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

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Killer cell inhibitory receptors (KIRs) 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 IIa 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 NKG2A (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 (ILTps) (21, 22) and leukocyte Ig-like receptors (LIRs) (23), which inhibit the activation of monocytes and dendritic cells; leukocyte-associated inhibitory receptors (LAIRs) (24), which inhibit the activation of lymphoid cells; and signal-regulatory proteins of the a type (SIRPs) (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). The structural basis
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, FcyRIIB was shown to selectively recruit SHP (30, 31), whereas most other negative receptors 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 (NFFT) 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 relative 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 FcyRIIB-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 IgG3a mAb 2682-I was used as culture supernatant of a subclone of DNP-H1-e-26 hybridoma cells (40). The rat anti-mouse FcyRIIB 2.4G2 mAb (Ab) (41) was purified by affinity-chromatography on protein G-Sepharose from ascitic fluid of nude mice inoculated i.p. with 2.4G2 hybridoma fluids, 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) in a phosphate-buffered saline (pH 8). Trinitrophenylated (TNP)-MAR F(ab)2 was obtained after purification on Sephadex G25 (Pharmacia, Uppsala, Sweden). Rabbit Abs against recombinant extracellular domains of FcyRIIB were kind gifts of Dr. Catherine Sautès (Institut Curie, Paris, France). Horse-radish peroxidase (HRP)-conjugated anti-phosphotyrosine (PY) mouse mAbs PY-20 were purchased from Chemicon (Temecula, CA). Mouse monoclonal anti-SHP-1 and anti-SHP-2 were purchased from Transduction Laboratories (Lexington, KY). Rabbit anti-SHP Abs were purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal goat anti-rabbit and goat anti-mouse IgAbs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-mouse Ig (RAM) IgG and F(ab)2 used for IL-2 release were purchased from Jackson ImmunoResearch Laboratories (West Chester, PA).

Expression constructs

cDNA of mouse FcyRIIB was modified by a point mutation of the codon coding for V_e1 (GTTAGTA), which induced no amino acid change but created a KpnI restriction site. This cDNA encoding the entire extracellular and transmembrane domains and the first six I C amino acids of FcyRIIB 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/Cys204) (N-KIR), or the C-terminal half (Val110-Pro143) (C-KIR) of the IC domain of p58.183 KIR (43) were fused to FcyRIIB cDNA by cloning them into the created KpnI and XbaI sites of the SacI site of the vector after PCR with the following primers: sense primers, 5'-TCGCTGTTGGGTACCACAAAATT5'-CGTGT3't (for N+C-KIR and N-KIR) and 5'-CAGTTGAGAGTGACCCCATACAGAGA3'- (for C-KIR); antisense primers, 5'-CTGACGTGTCAGCCATGCCCAGG3'- (for N+C-KIR and C-KIR) and 5'-TCTGGTGGAGCTCAGCGTACTCGGTTTCACACAGAGA3'- (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 FcyRIIB-deficient murine cell line, IIA1.6. RBL-2H3 and IIA1.6 transfectants were selected by culture with 0.5 and 1 mg/ml neomycin, respectively (Cayla, Toulouse, France). RBL transfectants 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% Seaplaque 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 recombinant receptors on clones remained stable over the duration of experiments. Several clones of each transfectant 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 FACScanLibur (Becton Dickinson, Mountain View, CA).

Serotonin release

Transfected RBL cells (resuspended in RPMI 1640 medium supplemented with 10% FCS (RPMI-FCS) at 1 × 10^6 cells/ml) were incubated at 37°C for 1 h with 2 µCi/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 × 10^3 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-plate counter (Wallac, Turku, Finland). The percentage of [3H]serotonin released was calculated using 0.100, 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 × 10^5 IIA1.6 transfectants, 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 Ca^{2+} mobilization

The intracellular free calcium concentration was determined by preloading 1 × 10^6 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 1.29 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 IgG 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 × 10^7 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 × 10^3 cells/ml in lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM NaF, 5 mM Na_2VO_4, 5 mM sodium pyrophosphate, 0.1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM PMSF. 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 (3 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 (Réglait, Saint-Martin-Belle-Roche, France) diluted in 10 mM Tris buffer (pH 7.4) containing 0.5% Tween 20 (Merck, Schuchardt, Germany) (Western blots) 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 consensus inhibitory motifs are indicated. The length of the IC domains of the KIR chimeras is proportional to the number of amino acids. The N+C-KIR chimera had the whole IC domain of a human p58 KIR, the N-KIR chimera had the N-terminal half, and the C-KIR chimera had the C-terminal half of the same p58 KIR (open boxes). The amino acid sequences of consensus inhibitory motifs are indicated. The length of the IC domains of the KIR chimeras is proportional to the number of amino acids.

B. Inhibition of serotonin release. Histograms show the expression levels of the KIR chimeras as assessed by indirect immunofluorescence using 2.4G2 and FITC-MAR F(ab’)2 in RBL-2H3 cells. Dotted histograms show the fluorescence of cells incubated with FITC-MAR F(ab’)2 only. Transfectants expressing KIR chimeras were sensitized with IgE anti-DNP and preincubated (●) or not (○) with 2.4G2 F(ab’)2 before they were challenged for serotonin release with 10 μg/ml TNP-MAR F(ab’)2. The figure represents the percentage of serotonin released as a function of the concentration of IgE.

**FIGURE 1.** Structure and expression of the KIR chimeras and their inhibitory properties on IgE-induced mast cell activation. A. Structure of the KIR chimeras. KIR chimeras were made of the extracellular and the transmembrane domains of FcγRIIB as well as the first six IC amino acids encoded by the transmembrane exon of FcγRIIB (shaded boxes). The N+C-KIR chimera had the whole IC domain of a human p58 KIR, the N-KIR chimera had the N-terminal half, and the C-KIR chimera had the C-terminal half of the same p58 KIR (open boxes). The amino acid sequences of consensus inhibitory motifs are indicated. The length of the IC domains of the KIR chimeras is proportional to the number of amino acids. B. Inhibition of serotonin release. Histograms show the expression levels of the KIR chimeras as assessed by indirect immunofluorescence using 2.4G2 and FITC-MAR F(ab’)2 in RBL-2H3 cells. Dotted histograms show the fluorescence of cells incubated with FITC-MAR F(ab’)2 only. Transfectants expressing KIR chimeras were sensitized with IgE anti-DNP and preincubated (●) or not (○) with 2.4G2 F(ab’)2 before they were challenged for serotonin release with 10 μg/ml TNP-MAR F(ab’)2. The figure represents the percentage of serotonin released as a function of the concentration of IgE.

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
The N+C-KIR chimera and the N-KIR chimera, but not the C-KIR chimera, become tyrosyl-phosphorylated upon coaggregation with FcεRI or BCR

KIR-dependent inhibition of cell activation is correlated with the tyrosyl-phosphorylation of KIR ITIMs, and the double mutation of the two ITIM tyrrosines abolished inhibition (34). Therefore, we examined the tyrosyl-phosphorylation of KIR chimeras immunoprecipitated from RBL and from IIA1.6 transfecants, 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 transfecants 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 transfecants. 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

the C-KIR chimera, inhibited BCR-induced phosphorylation (Fig. 2C).

Taken together, these results indicate that to fully inhibit FcεRI-induced mast cell activation and BCR-induced B cell activation, KIRs require the conservation of sequences containing the two tandem ITIMs. Although both contain one ITIM, the N-terminal and C-terminal halves of the KIR IC domain do not appear to exert redundant functions, because the N-KIR chimera still inhibited cell activation, whereas the C-KIR chimera did not.

FIGURE 2. Inhibitory properties of KIR chimeras on B cell activation. A. Inhibition of BCR-dependent IL-2 secretion by IIA1.6 transfecants. Histograms show the expression levels of the KIR chimeras as assessed by indirect immunofluorescence using 2.4G2 and FITC-MAR F(ab')2 in IIA1.6 cells. Dotted histograms show the fluorescence of cells incubated with FITC-MAR F(ab')2 only. IIA1.6 transfecants were stimulated with either 0.1 or 3.0 μg/ml RAM F(ab')2 (thin lines) or 45 μg/ml RAM IgG (bold lines). The IL-2 released in 18-h culture supernatants was assayed for [3H]thymidine incorporation in CTLL-2 cells. The figure represents the radioactivity incorporated in CTLL-2 cells as a function of the concentration of RAM Abs or fragments. B, Inhibition of Ca2+ mobilization. IIA1.6 transfecants expressing KIR chimeras were loaded with Fluo-3 and stimulated with either 30 μg/ml RAM F(ab')2 (thin lines) or 45 μg/ml RAM IgG (bold lines). The intracellular Ca2+ concentration was measured after the resuspension of cells in 60 mM [Ca2+]0 buffered medium, followed by the replenment of ambient Ca2+ to 1.3 mM. The figure represents the mean fluorescence of the entire population, based on the analysis of ~1 x 104 cells/s, as a function of time. C, Inhibition of tyrosine phosphorylation of intracellular proteins. IIA1.6 transfecants (5 x 105) expressing KIR chimeras were challenged or not with RAM F(ab')2 or RAM IgG for 3 min and lysed. Whole cell lysates were fractionated by SDS-PAGE, transferred onto Immobilon-P membranes, and Western blotted with anti-PY Abs.
Abs.

bilon-P membranes, and Western blotted with anti-PY and anti-Fc

tated material was fractionated by SDS-PAGE, transferred onto Immo-

were lysed, and KIR chimeras were immunoprecipitated. Immunoprecipi-

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.

with the C-KIR chimera (Fig. 5A). To determine whether the differential recruitment of SHP-1 by the N+C-KIR and N-KIR chimeras might be explained by differences in the kinetics of recruitment, phosphatases coprecipitated with the two chimeras were examined following coaggregation with FcεRI for various periods of time. Both SHP-1 and SHP-2 were coprecipitated with the N+C-KIR chimera as early as 15 s after coaggregation, and the amount of coprecipitated phosphatases remained constant over a 30-min period. SHP-2 was coprecipitated with the N-KIR chimera with the same kinetics, but no SHP-1 was coprecipitated at any timepoint (Fig. 5B).

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 Ick 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. Ick 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.

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 or anti-FcγRIIB Abs. B, Kinetics of N+C-KIR and N-KIR phosphorylation. RBL-2H3 transfectants (1 × 10⁷) expressing KIR chimeras were incubated with 2.4G2 F(ab')₂, sensitized or not with IgE anti-DNP, challenged or not with TNP-MAR F(ab')₂ for indicated periods of time, and lysed. KIR chimeras were immunoprecipitated and fractionated by SDS-PAGE, transferred onto Immobilon-P membranes, and Western blotted with anti-PY Abs.

FIGURE 4. Pervanadate-induced tyrosine phosphorylation of KIR chimeras. RBL-2H3 transfectants (1 × 10⁷) expressing KIR chimeras were incubated with 2.4G2 F(ab')₂ and treated with 90 μM pervanadate (1×) or with 450 μM pervanadate (5×) for the indicated periods of time. Cells were lysed, and KIR chimeras were immunoprecipitated. Immunoprecipitated material was fractionated by SDS-PAGE, transferred onto Immobilon-P membranes, and Western blotted with anti-PY and anti-FcγRIIB Abs.
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 FcyRIIB (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 SHP-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 phosphorylated; comparable amounts of SHP-2 were recruited by the N-KIR chimera which would account for inhibition. Further, the heavily phosphorylated N-KIR chimera, which recruited both SHP-1 and SHP-2, induced more prominent phosphatase activity 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-2 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 FcyRIIB 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 Ca\(^{2+}\) 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
Differential roles of KIR ITIMs


