Expression and Function of the Mouse Collagen Receptor Glycoprotein VI Is Strictly Dependent on Its Association with the FcRγ Chain*

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Platelet glycoprotein (GP) VI has been proposed as the major collagen receptor for activation of human platelets. Human GPVI belongs to the immunoglobulin superfamily and is noncovalently associated with the FcRγ chain that is involved in signaling through the receptor. In mice, similar mechanisms seem to exist as platelets from FcRγ chain-deficient mice do not aggregate in response to collagen. However, the activating collagen receptor on mouse platelets has not been definitively identified. In the current study we examined the function and in vivo expression of GPVI in control and FcRγ chain-deficient mice with the first monoclonal antibody against GPVI (JAQ1). On wild type platelets, JAQ1 inhibited platelet aggregation induced by collagen but not PMA or thrombin. Cross-linking of bound JAQ1, on the other hand, induced aggregation of wild type but not FcRγ chain-deficient platelets. JAQ1 stained platelets and megakaryocytes from wild type but not FcRγ chain-deficient mice. Furthermore, JAQ1 recognized GPVI (approximately 60 kDa) in immunoprecipitation and Western blot analysis with a newly expressed mouse antibody (JAQ1). JAQ1 completely inhibited collagen-induced aggregation in mice and demonstrate that the association with the FcRγ chain is critical for its expression and function.

Collagen is one of the major components of the vessel wall responsible for platelet adhesion and activation at sites of vascular injury (1). A variety of collagens have been identified, seven of which are found in the subendothelial layer. The interaction between platelets and collagen can either occur indirectly via intermediary proteins like von Willebrand factor, which complexes to collagen(s) in the vessel wall and concomitantly binds to the platelet receptors glycoprotein (GP)1 Ib-V-IX and activated GPIIb/IIIa, or by direct recognition of collagen by specific receptors. Several different receptors for collagen have been identified on platelets including CD36 (2), a p65 collagen type I-specific receptor (3), integrin αβ (4), and the non-integrin GPVI (5). In humans, there is growing evidence for GPVI as the major collagen receptor for platelet activation (6). Very recently, molecular cloning of GPVI cDNA demonstrated this receptor as a type I transmembrane protein belonging to the immunoglobulin (Ig) superfamily (7). Its sequence is most closely related to human FcαR and natural killer cell receptors as well as to polymorphic mouse receptors known as paired Ig-like receptors (PIR) (8, 9). GPVI (62–65 kDa) contains two Ig-C2-like extracellular domains formed by disulfide bonds and a 51-amino acid cytoplasmic tail that is free of any consensus sequence motifs for activation. Instead, GPVI harbors a positively charged arginine in its transmembrane region. The presence of transmembrane-charged residues is a typical feature seen in a variety of stimulatory receptors, such as Fc receptors for IgG (FcγRI and FcγRIII) (10), IgE (FcεRI) (10), and IgA (FcαR) (11), as well as PIR-A (8) and natural killer receptor-P1 (CD161) (12). All of them lack amino acid activation motifs but can associate with the FcRγ signaling subunit to trigger activation in response to receptor aggregation. According to in vitro studies on FcεR and mouse PIR-A, it has been suggested that the transmembrane arginine of GPVI is similarly required for its function and association with the FcRγ chain (7, 8, 11). Findings by Gibbins and co-workers (13, 14) support a model where human GPVI couples collagen stimulation of platelets to phosphorylation of the FcRγ chain leading to activation of Syk and phospholipase Cγ2.

Activation of mouse platelets by collagen is also found to be associated with tyrosine phosphorylation of multiple proteins including the FcRγ chain, Syk, and phospholipase Cγ2 (15). However, identification of the mouse collagen receptor coupled to the FcRγ chain has not been established due to the lack of antibodies recognizing GPVI. Here we report that GPVI expression on mouse platelets is strictly dependent on the presence of the FcRγ chain as demonstrated by flow cytometry, immunoprecipitation, and Western blot analysis with a newly developed mAb (JAQ1) against GPVI in FcRγ chain-deficient and control mice. Moreover, JAQ1 completely inhibited collagen-induced aggregation of mouse platelets. Therefore, it is concluded that GPVI represents the major collagen receptor for platelet activation in both mice and humans.

EXPERIMENTAL PROCEDURES

Animals—Specific pathogen-free mice (NMRI, C57Bl/6) 6–10 weeks of age were obtained from Charles River Breeding Laboratories, Sulzfeld, Germany, and kept in our animal facilities. C57Bl/6 mice deficient in the Fcγ chain (16) were obtained from Taconics, Germantown,
NY, and kept under dry barrier conditions in the animal facilities at the Hannover Medical School (Hannover, Germany) until usage at 2–4 months of age.

Reagents—
EZ-Link™ sulfo-NHS-LC-biotin (Pierce), ADP, phorbol 12-myristate 13-acetate (PMA), high molecular weight heparin (all from Sigma, Deisenhofen, Germany), α-thrombin (Roche Molecular Biochemicals), collagen (Nycomed GmbH, Munich, Germany), and streptavidin-HRP (Dako, Denmark) were purchased.

Antibodies—
The rat anti-mouse P-selectin mAb RB40.34 was kindly provided by D. Vestweber, Münster, and modified in our laboratories. FITC-labeled rabbit anti-rat Ig, HRP-labeled rabbit anti-FITC, and rabbit anti-fibrinogen were purchased from Dako. All the following antibodies were generated, produced, and modified in our laboratories: MWReg30 (17) (anti-GPIIb/IIIa), EDL1 (anti-GPIIIa), p0p1 (18) (anti-GPIb-IX), DOM1 (anti-GPV), and LEN1 (anti-GPIaIIa).

Platelet Preparation—Mice were bled under ether anesthesia from the retro-orbital plexus. Blood was collected in a tube containing 10% (v/v) 0.1 mol/liter sodium citrate or 7.5 units/ml heparin, and platelet-rich plasma (prp) was obtained by centrifugation at 300 g for 10 min at room temperature. The platelets were washed twice with Tris buffer (TB, 20 mmol/liter Tris-HCl, pH 7.3, 0.9% NaCl) by centrifugation at 1,300 g for 10 min and used immediately. Isolated platelets did not show any signs of activation as shown by flow cytometry (staining for P-selectin and surface-expressed fibrinogen).

Production of Monoclonal Antibodies (mAbs)—Female Wistar rats, 6–8 weeks of age, were immunized repeatedly with mouse platelets. The rat spleen cells were then fused with mouse myeloma cells (Ag8.653), and hybridomas were selected in HAT medium. Hybridomas secreting mAbs directed against platelet receptors were identified by flow cytometry. Briefly, a 1:1 mixture of resting and thrombin-activated

![Figure 1](https://example.com/fig1.png)

**FIG. 1.** Platelet aggregation by collagen, PMA, and α-thrombin in the presence of JAQ1. Heparinized prp was incubated with JAQ1 (20 µg/ml) or irrelevant rat IgG2a (20 µg/ml) before addition of collagen (5 µg/ml) or PMA (50 ng/ml). Thrombin-induced aggregation (0.1 unit/ml) was performed with washed platelets.

![Figure 2](https://example.com/fig2.png)

**FIG. 2.** JAQ1 binds to mouse GPVI.
(a) flow cytometric detection of GPVI on mouse platelets. Platelets were incubated with JAQ1 (10 µg/ml, solid line) or irrelevant rat IgG2a (10 µg/ml, shaded area) for 30 min at room temperature. Bound JAQ1 was detected with rabbit anti-rat IgG-FITC.
(b) immunoprecipitation of GPVI from surface-biotinylated mouse platelets. Nonidet P-40 lysates were incubated with 10 µg/ml nonimmune rat IgG2a (control, contr.) or JAQ1, followed by protein G-Sepharose. Proteins were separated on a 9–15% gradient SDS-PAGE gel under reducing (red.) conditions, transferred onto a PVDF membrane, and detected by streptavidin-HRP and ECL.
(c) platelet proteins were separated by SDS-PAGE and immunoblotted with FITC-labeled JAQ1. Bound JAQ1 was detected by HRP-labeled rabbit anti-FITC.
(d) heparinized prp was incubated with JAQ1 (20 µg/ml) or irrelevant rat IgG2a (20 µg/ml) followed by addition of polyclonal rabbit anti-rat IgG antibodies (10 µg/ml). nonred., non-reduced.
A new mAb against mouse GPVI (JAQ1, rat IgG2a) was generated. JAQ1 blocked collagen-induced platelet aggregation in pp and on washed platelets in a dose-dependent manner, whereas it had no effect on aggregation induced by PMA (in pp) or α-thrombin (washed platelets) (Fig. 1). Incubation of pp with varying concentrations (3–30 μg) of JAQ1 under stirring (1000 rpm, 37 °C) or static conditions did not induce any signs of platelet activation (surface expression of P-selectin or fibrinogen). Consequently, no aggregation was induced by the mAb itself.

JAQ1 bound to mouse platelets (Fig. 2a) and precipitated a single chain protein of an apparent molecular weight of approximately 60 kDa under nonreducing conditions (Fig. 2b). The molecular weight slightly shifted to approximately 65 kDa under reducing conditions, demonstrating that the apparent molecular weight of mouse GPVI is very similar to its human homolog (7, 20). Furthermore, JAQ1 recognized GPVI as a single band of approximately 60 kDa under nonreducing conditions in a Western blot analysis of platelet lysates (Fig. 2c), whereas no band was detected under reducing conditions (not shown) indicating that JAQ1 binds to a conformational epitope on the receptor. On human platelets, polyclonal antibodies against GPVI have been reported to induce platelet aggregation when used as IgG or F(ab')2 fragments (7, 21), whereas monovalent Fab fragments had no activator effect, suggesting that clustering of GPVI is required for signal transduction. To induce clustering of GPVI, we cross-linked surface-bound JAQ1 with maleimide-conjugated JAQ1 mAb for 10 min at room temperature, washed with PBS (1300 × g, 10 min), and stained with FITC-labeled rabbit anti-rat IgG (Dako) for 15 min.

RESULTS AND DISCUSSION

GPVI is Not Detectable on Platelets and Megakaryocytes from FeRγ Chain-deficient Mice—Human GPVI has been described to be associated physically and functionally with the FeRγ chain which seems to be critical for platelet activation through
the receptor (13, 22). Furthermore, it is well recognized that platelets from FcRγ chain-deficient mice do not aggregate in response to collagen (15). Based on these findings, we expected JAQ1 not to induce platelet aggregation upon cross-linking on platelets from FcRγ chain-deficient mice. Indeed, addition of increasing concentrations of JAQ1 (3–30 μg/ml) followed by polyclonal rabbit anti-rat IgG antibodies (10 μg/ml) had no effect on FcRγ chain-deficient platelets (Fig. 3). In parallel experiments with platelets from C57Bl/6 control mice, the same procedure always resulted in platelet aggregation. This finding again demonstrated that the antigen recognized by JAQ1 is mouse GPVI and further suggested that function through mouse GPVI requires the association with the FcRγ chain.

Therefore, we assessed the expression of GPVI in platelets and megakaryocytes from C57Bl/6 mice in comparison to FcRγ chain-deficient mice. JAQ1 did not bind to FcRγ chain-deficient platelets as determined by flow cytometry (Fig. 4a). Immunohistochemical examination of splenic megakaryocytes from FcRγ chain-deficient mice demonstrated no specific binding of JAQ1, whereas megakaryocytes from C57Bl/6 control mice were clearly stained (Fig. 4b).

Moreover, in the absence of the FcRγ chain, GPVI-specific bands were not detectable in JAQ1 immunoprecipitates from surface-biotinylated platelets nor in a Western blot analysis of whole platelet lysate. These data strongly suggest that GPVI is not expressed in vivo in the absence of the FcRγ chain, although we cannot definitively rule out the possibility that the protein is expressed but fails to undergo the folding necessary for JAQ1 to bind. In contrast to GPVI, similar amounts of GPIIb/IIIa were immunoprecipitated from control and FcRγ chain-deficient platelets, and GPIIIa was detected in both platelet preparations by Western blot analysis (Fig. 4, c and d).

Taken together, our results give strong evidence that GPVI is the dominant receptor for collagen-induced activation of platelets in mice. We have used FcRγ chain-deficient mice in combination with the first monoclonal antibody against mouse GPVI to demonstrate that correct expression and function of mouse GPVI is strictly dependent on the presence of the FcRγ chain.

**Fig. 4. Mouse GPVI is not expressed in the absence of the FcRγ chain.** a, flow cytometric analysis of FcRγ chain-deficient platelets stained with JAQ1. Platelets were incubated with JAQ1 (10 μg/ml, solid line) or irrelevant rat IgG2a (10 μg/ml, shaded area) for 30 min at room temperature, followed by rabbit anti-rat IgG-FITC. b, immunohistochemical detection of GPVI on acetone-fixed frozen sections. Unstained megakaryocytes are indicated by arrows. c, immunoprecipitation of GPVI from surface-biotinylated control and FcRγ chain-deficient platelets. Nonidet P-40 lysates were incubated with 10 μg/ml JAQ1 or MWReg33 (anti-GPIIb/IIIa), followed by protein G-Sepharose (Amersham Pharmacia Biotech). Proteins were separated on a 9–15% gradient SDS-PAGE gel under non-reducing conditions, transferred onto a PVDF membrane, and detected by streptavidin-HRP and ECL. d, whole platelet proteins were separated by SDS-PAGE and immunoblotted with FITC-labeled JAQ1 or EDL1 (anti-GPIIIa). Bound mAb was detected by HRP-labeled rabbit anti-FITC.
subunit. These observations, combined with reports that human GPVI is also physically and functionally associated with the FcRγ chain (13, 14), indicate that the mechanisms leading to collagen-induced platelet activation are similar in mice and humans.

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