Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor γ-chain

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Abstract We have recently shown that collagen activates platelets through a pathway dependent on the Fc receptor γ-chain and the tyrosine kinase Syk. We report here that the Fc receptor γ-chain and the candidate collagen receptor glycoprotein VI (GPVI) co-associate. Furthermore, cross-linking GPVI stimulates a similar pattern of tyrosine phosphorylation to that stimulated by collagen, including tyrosine phosphorylation of Fc receptor γ-chain. These results support a model where GPVI couples collagen-stimulation of platelets to phosphorylation of the Fc receptor γ-chain leading to activation of Syk and phospholipase Cγ2.

Key words: Collagen receptor; Human platelet; Glycoprotein VI; Fc receptor γ-chain

1. Introduction

Collagens are major components of extracellular matrix and connective tissue. On damage to the vascular endothelium, platelets adhere to newly exposed collagen fibres of the blood vessel wall leading to the secretion of various mediators and platelet aggregation, contributing to the formation of a haemostatic plug. The size and complex structure of collagen gives the potential to bind and consequently cross-link multiple receptors on platelets. Several potential collagen receptors on the platelet surface have been proposed. It is generally recognised that the integrin $\alpha_2\beta_1$ plays a major role in the initial adhesion to collagen, and a growing body of evidence supports a second receptor site underlying secretion and aggregation [1–5]. Candidates for this second receptor include glycoprotein IV (GPIIIb, CD36) [6], glycoprotein VI [7–10], the C1q receptor [11] and uncharacterised 65-kDa [12] and 85–90-kDa glycoproteins [13]. Activation of platelets by collagens is mediated via a tyrosine kinase-dependent pathway resulting in phosphorylation of the FcR γ-chain [4,5], the tyrosine kinase Syk [4,5,14] and culminates in the phosphorylation of phospholipase Cγ2 (PLCγ2) [15,16]. Using genetically modified mice we have demonstrated that the FcR γ-chain and Syk are absolutely required for collagen-stimulated secretion and aggregation and that tyrosine phosphorylation of the FcR γ-chain lies upstream of Syk, and that Syk lies upstream of PLCγ2 [5]. Collagen-stimulated signalling in platelets therefore resembles signalling mechanisms induced by immune receptors such as Fc and antigen receptors [17–20]. This identifies collagen as a unique physiological platelet agonist which induces activation through a pathway that was originally thought to be used only by immune receptor stimuli. Of the proteins proposed as collagen receptors underlying activation through a pathway of this kind, an increasing amount of evidence favours GPVI. Deficiency in expression of GPVI results in impaired aggregation responses to collagen, and antibodies which recognise GPVI have been shown to inhibit collagen-stimulated aggregation [7,8,10,21]. Cross-linking GPVI results in phosphorylation and activation of Syk [9] and activation of Syk by collagen is severely compromised in platelets which do not express GPVI [22]. It has also been reported that under conditions of flow, GPVI contributes to platelet adhesion to a collagen-coated surface [23]. In this report we examine the roles of GPVI and the FcR γ-chain as components of a multimeric receptor complex which underlies collagen-stimulated activation of platelets.

2. Materials and methods

2.1. Materials

Anti-FcR γ-chain antiserum [24] was kindly provided by Dr. J.-P. Kinet (Beth Israel Hospital, Boston, MA, USA). IgG was extracted using protein A-Sepharose from the plasma of a patient with autoimmune thrombocytopenia and whose serum contains autoantibodies specific for GPVI [21]. Control IgG was extracted in the same way from normal human plasma. Anti-phosphotyrosine monoclonal antibody (mAb) 4G10 was from Upstate Biotechnology (TCS Biologicals Ltd., Botolph Claydon, Bucks, UK) and anti-FcγRII mAb IV.3 was from Medarex Inc. (Annandale, NJ, USA). Collagen fibres, as Horm collagen, a suspension of type I fibres from equine tendon, were obtained from Nycomed (Munich, Germany). Collagen-related peptide (CRP: GCP*(GPP*)$_{10}$GCP*G; single amino acid code P* = hydroxyproline) was synthesised and cross-linked (CRP-XL) as previously described [25]. NHS-LC-Biotin was from Pierce and Warriner (Chester, Cheshire, UK), immobilised pepsin was from Sigma (Poole, Dorset, UK) and all other reagents were from previously described sources [14].

2.2. Preparation and stimulation of platelets

Blood was taken with informed consent from drug-free volunteers. Washed platelets were prepared as described previously [14] and suspended in modified Tyrodes-HEPES buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, pH 7.3). Platelets were resuspended at densities of 2×10^8 cells/ml and 8×10^8 cells/ml for aggregation and protein precipitation studies respectively. Platelets were stimulated at 37°C with continuous stirring with collagen (100 μg/ml, 90 s) or CRP-XL (1 μg/ml, 90 s) in the presence of indomethacin (10 μM) and EGTA (1 mM), with the exception of the aggregation studies in which these

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reagents were omitted. Cross-linking of the platelet IgG receptor, FcγRIIA, was performed by pre-incubation of platelets for 60 s with mAb IV.3 (1 μg/ml) followed by stimulation for 60 s by addition of anti-mouse IgG $F(ab')_2$ (30 μg/ml). Where surface labelling of platelets was required, platelets isolated from platelet-rich plasma were resuspended in 1 ml of a 10 mM solution of the water soluble biotinylation reagent sulphosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin) prepared in modified Tyrodes-HEPES buffer. Platelets were incubated in this buffer for 30 min at 30°C and then diluted in modified Tyrodes-HEPES buffer to 25 ml. Platelets were pelleted by centrifugation at $1000 \times g$ for 10 min and resuspended for use as described above.

2.3. Generation of $F(ab')_2$ fragments

IgG solutions were dialysed and concentrated to give 20 mg/ml IgG in 20 mM CH_3COONa pH 4. To the IgG was added pepsin (250–500 U) immobilised on agarose which was then mixed for 15 h at 37°C. Digestion was terminated by the addition of an equal volume of 10 mM Tris-HCL pH 7.5, the antibody solution was removed and remaining whole antibody and Fc fragments removed by incubation with protein-A Sepharose. The purity of the $F(ab')_2$ preparations were checked by SDS-PAGE and protein concentration determined using a bicinchoninic acid protein assay.

2.4. GPVI cross-linking studies

Control and anti-GPVI $F(ab')_2$ fragments were incubated with washed platelets for 5 min under stirring conditions and aggregation measured as an increase in optical density. Stimulation was terminated by the addition of Laemmli sample treatment buffer. Proteins were separated by SDS-PAGE and immunoblotted for tyrosine phosphorylation using mAb 4G10. A GST-fusion protein containing the tandem SH2 domains of Syk was prepared, immobilised on agarose and used for the precipitation of the FcR γ-chain as described previously [4]. Platelets were stimulated as above and tyrosine phosphorylated FcR γ-chain precipitated from lysates using GST-Syk SH2 fusion protein (10 μg/ml lysate). Proteins were separated by SDS-PAGE and immunoblotted for tyrosine phosphorylation.

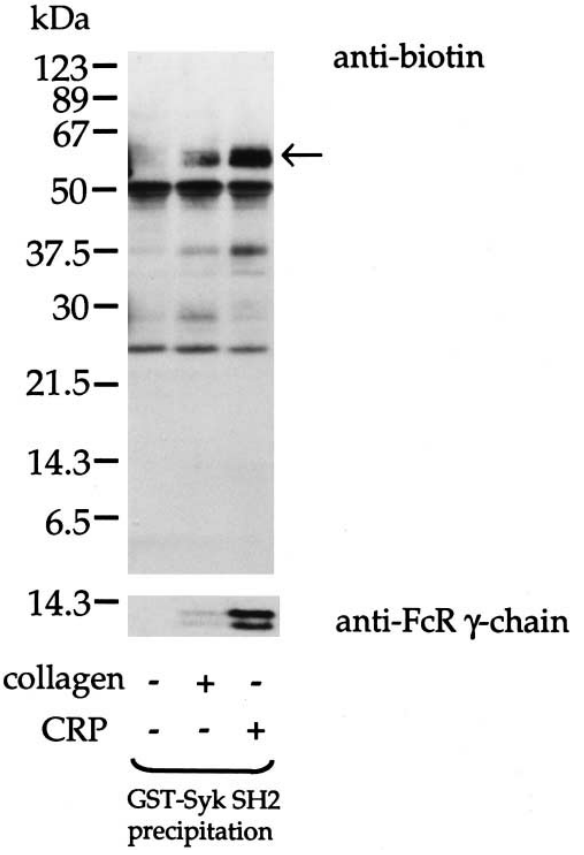

Fig. 1. The FcR γ-chain co-precipitates with a 60-kDa cell-surface protein. Tyrosine phosphorylated FcR γ-chain was precipitated from collagen- (100 μg/ml, 90) and CRP-XL- (1 μg/ml, 90 s) stimulated surface-biotinylated platelets using a GST fusion protein containing the tandem SH2 domains of Syk. Proteins were separated by SDS-PAGE, transferred to a PVDF membrane and surface proteins detected by probing with a streptavidin-horseradish peroxidase conjugate and developed using an ECL detection system. The position of a surface protein of approximately 60 kDa which co-precipitates with the FcR γ-chain. Similar results were observed in 6 studies.

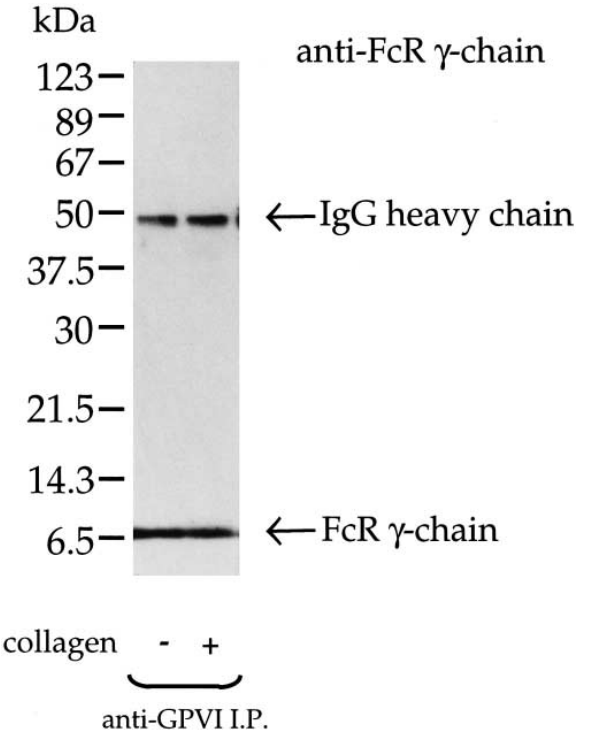

Fig. 2. The FcR γ-chain co-associates with GPVI. GPVI was immunoprecipitated from lysates from basal or collagen-stimulated (100 μg/ml, 90 s) platelets using anti-GPVI IgG (10 μg/ml lysate) extracted from human plasma. Proteins were separated by SDS-PAGE and immunoblotted to detect the FcR γ-chain. The position of non-tyrosine phosphorylated FcR γ-chain which co-immunoprecipitates with GPVI is shown. Similar results were observed in 4 experiments.

2.5. Immunoprecipitation and immunoblotting studies

Immunoprecipitation and immunoblotting studies were performed as described previously [4]. Anti-GPVI IgG was used at a concentration of 10 μg/ml platelet lysate for immunoprecipitation of GPVI. Detection of phosphotyrosine residues was performed using mAb 4G10 (1 μg/ml) and anti-mouse Ig–horseradish peroxidase conjugated secondary antibody used at a dilution of 1:10000. For detection of the FcR γ-chain, anti-FcR γ-chain rabbit antiserum was used diluted 1:10000 and anti-rabbit Ig–horseradish peroxidase conjugated secondary antibody used at a dilution of 1:10000. All immunoblots were developed using an enhanced chemiluminescence (ECL) detection system. Platelet surface proteins labelled with biotin were detected on western blots by incubation with a streptavidin-horseradish peroxidase conjugate diluted 1:50000 in Tris-buffered saline (20 mM Tris, 137 mM NaCl) containing 0.1% (v/v) Tween 20 (TBS-T) and 2% (w/v) BSA. Blots were washed in TBS-T for 2 h and developed using an ECL detection system.

3. Results and discussion

We have shown that the FcR γ-chain is essential for collagen-stimulated activation of platelets and have proposed that

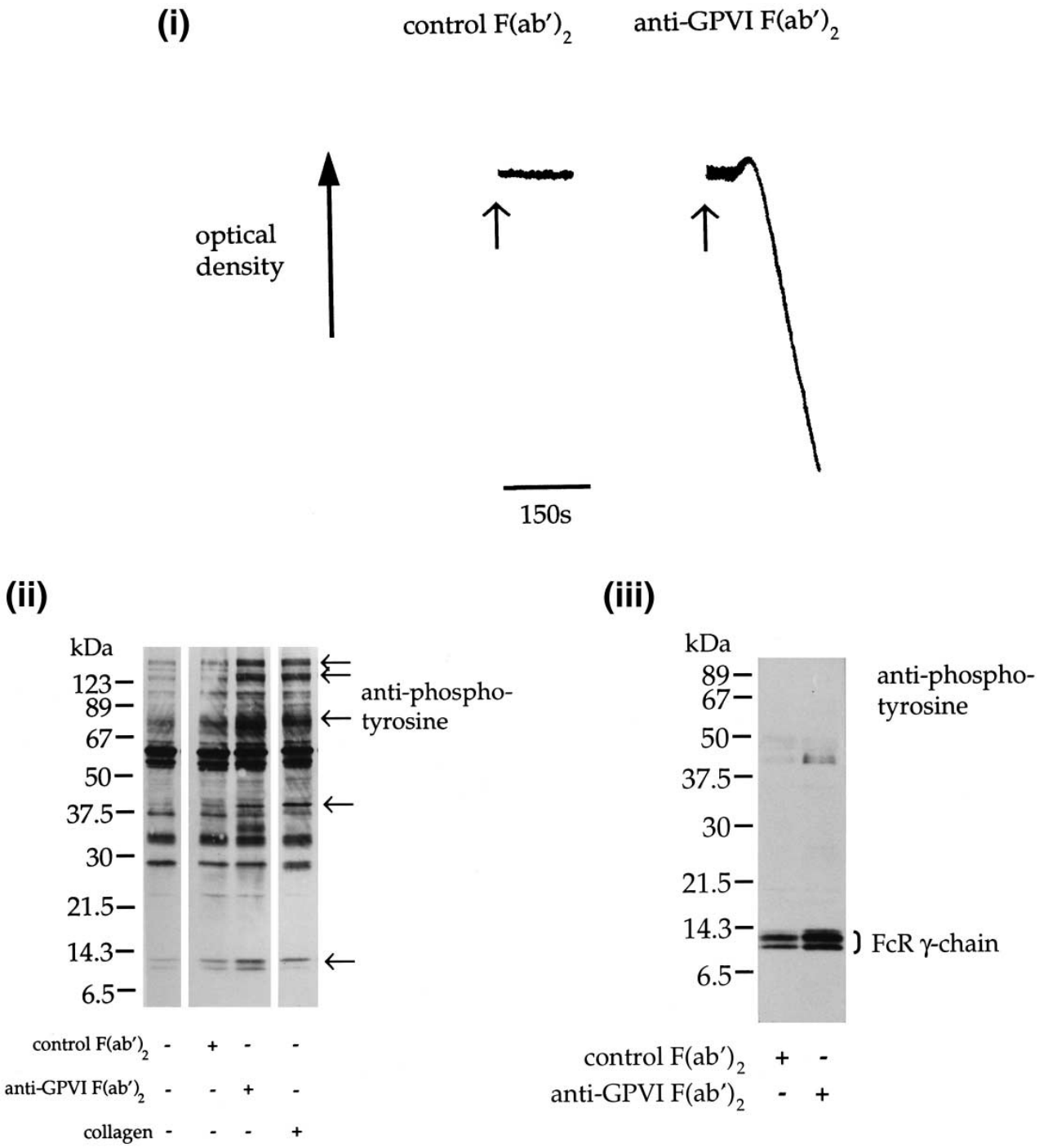

Fig. 3. Cross-linking GPVI causes tyrosine phosphorylation of the FcR γ-chain. (i) Washed platelets were incubated with control or anti-GPVI $F(ab')_2$ fragments (750 μg/ml) at 37°C under stirring conditions. The level of aggregation was monitored by measuring the increase in optical density. (ii) Platelets incubated with control or anti-GPVI $F(ab')_2$ fragments for 150 s or stimulated with collagen (100 μg/ml, 90 s) were treated with Laemmli sample treatment buffer. Following SDS-PAGE and transfer to PVDF membrane, levels of tyrosine phosphorylation were assessed by immunoblotting using mAb 4G10. Arrows indicate prominent proteins of 145, 130, 80, 40 and 12 kDa which become tyrosine phosphorylated following treatment with anti-GPVI $F(ab')_2$ fragments and collagen-stimulation. (iii) Platelets were incubated with control or anti-GPVI $F(ab')_2$ fragments for 150 s and then lysed. Tyrosine phosphorylated FcR γ-chain was precipitated from platelet lysates with an immobilised GST fusion protein containing the tandem SH2 domains of Syk. Precipitated proteins were separated by SDS-PAGE and immunoblotted to detect phosphotyrosine residues using mAb 4G10. Results are representative of 2 separate experiments.

this protein may be associated with the collagen receptor which underlies platelet activation. To examine this, tyrosine phosphorylated FcR γ-chain was precipitated from lysates from biotin surface-labelled platelets using a GST fusion protein containing the tandem SH2 domains of Syk a described previously [4]. Co-associated surface proteins were detected on western blots by probing for biotin. Fig. 1 shows that stimulation with collagen or CRP-XL leads to an increase in the

level of tyrosine phosphorylation and precipitation of the FcR γ-chain and co-precipitation of a membrane protein with a molecular mass of approximately 60 kDa. This protein migrates on reducing SDS-PAGE to a similar position to GPVI.

The interaction of GPVI with the FcR γ-chain was examined further by immunoprecipitation of GPVI. Proteins were separated by SDS-PAGE and immunoblotted to detect the FcR γ-chain. Fig. 2 shows the presence of a similar level of the FcR γ-chain in GPVI immunoprecipitates under basal and collagen-stimulated conditions. This suggests that GPVI and the FcR γ-chain co-associate in the collagen receptor complex. Tyrosine phoshorylated FcR γ-chain is distinguished from the non-phosphorylated form due to a shift in migration on SDS-PAGE under reducing conditions from an apparent molecular weight of 6–8 kDa to 12 kDa. However, collagen-stimulation results in only a small proportion of the total cellular FcR γ-chain becoming tyrosine phosphorylated and is normally only detectable following precipitation using a fusion protein containing the SH2 domains of Syk. The position of the migration in Fig. 2 indicates that the detectable FcR γ-chain which is co-precipitated with GPVI is not tyrosine phosphorylated.

Reports have demonstrated that platelets can be activated by incubation with anti-GPVI antibodies [7,9,21]. This is associated with an increase in protein tyrosine phosphorylation, including several key components of the pathway which is activated by collagen. Amongst these is Syk, which we have shown to be essential for collagen-mediated activation [5], whereupon it binds to tyrosine phosphorylated FcR γ-chain [4] and becomes phosphorylated and activated. We therefore investigated the effect of cross-linking GPVI on the level of tyrosine phosphorylation of the FcR γ-chain. GPVI cross-linking studies were performed using $F(ab')_2$ fragments generated from anti-GPVI IgG to avoid antibody mediated activation. Anti-GPVI $F(ab')_2$ fragments caused rapid and complete aggregation whereas treatment with the same concentration of $F(ab')_2$ fragments prepared from control plasma had no effect (Fig. 3i). The level of whole cell protein tyrosine phosphorylation in these samples was assessed by immunoblotting. The profile of tyrosine phosphorylation induced following cross-linking of GPVI is indistinguishable from that of collagen-stimulated platelets; in contrast no significant change in the level of tyrosine phosphorylation was observed following treatment with the control $F(ab')_2$ (Fig. 3ii). Arrows indicate the positions of several prominent proteins of approximately 145, 130, 80, 40 and 12 kDa which become heavily tyrosine phosphorylated in both collagen-stimulated and GPVI cross-linked cells. The smallest of these proteins migrates on SDS-PAGE under reducing conditions to a similar position to tyrosine phosphorylated FcR γ-chain. To confirm the identity of this protein, tyrosine phosphorylated FcR γ-chain was precipitated from platelets lysates using a fusion protein containing the tandem SH2 domains of Syk. Precipitated proteins were separated by SDS-PAGE and immunoblotted for phosphotyrosine residues. Fig. 3iii shows that incubation of platelets with anti-GPVI $F(ab')_2$ induces an increase in the level of tyrosine phosphorylation of the FcR γ-chain resulting in the precipitation of more of the protein. To check that this increase was not due to whole IgG contamination of the anti-GPVI $F(ab')_2$ preparation, the effect of cross-linking the platelet IgG receptor, FcγRIIA, on the level of phosphorylation of the FcR γ-chain was examined and found to be similar to that following treatment with the control $F(ab')_2$ preparation (not shown). This is in accordance with our previous findings [4].

Our results indicate that GPVI is the signalling collagen receptor whose activation results in the tyrosine phosphorylation of the FcR γ-chain. This supports the recent conclusion of Polgar et al. [26] who identified a component of the venom of the tropical rattlesnake (*Crotalus durissus terrificus*) which binds to GPVI. Exposure of platelets to this C-type lectin results in tyrosine phosphorylation of the FcR γ-chain amongst many other proteins, leading to platelet activation. The present results strengthen the proposal that effect of the venom is due to its ability to bind and cross-link GPVI.

The importance of the collagen-stimulated tyrosine kinase-dependent pathway and its stimulation via GPVI is demonstrated by the mild bleeding problems observed in patients who lack this protein or who express autoantibodies to it [7,8,10,22]. Given the potential importance of collagen in thrombotic disorders, the receptor described here, or an additional component of it, may provide an important target in the development of anti-thrombotic drugs. GPVI is the first example of a non-Fc receptor to couple to the FcR γ-chain.

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