

The C-type Lectin Receptors CLEC-2 and Dectin-1, but Not DC-SIGN, Signal via a Novel YXXL-dependent Signaling Cascade*

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The two lectin receptors, CLEC-2 and Dectin-1, have been shown to signal through a Syk-dependent pathway, despite the presence of only a single YXXL in their cytosolic tails. In this study, we show that stimulation of CLEC-2 in platelets and in two mutant cell lines is dependent on the YXXL motif and on proteins that participate in signaling by immunoreceptor tyrosine-based activation motif receptors, including Src, Syk, and Tec family kinases, and on phospholipase C γ . Strikingly, mutation of either Src homology (SH) 2 domain of Syk blocks signaling by CLEC-2 despite the fact that it has only a single YXXL motif. Furthermore, signaling by CLEC-2 is only partially dependent on the BLNK/SLP-76 family of adapter proteins in contrast to that of immunoreceptor tyrosine-based activation motif receptors. The C-type lectin receptor, Dectin-1, which contains a YXXL motif preceded by the same four amino acids as for CLEC-2 (DEDG), signals like CLEC-2 and also requires the two SH2 domains of Syk and is only partially dependent on the BLNK/SLP-76 family of adapters. In marked contrast, the C-type lectin receptor, DC-SIGN, which has a distinct series of amino acids preceding a single YXXL, signals independent of this motif. A mutational analysis of the DEDG sequence of CLEC-2 revealed that the glycine residue directly upstream of the YXXL tyrosine is important for CLEC-2 signaling. These results demonstrate that CLEC-2 and Dectin-1 signal through a single YXXL motif that requires the tandem SH2 domains of Syk but is only partially dependent on the SLP-76/BLNK family of adapters.

The C-type lectin superfamily of transmembrane proteins consists of at least 70 members in the human genome (1). The superfamily can be divided into “classical” C-type lectins, which contain a carbohydrate recognition domain and bind sugars in a calcium-dependent manner, and the “nonclassical” C-type lectin-like proteins, which contain a C-type lectin-like domain, homologous to a carbohydrate recognition domain, but lacks the consensus sequence for binding sugars and calcium (2). Protein ligands for a number of classical and nonclassical C-type lectin receptors have been described.

C-type lectin-like receptor 2 (CLEC-2)⁶ is a type II transmembrane protein and a nonclassical C-type lectin (3). The C-type lectin-like domain in CLEC-2 is supported by a 41-amino acid neck region, a single transmembrane domain, and 31-amino acid cytoplasmic domain (3). CLEC-2 mRNA has been identified in liver and in blood cells, mostly of myeloid origin, including monocytes, granulocytes, and dendritic cells (3). Recently, we have identified expression of CLEC-2 in platelets and have shown that it functions as a receptor for the snake venom toxin rhodocytin (also known as aggretin), which elicits powerful platelet activation (4). Rhodocytin, however, also binds to several other platelet receptors (5, 6), making it unclear whether CLEC-2 is sufficient to mediate activation alone and thereby hampering analysis of the mechanism of activation.

The cytosolic domain of CLEC-2 contains a single tyrosine residue within a YXXL motif, a consensus sequence for phosphorylation by Src family kinases in immunoreceptor tyrosine-based activation motifs (ITAMs) and immunoreceptor tyrosine-based inhibitory motifs (ITIMs). ITAMs have the sequence YXX(L/I)X_{6–12}YXX(L/I), and ITIMs have the sequence (L/I/V)XYXX(L/I/V). Phosphorylation of the two tyrosine residues within an ITAM leads to recruitment of the tyrosine kinases Syk and Zap-70 via their tandem Src homology 2 (SH2) domains, leading to cellular activation (7, 8).

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⁶ The abbreviations used are: CLEC-2, C-type lectin-like receptor 2; SH, Src homology; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; GST, glutathione S-transferase; PMA, phorbol 12-myristate 13-acetate; PLC γ , phospholipase C γ ; WT, wild type; IL, interleukin; FWD, forward; REV, reverse; NFAT, nuclear factor of activated T cells.

Phosphorylated ITIMs binds to the SH2 domain-containing tyrosine phosphatases, SHP-1 and SHP-2, or the lipid phosphatases SHIP1 and SHIP2, in most cases leading to cellular inhibition (9).

Signaling by ITAM receptors, such as the platelet collagen receptor complex, GPVI/FcR γ -chain, or the B and T cell antigen receptors, is mediated via members of the Src, Syk, Tec, Vav, SLP-76/BLNK, and PLC γ families of signaling proteins (reviewed in Refs. 10–12). The specific members of each family that mediate ITAM signaling are cell-dependent. For example, SLP-76 is used by the T cell receptor (13) and the platelet collagen receptor GPVI (14), although B cells use the homologous protein BLNK (15).

We have shown that activation of platelets by rhodocytin is critically dependent on the tyrosine kinase Syk and many of the proteins that participate in ITAM signaling in platelets (4). This has led us to propose that the snake venom toxin signals through a similar pathway to that of ITAM receptors, with Syk being recruited via the phosphorylated YXXL sequence in the cytosolic tail of the lectin-like receptor. A similar coupling to Syk has been proposed for a second C-type lectin receptor, Dectin-1, which mediates activation of dendritic cells by zymosan (16). A third YXXL-containing member of the C-type lectin superfamily, DC-SIGN, has also been reported recently to signal to PLC γ in dendritic cells, although the role of Syk in signaling by this receptor is not known (17).

The aim of this study was to characterize the mechanism of CLEC-2 signaling in platelets and in two hematopoietic-derived cell line model systems and to compare this to signaling by Dectin-1 and DC-SIGN. The results demonstrate that signaling by CLEC-2 is completely dependent on the cytoplasmic YXXL motif and requires both SH2 domains of Syk. The signaling pathway activated by CLEC-2 involves Src, Syk, and Tec family kinases and PLC γ , but it is distinct from that of ITAM signaling in that it has a partial rather than absolute dependence on the SLP-76/BLNK family of adapter proteins. Dectin-1 signals in a similar way to CLEC-2, whereas the mechanism of signaling by DC-SIGN is distinct. The results demonstrate that some but not all lectin receptors signal through a single YXXL motif leading to activation of PLC γ .

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Polyclonal goat α -human CLEC-2, α -mouse Dectin-1, and normal goat IgG were purchased from R & D Systems Inc. (Minneapolis, MN). Monoclonal α -CD209 (DC-SIGN) was purchased from Pharmingen. Rabbit polyclonal antibodies α -Syk, α -PLC γ 2, and α -Btk have been described previously (18, 19). Anti-phosphotyrosine monoclonal antibody 4G10, α -SLP-76 polyclonal antibody, and α -LAT polyclonal antibody were purchased from Upstate Biotechnology Inc. (TCS Biologicals Ltd., Bucks, UK). Anti-human Vav3 antibody was a kind gift from Dr. Daniel Billadeau and was raised in rabbits as described previously (20). Anti-MYC antibody was purchased from Cell Signaling Technology (New England Biolabs, Herts, UK). F(ab')₂ fragments of the anti-human Fc γ RIIA antibody IV.3 were generated as described previously (21). Fluorescein isothiocyanate-conjugated donkey anti-goat IgG secondary antibody was from Jackson ImmunoResearch.

Horseradish peroxidase-conjugated sheep anti-mouse secondary antibody, horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody, and enhanced chemiluminescence reagents (ECL) were purchased from Amersham Biosciences. GST fusion proteins corresponding to single or tandem SH2 domains of Syk were prepared as described previously (22). Rhodocytin was purified by Dr. Johannes Eble as described previously (23). The GPIIb/IIIa antagonist lotrafiban was a gift from GlaxoSmithKline (King of Prussia, PA), and the Gly-Arg-Gly-Asp-Ser (GRGDS) peptide was obtained from Peptide Institute (Osaka, Japan). The Src kinase inhibitor PD0173952 was a gift from Pfizer Global Research and Development (Ann Arbor, MI). The Src kinase inhibitor PP2 was purchased from Calbiochem. All other reagents were purchased from Sigma or from previously described sources (18, 24).

Platelet Studies—Blood was drawn on the day of experiment from healthy, drug-free volunteers into 1:10 (v/v) sterile sodium citrate and 1:9 (v/v) acid citrate dextrose (ACD: 120 mM sodium citrate, 110 mM glucose, 80 mM citric acid). Washed platelets were prepared as described previously (18).

Platelet aggregation studies were carried out using washed platelets at a concentration of 2×10^8 /ml in a Born aggregometer (ChronoLog, Havertown, PA) at 37 °C with continuous stirring at 1200 rpm for 5 min. Aggregation of platelets in response to rhodocytin (300 nM) or α -CLEC-2 antibody (10 μ g/ml) was recorded by measuring change in optical density. Platelets were preincubated with IV.3 F(ab')₂ (12 μ g/ml), PP2 (10 μ M), or PD0173952 (25 μ M) for 10 min prior to stimulation where indicated. Platelets were used at 1×10^9 /ml for protein studies. Lotrafiban (10 μ M) or GRGDS peptide (1 mM) was included in the resuspension buffer to block aggregation and signaling through GPIIb/IIIa. Stimulations were carried out in a Born aggregometer for the times shown. Following stimulation platelets were lysed with an equal volume of 2 $\times$ lysis buffer (300 mM NaCl, 20 mM Tris, 2 mM EGTA, 2 mM EDTA, 2% Nonidet P-40, pH 7.4, with 2.5 mM Na₃VO₄, 100 μ g/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin, and 0.5 μ g/ml pepstatin).

Constructs—Human CLEC-2 cloned into pcDNA3 has been described previously (25). For these experiments CLEC-2 was subcloned into pEF6 vector with a C-terminal MYC tag, pEF6/MyC-His A (Invitrogen). A point mutation of the cytoplasmic tyrosine residue of CLEC-2 to a phenylalanine (Y7F) was generated by a two-step PCR method (26). The mutating primers CLEC-2-Y7F-REV (5'-GTT TTA ATA TTT AAG GTG ATG AAT CCA TCT TCA TCC TG-3') and CLEC-2-Y7F-FWD (5'-CAG GAT GAA GAT GGA TTC ATC ACC TTA AAT ATT AAA AC-3') were used along with vector specific primers T7 and 4150 (5'-AGG CAC AGT CGA GGC TGA TC-3'). D3A, E4A, D5A, and G6A CLEC-2 were generated by a single step PCR approach. The mutating primers were D3A-FWD (5'-TAG TAG GGA TCC ATG CAG GCT GAA GAT GGA TAC-3'), E4A-FWD (5'-TAG TAG GGA TCC ATG CAG GAT GCA GAT GGA TAC-3'), D5A-FWD (5'-TAG TAG GGA TCC ATG CAG GAT GAA GCT GGA TAC ATC-3'), G6A-FWD (5'-TAG TAG GGA TCC ATG CAG GAT GAA GAT GCA TAC ATC ACC-3'), and hCLEC-2-REV (5'-TAG TAG GCG GCC GCA GGT AGT TGG TCC ACC TTG GTC-

3'). Porcine Syk cloned into pcDNA3 has been described previously (27). Inactivating point mutants of each SH2 domain of Syk were made by mutating Arg-37 or Arg-190 to Ala. In both cases T7 and BGH were used as outside primers. Specific primers for the point mutants were Syk-R37A-FWD (5'-GGG CTC TAC CTG CTT GCC CAG AGC CGC AAC TAC-3'), Syk-R37A-REV (5'-GTA GTT GCG GCT CTG GGC AAG CAG GTA GAG CCC-3'), Syk-R190A-FWD (5'-GGG AAG TTT TTG ATC GCG GCC AGG GAC AAC GGG-3'), Syk-190A-REV (5'-CCC GTT GTC CCT GGC CGC GAT CAA AAA CTT CCC-3'). Human GPVI cloned into pRC plasmid has been described previously (24). For these experiments hGPVI was subcloned into pcDNA3 with a C-terminal MYC tag (Invitrogen). Human FcR γ -chain DNA was amplified from HEL cell cDNA by PCR (hFcR γ -FWD (5'-TAG TAG GGA TCC CAG CCC AAG ATG ATT CCA GC-3') and hFcR γ -REV (5'-TAG TAG GCG GCC GCC TAC TGT GGT GGT TTC TCA TG-3')) and cloned into pEF6 vector with no tag (Invitrogen). Murine Dectin-1 was amplified from cDNA prepared from murine spleen by PCR (mDectin-1-FWD (5'-TAG TAG GGA TCA TGA AAT ATC ACT CTC ATA TAG-3') and mDectin-1-REV (5'-TAG TAG GCG GCC AGT TCC TTC TCA CAG ATA C-3')) and cloned into pEF6 vector with a C-terminal MYC tag, pEF6/*Myc*-His. All sequences were verified by sequencing. Wild type DC-SIGN in pcDNA3 has been described previously (28). A point mutation of the YXXL tyrosine (Tyr-31) to phenylalanine was generated by a two-step PCR method. The mutating primers were hDCSIGN31F-FWD (5'-CGA CAG ACT CGA GGA TTC AAG AGC TTA GCA GGG-3') and hDCSIGN31F-REV (5'-CCC TGC TAA GCT CTT GAA TCC TCG AGT CTG TCG-3'). The NFAT luciferase reporter contains three copies of the distal NFAT site from the IL-2 promoter (29) and was kindly provided by Prof. A. Weiss. The pEF6-*lacZ* expression construct was obtained from Invitrogen.

Cell Culture and Transfection—DT40 chicken B cells were grown in RPMI supplemented with 10% fetal bovine serum, 1% chicken serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, 50 μ M mercaptoethanol, and 20 mM GlutaMAX. DT40 cells rendered deficient for *SYK* (30), *LYN* (30), *LYN*/*SYK* (31), *BLNK* (32), *BTk* (31), and *PLC γ 2* (33) were described previously and kindly provided by Dr. T. Kurosaki (Kansai Medical University, Moriguchi, Japan). Jurkat T cells were grown in RPMI supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 20 mM GlutaMAX. Jurkat derivatives JCaM1 and JCaM1/Lck and J14 and J14/SLP-76 (J14-76) were kindly provided by Dr. A. Weiss (University of California, San Francisco) and have been described previously (34, 35). Cells were transfected in a volume of 400 μ l of nonsupplemented RPMI by electroporation using a GenePulser II (Bio-Rad) set at 350 V and 500 microfaradays for DT40 and 250 V and 950 microfaradays for Jurkat cells. 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 20 mM Glutamax. 293T cells were transfected with 5 μ g of DNA of each DNA construct by calcium phosphate precipitation.

Cell Line Protein Studies—Cells were transfected as described above with 10 μ g of CLEC-2 or 10 μ g of Y7F CLEC-2. Twenty hours following transfection cells were washed and resuspended in nonsupplemented RPMI. Cells were stimulated with 500 nM rhodocytin at room temperature for 10 min. Where indicated, inhibitors were preincubated with the cells for 10 min prior to stimulation. Following stimulation cells were lysed with an equal volume of 2 $\times$ lysis buffer. 293T cells were harvested 48 h after transfection, washed once in phosphate-buffered saline, and lysed in 500 μ l of 1 $\times$ lysis buffer.

Luciferase Assay—Cells were transfected as described above with either 10 μ g of CLEC-2, 10 μ g of Y7F CLEC-2, 10 μ g of Dectin-1, or 2 μ g of GPVI and 2 μ g of FcR γ -chain constructs, in addition to 15 μ g of the luciferase reporter construct and 2 μ g of pEF6-*lacZ* to control for transfection efficiency. Where indicated the receptor of interest was cotransfected along with 5 μ g of wild type Syk, R37ASyk, or R190ASyk into Syk-deficient DT40 cells. Twenty hours after transfection, live cells were counted by trypan blue exclusion, and samples were divided for luciferase assay, β -galactosidase assay, and flow cytometry. Luciferase assays were as described previously (36). For luciferase assays, rhodocytin was used at 50 nM, α -CLEC-2 antibody at 40 μ g/ml, α -DC-SIGN at 10 μ g/ml cross-linked with sheep α -mouse F(ab')₂ fragments at 30 μ g/ml, zymosan at 250 μ g/ml, and convulxin at 10 μ g/ml. Luciferase activity was measured with a Centro LB 960 microplate luminometer (Berthold Technologies, Germany). Data are expressed either as luminescence units normalized to β -galactosidase activity or as fold increase in luminescence units over basal as indicated. All luciferase data are averaged from three readings. Data are represented as one experiment representative of three $\pm$ S.E. for the three readings of the experiment. β -Galactosidase assays were performed with half a million cells using the Galacto-Light chemiluminescent reporter assay, according to the manufacturer's instructions (Applied Biosystems, Bedford, MA). β -Galactosidase activity was measured in triplicate using a microplate luminometer. All luciferase assay data were normalized to β -galactosidase values.

Flow Cytometry—Expression of each receptor was confirmed by flow cytometry. For CLEC-2 or Dectin-1 detection, 5×10^5 cells were stained in 50- μ l volume for 20 min with either 10 μ g/ml goat α -CLEC-2 or 10 μ g/ml goat α -Dectin-1 antibody alongside goat IgG as a negative control. Cells were then washed and incubated for 20 min with 15 μ g/ml fluorescein isothiocyanate-conjugated α -goat IgG secondary antibody. Stained cells were analyzed using a FACSCalibur (BD Biosciences). Data were collected and analyzed using Cellquest software.

Immunoprecipitation, Pulldowns, and Western Blotting—Cell lysates were precleared, and detergent-insoluble debris was removed as described (37). Following preclearing, 50- μ l aliquots of the stimulation were added to an equal volume of 2 $\times$ Laemmli sample buffer for whole cell phosphorylation studies. For immunoprecipitation and pulldown studies, lysates were incubated with the indicated antibodies and a mixture of Protein A-Sepharose and Protein G-Sepharose or GST fusion proteins corresponding to single or tandem SH2 domains of Syk associated with glutathione-Sepharose. Following immunopre-

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cipitation, the Sepharose beads were washed, and resulting protein complexes were eluted with 2× Laemmli sample buffer. The resulting whole cell lysates and immunoprecipitates were resolved by SDS-PAGE, and Western blotting was carried out as described previously (18).

Analysis of Data—Experiments were performed on at least three occasions and are shown as representative data from one experiment. Where experiments were carried out in triplicate, results are presented as the mean of the data.

RESULTS

Anti-CLEC-2 Antibody and Rhodocytin Stimulate Similar Patterns of Tyrosine Phosphorylation in Platelets—The snake toxin rhodocytin binds to multiple receptors on the platelet surface therefore making it unclear which signaling events are mediated through CLEC-2 or by other receptors for the snake toxin. To address this, we used an α -CLEC-2 antibody, which we have shown previously is able to induce platelet aggregation and phosphorylation of CLEC-2 in platelets independent of the low affinity immune receptor, Fc γ RIIA (4). Experiments were carried out in the presence of F(ab')₂ fragments of the antibody IV.3 to block the Fc γ RIIA receptor on platelets. The antibody to CLEC-2 (10 μ g/ml) stimulated platelet shape change and aggregation, whereas a nonspecific goat IgG control antibody had no effect (Fig. 1A, *panel i*). The onset of aggregation in response to the CLEC-2 antibody occurs after a lag time that is characteristic of platelet aggregation to rhodocytin (Fig. 1A, *panel i*). Aggregation to the CLEC-2 antibody is completely inhibited by the Src family kinase inhibitor, PP2 (Fig. 1A, *panel ii*), as is also the case for rhodocytin (4). The same result was observed with the structurally distinct Src kinase inhibitor PD0173952 (data not shown). Tyrosine phosphorylation of platelet lysates induced by rhodocytin and the CLEC-2 antibody was compared by Western blotting with the anti-phosphotyrosine antibody 4G10. The two agonists stimulated a similar pattern of tyrosine phosphorylation suggesting that the increase in tyrosine phosphorylation induced by rhodocytin is mediated through CLEC-2 (Fig. 1B). No increase in whole cell tyrosine phosphorylation was observed in platelets incubated with nonspecific goat IgG antibody (Fig. 1B). In addition, immunoprecipitation studies confirmed that the CLEC-2 antibody induced tyrosine phosphorylation of the same set of proteins that are regulated by rhodocytin in platelets, namely Syk, PLC γ 2, Vav3, LAT, SLP-76, and Btk (Fig. 1C). The greater level of tyrosine phosphorylation of Syk, LAT, and PLC γ 2 induced by the CLEC-2 antibody may reflect slight differences in the kinetics of activation or differences in the level of stimulation. Importantly, none of these proteins became phosphorylated following stimulation with control goat IgG. These results demonstrate that CLEC-2 is sufficient to cause platelet activation and suggest that the major mechanism of platelet activation by rhodocytin is through the lectin receptor.

CLEC-2 Expressing DT40 Cells and Jurkat Cells Are Activated by Stimulation of the Receptor—To further investigate the mechanism of CLEC-2 signaling, CLEC-2 was cloned into an expression vector and transiently transfected into cell lines. DT40 B cells and Jurkat T cells were used as model systems for

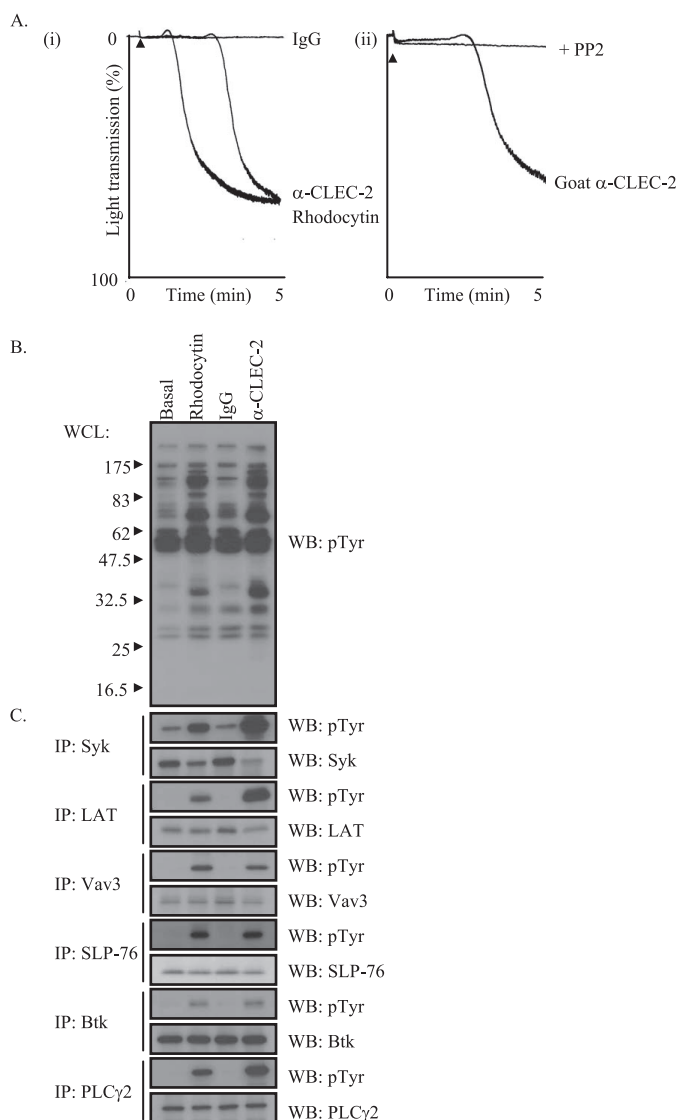


FIGURE 1. Platelet activation by rhodocytin and a specific antibody to CLEC-2. *A, panel i*, washed platelet aggregation was measured following addition of 300 nm rhodocytin, 10 μ g/ml goat α -CLEC-2, or 10 μ g/ml goat IgG. Addition of agonist is indicated by an arrowhead. *Panel ii*, where indicated washed platelets were preincubated with the Src family kinase inhibitor PP2 (10 μ M) for 10 min prior to addition of agonist. In all experiments platelets were preincubated with IV.3 F(ab')₂ fragments (10 μ g/ml) for 5 min to block the Fc γ RIIA receptor. *B*, washed platelets were stimulated with 300 nm rhodocytin, 10 μ g/ml goat α -CLEC-2, or 10 μ g/ml goat IgG for 5 min in the presence of Itrafiban. Whole cell lysates (WCL) of unstimulated (*basal*) and stimulated platelets were prepared by addition of ice-cold lysis buffer. Proteins in whole cell lysates were visualized by SDS-PAGE and Western blotting (WB). The membrane was immunoblotted with α -phosphotyrosine antibody (pTyr). *C*, individual signaling proteins were immunoprecipitated (IP) from platelet whole cell lysates as indicated. The resulting immunoblots were probed for phosphotyrosine (pTyr, upper panel), stripped, and reprobed for corresponding protein (lower panel). The α -Syk antibody does not recognize the phosphorylated form of Syk. Data are representative of at least three experiments.

studying CLEC-2 signaling because B cells and T cells express many of the same signaling proteins as platelets, and mutants of both cell lines are available with deficiencies in the key signaling proteins. Transfection of CLEC-2 into DT40 cells and Jurkat cells led to expression at the cell surface as measured by flow cytometry, although it was absent from mock-transfected cells (Fig. 2A).

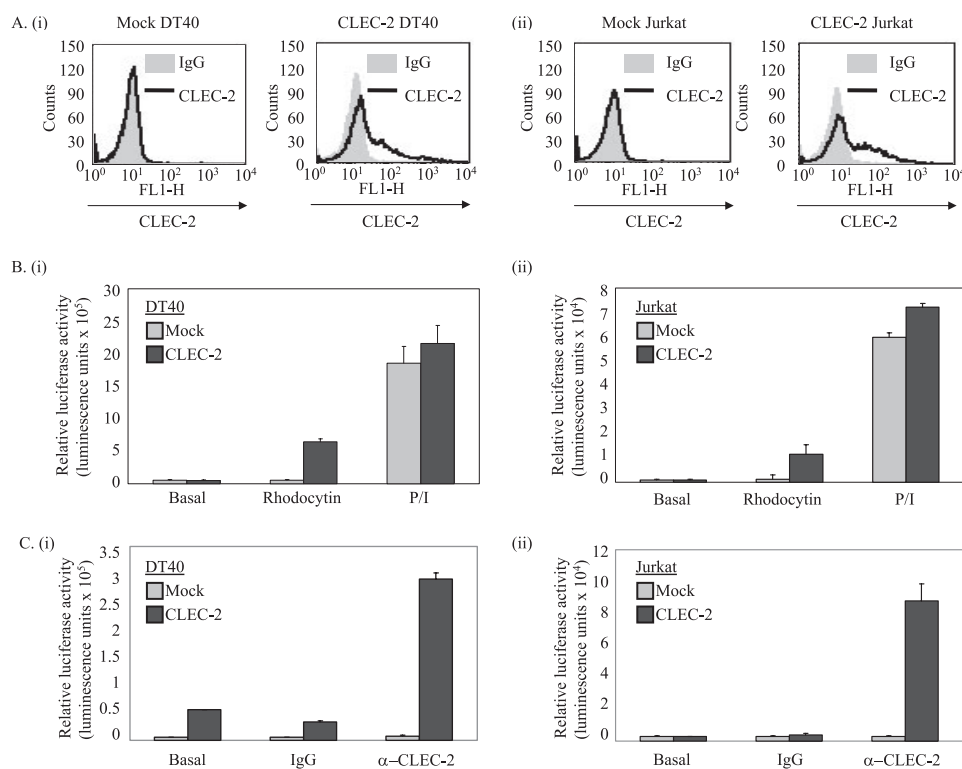


FIGURE 2. CLEC-2-expressing DT40 and Jurkat cells are activated by rhodocytin and anti-CLEC-2 antibody. Wild type DT40 chicken B cells and Jurkat T cells were transiently transfected with NFAT-luciferase reporter construct, β -galactosidase construct, and either pEF6-MYC-CLEC2 or empty pEF6 vector (*mock*). A, CLEC-2 expression in DT40 (*panel i*) and Jurkat cells (*panel ii*) was confirmed by flow cytometry using goat α -CLEC-2 antibody relative to a control goat IgG. B, mock- and CLEC-2-transfected DT40 (*panel i*) and Jurkat cells (*panel ii*) were stimulated with media alone (*Basal*), 50 nM rhodocytin, or 50 ng/ml PMA plus 1 μ M ionomycin (*P/I*). NFAT-luciferase activity was measured as a marker of PLC γ activation in a luminometer. All luciferase values were normalized to β -galactosidase values to control for transfection efficiency. Data are represented as mean luciferase value from one experiment. C, mock- and CLEC-2-transfected DT40 (*panel i*) and Jurkat cells (*panel ii*) were stimulated with media alone (*Basal*), 40 μ g/ml normal goat IgG, or 40 μ g/ml goat α -CLEC-2, and luciferase activity was measured as described above. Data are representative of at least three experiments.

The ability of CLEC-2 to activate PLC γ in DT40 and Jurkat cells was investigated by cotransfection with a luciferase reporter construct encoding the luciferase enzyme under control of NFAT/AP1 sites from the IL-2 promoter. This reporter construct is activated by protein kinase C and Ca²⁺, and the amount of luciferase activity is therefore a measure of PLC γ activity. Transfection of CLEC-2 conferred activation of luciferase in response to the CLEC-2 antibody and to rhodocytin (Fig. 2, B and C). Cells cotransfected with the reporter construct and empty pEF6 vector showed no response to rhodocytin or the CLEC-2 antibody, thereby confirming that activation is mediated by expression of CLEC-2 (Fig. 2, B and C). All cells exhibited a marked increase in expression of luciferase in response to the protein kinase C-activating phorbol ester PMA and the calcium ionophore ionomycin, which served as a positive control (Fig. 2B). These results demonstrate that neither DT40 nor Jurkat cells express functional, endogenous CLEC-2, and that expression of CLEC-2 is sufficient to confer responses to rhodocytin and the CLEC-2 antibody.

Tyrosine Phosphorylation of CLEC-2 Is Critical for Activation—We have shown previously that CLEC-2 becomes tyrosine-phosphorylated in platelets in a Src kinase-dependent manner following stimulation with rhodocytin (4). To investi-

gate the functional significance of CLEC-2 phosphorylation, we have expressed wild type CLEC-2 and a mutant of CLEC-2, Y7F, in which the cytosolic tyrosine has been replaced by phenylalanine, in DT40 cells. Rhodocytin stimulated an increase in whole cell tyrosine phosphorylation in DT40 cells expressing the wild type receptor through a pathway that was blocked by the Src kinase inhibitor PP2 (Fig. 3A). In contrast, no increase in tyrosine phosphorylation was seen in mock-transfected DT40 cells or in DT40 cells expressing the Y7F mutant of CLEC-2 in response to rhodocytin. Furthermore, rhodocytin was unable to support activation of PLC γ 2 in cells expressing the Y7F mutant, as measured in the luciferase assay (Fig. 3B). Importantly, in all of these studies, wild type and Y7F-CLEC-2 were expressed on the cell surface at a similar level (Fig. 3C). These results demonstrate that CLEC-2 requires the cytoplasmic tyrosine to mediate activation of PLC γ .

CLEC-2 Signals via Src, Syk, and Tec Family Kinases and PLC γ —The signaling pathway used by CLEC-2 was further investigated using mutant DT40 cells lacking key signaling proteins (30–33). In each

case CLEC-2 was transfected into mutant DT40 cells along with the luciferase reporter construct described above. CLEC-2 was expressed on the surface at a similar level in all of the mutant cells as confirmed by flow cytometry (data not shown). The ability of each transfectant to respond to PMA/ionomycin within the expected range was also confirmed in each experiment (data not shown). The results are shown as the fold increase over basal luciferase levels in each cell line.

CLEC-2-expressing wild type DT40 cells exhibited a robust increase in luciferase activity in response to rhodocytin, whereas cells deficient in Syk, Btk, or PLC γ 2 failed to respond (Fig. 4A), demonstrating a critical role for these proteins in mediating CLEC-2 signaling. In contrast, CLEC-2-expressing DT40 cells deficient in the major Src family kinase that is expressed in these cells, Lyn, exhibited a potentiated response to rhodocytin (Fig. 4B, *panel i*). This activation is also dependent on Syk because DT40 cells deficient in both Lyn and Syk do not respond to rhodocytin (Fig. 4B, *panel i*). This result may be due to a negative feedback role of Lyn, as described previously for the regulation of B cell receptor signaling (30, 38). Interestingly, a similar negative feedback role for Lyn has also been proposed in platelets stimulated by the ITAM receptor GPVI (39).

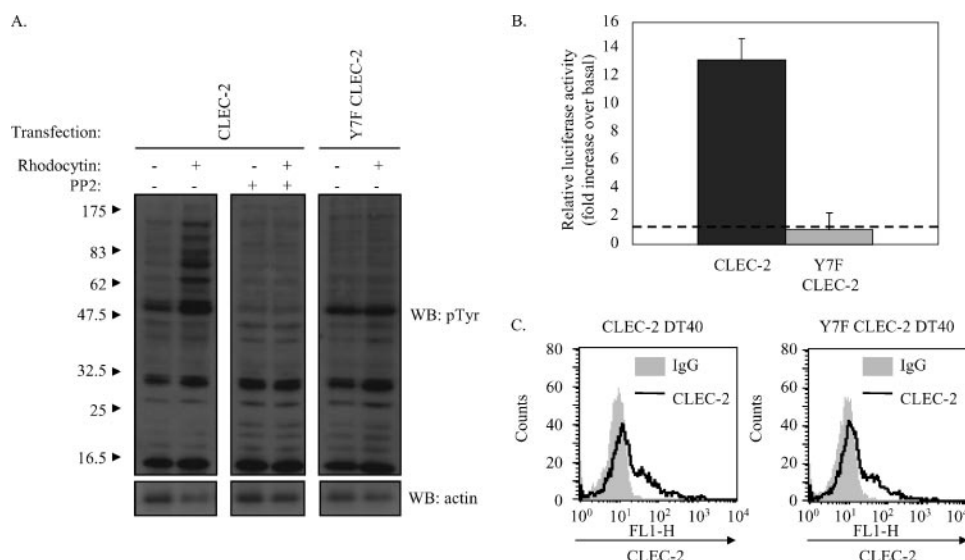


FIGURE 3. Role of CLEC-2 cytoplasmic tyrosine residue in signaling by the receptor. *A*, wild type DT40 cells were transfected with pEF6-MYC-CLEC-2 or pEF6-MYC-Y7FCLEC-2 and were stimulated with 500 nM rhodocytin for 10 min. Where indicated cells were preincubated with the Src kinase inhibitor PP2 (10 μ M) for 10 min. Basal and stimulated whole cell lysates were prepared by addition of ice-cold lysis buffer. Proteins were visualized by SDS-PAGE and Western blotting (WB). The membrane was immunoblotted with α -phosphotyrosine (pTyr). Membranes were stripped and re-probed with α -actin to confirm equal loading. *B*, luciferase assay was carried out on CLEC-2- and Y7FCLEC-2-expressing DT40 cells. Cells were stimulated with 50 nM rhodocytin. Data are expressed as fold increase over basal. *C*, expression of CLEC-2 in CLEC-2- and Y7F-CLEC-2-transfected cells was confirmed by flow cytometry with goat α -CLEC-2 antibody relative to a control goat IgG. Data are representative of at least three experiments.

cell Src family kinase Lck (34). Importantly, Lck is believed to be the only member of the Src family of tyrosine kinases in these cells and does not mediate feedback inhibition of PLC γ regulation. As a control for Lck deficiency, we have used JCaM1 cells stably transfected with wild type Lck (JCaM1/Lck(+)) (34). It was necessary to use Lck-transfected JCaM1 cells as a control in view of the possibility that these cells, which were originally made by chemical mutagenesis (34), have additional unidentified defects. JCaM1/Lck(+) cells transfected with CLEC-2 exhibited an increase in luciferase activation in response to rhodocytin, whereas JCaM1 cells transfected with CLEC-2 failed to respond (Fig. 4*B*, panel ii). Together these results demonstrate that CLEC-2 signaling is critically dependent on Src, Syk, and Tec family kinases and on PLC γ .

Both SH2 Domains of Syk Are

Required for Binding and Signaling Downstream of CLEC-2—We have previously reported that fusion proteins corresponding to the tandem SH2 domains of Syk associate with phosphorylated CLEC-2 in platelet lysates (4). Similarly, a phosphopeptide corresponding to the CLEC-2 cytoplasmic tail is able to pull down Syk from platelet lysates (4). To investigate whether this interaction requires either or both of the SH2 domains of Syk, we have used recombinant N-terminal, C-terminal, and tandem SH2 domains of Syk to precipitate CLEC-2 from platelet lysates. Strikingly, both SH2 domains were required for precipitation of CLEC-2 as shown in Fig. 5*A*, thereby suggesting that both SH2 domains of Syk are required for the interaction with the lectin receptor. To confirm this conclusion, we introduced inactivating point mutations into the SH2 domains of Syk by mutating either Arg-37 or Arg-190 of porcine Syk to alanine residues. The residues were identified by alignment with rat, murine, and human Syk sequences (Fig. 5*B*). In addition, mutation of these residues, in combination with neighboring amino acids, has been demonstrated previously to inactivate the SH2 domains of porcine Syk and to block the ability of porcine Syk to reconstitute signaling in the chicken cell line DT40 (30). Mutation of the corresponding residues in rat Syk has been reported previously to be required for Syk binding to phosphorylated tyrosine residues in ITAMs. In both cases these mutations have been demonstrated to render the SH2 domains inactive without affecting the autoactivated kinase activity of the enzyme (30, 40–42). In agreement with this, constructs for R37A and R190A porcine Syk translate a full-length Syk (Fig. 5*C*) that induces a degree of basal signaling when overexpressed in DT40 cells (data not shown). Syk-deficient DT40 cells were cotransfected with wild type or mutant Syk and CLEC-2. In each case, expression of CLEC-2 was confirmed by flow cytometry and was of an equiv-

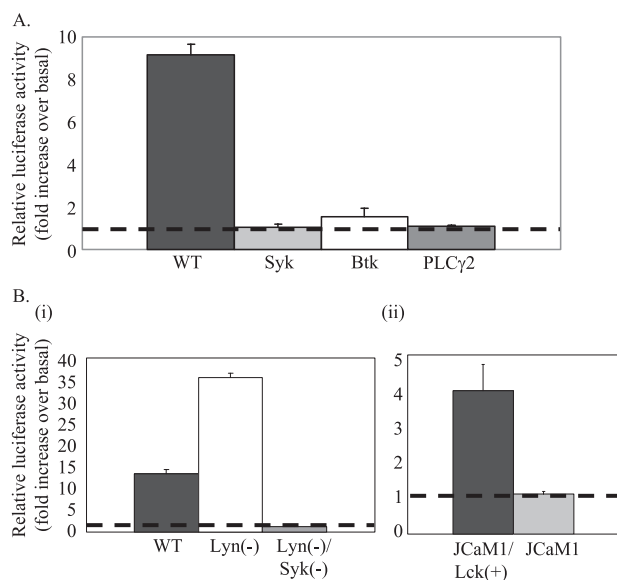
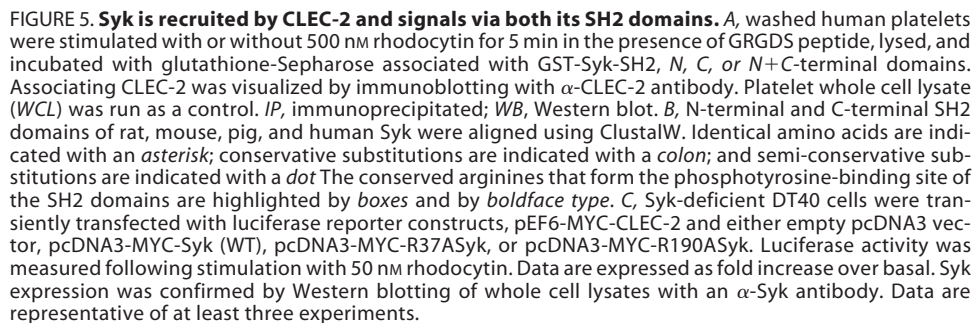


FIGURE 4. Role of Src, Syk, and Btk family kinases downstream of CLEC-2. *A*, wild type (WT) DT40 cells or DT40 cells engineered to lack indicated signaling proteins (Syk, Btk, or PLC γ 2) were transiently transfected with pEF6-MYC-CLEC-2. Signaling to PLC γ was assessed by measuring luciferase activity following stimulation with 50 nM rhodocytin. Data are expressed as fold increase over basal. *B*, panel i, wild type DT40 cells and cells deficient in Lyn (Lyn(-)) or Lyn and Syk (Lyn(-)/Syk(-)) were transiently transfected with pEF6-MYC-CLEC-2, and luciferase activity was measured as described above following stimulation with 50 nM rhodocytin. Data are expressed as fold increase over basal. *Panel ii*, Jurkat cells deficient in Lck (JCaM1) and JCaM1 cells stably expressing Lck (JCaM1/Lck(+)) were transiently transfected with pEF6-MYC-CLEC-2, and luciferase activity was measured following stimulation with 50 nM rhodocytin. Data are expressed as fold increase over basal. Data are representative of at least three experiments.

In view of the potentiation observed in the absence of the Src family kinase Lyn in DT40 cells, we extended these studies to the Jurkat-derived cell line JCaM1, which is deficient in the T



Differential Regulation of BLNK/SLP-76 Downstream of GPVI and CLEC-2—Signaling downstream of ITAM receptors is critically dependent on the SLP-76/BLNK family of adapter proteins (43–46). A notable difference between the signaling cascade used by the platelet ITAM receptor GPVI and by the CLEC-2 ligand rhodocytin is the ability of high concentrations of rhodocytin to overcome the blockade caused by the absence of SLP-76 (4). This difference could be due to the ability of rhodocytin to activate other receptors in platelets, such as integrin $\alpha 2\beta 1$ or GPIIb α (47) or because the adapter plays a partial role in signaling by the lectin receptor. Thus there may be a fundamental difference between the contribution of the SLP-76/BLNK family of adapter proteins to signaling downstream of CLEC-2 relative to ITAM receptors.

To compare the role of BLNK and SLP-76 in signaling by GPVI and CLEC-2, we have transfected CLEC-2 or GPVI and Fc γ -chain into mutant DT40 and Jurkat cells lacking BLNK and SLP-76, respectively (32, 35). Stimulation of wild type DT40 cells expressing the GPVI/FcR γ -chain complex with the snake venom toxin convulxin resulted in a robust increase in luciferase activity, which was totally abrogated in the absence of BLNK (Fig. 6A). In comparison, rhodocytin generated a significant but diminished ($\sim 30\%$ of wild type) response in the absence of BLNK

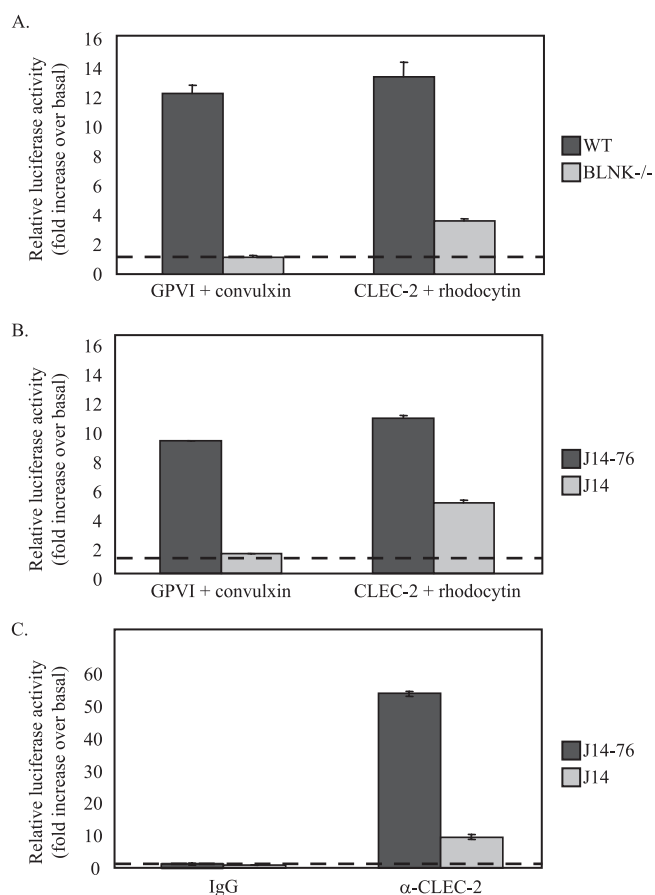


FIGURE 6. BLNK/SLP-76 plays a differential role in signaling by GPVI and CLEC-2. A, wild type (WT) or BLNK-deficient (BLNK^{-/-}) DT40; B, SLP-76-deficient Jurkat (J14) or J14 reconstituted with SLP-76 (J14-76) cells were transfected with either pcDNA3-GPVI and pEF6-FcR γ -chain or pEF6-MYC-CLEC-2 along with the luciferase reporter construct. GPVI-expressing cells were stimulated with 10 μ g/ml convulxin and CLEC-2-expressing cells with 50 nm rhodocytin. C, SLP-76-deficient (J14) or J14 reconstituted with SLP-76 (J14-76) cells were transfected with pEF6-MYC-CLEC-2 and the luciferase reporter construct and stimulated with 40 μ g/ml goat α -CLEC-2 antibody or negative control goat IgG. Luciferase activity was measured following stimulation and is expressed as fold increase over basal. Data are representative of at least three experiments.

even though it stimulated a similar increase to that induced by convulxin in wild type cells (Fig. 6A). A similar set of observations was made in the Jurkat-derived cell line J14, which lacks SLP-76. J14 cells stably transfected with SLP-76 were used as a control (J14-76) for these studies as these cells were also made by chemical mutagenesis, which could have introduced additional genetic modifications. SLP-76-expressing cells transfected with CLEC-2 or GPVI/FcR γ -chain give similar robust responses to rhodocytin and convulxin, respectively (Fig. 6B). The response to convulxin was abolished in the absence of SLP-76, whereas the response to rhodocytin was reduced by 60%. To further confirm that the partial dependence on BLNK/SLP-76 was not because of rhodocytin binding to another receptor on the cell lines, we have investigated whether CLEC-2 signaling induced by a CLEC-2-specific antibody was able to bypass SLP-76 in Jurkat cells. J14 and J14-76 cells were transfected with CLEC-2 and stimulated with the anti-CLEC-2 antibody. In response to CLEC-2 antibody, CLEC-2 signaling is significantly reduced in the absence of SLP-76, but the receptor is still able to

signal without this adapter (Fig. 6C). These data therefore demonstrate a fundamental difference in signaling by CLEC-2 and the ITAM receptor, GPVI-FcR γ -chain complex, in that activation by the lectin receptor is only partially dependent on the SLP-76/BLNK family of adapter proteins.

The Lectin Receptor Dectin-1 Signals through a Similar Pathway to CLEC-2—Dectin-1 is also a member of the C-type lectin receptor family, which has recently been shown to activate Syk via a single YXXL motif in its cytosolic tail in both dendritic cells and in macrophages (16, 48). However, the signaling events downstream of Syk activation in response to Dectin-1 stimulation have not been characterized. A series of studies was therefore undertaken in transfected DT40 and Jurkat cells to compare the signaling pathway used by Dectin-1 to that used by CLEC-2 using zymosan as the activating ligand.

Transfection of DT40 and Jurkat cells with Dectin-1 leads to expression of the lectin receptor on the cell surface as measured by flow cytometry using a goat α -Dectin-1 antibody (Fig. 7A). In comparison, there was no specific binding of the antibody to mock-transfected cells suggesting that Dectin-1 is not endogenously expressed on either cell line (Fig. 7A). Transfection of Dectin-1 conferred marked activation of luciferase in response to zymosan in DT40 cells (Fig. 7B) and in Jurkat T cells (data not shown), although there was no response in mock-transfected cells confirming the absence of expression of endogenous receptor (Fig. 7B and data not shown). Stimulation with PMA and ionophore stimulated robust activation of luciferase in Dectin-1 and mock-transfected DT40 (Fig. 7B) and Jurkat cells (data not shown), thereby confirming cell viability. These observations demonstrate that expression of Dectin-1 confers signaling responses to zymosan in both DT40 and Jurkat cell lines.

To investigate if the Dectin-1 signaling pathway shares the same characteristics as for CLEC-2, the lectin receptor was transfected into DT40 cells deficient in Syk, BLNK, Btk, or PLC γ 2 and in Jurkat cells deficient in the Src kinase Lck (JCaM1) or SLP-76 (J14). In all cases, Dectin-1 was cotransfected with the luciferase reporter construct, and luciferase activity was measured following stimulation with zymosan. Flow cytometry studies confirmed that similar levels of Dectin-1 were expressed in each cell line (data not shown).

As observed with CLEC-2, Dectin-1 signaling was completely inhibited in the absence of Syk, Btk, and PLC γ 2 in DT40 cells (Fig. 7C, panel i) and in the absence of Lck in JCaM1 cells (Fig. 7C, panel ii). Furthermore, Dectin-1 signaling was partially but not fully dependent on the adapters BLNK and SLP-76 in DT40 and Jurkat cells, respectively (Fig. 7C, panels i–iii). In both cases, the response to zymosan was ~40% of that in the control (Fig. 7C, panels i–iii). Dectin-1 signaling was also markedly inhibited in Syk-deficient DT40 cells transfected with either of the SH2 domain mutants of Syk described above relative to the response seen with transfection of wild type Syk (Fig. 7D, panel i). Mutation of the N-terminal (R37A) or C-terminal (R190A) SH2 domain of Syk reduced the response to Dectin-1 by ~80% in both cases (Fig. 7D, panel i). Expression of similar amounts of Syk and the two Syk mutants in the DT40 cells was confirmed by Western blotting (Fig. 7D, panel ii). The marked but partial

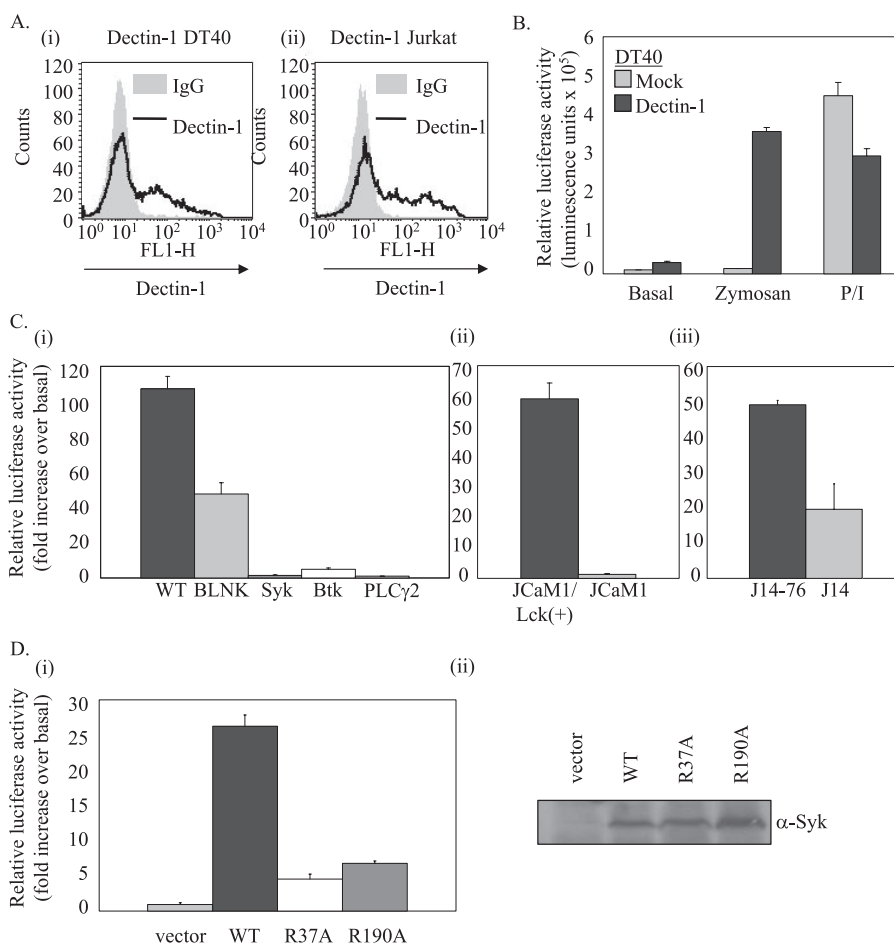


FIGURE 7. Role of Src, Syk, and Tec kinases, PLC γ , and SLP-76/BLNK in Dectin-1 signaling. Wild type DT40 chicken B cells and Jurkat T cells were transiently transfected with NFAT-luciferase reporter construct, β -galactosidase construct, and either pEF6-MYC-Dectin-1 or empty pEF6 vector (*mock*). **A**, Dectin-1 expression in DT40 (*panel i*) and Jurkat cells (*panel ii*) was confirmed by flow cytometry using a goat α -Dectin-1 antibody relative to a negative control goat IgG. **B**, mock- and Dectin-1-transfected DT40 cells were stimulated with media alone (*Basal*), 250 μ g/ml zymosan, or 50 ng/ml PMA plus 1 μ M ionomycin (*P/I*). NFAT-luciferase activity was measured. All luciferase values were normalized to β -galactosidase values. Data are represented as mean luciferase value. **C**, *panel i*, wild type DT40 cells or DT40 cells deficient in BLNK, Syk, Btk, or PLC γ 2; *panel ii*, Jurkat cells deficient in Lck (*JCaM1*) and JCaM1 cells stably expressing Lck (*JCaM1/Lck(+)*); or *panel iii*, SLP-76-deficient Jurkat (*J14*) or J14 reconstituted with SLP-76 cells (*J14-76*) were transfected with pEF6-MYC-Dectin-1 along with the luciferase reporter construct. Cells were stimulated with 250 μ g/ml zymosan. Luciferase activity was measured following stimulation and is expressed as fold increase over basal. **D**, *panel i*, Syk-deficient DT40 cells were transiently transfected with luciferase reporter constructs, pEF6-Dectin-1, and either empty pcDNA3 vector, pcDNA3-MYC-Syk (WT), pcDNA3-MYC-R37ASyk, or pcDNA3-MYC-R190ASyk. Luciferase activity was measured following stimulation with zymosan. Data are expressed as fold increase over basal. *Panel ii*, Syk expression was confirmed by Western blotting of whole cell lysates with an α -Syk antibody. Data are representative of at least three experiments.

inhibition of response to Dectin-1 observed with the Syk SH2 domain mutants contrasts with the complete abolition of response to CLEC-2 (Fig. 5C).

These results demonstrate that CLEC-2 and Dectin-1 signal through a similar signaling cascade, which can be distinguished from that used by ITAM receptors through the partial dependence on the SLP-76/BLNK family of adapter proteins. The CLEC-2 and Dectin-1 signaling pathways can be distinguished from each other by their complete or partial dependence on the phosphotyrosine-binding properties of the Syk SH2 domains.

DC-SIGN Cross-linking Results in PLC γ Activation, Independent of Tyrosine Phosphorylation of its YXXL Motif—Activation of a third member of the C-type lectin family, DC-SIGN, with specific antibodies has been reported to lead to ERK1/2,

Akt, and PLC γ phosphorylation and increases in intracellular calcium in dendritic cells (17). DC-SIGN also contains a single YXXL motif in its cytoplasmic tail, although it is not known whether this undergoes tyrosine phosphorylation upon activation. Experiments were therefore designed to compare the signaling pathway used by DC-SIGN with that of Dectin-1 and CLEC-2 and to investigate whether this requires the YXXL motif of DC-SIGN.

Jurkat cells cotransfected with the luciferase reporter construct and DC-SIGN conferred a marked increase in luciferase activity over mock-transfected cells in response to cross-linking with an antibody to DC-SIGN, whereas there was no increase in response to an isotype-matched negative control (Fig. 8A). Strikingly, cells transfected with a mutant form of DC-SIGN, in which the Tyr residue of the YXXL sequence has been replaced by phenylalanine (Y31F DC-SIGN), gave a slightly greater response upon cross-linking of DC-SIGN relative to cells transfected with wild type receptor (Fig. 8A). Expression of similar levels of the YXXL mutant of DC-SIGN and wild type DC-SIGN was confirmed by flow cytometry (data not shown). All cells exhibited a similar increase in expression of luciferase in response to PMA and ionomycin (data not shown). These results demonstrate that DC-SIGN signals through a distinct pathway to that used by CLEC-2 and Dectin-1.

To investigate the role of Src kinases in signaling by DC-SIGN, we transfected the Lck-deficient Jurkat cell line JCaM1 with DC-SIGN and stimulated them with α -DC-SIGN antibody as above. JCaM1/Lck(+) cells were used as a control. DC-SIGN antibody induced a robust signal in JCaM1/Lck(+) cells, whereas in JCaM1 cells DC-SIGN was unable to signal (Fig. 8B). Treatment of either cell line with a nonspecific IgG did not induce signaling. These results demonstrate that although DC-SIGN signaling is independent of the YXXL motif of the receptor, it does require Src family kinases. Together these data demonstrate a marked difference in the mechanism of signaling of the C-type lectin receptor DC-SIGN relative to that used by the C-type lectin receptors CLEC-2 and Dectin-1 in that signaling by the former is independent of its YXXL motif.

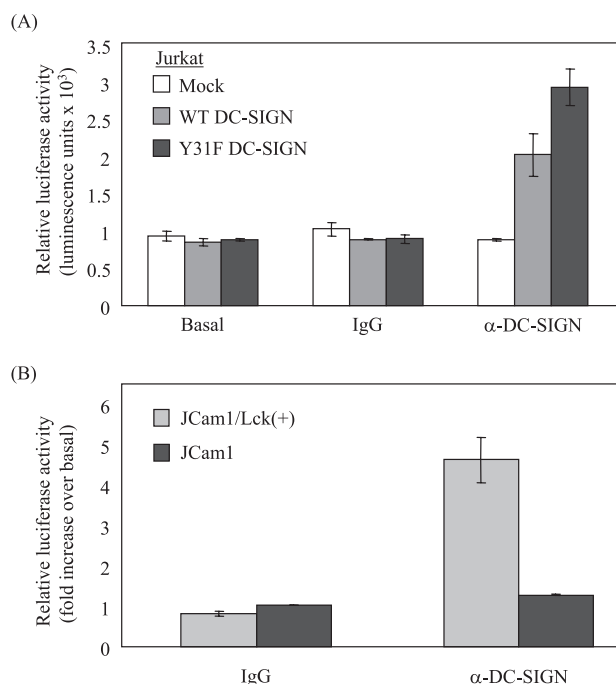


FIGURE 8. DC-SIGN signaling is not dependent on the YXXL motif. A, Jurkat cells were transiently transfected with NFAT-luciferase reporter construct, β -galactosidase construct, and either pcDNA3-DC-SIGN, pcDNA3-Y31FDC-SIGN, or empty pcDNA3 vector (*mock*). Cells were stimulated with media alone (*Basal*), α -DC-SIGN (10 μ g/ml) cross-linked with sheep α -mouse F(ab')₂ fragments (α -DC-SIGN), or control mouse IgG (10 μ g/ml) cross-linked with sheep α -mouse F(ab')₂ fragments (*IgG*). NFAT-luciferase activity was measured. Data are represented as mean luciferase value from one experiment, representative of at least three experiments. B, JCam1/Lck(+) cells and JCam1 cells were transfected with NFAT-luciferase reporter construct, β -galactosidase construct, and pcDNA3-DC-SIGN. Cells were stimulated with media alone (*Basal*), α -DC-SIGN, or control mouse IgG as above, and NFAT-luciferase activity was measured. All luciferase values were normalized to β -galactosidase values. Data are represented as fold increase over basal from one experiment and representative of at least three experiments.

CLEC-2 Signaling Is Dependent on the DEDG Sequence Preceding Its YXXL Motif—Because CLEC-2 and Dectin-1 are both able to signal via their YXXL motifs and DC-SIGN is not, we have compared the sequences flanking the YXXL in all three receptors (Table 1). CLEC-2 and Dectin-1 have four identical amino acids preceding their YXXL motifs, DEDG, whereas DC-SIGN does not have this sequence. To investigate the role of these amino acids in signaling by CLEC-2, we have made a series of mutants, substituting each amino acid in turn for alanine. These mutants were cotransfected into DT40 cells with the NFAT-luciferase reporter construct. Cells were stained with α -CLEC-2 antibody and analyzed for receptor expression by flow cytometry. They were then stimulated with rhodocytin and signaling measured by luciferase assay. D3A, E4A, and G6A were expressed at the surface of the cells at similar levels to WT CLEC-2 (Fig. 9A). However, D5A CLEC-2 was not detected at the cell surface (Fig. 9A). In response to rhodocytin, D3A and E4A CLEC-2 responded to the same degree as WT CLEC-2 (Fig. 9B). D5A CLEC-2-transfected cells did not respond to rhodocytin consistent with the lack of surface expression of this mutant. Strikingly, signaling by the G6A mutant of CLEC-2 is significantly reduced to ~25% of WT CLEC-2.

To investigate if D5A CLEC-2 is retained in an intracellular compartment, WT CLEC-2 and D5A CLEC-2 were transfected

TABLE 1

C-type lectin receptors containing YXXL motifs

Signaling	Name	Sequence	Cell types ^a
Activatory	CLEC-2	DEDGYITL	Neutrophils, Mo, DC, platelets
	Dectin-1	DEDGYTQL	DC, M ϕ
Inhibitory	MAFA-L	DSVTYSML	Basophils, NK
	MICL	EEVTYADL	Neutrophils, Mo
	CD72	EAITYADL	B
Unknown	CD23	EEGQYSEI	B, Mo, T, DC, NK, LC, eosinophils, platelets
	NKp80/KLRF1	DEERYMTL	NK, T
	DC-SIGN	QTRGYKSL	DC
	ASGPR	MTKEYQDL	iDC, M ϕ
	HNKR-P1A/CD161	QQATYAEI	NK, T

^a The abbreviations used are as follows: Mo, monocytes; DC, dendritic cells (iDC, immature); M ϕ , macrophages; NK, natural killer cells; T, T cells; B, B cells; LC, Langerhans cells.

into 293T cells, and whole cell lysates were Western-blotted for CLEC-2. 293T cells were used for these experiments because they tolerate high levels of exogenous protein expression therefore facilitating analysis by Western blotting. Consistent with the results in DT40 cells, CLEC-2 was detectable on the surface of 293T cells transfected with WT CLEC-2 but not on the surface of mock-transfected or D5A CLEC-2-transfected cells. CLEC-2 was detectable by Western blot in 293T cells transfected with WT CLEC-2 but not in cells transfected with empty vector or D5A CLEC-2. The multiple immunoreactive bands in the cells transfected with WT CLEC-2 likely reflect a combination of different glycosylation states (4) and breakdown products of the receptor.

Together, these results demonstrate that aspartate 5 and glycine 6 are important for CLEC-2 signaling. Whether the analogous residues of Dectin-1 are important for signaling and how these residues effect CLEC-2 and Dectin-1 signaling are worthy of further investigation.

DISCUSSION

In this study, we have used a specific antibody to CLEC-2 and the snake venom toxin rhodocytin to dissect the signaling pathway used by the lectin receptor in a physiologically relevant system, the platelet, and in two transfected cell lines. We have further compared the molecular basis of signaling by CLEC-2 with that of the two C-type lectin receptors Dectin-1 and DC-SIGN, both of which have a YXXL motif in their cytosolic tail. The results support a model in which CLEC-2 and Dectin-1 signal through Src, Syk, and Tec tyrosine kinases leading to activation of PLC γ downstream of tyrosine phosphorylation of the YXXL motif. In striking contrast, signaling by DC-SIGN is independent of its YXXL motif.

The signaling pathway used by CLEC-2 and Dectin-1 resembles that regulated by ITAM receptors in hematopoietic cells. However, the signaling pathway used by the two lectin receptors can be distinguished from that used by ITAM receptors by its partial rather than absolute dependence on the SLP-76/BLNK family of adapter proteins and by the presence of a single rather than dual YXXL motif within their cytoplasmic tail. Many of the signaling proteins used by CLEC-2 and Dectin-1 also participate in the regulation of PLC γ 2 by integrin receptors in hematopoietic cells, but the integrin pathway can be distinguished by the complete dependence on activation of the

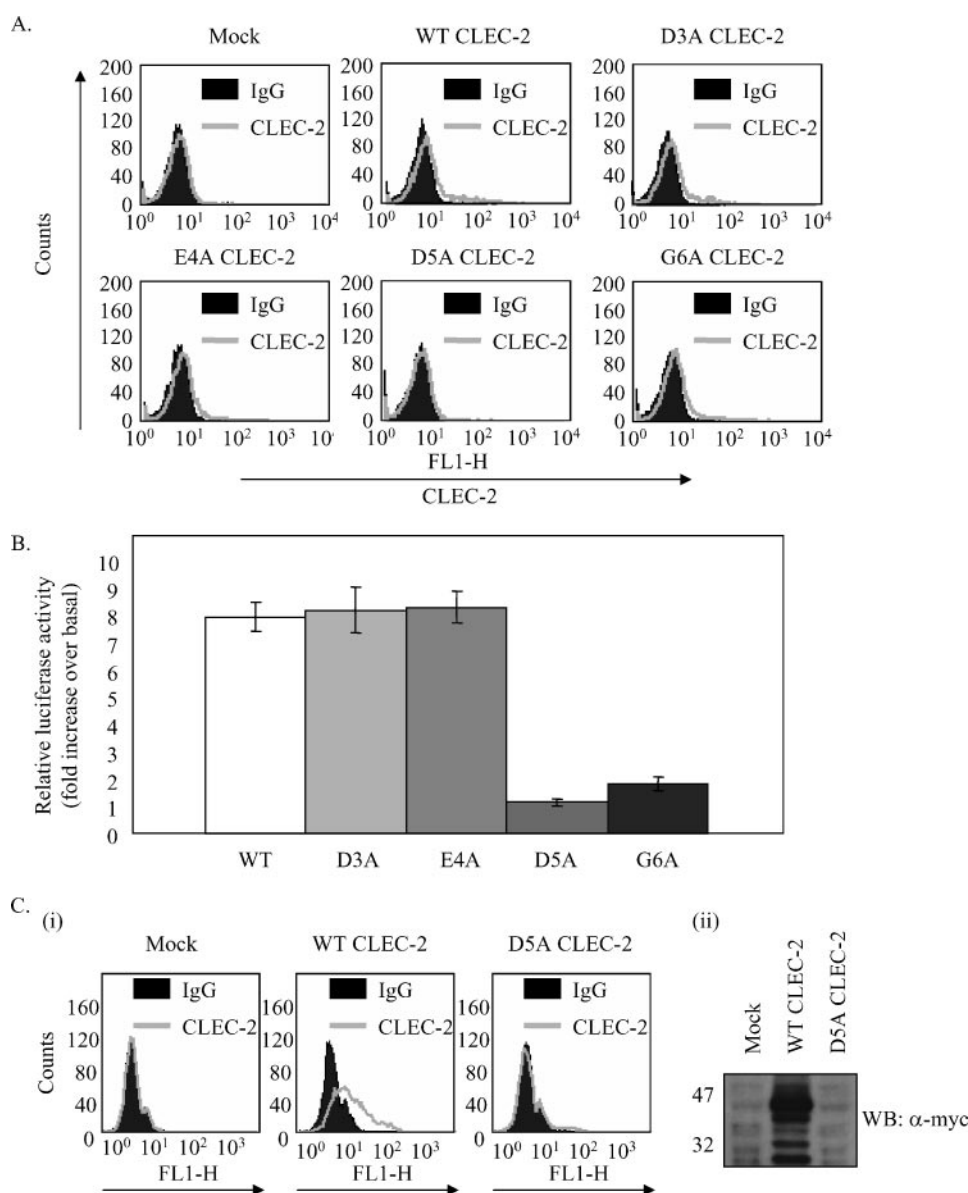


FIGURE 9. CLEC-2 signaling is dependent on the DEDG sequence preceding its YXXL motif. Wild type DT40 cells were transfected with empty pEF6 vector (*mock*), pEF6-MYC-CLEC-2, pEF6-MYC-D3ACLEC-2, pEF6-MYC-E4ACLEC-2, pEF6-MYC-D5ACLEC-2, or pEF6-MYC-G6ACLEC-2 together with the NFAT-luciferase reporter and β -galactosidase constructs. *A*, CLEC-2 expression was assessed by flow cytometry with goat α -CLEC-2 antibody relative to a negative control goat IgG. *B*, luciferase assay was carried out on the transfected cells stimulated with 50 nM rhodocytin. Data are expressed as fold increase over basal. *C*, 293T cells were transfected with empty pEF6 vector (*mock*), pEF6-MYC-CLEC-2, or pEF6-MYC-D5ACLEC-2. Expression of CLEC-2 was assessed by flow cytometry (*panel i*) using goat α -CLEC-2 antibody relative to negative control goat IgG or by Western blotting (WB) (*panel ii*) whole cell lysates with α -MYC monoclonal antibody. Data are representative of at least three experiments.

TABLE 2
ITAM receptor motifs

Group ^a	Name	Sequence
BCR	CD79a	DENLYEGL-----NLDDCSMYEDI
	CD79b	EDHTYEGL-----DIDQTATYEDI
TCR	ζ chain 1	QNQLYNEL-----NLGRREEYDVL
	ζ chain 2	QEGLYNEL-----QKDKMAEAYSEI
	ζ chain 3	HDGLYQGL-----STARKDTYDAL
	CD3 γ	NDQLYQPL-----KDREDDQYSHL
FcR	CD3 δ	NDQVYQPL-----RDRDDAQYSHL
	CD3 ϵ	PNPDYEP-----RKGQRDLVSGL
	FcR γ -chain	SDGVYTG-----STRNQETVETL
Others	Fc γ RIIA	ADGGYMTLNPRAPTDKNNIYLT
	Dap-12	TESPYQEL-----QQRSDVYSDL

^a The abbreviations used are as follows: BCR, B cell receptor; TCR, T cell receptor; FcR, Fc receptor.

BLNK/SLP-76 family of adapters and by the fact that integrin receptors regulate Syk independent of a YXXL or an equivalent motif (49).

For ITAM receptors, phosphorylation of the two tyrosines in the dual YXXL motif is essential for activation of the Syk/ZAP-70 family of tyrosine kinases by virtue of binding to their tandem SH2 domains. Mutagenesis studies in T cells have shown that activation of the Syk family kinase ZAP-70 by the T cell receptor requires a doubly phosphorylated ITAM to bind the tandem SH2 domains of ZAP-70 (7, 8, 50). The individual SH2 domains of ZAP-70 do not bind appreciably with phosphorylated ITAM peptides (51) and the tandem SH2 domains of ZAP-70 bind to mono-phosphorylated ITAM peptides with a 100–1000-fold lower affinity than to the corresponding doubly phosphorylated peptides (52). Similar studies in B cells have shown that a small degree of Syk activation can be seen following phosphorylation of the N-terminal ITAM tyrosine alone, but marked activation of Syk requires phosphorylation of both ITAM tyrosine residues and the integrity of both Syk SH2 domains (40, 41, 53). The coupling of CLEC-2 and Dectin-1 to Syk is also dependent on tyrosine phosphorylation of the YXXL motif within their cytosolic tail (present study and see Refs. 16 and 54), but it is not known how a single YXXL motif is sufficient to cause robust activation of the Syk family of tyrosine kinases.

One of the striking features of the YXXL motifs in the cytosolic tails of CLEC-2 and Dectin-1 is conservation of the four upstream amino acids, namely DEDG. Moreover, although eight other C-type lectin receptors contain a cytosolic YXXL sequence, none of these contain the preceding sequence DEDG (Table 1). The sequence DEDG YXXL is also absent from ITAM receptors, although an acidic residue is often found three amino acids upstream of the first ITAM tyrosine (Table 2), and from ITIM receptors (not shown).

The alanine scan of the DEDG sequence in CLEC-2 in this study implicates the Asp-5 and Gly-6 residues as playing an important role in CLEC-2 stability and signaling. These studies suggest that the upstream amino acids are important in allowing the YXXL to confer signaling and that the YXXL motif alone

is not sufficient to confer the ability to signal. Consistent with this is the observation in this study that DC-SIGN does not signal via its YXXL motif. These observations raise the possibility that the sequence DEDGYXXL is sufficient to activate the Syk family of tyrosine kinases. Studies are currently underway to investigate this possibility.

An unexpected observation in this study was that only the Syk tandem SH2 domains and not the single SH2 domains were able to precipitate CLEC-2 from stimulated platelets and that site-directed mutagenesis of the single SH2 domains in Syk abrogated signaling by CLEC-2 and inhibited that of Dectin-1 by ~80%. The crystal structure of ZAP-70 interacting with an ITAM peptide maps the binding sites for the ITAM tyrosine residues to the ZAP-70 SH2 domains (55). The binding site for one of the tyrosines of the ITAM lies within the C-terminal SH2 domain of the kinase, whereas the binding site for the other ITAM-tyrosine lies in the interface between the two SH2 domains. The observation that association of the CLEC-2 YXXL motif with Syk requires both SH2 domains could be explained by the binding taking place in the interface between the two SH2 domains of Syk, as is the case for ZAP-70. However, this does not explain the abolition or marked reduction in signaling observed following the introduction of point mutations into the N-terminal and C-terminal SH2 domains of Syk. It is possible that the two SH2 domains of Syk bind to two CLEC-2 receptors and that this is necessary for its activation. However, if this were the case it would be expected that either SH2 domain of Syk alone would bind CLEC-2, and this does not seem to be the case. Crystallization and structural studies of the signaling complex between Syk and tyrosine-phosphorylated CLEC-2 or Dectin-1 would provide valuable information on this.

A further surprising difference between the downstream signaling pathway of the lectin receptors and that used by ITAM receptors is their dependence on the SLP-76/BLNK adapter family. Signaling through the GPVI/FcR γ -chain complex is completely abrogated in SLP-76- and BLNK-deficient Jurkat and DT40 cells, respectively, whereas the response to CLEC-2 or Dectin-1 is only reduced by 60–70% in both cases. Further examples of the absolute dependence of ITAM signaling on SLP-76/BLNK include the complete block in T cell development in SLP-76-deficient mice as a result of a failure of signal transduction through the pre-TCR (44, 45) and abrogation of B cell signaling in the absence of the SLP-76 homologue BLNK (46). It is therefore of interest to establish the way in which the signaling pathway used by CLEC-2 and Dectin-1 differs from that used by ITAM receptors. It is possible that spatial separation of signaling pathways in membrane microdomains such as membrane rafts or tetraspanin webs (56) may be responsible for this difference. Alternatively, CLEC-2 and Dectin-1 may be capable of using one or more adapter proteins that function as alternatives to SLP-76 or BLNK, such as Clnk or Slnk (57). Interestingly, a very recent study has also highlighted a difference in the proteins used by ITAM receptors and Dectin-1 in regulating NF- κ B in immune cells (58). The caspase recruitment domain family adapter protein, Card9, plays a critical role in the activation of NF- κ B by Dectin-1 but is dispensable for NF- κ B regulation by the T cell and B cell antigen receptors (58).

Further analysis of the signaling pathways activated downstream of these receptors may lead to identification of other differences in signaling by this class of receptor and novel signaling proteins.

The ability of CLEC-2 to activate platelets represents a novel role for this family of proteins in platelet function. To date, C-type lectin receptors are known to play important roles in pathogen recognition within the immune system. For example, Dectin-1 is known to recognize β -glucan-bearing pathogens and is responsible for phagocytosis of various fungi into macrophages (59–61). In addition to its role in pathogen internalization, Dectin-1 has been shown to initiate signaling responses leading to production of cytokines IL-2 and IL-10 in dendritic cells and reactive oxygen species in macrophages (16, 48). Therefore, the possibility that CLEC-2 may also bind one or more exogenous ligands such as bacterial or yeast envelope proteins or viral coat proteins, as well as rhodocytin, is worthy of consideration. Indeed we have recently reported a role for CLEC-2 in platelet binding to human immunodeficiency virus and transmission of the virus to permissive cells in culture (62). It is noteworthy that many systemic infections such as sepsis or human immunodeficiency virus result in platelet-based complications such as thrombocytopenia or thrombosis (63, 64). The role of CLEC-2 in mediating responses of platelets and other blood cells to pathogens and its role in infection-induced hemostatic complications is therefore worthy of investigation.

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