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Brief Definitive Report

Phosphotyrosines in the Killer Cell Inhibitory Receptor Motif of NKB1 Are Required for Negative Signaling and for Association with Protein Tyrosine Phosphatase 1C

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Summary

NKB1 is one member of a growing family of killer cell inhibitory receptors (KIR). It is expressed on natural killer (NK) cells and T cells, and has been shown to inhibit cytolytic functions of these cells upon interacting with its ligand, HLA-B (Bw4). We demonstrate here that the cytoplasmic region of NKB1 is capable of inhibiting T cell activation in Jurkat cells. The tyrosine phosphorylation of the NKB1 KIR consensus motif, $YxxL(x)_{26}YxxL$, induces an association with the protein tyrosine phosphatase 1C (PTP1C). Importantly, mutation of both tyrosines in the motif abolished the inhibitory functions of NKB1 and abrogated PTP1C association. Mutational analysis of the individual tyrosines suggest that the membrane proximal tyrosine may play a crucial role in mediating the inhibitory signal. These results demonstrate that KIR can not only inhibit cytolytic activity, but can also negatively regulate T cell receptor activation events that lead to downstream gene activation, and further supports a model that implicates PTP1C as a mediator in the KIR inhibitory signal.

Killer cell inhibitory receptors (KIR), a growing family of receptors that are present on NK cells and on subsets of peripheral T cells, inhibit cytolytic activity by recognizing polymorphic MHC class I molecules on target cells (1). NK cells, important in innate immune responses, recognize and kill tumor cells, virus-infected cells, and cells lacking MHC class I antigens. The effector functions of NK cells, cytolytic activity and cytokine secretion, appear to be the result of a finely regulated balance between positive signals that initiate the response and negative signals that inhibit the activated state (1). KIR appear to be important regulatory molecules of NK cell effector functions.

The KIR present on peripheral T cells also inhibit killing of target cells expressing an appropriate MHC class I ligand (2, 3). Although the positive signals through the antigen receptor resulting in T cell activation are well defined (4), little is known about the signals that may regulate the extent or degree of T cell activation. The presence of KIR on T cells offers an intriguing candidate for a negative regulatory molecule of cytolytic and noncytolytic functions.

The cytoplasmic tails of the KIR contain the consensus motif $D/E(x)_2YxxL(x)_{26}YxxL$ (5–7), which is reminiscent of the immunoreceptor tyrosine-based activation motif (ITAM) ($D/E(x)_2YxxL(x)_{7-8}YxxL$) present in TCR and BCR subunits (4). In T and B cells, the tyrosines (Y) in the ITAM motif become phosphorylated after receptor stimulation, and they mediate interaction with the Src homology 2 (SH2) domains of the ZAP-70/Syk family of protein tyrosine kinases (PTK; 8). The tyrosines in the cytoplasmic region of the KIR may also be involved in SH2 interactions. Recently, a study by Burshtyn et al. (9) showed that the KIR p58 can bind to the protein tyrosine phosphatase 1C (PTP1C). PTP1C (SHP-1, HCP, SH-PTP1) is a 66-kD protein and a member of the cytoplasmic, SH2 domain–containing family of phosphatases, which includes PTP1D (SHP-2, Syp, SH-PTP2) and the *Drosophila* protein corkscrew (10). PTP1C has been shown to become inducibly associated with the erythropoietin receptor (EpoR), FcγIIB1, CD22, *c-kit*, and the IL-3 receptor β chain after stimulation of these receptors (11–15). Recent studies correlate the binding of PTP1C with the negative regulation of the EpoR and the negative regulation of BCR-mediated activation by FcγRIIB1 (11, 12). Additionally, mutations in the PTP1C gene have been linked to the severe hematopoietic defects in motheaten (*me*) and motheaten viable (*mev*) mice (16–18).

In this report, we study the inhibitory function of the KIR NKB1 in T cells. Transient expression of a CD8/NKB1 chimera in Jurkat T cells allowed analyses of the structural requirements of NKB1 necessary for the inhibi-

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tion of TCR activation. We demonstrate that the cytoplasmic region of NKB1 inhibited TCR-mediated downstream gene activation in Jurkat T cells, and that tyrosine phosphorylation of NKB1 induced an association between PTP1C and the chimeric receptor. Also, mutational analysis of the tyrosines in the KIR consensus motif established the requirement of phosphotyrosines for the inhibitory functions of NKB1 and for association with PTP1C.

Materials and Methods

Cells. Jurkat cells and Raji cells were maintained in RPMI 1640 medium supplemented with 5% FCS, penicillin, streptomycin, and glutamine, as previously described (19).

Plasmids. The CD8/T construct was described previously (19). The CD8/NKB1 plasmid was made by digesting CD8/T with BglII and BamH1 and ligating in a cytoplasmic fragment of NKB1 (basepairs 1114–1865), in which these enzyme sites were introduced by PCR mutagenesis or through shuttle vectors. A BglII site was created at amino acid (aa) 364/365 (C/R mutation) in NKB1. Primers 5′ and 3′ of the Bsu36 and Esp1 sites, respectively, which mutated Y to F, were used to make the F1, F2, and F1F2 constructs. A primer 5′ of the Bsu36 site, deleting 18 aa between the two Y in the KIR motif, was used to create the 18-aa del construct. All constructs were sequenced using the Sanger dideoxy-nucleotide technique and then subcloned into the expression vector pEF Bos. The NFAT-luciferase reporter construct was a generous gift from Dr. G. Crabtree (Stanford University, Stanford, CA).

Transfections, Stimulations, and NFAT-Luciferase assays. 10^7 Jurkat cells were transfected by electroporation, as previously described (20), with 20 μg of the NFAT reporter plasmid and 40 μg of the CD8 chimeric plasmids. 24–40 h after transfection, 2×10^5 cells were aliquoted into 96-well plates (Corning Glassware, Corning, NY) and cultured in a final volume of 90 μl. The remaining cells were analyzed by flow cytometry for CD8 surface expression. For stimulation, equal numbers of Raji cells and Streptococcus enterotoxin D (SED; Toxin Technology Inc., Sarasota, FL) in the concentrations indicated were added to each well. As a control for NFAT activation, cells were stimulated with 50 ng/ml PMA and 1.0 μM ionomycin. The average value of the PMA and ionomycin stimulation was used as a maximum stimulation value. After 6–8 h at 37°C, cells were lysed and luciferase activation was determined as described previously (20). Activation for each condition was determined in duplicate, and each experiment was repeated at least three times. The percent maximum stimulation value achieved with 300 ng/ml SED plus Raji was averaged from three independent experiments for each plasmid.

Antibodies and Flow Cytometry. 4G10, an antiphosphotyrosine mAb, was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-PTP1C is a rabbit anti–mouse serum (generously provided by Dr. J. Schlessinger, New York University, New York). OKT8 mAb recognizes an extracellular epitope of CD8 and was acquired from American Type Culture Collection (Rockville, MD). Anti–Zap-70 mAb has been described previously (21). FITC-conjugated mouse anti-Leu2a (anti-CD8) and FITC-conjugated mouse IgG1 were purchased from Becton Dickinson & Co. (Mountain View, CA). For flow cytometry, 5×10^5 to 10^6 cells were stained with saturating concentrations of antibody and then analyzed using a FACScan® (Becton Dickinson), as previously described (22).

Peptides and Peptide-binding Assays. The peptides were synthesized in a peptide synthesizer (Applied Biosystems, Inc., Foster City, CA), as previously described (21). The peptide sequence is: DPQEVT(Y)1TQLNHCVFTQRKITRPSQRPKTPPTDIIV(Y)2TELPNAESR. Peptides were phosphorylated as follows: P1 peptide on Y1; P2 peptide on Y2; P1P2 peptide on Y1 and Y2. P0 was unphosphorylated. The ζ peptides are described elsewhere (21). Peptide precipitation was performed with avidin-conjugated beads (Vector Laboratories, Burlingame, CA), as previously described (21). Methods for [^{35}S]methionine biosynthetic labeling were described previously (23).

Western Blotting and Immunoprecipitation. 2×10^7 Jurkat cells were transiently transfected with 40 μg of the CD8/NKB1 or 20 μg of the F1F2 (plus 20 μg pEF Bos with no insert). 24–40 h later, 10^6 cells were analyzed by flow cytometry for surface CD8 expression, and the remaining cells were stimulated with pervanadate (10 mM Na-orthovanadate, 3.36% H_2O_2) for 10 min at 37°C. The cells were then lysed in buffer containing 1% NP-40, 10 mM Tris (pH 7.8), 150 mM NaCl, 2 mM EDTA, and protease and phosphatase inhibitors, as previously described (20). Lysates were immunoprecipitated with 2 μl OKT8 ascites or 4 μl anti-PTP1C serum and protein G–Sepharose (Pharmacia, Alameda, CA). The precipitates were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). The blots were blocked, as described previously (20) and then incubated with primary antibody, followed by secondary step reagent conjugated with horseradish peroxidase (protein A-HRP; Amersham, Arlington Heights, IL) or goat anti–mouse IgG-HRP (Southern Biotechnology Associates, Birmingham, AL). Proteins were detected by enhanced chemiluminescence (ECL kit; Amersham).

Results and Discussion

PTP1C-1 Associates with Doubly Tyrosine-phosphorylated KIR Peptides. To identify proteins associated with the cytoplasmic region of the KIR that may be involved in signal transduction, we generated biotinylated peptides corresponding to the KIR consensus motif of NKAT1 (aa 295–341 [5]) and used these peptides to precipitate proteins from metabolically labeled cells. The peptides were either unphosphorylated (P0) or, phosphorylated on the first (membrane proximal) (P1), the second (carboxy-terminal) (P2), or both tyrosines (P1P2). Only the P1P2 peptide was found to bind to a distinct 66-kD band in ^{35}S-metabolically labeled lysates from Jurkat cells (data not shown). Western blot analyses using antisera against known SH2 domain–containing proteins in this molecular mass range identified PTP1C as one 66-kD protein that could bind to the doubly phosphorylated peptide (Fig. 1). The P1P2 peptide bound to PTP1C in a dose-dependent manner. Conversely, the doubly phosphorylated ζ peptide bound to ZAP-70, but not to PTP1C, confirming the specificity of the interaction. Phosphorylation of both tyrosines was required for in vitro binding, since the single phosphotyrosine peptides did not bind to PTP1C. Therefore, PTP1C associated with the doubly tyrosine phosphorylated KIR peptide but not to the unphosphorylated or singly tyrosine phosphorylated peptides.

These peptide binding study results differ from those by Burshtyn et al., who showed PTP1C association with single phosphotyrosine peptides (9). This may represent dif-

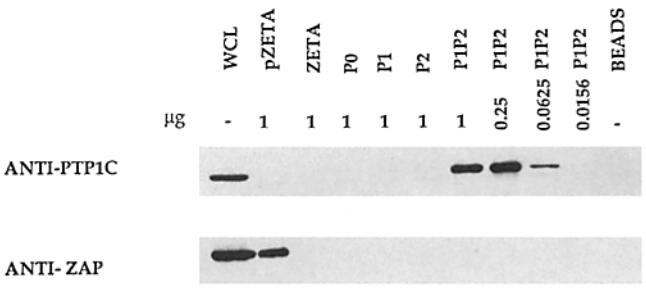

Figure 1. Binding of PTP1C to phosphorylated KIR peptides. Total cell lysates from unstimulated Jurkat cells were mixed with the indicated biotinylated peptides. pζ is a doubly tyrosine-phosphorylated ζITAM peptide. ζ is an unphosphorylated ζITAM peptide. The ζ peptides are described elsewhere (21). The remaining peptides are based on the NKAT1 KIR motif, DPQEVT(Y)1TQLNHCVFTQRKITRPSQRPKTPPTDIIV-(Y)2TELPNAESR. P0 is unphosphorylated. P1, P2, and P1P2 are phosphorylated on the first, second, and both tyrosines, respectively. The amount of peptide is given in micrograms. Peptides were isolated with avidin-conjugated beads, and the complexes were analyzed by immunoblot analysis with the antibodies shown on the left. Lysates from 4.5×10^7 cells were used for each peptide and samples were split and loaded on two separate gels. Whole-cell lysate (*WCL*) on the left is a positive control for the protein detected by the antibody. Avidin-conjugated beads (*BEADS*) with no peptide is shown on the right.

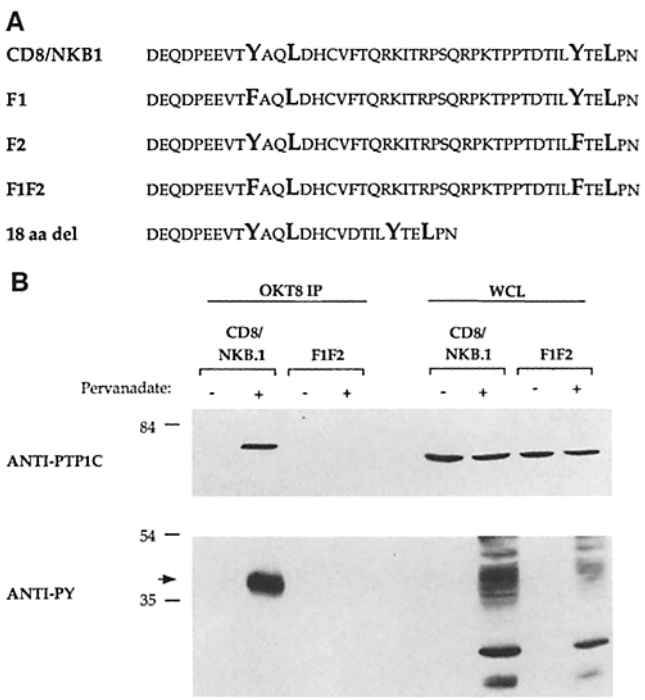

Figure 2. PTP1C binds to the phosphorylated CD8/NKB1 chimera. (*A*) Amino acid sequence of the cytoplasmic region of NKB1 highlighting tyrosines in the KIR motif that were mutated. F1 mutated the first Y to F, F2 the second, Y and F1F2 mutated both. 18-aa del deleted 18 amino acids between the two tyrosines. (*B*) Jurkat T cells were transfected with plasmids encoding CD8/NKB1 or the F1F2 mutant. 40 h after transfection, cells were left either unstimulated or stimulated with pervanadate for 10 min and then lysed. The lysates were immunoprecipitated with anti-CD8 (*OKT8*) and blotted with anti-PTP1C (*upper panel*). The blot was then stripped and reblotted with antiphosphotyrosine mAb (*4G10*; *lower panel*). The arrow corresponds to the chimera. Each lane represents 4.5×10^7 cells. CD8 surface expression for CD8/NKB1 and F1F2 were 5 and 41% positive cells, respectively. Whole-cell lysates (*WCL*) representing 10^6 cells are shown on the right. Similar results with equivalent tyrosine phosphorylation patterns were seen in three other experiments.

ferences in the peptide-binding assays. For example, Burshtyn et al. used peptides covalently conjugated to Affi-gel 10 containing only a partial KIR p58 consensus motif. The high local concentration of the phosphorylated peptide bound to Affi-gel 10 may have facilitated detection of the interaction and involved both SH2 domains binding to multiple peptides. However, the solution binding studies reported here show that a high affinity interaction is favored when both tyrosines of a single KIR peptide are phosphorylated.

PTP1C Associates with the Cytoplasmic Region of NKB1. To further study the mechanisms by which NKB1 mediates negative signaling in T cells and whether this involves PTP1C binding, we constructed a CD8/NKB1 chimeric expression vector. This construct, containing the extracellular and transmembrane region of the CD8 molecule and the intracellular region of NKB1, was transiently transfected into Jurkat T cells. We also mutated the first (F1), the second (F2), or both tyrosines to phenylalanine (F1F2) in the KIR consensus motif of NKB1 (Fig. 2 *A*) and transfected these into Jurkat cells.

The transfected cells were stimulated with pervanadate, a phosphatase inhibitor that induces maximal tyrosine phosphorylation (24), lysed, and the CD8/NKB1 chimera proteins precipitated. The CD8/NKB1 proteins were resolved by SDS-PAGE and blotted with anti-PTP1C. As shown in Fig. 2 *B* (*upper panel*), PTP1C associated with the CD8/NKB1 chimera expressed in Jurkat T cells after tyrosine phosphorylation of NKB1 by pervanadate. Despite a higher expression level, the mutant lacking both tyrosines (F1F2), did not associate with PTP1C. Stripping and reblotting the wild-type CD8/NKB1 chimera protein with an antiphosphotyrosine mAb 4G10 demonstrated a 40-kD phosphorylated protein in the pervanadate-stimulated cell lysate, consistent with the migration of the chimera protein (Fig. 2 *B*, *lower panel*). Similarly, the CD8/NKB1 chimera was detected in PTP1C immunoprecipitates (data not shown). Therefore, the phosphotyrosines in the KIR motif of NKB1 are necessary for PTP1C association. These results are consistent with the in vitro peptide-binding studies presented above.

The Cytoplasmic Domain of NKB1 Is Capable of Inhibiting TCR Activation. The in vivo transient expression system described above also allowed us to assess the functional effect of NKB1 expression on TCR-mediated signal transduction. Activation through the TCR was achieved by stimulation with the superantigen, SED, presented by the Raji B cell line, and was monitored by transcriptional activation of the NFAT-luciferase reporter. As shown in Fig. 3 *A*, we observed a striking inhibition in NFAT activation in T cells expressing the chimera containing NKB1 when compared to a truncated CD8 molecule (CD8/T). Thus,

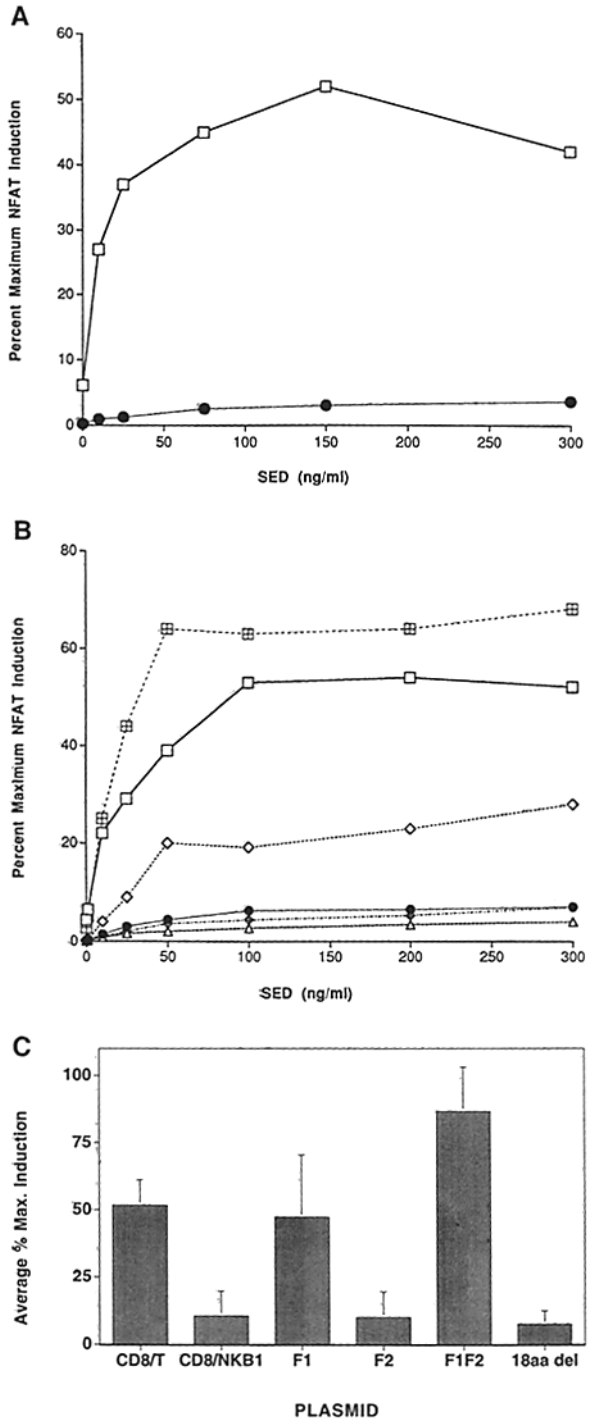

Figure 3. Inhibition of superantigen-induced Jurkat activation by the CD8/NKB1 chimera is dependent on cytoplasmic tyrosines. (*A*) Jurkat T cells were cotransfected with plasmids encoding CD8/T or CD8/NKB1 and NFAT-luciferase. Transfected cells were stimulated with equal numbers of Raji cells plus SED at the concentrations indicated and then assayed for luciferase activity. The results are shown as the percent of PMA plus ionomycin stimulation. PMA plus ionomycin values for CD8/T and CD8/NKB1 were 40,602, and 120,675 light units, respectively. CD8 surface expression levels for CD8/T and CD8/NKB1 were 25 and 12% positive cells, respectively. –□–, CD8/T; –●–, CD8/NKB.1. (*B*) Jurkat T cells were cotransfected with NFAT-luciferase and plasmids encoding CD8/T, CD8/NKB1, F1, F2, F1F2, and 18-aa del. Transfected cells were then stimulated with Raji cells and SED in the concentrations indicated and assayed for luciferase activity. The results are shown as percent of PMA plus ionomycin stimulation. PMA plus ionomycin values for CD8/T, CD8/NKB1, F1, F2, F1F2, and 18-aa del were 78,358, 116,664, 141,725, 131,383, 79,636, and 78,840 light units, respectively. The percent of CD8-positive cells were: CD8/T, 33%; CD8/NKB1, 9%; F1, 33%; F2, 22%; F1F2, 30%, and 18-aa del, 23%. Vector without insert gave luciferase activity that was 25–50% higher than CD8/T. –□–, CD8/T; –●–, CD8/NKB.1; ··◇··, F1; –△–, F2; ··⊞··, F1F2; ··⊕··, 18-aa del. *A* and *B* each represent individual experiments. (*C*) The results are expressed as the mean maximum stimulation from three independent experiments (300 ng/ml SED). Error bars represent SD from the mean.

the cytoplasmic region of NKB1 is sufficient to inhibit TCR-mediated downstream gene activation in Jurkat T cells. NKB1 has also been implicated in the negative regulation of IFN-γ and TNF-α production in NKB1$^+$ T cell clones (25). Therefore, NKB1 appears to regulate both cytolytic and noncytolytic effector functions in T cells.

Inhibition by NKB1 Is Mediated by the Tyrosines in the KIR Consensus Motif. To further study the structural importance of phosphotyrosines in the inhibitory signal of NKB1, we transiently expressed the tyrosine mutant constructs (Fig. 2 *A*) and assessed their ability to inhibit TCR-mediated NFAT activation. The mutation of both tyrosines (F1F2) completely abolished the inhibitory effect of NKB1 (Fig. 3 *B*). This effect was not caused by expression levels because the F1F2 mutant was present at much higher levels than the CD8/NKB1 chimera, based on flow cytometry analysis. Therefore, loss of inhibition must be a consequence of the tyrosine mutations. The loss of inhibition by the F1F2 mutant correlates with the absence of PTP1C association demonstrated in Fig. 2. Therefore, PTP1C is likely to be important in mediating the inhibitory signal of the NKB1 KIR.

Subsequent analysis of the single tyrosine mutants, F1 and F2, suggest that the tyrosines may have different degrees of importance in the inhibitory signal mediated by NKB1. The F2 mutant inhibited NFAT activation to a similar degree as the wild-type CD8/NKB1 chimera, whereas the F1 mutant inhibited it to a much lesser extent (~50% of CD8/NKB1). Thus, both tyrosines contribute to the inhibitory effect, but the first tyrosine (membrane proximal) appears crucial. Interestingly, Burshtyn et al. (9) found a greater enhancement of PTP1C enzyme activity with a phosphorylated peptide containing the first tyrosine (pY1) from the KIR motif than with a peptide containing the second tyrosine (pY2). The difference in inhibition between F1 and F2 detected in our in vivo assay may reflect either differential binding to PTP1C or differences in phosphatase activation.

The KIR consensus motif is very similar to the ITAM motif present in TCR and BCR subunits. The two tyrosines in the ITAM motif are important for ZAP-70/Syk binding (8). In the crystal structure of ZAP-70 bound to a phosphorylated peptide, the SH2 domains are in a fixed position and oriented in such a way as to place strict spatial constraints on the spacing between tyrosines in the ITAM (26). We tested whether the tyrosines in the KIR motif

were also spaced at a sterically important distance. We deleted 18 amino acids between the two tyrosines in the NKB1 KIR motif (Fig. 2 *A*) and tested the mutant for its ability to inhibit TCR-mediated NFAT activation. As Fig. 3 *B* demonstrates, the expression of this mutant (18-aa del) inhibited NFAT activation to the same degree as the wild-type CD8/NKB1 chimera. Thus, altering the spacing between tyrosines did not affect the inhibitory effect of NKB1, suggesting that the spacing is not essential in the protein interactions that mediate inhibition. The crystal structure of the SH2 domains of the related PTP PTP1D bound to phosphotyrosine peptides may offer an explanation for these findings. The SH2 domains of PTP1D are fixed in orientation, like the ZAP-70 SH2 domains, but in contrast to ZAP-70, they are oriented such that the phosphotyrosine peptides are roughly antiparallel relative to each other (27). This suggests that the binding of the SH2 domains of PTP1D and related phosphatases like PTP1C to phosphotyrosines may not occur in tandem, like ZAP-70, but on two separate proteins in *trans*. Thus, alteration of the spacing between the two tyrosines in the KIR motif might not affect PTP1C binding.

In summary, we demonstrate that expression of the cytoplasmic domain of the KIR NKB1 in a chimeric receptor inhibits TCR-mediated downstream gene activation in Jurkat T cells. Tyrosine phosphorylation of the NKB1 chimera in Jurkat cells induced association between PTP1C and the chimeric receptor. Mutations of both tyrosines abolished the ability of NKB1 to both inhibit TCR activation and to bind PTP1C. The in vitro peptide-binding studies suggest that optimal binding of PTP1C to the KIR motif occurs when both tyrosines are phosphorylated. However, the single tyrosine mutants (F1 or F2) retained their ability to inhibit TCR activation, suggesting that PTP1C association with KIR proteins may occur in *cis* or *trans*. The membrane-proximal tyrosine plays a crucial role in mediating the inhibitory signal. In contrast to the tyrosine mutations, alteration of the spacing between tyrosines in the KIR consensus motif did not affect the inhibitory signal. These results support a model in which PTP1C plays a role in mediating the negative signal transmitted by NKB1 and are consistent with studies by Burshtyn et al. (9). Future studies on the targets of NKB1 will further clarify its role in modulating TCR-mediated signals.

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