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ORIGINAL ARTICLE

The novel Syk inhibitor R406 reveals mechanistic differences in the initiation of GPVI and CLEC-2 signaling in platelets

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Summary. *Background*: Syk is a key mediator of signaling pathways downstream of several platelet surface receptors including GPVI/FcRγ collagen receptor, the C-type lectin receptor CLEC-2, and integrin αIIbβ3. A recent study identified the novel small molecule R406 as a selective inhibitor of Syk. *Objectives*: The present study evaluates the role of Syk in human platelets using the novel inhibitor R406. *Methods*: Agonist-induced GPVI and CLEC-2 signaling were assessed using aggregometry, immunoprecipitation and western blotting to determine the effects of R406 on platelet activation. *Results*: We demonstrate R406 to be a powerful inhibitor of Syk in human platelets. R406 abrogated shape change and aggregation induced by activation of GPVI and CLEC-2, and reduced platelet spreading on fibrinogen. The inhibitory effect of R406 was associated with inhibition of tyrosine phosphorylation of signaling proteins that lay downstream of Syk for all three receptors, including PLCγ2. Strikingly, R406 markedly inhibited tyrosine phosphorylation of CLEC-2 and Syk downstream of CLEC-2 activation, whereas phosphorylation of Syk downstream of GPVI and integrin αIIbβ3 was unaffected. *Conclusions*: The inhibitory effect of R406 provides direct evidence of a role for Syk in GPVI, CLEC-2 and integrin αIIbβ3 signaling in human platelets. Further, the results demonstrate a critical role for Syk in mediating tyrosine phosphorylation of CLEC-2, suggesting a novel model in which both Src and Syk kinases mediate tyrosine phosphorylation of the C-type lectin receptor leading to platelet activation.

Keywords: CLEC-2, GPVI, integrin αIIbβ3, platelets, Syk.

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Introduction

Spleen tyrosine kinase (Syk) and ζ-associated protein of 70 kDa (Zap-70) are the only two members of the Syk family of non-receptor tyrosine kinases, which is characterised by two N-terminal SH2 domains and a C-terminal catalytic domain. Both members are regulated by binding to two phosphorylated tyrosines in a sequence known as an immunoreceptor tyrosine-based activation motif (ITAM), which has two YXXL groups, separated by 6–8 amino acids for Zap-70, or 6–12 amino acids for Syk. Zap-70 is expressed in T cells and natural killer cells, whereas Syk is expressed in most cells in the hematopoietic lineage [1]. Studies using Syk-deficient mice have demonstrated a pivotal role for the kinase in signaling downstream of ITAM receptors, as illustrated by its role in activation of platelets by the collagen receptor complex, GPVI/FcR γ-chain (FcRγ), and in the mast cell by FcεRI [2]. Syk has also been shown to play a key role in activation of mouse platelets by integrins α2β1 and αIIbβ3, GPIb-IX-V, and the C-type lectin receptor CLEC-2 [3–5].

The involvement of Syk in immunoreceptor signaling has been studied extensively. On immunoreceptor activation, Src family kinases (SFKs) phosphorylate two tyrosine residues within the consensus ITAM, facilitating recruitment of Syk to the plasma membrane through its tandem SH2 domains [6]. On relocalization, Syk undergoes both autophosphorylation and phosphorylation by SFKs, leading to its activation, interaction with adaptor and effector proteins, and tyrosine phosphorylation of downstream substrates, including PLCγ2, leading to cell activation [7,8]. In contrast, the mechanism of activation of Syk by integrin αIIbβ3, which lacks an ITAM, is controversial [9]. It was originally proposed that integrin αIIbβ3 signaling proceeds independently of receptor tyrosine phosphorylation [9,10], but a subsequent study provided evidence that the phosphotyrosine-binding capacity of Syk is required for activation by integrins, possibly via an unidentified ITAM-containing protein [11]. Indeed, it has recently been shown that the low affinity Fc receptor, FcγRIIA, couples integrin αIIbβ3 to downstream signaling events in human platelets [12]. It thus seems that αIIbβ3 signals through both ITAM-dependent and ITAM-independent regulation of Syk.

CLEC-2 signals through a similar pathway to that of immunoreceptors, including a critical role for sequential activation of SFKs and Syk, and phosphorylation of common downstream proteins including PLCγ2 [13]. However, in contrast to immunoreceptors, the C-type lectin receptor contains a single cytoplasmic YXXL sequence, which we have shown to be essential for activation [14]. Studies using mouse models and cell lines deficient in key signaling proteins have demonstrated that CLEC-2 signaling can be further distinguished from that of the GPVI-FcRγ pathway through a partial dependence on the adapter SLP-76 [13,14].

There is considerable interest in the role of Syk in platelets in view of its potential as an anti-thrombotic target. Investigation into the role of Syk, however, has been hampered by the perinatal lethality of homozygous Syk-deficient mice [15] and the absence of a truly specific inhibitor to verify its function in human platelets. For example, piceatannol has been used extensively as a Syk inhibitor, but a study comparing the effects of this inhibitor with Syk-deficient mouse platelets showed differences in functional responses, suggesting that piceatannol has off-target effects, including inhibition of SFKs [16].

A novel small molecule inhibitor, R406, was recently identified and shown to inhibit Syk kinase activity both *in vitro* and *in vivo* in an ATP-competitive manner [17]. The availability of R406 enables testing of the role for Syk in mediating activation of human platelets. In the present study, we demonstrate that R406 is a powerful and specific inhibitor of Syk and that the tyrosine kinase plays a critical but differential role in the activation of human platelets by GPVI, CLEC-2 and integrin αIIbβ3.

Experimental procedures

Reagents

Rhodocytin and CRP were obtained as previously described [18–20]. R406 was a gift from Dr David Simmons, Cellzome Ltd (Cambridge, UK). Phospho-Syk antibodies were from NEB (Hertfordshire, UK). GST-Igα was expressed as a GST fusion protein and donated by Dr Mike Tomlinson (University of Birmingham, Birmingham, UK). All other reagents were from Sigma Ltd (Poole, UK) or previously published sources [5,8,13].

Platelet preparation and functional studies

Washed platelets (WP) were prepared as previously described [3,14] and treated with inhibitor for 60 s prior to stimulation with agonists. Aggregation was monitored by light transmission using a Born aggregometer (Chronolog, Havertown, PA) [14]. Spreading on fibrinogen was performed as previously described [5]. Images were captured using differential interference contrast (DIC) microscopy and five fields per image analysed using IMAGE J software.

Western blotting

WP were pretreated with 9 μM integrilin to block aggregation, and stimulations were carried out at 37 °C with stirring. Immunoprecipitation and western blotting were carried out as previously described [13].

In vitro *kinase assay*

Syk was immunoprecipitated from platelet lysates as above, resuspended in 40 μL kinase buffer (50 mM MOPS, 150 mM NaCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$), containing 5 μg GST-Igα, 1 mM DTT and 35 nM ATP, and incubated for 30 s at 37 °C. Where stated, samples were pretreated with inhibitor for 1 min. The assay was stopped by the addition of SDS sample buffer, and samples analysed by western blotting.

Statistical analysis

Results are shown as mean ± SE mean of three experiments. Statistical analysis was determined by Student's *t*-test.

Results

R406 inhibits GPVI signaling

We initially examined the effect of R406 on aggregation of human platelets downstream of GPVI activation. Shape change and aggregation induced by collagen related peptide (CRP) (3 μg/mL), collagen (1 μg/mL) and convulxin (1 μg/mL) were greatly reduced in the presence of 0.1–0.3 μM R406 and abrogated at 1 μM R406 (Fig. 1A,B, and not shown). In contrast, R406 (1 μM) had a minor effect on aggregation

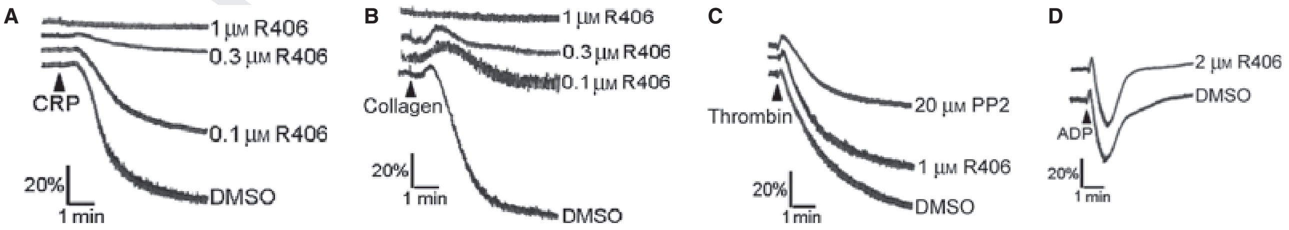

Fig. 1. GPVI-induced aggregation is inhibited by R406. Washed platelets pretreated with DMSO, R406 or PP2 for 60 s were stimulated with (A) 3 μg/mL CRP, (B) 1 μg/mL collagen, or (C) 0.03 U/mL thrombin. (D) Platelet-rich plasma was pretreated with 2 μM R406 prior to stimulation with 3 μM ADP. Aggregation was determined by change in light transmission. Arrows mark addition of agonist. Data are representative of three independent experiments.

induced by a low concentration of the G protein-coupled receptor agonist, thrombin (0.03 U/mL), but had no effect on the response to ADP (3 μm) (Fig. 1C,D). In the latter study, a slightly higher concentration of R406 was used to overcome protein binding in the plasma as monitored by inhibition of the response to CRP (not shown). The SFK inhibitor PP2 (20 μm) had a slightly greater inhibitory effect against thrombin in washed platelets relative to that of R406 (Fig. 1C).

Having established that R406 has a powerful and potent inhibitory effect on aggregation induced through the GPVI/FcRγ complex, we examined the underlying signaling events. R406 (1 μm) inhibited tyrosine phosphorylation of several protein bands in platelets stimulated by collagen, convulxin or CRP, whilst having a negligible effect on others (Fig. 2A,B). R406 had a more powerful inhibitory effect against low concentrations of all three agonists, with partial recovery observed in response to higher concentrations [Fig. 2A(i) and not shown], most likely due to incomplete blockade of Syk. The reduction of some but not all bands by R406 is in stark contrast to the complete abolition of tyrosine phosphorylation to all three agonists in the presence of the SFK inhibitor, PP2, as illustrated for CRP in Fig. 2(B), and in line with previous studies [21–23]. The difference in the pattern of inhibition confirms that over the concentration range of 0.1–1 μm, R406 is selective for Syk over SFKs. A 10-fold higher concentration of R406 induced a similar pattern of inhibition of tyrosine phosphorylation to PP2, demonstrating loss of selectivity to Syk (data not shown).

The reduction in tyrosine phosphorylation was investigated further by immunoprecipitation of signaling proteins combined with western blotting. R406 (1 μm) markedly inhibited tyrosine phosphorylation of LAT, SLP-76 and PLCγ2 induced by collagen, convulxin and CRP (Fig. 2 and not shown), consistent with previous results in Syk-deficient mice. In contrast, tyrosine phosphorylation of FcRγ and Syk by the three agonists was not significantly altered, confirming that SFK activity is not affected (Fig. 2). To investigate phosphorylation of Syk in further detail, we used phospho-specific antibodies against residues within the linker region (Y352) and kinase domain (Y525/526) of Syk. Phosphorylation of these residues increased in a concentration-dependent manner and was not altered in the presence of R406 (Fig. 2A).

An *in vitro* kinase assay was performed on Syk immunoprecipitates to further investigate the specificity of R406 for Syk. Syk underwent autophosphorylation and stimulated tyrosine phosphorylation of Igα following immunopreciptiation from non-stimulated platelets. Phosphorylation was inhibited by direct addition of R406 but not PP2 to the kinase assay (Fig. 3A). In contrast, stimulation of platelets by CRP induced a marked increase in Syk autophosphorylation and Igα phosphorylation, neither of which was altered by pretreatment of intact platelets with R406 (Fig. 3B). This is consistent with the above observation that tyrosine phosphorylation of Syk is not altered in CRP-stimulated platelets in the presence of R406, assuming removal of the kinase inhibitor during

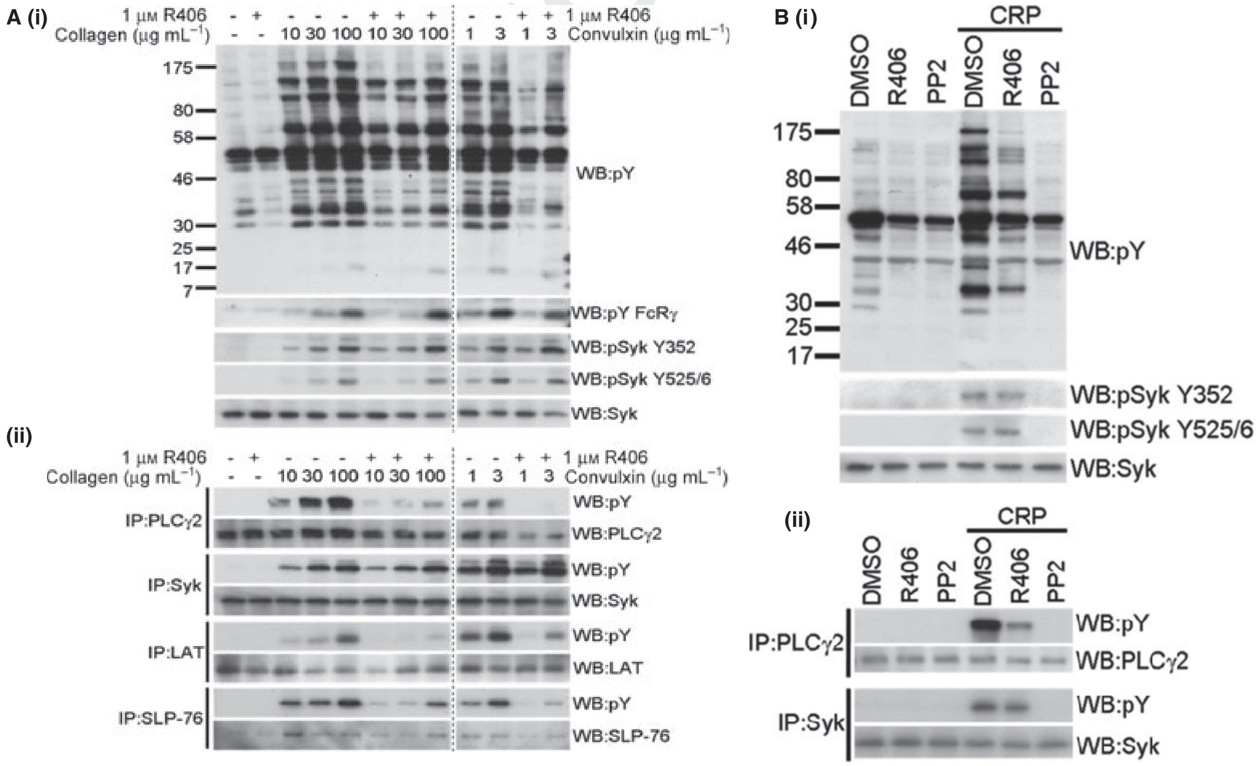

Fig. 2. R406 reduces tyrosine phosphorylation downstream of GPVI. Platelets pretreated with 1 μm R406, 20 μm PP2 or DMSO for 1 min were stimulated with collagen, convulxin or CRP (3 μg/mL) for 90 s. Whole cell lysates [A(i), B(i)] and immunoprecipitates [A(ii), B(ii)] were immunoblotted as stated. Data are representative of three independent experiments.

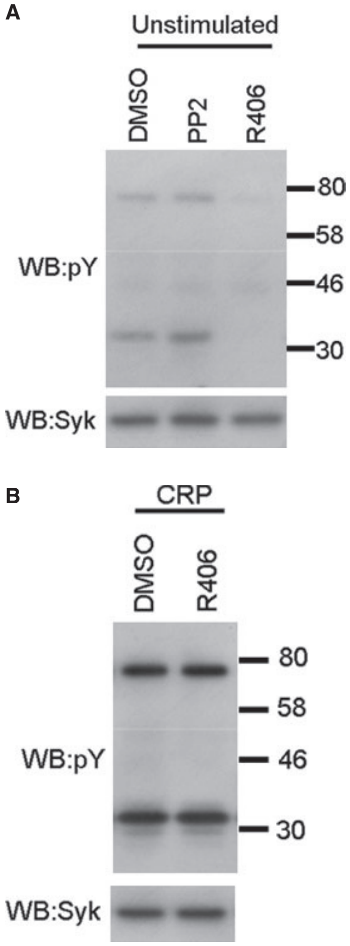

Fig. 3. Inhibition of Syk by R406 in an *in vitro* kinase assay. The ability of Syk immunoprecipitates to phosphorylate GST-Igα was determined by an *in vitro* kinase assay and samples were immunoblotted as stated. (A) 1 μM R406 or 20 μM PP2 was added directly to the kinase assay. (B) Platelets pretreated with 1 μM R406 or 20 μM PP2 for 60 s were stimulated with 1 μg/mL CRP for 90 s prior to the kinase assay. Data are from one experiment that is representative of four similar experiments.

preparation of the lysates. The above results demonstrate that R406 is a selective inhibitor of Syk and confirm a critical role for the kinase in mediating tyrosine phosphorylation of LAT, SLP-76 and PLCγ2, and platelet aggregation induced by GPVI agonists.

R406 inhibits CLEC-2 signaling

Syk has been shown to play a critical role in activation of mouse platelets induced by CLEC-2 using Syk-deficient mice [12]. In the present study, a role for Syk in mediating aggregation of human platelets in response to the snake venom toxin, rhodocytin, or an antibody to CLEC-2, has been shown using R406 (Fig. 4A). Significantly, the CLEC-2 antibody activates human platelets independent of the low affinity immune receptor, FcγRIIA [14]. Strikingly, and in marked contrast to results for GPVI, R406 (1 μM) dramatically inhibited the increase in tyrosine phosphorylation of all proteins in whole cell lysates induced by intermediate and high concentrations of rhodocytin (Fig. 4B) or by the CLEC-2 antibody (Fig. 4C). This was associated with inhibition of tyrosine phosphorylation of CLEC-2 and Syk, as well as the downstream proteins LAT, SLP-76 and PLCγ2 (Fig. 4B). The observation that CLEC-2 runs as a doublet is consistent with the findings of Suzuki-Inoue *et al.* [13], who identified that the pair of protein bands represent differentially *N*-glycosylated forms of CLEC-2. Inhibition of tyrosine phosphorylation of Syk was confirmed using phospho-specific antibodies to Y352 and Y525/526 following stimulation by R406 (Fig. 4B). Inhibition of CLEC-2 phosphorylation and downstream phosphorylation events was also observed in the presence of the SFK inhibitor PP2, in addition to the inhibition of both rhodocytin- and CLEC-2 antibody-induced aggregation ([24] and not shown). These data confirm that SFKs and Syk are key mediators of platelet activation by CLEC-2, but identify an unexpected role for Syk in mediating tyrosine phosphorylation of the C-type lectin receptor.

Syk is required for lamellipodia formation on fibrinogen

The effects of R406 were further investigated on adhesion and spreading of platelets on fibrinogen. Human platelets readily adhere to fibrinogen and undergo spreading characterized by sequential formation of filopodia and lamellipodia (Fig. 5). There was a marked inhibition of spreading on fibrinogen in the presence of R406, with only a small proportion of platelets generating filopodia, whereas the level of adhesion was not altered (Fig. 5A). Similar observations were made when platelets were treated with the SFK inhibitor PP2 (not shown), consistent with previous observations [4,25]. However, in contrast to the complete inhibition of tyrosine phosphorylation that is seen in the presence of the SFK inhibitor PP2 (20 μM), R406 (1 μM) caused a reduction in tyrosine phosphorylation of only a small subset of proteins, including a band of 150 kDa that co-migrates with PLCγ2 (Fig. 5B). Analysis of tyrosine phosphorylation of PLCγ2 following immunoprecipitation confirmed inhibition by both PP2 and R406. On the other hand, tyrosine phosphorylation of Syk was partially inhibited by R406 but abolished by PP2, a result that was confirmed using phospho-specific antibodies to Syk (Fig. 5B). These observations are consistent with previous studies in mouse platelets, and demonstrate a critical role for Src and Syk kinases in mediating tyrosine phosphorylation of PLCγ2 and spreading of human platelets on fibrinogen.

Discussion

The present findings demonstrate R406 to be a powerful and specific inhibitor of Syk in human platelets and enabled verification of a role for Syk in mediating activation of human platelets through GPVI, CLEC-2 and integrin αIIbβ3, consistent with previous studies in Syk-deficient mouse platelets. Strikingly, the present study further reveals an unexpected role for Syk in mediating tyrosine phosphorylation of CLEC-2,

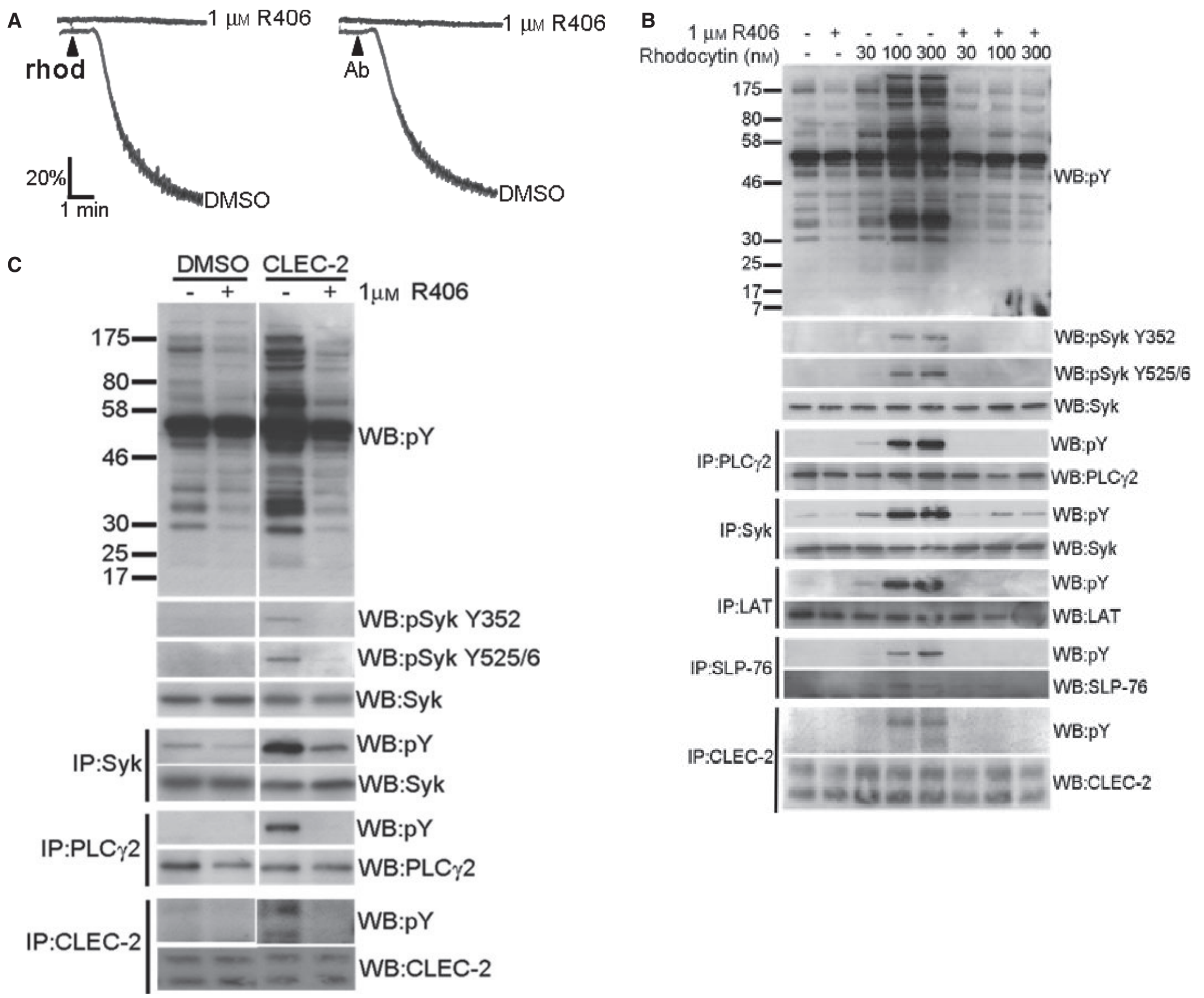

Fig. 4. R406 inhibits CLEC-2 signaling. Platelets pretreated with R406 or DMSO for 1 min were stimulated with 100 nM rhodocytin (rhod) or 20 µg/mL CLEC-2 antibody (Ab). (A) Aggregation was determined by change in light transmission. Whole cell lysates [B(i), C] and immunoprecipitates [B(ii), C] were immunoblotted as stated. Data are representative of three independent experiments.

thereby demonstrating a fundamental difference in the mechanism of signaling by the C-type lectin receptor in comparison with GPVI and integrin αIIbβ3.

The pivotal role of Syk in mediating signaling events downstream of GPVI/FcRγ is illustrated in functional studies. Shape change and aggregation induced by the GPVI-specific agonist CRP were completely abrogated by 1 µM R406, as was the case for a low concentration of the physiological agonist collagen. There was, however, a limited recovery in response to a 10-fold higher concentration (10 µg/mL) of collagen but not to CRP (Supplementary data, Fig. S1), which could reflect Syk-independent signaling through integrin α2β1 [26],and possibly activation of a third, unidentified, receptor [27]. In contrast, Braselmann *et al.* [17] reported no effect of R406 when given orally on collagen-induced aggregation in human subjects, although it is unclear why R406 is not effective against collagen when given in this way. We have also shown that Syk inhibition does not affect adhesion to fibrinogen but blocks platelet spreading through the inhibition of lamellipodia formation. This is consistent with observations in Syk-deficient mouse platelets that first demonstrated a role for Syk in mediating platelet spreading downstream of integrin αIIbβ3 [4] and the recent demonstration of a role for FcγRIIA in mediating signaling by the integrin [12].

R406 had a weak inhibitory effect on platelet aggregation induced by thrombin, which signals through the G_q-coupled family of G proteins. The inhibitory effect of R406 was slightly smaller than that of the SFK inhibitor, PP2, suggesting that SFKs may contribute at least partly to thrombin-induced aggregation through activation of Syk. On the other hand, R406 had no effect on the response to ADP, which signals through a synergistic interaction between the G_i-coupled $P2Y_{12}$ receptor and Gq-coupled $P2Y_1$ receptor. We have previously reported that aggregation induced by thrombin in mouse platelets is independent of SFK and Syk, suggesting a species difference [5]. In this context, it is noteworthy that the major receptor for thrombin in mouse platelets is PAR-4, whereas in humans it is PAR-1.

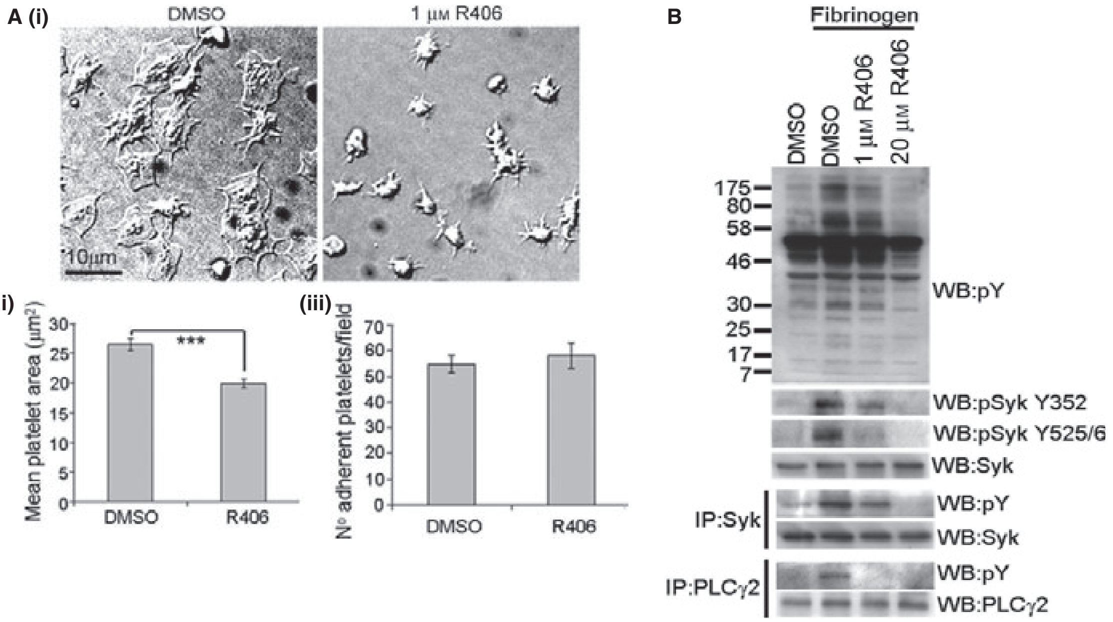

Fig. 5. R406 blocks lamellipodia formation on fibrinogen. (A) Platelets were treated as indicated before plating on fibrinogen-coated dishes. Fixed adherent platelets were imaged [A(i)] and analysed for mean platelet area ± SE [A(ii)] and adhesion ± SE [A(iii)]. (B) Whole cell lysates and immunoprecipitates were immunoblotted with stated antibodies; $^{***}P < 0.001$. Data are representative of three independent experiments.

Phospho-specific antibodies have proved to be a useful tool in measuring the tyrosine phosphorylation of Syk at specific residues, and have provided new information into the mechanism of Syk activation in human platelets. It has been shown that upon B cell receptor activation, the SFK Lyn phosphorylates Syk at position Y352 [28], thereby leading to phosphorylation at further sites, including autophosphorylation of tyrosines 525 and 526 within the kinase domain [29,30]. Tyrosine phosphorylation at Y525/526 has been used in several studies as a marker for Syk activity as it fits with the generic kinase regulatory mechanism in which activation loop phosphorylation causes a conformational change to an active state [31]. However, there has been much discussion regarding the mechanism of activation loop phosphorylation, with the proposal that the relative contribution of SFK and autophosphorylation varies with cell type [30,32]. The present study has resolved this issue in human platelets by reporting both a normal level of Syk phosphorylation as measured using phospho-specific antibodies in the presence of R406 and full kinase activity upon washout of R406. This suggests that Syk is phosphorylated downstream of Src kinases rather than through autophosphorylation. Consistent with this, R406 but not PP2, inhibited Syk autophosphorylation and phosphorylation of an exogenous substrate in an *in vitro* assay, in confirmation of the findings of Cha *et al.* [33], whereas R406 had no effect on Syk activation in CRP-stimulated platelets

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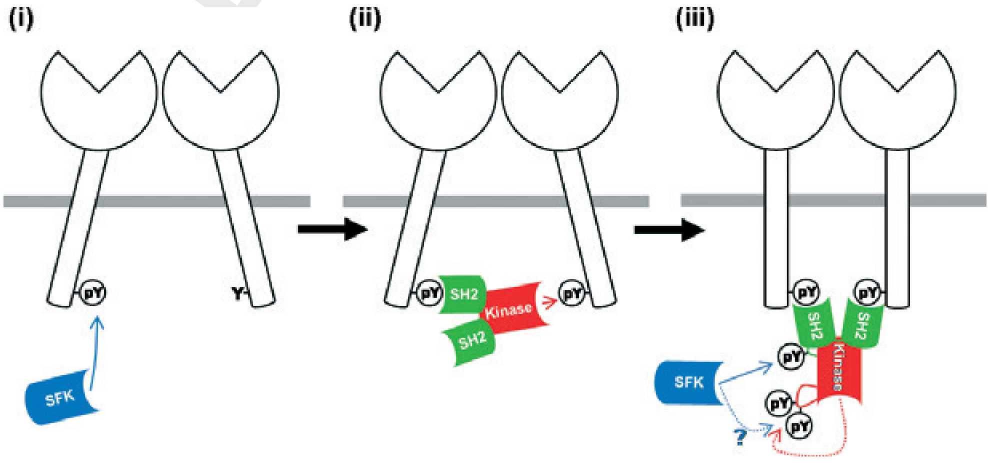

Fig. 6. Model of CLEC-2 activation. Src family kinase (SFK) phosphorylation of CLEC-2 allows recruitment of Syk via one of its SH2 domains (i). The opposing orientation of the kinase domain enables Syk to phosphorylate the second CLEC-2 receptor in the dimer, leading to a rearrangement in binding that allows cross-linking of two CLEC-2 receptors via its SH2 domains (ii). Syk is fully activated by SFK-mediated phosphorylation, and possibly autophosphorylation, initiating downstream signaling (iii).

pretreated with R406. This illustrates that Syk undergoes phosphorylation in intact platelets in the presence of Syk inhibition, and provides direct evidence of the inhibitory effect of R406 on Syk.

An unexpected finding from the present study is the critical role that Syk plays in mediating tyrosine phosphorylation of CLEC-2 induced by either rhodocytin or the CLEC-2 antibody. This is in marked contrast to stimulation of platelets through GPVI by CRP, collagen and the snake venom toxin, convulxin, in which phosphorylation of the FcRγ is minimally altered in the presence of R406. Thus, the results suggest that both SFKs and Syk play a role in mediating tyrosine phosphorylation of CLEC-2. Although the GPVI/FcRγ and CLEC-2 signaling pathways share common elements, CLEC-2 has a single YXXL motif in its cytoplasmic tail, compared with the tandem YXXL motif in FcRγ. Even so, point mutations that destroy phosphotyrosine binding in either of the two SH2 domains in Syk abrogate signaling by CLEC-2 [14], suggesting that Syk must crosslink phosphorylated YXXL motifs in two CLEC-2 receptors to mediate activation, consistent with the observation that rhodocytin causes clustering of the CLEC-2 receptor [34]. Together, these observations lay the foundation for the proposal of a novel pathway through which CLEC-2 initiates signaling, which is distinct from that used by GPVI.

In the model shown in Fig. 6, we propose that both SFKs and Syk directly mediate tyrosine phosphorylation of CLEC-2. In this model, receptor dimerisation leads to Src-dependent tyrosine phosphorylation of the YXXL motif of CLEC-2 and transient recruitment of Syk, via either of its SH2 domains, to the phosphorylated tyrosine [Fig. 6(i)]. The crystal structure shows that the tyrosine kinase domain of Syk is positioned in the opposite direction to the tandem SH2 domains [35]. Thus, binding of Syk brings its kinase domain into contact with the YXXL sequence in the second CLEC-2 receptor and allows subsequent phosphorylation of this motif [Fig. 6(ii)]. In turn, this leads to a rearrangement of binding such that Syk is able to crosslink two CLEC-2 receptors via its tandem SH2 domains [Fig. 6(iii)]. In this position, Syk is then activated by SFK phosphorylation at position 352, and possibly also at positions 525 and 526, although the latter could be mediated by autophosphorylation. Activation of Syk in this way initiates a series of downstream events leading to tyrosine phosphorylation and activation of PLCγ2. In this model, the initial transient phosphorylation of CLEC-2 by SFKs may be subject to rapid dephosphorylation, whilst binding of Syk would prevent protein tyrosine phosphatases from accessing and dephosphorylating the ITAM. Alternative models in which receptor phosphorylation is mediated solely by engagement of either SFKs or Syk would seem less likely because inhibition of either kinase type results in abrogation of receptor phosphorylation. Indeed, the interaction between the tyrosine phosphatase SHP2 and the PDGF receptor provides a precedent for the model shown in Fig. 5, as the tandem SH2 domains of SHP2 are believed to bridge PDGF receptor dimers by binding equivalent recognition sites on each monomer [36]. Further studies will enable more detailed analysis of the events involved in CLEC-2 receptor activation and downstream signaling, and will allow this model to be developed.

In summary, we have used the novel inhibitor R406 to demonstrate a role for Syk in GPVI-, CLEC-2- and αIIbβ3-signaling in human platelets. Further, we have identified a mechanistic difference between the CLEC-2 and GPVI signaling pathways that has led us to propose a model of CLEC-2 signaling in which SFKs and Syk combine together to directly phosphorylate the C-type lectin receptor.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Inhibition of aggregation by R406 can be overcome by a higher collagen concentration.

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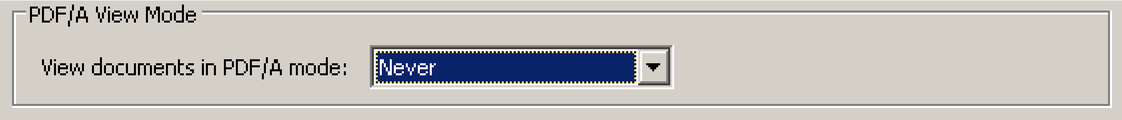
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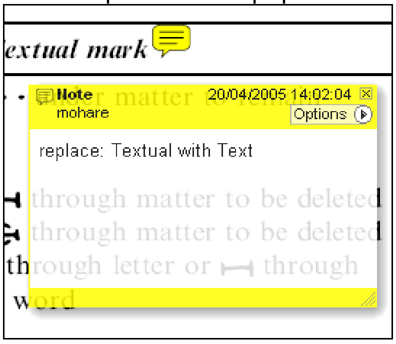

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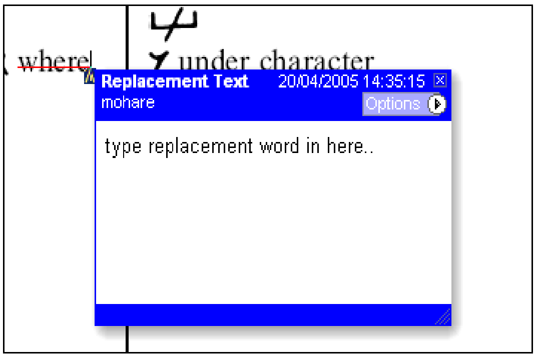

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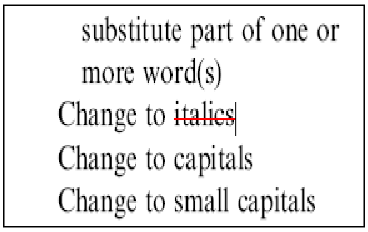
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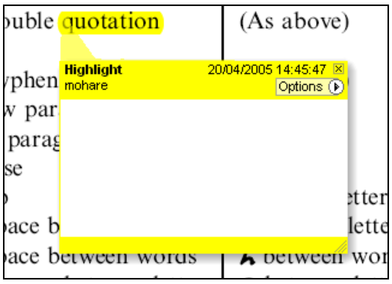

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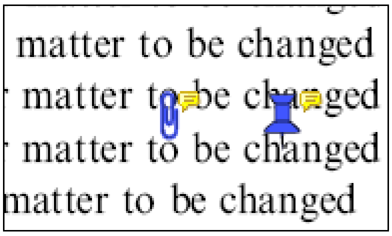

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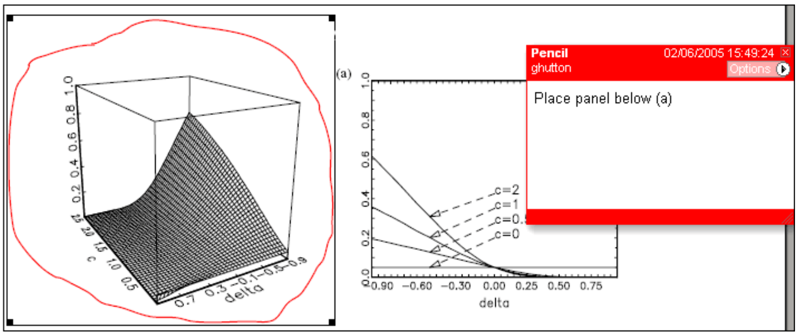

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