Missing self-recognition of Ocil/Clr-b by inhibitory NKR-P1 natural killer cell receptors

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The NKR-P1 family of C-type lectin-like receptors are expressed on natural killer (NK) cells and NKT cells. We report the cloning and characterization of a cognate ligand for the inhibitory mouse NK receptors (NKR)-P1B and NKR-P1D (CD161b/d). The NKR-P1B/D ligand is osteoclast inhibitory lectin (Ocil), also known as Clr-b, a member of a previously cloned group of C-type lectin-related (Clr) proteins linked to the NKR-P1 receptors in the mouse NK gene complex (NKC). Expression of Ocil/Clr-b on mouse tumor cell lines inhibits NK cell-mediated killing. Inhibition is blocked with a new mAb (4A6) specific for Ocil/Clr-b. By using 4A6 mAb, we demonstrate that Ocil/Clr-b is displayed at high levels on nearly all hematopoietic cells, with the exception of erythrocytes, in a pattern that is similar to that of class I MHC molecules. Remarkably, Ocil/Clr-b is frequently down-regulated on mouse tumor cell lines, indicating a role for this receptor–ligand system in a new form of “missing self-recognition” of tumor cells.

Methods

Mice. C57BL/6 (B6), BALB/c, and CD1 mice were maintained in our own facility. NIH Swiss and timed-pregnant B6 mice were purchased from the Animal Production Area at Charles River Breeding Laboratories.

Cell Preparations. Adult-cell suspensions were purified by using Lymphocyte–Mammal (Cedarlane Laboratories). Bone marrow macrophages were prepared as described (24). B6 total fetal cell populations (days 14–17 of gestation) include erythrocytes (no Lymphocyte gradient was applied). Fetal NK-enriched populations were described as prepared (19). Fetal NK lymphokine-activated killer (LAK) cells were prepared by culturing cells in 500 units/ml rhIL-2 for 5 days.

mAbs, Flow Cytometry, and Cell Sorting. All commercial mAbs were purchased from Pharmingen or eBioscience (San Diego). Streptavidin-r-phycocerythrin (Molecular Probes) was used as a secondary reagent. Cells were stained as described (19). Automated magnetic cell sorting was performed by using an autoMACS sorter according to the manufacturer’s instructions (Milenyi Biotec, Auburn, CA).

Generation of NKR-P1 Tetramers. The full extracellular domain or only the C-type lectin-like domain segment of NKR-P1B were isolated by PCR using the following primers: P1Btet, 5′-CTCGAGCTGAAACGACATCTTCTCGAGCTCAAAAAGATCAGTGGCAAGCAGCGGATGGTCA; P1Btet, 3′-CTCGAGTCGACATCTTCTCGAGCTCAAAAAGATCAGTGGCAAGCAGCGGATGGTCA; and P1Bcd, 5′-CATATGCTGAAACGACATCTTCTCGAGCTCAAAAAGATCAGTGGCAAGCAGCGGATGGTCA; and P1Bcd, 5′-CATATGCTGAAACGACATCTTCTCGAGCTCAAAAAGATCAGTGGCAAGCAGCGGATGGTCA. PCR products were cloned into pET15b (Novagen) and confirmed by sequencing. The expression plasmids were transformed into BL21 Star bacteria (Invitrogen) containing a Clr-b, we show that the ligand is induced by protein with 1 M isopropyl β-d-thiogalactoside at OD600 = 0.500, 50 μM d-biotin was added to allow biotinylation of proteins in vivo. Inclusion-body proteins were isolated by using BugBuster reagent and the nuclease Benzoxase (Novagen), and they were refolded by using condition 6 of the FoldIt Screen kit (Hampton Research, Riverside, CA). Equal amounts of the biotinylated C-type lectin-like domain and full extracellular domain were refolded together, and tetramers

Abbreviations: LAK, lymphokine-activated killers; NK, natural killer; NKR, NK receptor; NKC, NK gene complex.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY320031).

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were prepared as described (25). For staining, cells were incubated with ~4 μg of tetramer in staining buffer for 1 h on ice or at room temperature.

**Generation of CD3ζ/NKR-P1 Fusion Receptors and BWZ Reporter Cells.** The transmembrane and extracellular domains of mouse NKR-P1B and the cytoplasmic domain of CD3ζ were isolated by PCR using the following primers: P1BFF, ACCCTGGCCCTCGCT-GTGCTGTCCTCCTGGGCATC; P1BFR, GCCGCGGTCTC-AAGGACTTATTACTCGGGTCTTCC; CD3ZF, CTCAAGATGAGAAGCAAATTTAGCGAAGAGTTG; and CD3ZR, GCGGGGACCCGACGGAGGGGCGAGGTCTGC. The fusion receptor was generated by gene splicing by overlap extension by using CD3ZF and P1BFR, cloned into pcDNA3.1-

**Retroviral Library Screening.** Retroviral supernatants were generated by triple transfection of proviral vector into the pMSCV2.2-IRES-GFP proviral vector (gift from W. Sha, University of California, Berkeley). Retroviral supernatants were generated by triple transfection of proviral vector with VSV-G using TOPO (Invitrogen) and subcloned with the cytomegalovirus promoter into the pMSCV2.2-IRES-GFP proviral vector (gift from W. Sha, University of California, Berkeley). Retroviral supernatants were generated by triple transfection of proviral vector with VSV-G using TOPO (Invitrogen) and subcloned with the cytomegalovirus promoter into the pMSCV2.2-IRES-GFP proviral vector (gift from W. Sha, University of California, Berkeley). 

**Immunofluorescent Staining and Flow Cytometry.** Staining was performed as described (24). For flow cytometric analysis, cells were stained with mAbs conjugated with phycoerythrin, allophycocyanin, or fluorescein isothiocyanate and analyzed using a FACScalibur (Becton Dickinson) flow cytometer.
The transiently transfected cells used as stimulator cells in placed according to staining of untransfected 293T cells. after transfection with 4A6 mAb (10D7 mAb served as a control). Markers were expressing chimeric NKR-P1A, NKR-P1B, NKR-P1C, or NKR-P1D receptors. (Clr-b, Clr-f, or Clr-g to stimulate an equal number of BWZ indicator cells not Clr-f or Clr-g, is a ligand of NKR-P1B and NKR-P1D. We used 293T cells that mAbs.

Comparisons of BWZ cell lines expressing fusions of all four NKR-P1 proteins with CD3ε showed that MNK-1 cells expressed BWZ.CD3ε/NKR-P1A, BWZ.CD3ε/NKR-P1B, BWZ.CD3ε/NKR-P1C, or BWZ.CD3ε/NKR-P1D cells by both MNK-1 and NIH 3T3 cells (Figs. 1D and 7B), as did PK136 (Fig. 1D). Biotinylated 4A6 stained NIH 3T3 cells brightly, MNK-1 cells less intensely, and BWZ cells only weakly, and it did not stain 293 T cells (Fig. 7C), consistent with the NKR-P1B tetramer staining. Importantly, 4A6 mAb blocked staining of MNK-1 and NIH 3T3 cells by NKR-P1B tetramers (Fig. 1C Lower), providing direct evidence that 4A6 recognizes the NKR-P1B ligand.

Because we observed that NK cells stained with 4A6 mAb (see below), we transduced the pMXS-NK cell cDNA retroviral expression library into the BWZ cell line and selected 4A6+ BWZ clones. PCR of retroviral integrants from genomic DNA (27) revealed that most of the 4A6 clones lacked retroviral sequences and presumably expressed endogenous 4A6 antigen. However, one transductant (1B8) yielded a PCR product (see Fig. 8A, which is published as supporting information on the PNAS web site) that conferred 4A6 reactivity when transfected into 293T cells in the form of a new proviral construct (pMXS-1B8; Fig. 2A). Control DX5 mAb did not stain the transfectants. Furthermore, when virus obtained from these transfected cells was transduced into BWZ cells, we observed strong staining of

Results

To identify NKR-P1 ligands, we developed a rapid functional assay as well as a ligand-binding staining reagent. The functional assay was developed by transfecting the BWZ reporter cell line, which produces β-galactosidase in response to stimulation (26), with a chimeric cDNA encoding the extracellular domain of NKR-P1B fused to a modified inverted cytoplasmic domain of the stimulatory CD3ε subunit (28). The staining reagent consisted of a tetrameric form of the soluble extracellular domain of NKR-P1B, which had been expressed in bacteria and refolded in vitro.

The BWZ-transfectants (BWZ.CD3ε/P1B cells) responded well when stimulated on plates coated with anti-NKR-P1 mAb PK136, but they did not respond to other Abs (Fig. 1A). The indicator cells responded well also when stimulated with the MNK-1 immature NK cell line or with NIH 3T3 fibroblasts, gave intermediate responses with the C1498 leukemia cell line or bone marrow macrophages, and gave minimal responses with several other cell lines (Fig. 1B). A separate analysis revealed strong responses with bone marrow cells and spleen cells and lower but consistent responses with thymocytes (see Fig. 6F, which is published as supporting information on the PNAS web site). Comparisons of BWZ cell lines expressing fusions of all four NKR-P1 proteins with CD3ε showed that MNK-1 cells expressed ligands for NKR-P1B and NKR-P1D, the two inhibitory isoforms, but not NKR-P1A and NKR-P1C, two stimulatory isoforms (see Fig. 7A, which is published as supporting information on the PNAS web site). All four reporter cell lines responded when the fusion protein was crosslinked with the appropriate mAbs.

51Cr-Release Assays. LAK cells were generated, and lysis assays were performed as described (24).

NKR-P1B tetramers stained NIH 3T3 cells and, to a lower extent, MNK-1 cells, but they did not stain BWZ cells (Fig. 1C Upper). Notably, MNK-1 cells also express NKR-P1B receptor (13), which could interfere with tetramer binding.

To prepare an mAb specific for NKR-P1B ligands, Lewis rats were sequentially immunized with MNK-1, NIH 3T3, and B6 bone marrow macrophages to focus the Ab response on their shared antigens. Hybridoma supernatants were screened for inhibition of the response of BWZ.CD3ε/P1B cells to ligand-expressing cells. Hybridoma 4A6 (rat IgM isotype), blocked stimulation of BWZ.CD3ε/P1B cells by both MNK-1 and NIH 3T3 cells (Figs. 1D and 7B), as did PK136 (Fig. 1D). Biotinylated 4A6 stained NIH 3T3 cells brightly, MNK-1 cells less intensely, and BWZ cells only weakly, and it did not stain 293 T cells (Fig. 7C), consistent with the NKR-P1B tetramer staining. Importantly, 4A6 mAb blocked staining of MNK-1 and NIH 3T3 cells by NKR-P1B tetramers (Fig. 1C Lower), providing direct evidence that 4A6 recognizes the NKR-P1B ligand.

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a large subset of the cells with 4A6 mAb compared with control untransfected BWZ cells (Fig. 8B). Thus, 1B8 cDNA encodes the 4A6 antigen.

The 1B8 cDNA coding sequence was 100% identical to that of Ocil isolated from B6 mice (29), and it contained only a few BALB/c-specific substitutions in the untranslated regions. Ocil is also called Clr-b, a member of the Clr gene family, which includes Clr-f and Clr-g in B6 mice (30). Ocil/Clr-b transcripts were up-regulated in BWZ clones with endogenously encoded (nonretroviral) 4A6 antigen expression (Fig. 8C), supporting the conclusion that Ocil/Clr-b is the 4A6 antigen. Interestingly, the Clr genes are located in the NKC, closely linked to the Nkr-p1 genes (30).

We tested 293T cells transiently transfected with Ocil/Clr-b, Clr-f, and Clr-g/OcilrP2 (29–31) in the BWZ assay. Ocil/Clr-b transfectants stimulated BWZ.CD3/H9256/H20862 P1B and BWZ.CD3/H9256/H20862 P1D indicator cells but not the other indicator cells, providing direct functional evidence that Ocil/Clr-b is a ligand for NKR-P1B and NKR-P1D but not for NKR-P1A or NKR-P1C (Fig. 2B). All of the indicator cells responded to stimulation with antireceptor Abs (Fig. 7A). Furthermore, Clr-f and Clr-g/OcilrP2 transfectants failed to stimulate any of the indicator cells (Fig. 2B). Parallel analysis showed that a fraction of the Clr-b transfectants, but not Clr-f or Clr-g/OcilrP2 transfectants, were stained with 4A6 mAb (Fig. 2C). Thus, Ocil/Clr-b is a functional ligand for NKR-P1B and NKR-P1D but not for NKR-P1A or NKR-P1C.

Expression of Ocil/Clr-b Resembles That of Class I MHC but Is β2m-Independent. Ocil/Clr-b is broadly expressed by nearly all adult splenocytes, thymocytes, and lymph-node cells, as shown by 4A6 staining (Figs. 3A and B and 6). The positive cells included nearly all lymphocytes, granulocytes, and myeloid cells (Figs. 3 and 6). All four thymocyte subsets defined with CD4 and CD8 were mostly positive, although CD4+CD8+ thymocytes stained only ~1/10th as well as the other subsets (Fig. 3B). Nearly all CD45+ cells from fetal liver, fetal spleen, and fetal thymus expressed Ocil/Clr-b, including all subsets of CD3+CD4–CD8– triple-negative thymocytes (Figs. 3C and 6). Notably, Ocil/Clr-b expression diminishes during erythropoiesis, and terminally differentiated erythrocytes (CD45+TER-119+) in the fetal liver or fetal spleen did not stain detectably with 4A6 (Figs. 3C and 6A and data not shown). In contrast, various nonhematopoietic CD45– cells expressed high levels of Ocil/Clr-b, as shown by 4A6 staining of NIH 3T3 and other fibroblast cell lines (Fig. 7 and see below) and mRNA analysis (30). The broad pattern of expression with lower levels on CD4+CD8+ thymocytes and erythrocyte lineage cells is remarkably similar to that of classical MHC class I molecules. Furthermore, costaining with 4A6 and anti-class I (Kd) mAbs revealed a correlation between the relative levels of Ocil/Clr-b and Kd on various ex vivo BALB/c cell populations prepared without enrichment of lymphocytes (Fig. 6G). Staining and functional analyses showed that Ocil/Clr-b expression was normal in MHC class I-deficient (β2m–) mice, showing that it is not class I-dependent (Figs. 3D and 6F).

Ocil/Clr-b Is Frequently Down-Regulated in Mouse Tumor Cell Lines. Analysis of cell lines with 4A6 mAb demonstrated that numerous lymphoid tumor lines expressed dramatically lower cell-surface
levels of Ocil/Clr-b (Fig. 4), compared with the uniformly high levels detected on their ex vivo lymphocyte counterparts (Fig. 3). Several carcinoma cell lines also expressed low levels of Ocil/Clr-b (Fig. 4). Loss of ligand for the inhibitory NKR-P1 receptors would be expected to make the tumor lines more sensitive to NK cell-mediated killing.

Expression of Ocil/Clr-b Protects Target Cells from NK Cell-Mediated Lysis. To test whether Ocil/Clr-b expression in target cells inhibits NK cell function, we compared the sorted 4A6⁺ variants of BWZ (BWZ.Ocil⁺), which had up-regulated endogenous Ocil/Clr-b, with parental BWZ.36 cells, which expressed only low levels of Ocil/Clr-b (Figs. 7 and 8C). Unexpectedly, BWZ.Ocil⁺ variants stimulated BWZ.CD3⁺/P1D indicator cells much less well than they stimulated BWZ.CD3⁺/P1B cells (Fig. 54), possibly because of a modification of the endogenous Ocil/Clr-b (BWZ.36 cells were derived from AKR strain T lymphoma, BW5147α⁻β⁻). Presumably because B6 NK cells lack NKR-P1B and NKR-P1D fails to functionally engage Ocil/Clr-b on these target cells, B6 NK cells were not inhibited by the BWZ.Ocil⁺ cells and lysed them equally to control BWZ cells (Fig. 5B). However, NK cells from NIH Swiss or CD1 mice, which express NKR-P1B, were inhibited by BWZ.Ocil⁺ cells (Fig. 5B). The inhibitory effect was reversed by preincubating the effector cells with the 4A6 anti-Ocil mAb (Fig. 5C). Together, these data indicate that Ocil/Clr-b is a ligand for the inhibitory NKR-P1 receptors and that Ocil/Clr-b expression protects cells from NK cell-mediated killing.

Discussion

This article identifies Ocil/Clr-b as the cognate ligand for the inhibitory NKR-P1B and NKR-P1D receptors. As this article was in preparation, the identification of Clr family members as ligands for NKR-P1 receptors was reported by Yokoyama and colleagues (32). The NKR-P1 receptors are NKC-encoded lectin-like NRs that are shown to recognize other lectin-like molecules. All of the other lectin-like NRs studied have been shown to recognize class I MHC molecules or relatives of class I MHC molecules. Moreover, the fact that the Nkrp1 genes are intermingled within the NKC with the genes encoding their ligands is remarkable. It is interesting to speculate that the NKR-P1 proteins and their Clr ligands may be descended from a common C-type lectin-like ancestral protein that engaged in homophilic interactions.

Close genetic linkage of the genes encoding receptors and ligands in this system is unprecedented for NRs. In contrast, self-recognition systems in inbreds that impose barriers to inbreeding in plants such as crucifers consist of closely linked genes for a receptor and cognate ligand, which interact to prevent pollen tube formation (33). Because both molecules are polymorphic, close genetic linkage of receptor and ligand genes enhances the likelihood that the self receptor and self ligand will be coinherited. By analogy, close linkage of NKR-P1 and its Ocil/Clr ligands may also ensure coinheritance of cognate receptor and ligand genes.

These results demonstrate that the NKR-P1 receptors, like other lectin-like proteins encoded within the NKC, recognize proteins, in contrast to the earlier reports of oligosaccharide recognition by these receptors (20–23). It remains possible that glycans linked to Ocil/Clr-b play a role in binding by NKR-P1.

A significant finding with the 4A6 mAb was that Ocil/Clr-b is broadly expressed by hematopoietic cells, consistent with the finding that the transcripts are broadly expressed (29–31). In general, 4A6⁺ cells were stimulatory in our functional assay (Figs. 1, 6F, and 7). The few discrepancies may be accounted for by variable expression of accessory molecules by different cells, although it remains possible that modifications of Ocil/Clr-b occur in some cells. In contrast to our results, it was reported recently based on functional studies that Ocil/Clr-b is expressed predominantly by dendritic cells and macrophages (32). Furthermore, although it was suggested that the expression pattern of Ocil/Clr-b is dissimilar to that of MHC class I proteins (32), we found instead a remarkable similarity in their expression patterns, including the low expression on CD4⁺ CD8⁻ thymocytes and erythroid lineage cells and a correlation in the relative levels of expression in various ex vivo populations. The similarity in the expression patterns of ligands for NKR-P1B/D receptors and the MHC class I ligands for Ly49 inhibitory receptors suggests that low expression of ligands by these cell lineages may have significance in missing self-recognition systems. Notably, other Clr proteins, such as Clr-F and Clr-g, exhibit a much more restricted expression pattern (30), suggesting a different function for these proteins.

One of our most interesting findings was that many of the tumor cell lines we examined expressed notably low levels of Ocil/Clr-b on the cell surface. Comparisons of Ocil/Clr-b levels on lymphomas and other hematopoietic tumors with those on corresponding nontransformed lymphohematopoietic cell types.
suggested that Ocil/Clr-b down-regulation on tumor cell lines is often dramatic. These findings are consistent with the notion that down-regulation of Ocil/Clr-b on tumor cells contributes to their sensitivity to lysis by NK cells. Indeed, we observed reduced lysis of cells expressing Ocil/Clr-b that could be reversed with the 4A6 mAb. Hence, the NKR-P1B/D-Ocil/Clr-b receptor-ligand system appears to represent an independent mode of missing self-recognition that functions in parallel with missing self-recognition of class I MHC molecules.

The adaptive value to the animal of missing self-recognition of the Ocil/Clr-b molecules remains unclear, but the presence of the consensus casein kinase 2 (CK2) phosphorylation site in the cytoplasmic domain of Ocil/Clr-b (31) raises an interesting possibility. CK2 overexpression is common in tumor cell lines (34, 35), and active CK2 is known to regulate the NF-κB pathway, caspase activation, and Bel-4(X,L) levels (36), leading to lymphoproliferation and accelerated lymphomagenesis (34, 37).

Furthermore, endocytosis of certain other receptors that contain CK2 phosphorylation sites, such as the transferrin receptor and possibly the polymeric Ig receptor, depends on CK2-mediated receptor phosphorylation (38, 39). Hence, it is possible that the polymeric Ig receptor, depends on CK2-mediated CK2 phosphorylation sites, such as the transferrin receptor and Bcl-X(L) levels (36), leading to apoptosis, caspase activation, and Bcl-X(L) levels (36), leading to lymphoproliferation and accelerated lymphomagenesis (34, 37).

It is possible also that, under certain circumstances, expression of Ocil/Clr-b favors apoptosis over uncontrolled growth. For example, although we were successful in expression cloning of the Ocil/Clr-b cDNA by using a single cycle of cell sorting after transfection, these cells rapidly lost retrotrophic integrations when they were resorted (data not shown), possibly because the transduced cells died. Moreover, on transduction of the cloned IBS cDNA into BWZ cells, 4A6 staining waned over time, and a dramatic loss in viability of the 4A6+ (but not 4A6−) cells was observed during the 4A6 sorting procedure, as monitored by propidium iodide uptake (data not shown). Also, transient expression of Ocil/Clr-b in certain cell lines, such as COS cells, was inefficient, possibly because of induced cell death (data not shown). If Ocil/Clr-b expression normally favors the death of certain tumor cells, it is conceivable that loss of these molecules would be selected for during the evolution of a tumor. This mechanism could provide a rationale for missing self-recognition of Ocil/Clr-b molecules by NK cells. It remains a mystery why some cells, such as normal cells or BWZ cells expressing endogenous Ocil/Clr-b, display no ill effects.

In conclusion, we have demonstrated that the inhibitory NKR-P1B/D orphan NK cell receptors functionally interact with Ocil/Clr-b ligand and can mediate missing self-recognition of tumor cells by mouse NK cells. A recent study (40) identified a gene in the tunicate Botryllus schlosseri that encodes a lectin-like protein that is more similar in its extracellular domain to NKR-P1 than to other lectin-like genes. The possibility that the tunicate gene in the tunicate Botryllus schlosseri favors apoptosis over uncontrolled growth.
Fig. 6. Expression of Ocil/Clr-b by hematopoietic cells, including natural killer (NK) cells. (A) Fetal splenocytes (Left) and thymocytes (Right) at day 14 of gestation express high levels of Ocil/Clr-b ex vivo. Cells
were examined without Lympholyte enrichment. Among fetal splenocytes, the CD45+ population includes erythroid lineage cells. Similarly, a fraction of the DN IV fetal thymocytes represent erythroid lineage cells. DN I, CD44+CD25−; DN II, CD44+CD25+; DN III, CD44−CD25+; and DN IV, CD44−CD25−. (B) Fetal liver, bone marrow, splenocytes, and thymocytes at day 17 of gestation were analyzed by flow cytometry, gating on NK1.1+10D7+ cells. (C) Lymphokine-activated killer (LAK) cells were derived from fetal thymocytes (FT) or fetal liver (FL) cells by culturing cells isolated from day 14 embryos in IL-2 for 5 days. Gated NK1.1+ cells were analyzed by flow cytometry. (D) Nearly all adult lymph-node cells, including γδ T cells, express high levels of Ocil/Clr-b ex vivo. (E) Most adult bone marrow cells express high levels of Ocil/Clr-b ex vivo. Cells were examined after Lympholyte enrichment. (F) Functional expression of NKR-P1B ligand is unaltered in β2m−/− mice. Various cell types from β2m−/− or normal mice were used to stimulate BWZ.CD3ζ/P1B reporter cells. The β2m−/− and wild-type cells were equally stimulatory for each cell type. Media, reporter cells alone; P/I, PMA and ionomycin added. (G) 4A6 (Ocil/Clr-b) expression resembles class I MHC expression ex vivo. Total BALB/c bone-marrow cells, lymph-node cells, splenocytes, and thymocytes (without lympholyte enrichment) were stained with biotin-conjugated 4A6 plus streptavidin allophycocyanin versus H-2Kd-FITC and analyzed by flow cytometry. Markers were placed according to staining with streptavidin allophycocyanin alone. Cells were gated on live cells based on forward and side scatter and exclusion of propidium iodide.
Fig. 7. NKR-P1 specificity and the 4A6 mAb. (A) Functional specificity of NKR-P1A–D proteins assayed with BWZ indicator cells. Each type of indicator cell (NKR-P1A–D) was incubated with an equal number of MNK-1 cells, or with the indicated plate-bound mAbs (100 μg/ml). (B) 4A6, but not other hybridoma supernatants, blocks response of BWZ.CD3ζ/P1B cells to NIH 3T3 or MNK-1 cells. (C) Expression of the 4A6 antigen. Cells were stained with biotinylated 4A6 mAb and phycoerythrin-conjugated streptavidin (shaded region), or SA-PE alone (region outlined in black). RCN, relative cell number.
**Fig. 8.** cDNA cloning of the 4A6 antigen, a ligand for NKR-P1B. (A) PCR amplification of retroviral inserts from a 4A6-sorted pool (1B8) of BWZ cells that had been infected with the pMXS-NK retroviral expression library (see Methods). Genomic DNA from uninfected BWZ cells was used as a negative control. PCR was performed with MSCV primers (Left), followed by nested PCR of the PCR products by using PMX primers (Right). Indicated is the prominent 1.4-kb band (Left), which corresponds to the 1.3-kb band in the nested PCR (Right). Reverse-image photos of ethidium bromide-stained agarose gels are shown. (B) Retroviral transduction of 1B8 cDNA into BWZ cells generates 4A6⁺ transductants. Recombinant retrovirus was obtained by performing a triple-transfection of 293T cells with pMXS-1B8 and VSV-G gag-pol + env
constructs by using Lipofectamine Plus. Virus-containing cell supernatants were used to infect BWZ cells. After 3 days, BWZ transductants (shaded region) were compared with uninfected BWZ cells (region outlined by black line) by flow cytometry. (C) Selected BWZ cells that express endogenous 4A6 antigen (Left) contain increased levels of Ocil/Clr-b mRNA (Right). BWZ.36 cells were sorted for 4A6 expression and cloned by limiting dilution. A positive clone (BWZ.4A6+) was compared with control parental BWZ cells (BWZ Con). We subjected 3-fold dilutions of cDNA to PCR with primers specific for Ocil/Clr-b or control G3PDH primers.

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