Immunity, Vol. 12, 721–727, June, 2000, Copyright ©2000 by Cell Press

Retinoic Acid Early Inducible Genes Define a Ligand Family for the Activating NKG2D Receptor in Mice

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Summary

Here we describe a family of GPI-anchored cell surface proteins that function as ligands for the mouse activating NKG2D receptor. These molecules are encoded by the *retinoic acid early inducible* (*RAE-1*) and *H60* minor histocompatibility antigen genes on mouse chromosome 10 and show weak homology with MHC class I. Expression of the NKG2D ligands is low or absent on normal, adult tissues; however, they are constitutively expressed on some tumors and upregulated by retinoic acid. Ectopic expression of RAE-1 and H60 confers target susceptibility to NK cell attack. These studies identify a family of ligands for the activating NKG2D receptor on NK and T cells, which may play an important role in innate and adaptive immunity.

Introduction

NK cells are effector cells of the innate immune system, providing protection against certain tumors and microbial infections (Trinchieri, 1989; Biron et al., 1999). The mechanisms allowing NK cells to discriminate between normal versus abnormal (infected or transformed) cells are not well defined. It appears, however, that NK cell function is regulated by a delicate balance between the engagement of activating and inhibitory receptors (reviewed in Lanier, 1998; Long, 1999). Many of the activating NK cell receptors (e.g., CD16, KIR2DS, Ly49D/H, CD94/NKG2C) are noncovalently associated with membrane-anchored adaptors that possess immunoreceptor tyrosine-based activation motifs (ITAM) in their cytoplasmic domains (reviewed in Bakker et al., 2000). These adaptor proteins, which include FcεRIγ, CD3ζ, and DAP12, stimulate NK cells by recruiting and activating the Syk and ZAP70 protein tyrosine kinases (reviewed in Bakker et al., 2000).

Recently, we reported that another membrane adaptor molecule, DAP10, forms an activating NK cell receptor complex with the NKG2D glycoprotein (Wu et al., 1999). DAP10 contains a YxxM motif in its cytoplasmic domain (Songyang et al., 1996) that upon tyrosine phosphorylation recruits the p85 subunit of the PI3 kinase (Wu et al., 1999). Unlike most other NK cell receptors that are expressed on minor subsets of NK cells or T cells, the NKG2D/DAP10 receptor complex is on all NK cells, $CD8^+$ $\alpha\beta TCR^+$ T cells, and $\gamma\delta TCR^+$ T cells (Bauer et al., 1999), implying a broader role in immune responses. In humans, the NKG2D receptor binds to the polymorphic nonclassical MHC molecules MICA (MHC class I chain–related A) and MICB (Bahram and Spies, 1996; Bauer et al., 1999), which are frequently expressed on epithelial tumors (Groh et al., 1999). Because NK cells (Bauer et al., 1999) and $\gamma\delta TCR^+$ T cells (Groh et al., 1998, 1999) can kill MICA/B-bearing cells, expression of these molecules might be tightly regulated to avoid the inadvertent destruction of healthy tissues. Expression of MICA and MICB is under the control of a heat shock promoter element, ensuring that these molecules are present on "stressed" but not healthy cells (Groh et al., 1996).

MICA/B homologs have been found in primates (Steinle et al., 1998) but not rodents. Often mouse and human homologs within the MHC are divergent, with little structural similarity, but their functions and ligand binding sites are conserved. For example, HLA-E in humans and $Qa\text{-}1^b$ in mouse share little structural homology, but both bind similar peptides generated from other MHC class I proteins (Aldrich et al., 1994; Braud et al., 1997) and serve as ligands for the conserved mouse and human CD94/NKG2A receptors (Borrego et al., 1998; Braud et al.,1998; Lee et al., 1998; Vance et al., 1998). Because mouse, rat, and human NKG2D (Houchins et al., 1991; Vance et al., 1997; Berg et al., 1998; Ho et al., 1998) are evolutionary conserved, we assumed that NKG2D ligands exist in rodents and undertook studies to identify these molecules. Here we report the nature and function of a family of ligands for the activating NKG2D receptor in mice.

Results and Discussion

A mNKG2D-Ig Fusion Protein Binds to Mouse Tumor Cell Lines

In search for the ligands of mouse NKG2D (mNKG2D), we generated, expressed, and purified a soluble protein consisting of the extracellular domain of mNKG2D fused to the Fc portion of human IgG. Because the human NKG2D ligands MICA and MICB are frequently overexpressed on human carcinomas (Groh et al., 1999), we analyzed a panel of transformed mouse cell lines for reactivity with the mNKG2D-Ig fusion protein (FP). Positive staining was observed with the mouse lung fibroblast line MLg, the Lewis lung carcinoma line LL/2, and the rectum carcinoma line CMT, but not the pro-B cell line Ba/F3 (Figure 1). These results suggested the existence of mNKG2D ligands on MLg, LL/2, and CMT.

Expression Cloning of the mNKG2D Ligands by Retroviral Library Screening

Expression cloning of mNKG2D ligands was achieved by infecting Ba/F3 cells with a retroviral library prepared

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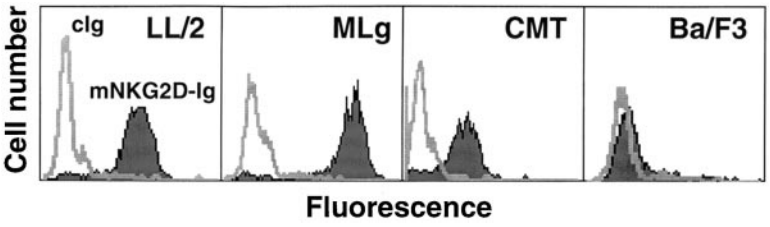

Figure 1. The mNKG2D-Ig FP Binds to Certain Mouse Tumor Lines but Not to the Pro-B Cell Line Ba/F3

The cell lines LL/2, MLg, CMT, and BA/F3 were stained with the mNKG2D-Ig FP (filled histograms) or the control human IgG (open histograms), followed by a biotinylated anti-human Ig second step and streptavidin-PE. Histograms (x axis, fluorescence, 4 decade log scale; y axis, number of cells) displaying the fluorescence of live cells are presented, and the results are representative of five independent experiments.

from the MLg cell line (Kitamura et al., 1995). After infection of Ba/F3 and selection of cells reactive with the mNKG2D-Ig FP by flow cytometry, we isolated several candidate Ba/F3 cell clones. Recovery of the integrated cDNA from these clones by RT–PCR using retroviral vector primers revealed several bands in the 0.7–1.4 kb range, all of which were sequenced. Three distinct cDNA encoding putative ligands of mNKG2D were identified.

Two cDNA were identical to the previously described retinoic acid inducible early transcript-1γ (RAE-1γ) (Zou et al., 1996). RAE-1γ is a member of the mouse *RAE-1* gene family, which was originally identified as a cDNA upregulated by retinoic acid (RA) treatment of the mouse carcinoma cell line F9 using differential plaque hybridization (Nomura et al., 1994). The family contains at least three distinct loci, designated *RAE-1*α, β, and γ, that encode polypeptides with ~92%–95% identity (Nomura et al., 1996). Previously, no function was attributed to these genes. Three cDNA cloned from the MLg retroviral library using mNKG2D-Ig FP encoded a novel polypeptide displaying ~92% amino acid identity with RAE-1γ, which we call RAE-1δ. This sequence may represent a fourth *RAE-1* locus; however, sequencing of genomic DNA from 129 strain mice, where *RAE-1*α, β, and γ were defined, will be required to verify this possibility. Alternatively, this may represent a polymorphic allele of *RAE-1*α, β, or γ in the random-bred ddY mouse strain from which MLg was established.

In addition to the RAE-1 molecules, we identified the minor histocompatibility antigen H60 (Malarkannan et al., 1998) as a ligand of mNKG2D. H60 was originally defined as an immunodominant autosomal minor H antigen differentially transcribed in BALB.B and B6 mice. When B6 mice were immunized with BALB.B splenocytes, CTL clones were isolated that were specific for a peptide derived from the H60 polypeptide associated with H-2K^b (Malarkannan et al., 1998). Previously, the only function attributed to the H60 protein was to serve as a precursor for an antigenic peptide bound to the H-2K^b class I molecule (Malarkannan et al., 1998).

RAE-1 Family Members and the Minor H Antigen H60 Are Ligands for mNKG2D

RAE-1α and RAE-1β (approximately 92%–95% identical to RAE-1γ) cDNA were obtained by RT–PCR from RA-induced F9 cells (Zou et al., 1996). RAE-1 α, β, γ, δ and H60 cDNA were subcloned into the pMX-pie retroviral vector, containing an IRES element followed by the eGFP gene. Viruses were produced and used to infect Ba/F3 cells. Ba/F3 cells infected with these retroviruses stained with the mNKG2D-Ig FP (Figure 2). The mNKG2D-Ig FP also specifically stained the BW5147 thymoma cell line transduced with retroviruses encoding the RAE-1 family members and H60; no staining was detected on uninfected or mock infected BW5147 cells (data not shown). These data confirm that RAE-1 α, β, γ, δ and the minor histocompatibility antigen H60 are ligands for mNKG2D.

Alignment of the predicted protein sequences of the RAE-1 family members revealed 92%–95% homology (Figure 3). As reported previously (Zou et al., 1996), the RAE-1 molecules show weak homology with MHC class I in the α1 and α2 regions but lack an α3 domain. In classical MHC class I molecules, the α3 domain contains the binding site for β_2 microglobulin; therefore, it is unlikely that RAE-1 molecules require β_2m for surface expression. The RAE -1 molecules are expressed as GPI-anchored membrane proteins (Nomura et al., 1996).

The H60 polypeptide demonstrates only ~24% homology with the RAE-1 molecules. However, like RAE-1, it has weak homology with classical MHC class I and is predicted to be a GPI-anchored membrane glycoprotein. Neither H60 nor RAE-1 demonstrates substantial homology (<20%) with human MICA and MICB. Moreover, the *RAE-1* (Nomura et al., 1996) and *H60* (Malarkannan et al., 1998) genes are not encoded within the MHC

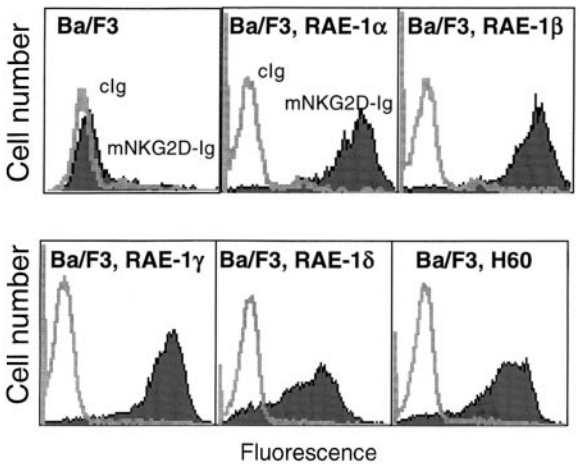

Figure 2. RAE-1 α, β, γ, δ, and H60 Are Ligands for mNKG2D

Ba/F3 were transduced with ecotropic retroviruses encoding RAE-1α, RAE-1β, RAE-1γ, RAE-1δ, and H60. Transduced cells were sorted by flow cytometry for high expression of mNKG2D ligands and stained with mNKG2D-Ig FP (filled histograms) or the control human Ig (open histograms), followed by a biotinylated anti-human Ig and streptavidin-PE. Data displayed are representative of results obtained in three independent experiments.

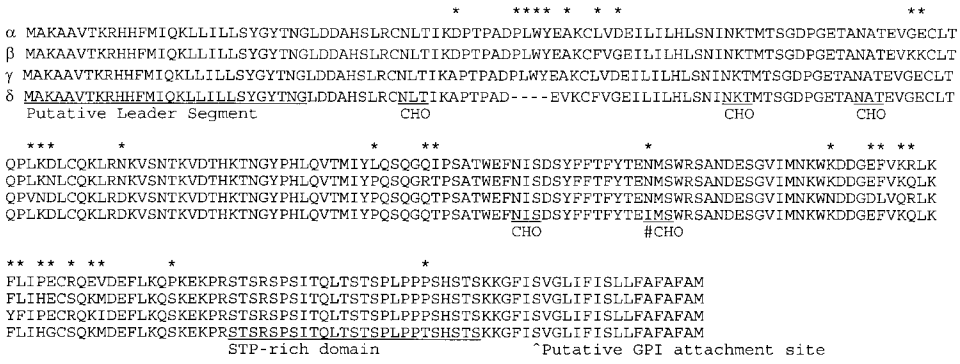

Figure 3. Alignment of RAE-1 α, β, γ, δ

Alignment of the predicted amino acid sequences of the RAE-1 α, β, γ, δ polypeptides. Sites where amino acids differ are denoted by an asterisk. Potential sites of N-linked glycosylation are indicated (CHO). "CHO#" designates a potential glycosylation site that is absent in the RAE-1δ isoform. The predicted sites of cleavage of the leader segments, the STP-rich domains, and for the predicted sites for the addition of GPI (caret) are noted. The sequence of RAE-1δ is deposited as GenBank AF257520.

but are localized on mouse chromosome 10. Preliminary findings indicate the existence of a human homolog of *RAE-1* (data not shown), suggesting that human NKG2D has both MHC and non-MHC encoded ligands. Therefore, in the mouse we cannot exclude the existence of nonclassical MHC class Ib ligands for NKG2D.

Expression of the RAE-1 Family Members and H60

Nomura et al. (1996) reported that RAE-1 transcripts were present in the brain of an 11-day-old mouse embryo but not in an 18-day-old embryo or adult tissues. These data indicated that RAE-1 expression is controlled in a developmental stage-specific and tissue-specific manner. We performed a more detailed analysis of RAE-1 expression on mouse tissues and cell lines by RT–PCR using primers which amplified RAE-1α, β, γ, and δ (Figure 4). RAE-1 transcripts were present in several transformed cell lines, including LL/2 lung carcinoma cells and MLg lung fibroblast cells, and were expressed in low but detectable amounts in CMT rectum carcinoma cells and YAC lymphoma cells (the prototypic NK-sensitive target cell line) (Figure 4). No RAE-1 transcripts were detected in Ba/F3 cells, which also did not surface stain with the mNKG2D-Ig FP. With regard to tissues, RAE-1 transcripts were abundant in the 7-day-old embryo, consistent with the findings of Nomura et al. (1996). Very low but detectable amounts of RAE-1 transcript were found in adult spleen and liver but were absent from adult brain and kidney (Figure 4). However, freshly isolated splenocytes, lymph node cells, and thymocytes from B6 mice did not surface stain with the mNKG2D-Ig FP (data not shown). Thus, the expression of *RAE-1* genes is restricted to certain transformed cells and selected tissues. This expression pattern fits very well with the concept that expression of a ligand for a widely expressed activating receptor in innate immunity must be tightly controlled to avoid attack of healthy cells.

Malarkannan et al. (1998) previously reported that H60 transcripts were detected in activated BALB.B splenocytes but were undetectable by Northern blot analysis of B6 splenocytes. Therefore, *H60* is polymorphic and is expressed in mice of the BALB/c background but not B6. When we analyzed the panel of cell lines, only the lung fibroblast line MLg (from the random-bred ddY mouse strain) and YAC lymphoma cells (from the AKR mouse strain) had H60 transcripts (Figure 4). The Lewis lung carcinoma LL/2 (B6 background) and the rectum carcinoma CMT (B6 background) were both negative, consistent with the prior finding that H60 transcripts were present in BALB.B but absent from B6 mice (Malarkannan et al., 1998). Low levels of H60 transcripts were detected in adult spleen and embryonic tissues from BALB/c mice but were absent in adult brain, liver, and kidney (Figure 4).

The Ligands for mNKG2D Are Induced on F9 Cells by Treatment with Retinoic Acid

RAE-1 family members, which have a retinoic acid–inducible element in their promoters (Nomura et al., 1996), were originally cloned from RA-induced F9 cells. Therefore, we investigated whether the treatment of F9 cells with RA results in upregulation on the cell surface of an antigen recognized by the NKG2D-Ig FP. F9 cells were cultured in the presence or absence of RA for 48 hr (Nomura et al., 1994) and then stained with the mNKG2D-Ig FP. mNKG2D-Ig FP binding was significantly upregulated by RA treatment, as determined by flow cytometry (Figure 5A). When the kinetics of induction of RAE-1 by retinoic acid treatment of F9 cells was investigated by quantitative PCR, we found a 6-fold increase in transcripts within 48–72 hr (Figure 5B). In contrast, neither treatment with mouse IFN-α, mouse IFN-γ, or heat shock (42°C for 90 min) elevated binding of the mNKG2D-Ig FP (data not shown). LL/2 and MLg cells did not show increased mNKG2D-Ig FP binding after RA treatment, possibly because they lack RA responsiveness or already constitutively express maximal amounts of the NKG2D ligands (data not shown).

The Ligands for mNKG2D Are GPI-Anchored Membrane Proteins

F9 cells, RA-induced F9 cells, LL/2 cells, and BW5147 cells transduced with RAE-1γ were treated with PI-PLC, an enzyme that cleaves GPI linkages. PI-PLC or untreated cells were stained with the mNKG2D-Ig FP and examined by flow cytometry (Figure 6). PI-PLC treatment

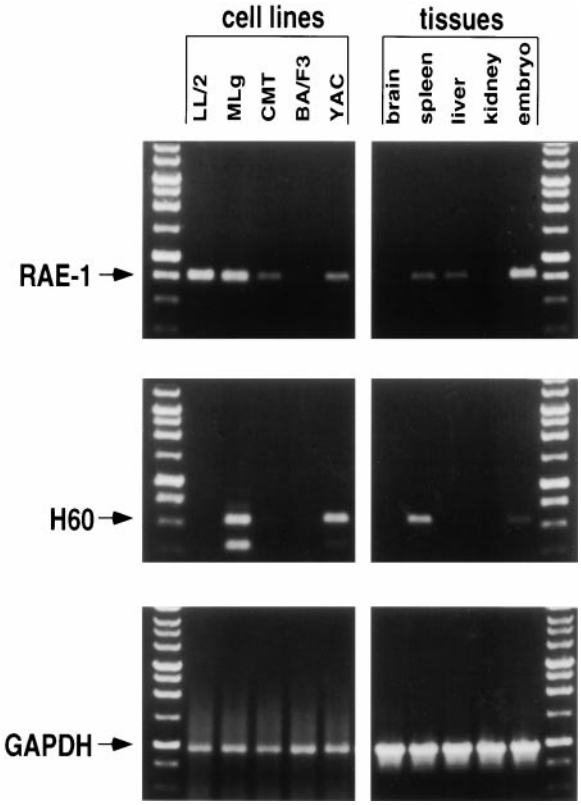

Figure 4. Expression Analysis of RAE-1 and H60

The following cell lines (left) were analyzed by RT–PCR (37 cycles at 94°C for 30 s and 68°C for 2 min) for expression of RAE-1 (upper panel) or H60 (middle panel): Lewis lung carcinoma LL/2, lung fibroblast MLg, rectum carcinoma CMT, pro-B cell line Ba/F3, and thymic lymphoma YAC. The following tissues from BALB/c mice (right) were analyzed: brain, spleen, liver, kidney, 7-day-old embryo. The integrity of the cDNA was assessed by PCR by using glyceraldehyde-3-phosphate dehydrogenase primers (lower panel). PCR products of the expected size for RAE-1, H60, and GAPDH are indicated by arrows. A smaller size H60 PCR product has been observed in MLg and at lesser amounts in other cells. Sequencing revealed that this likely represents a splice variant of H60.

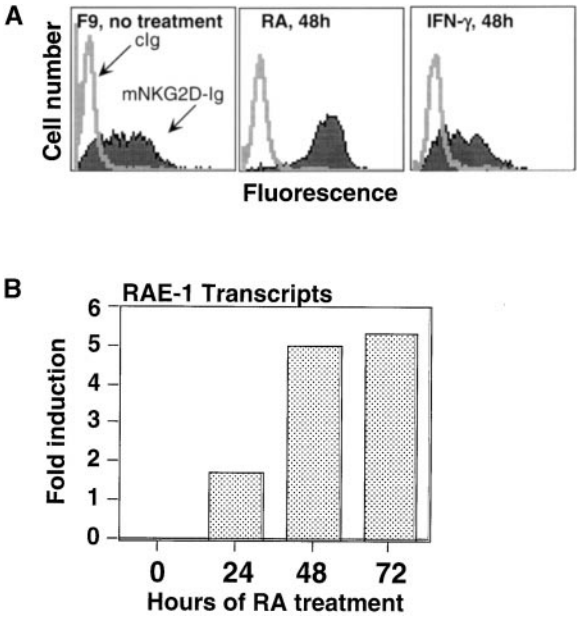

Figure 5. Retinoic Acid Treatment Induces Expression of the Ligand for mNKG2D on the Embryonic Carcinoma Cell Line F9

(A) F9 cells were mock-treated for 48 hr in the presence of the solvent (ETOH) alone (1:1000 final dilution) (upper panel) or treated with all-trans retinoic acid (1 μM) in ETOH or 20 ng/ml mouse IFN-γ. Cells were then stained with mNKG2D-Ig FP (filled histograms) or the control human Ig (open histograms), followed by a biotinylated anti-human Ig and streptavidin-PE. Histograms displaying the fluorescence of live cells are presented, and the results are representative of three independent experiments.

(B) F9 cells were treated with RA as described, and at the indicated time points, cell pellets were snap-frozen. RT–PCR was performed with RAE-1 primers that amplify all four family members and the amount of cDNA was quantified by quantitative PCR (TaqMan) analysis. Data are shown as fold induction over the mock-treated F9 cells.

resulted in complete abrogation of mNKG2D-Ig FP binding to F9 cells and RA-induced F9 cells and substantially removed mNKG2D-Ig FP binding to LL/2 cells and RAE-1γ-transfected BW5147 cells. These results confirmed that the ligands for mNKG2D on these cell lines are GPI-anchored membrane proteins.

RAE-1 and H60 Render Cells Susceptible to NK Cell–Mediated Cytotoxicity

Finally, we evaluated the functional significance of the interaction between NKG2D on mouse NK cells and RAE-1 or H60 on target cells. Polyclonal mouse NK cells prepared from B6 mice, which express NKG2D (Vance et al., 1997; Ho et al., 1998), were cultured in IL-2 and IL-15 for 10 days. These cells were used as effectors in a cytotoxicity assay using RAE-1γ-transduced Ba/F3 cells, H60-transduced Ba/F3 cells, or parental Ba/F3 cells as targets. The RAE-1γ and H60-transduced Ba/F3 cells but not NK-resistant Ba/F3 cells were specifically killed by the activated mouse NK cells (Figure 7A). Similar results were obtained when BW5147 thymoma cells were transduced with RAE-1γ or H60 (Figure 7B). Thus, the ectopic expression of the mNKG2D ligands RAE-1 and H60 rendered Ba/F3 cells susceptible to attack by NK cells and augmented killing of the BW5147 target cells.

Concluding Remarks

These studies identify the RAE-1 and H60 proteins as functional ligands of the activating mouse NKG2D/DAP10 receptor complex. However, because the activity of NK cells is regulated by both activating and inhibitory receptors, further studies will be necessary to determine how the inhibitory receptors for MHC class I (e.g., Ly49 and CD94/NKG2A) might modulate signals transmitted by NKG2D/DAP10. In this regard, it has previously been shown that human NK cells are able to kill tumors expressing MICA and MICB, despite the presence of conventional HLA class I molecules on these targets (Bauer et al., 1999).

An unanticipated result of these studies was the discovery of five mouse genes, none within the MHC complex, able to encode ligands recognized by NKG2D. Because preliminary studies indicate the existence of a

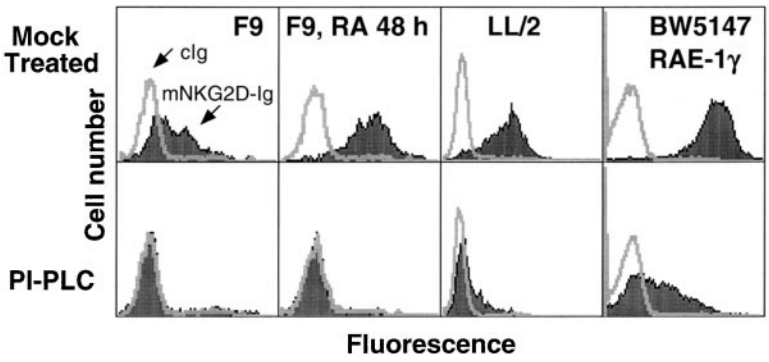

Figure 6. The Ligand for mNKG2D Is a GPI-Linked Cell Surface Protein

F9 cells, F9 cells treated with RA for 48 hr, LL/2 cells, and the BW5147 cells transduced with RAE-1γ were mock treated (upper panels) or treated with PI-PLC (lower panels). Subsequently, cells were stained with mNKG2D-Ig FP (filled histograms) or the control human Ig (open histograms), followed by a biotinylated anti-human Ig and streptavidin-PE. Histograms displaying the fluorescence of live cells are presented and the results are representative of three independent experiments.

RAE-1 homolog in humans, this raises the possibility that MHC class Ib ligands might also be expected in rodents. What is the biological rationale for multiple genes in mice (e.g., *RAE-1* α, β, γ, δ, and *H60*) and humans (*MICA* and *MICB*) that encode NKG2D ligands? Presently, very little is known about the physiological stimuli inducing these genes. It is possible that different types of "stress" will transcriptionally activate these loci in different tissues. For example, *MICA* and *MICB* are induced by heat shock (Groh et al., 1996), whereas the *RAE-1* genes apparently are not. Studies are needed to examine whether distinct environmental insults (e.g., cytokines, transformation, viral infection, etc.) can differentially induce these genes. Further insights about the regulation of these ligands might lead to potential therapeutic strategies against tumors and certain viral diseases. Finally, we cannot exclude the possibility that receptors other than NKG2D might use these glycoproteins as ligands, particularly since the *RAE-1* genes are active during fetal development (Nomura et al., 1996).

Experimental Procedures

Cell lines, Transduction, and Culture Conditions

The mouse Lewis lung carcinoma cell line LL/2, the mouse BW5147 thymoma cell line, the mouse fibroblast cell line MLg, and the mouse rectum carcinoma cell line CMT were obtained from the American Type Culture Collection (ATCC). Cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS). The mouse embryonic carcinoma cell line F9 (ATCC) was maintained in DMEM with 10% FCS on a 0.1% gelatin layer (Sigma), as described (Nomura et al., 1994). F9 cells were induced to differentiate by treatment with 1 μM all-trans retinoic acid (Sigma) (prepared as a 1 mM stock solution in ethanol) (Nomura et al., 1994). The pro-B cell line Ba/F3 was maintained in RPMI 1640 supplemented with 10% FCS and 10 ng/ml mouse IL-3 (kindly provided by Dr. K. Moore, DNAX). BW5147 and Ba/F3 cells were transduced with ecotropic retroviruses generated by using the pMX-pie vector, as described previously (Onihsi et al., 1996). Transduced cells expressing high levels of antigen on the cell surface were isolated by flow cytometry. Cells transduced with the pMX-pie retroviral vector were selected by growth in 1 μg/ml puromycin (Sigma).

Construction and Expression of the mNKG2D-Ig FP

A cDNA fragment corresponding to the extracellular domain of mNKG2D was amplified by PCR using a mouse NKG2D cDNA as template (sense primer, 5′-AACTCGAGTTTCAGCCAGTATTGTG CAACAAGG-3′; antisense primer, 5′-AACTCGAGCACCGCCCTTTT CATGCAGATGTA-3′). XhoI restriction enzyme sites used for cloning are underlined. This fragment was inserted into the XhoI cloning site of a modified CDM8 expression vector that contained a human CD8 leader segment followed by the Fc segment of human IgG at the N terminus (Phillips et al., 1996). The extracellular domain of mNKG2D was then placed, in frame, at the COOH terminus of the fusion protein to position it in the proper orientation for a type II protein. 293 T cells were transiently transfected with the plasmid, and the mNKG2D-Ig FP was purified by affinity chromatography using immobilized protein A (Phillips et al., 1996).

Flow Cytometry

Cells (2×10^5) were stained for 15 min on ice with 1 μg mNKG2D-Ig FP or control human IgG. A biotinylated donkey anti-human IgG antiserum (Jackson ImmunoResearch) was used as a second step reagent, followed by staining with phycoerythrin (PE)-conjugated streptavidin (PharMingen). Flow cytometry was performed using a FACScan (Becton Dickinson). Samples were analyzed and data displayed as described previously (Lanier and Recktenwald, 1991).

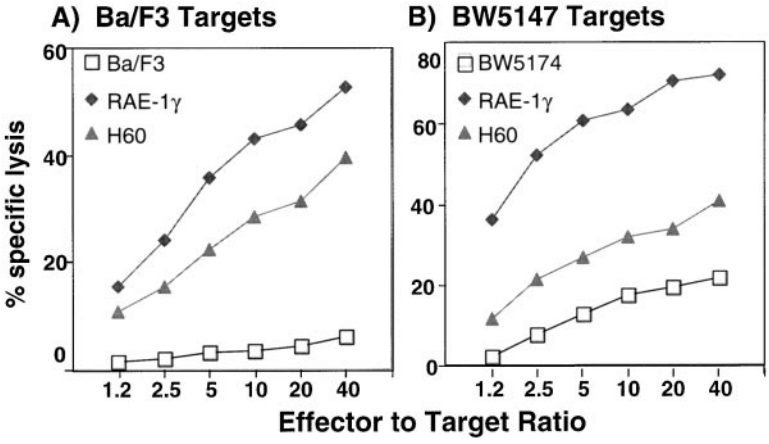

Figure 7. RAE-1- and H60-Transduced Cells Are Preferentially Killed by Mouse NK Cells

4h-^{51}Cr-release assays were performed using IL-2 and IL-15 activated NK cells from B6 mice at the indicated effector to target ratios.

(A) The following cell lines were used as targets: Ba/F3 cells (open squares), Ba/F3 cells transduced with RAE-1γ (filled diamonds), and Ba/F3 cells transduced with H60 (filled triangles).

(B) Target cells were BW5147 cells (open squares), BW5147 cells transduced with RAE-1γ (filled diamonds), and BW5147 cells transduced with H60 (filled triangles).

The data shown are representative for four (A) or two (B) independently performed experiments.

Expression Cloning Using Retroviral Libraries

A cDNA library from MLg cells containing 1.8×10^8 independent cDNA inserts was constructed using the retroviral expression vector pMX, as described (Kitamura et al., 1995; Onihsi et al., 1996). The library was transfected into the ecotropic packaging cell line Phoenix-E (kindly provided by Dr. G. Nolan, Stanford, Palo Alto, CA) using lipofectamine (GIBCO). Infectious retroviruses were harvested in the supernatant after 48 hr. Ba/F3 cells (5×10^7) were infected with 100 ml of virus stock in the presence of polybrene (Sigma) (8 μg/ml). After two rounds of enrichment for mNKG2D-Ig FP-positive cells by flow cytometry, single-positive cells were cloned. Total RNA from several different Ba/F3 cell clones (all positive for mNKG2D-Ig FP binding) were prepared using the RNA-easy kit (Qiagen). Reverse transcription was performed by using AMV reverse transcriptase (GIBCO). cDNA inserts were amplified by PCR with AmpliTaq Gold polymerase (Perkin-Elmer) with the pMX vector primers (sense, 5′- CCCGGGGGTGGACCATCCTCT-3′; antisense, 5′-CTACAGGTG GGGTCTTTCATTCC-3′). The amplified cDNA fragments were subcloned into the TA vector (InVitrogen) and sequenced.

RT–PCR Expression Analysis

For expression studies, the following oligonucleotide primers for RAE-1 were used: sense, 5′-ATAATGGATCCATGGCCAAGGCAG CAGTGACCAA-3′; antisense 5′-ATATTATAGCGGCCGCTCACATC GCAAATGCAAATGCAAATAAT-3′. The BamHI and NotI restriction enzyme sites, respectively, used for cloning the PCR products are underlined. These primers efficiently amplified cDNA of RAE-1 α, β, γ, and δ. Primers used for analysis of H60 expression were sense, 5′-ATT GCCTCGAGGATGGTACAGACTCTCTAAGT-3′; antisense, 5′-TATC CTCGAGCAGACCCTGGTTGTCAGAATTATGTC-3′. The XhoI restriction enzyme sites used for cloning are underlined. The integrity of the cDNA was assessed by PCR by using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (sense, 5′-TGAAGGTCGGTGTGA ACGGATTTGGC-3′; antisense, 5′-CATGTAGGCCATGAGGTCCAC CAC-3′) under identical conditions (37 cycles at 94°C for 30 s and 68°C for 2 min). The panel of cDNA from mouse tissues was purchased from Clontech. Quantitative PCR was performed by using a TaqMan model 5700 (Perkin Elmer). Primers used for amplification of RAE-1 transcripts for a TaqMan analysis were sense, 5′-TGGCCAAGGCAG CAGTG-3′; antisense, 5′-CGTTGGTGTATCCATAGCTCAGTAG-3′. PCR products were detected by SYBR green dye (Perkin Elmer).

Phosphatidyl-Inositol-Specific Phospholipase C Treatment

To obtain evidence for GPI linkage of RAE-1 proteins, cells were treated with 0.8 U/ml phosphatidyl-inositol-specific phospholipase C (PI-PLC) (Molecular Probes) at 37°C for 45 min. Subsequently, cells were washed in PBS with 0.05% sodium azide and stained on ice by indirect immunofluorescence using mNKG2D-Ig FP or control human IgG, as described above.

^{51}Cr Release Assay

NK cells used as effectors were prepared from B6 splenocytes. B cells were removed by negative depletion using anti-Ig-coated magnetic beads (Dynal). Subsequently, NK cells were positively selected by using DX5 mAb–coupled beads with the corresponding column system (MACS). Polyclonal mouse NK cell lines were cultured in 1000 U/ml mouse IL-2 (generously provided by Dr. D. Rennick) and 1 ng/ml human IL-15 (Peprotech) for at least 10 days before being used as effectors in a cytotoxicity assay. The ^{51}Cr release assay was performed using 3000 ^{51}Cr-labeled targets per well. Effectors were added at the indicated concentration, plates were briefly centrifuged, incubated at 37°C for 4 hr, and the released radioactivity was measured with a γ-counter (Lanier et al., 1983). Specific lysis was calculated by the standard formula: (experimental-spontaneous release)/(total-spontaneous release) × 100.

Acknowledgments

We are grateful to T. Kitamura and G. Nolan for retroviruses; D. Rennick for IL-2; R. de Waal Malefyt and K. Smith for expert assistance with TaqMan analysis; M. Bigler for expert help with cell culture; and J. Katheiser, G. Burget, and M. Andonian for graphics. We also thank S. Zurawski, T. Hauser, K. Moore, H. Strobl, C. Parham, C. O'Callaghan, and S. Tangye for helpful discussion and technical advice. Schering Plough Corporation supports the DNAX Research Institute. The Sandler Family Supporting Foundation funded studies at UCSF.

Received April 20, 2000; revised May 25, 2000.

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