Evidence for the involvement of p59^{fyn} and p53/56^{lyn} in collagen receptor signalling in human platelets

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The binding of collagen to platelet glycoprotein VI (GPVI) leads to the subsequent activation of phospholipase Cγ2 through a pathway that is dependent on the Fc receptor γ (FcR γ) chain and the tyrosine kinase p72^{syk}. We have investigated the role of platelet Src-family kinases in this signalling pathway. The selective Src-family kinase inhibitor PP1 prevented collagen-stimulated increases in whole-cell tyrosine phosphorylation and tyrosine phosphorylation of the FcR γ chain and p72^{syk}. A similar set of observations was made for a collagen-related peptide (CRP), which binds to GPVI but not to the integrin $\alpha_2\beta_1$ (GPIa/IIa). These effects were seen at a concentration of PP1 that inhibited platelet aggregation, dense granule release and Ca^{2+} mobilization induced by CRP, but not aggregation and Ca^{2+} mobilization mediated by the G-protein-coupled receptor agonist thrombin. After stimulation by CRP or collagen, the Src-family kinases p59^{fyn} and p53/56^{lyn} became associated with several tyrosine-phosphorylated proteins including the FcR γ chain. This was not true of the other platelet Src-family kinases. The association between the FcR γ chain and p59^{fyn} was also seen under basal conditions, and was stable only in the weak detergent Brij96 but not in Nonidet P40, suggesting a non-SH2-dependent interaction. These results provide strong evidence for the involvement of p59^{fyn} and p53/56^{lyn} in signalling via GPVI, with p59^{fyn} possibly acting upstream of FcR γ chain phosphorylation.

Key words: immunoreceptor tyrosine-based activation motif, Src kinase, tyrosine phosphorylation.

INTRODUCTION

After damage to blood vessels, the formation of a haemostatic plug is initiated by the adhesion of platelets to subendothelial collagens and their subsequent activation. Of several proposed platelet receptors for collagen, the integrin $\alpha_2\beta_1$ has a major role in platelet adhesion to collagen, with activation being mediated largely by the cell surface receptor, platelet glycoprotein VI (GPVI) [1–3]. The signalling pathways activated downstream of GPVI require the Fc receptor γ chain (FcR γ chain) and the tyrosine kinase p72^{syk} [3–5]. Binding of collagen to GPVI leads to the phosphorylation of the FcR γ chain on two tyrosine residues within a conserved signalling motif known as the immunoreceptor tyrosine-based activation motif (ITAM). These phosphotyrosine residues are bound by the tandem Src homology 2 (SH2) domains of the tyrosine kinase p72^{syk} [6]. Subsequent activation of p72^{syk} leads to the activation of phospholipase Cγ2 and the generation of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, which induce Ca^{2+} mobilization and the activation of protein kinase C respectively [7,8].

There is a strong similarity between the GPVI signalling pathway in platelets and that used by receptors for immune complexes, such as the high-affinity and low-affinity receptors for IgG (FcγRI and FcγRIII) and the high-affinity receptor for IgE (FcϵRI), which also signal via the FcR γ chain and p72^{syk} ([9], and references therein). For these receptors, Src-family kinases have a crucial and initiating role in receptor signalling [10,11]. For instance, ITAM phosphorylation seems to be mediated by p53/56^{lyn} in FcϵRI signalling [10] and by p54/56^{hck} and p53/56^{lyn} in FcγRI signalling [12]. The Src-family kinases are well placed to perform this early task in signalling through their association with non-phosphorylated ITAMs, as illustrated by the interaction of p59^{fyn} with the ζ chain of the T-cell antigen receptor [13,14] and of p53/56^{lyn} with the FcϵRI and B-cell receptors [15,16].

We have investigated whether one or more of the platelet Src-family kinases, namely p60$^{c\text{-}src}$, p53/56^{lyn}, p59^{fyn}, p54/56^{hck} and p62^{yes} [17], participate in signalling via GPVI, and whether they might be responsible for phosphorylation of the FcR γ chain. We show that the selective Src-family kinase inhibitor PP1 (CP118,556) [18] can inhibit both collagen-mediated and collagen-related-peptide (CRP)-mediated signalling in human platelets and that both p59^{fyn} and p53/56^{lyn}, through their association with the FcR γ chain, are involved in signalling via GPVI.

EXPERIMENTAL

Materials

CRP [GCP*(GPP*)$_{10}$GCP*G, single letter amino acid code; P* = hydroxyproline] was from Dr. M. Barnes (Department of Biochemistry, Cambridge, U.K.) and was cross-linked as described [19]. Collagen fibres (predominantly type I) from equine Achilles tendon were obtained from Nycomed (Munich, Germany). PP1 was provided by Dr. J. Hanke (Pfizer Central Research, Groton, CT, U.S.A.) [18], and PD173956 was a gift from Dr. A. J. Kraker (Parke-Davis, Holland, MI, U.S.A.). Monoclonal antibody 327

Abbreviations used: $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; CRP, collagen-related peptide; FcϵRI, high-affinity receptor for IgE; FcγRI, high-affinity receptor for IgG; FcR γ chain, common Fc receptor γ chain; GPVI, platelet glycoprotein VI; 5-[^{3}H]HT, 5-hydroxy[1,2(*N*)-^{3}H]tryptamine; ITAM, immunoreceptor tyrosine-based activation motif; NP40, Nonidet P40; PP1, CP118,556 (Src-family kinase inhibitor); SH2, SH3 or SH4, Src homology 2, 3 or 4 domain respectively.

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against human p60$^{c\text{-}src}$ was a gift from Dr. J. Brugge, the antibody against human p62^{yes} was a gift from Dr. S. Courtneidge and rabbit antiserum to the FcR γ chain was from Dr. J. P. Kinet. The anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology (TCS, Buckingham, Bucks., U.K.) and rabbit polyclonal antibodies against human p59^{fyn}, p53/56^{lyn} and p54/56^{hck} were obtained from Santa Cruz (Autogen Bioclear, Devises, Wiltshire, U.K.). Rabbit anti-(mouse IgG) was from Sigma (Poole, Dorset, U.K.). [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham (Little Chalfont, Bucks., U.K.) and 5-hydroxy[1,2(*N*)-^{3}H]tryptamine (5-[^{3}H]HT) (30 Ci/mmol) was from DuPont–NEN (Boston, MA, U.S.A.). Other reagents were from sources previously described [6,20].

Platelet preparation, aggregation studies and 5-[^{3}H]HT release

Platelets were prepared as described previously [20], resuspended at a concentration of 2×10^8 cells/ml in Tyrode's buffer and left for 30 min before experimentation. Stimulation was performed in a Chrono-Log four-channel aggregometer at 37 °C with stirring (1200 rev./min). For inhibitor studies, PP1 and PD173956 were dissolved in DMSO and added such that the final solvent concentration was 0.2 %; an equivalent concentration of solvent was added to control platelets. PP1 was added to continuously stirred platelets 5 min before stimulation, and PD173956 for 10 min. For measurement of 5-[^{3}H]HT release, platelets were loaded with 10 μCi of 5-[^{3}H]HT in platelet-rich plasma for 1 h at 37 °C before being washed and final resuspension in Tyrode's buffer. Stimulations were stopped after 90 s by the addition of glutaraldehyde [3 % (w/v) final concentration], and release was quantified as described previously [20].

Measurement of platelet cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$)

For the measurement of $[Ca^{2+}]_i$, platelets were isolated from platelet-rich plasma, resuspended in Tyrode's buffer and incubated with fura 2 acetoxymethyl ester (5 μM, 30 min, 37 °C). After being washed in 25 ml of Tyrode's buffer, platelets were resuspended at 2×10^8 cells/ml. Stimulation was performed in a Perkin–Elmer LS50B spectrofluorimeter at 37 °C with agitation in the presence of 1 mM EGTA. $[Ca^{2+}]_i$ was calculated as the ratio of fluorescence of fura 2, with excitation at 340 and 380 nm and measurement at 510 nm (FLWinLab; Perkin–Elmer).

Immunoprecipitations, whole-cell phosphorylation studies and immunoblotting

Platelets were prepared, resuspended at a final concentration of 4.5×10^8 cells/ml and stimulated as described in the presence of 10 μM indomethacin and 1 mM EGTA. For whole-cell phosphorylation studies, the reaction was terminated with an equal volume of 2 × Laemmli sample buffer and heated at 95 °C for 5 min. For immunoprecipitations, reactions were stopped with an equal volume of 2 × lysis buffer [2 % (v/v) detergent/300 mM NaCl/20 mM Tris/2 mM EDTA/2 mM EGTA/2 mM PMSF/5 mM sodium orthovanadate/10 μg/ml pepstatin A/10 μg/ml leupeptin/10 μg/ml aprotinin], and antibody precipitation was performed as described previously [6]. For precipitation with monoclonal antibodies, 0.5 μg of rabbit anti-(mouse IgG) was included to enhance binding to Protein A. Proteins were separated by SDS/PAGE [10 % or 10–18 % (w/v) gel], and transferred to PVDF membranes. Immunoblotting, stripping and reprobing of membranes was performed by using a previously published protocol [20].

Immune-complex kinase assays and reprecipitations

Immunoprecipitations were performed and the final pellet was washed in kinase buffer [105 mM NaCl/20 mM Hepes/5 mM $MgCl_2$/5 mM $MnCl_2$ (pH 7.4)]. Kinase reactions were initiated by the addition of 20 μl of kinase buffer containing 5 μCi of [γ-^{32}P]ATP and incubated for 10 min at room temperature. Reactions were stopped by the addition of 500 μl of ice-cold 50 mM EDTA; subsequently 20 μl of 2 × Laemmli sample buffer was added to the final pellet before being boiled for 5 min. For reprecipitation analysis, after reactions had been stopped with 50 mM EDTA, Protein A–Sepharose pellets were heated at 95 °C for 15 min in 1 × lysis buffer containing 2 % (w/v) SDS. Subsequently, the SDS concentration was decreased to 0.1 % by the addition of 1 × lysis buffer; the second immunoprecipitation was performed as described above. In each case, proteins were separated by SDS/PAGE; PVDF membranes were exposed to film at −70 °C.

Statistical analysis

All results are quoted as means ± S.E.M. for the number of experiments indicated. Statistical significance was assessed by a single sample *t*-test and in each case $P < 0.05$ was taken to indicate a statistically significant difference.

RESULTS

Effects of PP1 on collagen receptor signalling in platelets

The role of Src-family kinases in platelet collagen receptor signalling was investigated with the use of the selective Src-family kinase inhibitor PP1. PP1 is a potent inhibitor of p59^{fyn} and p56^{lck} (which is not present in platelets) and is selective over p72^{syk} by 10000-fold in both studies *in vitro* and functional studies [18,21]. Platelet aggregation induced by threshold concentrations of collagen (0.5 μg/ml) (results not shown) and CRP (0.1 μg/ml), which binds to GPVI but not $\alpha_2\beta_1$ [20], was inhibited by PP1 in a concentration-dependent manner, with 50 % inhibition at 300 ± 50 nM ($n = 3$) (Figure 1a). Full inhibition of aggregation was seen with 1 μM PP1; inhibitions of both shape change and aggregation were seen at 10 μM. Similar results were seen with maximal concentrations of collagen (10 μg/ml) and CRP (1 μg/ml). Aggregation in response to a threshold concentration of the G-protein-coupled receptor agonist thrombin (0.025 i.u./ml) (Figure 1b), or a higher concentration (0.1 i.u./ml), was not significantly decreased by PP1 (10 μM). The structurally different Src-family kinase inhibitor PD173956 (20 μM) also inhibited collagen- and CRP-mediated platelet aggregation (results not shown). PP1 (10 μM) inhibited the release of 5-[^{3}H]HT from platelet dense granules mediated by CRP (1 μg/ml, 90 s) (43.1 ± 2.4 % compared with 5.2 ± 2.4 %, control and PP1-treated respectively; $P < 0.05$, $n = 3$). Similar results were obtained for collagen (10 μg/ml, 90 s). Studies on platelets loaded with the fluorescent Ca^{2+} dye fura 2 showed that the elevation of $[Ca^{2+}]_i$ in response to 1 μg/ml CRP (Figure 1c) or 30 μg/ml collagen (results not shown) was completely prevented by preincubation with 10 μM PP1 in both the presence and the absence of indomethacin. However, the mobilization of $[Ca^{2+}]_i$ in response to thrombin at 1 i.u./ml (Figure 1d) and 0.1 i.u./ml (results not shown) was not affected by the treatment of platelets with PP1.

In addition to its functional effects, PP1 (0.1–10 μM) caused a concentration-dependent decrease in CRP- and collagen-induced whole-cell tyrosine phosphorylation. In the presence of 10 μM

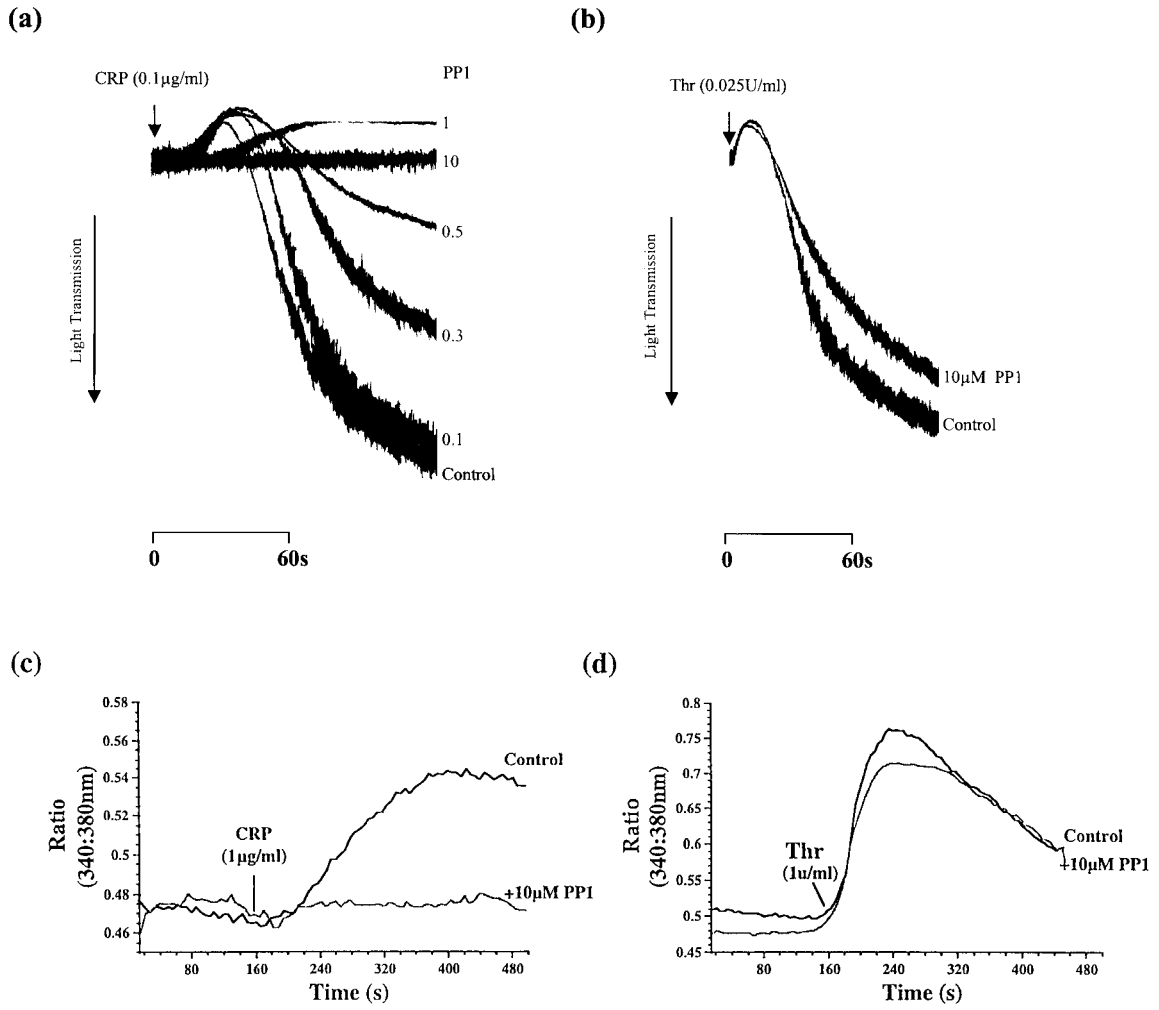


Figure 1 Inhibition of CRP-mediated platelet aggregation and $[Ca^{2+}]_i$ mobilization by PP1

Platelets were prepared as described and stimulated with threshold concentrations of CRP (0.1 μg/ml) (**a**) or thrombin (Thr) (0.025 i.u./ml) (**b**) in the presence of the indicated concentrations of PP1, added 5 min before agonist. Platelets loaded with the fluorescent Ca^{2+} indicator fura 2 were also stimulated in a spectrofluorimeter with either CRP (1 μg/ml) (**c**) or thrombin (1 i.u./ml) (**d**) in the presence or absence of 10 μM PP1. Results in (**c**) and (**d**) are shown as the ratio of the measurements at 510 nm for excitations at 340 and 380 nm. For all experiments, results shown are representative of at least three experiments performed.

PP1, CRP (1 μg/ml) was unable to increase cellular levels of tyrosine phosphorylation above basal levels, which were also decreased (Figure 2, upper panel). Immunoprecipitation experiments showed that, under these conditions, CRP-mediated increases in tyrosine phosphorylation of the FcR γ chain and the tyrosine kinase $p72^{syk}$ were prevented, although a small degree (less than 15%) of $p72^{syk}$ phosphorylation remained (Figure 2, lower panels). In addition, tyrosine-phosphorylated $p72^{syk}$ was absent from FcR γ chain immunoprecipitates after pretreatment with PP1 and vice versa. This suggests that inhibition of Src-family kinases prevented ITAM phosphorylation, and also binding of the tandem SH2 domains of $p72^{syk}$.

$p59^{fyn}$ and $p53/56^{lyn}$ associate with FcR γ chain and other tyrosine-phosphorylated proteins during CRP-mediated signalling

The role of individual Src-family kinases in the response to CRP was further investigated. After stimulation with CRP (1 μg/ml), both $p53/56^{lyn}$ and $p59^{fyn}$ co-precipitated with several phosphotyrosine-containing proteins in a time-dependent manner, consistent with their participation in a protein signalling complex downstream of GPVI [Figure 3, top panel (i) and Figure 3, middle panel (i)]. The major tyrosine-phosphorylated protein identified in $p59^{fyn}$ immunoprecipitates was a 14 kDa phosphoprotein that co-migrated with the FcR γ chain and was identified as such by immunoblotting (results not shown). Other tyrosine-phosphorylated proteins of 38, 55 and 72 kDa were detected at a lower level. The phosphorylated FcR γ chain also co-precipitated with $p53/56^{lyn}$ along with several other tyrosine-phosphorylated proteins, including those of 36, 60, 72 and 120 kDa. The identities of these proteins are not known, although immunoblots for $p72^{syk}$ were negative. Stimulation with collagen also resulted in the co-precipitation of a similar pattern of tyrosine-phosphorylated proteins with both $p59^{fyn}$ and $p53/56^{lyn}$, including the FcR γ chain (results not shown). Over the time course of the experiment, there was little change in the tyrosine phosphorylation levels or the kinase activity *in vitro* of either $p59^{fyn}$ or $p53/56^{lyn}$ (Figure 3, top panel, and Figure 3, middle panel). In contrast with $p59^{fyn}$ and $p53/56^{lyn}$, immunoprecipitated $p60^{c\text{-}src}$ (Figure 3, bottom panel), $p62^{yes}$ and $p54/56^{hck}$

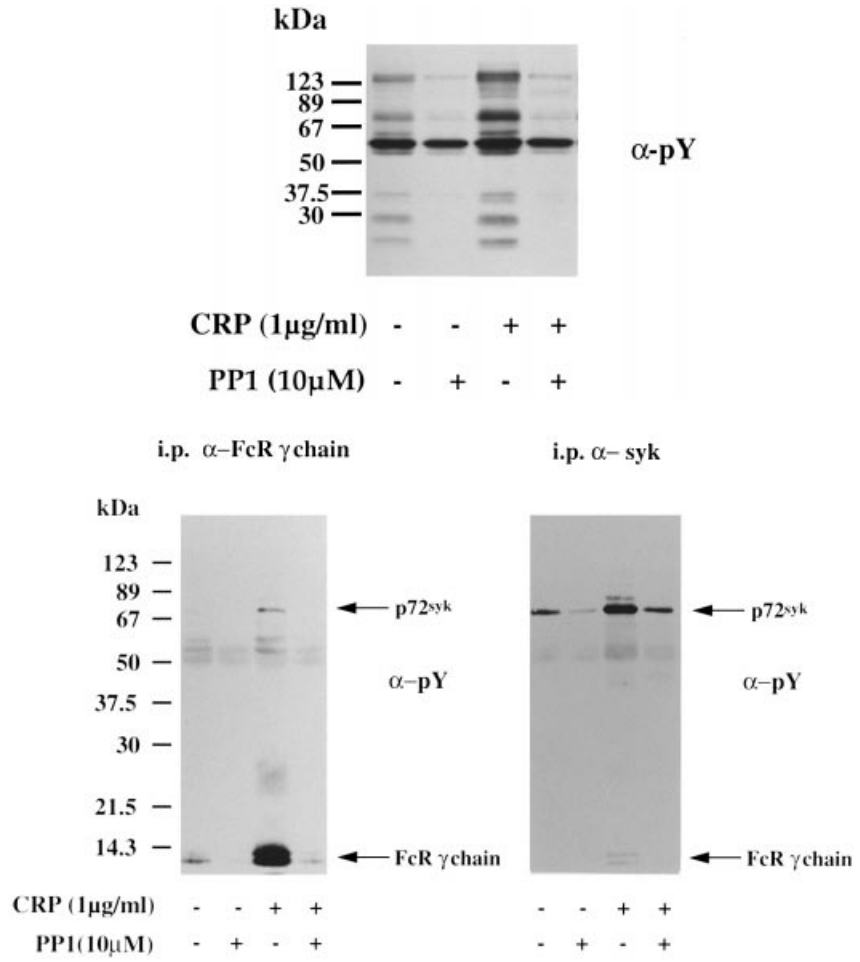


Figure 2 Effect of PP1 on CRP-mediated changes in whole-cell tyrosine phosphorylation and tyrosine phosphorylation of the FcR γ chain and p72^{syk}

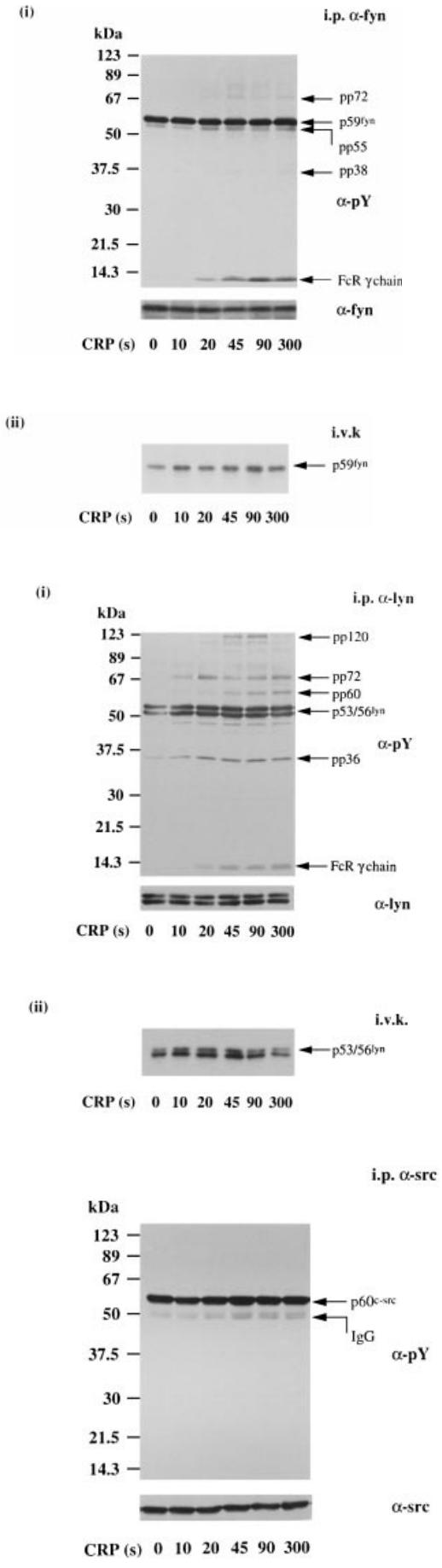
Platelets were stimulated with CRP (1 μg/ml) for 60 s in the presence or absence of 10 μM PP1 as indicated. Upper panel: the reaction was stopped with 2 × Laemmli sample buffer and whole-cell tyrosine phosphorylation was assessed by immunoblotting with the anti-phosphotyrosine (α-pY) monoclonal antibody 4G10 as described in the Experimental section. Lower panels: either FcR γ chain or p72^{syk} was then immunoprecipitated (i.p.). Proteins were separated by SDS/PAGE [10–18% (w/v) gradient gel] and immunoblotted for phosphotyrosine as described. The positions of the FcR γ chain and p72^{syk} are indicated at the right. The positions of molecular mass standards are indicated at the left. Abbreviations: α-FcR γ chain, anti-(FcR γ chain); α-syk, anti-p72^{syk}; α-pY, anti-phosphotyrosine.

(results not shown) showed no association with other phosphotyrosine-containing proteins after stimulation with CRP for up to 300 s.

Further investigation of the interaction between p59^{fyn}, p53/56^{lyn} and the FcR γ chain

Src-family kinases can bind constitutively to components of immune receptors through interactions involving their unique N-terminal region, or SH4 domain [13,16]. These interactions are preserved in weaker detergents such as Brij96, but not in stronger detergents such as Nonidet P40 (NP40) [15]. When Brij96 was used as detergent, the amount of phosphorylated 14 kDa protein present in p59^{fyn} immunoprecipitates from CRP-stimulated platelets was significantly greater than that in NP40 immunoprecipitates (Figure 4, top panel). The kinetics of phosphorylation were also changed, with an earlier peak at 45 s and a loss by 300 s. Identification of this protein as the FcR γ chain was confirmed by immunoblotting. A phosphoprotein of 72 kDa, which underwent a transient tyrosine phosphorylation, also co-precipitated with p59^{fyn} in Brij96 more strongly than in NP40 lysates. Immunoblotting failed to identify this protein as p72^{syk}. In contrast with p59^{fyn}, association of the FcR γ chain with p53/56^{lyn} was lost when it was precipitated in Brij96 (Figure 4, middle panel). In addition, a further unidentified protein with an apparent molecular mass of 125 kDa was present after 90 and 300 s when Brij96 was used as detergent, which was not present in NP40. This protein was not identified.

To determine whether there was a constitutive association between the FcR γ chain and its phosphorylating kinase, kinase assays were carried out *in vitro* on FcR γ chain immunoprecipitates under basal conditions. Figure 4 [bottom panel (i)] shows that, with Brij96 as a detergent, the FcR γ chain co-precipitated with a kinase that was capable of phosphorylating it *in vitro*. A significant decrease in associated kinase activity was seen in NP40 lysates. To further investigate the nature of the interaction between Src-family kinase and FcR γ chain, p59^{fyn} and p53/56^{lyn} were reprecipitated from these kinase assays *in vitro* [Figure 4, bottom panel (ii)]. These experiments confirmed that p59^{fyn} associated with the FcR γ chain under basal con-



ditions but that this association was decreased in NP40 immunoprecipitates. Stimulation with CRP caused an increase in the amount of ^{32}P-labelled p59^{fyn} co-precipitating with the FcR γ chain under both conditions, which could represent either an increase in the quantity of p59^{fyn} present or, alternatively, an increase in the activity of the p59^{fyn} present in FcR γ chain immunoprecipitates from CRP-stimulated platelets. This result is consistent with a non-SH2 interaction of p59^{fyn} with the unphosphorylated FcR γ chain ITAM under basal conditions. In contrast, significantly less p53/56^{lyn} was found in FcR γ chain immunoprecipitates under basal conditions; the amount was unaffected by the detergent used, suggesting a more stable SH2-mediated interaction. These results are consistent with p59^{fyn} rather than p53/56^{lyn} being responsible for the kinase activity in FcR γ chain immunoprecipitates in basal conditions, and therefore ITAM phosphorylation.

DISCUSSION

We have investigated whether platelet Src-family kinases have a role in signalling by the collagen receptor, GPVI. This was done initially by using the selective Src-family kinase inhibitor PP1 and the novel inhibitor PD173956. Both compounds prevented collagen- and CRP-mediated functional responses; PP1 blocked increases in cellular tyrosine phosphorylation after stimulation by these agonists. The inhibition of FcR γ chain and p72^{syk} phosphorylation by PP1 suggests that this signalling pathway was being blocked at an early stage. Additionally, the selectivity of PP1 implies that the phosphorylation of the FcR γ chain is unlikely to be mediated by p72^{syk}, a conclusion supported by the maintained phosphorylation of the FcR γ chain after the collagen stimulation of platelets from mice lacking p72^{syk} [4].

After stimulation by collagen and CRP, both p59^{fyn} and p53/56^{lyn} became associated with other tyrosine-phosphorylated proteins, the major one of which was the FcR γ chain. This strongly implies their involvement in a signalling complex downstream of GPVI. In contrast, the lack of these changes for p62^{yes}, p54/56^{hck} and p60$^{c\text{-}src}$ suggest that they have little if any function in this signalling pathway, although this cannot be ruled out completely. The interaction of p59^{fyn} and p53/56^{lyn} with the FcR γ chain was further investigated by using the milder detergent Brij96, which, unlike NP40, maintains the association of p59^{fyn} and p53/56^{lyn} with non-phosphorylated ITAMs [13,17,22]. In Brij96, the amount of tyrosine-phosphorylated FcR γ chain was significantly increased relative to that in experiments performed in NP40. This result is consistent with the greater kinase activity precipitated with the FcR γ chain, and the greater amount of p59^{fyn} in FcR γ chain immunoprecipitates under these conditions. These results suggest that p59^{fyn} is constitutively associated

Figure 3 Immunoprecipitation (i.p.) and kinase assays *in vitro* of p53/56^{lyn}, p59^{fyn} and p60$^{c\text{-}src}$ after stimulation of platelets with CRP

Platelets were stimulated with CRP (1 μg/ml) for the times indicated. Top panels: (i) the reaction was stopped with lysis buffer containing 1% (v/v) NP40 (final concentration) and p59^{fyn} was immunoprecipitated; proteins were separated by SDS/PAGE. In the upper panel in (i), the membrane was immunoblotted for phosphotyrosine; in the lower panel in (i), the same membrane was stripped and immunoblotted for p59^{fyn}. (ii) Kinase assays were performed *in vitro* (i.v.k) on p59^{fyn} immunoprecipitates as described. Middle panels: similar experiments to those shown in the top panel were performed on p53/56^{lyn} immunoprecipitates and (i) blotted for phosphotyrosine (upper panel) or stripped and re-probed for p53/56^{lyn} (lower panel), or (ii) kinase assays were performed *in vitro* on p53/56^{lyn} immunoprecipitates. Bottom panels: similar experiments to those shown in the top panel were performed on p60$^{c\text{-}src}$ immunoprecipitates and blotted for phosphotyrosine (upper panel) or the same membrane was stripped and re-probed for p60$^{c\text{-}src}$ (lower panel). The positions of molecular mass standards are indicated at the left. Abbreviations: α-fyn, anti-p59^{fyn}; α-lyn, anti-(p53/p56^{lyn}); α-src, anti-p60$^{c\text{-}src}$; α-pY, anti-phosphotyrosine.

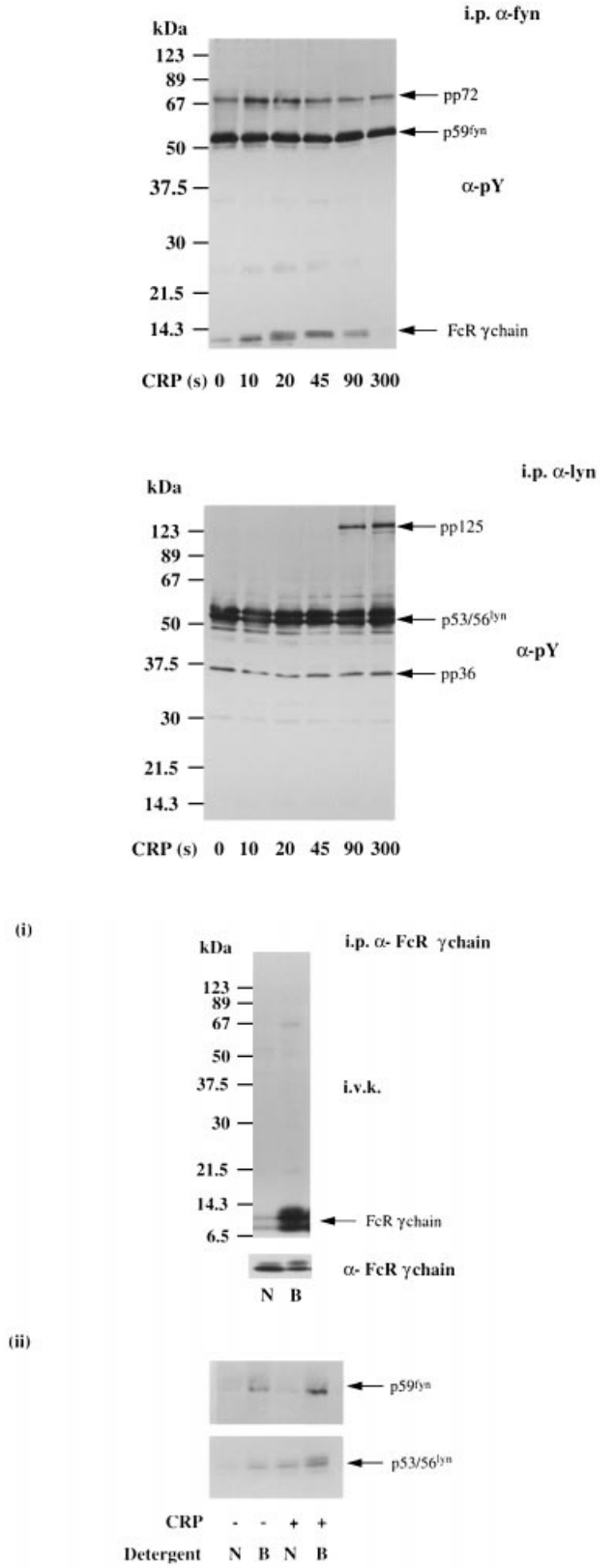


Figure 4 Effect of detergents on p59^{fyn}- and p53/56^{lyn}-associated proteins, and on kinase activity associated with the FcR γ chain

Platelets were stimulated with CRP (1 μg/ml) for the durations indicated and the reaction was stopped with lysis buffer containing 1% (v/v) Brij96 (final concentration). Either p59^{fyn} (top panels) or p53/56^{lyn} (middle panels) was then immunoprecipitated (i.p.) from lysates, samples were separated by SDS/PAGE and membranes were immunoblotted for phosphotyrosine. Bottom panels: (i) in the upper panel, the FcR γ chain was immunoprecipitated from unstimulated platelets in the presence of either 1% (v/v) NP40 (lane N) or 1% (v/v) Brij96 (lane B) and kinase assays were performed *in vitro*; in the lower panel, the same membrane was immunoblotted for the FcR γ chain. (ii) Platelets were stimulated with CRP (1 μg/ml) for 60 s and the FcR γ chain was precipitated from lysates in the presence of either 1% (v/v) NP40 (lane N) or 1% (v/v) Brij96 (lane B). Kinase assays were performed *in vitro*, and subsequently either p59^{fyn} (upper panel) or p53/56^{lyn} (lower panel) was re-precipitated. The positions of molecular mass standards are indicated at the left. Abbreviations: α-fyn, anti-p59^{fyn}; α-lyn, anti-(p53/p56^{lyn}); α-FcR γ chain, anti-(FcR γ chain); α-pY, anti-phosphotyrosine.

either directly with the FcR γ chain or with another component of the collagen receptor complex. A role for p59^{fyn} in collagen receptor signalling is supported by the inhibition of CRP-mediated increases in intracellular Ca^{2+} in megakaryocytes lacking this protein [23].

Our evidence points to a role for p53/56^{lyn} downstream of the FcR γ chain in this signalling pathway, because it associates strongly with several other tyrosine-phosphorylated proteins, suggesting that it is part of a much larger signalling complex. This is supported by work demonstrating an association of phospholipase Cγ2 with p53/56^{lyn}, but not p59^{fyn}, after CRP stimulation of platelets (B. Gross and S. P. Watson, unpublished work). There is also significantly less association of p53/56^{lyn} with the FcR γ chain under basal conditions than with p59^{fyn}. Because this association is unaffected by the type of detergent used, it is likely to be an SH2-mediated interaction rather than the predicted SH4-mediated interaction seen for p59^{fyn}, and is consistent with the small degree of tyrosine phosphorylation of the FcR γ chain under basal conditions. The lack of association of FcR γ chain and p53/56^{lyn} under basal conditions might reflect the fact that the involved pool of this kinase is compartmentalized in detergent-resistant compartments, such as caveoli. Similar findings have been demonstrated in B cells [24] and mast cells [25], in which active p53/56^{lyn} was found only in the detergent-insoluble fraction of immunoprecipitates, in association with FcϵRI.

The small changes seen in the tyrosine phosphorylation levels and activity of p59^{fyn} and p53/56^{lyn} after stimulation with CRP are not unusual and do not preclude their involvement in this pathway. Src-family kinases are constitutively phosphorylated on an inhibitory tyrosine residue in their N-terminal region [26]. Activation is often accompanied by sequential dephosphorylation and autophosphorylation in their kinase domain, or by binding of their SH2 or SH3 domains to other substrates [27]. In both cases there is little net change in phosphorylation state. In addition, only a small proportion of cellular kinase is likely to be involved in this particular pathway. Because basal levels of tyrosine phosphorylation and activities of platelet Src-family kinases are high, small changes in these parameters might be difficult to detect. It seems more likely that their activation in collagen receptor signalling is achieved by translocation to their site of action or by receptor clustering, thereby bringing them into close association with their substrates.

In conclusion, the association of p59^{fyn} and p53/56^{lyn} with the phosphorylated FcR γ chain, in conjunction with the functional effects of PP1, suggests their involvement in collagen-mediated activation of platelets. Our evidence shows that p59^{fyn} constitutively associates with the FcR γ chain, most probably via a non-SH2 interaction, and that it has an early role in the signalling pathway. However, p53/56^{lyn} might act predominantly downstream of FcR γ chain phosphorylation. Further studies will be necessary to clarify the more specific roles of these two Src-family kinases in signalling via GPVI and the FcR γ chain.

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REFERENCES

1 Arai, M., Yamamoto, N., Moroi, M., Akamatsu, N., Fukutake, K. and Tanoue, K. (1995) Br. J. Haematol. **89**, 124–130
2 Ichinihoe, T., Takayama, H., Wzumi, Y., Yamamoto, N., Takahashi, H. and Okuma, M. (1997) J. Biol. Chem. **272**, 63–68
3 Gibbins, J., Okuma, M., Farndale, R., Barnes, M. and Watson, S. (1997) FEBS Lett. **413**, 255–259
4 Poole, A., Gibbins, J., Turner, M., van Vugt, M., van de Winkel, J., Saito, T., Tybulewicz, V. and Watson, S. (1997) EMBO J. **16**, 2333–2341
5 Tsuji, M., Ezumi, Y., Arai, M. and Takayama, H. (1997) J. Biol. Chem. **272**, 23528–23531
6 Gibbins, J., Asselin, J., Farndale, R., Barnes, M., Law, C.-L. and Watson, S. (1996) J. Biol. Chem. **271**, 18095–18099
7 Blake, R. A., Schieven, G. L. and Watson, S. P. (1994) FEBS Lett. **353**, 212–216
8 Yanaga, F., Poole, A., Asselin, J., Blake, R., Schieven, G. L., Clark, E. A., Law, C. L. and Watson, S. P. (1995) Biochem. J. **311**, 471–478
9 Bolen, J. B. (1995) Curr. Opin. Immunol. **7**, 306–311
10 Costello, P., Turner, M., Walters, A. E., Cunningham, C. N., Bauer, P., Downward, J. and Tybulewicz, V. J. L. (1997) Oncogene **13**, 2595–2605
11 Duchemin, A. M. and Anderson, C. L. (1997) J. Immunol. **158**, 865–871
12 Wang, A. V. T., Scholl, P. R. and Geha, R. S. (1994) J. Exp. Med. **180**, 1165–1170
13 Gauen, L. K. T., Kong, A. N., Samelson, L. E. and Shaw, A. S. (1992) Mol. Cell. Biol. **12**, 5438–5446
14 Gauen, L. K. T., Zhu, Y., Letourneur, F., Hu, Q., Bolen, J. B., Matis, L. A., Klausner, R. D. and Shaw, A. S. (1994) Mol. Cell. Biol. **14**, 3729–3741
15 Burg, D. L., Furlong, M. T., Harrison, M. L. and Geahlen, R. L. (1994) J. Biol. Chem. **269**, 28136–28142
16 Vonakis, B. M., Chen, H., Haleem-Smith, H. and Metzger, H. (1997) J. Biol. Chem. **272**, 24072–24080
17 Jackson, S. P., Schoenwaelder, S. M., Yuan, Y., Salem, H. H. and Cooray, P. (1996) Thromb. Haemostasis. **76**, 640–650
18 Hanke, J., Gardner, J., Dow, R., Changelian, P., Brissette, W., Weringer, E., Pollok, B. and Connelly, P. (1996) J. Biol. Chem. **271**, 695–701
19 Morton, L. F., Hargreaves, P. G., Farndale, R. W., Young, R. D. and Barnes, M. J. (1995) Biochem. J. **306**, 337–344
20 Asselin, J., Gibbins, J., Achison, M., Lee, Y. H., Morton, L., Farndale, R., Barnes, M. and Watson, S. (1997) Blood **89**, 1235–1242
21 Amoui, M., Draber, P. and Draberova, L. (1997) Eur. J. Immunol. **27**, 1881–1886
22 Gauen, L. K. T., Linder, M. E. and Shaw, A. S. (1996) J. Cell Biol. **133**, 1007–1015
23 Melford, S. K., Turner, M., Briddon, S. J., Tybulewicz, V. L. J. and Watson, S. P. (1997) J. Biol. Chem. **272**, 27539–27542
24 Field, K. A., Holowka, D. and Baird, B. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 9201–9205
25 Field, K. A., Holowka, D. and Baird, B. (1997) J. Biol. Chem. **272**, 4276–4280
26 Brown, M. T. and Cooper, J. A. (1996) Biochim. Biophys. Acta **1287**, 121–149
27 Qian, D. and Weiss, A. (1997) Curr. Opin. Cell Biol. **9**, 205–212

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