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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 7, pp. 5127–5135, February 15, 2013

Critical Role for an Acidic Amino Acid Region in Platelet Signaling by the HemITAM (Hemi-immunoreceptor Tyrosine-based Activation Motif) Containing Receptor CLEC-2 (C-type Lectin Receptor-2)*[S]

Received for publication, August 17, 2012, and in revised form, December 12, 2012 Published, JBC Papers in Press, December 21, 2012, DOI 10.1074/jbc.M112.411462

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Background: CLEC-2 signals through a cytosolic Y*XX*L downstream of a triacidic amino acid sequence (hemITAM).
Results: We show a critical role for the triacidic amino acid in phosphorylation of the cytosolic tyrosine and that CLEC-2 signals via Syk but not Zap-70.
Conclusion: CLEC-2 signaling requires a cytosolic triacidic amino acid sequence and Syk.
Significance: Proximal signaling events by hemITAM receptors are distinct to those in ITAM receptors.

CLEC-2 is a member of new family of C-type lectin receptors characterized by a cytosolic Y*XX*L downstream of three acidic amino acids in a sequence known as a hemITAM (hemi-immunoreceptor tyrosine-based activation motif). Dimerization of two phosphorylated CLEC-2 molecules leads to recruitment of the tyrosine kinase Syk via its tandem SH2 domains and initiation of a downstream signaling cascade. Using Syk-deficient and Zap-70-deficient cell lines we show that hemITAM signaling is restricted to Syk and that the upstream triacidic amino acid sequence is required for signaling. Using surface plasmon resonance and phosphorylation studies, we demonstrate that the triacidic amino acids are required for phosphorylation of the Y*XX*L. These results further emphasize the distinct nature of the proximal events in signaling by hemITAM relative to ITAM receptors.

The C-type lectin receptor CLEC-2[4] is highly expressed on platelets and at lower levels on subpopulations of hematopoietic cells, including mouse neutrophils (1–3). Platelet CLEC-2 is required for the separation of the vascular and lymphatic systems during development (4–6). The only known endogenous ligand for CLEC-2 is podoplanin, which is expressed at high levels in several cell types including lymphatic endothelial cells, kidney podocytes, choroid plexus, and type I lung alveolar cells. Podoplanin-deficient mice also have defects in the separation of the vascular and lymphatic systems that phenocopy those in the CLEC-2-deficient mice (7, 8).

CLEC-2 signals through a cytosolic Y*XX*L sequence which is downstream of three acidic amino acids in a motif known as a hemITAM (9). Signaling takes place in lipid rafts and is mediated through activation of Src and Syk tyrosine kinases and phosphorylation of key adapter proteins and PLCγ isoforms (9, 10). The CLEC-2 signaling pathway is similar to that of ITAM receptors which have two Y*XX*Ls separated by 6–12 amino acids and which include GPVI, the platelet collagen receptor, in complex with the FcR γ-chain (2, 3, 9–11). The proximal events in signaling by hemITAM and ITAM receptors are thought to be distinct; phosphorylation of the CLEC-2 hemITAM is dependent on Syk tyrosine kinase whereas phosphorylation of ITAM receptors is mediated by Src family tyrosine kinases (12, 13). Strikingly, mice deficient in Syk, the adapter SLP-76 and PLCγ2, have defects in the separation of the lymphatic and blood vasculatures, demonstrating a requirement for platelet activation downstream of podoplanin and CLEC-2 in this developmental process (6, 14–16).

CLEC-2 (2) and Dectin-1 (17) were the first two members to be identified of the hemITAM family of C-type lectin receptors which are characterized by a triacidic amino acid sequence upstream of a Y*XX*L (Fig. 1). Both receptors, and the third hemITAM receptor to be identified, CLEC9A (18), have three acidic amino acids preceding the Y*XX*L at the −2, −3, and −4 positions. A further three C-type lectins found in NK cells were recently identified among which NKp80 also adheres to the consensus sequence (19). In contrast, NKp65 and NKR-P1A are increasingly divergent in terms of the conserved triacidic amino

* This work was supported by the Wellcome Trust Grants 073107 and 088410 and by British Heart Foundation Grants PG/07/116 and PG/05/134. U. S. and A. P. are employees of Portola Pharmaceuticals.
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[S] This article contains Supplemental Table 1.
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[4] The abbreviations used are: CLEC-2, C-type lectin receptor-2; hemITAM, hemi-immunoreceptor tyrosine-based activation motif; NFAT, nuclear factor of activated T-cells; NK, natural killer; PLC, phospholipase C; SH2, Src homology 2.

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hemITAM
CLEC-2    --------MQDEDGYITLNIKTRKPALISVGS------ASSSWWR      31
Dectin-1  MEYHPDLENLDEDGYTQLHFDSQSNTRIAVVSEK-GSCAASPP      42
CLEC9A    --------MHEEEIYTSLQWDSPAPDTYQKCLSSNKCSGAC        33
NKp80     --------MQDEERYMTLNVQSKKRSSAQTSQLTFKDYSVTLHWYKI  39

Partial hemITAM
NKp65     --------MENEDGYMTLSFKNRCKSKQKS-----KDFSLYPQ      30
NKR-P1A   --------MDQQAIYAELNLPTDSGPESSSPSSLPRDVCQGSPWHQFALK 42
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FIGURE 1. **Sequence alignment of hemITAM receptors.** The intracellular tails of CLEC-2, Dectin-1, CLEC9A, NKp80, NKp65, and NKR-P1A were aligned using ClustalW software. The conserved Y*XX*L and upstream acidic amino acids are indicated in *bold* and *underlined*.

acids but retain the Y*XX*L. It is not yet known whether these NK C-type lectin receptors also signal through Syk (19).

CLEC-2 and CLEC9A are expressed as dimers on the cell surface. It is proposed that ligand engagement leads to further clustering with the tandem SH2 domains of Syk bridging two phosphorylated hemITAMs in two separate dimer combinations (3, 18, 20). Subsequently, a combination of Src kinase phosphorylation and autophosphorylation leads to further activation of Syk and initiation of a signaling pathway culminating in phospholipase Cγ2 (PLCγ2) activation (2, 3, 9–11).

To date, the publications on this novel family of receptors have described a dependence on Syk for signaling (18, 19, 21) but have not investigated the dependence on the other member of the Syk family of tyrosine kinases, Zap-70. Both kinases possess tandem SH2 domains required for ITAM signaling, but they differ in many ways including a much lower intrinsic kinase activity and conformational restraints in Zap-70 which may preclude the bridging model described above. On the other hand, there are several reports of signaling by CLEC-2 in transfected Jurkat T cells which express Zap-70 at high levels, but little or no Syk (2, 9, 21).

In this study we have investigated the role of the triacidic amino acid sequence and the ability of Zap-70 to mediate signaling by CLEC-2. We show that the triacidic amino acid sequence is critical for hemITAM phosphorylation but that CLEC-2 is unable to signal via Zap-70.

EXPERIMENTAL PROCEDURES

Reagents—Rhodocytin was purified from *Calloselasma rhodostoma* venom (22). A rabbit α-Syk polyclonal antibody (pAb) was used as reported (23). The goat α-human CLEC-2 pAb was purchased from R&D Systems. The α-Myc monoclonal antibody (mAb) 9B11 was from Cell Signaling Technology. The mouse α-FcγRIIA mAb (IV.3) was kindly provided by Dr. Y. Senis. Horseradish peroxidase-conjugated α-rabbit and α-goat secondary antibody and enhanced chemiluminescence reagents were purchased from Amersham Biosciences. GST fusion proteins corresponding to single or tandem SH2 domains of Syk were prepared as described previously (24, 25). Biotinylated peptides were synthesized by Severn Biotech. All other reagents were purchased from Sigma-Aldrich or from described sources (26).

Platelet Preparation—Venous blood from healthy drug-free volunteers was taken into 10% sodium citrate. Washed platelets were obtained by centrifugation using prostacyclin to prevent activation during the isolation procedure (3). Platelets were resuspended in modified Tyrode's buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$; pH 7.3) as described (3). Platelets were used at a cell density of 5×10^8/ml unless stated otherwise.

Immunoprecipitation, Pulldowns, and Western Blotting—Washed platelets were pretreated with 9 μM Integrilin to inhibit platelet aggregation through integrin αIIbβ3. Stimulations with collagen-related peptide (CRP) or mAb IV.3 were pretreated with 10 μM indomethacin and 2 units/ml apyrase to inhibit thromboxane production and block ADP, respectively. Platelets were stimulated with agonists at 37 °C with stirring at 1200 rpm in a Born lumiaggregometer. Reactions were terminated by addition of 2× ice-cold Nonidet P-40 lysis buffer. Platelet lysates were precleared, and detergent-insoluble debris was discarded. An aliquot was dissolved with SDS sample buffer for detection of total tyrosine phosphorylation. Lysates were incubated with either the indicated antibodies and protein G- or protein A-Sepharose. Precipitated proteins and whole cell lysates were separated by reducing SDS-PAGE, electrotransferred, and Western blotted.

Constructs—Wild type CLEC-2 cloned into pEF6 has been described (9, 27). Further mutations were generated by PCR using the mutating primers **CLEC-2 Δ2–5** (5′-TAG-GGA-TCC-ACC-ATG-GGA-TAC-ATC-ACC-TTA-AAT-ATT-AAA-ACT-CGG-3′), **CLEC-2 2–5 Ala** (5′-TAG-GGA-TCC-ACC-ATG-GCG-GCT-GCA-GCT-GGA-TAC-ATC-ACC-TTA-AAT-ATT-AAA-ACT-CGG-3′), **CLEC-2 2–5 Arg** (5′-TAG-GGA-TCC-ACC-ATG-CGG-CGT-CGA-CGT-GGA-TAC-ATC-ACC-TTA-AAT-ATT-AAA-ACT-CGG-3′), **CLEC-2 3–5 Ala** (5′-TAG-GGA-TCC-ACC-ATG-CAG-GC-T-GCA-GCT-GGA-TAC-ATC-ACC-TTA-AAT-ATT-AAA-ACT-CGG-3′) along with vector specific primer 4150. CLEC-2/FcRγ chimeras were generated by a two-step PCR method using WT CLEC-2 and the previously described FcRγ point mutants as templates (3). The mutating primers **CLEC-2/FcRγ FWD** (5′-GAA-GCA-TGA-GAA-ACC-ACC-ACA-GTG-GTG-GCG-TGT-GAT-GGC-TTT-G-3′), **CLEC-2/FcRγ REV** (5′-CAA-AGC-CAT-CAC-ACG-CCA-CCA-CTG-TGG-TGG-TTT-CTC-ATG-CTT-C-3′), **FcRγ Y*XX*L** (5′-TAG-GGA-TCC-ACC-ATG-AAA-TCA-GAT-GGT-GTT-TAC-ACG-GGC-3′), and **FcRγ F*XX*L** (5′-TAG-GGA-TCC-ACC-ATG-AAA-TCA-GAT-GGT-GTT-TTC-ACG-GGC-3′) were used along with vector-specific primer 4150, and further mutation of the chimera was generated by PCR using the mutating primer **CLEC-2/FcRγ DED Y*XX*L** (5′-TAG-GGA-TCC-ACC-ATG-CAG-GAT-GAA-GAT-GGA-TAC-ACG-GGC-CTG-AGC-ACC-AGG-3′) with vector-specific primer 4150. The nuclear

factor of activated T-cells (NFAT) luciferase reporter contains three copies of the distal NFAT site from the IL-2 promoter (28) and was provided by Prof. A. Weiss.

Cell Culture and Transfection—DT40 chicken B cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% chicken serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol, and 20 mM GlutaMAX. Cells were transfected in 400 μl of Cytomix by electroporation using a GenePulser II (Bio-Rad) set at 350 V and 500 microfarads.

Luciferase Assay—Cells were transfected as described above with 10 μg of the indicated Myc-tagged CLEC-2 constructs, 15 μg of the luciferase reporter construct (9, 27), and where stated, 5 μg of Myc-tagged Syk or Myc-tagged Zap-70. Twenty hours after transfection, live cells were counted by trypan blue exclusion and samples divided for luciferase assay (1×10^5/well) and flow cytometry (5×10^5). Luciferase assays were carried out as described previously (29). Luciferase activity was measured with a Centro LB 960 microplate luminometer (Berthold Technologies). All luciferase data were averaged from triplicate readings.

Flow Cytometry—Surface expression of Myc-tagged CLEC-2 was measured by flow cytometry. Cells (5×10^5) were stained in a 25 μl volume for 20 min on ice with 10 μg/ml α-Myc antibody. Cells were then washed and incubated for 20 min on ice with 15 μg/ml FITC-conjugated α-mouse IgG secondary antibody. Stained cells were analyzed using a FACSCalibur (BD Biosciences). Data were collected and analyzed using Cellquest software (BD Biosciences).

Surface Plasmon Resonance—Surface plasmon resonance experiments were performed using a Biacore T100 machine (Biacore, GE Healthcare). Biotin-tagged CLEC-2 peptides were attached to carboxymethylated dextran-coated CM5 research grade sensor chips (Biacore) following attachment of streptavidin to the chip surface using amine coupling. All experiments were performed at 25 °C in 10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.005% (v/v) polysorbate 20. GST-tagged recombinant Syk domains were expressed and purified as described previously (3). The domains expressed were the N-terminal SH2 domain (N-SH2), the C-terminal SH2 domain (C-SH2), and both SH2 domains with the linker region, referred to as the tandem SH2 domains (tSH2). A range of concentrations (0–250 μM) of N-SH2, C-SH2, and tSH2 domains were injected over all peptide surfaces to determine equilibrium dissociation constants (K_D). Specific binding was identified by subtraction of the signal from an appropriate reference flow cell. K_D values were derived by nonlinear fitting using the Levenberg-Marquardt algorithm as implemented in the program Origin (OriginLab).

Statistical Analysis—NFAT-luciferase data are expressed as means ± S.E. Statistical analysis was carried out using unpaired Student's *t* test. Significance was taken for $p < 0.05$.

RESULTS

HemITAM Signaling Is mediated by Syk but Not Zap-70—Zap-70 and Syk are the only two members of a family of tyrosine kinases characterized by the presence of tandem SH2 domains and shown to mediate signaling by ITAM receptors. To date, there has been no comparison of the ability of Zap-70 and Syk to mediate signaling by hemITAM receptors. To address this, $Syk^{-/-}$ DT40 cells were transiently transfected with CLEC-2 and either Syk or Zap-70, and activation was monitored using a highly sensitive NFAT reporter assay. Transfection of CLEC-2 alone was insufficient to reconstitute signaling to the snake venom ligand, rhodocytin (Fig. 2*A*). Signaling in response to rhodocytin was restored upon co-transfection of CLEC-2 with Syk but not with Zap-70 despite similar levels of expression as shown by Western blotting for a Myc tag which was present at the C terminus of both kinases (Fig. 2*A*). Importantly, tagging with Myc at this position does not interfere with activation of Zap-70 by ITAM receptors (30). To confirm this observation, we expressed Syk and Zap-70 in a Zap-70-deficient Jurkat cell line (P116) that had been generated by chemical mutagenesis. This cell line also lacks Syk as shown by Western blotting and its failure to support signaling through the T-cell receptor (30, 31). CLEC-2 signaling could only be detected in cells co-transfected with both CLEC-2 and Syk, but not with CLEC-2 and Zap-70 (Fig. 2*B*). It is noteworthy that the level of signaling in these cells is low, which may reflect the presence of other uncharacterized mutations produced by the chemical mutagenesis strategy.

Together these data demonstrate that hemITAM signaling can be mediated by Syk but not Zap-70. However, we have previously reconstituted signaling by CLEC-2 in a Jurkat T cell line which is contrary to the present findings (9). One explanation for this would be the residual presence of a low level of Syk in the Jurkat T cell line used in the original study. To investigate this possibility, we expressed CLEC-2 in the same Jurkat cell line and confirmed activation of the NFAT reporter (Fig. 2*C*). Strikingly, however, this signal was completely inhibited in the presence of a low concentration of the highly selectively Syk inhibitor, PRT318 (0.5 μM) (32). At this concentration, PRT318 inhibits Syk by >99% but shows only weak inhibition of Zap-70 (32). This demonstrates that the previous report of CLEC-2 signaling in the Jurkat cell line was mediated by endogenous Syk.

A Differential Role for Syk in HemITAM versus ITAM Signaling—A previous study using the Syk inhibitor R406 (12) revealed an unexpected role for Syk in mediating phosphorylation of the hemITAM receptor in human platelets. Because of concerns over the selectivity of R406 toward Syk relative to Src kinases, we have extended these studies to the highly selective Syk inhibitor, PRT318. As shown in Fig. 3, a low concentration of PRT318 (0.25 μM) inhibited phosphorylation of PLCγ2 in platelets by the two ITAM receptors, GPVI and FcγRIIA, and also by the hemITAM receptor CLEC-2. Phosphorylation of PLCγ2 is fully inhibited in the presence of a high concentration of PRT318 (2.5 μM) which is consistent with Syk activity modulating downstream phosphorylation events. In the case of the ITAM receptors, phosphorylation of Syk and the two receptor ITAMs, FcRγ and FcγRIIA, is not affected by PRT318 but is completely blocked by the Src kinase inhibitor PP2, consistent with a model in which Src family kinases phosphorylate both the ITAM and Syk. In the case of CLEC-2, however, PRT318 completely blocks CLEC-2 phosphorylation and partially blocks Syk phosphorylation, both of which are also completely blocked by PP2. The observation of partial Syk phosphorylation despite the complete abolition of CLEC-2 phosphorylation is

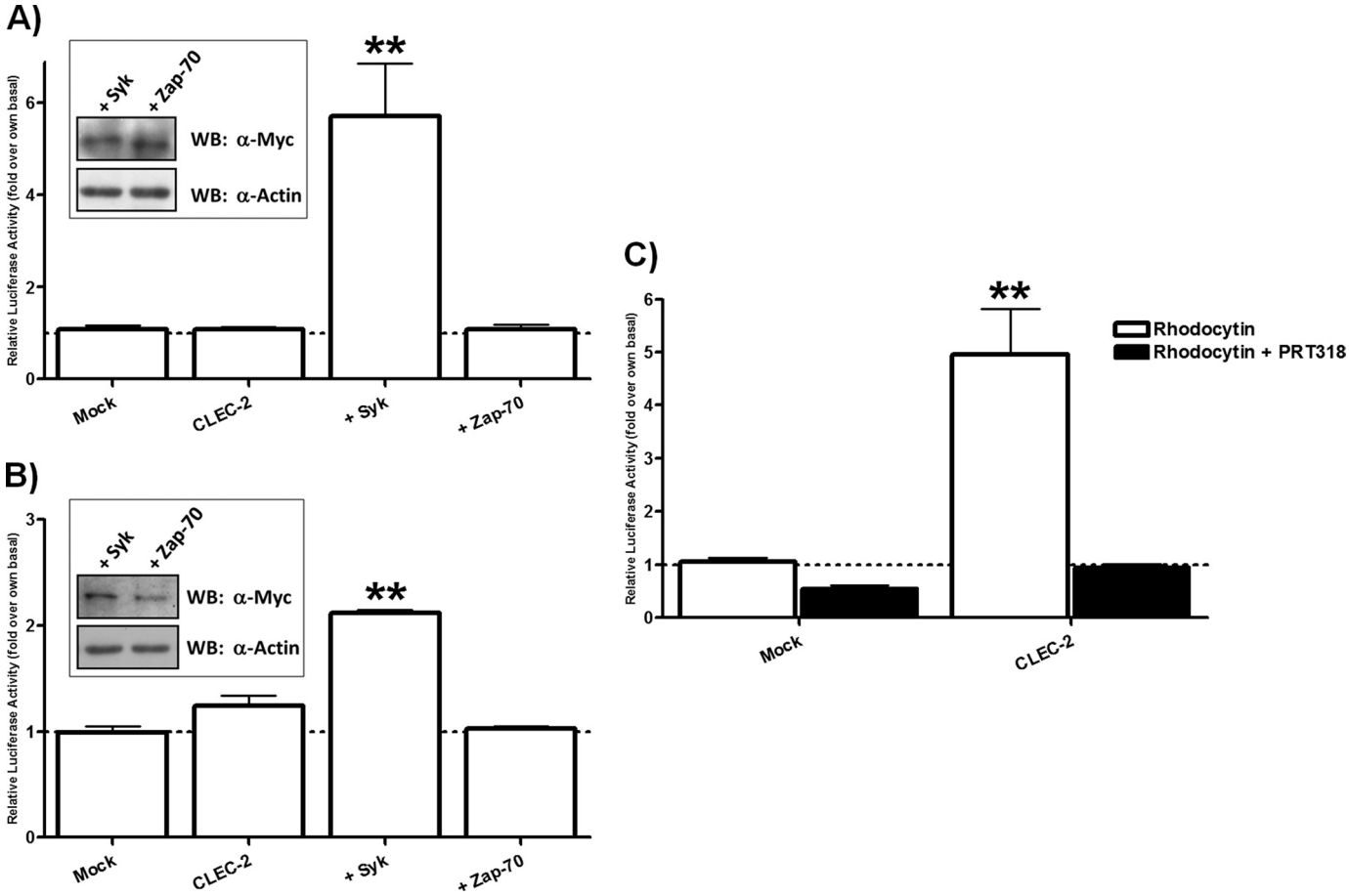

FIGURE 2. **CLEC-2 signaling is mediated by Syk and not Zap-70.** Syk$^{-/-}$ DT40 cells (*A*) or Zap-70^{-} Jurkat cells (*B*) or WT Jurkat cells (*C*) were transfected with CLEC-2 and the NFAT-luciferase reporter gene, and where indicated, with Syk or Zap-70. Transfected cells were stimulated with 50 nM rhodocytin, and where indicated, with the addition of 0.5 μM PRT318, for 6 h at 37 °C, after which time luciferase activity was measured as a readout of signaling. Results were normalized to -fold increases over the basal response. *Error bars* represent the means ± S.E. of at least three separate experiments. Cell lysates were analyzed by SDS-PAGE and Western blotting (WB) for Myc to demonstrate similar levels of Syk and Zap-70 expression (*insets*). Equal loading was confirmed by Western blotting for actin.

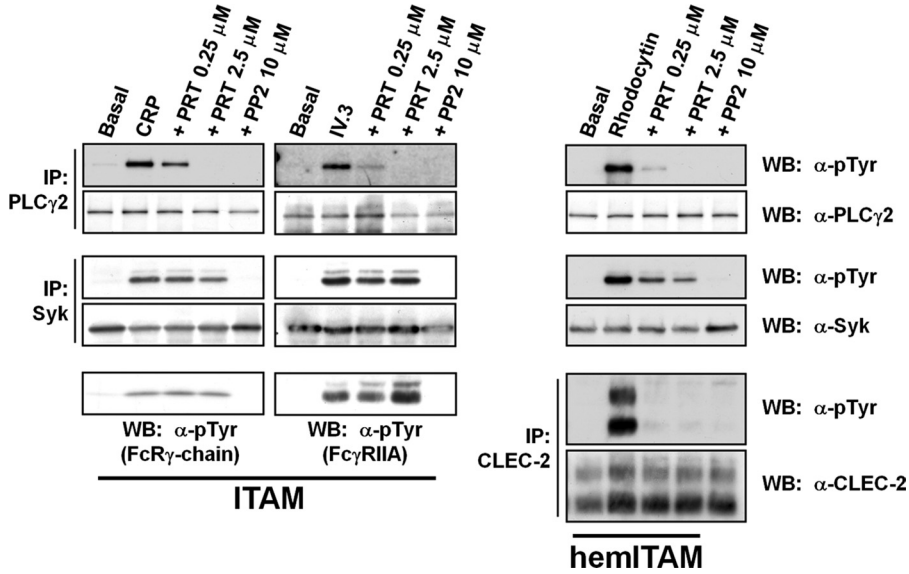

FIGURE 3. **Differential role for Syk in mediating ITAM and hemITAM phosphorylation.** Washed platelets (5 × 10^8/ml) were stimulated with collagen-related peptide (*CRP*; 3 μg/ml; 90 s), IV.3 antibody (2 μg/ml; 120 s) (+ secondary cross-linker (sheep α-mouse F(ab)$_2$; 30 μg/ml; 300 s)) or rhodocytin (300 nM; 180 s) in the presence of PRT318 (0.25 μM or 2.5 μM) or PP2 (10 μM) and subsequently lysed. Proteins were immunoprecipitated (*IP*) with specific pAbs for PLCγ2, Syk, and CLEC-2 and then separated by SDS-PAGE and Western blotted for phosphotyrosine. Membranes were then reprobed with the immunoprecipitated antibodies to measure equal loading. Images are representative of two separate experiments.

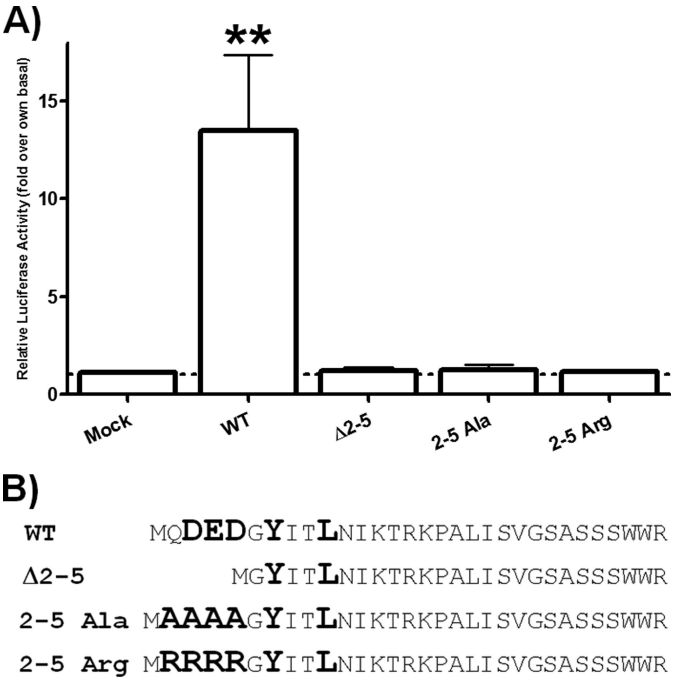

FIGURE 4. **The amino acids upstream of the *YXXL* are required for CLEC-2 signaling.** DT40 cells (*A*) were transfected with the stated CLEC-2 mutants (*B*) and the NFAT-luciferase reporter gene. Transfected cells were stimulated with 50 nM rhodocytin for 6 h at 37 °C after which time luciferase activity was measured as a readout of signaling. Results were normalized to -fold increase over the basal response. *Error bars* represent the means ± S.E. of at least three separate experiments.

consistent with a model in which Src kinase-mediated Syk activation precedes an initial round of phosphorylation of the hemITAM receptor. This is followed by further phosphorylation of Syk as a consequence of binding to the phosphorylated hemITAM, a proportion of which may be mediated by autophosphorylation. This adds to the growing evidence for a key mechanistic difference between the proximal events in the GPVI and CLEC-2 signaling cascades and extends this observation to a second platelet ITAM receptor, FcγRIIA.

The Triacidic Amino Acid Sequence Is Required for Activation of Syk by CLEC-2—It has previously been shown that the three acidic amino acids upstream of the *YXX*L in Dectin-1 play a role in phagocytosis (21). The equivalent sequence in CLEC-2 has been studied in part by single point mutations with inconclusive results (9). We therefore extended this work using two approaches. First, deletion of the triacidic amino acid region and upstream glutamine residue and second, replacement of all four residues with either uncharged alanine or basic arginine residues (Fig. 4). We chose to mutate all four residues because the triacidic amino acid sequence is spread over these four residues in the hemITAM receptor, NKp65. The rationale for the arginine mutant was to investigate whether the requirement for the acidic amino acid region is simply to provide an opposing charge that helps to separate the cytoplasmic tails in the CLEC-2 dimer. All three mutations resulted in a complete abrogation of CLEC-2 signaling in transfected DT40 cells in response to rhodocytin even though there was still surface expression of the mutants (see Supplemental Table 1).

We designed short, biotinylated, tyrosine-phosphorylated peptides to mimic the hemITAM and surrounding residues of wild type CLEC-2 and the deletion and alanine substitution mutants described above. We have shown previously that association between CLEC-2 and Syk does not occur in the absence of phosphorylation of the hemITAM (3). The interaction of Syk with these mutant hemITAM sequences was analyzed by surface plasmon resonance. The peptides were immobilized on streptavidin-coated sensor chips, and recombinant proteins of the Syk N-terminal SH2 domain (N-SH2), C-terminal SH2 domain (C-SH2) or tandem SH2 domains (tSH2) were flowed over. None of the recombinant proteins exhibited detectable binding to the Δ2–5 peptide (Fig. 5). On the other hand, the Syk N-SH2 domain had a similar affinity for wild type and the alanine mutant peptides, whereas there was a 4-fold decrease in the affinity of the alanine mutant peptide for the C-SH2 domain, which presumably accounts for the 3-fold decrease in affinity for the tSH2 domains (Fig. 5). Thus, these results demonstrate that the mutation of the upstream triacidic amino acids has only a minor effect on the binding of Syk to phosphorylated peptides based on the CLEC-2 cytoplasmic tail and that they are therefore not essential for the interaction.

The DED Sequence Is Required for HemITAM Phosphorylation—Based on the above results, we made a further alanine CLEC-2 mutant to confirm that it was solely the negatively charged DED sequence that was required for CLEC-2 signaling and tested it in the NFAT reporter assay. This additional mutant (3–5 Ala) was also unable to signal (Fig. 6). To investigate the possibility that the abrogation of signaling was mediated by loss of phosphorylation of the mutant CLEC-2, we stimulated transfected DT40 cells with rhodocytin and measured phosphorylation following immunoprecipitation. Rhodocytin stimulated tyrosine phosphorylation of wild type CLEC-2 but induced no significant phosphorylation of the 3–5 Ala mutant. Therefore, the loss of activity in the NFAT assay is explained by loss of phosphorylation of the mutant CLEC-2.

The HemITAM DED Can Confer Signaling to a Mutant ITAM—We have reported previously that signaling by the GPVI/FcRγ ITAM receptor is either ablated or inhibited by >95% following mutation of either of the conserved ITAM tyrosines thereby demonstrating that it is unable to signal in the same way as a hemITAM receptor (3). To ascertain whether this result is because of the requirement for the triacidic amino acid sequence upstream of the *YXX*L, we engineered a CLEC-2 chimera expressing the extracellular and transmembrane domains of CLEC-2 with some intracellular sequence surrounding the FcRγ ITAM. Rhodocytin stimulated a marked increase in NFAT activity in a chimera expressing the wild type FcRγ ITAM (Fig. 7). In contrast, point mutations of either of the ITAM tyrosines completely inhibited the response to rhodocytin (Fig. 7) even though both mutants were expressed at a level similar to the wild type receptor (see Supplemental Table 1). We then investigated whether the placement of the triacidic amino acid sequence from CLEC-2 upstream of the N-terminal *YXX*L sequence was able to restore signaling to rhodocytin in the CLEC-2/FcRγ chimera. Strikingly, rhodocytin was able to induce an increase in NFAT activity in the DED-Y2F chimera similar to that seen in wild type CLEC-2, demonstrating that the DED sequence was sufficient to convert activation by the hybrid receptor.

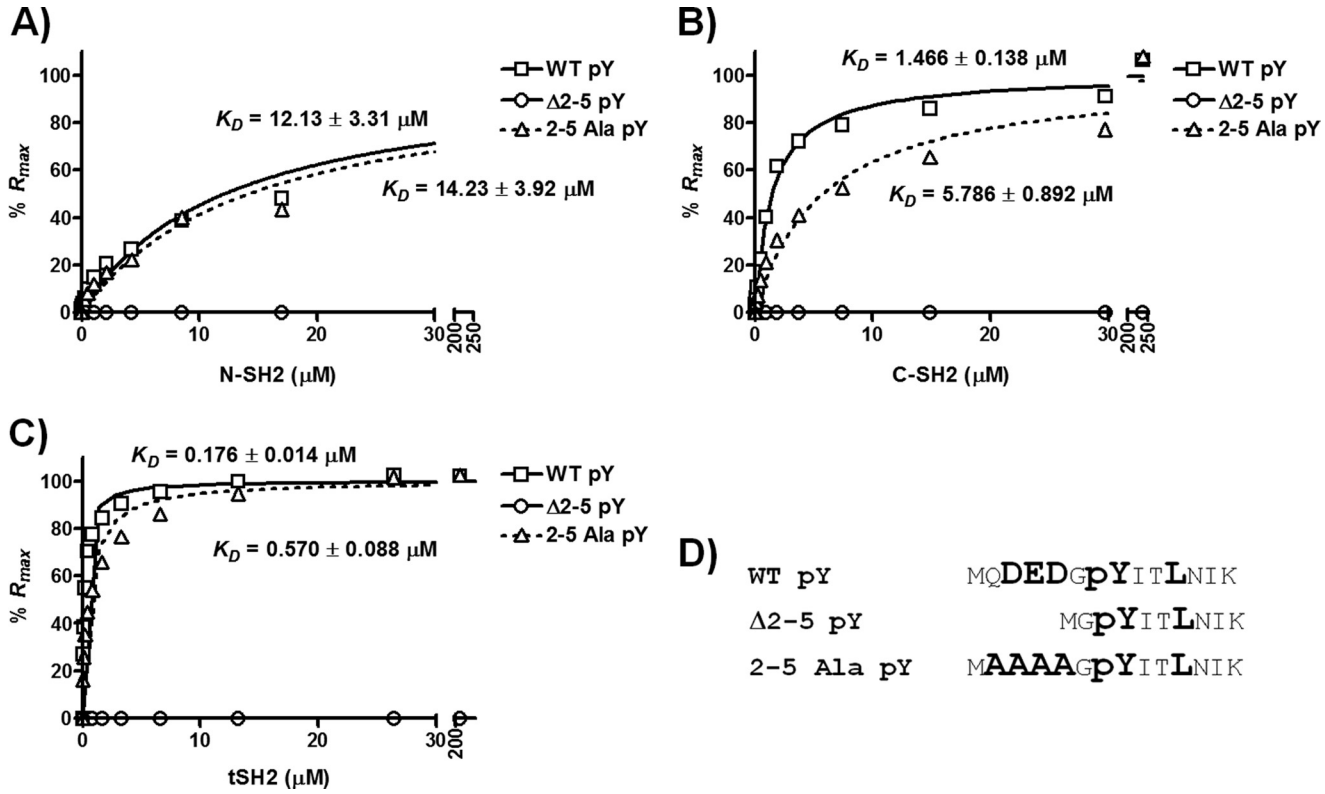

FIGURE 5. **Surface plasmon resonance measurements of the interaction of Syk with CLEC-2 mutants.** Biotinylated CLEC-2 peptides (*D*) were bound to streptavidin-coated biosensor chips surfaces. The N-SH2 (*A*), C-SH2 (*B*), or the tSH2 domains (*C*) of Syk were expressed as recombinant protein, purified, and flowed over the chip at a range of concentrations (0–250 μM). Nonlinear curve fitting was used to derive K_D values.

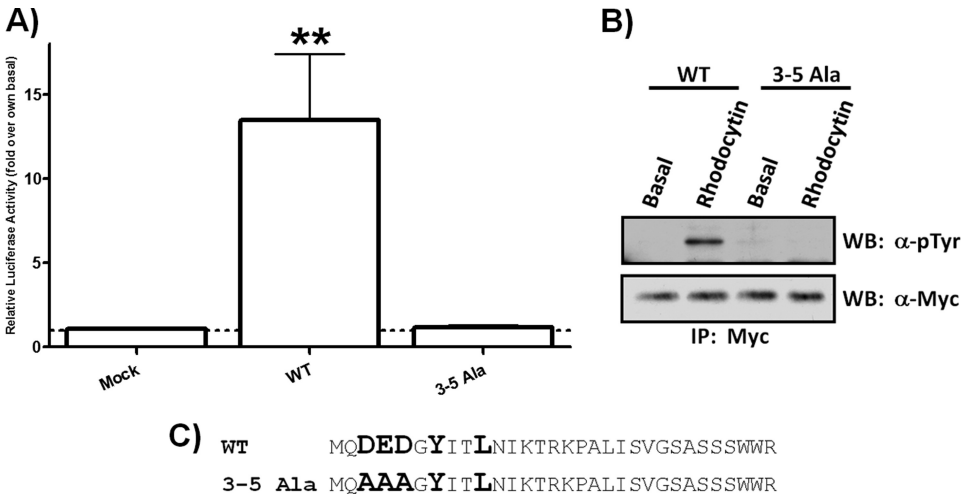

FIGURE 6. **The DED sequence is required for CLEC-2 signaling and phosphorylation.** *A* and *C*, DT40 cells (*A*) were transfected with the stated CLEC-2 mutants (*C*) and the NFAT-luciferase reporter gene. Transfected cells were stimulated with 50 nM rhodocytin for 6 h at 37 °C after which time luciferase activity was measured as a readout of signaling. Results were normalized to -fold increase over the basal response. *Error bars* represent the means ± S.E. of three separate experiments. *B* and *C*, DT40 cells (*B*) were transfected with the stated CLEC-2 mutants (*C*). Transfected cells were stimulated with 50 nM rhodocytin for 5 min at 37 °C followed by lysis. CLEC-2 was immunoprecipitated (*IP*), and the proteins were separated by SDS-PAGE and Western blotted (*WB*) for phosphotyrosine. Membranes were then reprobed with the immunoprecipitated antibody to measure equal loading. *Error bar* is means ± S.E. of two separate experiments.

DISCUSSION

The present study was designed to further dissect the proximal events in the CLEC-2 signaling pathway which signals through a single Y*XX*L sequence downstream of an acidic amino acid region. This work has shown the following: (i) CLEC-2 can signal through Syk but not Zap-70; (ii) the upstream triacidic amino acid sequence regulates hemITAM phosphorylation but is not required for binding to either of the Syk SH2 domains; (iii) Src-dependent phosphorylation of Syk precedes phosphorylation of the hemITAM sequence; and (iv) Syk is the likely mediator of hemITAM phosphorylation. These results provide further evidence that the proximal events in hemITAM signaling are distinct from those in ITAM signaling with a critical difference being that Syk mediates hemITAM phosphorylation following activation by a Src kinase-dependent pathway that leads to activation of Syk. In contrast, ITAM

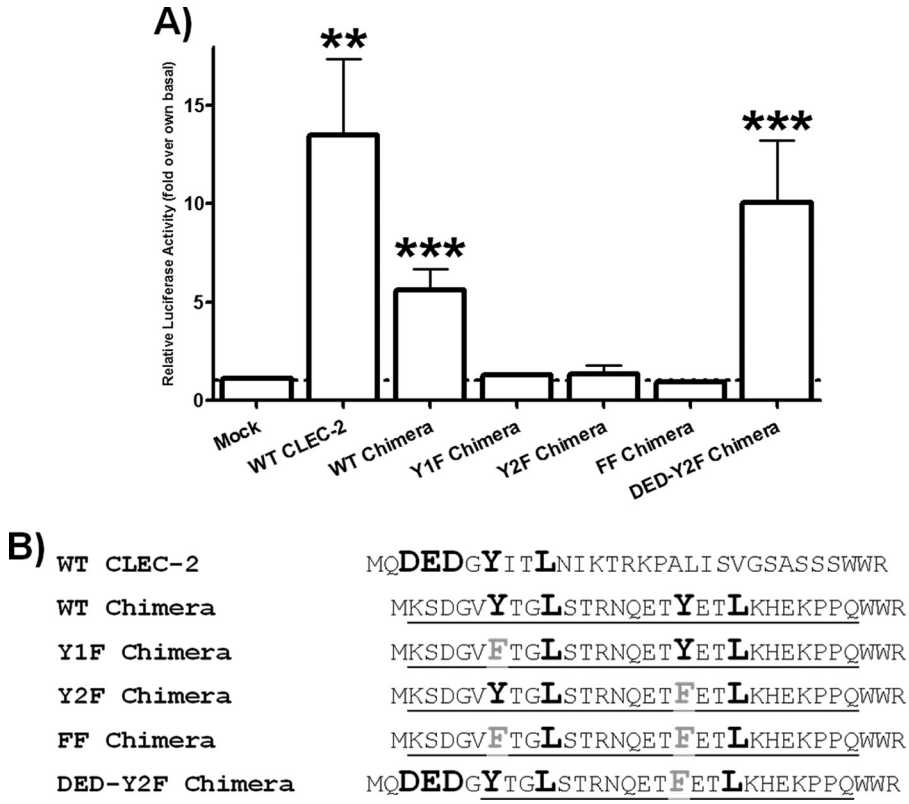

FIGURE 7. **The DED sequence confers hemITAM signaling to an ITAM.** DT40 cells (*A*) were transfected with CLEC-2 or the stated CLEC-2/FcRγ chimeras ((*B*) FcRγ sequence is *underlined*) and the NFAT-luciferase reporter gene. Transfected cells were stimulated with 50 nM rhodocytin for 6 h at 37 °C after which time luciferase activity was measured as a readout of signaling. Results were normalized to -fold increase over the basal response. *Error bars* represent the means ± S.E. of at least three separate experiments.

receptors are proposed to signal through the sequential activation of Src and Syk family kinases with phosphorylation of the ITAM preceding recruitment and activation of Syk or Zap-70, although there is still debate over whether ITAM phosphorylation is entirely Src kinase-dependent (see below).

A critical role for Syk in mediating hemITAM phosphorylation is consistent with two recent studies using a different Syk inhibitor, R406, which has a reduced selectivity for Syk over Src kinases (12), and mutant mice deficient in Src and Syk kinases (13). In both of these studies, and in the present paper, evidence is presented that Syk mediates phosphorylation of the CLEC-2 hemITAM. The present study, however, also shows that Syk is phosphorylated prior to phosphorylation of CLEC-2 by a Src kinase-dependent pathway. Thus, although the proximal events in hemITAM and ITAM signaling are distinct, both sets of receptors signal through the recruitment/activation of one or more Src kinases, and this is essential for subsequent receptor phosphorylation. It is interesting to note that the previous study in mouse platelets (13) showed that CLEC-2 phosphorylation in response to rhodocytin but not to a CLEC-2-specific monoclonal antibody was not blocked by Src kinase inhibition, which contrasts with the present results seen in human platelets. This suggests that there is a difference between species and ligands in the relative contribution of Src kinases to Syk activity, which may be a function of receptor density or agonist-induced cross-linking or a functional difference in Src or Syk kinase activity.

A key question is the molecular basis of the differential role of Src and Syk tyrosine kinases in hemITAM and ITAM phosphorylation. In this context it should be emphasized that although there is strong evidence that phosphorylation of the first tyrosine in an ITAM is mediated by a Src kinase, there is evidence that for some ITAM receptors, the second may be phosphorylation by a Syk kinases (33). The interplay of these kinases is therefore governed by their relative levels and localization within a cell (34, 35). It is likely that differences in kinase levels and localization and the differences in clustering of the receptors explains why Syk phosphorylation is reduced in the presence of PRT318 downstream of CLEC-2 but not following activation of the two ITAM receptors, GPVI-FcRγ-chain and FcγRIIA. The reduction in Syk phosphorylation observed in the presence of PRT318 could be due either to loss of autophosphorylation or to the feedback role of secondary agonists and actin polymerization in CLEC-2 signaling (10).

A further key question is the mechanism through which receptor dimerization regulates Src- or Syk-dependent phosphorylation of hemITAM and ITAM receptors. One explanation is that the kinases are constitutively associated with the nonphosphorylated ITAM/hemITAM sequence in a resting cell and that receptor ligation enables the kinases to mediate *cis* phosphorylation of an adjacent ITAM/hemITAM. An example of this is the constitutive association of Src and Syk kinases with the resting B cell receptor (35, 36). In the case of a hemITAM receptor, it may be optimal for the Src kinase to phosphorylate Syk rather than an adjacent hemITAM sequence, and this in turn mediates phosphorylation of the hemITAM. Either of the SH2 domains of Syk could then bind with high affinity to the

phosphorylated hemITAM, allowing phosphorylation of the other hemITAM in the dimeric receptor and binding of the second SH2 domain, thereby leading to Syk activation.

The present study has shown that, in contrast to many ITAM receptors, CLEC-2 is unable to signal through the second member of the Syk family of tyrosine kinases, Zap-70. This may reflect the structural differences in the two kinases in that the N-SH2 domain in Zap-70, but not the equivalent in Syk, requires part of the C-SH2 domain for the formation of the phosphotyrosine binding pocket (37). Thus, Zap-70 may be conformationally constrained from interacting with two phosphorylated hemITAM cytosolic chains. Alternatively, the much lower intrinsic kinase activity of Zap-70 may prevent significant hemITAM phosphorylation. The kinase activity of Syk has been estimated to be ~100 times that of Zap-70 (38), although other studies have shown the difference to be as modest as 3–4-fold (30). The inability of Zap-70 to reconstitute signaling by CLEC-2 is consistent with a recent study that was published during the writing of the present study which describes a novel mutant mouse in which Zap-70 has been knocked into the Syk locus. Platelets from the Zap-70-transgenic mouse are unable to signal in response to activation of CLEC-2 receptor agonists (39). However, the authors also reported that the platelets did not respond to GPVI activation, which may reflect the low intrinsic kinase activity of Zap-70, especially when considered in light of the rapid activation of platelets that occurs following activation of the collagen receptor. Therefore, the cell type as well as the nature of the signaling motif governs the interplay of Src and Syk family kinases.

The present study has further emphasized the significance of the triacidic amino acid sequence that lies upstream of the conserved Y*XX*L in CLEC-2. The results show that this sequence is required for phosphorylation of the conserved tyrosine by Syk but does not mediate binding to the SH2 domains of Syk, as mutation of this region has a negligible or minor inhibitory role on the binding of phosphorylated peptides to the N- and C-terminal SH2 domains of Syk. These observations are also unlikely to be due to an effect of the mutations on ligand binding and receptor dimerization, as it has been shown previously that CLEC-2 monomers can bind to rhodocytin on their own (40), and the model suggests that rhodocytin binding has stabilizing effects on dimerization. Therefore, the loss in signal of the mutants is most likely due to the lack of phosphorylation.

The molecular explanation for the loss of phosphorylation is unclear. The hemITAM sequence is not optimal for phosphorylation by either Syk or Src (Src, Fyn, Lyn, or Blk) (41, 42). We propose that phosphorylation of the hemITAM is facilitated by the preceding triacidic amino acid sequence. In support of this, we show that the introduction of a triacidic amino acid sequence upstream of one of the two Y*XX*L sequences in the FcRγ-chain ITAM (and mutation of the other conserved tyrosine to phenylalanine) is sufficient to restore signaling. The lack of phosphorylation could also be a consequence of altered localization in the surface membrane such that phosphorylation by Syk is not possible. In particular, triacidic amino acids would be repelled by the surface membrane, and so this may place the hemITAM in the vicinity of Syk.

In conclusion, the present study provides further evidence for key differences in the proximal events in signaling by the hemITAM receptor CLEC-2 relative to those of ITAM receptors with a critical role for a triacidic amino acid sequence in supporting phosphorylation by Syk but not Zap-70.

Acknowledgment—We thank Dr. Alice Pollitt for critical reading of the manuscript.

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