Facilitating roles of murine platelet glycoprotein Ib and αIIbβ3 in phosphatidylserine exposure during vWF–collagen-induced thrombus formation

Marijke J. E. Kuijpers[1], Valerie Schulte[2], Cécile Oury[3], Theo Lindhout[1], Jos Broers[4], Marc F. Hoylaerts[3], Bernhard Nieswandt[2] and Johan W. M. Heemskerk[1]

Departments of [1]Biochemistry, Cardiovascular Research Institute Maastricht, and [4]Molecular Cell Biology & Genetics, University of Maastricht, Maastricht, The Netherlands
[2]Rudolf Virchow Center for Experimental Biomedicine, University of Würzburg, Germany
[3]Center for Molecular and Vascular Biology, KU Leuven, Belgium

Vessel wall damage exposes collagen fibres, to which platelets adhere directly via the collagen receptors glycoprotein (GP) VI and integrin $\alpha_2\beta_1$ and indirectly by collagen-bound von Willebrand factor (vWF) via the GPIb-V-IX and integrin αIIbβ3 receptor complexes. Platelet–collagen interaction under shear stimulates thrombus formation in two ways, by integrin-dependent formation of platelet aggregates and by surface exposure of procoagulant phosphatidylserine (PS). GPVI is involved in both processes, complemented by α2β1. In mouse blood flowing over collagen, we investigated the additional role of platelet–vWF binding via GPIb and αIIbβ3. Inhibition of GPIb as well as blocking of vWF binding to collagen reduced stable platelet adhesion at high shear rate. This was accompanied by delayed platelet Ca^{2+} responses and reduced PS exposure, while microaggregates were still formed. Inhibition of integrin αIIbβ3 with JON/A antibody, which blocks αIIbβ3 binding to both vWF and fibrinogen, reduced PS exposure and aggregate formation. The JON/A effects were not enhanced by combined blocking of GPIb–vWF binding, suggesting a function for αIIbβ3 downstream of GPIb. Typically, with blood from FcR γ-chain +/− mutant mice, expressing 50% of normal platelet GPVI levels, GPIb blockage almost completely abolished platelet adhesion and PS exposure. Together, these data indicate that, under physiological conditions of flow, both adhesive receptors GPIb and αIIbβ3 facilitate GPVI-mediated PS exposure by stabilizing platelet binding to collagen. Hence, these glycoproteins have an assistant procoagulant role in collagen-dependent thrombus formation, which is most prominent at reduced GPVI activity and is independent of the presence of thrombin.

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Corresponding author J. W. M. Heemskerk: Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht, The Netherlands. Email: jwm.heemskerk@bioch.unimaas.nl

Collagen fibres, exposed upon vessel wall damage, are strongly platelet adhesive. Binding of platelets to collagen triggers a chain of activating events and leads to the assembly of platelet aggregates and the formation of fibrin-containing thrombi. The thrombus-forming reaction is essential in haemostasis, but detrimental in the progression of atherothrombosis and plaque rupture. This process of thrombus formation has been widely studied *in vitro*, using flow chambers where whole blood is perfused over a collagen-containing surface under (patho)physiological shear conditions, even in the absence of coagulation. From this work, it has appeared that multiple receptors are involved in the interaction of platelets with collagen.

Collagen rapidly adsorbs von Willebrand factor (vWF), which is present in plasma as a multimeric protein (Novak *et al.* 2002). vWF acts as a bridging molecule in platelet–collagen interactions, since it can bind to both collagen and the receptor complexes glycoprotein (GP)Ib-V-IX and integrin αIIbβ3 through its A3, A1 and C1 domains, respectively. High shear stress induces conformational changes of vWF, which result in a

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reversible interaction with GPIb (Huizinga *et al.* 2002). This reduces the velocity of platelets flowing over collagen-bound vWF and results in transient attachment to the collagen surface (Savage *et al.* 1996). Subsequent, irreversible binding to vWF is mediated by the αIIbβ3 integrin. This integrin also needs conformational changes for ligand interaction (inside-out signalling), which can be achieved, for example, by vWF–GPIb binding (Nesbitt *et al.* 2002; Arya *et al.* 2003) or following stimulation of the ADP, thromboxane A_2 or collagen receptors (Shattil & Ginsberg, 1997; Jung & Moroi, 2001). Both GPIb and αIIbβ3 also mediate vWF/fibrinogen dependent platelet aggregate formation under shear (Shattil & Ginsberg, 1997; Savage *et al.* 2002).

Direct platelet–collagen contact is established by the collagen receptors GPVI and integrin $\alpha2\beta1$ (Jung & Moroi, 2000; Savage *et al.* 2002; Nieswandt & Watson, 2003). GPVI acts as a major signalling receptor, while $\alpha2\beta1$ is required for stable adhesion to collagen. Ligand-induced clustering of GPVI results in its non-covalent association with the Fc receptor (FcR) γ-chain, which leads to signalling via tyrosine phosphorylation (Gibbins *et al.* 1997; Tsuji *et al.* 1997). As a result, phospholipase Cγ2 becomes phosphorylated and activated, which causes a prolonged increase in cytosolic $[Ca^{2+}]_i$ (Watson *et al.* 2001). This Ca^{2+} response contributes to the release of feedback agonists such as ADP and thromboxane A_2, which sustain platelet aggregate formation.

Previous *in vivo* and *in vitro* flow studies with mice have indicated that the $\alpha2\beta1$ integrin is dispensable for platelet-collagen adhesion and subsequent thrombus formation (Nieswandt *et al.* 2001*a*,*b*). Using mice deficient in $\alpha2\beta1$ or GPVI, it was furthermore shown that under high, arterial shear conditions this integrin enhances GPVI signalling and thereby stabilizes the platelet aggregates on collagen (Kuijpers *et al.* 2003). This has led to a model of interplay between the collagen receptors in which the $\alpha2\beta1$ integrin supported by release products functions to enhance GPVI-induced platelet activation. Such a model is now proposed by several groups (Atkinson *et al.* 2003; Chen & Kahn, 2003; Nieswandt & Watson, 2003; Siljander *et al.* 2004), although it is still unclear to what extent the synergistic effect of $\alpha2\beta1$ on GPVI is due to intracellular signalling by the integrin itself (Jung & Moroi, 2000; Inoue *et al.* 2003) or to stabilization of collagen–GPVI contact by an activated integrin form.

Apart from aggregate formation, platelet–collagen interaction stimulates the coagulation process. Collagen or collagen-related peptide provokes, in a Ca^{2+}-dependent way, exposure of phosphatidylserine (PS) at the platelet outer membrane surface (Heemskerk *et al.* 1997; Siljander *et al.* 2001). The availability of PS greatly potentiates the conversion of prothrombin into coagulant thrombin and thus enhances thrombin generation (Bevers *et al.* 1982; Béguin & Kumar, 1997; Heemskerk *et al.* 2002). PS exposure is one of the early platelet responses in shear-dependent thrombus formation upon perfusion of human or murine blood over vWF–collagen (Kuijpers *et al.* 2003; Siljander *et al.* 2004). In both species, it is a consequence of GPVI activity, while $\alpha2\beta1$ mainly potentiates the GPVI effect. There is evidence, mostly from experiments with coagulating plasma, that GPIb, αIIbβ3 and also vWF have discrete functions in stimulating platelet-dependent thrombin generation (Béguin *et al.* 1999; Keuren *et al.* 2003). However, whether these factors also contribute to the PS-exposing, procoagulant response of platelets interacting with vWF–collagen is still unresolved.

Since platelet–collagen interaction under high shear is dependent on vWF, GPIb and αIIbβ3, we focused here on the contribution of these proteins in the generation of the platelet procoagulant response under flow conditions. Using real-time video imaging techniques, we show that at high shear rates GPIb and vWF – and downstream of them αIIbβ3 – enhance GPVI-mediated PS exposure, especially under conditions of reduced GPVI expression and activity. This novel procoagulant effect of GPIb is independent of thrombin action and is additional to its function in platelet aggregation. It is likely to be of physiological relevance, given the large variation in GPVI expression between humans. As $\alpha2\beta1$ also stimulates GPVI activity, our results reveal receptor interplay in the regulation of platelet procoagulant activity that is not limited to collagen receptors, but extends to the GPIb-V-IX complex.

Methods

Animals

Pathogen-free C57Bl/6 mice were obtained from Charles River (Maastricht, the Netherlands); mice deficient in FcR γ-chain (Takai *et al.* 1994) were obtained from Taconics (Germantown, NY, USA). Heterozygous FcR γ-chain deficient mice were generated by crossing wild-type mice with homozygous deficient mice of C57Bl/6 background (Nieswandt *et al.* 2001*a*). Reduced expression of both GPVI and FcR γ-chain was checked at the protein level by Western blots, and by flow cytometric measurement of surface expression of GPVI, as described (Snell *et al.* 2002). Experimental protocols were ethically approved by the animal care and use committee of the University of Maastricht.

Materials

Fab fragments of rat anti-mouse antibodies were produced and modified in our laboratories (Bergmeier *et al.* 2000). This involved the p0p/B antibody, which binds to the vWF binding site on GPIbα, and the JON/A antibody, which selectively binds to activated integrin αIIbβ3 and inhibits integrin interaction with both vWF and fibrinogen. Polyclonal rabbit anti-human vWF antibody, cross-reacting with mouse vWF, was obtained from Dako (Glostrup, Denmark), and fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG (GAR-FITC) was from Southern Biotech (Birmingham, AL, USA).

Fibrillar Horm collagen from equine tendon (Horm type-I) was purchased from Nycomed (Munich, Germany). Apyrase, high molecular weight heparin and MRS2179, an antagonist of the $P2Y_1$ purinergic receptor, came from Sigma (St Louis, MO, USA). Annexin V (annexin A5) labelled with FITC was from Nexins Research (Hoeven, The Netherlands); calcein and fluo-3 acetoxymethyl esters, Pluronic F-127, and phalloidin labelled with Texas Red X were from Molecular Probes (Leiden, The Netherlands); H-Phe-Pro-Arg chloromethyl ketone (PPACK) was from Calbiochem (La Jolla, CA, USA). AR-C69931MX, an antagonist of the $P2Y_{12}$ purinergic receptor, was kindly provided by AstraZeneca R&D (Charnwood, UK). Recombinant saratin was produced in the yeast *Hansenula polymorpha,* as described (Barnes *et al.* 2001). Sources of other chemicals are mentioned elsewhere (Heemskerk *et al.* 1999).

Platelet preparation and labelling

Under full anaesthesia, mice were bled retro-orbitally and killed by cervical dislocation. The blood (1 volume) was collected in 0.5 volumes of saline containing 5 units ml^{-1} heparin and 40 μM PPACK. Anticoagulated blood (1 volume) was diluted in Hepes buffer (pH 7.4; 0.5 volumes) containing 137 mM NaCl, 5.6 mM glucose, 5 mM Hepes, 2.7 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.42 mM NaH_2PO_4, 0.1% (w/v) bovine serum albumin (BSA), which was supplemented with 1 unit ml^{-1} heparin. The blood was used within 2 h. Where indicated, blood was incubated with 2.5 μM calcein acetoxymethyl ester for 30 min, as described (Nieswandt *et al.* 2001*a*).

Washed murine platelets in Hepes buffer (pH 7.45) were prepared from platelet-rich plasma, as described for rat blood (Heemskerk *et al.* 1994). For Ca^{2+} measurements, washed platelets were incubated with fluo-3 acetoxymethyl ester (5 μM) in the presence of Pluronic F-127 (0.2 mg ml^{-1}) and apyrase (0.1 units ml^{-1} ADPase) at room temperature for 45 min.

Real-time video imaging

Adhesion experiments under flow conditions were performed with mouse blood anticoagulated with PPACK and heparin, basically as described earlier (Nieswandt *et al.* 2001*a*). Briefly, coverslips (24 × 60 mm) were partly coated with Horm collagen type I fibres (1.25 mg per 50 mm^2), rinsed with saline, and blocked with Hepes buffer containing 1% (w/v) BSA. Coverslips were placed in a parallel-plate transparent flow chamber (slit depth of 50 μm), which was connected by polyethylene tubing (diameter 1 mm), and mounted on an inverted Nikon (Tokyo, Japan) microscope. To prevent coagulation, the chamber and tubing were pre-washed with Hepes buffer containing 1 unit ml^{-1} heparin. Blood was perfused through the flow chamber using a 1 ml syringe and a pulse-free pump, at the desired shear rate (1000 or 1500 s^{-1}) for 4 min. During perfusion, high-resolution microscopic images of transmission or fluorescence (fluo-3 or calcein) were recorded in real-time with a Visitech imaging system (Sunderland, UK). Digital images were captured with two parallel-placed intensified, CCD cameras, recording infrared (0.3 Hz) and epifluorescence (5 Hz) light at the desired wavelengths (Heemskerk *et al.* 1997). After perfusion, the flow chamber was rinsed with Hepes buffer supplemented with 1 unit ml^{-1} heparin at the desired shear rate. Exposure of PS was detected with FITC-labelled annexin A5 (0.5 μg ml^{-1}) added to the rinse buffer.

Where indicated, blood was incubated for 10 min prior to perfusion with a saturating concentration of p0p/B or JON/A Fab fragment (40 μg ml^{-1}), saratin (10 μg ml^{-1}) or MRS2179 (20 μM) plus AR-C69931MX (50 μM). These antagonists were also added to the rinse buffer. To measure changes in cytosolic $[Ca^{2+}]_i$, fluo-3-labelled platelets from mice of the same genotype were added to the anticoagulated blood, so that 5–10% of total platelets were labelled. Labelling a small fraction of the platelets in blood had the advantage that individual, collagen-bound platelets could be distinguished from each other, even when trapped in multiplatelet aggregates.

Real-time changes in fluo-3 fluorescence from single platelets, recorded during the flow experiment, were analysed with Quanticell software. Changes in fluo-3 fluorescence of individual platelets were converted into levels of $[Ca^{2+}]_i$ using a pseudo-ratio calibration procedure, exactly as described (Heemskerk *et al.* 2001). Traces representing averaged Ca^{2+} responses were

constructed from time-adjusted responses of at least 13 single platelets that could be observed for at least 60 s (2–4 mice per condition).

Confocal fluorescence microscopy

Immediately after perfusion, collagen-coated coverslips with aggregates were removed from the flow chamber, fixed with 2% formaldehyde for 15 min, and rinsed with tap water. Further procedures were performed in a humid chamber at room temperature. The samples were first blocked with 15% (w/v) BSA in phosphate-buffered saline (PBS) for 30 min. Samples were then incubated with rabbit anti-human vWF antibody ($10\,\mu g\,ml^{-1}$) for 1 h, washed in PBS 3 times for 5 min, and stained with GAR-FITC ($10\,\mu g\,ml^{-1}$) for 1 h. Samples were counter-stained for actin to stain all platelets. After a triple wash in PBS and permeabilization with 0.005% SDS in PBS for 10 min, they were blocked with 1% BSA in PBS. The samples were then incubated with Texas Red-labelled phalloidin (3 units ml^{-1}) in blocking buffer for 1 h and, after another wash, they were mounted in 9 volumes of glycerol plus 1 volume of 0.2 M Tris-HCl, 0.02% NaN_3 with 2% 1,4-diazabicyclo(2,2,2)-octane (Dabco) (pH 8.0). Double-stained aggregates were observed with a Bio-Rad MRC-600 confocal scanning laser microscope (Bio-Rad, Richmond, CA, USA) equipped with a krypton–argon mixed gas laser and a red diode laser (Ion Laser Technology, Salt Lake City, UT, USA) with two separate wavelengths for excitation of fluorescein (488 nm) and Texas Red X (568 nm).

Image analysis

Data were compared off-line from at least nine different randomly chosen, microscopic images, taken at the collagen surface. Platelet deposition was determined from phase-contrast images, as described before (Kuijpers *et al.* 2003). Area coverage by platelets staining with calcein or FITC-annexin A5 fluorescence was determined with Quanticell software (Visitech). Procoagulant area was corrected for fluorescent glare in the optics by comparison of camera images in fluorescence and transmission modes.

Statistical analysis

Data were tested for significant differences using the statistical package for social sciences (SPSS 11.0, Chicago, IL, USA). Significant differences compared to control condition were evaluated with a Mann–Whitney *U* test.

Results

Roles of GPIb and vWF in PS exposure in platelets adhering to collagen under shear

Whole blood from wild-type mice, anticoagulated with PPACK–heparin, was used to investigate the contribution of the GPIb–vWF interaction to platelet deposition on collagen and subsequent PS exposure. The blood was perfused over immobilized type-I collagen at an intermediate, arterial wall shear rate ($1000\,s^{-1}$) to achieve GPIb dependency (Kuijpers *et al.* 2003). Video microscopy showed that most wild-type platelets firmly adhered to the collagen fibres. Some of these platelets assembled to form platelet aggregates, while others remained single and transformed into round, blebbing structures. The latter morphological change is an indicator of PS exposure and procoagulant activity (Siljander *et al.* 2001; Kuijpers *et al.* 2003). The deposition of platelets, measured as surface area coverage, increased about linearly for at least 6 min. After 4 min perfusion, $25 \pm 2\%$ (mean ± S.E.M., $n = 32$) of the surface was covered with platelets (Fig. 1*A*). A similar, linear increase in platelet deposition was observed, when blood containing calcein-labelled platelets was perfused over the collagen surface (data not shown, but see Nieswandt *et al.* 2001*a*). Staining with FITC-labelled annexin A5 showed that, at the 4-min end point, a considerable proportion of single, collagen-binding platelets had exposed PS, with an area coverage of fluorescence of $9 \pm 1\%$ ($n = 20$) (Fig. 1*B*). Experiments where FITC–annexin A5 was added to the blood indicated that PS exposure in deposited platelets started after a lag time of 2 min and then increased continuously up to 10 min.

To block the GPIb–vWF interaction, the wild-type blood was pre-incubated with a saturating concentration of anti-GPIbα p0p/B Fab fragment ($40\,\mu g\,ml^{-1}$), which specifically abolishes GPIb binding to mouse vWF (Bergmeier *et al.* 2000). This treatment led to an increased translocation velocity of platelets over the collagen surface and to diminished stable adhesion, while microaggregates were still formed (Fig. 1*C*). Platelet deposition was reduced by p0p/B to $66 \pm 13\%$ ($n = 5$) of the control condition. The number of single, PS-exposing platelets (Fig. 1*D*) was more strongly reduced to about 20% ($P = 0.02$) of control (Fig. 2*A*). As an alternative way to eliminate the contribution of vWF, blood was treated with $10\,\mu g\,ml^{-1}$ saratin, a leech protein which blocks vWF binding to collagen (Barnes *et al.* 2001). Again, platelets were moving over the collagen surface and made fewer stable contacts (Fig. 1*E*). With some of the platelets still forming

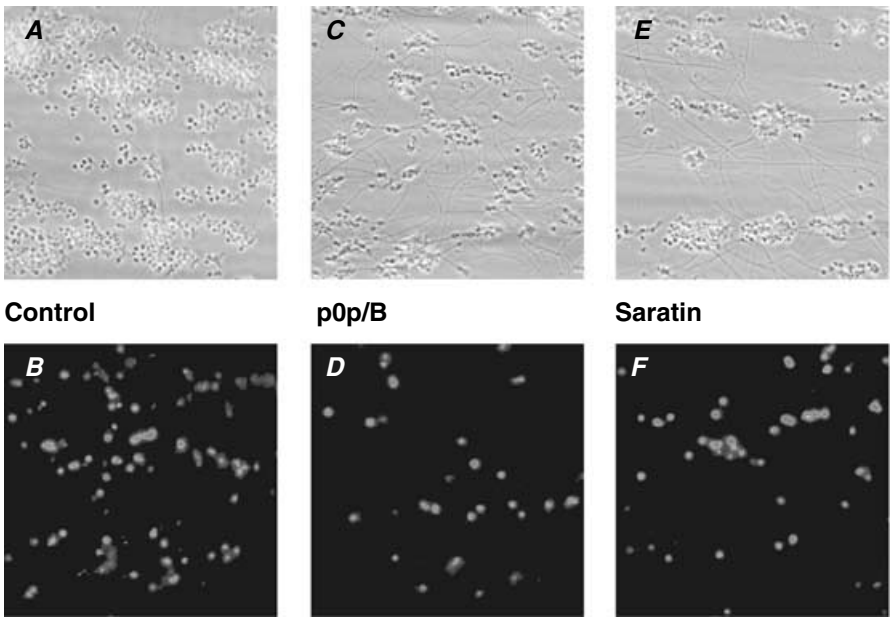

Figure 1. Effect of blocking GPIb on platelet deposition and PS exposure upon flow over vWF/collagen
Whole blood from C57Bl/6 wild-type mice was perfused over type-I collagen at a wall shear rate of 1000 s^{-1}. *A, C* and *E,* phase-contrast microscopic images (120 × 120 μm) after 4 min of perfusion. *B, D* and *F,* fluorescence images (150 × 150 μm) after staining with FITC-labelled annexin A5. *A* and *B*, untreated control blood. *C* and *D*, blood pre-treated with 40 μg ml^{-1} anti-GPIb p0p/B Fab fragment (10 min). *E* and *F,* blood pre-treated with 10 μg ml^{-1} saratin (10 min).

aggregates, the total area coverage was 34 ± 6% ($n = 11$) of control. The number of PS-exposing platelets (Fig. 1*F*) was similarly reduced to 30 ± 13% of control (Fig. 2*A*).

To achieve higher GPIb dependency, additional experiments were performed at a higher shear rate of 1500 s^{-1}. In this case, blocking GPIb with p0p/B further reduced the area coverage of platelets to 15 ± 6% and of annexin A5-binding platelets to 26 ± 7% of the control (Fig. 2*B*). Similarly, the presence of saratin decreased the coverage of platelets to 21 ± 5% and of annexin A5 fluorescence to 36 ± 17% of control. Thus, inhibition of the GPIb–vWF interaction by GPIb blocking (p0p/B) or the absence of vWF (saratin) caused a substantial but incomplete reduction in platelet deposition and PS exposure at shear rates up to 1500 s^{-1}.

The similarities in effects of p0p/B and saratin treatment suggested that the GPIb interaction with collagen-bound vWF could control PS exposure by allowing stable platelet adhesion. To confirm this, platelets on collagen-coated coverslips were stained post perfusion for vWF and then counterstained with phalloidin to visualize actin filaments. In the control situation, confocal microscopy showed bright vWF staining on the collagen fibres as well as on platelet aggregates, where it appeared as clustered spots (Fig. 3*A*). With saratin present, the collagen fibres no longer stained for vWF, whereas the platelets in aggregates had unaltered vWF staining (Fig. 3*B*).

Role of GPIb in Ca^{2+} responses of platelets adhering to vWF–collagen

The activation state of platelets interacting with vWF–collagen was investigated by adding 10% fluo-3-labelled murine platelets to the blood. This labelling of

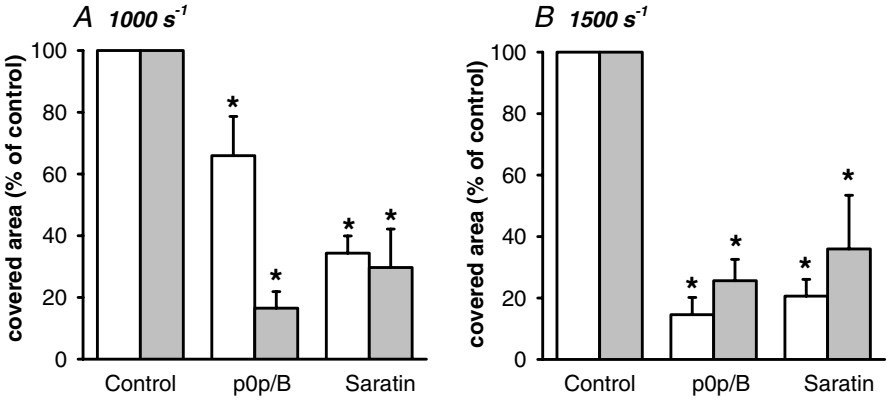

Figure 2. Roles of GPIb and shear stress in platelet deposition and PS exposure
Whole blood from wild-type mice was perfused over collagen for 4 min at a shear rate of 1000 or 1500 s^{-1}. Effect of pre-treatment with p0p/B Fab (40 μg ml^{-1}) or saratin (10 μg ml^{-1}) on area coverage of all platelets (white bars) and PS-exposing platelets (grey bars). Data are percentages of control condition at indicated shear rate (mean ± S.E.M., $n = 6$–20). $^*P < 0.05$ compared to control.

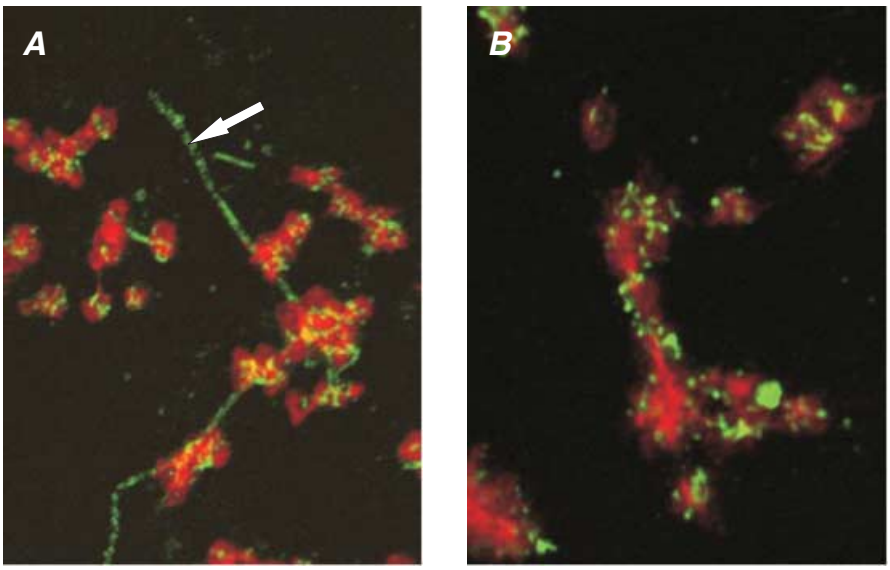

Figure 3. Effect of saratin on vWF binding to collagen and platelets
Blood from wild-type mice was perfused over collagen in the absence (*A*) or presence (*B*) of saratin (10 μg ml^{-1}) at 1000 s^{-1} for 4 min. Representative confocal fluorescence images are given after fixation with 2% formaldehyde and staining for actin and vWF. Micrographs show contours of platelets (actin) and deposited vWF as brighter punctuated spots; arrow indicates vWF-covered collagen fibre. Image sizes are 20 μm × 25 μm.

platelets did not influence collagen-induced aggregate formation *in vitro*, as measured by aggregometry (data not shown). Under control conditions of flow (1000 s^{-1}), the large majority of fluo-3-labelled platelets stably attached to collagen fibrils. The platelets showed a potent and prolonged Ca^{2+} response (Fig. 4*A*), as is required for PS exposure (Heemskerk *et al.* 1997). Traces from single platelets indicated a rapid, prolonged rise in $[Ca^{2+}]_i$ that was occasionally preceded by one high spike in $[Ca^{2+}]_i$ (Fig. 4*B*; see also 'control' movie in Supplementary material, available online only). As we have shown before, this Ca^{2+} signal under flow conditions is almost completely dependent on GPVI signalling, with minute, spiking $[Ca^{2+}]_i$ increases remaining in the absence of GPVI (Kuijpers *et al.* 2003).

When the blood was treated with anti-GPIb p0p/B, many of the fluo-3-labelled platelets moved over the surface before stopping. Once they had adhered, the Ca^{2+} signals of many of the p0p/B-treated platelets were reduced in comparison to the control (Fig. 4*C* and *D*). Individual cells often showed a series of Ca^{2+} spikes before reaching a maximal level, which was on average reduced by 35% ($n = 13$–26 platelets, $P = 0.02$, unpaired t test, measured after 60 s). This is illustrated in the 'p0p/B' movie (see Supplementary material). Treatment of the blood with saratin caused similar but not identical effects.

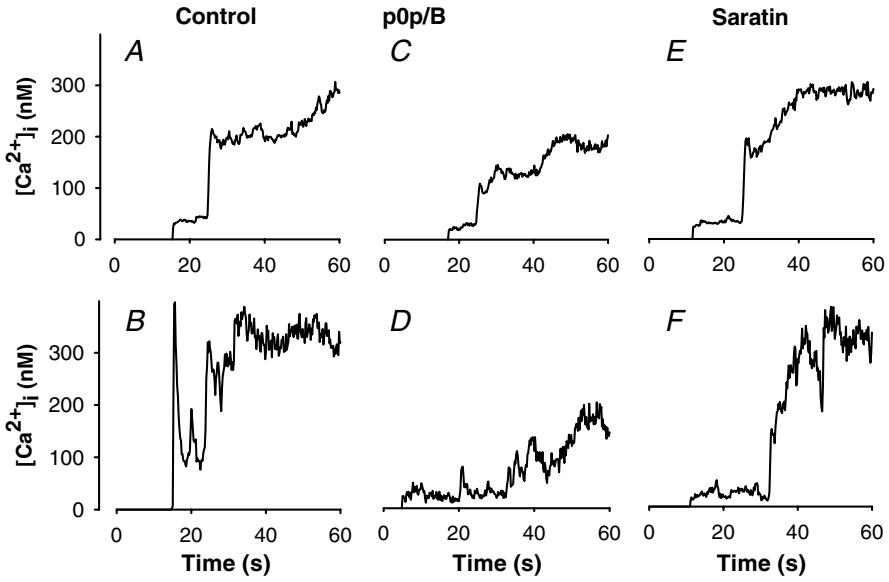

Figure 4. Potentiating role of GPIb in Ca^{2+} response of collagen-adhering platelets under shear
Whole blood was supplemented with 10% fluo-3-loaded platelets, and perfused over collagen as indicated for Fig. 1. Upper panels show averaged changes in $[Ca^{2+}]_i$ of 13–32 platelets, observed during at least 60 s, from at least two different mice. Lower panels show changes in $[Ca^{2+}]_i$ of representative single platelets. Blood was either untreated (*A, B*) or incubated for 10 min prior to perfusion with 40 μg ml^{-1} p0p/B Fab (*C, D*) or 10 μg ml^{-1} saratin (*E, F*).

Again, many of the fluo-3-labelled platelets moved over the surface before adhering stably. The Ca^{2+} responses of these platelets were typically delayed (Fig. 4*E* and *F*). The median lag time (time measured from first collagen contact until $[Ca^{2+}]_i$ rise) was notably increased from 9 to 13 s compared to control platelets. Maximal $[Ca^{2+}]_i$ rises after the delay were unchanged with saratin. These data indicated that the blocking of GPIb and the absence of vWF delayed and destabilized platelet–collagen contact and subsequent signalling. This points to a stimulating role of GPIb and vWF in GPVI-induced Ca^{2+} signalling. Since PS exposure relies on a persistent $[Ca^{2+}]_i$ rise, the reduced or delayed Ca^{2+} signal with p0p/B or saratin can explain the lower PS exposure under these flow conditions (see Fig. 2*A*).

Role of integrin αIIbβ3 and GPIb in PS exposure in platelets adhering to vWF–collagen

Stable adhesion to vWF requires platelet interaction with both GPIb-V-IX and activated integrin αIIbβ3 (Savage *et al.* 1998; Wu *et al.* 2000; Nesbitt *et al.* 2002; Patel *et al.* 2003). To interfere with the contribution of αIIbβ3, the murine blood was incubated with Fab fragments (40 μg ml^{-1}) of JON/A, which is a unique, recently characterized antibody that blocks αIIbβ3 binding sites for vWF and fibrinogen (Bergmeier *et al.* 2002; Grüner *et al.* 2003). This is in contrast to anti-aggregatory peptide inhibitors of αIIbβ3, which only react with the fibrinogen binding site. Upon perfusion of blood in the presence of JON/A, platelets adhered to vWF–collagen as single cells or two-layered microaggregates (Fig. 5*A–D*). Surface coverage with platelets was reduced to 44 ± 15% ($n = 7$) of control (Fig. 5*I*). PS exposure in platelets on collagen was greatly lowered to 7 ± 3% of control. In comparison, when effects of autocrine ADP were blocked with the $P2Y_1$ and $P2Y_{12}$ receptor antagonists MRS2179 (20 μM) and AR-C69931MX (50 μM), respectively, this gave a similar decrease in platelet deposition (area coverage with platelets decreased to 48 ± 9% of control; not shown), but a weaker decrease in PS exposure (38 ± 17% of control).

To test whether the inhibitory effects of JON/A were dependent on GPIb and vWF, combined treatments were carried out with either p0p/B or saratin (Fig. 5*E–H*). The addition of p0p/B did not further reduce platelet deposition nor PS exposure in comparison to JON/A alone (Fig. 5*I*). Perfusion with saratin plus JON/A resulted in a somewhat lower platelet deposition, and an unchanged reduction in PS-exposing platelets. These data suggest that GPIb and integrin αIIbβ3 influenced platelet activation at least in part via a common route involving vWF.

Increased role of GPIb in PS exposure by platelets with reduced expression of GPVI

Since GPVI activity is a limiting factor in collagen-dependent platelet activation and aggregation (Chen & Kahn, 2003), the effect of GPIb inhibition was also examined under conditions of reduced GPVI expression. Therefore, blood was used from mice which were partially or completely deficient in FcR γ-chain. As expected, homozygous deficiency resulted in undetectable levels of both FcR γ-chain and GPVI on platelets, while heterozygous deficiency gave a 50% reduction of both proteins (Table 1). Expression levels of GPIb-V-IX and αIIbβ3 were unchanged in these animals. When blood from heterozygous mice was perfused over vWF–collagen (1000 s^{-1}), platelet deposition was about linear with time. In a typical experiment, the increase in surface area coverage was 2.3% min^{-1} ($R^2 = 0.44$) in comparison to 5.5% min^{-1} ($R^2 = 0.93$) for wild-type blood. At the 4-min end-point, this resulted in an approximately 42% lower platelet adherence for the FcR γ-chain +/− mice (Fig. 6*A–D*). Exposure of PS was also about 40% reduced in comparison to wild-type (Fig. 6*I*). Strikingly, blockage of GPIb with p0p/B fragments in the heterozygous blood nearly completely abolished stable platelet adhesion, while PS-exposing platelets were hardly detectable (Fig. 6*E–F*). In agreement with earlier results (Nieswandt *et al.* 2001*a*; Kuijpers *et al.* 2003), perfusion of blood from homozygous FcR γ-chain −/− mice under these conditions resulted in diminished platelet adhesion to the collagen. During flow, most FcR γ-chain −/− platelets adhered only transiently to the surface (putatively via GPIb) and then moved on. After 4 min, a limited number of stably adherent platelets was detected (Fig. 6*G–H*), interestingly especially at sites with a higher density of collagen fibres. These platelets neither showed morphological signs of activation nor exposed PS, confirming the essential role of GPVI in these platelet responses.

Discussion

In this study with mouse blood, we have evaluated the contribution of GPIb and collagen-bound vWF to shear-dependent platelet adhesion and procoagulant activity during thrombus formation. The results extend earlier work, demonstrating that GPVI has a conditional role in platelet–collagen interaction and subsequent activation both *ex vivo* (Nieswandt *et al.* 2001*a*; Kuijpers *et al.* 2003) and in denuded artery *in vivo* (Massberg *et al.* 2003). In the present study we find that, at high shear rates up to 1500 s^{-1} (mimicking those at the arterial wall), the

GPIb interaction with vWF deposited on collagen enhances irreversible platelet binding and GPVI-induced PS exposure, along with stimulation of the rise in $[Ca^{2+}]_i$, which is a prerequisite for exposure of procoagulant PS. This response must be regulated separately from platelet aggregate formation, as the PS-exposing cells are usually not found in aggregates. Importantly, at reduced levels of GPVI we find that the procoagulant response is highly dependent on GPIb. Inhibitor studies indicate that αIIbβ3, which also binds to vWF, has an overlapping role with GPIb, underlining the important adhesive function of this integrin, which is mostly considered subordinate to its function in platelet aggregation. As exposed PS potently enhances thrombin generation, in the platelet–collagen interaction either of the two adhesive glycoproteins appears to potentiate the two haemostatic processes contributing to thrombus formation: aggregate formation and coagulant activity.

Human and murine vWF and GPIb-V-IX play an initial role in tethering and adhesion to collagen under high shear flow conditions, and thereby mediate platelet aggregate formation on collagen (Wu *et al.* 2000; Goto *et al.* 2002; Savage *et al.* 2002). In addition, both vWF and the GPIb-V-IX complex are involved in thrombin generation and

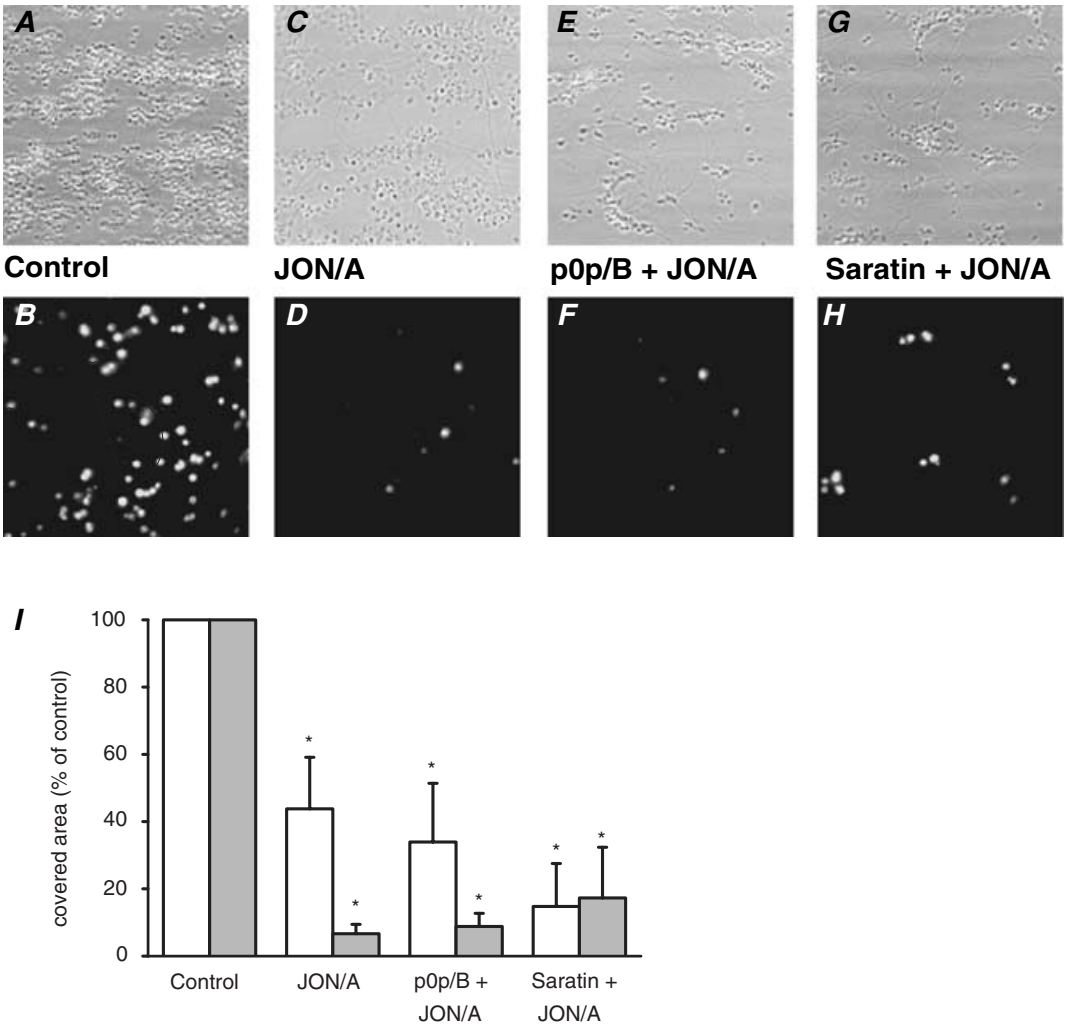

Figure 5. Role of integrin αIIbβ3 in platelet deposition and PS exposure
Whole blood from wild-type mice was perfused over type-I collagen as indicated for Fig. 1. Upper panels: representative phase contrast images (120 × 120 μm) after 4 min of perfusion. Lower panels: fluorescence images (150 × 150 μm) after staining with FITC-labelled annexin A5. Blood was untreated (*A, B*), or treated with 40 μg ml^{-1} anti-αIIbβ3 JON/A Fab fragment alone (*C, D*), with JON/A plus 40 μg ml^{-1} p0p/B Fab fragment (*E, F*), or with 10 μg ml^{-1} saratin (*G, H*) 10 min prior to perfusion. *I,* surface area coverage of all platelets (white bars) and PS-exposing platelets (grey bars) of conditions described in panels *A–H*. Data are given as percentages of control condition (mean values ± S.E.M., $n = 3$–11). *$P < 0.05$ compared to control.

Table 1. Surface expression of different glycoproteins on platelets from wild-type and FcR γ-chain deficient mice

Glycoprotein(s)	Wild-type	FcRγ +/−	FcRγ −/−
GPVI	48.3 ± 8.7	27.2 ± 9.3*	6.4 ± 1.9*
GPIIa (β1)	135.6 ± 11.2	131.7 ± 13.1	133.3 ± 12.8
GPIa (α2)	47.2 ± 8.5	45.5 ± 7.3	44.8 ± 7.9
GPIIb/IIIa (αIIbβ3)	395.8 ± 30.4	416.2 ± 32.5	399.4 ± 33.7
GPV	187.2 ± 19.3	179.4 ± 16.7	185.1 ± 20.5
CD9	532.3 ± 38.2	535.7 ± 41.4	528.7 ± 35.6

Expression levels were determined by flow cytometry using fluorophore-labelled monoclonal antibodies, as described (Nieswandt *et al.* 2000). Platelets were gated by their forward scatter/side scatter characteristics. Results are expressed as mean log fluorescence (arbitrary units) ± S.D. for 6 mice per group. *$P < 0.01$ compared to wild-type.

coagulation, perhaps because of the binding to multiple coagulation proteins, including factor VIII, factor XI, thrombin and fibrin (Beguin *et al.* 1999; Baird & Walsh, 2002; Andrews *et al.* 2003; Keuren *et al.* 2003). Here, we report on a procoagulant effect of vWF and GPIb, i.e. PS exposure, linked to collagen receptor stimulation, which is independent of the formation of thrombin. Whether vWF and GPIb may also contribute to platelet PS exposure in coagulating plasma is still unknown. However, recent data indicate that GPIb can stimulate thrombin formation (in the absence of collagen) in a shear-dependent way (Keuren *et al.* 2003). It is thus conceivable that the function of GPIb in thrombin formation also involves a shear-dependent stimulation of PS exposure, similar to its function we report here for platelet–collagen interaction.

The data with anti-GPIb p0p/B Fab fragment or saratin (which prevents vWF binding to collagen) show that platelets can still bind to collagen in the absence of the vWF–GPIb interaction up to a shear rate of 1500 s^{-1}. Others have described that GPIb, in the absence of vWF, is still free to bind thrombospondin, which also mediates platelet adhesion under flow (Jurk *et al.* 2003). Thus, GPIb can still have a role at intermediate shear rates independently of collagen-bound vWF. However, we find that residual, vWF-independent adhesion is mostly due to GPVI or integrin α2β1, as it is abolished in the absence of functional collagen receptors (M. Kuijpers, unpublished results). On the other hand, p0p/B or saratin treatment did lead to a decreased deposition of platelets (Fig. 1), and a reduced or delayed Ca^{2+} response and PS exposure (Fig. 4). This could indicate that the GPIb-dependent spiking elevations in $[Ca^{2+}]_i$ reported in platelets binding to immobilized vWF (Mazzucato *et al.* 2002; Nesbitt *et al.* 2002; Mangin *et al.* 2003) also contribute to the Ca^{2+} signal in the presence of collagen. We have shown previously that collagen-induced Ca^{2+} signalling in murine platelets is almost completely dependent on the presence of the GPVI–FcR γ-chain complex, with minute spiking $[Ca^{2+}]_i$ increases remaining in FcRγ −/− platelets (Kuijpers *et al.* 2003), which might be the result of the vWF–GPIb interaction. Accordingly, GPVI must be responsible for the majority of the Ca^{2+} signal, while GPIb makes only a small contribution. Taken together, these data point to an enhancement of GPVI signalling by GPIb–vWF, by direct signalling and/or allowing full engagement of the GPVI receptors.

The Fab fragment JON/A blocks binding of the αIIbβ3 to fibrinogen and vWF (Gruner *et al.* 2003), and thus provides a unique tool to study the role of this integrin in adhesion and aggregation. Incubation with JON/A resulted in a stronger inhibition of platelet deposition and PS exposure than the blocking of ADP function induced by receptor antagonists.This confirms the concept that αIIbβ3 is involved in stable adhesion to this surface (Savage *et al.* 1998). Furthermore, combined blockage of GPIb and integrin αIIbβ3 did not further reduce platelet activation in comparison to αIIbβ3 inhibition alone. This places the function of integrin αIIbβ3 downstream of GPIb, which is in good agreement with the current model of vWF–platelet interaction. This model presumes initial shear-dependent tethering via GPIb-V-IX, subsequent stable adhesion and enhanced signalling via activated integrin αIIbβ3 (Savage *et al.* 1998; Nesbitt *et al.* 2002). This is also in line with *in vivo* findings with mice that platelet adhesion to the vessel wall involves multiple integrin–ligand interactions, none of which are essential by themselves (Grüner *et al.* 2003), and that β3 deficiency results in defective stable platelet adhesion to the vessel wall (Wagner & Burger, 2003).

For mice with reduced levels of GPVI–FcR γ-chain it is demonstrated that, under static conditions, neither platelet adhesion to immobilized collagen nor protein tyrosine phosphorylation induced by collagen is affected compared to wild-type (Snell *et al.* 2002). Here we show, with mice expressing half of the normal GPVI–FcR γ-chain levels, that under flow conditions both platelet adhesion on collagen and platelet activation – as apparent from PS exposure – are reduced by about 50%. This indicates that the level of GPVI expression determines (and is a limiting factor for) signalling under shear. This conclusion is in agreement with the findings that collagen-induced Ca^{2+} responses of murine platelets are dependent on the GPVI expression level (Chen *et al.* 2002). Similarly, in human tissue, partial blockage of GPVI has been shown to suppress aggregate formation and PS exposure under flow (Siljander *et al.* 2004).

Thus, the regulatory function of this collagen receptor in platelet activation and procoagulant activity appears to be conserved between species.

Interestingly, in FcR γ-chain heterozygous mice, expressing normal GPIb and αIIbβ3 levels on platelets, the blockage of GPIb caused almost complete abolition of stable platelet adhesion and PS exposure under shear (Fig. 6). This indicates that GPIb plays a more prominent role in platelet–collagen interaction at reduced levels of GPVI/FcR γ-chain. Apparently, there is some redundancy in GPIb and GPVI receptor function. It is likely to be of physiological relevance, given the large variation in GPVI expression between humans (Chen *et al.* 2002; Best *et al.* 2003; Joutsi-Korhonen *et al.* 2003). As also $\alpha 2\beta 1$ stimulates GPVI activity, the present results point to receptor interplay in the regulation of platelet procoagulant activity that is not restricted to collagen receptors, but extends to the GPIb-V-IX complex.

Earlier it has been proposed that the FcR γ-chain mediates GPIb-induced platelet activation (Falati *et al.* 1999; Canobbio *et al.* 2001). However, our data do not support this concept, as we find that partial knock-out

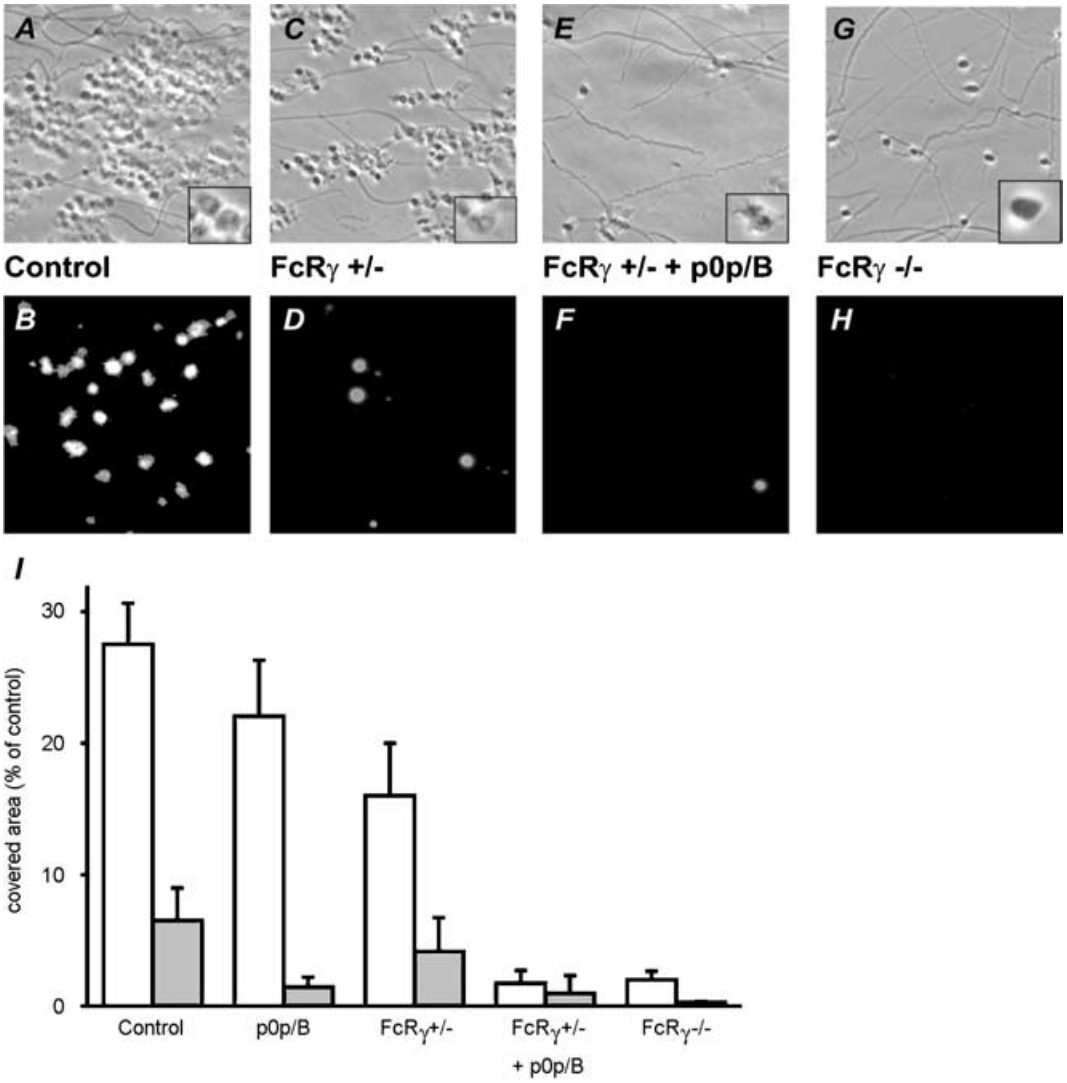

Figure 6. Increased role of GPIb in platelet deposition and PS exposure upon reduced expression of GPVI
Blood from wild-type and FcR γ-chain +/– and –/– mice was perfused over collagen (see Fig. 1). Wild-type and FcR γ-chain +/– blood were pre-incubated with 40 μg ml^{-1} p0p/B Fab as indicated. Upper panels (*A*, *C*, *E*, *G*): representative phase contrast images (48 × 48 μm) after 4 min perfusion. Inserts are 200% magnifications showing platelet morphology. Lower panels (*B*, *D*, *F*, *H*): fluorescence images (150 × 150 μm) after staining with FITC-labelled annexin A5. *I*, surface area coverage of all platelets (white bars) and PS-exposing platelets (grey bars). Data are mean values ± S.E.M. ($n = 3$–5).

of the γ-chain enhances the GPIb functioning. On the other hand, our findings are fully in line with those of Mangin *et al.* (2003), who propose that in mice GPIb signalling to Ca^{2+} flux and filopod extension relies on Src kinases via a pathway distinct from FcR γ-chain derived signalling.

Perfusion of blood from homozygous FcR γ-chain deficient mice resulted in only few adhering platelets, especially at sites where the collagen density was relatively high (Fig. 6). This is in apparent contrast to recent findings with GPVI knockout mice, where full adhesion to collagen was detected under flow conditions (Kato *et al.* 2003). The difference with the present data is likely explained by differences in methodology (collagen surface, anti-coagulant, mepacrine labelling, etc.). Preliminary data in our laboratory indicate that the few GPVI/FcRγ −/− platelets adhere in a GPIb/α2β1-dependent way (unpublished results). This is similar to the situation with human platelets, where it also has been shown that α2β1 is adhesive in the absence of GPVI signalling (Siljander *et al.* 2004).

In summary, the current data, together with the earlier established role of integrin α2β1 (Nieswandt *et al.* 2001*a*; Kuijpers *et al.* 2003; Nieswandt & Watson, 2003), confirm that murine GPVI is the primary trigger of activation in platelet–collagen interaction under shear. Further, it is shown that GPVI is facilitated by GPIb-V-IX in addition to integrins α2β1 and αIIbβ3. Platelet adhesion and subsequent activation evolves as a series of multireceptor events with redundancy, facilitation and mutual interactions, rather than as a linear process. Specifically, concerning the procoagulant platelet reaction, both GPIb and αIIbβ3 appear to control PS exposure under these shear conditions, likely by enhancing GPVI-dependent activation. The finding that these adhesive receptors in concert with GPVI have a procoagulant role in collagen-dependent thrombus formation indicates that interplay does not only occur between the collagen receptors, but also extends to the GPIb-V-IX complex.

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Supplementary material

The online version of this paper can be accessed at:
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