Mouse NKR-P1B, a Novel NK1.1 Antigen with Inhibitory Function[1]

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The mouse NK1.1 Ag originally defined as NK cell receptor (NKR)-P1C (CD161) mediates NK cell activation. Here, we show that another member of the mouse CD161 family, NKR-P1B, represents a novel NK1.1 Ag. In contrast to NKR-P1C, which functions as an activating receptor, NKR-P1B inhibits NK cell activation. Association of NKR-P1B with Src homology 2-containing protein tyrosine phosphatase-1 provides a molecular mechanism for this inhibition. The existence of these two NK1.1 Ags with opposite functions suggests a potential role for NKR-P1 molecules, such as those of the Ly-49 gene family, in regulating NK cell function. ***The Journal of Immunology*, 1999, 162: 5917–5923.**

The nature of how NK cells distinguish between self and nonself and the molecules that are responsible for this determination remain largely unknown. The killer cell activating receptors and killer cell inhibitory receptors expressed on NK cells have been suggested to allow for this discrimination. Members of the NK cell receptor (NKR)[3]-P1 (CD161) family of molecules are type II transmembrane C-type lectin receptors found on the surface of NK cells and a subset of T cells (1–5). These molecules have been shown to activate NK cell cytotoxicity (2, 6–8), and thus function as killer cell activating receptors (1, 3). The mouse NK1.1 Ag (9) originally defined as NKR-P1C (8, 10) belongs in this family (5). Recently, it was shown that NK1.1 (NKR-P1C)-mediated activation occurs through association with the FcRγ chain (5), which in turn signals through the protein tyrosine kinase Syk (11). Although the NKR-P1 molecules may recognize carbohydrate ligands on target cells (12, 13), cognate protein ligands for these receptors remain unknown.

Killer cell inhibitory receptors expressed on NK cells prevent activation and lysis of class I MHC-bearing cells (14), providing a mechanism for the "missing self" model of NK cell function (15–17). Members of the mouse Ly-49 family of receptors, which are structurally related to the NKR-P1 molecules, are included in this category (18). The ability of these receptors to inhibit NK cell function seems to be dictated by the presence in their cytoplasmic domain of a conserved immunoreceptor tyrosine-based inhibitory motif (ITIM) (single-letter amino acid code I/V/LxYxxL/V (19, 20)) (3, 21, 22). However, not all Ly-49 members are inhibitory in function, as Ly-49D and Ly-49H, which lack consensus ITIMs, have been shown to activate NK cell function (22, 23). In keeping with this, the mouse NKR-P1A and NKR-P1C molecules lack consensus ITIMs (3, 10). Alternatively, the closely related mouse NKR-P1B molecule possesses a consensus ITIM (LxYxxL) in its cytoplasmic domain (3, 10). However, due to a lack of available specific Abs, this molecule has remained relatively uncharacterized to date.

Here we show that the mouse NKR-P1B gene product serves as a ligand for the anti-NK1.1 mAb PK136, and thus represents a novel NK1.1 Ag (8, 10). Surprisingly, NKR-P1B fails to activate NK cell cytotoxicity. Moreover, in NK cells expressing both the NKR-P1B and NKR-P1C molecules, NK1.1-mediated redirected lysis of target cells is abrogated. Taken together, these data indicate that NKR-P1B functions as a killer cell inhibitory receptor (3, 14). Indeed, like other ITIM-bearing receptors expressed by NK cells, NKR-P1B binds Src homology 2 (SH2)-containing protein tyrosine phosphatase-1 (SHP-1) in a phosphorylation-dependent manner (14, 24, 25). This provides a molecular mechanism for the inhibition of NK cell cytotoxicity through this novel NK1.1 Ag and demonstrates that NKR-P1B is an inhibitory member of the CD161 family. The existence of two closely related NK1.1 Ags with opposite regulatory function suggests a role for NKR-P1 molecules, such as those of the Ly-49 family, in regulating NK cell-mediated functions.

Materials and Methods

Mice

C57BL/6 (B6) and Swiss.NIH (Sw) mice were obtained from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD). (B6 $\times$ Sw)F_1 mice were bred and maintained in our own animal facility.

Cell lines

P815 target cells were obtained from Dr. Brian Barber (Department of Immunology, University of Toronto, Toronto, Ontario, Canada); Jurkat cells were obtained from Dr. Neil Berinstein (Department of Immunology, University of Toronto, Toronto, Ontario, Canada). An Sw-derived pre-NK cell line, MNK-1, was derived in our own laboratory. Briefly, day 15 fetal thymocytes were sorted for NK1.1$^+$ cells, infected overnight with a Bcl-2 retroviral producer cell line, and transfected with replication-deficient SV40 and human c-*myc* constructs by electroporation. Cells were then maintained in complete medium (DMEM supplemented with 12% FCS, 2 mM glutamine, 10 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, 110 μg/ml sodium pyruvate, 50 μM 2-ME, and 10 mM

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[3] Abbreviations used in this paper: NKR, NK cell receptor; ITIM, immunoreceptor tyrosine-based inhibitory motif; ITAM, immunoreceptor tyrosine-based activation motif; B6, C57BL/6; Sw, Swiss.NIH; AIRL, Ab-induced redirected lysis; GFP, green fluorescent protein; SH2, Src homology 2; KIR, killer cell Ig-like receptor; SHP, SH2-containing protein tyrosine phosphatase; SHIP, SH2-containing inositol phosphatase; NKR, ???.

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HEPES (pH 7.4)) plus human rIL-2 (10 U/ml) for 6 wk before adherent colonies formed. MNK-1 cells have the following phenotype: NK1.1$^+$. In addition, they are FcγRII/III$^+$, CD90$^+$, CD117$^+$, CD122$^+$, Lin$^-$, TCR/CD3$^-$, Ig$^-$, and DX5$^-$ by flow cytometric analysis; do not express Ly-49A, Ly-49C, and perforin by RT-PCR analysis; and fail to display cytotoxic activity. These characteristics indicate that the resulting MNK-1 cell line possesses a pre-NK cell phenotype (26).

Flow cytometric analysis

Fetal blood cells were prepared as described previously (27). FITC-, PE-, and biotin-conjugated mAbs as well as streptavidin APCs, were obtained from PharMingen (San Diego, CA). Cell suspensions were stained in 50 μl of staining buffer (HBSS, without phenol red, plus 1% BSA and 0.05% NaN_3) for 20 min on ice and washed twice before analysis. Intracellular staining was performed as directed by the supplier using the Cytofix/Cytoperm staining kit (PharMingen). Stained cells were analyzed with a FACScalibur flow cytometer using CellQuest software (Becton Dickinson, Mountain View, CA); data were live-gated by forward/side light scatter and a lack of propidium iodide uptake (except propidium iodide was omitted for intracellular stains). All plots display 10,000 events; frequencies in each quadrant are given as the percentage of the total in the upper right corner.

DNA transfections

Jurkat cells were electroporated using a BTX (San Diego, CA) ECM600, under the following conditions: 300 V, 186 Ω, and 1600 μF in 4-mm gap cuvettes. Cells were resuspended in 250 μl of RPMI 1640 with 20% FCS before electroporation and subsequently cotransfected with 20 μg of the NKR-P1 plasmid DNA, as indicated, and with 6 μg of the CMV-green fluorescent protein (GFP) plasmid DNA (CMV enhancer/promoter driving the expression of enhanced GFP from the pEGFP-1 plasmid (Clontech, Palo Alto, CA)). Transfected cells were then cultured for an additional 6 h before analysis by flow cytometry.

RT-PCR

RNA was prepared from B6 and Sw spleen cell suspensions depleted of CD24$^+$ cells by Ab/complement-mediated lysis, as described previously (28), and from the CTLL-2 (B6) and MNK-1 (Sw) cell lines. Total RNA was isolated using the Trizol RNA isolation protocol (Life Technologies, Gaithersburg, MD). cDNA was prepared from 1 μg of each RNA using random hexamer primers and the cDNA Cycle kit (Invitrogen, San Diego, CA). Subsequent PCR analysis was performed using Expand DNA polymerase (Boehringer Mannheim, Indianapolis, IN) on an automated GeneAmp 9600 thermocycler (Perkin-Elmer, Norwalk, CT) with 10 s of denaturation at 94°C, 30 s of annealing at 55°C, and 2-min extensions at 72°C for 35 cycles, with a hot start at 94°C for 2 min and a final extension at 72°C for 6 min. The gene-specific primers used for PCR were as follows: (5′→3′): NKR-P1A (5′), GCA CAA TGG ACA CAG CAA; NKR-P1A (3′), GTA GAC ATG GCT CAG TGA TTG; NKR-P1B (5′), CAA TGG ATT CAA CAA CAC TGG TC; NKR-P1B (3′), GGA CAG GGG AGA GAT GGA GAT; NKR-P1C (5′), TGA AAT GGA CAC AGC AAG TAT C; and NKR-P1C (3′), GAG TCA ACG AAT GGA AAG GAA. Inverse photo images of ethidium bromide-stained gels are shown. PCR products were cloned using a mammalian expression vector, a eukaryotic TOPO TA cloning kit (Invitrogen), and were sequenced before analysis.

^{51}Cr release Ab-induced redirected lysis (AIRL) assay

Spleen cell suspensions from B6, Sw, and (B6 × Sw)F$_1$ mice were depleted of CD3$^+$, CD4$^+$, CD8$^+$, and CD24$^+$ cells by Ab/complement-mediated lysis and subsequently sorted for DX5$^+$ cells using Midi-MACS (Miltenyi Biotech, Auburn, CA). Sorted cells were grown for 1 wk in complete medium containing 100 U/ml of human rIL-2. Effector cells were prelabeled with 10 μg/ml Ab (NK1.1, PK136; CD16/32, 2.4G2) for 15 min in complete medium and washed once before use. Target P815 cells were labeled with ^{51}Cr for 1 h and used at 2–3 × 10^3 cells in 100 μl per well (V-bottom, 96-well plates). Cells were mixed at different E:T ratios, and plates were centrifuged for 1 min at 200 × g and placed in culture for 4 h at 37°C. After lysis, 100 μl of each culture supernatant was collected, and radioactivity was measured in a gamma counter. Supernatant from target cells cultured alone or target cells alone plus 1% SDS gave the spontaneous or maximal release counts, respectively. The counts obtained from the culture supernatant of different E:T ratios (experimental) were used to determine the percent specific lysis (%SL), as described previously (29). Percent specific lysis values for anti-NK1.1-mediated AIRL were indexed relative to those for anti-FcγRIII/II AIRL (anti-CD16/32) and spontaneous lysis (no Ab) controls for each effector subset and ratio tested, according to the following formula: % NK1.1 cytotoxicity = ([%SL (NK1.1) − %SL (no mAb)]/[%SL (CD16/32) − %SL (no mAb)]) × 100%. The data shown represent the mean (± SEM) of five independent experiments performed at a 30:1 E:T ratio; similar results were obtained with 10:1 and 3:1 E:T ratios.

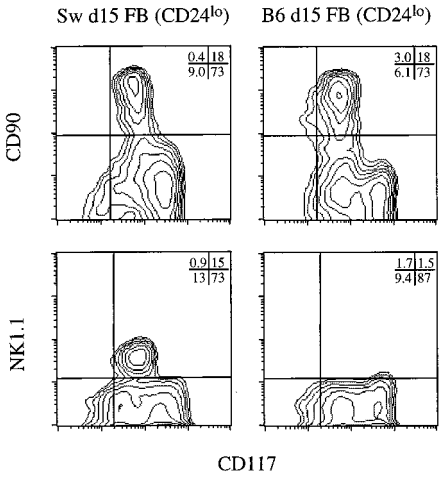

FIGURE 1. Expression of NK1.1 on CD90$^+$ fetal blood cells is strain-dependent. Fetal blood cells derived from Sw and B6 mice were depleted for CD24^{high} cells by Ab/complement-mediated lysis and analyzed for expression of CD117, CD90, and NK1.1 by flow cytometry.

Immunoprecipitation and Western blotting

A total of 50–100 × 10^6 MNK-1 cells were left unstimulated or stimulated with pervanadate for 20 min, as described previously (30), washed once in serum-free medium, and lysed for 15 min in 0.5 ml of ice-cold lysis buffer (0.5% TNTE (0.5% Triton X-100, 150 mM NaCl, and 20 mM Tris-Cl (pH 7.4)) plus 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and protease inhibitors (pepstatin, leupeptin, aprotinin, and trypsin inhibitor)). Lysates were centrifuged at 14,000 × g for 10 min to remove cell debris; next, 5 μg of anti-NK1.1 (PK136) or isotype control mouse IgG2aκ (anti-trinitrophenyl, G155–178) Ab was added to precleared lysates and incubated with gentle agitation for 1 h. A total of 50 μl of protein G-Sepharose beads (1:5 (w/v) in 0.1% Triton X-100 lysis buffer) were added to lysates and incubated for an additional 1 h with gentle agitation. Beads were washed six times with 1 ml of wash buffer (0.1% TNTE plus 1 mM sodium orthovanadate). Beads and lysate supernatant were boiled for 2 min in protein-loading buffer containing 2-ME, and proteins were resolved on 10% SDS-PAGE gels and electroblotted onto membranes. Membranes were blocked with 3% BSA in TBST (50 mM Tris-Cl (pH 8.0), 500 mM NaCl, and 0.1% Tween 20) for 1 h, and analyzed by Western blotting using polyclonal rabbit anti-SHP-1 Ab (Upstate Biotechnology, Lake Placid, NY), affinity-purified rabbit anti-SHP-2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit anti-SH2-containing inositol phosphatase (SHIP) Ab (a kind gift of Dr. D. J. Dumont (Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada) at 1:2000 dilutions in 1% BSA-TBST. All blots were visualized using 1:10,000 anti-rabbit HRP in 1% BSA-TBST and an enhanced chemiluminescence Plus kit (Amersham, Arlington Heights, IL).

Results

Expression of NK1.1 on fetal blood cells is strain-dependent

Molecular cloning of the mouse NKR-P1C gene served to identify it as the NK1.1 Ag (8). However, during the course of studying two NK1.1-expressing strains of mice, we obtained evidence suggesting that NKR-P1C may not be the only NK1.1 Ag. Specifically, we observed that NK1.1 is differentially expressed on a subset of fetal blood CD117$^+$ (c-*kit*) progenitors for T/NK cells derived from two NK1.1-expressing mouse strains, Sw and B6 (26, 27). Fig. 1 shows Sw and B6 day 15 fetal blood cells depleted for CD24^{high} cells by Ab/complement-mediated lysis, which enriches for CD117$^+$ cells (27). As demonstrated previously (27, 31), a subset of CD117^{low} fetal blood cells derived from both Sw and B6 mice expresses CD90 (Thy-1) (Fig. 1). However, although the majority of CD90$^+$/CD117^{low} fetal blood cells from Sw mice display

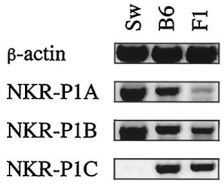

FIGURE 2. Expression of NKR-P1 family members is strain-specific. Total RNA was isolated from Sw, B6, and (B6 × Sw)F_1 splenocytes and analyzed by RT-PCR for expression of NKR-P1 gene family members.

the NK1.1 marker (27), the identical subset from B6 mice lacks NK1.1-expression (Fig. 1). As both subsets of cells express high levels of CD16/32 and were found to be functionally identical (26), this suggested either that the developmental regulation of NKR-P1C is strain-specific, even among NK1.1-expressing strains, or that the anti-NK1.1 mAb PK136 recognizes a different ligand in each of the strains. To address this issue, we analyzed the expression of NKR-P1 family members by RT-PCR.

Expression of NKR-P1 family members is strain-specific

To investigate the expression of NKR-P1 genes in different strains of mice, we employed RT-PCR on RNA isolated from spleen cells of adult mice. Fig. 2 shows RT-PCR for the expression of full-length NKR-P1A/B/C transcripts in spleen cells from Sw, B6, and (B6 × Sw)F_1 mice. Surprisingly, although all three strains possessed similar populations of $NK1.1^+$ splenocytes (data not shown), the Sw strain lacked transcripts for NKR-P1C (Fig. 2). This finding suggested that the NK1.1 Ag expressed by Sw NK cells and fetal blood cells might represent the product of a closely related gene in the NKR-P1 family. As the NKR-P1A gene product was shown previously not to encode an NK1.1 Ag (8), and because Sw mice express abundant mRNA for the NKR-P1B gene (Fig. 2) (28), these data suggested that the NKR-P1B molecule might represent a novel NK1.1 Ag in Sw mice. Notably, NKR-P1B shares ~96% amino acid identity to NKR-P1C in its extracellular C-type lectin domain (10).

Mouse NKR-P1B represents a novel NK1.1 Ag

To test whether the NKR-P1B molecule might represent a second NK1.1 Ag, we cloned the NKR-P1B cDNA from Sw-derived NK cells into a mammalian expression vector for use in transfection experiments. As controls, we also cloned the NKR-P1A and NKR-P1C cDNAs from B6-derived NK cells (8). These constructs were transiently transfected into Jurkat cells. To identify transfected cells, we cotransfected a plasmid construct encoding an enhanced mammalian version of jellyfish GFP. Fig. 3*a* shows a representative GFP fluorescence profile of transfected Jurkat cells (Fig. 3*a*, solid dark line). Fig. 3*b* shows sequential extracellular vs intracellular staining using the anti-NK1.1 mAb, PK136, after gating on GFP^+ Jurkat transfectants. We performed this dual analysis because we were not certain whether mouse NKR-P1 molecules would be efficiently expressed on the cell surface of transfected human Jurkat cells. As shown previously (8), the B6-derived NKR-P1C construct encodes an NK1.1 Ag, whereas the NKR-P1A molecule does not (Fig. 3*b*, NKR-P1C vs NKR-P1A). Furthermore, as predicted by our previous data (Figs. 1 and 2) (26, 27), the Sw-derived NKR-P1B molecule also binds the anti-NK1.1 Ab (Fig. 3*b*, NKR-P1B). Thus, the mouse NKR-P1B molecule represents a second NK1.1 Ag.

Interestingly, although nucleotide sequencing of each clone confirmed its identity, and although the Sw NKR-P1B cDNA is indeed identical with the NKR-P1B sequence published previously (10), analysis of the resulting NKR-P1B PCR product from B6 mice revealed that it does not correspond to the previously published sequence (data not shown). Moreover, in transfection experiments, this molecule did not bind the anti-NK1.1 mAb and thus does not represent an NK1.1 Ag (data not shown). Using multiple primer pairs for RT-PCR, we and others have repeatedly failed to detect the expression of an NKR-P1B gene product that represents an NK1.1 Ag in B6 mice (Richard G. Miller, unpublished observations). Therefore, it is possible that this PCR product represents the product of another closely related gene, and B6 mice do not express detectable mRNA for NKR-P1B. This would be consistent with our previous finding that NKR-P1B expression is undetectable in fetal B6 mice using alternate PCR primers for the mouse NKR-P1 genes (28). Notably, alternate PCR primers have also detected a product with the expected molecular size of NKR-P1C in Sw mice; however, the sequence of this product more closely resembles NKR-P1A than NKR-P1C, and it does not serve as an NK1.1 Ag upon transfection (data not shown). In any case, it remains likely that the only NK1.1 Ag expressed in B6 NK cells

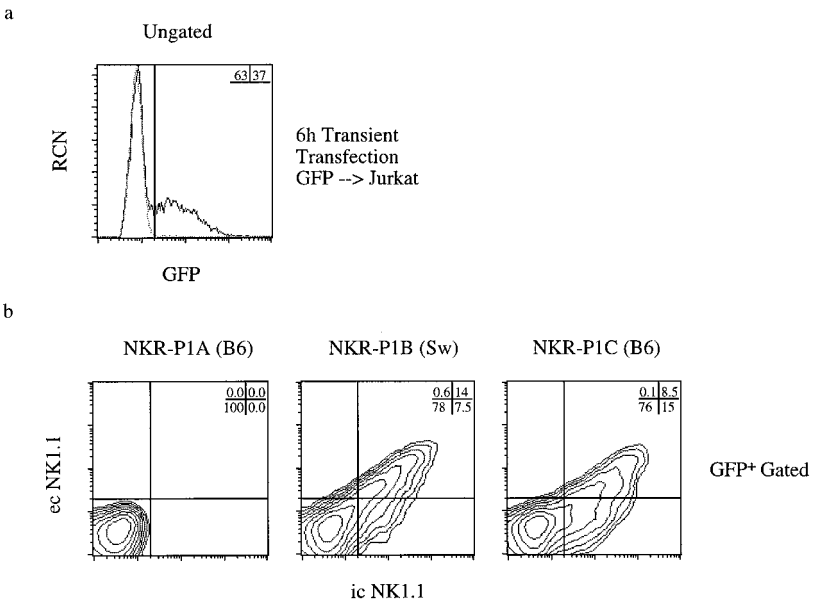

FIGURE 3. Mouse NKR-P1B represents a novel NK1.1 Ag. Full-length cDNA clones of B6 and Sw NKR-P1 genes were cloned into mammalian expression vectors and transiently cotransfected (20 μg) into Jurkat cells with a second vector (6 μg) containing an enhanced GFP construct. *a*, GFP fluorescence, which was used to monitor transfection efficiency, was analyzed by flow cytometry at 6 h posttransfection. *b*, Surface NK1.1 expression on transfected cells (GFP^+ gated) was analyzed by flow cytometry using the anti-NK1.1 mAb PK136. Transfectants of B6-derived NKR-P1(A/C) and Sw-derived NKR-P1B constructs are shown.

represents the product of the NKR-P1C gene, whereas that expressed in Sw NK cells represents the product of the NKR-P1B gene. Thus, each NKR-P1 gene appears to be expressed in a mutually exclusive fashion, at least between these two strains, whereas (B6 × Sw)F_1 NK cells express the products of both genes (Fig. 2; data not shown). Therefore, to assess the function of each NKR-P1 molecule independently, we tested the capacity of B6, Sw, and F_1 NK cells to mediate redirected lysis using the PK136 mAb.

NKR-P1B functions as an inhibitory receptor

To purify NK cells for use in functional assays, splenocytes from each mouse strain were depleted of $CD3^+$, $CD4^+$, $CD8^+$, and $CD24^+$ cells and subsequently sorted for $DX5^+$ cells using magnetic beads. These cells were then expanded in the presence of IL-2 for 1 week in culture. As shown in Fig. 4*a*, the resulting NK cells from each of these strains expressed the NK1.1 Ag on the cell surface. Therefore, to analyze the function of the Sw NK1.1 Ag, Sw-derived NK cells were tested in AIRL assays using the anti-NK1.1 mAb, PK136. In AIRL assays, an activating molecule on the surface of an NK cell augments cytotoxicity toward $Fc\gamma R^+$ target cells, such as mouse P815 mastocytoma cells, whereas inhibitory and nonstimulatory receptors do not significantly affect target cell lysis. As a positive control for lysis, we performed the AIRL assay using the anti-CD16/32 ($Fc\gamma RIII/II$) mAb 2.4G2, which activates NK cells (11). In addition, spontaneous cytotoxicity in the absence of redirecting Ab was used to determine background levels of natural killing. To simplify these data, the percent specific lysis values for the NK1.1-mediated AIRL were indexed relative to the CD16/32-mediated AIRL and spontaneous lysis values (a product termed the percentage of anti-NK1.1 cytotoxicity; see *Materials and Methods*). As shown in Fig. 4*b*, NK1.1-mediated cytotoxicity was high using B6-derived NK cells, confirming an activating role for the B6 NKR-P1C molecule. In contrast, NK1.1-mediated cytotoxicity was insignificant using Sw-derived NK cells (Fig. 4*b*). As this suggested that NKR-P1B might function as an inhibitory receptor, we subsequently assessed NK1.1-mediated AIRL using (B6 × Sw)F_1 NK cells, which express both NK1.1 Ags, NKR-P1B and NKR-P1C (Figs. 2 and 4*a*). Fig. 4*b* demonstrates that the presence of the NKR-P1B molecule on (B6 × Sw)F_1 NK cells exerted a dominant inhibitory effect over the ability of NKR-P1C to augment AIRL cytotoxicity.

To obtain further evidence of NKR-P1B function, we tested the ability of the NKR-P1B molecule to inhibit CD16/32-mediated activation. To this end, we performed AIRL assays using Sw-derived NK cells in the presence of different combinations of the anti-NK1.1 and anti-CD16/32 mAbs. As shown in Fig. 4*c*, the addition of both anti-NK1.1 and anti-CD16/32 significantly reduced cytotoxicity relative to AIRL in the presence of anti-CD16/32 alone ($p < 0.05$). Notably, this effect was not seen in assays using B6 or (B6 × Sw)F_1 NK cells (data not shown). However, NKR-P1B-mediated inhibition of CD16/32 AIRL (Fig. 4*c*) was not as complete as that of NKR-P1C AIRL (Fig. 4*b*). There may be a number of reasons for this apparent difference. First, the anti-CD16/32 mAb is capable of binding to the $CD16/32^+$ P815 target cells both indirectly (by means of AIRL) and directly (by means of Ab-dependent cellular cytotoxicity). This may lead to a stronger association and activation of NK cells through CD16/32 relative to other activating receptors, and might explain why cytotoxicity values were always higher for CD16/32 than NK1.1 (Fig. 4*b*). In addition, NK1.1 expression levels on a population basis were lower and more variable on Sw NK cells than on B6 or (B6 × Sw)F_1 NK cells (Fig. 4*a*), whereas expression levels of CD16/32 were consistently high in all strains tested (data not shown). In such a case, a higher number of activating receptors in the presence of fewer inhibitory signals might lead to an activation bias, either in individual NK cells or generally among a population. Indeed, in contrast to the inhibition observed for Sw NK cells

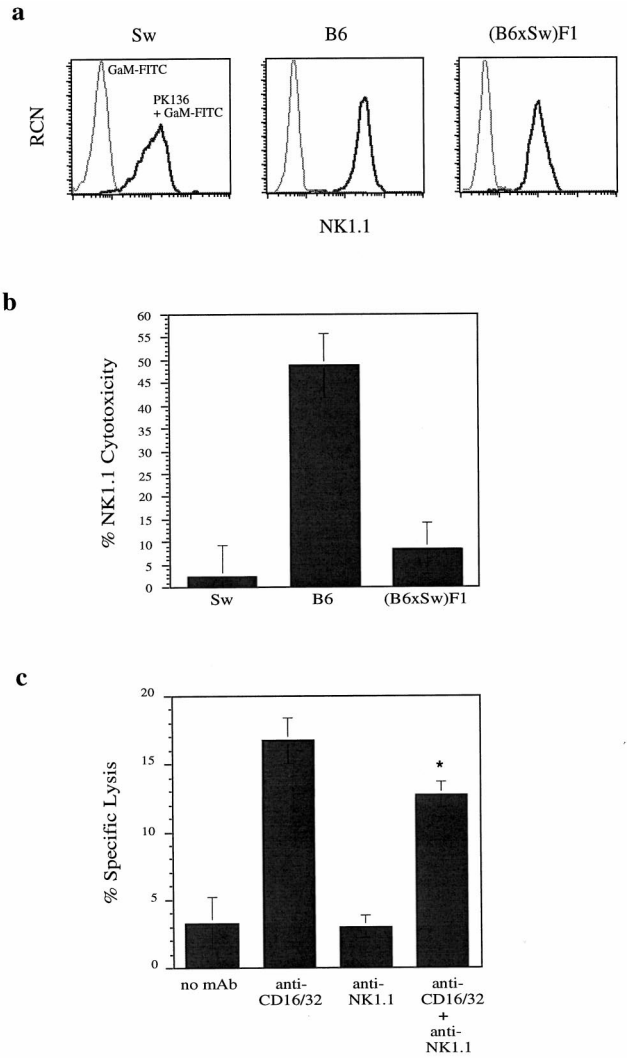

FIGURE 4. NKR-P1B inhibits NK cell function in AIRL assays. *a*, Purified NK cells were prepared from B6, Sw, and (B6 × Sw)F_1 mice and analyzed for NK1.1 surface expression by flow cytometry (PK136 plus anti-mouse-IgG, dark line; anti-mouse-IgG alone, light line). The mean fluorescence intensity values for NK1.1 staining are: 107 for Sw, 316 for B6, and 118 for F_1. *b*, NK cells were tested for cytotoxicity in AIRL assays using $CD16/32^+$ ($Fc\gamma RIII/II^+$) P815 targets. The data shown are those of a 30:1 E:T ratio (similar data were obtained for 10:1 and 3:1 ratios). The percentage of NK1.1 cytotoxicity is defined as the indexed percent specific lysis observed with anti-NK1.1 (PK136) mAb relative to the percent specific lysis induced by anti-CD16/32 (2.4G2) mAb and without added mAb. Error bars indicate the SEM (± SEM) of five independent experiments ($n = 5$). The percent specific lysis values for the AIRL assay are: B6 NK cells: no Ab = 20% (± 9.2), with PK136 = 45% (± 7.4), with 2.4G2 = 62% (± 6.9); Sw NK cells: no Ab = 7.7% (± 3.1), with PK136 = 7.7% (± 2.1), with 2.4G2 = 25% (± 3.5); (B6 × Sw)F_1 NK cells: no Ab = 19% (± 2.2), with PK136 = 22% (± 4.2), with 2.4G2 = 70% (± 9.6). *c*, Sw NK cells were tested for cytotoxicity in AIRL assays using P815 targets in the presence of different redirecting mAbs. Percent specific lysis values are shown for Sw NK cells in the absence of redirecting mAb or in the presence of anti-CD16/32 (2.4G2), anti-NK1.1 (PK136), or both anti-CD16/32 and anti-NK1.1 mAbs. The data shown are those of a 10:1 E:T ratio. Error bars indicate the SEM (± SEM) of three independent experiments ($n = 3$). The asterisk (*) indicates a significant difference ($p < 0.05$).

in the presence of both anti-NK1.1 and anti-CD16/32, the additional presence of the activating NKR-P1C molecule on (B6 × Sw)F_1 NK cells (Fig. 2) may explain why these cells were not inhibited (data not shown). Notably, strong or numerous activating signals have been shown to be capable of overriding the negative signals induced by inhibitory NK cell receptors (32, 33). Taken together, these data indicate that the NKR-P1B molecule functions as a killer cell inhibitory receptor.

NKR-P1B binds SHP-1 in a phosphorylation-dependent manner

A number of different inhibitory receptors in both humans (killer cell Ig-like receptor (KIR)-2DL/3DL; CD94/NKG2A) and mice (Ly-49A/G2) have been shown to exert their inhibitory effects on NK cell function by interrupting the early tyrosine phosphorylation pathways responsible for NK cell activation (33). This was first shown at the molecular level by the demonstration that KIR cross-linking recruits SHP-1 to the intracellular ITIM in a phosphorylation-dependent manner (19, 24, 34–36). Another structurally related tyrosine phosphatase, SHP-2, was found to function in similar capacity to SHP-1 (36), although SHP-2 may also be involved in transducing activating signals (37). Alternatively, SHIP, which is involved in mediating inhibition through the FcγRIIB pathway (38), does not associate with KIRs (25, 39). Notably, the mouse NKR-P1B molecule possesses a consensus ITIM (LxYxxL) in its intracellular domain (3, 10). Therefore, to determine the molecular mechanism for inhibition of cytotoxicity through the NKR-P1B molecule (Fig. 4*b*), we tested whether SHP-1 is recruited to the ITIM of NKR-P1B upon phosphorylation of the receptor. To achieve this, we used an NK1.1$^+$ Sw-derived pre-NK cell line, MNK-1 (see *Materials and Methods*), which expresses abundant NKR-P1B mRNA but no NKR-P1C (data not shown). Phosphorylation of the ITIM-based tyrosine was accomplished in intact cells by the disruption of endogenous tyrosine phosphatase activity using pervanadate stimulation (40), as described previously for the human CD94/NKG2A (41) and murine Ly-49A and Ly-49G2 molecules (21, 30).

As shown in Fig. 5*a*, Western blot analysis for SHP-1 on anti-NK1.1 immunoprecipitates indicates that the NKR-P1B molecule associates with SHP-1 upon pervanadate stimulation (Fig. 5*a*, VO_5+/IPαNK1.1+). This association is phosphorylation-dependent (21, 30), as SHP-1 does not coimmunoprecipitate with NKR-P1B in unstimulated cells (Fig. 5*a*, VO_5−/IPαNK1.1+). The total cell lysate supernatant (shown on the right of Fig. 5*a*, S) indicates that ~0.4% of total intracellular SHP-1 coimmunoprecipitates with the NKR-P1B molecule in response to pervanadate stimulation. The additional bands observed in the total lysate supernatant are due to anti-SHP-1 cross-reactivity (data not shown) (24) and are absent from anti-NK1.1 immunoprecipitations. Importantly, the association of NKR-P1B with SHP-1 is specific, as Western blots for SHP-2 (Fig. 5*b*, anti-SHP-2 blot) and SHIP (Fig. 5*c*, anti-SHIP blot) revealed that these phosphatases do not significantly coimmunoprecipitate with NKR-P1B upon pervanadate stimulation. Moreover, coimmunoprecipitating SHP-1 was also detected in pervanadate-stimulated Sw and (B6 × Sw)F_1 NK cells, although SHP-1 could not be detected in anti-NK1.1 immunoprecipitates from B6 NK cells or the NK1.1$^+$ (NKR-P1C) B6-derived cell line CTLL-2 (data not shown) (3). These data support initial studies demonstrating that the cross-linking of NKR-P1C on B6-derived cells leads to transient increases in the intracellular calcium concentration and tyrosine phosphorylation of downstream substrates (3), whereas similar responses were not observed upon NKR-P1B cross-linking using Sw-derived cells (data not shown). Taken together, these data indicate that the NKR-P1B molecule binds to SHP-1 in a phosphorylation-dependent manner, confirming a functional role for the NKR-P1B consensus intracellular ITIM.

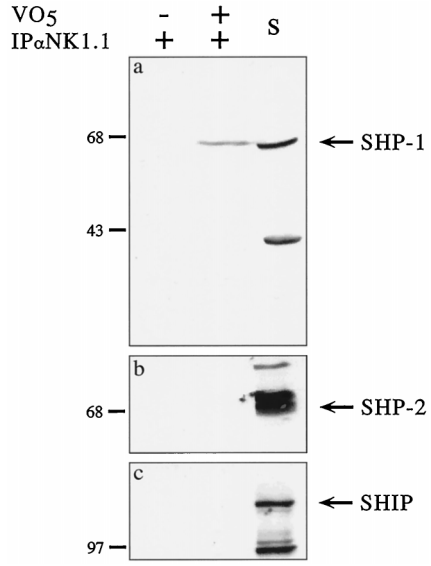

FIGURE 5. Mouse NKR-P1B recruits SHP-1 in a phosphorylation-dependent manner. An Sw-derived pre-NK cell line, MNK-1, was stimulated for 20 min using pervanadate. Cell lysates were immunoprecipitated with anti-NK1.1 (PK136) mAb, and coprecipitating SHP-1 (*a*), SHP-2 (*b*), and SHIP (*c*) proteins were visualized by Western blotting using enhanced chemiluminescence. Control immunoprecipitations using a mouse IgG2aκ mAb (anti-trinitrophenyl, G155–178) did not yield significant signal upon Western blotting (data not shown). VO_5, pervanadate stimulation; IPαNK1.1, anti-NK1.1 immunoprecipitation; S, total cell lysate supernatant.

Discussion

The finding that NKR-P1B functions as an inhibitory receptor and associates with the SHP-1 phosphatase (Fig. 5), whereas the activating mouse NKR-P1A and NKR-P1C molecules do not, can be explained in the latter cases by their lack of consensus ITIMs (I/V/LxYxxL/V; Fig. 6*a*). Both NKR-P1A and NKR-P1C possess hydrophilic amino acid residues (R and S, respectively) at the −2 position relative to their YxxL motif, a position that has been shown to be critical for SHP-1 binding (19). Like the inhibitory Ly-49 family members, NKR-P1B possesses a hydrophobic amino acid at this position (L for NKR-P1B, V/I for Ly-49 molecules). However, unlike the activating Ly-49 family of molecules, which possess a phenylalanine residue in substitution of the ITIM-based tyrosine, NKR-P1A and NKR-P1C retain the YxxL motif. This may have important implications for the respective signaling capacities of the Ly-49 vs the NKR-P1 families of molecules.

Interestingly, both NKR-P1A and NKR-P1C possess a positively charged arginine residue near the extracellular region of their transmembrane domains (Fig. 6*a*). The existence of similar residues in the activating human CD94/NKG2C and murine Ly-49D and Ly-49H molecules has been shown to be necessary for the association of these molecules with the immunoreceptor tyrosine-based activation motif (ITAM)-containing DAP-12 immunoreceptor (22, 42, 43). Furthermore, similar residues are important for the association of the CD3 subunits with the TCR and the CD79 subunits with the B cell receptor (42). The finding that NKR-P1C, like CD16 (FcγRIII), functionally associates with the Fc$R\gamma$ chain (FcϵRIγ) (5), suggests that this residue may be important for NKR-P1-mediated activation. Similar to DAP-12 and the CD3 subunits, Fc$R\gamma$ possesses a negatively charged aspartic acid residue in its transmembrane domain (42). Thus, the absence of a positively charged amino acid in the NKR-P1B transmembrane domain supports its inability to activate NK cell activity, as association with FcϵRIγ would be undesirable for mediating inhibitory signals.

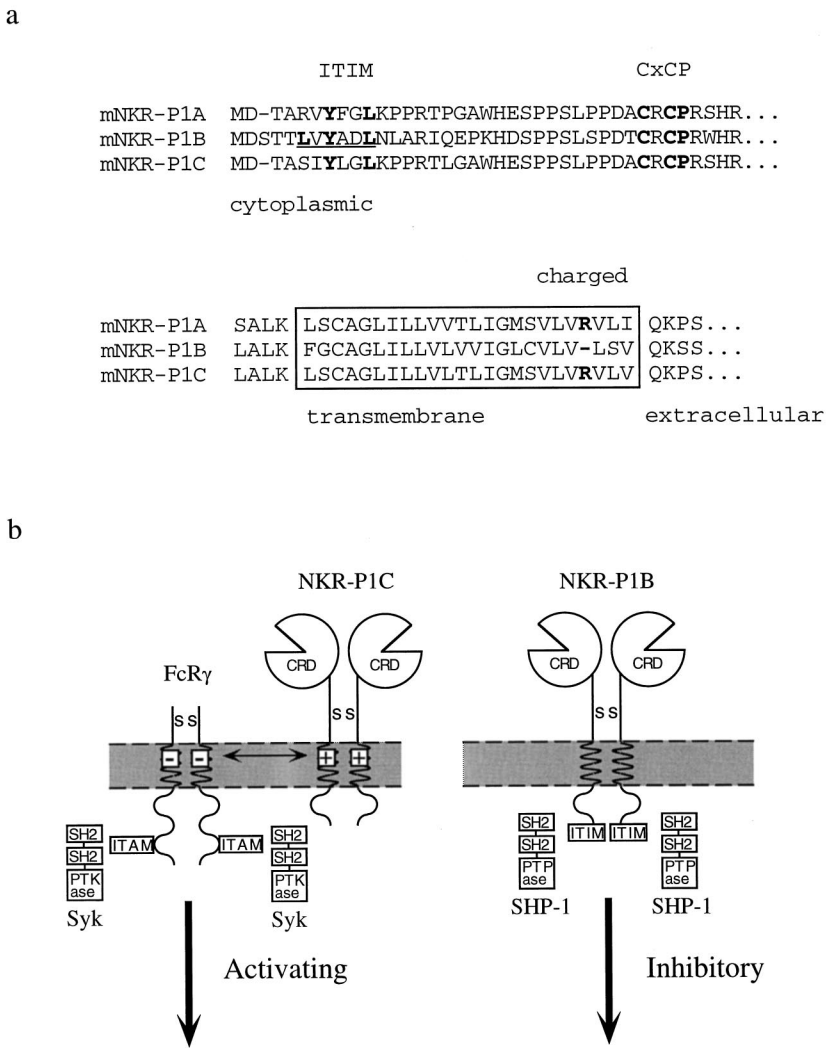

FIGURE 6. Proposed mechanism for signaling through the NKR-P1 molecules. *a*, Sequence analysis of the mouse NKR-P1 molecules. The cytoplasmic and transmembrane domains of the mouse NKR-P1A/B/C molecules, adapted from References 3 and 10, are shown. Amino acid residues of interest are shown in boldface type, the putative transmembrane domain is boxed, and the ITIM motif of NKR-P1B is underlined. A dash represents the absence of an amino acid in the sequence. *b*, Model for NKR-P1 signaling. The NKR-P1C and NKR-P1B receptors and putative associated signaling molecules are shown. Plus and minus signs represent charged amino acid residues in the transmembrane domains; PTKase, protein tyrosine kinase domain; PTPase, protein tyrosine phosphatase domain; SS, intermolecular disulfide bond; CRD, C-type lectin carbohydrate recognition domain.

Taken together, the findings reported here and elsewhere (3, 5, 11) suggest a model for the mechanism of NKR-P1 signaling (Fig. 6*b*). Association of the activating NKR-P1 family members (NKR-P1A/C) with the ITAM-containing FcRγ chain induces tyrosine phosphorylation of downstream substrates, including the SH2-containing tyrosine kinase Syk, resulting in the activation of NK cell function. Other motifs present in the intracellular domains of these receptors may be responsible for mediating increases in intracellular calcium concentration and phosphatidylinositol turnover. Alternatively, phosphorylation of the cytoplasmic ITIM of inhibitory NKR-P1 molecules (NKR-P1B) recruits SHP-1, resulting in the disruption of proximal tyrosine phosphorylation pathways and in an inhibition of NK cell function. The signaling molecules responsible for inducing phosphorylation of the ITAM and ITIM motifs remain unknown, although the presence of a conserved CxCP motif in the NKR-P1 molecules (Fig. 6*a*) suggests that Src family tyrosine kinases may play a role in this event (3, 10, 44). Additional experiments are required to elucidate the details of the molecular cascades involved in NKR-P1 signaling.

Over 20 years after its original identification (9), the mouse NK1.1 Ag is still incompletely characterized. Our data demonstrate that the anti-NK1.1 mAb recognizes the products of two distinct mouse genes, NKR-P1B and NKR-P1C (Fig. 3). Moreover, these two NKR-P1 molecules, which share a cluster of differentiation designation, CD161 (5), possess opposite roles in the regulation of NK cell function (Fig. 4*b*). Until now, members of the NKR-P1 family of molecules were thought to be responsible for transducing activating signals (2, 5). Here, we show that this is not necessarily the case, and that one member, NKR-P1B (3, 10), acts as a inhibitory receptor.

This finding highlights the increasingly similar features that these molecules share with the structurally related mouse Ly-49 family of type II transmembrane C-type lectin receptors (1, 2, 18). However, unlike the well-characterized MHC class I ligands that regulate the specificity of the Ly-49 family of molecules (18), cognate protein ligands for the NKR-P1 molecules have yet to be identified (3, 12). If the NKR-P1 molecules do not recognize specific self molecules, such as the MHC Ags, the existence of two NKR-P1 receptors with possibly identical ligands and yet opposite regulatory functions presents a potential paradox for NK cell biology. More likely, these data suggest that the cognate ligands for the NKR-P1 molecules are similar in form and function to the MHC class I alleles specifically recognized by members of the Ly-49 family (3, 18) or to the related nonclassical MHC gene products such as human HLA-E (mouse H-2Qa) recognized by the heterodimeric CD94/NKG2 receptors (41, 43, 45–47).

In this regard, there is some emerging evidence that NK1.1 (NKR-P1C) may be involved in allogeneic target recognition in the NK cell-mediated F_1-antiparent "hybrid resistance" phenomenon (3, 48). The identification of a negative regulatory member of the NKR-P1 family, NKR-P1B, now suggests a role for these molecules in the missing self hypothesis (15, 16), in which the perception

of self vs nonself appears to rely on the presence or absence on the target cell surface of MHC or MHC-like gene products (17). In a manner analogous to the recent discovery of MHC class I-related CD1 ligands recognized by the $\alpha\beta$TCR of mouse NK1.1$^+$ natural T cells (4), it seems that the variety of receptors and ligands involved in self/nonself recognition is still expanding. Interestingly, selective expression in the Sw (this report) and SJL/J (Richard G. Miller, unpublished observations) mouse strains of NKR-P1B but not NKR-P1C may explain the deficiency in NK1.1 expression on the surface of the $\alpha\beta$TCR$^+$ natural T cells derived from these mice; this defect has been reported to genetically segregate within the NK gene complex independently of defects in IL-4 production and IgE secretion (49). These findings raise some important questions as to the role of CD161 molecules in both mice and humans and suggest a previously unrecognized level of specificity in the regulation of the immune response.

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