Differential effects of reduced glycoprotein VI levels on activation of murine platelets by glycoprotein VI ligands

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We have investigated the effects of decreased levels of the complex between glycoprotein VI (GPVI) and the Fc receptor γ -chain (FcR γ) on responses to collagen and GPVI-specific ligands in murine platelets. We show that levels of GPVI–FcR γ of the order of 50 % and 20 % of wild-type levels caused 2- and 5-fold shifts to the right respectively in the dose–response curve for aggregation in response to collagen, the snake toxin convulxin and the monoclonal antibody JAQ1. In addition, there is a delay in the onset of aggregation in response to collagen. In contrast, the stimulation of protein tyrosine phosphorylation by collagen (as measured after 150 s) and adhesion to a collagen-coated surface under static conditions were unaffected in platelets with 50 % and 20 % of wild-type levels of GPVI. In contrast, responses to a collagen-related peptide (CRP), made up of repeat glycine-proline-hydroxyproline motifs, were markedly inhibited and

abolished in platelets expressing 50 % and 20 % of wild-type levels of GPVI respectively. We suggest that the marked effect of a reduction in GPVI levels on the CRP-induced activation of platelets is due to the multivalent nature of CRP and the fact that GPVI is its sole receptor on platelets. Thus it appears that the interaction of CRP with GPVI is determined by a combination of affinity and avidity. The observation that collagen does not behave like CRP in platelets expressing reduced levels of GPVI, even in the combined presence of blocking antibodies against integrin $\alpha 2\beta 1$ and GPV, suggests that collagen has a greater affinity than CRP for GPVI, and/or that other receptors are involved in its binding to platelets. The clinical significance of these results is discussed.

Key words: aggregation, collagen, FcR γ -chain, GPVI, platelets.

INTRODUCTION

Over the course of the last 20 years, a remarkable number of platelet surface proteins have been proposed as receptors for the subendothelial matrix protein collagen. Within this group, attention has focused on the integrin $\alpha 2\beta 1$ [also known as glycoprotein Ia–IIa (GPIa–IIa)] and the immunoglobulin-domain-containing GPVI as the major receptors underlying the interaction. It was originally proposed that these two receptors interact in a two-site/two-state model, with integrin $\alpha 2\beta 1$ being critical for adhesion and GPVI for activation. More recently, several lines of evidence have emerged which support a pivotal role for GPVI in both of these events [1–3]. In a revised two-site/two-state model, it is now proposed that the initial interaction of collagen with platelets is via GPVI, leading to activation of integrin $\alpha 2\beta 1$ and a strengthening of adhesion [1,2]. This model is supported by observations on platelets from integrin $\beta 1$ - and $\alpha 2$ -deficient mice which demonstrate that the integrin is not essential for platelet activation by collagen [2,4].

GPVI is present on the platelet surface in a complex with the Fc receptor γ -chain (FcR γ) [5,6]. The latter is necessary for the surface expression of GPVI in platelets and for the regulation of intracellular signalling events. Mice engineered to lack expression

of FcR γ fail to express GPVI on their platelets [7]. In line with this, a patient with a marked reduction in GPVI levels was found to have a corresponding reduction in FcR γ [6]. FcR γ is also required for expression of several other Ig-domain-containing receptors on the surface of other haematopoietic cells, including the Fc receptors for IgG (Fc γ RI and III) and IgE (Fc ϵ RI) [8] and the paired immunoglobulin-like receptor-A (PIR-A) [9,10]. Signalling via GPVI is believed to take place entirely through FcR γ . Activation of GPVI leads to tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) in FcR γ by a Src-like kinase [11,12] and subsequent recruitment of the tyrosine kinase Syk [13]. This initiates a series of events that culminate in activation of phospholipase C $\gamma 2$, phosphoinositide 3-kinase and other downstream effector enzymes.

The critical role of GPVI in platelet activation has been demonstrated using GPVI-deficient platelets and selective agonists [14]. A number of synthetic collagen-related peptides (CRPs) have been developed by the group of Barnes and Farndale [15] which activate GPVI but are inactive on integrin $\alpha 2\beta 1$. These peptides are based on a repeat glycine-proline-hydroxyproline (GPO) motif and are cross-linked by cysteine or lysine residues in their N- and C-termini. Several snake venom toxins activate platelets through GPVI [16]. The snake C-type lectin convulxin, from the tropical rattlesnake *Crotalus durissus terrificus*, was the

Abbreviations used: CRP, collagen-related peptide; FcR γ , Fc receptor γ -chain; FcR γ -Tg mice, transgenic mice expressing a transgene for FcR γ in an FcR γ -null background; FcR γ -Tg/KO mice, cross between FcR γ -Tg and FcR γ -deficient mice; GPO motif, glycine-proline-hydroxyproline motif; GPVI (etc.), glycoprotein VI (etc.); HRP, horseradish peroxidase; mAb, monoclonal antibody; PRP, platelet-rich plasma; TBS-T, Tris-buffered saline containing Tween.

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first member of this group to be identified and is the best characterized. A number of antibodies to GPVI have been developed, including the rat monoclonal antibody (mAb) JAQ1, which recognizes murine GPVI [7].

Recently, studies in GPVI-transfected cells have demonstrated that a high level of expression of the receptor, approaching that found in platelets, is necessary to confer adhesion and activation in response to collagen [17,18]. In the present study, we have investigated whether this relationship also holds for collagen in platelets, as well as for a number of GPVI-specific agonists. A reduction in expression of the GPVI-FcR γ complex has been achieved using mice that are heterozygous for FcR γ , or FcR $\gamma^{-/-}$ mice that express a transgene for FcR γ .

EXPERIMENTAL

Animals

C57Bl/6 mice deficient in FcR γ were obtained as previously described [19] or from Taconics (Germantown, NY, U.S.A.) FcR γ heterozygous mice were generated by crossing FcR γ -deficient mice with wild-type mice. C57Bl/6 FcR γ transgenic (FcR γ -Tg) mice express a transgene for FcR γ in an FcR γ -null background; the engineering of these mice will be described elsewhere (K. Arase, D. Sakurai and T. Saito, unpublished work; details on application to K.A.). The FcR γ -Tg mice were bred with FcR γ -deficient mice to reduce the level of expression of FcR γ ; these mice are termed FcR γ -Tg/KO mice. Specific-pathogen-free C57Bl/6 mice were obtained from Charles River (Sulzfeld, Germany) or Harlan SERA-LAB Ltd. (Belton, Leics., U.K.).

Materials

Horm collagen (predominately Type I; derived from equine tendon) was from Nycomed (Munich, Germany). Convulxin and FITC-labelled convulxin were kindly donated by Dr Mireille Leduc and Dr Cassian Bon (Unite des Venins, Institut Pasteur, Paris, France). CRP [GKO(GPO)₁₀GKOG; single-letter code, where O is hydroxyproline] was synthesized by Tana Laboratories (Houston, TX, U.S.A.); it was cross-linked with 0.25 % glutaraldehyde for 3 h on ice and then dialysed into PBS. All salts and Nonidet P-40 were purchased from BDH-Merck (Poole, Dorset, U.K.). The mAb JAQ1 was produced as described previously [7]. Polyclonal rabbit antibodies against murine FcR γ were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Hamster anti-(α 2 integrin) (Ha1/29) was purchased from BD Pharmingen (Oxford, U.K.). The anti-GPV antibody was kindly provided by Dr Francois Lanza (INSERM, Strasbourg, France).

Preparation of mouse platelets

Blood (750–1000 μ l) was taken into 300 μ l of heparin solution (10 units/ml) by cardiac puncture under terminal CO₂ narcosis. Alternatively, mice were bled under terminal ether anaesthesia from the retro-orbital plexus and blood was collected in a tube containing 7.5 units/ml heparin. Platelets obtained using either procedure gave the same results.

Platelet-rich plasma (PRP) was obtained by centrifugation at 300 *g* for 10 min at room temperature. PRP was centrifuged at 1000 *g* in the presence of prostacyclin (0.1 μ g/ml) for 6 min at room temperature. Pelleted platelets were resuspended in modified Tyrode's/Hepes buffer (134 mM NaCl, 0.34 mM Na₂HPO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM Hepes, 5 mM glucose, 1 mM MgCl₂, pH 7.3) to the required concentration and left for 30 min at 30 °C prior to stimulation. All experiments were

performed at 37 °C in siliconized glass tubes with continuous stirring. Agonists were added as 10–100-fold concentrates.

Aggregometry

Platelet aggregation was monitored in PRP. To determine aggregation, light transmission was measured relative to that in platelet-poor plasma. Transmission was recorded on a BioData PAP-4 (Alpha Laboratories, Eastleigh, Hants., U.K.) or a FibrinTimer 4-channel aggregometer (APACT Laborgeräte und Analysensysteme, Hamburg, Germany) over 10 min, and the transmission with PRP was expressed as a percentage of that with platelet-poor plasma.

Protein phosphorylation studies

Platelet stimulation was carried out using 500 μ l of platelet suspension containing 2×10^8 cells/ml. All experiments were carried out in the presence of the GPIIb–IIIa antagonist lotrafiban (10 μ M). For measurement of whole-cell tyrosine phosphorylation, reactions were stopped by the addition of an equal volume of Laemmli sample buffer, boiled for 5 min and separated by SDS/PAGE on 4–12 % (w/v) pre-cast Bis-Tris gels (NuPage; Invitrogen) under reducing conditions before transfer to a PVDF membrane. For measurement of GPVI and FcR γ , proteins were separated on 10 % (w/v) acrylamide gels under non-reducing and reducing conditions respectively. Membranes were blocked by incubation with TBS-T (Tris-buffered saline containing Tween; 20 mM Tris/137 mM NaCl/0.1 % Tween 20, pH 7.6) containing 10 % (w/v) BSA. Antibodies were diluted 1:1000 in blocking buffer and incubated with blots for 1 h at room temperature. Membranes were washed twice for 30 min in TBS-T before incubation for 1 h with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:10000 in TBS-T. Following washing in TBS-T as above, the membranes were developed using an enhanced chemiluminescence detection system.

Glycoprotein receptor densities

Flow cytometry was performed by incubating 10^6 washed platelets with 10 μ g/ml phycoerythrin-conjugated mAb JAQ1 for 20 min at room temperature. Samples were analysed on FACScalibur (Becton Dickinson, Heidelberg, Germany). Western blotting for GPVI was carried out by lysis of 10^8 unstimulated washed platelets in non-reducing Laemmli sample buffer. A volume of 20 μ l of platelet lysate was separated by SDS/PAGE as described above. GPVI was detected using HRP-conjugated JAQ1 (10 μ g/ml) and visualized by enhanced chemiluminescence as above.

Adhesion

Static adhesion assays were performed with washed platelets from the indicated mice in modified Tyrode's buffer containing 1 mM MgCl₂ and 1 mM CaCl₂ on 96-well plates (Nunc, Wiesbaden, Germany) coated with different concentrations of Horm collagen as described previously [14].

Data analysis

Results are shown as means $\pm$ S.D. Statistical analyses were performed using Student's *t* test, with *P* < 0.05 taken as the level of significance.

RESULTS

Modulation of GPVI–FcR γ expression in murine platelets

We used the observation that the expression of GPVI on murine platelets is dependent on the presence of FcR γ [7] to study platelets with decreased levels of GPVI. Platelets from mice that were heterozygous for FcR γ (+/–) had 50 % of wild-type levels of FcR γ and a comparable decrease in GPVI levels, as measured by flow cytometry and Western blotting (Figure 1). We also used platelets from mice that were deficient in FcR γ but which also expressed a transgene for FcR γ (FcR γ -Tg mice). Platelets from these mice expressed 40 % of the control level of FcR γ (results not shown). Platelets from a cross between the FcR γ -Tg and

FcR γ -deficient mice (FcR γ -Tg/KO mice) expressed 20 % of the wild-type level of FcR γ (Figure 1B). The level of GPVI on platelets from these mice was also 20 % of control, as revealed by Western blotting and flow cytometry (Figure 1). Importantly, the level of expression of the integrin $\alpha 2\beta 1$ was not altered in the platelets from heterozygote or FcR γ -Tg/KO mice, as measured by Western blotting (results not shown). The observation that the level of GPVI is directly related to the level of FcR γ is consistent with the need for formation of a complex between the two proteins to ensure stable expression in platelets, and suggests that all of the GPVI is associated with the FcR γ in the cell. Flow cytometric analysis showed that the level of the collagen receptor GPIa–IIa or of integrin $\alpha 2\beta 1$, as well as of several other surface glycoproteins, was unaffected by a decrease in the levels of the GPVI–FcR γ complex (Table 1).

Figure 1 Different levels of GPVI expression in platelets from FcR γ heterozygous and transgenic mice

(A) Flow cytometric analysis of GPVI expression levels using phycoerythrin-conjugated mAb JAQ1 on platelets from wild-type (wt), FcR γ heterozygous (+/–), FcR γ -Tg/KO (Tg) and FcR γ -deficient (–/–) mice. Results are shown as means $\pm$ S.D. for groups of six mice. (B) Platelet proteins were separated by SDS/PAGE, transferred to PVDF membranes and immunoblotted with HRP-labelled JAQ1 (anti-GPVI) or anti-FcR γ and anti-rabbit–HRP.

Table 1 Surface expression of glycoproteins on platelets from control (+/+), heterozygous (+/–) and FcR γ chain-null (–/–) mice

The surface expression of the indicated glycoproteins was detected by flow cytometry. Platelets were gated by FSC/SSC characteristics. Results are expressed as means $\pm$ S.D. for six mice per group.

Glycoprotein	log [Fluorescence (units)]		
	+/+	+/-	-/-
GPVI	51.6 $\pm$ 8.7	28.3 $\pm$ 10.8	6.6 $\pm$ 2.3
GPIa ($\alpha 2$)	42.3 $\pm$ 7.5	43.8 $\pm$ 8.3	41.7 $\pm$ 6.5
GPIIa ($\beta 1$)	133.7 $\pm$ 17.6	130.2 $\pm$ 14.1	130.3 $\pm$ 12.2
GPIIb/IIIa	350.8 $\pm$ 19.5	358.5 $\pm$ 21.4	355.6 $\pm$ 25.8
GPIb–IX	288.3 $\pm$ 26.1	279.7 $\pm$ 22.8	280.3 $\pm$ 30.4
GPV	145.4 $\pm$ 13.2	150.4 $\pm$ 15.3	149.7 $\pm$ 15.5
CD9	549.5 $\pm$ 42.1	541.7 $\pm$ 49.6	551.3 $\pm$ 46.6

Influence of the level of GPVI–FcR γ expression on responses to collagen

The dose–response curve for stimulation of aggregation by collagen was right-shifted by approx. 2- and 5-fold in platelets expressing levels of 50 % and 20 % of GPVI–FcR γ complex respectively relative to wild-type controls (Figure 2A). In all cases, a high concentration of collagen induced a maximal aggregation response. However, there was a clear delay in the onset of aggregation in response to low concentrations of collagen in the platelets from FcR γ (+/–) and FcR γ -Tg/KO mice. This is illustrated in Figure 2(B) for a concentration of 2 μ g/ml collagen, whereby a 50 % reduction in the level of GPVI–FcR γ complex led to a significant increase in the time taken for the aggregation trace to fall below the basal level after the initial shape change response, namely from 59 $\pm$ 15 to 103 $\pm$ 30 s (n = 10 per group; P < 0.05). We also investigated whether the dose–response curve for tyrosine phosphorylation was also right-shifted following a decrease in GPVI levels. Due to the varying delay in activation, tyrosine phosphorylation was measured at 150 s, at which time the aggregation response had reached a plateau in all groups of platelets. There was no detectable reduction in tyrosine phosphorylation in platelets expressing 50 % and 20 % levels of GPVI as compared with that in platelets expressing control (100 %) GPVI levels (Figure 3), although phosphorylation was blocked in FcR γ -deficient platelets (results not shown). In all cases, aggregation in response to thrombin was not altered in platelets expressing a reduced level of GPVI (results not shown).

The effect of reduced GPVI levels on platelet adhesion to collagen was also investigated under static conditions. Adhesion to collagen was dose-related over the concentration range of 0.05–10 μ g of collagen per well. Platelets with 50 % and 20 % of control levels of GPVI–FcR γ adhered to collagen to the same extent as control platelets at all concentrations when measured at 15 min (results not shown) and 60 min (Figure 2C). FcR γ -null platelets did not adhere to the collagen-coated surface (results not shown), as reported previously [2].

Effects of blocking antibodies to other collagen receptors on platelets expressing a reduced level of GPVI

It has been proposed that the interaction of collagen with GPVI–FcR γ is supported by other receptors, including integrin $\alpha 2\beta 1$ and GPV. We used blocking antibodies to integrin $\alpha 2\beta 1$ and GPV to investigate their importance in platelets expressing a reduced level of GPVI. An integrin $\alpha 2\beta 1$ blocking antibody delayed the onset of aggregation in response to collagen in wild-type platelets, and shifted the curve to the right by approx. 2-fold

Figure 2 Influence of the level of GPVI-FcR γ expression on platelet responses to collagen

(A) Heparinized PRP from wild-type (circles; +/+), FcR γ heterozygous (triangles; +/-) or FcR γ -Tg/KO (squares) mice was incubated with stirring in the presence of different concentrations of collagen. Dose-responses to collagen are shown as means $\pm$ S.D. ($n = 6-10$). (B) Aggregation traces for the indicated concentration of collagen. (C) Washed platelets from wild-type (circles), FcR γ heterozygous (triangles) or FcR γ -Tg/KO (squares) mice were incubated for 60 min in microtitre plates coated with the indicated collagen concentrations, and adherent platelets were quantified fluorimetrically (OD = absorbance). The data shown are representative of three identical experiments and are expressed as means $\pm$ S.D. of triplicate readings.

Figure 3 Effect of a decrease in GPVI-FcR γ levels on protein tyrosine phosphorylation in response to collagen

Washed platelets from mice expressing 100%, 50% and 20% of control levels of GPVI were incubated at 37 °C and stimulated with the indicated concentrations of collagen for 150 s. Aliquots were lysed by sample buffer and dissolved in reducing SDS buffer. After separation by SDS/4–12%-PAGE and transfer to PVDF membranes, blots were incubated with the anti-phosphotyrosine antibody 4G10, and proteins were detected by anti-mouse IgG–HRP and enhanced chemiluminescence. Results are representative of four experiments. MW, molecular mass; LAT, Linker for Activation of T-cells.

(Figure 4A). In the presence of the blocking antibody, the onset of aggregation was further delayed in the platelets from FcR γ (+/-) mice (results not shown), and the curve was shifted a further 2-fold to the right (Figure 4A). The integrin $\alpha 2\beta 1$ blocking antibody induced an even greater delay in aggregation in the platelets from FcR γ -Tg/KO mice (results not shown), and again shifted the dose-response curve 2-fold further to the right (Figure 4A). A blocking antibody to GPV did not cause an obvious delay in the onset of aggregation and caused a shift of less than 2-fold in the dose-response curve for aggregation in platelets from wild-type, FcR γ (+/-) and FcR γ -Tg/KO mice (Figure 4A). The combination of antibodies to integrin $\alpha 2\beta 1$ and GPV caused a similar delay in the onset of aggregation and shift in the dose-response curve to collagen to those caused by the integrin $\alpha 2\beta 1$ blocking antibody alone (results not shown).

We have previously reported that the anti-GPVI mAb JAQ1 prevents the aggregation of platelets induced by low concentrations of collagen and reduces the rate of response to concen-

trations of 5 $\mu\text{g/ml}$ collagen and above. On the other hand, it fully inhibits the response to CRP [21]. We have interpreted these results to suggest the presence of two epitopes on GPVI which support binding to collagen, and that JAQ1 blocks the site for interaction with CRP. We investigated here whether platelet aggregation shows a greater dependency on the levels of GPVI following inhibition of the CRP binding site with JAQ1. As shown in Figure 4(B), JAQ1 induced a similar shift to the right in the dose-response curve to collagen in platelets expressing 100%, 50% and 20% levels of GPVI.

Effects of a reduction in GPVI-FcR γ levels on platelet responses to GPVI-specific ligands

The effects of a reduction in the level of the GPVI-FcR γ complex on responses to a number of GPVI-specific ligands were investigated. The dose-response curves for aggregation in response to the snake venom toxin convulxin and cross-linked mAb JAQ1

Figure 4 Effects of blocking antibodies against integrin $\alpha 2\beta 1$, GPV and GPVI on aggregation in platelets expressing a reduced level of GPVI

(A) Heparinized PRP from wild-type (+/+), FcR γ heterozygous (+/-) or FcR γ -Tg/KO (Tg -/-) mice was incubated with stirring with control IgG (circles), anti-GPV (squares) or anti- $\alpha 2$ (triangles) monoclonal antibodies (all 20 μ g/ml) for 5 min before addition of collagen (0.1–10 μ g/ml). (B) Heparinized PRP from wild-type (+/+), FcR γ heterozygous (+/-) or FcR γ -Tg/KO (Tg -/-) mice was incubated under stirring conditions in the absence (black symbols) or presence (grey symbols) of mAb JAQ1 (20 μ g/ml) for 5 min before addition of collagen (0.5–20 μ g/ml). Results are shown as means $\pm$ S.D. (n = 6–10).

Figure 5 Effect of a decrease in GPVI-FcR γ levels on aggregation in response to GPVI-specific ligands

Heparinized PRP from wild-type (circles), FcR γ heterozygous (triangles) or FcR γ -Tg/KO (squares) mice was stimulated with different concentrations of convulxin (A), cross-linked mAb JAQ1 (JAQ1 xl) (B) or CRP (C). Results are shown as means $\pm$ S.D. (n = 6–10).

were shifted approx. 2- and 5-fold to the right in platelets expressing 50 % and 20 % respectively of the endogenous level of GPVI-FcR γ (Figures 5A and 5B). In marked contrast, the dose-response curve for aggregation to the synthetic GPVI

ligand CRP was shifted 20-fold to the right in the FcR γ (+/-) platelets and abolished in cells expressing 20 % of the endogenous level of GPVI (Figure 5C). Consistent with these observations, the dose-response curve for tyrosine phosphorylation to con-

Figure 6 Effect of a decrease in GPVI-FcR γ levels on protein tyrosine phosphorylation in response to convulxin and CRP

Washed platelets were stimulated with the indicated concentrations of convulxin (CVX) or CRP for 150 s and reactions were stopped by the addition of reducing sample buffer. After separation by SDS/4–12%-PAGE and transfer to PVDF membranes, blots were incubated with the anti-phosphotyrosine antibody 4G10, and proteins were detected by anti-(mouse IgG)-HRP and enhanced chemiluminescence. Basal phosphorylation for the convulxin samples (from the same experiment) are shown in (B). Results are representative of three experiments. MW, molecular mass.

convulxin was right-shifted in platelets expressing 50 % of the endogenous level of GPVI, and right-shifted further in platelets with 20 % of the control level (Figure 6A). High concentrations of CRP stimulated protein tyrosine phosphorylation in the FcR γ (+/–) platelets (results not shown), but had no effect in platelets expressing 20 % of the control level of the GPVI-FcR γ complex (Figure 6B).

DISCUSSION

The GPVI-FcR γ complex plays a pivotal role in platelet activation by collagen. In the present study, we have investigated the effects of 2- and 5-fold decreases in the level of GPVI-FcR γ in platelets on responses to collagen and GPVI-specific ligands. The results show that functional events induced by collagen, convulxin and cross-linked mAb JAQ1 are inhibited in parallel with a decrease in GPVI levels. Thus platelets expressing 50 % or 20 % of control levels of GPVI-FcR γ exhibit 2- and 5-fold shifts to the right respectively of the dose-response curves to all three agonists. The dose-response curve to collagen, but not those for convulxin or JAQ1, was also shifted to the right by approx. 2-fold in the presence of a blocking antibody to integrin $\alpha 2\beta 1$, and this shift was additive with that induced by a reduction in expression of GPVI-FcR γ . In contrast, a blocking antibody to GPV had little effect on the dose-response curve to collagen in platelets with a reduced level of GPVI, even in the presence of the

integrin $\alpha 2\beta 1$ blocking antibody. Thus these results demonstrate that the sensitivity of platelets to collagen, in terms of aggregation, is related to the density of GPVI, and that this relationship is maintained in the presence of blockers of integrin $\alpha 2\beta 1$ and GPV.

We previously reported that GPVI is essential for platelet adhesion to collagen under static conditions [2]. Interestingly, however, adhesion to a collagen-coated surface was not affected by a decrease of GPVI levels of up to 80 % over a 200-fold range of collagen concentrations. Several factors may contribute to this difference in dependency on GPVI levels with respect to aggregation. A decrease in the level of GPVI causes a delay in the onset of aggregation as a consequence of the decrease in intracellular signalling at the initial stages of the response. This, in turn, would reduce the positive feedback signals from ADP and thromboxanes, by spreading them out over a longer period of time. Aggregation in response to collagen is dependent on the release of secondary mediators [23]. While it seems highly probable that adhesion would also have been impaired at early times, this could not be monitored because of limitations in sensitivity. Such a difference, however, would be enhanced under flow conditions, where initial events are key to thrombus formation. The observation that static adhesion is not impaired in platelets when measured at later times is consistent with the observation that tyrosine phosphorylation was not altered in platelets from FcR γ (+/–) and FcR γ -Tg/KO mice at 150 s.

GPVI is critical for platelet adhesion to collagen [2]. The observation, therefore, that the level of adhesion was not altered by a 5-fold reduction in the level of GPVI indicates that additional receptors must also support the adhesion of platelets to collagen. Moreover, the critical role of GPVI in the adhesion process means that binding of collagen to the other receptors is GPVI-dependent. For example, signals from GPVI increase the affinity of integrin $\alpha 2\beta 1$ and thereby enable it to bind to collagen [3].

A marked difference in the effect of a reduction in GPVI-FcR γ was seen between the synthetic peptide CRP, which consists of GPO repeat sequences, and collagen. Aggregation to CRP was shifted 20-fold to the right in platelets with 50 % of control levels of the GPVI-FcR γ complex and abolished in platelets with 20 % of endogenous levels. This profound effect on the dose-response curve to the multivalent CRP indicates that the response is determined by a combination of receptor affinity and avidity, such that the ‘apparent affinity’ of CRP for the platelet surface is increased through multiple interactions with GPVI. In essence, the binding of one GPO motif of CRP to GPVI facilitates the interaction of a second GPO motif with the glycoprotein as a consequence of proximity, and so on. In contrast, the effect of a reduction in the level of GPVI on aggregation to convulxin and cross-linked mAb JAQ1 was similar to that seen with collagen, despite the fact that both ligands interact with multiple GPVI receptors. This difference is explained by the fact that CRP has many more binding motifs for GPVI than convulxin and JAQ1.

Interestingly, a decrease in the level of GPVI had a differential effect on the dose-response curves for tyrosine phosphorylation to collagen and convulxin. Whereas the response to collagen was not altered, the curve for convulxin was right-shifted by the reduction in GPVI levels. The absence of a shift in the collagen dose-response curve demonstrates that the level of GPVI is not rate limiting for tyrosine phosphorylation. This is consistent with the observation that collagen stimulates a much lower level of tyrosine phosphorylation of the FcR γ and downstream proteins relative to convulxin [22]. Further, this also suggests that the degree of tyrosine phosphorylation is not related to the extent of aggregation, consistent with the dependency of the aggregation

response to collagen on the release of ADP and thromboxanes [23,24]. In contrast, ADP and thromboxanes play only a minimal role in aggregation in response to convulxin [23].

The question emerges as to what are the key differences between collagen and CRP that influence their differential dependency on the level of expression of GPVI. The ability of collagen to bind to other surface receptors is likely to be a major factor. Blocking antibodies to two collagen receptors on the platelet surface, integrin $\alpha 2\beta 1$ and GPV, however, caused no more than a 2-fold shift to the right of the dose-response curve to collagen in wild-type platelets and cells expressing 20% or 50% of endogenous levels of GPVI, even when given in combination. This demonstrates that the difference between collagen and CRP is not due solely to the ability of collagen to bind to integrin $\alpha 2\beta 1$ and GPV. Other receptors for collagen on the platelet surface have been proposed, although their significance remains unclear. For example, responses to collagen types I–V are not altered in mouse platelets deficient in CD36, a putative collagen receptor, arguing against a critical role for this protein in platelet–collagen interactions [25]. A critical role for a further receptor in supporting the interaction with collagen is consistent with the data reported in the present work.

An additional explanation for the difference between CRP and collagen is the affinity of the interaction with GPVI. CRP contains the minimal sequence information for recognition of GPVI by collagen, i.e. the GPO motif. It is not known whether additional amino acids surrounding this sequence in collagen influence its affinity for the glycoprotein. Interestingly, we have reported evidence for two epitopes in collagen that support binding to GPVI, only one of which is blocked by mAb JAQ1 [21]. In the present study, we have shown that aggregation of platelets with reduced levels of GPVI was maintained in the presence of JAQ1, whereas it has been reported previously that the combination of JAQ1 and a blocking antibody to integrin $\alpha 2\beta 1$ abolished responses to high concentrations of collagen [2]. This demonstrates that the second epitope on GPVI is unable to support aggregation in response to collagen in the presence of the integrin $\alpha 2\beta 1$ blocking antibody, despite being able to support responses in platelets expressing a reduced level of GPVI. These results argue for a critical role for at least three sites of interaction of collagen with platelets, namely the two sites on GPVI and a site on integrin $\alpha 2\beta 1$. Additional receptors may also help to maintain the affinity of collagen for platelets, as described above.

Collagen is unable to activate GPVI in megakaryocytic and GPVI-transfected cell lines which express low levels of GPVI and often other collagen receptors, notably integrin $\alpha 2\beta 1$ [26–28]. This is exemplified in the original cloning study of Clemetson et al. [17], who demonstrated that DAMI cells could be induced to respond to collagen only following transfection with GPVI, whereas control cells do express a low level of GPVI and can be activated by the snake toxin convulxin. Consistent with this, Chen et al. [18] recently reported that a level of expression of GPVI in transfected RBL-2H3 cells and Jurkat T cells comparable with that in human platelets is essential for functional responses and/or adhesion to collagen. These observations strengthen the conclusion that the presence of a second receptor is necessary to support the interaction of collagen with GPVI in platelets, as described above. The present findings also explain reports that platelets from GPVI-deficient patients are unresponsive to CRP, but are partially activated by collagen [29,30]. The residual level of GPVI expression on the patient's platelets is likely to be sufficient to support collagen- but not CRP-induced responses.

In conclusion, the present study highlights important differences between collagen and CRP with respect to dependence on

GPVI. This is most probably explained by the involvement of one or more additional receptors in platelet activation by collagen, as well as a possible differential binding to GPVI. The additional receptors include integrin $\alpha 2\beta 1$ and possibly an unidentified third receptor for collagen. Finally, it should be emphasized that while a reduction in the level of GPVI produces only a small, albeit predictable, shift in the dose–response curve to collagen, it also induces a significant delay in activation. This is likely to have considerable significance *in vivo* under conditions of high shear, thereby explaining the increased bleeding problems experienced by individuals with a low level of GPVI.

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