Glycoprotein VI/Fc receptor γ chain-independent tyrosine phosphorylation and activation of murine platelets by collagen

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We have investigated the ability of collagen to induce signalling and functional responses in suspensions of murine platelets deficient in the FcR γ (Fc receptor γ) chain, which lack the collagen receptor GPVI (glycoprotein VI). In the absence of the FcR γ chain, collagen induced a unique pattern of tyrosine phosphorylation which was potentiated by the thromboxane analogue U46619. Immunoprecipitation studies indicated that neither collagen alone nor the combination of collagen plus U46619 induced phosphorylation of the GPVI-regulated proteins Syk and SLP-76 (Src homology 2-containing leucocyte protein of 76 kDa). A low level of tyrosine phosphorylation of phospholipase C γ 2 was observed, which was increased in the presence of U46619, although the degree of phosphorylation remained well below that observed in wild-type platelets (~10%). By contrast, collagen-induced phosphorylation of the adapter ADAP (adhesion- and degranulation-promoting adapter protein) was substantially po-

tentiated by U46619 to levels equivalent to those observed in wild-type platelets. Collagen plus U46619 also induced significant phosphorylation of FAK (focal adhesion kinase). The functional significance of collagen-induced non-GPVI signals was highlighted by the ability of U46619 and collagen to induce the secretion of ATP in FcR γ chain-deficient platelets, even though neither agonist was effective alone. Protein tyrosine phosphorylation and the release of ATP were abolished by the anti-(α 2 integrin) antibodies Ha1/29 and HM α 2, but not by blockade of α IIB β 3. These results illustrate a novel mechanism of platelet activation by collagen which is independent of the GPVI–FcR γ chain complex, and is facilitated by binding of collagen to integrin α 2 β 1.

Key words: α 2 β 1 integrin, collagen, Fc receptor γ chain, glycoprotein VI, knock-out model, protein tyrosine phosphorylation.

INTRODUCTION

Collagen-induced activation of platelets is a key event in the pathogenesis of arterial thrombosis associated with myocardial infarction and thrombotic stroke. Collagen interacts with at least two receptors on the surface of the platelets: the immunoglobulin receptor GPVI (glycoprotein VI) and integrin α 2 β 1 [1–3].

GPVI is a well characterized signalling receptor which forms a complex with the FcR γ chain (Fc receptor γ chain) [4–7]. Interaction of collagen with the GPVI–FcR γ -chain complex initiates a signalling pathway via tyrosine phosphorylation of an ITAM (immunoreceptor tyrosine-based activation motif) present on the FcR γ chain [3]. The tyrosine kinase Syk [8,9], as well as the adapters LAT (linker for activation of T cells) [10] and SLP-76 (Src homology 2-containing leucocyte protein of 76 kDa) [11], form key elements of the GPVI/FcR γ chain pathway resulting in the phosphorylation and activation of PLC γ 2 (phospholipase C γ 2) [8]. PLC γ 2 mediates an increase in intracellular calcium and subsequent platelet activation. Platelet activation by the GPVI-selective agonists CRP (collagen-related peptide) [12] and convulxin [13] is mediated through this pathway.

Although GPVI is the primary signalling receptor for collagen in platelets, collagen signalling has been reported previously in both murine and human platelets deficient in GPVI/FcR γ chain [9,14], implicating regulation via an additional receptor. These non-GPVI-mediated signals have not been characterized in detail and their functional significance remains uncertain, in view of the

inhibition of collagen-induced platelet activation in the absence of the GPVI–FcR γ -chain complex [15].

The most studied collagen-binding protein apart from GPVI remains the integrin α 2 β 1 [16]. In the resting platelet, α 2 β 1 exists in a form with low affinity for collagen, but, on activation, so-called inside-out signals [17] result in the conversion of the integrin into a high-affinity conformation [18]. In this way, α 2 β 1 mediates firm adhesion of platelets to collagen [19], thereby increasing the net interaction with GPVI [20] and promoting thrombus formation [21]. However, in platelet suspensions, blockade or ablation of α 2 β 1 has a limited effect on collagen-induced activation, the most consistent effect being a delay in response, casting doubt on the role of α 2 β 1 as an activatory receptor [15,20,22].

Although collagen-induced α 2 β 1-mediated signalling has been demonstrated in various cell types, including smooth muscle cells [23] and mammary epithelial cells [24], a role for α 2 β 1 in collagen signalling in platelets has been less clearly established. Prior to the cloning of GPVI, several studies implicated α 2 β 1 in collagen signalling in platelet suspensions [14,25]. However, in the study of Ichinohe et al. [14] it is not clear that the patient platelets used were completely devoid of GPVI [3,14], and since low levels of GPVI can mediate significant responses [26], which are themselves more sensitive to blockade of α 2 β 1 [27], this undermines the implicit claim that the responses observed were not mediated by GPVI. In the paper by Keely and Parise [25], the signalling responses observed were dependent on Fc γ RII, and α 2 β 1 was characterized as a co-receptor and not definitively

Abbreviations used: ADAP, adhesion- and degranulation-promoting adapter protein; CRP, collagen-related peptide; FAK, focal adhesion kinase; FcR γ chain, Fc receptor γ chain; γ –/– platelets, FcR γ chain-deficient platelets; GPVI, glycoprotein VI; LAT, linker for activation of T cells; PLC, phospholipase C; SLP-76, Src homology 2-containing leucocyte protein of 76 kDa; WT, wild-type.

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as an independent signalling receptor. Evidence using the snake toxin rhodocytin suggested that $\alpha 2\beta 1$ mediates phosphorylation of Syk and PLC $\gamma 2$ [28,29], although doubt has been cast on the specificity of rhodocytin for $\alpha 2\beta 1$ [30]. Subsequent to the identification and characterization of GPVI, a role for $\alpha 2\beta 1$ in collagen signalling in suspensions of platelets has been robustly [15] and more cautiously [31] challenged. A role for $\alpha 2\beta 1$ in collagen signalling in adherent platelets, however, has been identified previously [32], and more recently, two reports have indicated that $\alpha 2\beta 1$ -dependent platelet adhesion to collagen [33] or decorin [34] leads to tyrosine phosphorylation of Syk and PLC $\gamma 2$.

Despite the uncertainty surrounding the role of $\alpha 2\beta 1$ as a signalling receptor in platelets, a role for integrin signalling in general is well established. For example, phosphorylation of FAK (focal adhesion kinase) has been shown to be mediated via activation of integrin $\alpha \text{IIb}\beta 3$ in platelets [35,36], and has also been associated with binding of platelets to collagen via integrin $\alpha 2\beta 1$ [37]. In addition, ADAP (adhesion- and degranulation-promoting adapter protein; also known as SLAP-130 or Fyb) has been implicated in the clustering of integrin LFA-1 (lymphocyte function associated antigen-1; $\alpha \text{L}\beta 2$) following T cell receptor activation [38,39] and in signalling from $\beta 1$ integrins [40], as well as in $\alpha \text{IIb}\beta 3$ [41] and collagen [11] signalling in platelets.

In the present study, we have investigated and characterized further the collagen-induced signalling in murine platelets deficient in GPVI/FcR γ chain. The advantage of the murine knock-out system is that the platelets are definitively devoid of FcR γ chain, thereby avoiding the problems associated with the study of Ichinohe et al. [14]. Our results show that collagen induces a novel profile of tyrosine phosphorylation independently of GPVI/FcR γ chain, and, in combination with the thromboxane mimetic U46619, mediates the release of platelet ATP. These responses are dependent on the engagement of $\alpha 2\beta 1$, although it remains unclear whether the integrin acts as a signalling molecule or as an affinity modulator for collagen.

EXPERIMENTAL

Materials

C57/BL6 mice deficient in FcR γ chain were obtained from Dr Takashi Saito (Chiba University Graduate School of Medicine, Japan) [42]. Age-, sex- and strain-matched WT (wild-type) mice were used as controls. Heparin sodium (Monoparin®; 25 000 units/ml) was from CP Pharmaceuticals (Wrexham, U.K.); BSA was from First Link (U.K.) Ltd. (Brierley Hill, U.K.); Horm® collagen (Nycomed, Munich, Germany) was from Axis Shield (Dundee, U.K.). CRP [GPC(GPO)₁₂GPC] was synthesized by Tana Peptides (Houston, TX, U.S.A.) and cross-linked as described below. The $\alpha \text{IIb}\beta 3$ antagonist lotrafiban was supplied by GlaxoSmithKline (King of Prussia, PA, U.S.A.). Two Armenian hamster antibodies against $\alpha 2$ integrin, Ha1/29 (IgG2, λ) and HM $\alpha 2$ (IgG1, κ), and an isotype control against $\alpha 1$ integrin, Ha31/8 (IgG2, λ), were from BD Pharmingen (Oxford, U.K.). Murine anti-phosphotyrosine antibody 4G10 was from Upstate (Milton Keynes, Bucks., U.K.). Anti-Syk (BR15) and anti-PLC $\gamma 2$ (DN84) antibodies were kindly donated by Dr M. Tomlinson and Dr J. Bolen (DNAX Research Institute, Palo Alto, CA, U.S.A.). Rabbit polyclonal anti-FAK antibody (C-903) was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Sheep polyclonal anti-ADAP and anti-SLP-76 antibodies were gifts from Dr G. Koretzky (University of Pennsylvania, PA, U.S.A.). Horseradish peroxidase-conjugated sheep anti-mouse, donkey anti-sheep and donkey anti-rabbit antibodies were obtained from Amersham

Biosciences (Little Chalfont, Bucks., U.K.). Other reagents were from Sigma (Poole, Dorset, U.K.) or BDH (Poole, Dorset, U.K.).

Preparation of cross-linked CRP

CRP was dissolved in 0.1 M NaHCO₃ at a concentration of 3 mM and cross-linked with 1.5 molar equivalents of SPDP [3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester] at room temperature for 1 h, after which the cross-linked peptide was dialysed against 0.01 M acetic acid for 4 h.

Preparation of washed murine platelets

Blood (750–1000 μl) was taken into 300 μl of heparin sodium (10 units/ml) from the hepatic portal vein under terminal CO₂ narcosis. Platelet-rich plasma was obtained by differential centrifugation at 300 *g* for 5 min at room temperature. Following the addition of prostacyclin (1 $\mu\text{g}/\text{ml}$), platelet-rich plasma was centrifuged at 1000 *g* for 6 min at room temperature. Pelleted platelets were resuspended at $200 \times 10^6/\text{ml}$ in modified Tyrode's solution (137 mM NaCl, 11.9 mM NaHCO₃, 0.4 mM NaH₂PO₄, 2.7 mM KCl, 1.1 mM MgCl₂, 5.6 mM glucose, pH 7.4) and left for approx. 1 h at room temperature prior to use.

Platelet aggregometry

Platelet aggregation was measured using an optical method [43] with a BioData PAP-4 aggregometer (Alpha Laboratories, Eastleigh, U.K.) and also with a single platelet counting technique [44]. Platelet stimulation was performed in siliconized glass cuvettes at 37 °C with a stir speed of 1000 rev./min. On completion, the aggregometer generated a value for the extent of aggregation, measured as percentage light transmission, where platelet-poor plasma represented 100 % transmission. At the same time, the platelets were fixed using formaldehyde (0.1 % final concentration) and EDTA (3 mM final concentration). Platelet counts of the fixed samples were performed using a Coulter Z2 particle count and size analyser. Only data for optical aggregation are shown, since the results with the platelet counting method revealed a similar pattern.

Measurement of released ATP

Following activation, platelets were fixed as described above. A 100 μl aliquot of each fixed sample was centrifuged at 13 000 *g* for 1 min, and 50 μl of the supernatant was frozen prior to analysis. Levels of ATP were determined using a luminometric assay [45]. Levels of luminescence were measured using an EG&G Berthold Lumat LB9507 luminometer (PerkinElmer Life Sciences, Cambridge, U.K.). Results are expressed as the concentration of ATP (μM) present in the sample following stimulation.

Protein phosphorylation studies

For signalling studies, platelets were stimulated in suspension at 37 °C under stirring conditions, as for aggregation and ATP release. For whole-cell tyrosine phosphorylation, stimulation was stopped by the addition of Laemmli's sample buffer, boiled for 5 min and separated by SDS/PAGE on 4–12 % (w/v) pre-cast NuPage Bis-Tris gels (Invitrogen, Paisley, U.K.) under reducing conditions before transfer to a PVDF membrane (Bio-Rad, Hemel Hempstead, U.K.).

For measurement of tyrosine phosphorylation of individual proteins, stimulation was stopped by the addition of an equal volume of ice-cold lysis buffer (2 % Nonidet P40, 300 mM

NaCl, 20 mM Tris, 10 mM EDTA, 2 mM Na_3VO_4 , 1 mM PMSF, 10 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ pepstatin A, pH 7.4). Samples were pre-cleared using combinations of Protein G-Sepharose and Protein A-Sepharose, before the addition of specific immunoprecipitating antibodies. Rotating samples were incubated with antibodies overnight at 4 °C in the presence of either Protein A-Sepharose (for rabbit antibodies) or a combination of Protein A-Sepharose and Protein G-Sepharose (for sheep antibodies). Immunoprecipitated proteins were washed in ice-cold PBS before being boiled in Laemmli sample buffer for 5 min. Proteins were separated by SDS/PAGE on 10 % (w/v) acrylamide gels under reducing conditions before transfer to a PVDF membrane.

Membranes were blocked for at least 1 h in 10 % (w/v) BSA in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.6) and then incubated for at least 1 h with primary antibody. The membranes were then washed for 30 min in TBS-T before being incubated for a further 1 h with an appropriate horseradish peroxidase-conjugated secondary antibody diluted in TBS-T. Following washing in TBS-T, the membranes were developed using an enhanced chemiluminescence detection system. Levels of phosphorylation were quantified densitometrically using a Leica Q500C image analyser.

Data analysis and statistics

Phosphorylation data were normalized between datasets from different experiments so that the standardized value for the response induced by collagen in WT platelets was unity and the estimated variance was directly proportional to the estimated mean value within each treatment group. Levels of phosphorylation are expressed as relative units of integrated absorbance.

Functional and phosphorylation data were analysed using one-way ANOVA and Waller–Duncan post-hoc testing with a Type I/Type II error seriousness ratio of 100. This method groups different treatments into subsets, treatments within which are not significantly different at a level of $P = 0.05$.

All data are expressed as means $\pm$ S.E.M. Analysis was performed with Microsoft® Excel 2000 and SPSS for Windows (Version 10).

RESULTS

Collagen induces tyrosine phosphorylation in γ -/- (FcR γ chain-deficient) platelets

Washed suspensions of WT and γ -/- platelets were stimulated with CRP (30 $\mu\text{g/ml}$) or collagen (30 $\mu\text{g/ml}$) for 2 min in the presence of the $\alpha\text{IIb}\beta_3$ integrin inhibitor lotrafiban (10 μM). CRP and collagen induced strong signalling responses in WT platelets (Figure 1A). Bands corresponding to the FcR γ chain (15 kDa), LAT (36 kDa) and PLC γ_2 (150 kDa) showed increased phosphorylation, as did bands at 23, 75, 100 and 130 kDa. In γ -/- platelets, there was no CRP-induced increase in tyrosine phosphorylation (Figure 1A), whereas collagen stimulated a qualitatively distinct, albeit markedly reduced, pattern of tyrosine phosphorylation that included bands at 23, 50–60, 75, 130 and 150 kDa. However, there was no discernible phosphorylation of LAT or the FcR γ chain. In some experiments, a constitutively phosphorylated band of 25 kDa was observed in the γ -/- platelets, which was not altered upon activation by collagen and was not seen in all studies. WT and γ -/- platelets were responsive over similar concentration ranges of collagen (> 1 $\mu\text{g/ml}$) (Figure 1B).

Figure 1 Whole-cell tyrosine phosphorylation of WT and γ -/- platelets

Tyrosine phosphorylation in whole-cell lysates was detected using the anti-phosphotyrosine antibody 4G10. (A) Collagen (30 $\mu\text{g/ml}$) and CRP (30 $\mu\text{g/ml}$) both induced tyrosine phosphorylation in WT platelets, but only collagen induced a response in γ -/- platelets. Consistent increases in phosphorylation were observed in bands of 150, 130, 75 and 50–60 kDa. No LAT phosphorylation was observed in γ -/- platelets, confirming the lack of GPVI signalling. (B) Concentration–response data for collagen-induced whole-cell tyrosine phosphorylation in WT and γ -/- platelets, showing that the range of active concentrations is the same in both sets of platelets. The apparent lack of phosphorylation of the 130 kDa band in WT platelets stimulated with 1 $\mu\text{g/ml}$ collagen was caused by a localized failure of transfer of protein from the gel to the PVDF membrane. MM, molecular mass (kDa).

Role of integrins $\alpha 2\beta 1$ and $\alpha\text{IIb}\beta_3$

The role of integrins $\alpha 2\beta 1$ and $\alpha\text{IIb}\beta_3$ in collagen-induced tyrosine phosphorylation in γ -/- platelets was investigated. Platelets were stimulated for 2.5 min with collagen (50 $\mu\text{g/ml}$) and the effects of the $\alpha\text{IIb}\beta_3$ antagonist lotrafiban (10 μM) and the anti-(murine $\alpha 2$ integrin) monoclonal antibodies Ha1/29 (20 $\mu\text{g/ml}$) and HM $\alpha 2$ (20 $\mu\text{g/ml}$) were investigated. Both Ha1/29 and HM $\alpha 2$ have been used previously to block the collagen– $\alpha 2\beta 1$ interaction in mice [26,33]. The effects of the anti-($\alpha 1$ integrin) antibody Ha31/8 (20 $\mu\text{g/ml}$), an isotype control for Ha1/29 (Armenian hamster IgG2, λ), are also shown. Collagen-induced phosphorylation of γ -/- platelets was not altered in the presence of the $\alpha\text{IIb}\beta_3$ antagonist lotrafiban (Figure 2A). In contrast, the anti-(murine $\alpha 2$ integrin) monoclonal antibodies Ha1/29 and HM $\alpha 2$ substantially inhibited tyrosine phosphorylation induced by collagen. The isotype control Ha31/8 had no effect on the response. These data indicate that $\alpha 2\beta 1$ facilitates the

Figure 2 Inhibition and potentiation of collagen-induced whole-cell tyrosine phosphorylation in γ -/- platelets

Washed platelets were stimulated with collagen (50 μ g/ml) plus U46619 (1 μ M) in the presence or absence of the α IIb β 3 antagonist lotrafiban (10 μ M), the anti- α 2 antibodies Ha1/29 (20 μ g/ml) or HM α 2 (20 μ g/ml), or the anti- α 1 antibody Ha31/8 (20 μ g/ml). Tyrosine phosphorylation was measured in whole-cell lysates using the anti-phosphotyrosine antibody 4G10. **(A)** Collagen-induced tyrosine phosphorylation was unaffected by lotrafiban, whereas it was substantially inhibited in the presence of Ha1/29 or HM α 2. The isotype control Ha31/8 (Armenian hamster IgG2, λ) had no effect on the response. **(B)** In the presence of lotrafiban, the thromboxane A_2 analogue U46619 induced a weak response when added alone. However, when added in combination with collagen, the response was greatly potentiated, in particular for a band at 100 kDa. Both Ha1/29 and HM α 2 inhibited the response, although the former antibody was slightly more effective. Ha31/8 had no effect. In order to highlight the increase in response in the presence of U46619 in particular, the films shown in this Figure were exposed to a much lesser extent than those shown in Figure 1. This accounts for the apparently lower levels of phosphorylation in basal and collagen-stimulated lanes, and the lighter background in this Figure compared with Figure 1. MM, molecular mass (kDa).

collagen-induced response in γ -/- platelets, but does not support a significant role for α IIb β 3.

Synergy with U46619 in γ -/- platelets

We investigated the ability of the thromboxane A_2 analogue U46619 (1 μ M) to augment the signalling response induced by collagen (50 μ g/ml). In the presence of lotrafiban (10 μ M), U46619 and collagen in combination induced substantially more tyrosine phosphorylation than was induced by either agonist alone, as illustrated in particular by the greatly increased phosphorylation of a band at 100 kDa. In the presence of the α 2 β 1 blocking antibody Ha1/29, the response induced by collagen and U46619 in γ -/- platelets was substantially inhibited. The α 2-blocking antibody HM α 2 also inhibited the response, but was

Figure 3 Functional responses of γ -/- platelets

Release of ATP **(A)** and aggregation [measured using absorbance ('optical density')] **(B)** in WT and γ -/- platelets. Platelets were stimulated with collagen (50 μ g/ml), CRP (5 μ g/ml), U46619 (1 μ M), thrombin (1 units/ml) or a combination of collagen and U46619. The effects of the α IIb β 3 antagonist lotrafiban (10 μ M) and the anti- α 2 antibody Ha1/29 (20 μ g/ml) are shown. Negative values for aggregation indicate shape change. The apparent inability of lotrafiban to inhibit thrombin-induced aggregation is due to thrombin-induced release of fibrinogen from alpha granules and subsequent generation of fibrin, which causes platelet trapping. This can be blocked by the fibrin polymerization inhibitor Gly-Pro-Arg-Pro (results not shown). The apparent residual CRP-induced aggregation in the presence of lotrafiban is caused by the release of platelet granules, which reduces the absorbance of suspensions of platelets [43]. The unexpectedly weak aggregation induced by the combination of U46619, collagen and Ha1/29 in γ -/- platelets was caused by a non-specific inhibitory effect of Ha1/29 on aggregation induced by U46619 alone. A similar effect was observed with the antibodies HM α 2 and Ha31/8 (results not shown). Data are means $\pm$ S.E.M. for responses measured 6 min after addition of the agonist ($n = 3-6$).

not as effective as Ha1/29. The anti- α 1 integrin) isotype control Ha31/8 had no effect on the response (Figure 2B).

Functional synergy with U46619 in γ -/- platelets

We investigated platelet aggregation (measured optically and by single platelet counting) and the release of ATP induced by collagen (50 μ g/ml), CRP (5 μ g/ml) and U46619 (1 μ M). In γ -/- platelets, CRP had no effect, whereas collagen induced a slow, continuous decrease in absorbance that was not significantly inhibited by lotrafiban but was abolished by the antibody Ha1/29 (20 μ g/ml) (Figure 3B). This pattern was paralleled by the platelet count data (results not shown). These data suggest that the small aggregation responses were mediated predominantly by adhesion of platelets to collagen via α 2 β 1 and not as a result of α IIb β 3-mediated aggregation. Collagen induced no significant release of ATP in γ -/- platelets (Figure 3A).

U46619 (1 μ M) consistently induced maximal α IIb β 3-dependent aggregation, but, in the presence of lotrafiban, it induced only shape change, and had no significant effect on the platelet count. In γ -/- platelets with lotrafiban present, however, U46619 enhanced the collagen-induced reduction in absorbance (Figure 3B), and together they caused a near-maximal reduction in the platelet count (results not shown). Both responses were blocked by Ha1/29, suggesting that U46619 had induced the

activation of $\alpha 2\beta 1$, thereby promoting greater binding of collagen to the platelets.

U46619 caused no significant release of ATP, in either the presence or the absence of lotrafiban, in WT or $\gamma^{-/-}$ platelets (Figure 3A). These data are consistent with other studies in mice which indicate that there is no measurable ATP release by 1 μ M U46619 [46]. Strikingly, however, in $\gamma^{-/-}$ platelets, U46619 plus collagen induced significant ATP release, equivalent to approx. 50% of that seen when platelets were activated with thrombin (1 unit/ml). This release was unaffected by lotrafiban, but was abolished by Ha1/29, indicating the involvement of $\alpha 2\beta 1$. These results reveal a previously unrecognized synergy between collagen and U46619 in the absence of the GPVI-FcR γ -chain complex. This synergy is independent of α Ib β 3, but is critically dependent on the binding of collagen to integrin $\alpha 2\beta 1$.

Identification of signalling proteins

Candidate proteins that co-migrate with the phosphorylated bands in whole-cell lysates were analysed by immunoprecipitation. PLC γ 2, Syk and SLP-76 are well characterized participants in the GPVI-induced signalling pathway [8,9,11] and have been implicated in signalling mediated by α Ib β 3 [41,47] and $\alpha 2\beta 1$ [25,33]. FAK [35] and ADAP [11] have also been implicated in collagen-induced signalling in platelets. Each protein was immunoprecipitated following stimulation of platelets for 2.5 min with collagen (50 μ g/ml), CRP (5 μ g/ml), U46619 (1 μ M) or combined collagen (50 μ g/ml) plus U46619 (1 μ M). All studies were performed in the presence of lotrafiban (10 μ M).

The tyrosine kinase Syk, the adapter SLP-76 and PLC γ 2 were phosphorylated in WT platelets stimulated by CRP and collagen, but not by in those treated with U46619 (Figures 4A–4C). Neither CRP nor collagen induced phosphorylation of Syk or SLP-76 in $\gamma^{-/-}$ platelets. Similarly, CRP induced no tyrosine phosphorylation of PLC γ 2 in $\gamma^{-/-}$ platelets, whereas collagen stimulated weak tyrosine phosphorylation of PLC γ 2, equivalent to approx. 4% of the level observed in WT platelets. Collagen and U46619 together induced increased phosphorylation of PLC γ 2, which was approx. 10% of the response to collagen in WT platelets (Figure 4A). Collagen and U46619 together induced no detectable increase in phosphorylation of SLP-76 or Syk in $\gamma^{-/-}$ platelets (Figures 4B and 4C).

Blockade of $\alpha 2\beta 1$ by Ha1/29 inhibited PLC γ 2 phosphorylation in $\gamma^{-/-}$ platelets, implicating the integrin in this response. The attenuation of the collagen-induced phosphorylation of PLC γ 2, Syk and SLP-76 in WT platelets by Ha1/29 is consistent with previous findings indicating that the binding of collagen to $\alpha 2\beta 1$ promotes its interaction with GPVI and subsequent signalling [20]. The ability of U46619 to restore the response in the presence of Ha1/29 in WT platelets suggests that signals induced by U46619, while unable to induce significant phosphorylation alone, are able to synergize with those from GPVI, as suggested previously [46].

In WT platelets, collagen induced substantial phosphorylation of both the adapter ADAP and FAK, in contrast with CRP, which induced a much lower level of phosphorylation (Figure 5). In $\gamma^{-/-}$ platelets, collagen induced the tyrosine phosphorylation of ADAP; this was increased in the presence of U46619 to a level equivalent to that observed with collagen in WT platelets (Figure 5A). Collagen alone induced no discernible phosphorylation of FAK, but in combination with U46619 it induced a significant response, equivalent to 50% of that seen with collagen in WT platelets (Figure 5B). This is consistent with previous findings that FAK phosphorylation is dependent on co-ordinated signalling through integrin and agonist receptors [48]. Ha1/29 abolished the

Figure 4 Roles of PLC γ 2, Syk and SLP-76 in collagen-induced signalling in WT and $\gamma^{-/-}$ platelets

Tyrosine phosphorylation of (A) PLC γ 2, (B) Syk and (C) SLP-76 in WT and $\gamma^{-/-}$ platelets is shown. The proteins were immunoprecipitated (IP) from lysed aliquots of platelets following stimulation for 2.5 min with collagen (50 μ g/ml), CRP (5 μ g/ml), U46619 (1 μ M), or a combination of collagen and U46619 in the absence or presence of Ha1/29 (20 μ g/ml). Lotrafiban (10 μ M) was present in all cases. Densitometric data are shown for PLC γ 2 ($n = 4$), and expressed as means $\pm$ S.E.M. of relative integrated absorbance (IOD) units. No densitometric data are shown for Syk or SLP-76, since no consistent measurable phosphorylation above background was ever observed in the $\gamma^{-/-}$ platelets. Representative Western blots (WB) are shown for PLC γ 2, Syk ($n = 3$) and SLP-76 ($n = 2$).

phosphorylation of ADAP and FAK observed in $\gamma^{-/-}$ platelets, emphasizing the critical role of $\alpha 2\beta 1$.

Ha1/29 also inhibited ADAP and FAK phosphorylation in WT platelets. However, in contrast with the phosphorylation of

A IP ADAP

B IP FAK

Figure 5 Roles of ADAP and FAK in collagen-induced signalling in WT and γ -/- platelets

Tyrosine phosphorylation of (A) ADAP and (B) FAK in WT and γ -/- platelets is shown. Platelet stimulation and protein immunoprecipitation (IP) were conducted as described in the legend to Figure 4. Densitometric data are shown for ADAP ($n=5$) and FAK ($n=3$), and are expressed as means $\pm$ S.E.M. of relative integrated absorbance (IOD) units. Representative Western blots (WB) are also shown.

PLC γ 2, Syk and SLP-76, the further addition of U46619 did not significantly restore the response in the presence of α 2 β 1 blockade. This distinction between PLC γ 2, Syk and SLP-76 on the one hand and FAK and ADAP on the other is emphasized further in the comparison of collagen and CRP signalling. Tyrosine phosphorylation of ADAP and FAK induced by collagen plus U46619 in γ -/- platelets was substantially greater than that induced by CRP in WT platelets, whereas the CRP-induced phosphorylation of PLC γ 2, SLP-76 and Syk in WT platelets

was substantially greater than that induced by collagen plus U46619 in γ -/- platelets. Therefore these findings highlight the distinctive pattern of collagen-induced signalling in γ -/- platelets as compared with the CRP-induced activation of the GPVI/FcR γ -chain pathway in WT platelets.

DISCUSSION

In the present study we have demonstrated that collagen induces a unique pattern of tyrosine phosphorylation in platelets in suspension independently of the GPVI-FcR γ -chain complex. This phosphorylation occurs within a time scale that is of functional relevance and at concentrations of collagen that induce responses in WT platelets. The combination of collagen and U46619 resulted in a greatly increased phosphorylation response and also in release of ATP. We have shown that integrin α 2 β 1 plays a critical role in these phenomena, whereas α IIb β 3 does not.

The GPVI-FcR γ -chain complex mediates platelet activation by the selective GPVI agonist CRP and the native ligand collagen. Platelets deficient in the FcR γ chain do not express GPVI [5] and do not respond to CRP [22]. The functional deficit in platelets lacking GPVI also highlights its importance for collagen-induced aggregation and release [9,15,22].

In the present study, we investigated collagen-induced responses and signalling in γ -/- platelets. In suspensions of washed γ -/- platelets, collagen induced a weak optical response and a partial loss of single platelets. These responses were all abolished by the anti- α 2 antibody Ha1/29, suggesting that the small increase in light transmission and the loss of single platelets were mediated by the adhesion of platelets to collagen fibres via α 2 β 1. These responses were accompanied by phosphorylation, which was also abolished by Ha1/29. This may indicate that these signals are mediated directly by α 2 β 1 or, alternatively, that binding of collagen to platelets via α 2 β 1 facilitates its interaction with another, as yet unidentified, signalling receptor in a manner similar to that by which it facilitates the interaction of collagen with GPVI in WT platelets [20].

U46619, an analogue of thromboxane A₂, potentiated these responses and signals induced by collagen in γ -/- platelets. At 1 μ M, U46619 consistently induced lotrafiban-sensitive aggregation, and hence α IIb β 3 activation, and has been shown previously to activate α 2 β 1 [18]. Hence the potentiation observed may have arisen simply as a result of increased binding to activated α 2 β 1. Indeed, 0.1 μ M U46619 induced only shape change, and did not potentiate collagen-induced phosphorylation (results not shown). However, there may also have been synergism between U46619-induced signals and the non-GPVI-mediated collagen signals.

It is noteworthy in this regard that, while U46619 induced an increase in tyrosine phosphorylation of all the bands that were phosphorylated by collagen in γ -/- platelets, U46619 and collagen together induced substantial phosphorylation of a further band at 100 kDa that was barely affected by either agonist alone (Figure 2B). Furthermore, neither U46619 nor collagen alone induced release of ATP, whereas together they induced substantial release. These observations suggest that collagen and U46619 may generate signals which synergize independently of GPVI. Irrespective of the mechanism of potentiation of the collagen signal, it is possible that the functional activation induced by collagen and U46619 observed in the present study may be of physiological significance *in vivo*, and may help to explain why GPVI-deficient mice do not manifest a severe bleeding tendency [49].

Previous studies in human platelets have implicated PLC γ 2 and Syk in α 2 β 1-mediated signalling [25,28,33,34]. In the present study, we also observed phosphorylation of PLC γ 2 in murine platelets, although we were unable to demonstrate phosphorylation of Syk even with concomitant activation with U46619. Since PLC γ 2 phosphorylation was weak in our study, it is possible that levels of Syk phosphorylation were simply below the limit of detection. However, it is also possible that this apparent discrepancy with previous reports reflects a species difference.

The pattern of collagen signalling in the γ -/- platelets reflected a radical reduction in the phosphorylation of proteins associated with the GPVI/FcR γ chain, namely PLC γ 2, Syk, SLP-76 and LAT, and a substantial participation of two proteins implicated previously in integrin signalling, namely FAK and ADAP. In WT platelets, as expected, CRP induced substantial phosphorylation of PLC γ 2, Syk and SLP-76, but minimal phosphorylation of either FAK or ADAP, most notably as compared with the collagen-induced response in WT platelets. By contrast, in γ -/- platelets stimulated simultaneously with collagen and U46619, there was substantial phosphorylation of FAK and ADAP, no detectable phosphorylation of Syk, SLP-76 or LAT, and minimal phosphorylation of PLC γ 2. These data suggest that the phosphorylation of FAK and ADAP induced by collagen in WT platelets may be mediated to a significant extent by receptors other than GPVI/FcR γ chain. Hence these results suggest the existence of a novel pathway of collagen-induced platelet activation, distinct from GPVI.

The failure of collagen alone to induce phosphorylation of FAK in the γ -/- platelets is consistent with the findings of Shattil et al. [48] who showed that concomitant signalling from U46619 and α IIB β 3 was required for FAK phosphorylation in human platelets. Our study parallels this result by showing a requirement for U46619 and the integrin α 2 β 1. In contrast with this, Achison et al. [50] showed that collagen-induced FAK phosphorylation in human platelets was mediated primarily by GPVI and secondarily by α IIB β 3, but not by α 2 β 1. Given that the response in the present study is clearly not mediated by either GPVI or α IIB β 3, this may also reflect a species difference.

In conclusion, we have demonstrated that collagen induces functional responses in suspensions of mouse platelets in the presence of the thromboxane mimetic U46619 which are independent of GPVI/FcR γ chain. These responses are induced by concentrations of collagen that activate WT platelets, and the response occurs within a short and relevant period of time. Further work is in progress to confirm whether the observed synergy is simply a function of increased collagen binding or is indicative of genuine signalling synergy. Although GPVI is clearly of fundamental importance in initiating collagen-induced activation, our study shows that other signalling responses that arise as a result of collagen binding to α 2 β 1 are of potential significance in platelet physiology. This study confirms and adds significantly to the growing body of evidence that shows that α 2 β 1 is a key regulatory molecule in collagen-induced platelet signalling and activation. Whether its role is to mediate activation directly or to present collagen to a further, as yet uncharacterized, signalling receptor is the subject of ongoing studies.

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