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Two Novel NKG2D Ligands of the Mouse H60 Family with Differential Expression Patterns and Binding Affinities to NKG2D

Akio Takada,2*,† Shigeru Yoshida,2*,‡ Mizuho Kajikawa,§ Yukiko Miyatake,* Utano Tomaru,* Masaharu Sakai,‡ Hitoshi Chiba,† Katsumi Maenaka,§ Daisuke Kohda,§ Kazunori Fugo,* and Masanori Kasahara3*

H60, originally described as a dominant minor histocompatibility Ag, is an MHC class I-like molecule that serves as a ligand for the NKG2D receptor. In the present study, we identified two novel mouse chromosome 10-encoded NKG2D ligands structurally resembling H60. These ligands, which we named H60b and H60c, encode MHC class I-like molecules with two extracellular domains. Whereas H60b has a transmembrane region, H60c is a GPI-anchored protein. Recombinant soluble H60b and H60c proteins bound to NKG2D with affinities typical of cell–cell recognition receptors ($K_d = 310$ nM for H60b and $K_d = 8.7$ μM for H60c). Furthermore, expression of H60b or H60c rendered Ba/F3 cells susceptible to lysis by NK cells, thereby establishing H60b and H60c as functional ligands for NKG2D. H60b and H60c transcripts were detected only at low levels in tissues of healthy adult mice. Whereas H60b transcripts were detectable in various tissues, H60c transcripts were detected mainly in the skin. Infection of mouse embryonic fibroblasts with murine cytomegalovirus induced expression of H60b, but not H60c or the previously known H60 gene, indicating that transcriptional activation of the three types of H60 genes is differentially regulated. The present study adds two new members to the current list of NKG2D ligands. The Journal of Immunology, 2008, 180: 1678–1685.

Cytolytic activities of NK cells are controlled by the interplay of a multitude of inhibitory and activating receptors expressed on their surfaces (1, 2). Inhibitory receptors of the members of the mouse Ly49 and human killer Ig-related receptor families recognize self-MHC class I molecules on target cells (3, 4). Abnormal cells, whether transformed or infected, frequently lose expression of class I molecules. Because these cells are unable to engage inhibitory receptors, they become susceptible to lysis by NK cells. This mode of recognition by NK cells forms the basis of the “missing-self” phenomenon (5).

In comparison to inhibitory receptors, less is known about the ligands and biologic functions of activating receptors (6). Among the known activating receptors, the best characterized is NKG2D, a homodimeric C-type, lectin-like receptor encoded by the NK gene complex (7–10). In mice, NKG2D is expressed on NK cells, activated CD8+ αβ T cells, subsets of γδ T cells, subsets of NK T cells, macrophages, and IFN-producing killer dendritic cells (11–14). The ligands for NKG2D are usually expressed only poorly or not at all by normal cells, but their expression is up-regulated in response to cellular distress such as transformation (15), infection (16, 17), heat shock (18), and DNA damage (19). This up-regulation alerts the immune system to the presence of damaged and potentially dangerous cells via NKG2D receptors. Whereas NKG2D is a direct activating receptor in NK cells and macrophages, it acts as a costimulatory receptor in T cells (20).

A unique property of the NKG2D receptor-ligand system is the presence of multiple ligands for a single receptor. In humans, known ligands include MHC class I-related chains A and B (MICA and MICB)3 molecules encoded in the MHC (20), as well as a total of five retinoic acid early inducible-1 (RAEI1)/UL16-binding protein (ULBP) molecules encoded outside the MHC (21, 22). Similarly, seven ligand molecules for NKG2D have been identified in mice: RAE1α, RAE1β, RAE1γ, RAE1δ, RAE1ε, H60, and MULT1 (murine ULBP-like transcript 1) (11, 23–25). All of these ligands are encoded outside the MHC and are more closely related to human retinoic acid early transcript (RAET)/ULBP than to MICA and MICB. Thus far, no ligands corresponding to human MICA/MICB have been identified in mice.

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Although all known NKG2D ligands are members of the MHC class I family, they exhibit remarkable structural diversity. Thus, MICA shows only ~25% amino acid sequence identity to human HLA-E/UBLP molecules. NKG2D ligands are also heterogeneous in domain organization; that is, whereas most NKG2D ligands have two extracellular domains (α1 and α2) (11, 22, 26, 27), MICA and MICB have three extracellular domains (α1–α3) (28). Furthermore, NKG2D ligands can be an integral membrane protein or a GPI-anchored protein. It has been suggested that the ability of the NKG2D receptor to interact with diverse ligands is achieved through rigid adaptation rather than induced fit (29, 30).

In the present study, we describe the identification and functional characterization of two novel mouse NKG2D ligands structurally resembling H60. These ligands, which we named H60b and H60c, encode MHC class I-like molecules with two extracellular domains. To distinguish H60b and H60c from the previously described H60, we call the latter H60a.

Materials and Methods
Cell lines, abs, and reagents
The pro-B cell line Ba/F3 was obtained from RIKEN BioResource Center. Ba/F3 cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated FBS and 10% WEHI-3 cell-conditioned medium at 37°C under 5% CO2. The mouse T lymphoma cell line RMA was obtained from Dr. Kärre (Kolaringska Institute, Stockholm, Sweden). These cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS at 37°C and 5% CO2.

Rabbit anti-FLAG polyclonal Ab (F7425), human IgG (I4506), and rabbit IgG (I5006) were purchased from Sigma-Aldrich. Recombinant mouse NKG2D-Fc fusion proteins (139-NK; referred to as NKG2D-Ig hereafter) and anti-mouse NKG2D mAb (MAB1547) were from R&D Systems. Rat anti-mouse GM-CSF mAb (1723-01) was purchased from Genzyme. The Abs used as secondary reagents were FITC-conjugated goat anti-rabbit IgG, F(ab')2 (F1262, Sigma-Aldrich) and FITC-conjugated goat anti-human IgG, F(ab')2 (109-096-008, Jackson ImmunoResearch).

Isolation of H60b and H60c cDNA by 5’- and 3’- RACE
The expressed sequence tag (EST) database of GenBank was searched using the mouse H60a amino acid sequence (accession number: NP_034530.1) as a query. This search resulted in the identification of two H60-like sequences distinct from the previously known H60a sequence. Based on these newly identified EST sequences, we designed primers for 5’- and 3’-RACE with the sequence similarity. Reliable alignment was possible only in the α1–α3 domain (or the α1–α2 domains for class I-like molecules lacking the α3 domain). We therefore excluded the remaining regions from the analysis. The distance matrix was obtained by calculating p-distances for all pairs of sequences. Sites containing gaps were excluded from the analysis using the pairwise deletion option. Neighbor-joining trees were constructed using the MEGA version 3.0 (35). The reliability of branching patterns was assessed by bootstrap analysis (5,000 replications).

Mapping and structural analysis of the H60 genes
BLAST searches of the mouse genome sequence were conducted using H60a, H60b, or H60c cDNA sequences as queries. Chromosomal localization of H60b and H60c was deduced based on the mouse genome assembly (NCBI Build 37.1). We deduced the exon-intron structure of the three H60 genes by comparing respective cDNA and genomic sequences.

Construction of mammalian expression plasmids
The coding regions of mouse H60 molecules excluding the signal peptide were obtained by PCR using the plasmid cDNA isolated above as templates. The primer sequences that incorporated HindIII and BamHI sites for cloning purpose were 5’-CCAAGCTTGAAGACCTCAGACCTCTAAG-3’ and 5’-GGTGAATTCCTATAGTGGAAAGTTCGAGC-3’. For H60a, 5’-AGTAGCTTCTTGGTGAGGAGTGAGCA-3’ and 5’-GGAGATTGGACTGGAAGCGACG-3’ were used. For H60b, 5’-GGTGTAGAGGAAGAGGAGCA-3’ and 5’-GGAGATTGGACTGGAAGCGACG-3’ were used. For H60c, 5’-GGTGTAGAGGAAGAGGAGCA-3’ and 5’-GGAGATTGGACTGGAAGCGACG-3’ were used. For H60b, 5’-GGTGTAGAGGAAGAGGAGCA-3’ and 5’-GGAGATTGGACTGGAAGCGACG-3’ were used. For H60c, 5’-GGTGTAGAGGAAGAGGAGCA-3’ and 5’-GGAGATTGGACTGGAAGCGACG-3’ were used.

Stable RMA transfectants expressing H60 molecules were established according to the method described previously (36). These plasmids, designated H60a-pFLAG-CMV-3, H60b-pFLAG-CMV-3, and H60c-pFLAG-CMV-3, respectively, were used to establish RMA cell lines stably expressing N-terminal FLAG-tagged H60 molecules. For stable expression of H60 molecules on Ba/F3 cells, we resorted to a pBabe-puro retroviral vector system (Addgene). Briefly, the regions coding for the preprotrypsin signal sequence, FLAG, and the open reading frame of each H60 molecule were amplified by PCR using H60a-pFLAG-CMV-3, H60b-pFLAG-CMV-3, and H60c-pFLAG-CMV-3 as templates. The PCR products were then ligated to the pBabe-puro vector puro constructs described above using the FuGENE6 transfection reagent (Roche). Ba/F3 cells (1 × 10^6) were then infected with packaged virus particles for 2 h in the presence of 8 μg/ml polybrene. To select puromycin-resistant cells, infected Ba/F3 cells were cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated FBS, 3 μg/ml puromycin, and 10% WEHI-3 cell-conditioned medium for 14 days. Clones expressing high levels of H60 were selected by flow cytometry with anti-FLAG Abs.

Flow cytometry analysis
For cell surface staining, single-cell suspensions (1 × 10^6) were washed with PBS (pH 7.4) and incubated in 100 μl PBS (pH 7.4) containing 0.1% NaN3 with 1 μg rabbit anti-FLAG polyclonal Ab (1:250 dilution) for 20 min at room temperature. After washing with PBS (pH 7.4), cells were incubated in 100 μl PBS (pH 7.4) containing 0.1% NaN3 with the FITC-conjugated F(ab’)2 of goat anti-rabbit IgG (1:200 dilution). Subsequently, cells were washed with PBS (pH 7.4) and analyzed by FACSCalibur (BD Biosciences). Data were analyzed with CellQuest software (BD Biosciences). Staining with mouse NKG2D-Ig (500 ng/ml) was conducted as recommended by the manufacturer (R&D Systems).
Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment

H60 RNA transfectants were washed with ice-cold PBS (pH 7.4) and treated with 1 U/ml PI-PLC (Sigma-Aldrich) in PBS (pH 7.4) or PBS alone at 37°C under 5% CO2 for 1 h. Subsequently, cells were washed with ice-cold PBS (pH 7.4) and used for flow cytometric analysis.

Preparation of recombinant soluble H60 proteins

The N-terminal extracellular domains of H60 proteins (amino acid residues 1–186 for H60a and H60b, and amino acid residues 1–159 for H60c) were expressed in Escherichia coli strain BL21 (DE3) pLysS using the pGTM7 expression vector (38). The DNA fragments for expression were PCR amplified by using the respective H60 plasmid cDNAs as templates. The primer sequences that incorporated Ndel and HindIII sites to facilitate cloning were 5’-TTCAGCGATATGAGTGTACGACTCTTCAAG-3’ and 5’-CC AGACTTACACACTGACAGCTTGTTGAC-3’ for H60a, 5’-TTCAG CGCATATGAGTGTACGACTCTTCAAG-3’ and 5’-CCAGCTTAT CAACTGACAGCGTTGTCAG-3’ for H60b, and 5’-TTCAGCGCAT ACGTGTACGACTCTTCAAG-3’ and 5’-CCAGCTTATCCAACATTACAGAGGAT-3’ for H60c. The mature H60b molecule, made up of 180 amino acids, has two extracellular domains comprised the signal peptide (Fig. 1A). The deduced H60b polypeptide is made up of 251 amino acids, of which the N-terminal 17 residues were predicted to comprise a signal peptide (33). The extracellular domains of H60b and H60c contain six and five potential glycosylation sites, respectively. Of H60b and H60c contain six and five potential glycosylation sites, respectively.

Surface plasmon resonance (SPR)

SPR experiments were performed using Biacore 2000 (Biacore Life Sciences). Recombinant mouse NKG2D-Ig and BSA were coupled to the sensor surface CM5 sensor chip via primary amines using the standard amine coupling kit (Biacore Life Sciences). After buffer exchange to HBS-EP (10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% Surfactant P20), H60 proteins or BSA (as a control soluble protein) were injected over the immobilized NKG2D and BSA at 25°C. The binding response at each concentration of H60 proteins or at 1 μM HBS-EP (10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% Surfactant P20), H60 proteins or BSA (as a control soluble protein) were injected over the immobilized NKG2D and BSA at 25°C. The binding response at each concentration of H60 proteins or at 1 μM HBS-EP was calculated by subtracting the equilibrium response measured in the BSA flow cell from the response in the NKG2D flow cell. Dissociation constants were calculated by subtracting the equilibrium response measured in the BSA flow cell from the response in the NKG2D flow cell. Dissociation constants were calculated by subtracting the equilibrium response measured in the BSA flow cell from the response in the NKG2D flow cell.

Cytotoxicity assays

NK cells used as effectors were prepared from BALB/c splenocytes using the MACS NK cell isolation kit (Miltenyi Biotec). NK cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 100 ng/ml recombinant mouse IL-2 (R&D Systems) for 48 h at 37°C under 5% CO2 for 1 h. Subsequently, cells were washed with ice-cold PBS (pH 7.4) and used for flow cytometric analysis.

Measurement of H60 mRNA levels in mouse embryonic fibroblasts (MEF) infected with murine CMV (MCMV)

MEF cells prepared from BALB/c mice (40) were cultivated in DMEM supplemented with 10% FBS. The Smith strain of MCMV (ATCC VR-1399) was propagated on MEF. Titer of virus stocks were determined by a standard plaque assay on MEF. MEF cells were infected with MCMV at a multiplicity of infection of 1. At 24 and 48 h postinfection, MEF cells were collected and total RNA was isolated by RNeasy Plus Mini Kit (Qiagen) according to the manufacturer’s instruction. Measurement of H60 mRNA was conducted as described in the preceding section except that expression levels of H60 mRNA were normalized to GAPDH mRNA levels. The primer sequences for GAPDH were 5’-GAAGTCGTGGTGAACGA-3’ and 5’-GTTAATGGGCTCTGCTCCTC-3’. Relative mRNA expression levels of H60 in MCMV-infected vs uninfected control cells were calculated with the ΔΔCT method (41).

Results

Identification of two novel cDNA sequences resembling H60a

We searched the mouse EST database using the amino acid sequence of mouse H60a as a query. This search identified two types of EST clones assumed to encode MHC class I-like molecules similar to, but distinct from, the previously known H60. One type was represented by at least seven EST clones (accession numbers: BY755830.1, BB831986.1, BB833604.1, BB836078.1, BY377500.1, BB837506.1, and BB828914.1) and another type by one EST clone (accession number: BY714012.1). The proteins specified by the former and latter types of EST clones were named H60b and H60c, respectively. We obtained full-length cDNA sequences encoding H60b and H60c by 5’- and 3’-RACE. The cDNA sequences thus obtained were deposited in GenBank under accession numbers AB284505 (H60b) and AB284506 (H60c).

The deduced H60b polypeptide is made up of 251 amino acids, of which the N-terminal 24 residues were predicted to comprise the signal peptide (Fig. 1A). The mature H60b molecule, made up of 227 amino acids, has two extracellular domains corresponding to the α1 and α2 domains of MHC class I molecules. Residues 188–210 of H60b were predicted to constitute a transmembrane region. In contrast, the deduced H60c molecule has 197 amino acids, of which the N-terminal 17 residues were predicted to comprise the signal peptide (Fig. 1A). The mature H60c polypeptide, made up of 180 amino acids, has two extracellular domains corresponding to the α1 and α2 domains of MHC class I molecules. Unlike H60b, no transmembrane region was predicted for H60c; instead, H60c was predicted to encode a GPI-anchored protein with the big-PI Predictor program (33). The extracellular domains of H60b and H60c contain six and five potential N-linked glycosylation sites, respectively.

The overall amino acid sequence identities of the two extracellular domains of H60b and H60c to those of H60a were 86% and 75%, respectively. When the entire coding sequences including the signal peptides were subjected to comparison, the corresponding figures were 73% and 44%, respectively. Thus, H60b is more closely related to H60a than is H60c. To examine the relationship of H60b and H60c to known human and mouse NKG2D ligands, we constructed the phylogenetic tree using the amino acid sequences of...
FIGURE 1. A. Deduced amino acid sequences of H60a, H60b, and H60c. N-terminal sequences predicted to function as signal peptides (SP) are indicated by dotted lines. Amino acids are numbered from the first residue of mature proteins. The octamer peptide of H60a, which is presented by H2-K\(^\alpha\) and functions as a minor histocompatibility Ag, is boxed. Underlines indicate potential N-linked glycosylation sites. Filled triangles indicate exon-intron boundaries. A star indicates the \(\omega\)-site for GPI anchorage. The signs “…”, “-” and “…” indicate identity with the top sequence and absence of residues, respectively. TM/CYT stands for the connecting peptide/transmembrane region/cytoplasmic tail. TM is doubly underlined. GenBank accession numbers for H60a, H60b, and H60c are NM_010400, AB284505, and AB284506, respectively. B. Neighbor-joining tree of the heavy chains of human and mouse MHC class I family proteins. Numbers at the nodes represent the bootstrap confidence levels in percentages. Only bootstrap values over 90 are indicated. M_ and H_ stand for mouse and human, respectively. Database accession numbers are: HLA-A, P18462; HLA-B, HL-HUB7; HLA-C, NP_001001788.1; M_RAE1, NP_033043.1; M_RAE1L, AAK91503.1; H_RAET1E, AAP12008.1; H_RAET1G, NP_001001788.1; M_RAE1, NP_033043.1; M_RAE1L, AAK91503.1; H_RAET1E, AAP12008.1; H_RAET1G, NP_001001788.1; M_RAE1, NP_033043.1; M_RAE1L, AAK91503.1; H_RAET1E, AAP12008.1; H_RAET1G, NP_001001788.1; M_RAE1, NP_033043.1; M_RAE1L, AAK91503.1; H_RAET1E, AAP12008.1; H_RAET1G, NP_001001788.1; M_RAE1, NP_033043.1; M_RAE1L, AAK91503.1; H_RAET1E, AAP12008.1; H_RAET1G, NP_001001788.1; M_RAE1, NP_033043.1; M_RAE1L, AAK91503.1; H_RAET1E, AAP12008.1; H_RAET1G, NP_001001788.1; M_RAE1, NP_033043.1; M_RAE1L, AAK91503.1; H_RAET1E, AAP12008.1; H_RAET1G, NP_001001788.1;

We deduced the exon-intron organization of the three H60 genes by comparing the corresponding cDNA and genomic sequences. The genes coding for H60a, H60b, and H60c have a similar organization, with six exons and five introns (Fig. 2). The 5’-untranslated (UT) region is encoded by exons 1 and 2, and the signal peptide is encoded by exon 2. Exons 3 and 4 encode the \(\alpha_1\) and \(\alpha_2\) domains, respectively. In H60a and H60b, exon 5 encodes the transmembrane region and part of the cytoplasmic tail; exon 6 encodes the remaining part of the cytoplasmic tail and the 3’-UT region. In H60c, the stop codon occurs in exon 5, and exon 6 encodes only the 3’-UT region. In all cases, the nucleotide sequences surrounding the exon-intron boundaries conformed to the GT/AG rule (42).

NKG2D binds to cells expressing H60b or H60c

The overall sequence similarity of the three H60 molecules and their positions in the phylogenetic tree (Fig. 1, A and B) suggested that H60b and H60c could function as NKG2D ligands.

the extracellular domains (Fig. 1B). This analysis confirmed the close relationship of the three H60 molecules and provided convincing evidence that H60 is a multigene family made up of at least three members.

Genes coding for H60b and H60c map to mouse chromosome 10

The gene coding for H60a has been localized to band A3 of chromosome 10 by interspecific backcross mapping (27). To examine the chromosomal localization of H60b and H60c, we conducted basic local alignment search tool searches of the mouse genome assembly using the respective cDNA sequences as queries. This analysis localized H60b to band A3 and H60c to band A1 of chromosome 10 (Fig. 2). In NCBI Build 37.1, the contiguous encoding H60a (Mm5_11165558_37: accession number: NW_001030795.1) is misassigned to chromosome 5. Thus, it was not possible to determine the precise location of H60a on band 10A3.
FIGURE 2. H60b and H60c map to mouse chromosome 10. H60b and H60c are encoded by contigs Mm10_39532_37 (accession number: NT_039492.7) and Mm10_39530_37 (accession number: NT_039490.7), respectively. Because the contig coding for H60a (accession number: NW_001030795.1) is misassigned to chromosome 5 in NCBI Build 37.1, the precise location of H60a on band A3 is not known. The relative order of H60a and H60b shown here is tentative. Exons are indicated by boxes. Open boxes indicate 5′- and 3′-UTR regions; filled boxes indicate coding sequences. Domains are abbreviated as in Fig. 1. In NCBI Build 37.1, the sequence predicted to represent exon 1 of H60b and H60c occurs ~120 kb upstream of exon 2; the genomic region between these exons contains the Ulbp1 gene coding for MULT1.

To test this possibility, we established Ba/F3 cell lines stably expressing high levels of H60a, H60b, or H60c using a retroviral expression system. Ba/F3 transductants expressing H60a, H60b, or H60c (confirmed by staining with anti-FLAG Ab) were specifically stained with NKG2D-Ig (Fig. 3, A and B), whereas mock-infected Ba/F3 cells were stained only weakly with NKG2D-Ig. These results indicate that H60b and H60c are ligands for NKG2D.

H60c is attached to the plasma membrane with a GPI anchor

To examine whether H60c is a GPI-anchored protein as predicted from its sequence, RMA transfectants stably expressing H60a, H60b, or H60c were treated with PI-PLC. The cells were then stained with anti-FLAG Ab and examined by flow cytometry. In RMA cells expressing H60c, cell-surface staining was reduced by 63% after PI-PLC treatment (Fig. 3C). By contrast, similar treatment did not alter cell-surface staining in RMA cells expressing H60a or H60b, consistent with the fact that the transmembrane regions are predicted for H60a and H60b. These results indicate that, whereas H60a and H60b are integral membrane proteins, H60c is a GPI-anchored protein.

Binding of recombinant soluble H60b and H60c to NKG2D

To confirm the ability of H60b and H60c to bind to NKG2D and measure their binding affinities, the extracellular domains of three types of H60 molecules were obtained as inclusion bodies from bacteria and then solubilized by 6 M guanidine buffer. Refolded recombinant soluble H60 proteins were purified by size-exclusion chromatography.

SPR experiments were done using the Biacore 2000 instrument. Each soluble H60 protein was injected over sensor surfaces to which NKG2D or BSA had been immobilized. All types of H60 proteins bound to NKG2D but not to BSA (Fig. 4). When BSA (1 μM) was injected as a control soluble protein, no binding response to immobilized NKG2D or BSA was observed (data not shown). To determine Kd, equilibrium binding analysis was performed by injecting varying amounts of soluble H60 proteins (Fig. 4, insets). Each binding response was calculated by subtracting the equilibrium response measured in the BSA flow cell from the response in the NKG2D flow cell. Kd values derived from nonlinear curve fitting of the standard Langmuir binding isotherm were 34 ± 1.7 nM for H60a, 310 ± 7 nM for H60b, and 8.7 ± 3.1 μM for H60c. These results indicate that H60b and H60c bind to NKG2D with considerably lower affinity than does H60a.
Expression of H60b and H60c renders Ba/F3 cells susceptible to lysis by NK cells

To examine whether H60b and H60c can activate NK cells, Ba/F3 cells stably expressing H60a, H60b, or H60c were used as target cells in cytotoxicity assays (Fig. 5). Ba/F3 cells expressing H60 molecules were consistently more susceptible to lysis by NK cells than were mock-transduced or nontransduced Ba/F3 cells. When the NKG2D receptor was blocked by pretreatment of NK cells with anti-NKG2D mAb, H60b and H60c transductants and mock transductants became almost equally susceptible to lysis by NK cells (Fig. 5), confirming that recognition of H60b and H60c on target cells is mediated by NKG2D. These results indicate that H60b and H60c are functional ligands for NKG2D.

H60c transcripts show a more restricted tissue distribution than do H60a and H60b transcripts

We compared tissue distributions of H60a, H60b, and H60c transcripts in adult healthy mice by real-time RT-PCR (Fig. 6). Overall expression levels of H60a, H60b, and H60c were quite low when compared with that of the β-actin gene. Among the three H60 genes, H60a was most abundantly expressed, with relative expression levels of H60a, H60b, and H60c (averaged for all organs) being roughly 80:1:5. B6 mice expressed the H60b and H60c genes; however, in this mouse strain, H60a transcripts were not detected in any of the tissues examined as reported previously (27). By contrast, BALB/c mice expressed all three H60 genes. Basal expression levels of the H60b and H60c genes tended to be higher in B6 mice than in BALB/c mice. H60c transcripts were detected mainly in the skin, whereas H60a and H60b transcripts had a broader tissue distribution.

MCMV infection induces expression of H60b, but not H60a or H60c, in MEF

To examine whether MCMV infection can induce transcription of the newly discovered H60 genes, we infected MEF with MCMV and monitored the expression levels of H60a, H60b, and H60c mRNA by real-time PCR (Fig. 7). Low levels of H60a and H60b mRNA were detected in uninfected MEF cells. MCMV infection induced transcription of H60b, but not H60a. The failure of MCMV infection to induce transcription of H60a is consistent with the previous observations (43). H60c transcripts were not detected in any of the tissues examined as reported previously (27). By contrast, BALB/c mice expressed all three H60 genes. Basal expression levels of the H60b and H60c genes tended to be higher in B6 mice than in BALB/c mice. H60c transcripts were detected mainly in the skin, whereas H60a and H60b transcripts had a broader tissue distribution.
detectable in uninfected MEF cells, and MCMV infection did not induce transcription of H60c (data not shown).

Discussion

When B6 mice are immunized with spleen cells of MHC-identical BALB.B mice, CTLs are generated against BALB.B minor histocompatibility Ags (44). Of many such histocompatibility Ags, H60 is highly immunodominant and dominates the allelogeneic CTL response (45–47). Molecular cloning of H60 Ag by Malarkannan et al. (27) revealed that it is derived from an MHC class I-like integral membrane protein with α1 and α2 domains. Subsequently, H60 molecules (referred to as H60a in this paper) were shown to function as a ligand for the mouse NKG2D receptor (11, 23). Our present work demonstrates that mice have two additional chromosome 10-encoded NKG2D ligands that structurally resemble H60a (Figs. 1–4). These ligands not only bind to NKG2D (Figs. 3 and 4), but they also have the ability to activate NK cells (Fig. 5). These results establish H60b and H60c as functional ligands for NKG2D.

Although it is still poorly understood why H60 of BALB.B mice dominates over other minor histocompatibility Ags and elicits particularly strong CTL responses in B6 mice (46, 48), the reason why H60 functions as a minor histocompatibility Ag is well understood: the gene for H60 (H60a) is transcribed in BALB.B but not in B6 mice (27). When immunized with BALB.B splenocytes, B6 mice generate CTLs that recognize an H60-derived octamer (LTFNYRNL) presented by H2-Kb. Our work confirms the absence of H60a transcripts in tissues of B6 mice and shows that the H60b and H60c genes are expressed in B6 mice (Fig. 6). Interestingly, B6 mice tended to express H60b and H60c mRNA at higher levels than did BALB/c mice, suggesting that the newly discovered H60 genes may play a compensatory role in B6 mice.

The interactions of NKG2D and its ligands are generally of higher affinity than are those of other immunoreceptors and their ligands (8). Of all known mouse ligands, MULT1 binds to NKG2D with the highest affinity, with a \( K_d \) value of 6 nM (24). This is followed by H60a with \( K_d \) in the 20–30 nM range (49, 50). The \( K_d \) value we obtained for H60a (34 ± 1.7 nM) is in good agreement with those obtained by other investigators (49, 50). Compared with MULT1 and H60a, RAE1 ligands bind to NKG2D with lower affinity, with \( K_d \) values in the range of 300–800 nM (49, 50). The binding affinity of H60b (310 ± 7 nM) was comparable to that of RAE1 ligands (Fig. 4). In contrast, H60c bound to NKG2D with lower affinity as compared with RAE1 ligands (Fig. 4). Indeed, of all known NKG2D ligands, H60c binds to NKG2D with the lowest affinity. H60c was unique in that its basal transcription was largely restricted to the skin (Fig. 6). These observations suggest that H60c may play a more specialized role in immune surveillance.

The present study adds two new members to the current list of mouse NKG2D ligands. The existence of multiple NKG2D ligands is thought to be beneficial to the host for at least two reasons. First, individual ligands appear to be differentially regulated by different stimuli. Thus, whereas transcription of MICA and MICB is induced by heat shock (18), this is not necessarily the case with other ligands. Furthermore, signaling through TLR is known to induce transcription of RAE1, but not that of H60a or MULT1 (51). Our observation that MCMV infection induces H60b, but not H60a or H60c, mRNA in MEF (Fig. 7) provides another example of differential regulation of NKG2D ligands. Second, microbes such as MCMV encode proteins that down-regulate expression of NKG2D ligands (52, 53), thereby allowing MCMV-infected cells to escape from attack by NKG2D-expressing immune cells. Thus, the MCMV gene m152 encoding gp40 down-regulates expression of RAE1 (54). Similarly, the MCMV genes m155 and m145 specifically inhibit cell-surface expression of H60a (55, 56) and MULT1 (57), respectively. Thus, in defense against viruses, the existence of multiple NKG2D ligands is likely to be beneficial to the host, because it increases the probability that some ligands are not antagonized by virus-encoded genes. It remains to be determined whether the newly identified NKG2D ligands are targeted by viral immune evasion tactics.

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Disclosures

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References
