Evidence for Two Distinct Epitopes within Collagen for Activation of Murine Platelets*

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It has recently been shown that the monoclonal antibody JAQ1 to murine glycoprotein VI (GPVI) can cause aggregation of mouse platelets upon antibody cross-linking and that collagen-induced platelet aggregation can be inhibited by preincubation of platelets with JAQ1 in the absence of cross-linking (Nieswandt, B., Bergmeier, W., Schulte, V., Rackebrandt, K., Gessner, J. E., and Zirnbibl, H. (2000) J. Biol. Chem. 275, 23998–24002). In the present study, we have shown that cross-linking of GPVI by JAQ1 results in tyrosine phosphorylation of the same profile of proteins as that induced by collagen, including the Fc receptor (FcR) γ-chain, Syk, LAT, SLP-76, and phospholipase Cγ2. In contrast, platelet aggregation and tyrosine phosphorylation of these proteins were inhibited when mouse platelets were preincubated with JAQ1 in the absence of cross-linking and were subsequently stimulated with a collagen-related peptide (CRP) that is specific for GPVI and low concentrations of collagen. However, at higher concentrations of collagen, but not CRP, aggregation of platelets and tyrosine phosphorylation of the above proteins (except for the adapter LAT) is re-established despite the presence of JAQ1. These observations suggest that a second activatory binding site, which is distinct from the CRP binding site on GPVI on mouse platelets, is occupied in the presence of high concentrations of collagen. Although this could be a second site on GPVI that is activated by a novel motif within the collagen molecule, the absence of LAT phosphorylation in response to collagen in the presence of JAQ1 suggests that this is more likely to be caused by activation of a second receptor that is also coupled to the FcR γ-chain. The possibility that this response is mediated by a receptor that is not coupled to FcR γ-chain is excluded on the grounds that aggregation is absent in platelets from FcRγ-chain-deficient mice.

The platelet collagen receptor GPVI1 plays a pivotal role in platelet activation following receptor cross-linking by collagen. This is highlighted by the impaired response to collagen in GPVI-deficient patients (2–4) and the bleeding disorders associated with this. GPVI is a 60–65-kDa type I transmembrane glycoprotein belonging to the immunoglobulin superfamily (5), which forms a complex with the FcR γ-chain at the cell surface in human and mouse platelets (1, 6, 7). Signaling through GPVI occurs via a similar pathway to that used by immunoreceptors (8) as revealed by the tyrosine phosphorylation of the FcR γ-chain immunoreceptor tyrosine-based activation motif (ITAM) by a Src-like kinase (9, 10). The Src-like kinases Fyn and Lyn are believed to be involved in this phosphorylation (9–11). Syk is able to bind to the tyrosine-phosphorylated ITAM of the FcR γ-chain via its tandem SH2 domains (12) leading to autophosphorylation and activation. Syk plays a critical role in the regulation of PLCγ2 through phosphorylation of a number of key proteins including the adapters LAT and SLP-76 (13–15).

These signaling events occur upon collagen-induced GPVI cross-linking and following GPVI binding to collagen-related peptides (CRPs) (15–17). CRPs contain Gly-Pro-Hyp triplet repeats (where Hyp is hydroxyproline) in a triple-helical conformation, which are formed into a quaternary structure by cysteine or lysine cross-linking at the N- and C-terminal ends (18). Platelets can also be activated through GPVI via interaction with the snake venom toxin convulxin (Cvx), isolated from the venom of the tropical rattlesnake Crotalus durissus terrificus (19, 20). Cvx-induced platelet activation is associated with a similar pattern of tyrosine phosphorylation as that induced by collagen (20).

We have recently shown that a monoclonal antibody (JAQ1) to murine GPVI can cause aggregation of mouse platelets upon antibody cross-linking and that collagen-induced platelet aggregation can be inhibited by preincubation of platelets with JAQ1 (1). In the present study, we demonstrate that JAQ1 inhibits aggregation by low concentrations of collagen and CRP, and this effect is overcome by high concentrations of collagen, but not CRP. This study presents a novel motif within collagen that is mediating platelet activation.

EXPERIMENTAL PROCEDURES

Animals—Specific pathogen-free mice (NMRI strain) 6–10 weeks of age were obtained from Charles River, Sulzfeld, Germany or (CD1 strain) from Harlan SERA-LAB Ltd (Belton, Leics., UK).

Materials—Convulxin was obtained from the venom of the tropical rattlesnake Crotalus durissus terrificus, which was kindly donated by Drs. Mireille Leduc and Cassian Bon (Unite des Venins, Insitut Pasteur, Paris, France). Collagen (predominantly type I, derived from Cyt2; PRP, platelet-rich plasma; PVDF, polyvinylidene difluoride; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis.
Equine achilles tendon) was from Nycomed (Munich, Germany). CRP (GKO/GP01GKOG, single letter amino acid code where O is hydroxyproline) was synthesized by Tanaka Laboratories, L.C., TX and was cross-linked with 0.25% glutaraldehyde for 3 h on ice and then dialysed into phosphate-buffered saline. All salts and Nonidet P-40 were purchased from BDH-Merck. Protein G-Sepharose was purchased from Calbiochem-Novabiochem (Nottingham, UK). The mAb JAQ1 was produced as described previously (1). Anti-SLP-76 sheep polyclonal anti-serum was generously provided by Dr. Gary Koretsky (Abramson Family Cancer Center, University of Pennsylvania). All other reagents were from previously described sources (15). Modification of antibodies was performed as follows. Fab fragments from JAQ1 were generated by 12-hour incubation with 10 mg/ml mAb with immobilized papain (Pierce, Rockford, IL), and the preparations were then applied to an immobilized protein A column followed by an immobilized protein G column (Amersham Pharmacia Biotech) to remove Fc fragments and any undigested IgG. The purity of the Fab fragments was checked by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining of the gel.

Preparation of Platelets—Mice were bled under ether anesthesia from the retro-orbital plexus. Blood was collected in a tube containing 7.5 units/ml heparin, and platelet-rich plasma (PRP) was obtained by centrifugation at 300 × g for 10 min at room temperature. Isolated platelets did not show any signs of activation as shown by flow cytometry (staining for P-selectin and surface-bound fibrinogen). Alternatively, blood was taken by cardiac puncture following carbon dioxide asphyxiation using acidic citrate dextrose (120 mM sodium citrate, 110 mM glucose, 80 mM citric acid) as anticoagulant. Blood was diluted with 200 μl of modified Tyrodes-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 6.6) and centrifuged at 200 × g for 8 min at room temperature. Platelets were removed from PRP by pipetting the cells above the plasma/red blood cell interface. PRP was centrifuged at 1000 × g for 10 min at room temperature. Isolated platelets were resuspended in modified Tyrodes-HEPES buffer 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 and in washed platelets. Similar results were obtained in both cases. To determine aggregation, light transmission (200 μl with 0.5 × 108 platelets/μl) was measured relative to platelet-poor plasma (PPP). Transmission was recorded on a Fibrintimer 4-channel aggregometer (APACT Systems GmbH, Germany) over 30 min and was expressed as percent transmission relative to PPP. Platelet aggregation was induced by addition of collagen (1–100 μg/ml) or CRP (1–100 μg/ml).

Protein Phosphorylation Studies—Platelet stimulation was carried out using 500 μl of platelet suspension containing between 7.1 × 107 to 1 × 108 cells/ml. All experiments were performed in the presence of EGTA (100 μM) and dithiothreitol (10 μM) to prevent secondary events and facilitate protein isolation. Reactions were stopped by addition of an equal volume of ice-cold Nonidet P-40 lysis buffer (20 mM Tris, 300 mM NaCl, 10 mM EDTA, 2% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, pH 7.5). Antibody (4 μl of anti-LAT antibody or 1 μl of anti-SLP-76 antisera or 2 μl of anti-PLC-γ2 antisera or 2 μl of anti-Syk antibody per sample) was added to the lysate followed by 20 μl of protein A-Sepharose CL-4B. The mixture was incubated overnight at 4 °C on a rotating carousel and centrifuged to pellet the protein A-Sepharose. The pellets were washed in lysis buffer followed by Tris-buffered saline (TBS-T; 20 mM Tris, 137 mM NaCl, 0.1% (v/v) Tween 20, pH 7.6) before the addition of Laemml sample treatment buffer in preparation for SDS-PAGE. A GST fusion protein containing the tandem SH2 domains of Syk was prepared and used to precipitate the FcR γ-chain as described previously (12).

Proteins were separated by SDS-PAGE on 10% acrylamide gels under reducing or nonreducing conditions and transferred to PVDF membrane. Membranes were blocked by incubation with TBS-T containing 0.1% (v/v) Tween 20 and 5% (w/v) skimmed milk. Membranes were washed for 5 min with TBS-T and incubated for 1 h with an appropriate HRP-conjugated secondary antibody diluted 1:10,000 in TBS-T. Following incubation in TBS-T as above, the membranes were developed using an enhanced chemiluminescence (ECL) detection system. Densitometric analysis of the results was performed using Quantity One (version 4) densitometry software (Bio-Rad).

RESULTS AND DISCUSSION

The Inhibitory Effect of JAQ1 Is Overcome by High Concentrations of Collagen—We recently reported the generation of the first mAb (JAQ1, rat IgG2a) against mouse GPVI and demonstrated that JAQ1 inhibits platelet aggregation in response to collagen (5 μg/ml). Whereas binding of JAQ1 alone did not induce platelet activation, cross-linking of the surface-bound antibody induced irreversible platelet aggregation (1).

Subsequent to this first report, we performed a series of studies to further characterize the interaction of JAQ1 with GPVI on platelets. To our surprise, we found that the inhibitory effect of JAQ1 against collagen could be overcome by increasing the concentration of the adhesion molecule. Thus, in the presence of intact JAQ1 (10 μg/ml) or monovalent Fab fragments of the mAb (10 μg/ml), collagen at a concentration of 10 μg/ml stimulated partial aggregation, which reached almost maximal levels at 20 μg/ml (Fig. 1A and B). Flow cytometric preincubation studies demonstrated that bound fluorescent isothiocyanate-labeled JAQ1 (JAQ1FITC) was not displaced by a 50-fold excess of unlabeled JAQ1 for at least 30 min (not shown), indicating that JAQ1 binds essentially irreversibly to GPVI over the time course of this study. Therefore, collagen must be able to bind either to a second site on GPVI or to a second...
higher concentrations of collagen because of the weak stimulatory effect of the antibody itself. Nevertheless, higher concentrations of collagen (10 and 30 μg/ml) were able to stimulate significant tyrosine phosphorylation of SLP-76 and PLCγ2 in the presence of JAQ1, consistent with the restoration of aggregation to collagen under these conditions (Fig. 1, A and B). Densitometric analysis demonstrated that the increase in tyrosine phosphorylation of PLCγ2 and SLP-76 induced by higher concentrations of collagen was similar in the presence or absence of JAQ1 (not shown). However, this was not the case for LAT, which did not increase in phosphorylation in response to higher concentrations of collagen in the presence of JAQ1 (Fig. 1, C and D). These observations demonstrate that higher concentrations of collagen are able to overcome the inhibitory effect of JAQ1 on aggregation and phosphorylation. The minimal tyrosine phosphorylation of LAT, however, in contrast to the substantial phosphorylation of SLP-76 and PLCγ2 that is observed in the presence of JAQ1, raises the possibility that this is mediated by a receptor other than GPVI. In this context, it is noteworthy that tyrosine phosphorylation of PLCγ2 and platelet activation in response to CRP are heavily reduced but not abolished in LAT-deficient platelets (15). Thus, there is a pathway of regulation of PLCγ2 and platelet activation by collagen which is independent of LAT.

JAQ1 Completely Inhibits Platelet Activation to CRP—These results provide evidence for a second epitope on the collagen molecule that is capable of binding to GPVI in the presence of JAQ1 or that the adhesion molecule is binding to a second activatory receptor. In the latter case, it is possible that binding is mediated through the same epitope in the collagen molecule.
completely inhibited in mice deficient in the FcRγ-chain. A second site in GPVI or to a second receptor for collagen. This demonstrates the presence of a second epitope as a stronger agonist than collagen (16). This result rules out the effect of cross-linking JAQ1 through interaction of the antibody (Fig. 2A) and that tyrosine phosphorylation is predominantly blocked platelet aggregation in response to CRP at concentrations up to 100 μg/ml (Fig. 2, A and B). CRP stimulated a marked increase in tyrosine phosphorylation in the whole cell lysate, which was reduced to basal in the presence of the antibody (Fig. 2C). These results demonstrate that JAQ1 is able to fully inhibit responses to CRP, in contrast to the result for collagen, despite the fact that CRP is generally recognized as a stronger agonist than collagen (16). This result rules out the possibility that the high concentrations of collagen overcome the inhibitory effect of JAQ1 through interaction of the GPO motif with a second site on GPVI or on a receptor other than GPVI. This demonstrates the presence of a second epitope in collagen that mediates activation in the presence of a saturating concentration of JAQ1. This epitope could bind either to a second site in GPVI or to a second receptor for collagen. Bearing in mind that aggregation in response to collagen is completely inhibited in mice deficient in the FcRγ-chain (not shown) and that tyrosine phosphorylation is predominantly inhibited (8), it would appear that this second receptor is also associated with the FcRγ-chain.

Cross-linking of GPVI by JAQ1 Stimulates Aggregation and Protein Phosphorylation—We have previously reported that cross-linking of JAQ1 by addition of anti-rat IgG antibodies (10 μg/ml) stimulates platelet aggregation (1). In view of the ability of JAQ1 alone to stimulate a minimal increase in protein-tyrosine phosphorylation and the absence of tyrosine phosphorylation of LAT in response to high concentrations of collagen in the presence of JAQ1, we were interested in examining the effect of cross-linking JAQ1 on phosphorylation. The aim of this set of studies was to examine whether cross-linking JAQ1 induces a similar pattern of tyrosine phosphorylation to that seen in response to classical activation of GPVI, and specifically whether LAT is phosphorylated.

Cross-linking of surface-bound JAQ1 on platelets stimulated a similar pattern and time course of tyrosine phosphorylation in whole cell lysates to those detected in platelets activated by the GPVI selective agonist, convulxin (5 μg/ml), albeit with a lower intensity of phosphorylation (Fig. 3A). Phosphorylation in response to convulxin was maintained for all proteins up to 90 s, with the exception of a band of 25 kDa (nonreducing conditions), which comigrates with FcRγ-chain. In contrast, tyrosine phosphorylation in response to cross-linking of JAQ1 declined more rapidly, most notably for protein bands of 25 and 36 kDa (Fig. 3, B and C). Immunoprecipitation studies confirmed that cross-linking of JAQ1 stimulated tyrosine phosphorylation of the same pattern of proteins as seen with convulxin, including namely FcRγ-chain, Syk, SLP-76, LAT, and PLCγ2 (Fig. 4). Tyrosine phosphorylation of all of these proteins was less than that seen in response to convulxin and was particularly weak for FcRγ-chain, which may partly be explained by the more rapid decline in tyrosine phosphorylation in response to cross-linking of JAQ1. Aggregation and protein phosphorylation in response to cross-linking of JAQ1 were completely inhibited in the presence of the Src family kinase inhibitor, PP1 (not shown), as previously reported for activation by CRP (10). These results confirm that cross-linking of GPVI by JAQ1 stimulates a similar pattern of protein-tyrosine phosphorylation and aggregation to that seen in response to activation of GPVI by convulxin.

Conclusion—The present results demonstrate that high concentrations of collagen induce platelet aggregation in the presence of an antibody to GPVI, which completely blocks aggregation in response to the selective agonist, CRP. This strongly suggests that there is a second activatory sequence within collagen, which is able to mediate platelet activation. It appears that the binding site for this novel epitope within collagen is located on a receptor that is coupled to the FcRγ-chain bearing in mind that aggregation to collagen is completely inhibited in FcRγ-chain-deficient platelets. This site could be located on GPVI or on a novel receptor, which also signals through the FcRγ-chain. Evidence for the latter is provided by the distinct profile of tyrosine phosphorylation, characterized by the absence of phosphorylation of LAT that is seen in response to high concentrations of collagen in the presence of JAQ1. Studies on mice platelets in which GPVI has been depleted through gene targeting are required to confirm this conclusion.

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Fig. 4. JAQ1 and convulxin induce tyrosine phosphorylation of FcRγ-chain, Syk, SLP-76, LAT, and PLCγ2. Washed platelets were incubated as described in the legend to Fig. 3. Samples for immunoprecipitations (IP) were lysed after 2 min by addition of an equal volume of ice-cold Nonidet P-40 buffer and were immunoprecipitated for FcRγ-chain, Syk, SLP-76, LAT, and PLCγ2 as described under “Experimental Procedures.” After separation by SDS-PAGE (12.5% acrylamide, nonreducing conditions) and transfer to PVDF membranes, the proteins were incubated with the anti-phosphotyrosine antibody 4G10 (anti-PY) and were detected by anti-mouse IgG-HRP and ECL. The membranes were stripped and reprobed for the immunoprecipitated proteins. Results are from a representative experiment (n = 3).
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