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Role That Each NKG2A Immunoreceptor Tyrosine-Based Inhibitory Motif Plays in Mediating the Human CD94/NKG2A Inhibitory Signal

Juraj Kabat, Francisco Borrego, Andrew Brooks, and John E. Coligan

The human NKG2A chain of the CD94/NKG2A receptor contains two immunoreceptor Tyr-based inhibitory motifs (ITIMs) in its cytoplasmic tail. To determine the relative importance of membrane-distal (residues 6–11) and membrane-proximal (residues 38–43) ITIMs in mediating the inhibitory signal, we made site-directed mutants of NKG2A at the Y (Y8F, Y40F, Y8F/Y40F) and the residues two positions N-terminal (Y−2) of Y (V6A, I38A, V6A/I38A) in each motif. Wild-type (wt) and mutated NKG2A were then cotransfected with CD94 into rat basophilic leukemia 2H3 cells. Immunochemical analyses after pervanadate treatment showed that each of the mutant molecules could be phosphorylated to expected levels relative to wt NKG2A and that all the mutations significantly reduced the avidity of SH2 domain-bearing tyrosine phosphatase-1 for NKG2A. Confocal microscopy was used to determine whether SH2 domain-bearing tyrosine phosphatase-1 and CD94/NKG2A colocalized intracellularly after receptor ligation. Only the Y8F/Y40F and Y8F mutant NKG2A molecules failed to show a dramatic colocalization. In agreement with this result, the Y8F/Y40F mutant was unable to inhibit FcεRI-mediated serotonin release and the Y8F mutant was relatively ineffective compared with wt NKG2A. In contrast, the Y40F mutant was 70% as effective as wt in mediating inhibition, and the Y−2 mutations did not remarkably affect inhibitory function. These results show that, like KIR, both NKG2A ITIMs are required for mediating the maximal inhibitory signal, but opposite to KIR, the membrane-distal ITIM is of primary importance rather than the membrane-proximal ITIM. This probably reflects the opposite orientation of the ITIMs in type II vs type I proteins. The Journal of Immunology, 2002, 169: 1948–1958.
PIR-B has paired ITIMs (Y794; VTYAQL and Y824; SVYATL) that are responsible for a significant portion of its inhibitory function (23, 24). It is clear from both studies that inactivation of the membrane-distal, C-ter ITIM (Y824) alone has little effect on inhibitory function. Also both groups of investigators agreed that simultaneous inactivation of both ITIMs drastically reduced SHP-1/SHP-2-mediated inhibitory function, but they somewhat disagreed on the independent role of the N-ter ITIM (Y794). Maeda et al. (23) found that inactivation of this ITIM alone gave loss of function approaching dual inactivation, whereas Béry et al. (24) found that inactivation of the Y794 ITIM alone had less of an effect on inhibition, but it was clearly more significant than the level of inhibition obtained with the Y824 mutant.

Studies on KIR ITIMs provide further evidence that ITIMs vary in functional capability. KIR inhibitory receptors, except for KIR2DL4 and KIR2DL5, harbor two ITIMs. Studies with peptides containing both ITIM sequences showed that a peptide with only a functional N-ter ITIM could activate SHP-1, whereas a peptide with only a functional C-ter ITIM could not; however, both ITIMs intact were required for full SHP-1 activation (16). This result is supported by receptor ligation studies (14, 25) that showed that the membrane-distal ITIM of KIR cannot function independently of the membrane-proximal ITIM, but that the membrane-distal ITIM can serve to enhance the function of the membrane-proximal ITIM. Fry et al. (26), using a chimeric molecule consisting of the extracellular and transmembrane domain of CD8 and a KIR cytoplasmic tail, also found that the membrane-proximal ITIM was nearly as effective as the combined ITIMs for inhibition of TCR activation, but in contrast, found that the membrane-distal ITIM of KIR2DL4 and KIR2DL5, harbor two ITIMs. Studies with peptides containing both ITIM sequences showed that a peptide with only a functional N-ter ITIM could activate SHP-1, whereas a peptide with only a functional C-ter ITIM could not; however, both ITIMs intact were required for full SHP-1 activation (16). This result is supported by receptor ligation studies (14, 25) that showed that the membrane-distal ITIM of KIR cannot function independently of the membrane-proximal ITIM, but that the membrane-distal ITIM can serve to enhance the function of the membrane-proximal ITIM. Fry et al. (26), using a chimeric molecule consisting of the extracellular and transmembrane domain of CD8 and a KIR cytoplasmic tail, also found that the membrane-proximal ITIM was nearly as effective as the combined ITIMs for inhibition of TCR activation, but in contrast, found that the membrane-distal ITIM alone retained partial inhibitory capacity.

Human NKG2A has two ITIMs that have been shown to be capable of interacting with SHP-1 and SHP-2 (27, 28). We set out to determine the relative importance of each of the ITIMs present in the intracytoplasmic region of NKG2A for mediating an inhibitory signal. We were particularly interested in determining the membrane-distal, C-ter ITIM being Y794; VTYAQL and Y824; SVYATL, with the two ITIMs in each molecule apart. To examine the role of these ITIMs in CD94/NKG2A signaling, we generated a series of mutants within the ITIMs of NKG2A and determined their ability to mediate inhibition of activation signals.

### Materials and Methods

#### Cells and Abs

The rat basophilic leukemia 2H3 (RBL) cell line and clones transfected with CD94 plus NKG2A cDNAs were grown in complete RPMI 1640 media supplemented with 10% heat-inactivated FBS and 4 mM glutamine, and maintained in a humidified atmosphere of 5% CO2 at 37°C. The cell lines adherent and are recovered by treatment with trypsin-EDTA purchased from Invitrogen (Rockville, MD). The human NK cell line, NKL, was grown in RPMI 1640 media supplemented with 10% human serum, 4 mM glucose, 1 mM sodium pyruvate, and 200 U/ml of rIL-2 (TECIN-recombinant human IL-2 from National Cancer Institute-Cancer Research and Development Center, Frederick, MD).

Sources for each Ab are indicated: anti-NKG2A mAb (Z199, IgG2b), anti-CD94 mAb (HP-3B1, IgG2a) with and without PE conjugation, and anti-CD56 mAb (C218) from Beckman Coulter (Fullerton, CA); PE-conjugated isotype-matched control Ab anti-dansyl IgG2b, isotype matched for Z199 from BD PharmMingen (San Diego, CA); Fab′1 donkey anti-mouse IgG from Jackson ImmunoResearch Laboratories (West Grove, PA); anti-phosphotyrosine mAb 4G10 (IgG2b) and anti-SHP-1 panto- clonal IgG from Upstate Biotechnology (Lake Placid, NY); anti-SHP-2 mAb (PTP1D/SHP2) from BD Transduction Laboratories (San Diego, CA); anti-SHP-2 rabbit polyclonal IgG from Upstate Biotechnology; and anti-SH2-containing inositol (poly)phosphate 5′ phosphatase (SHIP) rabbit polyclonal IgG (N-1) from Santa Cruz Biotechnology (Santa Cruz, CA). Western blots were developed with HRP-conjugated anti-mouse and anti-rabbit IgG from Amersham Pharmacia Biotech (Piscataway, NJ). Mouse anti-DNP IgE mAb was purchased from Sigma-Aldrich (St. Louis, MO). The NKG2-specific mAb 8E4 was derived by Houchins et al. (29).

#### cDNA constructs

Mutagenesis was performed with sets of mutagenic primers (Table I) with a QuickChange Site-directed mutagenesis kit (Stratagene, La Jolla, CA) starting with wild-type (wt) NKG2A cloned in the plasmid pFneo-MCS. Mutation of A for T was used to replace Tyr by Phe in generating the Y8F and Y40F mutants. Silent mutations were also created in both cases to generate restriction sites for BglII (Y8F) and SacI (Y40F) restriction endonucleases to facilitate selection of the mutant clones. The Tyr→Phe mutant Y8F was used to generate the double mutant Y8F/Y40F using the primers shown in Table I. The V6A and I38A mutants were generated using double mutations of AT for GC and TA for CC to replace Val and Ile, respectively, by Ala. Silent mutations were used to generate restriction sites for SacI in the V6A mutant and NcoI in the I38A mutant to enhance selection of positive mutant clones. The V6A mutant was used to generate V6A/I38A double mutant.

Dideoxy sequencing analyses were performed on selected clones for each mutant to ensure that no other mutations were introduced into the nucleotide sequences during the polymerase chain reactions.

#### Transfection of RBL cells

The wt or mutant forms of NKG2A cDNA in the mammalian expression vector pFneo-MCS and CD94 cDNA in the mammalian expression vector pFHM-MCS (the vectors were the gift of K. Sturmhoefel) were cotransfected into RBL cells using LipofectAMINE 2000 (Invitrogen) as follows. The day before transfection, RBL cells were trypsinized and counted, followed by plating at 5 × 10⁵ cells/well in a 6-well plate (flat-bottom tissue culture and Development Center, Frederick, MD).

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**Table I. Primers used for mutagenesis**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Restriction Enzyme</th>
</tr>
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<tbody>
<tr>
<td>Y8F</td>
<td>CCAAGGAGTAAATCTTCGAGATGACTGGAATCTGCCACC</td>
<td>BglII</td>
</tr>
<tr>
<td>Y40F</td>
<td>CCGGGCCAGATTCAGCTGACAAGATATCCCTTGGG</td>
<td>SacI</td>
</tr>
<tr>
<td>I38A</td>
<td>GAGAATACCGGGCCATCTAACACAGGCTCCTGACCTTACAAAGCC</td>
<td>NcoI</td>
</tr>
<tr>
<td>V6A</td>
<td>GAGAATACCGGGCCATCTAACAGGCTCCTGACCTTACAAAGCC</td>
<td>SacI</td>
</tr>
</tbody>
</table>

* Nucleotide changes introduced for changing tyrosine—phenylalanine, valine—alanine and isoleucine—valine are in bold and underlined. Coding strand primers are shown on top.

* Nucleotide changes introduced as silent mutations for restriction enzyme cleavages to enhance selection of mutant clones are shown in bold.
culture plate; Costar, Corning, NY). Each well contained 2.5 ml of complete RPMI 1640 medium and cells were grown overnight to allow attachment to the plate at 90–95% confluence. For transfection of the cells in each well, 4 μg each of the NGK2A and CD94 cDNA constructs were mixed with 250 μl of Opti-MEM medium (Opti-MEM reduced serum medium; Invitrogen). A total of 15 μl of LF2000 Reagent was added to another 250 μl of Opti-MEM medium and allowed to set for 5 min. This was then combined with the DNA suspension, and the mixture was incubated for 20 min at room temperature (RT) to allow DNA-LF2000 Reagent complex to form. The cells were washed with complete RPMI 1640 medium and a fresh 2.5 ml of complete RPMI 1640 medium was then added to each well together with 0.5 ml of the DNA-LF2000 Reagent complexes. After a 24-h incubation in a humidified CO2 incubator, the complete RPMI 1640 was replaced and, after another 24 h, the cells were washed and fresh complete RPMI 1640 medium containing 1 mg/ml of G418 (Invitrogen) and 0.6 mg/ml of hygromycin B (Invitrogen) were added. Clones obtained by cultivating under limiting dilution conditions were examined for surface expression levels of NGK2A/CD94 by flow cytometric analyses.

Flow cytometry
Flow cytometric analyses were performed on a FACSort cytofluorometer (BD Immunocytes, San Jose, CA). Direct immunofluorescent staining was performed using PE-conjugated anti-NGK2A (Z199) or anti-CD94 (HP-3B1) mAb (Beckman Coulter). PE-conjugated isotype-matched control Abs were used to monitor background staining levels.

Pervanadate treatment and immunoprecipitation
A total of 1 × 10⁶ RBL cells were plated in a petri tissue culture dish (10-cm diameter) in 20 ml of complete RPMI 1640 medium and incubated overnight at 37°C. After washing with serum-free medium, 10 ml of serum-free medium preheated to 37°C containing freshly prepared sodium pervanadate (0.1 mM sodium orthovanadate and 10 mM H₂O₂) was added and cells were incubated at 37°C for 20 min. The medium was removed by aspiration, plates were placed on ice, and the RBL cells were lysed in 1 ml of lysis buffer (PBS with 0.5% Triton X-100, 50 mM NaF, 1 mM NaVO₃ and protease inhibitors). Protease inhibitors were diluted according to the manufacturer’s instructions (protease inhibitor mixture for mammalian cell extracts; Sigma-Aldrich). Pervanadate-treated cells were included as a control. After 30 min on ice, lysates were cleared of debris by centrifugation at 16,000 × g for 15 min at 4°C. Immunoprecipitations were done with anti-NGK2A or anti-CD94 mAb that were preattached to protein A magnetic beads (Dynal Biotech, Lake Success, NY) according to the manufacturer’s protocol.

Immunoblotting
Immunoprecipitates and lysates, precleared by centrifugation at 16,000 × g, were boiled for 5 min in reducing (50 mM Tris-Cl (pH 6.8), 2% (v/v) SDS, 0.1% (v/v) bromophenol blue, 10% (v/v) glycerol, and 100 mM 2-ME) or nonreducing (the same as reducing, but without 2-ME) sample buffer. Samples were then fractionated by SDS-PAGE (10% acrylamide) and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were then saturated/blockd with 4% (w/v) BSA (catalog no. A9647; Sigma-Aldrich) in PBS for 1 h and then probed with either anti-phosphotyrosine, anti-SHP-1, anti-SHP-2, or anti-SHP-Ab appropriately diluted in 4% (w/v) BSA in PBS for 16 h at 4°C, followed by a 1 h incubation with HRP-conjugated goat anti-rabbit or goat anti-mouse IgG diluted in 10% nonfat milk powder in PBS. Membranes were washed between each step with washing buffer (10 mM Tris-Cl (pH 7.5), 100 mM NaCl, 0.1% Tween 20). Peroxidase-labeled Ab were detected using an ECL Western blotting kit (Amersham Pharmacia Biotech). After blocking, NGK2A membranes were stripped with 10 mM 2-ME, 2% SDS, 62.5 mM Tris-HCl (pH 6.7) at 50°C for 30 min, and after blocking nonspecific reactive by saturation of the membrane with 4% (w/v) BSA in PBS, were immunoblotted with anti-NGK2A mAb (8E4). Densitometry measurements of bands detected with anti-phosphotyrosine, anti-SHP-1, and anti-NGK2A were conducted using a Scion Image 4.0.2.2 (Scion, Frederick, MD). Figures: For compensations for background, the phosphotyrosine and SHP-1 band intensities were adjusted by the level of the NGK2A band intensity for each immunoblot. Band intensities relative to wt NGK2A were determined and plotted as a bar graph.

Serotonin release
RBL cells transfected with wt or mutant NGK2A plus CD94 cDNAs were suspended in 10 ml of RPMI 1640 medium supplemented with 10% FCS at 1 × 10⁶ cells/ml and incubated at 37°C for 1 h with 2 μCi/ml [3H]hydroxytryptamine creatinine sulfate (NEN, Boston, MA). After washing three times with 50 ml of PBS and once with 50 ml of RPMI 1640 medium, the cells were incubated for 1 h at 37°C in RPMI 1640 medium supplemented with 10% FCS. They were washed again with 50 ml of RPMI 1640 medium, followed by resuspending in the same medium and distribution in 96-well microculture plates at 2 × 10³ cells/well/50 μl. The wells were precoated with either anti-CD94 (or anti-NGK2A) mAb or IgE, or a mixture of the Abs plus IgE. For titration analyses, we used a constant concentration of IgE anti-DNP mAb (20 μg/ml) and various concentrations of anti-CD94 mAb (0, 0.2, 2, and 20 μg/ml). The plates with the cells were then incubated for 30 min at 37°C and the reactions were stopped by adding 150 μl of ice-cold medium and by placing the plates on ice for 5 min. Plates were centrifuged for 1 min at 3,000 × g at 4°C and 100 μl of each supernatant was mixed with 5 ml of scintillation fluid (Bio-Safe II; Research Products, Mount Prospect, IL) for determination of radioactivity levels in a Beckman LS6000 counter (Beckman Coulter). For determination of the total amount of serotonin, 100 μl of 1% SDS and 1% IGEPAL CA-630 (Sigma-Aldrich) were added to the remaining cell suspensions in each well, and then, after a 10 min incubation at RT, 100 μl of lysed cell suspension was counted as described above. Triplicate samples were used for all experiments. Percentage of specific release was calculated as follows: (cpm in supernatant – cpm spontaneous release)/total cpm × 100, where total cpm is cpm in supernatant + cpm in lystate.

Confocal microscopy
Anti-NGK2A mAb, anti-SHPI-1 Ab, and anti-SHPI-2 Ab were labeled with the Alexa Fluor 488 and Alexa Fluor 594 mAb Labeling kits (Molecular Probes, Eugene OR), respectively, according to the manufacturer’s instructions. Anti-CD94 mAb at 20 μg/ml in 200 μl of PBS was added to cell culture vessel wells with coverglass growth surfaces (Lab-Tek Chambered Coverglass system; PGC Sciences, Frederick, MD) and incubated overnight at 4°C. The chambers were washed three times with PBS before addition of RBL cells to each chamber at 2 × 10⁵ cells in 200 μl of complete RPMI medium. The vessels were spun for 3 min at 117 × g, followed by incubation for 10 min at 37°C. The medium was removed by aspiration and the cells were permeabilized and fixed with 0.1% Triton X-100 in 4% paraformaldehyde/glutaraldehyde for 1 h at 4°C. The plates with the cells were washed again with 4% paraformaldehyde/glutaraldehyde in the dark in 200 μl of 4% paraformaldehyde until analysis by confocal microscopy. Images were collected on a Leica TCS-SP2 confocal microscope (Leica Microsystems, Exton, PA) equipped with argon (488 nm) and krypton (568 nm) lasers, using ×40 and ×63 oil immersion objectives NA 1.25. Detector slits were adjusted to optimize signal and simplify overlap between the channels. Images were processed with Imaris software v3.1.2 (Bitplane, Zürich, Switzerland) without filtering. Optical sections of the cells (21 images each) were taken. For illustration, the sections, comprising the one-third of the cells (7 images) on the side attached to the coverslip (cs) and the one-third of the cells at the opposite side are shown as a maximum projection. For three-dimensional (3D) reconstruction of single cells, ×63 oil immersion objective (NA 1.32) and confocal area 3.00 was used and images were taken with a z-axis interval of 0.35 μm. A composite of these images was then projected as a 3D model of the cell. Multiple RBL cells of each type were imaged in this manner. Vertical segments of each from this 3D model were then made by using Imaris software v3.1.2.

Results
Expression of CD94/NGK2A by RBL cells
To examine the function of individual ITIMs within the cytoplasmic tail of NGK2A, we generated Tyr→Phe mutations by site-directed mutagenesis in both the membrane-distal and membrane-proximal ITIMs either individually or in combination. Likewise, as the alphatic residues N-ter to the Tyr (Y-2) residues have been shown to be important for ITIM function, in particular SHP-1 binding (14–16), these residues were changed to Ala either individually or in combination (Fig. 1A). cDNAs encoding the various forms of NGK2A were stably cotransfected with the cDNA encoding the various forms of NGK2A were stably cotransfected with the cDNA-
coding CD94 into RBL cells and clones expressing comparable levels of each CD94/NKG2A receptor (Fig. 1B) were selected for comparative analyses.

**Effect of ITIM mutations on the ability to phosphorylate NKG2A**

The recruitment of PTPs by ITIM containing proteins is dependent on phosphorylation of the ITIM Tyr residues by kinases. To insure that the ITIM Tyr residues could be phosphorylated, CD94/NKG2A was isolated from cells that had been treated with pervanadate to inhibit endogenous phosphatase activity (30). CD94/NKG2A was immunoprecipitated using the NKG2A-specific mAb Z199 and analyzed for phosphorylation by immunoblotting (Fig. 2A). As expected, no Tyr phosphorylation was detected for the double Tyr mutant Y8F/Y40F. The Y40F mutation decreased the amount of phosphorylation detectable by immunoblotting roughly by one-half compared with the wt NKG2A. The Y8F mutant NKG2A was routinely less phosphorylated than the Y40F mutant. The mutations at the Y-2 positions (V6A, I38A, V6A/I38A) had little or no effect on phosphorylation levels. Similar data was obtained when CD94-specific mAb was used to precipitate the CD94/NKG2A receptors (data not shown).

**Effect of ITIM mutations on the ability of CD94/NKG2A to associate with phosphatases**

Phosphorylated NKG2A has been shown to primarily recruit the SHP-1 PTP and to interfere with activation signals generated by stimulatory receptors (27, 28). To identify the PTPs capable of being recruited by the CD94/NKG2A receptors expressed in the RBL transfectants, the cells were treated with pervanadate to insure maximal phosphorylation and the PTPs coimmunoprecipitated with anti-CD94 or -NKG2A mAb were examined by immunoblotting with anti-SHP-1, -SHP-2, or SHIP Ab. As expected, SHP-1 was found to coprecipitate with the wt CD94/NKG2A receptor (Fig. 2B); however, all of the ITIM mutations interfered to some degree with the amount of SHP-1 coprecipitated with CD94/NKG2A. The immunoprecipitates of the Tyr single mutants Y8F or Y40F had markedly reduced levels of SHP-1 compared with wt, and no detectable SHP-1 was coprecipitated with the double Tyr mutant Y8F/Y40F. Both of the single Y-2 mutants, V6A and I38A, coprecipitated roughly 50% of the amount of SHP-1 compared with wt CD94/NKG2A and the double Y-2 mutant V6A/I38A was even less effective in coprecipitating SHP-1. Only wt

**FIGURE 1.** NKG2A mutations and surface expression. A, NKG2A ITIM sequences are shown for wt with amino acid substitutions shown for the mutants. B, Flow cytometric analyses of CD94/NKG2A cell surface expression levels by RBL cells. Solid line histograms show the expression levels of the CD94/NKG2A receptor as assessed by direct immunofluorescence using PE-conjugated anti-NKG2A-specific mAb (Z199). Dotted line histograms show the fluorescence of cells incubated with PE-conjugated isotype control Ab (anti-dansyl IgG2b). Numbers are the mean values.
CD94/NKG2A showed any detectable association with SHP-2 and in no case was SHIP detected in the CD94/NKG2A immunoprecipitates (data not shown).

**Analysis of SHP-1 and NKG2A cellular localization by confocal microscopy**

Except for the Y8F/Y40F double mutant, all of the CD94/NKG2A receptors clearly can associate with SHP-1 after hyperphosphorylation induced by pervanadate treatment (Fig. 2B). We used confocal microscopy to determine whether receptor ligation leads to the colocalization of SHP-1 with each of the CD94/NKG2A receptors. RBL cells transfected with CD94 plus wt or mutant NKG2A were plated on cs coated with anti-CD94 mAb. The cells were permeabilized and stained with Alexa Fluor 594 (red) tagged anti-SHP-1 or anti-SHP-2 and Alexa Fluor 488 (green) tagged anti-NKG2A. Fig. 3, A and B, show the results of anti-SHP-1 staining for cells transfected with wt, Y8F/Y40F, Y8F, and Y40F NKG2A. For each type of transfectant, the staining for the one-third portion of the cells on the side of cs attachment and the one-third opposite this is shown. The cartoon in Fig. 3 illustrates the portions of the cells depicted in Fig. 3, A and B. Cross-linking of CD94/NKG2A receptors leads to an accumulation of receptor clusters within the portion of the cells where CD94/NKG2A attaches to the anti-CD94 on the cs (data not shown; see Fig. 4). In cells expressing wt NKG2A, SHP-1 also tends to localize to this region. The colocalization of SHP-1 with wt NKG2A is shown in the Fig. 3, C–E, top panels. The green color shows the location of NKG2A in the cells (Fig. 3C) and the red staining in the same cells shows the location of SHP-1 (Fig. 3D). Fig. 3E shows these same cells with visualization of SHP-1 and NKG2A merged. The yellow color is indicative of regions within the cell where SHP-1 and NKG2A are colocalized. Similar studies done with Alexa Fluor 594-tagged anti-SHP-2 Ab revealed positive staining, but did not reveal colocalization of this PTP with CD94/NKG2A. In contrast, there is a marked reduction in the colocalization of SHP-1 with the Y8F/Y40F mutant (Fig. 3, upper middle panels), which cannot be phosphorylated and does not coprecipitate SHP-1. In contrast, the staining pattern observed with the Y40F NKG2A mutant approximates that observed for wt NKG2A. Similar results were obtained with the V6A, I38A, and V6A/I38A NKG2A mutants (data not shown). This colocalization of SHP-1 and NKG2A in the portion of the cells (wt, V6A/I38A, Y40F, V6A, Y40F, I38A) where cs attachment occurs is more clearly shown in the vertical cross-sections of representative cells in which both SHP-1 and NKG2A are visualized (Fig. 4). The cartoon illustrates the stained portions of the cells shown in Fig. 4. Furthermore, this figure shows that this colocalization of SHP-1 and CD94/NKG2A also occurs in NKL cells that naturally express the CD94/NKG2A receptor.

**Analysis of the RBL cells expressing the Y8F NKG2A mutant molecules** showed that similar to the other transfectants, CD94/NKG2A clearly accumulates on the side of cs attachment (Fig. 4). However, similar to the Y8F/Y40F double mutant, SHP-1 is more evenly dispersed throughout the cell, as indicated by the near equal staining intensity for SHP-1 in the two portions of the cell (Fig. 3.
lower middle panels, compare A and B). This nearly even distribution of SHP-1 within the Y8F transfectant cells is further emphasized in the single-cell cross-section depicted in Fig. 4. As shown in Fig. 3E, cells containing the Y8F mutant NKG2A molecules show significantly less colocalization of SHP-1 with NKG2A than the other transfectants, other than the Y8F/Y40F double mutant.

**Effect of ITIM mutations on the ability of CD94/NKG2A to inhibit mast cell degranulation**

It was shown for KIR and other inhibitory receptors that their ability to inhibit transmission of activation signals requires coaggregation with the activation receptors (31). We attached IgE to the culture plate to generate stimulatory signals through ligation of the FcεRI receptor present on RBL cells either in the presence or absence of plate-bound anti-CD94 or anti-NKG2A. With the maximal concentration of anti-CD94 (20 μg/ml), the coligation of CD94/NKG2A possessing wt ITIMs simultaneously with the cross-linking of FcεRI resulted in a 79 ± 5% (n = 6) inhibition of serotonin release compared with cells activated by FcεRI cross-linking alone (Fig. 5). In contrast, consistent with its inability to recruit SHP-1 to sites of receptor ligation, coligation of the Y8F/Y40F CD94/NKG2A mutant receptor had no effect on FcεRI-mediated degranulation. As indicated in Fig. 5, the V6A mutation had essentially no impact on the level of inhibition relative to wt, whereas the double Ala mutant (V6A/I38A) and the other single Ala mutant (I38A) were slightly less effective than the wt CD94/NKG2A receptor in inhibition of degranulation. Consistent with the fact that it was able to colocalize SHP-1, the Y40F NKG2A was 70% as effective as the wt receptor. The CD94/NKG2A receptor containing the Y8F mutation was markedly less effective at transducing an inhibitory signal. As can be seen, the relative ability of the various CD94/NKG2A receptors to inhibit serotonin release does not change with different Ab concentrations. The ability of the Y8F mutant to generate a relatively weak inhibitory signal is consistent with a hint of SHP-1 colocalization (Fig. 4) and its ability to coprecipitate SHP-1 (Fig. 2B). However, it is clear from these results that the mutant NKG2A molecules (Y8F/Y40F and Y8F) that fail to generate CD94/NKG2A receptors that efficiently inhibit serotonin release.
Discussion

Our data indicate that both NKG2A ITIMs contribute to inhibitory function as maximal inhibitory activity is only achieved when both ITIMs are intact. These results with NKG2A agree with previous studies using KIR constructs showing that while both ITIMs contributed in mediating inhibitory function, one clearly was more crucial for mediating the inhibitory signal (25, 26). The results obtained by Burshtyn et al. (14) using KIR to inhibit Ab-dependent cellular cytotoxicity mediated by the FcγRIII receptor in NK cells differ in that they showed that a single intact ITIM could mediate a maximal inhibitory signal. In this case, inhibition was almost completely abrogated by substitution of the Tyr in the membrane-distal ITIM, whereas mutation of the membrane-proximal ITIM had little effect. As each of the above results were obtained in different experimental systems, the more potent single ITIM signal obtained by these investigators may be related to the degree of inhibitory signal required to block different activation signals in different cell types. Nonetheless, these data clearly indicate that for KIR the membrane-proximal (N-ter), ITIM is essential for function, whereas the membrane-distal (C-ter) ITIM plays at best a supportive role. As with KIR (14–16, 25, 26), our data show that one NKG2A ITIM plays a more critical role in CD94/NKG2A inhibitory function than the other (Fig. 5), but unlike KIR, the more important ITIM is the membrane-distal one rather than the proximal one. However, because KIR and NKG2A are type I and II membrane proteins, respectively, for both molecules, it is the N-ter ITIM that is most crucial.

A comparison of the human (32) and mouse (33) NKG2A sequences shown below (ITIMs underlined) offers further support that the membrane-distal (N-ter) ITIM is most critical for NKG2A inhibitory function:

Human, NKG2A (32):

\[
\text{NKG2A} \quad \text{Y6V/A38I} \\
\text{NKG2A} \quad \text{Y6A/V40F} \\
\text{NKG2A} \quad \text{Y6A/I38A} \\
\text{NKG2A} \quad \text{Y8F/A40F} \\
\text{NKG2A} \quad \text{Y8F/Y40F} \\
\text{NKG2A} \quad \text{Y8F/V6A} \\
\text{NKG2A} \quad \text{Y8F/V6A/I38A} \\
\]

Mouse, NKG2A (33):

\[
\text{NKG2A} \quad \text{Y6V/A38I} \\
\text{NKG2A} \quad \text{Y6A/V40F} \\
\text{NKG2A} \quad \text{Y6A/I38A} \\
\text{NKG2A} \quad \text{Y8F/A40F} \\
\text{NKG2A} \quad \text{Y8F/Y40F} \\
\text{NKG2A} \quad \text{Y8F/V6A} \\
\text{NKG2A} \quad \text{Y8F/V6A/I38A} \\
\]

As can be seen, mouse NKG2A lacks a functional membrane-proximal ITIM due to the presence of a Phe residue at position 43 (bold above) in its intracytoplasmic amino acid sequence, yet it is a fully functional inhibitory receptor (33, 34). This agrees with data discussed below that proposes that the ITIM that interacts with the N-ter SH2 domain (N-SH2) of SHP is the only ITIM essential for function. Rat NKG2A also lacks a functional membrane-proximal ITIM (35).

Previous investigators (14–16) have shown that the residues at position Y-2 in ITIMs contribute to the strength of the inhibitory signal delivered by KIR, but are not an absolute requirement for inhibition. The V and I residues at this position in the respective ITIMs were shown to be important for maintaining the association of KIR and SHP-1 in detergent lysates (14). In agreement with this, we show that mutations at the Y-2 positions (V6A, I38A, V6A/I38A) greatly reduce SHP-1 association relative to wt NKG2A (Fig. 2B). However, these mutations did not impact the ability of the ITIM Tyr residues to be phosphorylated (Fig. 2A) and, despite the reduction in affinity for SHP-1, these mutations had little effect on the ability of NKG2A to convey inhibitory signals (Fig. 5) in agreement with analogous studies on KIR (14).

The fact that SHP-1 association can still be detected with the V6A and I38A mutant forms of NKG2A suggests that SHP-1 is still mediating the inhibitory signal for the Y-2 position mutants. This is supported by the observation that SHP-1 colocalizes with ligated CD94/NKG2A containing Y-2 mutations as well as it does with wt CD94/NKG2A (Figs. 3 and 4).

NKG2A clearly reacts with SHP-1 (Figs. 2B, 3, and 4) and, as with other ITIM-bearing molecules (1, 27), this association likely leads to activation of this PTPase, which mediates its inhibitory effect. SHP-1 (16) and SHP-2 (36) catalytic activity is enhanced by the simultaneous interaction of its two SH2 domains with phosphorylated ITIMs. In agreement, our data (Fig. 5) show that the most effective inhibition occurs with NKG2A molecules that retain two intact phosphorylation sites (wt and the Y-2 mutants). SHP-1 has been shown to dephosphorylate phosphotyrosine proteins like CD3ζ in the CD3/TCR and CD16 signaling pathways (37), as well as tandem SH2 containing protein tyrosine kinases like Syk and ZAP-70 (38, 39), and adaptor proteins like linker for activation of T cells (LAT) (40) and SLP-76 (41). This likely accounts for the ability of CD94/NKG2A to inhibit signal transduction by the FcεRI receptor in RBL cells, as phosphorylation of Lyn and Syk are primary events in the FcεRI signaling cascade (42).

The domain organization of many signaling proteins facilitates the segregation of binding, catalytic, and regulatory functions. Intracellular targeting and enzyme activation are usually intimately
coupled. The mammalian SH2 domain PTPs contain tandem SH2 domains (N-SH2 and C-ter SH2 domain (C-SH2)) and a single C-ter catalytic domain (PTPase) (Ref. 43; see Fig. 6A). As well as directing subcellular localization by binding receptors with specific phosphorylated amino acid sequences, the two SH2 domains function together to regulate catalysis (16, 36, 44). The crystal structure of SHP-2 has been determined, as well as the binding and functional properties of its individual domains determined (44, 45). In the basal state, the N-SH2 domain of SHP-2 is bound to the PTPase domain by a series of hydrogen bond and charge-charge interactions (44–47), which serve to inactivate PTPase activity (Fig. 6A). In comparing the amino acid sequences of SHP-1 and SHP-2, which share 60% overall sequence identity (47), almost all of the interactions between the N-SH2 and PTPase domains of SHP-2 are conserved in SHP-1 (45), suggesting that these PTPs share a similar mechanism of regulation.

The interaction between the N-SH2 domain and the PTPase domain occludes the phosphopeptide binding groove of the N-SH2 domain (45). In contrast, the C-SH2 domain makes only weak contacts with the PTPase domain and its binding pocket is unper-

FIGURE 5. Effect of ITIM mutations on CD94/NKG2A-mediated inhibition of serotonin release by RBL cells. The graphs show the percentage of serotonin release (±SD) after coligation of FcεRI and CD94/NKG2A for each CD94/NKG2A molecule (n = 6). A constant concentration of IgE anti-DNP mAb (20 μg/ml) was used with increasing concentrations of anti-CD94 mAb. Percentage of specific release was calculated as follows: (cpm in supernatant – cpm spontaneous release)/(total cpm – cpm spontaneous release) × 100, where total cpm is cpm in supernatant + cpm in lysate+2. The percentage of serotonin released obtained by cross-linking IgE without CD94/NKG2A coligation was: wt (20 ± 1.5), V6A (21 ± 0.7), I38A (15 ± 2.9), V6A/I38A (26 ± 0.6), Y40F (25 ± 1), Y8F (29 ± 0.4), and Y8F/Y40F (18 ± 1). For each CD94/NKG2A receptor, the plotted values are normalized with maximal release as 100%. The table under the graphs shows the percentage of inhibition of serotonin release after coligation of FcεRI (20 μg/ml of IgE anti-DNP mAb) and CD94/NKG2A (20 μg/ml of anti-CD94 mAb). The inhibition values are normalized to wt as 100% of maximal inhibition.
mutants (V6A, I38A, V6A/I38A), it is clear that all of these mutations interfere with the affinity of SHP-1 for NKG2A, as indicated by the diminished coprecipitation of SHP-1 compared with wt (Fig. 2B). However, this diminished affinity for SHP-1 does not significantly impact on the inhibitory function of the mutants.

It was shown for insulin receptor substrate-1 (36, 44, 51) and Gab1 (52) that the order and orientation of tandem ITIMs is very important for SHP-2 activation. This observation, coupled with the facts that N-SH2 interaction with ITIM is critical for PTP activation (45) and that maximum PTP activation is achieved only when both N-SH2 and C-SH2 are engaged (16, 36) supports the model for NKG2A ITIM interactions with the SHP-1 SH2 domains depicted in Fig. 6C. This model, which shows the interaction of the NKG2A N-ter and C-ter ITIMs with the N-SH2 and C-SH2 SHP-1 domains, respectively, is supported by the fact that the Y40F NKG2A mutant retains significantly more inhibitory function than the Y8F mutant (Fig. 5). Data obtained with KIR (14) support an identical orientation (Fig. 6C). The observations that for KIR the membrane-proximal ITIM is more critical for function (14, 26), whereas our data indicate that for NKG2A the membrane-distal is more critical, are explained by the fact that KIR and NKG2A are type I and II membrane proteins, respectively. Other studies that
have investigated the interaction between SHP-1/2 and proteins containing multiple ITIMs also support this view of the orientation of the interaction (15, 16, 25, 53). In summary, it is clear from our data on CD94/NKG2A and for that on KIR ITIMs (14–16, 25, 26) that one of the two ITIMs present in these molecules is of primary importance for generating inhibitory activity and in both cases this is the N-ter ITIM.

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References


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