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Differential Roles of N- and C-Terminal Immunoreceptor Tyrosine-Based Inhibition Motifs During Inhibition of Cell Activation by Killer Cell Inhibitory Receptors

Pierre Bruhns,* Philippe Marchetti,* Wolf H. Fridman,* Eric Vivier,† and Marc Dae¨ron 2*  

Killer cell inhibitory receptors (KIRs)3 are a large multi-gene family of receptors for MHC class I molecules that belong to the Ig superfamily (1–3). They are expressed by human NK cells and by a subset of T cells. When engaged by MHC class I molecules expressed on target cells, KIRs inhibit NK cell cytotoxicity, Ab-dependent cell-mediated cytotoxicity induced by type II stress receptors for the Fc portion of IgG, and TCR-dependent cytotoxicity (4). KIRs were also shown to negatively regulate the cell activation induced by receptors bearing immunoreceptor tyrosine-based activation motifs (ITAMs), such as B cell receptors (BCRs) (5) or mast cell high-affinity IgE receptors (FceRI) (6). KIRs belong to a wider group of negative receptors that possess immunoreceptor tyrosine-based inhibition motifs (ITIMs) in their intracytoplasmic (IC) domain (7). In addition to KIRs (8, 9), these receptors include type IIB receptors for the Fc portion of IgG (FcγRIIB), which inhibit BCR- (10, 11), TCR-, and FcR-dependent cell activation (12); Ly49 (13) and NK2G2A (14), which inhibit cell-mediated cytotoxicity; CD22 (15) and CD72 (16, 17), which inhibit B cell activation; paired Ig-like receptors of the B type (PIR-B) (18) which inhibit the activation of B cells and myeloid cells; gp49B1 (19) and mast cell function-associated Ag (MAFA) (20), which inhibit IgE-induced mast cell activation; products of Ig-like transcripts (ILTs) (21, 22) and leucocyte Ig-like receptors (LIRs) (23), which inhibit the activation of monocytes and dendritic cells; leucocyte-associated inhibitory receptors (LAIRs) (24), which inhibit the activation of lymphoid cells; and signal-regulatory proteins of the α type (SIRPα) (25, 26), which inhibit the proliferation of hemopoietic and nonhemopoietic cells induced by hormones and growth factors via protein tyrosine kinase receptors.

ITIMs were structurally defined as a consensus sequence made of a tyrosine residue, an N-terminal isoleucine, valine, leucine, or serine at position Y-2, and a C-terminal leucine or valine at position Y + 3 (I/V/L/SxYxxL/V) (27, 28). ITIMs were functionally defined as specifically binding Src homology 2 (SH2) domain-bearing cytoplasmic phosphatases when tyrosyl-phosphorylated (7). Phosphorylated peptides corresponding to ITIMs of most ITIM-bearing receptors, including KIRs, were found to bind in vitro to the two protein tyrosine phosphatases SHP-1 and SHP-2 with variable affinities (27–29). Phosphorylated peptides corresponding to ITIMs of a few ITIM-bearing receptors, including FcγRIIB, bound to SHP-1 and SHP-2 and also to the inositol 5-phosphatase SHIP (30, 31). It was demonstrated that the binding of SHP-1 and SHP-2 to KIR and FcγRIIB ITIM phosphopeptides was dependent upon the Y-2 residue (27, 28).
of the binding of ITIM phosphopeptides to SHIP is not known. Interestingly, ITIM-bearing receptors were found to differentially recruit phosphatases in vivo among those that they bind in vitro. Thus, FcγRIIB were reported to recruit SHP-1. KIRs were reported to recruit SHP-1 following pervanadate treatment of KIR-expressing cells (28, 29), following KIR aggregation with specific Abs (32), following coaggregation of KIRs with BCR in KIR-transfected B cells (5), and during cell-cell interactions (33). The molecular basis of the selective in vivo recruitment of phosphatases by ITIM-bearing receptors is not known.

Noticeably, most SHP-1-recruiting receptors possess two or more ITIMs, and one may wonder whether these play redundant roles. Supporting this possibility, the mutation of the ITIM C-terminal tyrosine in a human KIR-based chimeric molecule had no effect, and the mutation of the ITIM N-terminal tyrosine only partially suppressed inhibition of the NF of activated T cells (NFTA) promoter activity (34). However, these chimeras, which comprised the extracellular and the transmembrane domains of CD8, were expressed as dimeric molecules, and one may argue that the mutated cis ITIM may have been replaced by an identical ITIM in trans. Against a redundant role of ITIMs, SHP-1 and SHP-2 have two tandem SH2 domains that have different affinities for ITIMs (28, 29) and which are thought to differentially mediate the interaction of these phosphatases with ITIM-phosphorylated receptors (35, 36). The aim of our work was to determine the respective contributions of the two KIR ITIMs during the inhibition of cell activation. To address this issue, we analyzed the properties of chimeric molecules whose IC domain contained the N-terminal and/or the C-terminal KIR ITIMs in their polypeptidic environment. We found that the two KIR ITIMs play distinct roles that may synergize to inhibit cell activation.

Materials and Methods

Cells

RBL-2H3 cells (37) were cultured in DMEM supplemented with 10% FCS, 100 international units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine; only adherent cells (recovered with trypsin-EDTA) were used. The FcγRIIB-negative variant (38) of the A20/2J lymphoma B cell line IIA1.6 (39) and CTLL-2 cells were cultured in RPMI 1640 supplemented with 10% FCS, 0.5 μM 2-ME, 2 mM sodium pyruvate, 100 international units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine; nine U/ml murine rIL-2 (R&D Systems, Minneapolis, MN) were added to CTLL-2 cultures. All culture reagents were obtained from Life Technologies (Paisley, U.K.).

Antibodies

The mouse IgM mAb 2682-I was used as culture supernatant of a subclone of DNP-H1-E-26 hybridoma cells (40). The rat anti-mouse FcγRIIB 2.4G2 IgG (41) was purified by affinity-chromatography on protein G-Sepharose from ascitic fluid of nude mice inoculated i.p. with 2.4G2 hybridoma cells. F(ab')2 fragments were obtained by pepsin digestion for 48 h. The purity of IgG and F(ab')2 fragments was assessed by SDS-PAGE. F(ab')2 fragments and intact IgG of polyclonal mouse anti-rat Ig (MAR) were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). MAR F(ab')2 was trinitrophenylated by incubation for 1 h at room temperature with trinitrobenzene sulfonic acid (Eastman Kodak, Rochester, NY). The protein was dialyzed against PBS. MAR F(ab')2 was added to CTLL-2 cultures. All culture reagents were obtained from Life Technologies (Paisley, U.K.).

Expression constructs

cDNA of mouse FcγRIIB was modified by a point mutation of the codon coding for Val212 (GTG → GTA), which induced no amino acid change but created a PstI restriction site. This cDNA encoding the entire extracellular and transmembrane domains and the first six IC amino acids of FcγRIIB encoded by the transmembrane exon was inserted into an expression vector under the control of the SRα promoter in pBR322 (42) and in which a resistance gene to neomycin was introduced (NT-neo). The cDNAs encoding the entire N-(C-KIR), the N-terminal half (Ly52-Cys90) (N-KIR), or the C-terminal half (Val110-Pro131) (C-KIR) of the IC domain of pSL8.13 KIR (43) were fused to FcγRIIB cDNA by cloning them into the created EcoRI and SacI site of the vector after PCR with the following primers: sense primers, 5′-TCGCTGTTGGTGGTACCAACTGTC-3′ (for N-C-KIR and N-KIR) and 5′-CAGTTGAGAGGATACCGTTTTCACACAGAGA-3′ (for C-KIR); antisense primers, 5′-CTGACGTGGAGCTCATGGCGGACG-3′ (for N-C-KIR and C-KIR) and 5′-TCGGTGGAGCTCAGCAGTGATTCAACTGTC-3′ (for N-KIR).

Transfectants

Fifty micrograms of cDNA were transfected by electroporation at 260 V and 960 μF into RBL-2H3 cells or into the FcγRIIB-deficient murine cell line, IIA1.6. RBL-2H3 and IIA1.6 transfected cells were selected by culture with 0.5 and 1 mg/ml neomycin, respectively (Cayla, Toulouse, France). RBL transfected recovered after selection were cloned as described previously (44). After selection, IIA1.6 cells were cultured for 10 days in RPMI 1640 supplemented with 1 mg/ml neomycin and 0.07% SeptaPlaque agarose (FMC Corporation, Rockland, ME) in the presence of feeder cells (rat embryonic cells). The clones, grown up in soft agar, were picked up with a Pasteur pipette. The expression of recombinant receptors by cloned cells was assessed by indirect immunofluorescence. The expression of recombiant receptors on clones remained stable over the duration of experiments. Several clones of each transfected were used for experiments and gave similar results.

Indirect immunofluorescence

Cells were incubated for 1 h at 0°C with 10 μg/ml 2.4G2 IgG in BSS containing 5% FCS. Cells were washed and stained by being incubated for 30 min at 0°C with 50 μg/ml FITC-labeled MAR F(ab')2. Fluorescence was analyzed by flow cytometry using a FACScalibur (Becton Dickinson, Mountain View, CA).

Serotonin release

Transfected RBL cells (resuspended in RPMI 1640 medium supplemented with 10% FCS (RPMI-FCS) at 1 × 106 cells/ml) were incubated at 37°C for 1 h with 2 μg/ml [3H]serotonin (Amersham, Les Ulis, France), washed, resuspended in RPMI-FCS, incubated for another hour at 37°C to remove excess [3H]serotonin, washed again, resuspended in the same medium, distributed in 96-well microculture plates at 2 × 104 cells/well, and incubated for 1 h at 37°C in the presence or absence of IgE anti-DNP and/or 2.4G2 F(ab')2 in a final volume of 50 μl. Adherent cells were washed four times with 200 μl HBSS; next, 25 μl of culture medium was added to each well, and cells were warmed at 37°C for 15 min before challenge. Cells were challenged for 30 min at 37°C with 25 μl TNP-MAR F(ab')2 previously warmed at 37°C for 15 min. Reactions were stopped by the addition of 50 μl of ice-cold medium and by placing plates on ice. Fifty microliters of supernatants was mixed with 200 μl of Aqualuma-Plus scintillation fluid (Wallac, Turku, Finland) and counted in a beta-counter (Packard). The percentage of [3H]serotonin released was calculated using as 100%, cpm in 50 μl harvested from wells containing the same number of cells that were lysed in 100 μl of 0.5% SDS and 0.5% Nonidet P-40.

IL-2 secretion

Aliquots of 5 × 103 IIA1.6 transfecants, resuspended in culture medium and distributed in 96-well microculture plates, were incubated with various concentrations of intact IgG or F(ab')2 fragments of RAM for 18 h at 37°C. Cell-free supernatants were harvested and assayed for IL-2 on CTLL-2 cells as described previously (45).

Flow cytometric analysis of Ca2+ mobilization

The intracellular free calcium concentration was determined by preloading 1 × 106 IIA1.6 cells with 5 mM Fluo-3 AM (Molecular Probes, Eugene, OR) in the presence of 0.2% Pluronic F-127 (Sigma, St. Louis, MO) for 30
min at room temperature. Cells were washed three times in RPMI 1640 and resuspended at 1 × 10^6 cells/ml in complete medium; the intracellular free calcium concentration was monitored with a flow cytometer. The mean [Ca^{2+}]_i was evaluated with FCS assistant 1.2.9 β software (Becton Dickinson). In these experiments, the intracellular Ca^{2+} mobilization and the extracellular Ca^{2+} influx were recorded separately, as described previously (46). To detect intracellular Ca^{2+} mobilization, cells were suspended in medium in which calcium was buffered to 60 nM (equivalent to [Ca^{2+}]_i in B cells) using EGTA. Cells were immediately (within 1 min) stimulated with RAM IgG or F(ab')_2, and [Ca^{2+}]_i was measured. To detect extracellular Ca^{2+} influx, the extracellular Ca^{2+} concentration ([Ca^{2+}]_o) was raised to a final concentration of 1.3 mM with CaCl_2.

***Immunoprecipitation and Western blot analysis***

Transfected RBL cells, resuspended at 1 × 10^6/ml, were incubated for 1 h at 37°C with IgE anti-DNP (culture supernatant diluted 1/10) and 2.4G2 F(ab')_2 (3 μg/ml) in complete culture medium, washed three times, resuspended in the same medium at 1 × 10^6 cells/ml, and challenged for various periods of time at 37°C with 10 μg/ml of TNP-MAR F(ab')_2. Cells were centrifuged, and pellets were lysed for 10 min at 0°C at 8 × 10^7 cells/ml in lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM NaVO_4, 5 mM NaF, 5 mM sodium pyrophosphate, 0.4 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM PMFS. Lysates were centrifuged at 12,000 rpm for 10 min at 4°C. Protein G-Sepharose (Pharmacia) (50 μl beads diluted 1/2) was used to precipitate 2.4G2-bound FcγRIIB–KIR chimeras.

Transfected IIA1.6 cells, resuspended at 3 × 10^7 /ml, were stimulated at 37°C for 3 min in complete culture medium with 45 μg/ml IgG or 30 μg/ml F(ab')_2. The cells were centrifuged, and pellets were lysed for 10 min at 0°C at 8 × 10^7 cells/ml in lysis buffer. We used 2.4G2-coated protein G-Sepharose (5 μg of purified Ab per 50 μl beads diluted 1/2) to precipitate FcγRIIB–KIR chimeras. Immunoadsorbents were washed three times in 1 ml of lysis buffer and boiled for 3 min in reducing or nonreducing sample buffer. Eluted material was fractionated by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Membranes were saturated with either 5% BSA (Sigma) or 5% skimmed milk (Régilait, Saint-Martin-Belle-Roche, France) diluted in 10 mM Tris buffer (pH 7.4) containing 0.5% Tween 20 (Merck, Schuchardt, Germany) (Western buffer) and blotted with appropriate dilutions of either HRP-conjugated anti-PY Abs or anti-FcγRIIB, anti-SHP-1, anti-SHP-2, and anti-SHIP, followed by HRP-conjugated goat anti-rabbit or goat anti-mouse IgG. Peroxidase-labeled Abs were detected using the Amersham enhanced chemiluminescence kit (Amersham, Little Chalfont, U.K.).

### Results

To determine the respective contributions of the two tandem ITIMs in the inhibition of cell activation induced by KIRs, we constructed cDNAs encoding chimeric molecules having the extracellular and transmembrane domains of murine FcγRIIB and an IC domain consisting of the N-terminal half, the C-terminal half, each containing one ITIM, or the whole IC domain, containing both ITIMs of human p58 KIRs (N-KIR chimera, C-KIR chimera, and N+C-KIR chimera, respectively) (Fig. 1A). cDNAs encoding the three KIR chimeras were stably transfected in the RBL-2H3 rat mast cells, which constitutively express FcγRII, and in the FcγRIIB-deficient IIA1.6 mouse lymphoma B cells (38), which constitutively express BCRs composed of IgG2α of an unknown specificity (39). Clones expressing comparable levels of recombining chimeric molecules in each cell type were selected and used for the experiments (Figs. 1B and 2A).

The N+C-KIR chimera and the N-KIR chimera, but not the C-KIR chimera, inhibit cell activation

KIR chimeras were coaggregated with FcεRI in RBL transfectants sensitized with mouse IgE anti-DNP, incubated with F(ab')_2 fragments of the rat anti-mouse FcγRIIB mAb 2.4G2, and challenged with TNP-MAR F(ab')_2, as described previously (47). Serotonin release induced under these conditions was compared with serotonin release induced by aggregating FcεRI with TNP-MAR F(ab')_2 in the same transfectants sensitized with mouse IgE anti-DNP but not incubated with 2.4G2 F(ab')_2. When coaggregated with FcεRI, the N+C-KIR chimera virtually abolished serotonin release, the N-KIR chimera induced partial inhibition, and the C-KIR chimera induced no inhibition (Fig. 1B).

KIR chimeras were coaggregated with BCR in IIA1.6 transfectants challenged with intact RAM IgG. IL-2 secretion induced under these conditions was compared with IL-2 secretion induced by aggregating BCR with RAM F(ab')_2 fragments in the same transfectants. When coaggregated with BCR, the N+C-KIR chimera and the N-KIR chimera abolished IL-2 secretion, whereas the C-KIR chimera induced no inhibition (Fig. 2A).

Ca^{2+} responses were monitored in IIA1.6 transfectants challenged under the same conditions with the same two ligands in the presence and the absence of extracellular Ca^{2+}. The N+C-KIR chimera abolished both the intracellular Ca^{2+} mobilization and the extracellular Ca^{2+} influx induced upon BCR aggregation, the N-KIR chimera partially inhibited the intracellular Ca^{2+} mobilization and abolished the extracellular Ca^{2+} influx, and the C-KIR chimera inhibited neither the intracellular Ca^{2+} mobilization nor the extracellular Ca^{2+} influx (Fig. 2B).

Finally, we examined the tyrosyl-phosphorylation of intracellular proteins in IIA1.6 transfectants following the aggregation or coaggregation of BCR with the three KIR chimeras. The N+C-KIR chimera and, to a lower extent, the N-KIR chimera, but not
KIR-dependent inhibition of cell activation is correlated with the tyrosyl-phosphorylation of KIR ITIMs, and the double mutation of the two ITIM tyrosines abolished inhibition (34). Therefore, we examined the tyrosyl-phosphorylation of KIR chimeras immunoprecipitated from RBL and from IIA1.6 transfectants, following their coaggregation with FcεRI or BCR, respectively (Fig. 3A), using the same ligands as in Fig. 1. Phosphorylation of the N+C-KIR and, with a lower intensity, of the N-KIR chimera, but not of the C-KIR chimera, was observed in RBL cells incubated with 2.4G2 F(ab')2 only and in resting IIA1.6 cells. Following coaggregation for 3 min with FcεRI in RBL cells or with BCR in IIA1.6 cells, phosphorylation of the N+C-KIR and N-KIR chimeras increased. No phosphorylation of the C-KIR chimera was induced (Fig. 3A). Phosphorylated proteins coprecipitated with the three chimeras when coaggregated with the BCR, particularly a 77-kDa molecule that was heavily phosphorylated in cells expressing the C-KIR chimera. To determine whether the differences in phosphorylation seen in the N+C-KIR and N-KIR chimeras might be due to differences in phosphorylation kinetics, the two chimeras were coaggregated with FcεRI for various periods of time before immunoprecipitation and Western blot analysis with anti-PY Abs. The induced phosphorylation of both the N+C-KIR and the N-KIR chimeras was detectable as early as 30 s and slightly increased until 15 min. The difference between the phosphorylation of the two chimeras remained constant with time (Fig. 3B).

To exclude that the C-KIR chimera might be refractory to in vivo phosphorylation, RBL transfectants expressing each of the three KIR chimeras were treated with two concentrations of pervanadate for 15 or 30 min. Under these conditions, both the N-KIR and the C-KIR chimeras were phosphorylated after treatment with the highest concentration of pervanadate. Phosphorylation of the C-KIR chimera, however, was of a lower intensity than that of the N-KIR chimera. Phosphorylation of the N-KIR chimera was of a lower intensity than phosphorylation of the N+C-KIR chimera in cells treated with a five times lower concentration of pervanadate (Fig. 4).

The N-KIR chimera recruits SHP-2, whereas the N+C-KIR chimera recruits SHP-1 and SHP-2

Once they have been tyrosyl-phosphorylated, KIRs recruit the protein tyrosine phosphatase SHP-1 (32), which was shown to be necessary and sufficient to account for a KIR-mediated inhibition of B cell activation (5). SHP-1 is thought to dephosphorylate proteins that become tyrosyl-phosphorylated upon aggregation of ITAM-bearing receptors (48). To identify the protein tyrosine phosphatase(s) recruited by the KIR chimeras, these chimeras were coaggregated with FcεRI for 5 min in RBL transfectants. Phosphatases coprecipitated with the chimeras were examined by Western blotting with anti-SHP-1 and anti-SHP-2 Abs. Detectable amounts of SHP-1 and SHP-2 were coprecipitated with the N+C-KIR chimera but not with the N-KIR or the C-KIR chimeras in unstimulated cells. Upon coaggregation with FcεRI, higher amounts of both SHP-1 and SHP-2 were coprecipitated with the N+C-KIR chimera. SHP-2, but not SHP-1, was coprecipitated with the N-KIR chimera. Comparable amounts of SHP-2 were recruited by the N+C-KIR and N-KIR chimeras. Under the same conditions, no phosphatase was coprecipitated.
FIGURE 3. Phosphorylation of KIR chimeras upon coaggregation with FcεRI or BCR. A, Phosphorylation of KIR chimeras. RBL-2H3 transfectants (1 × 10⁷) expressing KIR chimeras were incubated with 2.4G2 F(ab’)², sensitized or not with IgE anti-DNP, and challenged or not with TNP-MAR F(ab’)² for 3 min. IIA1.6 transfectants (1 × 10⁷) were stimulated or not with RAM IgG for 3 min. BCR was coaggregated with the KIR chimeras in cells challenged with RAM IgG. Cells were lysed, and KIR chimeras were immunoprecipitated, fractionated by SDS-PAGE, transferred onto Immobilon-P membranes, and Western blotted with anti-PY Abs.

Discussion

We show here that, although they both contain an ITIM which binds the same two phosphatases in vitro (28, 29), the two halves of the KIR IC domain differentially contribute to the KIR-dependent inhibition of cell activation in vivo. The respective roles of the two ITIMs were examined in chimeric molecules whose IC domain consisted of an intact IC KIR domain bearing both ITIMs (N+C-KIR), or a deleted domain containing the N-terminal (N-KIR) or the C-terminal ITIM (C-KIR) only. All three chimeras could be phosphorylated following pervanadate treatment. However, the N+C-KIR and N-KIR chimeras, but not the C-KIR chimera, were tyrosyl-phosphorylated upon coaggregation with FcεRI in RBL cells or with BCR in IIA1.6 cells. As a consequence, the N+C-KIR and N-KIR chimeras, but not the C-KIR chimera, recruited SH2-domain-bearing phosphatases and inhibited cell activation. Surprisingly, the N+C-KIR chimera recruited both SHP-1 and SHP-2, whereas the N-KIR chimera, recruited SHP-2 only. Therefore, the N-terminal half of the KIR IC domain is sufficient for the in vivo recruitment of SHP-2, whereas the recruitment of SHP-1 requires the whole KIR IC domain.

One unexpected finding of our work was that the three KIR chimeras were differentially phosphorylated under several conditions. The N+C-KIR and N-KIR chimeras, but not the C-KIR chimera, were constitutively phosphorylated in unstimulated cells. The N+C-KIR chimera was more phosphorylated than the N-KIR chimera, probably because they possess two and one tyrosine residues, respectively. Phosphorylation of the N+C-KIR chimera dramatically increased following pervanadate treatment. The phosphorylation of the N-KIR chimera also increased, and the C-KIR chimera became phosphorylated. This finding indicates that the three KIR chimeras are constitutively associated with protein tyrosine kinases. Supporting this conclusion, KIRs were reported to be constitutively associated with lck in NK cells (48, 49), and the aggregation of KIRs by anti-KIR Abs, in NK cells (32), or in RBL cells (28) induced their phosphorylation. lck is not expressed in RBL or in IIA1.6 cells, but it could possibly be replaced by another src family protein tyrosine kinase such as lyn. Phosphorylation of the N+C-KIR and N-KIR chimeras, but not of the C-KIR chimera, was enhanced following coaggregation with BCR in IIA1.6 cells.
or with FcεRI in RBL cells. The phosphorylation induced under these conditions is most likely due to kinases associated with BCR and FcεRI, as was shown for FcγRIIB (47). The reason why these kinases failed to phosphorylate the C-KIR chimera is unclear.

We provide here the first evidence that KIRs can recruit SHP-2 in addition to SHIP-1 in vivo. SHP-2 is a ubiquitous protein tyrosine phosphatase that is expressed by human NK cells (29). Interestingly, the N-KIR chimera was sufficient to recruit SHP-2, indicating that a single ITIM is sufficient. Comparable amounts of SHP-2 were recruited by the N-KIR and N+C-KIR chimeras, suggesting that the C-terminal ITIM did not contribute to SHP-2 recruitment. Thus, SHP-2 might bind to molecules bearing a single ITIM, possibly through an interaction of its two tandem SH2 with ITIMs in trans borne by adjacent phosphorylated molecules. This hypothesis is supported by a mutational analysis showing that SHP-2 binds to a single tyrosine-containing sequence of the IC domain of the platelet-derived growth factor receptor (PDGF-R) in vivo (50) and by the loss of catalytic activity of SHP-2 when binding to two different tandem tyrosine-based motifs on a doubly phosphorylated PDGF-R-derived peptide in vitro (35). Surprisingly, the N-KIR chimera selectively recruited SHP-2 in vivo. This finding is in contrast to the fact that phosphopeptides corresponding to the N-terminal KIR ITIM bind SHP-1 with higher affinity than SHP-2 in vitro (28, 29). The selective recruitment of SHP-2 by the N-KIR chimera is reminiscent of the selective recruitment of SHIP by single-ITIM FcγRIIB (31, 47), when corresponding phosphopeptides bind SHP-1 and SHP-2 as well as SHIP in vitro (30, 31, 47). The structural basis for such a selectivity is unknown.

Like the N-KIR chimera, the N+C-KIR chimera recruited SHP-2 when phosphorylated; comparable amounts of SHP-2 were coprecipitated with the two chimeras. Unlike the N-KIR chimera, the N+C-KIR chimera also recruited SHP-1. Whether SHP-1 was recruited by the C-terminal ITIM in the N+C-KIR chimera could be neither ascertained nor excluded in the absence of detectable phosphorylation of the C-KIR chimera. Alternatively, two tandem ITIMs may be required for the recruitment of SHP-1. That 13-aa long phosphopeptides containing one ITIM can bind SHP-1 apparently does not favor this hypothesis. However, the in vitro binding of phosphopeptides heavily coupled to beads may permit a binding that cannot occur in vivo. Moreover, the phosphorylation of both tyrosines, in a 46-aa long peptide encompassing the two ITIMs of a KIR molecule, was found to be mandatory for the binding of SHP-1 (34). The binding of phosphopeptides with two KIR ITIMs was also shown to increase the phosphatase activity of SHP-1 (27). Finally, with the exception of Ly49, other molecules that recruit SHP-1 in vivo (i.e., PIR-B, CD22, ILT-3, LAIRs, and NKG2A) have several ITIMs, and one can notice a C-terminal tyrosine in Ly49, distant of 26 residues from the ITIM tyrosine. This finding suggests the existence of cooperative rather than additive interactions between the N-terminal and the C-terminal ITIMs in KIRs and implies that the C-terminal ITIM is phosphorylated in the N+C-KIR chimera.

The differential phosphorylation and the differential recruitment of phosphatases by the three chimeras can be correlated with their biological properties. The heavily phosphorylated N+C-KIR chimera, which recruited both SHP-1 and SHP-2, induced more profound inhibitions in both RBL and IIA1.6 cells than the more lightly phosphorylated N-KIR chimera, which recruited SHP-2 only. SHP-1 is thought to dephosphorylate the phosphorylated substrates of protein tyrosine kinases, which are associated and/or recruited by ITAM-bearing receptors, and to abort early phosphorylation signals (48). SHP-1 was shown to be sufficient to inhibit B cell activation by coaggregating BCR with a chimeric molecule made of the extracellular and transmembrane domains of FcγRIIB and the catalytic domain of SHP-1 as an IC domain in IIA1.6 cells (5). The role of SHP-2 is less well known. Our results suggest that SHP-1 can amplify the inhibitory effects of SHP-2. The fact that the N-KIR chimera could inhibit serotonin release in mast cells and IL-2 secretion and Ca2+ mobilization in B cells suggests that SHP-2 may be sufficient for inhibition. However, one cannot exclude the possibility that not yet known phosphatases may be recruited by the N-KIR chimera which would account for inhibition. Unidentified phosphorylated proteins were indeed coprecipitated with phosphorylated chimeras, the most prominent of which was a
77-kDa molecule (Fig. 3A). Noticeably, however, the intensity of phosphorylation of these proteins was correlated with that of the total proteins seen in whole cell lysates, including lysates from cells stimulated with RAM F(ab')2 (Fig. 2C), suggesting that these molecules were not directly involved in inhibition. In apparent contradiction to our data, SHP-1 was reportedly necessary to inhibit B cell activation. Indeed, the same N+C-KIR chimera as the one used in this study had markedly reduced inhibitory properties in SHP-1-deficient chicken DT40 cells (5). However, one may notice that inhibition of Ca2+ responses and inhibition of NFAT activity were not completely abolished in these cells. Direct evidence that SHP-2 can inhibit is still lacking, and the inhibitory role of SHP-2 (51, 52) is controversial, as SHP-2 has been reported to positively regulate cell activation (53).

In conclusion, our results provide evidence that the two tandem ITIMs in KIRs may differentially contribute to the inhibition of cell activation. Our study suggests that two types of cooperative events may occur during KIR-mediated inhibition. Cooperative effects may be required for the phosphorylation of the C-terminal ITIMs in KIRs may differentially contribute to the inhibition of cell activation. Our study suggests that two types of cooperative events may occur during KIR-mediated inhibition. Cooperative effects may be required for the phosphorylation of the C-terminal ITIMs in KIRs may differentially contribute to the inhibition of cell activation. Our study suggests that two types of cooperative events may occur during KIR-mediated inhibition. Cooperative effects may be required for the phosphorylation of the C-terminal ITIMs in KIRs may differentially contribute to the inhibition of cell

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References


Differential Roles of KIR ITIMs

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