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The Fc receptor γ-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen

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Activation of mouse platelets by collagen is associated with tyrosine phosphorylation of multiple proteins including the Fc receptor γ-chain, the tyrosine kinase Syk and phospholipase Cγ2, suggesting that collagen signals in a manner similar to that of immune receptors. This hypothesis has been tested using platelets from mice lacking the Fc receptor γ-chain or Syk. Tyrosine phosphorylation of Syk and phospholipase Cγ2 by collagen stimulation is absent in mice lacking the Fc receptor γ-chain. Tyrosine phosphorylation of phospholipase Cγ2 by collagen stimulation is also absent in mice platelets which lack Syk, although phosphorylation of the Fc receptor γ-chain is maintained. In contrast, tyrosine phosphorylation of platelet proteins by the G protein-coupled receptor agonist thrombin is maintained in mouse platelets deficient in Fc receptor γ-chain or Syk. The absence of Fc receptor γ-chain or Syk is accompanied by a loss of secretion and aggregation responses in collagen- but not thrombin-stimulated platelets. These observations provide the first direct evidence of an essential role for the immunoreceptor tyrosine-based activation motif (ITAM) in signalling by a non-immune receptor stimulus.

Keywords: collagen/Fc receptor γ-chain/ITAM/platelets/Syk

Introduction

Collagen is an abundant extracellular matrix protein present in blood vessel subendothelium. Upon damage to the endothelial lining, platelets adhere to collagen fibres and undergo activation through a tyrosine kinase-dependent mechanism leading to secretion of granule contents and platelet aggregation. The molecular mechanism underlying these activation events by collagen is not established, with several platelet surface glycoproteins implicated as collagen receptors, including the integrin $\alpha_2\beta_1$ (Santoro *et al.*, 1988), glycoprotein IV (GPIIIb, CD36) (Tandon *et al.*, 1989), glycoprotein VI (Ryo *et al.*, 1992) and uncharacterized 65 (Chiang and Kang, 1982) and 85–90 kDa glycoproteins (Deckmyn *et al.*, 1992). Patients with defects in expression of these proteins, or who possess autoantibodies to them, display mild bleeding disorders (Moroi *et al.*, 1989; Deckmyn *et al.*, 1992; Ryo *et al.*, 1992; Kehrel *et al.*, 1993; Daniel *et al.*, 1994b; McKeown *et al.*, 1994).

Collagen induces phosphorylation of multiple platelet proteins on tyrosine, including the Fc receptor γ-chain (FcR γ-chain), the non-receptor tyrosine kinase Syk and phospholipase Cγ2 (PLCγ2) (Blake *et al.*, 1994; Daniel *et al.*, 1994a; Fujii *et al.*, 1994; Yanaga *et al.*, 1995; Asazuma *et al.*, 1996; Gibbins *et al.*, 1996). Syk assembles into signalling complexes at the plasma membrane via interaction between its tandem Src homology 2 (SH2) domains and a tyrosine-phosphorylated immunoreceptor tyrosine-based activation motif (ITAM). We recently proposed a model for collagen-induced signalling in human platelets in which receptor clustering induced by collagen leads to tyrosine phosphorylation of the FcR γ-chain, possibly by a Src family kinase, allowing binding of Syk, which becomes tyrosine phosphorylated and activated (Gibbins *et al.*, 1996). This initiates a series of events which may involve other kinases and adapter proteins leading to tyrosine phosphorylation and activation of PLCγ2. This model has been evaluated in the present study using platelets from genetically modified mice which lack the FcR γ-chain or Syk.

Results

Tyrosine phosphorylation in collagen- and thrombin-stimulated platelets

Collagen and thrombin stimulated distinct but overlapping increases in whole cell tyrosine phosphorylation in platelets (Figures 1 and 2A). Both agonists stimulated increases in tyrosine phosphorylation of proteins of ~42, 70–75, 100, 110 and 130 kDa in platelets from B6 mice; the 42 and 110 kDa proteins were more heavily phosphorylated in thrombin-stimulated cells. The largest increase in tyrosine phosphorylation was in the 70–75 kDa proteins. Collagen but not thrombin also stimulated marked tyrosine phosphorylation of a 36 kDa protein. A candidate protein for this is p36-38 which is tyrosine phosphorylated in collagen-stimulated human platelets and associates with the SH2 domains of Grb2 and PLCγ1 (Robinson *et al.*, 1996).

The concentration–response curve for the increase in tyrosine phosphorylation in response to collagen occurred over a similar range (3–100 μg/ml) to that in human platelets (not shown). For the purpose of studies in which tissue was limiting, 10 and 60 μg/ml were chosen to represent low and high concentrations of collagen,

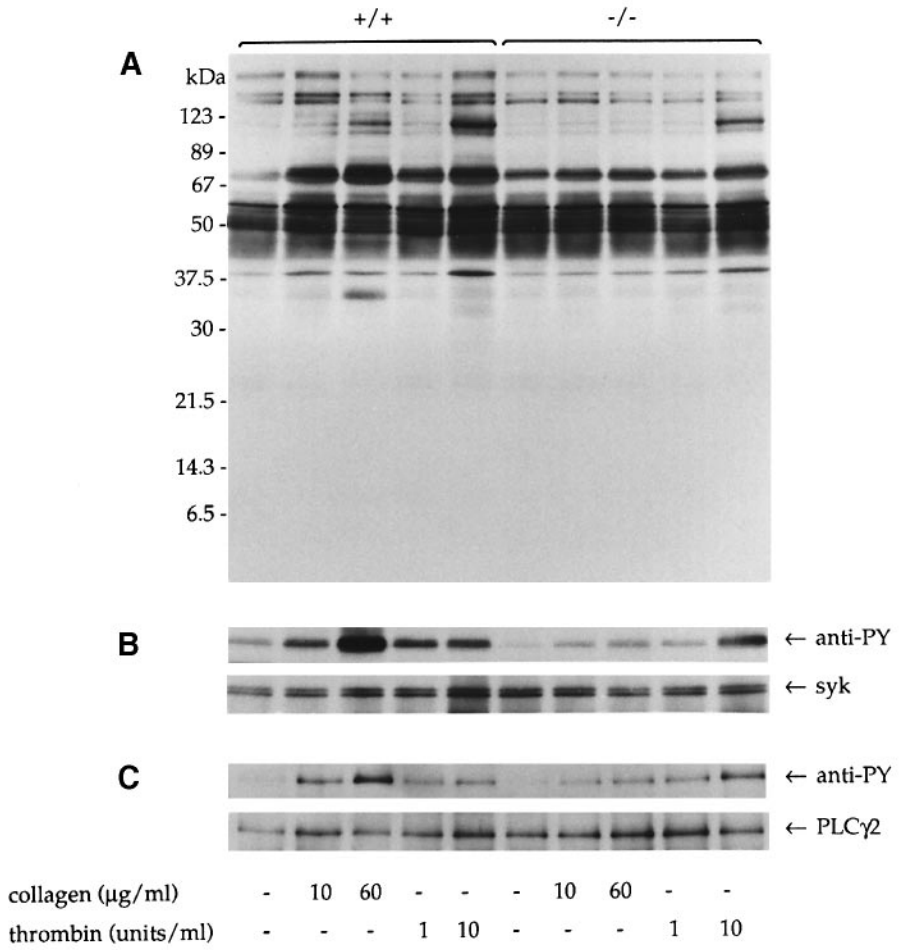

Fig. 1. Absence of tyrosine phosphorylation of Syk and PLCγ2 in collagen-stimulated platelets which lack the FcR γ-chain. Platelets prepared from control B6 and FcR γ-chain-deficient mice were stimulated with collagen (10 or 60 μg/ml for 120 s) or thrombin (1 or 10 U/ml for 60 s). For whole cell lysates (A), stimulation was terminated by addition of Laemmli sample treatment buffer and samples immunoblotted for phosphotyrosine residues. Tyrosine-phosphorylated bands corresponding to Syk (72 kDa), PLCγ2 (140–150 kDa) and the FcR γ-chain (14 kDa) cannot be resolved from other tyrosine-phosphorylated proteins in whole cell lysates or are absent. For immunoprecipitations of Syk (B) and PLCγ2 (C), stimulation was terminated by addition of NP-40 lysis buffer and precipitated proteins were immunoblotted for phosphotyrosine residues (upper immunoblot in each panel). Immunoprecipitations were verified by reprobing for the relevant protein [lower immunoblot of (B) and (C)]. Results are representative of two FcR γ-chain –/– mutants and two control mice.

respectively. Thrombin stimulated tyrosine phosphorylation at a threshold of 1 U/ml and produced a maximal effect at 10 U/ml. The relative insensitivity to thrombin compared with human platelets is consistent with a novel receptor as demonstrated in mutant mice lacking the 'classical' thrombin receptor (Connolly *et al.*, 1996). Thrombin stimulated a rapid onset of tyrosine phosphorylation which was detectable within 5 s of stimulation. In contrast, the response to collagen is delayed by ~15 s, as with human platelets. The increase in phosphorylation was maintained for up to 300 s for both agonists (not shown).

Antibodies and a GST fusion protein were used to precipitate and identify specific proteins which undergo tyrosine phosphorylation. Collagen stimulated tyrosine phosphorylation of Syk and PLCγ2, with marked phosphorylation observed at 60 μg/ml (Figures 1 and 2B). No tyrosine-phosphorylated proteins co-immunoprecipitated with PLCγ2. An unidentified tyrosine-phosphorylated protein of 80 kDa co-immunoprecipitated (not shown) with Syk, which may be the same protein as that seen in human platelets in Syk immunoprecipitates (Asselin *et al.*, 1997). Thrombin also stimulated tyrosine phosphorylation of Syk and PLCγ2, although to a much lesser extent, with phosphorylation of PLCγ2 being particularly weak (Figure 1). Collagen stimulated tyrosine phosphorylation of the FcR γ-chain (Figure 2C) which was precipitated by association with the GST fusion protein of the tandem SH2 domains of Syk. These results agree with previous observations in human platelets (Blake *et al.*, 1994; Daniel *et al.*, 1994a; Fujii *et al.*, 1994; Yanaga *et al.*, 1995; Asazuma *et al.*, 1996; Gibbins *et al.*, 1996).

Collagen-stimulated tyrosine phosphorylation of Syk and PLCγ2 is absent in platelets lacking the FcR γ-chain

Collagen did not stimulate an increase in whole cell tyrosine phosphorylation in platelets from mutant mice lacking the FcR γ-chain (Figure 1). Immunoprecipitation of Syk and PLCγ2 demonstrated that tyrosine phosphorylation of these proteins in response to collagen was almost completely absent in FcR γ-chain-deficient platelets (Figure 1). The small increase in phosphorylation of both proteins observed was reproducible, suggesting the existence of an alternative pathway of collagen receptor

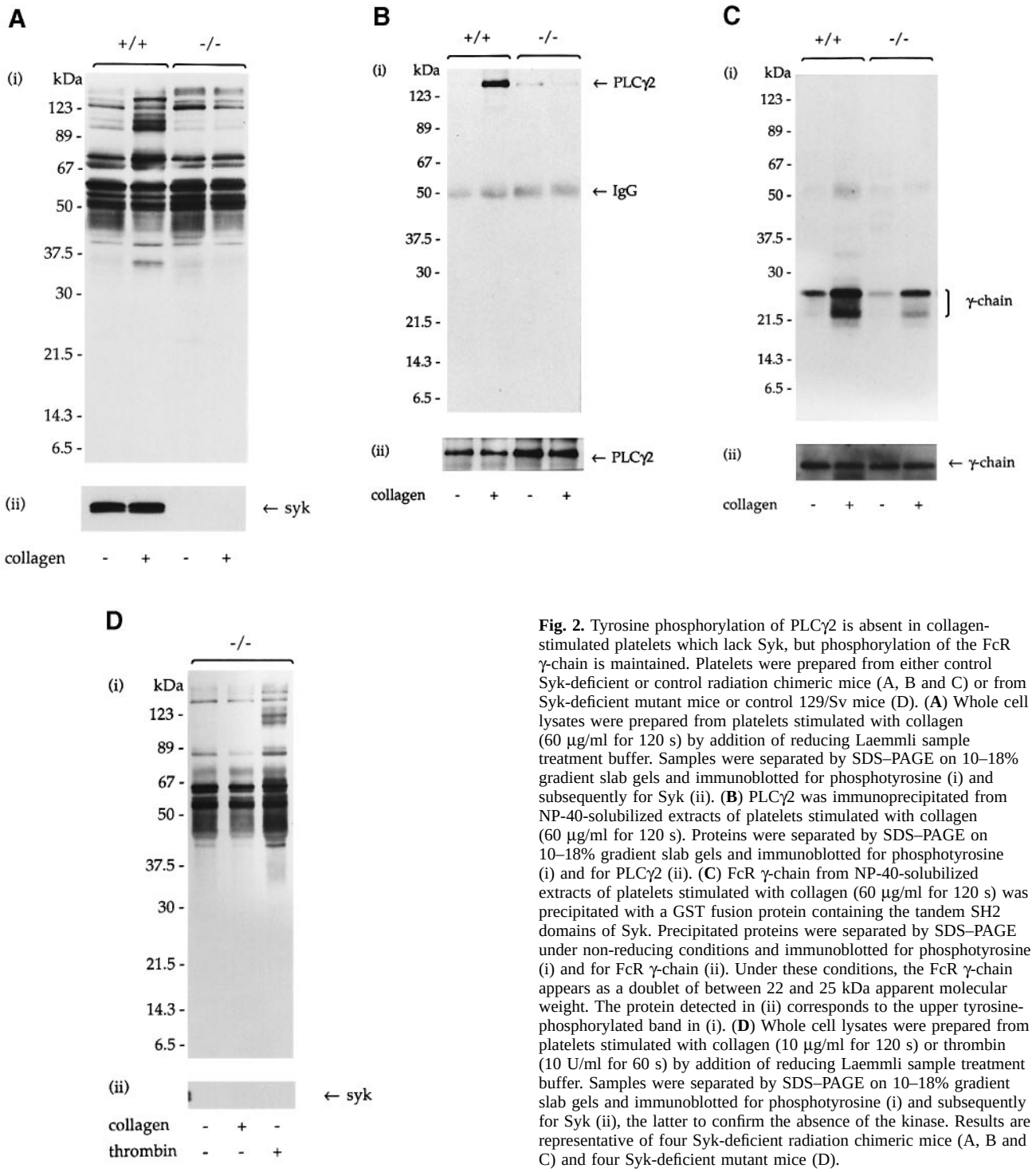

Fig. 2. Tyrosine phosphorylation of PLCγ2 is absent in collagen-stimulated platelets which lack Syk, but phosphorylation of the FcR γ-chain is maintained. Platelets were prepared from either control Syk-deficient or control radiation chimeric mice (A, B and C) or from Syk-deficient mutant mice or control 129/Sv mice (D). (**A**) Whole cell lysates were prepared from platelets stimulated with collagen (60 μg/ml for 120 s) by addition of reducing Laemmli sample treatment buffer. Samples were separated by SDS–PAGE on 10–18% gradient slab gels and immunoblotted for phosphotyrosine (i) and subsequently for Syk (ii). (**B**) PLCγ2 was immunoprecipitated from NP-40-solubilized extracts of platelets stimulated with collagen (60 μg/ml for 120 s). Proteins were separated by SDS–PAGE on 10–18% gradient slab gels and immunoblotted for phosphotyrosine (i) and for PLCγ2 (ii). (**C**) FcR γ-chain from NP-40-solubilized extracts of platelets stimulated with collagen (60 μg/ml for 120 s) was precipitated with a GST fusion protein containing the tandem SH2 domains of Syk. Precipitated proteins were separated by SDS–PAGE under non-reducing conditions and immunoblotted for phosphotyrosine (i) and for FcR γ-chain (ii). Under these conditions, the FcR γ-chain appears as a doublet of between 22 and 25 kDa apparent molecular weight. The protein detected in (ii) corresponds to the upper tyrosine-phosphorylated band in (i). (**D**) Whole cell lysates were prepared from platelets stimulated with collagen (10 μg/ml for 120 s) or thrombin (10 U/ml for 60 s) by addition of reducing Laemmli sample treatment buffer. Samples were separated by SDS–PAGE on 10–18% gradient slab gels and immunoblotted for phosphotyrosine (i) and subsequently for Syk (ii), the latter to confirm the absence of the kinase. Results are representative of four Syk-deficient radiation chimeric mice (A, B and C) and four Syk-deficient mutant mice (D).

signalling independent of the FcR γ-chain. The relatively small increase in tyrosine phosphorylation induced by thrombin (10 U/ml) in FcR γ-chain-deficient mice was comparable with that in control animals, demonstrating that the FcR γ-chain is not essential for phosphorylation of Syk and PLCγ2 by the G protein receptor-coupled agonist.

Collagen-stimulated tyrosine phosphorylation of FcR γ-chain, but not PLCγ2, in Syk-deficient platelets

As reported previously, the survival rate of Syk-deficient mutant mice to adulthood is extremely low (Cheng *et al.*, 1995; Turner *et al.*, 1995). Four Syk-deficient animals on an outbred genetic background survived to adulthood and were used for experimentation. None of the four animals exhibited an increase in whole cell tyrosine phosphorylation in response to collagen (Figure 2D). In contrast, thrombin stimulated a similar pattern of tyrosine phosphorylation to that in control cells, possibly with a small reduction in the 72 kDa region, the position of Syk (Figure 2D and data not shown).

In order to extend these findings, further experiments were performed using radiation chimeric animals which had been reconstituted with fetal liver lacking Syk; con-

firmation of successful reconstitution was obtained by immunoblotting for Syk [Figure 2A(ii)]. As found in platelets from Syk –/– mutants, collagen also failed to stimulate tyrosine phosphorylation in chimeric Syk-deficient animals [Figure 2A(i)]. We have shown previously in human platelets that only a small proportion of total cellular FcR γ-chain becomes tyrosine phosphorylated on collagen stimulation and, consequently, it is difficult to detect on anti-phosphotyrosine immunoblots of whole cell lysates (Gibbins *et al.*, 1996). This was also the case with mouse platelets, although overexposure of immunoblots from collagen-stimulated platelets allowed detection of an increase in tyrosine phosphorylation of a protein of 10–14 kDa under reducing conditions and at ~25 kDa under non-reducing conditions, the expected position of the FcR γ-chain under the respective conditions. Tyrosine phosphorylation of the FcR γ-chain was investigated by precipitation with a GST fusion protein containing the tandem SH2 domains of Syk, followed by immunoblotting (Figure 2C). Collagen stimulated tyrosine phosphorylation of the FcR γ-chain in Syk-deficient mice, with the relative increase in tyrosine phosphorylation over basal being similar to that in platelets from control mice; however, the absolute level of tyrosine phosphorylation of the FcR γ-chain in the control and stimulated samples was lower than that in control mice [Figure 2C(i)] despite a similar level of expression of the FcR γ-chain [Figure 2C(ii)]. These data demonstrate that tyrosine phosphorylation of the FcR γ-chain in collagen-stimulated platelets is independent of Syk. The lower level of tyrosine phosphorylation of the FcR γ-chain in control and stimulated samples could be due to a protective action of Syk against dephosphorylation mediated through binding of its tandem SH2 domain to the phosphotyrosine residues in the ITAM of the FcR γ-chain. Tyrosine phosphorylation of PLCγ2 induced by collagen was absent in Syk-deficient animals, suggesting that its phosphorylation occurs downstream of Syk (Figure 2B).

Release of 5-HT following collagen stimulation is absent in platelets deficient in FcR γ-chain or Syk, whereas the response to thrombin is maintained

Collagen stimulated the release of [^{3}H]5-hydroxytryptamine (5-HT) over the same concentration range as that for whole cell tyrosine phosphorylation (3–100 μg/ml), with maximal release occurring at 100 μg/ml (49.9 ± 6.6% of tissue [^{3}H]5-HT content, n = 4, 129/Sv mice). Thrombin also stimulated release over the same concentration range as that for tyrosine phosphorylation, with a threshold at 1 U/ml and maximal release at 10 U/ml (73.6 ± 2.4% of tissue [^{3}H]5-HT content, n = 4, 129/Sv mice).

Stimulation of [^{3}H]5-HT release by collagen (60 μg/ml) was inhibited completely in mutants deficient in FcR γ-chain or Syk relative to litter-matched controls (Figure 3). The response to collagen (60 μg/ml) is also inhibited completely in chimeric Syk-deficient mice (–2.1 ± 0.8% of tissue [^{3}H]5-HT content, n = 4). The response to collagen (10 and 60 μg/ml) in mice heterozygous for Syk (+/–) was ~50% of that in controls (not shown). In contrast, the response to thrombin (10 U/ml) was not altered in mutants deficient in FcR γ-chain or Syk (Figure 3).

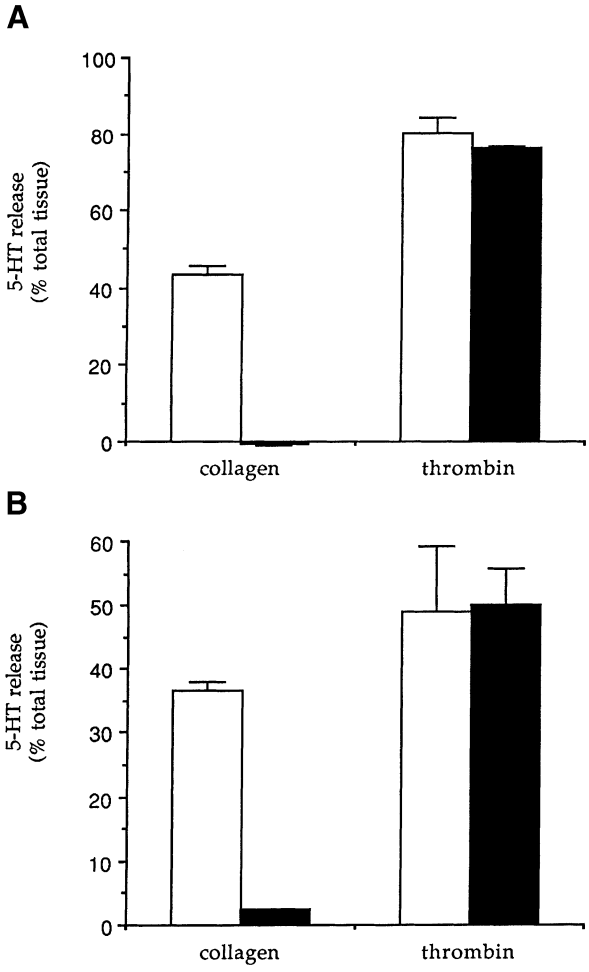

Fig. 3. Release of 5-HT from platelets stimulated with collagen is absent in mice deficient in FcR γ-chain or Syk, but the response to thrombin is maintained. (**A**) Platelets were prepared from control mice (□) and FcR γ-chain-deficient mutant mice (■), loaded with [^{3}H]5-HT and stimulated with either collagen (60 μg/ml for 120 s) or thrombin (10 U/ml for 60 s). Stimulation was terminated by centrifugation and the amount of [^{3}H]5-HT released into the supernatant measured by scintillation spectrometry. [^{3}H]5-HT release is expressed as a percentage of total tissue content (basal release = 0%). Results represent mean ± SE from three FcR γ-chain-deficient mutants and three control mice. (**B**) Platelets were prepared from control mice (□) and from Syk-deficient mutant mice (■), loaded with [^{3}H]5-HT and stimulated with either collagen (60 μg/ml for 120 s) or thrombin (10 U/ml for 60 s). Stimulation was terminated by centrifugation to pellet platelets, and the amount of [^{3}H]5-HT released into the supernatant was measured by scintillation spectrometry. [^{3}H]5-HT release is expressed as a percentage of total tissue content (basal release = 0%). Results represent mean ± SE from Syk-deficient mutants and control mice.

Aggregation and release of arachidonic acid in response to collagen are absent in platelets deficient in Syk, whereas responses to thrombin are maintained

The above data are consistent with an essential role for the FcR γ-chain and Syk in collagen receptor signalling in platelets. The functional significance of the increase in tyrosine phosphorylation of Syk in thrombin-stimulated platelets, however, is not clear. The role of Syk in receptor signalling by collagen and thrombin was therefore extended to further functional responses, namely aggregation, adhesion and release of arachidonic acid, in chimeric Syk-deficient animals.

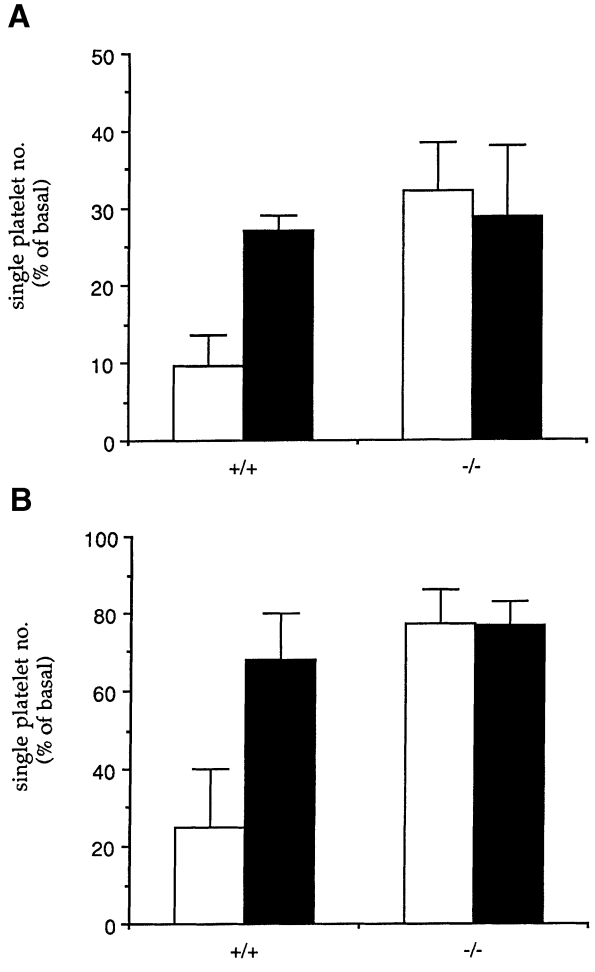

Fig. 4. Collagen- and CRP-induced platelet aggregation, but not adhesion, is reduced in platelets which lack Syk. Platelets were prepared from control and Syk-deficient radiation chimeric mice and stimulated with (**A**) collagen (60 μg/ml for 120 s) or (**B**) CRP (3 μg/ml for 120 s) and in the absence (□) or presence (■) of the tetrapeptide RGDS (1 mM). Aggregation was halted by addition of glutaraldehyde (see Materials and methods) and the level of aggregation determined by measuring the reduction in single platelets in suspension. The platelet count in stimulated samples is expressed as a percentage of the platelet count in unstimulated samples. Results represent mean ± SE from four Syk-deficient radiation chimeric mice and three control mice.

Aggregation was assessed by the reduction in single platelet number. This requires less tissue than methods that measure changes in optical density such as Born aggregometry, and is highly sensitive as it detects formation of microaggregates. Studies of aggregation induced by collagen, however, are complicated by the decrease in platelet count which occurs as a result of adhesion to the extracellular matrix protein. This is illustrated by the use of the fibrinogen receptor antagonist Arg–Gly–Asp–Ser (RGDS) which prevents platelet aggregation. Collagen (60 μg/ml) reduces the platelet count to 9.5 ± 4.1% (n = 3) of non-stimulated samples, while this is increased to 27 ± 2.0% (n = 3) in the presence of RGDS (Figure 4A). The reduction in platelet count in the presence of RGDS reflects adhesion, while the difference between the two values is due to aggregation. This is illustrated further by the use of a collagen-related peptide (CRP) which supports a much lower level of platelet adhesion than that by

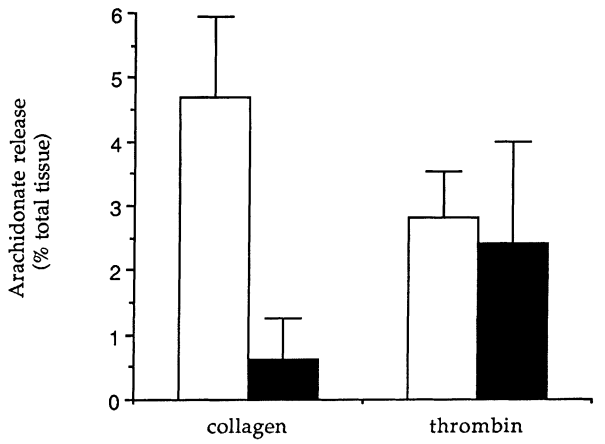

Fig. 5. Release of arachidonic acid in response to collagen stimulation is reduced in platelets which lack Syk, but their response to thrombin stimulation is maintained. Platelets were prepared from control mice and from Syk-deficient radiation chimeric mice and loaded with [^{3}H]arachidonic acid. Samples were stimulated with collagen (60 μg/ml for 120 s) or thrombin (10 U/ml for 60 s). Stimulation was terminated by centrifugation to pellet platelets, and the amount of [^{3}H]arachidonic acid released into the supernatant was measured by scintillation spectrometry. [^{3}H]Arachidonic acid release is expressed as a percentage of total tissue content (basal release = 0%). Results represent mean ± SE from four Syk-deficient radiation chimeric mice (■) and three control mice (□).

collagen as it does not bind to the integrin $\alpha_2\beta_1$ (Morton *et al.*, 1995). CRP stimulates a marked decrease in platelet count in the absence of RGDS, but induces a much lower decrease in platelet count in its presence (Figure 4B).

The reduction in single platelet count induced by collagen or CRP in the Syk-deficient chimeric mice is not significantly different from that in control mice in the presence of RGDS (Figure 4), consistent with a lack of aggregation. Further, the response to collagen or CRP is not altered by addition of RGDS in the Syk-deficient chimeric mice, confirming the lack of aggregation (Figure 4). In contrast, the reduction in single platelet count by thrombin (10 U/ml) was not altered significantly in the Syk-deficient chimeric mice. Thrombin decreased the platelet count to 4.6 ± 2.3% and 10.7 ± 4.7% in control (n = 3) and chimeric mice (n = 4), respectively.

Collagen (3–100 μg/ml) and thrombin (1–10 U/ml) induce release of [^{3}H]arachidonic acid from platelets over the same concentration range as that for stimulation of tyrosine phosphorylation. Collagen induced a greater release than thrombin in control mice. Release of [^{3}H]arachidonic acid by collagen was completely inhibited in the Syk-deficient chimeric mice whereas, once again, the response to thrombin was not altered (Figure 5).

Platelet number and bleeding times

In view of the critical role of Syk in B-cell development (Cheng *et al.*, 1995; Turner *et al.*, 1995), it was of interest to investigate whether Syk has a role in platelet formation. The platelet count was comparable in control (1.01 ± 0.14×10^8/ml; n = 5) and chimeric Syk-deficient mice (1.22 ± 0.11×10^8/ml; n = 4), and also in Syk-deficient mutants (not shown). The platelet number was also similar in FcR γ-chain-deficient mutants (1.50 ± 0.12×10^8/ml; n = 3) relative to controls (1.50 ± 0.33×10^8/ml; n = 3). Bleeding times were not significantly different between

control (155 ± 15 s; $n = 4$) and chimeric Syk-deficient mice (184 ± 61 s; $n = 4$).

Discussion

There is mounting evidence in support of the two-step, two-site model of collagen signalling in human platelets (Morton *et al.*, 1989, 1995; Santoro *et al.*, 1991). The initial interaction of collagen fibres with the platelet surface is mediated through the integrin $\alpha_2\beta_1$, an Mg^{2+}-dependent process (Santoro *et al.*, 1988). This is believed to facilitate interaction of collagen with an uncharacterized low-affinity signal-transducing receptor coupled to the FcR γ-chain. Phosphorylation of the FcR γ-chain on its ITAM tyrosine residues and its association with Syk leads to tyrosine phosphorylation and activation of PLCγ2, possibly via adapter proteins and additional tyrosine kinases. Binding of collagen to the integrin $\alpha_2\beta_1$ is not essential for stimulation; collagen induces tyrosine phosphorylation of the FcR γ-chain, Syk and PLCγ2 in the absence of Mg^{2+} or in the presence of antibodies to the integrin, albeit to a lower level (Gibbins *et al.*, 1996; Asselin *et al.*, 1997). CRP, a synthetic peptide based on the triple-helical structure of collagen, also stimulates phosphorylation of these three proteins but does not support adhesion to $\alpha_2\beta_1$ (Gibbins *et al.*, 1996; Asselin *et al.*, 1997).

The roles of the FcR γ-chain and Syk in collagen signalling have been tested in the present study using platelets from genetically modified mice. It is not possible to perform similar studies on human platelets because of the lack of a nucleus and the absence of established procedures for platelet formation *in vitro*. Mouse platelets respond in a similar manner to human platelets when stimulated with collagen, but are less sensitive to thrombin. As in human platelets, collagen induces tyrosine phosphorylation of multiple platelet proteins including the FcR γ-chain, Syk and PLCγ2. These proteins are phosphorylated to a much smaller extent, or not at all, in thrombin-stimulated platelets.

Whole cell protein tyrosine phosphorylation induced by collagen is abrogated in platelets lacking the FcR γ-chain and Syk, whereas the response to thrombin is similar to that in control platelets. In particular, tyrosine phosphorylation of PLCγ2 induced by collagen is blocked in mice lacking either protein. Tyrosine phosphorylation of Syk is also absent in mice lacking the FcR γ-chain, whereas tyrosine phosphorylation of the FcR γ-chain is maintained in mice lacking Syk. These results place tyrosine phosphorylation of Syk downstream of the FcR γ-chain and tyrosine phosphorylation of PLCγ2 downstream of both the FcR γ-chain and Syk, consistent with the above model. This sequence is the same as that established previously for signalling by immune receptors. For example, tyrosine phosphorylation of the FcR γ-chain by cross-linking of the mast FcεRI receptor is mediated upstream of the tyrosine kinase Syk and is essential for mast cell activation (Jouvin *et al.*, 1994; Oliver *et al.*, 1994; Costello *et al.*, 1997).

It is likely that this pathway is also important in the activation of human platelets by collagen. Okuma's group has identified an individual deficient in glycoprotein VI, a candidate collagen receptor, whose platelets do not undergo aggregation or exhibit increased tyrosine phosphorylation of Syk in response to collagen whereas activation of c-src is maintained (Ichinohe *et al.*, 1997). Glycoprotein VI also co-immunoprecipitates with the FcR γ-chain, providing evidence for their association *in vivo* (J.M.Gibbins, M.Okuma and S.P.Watson, unpublished). These observations suggest a pathway of platelet activation by collagen mediated through glycoprotein VI, the FcR γ-chain and the tyrosine kinase Syk. The FcR γ-chain also associates with FcγRI, FcγRIII and FcεRI, and is required for their cell surface expression (Takai *et al.*, 1994; van Vugt *et al.*, 1996). It may have a similar role in the regulation of the expression of the collagen receptor. This could not be tested in the present study because of the absence of antibodies which recognize glycoprotein VI in the mouse.

Tyrosine phosphorylation of PLCγ2 by collagen is believed to be essential for phosphoinositide hydrolysis and the onset of secretion and aggregation responses. Consistent with this, collagen-induced dense granule secretion, measured by release of [^{3}H]5-HT, is absent in mice deficient in the FcR γ-chain and Syk whereas the response to thrombin is not altered. The release of arachidonic acid and onset of aggregation induced by collagen were also inhibited in mice deficient in Syk (not studied for the FcR γ-chain-deficient mice). Adhesion of platelets to collagen fibres is not altered in the chimeric Syk-deficient mice, demonstrating independence from the tyrosine kinase. Activation-independent adhesion to collagen via $\alpha_2\beta_1$ has been described in human platelets (Morton *et al.*, 1994).

Role of FcR γ-chain and Syk in thrombin-stimulated platelets

Tyrosine phosphorylation of Syk in thrombin-stimulated platelets has been reported previously (Taniguchi *et al.*, 1993; Sada *et al.*, 1994), although the mechanism underlying phosphorylation and its significance are not known. Syk is also phosphorylated in mouse platelets stimulated by thrombin, but to a lesser degree than following collagen stimulation. Mouse platelets deficient in Syk undergo full aggregation and secretion of arachidonic acid and 5-HT in response to a maximal concentration of thrombin, demonstrating that the tyrosine kinase does not have an essential role in these events. Syk may play a role, however, in other platelet responses stimulated by thrombin, e.g. shape change or clot retraction, or it may contribute to the above responses induced by intermediate concentrations of thrombin. The latter is difficult to test in view of the steep nature of the thrombin concentration response curve and limited amount of tissue.

Phenotypes of FcR γ-chain-deficient and chimeric Syk-deficient mice

Three or more months following reconstitution, the chimeric Syk-deficient mice developed severe abdominal chylous/haemorrhagic ascites coupled with marked anaemia (not shown). In the adult chimeras, the bleeding into the peritoneal cavity leads to severe anaemia, in some cases enough to fill the abdomen with 4–5 ml of ascitic fluid, greatly exceeding the circulating blood volume. These mice also showed petechiael haemorrhages throughout the gut. A consistent feature of neonatal Syk-deficient mice is also development of chylous ascites and petechiae

at approximately day 16 *in utero*, although the latter fully recovers by the time of birth (Cheng *et al.*, 1995; Turner *et al*., 1995); there is, however, no associated haemorrhaging into the gut. In contrast, mice lacking the FcR γ-chain showed no such development of ascites, or any other sign of bleeding disorder. Since both Syk and the FcR γ-chain are essential for collagen-stimulated signalling in platelets, it is clear that a defect in collagen signalling alone does not explain the development of ascites. This would indicate that Syk might play another role in haematopoietic cells in the prevention of bleeding unrelated to its role in collagen receptor signalling. Alternatively, the defect could be related to platelet adhesion to collagen fibres in the absence of activation. This may not be seen in the FcR γ-chain-deficient mice if they lack expression of the associated collagen receptor.

The apparent lack of an effect on the bleeding time response in the Syk-deficient mice is consistent with the relatively mild bleeding problems observed in humans whose platelets exhibit impaired responses to collagen. This can be explained by the involvement of multiple stimuli in the activation of platelets at sites of blood vessel damage. This may also account for the ability of mice deficient in the portion of the fibrinogen molecule which binds to integrin $\alpha_{IIb}\beta_3$ to effectively control bleeding at most, but not all, sites of injury (Holmbäck *et al.*, 1996). It can be speculated that collagen receptor activation may be of particular importance at particular sites or under certain pathological conditions.

Conclusion

This study provides direct evidence for the essential role of the FcR γ-chain and Syk in the activation of PLCγ2 by the adhesion molecule collagen in mouse platelets. Loss of either protein abolishes collagen-induced secretion and platelet aggregation responses. Tyrosine phosphorylation of the FcR γ-chain lies upstream of tyrosine phosphorylation of Syk, and tyrosine phosphorylation of Syk lies upstream of tyrosine phosphorylation of PLCγ2. A similar sequence of events is predicted to underlie collagen receptor signalling in human platelets, although verification of this will require identification of the collagen receptor underlying activation and its relationship with the FcR γ-chain and Syk.

This pathway of collagen receptor signalling is the same as that established for signalling by immune receptors in which tyrosine phosphorylation of an ITAM is essential for activation of Syk or Zap-70. The present study therefore extends the role of the ITAM to signalling by a non-immune receptor stimulus and demonstrates that this motif (in which the letter I represents immunoreceptor) has a wider role than is suggested by its name.

Materials and methods

Materials

All salts and NP-40 were purchased from BDH-Merck. Collagen (derived from equine achilles tendon) was from Nycomed (Munich, Germany) and CRP was a kind gift of Dr Michael Barnes (Strangeways Laboratory, Cambridge). [^{3}H]5-HT was from DuPont NEN (Boston, MA). [^{3}H]Arachidonic acid was from Tocris Cookson (Bristol, UK). The anti-phosphotyrosine monoclonal antibody 4G10 was purchased from Upstate Biotechnology Inc. (TCS Biologicals Ltd., Buckinghamshire, UK). Polyclonal anti-Syk rabbit antiserum, raised against the peptide EPTGGPWGPDGRL corresponding to amino acid residues 318–330 in murine Syk, has been described previously (Turner *et al.*, 1995). Rabbit antiserum generated against whole human PLCγ2 was a gift from Dr Young Han Lee (Lee *et al.*, 1995). Anti-FcR γ-chain antiserum was a gift from Dr R.Siraganian (Benhamou *et al.*, 1993). Polyvinylidene difluoride (PVDF) Western blotting membrane was from Bio-Rad (Hertfordshire, UK). Tween-20, protein A–Sepharose CL-4B, phenylmethylsulfonyl fluoride (PMSF) and thrombin (bovine) were purchased from Sigma (Poole, Dorset, UK). Prostacyclin was a gift from The Wellcome Foundation (Beckenham, UK). Horseradish peroxidase (HRP)-conjugated anti-immunoglobulin antibodies, ECL reagents and Hyperfilm were from Amersham International (Buckinghamshire, UK).

FcR γ-chain-deficient and Syk-deficient mice

The FcR γ-chain-deficient C57BL/6 (B6) mice have been described previously (van Vugt *et al.*, 1996) and their generation will be described elsewhere (S.A.Park, S.Ueda, H.Ohno, M.Tanaka, T.Shiratori, T.Yamazaki, Y.Hamano, K.Yao, S.Satoh, B.Ledermann, K.Okumura, C.Ra and T.Saito, in preparation). B6 mice were used as controls in this series of experiments.

The generation of Syk-deficient mice has been described previously (Turner *et al.*, 1995). 129/Sv inbred mice were used as controls in this series of experiments.

Syk-deficient radiation chimeric mice were generated as follows. Eight- to ten-week-old Balb/c female mice, which had been kept on acidified water for a week, were given two irradiations of 500 rads from a ^{60}Co source 3 h apart (Turner *et al.*, 1995). The mice were then reconstituted by intravenous injection of 1.5×10^6 fetal liver cells from Syk –/– or control fetuses at 16.5 days of gestation. These fetuses had been back-crossed at least five times onto the B10.D2 genetic background. The reconstituted mice were given neomycin sulfate (0.16%) for 4 weeks following irradiation and were used for experiments no less than 8 weeks following irradiation.

Preparation and stimulation of mouse platelets

Blood (500–750 μl) was taken into heparin solution (30 u.s.p. units/ml) by cardiac puncture immediately following death. Blood was diluted with 250 μl of 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) and centrifuged at 200 *g* for 8 min at room temperature. Platelet-rich plasma (PRP) was removed with platelets which concentrate at the plasma–red blood cell interface. PRP was centrifuged at 1000 *g* in the presence of prostacyclin (0.1 μg/ml) for 5 min at room temperature. Pelleted platelets were resuspended in 250 μl of modified Tyrodes-HEPES buffer and rested for 30 min at 37°C prior to stimulation.

All experiments were performed at 37°C in siliconized glass tubes with continuous stirring. Agonists were added as 10-fold concentrates to 40 μl of platelet suspension containing between 1.5 and 2.0×10^7 cells. For protein precipitation studies, cells were stimulated in the presence of EGTA (100 μM) and indomethacin (10 μM) to prevent aggregation and maximize protein yields.

Immunoprecipitation and fusion protein precipitation

Stimulation of platelets was terminated by the addition of an equal volume of lysis buffer [20 mM Tris, 300 mM NaCl, 10 mM EDTA, 2% (v/v) NP-40, 1 mM PMSF, 2 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, pH 7.3] and lysates incubated for 30 min on ice. Insoluble debris was removed by centrifugation and the supernatant pre-cleared by incubation with protein A–Sepharose CL-4B for 1 h at 4°C. Antibody (2 μl of anti-Syk antiserum or 2 μl of anti-PLCγ2 antiserum per sample) was added to the supernatant which was mixed for 1 h before the addition of protein A–Sepharose CL-4B and incubation for 1 h at 4°C. The protein A–Sepharose CL-4B 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 Laemmli sample treatment buffer in preparation for SDS–PAGE under reducing conditions.

A GST fusion protein containing the tandem SH2 domains of Syk was prepared as described previously (Yanaga *et al.*, 1995). Precipitation with the GST fusion protein (2.5 μg) immobilized on agarose was performed on samples prepared as above, and lysates were pre-cleared using GST–agarose. Samples were incubated for 2 h at 4°C. Proteins isolated with GST fusion protein were separated by SDS–PAGE under non-reducing conditions.

Immunoblotting

Proteins were separated by SDS–PAGE on gradient (10–18%) slab gels and transferred to PVDF membrane. Membranes were blocked by incubation with TBS-T containing 10% (w/v) bovine serum albumin (BSA). Antibodies were diluted in TBS-T containing 2% (w/v) BSA (dilutions: anti-Syk, 1:1000; anti-PLCγ2, 1 μg/ml; anti-FcR γ-chain, 1:200) and incubated with blots for 1 h at room temperature. Membranes were washed twice for 1 h in TBS-T before incubation for 1 h with an appropriate HRP-conjugated secondary antibody diluted 1:10 000 in TBS containing 2% (w/v) BSA. Following washing in TBS-T as above, immunoblots were developed using an enhanced chemiluminescence detection system. Equivalent protein loading for immunoprecipitated proteins was verified by reprobing with a relevant antibody.

5-HT secretion assay

Platelets were loaded with [^{3}H]5-HT by incubation with 0.2 μCi/ml of PRP for 1 h at 37°C. Platelets were prepared from the PRP as described above. Stimulation of platelets was terminated by brief microcentrifugation, and the level of [^{3}H]5-HT release into the supernatant was determined by scintillation spectrometry. [^{3}H]5-HT release was expressed as a percentage of the total tissue content following subtraction of release under basal conditions as described previously (Nunn and Watson, 1987).

Aggregation assay

An aggregation assay was performed on samples which were also analysed for arachidonic acid release. For this reason, stimulation was perfomed in the presence of indomethacin (10 μM) and the lipoxygenase inhibitor BW4AC (3 μM). Stimulation of platelets was terminated by the addition of 4 volumes of glutaraldehyde solution (6% v/v). Platelets fixed in glutaraldehyde were diluted 1:40 000 and the level of aggregation determined by counting single platelets remaining in suspension using a Thrombocounter-C (Coulter Electronics Ltd.). In order to distinguish aggregation from adhesion to collagen or CRP, samples were pre-incubated for 1 min with the tetrapeptide RGDS (1 mM). This peptide blocks fibrinogen binding which is required to support platelet aggregation following activation; reduction in the number of single platelets in suspension following addition of collagen or CRP is due to adhesion to the agonists.

Release of arachidonic acid

Platelets were loaded in PRP with [^{3}H]arachidonic acid by incubation with 5 μCi/ml for 2 h at 37°C. Platelets were prepared from the PRP as described above, and stimulated in the presence of indomethacin (10 μM) and the lipoxygenase inhibitor BW4AC (3 μM). Stimulation of platelets was terminated by brief microcentrifugation, and the level of [^{3}H]arachidonic acid release into the supernatant was determined by scintillation spectrometry. This was expressed as a percentage of the total tissue [^{3}H]arachidonic acid content following subtraction of release under basal conditions.

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References

Asazuma,N., Yatomi,Y., Ozaki,Y., Qi,R., Kuroda,K., Satoh,K. and Kume,S. (1996) Protein-tyrosine phosphorylation and p72^{Syk} activation in human platelets stimulated with collagen is dependent upon glycoprotein Ia/IIa and actin polymerisation. *Thromb. Haemostasis*, **75**, 648–654.

Asselin,J., Gibbins,J.M., Achison,M., Lee,Y.-H., Morton,L.F., Farndale,R.W., Barnes,M.J. and Watson,S.P. (1997) A collagen-like peptide stimulates tyrosine phosphorylation of Syk and phospholipase Cγ2 in platelets independent of the integrin $\alpha_2\beta_1$. *Blood*, **89**, 1235–1242.

Benhamou,M., Ryba,N., Kihara,H., Nishikata,H. and Siraganian,R. (1993) Protein-tyrosine kinase p72^{Syk} in high affinity IgE receptor signaling. Identification as a component of pp72 and association with the receptor gamma chain after receptor aggregation. *J. Biol. Chem.*, **268**, 23318–23324.

Blake,R.A., Schieven,G.L. and Watson,S.P. (1994) Collagen stimulates tyrosine phosphorylation of phospholipase C-γ2 but not phospholipase C-γ1 in human platelets. *FEBS Lett.*, **353**, 212–216.

Cheng,A.M., Rowley,B., Pao,W., Hayday,A., Bolen,J.B. and Pawson,T. (1995) Syk tyrosine kinase required for mouse viability and B-cell development. *Nature*, **378**, 303–306.

Chiang,T.M. and Kang,A.H. (1982) Isolation and purification of collagen α1(I) receptor from human platelet membrane. *J. Biol. Chem.*, **257**, 7581–7586.

Connolly,A., Ishihara,H., Kahn,M., Farese,R. and Coughlin,S. (1996) Role of the thrombin receptor in development and evidence for a second receptor. *Nature*, **381**, 516–519.

Costello,P.S., Turner,M., Walters,A.E., Cunningham,C.N., Bauer,P.H., Downward,J. and Tybulewicz,V.L.J. (1996) Critical role for the tyrosine kinase Syk in the signalling through the high affinity IgE receptor of mast cells. *Oncogene*, **13**, 2595–2605.

Daniel,J.L., Dangelmaier,C. and Smith,J.B. (1994a) Evidence for a role for tyrosine phosphorylation of phospholipase Cγ2 in collagen-induced platelet cytosolic calcium mobilisation. *Biochem. J.*, **302**, 617–622.

Daniel,J.L., Dangelmaier,C., Strouse,R. and Smith,J.B. (1994b) Collagen induces normal signal transduction in platelets deficient in CD36 (platelet glycoprotein IV). *Thromb. Haemostasis*, **71**, 353–356.

Deckmyn,H., Van Houtte,E. and Vermylen,J. (1992) Disturbed platelet aggregation to collagen associated with an antibody against an 85- to 90Kd platelet glycoprotein in a patient with prolonged bleeding time. *Blood*, **79**, 1466–1471.

Fujii,C., Yanagi,S., Sada,K., Nagai,K., Taniguchi,T. and Yamamura,H. (1994) Involvement of protein-tyrosine kinase p72syk in collagen-induced signal transduction in platelets. *Eur. J. Biochem.*, **226**, 243–248.

Gibbins,J., Asselin,J., Farndale,R., Barnes,M., Law,C.-L. and Watson,S.P. (1996) Tyrosine phosphorylation of the Fc receptor γ-chain in collagen-stimulated platelets. *J. Biol. Chem.*, **271**, 18095–18099.

Holmbäck,K., Danton,M.J.S., Suh,T.T., Daugherty,C.C. and Degen,J.L. (1996) Impaired platelet aggregation and sustained bleeding in mice lacking the fibrinogen motif bound by integrin αIIbβ3. *EMBO J.*, **15**, 5760–5771.

Ichinohe,T., Takayama,H., Ezumi,Y., Arai,M., Yamamoto,N., Takahashi,H. and Okuma,M. (1997) Collagen-stimulated activation of syk but not c-src is severely compromised in human platelets lacking membrane glycoprotein VI. *J. Biol. Chem.*, **272**, 63–68.

Jouvin,M.H., Adamczewski,M., Numerof,R., Letourneur,O., Valle,A. and Kinet,J.-P. (1994) Differential control of the tyrosine kinases Lyn and Syk by the two signaling chains of the high affinity immunoglobulin E receptor. *J. Biol. Chem.*, **269**, 5918–5925.

Kehrel,B. *et al.* (1993) Platelets deficient in glycoprotein IIIb aggregate normally to collagens type I and III but not to collagen type V. *Blood*, **82**, 3364–3370.

Lee,Y., Lee,H., Lee,S., Min,D., Baek,S., Kim,Y., Ryu,S. and Suh,P. (1995) Down-regulation of phospholipase C-gamma 1 during the differentiation of U937 cells. *FEBS Lett.*, **358**, 105–108.

McKeown,L., Vail,M., Williams,S., Kramer,W., Hansmann,K. and Gralnick,H. (1994) Platelet adhesion to collagen in individuals lacking glycoprotein IV. *Blood*, **83**, 2866–2871.

Moroi,M., Jung,S.M., Okuma,M. and Shinmyozu,K. (1989) A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. *J. Clin. Invest.*, **84**, 1440–1445.

Morton,L.F., Peachey,A.R. and Barnes,M.J. (1989) Platelet-reactive sites in collagens type I and type III. Evidence for separate adhesion and aggregatory sites. *Biochem. J.*, **258**, 157–163.

Morton,L.F., Peachey,A.R., Zijenah,L.S., Goodall,A.H., Humphries,J.J. and Barnes,M.J. (1994) Conformation-dependent platelet adhesion to collagen involving integrin α2β1-mediated and other mechanisms: multiple α2β1-recognition sities in collagen type 1. *Biochem. J.*, **299**, 791–797.

Morton,L.F., Hargreaves,P.G., Farndale,R.W., Young,R.D. and Barnes,M.J. (1995) Integrin alpha 2 beta 1-independent activation of platelets by simple collagen-like peptides: collagen tertiary (triple-helical) and quaternary (polymeric) structures are sufficient alone for alpha 2 beta 1-independent platelet reactivity. *Biochem. J.*, **306**, 337–344.

Nunn,D.L. and Watson,S.P. (1987) A diacylglycerol kinase inhibitor, R59022, potentiates secretion by and aggregation of thrombin-stimulated human platelets. *Biochem. J.*, **243**, 809–813.

Oliver,J.M., Burg,D.L., Wilson,B.S., McLaughlin,J.L. and Geahlen,R.L. (1994) Inhibition of mast cell FcεRI-mediated signaling and effector function by the Syk-selective inhibitor, piceatannol. *J. Biol. Chem.*, **269**, 29697–29703.

Robinson,A., Gibbins,J., Rodriguez-Linares,B., Finan,P.M., Wilson,L., Kellie,S., Findell,P. and Watson,S.P. (1996) Characterisation of Grb2-binding proteins in human platelets activated by FcγRIIA cross-linking. *Blood*, **88**, 522–530.

Ryo,R., Yoshida,A., Sugano,W., Yasunaga,M., Nakayama,K., Saigo,K., Adachi,M., Yamaguchi,N. and Okuma,M. (1992) Deficiency of P62, a putative collagen receptor, in platelets from a patient with defective collagen-induced platelet aggregation. *Am. J. Hematol.*, **39**, 25–31.

Sada,K., Yanagi,S. and Yamamura,H. (1994) Activation of p72Syk by thrombin in a cell-free system. *Biochem. Biophys. Res. Commun.*, **200**, 1–7.

Santoro,S.A., Rajpara,S.M., Staatz,W.D. and Woods,V.,Jr (1988) Isolation and characterization of a platelet surface collagen binding complex related to VLA-2. *Biochem. Biophys. Res. Commun.*, **153**, 217–223.

Santoro,S.A., Walsh,J.J., Staatz,W.D. and Baranski,K.J. (1991) Distinct determinants on collagen support alpha 2 beta 1 integrin-mediated platelet adhesion and platelet activation. *Cell Regul.*, **2**, 905–913.

Takai,T., Li,M., Sylvestre,D., Clynes,R. and Ravetch,J.V. (1994) FcR γ chain deletion results in pleiotrophic effector cell defects. *Cell*, **76**, 519–529.

Tandon,N.N., Kralisz,U. and Jamieson,G.A. (1989) Identification of glycoprotein IV (CD36) as a primary receptor for platelet–collagen adhesion. *J. Biol. Chem.*, **264**, 7576–7583.

Taniguchi,T., Kitagawa,H., Yasue,S., Sakai,K., Asahi,M., Ohta,S., Takeuchi,F., Nakamura,S. and Yamamura,H. (1993) Protein-tyrosine kinase p72Syk is activated by thrombin and is negatively regulated through Ca^{2+} mobilisation in platelets. *J. Biol. Chem.*, **268**, 2277–2279.

Turner,M., Mee,P.J., Costello,P.S., Williams,O., Price,A.A., Duddy,L.P., Furlong,M.T., Geahlen,R.L. and Tybulewicz,V.L.J. (1995) Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature*, **378**, 298–302.

van Vugt,M.J., Heijnen,I.A.F.M., Capel,P.J.A., Park,S.Y., Ra,C., Saito,T., Verbeek,J.S. and van de Winkel,J.G.J. (1996) FcR γ-chain is essential for both expression and function of human FcγRI (CD64) *in vivo*. *Blood*, **87**, 3593–3599.

Yanaga,F., Poole,A., Asselin,J., Blake,R., Schieven,G., Clark,E.A., Law,C.-L. and Watson,S.P. (1995) Syk interacts with tyrosine-phosphorylated proteins in human platelets activated by collagen and cross-linking of the Fcγ -IIA receptor. *Biochem. J.*, **311**, 471–478.

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