SYK DEPENDENT PHOSPHORYLATION OF CLEC-2:
A NOVEL MECHANISM OF HEMITAM SIGNALLING

Sonia Séverin1*, Alice Y. Pollitt1*, Leyre Navarro-Nuñez1, Craig A. Nash1, Diego Mourão-Sá2,
Johannes A. Eble3, Yotis A. Senis1, Steve P. Watson1

1Centre for Cardiovascular Sciences, Institute of Biomedical Research, College of Medical and Dental
Sciences, University of Birmingham B15 2TT, UK, 2Immunobiology Laboratory, Cancer Research
UK, London Research Institute, Lincoln’s Inn Fields Laboratories, 44 Lincoln’s Inn Fields, London
WC2A 3PX, UK, 3Centre for Molecular Medicine, Excellence Cluster Cardio-Pulmonary System,
Frankfurt University Hospital, Frankfurt am Main, Germany.

*The authors contributed equally to this work

Address correspondence to: Sonia Séverin, Centre for Cardiovascular Sciences, Institute of
Biomedical Research, College of Medical and Dental Sciences, University of Birmingham B15 2TT,
UK; Tel: 0044(0)1214158679; Fax: 0044(0)1214158817; Email: s.severin@bham.ac.uk

The C-type lectin-like receptor CLEC-2 signals via phosphorylation of a single
cytoplasmic YxxL sequence known as a hem-
immunoreceptor tyrosine-based activation
motif (hemITAM). In this study, we show
that phosphorylation of CLEC-2 by the snake
toxin rhodocytin is abolished in the absence of
the tyrosine kinase Syk but is not altered in
the absence of the major platelet Src family
kinases, Fyn, Lyn and Src, or the tyrosine
phosphatase CD148 which regulates the basal
activity of Src family kinases. Further,
phosphorylation of CLEC-2 by rhodocytin is
not altered in the presence of the Src family
kinase inhibitor PP2 even though PLCγ2
phosphorylation and platelet activation are
abolished. A similar dependency of
phosphorylation of CLEC-2 on Syk is also
seen in response to stimulation by an IgG
monoclonal antibody (mAb) to CLEC-2,
although interestingly CLEC-2
phosphorylation is also reduced in the
absence of Lyn. These results provide the
first definitive evidence that Syk mediates
phosphorylation of the CLEC-2 hemITAM
receptor with Src family kinases playing a
critical role further downstream through the
regulation of Syk and other effector proteins,
providing a new paradigm in signalling by
YxxL-containing receptors.

The C-type lectin-like receptor CLEC-2 is a type
II transmembrane protein that is highly expressed on the surface of platelets and
megakaryocytes (1,2) and at a lower level on
several other hematopoietic lineages, including
monocytes and dendritic cells (3-5). The first
ligand to be identified for CLEC-2 was the
powerful snake venom toxin, rhodocytin,
purified from the Malayan pit viper,
Calloselasma rhodostoma (2). F(ab)2 antibody
fragments of CLEC-2 antibodies were
subsequently shown to mediate activation
of human platelets (2,6). The transmembrane
protein, podoplanin, was identified as an
endogenous ligand, inducing powerful activation
of platelets and CLEC-2-expressing cell lines
(2,6-8). Podoplanin is expressed on the surface
of a wide variety of cells such as kidney
podocytes, lung type I alveolar cells and
lymphatic endothelial cells but is absent from
vascular endothelial cells and platelets. The
activation of CLEC-2 by podoplanin is essential
for the separation of the lymphatic and blood
vasculatures (9-12). In addition, expression of
podoplanin on the surface of certain tumours is
implicated in the process of tumour metastasis
through activation of CLEC-2 (8,13). CLEC-2
has recently reported to play a role in supporting
platelet activation at arteriolar rates of flow on
collagen in some (6,11) but not all studies (14).

CLEC-2 is expressed on resting platelets as a
non-covalent homodimer (15,16) and signals
through a single YxxL sequence in its
cytoplasmic tail known as a hem-
immunoreceptor tyrosine-based activation motif
(hemITAM) (2,15-17). The minimal signalling
unit for activation of Syk by CLEC-2 has been proposed to be the crosslinking of two CLEC-2 dimers (15,16), activating a similar signalling cascade to that of the platelet collagen receptor GPVI-FcRγ-chain complex, with critical roles for linker for activation of T cells (LAT), Src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa (SLP-76), phosphoinositide (PI) 3-kinases and phospholipase C (PLC) γ2 (2,17). CLEC-2 signalling occurs in lipid rafts and is dependent on actin polymerisation, release of the secondary mediators, adenosine diphosphate (ADP) and thromboxane (TX) A2, and activation of the small G protein Rac (18).

Phosphorylation of the GPVI-FcRγ-chain ITAM is mediated by Src family kinases leading to subsequent binding and activation of Syk. Thus, ITAM phosphorylation is preserved in the absence of Syk or the Syk inhibitor R406 (19,20). The Src family kinases, Lyn and Fyn, mediate phosphorylation of the GPVI-FcRγ-chain ITAM (21-23). Fyn and Lyn bind to a proline rich region in the cytosolic tail of GPVI via their SH3 domains, allowing for rapid initiation of platelet activation (21,24). In addition, they also support activation by a second pathway of activation that is independent of the GPVI proline-rich motif (25). Recently, it has been proposed that GPVI-associated Lyn is in an active conformation suggesting that Lyn initiates GPVI-induced platelet activation, with Fyn supporting sustained GPVI signalling (23).

Src family kinases also play a role in CLEC-2 mediated signalling, as structurally distinct broad range Src family kinase inhibitors such as PP2 and PD 173956 inhibit human platelet activation (2,17). However, it has recently been reported that phosphorylation of CLEC-2 in human platelets stimulated by rhodocytin is blocked by selective inhibitors of Src family or Syk tyrosine kinases implicating these kinases in hemITAM phosphorylation (20). One potential explanation for these observations is that Syk mediates CLEC-2 phosphorylation and that Src family kinases regulate Syk. Thus, the proximal events in signalling by hemITAM receptors appear to be distinct to those used by ITAM receptors.

In the present study, we have investigated the molecular events underlying CLEC-2 phosphorylation in mouse platelets using mutant mice deficient in the major platelet Src family kinases (Fyn, Lyn and Src), the tyrosine kinase Syk and the global regulator of Src family kinases in platelets, CD148. The results provide the first demonstration that Syk and not Src family kinases mediate hemITAM phosphorylation and identify Src family kinases regulating platelet activation downstream of Syk.

**EXPERIMENTAL PROCEDURES**

**Reagents.** Rhodocytin was purified from *Calloselasma rhodostoma* venom as previously described (2). Rat anti-mouse CLEC-2 IgG monoclonal antibody (mAb) and rabbit anti-Syk (BR15) polyclonal antibody were from previously described sources (4,26). CLEC-2 IgM antibody was a gift from Dr Caetano Reis e Sousa. Hamster anti–mouse CD148 antibody (8A-1) was generated as described (27). Mouse anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology Inc. pY1217 PLCγ2 antibody was from Cell Signaling Technology. Horseradish peroxidase (HRP) conjugated secondary anti-rabbit IgG and γ-bind protein G Sepharose were from GE Healthcare. Anti-rat Fc antibody was from Abcam. All other reagents were purchased from Sigma-Aldrich.

**Animals.** *fyn*<sup>−/−</sup>, *lyn*<sup>−/−</sup>, *src*<sup>−/−</sup> breeding pairs were from Jackson Laboratories (Bar Harbor, ME). *fyn*<sup>−/−</sup>*lyn*<sup>−/−</sup> double knockout mice were raised from mixed breeding of *fyn*<sup>−/−</sup> and *lyn*<sup>−/−</sup> mice. *CD148*<sup>−/−</sup> mice were generated as previously described (28). Experimental controls were wild-type littermates. No differences were observed in platelet function between the different wild-type controls. Mice were genotyped by polymerase chain reaction (PCR) amplification of mouse tail genomic DNA. Lack of protein expression was confirmed by Western blotting platelet lysates. Mice were housed under specific pathogen-free conditions in accordance with institutional guidelines approved by United Kingdom Home Office and were used between 8 and 12 weeks of age.

**Generation of chimera mice.** Radiation chimeras were generated as previously described (19). Briefly, 8 week-old C57BL/6 mice received two doses of irradiation each of 500 Gy 3 hours apart. The mice were then reconstituted with an intravenous injection of 1-2 x10<sup>6</sup> foetal liver cells obtained from E14-16 days *lyn*<sup>−/−</sup> *src*<sup>−/−</sup>, *lyn*<sup>−/−</sup> *src*<sup>−/−</sup>, *fyn*<sup>−/−</sup> *src*<sup>−/−</sup>, *fyn*<sup>−/−</sup> *src*<sup>−/−</sup>, *syk*<sup>−/−</sup>, *fyn*<sup>−/−</sup>*lyn*<sup>−/−</sup>*src*<sup>−/−</sup>*syk*<sup>−/−</sup>
syk<sup>−/−</sup>, clec-2<sup>−/−</sup> or clec-2<sup>−/−</sup> mouse livers. The genotype of the reconstituting foetal liver cells was confirmed in each case by PCR. Lack of protein expression was confirmed by Western blotting platelet lysates. Reconstituted mice were used for experiments at 8 weeks after irradiation. 

**Preparation of mouse platelets.** Whole blood was drawn from the inferior vena cava into acid citrate dextrose (1/9 vol) from CO<sub>2</sub>-asphyxiated mice following isofluorane anaesthesia. Washed mouse platelets were obtained as previously described (29). Briefly, platelets were obtained by centrifugation using prostaglandin (PG) I<sub>2</sub> to prevent activation during the isolation procedure. Washed platelets were then resuspended in modified HEPEs Tyrode buffer (134 mM NaCl, 2.9 mM KCl, 12 mM NaHCO<sub>3</sub>, 0.34 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 5.5 mM glucose, 1 mM MgCl<sub>2</sub>, 20 mM HEPEs and 5 mM glucose [pH 7.3]) at a density of 2x10<sup>8</sup> platelets/ml. Aggregation was monitored by light transmission using a Born lumi-aggregometer (Chronolog, Havertown, PA).

**Immunoprecipitation studies.** Washed resting or stimulated platelets (300µl at 2x10<sup>8</sup>platelets/ml) were lysed with an equal volume of 2x NP-40 lysis buffer (20mM TrisHCl pH 7.6, 300mM NaCl, 2mM EGTA, 2mM EDTA, 2% NP-40, 2mM PMSF, 5mM Na<sub>2</sub>VO<sub>4</sub>, 10µg/ml leupeptin, 10µg/ml aprotinin, 1µg/ml pepstatin). Cell debris was removed by centrifugation at 15000g for 10 min. An aliquot of whole cell lysate (WCL) was dissolved in reducing Laemmli buffer. Syk immunoprecipitation (IP) was carried out as standard protocol (30,31). Platelet lysate was precleared and 2µl of anti-Syk antibody and 15µl bed volume of protein A-Sepharose were added and each sample rotated at 4°C for 2h. The pellet was washed four times sequentially in lysis buffer before the addition of 20µl of reducing Laemmli sample buffer. Modifications from standard IP protocols were made for mouse CLEC-2 IPs. The amount of CLEC-2 mAb in each sample was normalised to 3µg. 20µl bed volume of γ-bind protein G Sepharose was added to each sample and allowed to capture the antibody for 1 hour with rotation at 4°C. Following four washes with 1x lysis buffer, 20µl of non-reducing Laemmli sample buffer was added. 75% of the sample proteins was separated on a SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking in 2% BSA, the membranes were incubated with 4G10 antibody overnight, washed and then incubated with HRP-conjugated secondary antibody. Immunoprecipitated proteins were visualised by chemiluminescence (ECL; Pierce). For CLEC-2 IPs, the remaining 25% was treated in the same way but incubated with CLEC-2 mAb overnight.

**Platelet surface protein cross-linking.** After platelet stimulation, 1.5mM Sulfo-EGS was added and allowed to incubate at room temperature for 30 min. The reaction was quenched with the addition of Tris-HCl (pH 7.5; 25mM), and allowed to incubate for a further 20 min at room temperature. The samples were lysed with the addition of an equal volume of 2x ice-cold NP-40 lysis buffer.

**Cell lines.** Chinese hamster ovary (CHO) cells were transfected with pcDNA3 containing full length mouse podoplanin using a calcium phosphate transfection method. Stable transfectants were obtained using media containing 1mg/ml genetin (G418) and clonal cell populations were obtained following serial dilutions into 96 well plates. Primary human lymphatic endothelial cells (hLEC) were obtained from Promocell GmbH (Heidelberg, Germany) and cultured in endothelial cell growth medium according to manufacturer instructions. Surface expression of podoplanin was assessed by flow cytometry.

**Statistical Analysis.** Statistical significance was evaluated using 2-tailed Student’s t test. P value <0.05 was considered statistically significant.

**RESULTS**

**Differential role of Src family kinases in platelet activation by a CLEC-2 monoclonal antibody and by rhodocytin.** A rat anti-mouse IgG CLEC-2 monoclonal antibody (CLEC-2 mAb) induces concentration-dependent CLEC-2 phosphorylation and platelet aggregation which is abolished in CLEC-2 deficient mouse platelets (Fig. 1A-C). The time to onset of aggregation decreases with increasing concentrations of antibody (Fig. 1B). Rhodocytin also stimulated concentration dependent phosphorylation with the delay in onset also decreasing with increasing concentrations (Fig. 1B and C). Concentrations of CLEC-2 mAb (10µg/ml) and
rhodocytin (30nM) that were just sufficient to induce maximal CLEC-2 phosphorylation and aggregation were chosen for further experiments, unless stated.

We have previously reported that CLEC-2 mediated activation of human platelets is abolished in the presence of Src family kinase inhibitors (2,17). Consistent with this, we show that the Src family kinase inhibitor PP2 also blocks aggregation of mouse platelets stimulated by CLEC-2 mAb and by rhodocytin (Fig. 2A), confirming a critical role for Src family kinases in CLEC-2 induced platelet activation. Tyrosine phosphorylation of both CLEC-2 and the downstream kinase Syk is completely blocked by PP2 in response to CLEC-2 mAb. Strikingly, however, phosphorylation of CLEC-2 by rhodocytin is not altered in the presence of PP2 (Fig. 2B). The inhibition of aggregation to the snake toxin by PP2 (Fig. 2A) is explained by the partial reduction in Syk phosphorylation and inhibition of tyrosine phosphorylation of residue Y1217 of the downstream protein PLCγ2 (Fig. 2B), a residue shown to be correlated with its activity (32). These results demonstrate that Src family kinases do not mediate phosphorylation of CLEC-2 in mouse platelets stimulated by rhodocytin, but that they play a role in the hemITAM phosphorylation induced by a CLEC-2 mAb.

Distinct roles for Fyn, Lyn and Src in platelet activation by CLEC-2. The major Src family kinases known to be expressed in mouse platelets are Fyn, Lyn and Src, although there is evidence for the presence of other isoforms (33,34). To dissect the role of individual Src family kinases in CLEC-2 mediated signalling, we used mice deficient in the three major platelet Src family kinases, Fyn, Lyn or Src after first establishing that the absence of one Src family kinase did not alter expression of the others (Supplemental Fig. 1). CLEC-2 and αιβ3 surface expression in platelets deficient in each of the Src family kinases were similar to their littermatched controls (not shown).

Aggregation of platelets deficient in Src or Fyn induced by CLEC-2 mAb (3μg/ml and 10μg/ml) or by rhodocytin (10nM and 30nM) were similar to those of littermatched controls (Fig. 3A-C and not shown). In contrast, there was a marked delay in the onset of aggregation to 10μg/ml CLEC-2 mAb in Lyn-deficient platelets, with 50% aggregation being reached at 250 ± 18 sec compared to 140 ± 6 sec for control platelets (Fig. 3A and C). There was however no significant change in the time of onset or magnitude of aggregation induced by rhodocytin (10nM and 30nM) in Lyn-deficient platelets (Fig. 3B and C and not shown).

Phosphorylation of CLEC-2 and Syk in response to the CLEC-2 mAb was abolished at 180 sec in the absence of Lyn in contrast to the robust response observed in control platelets. This is consistent with the delay in platelet activation observed in Lyn-deficient platelets. Phosphorylation of CLEC-2 can however be seen following full aggregation of Lyn-deficient platelets (not shown). In contrast, a small reduction in CLEC-2 and Syk tyrosine phosphorylation was observed in response to the CLEC-2 mAb in Lyn-deficient platelets in some but not all experiments, although this did not reach statistical significance (Fig. 3D). There was no change in phosphorylation of CLEC-2 following CLEC-2 mAb stimulation in the absence of Src (not shown). Consistent with the results obtained with PP2 (Fig. 2B), phosphorylation of CLEC-2 induced by rhodocytin was not altered in the absence of Src, Lyn or Lyn (Fig. 3B and C and not shown). These data demonstrate that Lyn is the major kinase involved in CLEC-2 platelet activation following CLEC-2 mAb ligation.

To investigate if platelet activation is further delayed in the absence of more than one Src family kinases, we generated mice double deficient in Fyn/Lyn, Lyn/Src and Fyn/Src. Mice double deficient in Fyn/Lyn were generated by breeding of heterozygotes, while the other two sets of double knockouts were generated as radiation chimeras as described in the methods. As shown in Fig. 4A, the loss of two of the major platelet Src family kinases did not alter expression of the remaining major Src family kinases. Interestingly, a further delay in the onset of aggregation in platelets from mouse double deficient in Fyn/Lyn or Lyn/Src stimulated with CLEC-2 mAb was observed (Fig. 4B and D) when compared to Lyn-deficient platelets (Fig. 3A and C). In contrast, platelets double-deficient in Fyn/Src exhibited only a very minor delay in onset of platelet aggregation (Fig. 4B and D). CLEC-2 phosphorylation was abolished in platelets deficient in Fyn/Lyn and Lyn/Src at 180 sec (not shown), as was the case
for Lyn-deficient platelets (Fig. 3D), but was unchanged in platelets deficient in Fyn/Src (Fig. 4E and not shown). These data demonstrate that Fyn and Src contribute to platelet activation by the CLEC-2 mAb in the absence of Lyn but that neither plays a significant role in the presence of Lyn. In contrast, the response to rhodocytin was not altered in mouse platelets double deficient in Fyn/Lyn, Lyn/Src or Fyn/Src. Given that platelet activation by both CLEC-2 mAb and by rhodocytin is abolished in the presence of the broad spectrum inhibitor PP2, these results demonstrate that there are other Src family kinases that contribute to platelet activation downstream of CLEC-2 and that there may be considerable redundancy between Src family kinases in mediating this effect.

**CD148 is dispensable for platelet activation by CLEC-2 ligation.** Src family kinases are regulated by phosphorylation on excitatory and inhibitory tyrosines. Recently, we have demonstrated a reciprocal increase in phosphorylation of the inhibitory site and a decrease in phosphorylation of the activatory site of all Src family kinases in platelets deficient in the receptor-like protein tyrosine phosphatase CD148. This has a net effect of greatly reducing the activity of Src family kinases leading to a marked inhibition of platelet activation by the ITAM receptor GPVI and by integrin αIIbβ3 (35). In contrast, activation of mouse platelets by CLEC-2 mAb (3μg/ml and 10μg/ml) or by rhodocytin (10nM and 30nM) is not significantly altered in the absence of CD148 (Fig. 5 and not shown) demonstrating that Src family kinase activity is not rate-limiting for platelet activation by either of the CLEC-2 receptor ligands. This therefore provides further evidence for a fundamental difference in the proximal events underlying platelet activation by CLEC-2 to those of the platelet ITAM receptor GPVI.

**The tyrosine kinase Syk underlies phosphorylation of CLEC-2.** The role of the tyrosine kinase Syk in mediating CLEC-2 phosphorylation and platelet aggregation was investigated using Syk-deficient chimeric mice (Fig. 6A). Phosphorylation of CLEC-2 and aggregation of platelets induced by the CLEC-2 mAb or by rhodocytin were completely blocked in Syk-deficient platelets (Fig. 6B and C) demonstrating that Syk is essential for phosphorylation of the CLEC-2 hemITAM. Thus, the role of Lyn in mediating phosphorylation of CLEC-2 in response to the CLEC-2 mAb may be mediated via phosphorylation of Syk (Fig. 3D), which is regulated by both autophosphorylation and phosphorylation by Src family kinases (36).

**Role of Src family kinases in CLEC-2 signalling induced by podoplanin-expressing cell lines and other multivalent ligands.** The differential role of Src family kinases in mediating phosphorylation of CLEC-2 by CLEC-2 mAb and by rhodocytin could reflect the degree of crosslinking of CLEC-2 (37,38). Thus, the tetrameric rhodocytin (37) is able to cluster CLEC-2 to a much greater degree that the dimeric IgG mAb. This is illustrated in platelets treated with the chemical crosslinker SulfoEGS (Fig. 7A). Rhodocytin induced a much greater reduction in the monomeric form of CLEC-2 relative to the CLEC-2 mAb, whereas neither ligand induced clustering of the membrane tyrosine phosphatase CD148 (not shown) thereby demonstrating that this effect was not due to non-specific crosslinking.

In light of this result, we asked whether crosslinking of the CLEC-2 mAb using an anti-rat Fc antibody would induce a more powerful degree of platelet activation and whether this would reduce the dependency on Lyn. The addition of a crosslinking anti-rat Fc antibody to a concentration of CLEC-2 mAb that induced shape change converted the response to a rapid aggregation. Interestingly, a delay in aggregation was still observed in the absence of Lyn following crosslinking by an anti-rat Fc antibody (Fig. 7B), and also in response to a novel pentavalent IgM anti-CLEC-2 antibody (Fig. 7C), although in both cases this was less than in response to the IgG antibody (Fig. 3A). Aggregation to the IgM antibody was abolished in the presence of the Src family kinase inhibitor, PP2 (Fig. 7C).

In order to investigate the dependency of platelet aggregation to podoplanin on Lyn, we used two podoplanin-expressing cell lines, namely mouse podoplanin-transfected CHO cells and human lymphatic endothelial cells (hLEC). Neither cell line induced activation of platelets deficient in CLEC-2 confirming that activation was via CLEC-2 (not shown). The two cell lines induce rapid activation of mouse platelets which was blocked in the presence of PP2, confirming an essential role for Src family kinases in mediating aggregation.

In conclusion, these findings have important implications for our understanding of platelet activation and suggest that Src family kinases play a significant role in this process. Further study is required to determine the specific role of each Src family kinase in mediating platelet activation and the molecular mechanisms by which they contribute to this process.
kinases in mediating activation. However, the response to either of the cell lines was not altered in the absence of Lyn (Fig. 7D).

These data show that the dependency on Lyn is agonist dependent, with the dependency being reduced or abolished for ligands that induce oligomerisation of CLEC-2.

**DISCUSSION**

The present study provides an unequivocal demonstration that the tyrosine kinase Syk mediates CLEC-2 phosphorylation following stimulation by rhodocytin and that this is independent of Src family kinase activation. Phosphorylation of CLEC-2 in response to the snake toxin is unaltered in platelets deficient or double-deficient in the major platelet Src family kinases, namely Fyn, Lyn and Src, or in the global regulator of Src kinases, the membrane tyrosine phosphatase, CD148. The ability of broad spectrum Src family kinase inhibitors such as PP2 to block platelet aggregation by rhodocytin is explained by partial or complete inhibition of phosphorylation of Syk and downstream proteins such as PLCγ2 respectively. The fact that platelet aggregation by rhodocytin is not altered in mice deficient or double-deficient in the above Src family kinases demonstrates that one or more other Src family kinases must play a role in mediating aggregation or that there is considerable redundancy between the Src family kinases in mediating activation. These results reveal a new pathway of platelet activation by the hemITAM receptor CLEC-2 which is distinct from that mediated by the ITAM receptor, GPVI. CLEC-2 signals through the sequential action of Syk and Src family kinases, while GPVI induces sequential activation of Src family and Syk kinases, with Src family kinases playing a further role downstream of Syk.

The present study also shows that the role of individual members of Src family kinases in CLEC-2 signalling is ligand dependent. Thus, phosphorylation of CLEC-2 in response to the IgG CLEC-2 antibody is markedly delayed in the absence of Lyn and further delayed in the combined absence of Fyn/Lyn or Src/Lyn. On the other hand, phosphorylation is not altered in mice double deficient in Fyn/Src demonstrating that neither of these Src family kinases plays a critical role in the presence of Lyn. The delay in activation seen in the absence of Lyn is likely to be mediated by loss of phosphorylation of Syk, which is regulated by Src family kinases and regulates hemITAM phosphorylation. In contrast, activation of platelets by rhodocytin is not altered in mice that are either single or double deficient in Fyn, Lyn or Src whereas the response is abolished in the absence of Syk. Platelet activation by two podoplanin-expressing cell lines resembles the mechanism underlying activation by rhodocytin in that activation is also independent of Lyn but blocked by the Src kinase inhibitor PP2.

The differential role of Lyn and other Src family kinases in mediating platelet activation by rhodocytin, podoplanin-expressed cell lines and the CLEC-2 mAb may be related to the degree of clustering of CLEC-2. The antibody to CLEC-2 is an IgG and thus induces dimerisation of the receptor generating a weak intracellular signal. The further clustering of CLEC-2 with a secondary antibody induces rapid and powerful activation. In contrast, the tetrameric rhodocytin and polymeric podoplanin-expressing cells induce a much greater degree of receptor clustering. The differential dependency on Lyn may be also due to spatial aspects of the ligand–CLEC-2 interaction, as this may influence the degree of Syk recruitment.

We have recently reported that phosphorylation of CLEC-2 in human platelets in response to rhodocytin is abolished in the presence of inhibitors of Src or Syk kinases (20). It was unclear however from this study which of these kinases mediated receptor phosphorylation. The demonstration that phosphorylation of CLEC-2 in mouse platelets by rhodocytin is abolished in the absence of Syk but not in the presence of the Src kinase inhibitor PP2 demonstrates that the hemITAM phosphorylation is mediated by Syk. The explanation for the differing dependency of CLEC-2 phosphorylation on Src family kinases in the two species could reflect, for example, a difference in the expression levels of CLEC-2 or of Src family and Syk kinases.

An important question that emerges from this work is how receptor clustering of CLEC-2 brings about activation of Syk. One explanation is that it is brought about as a result of mass action as modelled by Cooper & Qian (39). The key feature of this model is that a constitutively active kinase is associated with the receptor in
the absence of agonist stimulation. Consistent with this, we have previously reported constitutive activation of Syk in non-stimulated platelets and transfected cell lines (40). Thus, although there may be other components that regulate Src family and Syk kinases upon ligand engagement, this model predicts that clustering alone is sufficient to mediate receptor phosphorylation. The reason why phosphorylation of CLEC-2 is mediated by Syk rather than by one or more other Src family kinases, as is the case for GPVI-FcRγ-chain ITAM, may be related to their juxtaposition in the cell. For example, we have recently shown that Syk is localised to plasma membrane in platelets by binding to filamin A and that loss of filamin A markedly impairs signalling by the tyrosine kinase (41).

In summary, this study demonstrates for the first time that Syk mediates phosphorylation of the CLEC-2 hemITAM and that Src family kinases play a critical role further downstream through regulation of Syk and other effector proteins including PLCγ2. It is now of considerable interest to establish whether this pathway is unique to CLEC-2 or whether it is also used by other hemITAM receptors.
REFERENCES

FOOTNOTES

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Abbreviations: ITAM, immunoreceptor tyrosine-based activation motif; GP, glycoprotein; Ig, immunoglobulin; mAb, monoclonal antibody; ADP, adenosine diphosphate; TX, tromboxane; LAT, linker for activation of T cells; SLP-76, Src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa; PI, phosphoinositide; PLC, phospholipase; HRP, horseradish peroxidase; PCR, polymerase chain reaction; PG, prostaglandin; FITC, fluorescein isothiocyanate; SDS, sodium dodecyl sulfate; IP, immunoprecipitation; PVDF, polyvinylidene difluoride; CHO, Chinese hamster ovary; WCL, whole cell lysate.

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**FIGURE LEGENDS**

**Fig. 1.** Dose response curves in response to CLEC-2 mAb and rhodocytin. (A) CLEC-2 deficient and littermatched wild-type washed platelets (2x10^8 platelets/ml) were stimulated with 10μg/ml CLEC-2 mAb. (B) Murine washed platelets (2x10^8 platelets/ml) were stimulated with increasing concentrations of CLEC-2 mAb or rhodocytin. Platelet aggregation was measured as a change in light transmission, using a lumi-aggregometer. Representative aggregation traces are shown. Addition of the agonist is indicated by an arrowhead. Data represent the means and standard error of at least 8 independent experiments at 3 min of stimulation. (C) CLEC-2 was immunoprecipitated and immunoprecipitates were immunoblotted with an anti-phosphotyrosine antibody and anti-CLEC-2 mAb as described in the Methods. The percentage of tyrosine phosphorylation was measured at 3 min of stimulation and represented as means and standard error of 4 independent experiments.

**Fig. 2.** Effect of Src family kinase inhibition on murine platelet in response to CLEC-2 mAb and rhodocytin. (A) Murine washed platelets (2x10^8 platelets/ml) were incubated for 10 min with or without 20μM PP2 and then stimulated with 10μg/ml CLEC-2 mAb or 30nM rhodocytin. Platelet aggregation was measured as a change in light transmission, using a lumi-aggregometer. Addition of the agonist is indicated by an arrowhead. Representative aggregation traces from 3-6 independent experiments are shown. (B) CLEC-2 and Syk were immunoprecipitated and immunoprecipitates were immunoblotted with an anti-phosphotyrosine antibody. CLEC-2 immunoprecipitates were also immunoblotted with anti-CLEC-2 mAb as described in the Methods. Whole cell lysates (WCL) were probed with pY1217 PLCγ2 antibody and reprobed with PLCγ2 antibodies. The percentage of tyrosine phosphorylation was measured at 3 min of stimulation and represented as means and standard error of 3-6 independent experiments. Significant difference (* P < 0.05 and ** P < 0.005) according to 2-tailed Student’s t test.

**Fig. 3.** Lyn plays a critical role in CLEC-2 mediated signalling by antibody ligation but is not involved in response to rhodocytin. Fyn or Lyn or Src deficient washed platelets and their littermatched wild-type platelets (2x10^8 platelets/ml) were stimulated with (A) 10μg/ml CLEC-2 mAb or (B) 30nM rhodocytin. Platelet aggregation was measured as a change in light transmission, using a lumi-aggregometer. Representative aggregation traces are shown. Addition of the agonist is indicated by an arrowhead. (C) Data represent the means of the time to get 50% of aggregation and standard error of 3-6 independent experiments. Significant difference (**) P < 0.005) versus wild-type, according to 2-tailed Student’s t test. (D) Fyn or Lyn deficient washed platelets and their littermatched wild-type platelets (2x10^8 platelets/ml) were stimulated with 10μg/ml CLEC-2 mAb or 30nM rhodocytin for 3 min. CLEC-2 and Syk were immunoprecipitated and immunoprecipitates were immunoblotted with an anti-phosphotyrosine antibody. CLEC-2 immunoprecipitates were also immunoblotted with anti-CLEC-2 mAb as described in Methods. The percentage of tyrosine phosphorylation was measured at 3 min of stimulation and represented as means and standard error of 3-6 independent experiments. Significant difference (** P < 0.005) versus wild-type, according to 2-tailed Student’s t test.

**Fig. 4.** (A) Whole cell lysates prepared from deficient platelets and their littermatched wild-type platelets were immunoblotted with anti-Lyn, anti-Fyn and anti-Src and anti-tubulin antibodies. Washed platelets deficient in Fyn/Lyn, Fyn/Src and Lyn/Src (2x10^8 platelets/ml) were stimulated with (B) 10μg/ml CLEC-2 mAb or (C) 30nM rhodocytin. Platelet aggregation was measured as a change in light transmission, using a lumi-aggregometer. Representative aggregation traces from 3 independent experiments are shown. Addition of the agonist is indicated by an arrowhead. (D) Data represent the means of the time to get 50% of aggregation and standard error of 3 independent experiments. Significant difference (**) P < 0.005) versus wild-type, according to 2-tailed Student’s t test. (E) Fyn/Src deficient washed platelets and their littermatched wild-type platelets (2x10^8 platelets/ml) were stimulated with 10μg/ml CLEC-2 mAb for 3 min. CLEC-2 was immunoprecipitated and immunoprecipitates were immunoblotted with an anti-phosphotyrosine antibody. CLEC-2
immunoprecipitates were also immunoblotted with anti-CLEC-2 antibody as described in Methods. Representative data from 2 independent experiments are shown.

**Fig. 5.** CD148 deficiency does not impair CLEC-2 signalling. (A) CD148-deficient washed platelets and their littermatched wild-type platelets (2x10^8 platelets/ml) were stimulated with 10μg/ml CLEC-2 mAb or 30nM rhodocytin. Platelet aggregation was measured as a change in light transmission, using a lumi-aggregometer. Representative aggregation traces of 4 independent experiments are shown. Addition of the agonist is indicated by an arrowhead. (B) CD148-deficient washed platelets and their littermatched wild-type platelets (2x10^8 platelets/ml) were stimulated with 10μg/ml CLEC-2 mAb or 30nM rhodocytin for 3 min. CLEC-2 and Syk were immunoprecipitated and immunoprecipitates were immunoblotted with an anti-phosphotyrosine antibody. CLEC-2 immunoprecipitates were also immunoblotted with anti-CLEC-2 mAb as described in Methods. The percentage of tyrosine phosphorylation was measured at 3 min of stimulation and represented as means and standard error of 4 independent experiments.

**Fig. 6.** Syk is a key regulator of CLEC-2 signalling. (A) Whole cell lysates prepared from wild-type and Syk-deficient platelets were immunoblotted with anti-Syk and anti-tubulin antibodies. (B) Syk-deficient washed platelets and their littermatched control platelets (2x10^8 platelets/ml) were stimulated with 10μg/ml CLEC-2 mAb. Platelet aggregation was measured as a change in light transmission, using a lumi-aggregometer. Representative aggregation traces of 3 independent experiments are shown. Addition of the agonist is indicated by an arrowhead. (C) After 3 min of stimulation with 10μg/ml CLEC-2 mAb, CLEC-2 was immunoprecipitated from Syk-deficient platelets and their littermatched control platelets (2x10^8 platelets/ml) lysates, and immunoblotted with an anti-phosphotyrosine antibody and anti-CLEC-2 mAb as described in Methods. Representative blot of 3 independent experiments is shown.

**Fig. 7.** Role of Src family kinases in CLEC-2 mediated aggregation following multivalent ligands and podoplanin ligation. (A) Washed platelets (2 x 10^8/ml) under basal, CLEC-2 mAb (10μg/ml) or rhodocytin (30nM)-stimulated conditions had their surface proteins cross-linked with the addition of 1.5mM Sulfo-EGS cross-linking reagent, with a linker length of 1.6 nm (16 Å) as described in Methods. Monomeric CLEC-2 has been quantified and data represent the mean and standard error of 3 independent experiments. Significant difference (***P < 0.005) versus wild-type, according to 2-tailed Student’s t test. mAb, CLEC-2 mAb; Rh, rhodocytin (B) Lyn-deficient washed platelets and their littermatched control platelets (2x10^8 platelets/ml) were stimulated with 2μg/ml CLEC-2 mAb for 2 min and then with 10μg/ml anti-rat Fc antibody for 5 min. Representative aggregation traces of 3 independent experiments are shown. Additions of the agonists are indicated by arrowheads. (C) Lyn-deficient washed platelets and their littermatched wild-type platelets (2x10^8 platelets/ml) incubated with or without 20μM PP2 were stimulated with IgM CLEC-2 antibody. Data represent the means of the time to get 50% of aggregation and standard error of 3-6 independent experiments. Significant difference (*P < 0.05) versus wild-type, according to 2-tailed Student’s t test. (D) Lyn-deficient platelets and their littermatched control platelets (2x10^8 platelets/ml) incubated with or without 20μM PP2 were stimulated in plasma with 1.5x10^5 lymphatic endothelial cells (hLEC) or mouse podoplanin expressing CHO cells (mPodoCHO). Platelet aggregation was measured as a change in light transmission, using a lumi-aggregometer. Representative aggregation traces of 3 independent experiments are shown. Addition of the agonist is indicated by an arrowhead.
Fig. 1
Fig. 3

A. CLEC-2 mAb

WT

fyn⁻

lyn⁻

src⁻

B. Rhodocytin

WT

fyn⁻

lyn⁻

src⁻

C. Time to 50% aggregation (Sec)

CLEC-2 mAb

Rhodocytin

**

WT

src⁻

fyn⁻

lyn⁻

D. 30nM Rhodocytin

10µg/ml CLEC-2 mAb

IP: CLEC-2

WB: 4G10

IP: Syk

WB: CLEC-2

WB: 4G10

Tyr Phosphorylation (%)

**
Fig. 6

A

WT  syk<sup>−</sup>

WB: Syk

WB: Tubulin

B

CLEC-2 mAb

WT

syk<sup>−</sup>

C

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>syk&lt;sup&gt;−&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>30mM Rhodocytin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>10µg/ml CLEC-2 mAb</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

IP: CLEC-2

WB: 4G10

WB: CLEC-2
Fig. 7

A: Fold change over basal

B: WT, lyn^-;

C: CLEC-2 IgM Ab, lyn^-;

D: hLEC, lyn^-;

WT, lyn^-;
Syk dependent phosphorylation of CLEC-2: a novel mechanism of hemitam signalling
Sonia Séverin, Alice Y. Pollitt, Leyre Navarro-Nuñez, Craig A. Nash, Diego Mourão-Sá, Johannes A. Eble, Yotis A. Senis and Steve P. Watson

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