G6b-B Inhibits Constitutive and Agonist-induced Signaling by Glycoprotein VI and CLEC-2*[S]

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Platelets play an essential role in wound healing by forming thrombi that plug holes in the walls of damaged blood vessels. To achieve this, platelets express a diverse array of cell surface receptors and signaling proteins that induce rapid platelet activation. In this study we show that two platelet glycoprotein receptors that signal via an immunoreceptor tyrosine-based activation motif (ITAM) or an ITAM-like domain, namely the collagen receptor complex glycoprotein VI (GPVI)-FcR γ-chain and the C-type lectin-like receptor 2 (CLEC-2), respectively, support constitutive (*i.e.* agonist-independent) signaling in a cell line model using a nuclear factor of activated T-cells (NFAT) transcriptional reporter assay that can detect low level activation of phospholipase Cγ (PLCγ). Constitutive and agonist signaling by both receptors is dependent on Src and Syk family kinases, and is inhibited by G6b-B, a platelet immunoglobulin receptor that has two immunoreceptor tyrosine-based inhibitory motifs in its cytosolic tail. Mutation of the conserved tyrosines in the two immunoreceptor tyrosine-based inhibitory motifs prevents the inhibitory action of G6b-B. Interestingly, the inhibitory activity of G6b-B is independent of the Src homology 2 (SH2)-domain containing tyrosine phosphatases, SHP1 and SHP2, and the inositol 5′-phosphatase, SHIP. Constitutive signaling via Src and Syk tyrosine kinases is observed in platelets and is associated with tyrosine phosphorylation of GPVI-FcR γ-chain and CLEC-2. We speculate that inhibition of constitutive signaling through Src and Syk tyrosine kinases by G6b-B may help to prevent unwanted platelet activation.

The GPVI-FcR γ-chain complex is the major signaling receptor for collagen on platelets and megakaryocytes (1, 2). Cross-linking of GPVI leads to Src-dependent phosphorylation of a tandem YXXL sequence on the FcR γ-chain known as an immunoreceptor tyrosine-based activation motif (ITAM).[4] In turn, this leads to recruitment and activation of the tyrosine kinase Syk through its tandem SH2 domains. Syk initiates a signaling cascade that generates a linker for activation of T (LAT) cell-dependent signalosome, which mediates activation of phospholipase Cγ2 (PLCγ-2) (3, 4). This pathway initiates a rise in intracellular Ca^{2+} and activation of protein kinase C leading to platelet aggregation.

CLEC-2 is a 32-kDa C-type lectin-like receptor that functions as a platelet receptor for the snake venom toxin rhodocytin and the lymphatic endothelial marker, podoplanin (5–7). Like glycoprotein (GP) VI, CLEC-2 signals via sequential activation of Src and Syk tyrosine kinases leading to activation of PLCγ2 (5). The regulation of PLCγ2 by CLEC-2 is distinct from that of GPVI in that it uses a single YXXL sequence and is only partially dependent on the adapter SLP-76 (5, 8).

G6b is a recently identified member of the immunoglobulin superfamily that exists in several splice variants (9). G6b-B is the only one of these variants to contain both a transmembrane region and two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that support binding to the two SH2 domain-containing protein tyrosine phosphatases, SHP1 and SHP2 (9, 10). ITIMs are defined by the consensus sequence (L/I/V/S)-X-Y-X-X-(L/V) and are commonly found in pairs separated by 15 to 30 amino acid residues (11, 12). The second ITIM in G6b-B is located ∼20 amino acids downstream of the first ITIM and has a slightly different sequence to the above, T$XY XX$V. G6b-B is the third ITIM-containing protein to be identified on platelets (10, 13). The other two platelet ITIM receptors are platelet/endothelial cell adhesion molecule-1 (PECAM-1) and TREM-like transcript-1 (14–16).

ITIM-containing receptors were originally identified by their ability to inhibit signaling by ITAM receptors, as demonstrated by the selective inhibition of the B-cell receptor when cross-linked by surface immunoglobulin to Fcγ receptor IIb (FcγRIIb) (17, 18). However, it is now recognized that ITIM-containing receptors can also generate stimulatory signals or

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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[4] The abbreviations used are: ITAM, immunoreceptor tyrosine-based activation motif; PLCγ2, phospholipase γ2; CLEC-2, C-type lectin-like receptor 2; ITIM, immunoreceptor tyrosine-based inhibitory motif; SH2, Src homology domain 2; PECAM-1, platelet/endothelial cell adhesion molecule-1; NFAT, nuclear factor of activated T-cells; mAb, monoclonal antibody; WT, wild type; SHIP, SH2-domain-containing inositol 5′-phosphatase; GP, glycoprotein; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

inhibit activation by G protein-coupled receptors. For example, PECAM-1 has been reported to induce integrin activation in T cells (19), whereas it causes mild inhibition of platelet activation by the ITAM and ITAM-like receptors, GPVI and CLEC-2, and by the G protein-coupled receptor agonist, thrombin (20–23). The latter action is similar to that of G6b-B, which upon cross-linking by a specific antibody inhibits activation of platelets by the GPVI-specific agonist, collagen-related peptide, and the G protein-coupled receptor agonist ADP (13). In contrast, the ITIM receptor, TREM-1, which is present on platelet intracellular granules, is translocated to the surface of activated platelets and reinforces platelet activation (16).

In this study we have used a sensitive NFAT reporter assay that monitors activation of PLCγ in DT40 B cells to investigate the effect of G6b-B on signaling by the GPVI-FcR γ-chain complex and CLEC-2. The NFAT reporter assay monitors weak, sustained signaling events and gives robust detection of activation of GPVI by collagen and of CLEC-2 by rhodocytin and podoplanin (7, 8, 24). The availability of variants of the DT40 cell line deficient in key signaling proteins allows dissection of the signaling pathways used by the above receptors.

Here we show that expression of the GPVI-FcR γ-chain complex and CLEC-2 in DT40 cells leads to the generation of both constitutive and agonist-induced signals that are inhibited by G6b-B. This effect is dependent on the two ITIMs in the cytosolic tail of G6b-B, although it is independent of the two SH2-domain containing tyrosine phosphatases, SHP1 and SHP2, and of the inositol lipid 5′-phosphatase, SHIP. Significantly, we also provide evidence for constitutive signaling through Src and Syk-dependent kinases in platelets, thereby raising the possibility that inhibition of this by G6b-B could help to prevent unwanted platelet activation in the vasculature.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-human GPVI monoclonal antibody (mAb) 204-11 has been described previously (25). Anti-Syk polyclonal Ab (pAb) (26) was kindly provided by J. B. Bolen (DNAX, CA). Anti-phospho-Syk (Tyr$^{525/526}$) pAb and anti-Myc mAb were purchased from Cell Signaling Technology (New England Biolabs UK Ltd., Herts, UK). T7-Tag mAb was purchased from Novagen (Nottingham, UK). Anti-phosphotyrosine mAb 4G10, anti-FcR γ-chain pAb, and normal rabbit IgG were purchased from Upstate Biotechnology (Milton Keynes, UK). Anti-human CLEC-2 mAb was purchased from R & D Systems Inc. (Minneapolis, MN). Anti-PECAM-1 mAb AB468 was from Autogen-Bioclear (Wiltshire, UK). Horseradish peroxidase-conjugated donkey anti-rabbit secondary Ab and enhanced chemiluminescence reagents (ECL) were purchased from Amersham Biosciences. Mouse IgG1 monoclonal was purchased from Abcam (Cambridge, UK). Fluorescein isothiocyanate-conjugated anti-mouse IgG secondary antibody was purchased from Sigma. Collagen was obtained from Nycomed Austria GmbH (Linz, Austria). Rhodocytin was purified from the venom of *Calloselasma rhodostoma* (27). The Src kinase inhibitors used were, PP1, purchased from BioSource Europe (Nivelles, Belgium); PP2, purchased from Calbiochem (Nottingham, UK); and PD0173952, a gift from Pfizer Global Research and Development (Ann Arbor, MI). The Syk kinase

inhibitor, R406, was a kind gift of Dr. D. Simmons (Cellzome UK Ltd., Cambridge). FcR γ-chain "knock-out" mice were bred as heterozygotes as described (28). 10 mM Pervanadate was freshly prepared on the day for use by mixing sodium orthovanadate and hydrogen peroxide in phosphate-buffered saline to final concentrations 10 mM, then left for 5 min at room temperature and kept on ice. Other reagents were from previously described sources (6, 8, 24).

Constructs—The human pRc/GPVI, pEF6/FcR γ-chain, pEF6/CLEC-2, and mutant CLEC-2 (Y7F) expression plasmids have been previously described (8). The human pcDNA3/-G6b-B (kindly given by Prof R. D. Campbell, Oxford, UK) has been described (9) and the human pcDNA3/PECAM-1 (kindly given by C. D. Buckley, Birmingham, UK) has also been described (29). The NFAT luciferase reporter containing three copies of the distal NFAT site from the interleukin-2 promoter has been described (30).

Making Myc-tagged FcR γ-Chain—The c-Myc epitope tag (EQKLISEEDL) was fused at the amino terminus of FcR γ-chain by using pEF6/FcR γ-chain as a template and the primers: forward (5′-GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG CTG GGA GAG CCT CAG CTC-3′) and reverse (5′-CAG ATC CTC TTC TGA GAT GAG TTT TTG TTC GGC CGC TGC TTG TTC AAC-3′), and subcloned into pEF6.

Site-directed Mutagenesis of G6b-B—Site-directed mutagenesis of G6b-B was performed by a QuikChange® Site-directed Mutagenesis Kit (Stratagene, Cambridge, UK). The primers G6b-B-Y211F-forward (5′-CCG AGC CTG CTC TTT GCG GAT CTG GAC-3′) and G6b-B-Y211F-reverse (5′-GTC CAG ATC CGC AAA GAG CAG GCT CGG-3′) were used for tyrosine to phenylalanine mutation in G6b-B (Y211F) using wild-type human pcDNA/G6b-B as a template. The primers G6b-B-Y237F-forward (5′-GAT GCC TCC ACC ATC TTT GCA GTT GTA GTT TG-3′) and G6b-B-Y237F-reverse (5′-CAA ACT ACA ACT GCA AAG ATG GTG GAG GCA TC-3′) were used for tyrosine to phenylalanine mutation in G6b-B (Y237F) using wild-type human pcDNA/G6b-B as a template and they were also used for tyrosine to phenylalanine mutation in G6b-B (Y211F/Y237F) using mutant pcDNA/G6b-B (Y211F) as a template. All sequences were verified by sequencing.

Cell Culture—Wild-type (WT), Syk-deficient (31), SHP1 and SHP2 double-deficient (32) (kindly donated by L. Meyaard, Utrecht, The Netherlands), SHIP-deficient (33) (kindly donated by D. K. Newman, Milwaukee, WI) DT40 chicken B cells were grown in RPMI supplemented with 10% fetal bovine serum, 1% chicken serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol, and 20 mM glutamine.

Luciferase Assay—The NFAT reporter assay was performed as described (8, 24). The indicated amount of DNA of each construct and 15 μg of NFAT-luciferase reporter construct were transfected by electroporation at 350 V and 500 microfarads into 2×10^7 cells of WT, Syk-deficient, and SHP1/SHP2-deficient DT40 cells. Twenty hours after transfection, live cells were counted by trypan blue exclusion, and samples divided for luciferase assay (2×10^6 cells/ml), flow cytometry (5×10^5 cells/sample), and Western blotting (1×10^6 cells/sample). Collagen was used at 10 μg/ml and rhodocytin was used at 50

nM. Luciferase activity was measured with a Centro LB960 microplate luminometer (Berthold Technologies, Germany). All results were compared with basal in mock-transfected cells.

Flow Cytometry—Cell surface expression of transfected cells was analyzed by flow cytometry using 1 μg/ml, GPVI mAb, PECAM-1 mAb, and Myc mAb to detect CLEC-2 and FcR γ-chain, T7-Tag mAb to detect G6b-B, or mouse IgG followed by staining with 4 μg/ml fluorescein isothiocyanate-conjugated anti-mouse IgG secondary antibody, and assessed on a FACScalibur (Becton Dickinson, San Jose, CA). Data were analyzed using CellQuest software.

Human and Mouse Platelets—Washed preparations of human and mouse platelets were prepared as previously described (5, 8). Platelets were resuspended in a modified Tyrodes-HEPES buffer at concentrations of 4×10^8/ml (human) or 2×10^8/ml (mouse). Platelets were prewarmed to 37 °C for 5 min and incubated with inhibitors or solvent controls for up to 10 min.

Immunoprecipitation and Western Blotting—Transfected cells (1×10^8/ml) were incubated for 30 min in RPMI at 37 °C before stimulating. After stimulation, transfected cells or platelets were lysed with ice-cold $2\times$ lysis buffer (2% Triton X-100, 2% dodecyl maltoside, 4 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 2 μg/ml pepstatin, 10 mM sodium orthovanadate, pH 7.5) and insoluble material was removed by centrifugation. For immunoprecipitation, lysates were precleared with protein A (G)-Sepharose beads for 30 min at 4 °C and mixed with 2 μg of the indicated antibodies and protein A-Sepharose beads (protein G-Sepharose). The mixture was rotated for 2 h at 4 °C. Whole cell lysates or immunoprecipitated lysates were added to $2\times$ Laemmli sample buffer. Samples were separated by SDS-PAGE on 10 or 4–12% BisTris gels (Invitrogen) and transferred to polyvinylidene difluoride membrane. Western blotting was carried out as described previously (24).

Statistical Analysis—Experiments were performed on at least three occasions and results are shown as mean ± S.E. with the exception of the representative Western blots and flow cytometry histograms. Statistical significance was determined using Student's *t* test.

RESULTS

Constitutive Signaling of GPVI-FcR γ-Chain in DT40 Cells— We set out to extend our previous characterization of the GPVI-FcR γ-chain signaling pathway in the DT40 B cell line (24) by testing whether activation of GPVI by collagen is dependent on its associated FcR γ-chain. Activation was monitored in the absence and presence of collagen and compared with mock-transfected cells that had been allowed to settle on fibronectin using a NFAT-luciferase reporter assay. Adhesion to fibronectin alone did not induce NFAT activation (not shown). As anticipated, collagen stimulated NFAT activation in DT40 cells expressing both GPVI and an NH$_2$-terminal Myctagged version of FcR γ-chain, whereas it had no effect when either subunit was transfected on its own (Fig. 1*A* and not shown). The Myc-tagged version of FcR γ-chain supports a similar level of activation to that of the wild-type protein (not shown) and has the advantage that it can be used to monitor surface expression by flow cytometry, which is not altered by

expression of GPVI (Fig. 1*A*, *panel ii*). In contrast, GPVI was not expressed on the surface of DT40 cells in the absence of the FcR γ-chain (not shown), as is the case for platelets (34). These results are consistent with a model in which cross-linking of GPVI by collagen leads to tyrosine phosphorylation of the FcR γ-chain and activation of the tyrosine kinase Syk.

These studies further revealed the unexpected observation that expression of the Myc-tagged or wild-type FcR γ-chain caused a significant increase in luciferase activity that approached almost 10% of the response to collagen (Fig. 1, *A, panel i* and *B, panel i*). Furthermore, the constitutive signal from the FcR γ-chain was not altered by co-expression with GPVI (Fig. 1*A*, *panel i*). Thus the increase in luciferase activity that was observed in the absence of a stimulatory agonist reflects signaling by the ITAM-containing protein.

To investigate constitutive signaling in further detail, DT40 cells were transfected with varying concentrations of the FcR γ-chain plasmid and the level of NFAT activation monitored. Increasing levels of transfection of FcR γ-chain led to a corresponding increase in both NFAT activation (Fig. 1*B*, *panel i*) and FcR γ-chain expression as shown by Western blotting (Fig. 1*B*, *panel ii*). Furthermore, Western blotting of GPVI-FcR γ-chain-transfected cells demonstrated an increase in tyrosine phosphorylation of a band of 72 kDa that comigrates with Syk, along with constitutive tyrosine phosphorylation of FcR γ-chain (Fig. 1*C*, *panel i*). Confirmation that the 72-kDa protein corresponds to Syk was confirmed using a phosphospecific antibody (phospho-Syk Tyr$^{525/526}$) that binds to the activated tyrosine kinase (not shown). Tyrosine phosphorylation of Syk and FcR γ-chain were inhibited in the presence of the Src family kinase inhibitor PD0173952 (Fig. 1*C*, *panel i*, and not shown). These results demonstrate that the FcR γ-chain generates constitutive signals in DT40 cells that lead to tyrosine phosphorylation of Syk and NFAT activation. Stimulation of GPVI-FcR γ-chain-transfected cells with collagen leads to a further increase in tyrosine phosphorylation of Syk (Fig. 1*C*, *panel ii*), consistent with the activation of Syk by the collagen receptor complex, as described in earlier studies.

Constitutive Signaling of CLEC-2 in DT40 Cells—The above studies were extended to CLEC-2, which is a recently identified platelet glycoprotein receptor that mediates activation through a single Y*XX*L motif in its cytosolic tail. CLEC-2 is a receptor for the snake venom toxin, rhodocytin, and the lymphatic marker podoplanin. Both agonists induce activation of CLEC-2-transfected DT40 cells, with the response being dependent on the conserved tyrosine in the cytoplasmic Y*XX*L motif (7, 8).

In the present study, we have confirmed that rhodocytin stimulates NFAT activation in CLEC-2-transfected DT40 cells, with the level of response increasing with the level of surface expression of CLEC-2 (Fig. 2*A*, *panels i* and *ii*). Moreover, as is the case with the FcR γ-chain, expression of CLEC-2 was sufficient to increase NFAT activity in the absence of agonist stimulation, with the level of response increasing in parallel with that of CLEC-2 (Fig. 2*A*, *panels i* and *ii*). The level of constitutive activity approached between 10 and 20% the response to rhodocytin. Constitutive signaling by CLEC-2 is abolished following mutation of the tyrosine in the CLEC-2 Y*XX*L sequence to a phenylalanine (Y7F) (Fig. 2*B*, *panel i*), as is the case for
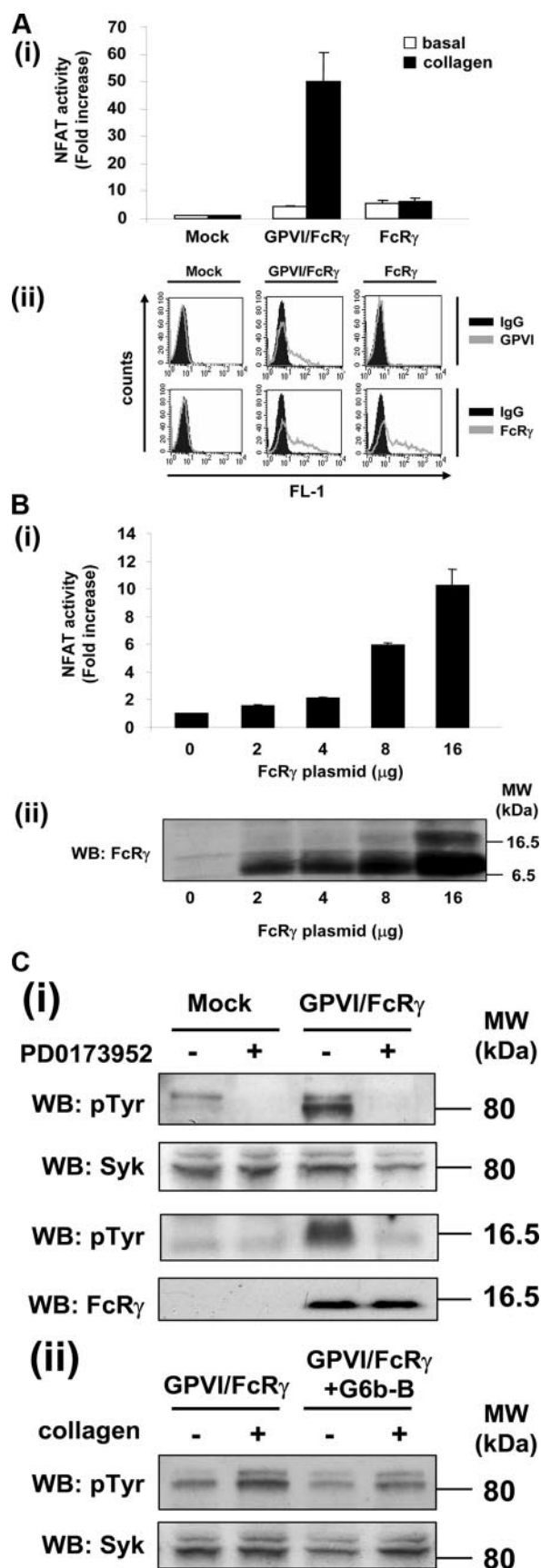
stimulation by rhodocytin (8). Flow cytometry confirmed a similar level of expression of wild-type and the Y7F mutant of CLEC-2 in these studies (Fig. 2B, panel ii). These results demonstrate that constitutive and agonist-induced signaling by CLEC-2 is dependent on the YXXL motif.

Constitutive Signaling by GPVI-FcR γ-Chain and CLEC-2 Is Mediated by Src and Syk Tyrosine Kinases—We and others have previously reported that collagen stimulates phosphorylation of the FcR γ-chain ITAM in platelets through the Src kinases, Fyn and Lyn, leading to recruitment and tyrosine phosphorylation of Syk (35–37). Similarly, phosphorylation of CLEC-2 and Syk by rhodocytin in platelets is mediated through a Src kinase pathway, although the identity of the Src kinase is not known (5). Consistent with this, we observed constitutive and collagen-stimulated tyrosine phosphorylation of Syk in GPVI-FcR γ-chain-transfected DT40 cells (Fig. 1C). In comparison, we have not been able to detect constitutive phosphorylation of CLEC-2 in our studies, possibly because of a lower level of expression relative to that of FcR γ-chain, as shown using a Myc-tagged version of both proteins (supplemental Fig. S1), and because the C-type lectin receptor has a single YXXL motif. However, we have observed a small increase in constitutive phosphorylation of Syk in DT40 cells expressing CLEC-2, which was further increased in the presence of rhodocytin (not shown).

Experiments were designed to investigate whether NFAT signaling by the GPVI-FcR γ-chain complex and CLEC-2 in transfected DT40 cells is mediated through Src and Syk kinases. As shown in Fig. 3A, constitutive and agonist-induced NFAT activation by both receptors is blocked in the presence of the Src family kinase inhibitor PD0173952. Furthermore, constitutive and agonist-induced signaling by the two receptors was also markedly inhibited in the absence of Syk (Fig. 3B and data not shown). Thus, these results demonstrate that constitutive and agonist-induced signaling by GPVI-FcR γ-chain and CLEC-2 are both dependent on Src family and Syk tyrosine kinases.

G6b-B Inhibits Constitutive and Agonist-induced Signaling by GPVI-FcR γ-Chain and CLEC-2 through Its Two ITIMs—A series of experiments were undertaken to investigate the possible regulation of constitutive and agonist-induced signaling

FIGURE 1. **Constitutive signaling by the FcR γ-chain in DT40 cells.** A, panel i, constitutive and collagen-induced signaling in DT40 cells transfected with plasmids (10 μg) encoding GPVI and/or Myc-FcR γ-chain (FcRγ) was analyzed using an NFAT-luciferase reporter assay. Cell lysates were analyzed for luciferase activity following incubation with media or collagen (10 μg/ml) for 6 h. Data are expressed as -fold increase over the basal of mock-transfected cells. Values are mean ± S.E. of three independent experiments. Panel ii, GPVI and FcRγ surface expression was measured by flow cytometry following transfection with 10 μg of plasmid of GPVI/Myc-FcRγ or Myc-FcRγ only (corresponding to A, panel i) relative to a control IgG. Data are representative of three experiments. B, constitutive signaling by the FcRγ alone. Panel i, concentration-dependent constitutive signaling by FcRγ in DT40 cells. Values are mean ± S.E. of three independent experiments. Panel ii, Western blotting (WB) for FcR γ-chain in DT40 cells transfected with increasing amounts of FcR γ-chain plasmid. Data are representative of three experiments. C, panel I, constitutive tyrosine phosphorylation of Syk and FcRγ. Mock and GPVI-FcRγ-transfected DT40 cells were pre-treated with PD0173952 (20 μM) for 5 min. Whole cell lysates were Western blotted for tyrosine phosphorylation using mAb 4G10 and then reprobed for Syk and FcRγ. Tyrosine phosphorylated bands that comigrate with Syk and FcRγ are shown. Panel ii, tyrosine phosphorylation of Syk in GPVI-FcRγ-transfected DT40 cells lysates by collagen (30 μg/ml) is partially inhibited by cotransfection with G6b-B. Cells were stimulated by collagen for 90 s. Experimental conditions were as for panel i. Data are representative of three experiments.
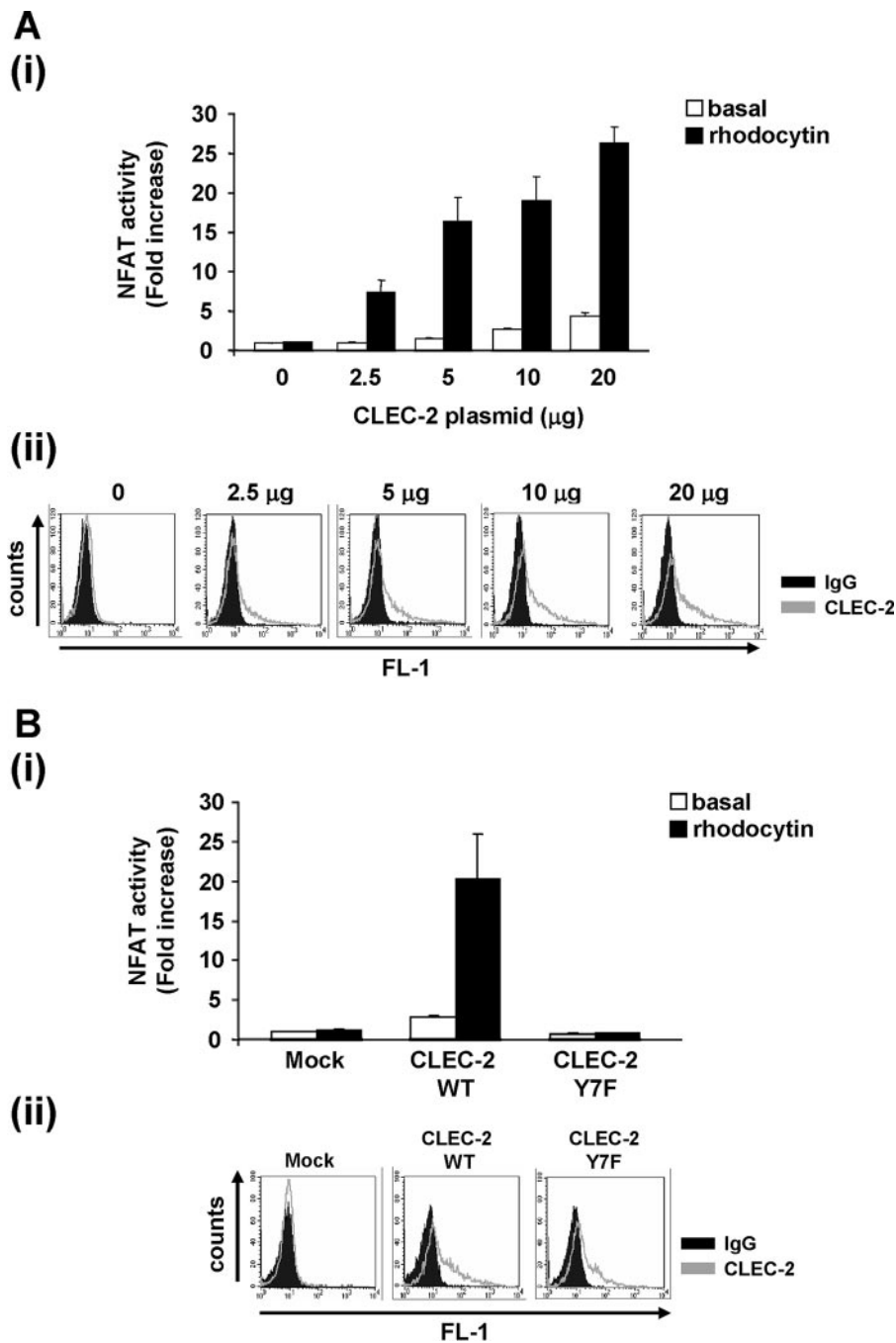
A
(i)

(ii)

B
(i)

(ii)

FIGURE 2. **Constitutive signaling by CLEC-2 in DT40 cells is dependent on its Y***XX***L motif.** *A, panel* i, constitutive and rhodocytin-induced signaling in DT40 cells transfected with varying amounts of the CLEC-2 plasmid was analyzed by NFAT-luciferase reporter assay. Cells were stimulated with 50 nM rhodocytin for 6 h. Values are mean ± S.E. of three independent experiments. *Panel ii*, CLEC-2 surface expression following transfection with varying amounts of CLEC-2 plasmid was assessed by flow cytometry using anti-Myc tag mAb relative to a control IgG. Data are representative of three experiments. *B, panel* i, constitutive and rhodocytin-induced signaling in cells transfected with wild-type (*WT*) and mutant (Y7F) CLEC-2. Values are mean ± S.E. of three independent experiments. *Panel ii*, CLEC-2 surface expression (corresponding to *panel i*) was assessed by flow cytometry using anti-Myc tag mAb relative to a control IgG. Data are representative of three experiments.

rhodocytin may reflect the lower level of expression of CLEC-2 relative to GPVI-FcR γ-chain (supplemental Fig. S1). Expression of G6b-B had no effect on the level of expression of either CLEC-2 or GPVI (supplemental Fig. S2). Expression of G6b-B also partially inhibited collagen-induced tyrosine phosphorylation of Syk (Fig. 1*C, panel ii*), demonstrating a molecular basis for its inhibitory action. These results demonstrate that G6b-B inhibits constitutive and agonist-induced signaling induced by GPVI-FcR γ-chain and CLEC-2.

G6b-B contains two ITIMs in its cytosolic tail, which independently mediate binding to SHP1 and SHP2 following incubation with the protein-tyrosine phosphatase inhibitor, pervanadate (9). Mutation of the two conserved tyrosines at positions 211 and 237 to phenylalanine inhibits association with the two SH2 domain-containing protein-tyrosine phosphatases (9). To investigate whether either or both of the ITIMs mediate the inhibitory effect of G6b-B, the Y211F/Y237F double mutant was expressed in DT40 cells together with the GPVI-FcR γ-chain complex and CLEC-2. Flow cytometry was used to confirm similar levels of expression of the G6b-B mutant, GPVI and CLEC-2 in the transfected cell lines to that in cells transfected with wild-type G6b-B and the two Y*XX*L-containing receptors (supplemental Fig. S2).

Mutation of the two conserved tyrosines in the G6b-B ITIMs abolished the inhibitory effect of G6b-B against constitutive and agonist-induced activation of NFAT induced by GPVI-FcR γ-chain and CLEC-2 (Fig. 4), whereas mutation of either of the two tyrosines alone had a partial or neglible effect (not shown). In line with this, we were able to detect weak tyrosine phosphorylation of G6b-B in the DT40-transfected cells (not shown). These results demonstrate that G6b-B inhibits constitutive and agonist-induced signaling through the conserved tyrosines at positions 211 and 237.

The ability of G6b-B to inhibit constitutive and agonist-induced responses mediated through GPVI-FcR γ-chain and CLEC-2 was further investigated in the combined absence of the two SH2 domain-containing tyrosine phosphatases, SHP1

through the GPVI-FcR γ-chain complex and CLEC-2 by the platelet immunoglobulin receptor G6b-B, which contains two ITIMs in its cytosolic tail (9, 10). xpression of G6b-B significantly inhibited constitutive signaling by both receptors by more than 60% (Fig. 4, *panel i*), as well as inhibiting the response to collagen and rhodocytin by ~35 and 60%, respectively (Fig. 4, *panel ii*). The greater degree of inhibition of the response to
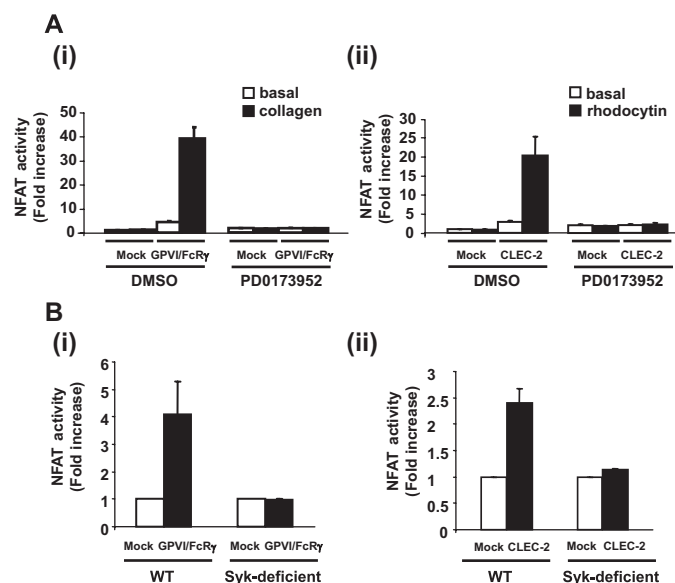
FIGURE 3. **Constitutive and agonist-induced signaling by GPVI-FcR γ-chain and CLEC-2 is dependent on Src and Syk tyrosine kinases.** Constitutive and agonist-induced signaling in wild-type (*WT*) and Syk-deficient DT40 cells transfected with GPVI-FcR γ-chain or CLEC-2 was analyzed using an NFAT-luciferase reporter assay. Cell lysates were analyzed for luciferase activity following agonist stimulation for 6 h. *A*, WT DT40 cells were either mock-transfected or transfected with (*panel i*) GPVI-FcR γ-chain (10 μg of each plasmid) or (*panel ii*) CLEC-2 (10 μg) and stimulated with 10 μg/ml collagen or 50 nM rhodocytin in the presence and absence of the Src kinase inhibitor, PD0173952 (20 μM). *B*, basal signaling in WT or Syk-deficient DT40 cells transfected with mock or transfected with (*panel i*) GPVI-FcR γ-chain (10 μg of each plasmid) or (*panel ii*) CLEC-2 (10 μg) and incubated for 6 h to monitor the degree of constitutive signaling. Values are mean ± S.E. of three independent experiments.
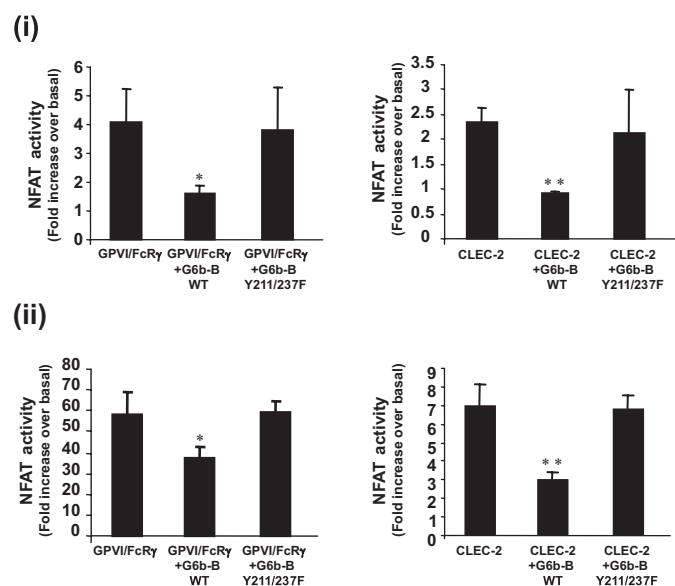

FIGURE 4. **The novel platelet ITIM transmembrane protein G6b-B inhibits constitutive and agonist-induced signaling through GPVI and CLEC-2.** The effect of the wild-type (*WT*) and double ITIM-mutant transmembrane protein G6b-B (Y211F/Y237F) on (*panel i*) constitutive and (*panel ii*) agonist-induced signaling by GPVI-FcR γ-chain and CLEC-2 in transfected (10 μg of each plasmid) DT40 cells, measured using an NFAT-luciferase reporter assay. Cells were stimulated with 10 μg/ml collagen or 50 nM rhodocytin as appropriate. The double ITIM mutant contained inactivating point mutations (Y/F) at tyrosines 211 and 237. The results are mean ± S.E. of between 4 and 7 independent experiments. Statistical significance was indicated by Student's *t* test. *, $p < 0.05$; and **, $p < 0.01$ compared with the GPVI-FcR γ-chain or CLEC-2-transfected samples.
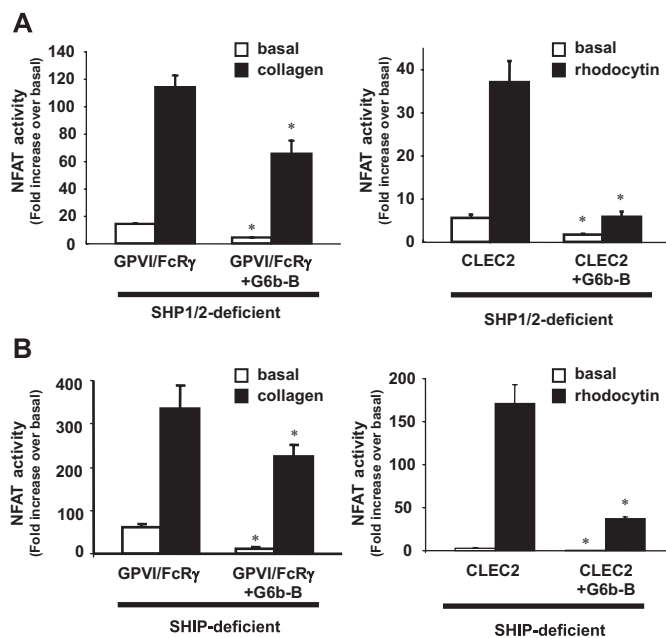

FIGURE 5. **The inhibitory effect of G6b-B is independent of SHP1, SHP2, and SHIP.** Constitutive and agonist-induced signaling in SHP1/SHP2 double-deficient (*A*) and SHIP-deficient (*B*) DT40 cells transfected with GPVI-FcR γ-chain, CLEC-2, and G6b-B was analyzed using a NFAT-luciferase reporter assay. Cells were stimulated with 10 μg/ml collagen or 50 nM rhodocytin for 6 h. *, $p < 0.05$ relative to response in the absence of G6b-B. Values are mean ± S.E. of three independent experiments.

and SHP2, or in the absence of the 5′-inositol phosphatase, SHIP. Unexpectedly, the absence of the two protein-tyrosine phosphatases led to an increase in the level of constitutive and agonist-induced NFAT activation through GPVI-FcR γ-chain and CLEC-2, suggesting that both receptors are under dynamic inhibitory feedback through the two tyrosine phosphatases (Fig. 5*A*). Nevertheless, G6b-B was still able to inhibit constitutive and agonist-induced signaling through GPVI-FcR γ-chain and CLEC-2 in the absence of SHP-1 and SHP-2 (Fig. 5*A*). Indeed, the inhibitory action against rhodocytin was enhanced in the combined absence of SHP1 and SHP2 (Fig. 5*A*), although this may reflect the increased response to rhodocytin that is observed in the absence of the two protein-tyrosine phosphatases. G6b-B also inhibits constitutive and agonist-induced NFAT activation by GPVI-FcR γ-chain and CLEC-2 in DT40 cells deficient in the SH2-domain-containing inositol 5′-phosphatase (SHIP) (Fig. 5*B*). Indeed the inhibitory action of G6b-B against CLEC-2 was also enhanced by the absence of the 5′-inositol phosphatase (Fig. 5*B*), as was the case in the absence of SHP1 and SHP2. These results demonstrate that G6b-B inhibits GPVI and CLEC-2 signaling in DT40 cells by a SHP1/SHP2-independent and SHIP-independent mechanism.

PECAM-1 Does Not Inhibit Constitutive Signaling in DT40 Cells—A similar set of studies were undertaken to investigate whether the major platelet ITIM receptor, PECAM-1, also inhibits constitutive and agonist-induced activation of NFAT downstream of GPVI-FcR γ-chain and CLEC-2. As shown in supplemental Fig. S3*A*, PECAM-1 had no effect on constitutive or agonist-induced activation of either receptor in DT40 cells, possibly because of a low level of expression (supplemental Fig. S3*B*).
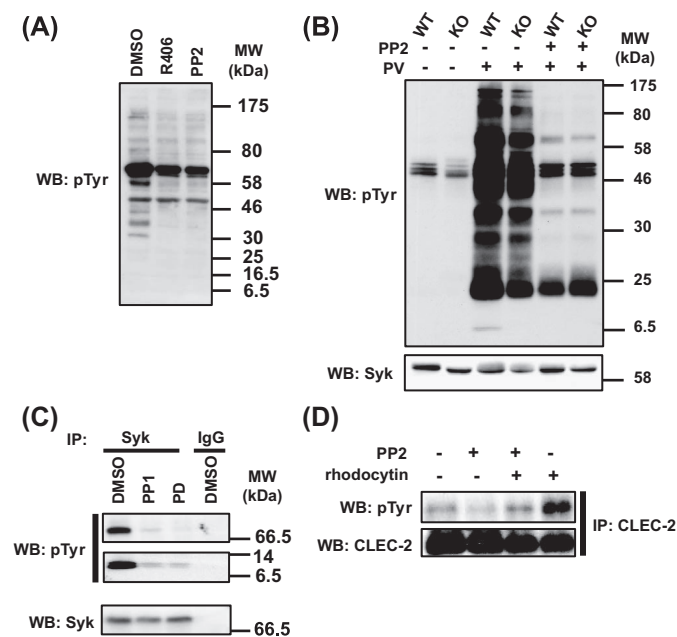
FIGURE 6. **Constitutive tyrosine phosphorylation of FcR γ-chain and Syk in human platelets.** *A*, human washed platelets were incubated at 37 °C with the Syk kinase inhibitor, R406 (1 μM), and Src inhibitor PP2 (20 μM) for 1 min. Whole cell lysates were probed for phosphotyrosine (*pTyr*). *B*, wild-type (*WT*) or FcR γ-chain deficient mouse (*KO*) platelets (pretreated with EGTA to block aggregation) were stimulated with 100 μM pervanadate (*PV*) for 90 s in the presence or absence of Src inhibitor PP2 (20 μM). *C*, human washed platelets were incubated at 37 °C with the Src kinase inhibitors PP1 (10 μM) or PD0173952 (10 μM) for 5 min. Cell lysates were immunoprecipitated with anti-Syk Ab or an IgG and probed for Tyr(P). Subsequently, they were stripped and reprobed for Syk. *D*, human washed platelets were stimulated with 30 nM rhodocytin for 5 min in the presence and absence of the Src kinase inhibitor, PP2 (10 μM). Cell lysates were immunoprecipitated (*IP*) with anti-CLEC-2 Ab and probed for Tyr(P). Subsequently, they were stripped and reprobed for CLEC-2. The results are representative of between 3 and 8 experiments. *WB*, Western blot.

Src and Syk Kinase-dependent Constitutive Signaling in Platelets—Experiments were designed to investigate whether there is evidence for constitutive signaling through Src and Syk kinases in platelets. In this context, it is important to emphasize that platelets express several classes of receptor that signal through Src and Syk tyrosine kinases, including the major platelet integrin, αIIbβ3, and the GPIb-IX-V complex, the levels of which are over 1 order of magnitude greater than that of GPVI-FcR γ-chain and CLEC-2.

We first asked the question as to whether the tyrosine phosphorylation that is seen in the absence of agonist stimulation in platelets is regulated by Src or Syk tyrosine kinases. Western blotting for phosphotyrosine using the monoclonal antibody 4G10 reveals the presence of a broad band of proteins in non-stimulated platelets of between 50 and 60 kDa (Fig. 6*A*), which covers the region of migration of Src family kinases. This region includes Src kinases, which are constitutively phosphorylated in resting platelets by the Src kinase-regulatory protein, Csk, at an inhibitory site that promotes intramolecular binding to the SH2 domain. In addition, several other proteins, which have been shown to be constitutively phosphorylated in nonstimulated platelets, migrate in this region, including Dok-2 (38). Additional phosphorylation bands that lie outside of this region can also be seen in non-stimulated platelets in longer exposures, as

illustrated in Fig. 6*A*. Tyrosine phosphorylation of the majority of these bands is markedly inhibited in the presence of the Src family kinase inhibitors, PP1 and PP2, and the Syk family kinase inhibitor, R406 (39), with the exception of the broad band of proteins that runs at 50–60 kDa, which is only partially reduced (Fig. 6*A* and not shown). The residual phosphorylation that is seen in this region is likely to reflect phosphorylation of Src kinases on their inhibitory site by Csk. Significantly, Csk is not known to be regulated downstream of Src and Syk kinases. The reduction in phosphorylation in this region is presumably due to loss of phosphorylation of proteins such as Dok-2, as discussed above. The similar pattern of reduction in tyrosine phosphorylation that is induced by R406 to that of PP2 cannot be due to direct inhibition of Src kinases, as R406 does not inhibit collagen-stimulated tyrosine phosphorylation of the FcR γ-chain,[5] which is mediated through Src kinase activation. These results demonstrate that constitutive signaling by Src and Syk family kinases underlies tyrosine phosphorylation of the majority of proteins in non-stimulated platelets, thereby providing evidence of constitutive signaling by Src and Syk kinases.

We used the protein-tyrosine phosphatase inhibitor, pervanadate, to further investigate the contribution of Src and Syk family kinases to constitutive tyrosine phosphorylation in human and mouse platelets, on the grounds that this would potentiate protein tyrosine phosphorylation by constitutively active tyrosine kinases. Results are shown for mouse platelets (Fig. 6*B*), with similar observations made for human platelets (not shown). As previously reported (40–42), pervanadate induces a marked increase in tyrosine phosphorylation of a large number of proteins in platelets (Fig. 6*B*). Strikingly, protein tyrosine phosphorylation induced by pervanadate was dramatically inhibited in the presence of the Src kinase inhibitor, PP2, confirming that Src family kinases are the major family of kinases that contribute to constitutive tyrosine phosphorylation in platelets. A small number of tyrosine-phosphorylated proteins are seen in longer exposures of pervanadate-treated platelets in the presence of PP2, including a band of 72 kDa (Fig. 6*B*) that was identified as Syk by immunoprecipitation of the kinase and Western blotting for phosphotyrosine (Fig. 6*B*). These data confirm that Src kinases are constitutively active in non-stimulated platelets and demonstrate that they underlie the major increase in tyrosine phosphorylation that is induced in the presence of protein-tyrosine phosphatase inhibition.

Further experiments were designed to investigate the possible dependence of constitutive signaling through Src and Syk family kinases on the GPVI-FcR γ-chain complex. For these experiments, Syk was immunoprecipitated from resting human platelets and Western blotted for phosphotyrosine in the absence and presence of the Src family kinase inhibitor, PD0173952. The results in Fig. 6*C* demonstrate association of constitutively tyrosine-phosphorylated Syk and constitutively tyrosine-phosphorylated FcR γ-chain, which is dramatically

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[5] J. C. Spalton and S. P. Watson, unpublished data.

reduced in the presence of the Src kinase inhibitor PD0173952. On the other hand, treatment of FcR γ-chain-deficient mouse platelets with pervanadate revealed that constitutive tyrosine phosphorylation in mouse platelets is minimally altered in platelets deficient in the ITAM-containing protein (Fig. 6*B*), demonstrating that the GPVI-FcR γ-chain complex makes a relatively minor contribution to the overall level of tyrosine phosphorylation.

Constitutive phosphorylation of CLEC-2 is observed in human platelets and is reduced in the presence of the Src kinase inhibitor, PP2 (Fig. 6*D*). The contribution of CLEC-2 to constitutive tyrosine phosphorylation in mouse platelets cannot be assessed, as mice lacking the C-type lectin receptor have not been made.

DISCUSSION

The present study demonstrates that the GPVI-FcR γ-chain complex and CLEC-2 generate constitutive signals in transfected DT40 cells leading to NFAT activation with the degree of signaling corresponding to the level of expression. Furthermore, the dependence on Src and Syk family kinases provides strong evidence that constitutive signaling by both receptors is mediated through the same pathway as used by agonists to mediate activation. In line with this, constitutive signaling by CLEC-2 is inhibited by mutation of the conserved tyrosine in the Y*XX*L sequence in its cytosolic tail. Furthermore, the response to the GPVI-FcR γ-chain complex is mediated by FcR γ-chain, and is independent of GPVI, consistent with it being generated through the tandem Y*XX*L sequence of the FcR γ-chain.

Constitutive and agonist-induced signaling through GPVI-FcR γ-chain and CLEC-2 is reduced by co-expression of the ITIM-containing platelet protein, G6b-B (9). The inhibitory effect of G6b-B is dependent on the conserved tyrosines in its two ITIMs, as shown by abolition of the inhibitory response upon mutation of the ITIM tyrosines to phenylalanine. However, unexpectedly, this inhibitory effect is retained in DT40 cells in the combined absence of the SH2 domain-containing tyrosine phosphatases, SHP1 and SHP2, which have been shown to bind to G6b-B (9, 10). The inhibitory effect of G6b-B is also retained in the absence of the SH2 domain-containing inositol phosphatase, SHIP, which has been shown to inhibit B cell receptor signaling through association with the FcγRIIb ITIM (43, 44). An alternative pathway of inhibition could be through recruitment of the SH2 domain-containing inhibitor of Src kinases, Csk, to the two ITIMs in G6b-B. Such a mechanism has been proposed to underlie the inhibition of FcεRI signaling by the ITIM-containing transmembrane protein, LAIR-1, in rat Rbl mast cells (45). However, we were unable to demonstrate association of Csk with G6b-B in transfected DT40 cells (not shown), possibly because of the low level of constitutive phosphorylation of the ITIM-containing protein. Access to Csk-deficient DT40 cells is required to directly test the role of the Src kinase regulatory protein in mediating the inhibitory effect of G6b-B. It is also possible that the inhibitory effect of G6b-B is mediated by association with several proteins, including a combination of SHP1, SHP2, SHIP, and

Csk, such that loss of any one is compensated by expression of the others. Such a mechanism would explain why G6b-B is still able to mediate inhibition in DT40 cells deficient in SHP1 and SHP2, which have both been shown to bind to its cytosolic ITIMs. Interestingly, we observed a small reduction in tyrosine phosphorylation of Syk by G6b-B in DT40 cells stimulated by collagen, suggesting that this underlies the inhibitory effect of the ITIM-containing protein.

The observation that the FcR γ-chain and CLEC-2 generate constitutive signals is in line with reports that ITAM receptors generate weak, constitutive signals in other hematopoietic cells. For example, in mouse thymocytes and peripheral T cells, there is limited constitutive phosphorylation of TCRζ chains leading to association with the Syk-related tyrosine kinase ZAP-70 (46, 47). This gives rise to constitutive signaling in resting mouse thymocyte and Jurkat T cell lines, as shown by suppression of *RAG* gene expression via extracellular signal-regulated kinase (ERK) and Abl kinases, and prevention of further recombination of T cell receptor genes (48). The B cell antigen receptor also signals independently of ligand engagement and this provides functionally relevant signals in immature B lymphocytes (49) and in diffuse large B-cell lymphoma (50). Significantly, this constitutive signaling is mediated through the B cell receptor ITAMs (51, 52). Thus, constitutive signaling appears to be a common feature of Y*XX*L-containing receptors.

Evidence of constitutive signaling through Src and Syk tyrosine kinases in platelets has been demonstrated in the present study in association with a low level of tyrosine phosphorylation of the FcR γ-chain and CLEC-2. Studies in mice deficient in the FcR γ-chain demonstrate that the GPVI receptor complex makes only a minimal contribution to this phosphorylation, most likely because of the association of Src kinases with other surface receptors, including the αIIbβ3 complex that is expressed at over 20 times the level of GPVI.

The demonstration of constitutive signaling via Src and Syk kinases in platelets emphasizes the need for inhibitory signals to oppose activation occurring in healthy vessels. We speculate that this function may be mediated, at least in part, by the ITIM receptor, G6b-B, which is markedly phosphorylated in resting platelets (10). The ligand for G6b-B is not known, but if this is expressed on platelets, it is possible that this may account for the high level of constitutive phosphorylation of G6b-B through a cis-interaction.

A similar function to that of G6b-B may be mediated in platelets by the ITIM-containing receptor, PECAM-1. This is in line with studies that have reported inhibition of agonist-induced platelet activation by the immunoglobulin receptor (21–23). However, unexpectedly, we were unable to observe inhibition of constitutive and agonist-induced signaling by PECAM-1 in the DT40 cell line, possibly because of too low a level of expression.

In summary, the present study reports that both FcR γ-chain and CLEC-2 generate weak, sustained constitutive signals that are inhibited by co-expression with G6b-B. We speculate that this could represent an important physiological role of G6b-B

and other platelet ITIM proteins in helping to prevent platelet activation *in vivo*.

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