcR γ chain-deficient platelets strongly adhered to fibrillar collagen in the presence of Mn^{2+} and comparable results were obtained with GPVI-depleted platelets (not shown). This adhesion was nearly abolished when $\alpha 2$ integrin was blocked with an antibody, whereas blockage of $\alpha \text{IIb}\beta 3$ had no significant effect. A different picture was found when the collagen substrate had previously been exposed to vWF. Under these conditions, blockage of either $\alpha 2\beta 1$ or $\alpha \text{IIb}\beta 3$ only had minor effects, whereas concurrent blockage of both receptors abolished

adhesion, suggesting that $\alpha \text{IIb}\beta 3$ mediated adhesion via vWF. When the collagen substrate had been pre-exposed to plasma, even the blockage of both $\alpha 2\beta 1$ and $\alpha \text{IIb}\beta 3$ only had partial inhibitory effects, indicating that additional platelet receptors may interact with adhesive plasma proteins immobilized on collagen under these experimental conditions.

These data suggested that the GPVI-collagen interaction is essential for the activation of integrins, which then mediate firm adhesion directly or indirectly via adhesive ligands. To test this hypothesis further, we incubated normal, GPVI-depleted and FcR γ chain-deficient platelets with fibrillar collagen and determined activation of $\beta 1$ integrin with the fluorescein isothiocyanate (FITC)-conjugated mAb 9EG7, which specifically recognizes the activated form of the $\beta 1$ subunit (Lenter *et al.*, 1993). Indeed, activated $\beta 1$ integrin was detectable on (a subpopulation of) normal, but not GPVI-depleted or FcR γ chain-deficient platelets. In addition to $\beta 1$ integrin, fibrillar collagen also activated $\alpha \text{IIb}\beta 3$, as determined by measuring the amount of surface-bound fibrinogen (Figure 6B).

Adhesion assays were also performed on immobilized soluble collagen. This process has been reported to be entirely $\alpha 2\beta 1$ dependent (Morton *et al.*, 1994). Normal

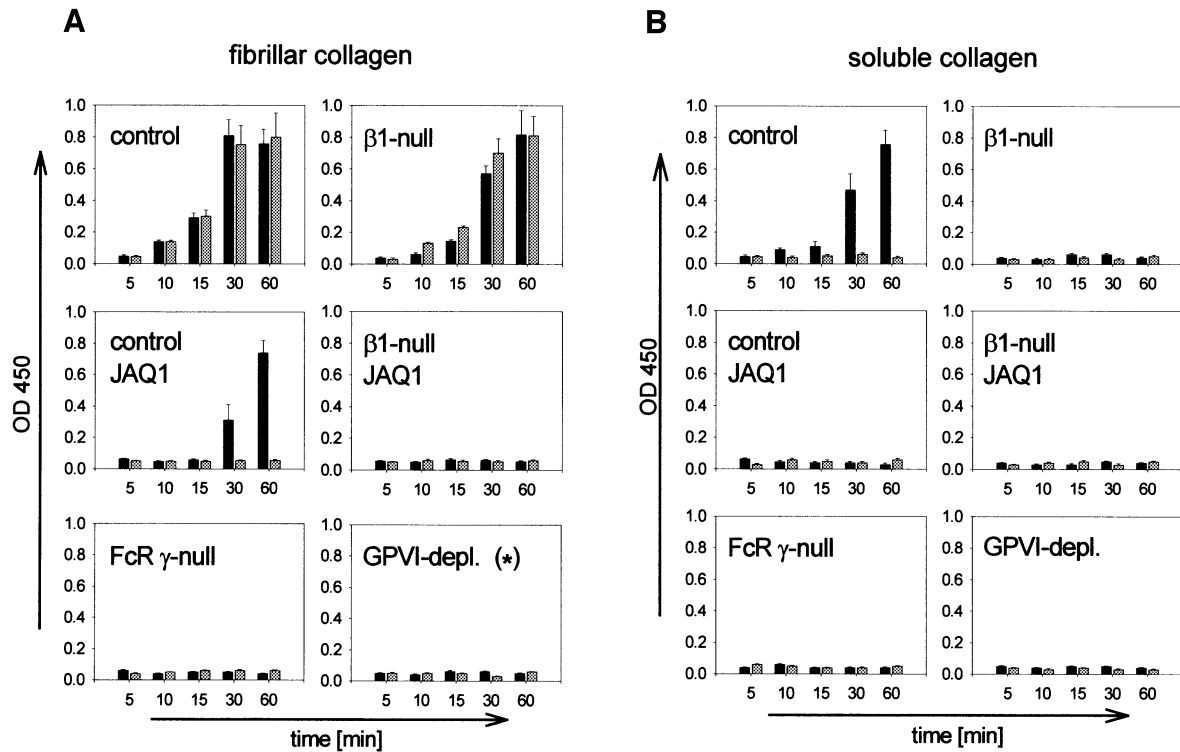


Fig. 5. Platelet adhesion to fibrillar and soluble collagen under static conditions. Washed platelets from the indicated mice were allowed to adhere under static conditions to fibrillar (A) or soluble (B) collagen immobilized in microtiter plates. The experiments were performed in the presence (black bars) or absence (gray bars) of Mg^{2+}/Ca^{2+} (1 mM each). Where indicated, platelets were pre-incubated with JAQ1 Fab fragments (20 $\mu g/ml$). Adherent platelets were quantitated fluorimetrically. The data shown are from a single experiment, representative of four identical experiments and expressed as the mean of triplicate readings \pm SD for the indicated times. (*) To exclude any residual GPVI activity, the platelets were used in the presence of (10 $\mu g/ml$) JAQ1.

platelets adhered to soluble collagen in the presence, but not in the absence of Mg^{2+}/Ca^{2+} , suggesting that the attachment is mediated by $\alpha 2\beta 1$ integrin (Figure 5B). This was confirmed by the abolished adhesion of $\beta 1$ -null platelets. However, normal platelets pre-treated with JAQ1, as well as GPVI-depleted and FcR γ chain-deficient platelets, also failed to adhere to soluble collagen, demonstrating that this interaction depends on both $\alpha 2\beta 1$ integrin and GPVI (Figure 5B).

Adhesion to fibrillar collagen at low and high shear is GPVI-, but not $\alpha 2\beta 1$ -dependent

To define further the role of the two collagen receptors under more physiological conditions, we used whole blood to study platelet adhesion to collagen under conditions of low and high shear stress (150 and 1000 s^{-1} , respectively). At low shear, normal and $\beta 1$ -null platelets formed large aggregates on fibrillar collagen within 5 min, indicating that integrin $\alpha 2\beta 1$ is not essential for adhesion under these conditions. Blocking the CRP binding site on GPVI by addition of 20 $\mu g/ml$ JAQ1 Fab fragments drastically reduced the adhesion of normal platelets and completely blocked the formation of aggregates (Figure 7). $\beta 1$ -null platelets did not adhere in the presence of JAQ1 (not shown). These data demonstrate that adhesion and subsequent thrombus formation are predominantly dependent on the CRP binding epitope on GPVI, and to a very minor part facilitated by $\alpha 2\beta 1$. Also, this minor $\alpha 2\beta 1$ -dependent adhesion required GPVI, since adhesion

of GPVI-depleted and FcR γ chain-deficient platelets was abolished (Figure 7).

At high shear stress (1000 s^{-1}), platelet adhesion greatly depends on interactions between GPIIb α and vWF (Savage *et al.*, 1998). Under these conditions, adhesion and thrombus formation occurred significantly faster than under low shear conditions (<2 min), and no significant difference was found between normal and $\beta 1$ -null platelets. In contrast, pre-incubation of platelets with JAQ1 Fab fragments almost completely abrogated adhesion and formation of aggregates. A comparable picture was found with GPVI-depleted and FcR γ chain-deficient platelets, confirming the critical role of GPVI in the adhesion process at high shear.

Adhesion under low and high shear flow conditions was also tested on soluble collagen. In agreement with the results obtained in the static assay, normal platelets adhered under both conditions in the absence but not in the presence of JAQ1 Fab fragments, whereas no adhesion occurred with $\beta 1$ -null, FcR γ -null or GPVI-depleted platelets, confirming the critical role of both $\alpha 2\beta 1$ and GPVI for adhesion to soluble collagen under flow conditions (not shown).

Discussion

The collagen receptor $\alpha 2\beta 1$ integrin on platelets was considered to play a pivotal role in hemostasis. In the present study, we established mice that lack $\beta 1$ integrins

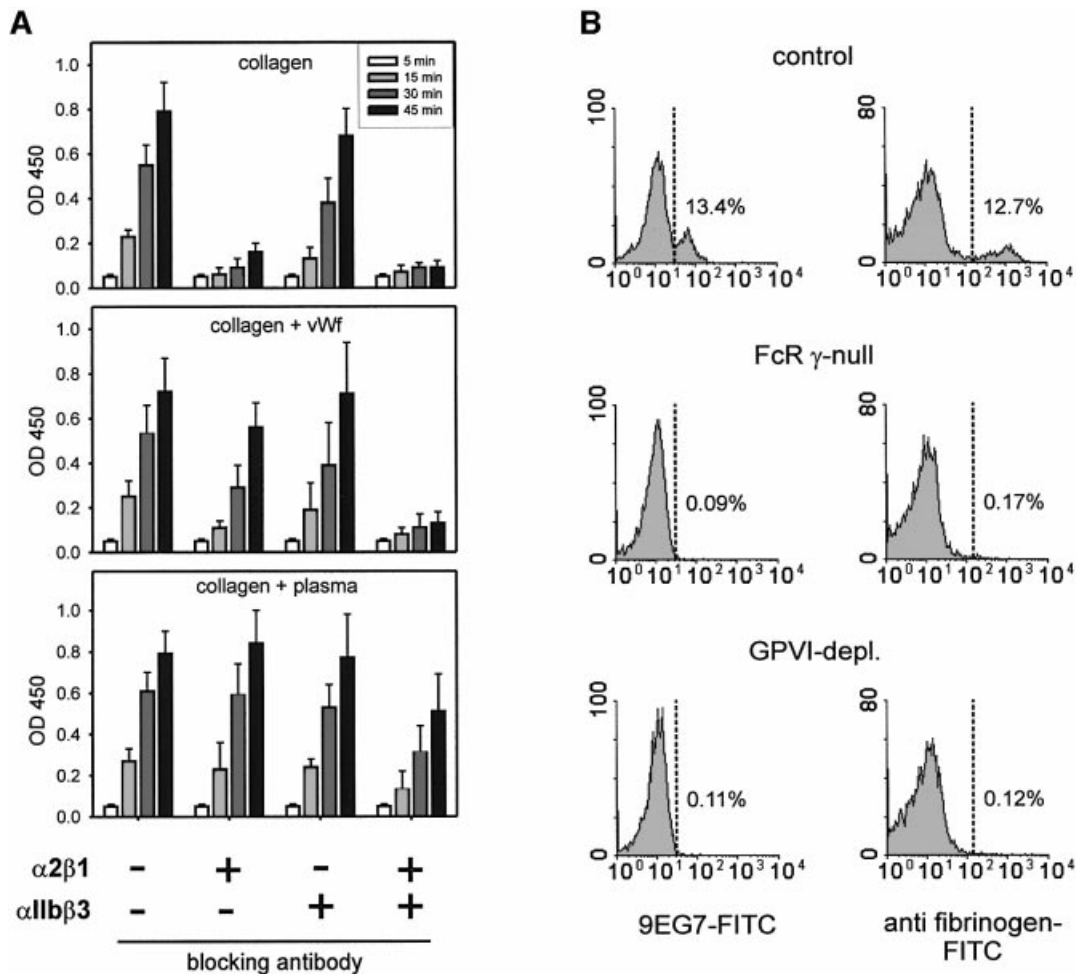


Fig. 6. GPVI-mediated integrin activation is a prerequisite for platelet adhesion to collagen. (A) Washed FcR γ chain-deficient platelets were allowed to adhere to fibrillar collagen immobilized in microtiter plates in the presence of Mn²⁺ with or without addition of blocking antibodies against $\alpha 2\beta 1$ or $\alpha \text{IIb}\beta 3$. Where indicated, the collagen substrate had been pre-exposed to vWf (20 $\mu\text{g}/\text{ml}$) or plasma for 30 min. Adherent platelets were quantitated fluorimetrically. The data shown are from a single experiment, representative of three identical experiments, and are expressed as the mean of triplicate readings \pm SD for the indicated times. (B) Flow cytometric analysis of collagen-induced integrin activation on control, FcR γ chain-deficient or GPVI-depleted platelets. Diluted prp was stimulated with 30 $\mu\text{g}/\text{ml}$ fibrillar collagen for 5 min at room temperature. Activated $\beta 1$ integrin was detected directly with 9EG7-FITC, and activated $\alpha \text{IIb}\beta 3$ indirectly by quantitation of surface-bound fibrinogen. Platelets were gated by FSC/SSC characteristics and F12 positivity (anti-CD9^{PE}). The results shown are representative of six individual experiments. Note that only a subpopulation of the control platelets is activated. This is based on the fact that fibrillar collagen is insoluble and, therefore, not all platelets come in contact with this agonist. The percentage of activated control platelets varied between 10.3 and 19.6 in these experiments.

on platelets and found, very unexpectedly, that they have no increased bleeding tendency. Furthermore, $\beta 1$ -null platelets can be efficiently activated with collagen *in vitro*, although the onset of activation is delayed. A similar delay has been observed using $\alpha 2\beta 1$ function-blocking antibodies (Coller *et al.*, 1989; Hers *et al.*, 2000). $\beta 1$ -null platelets can adhere to fibrillar collagen under static as well as low and high shear flow conditions. These findings are in sharp contrast to observations made in patients with low or no expression of $\alpha 2\beta 1$ on platelets. They suffered from severe bleedings and their platelets failed to respond to collagen *in vitro* (Nieuwenhuis *et al.*, 1985; Kehrel *et al.*, 1988). Possible explanations for the discrepancy could be species-specific differences or, more likely, that the few patients reported so far had additional defects on their platelets. Mice with $\beta 1$ -deficient platelets clearly demonstrate that $\alpha 2\beta 1$ integrin is dispensable for hemostasis under normal conditions *in vivo*. It is possible, however, that $\alpha 2\beta 1$ -mediated adhesion of platelets to

collagen is crucial if additional components such as vWf or fibronectin are absent.

We found, however, that $\alpha 2\beta 1$ integrin was essential for adhesion to soluble collagen under static and flow conditions (Figure 5 and data not shown). These results confirm the role of $\alpha 2\beta 1$ as a collagen receptor on platelets and may at least partly explain the conflicting data on the determinants of the platelet-collagen interaction reported in the literature. Numerous studies of platelet adhesion under both static and flow conditions were performed with type I collagen solubilized by pepsin, which cleaves collagen in the non-triple helical region where covalent cross-links are found that are essential for the assembly of collagen molecules into the typical banded structure as well as the insolubility of collagen (Kadler *et al.*, 1996; Savage *et al.*, 1999). Such soluble collagen has served as a powerful tool to characterize the recognition sites for individual collagen receptors within the molecule. Although collagens are degraded under certain

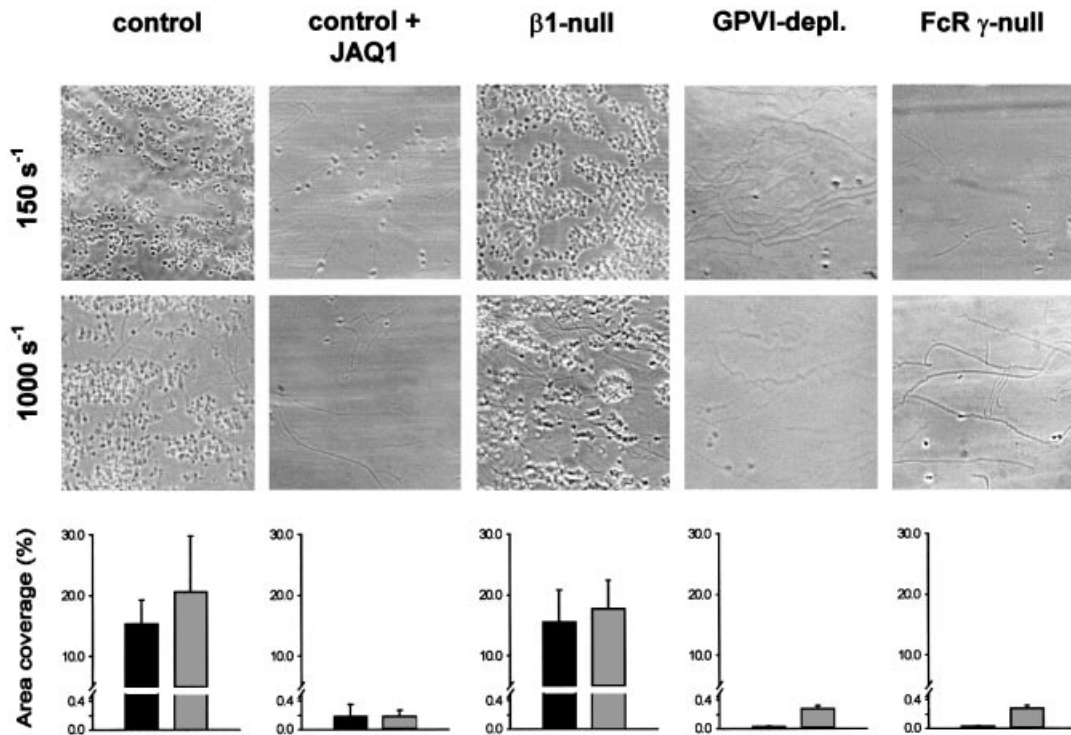


Fig. 7. GPVI, but not $\alpha 2\beta 1$, is essential for platelet adhesion to collagen under flow. Whole blood from the indicated mice was loaded with calcein and perfused at wall shear rates of 150 s^{-1} (10 min) or 1000 s^{-1} (4 min) over a collagen-coated surface. Blood from control mice was assessed in the absence or presence of $20 \mu\text{g/ml}$ JAQ1 Fab fragments. Upper panels: representative phase-contrast microscope images after perfusion. Lower panels: surface area coverage of calcein fluorescence at the end of the perfusion period (mean \pm SEM, $n = 3\text{--}6$). Similar results were obtained when analyzing surface area coverage of platelets from phase-contrast images.

pathological circumstances, they are still predominantly present in fibrillar form in normal vessels. These data suggest that platelet interactions with fibrillar collagen *in vivo* must be different and require investigation.

Since our results strongly suggested that $\alpha 2\beta 1$ plays a supportive rather than an essential role in platelet interactions with native, fibrillar collagen, we analyzed GPVI, which binds fibrillar collagens as a low affinity and signal transducing receptor. Importantly, we found that GPVI function is not impaired in the absence of $\alpha 2\beta 1$, which is in contrast to earlier reports showing that blockage of $\alpha 2\beta 1$ function reduced platelet responses to the GPVI-specific agonist convulxin and CRP (Jandrot-Perrus *et al.*, 1997; Hers *et al.*, 2000; Kamiguti *et al.*, 2000). An explanation for these different results could be that certain anti- $\alpha 2\beta 1$ agents may have impaired GPVI function by yet unidentified mechanisms (Saelman *et al.*, 1994; Kamiguti *et al.*, 1997; Verkleij *et al.*, 1998).

Interference with GPVI function revealed an essential role of this receptor in adhesion of normal as well as $\beta 1$ -null platelets to collagen. A function-blocking antibody to GPVI or the absence of the receptor on platelets efficiently inhibited platelet adhesion and aggregation to collagen under flow conditions. Under static conditions, GPVI was also essential for adhesion to fibrillar as well as soluble collagen, suggesting that GPVI-collagen interactions are a prerequisite for integrin-mediated adhesion.

We have recently shown that JAQ1 is unable to inhibit activation of platelets by high concentrations of fibrillar collagen, indicating the presence of a second, GPVI-independent collagen receptor (Schulte *et al.*, 2001). We

reasoned that $\alpha 2\beta 1$ could be this receptor since it is present on resting platelets in a low affinity binding state requiring high levels of collagens for interaction. Indeed, $\beta 1$ -null platelets fail to respond to high concentrations of fibrillar collagen in the presence of JAQ1. Interestingly, however, while this response is independent of the CRP binding site that is blocked by JAQ1, it is still dependent on the presence of GPVI, as high collagen concentrations are unable to aggregate GPVI-depleted as well as FcR γ chain-deficient platelets despite normal expression of $\alpha 2\beta 1$. A possible explanation for this finding could be that, in addition to the CRP binding site, GPVI has a second, low affinity binding site for fibrillar collagen mediating activation of $\alpha 2\beta 1$ integrin, which then contributes to signaling. Alternatively, binding of the multivalent fibrillar collagen to $\alpha 2\beta 1$ could co-aggregate $\alpha 2\beta 1$ and GPVI within lipid rafts. It has been shown recently that receptor aggregates in lipid rafts can signal in a ligand-independent manner (Harder and Kuhn, 2000; Janes *et al.*, 2000). In contrast, soluble monomeric collagen may not be able to mediate the clustering of its receptors in lipid rafts due to the lack of highly repetitive recognition sites within the molecule. It is important to note, however, that it is currently not clear whether $\alpha 2\beta 1$ integrin and GPVI are present in lipid rafts.

Resting platelets express integrins in a low affinity binding state to avoid interactions with fibrinogen or plasma fibronectin (Phillips *et al.*, 1991; Shattil *et al.*, 1998). When platelets become activated, their integrins shift to a high affinity state and bind ligands. In our present study, we provide multiple lines of evidence that

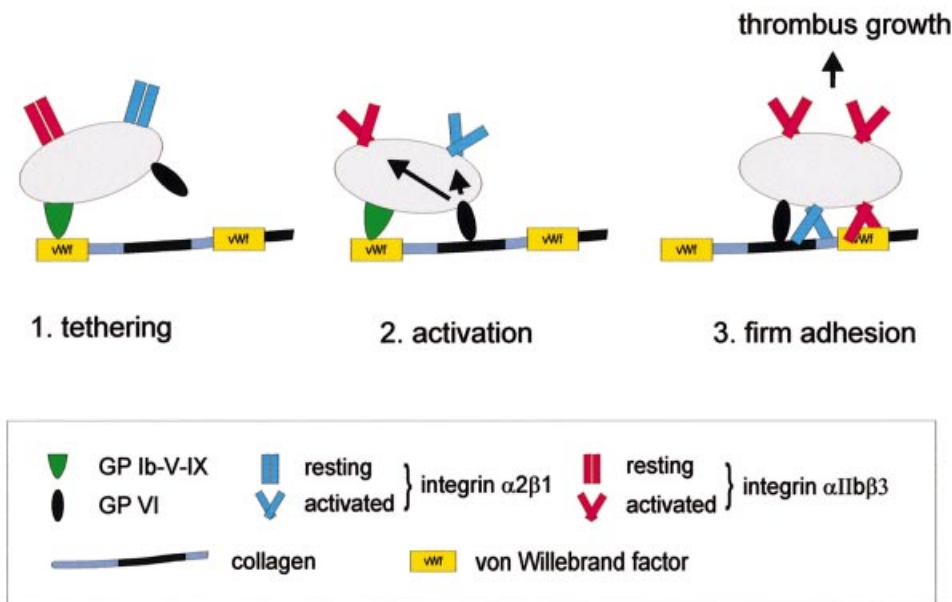


Fig. 8. Model for platelet adhesion to collagen. 1. The initial tethering to the reactive surface is mediated predominantly by GPIIb α -vWF interactions and is important at high shear rates ($>500\text{ s}^{-1}$), but may not be relevant at lower shear rates (Savage *et al.*, 1998). 2. GPIIb α -collagen interactions lead to cellular activation followed by shifting of integrins to a high affinity state. This activation step is proposed to be a strict prerequisite for adhesion. 3. Firm adhesion of platelets to collagen through activated $\alpha 2\beta 1$ (directly) and $\alpha \text{IIb}\beta 3$ (indirectly via vWF or other ligands). Integrin $\alpha 2\beta 1$ -collagen interactions are not essential for this process. This scheme only includes interactions examined in the current study (with the exception of GPIIb α -vWF) and does not exclude the involvement of other receptor-ligand interactions.

stimulation of GPVI can shift $\alpha 2\beta 1$ and $\alpha \text{IIb}\beta 3$ integrins from a low to high affinity state, thereby confirming and extending the observation of Moroi and co-workers (Jung and Moroi, 1998; Moroi *et al.*, 2000). First, GPVI- and Fc γ chain-deficient platelets failed to adhere to collagen despite normal expression of $\alpha 2\beta 1$ and $\alpha \text{IIb}\beta 3$ (Nieswandt *et al.*, 2000a, 2001), which are in a low affinity state, as demonstrated by fluorescence-activated cell sorting analysis (Figure 6B). Secondly, binding of these platelets to collagen via $\alpha 2\beta 1$ and $\alpha \text{IIb}\beta 3$ -vWf could be induced by Mn^{2+} , which is known to activate $\beta 1$ and $\beta 3$ integrins directly (Humphries, 2000). These findings, together with the observation that collagen induced the activation of both integrins in a GPVI-dependent manner (Figure 6C), demonstrate that GPVI triggers integrin-mediated platelet-collagen interactions.

A previous model incorporated the idea that $\alpha 2\beta 1$ integrin is essential for shear-resistant thrombus growth by mediating firm platelet adhesion to collagen. This first binding event would then allow a second step involving low affinity interactions of GPVI with collagen, leading to platelet activation and thrombus formation (Sixma *et al.*, 1997; Barnes *et al.*, 1998; Santoro, 1999). However, our analysis of $\beta 1$ -null platelets presented here demonstrates clearly that they can adhere to collagen under low as well as high shear stress. The number and size of platelet aggregates did not differ significantly between normal and $\beta 1$ -null platelets. In contrast, no adhesion occurred in the absence of GPVI. These findings establish a new sequence of events in the initial phase of hemostasis and thrombosis (Figure 8). First, platelets tether at sites of injury by GPIIb α -vWF interactions, which are essential under conditions of high shear (Savage *et al.*, 1998). In a second

step, the platelets use GPVI to bind to collagen and thereby become activated and upregulate the activity of integrins, including $\alpha 2\beta 1$ and $\alpha \text{IIb}\beta 3$. High affinity binding of $\alpha 2\beta 1$ to collagen strengthens firm adhesion without, however, being essential for this process. This revised model of platelet-collagen interactions clearly establishes GPVI as the central collagen receptor on platelets and may have important implications for the development of new anti-thrombotic therapies.

Materials and methods

Animals

Generation of mice with $\beta 1$ -null platelets. To produce mice carrying the $\beta 1$ -null allele in megakaryocytes, $\beta 1(\text{fl}/\text{fl})$ mice (Potocnik *et al.*, 2000) were crossed with transgenic mice carrying the Mx-cre transgene (mx-cre+) (Kühn *et al.*, 1995). Deletion of the $\beta 1$ gene was induced in 4- to 5-week-old [$\beta 1(\text{fl}/\text{fl})/\text{Mx-cre}+$] mice by three i.p. injections of 250 μg of pI-pC at 2-day intervals. Control $\beta 1(\text{fl}/\text{fl})$ mice received the same treatment and were derived from the same litters. For experiments, mice were used at least 2 weeks after pI-pC injection.

Bone marrow chimeras were generated by tail vein injection of 10^6 bone marrow cells derived from [$\beta 1(\text{fl}/\text{fl})/\text{Mx-cre}+$]c into lethally irradiated (6 MV X-rays, 10 Gy, 6.2 Gy/min) congenic B6.SJL mice. Control mice received bone marrow cells derived from [$\beta 1(\text{fl}/\text{fl})/\text{Mx-cre}$] mice. Four weeks after the transfer, deletion of the $\beta 1$ gene was induced by pI-pC injections as described above. Bleeding times were tested 3 weeks and 6 months later.

Generation of GPVI-deficient platelets. Wild-type mice were injected with 100 μg of JAQ1 i.p. and platelets were harvested on day 7. As reported previously, GPVI was not detectable on those platelets by flow cytometry and western blot analysis (Nieswandt *et al.*, 2001). For static adhesion assays on fibrillar collagen, these platelets were used in the presence of 10 $\mu\text{g}/\text{ml}$ JAQ1 to block any residual GPVI activity.

Fc γ chain-deficient mice. Fc γ chain-deficient mice (Takai *et al.*, 1994) were obtained from Taconics, Germantown.

Chemicals and antibodies

Fibrillar type I collagen from equine tendon (Horm; Nycomed, Munich, Germany), high molecular weight heparin, soluble, non-fibrillar type I collagen from rat tail (both from Sigma, Deisenhofen, Germany), calcein acetoxymethyl ester (Molecular Probes, Leiden, The Netherlands) and D-Phe-Pro-Arg chloromethyl ketone (PPACK; Calbiochem, San Diego, CA) were purchased. Purified human vWF was a generous gift from G. Dickneite (Marburg, Germany). All other reagents were from sources described previously (Nieswandt *et al.*, 2001).

The following antibodies were used: hamster anti- $\beta 1$ integrin (Ha31/8), hamster anti- $\alpha 2$ integrin (Ha1/29), FITC-anti- $\alpha 4$ integrin (R1-2), biotinylated anti- $\alpha 5$ integrin (5H10-27), FITC-anti- $\alpha 6$ integrin (GoH3), FITC-anti- $\alpha 1$ integrin (Ha2/5), rat anti- $\beta 1$ integrin (9EG7), rat anti- $\beta 4$ integrin (346-11A), biotinylated anti- $\beta 7$ integrin (M293) (all PharMingen), mouse hybridoma supernatant against $\alpha 7$ integrin (3C12; obtained from K. von der Mark, Erlangen, Germany), rat anti-mouse P-selectin mAb RB40.34 (from D. Vestweber, Münster, Germany) and polyclonal rabbit antibodies to human fibrinogen (Dako, Glostrup, Denmark). Rat anti-mouse $\alpha \text{IIb}\beta 3$ (JON1, JON/A), $\beta 3$ integrin (EDL4), GPIb-IX (p0p1), GPV (DOM1), CD9 (ULF1) and GPVI (JAQ1) were generated, produced and modified in our laboratories as described (Bergmeier *et al.*, 2000; Nieswandt *et al.*, 2000a,b). Fab fragments of JAQ1 were prepared as described (Nieswandt *et al.*, 2001). FITC-anti-hamster IgG (PharMingen), FITC-anti-mouse IgG (Jackson Laboratories), streptavidin-phycoerythrin (Southern Biotechnologies) and rabbit anti-FITC-horseradish peroxidase (Dako) were used as secondary reagents.

Platelet preparation and platelet aggregation

Mice were bled under ether anesthesia from the retro-orbital plexus. Platelet counting, preparation of platelet-rich plasma (prp) and determination of platelet aggregation were carried out as described (Nieswandt *et al.*, 2001).

Immunoblotting and flow cytometry

Platelet preparation, immunoblotting and flow cytometry were carried out as described (Nieswandt *et al.*, 2001). Tyrosine phosphorylation was determined as described elsewhere (Schulte *et al.*, 2001).

Bleeding time experiments

Mice were restrained and a 0.5 cm 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 s intervals, bleeding was determined to have ceased.

Static adhesion assay

The static adhesion assay was performed with washed platelets in the presence or absence of $\text{MgCl}_2/\text{CaCl}_2$ (each at 1 mM) on 96-well plates (Nunc, Wiesbaden, Germany) coated with fibrillar or soluble collagen (2 $\mu\text{g}/\text{well}$) as described (Nieswandt *et al.*, 2001). For adhesion in the presence of 0.5 mM MnCl_2 , the experiments were performed in the presence of apyrase (1 U/ml), aspirin (1 mM) and prostacyclin (1 μM) in order to minimize Mn^{2+} -induced platelet degranulation.

Adhesion under flow conditions

Mouse blood (1 vol.) was collected into 0.5 vols of HEPES buffer pH 7.45 containing 137 mM NaCl, 5.6 mM glucose, 5 mM HEPES, 2.7 mM KCl, 2 mM MgCl_2 , 0.42 mM NaH_2PO_4 , 0.1% bovine serum albumin (BSA), 120 μM PPACK and 15 U/ml heparin. The blood was incubated with 2.5 μM calcein acetoxymethyl ester for 30–60 min. Rectangular coverslips (24 \times 60 mm) were sprayed with 150 μl of fibrillar or non-fibrillar collagen (0.25 mg/ml), and blocked with HEPES buffer containing 1% BSA, as described previously (Heemskerk *et al.*, 1997).

Perfusion of whole blood was performed as described elsewhere (Briedé *et al.*, 2001). Briefly, transparent flow chambers with a slit depth of 50 μm , equipped with the collagen-coated coverslips, were rinsed with HEPES buffer supplemented with 2 mM CaCl_2 and 1 U/ml heparin, and connected to a syringe filled with the anti-coagulated blood. Perfusion was carried out at room temperature using a pulse-free pump under low shear stress conditions (10 min, flow rate of 1.12 ml/h, equivalent to a wall shear rate of 150 s^{-1}) or at high shear stress (4 min, flow rate of 7.53 ml/h, equivalent to a wall shear rate of 1000 s^{-1}). During perfusion, microscope phase-contrast images were recorded in real time. Thereafter, the chambers were rinsed by a 10 min perfusion with HEPES buffer pH 7.45 containing 2 mM CaCl_2 and 1 U/ml heparin at the same shear stress. Both phase-contrast and fluorescence images were recorded from

at least five different microscope fields (40 \times or 80 \times objectives), using a fluorescence digital imaging system equipped with two intensified, charge-coupled device cameras (Heemskerk *et al.*, 1997). Image analysis was performed off-line using Quanticell 700/900 software (Visitech, Sunderland, UK). The platelet adhesion results are expressed as the mean percentage of the total area covered by platelets or calcein fluorescence.

Statistical evaluation

Statistical analysis was performed using the unpaired Student's *t*-test.

Acknowledgements

We thank Michael Dictor and Kamin Johnson for discussion and critically reading the manuscript, U. Barnfred for constant support throughout the study and Per Nilsson for irradiating the mice used for bone marrow transplantations. The work was supported by grants from the Deutsche Forschungsgemeinschaft (to B.N.) and the Swedish National Research Foundation (to C.B. and R.F.).

References

- Arai, M., Yamamoto, N., Moroi, M., Akamatsu, N., Fukutake, K. and Tanoue, K. (1995) Platelets with 10% of the normal amount of glycoprotein VI have an impaired response to collagen that results in a mild bleeding tendency. *Br. J. Haematol.*, **89**, 124–130.
- Asselin, J., Gibbins, J.M., Achison, M., Lee, Y.H., Morton, L.F., Farndale, R.W., Barnes, M.J. and Watson, S.P. (1997) A collagen-like peptide stimulates tyrosine phosphorylation of syk and phospholipase C $\gamma 2$ in platelets independent of the integrin $\alpha 2\beta 1$. *Blood*, **89**, 1235–1242.
- Barnes, M.J., Knight, C.G. and Farndale, R.W. (1998) The collagen-platelet interaction. *Curr. Opin. Hematol.*, **5**, 314–320.
- Baumgartner, H.R. (1977) Platelet interaction with collagen fibrils in flowing blood. I. Reaction of human platelets with α chymotrypsin-digested subendothelium. *Thromb. Haemost.*, **37**, 1–16.
- Bergmeier, W., Rackeband, K., Schroder, W., Zirngibl, H. and Nieswandt, B. (2000) Structural and functional characterization of the mouse von Willebrand factor receptor GPIb-IX with novel monoclonal antibodies. *Blood*, **95**, 886–893.
- Briedé, J.J., Heemskerk, J.W.M., van't Veer, C., Hemker, H.C. and Lindhout, T. (2001) Contribution of platelet-derived factor Va to thrombin generation on immobilized collagen- and fibrinogen-adherent platelets. *Thromb. Haemost.*, in press.
- Clemetson, J.M., Polgar, J., Magnenat, E., Wells, T.N. and Clemetson, K.J. (1999) The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to Fc α R and the natural killer receptors. *J. Biol. Chem.*, **274**, 29019–29024.
- Coller, B.S., Beer, J.H., Scudder, L.E. and Steinberg, M.H. (1989) Collagen-platelet interactions: evidence for a direct interaction of collagen with platelet GPIa/IIa and an indirect interaction with platelet GPIIb/IIIa mediated by adhesive proteins. *Blood*, **74**, 182–192.
- Fässler, R., Georges-Labouesse, E. and Hirsch, E. (1996) Genetic analyses of integrin function in mice. *Curr. Opin. Cell Biol.*, **8**, 641–646.
- Gibbins, J.M., Okuma, M., Farndale, R., Barnes, M. and Watson, S.P. (1997) Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor γ -chain. *FEBS Lett.*, **413**, 255–259.
- Harder, T. and Kuhn, M. (2000) Selective accumulation of raft-associated membrane protein LAT in T cell receptor signaling assemblies. *J. Cell Biol.*, **151**, 199–208.
- Heemskerk, J.W., Vuist, W.M., Feijge, M.A., Reutelingsperger, C.P. and Lindhout, T. (1997) Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine and procoagulant activity of adherent platelets: evidence for regulation by protein tyrosine kinase-dependent Ca^{2+} responses. *Blood*, **90**, 2615–2625.
- Hers, I., Berlanga, O., Tiekstra, M.J., Kamiguti, A.S., Theakston, R.D. and Watson, S.P. (2000) Evidence against a direct role of the integrin $\alpha 2\beta 1$ in collagen-induced tyrosine phosphorylation in human platelets. *Eur. J. Biochem.*, **267**, 2088–2097.
- Humphries, M.J. (2000) Integrin structure. *Biochem. Soc. Trans.*, **28**, 311–339.
- Jandrot-Perrus, M., Lagrue, A.H., Okuma, M. and Bon, C. (1997) Adhesion and activation of human platelets induced by convulxin involve glycoprotein VI and integrin $\alpha 2\beta 1$. *J. Biol. Chem.*, **272**, 27035–27041.
- Jandrot-Perrus, M. *et al.* (2000) Cloning, characterization and functional studies of human and mouse glycoprotein VI: a platelet-specific

- collagen receptor from the immunoglobulin superfamily. *Blood*, **96**, 1798–1807.
- Janes, P.W., Ley, S.C., Magee, A.I. and Kabouridis, P.S. (2000) The role of lipid rafts in T cell antigen receptor (TCR) signalling. *Semin. Immunol.*, **12**, 23–34.
- Jung, S.M. and Moroi, M. (1998) Platelets interact with soluble and insoluble collagens through characteristically different reactions. *J. Biol. Chem.*, **273**, 14827–14837.
- Kadler, K.E., Holmes, D.F., Trotter, J.A. and Chapman, J.A. (1996) Collagen fibril formation. *Biochem. J.*, **316**, 1–11.
- Kamiguti, A.S., Markland, F.S., Zhou, Q., Laing, G.D., Theakston, R.D. and Zuzel, M. (1997) Proteolytic cleavage of the $\beta 1$ subunit of platelet $\alpha 2\beta 1$ integrin by the metalloproteinase jararhagin compromises collagen-stimulated phosphorylation of pp72. *J. Biol. Chem.*, **272**, 32599–32605.
- Kamiguti, A.S., Theakston, R.D., Watson, S.P., Bon, C., Laing, G.D. and Zuzel, M. (2000) Distinct contributions of glycoprotein VI and $\alpha(2)\beta(1)$ integrin to the induction of platelet protein tyrosine phosphorylation and aggregation. *Arch. Biochem. Biophys.*, **374**, 356–362.
- Kehrel, B., Balleisen, L., Kokott, R., Mesters, R., Stenzinger, W., Clemetson, K.J. and van de Loo, J. (1988) Deficiency of intact thrombospondin and membrane glycoprotein Ia in platelets with defective collagen-induced aggregation and spontaneous loss of disorder. *Blood*, **71**, 1074–1078.
- Kehrel, B., Wierwille, S., Clemetson, K.J., Anders, O., Steiner, M., Knight, C.G., Farndale, R.W., Okuma, M. and Barnes, M.J. (1998) Glycoprotein VI is a major collagen receptor for platelet activation: it recognizes the platelet-activating quaternary structure of collagen, whereas CD36, glycoprotein IIb/IIIa and von Willebrand factor do not. *Blood*, **91**, 491–499.
- Kühn, R., Schwenk, F., Aguet, M. and Rajewsky, K. (1995) Inducible gene targeting in mice. *Science*, **269**, 1427–1429.
- Lenter, M., Uhlig, H., Hamann, A., Jenö, P., Imhof, B. and Vestweber, D. (1993) A monoclonal antibody against an activation epitope on mouse integrin chain $\beta 1$ blocks adhesion of lymphocytes to the endothelial integrin $\alpha 6\beta 1$. *Proc. Natl Acad. Sci. USA*, **90**, 9051–9055.
- Moroi, M., Jung, S.M., Okuma, M. and Shinmyozu, K. (1989) A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. *J. Clin. Invest.*, **84**, 1440–1445.
- Moroi, M., Onitsuka, I., Imaizumi, T. and Jung, S.M. (2000) Involvement of activated integrin $\alpha 2\beta 1$ in the firm adhesion of platelets onto a surface of immobilized collagen under flow conditions. *Thromb. Haemost.*, **83**, 769–776.
- Morton, L.F., Peachey, A.R., Zijenah, L.S., Goodall, A.H., Humphries, M.J. and Barnes, M.J. (1994) Conformation-dependent platelet adhesion to collagen involving integrin $\alpha 2\beta 1$ -mediated and other mechanisms: multiple $\alpha 2\beta 1$ -recognition sites in collagen type I. *Biochem. J.*, **299**, 791–797.
- Nakamura, T., Jamieson, G.A., Okuma, M., Kambayashi, J. and Tandon, N.N. (1998) Platelet adhesion to native type I collagen fibrils. Role of GPVI in divalent cation-dependent and -independent adhesion and thromboxane A₂ generation. *J. Biol. Chem.*, **273**, 4338–4344.
- Nieswandt, B., Bergmeier, W., Rackebbrandt, K., Gessner, J.E. and Zirngibl, H. (2000a) Identification of critical antigen-specific mechanisms in the development of immune thrombocytopenic purpura in mice. *Blood*, **96**, 2520–2527.
- Nieswandt, B., Bergmeier, W., Schulte, V., Rackebbrandt, K., Gessner, J.E. and Zirngibl, H. (2000b) Expression and function of the mouse collagen receptor glycoprotein VI is strictly dependent on its association with the FcR γ chain. *J. Biol. Chem.*, **275**, 23998–24002.
- Nieswandt, B., Schulte, V., Bergmeier, W., Mokhtari-Nejad, R., Rackebbrandt, K., Cazenave, J.P., Ohlmann, P., Gachet, C. and Zirngibl, H. (2001) Long-term antithrombotic protection by *in vivo* depletion of platelet glycoprotein VI in mice. *J. Exp. Med.*, **193**, 459–469.
- Nieuwenhuis, H.K., Akkerman, J.W., Houdijk, W.P. and Sixma, J.J. (1985) Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. *Nature*, **318**, 470–472.
- Onley, D.J., Knight, C.G., Tuckwell, D.S., Barnes, M.J. and Farndale, R.W. (2000) Micromolar Ca²⁺ concentrations are essential for Mg²⁺-dependent binding of collagen by the integrin $\alpha 2\beta 1$ in human platelets. *J. Biol. Chem.*, **275**, 24560–24564.
- Phillips, D.R., Charo, I.F. and Scarborough, R.M. (1991) GPIIb–IIIa: the responsive integrin. *Cell*, **65**, 359–362.
- Poole, A., Gibbins, J.M., Turner, M., van Vugt, M.J., van de Winkel, J.G., Saito, T., Tybulewicz, V.L. and Watson, S.P. (1997) The Fc receptor γ -chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J.*, **16**, 2333–2341.
- Potocnik, A.J., Brakebusch, C. and Fässler, R. (2000) Fetal and adult hematopoietic stem cells require $\beta 1$ integrin function for colonizing fetal liver, spleen and bone marrow. *Immunity*, **12**, 653–663.
- Saelman, E.U. *et al.* (1994) Platelet adhesion to collagen types I through VIII under conditions of stasis and flow is mediated by GPIa/IIa ($\alpha 2\beta 1$ -integrin) *Blood*, **83**, 1244–1250.
- Santoro, S.A. (1986) Identification of a 160,000 dalton platelet membrane protein that mediates the initial divalent cation-dependent adhesion of platelets to collagen. *Cell*, **46**, 913–920.
- Santoro, S.A. (1999) Platelet surface collagen receptor polymorphisms: variable receptor expression and thrombotic/hemorrhagic risk. *Blood*, **93**, 3575–3577.
- Savage, B., Almus-Jacobs, F. and Ruggeri, Z.M. (1998) Specific synergy of multiple substrate–receptor interactions in platelet thrombus formation under flow. *Cell*, **94**, 657–666.
- Savage, B., Ginsberg, M.H. and Ruggeri, Z.M. (1999) Influence of fibrillar collagen structure on the mechanisms of platelet thrombus formation under flow. *Blood*, **94**, 2704–2715.
- Schulte, V., Snell, D., Bergmeier, W., Zirngibl, H., Watson, S.P. and Nieswandt, B. (2001) Evidence for two distinct epitopes within collagen for activation of murine platelets. *J. Biol. Chem.*, **276**, 364–368.
- Shattil, S.J., Kashiwagi, H. and Pampori, N. (1998) Integrin signaling: the platelet paradigm. *Blood*, **91**, 2645–2657.
- Siljander, P. and Lassila, R. (1999) Studies of adhesion-dependent platelet activation: distinct roles for different participating receptors can be dissociated by proteolysis of collagen. *Arterioscler. Thromb. Vasc. Biol.*, **19**, 3033–3043.
- Sixma, J.J., van Zanten, G.H., Huizinga, E.G., van der Plas, R.M., Verkley, M., Wu, Y.P., Gros, P. and de Groot, P.G. (1997) Platelet adhesion to collagen: an update. *Thromb. Haemost.*, **78**, 434–438.
- Takai, T., Li, M., Sylvestre, D., Clynes, R. and Ravetch, J.V. (1994) FcR γ chain deletion results in pleiotropic effector cell defects. *Cell*, **76**, 519–529.
- Tsuji, M., Ezumi, Y., Arai, M. and Takayama, H. (1997) A novel association of Fc receptor γ -chain with glycoprotein VI and their co-expression as a collagen receptor in human platelets. *J. Biol. Chem.*, **272**, 23528–23531.
- Verkleij, M.W., Morton, L.F., Knight, C.G., de Groot, P.G., Barnes, M.J. and Sixma, J.J. (1998) Simple collagen-like peptides support platelet adhesion under static but not under flow conditions: interaction via $\alpha 2\beta 1$ and von Willebrand factor with specific sequences in native collagen is a requirement to resist shear forces. *Blood*, **91**, 3808–3816.
- Watson, S.P. and Gibbins, J. (1998) Collagen receptor signalling in platelets: extending the role of the ITAM. *Immunol. Today*, **19**, 260–264.
- Weiss, H.J. (1975) Platelet physiology and abnormalities of platelet function (first of two parts). *N. Engl. J. Med.*, **293**, 531–541.

Received February 20, 2001; revised and accepted March 15, 2001