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Src Homology Region 2-Containing Protein Tyrosine Phosphatase-2 (SHP-2) Can Play a Direct Role in the Inhibitory Function of Killer Cell Ig-Like Receptors in Human NK Cells

Sei-ichi Yusa and Kerry S. Campbell

The inhibitory forms of killer cell Ig-like receptors (KIR) are MHC class I-binding receptors that are expressed by human NK cells and prevent their attack of normal cells. Substantial evidence indicates that the mechanism of KIR-mediated inhibition involves recruitment of the protein tyrosine phosphatase, Src homology region 2-containing protein tyrosine phosphatase (SHP)-1, to phosphorylated immunoreceptor tyrosine-based inhibitory motifs (ITIMs). However, the functional significance of parallel recruitment of a SHP-1-related phosphatase, SHP-2, to KIR ITIMs has not been addressed. In the present study, our results with mutant forms of a classical KIR, KIR3DL1, show a direct correlation between SHP-2 recruitment and functional inhibition of target cell conjugation and cytotoxicity. In addition, KIR3DL1 inhibition of target cell cytotoxicity is blocked by overexpression of a dominant-negative form of SHP-2. Finally, KIR3DL1 fused directly with the catalytic domain of SHP-2 inhibits both target cell conjugation and cytotoxicity responses. These results strongly indicate that SHP-2 catalytic activity plays a direct role in inhibitory KIR functions, and SHP-2 inhibits NK cell activation in concert with SHP-1.


natural killer cells play an important role in immune surveillance by detecting and spontaneously lysing MHC class I (MHC-I)−-deficient tumor cells and virus-infected cells that have escaped detection by CTL. NK cell activation is controlled by a dynamic balance of opposing signals from activating and inhibitory receptors (1, 2). Improved understanding of the receptors and intracellular pathways that control NK cell actions is important for developing methods to manipulate NK cells and enhance their therapeutic use in treating cancer, virus infection, and graft-vs-host disease (3, 4). NK cell activation is triggered by any one of several receptor-coupled pathways, including NKp46, NKp44, NKp30, NKG2D, 2B4, LFA-1, CD2, and CD69 (5). Thus, NK cell activation is not controlled by a single clonotypic Ag receptor, such as Ag receptors on T or B cells.

Conversely, NK cell activation is abrogated by MHC-I-binding inhibitory receptors. Inhibitory killer cell Ig-like receptors (KIR) are the major classical MHC-I-binding inhibitory receptors expressed on human NK cells and subsets of T cells (6–9). Engagement of these inhibitory receptors has been shown to block natural cytotoxicity, Ab-dependent cellular cytotoxicity (10, 11), and adhesion toward target cells (12).

KIR possess two immunoreceptor tyrosine-based inhibitory motifs (ITIMs; (I/V)xYxx (L/V)) (13, 14), which contain tyrosine residues that are critical elements for mediating inhibitory function (14–18). Phosphorylated tyrosines in the ITIMs of inhibitory receptors serve as docking sites to recruit Src homology region 2 (SH2) domain-containing phosphatases, such as the protein tyrosine phosphatases (PTP), SH2-containing PTP (SHP)-1 and SHP-2, and the SH2-containing inositol 5′-phosphatase (SHIP). Several groups have reported that tyrosine-phosphorylated KIR ITIMs recruit the PTP, SHP-1 (11, 15–20), SHP-2 (14), and SHP-2-containing inositol 5′-phosphatase (SHIP).

SHP-2 is a widely expressed cytoplasmic PTP, which shares high structural and primary sequence homology with SHP-1 (24). Interestingly, three previous reports have described recruitment of SHP-2 to phosphopeptides corresponding to the two ITIMs of KIR (19, 25) or to chimeric forms of KIR expressed in B cells and mast cells (15), while our group has recently demonstrated that SHP-2 binding to the single ITIM of the KIR2DL4 (2DL4) cytoplasmic domain correlates with strong inhibitory function (18). Paradoxically, SHP-2 has been thought to be primarily a positive regulator of cell proliferation when recruited to numerous receptors (26–31).
In contrast, some evidence supports an inhibitory role for SHP-2 upon binding certain ITIM-containing inhibitory receptors, such as CTLA4 (32, 33), platelet endothelial cell adhesion molecule/CD31 (34–36), SHP substrate-1/signal regulatory protein-ξ (37), FDF03 (38), and PZR (39).

Although previous biochemical evidence demonstrated that SHP-2 can be recruited to KIR, the functional significance of this recruitment has not been previously addressed. The current work was undertaken to evaluate whether SHP-2 plays a direct functional role in KIR-mediated inhibition. In addition to showing a correlation between inhibition and SHP-2 binding to mutant forms of KIR3DL1 (3DL1), we have observed that overexpression of functional KIR3DL1 (3DL1) and PZR (39).

Materials and Methods

Cell culture and Abs

The IL-2-dependent NK-like cell lines, NKL (a gift from Dr. M. Colonna, Washington University, St. Louis, MO) and NK-92 (a gift from Dr. C. Lutz, University of Iowa, Iowa City, IA), were cultured as previously described (18). Cells were passed with fresh IL-2 every 4 days. The murine mastocytoma, P815, was cultured as previously described (18). Abs were purified with protein G from the hybridomas for the anti-3DL1 mAb DX9 and the anti-CD56 mAb B159.2.5, which were kindly provided by Dr. L. L. Lanier (University of California, San Francisco, CA) and Dr. B. Perussia (Thomas Jefferson University, Philadelphia, PA), respectively. Hybridoma supernatant containing anti-NKp46 mAb (9E2) was kindly provided by Dr. M. Colonna.

cDNA constructs

KIR cDNA constructs were ligated into the retroviral expression vector, pBMN-NoGFP, derived from pBMN-ires-EGFP (40), using BamHI and NotI restriction sites as previously described (18). NotI restriction sites of 3DL1 were desig...

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>pBMN antisense</td>
<td>5’-GACCTTTGATCTCCTTCTTGGCG-3’</td>
</tr>
<tr>
<td>Y377F sense</td>
<td>5’-AGAAGTTGACATCCGACAGTGGTATGATC-3’</td>
</tr>
<tr>
<td>Y377F antisense</td>
<td>5’-TGACCTCAATCGGGAAATGTCACCTCCT-3’</td>
</tr>
<tr>
<td>Y407F sense</td>
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<td>Y407F antisense</td>
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that formed conjugates with target cells as calculated by the ratio of two-color events to total effector cell events using FlowJo software (Tree Star, Woburn, MA).

**Pervanadate treatment and cell lysis**

NK cells were washed three times in HBSS (Life Technologies), stimulated with pervanadate (final concentration: 100 μM Na2VO4 plus 0.015% H2O2), and lysed for 30 min on ice in 1 ml of 1% Triton X-100 lysis buffer per sample as previously described (18). Lysates were cleared by centrifugation at 20,800 × g for 15 min at 4°C.

**Immunoprecipitation and immunoblotting**

Lysates were precleared twice for 30 min each at 4°C with protein G-coupled agarose (Upstate Biotechnology, Lake Placid, NY). CD56 and KIR were sequentially immunoprecipitated for 90 min at 4°C with B159.5.2 and DX9 mAbs (5 μg each sample precoupled to 30 μl of protein G-agarose) as performed previously (18), separated on SDS-PAGE, transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), and probed either directly with HRP-coupled 4G10 mAb (anti-phosphotyrosine, 1:10,000; Upstate Biotechnology) or initially with rabbit polyclonal anti-SHP-1 (1 μg/ml; Upstate Biotechnology) or anti-SHP-2 (1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) Abs and secondarily with HRP-coupled protein G (1:10,000; Calbiochem, La Jolla, CA).

**Vaccinia virus infections**

Purified recombinant vaccinia virus preparations generated with the plasmid pSC65 containing cDNA of wild-type SHP-1 or DN-SHP-1 (C453S) (11) were kindly provided by Dr. E. O. Long (National Institutes of Health, Rockville, MD). Generation of the recombinant vaccinia virus encoding C459S SHP-2 cDNA was performed as described (41). Aliquots of the recombinant virus preparations were dispersed by water bath sonication and stored at −70°C before use. The titer of virus stocks was determined by plaque assay as described (41) (wild-type SHP-1, 1 × 109 PFU/ml; C453S SHP-1, 0.25 × 109 PFU/ml; C459S SHP-2, 1 × 109 PFU/ml). NK-92 cells were infected with the recombinant vaccinia virus as described previously (18, 42). Vaccinia virus infections were monitored for SHP-1 or SHP-2 protein expression by immunoblot analysis of the infected cells. Viability of the infected cells was monitored by trypan blue staining before mixing with target cells (>95% viable).

**Phosphatase assay**

NK-92 cells stably transduced with KIR/SHP1 or KIR/SHP2 were lysed with Triton X-100 lysis buffer (lacking sodium orthovanadate, sodium fluoride, and EDTA) and immunoprecipitated as described (18). Immunoprecipitates were washed three times with ice-cold 1% Triton X-100 buffer (same components as lysis buffer), washed again three times with 150 mM NaCl/50 mM Tris-HCl (pH 7.5) and resuspended in 100 μl of reaction buffer (12 mM p-nitrophenylphosphate (pNPP), 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA). After incubation at 37°C for 1 h, the reaction was stopped by adding 17 μl of 2.5 N NaOH, and the amount of p-nitrophenyl released was determined from absorbance at 410 nm.

**Results**

**The analysis of KIR with mutant ITIMs in human NK cells**

To determine the enzyme recruitment properties of and functional roles for the individual ITIM tyrosines on classical inhibitory KIR in human NK cells, we have used retroviral transduction to express wild-type and tyrosine to phenylalanine mutant forms of 3DL1 (WT) at the N-terminal (N-ITIM) (FY), C-terminal (C-ITIM) (YF), or both ITIMs (FF) (Fig. 1A, left panel). A truncated form of 3DL1, in which the cytoplasmic domain is deleted just before the N-ITIM (272Δ), was used as a negative control. These were expressed at comparable levels in the NK-like cell line, NK-92 (Fig. 1A, right panel), and tested for capacities to inhibit cytotoxicity in a redirected assay against P815 target cells designed to specifically engage only the 3DL1 inhibitory receptors. Retention of only the N-ITIM of 3DL1 was still strongly inhibitory (Fig. 1B, middle panel), in contrast to 3DL1/FF and 3DL1/FY, which demonstrated weak but highly reproducible inhibition (Fig. 1B and Table II). These results demonstrate that the N-ITIM is essential and sufficient to contribute strong inhibitory function to 3DL1, while residual inhibition remains, even when both ITIM tyrosines are mutated to phenylalanine.

The initial stage of NK cell-mediated cytotoxicity is the formation of stable conjugates between target and effector, and KIR engagement has been shown to disrupt conjugate formation (12). To determine whether the interaction of NK-92 with P815 cells was disrupted by engagement of the individual mutant KIR, we quantitated conjugate formation using two-colored flow cytometry. We found that engagement of the mutant retaining only the N-ITIM of 3DL1 (YF) still significantly reduced conjugate formation (Fig. 1C), although, as in the cytotoxicity experiments (Fig. 1B and Table II), the inhibition was not as strong as that of the wild-type receptor. In contrast, the other mutants, FY or FF, were inefficient in disrupting conjugate formation (Fig. 1C). These combined results demonstrate similar patterns of inhibitory capacities for the mutants in assays of both conjugate formation and redirected cytotoxicity, with WT (YY) > YF > FY > FF.

Biochemical analysis was next performed to assess PTP recruitment to phosphorylated 3DL1 ITIM mutants (Fig. 2). NK-92 cells expressing the wild-type and mutant forms of 3DL1 were treated with pervanadate to induce optimal tyrosine phosphorylation before 3DL1 immunoprecipitation. In these studies, both SHP-1 and SHP-2 were recruited to the phosphorylated wild-type form of the receptor, but SHP-1 was not recruited to any of the KIR with mutant ITIMs (Fig. 2), indicating that SHP-1 binding requires tyrosine phosphorylation of both ITIMs. In contrast, phosphorylation of only the N-ITIM (YF) of 3DL1 still recruited increased levels of SHP-2 (Fig. 2A), which correlated with the stronger inhibitory properties of that mutant KIR. In contrast, the receptor with mutation of the N-ITIM (FY) did not demonstrate increased SHP-2 binding in pervanadate-treated cells, despite strong tyrosine phosphorylation (Fig. 2A). Also, as we have previously observed with a phenylalanine mutant of the single N-ITIM in 2DL4 (18), the FY and FF mutants constitutively bound SHP-2, even in unstimulated cells, which was most evident in immunoprecipitates from greater numbers of cells (Fig. 2B). Interestingly, this constitutive SHP-2 association correlated with the weak inhibitory properties of the FY and FF mutant receptors in the redirected cytotoxicity assays (Fig. 1B). These results indicate that SHP-2 is constitutively associated with the unphosphorylated N-ITIM of KIR and may contribute to early negative signaling and/or allow rapid high avidity recruitment upon ITIM phosphorylation. Importantly, the common theme among these mutant receptors is a direct correlation between SHP-2 recruitment capacity and inhibitory functions (Fig. 1, B and C, and Table II). These results suggest that SHP-2 contributes substantially to the inhibitory properties of the mutant receptors and is likely also contributing to the inhibitory function of wild-type 3DL1.

**DN-SHP-2 blocks inhibition of natural cytotoxicity by 3DL1**

Because our experiments demonstrated efficient SHP-2 binding capacity by KIR and correlations between SHP-2 binding capacity and inhibitory function, we next tested whether expression of a DN-SHP-2 protein (catalytically inactive) could abolish KIR inhibitory function. Vaccinia virus-mediated expression of DN-SHP-1 has been shown to block inhibition through KIR, which provides convincing evidence for SHP-1 involvement in this process (10, 11, 23). Therefore, we compared the impacts of recombinant vaccinia virus preparations expressing DN-SHP-1 (C453S), wild-type SHP-1 (WT-SHP-1) (11), and DN-SHP-2 (C459S; with
a myc epitope tag) on 3DL1 inhibitory function. NK-92 cells expressing wild-type 3DL1 were infected with purified vaccinia preparations at PFU titers that produced robust levels of exogenous PTP (10- to 50-fold above endogenous; Fig. 3A and Ref. 18). The binding of the exogenous DN-SHP-2 protein to 3DL1 was demonstrated by immunoprecipitation and immunoblotting analysis, confirming that DN-SHP-2 is recruited to phosphorylated 3DL1 in vivo (Fig. 3B). In a redirected cytotoxicity assay against P815 target cells (Fig. 3C), KIR inhibition was unchanged by expression of WT-SHP-1 (Fig. 3C, upper panels). However, DN-SHP-1 completely abrogated wild-type KIR-mediated inhibition (Fig. 3C, middle panels). As predicted from our biochemical results, expression of DN-SHP-2 also completely blocked KIR-mediated inhibition (Fig. 3C, lower panels), providing direct evidence that SHP-2 is recruited to KIR during inhibitory signaling in vivo.

We next tested the impacts of the DN-PTP expression on inhibition through the mutant KIR (WT, YF, FY, and FF). We expected that the inhibitory function of these SHP-2-binding KIR would be blocked by DN-SHP-2, but not DN-SHP-1. Indeed, the DN-SHP-1 was not effective in blocking any of these modified inhibitory receptors (Fig. 4A and FF, data not shown). In agreement with our biochemical data, we therefore conclude that these mutant KIR lacking specific ITIMs inhibit NK cell cytotoxicity in a SHP-1-independent manner. Unexpectedly, we were unable to demonstrate blockade of inhibition through any of these mutant KIR by DN-SHP-2 (Fig. 4B); rather expression of the DN-SHP-2

Table II. Statistical analysis of the capacities of mutant KIR3DL1 receptors to inhibit cytotoxicity from multiple experiments

<table>
<thead>
<tr>
<th>Receptors</th>
<th>No. of experiments</th>
<th>Mean-Control (SD)</th>
<th>Mean-Anti-KIR3DL1 (SD)</th>
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<tr>
<td>KIR3DL1</td>
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<td>100 (3.2)</td>
<td>32.8 (21.4)*</td>
</tr>
<tr>
<td>FY</td>
<td>6</td>
<td>100 (8.5)</td>
<td>67.5 (10.3)*</td>
</tr>
<tr>
<td>YF</td>
<td>6</td>
<td>100 (3.8)</td>
<td>41.6 (13.4)*</td>
</tr>
<tr>
<td>FF</td>
<td>6</td>
<td>100 (3.7)</td>
<td>78.5 (9.3)*</td>
</tr>
<tr>
<td>272PΔ</td>
<td>4</td>
<td>100 (3.8)</td>
<td>93.2 (10.3)</td>
</tr>
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</table>

*Statistical analysis was performed on cytotoxicity data from multiple experiments by converting the data at a 10:1 E:T ratio within each experiment to percentage of control cytotoxicity (without Abs). Within each experiment the duplicate or triplicate data points in the absence of Abs were averaged, and the average was designated 100% of control cytotoxicity. Every data point for each transduced cell line within each experiment was converted to this percent of control cytotoxicity, and a Student’s t-test was performed to compare the aggregate data points from Abs untreated and anti-KIR3DL1 1-treated conjugation conditions for each transduced cell line. Statistical analysis was performed on the Excel X program for Macintosh (Microsoft, Redmond, WA).

p < 0.05 compared to nontreated controls.
dramatically reduced the basal cytolytic capacity of NK-92 cells expressing 3DL1/FY, 3DL1/YF, and 3DL1/FF (Fig. 3C and data not shown), but not those expressing wild-type 3DL1 (as in Fig. 3C). This is an intriguing observation, which may reflect the possibility that DN-SHP-2 interferes with a broad spectrum of functions in human NK cells, including positive roles of SHP-2, such as IL-2 receptor signaling (43–45). We cannot yet explain why cytolytic capacity was selectively disrupted by DN-SHP-2 expression only in cells expressing these mutant KIR, but the result prompted us to try an alternative approach to answer whether SHP-2 indeed contributes directly to mutant KIR inhibitory function.

The SHP-2 catalytic domain inhibits natural cytotoxicity

We demonstrated that KIR phosphorylated only at the N-ITIM strongly inhibit natural cytotoxicity and recruit only SHP-2 (Fig. 1B and 2). In addition, vaccinia virus-expressing DN-SHP-2 blocks normal 3DL1 inhibition of natural cytotoxicity (Fig. 3C).

FIGURE 2. Biochemical analysis of SHP-1 and SHP-2 recruitment to differentially phosphorylated KIR. A, SHP-2 recruitment can be seen in the mutant KIR with an intact tyrosine-phosphorylated N-terminal ITIM (Y407F) as well as with wild-type 3DL1. Receptor-transduced NK-92 cells (40 million cells/sample) were treated with pervanadate for 10 min, lysed, and sequentially immunoprecipitated with anti-CD56 as a control (data not shown) and then anti-3DL1 mAbs. Samples were analyzed on 10% SDS-PAGE gels under reducing conditions. Sequential immunoblot analysis was performed with anti-phosphotyrosine and anti-SHP-2. B, SHP-2 constitutively binds to the mutant ITIMs. Several forms of KIR were immunoprecipitated and processed (80 million/sample) as in A. Samples were sequentially immunoblotted with anti-SHP-1 and anti-SHP-2.

FIGURE 3. Transient expression of the DN forms of SHP-1 and SHP-2 blocks 3DL1 inhibition. A, Expression of the exogenous C459S SHP-2 using recombinant vaccinia virus. NK-92 cells were infected with the virus preparation at different PFU concentrations. The whole cell lysates (0.5 million cells/lane) were separated by 10% SDS-PAGE gels under reducing conditions and sequentially probed with anti-Myc followed by anti-SHP-2. B, The DN-SHP-2, C459S SHP-2, binding to 3DL1 in vivo. The 3DL1-transduced NK-92 (60 million) cells were infected with vaccinia virus possessing C459S SHP-2 cDNA at 10 PFU/cell, and stimulated without or with pervanadate for 10 min. KIR were immunoprecipitated with anti-KIR mAb (DX9), separated by 10% SDS-PAGE, and blotted sequentially with anti-phosphotyrosine (4G10), anti-Myc, and anti-SHP-2 Abs. C, Redirected cytotoxicity assay using P815 as a target. Recombinant vaccinia virus preparations encoding either wild-type SHP-1 (upper panels), C453S SHP-1 (middle panels), or C459S SHP-2 (lower panels) were used to infect 3DL1-expressing NK-92 cells at 10 PFU/cell (right panels). Aliquots of infected cells were used in a redirected cytotoxicity assay in the presence of either anti-CD56 (B159.5.2; ○) as a control or anti-3DL1(DX9; ●) mAbs (1 μg/ml).
Although these results strongly implicate SHP-2 in KIR inhibition, SHP-2 more commonly contributes to activation signals than inhibitory signals in other receptor systems (46–52). Therefore, to directly test whether the catalytic domain of SHP-2 can inhibit NK cell activation, we generated receptors in which we replaced the cytoplasmic domain of 3DL1 with the catalytic domains of SHP-1 or SHP-2. This is a strategy similar to that used to study the detrimental effect of SHP-1 on TCR signaling (53–55). Our chimeric receptors mimic conditions under which only a single effector enzyme is recruited, thereby excluding functional influences from the other effector enzyme (Fig. 5A). NK-92 cells were transduced with these chimeric receptors and cells expressing similar levels of receptor were sorted (Fig. 5B). We confirmed that immunoprecipitates of each chimera exhibit PTP catalytic activity, because they hydrolyzed pNPP to similar degrees (Fig. 5C). When tested for inhibitory capacity in redirected cytotoxicity experiments against P815 target cells, the chimeras containing the catalytic domains of both SHP-1 and SHP-2 demonstrated substantial inhibition (Fig. 5D). Engagement of the chimeras containing the catalytic domains of both SHP-1 and SHP-2 also significantly inhibited the conjugate formation between NK-92 and P815 cells (Fig. 5E), indicating that either PTP has the ability to regulate cell adhesion, when recruited to KIR ITIMs. These important results provide direct evidence that the PTP catalytic activities of either SHP-1 or SHP-2, when recruited to the cytoplasmic domain of KIR, can mediate negative signaling toward NK cell cytotoxicity responses. In addition, the chimeric receptor containing the phosphatase domain of SHP-2 also inhibited the cytotoxicity induced by NKP46 engagement on the surface of another NK-like cell line, NKL, in a redirected cytotoxicity assay (Fig. 5F), showing that the inhibitory role of SHP-2 is functional in the contexts of two different NK-like cell lines.

One readily apparent physical difference between SHP-2 recruitment to wild-type 3DL1 (with two ITIMs) and SHP-2 recruitment to 2DL4 or 3DL1/YF (with only the N-ITIM) is the likely existence of a free SH2 domain in the latter situations. We hypothesized that this free SH2 domain might recruit an additional negative effector enzyme to mediate inhibition. Therefore, we also generated 3DL1 chimeras in which the cytoplasmic domain consists of the two SH2 domains of SHP-2 (Fig. 5A) and expressed them in NK-92 cells as before (Fig. 5B). However, the chimera with SHP-2 SH2 domains did not exhibit any inhibitory (or activating) capacity in the redirected cytotoxicity assay, thereby indicating that a free SHP-2 SH2 domain does not provide adaptor function to recruit another negative effector enzyme (Fig. 5A, right panel).

**Discussion**

The majority of previously published reports have focused on the role of SHP-1 in KIR function, and that literature confirms that phosphorylation of both ITIMs is required for SHP-1 recruitment (10, 11, 14–20). Although two published reports have previously described SHP-2 binding to phosphorylated KIR ITIM peptides (19, 25), only the work of Bruhns et al. (15) has previously analyzed SHP-2 recruitment to KIR when expressed in rat mast cells or mouse B cells. In the current studies and our recent report (18), we have used retroviral transduction to express mutant and wild-type forms of 3DL1 and 2DL4 in NK cell lines to directly study their inhibitory functions on NK cell cytotoxicity and their SHP-1/SHP-2 recruitment capacities. Our results confirm and extend the work of Bruhns et al. (15) and our previous report (18) to show that phosphorylation of the N-ITIM of KIR is essential and sufficient for strong inhibitory function (Fig. 1) and SHP-2 recruitment (Fig. 2), but phosphorylation of both ITIMs is required to recruit SHP-1 (Fig. 2). Importantly, we have also shown that the capacities of mutant forms of 3DL1 to recruit SHP-2 correlate with their inhibitory functions. Interestingly, we have shown that SHP-2 associates weakly with mutant KIR in a phosphotyrosine-independent manner (Fig. 2). This supports our previous work (18), suggesting that SHP-2 is constitutively associated with the unphosphorylated N-ITIM and may be poised at the receptor for rapid high avidity binding upon ITIM phosphorylation. Previous work has ruled out a role for SHP in KIR function (14, 23), and we have not detected recruitment of SHIP to KIR in our studies (data not shown). Therefore, the phosphorylation status of KIR ITIMs determines SHP-1/SHP-2 recruitment capacities, which ultimately correlate with inhibitory function.

The most convincing evidence for SHP-1 involvement in KIR function is derived from experiments demonstrating that expression of DN-SHP-1 in NK cells blocks KIR-mediated inhibition of cytotoxicity responses (10, 11, 23). However, we have long considered that high avidity bivalent binding of DN-SHNP-1 to the phosphorylated KIR ITIMs would likely outcompete not only the functional endogenous SHP-1, but also any other negative effector protein that might be physiologically recruited to mediate inhibition. Because our biochemical evidence showed SHP-2 recruitment to KIR, we expressed DN-SHP-2 in NK cells and showed that it also effectively blocks the inhibitory function of wild-type KIR (Figs. 3 and 4). To our knowledge, this is the first report of the abrogation of function through any inhibitory receptor by expression of DN-SHP-2. Using DN-SHP-1, we also showed that inhibition by the mutant forms of KIR is SHP-1-independent (Fig. 4). Unfortunately, due to technical complications, we were unable to use DN-SHP-2 to block the inhibitory signal from mutant KIR (Fig. 4). Importantly, however, our results demonstrating the capacities of DN-SHP-1 and DN-SHP-2 to block wild-type 3DL1 function, strongly indicate that both SHP-1 and SHP-2 play roles in normal inhibitory KIR function.

Inhibition of cytotoxicity responses by the functionally related Ly-49A receptor is greatly impaired, but not completely abrogated, in SHP-1-deficient (motheaten) murine NK cells (56). It is possible that SHP-2 is mediating the residual inhibition through Ly-49 in...
motheaten NK cells. In contrast, it must be considered that the Ly-49 receptor is structurally very different from KIR, and therefore, a role for SHP-2 in its function awaits formal proof. Unfortunately, SHP-2-deficient mice are embryonic lethal, and embryonic stem cells from these mice are completely incapable of supporting lymphocyte development (57). Nonetheless, the motheaten results demonstrate that another effector enzyme is also mediating negative signaling through Ly-49 as well.

To directly address the capacity of SHP-2 catalytic activity to mediate KIR inhibition, we generated a chimeric KIR in which the cytoplasmic domain was substituted with the SHP-2 phosphatase domain, thereby mimicking only SHP-2 recruitment to KIR. Engagement of this KIR/SHP-2 chimeric receptor inhibited both target cell conjugation and cytotoxicity in a redirected assay in two NK-like cell lines (Fig. 5). A chimera with the catalytic domain of SHP-1 could also inhibit NK cell activation, while a chimera with the SH2 domains of SHP-2 exhibited no inhibitory capacity. These results demonstrate direct inhibitory capacity by both SHP-1 and SHP-2 phosphatases when recruited to the target cell interface in NK cells.

SHP-1 and SHP-2 share 60% sequence identity (amino acids) and very high homology in secondary and tertiary structure (24, 58). However, differential charge distribution in the catalytic clefts...
SHP-2 when recruited to KIR in the inhibitory immune synapse suggests that both PTPs have capacities to disrupt integrin-mediated adhesion. However, we further showed that the chimera containing SHP-1 and SHP-2 disrupts proximal Src family or Syk family protein tyrosine kinase functions while SHP-1 inhibits focal adhesion kinase in other cell types, which is believed to directly disrupt cell adhesion (65, 66). Because integrin-mediated signaling is a major activating pathway in NK cells during target cell conjugation (5), it is possible that SHP-2 attenuates integrin-mediated cell-cell adhesion, while SHP-1 inhibits proximal Src family or Syk family protein tyrosine kinase pathways, both of which are essential for “natural cytotoxicity.” Interestingly, we found that the SHP-2-binding YF mutant of 3DL1 and the chimera containing the catalytic domain of SHP-2 have the ability to inhibit conjugate formation (Fig. 1C and 5E), demonstrating a role for SHP-2 in regulating target cell adhesion. However, we further showed that the chimera containing the SHP-1 catalytic domain can also block target cell conjugation, suggesting that both PTPs have capacities to disrupt integrin-mediated target cell adhesion. Further study is required to address whether SHP-1 and SHP-2 disrupt distinct signaling pathways. Ultimately, the identification of specific substrates for SHP-1 and SHP-2 when recruited to KIR in the inhibitory immune synapse remains key to our future understanding.

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