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Cutting Edge: Murine UL16-Binding Protein-Like Transcript 1: A Newly Described Transcript Encoding a High-Affinity Ligand for Murine NKG2D

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Murine NKG2D is known to recognize H60 and five RAE1 variants. The human homologue recognizes both inducible MHC class I chain-related gene and constitutive (UL16-binding protein (ULBP)) ligands. Widely expressed, the latter are thought to mark transformed or infected cells for destruction by NK cells in the context of down-regulated cell surface class I (i.e., the “missing self”-response). Unlike MIC and ULBP, however, mRNA for the murine ligands appears only in very limited contexts in the mature animal. In this study, we describe a NKG2D ligand termed “murine ULBP-like transcript 1 (MULT1)” whose mRNA appears to be widely expressed in adult parenchyma. This molecule possesses MHC class I-like α1 and α2 domains as well as a large cytoplasmic domain. Recombinant MULT1 binds NKG2D with relatively high affinity (KD ~ 6 nM) and low koff (~ 0.006 s⁻¹). Expression of MULT1 by normally resistant RMA cells results in their susceptibility to lysis by C57BL/6 splenocytes. The Journal of Immunology, 2002, 169: 4079–4083.

A typical cells, whether transformed (1–3) or infected (4, 5), frequently demonstrate two phenomena: down-regulation of cell surface class I and up-regulation of NKG2D ligands. In innate responses, down-regulation of target cell class I releases NK cells from tyrosine phosphatase-mediated inhibition (6) and forms the basis of the “missing self”-phenomenon (7). Ligand recognition by the lectin-like immunoreceptor NKG2D provides at least a subset of the activation signals disinhibited by missing self (8). High levels of NKG2D ligand expression may even override normal levels of class I-mediated NK inhibition (2, 9, 10). In adaptive responses, down-regulation of class I on atypical cells would be expected to blunt CTL action; however, simultaneous engagement of NKG2D ligands provides compensatory costimulation (4) and enables activated CTL-mediated killing.

Two MIC and three or more ULBP molecules constitute the known human NKG2D ligand repertoire. mRNA for subsets of these proteins are widely expressed in resting nonlymphoid tissues (2, 11, 12). The known murine NKG2D-binding repertoire encompasses H60 and five RAE1 variants. Though expressed by numerous tumor cell lines (1, 13), mRNA for these molecules in normal tissues appear to be tightly restricted to the early embryo and selected adult cell types (1, 13–15).

Despite intense interest in this system, the murine ligands for NKG2D have not yet been shown to be expressed in nonlymphoid organs except following transformation (3), unlike their constitutively expressed and/or easily inducible human orthologs. The analogy between human and murine systems being accordingly unsatisfying, we suspected the existence of NKG2D-binding proteins that had eluded detection by the expression cloning systems previously used, both of which were based on transformed cell cDNA libraries (1, 13). In this study, we report the identification of a murine transcript (murine UL16-binding protein-like transcript 1 (MULT1)) encoding a high-affinity ligand for NKG2D with greater protein sequence similarity to human UL16-binding protein 3 (ULBP3) than to murine RAE1 or H60. Like ULBP, this transcript appears to be widely expressed in nonlymphoid tissues.

Materials and Methods

Production of RNA

C57BL/6NCR (B6) tissues were perfused free of blood, then organs were harvested and flash frozen in liquid nitrogen. RNA was isolated using TRIzol (Life Technologies, Rockville, MD) as per the instructions, treated with RQ1 DNase (Promega, Madison, W1), and re purified using RNeasy silica columns (Qiagen, Chatsworth, CA). RNA quality was verified by standard formaldehyde gel electrophoresis and RT-PCR of hypoxanthine guanine phosphoribosyltransferase.

Molecular cloning of MULT1 and generation of transduced cell lines

TBLASTn search (National Center for Biotechnology Information) of the murine expressed sequence tag (EST) database (16) was performed using the extracellular sequences of ULBP1–3 with the expected value set at 100.

Abbreviations used in this paper: MULT, murine UL16-binding protein-like transcript; ULBP, UL16-binding protein; EGFP, enhanced green fluorescent protein; EST, expressed sequence tag; SAFE, streptavidin-PE; rs, recombinant soluble; SPR, surface plasmon resonance.

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This yielded a match with a The Institute of Physical and Chemical Research neonatal thymus sequence (accession no. AK020784) herein termed “MULT1.” Pfu polymerase (Stratagene, La Jolla, CA) and oligonucleotides 5′-TTTGGCATGATCACGCCATGGA/G(A)CT(G/C)ACT(G/C)CCAGT-3′ and 5′-ACCGGTCAGCTTTGATGGTGAAGTGGTACGCCGGG ATCCCCATCAATATCGTC-3′ were used to amplify the corresponding sequence from B6 day 1 neonatal thymus RNA following reverse transcription with Superscript reverse transcriptase (Life Technologies). Primers were selected to amplify only MULT1 based on all available similar sequence fragments from the EST database and the Celera murine genome resource (Celera Genomics, Rockville, MD). The resultant amplicon was ligated into the BamHI and XhoI sites of pMXIRES-enhanced green fluorescent protein (EGFP; courtesy of Dr. T. Kitamura, University of Tokyo, Tokyo, Japan) (17). This plasmid was then used to generate RMA (American Type Culture Collection, Manassas, VA) and Ba/F3 cells retrovirally transduced with MULT1 as described elsewhere (18). Sequencing of constructs was performed using BigDye 2.0 (PerkinElmer, Norwalk, CT) exactly according to the manufacturer’s instructions. Sequence data were manipulated using VectorNTI 3.0 (Informax, Bethesda, MD). Secondary structure prediction was performed using 3D-PSSM (19). Alignments were performed using CLUSTALW. Signal and GPI predictions were performed using SignalP (20), and DGPI (21) and big-PI (22), respectively.

**Production of recombinant proteins**

Biotinylated recombinant soluble (rs) NKG2D ectodomains were produced in insect cells exactly as described previously (23). The rsMULT1 ectodomain sequence from (MG)IEETAS. . . to . . . GSFST was ligated into the NcoI and XhoI sites of pET-15b (Novagen, Madison, WI); the additional N-terminal methionine and glycine were a consequence of the NcoI cloning site. Expression, purification, and refolding of rsMULT1 and rsH60 was as described elsewhere (23). Mass spectrometric analysis (Keck Mass Spectrometry Resource, Yale University, New Haven, CT) of refolded rsMULT1 showed the N-terminal methionine to be removed. NKG2D tetramers were produced by dropwise addition of streptavidin-PE (SAPE; BD PharMingen, San Diego, CA) to biotin-rsNKG2D at a 1:4 molar ratio with gentle mixing. Standard amino acid analysis showed the A280/mass relationship for rsMULT1 to be: A280(1 mg/ml) = 1.34 in HEPES-buffered saline.

**Cell staining**

RMA and Ba/F3 cells infected with pMXIRES-EGFP, pMXIRES-RAE1-EGFP, or pMXIRES-MULT1-EGFP retrovirus were processed on a MoFlo sorter (Cytomation, Fort Collins, CO) to generate pure populations of green fluorescent protein-positive cells with similar geometric mean fluorescence intensity and scatter profiles. These were then stained with appropriate reagents as detailed below. Cytotoxic effectors were stained with 4 g/ml FIGURE 1. MULT1 possesses a similarity to other class I-like proteins and is widely expressed. A, MULT1 sequence was aligned to the ULBP3 and RAE1β sequences for which structural data exists. Gray shading denotes MULT1 cysteines; disulfide bonds are predicted at C44–C60 and at C99–C163. *, Potential N-linked glycosylation sites. Thin black bars mark the leader and transmembrane sequences. Open boxes enclose residues identically or strongly conserved between MULT1 and one of the other sequences; black shading marks residues common to all three sequences. Known secondary structure elements are schematized below the alignment while the 3D-PSSM-predicted secondary structural elements of MULT1 appear above. B, Total RNA (100 ng) from several tissues was subjected to RT-PCR using primers specific for MULT1. The 1.1-kb band corresponds to the full-length MULT1 coding sequence verified by sequence analysis. No bands were obtained in the absence of reverse transcriptase.
PK136 (24) conjugated to Alexa488 (Molecular Probes, Eugene, OR) and 4 μg/ml anti-CD3-PerCP (BD PharMingen) and then subjected to flow cytometry to verify phenotype.

Surface plasmon resonance (SPR) experimentation

SPR work, data interpretation, and quality control were performed exactly as described previously (23), except that kinetic and steady-state analyses were performed with \( R_{\text{max}} \) set at \(-40\) and \(-90\) response units, respectively. Kinetics experiments were performed four times at four concentrations on two separate surfaces. Steady-state experiments were performed twice, in duplicate, over two separate surfaces. Raw data were analyzed and graphed using BIAeval 3.1 (BIAcore, Piscataway, NJ) and Kaleidagraph 3.5 (Synergy Software, Reading, PA).

Cytotoxicity assay

B6 splenocytes were positively selected for DX5 expression using the MACS (Miltenyi Biotec, Auburn, CA) system, washed twice with RPMI 1640 supplemented with 10% FCS (R10), and incubated in R10 for 10 min with or without 1 μM rsH60 or 1 μM irrelevant recombinant protein purified the same way. These effector cells were then used in standard 4-h \(^{51}\)Cr release assays with 10,000 targets/well at 37°C and in 5% CO\(_2\). Percent specific lysis was defined as (observed dpm − spontaneous dpm)/(maximal detergent released dpm − spontaneous dpm) × 100. Data points were obtained in triplicate. Experiments were performed independently twice.

Results

Molecular cloning of MULT1

We observed staining of C1498 murine lymphoma cells with tetrameric NKG2D, yet could obtain neither RAE1 nor H60 sequences from C1498 RNA by RT-PCR (data not shown). We therefore searched the mouse EST database using ULBP1–3 query sequences; genomic correlations were obtained using the Celera Discovery Tool (Celera Genomics). Among several related sequences, an EST (accession no. AK020784) encoding a deduced 334-aa protein appeared most promising and was studied in greater detail. Based on its level of identity with ULBP3 (>20%), its placement on mouse chromosome 10 in a region syntenic to human chromosome 6q25, its appearance in several EST libraries, and its predicted MHC class I-like fold (3D-PSMM (19)), the sequence was termed MULT1. Distinct, highly related exons (within Loc 237247 on supercontig NW_000022) also exist nearby on B6 mouse chromosome 10. Examination of the Celera genome resource suggested three loci (mCG54954, mCG12640, and mCG51610) may encode similar genes in the DBA/2J mouse. Thus, sequence AK020784 was renamed MULT1 to reflect possible existence of a family of related sequences.

Full-length MULT1 cDNA was obtained from B6 neonatal thymus RNA by RT-PCR. Fig. 1A shows the protein sequence and secondary structural alignment with ULBP3 and RAE1β (25, 26). It encompasses α- and α2-like domains but no α3-like domain or predicted GPI transamidation site. Uniquely among known NKG2D ligands, MULT1 possesses an extensive intracellular domain. MULT1 has four possible N-linked glycosylation sites, two disulfide bonds, and two unpaired cysteines (verified by mass spectrometric analysis of the refolded, active protein; mass obs = 21011, mass pred/Reduced = 21015; data not shown).

RNA from several naive mouse tissues was screened by RT-PCR (34 cycles with Pfu of polymerase) for the presence of full-length MULT1 mRNA (Fig. 1B). Although several bands were obtained in most tissues, sequence analysis revealed the 1.1-kb fragment to represent the full-length MULT1 coding sequence. Similar to ULBP1–3 (2), MULT1 message was detectable in a wide variety of tissues; C1498 cells also expressed MULT1 mRNA (data not shown).

NKG2D binds to cells expressing full-length MULT1

The cell lines Ba/F3 and RMA were transduced with bicistronic vectors containing cDNA for EGFP alone, RAE1δ and EGFP, or MULT1 and EGFP. Transductants were stained with tetramerized NKG2D; results for RMA (Fig. 2) and Ba/F3 (data not shown) were identical. EGFP-alone transductants displayed no reactivity (Fig. 2D), while RAE1δ/EGFP and MULT1/EGFP transductants bound NKG2D tetramers in a manner linearly related to EGFP expression. Specificity of the interaction was confirmed both by the lack of staining with free SAPE and by the ability of 1 μM (100 × \( K_{D} \)) rsH60 to block binding (Fig. 2, H and J). Irrelevant recombinant protein purified identically from bacteria failed to block staining at the same concentration (data not shown). Thus, full-length MULT1, when expressed in cells, confers specific cell surface binding to NKG2D.

rsMULT1 binds the NKG2D ectodomain with high affinity

Ligands for murine NKG2D demonstrate two binding regimes—a lower affinity interaction demonstrated by RAE1α-δ \((K_{D} \approx 300–800 \text{nM})\) and a higher affinity interaction demonstrated by RAE1ε and H60 \((K_{D} \approx 10–30 \text{nM})\) (23, 27). To assess the properties of the MULT1-NKG2D interaction, the ectodomain of MULT1 was refolded from bacterial inclusion bodies. Integrity of the refolded protein was verified both by demonstration of a symmetric peak eluting at the volume expected for a 21-kDa monomer on gel filtration and by the ability of excess rsNKG2D to completely shift the migration of rsMULT1 in native polyacrylamide gels (data not shown).

The refolded rsMULT1 protein was applied to SPR experiments over a flowcell decorated with biotin-NKG2D. These studies revealed a \( K_{D} \) of 6 nM \((K_{D} \text{calc} = 2 \text{nM})\) and a \( K_{D} \) of \(-0.006 \text{s}^{-1}\), several times lower than that of H60 (Fig. 3). Thus, MULT1 binds NKG2D with the highest affinity of all known ligands and displays a \( t_{1/2} \) of \(-2\text{ min}, \text{ longer than either H60} (\sim 20 \text{ s}) \text{ or RAE-1α-δ} \sim (5 \text{ s})$.

Expression of MULT1 renders RMA cells susceptible to lysis by syngeneic fresh splenocytes

Neither fresh B6 splenocytes nor IL-2-activated killer cells can lyse RMA lymphoma cells due to syngeneic class I expression by RMA. To sensitize RMA cells to lysis by the CD94/NKG2D receptor, we introduced the MULT1 transgene into RMA cells by lentiviral transduction. The RMA-MULT1 cell line was selected by puromycin resistance and was shown to be more sensitive to lysis by fresh splenocytes than the RMA cell line. To confirm the role of the MULT1 transgene, a noncoding control virus (EcoGFP) was introduced into RMA cells to generate a parallel RMA-EcoGFP transductant. Both RMA-MULT1 and RMA-EcoGFP lines were then tested for their susceptibility to lysis by fresh splenocytes and to the NKG2D ligand RAE1δ.

Expression of MULT1 renders RMA cells susceptible to lysis by syngeneic fresh splenocytes

Neither fresh B6 splenocytes nor IL-2-activated killer cells can lyse RMA lymphoma cells due to syngeneic class I expression by RMA.
NKG2D) Langmuir model, incorporating the displayed K

The NKG2D signal (A) and representative net responses derived by subtraction of the control signal from biotin-antiCD19 (negative control) and biotin-rsNKG2D. Shown are representative independent experiments. Coefficients of variation of triplicate data points were 10% or less.

FIGURE 4. Overexpression of MULT1 sensitizes RMA lymphoma cells to lysis by fresh B6 splenocytes. Fresh splenocytes enriched for NK cells (70% NK1.1+CD3−, 5% NK1.1+CD3+) were applied as effectors to a 4-h 51Cr release assay at E:T ratios of 0.1, 1.1, 8:1, and 20:1 against RMA cells transduced with the following: EGFP alone ( ), RAE-1δ and EGFP ( ), and MULT1 and EGFP ( ). Open symbols represent, respectively, lysis of the same transductants by effectors pretreated for 15 min with 1 μM rsH60. This figure shows averaged triplicate data points from a representative independent experiment. Coefficients of variation of triplicate data points were <10%.

Discussion

MULT1 is a novel binding partner for mouse NKG2D, binding with the highest affinity yet described for an NKG2D ligand. Based on sequence conservation, it is most closely related to members of the human ULBP family. Akin to ULBP, MULT1 mRNA appears to be constitutively present in several adult tissues although demonstration of cell surface expression awaits specific serologic reagents. Thus, three distinct MHC class I-like molecules in the mouse, H60, RAE-1δ, and MULT1, bind NKG2D with high affinity despite low mutual sequence identity (<20%).

Evolutionary factors selecting for such a complicated receptor-ligand system are likely 2-fold. First, the functional consequences of NKG2D engagement are pleiotropic, involving T cell costimulation, NK cell activation, macrophage stimulation, and possibly regulation of fetal development (1, 4, 13, 14). Precise recruitment of these diverse functions to particular contexts may require multiple genes with distinct promoter/enhancer sequences, posttranslational controls, and even kinetics of binding. Second, microbes exert enormous selective pressure to diversify immune-related functions, albeit at differing rates (28, 29). Recent evidence suggests that human CMV interferes with the NKG2D system using the UL16 gene product to bind ULBP1 and ULBP2 (2); also, mouse CMV gp40 may down-regulate H60 (30). Pathogen-encoded factors such as these might have selected for NKG2D-binding partners which retain receptor specificity but lack susceptibility to interference or subversion (e.g., ULBP3, which does not bind to UL16), resulting in the current repertoire of dissimilar NKG2D ligands. However, a true understanding of this system awaits description of all of the NKG2D ligands, the mechanisms controlling their cell surface expression, and the functional consequences of the recognition event.

References


the latter (7). High-level expression of NKG2D ligands, however, appears to overcome class I-mediated inhibition of lymphokine-activated NK cells and NK cell lines, resulting in target lysis (2, 9, 10). We examined the ability of MULT1 overexpression to similarly render cells susceptible to lysis by fresh, unstimulated B6 splenocytes. RMA cells transduced with EGFP alone, RAE-1δ, and EGFP, or MULT1 and EGFP were used as targets for syngeneic fresh splenocytes in 4-h 51Cr release assays. As expected, EGFP-only transductants were not significantly lysed. MULT1-only transductants were lysed similarly to RAE-1δ transductants (Fig. 4). Killing of both transductants was greatly inhibited by excess rsH60 (open symbols) but not irrelevant recombinant protein (data not shown), verifying the role of NKG2D in mediating this effect. Similar results were obtained with IL-2-activated killer cells against Ba/F3 transductants (data not shown). These data demonstrate the ability of MULT1 to recruit NK-mediated killing via NKG2D.


