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Interactions of human NKG2D with its ligands MICA, MICB, and homologs of the mouse RAE-1 protein family

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Abstract NKG2D is an activating receptor that is expressed on most natural killer (NK) cells, CD8 $\alpha\beta$ T cells, and $\gamma\delta$ T cells. Among its ligands is the distant major histocompatibility complex class I homolog MICA, which has no function in antigen presentation but is induced by cellular stress. To extend previous functional evidence, the NKG2D-MICA interaction was studied in isolation. NKG2D homodimers formed stable complexes with monomeric MICA in solution, demonstrating that no other components were required to facilitate this interaction. MICA glycosylation was not essential but enhanced complex formation. Soluble NKG2D also bound to cell surface MICB, which has structural and functional properties similar to those of MICA. Moreover, NKG2D stably interacted with surface molecules encoded by three newly identified cDNA sequences (*N2DL-1*, -2, and -3), which are identical to the human ULBP proteins and may represent homologs of the mouse retinoic acid-early inducible family of NKG2D ligands. Because of the substantial sequence divergence among these molecules, these results indicated promiscuous modes of receptor binding. Comparison of allelic variants of MICA revealed large differences in NKG2D binding that were associated with a single amino acid

substitution at position 129 in the $\alpha 2$ domain. Varying affinities of MICA alleles for NKG2D may affect thresholds of NK-cell triggering and T-cell modulation.

Keywords NKG2D · Ligand · N2DL · MIC · Polymorphism

Introduction

Natural killer (NK) cell activity is regulated by signals from numerous activating and inhibitory receptors (Lanier 2001; Long 1999; Moretta et al. 2000). Some of these are also expressed on T cells and may modulate their antigen-specific responses (Mingari et al. 1998; Noppen et al. 1998). Among these receptors are inhibitory and activating isoforms of the killer cell Ig-like receptors (KIRs), which bind to HLA-A, -B or -C, and the C-type lectin-like inhibitory CD94-NKG2A and activating CD94-NKG2C heterodimers, which interact with HLA-E (Braud et al. 1998; Long et al. 1997). Inhibitory receptors contain immunoreceptor tyrosine-based inhibition motifs and recruit tyrosine phosphatases (Long 1999). Activating receptors associate with an adaptor molecule, DAP12, which contains an immunoreceptor tyrosine-based activation motif and recruits protein kinases (Lanier 2001; Lanier et al. 1998). Whereas the immunobiology of inhibitory receptors is well characterized, much less is known about activating receptors and their ligands. Among these is NKG2D, which shares little similarity with other NKG2 proteins and is not associated with CD94. It pairs with an adaptor molecule, DAP10, which can signal by recruitment of phosphatidylinositol 3-kinase (Wu et al. 1999). NKG2D is expressed on most NK cells, CD8 $\alpha\beta$ T cells, and $\gamma\delta$ T cells and thus is the most widely expressed “NK cell receptor” known (Bauer et al. 1999). Among its ligands is the major histocompatibility complex (MHC) class I-relat-

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ed, stress-inducible surface glycoprotein MICA (Bauer et al. 1999), which has a limited tissue distribution in gastrointestinal epithelium and diverse epithelial tumors and is induced by cytomegalovirus (CMV) infection (Groh et al. 1996, 1999, 2001). Engagement of NKG2D by MICA triggers NK cells and co-stimulates some $\gamma\delta$ T cells and antigen-specific CD8 $\alpha\beta$ T cells (Bauer et al. 1999; Groh et al. 1998, 2001).

The structure of MICA is similar to the protein fold of MHC class I, with an $\alpha 1\alpha 2$ platform domain and a membrane-proximal immunoglobulin-like $\alpha 3$ domain (Li et al. 1999). Unlike conventional class I molecules, however, MICA is not associated with β_2 -microglobulin (β_2m) and peptides (Groh et al. 1996; Li et al. 1999). As with MICA, functional observations imply that a closely related molecule, MICB, may also function as a ligand for NKG2D (Bahram and Spies 1996; Groh et al. 1998). Both MICA and MICB are polymorphic (Bahram et al. 1994; Fodil et al. 1996; Pellet et al. 1997; Visser et al. 1999); however, whether this is functionally significant is unknown.

Sequences directly related to *MIC* are conserved in the genomes of most mammals with the probable exception of rodents (Bahram et al. 1994; Steinle et al. 1998). In the mouse, which lacks the *MHC* interval that encodes *MIC* genes in other species, a heterologous family of proteins, the retinoic acid-early (*RAE-1*) molecules, function as ligands for NKG2D (Cerwenka et al. 2000; Diefenbach et al. 2000; Zou et al. 1996). These molecules share similarities with the recently described human CMV UL16-binding proteins (ULBPs), which also interact with NKG2D (Cosman et al. 2001). All of these molecules consist of an MHC class I $\alpha 1\alpha 2$ platform-like domain that is attached to the cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor; however, whether they correspond genetically is not clear.

To investigate the interactions of NKG2D with its ligands, we used recombinant soluble NKG2D to demonstrate that it forms homodimers that complex with monomeric MICA in solution. Moreover, NKG2D specifically bound to MICB and to three expressed human homologues of mouse *RAE-1* that were independently identified in this study. Comparison of different MICA alleles revealed that a single biallelic amino acid position is associated with weak or strong binding to NKG2D, which may affect thresholds of NK-cell triggering and T-cell modulation.

Materials and methods

Cell lines and antibodies

All human tumor cell lines were from the American Type Culture Collection (ATCC). The insect Sf9 and 'High Five' cell lines were from Invitrogen. The MICA-specific monoclonal antibodies (mAbs) 2C10, 6D4, and 6G6 have been described (Groh et al. 1996, 1999). W6/32 is an anti-pan-HLA class I mAb (ATCC). The anti-FLAG-tag mouse mAb M2 and biotinylated mAb Bio-M2 were from Sigma.

MICA and N2DL clones, cDNA constructs and transfectants

The *MICA**04, *07, and *08 cDNAs were generated by RT-PCR using mRNA from cell lines and cosmid transfectants expressing the corresponding genes. *MICA**04/*07 hybrid cDNAs were generated by exchange of cDNA restriction fragments *Sal*-*Eco*O109I (encoding amino acids 1–75; hybrid H1), *Eco*O109I-*Eco*RV (amino acids 76–170; hybrid H2), and *Eco*RV-*Bam*HI (amino acids 171–360; hybrid H3). A BLAST search of the human expressed sequence tag (EST) database (GenBank) with the mouse *RAE-1 α* sequence (Zou et al. 1996) led to the identification of three ESTs [I.M.A.G.E. Consortium (Lennon et al. 1996) clones 2406442, 2911855, and 1566539; GenBank accession numbers A1830832, AW510737, and A1091180, respectively; note that these sequences represent only partial sequence runs of the EST clones, excluding portions of the 3' ends]. These sequences were presumptive human *RAE-1* homologs and were termed *N2DL-1*, -2, and -3, respectively. EST clones 2406442 (*N2DL-1*) and 1566539 (*N2DL-3*) were purchased from the ATCC and fully sequenced using T7 and internal sequence primers (GenBank accession numbers AF346595 and AF346596, respectively). The *N2DL-2* sequence (GenBank accession number AF321606) was obtained by RT-PCR from HeLa cell mRNA using primers derived from the corresponding gene identified by alignment with the EST in the human high-throughput genomic sequence (HTGS) database (GenBank accession number AL355497), which also includes the sequences for the *N2DL-1* and *N2DL-3* genes. As determined by alignments with the genomic sequences, the open reading frames of all *N2DL* EST/cDNA sequences, which are complete at their 3' ends, lack 24–30 nucleotides encoding portions of the signal peptide sequences and the ATG start codon at their 5' ends. Thus, for transfection and expression, we amplified the *N2DL* sequences corresponding to exons 2–4 ($\alpha 1$, $\alpha 2$, and the serine-threonine-proline-rich domain) using the primers 5'-ACTCCGCGGACA CACTGTCTTTGCTATGAC-3' and 5'-ACTCTCGAGTC ATCTGCCAGCTAGAATGAAGC-3' (*N2DL-1*); 5'-AGT CCGCGGACCCTCACTCTCTTTGCTATGA-3' and 5'-ACT CTCCGAGTCAGATGCCAGGAGGATGAAGC-3' (*N2DL-2*); and 5'-ACTCCGCGGACGCTCACTCTCTCTGGT-3' and 5'-A CTCTCGAGTCAGATGCCAGGAGGATGA-3' (*N2DL-3*) from the EST/cDNA clones and fused these to the signal peptide sequence from a *MICA* cDNA, employing a 3-terminal *Sac*II restriction site, which was incorporated into the *N2DL* exon 2 primers (underlined). The *N2DL* exon 4 primers included a *Xho*I restriction site (italicized) following the authentic stop codon as a 3'-end cloning site. *MICA* and *N2DL* cDNAs in the RSV.5neo plasmid vector were stably transfected into the human C1R B-cell line as described elsewhere (Groh et al. 1998). Transfectants obtained after G418 selection were sorted using a Vantage cytometer (Becton Dickinson), if necessary. C1R transfectants expressing *MICA**01, *MICB**01, or *MICA*/D^b or C1R-*MICA*/K^b hybrids have been described (Groh et al. 1996, 1998).

Production of soluble human NKG2D and MICA*01 in insect cells

Recombinant human NKG2D lacking the amino-terminal cytoplasmic region and transmembrane domain was produced in insect cells using the BAC-to-BAC system (GIBCO-BRL). The expression constructs included amino-terminal heptahistidine (construct H7) or FLAG/hexahistidine (construct FH6) tags. For construct H7, the *MICA* signal peptide was used to direct NKG2D secretion. *NKG2D* (amino acids Asn80 through Val216) was amplified with oligonucleotides 5'-ACCCGCGG GAGCATCATCACCATCACCATCACAACCTATTATTCAA CCAAGAAGTTC-3' and 5'-GCTCTAGATCATTACACAGT CCTTTGCATGCAG-3' and co-ligated with the signal sequence of *MICA* into FASTBAC1. For construct FH6, a modified pFASTBAC1 vector was used. *NKG2D* was amplified with oli-

gonucleotides 5'-CATCCGCGGAGGGATCCGGAACATCAT TATTCAACCAAGGAG-3' and 5'-GATGAGCTCATTACAC AGTCCCTTTCATGCAG-3' and cloned into pBAC-3 (Novagen). Subsequently, the FLAG-tag was inserted into the *NcoI* site of pBAC-3-NKG2D using the oligonucleotides 5'-CATG GTCGACTACAAGGACGACGATGACAAGGG-3' and 5'-C ATGCCCTTGTTCATCGTCCTTGTAGTCGAC-3'. Finally, a *BglII-SacI* fragment containing the baculovirus gp64 promoter and signal sequence followed by the FLAG-tag, the hexahistidine-tag, and the *NKG2D* sequence of the pBAC-3-NKG2D construct was excised and ligated with linearized pFASTBAC1 replacing the 198-bp *SnaBI-SacI* polyhedrin promoter fragment of pFASTBAC1. Sequenced constructs were used to generate bacmids by transformation of DH10Bac host cells (GIBCO-BRL). Recombinant baculovirus was obtained by transfecting Sf9 cells with the bacmids and used for protein production in 'High Five' cells after several rounds of amplification as previously described for the production of soluble MICA*01 (Bauer et al. 1998). Soluble NKG2D was purified from culture supernatant by affinity chromatography on Ni²⁺-charged chelating sepharose (Pharmacia) and size-exclusion chromatography (SEC) (Bauer et al. 1998).

*Production of soluble MICA*01, MICA*04, and NKG2D in Escherichia coli*

The cDNAs of *MICA*01* and *MICA*04* encoding the extracellular $\alpha 1$ - $\alpha 3$ domains (amino acids Glu1 through Lys276) were amplified with oligonucleotides 5'-ACATGCATATGGAGC CCCACAGTCTTCG-3' and 5'-CATGGATCCCTACTAATG GTGGTGATGATG GTGTTTCCAGAGGGCACAGGGT G-3' and ligated into pET20b (Novagen). The cDNA fragment encoding the extracellular portion of NKG2D (Asn80 through Val216) was amplified with oligonucleotides 5'-TATACATAT GAACTCATTATTC AACCAAGAAG-3' and 5'-TATACCA TGGCTATCACACAGTCCCTTTCATG CAG-3' and cloned into pET22b (Novagen). Transformed *E. coli* cultures were induced for protein production with isopropyl thiogalactose for 2 h at 37 °C, harvested, and sonicated. Inclusion bodies were dissolved in 0.1 M Tris pH 8.0, 50 mM glycine and 8 M urea. Solubilized proteins were incubated with 5 mM reduced glutathione and 0.5 mM oxidized glutathione for 24 h at 5 °C and successively dialyzed against decreasing concentrations of urea (4 M, 2 M, 1 M, no urea) in 0.1 M Tris pH 8.0, 0.4 M L-arginine, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride. Refolded proteins were dialyzed against PNEA (50 mM PIPES pH 7.0, 0.15 M NaCl, 1 mM EDTA, 0.02% NaN₃) and purified by affinity chromatography on Ni²⁺-charged chelating sepharose and SEC. Protein preparations were examined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Size exclusion chromatography

Concentrated recombinant proteins were analyzed on Superdex 75 10/50 SEC columns on an FPLC system (LKB-Pharmacia). Columns and proteins were equilibrated with a buffer containing 25 mM sodium phosphate, 150 mM NaCl, 0.02% (w/v) NaN₃, pH 7.0. Approximately 50 μ g of each protein was loaded individually or mixed with a potential binding partner. A series of experiments was performed in which either one or the other component was added in increasing molar excess to confirm binding stoichiometries and test for potentially weak, secondary interactions. Columns were calibrated with a set of protein standards (BioRad). Binding is demonstrated by the appearance of a complex peak of higher molecular weight than the larger of the individual components (MICA) and by the reduction of the peak of the lower-molecular-weight component (NKG2D). HLA-E (a gift of M. Morris and D. Geraghty, Fred Hutchinson Cancer Research Center) was used as a negative control for

NKG2D binding. $\beta 2m$ was used as a negative control for MICA binding.

Flow cytometry

Cell surface expression of MICA and MICB was assayed with mAbs 2C10, 6D4, and 6G6 by indirect immunofluorescence using a FACScan flow cytometer (Becton Dickinson). For the NKG2D-binding assays, mAb M2 (final concentration 5 μ g/ml) was mixed with sNKG2D (construct FH6; final concentration 3 μ g/ml) prior to addition to cells. After incubation, cells were washed and stained with phycoerythrin (PE)-labeled goat anti-mouse Ig. For binding-inhibition assays, C1R-MICA*01 cells were stained first with MICA mAbs followed by fluoresceine isothiocyanate-labeled secondary reagents. Cells were then incubated with a mixture of mAb Bio-M2 and sNKG2D (construct FH6) and finally stained with PE-conjugated streptavidin. To evaluate the specificity of sNKG2D binding, sMICA*01, the NKG2D-specific mAb 1D11, or IgG1 control antibodies were added to sNKG2D-mAb M2 mixtures in separate experiments.

Results

Soluble NKG2D binds to cell surface MICA

To investigate its interaction with MICA, a soluble form of NKG2D (sNKG2D), which is a type II glycoprotein, was produced in insect cells. The baculovirus vector construct included an amino-terminal FLAG antibody-binding site followed by a hexahistidine-tag and the ectodomain of NKG2D (Asn80 through Val216; construct FH6; see Materials and methods). The recombinant protein was purified from supernatant of virus-infected insect cells using immobilized-metal chelate chromatography and analyzed by SDS-PAGE and immunoblot (data not shown). To assess binding to cell surface MICA, sNKG2D was preincubated with the anti-FLAG-tag mAb M2 and added to C1R-MICA transfectants or untransfected C1R cells. Staining with fluorochrome-labeled secondary antibodies and flow cytometry showed strong and specific staining of C1R-MICA cells with intensities that were similar to those seen with anti-MICA mAb, thus indicating a stable interaction between sNKG2D and MICA (Fig. 1A,B). Preincubation of sNKG2D with soluble MICA (sMICA), likewise produced in insect cells, abrogated binding as did preincubation with an anti-NKG2D mAb (mAb 1D11) (Fig. 1C; data not shown). Thus, these results show that sNKG2D binds to MICA in a specific and, apparently, high-affinity interaction.

With MHC class I molecules, T-cell antigen receptors (TCR) and KIRs bind to the top surface of the $\alpha 1\alpha 2$ platform domain where two α helices, overlying an eight-stranded β sheet, define the peptide-binding groove. The underside of the platform domain and one face of the $\alpha 3$ domain, which interacts with the CD8 co-receptor, are occluded by the association with $\beta 2m$ (Bjorkman and Parham 1990; Bjorkman et al. 1987; Boyington et al. 2000; Garboczi et al. 1996). In MICA, however, these surfaces are potentially avail-

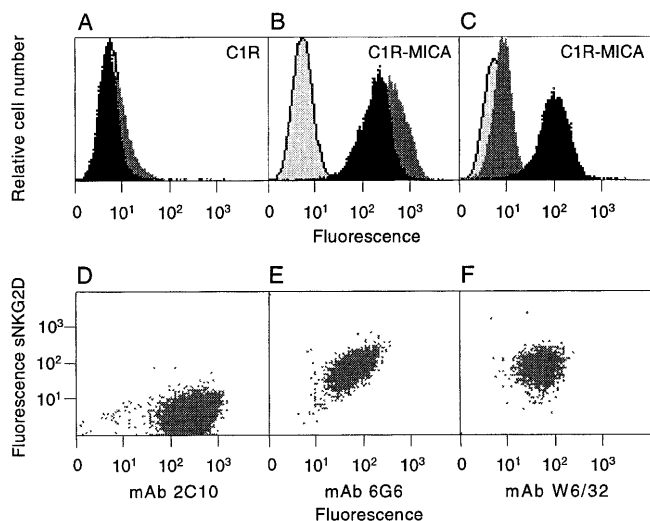


Fig. 1A–F Binding of sNKG2D to cell surface MICA. **A–C** Light-gray profiles show stainings with mAb M2 alone. **A,B** C1R-MICA*01 but not untransfected C1R cells were stained by sNKG2D/mAb M2 (black profiles) (see Materials and methods) and anti-MICA (mAb 2C10; dark-gray profiles) as shown by indirect immunofluorescence and flow cytometry. **C** Addition of sMICA (dark-gray profile) but not of bovine serum albumine (black profile) inhibited binding of sNKG2D. **D–F** By two-color FACS analysis, binding of sNKG2D was inhibited by a mAb specific for MICA $\alpha 1\alpha 2$ (mAb 2C10) but not by an anti- $\alpha 3$ mAb (6G6) or anti-HLA class I mAb (W6/32)

able for receptor interactions due to the lack of association with β_2m and considerable interdomain flexibility (Li et al. 1999). Defined anti-MICA mAbs were used to identify the MICA domain involved in NKG2D binding. mAb 2C10, which is specific for the $\alpha 1\alpha 2$ domain, blocked binding of sNKG2D, but the $\alpha 3$ domain-specific mAb 6G6 produced no inhibitory effect (Fig. 1D–F). Moreover, stainings of transfectants expressing hybrid molecules of MICA $\alpha 1\alpha 2$ and $\alpha 3$ in the context of mouse D^b and K^b, respectively (Groh et al. 1996), showed that sNKG2D binding was confined to the $\alpha 1\alpha 2$ domain (data not shown).

NKG2D binds MICA in solution

To study the NKG2D-MICA interaction in isolation, sNKG2D and sMICA were expressed as secreted proteins in insect cells or refolded from bacterially expressed inclusion bodies and analyzed by SEC. sNKG2D eluted at a volume consistent with the molecular mass of sNKG2D homodimers (M_r 35–40,000, depending on glycosylation state) (Fig. 2A,B). Soluble MICA eluted as a monomer. Mixtures of different molar ratios of sNKG2D and sMICA analyzed by SEC revealed maximal binding at a 2:1 ratio, consistent with the formation of complexes between sNKG2D homodimers and sMICA monomers (Fig. 2A, B). Bacterially produced MICA formed less

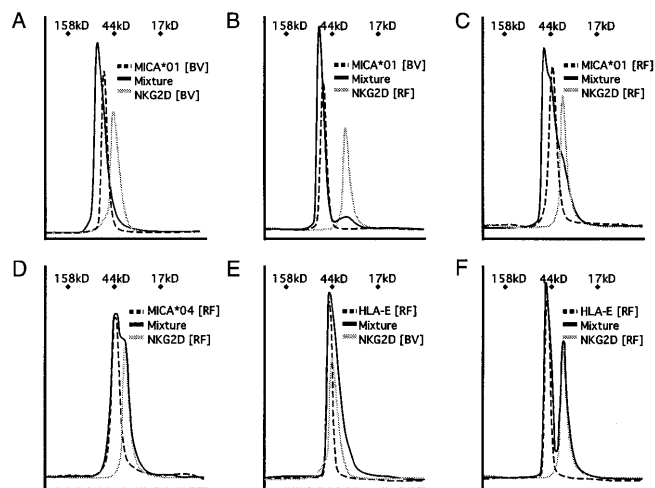


Fig. 2A–F NKG2D and MICA*01 form tight complexes in solution. **A–C** SEC analyses of individual components and mixtures of sNKG2D and sMICA*01 purified from recombinant baculovirus-infected insect cell culture supernatant [BV] (glycosylated) or refolded from bacterial inclusion bodies [RF] (unglycosylated). The elution volumes, calibrated with molecular-weight standards, of sNKG2D and sMICA were consistent with homodimers and monomers, respectively. Mixtures of NKG2D homodimers and MICA formed complexes that eluted at a volume consistent with a 1:1 binding stoichiometry (see Materials and methods). Complexes of NKG2D [RF] and MICA*01 [RF] were less stable than those formed with MICA*01 [BV]. **D** Soluble MICA*04 [RF] formed no complexes with sNKG2D [RF]. **E,F** Negative control experiments were done using HLA-E

stable complexes than MICA derived from insect cells, suggesting that glycosylation enhanced the interaction with NKG2D (Fig. 2C). In control experiments, sNKG2D formed no complex with soluble HLA-E- β_2m , the ligand for the NKG2A-CD94 receptor (Brooks et al. 1999; Lee et al. 1998) (Fig. 2E, F), and β_2m formed no complex with sMICA (data not shown). These results demonstrate that NKG2D can homodimerize in the absence of sequences outside of its ectodomain and can form stable complexes with MICA in solution, with no requirement for additional components.

NKG2D binds to MICB and to human homologues of the mouse RAE-1 family of NKG2D ligands

MICB is closely related to MICA, sharing 84% sequence identity in the extracellular domains (Bahram and Spies 1996). Similar to MICA, MICB contributes to activation of certain $\gamma\delta$ T cells, suggesting that it may also serve as a ligand for NKG2D (Bauer et al. 1999; Groh et al. 1998). As with C1R-MICA cells, C1R-MICB transfectants stained intensely with sNKG2D, which was inhibited by the addition of sMICA (Fig. 3; data not shown). Thus, both MIC molecules are physical ligands for NKG2D.

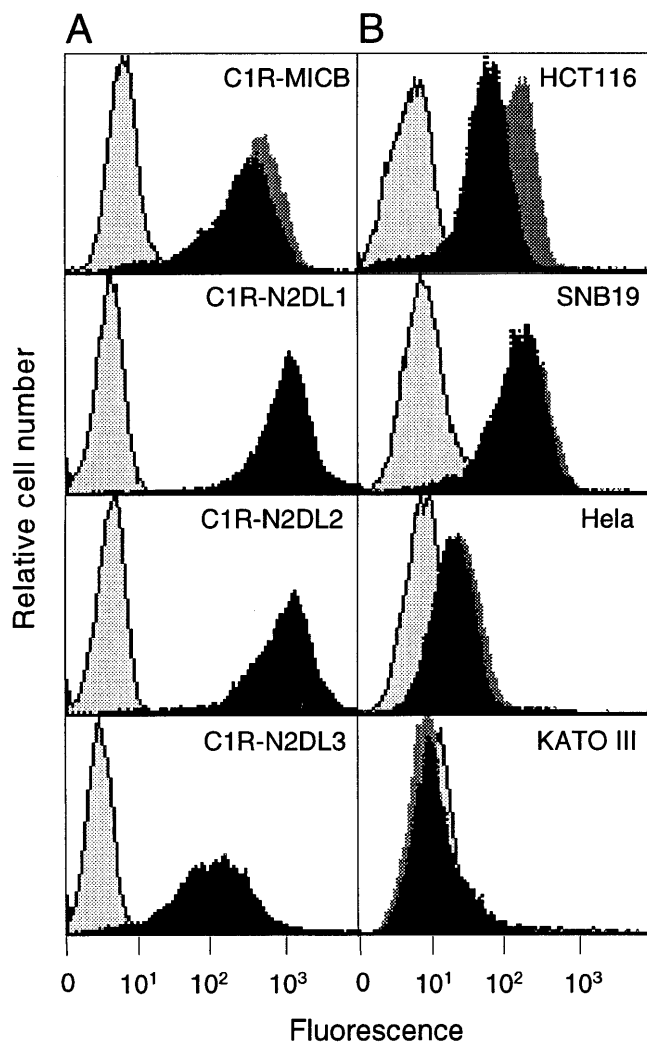


Fig. 3A,B sNKG2D binding to cell surface MICB and N2DL-1, -2, and -3 on transfected C1R cells and to various human cell lines in correlation with their expression of MIC. **A** Stainings and flow cytometry of C1R transfectants with sNKG2D/mAb M2 (black profiles) or mAb M2 alone (light-gray profiles). The MICB transfectants were also stained with mAb 6G6 (anti-MICA/B; dark-gray profile). **B** Stainings with the same reagents of the HCT116 (colon carcinoma), SNB19 (glioblastoma), HeLa (cervix carcinoma), and KATO III (gastric carcinoma) cell lines. The ranges of staining intensity were representative for many other cell lines of which none showed sNKG2D binding in the absence of at least similar and mostly higher staining for MIC

The existence of additional human NKG2D ligands was suggested by the recent isolation of a mouse family of NKG2D ligands, the RAE-1 proteins (Cervenka et al. 2000; Diefenbach et al. 2000). Screening the human EST and HTGS databases, we identified three related sequences encoding polypeptides of 215–218 amino acids which share about 23% sequence identity with the mouse *RAE-1* sequences (see Materials and methods). Provisionally, we refer to these molecules as NKG2D ligands (N2DL)-1, -2, and -3. The isolated EST/cDNAs lack portions of their leader peptide sequences, which were identifiable in genomic

DNA just downstream of respective ATG start codons (see Materials and methods). As with mouse RAE-1, all N2DL polypeptides comprise an isolated MHC class I-like $\alpha 1\alpha 2$ platform domain, lack an $\alpha 3$ -like domain, and have potential GPI anchor membrane attachment sites. Their $\alpha 1\alpha 2$ domains share 50–60% amino acid sequence identity and are equidistant from those of MHC class I and MIC with about 25% sequence identity (Fig. 4). The N2DL-1, -2, and -3 sequences are identical to those of the GPI-anchored ULBP1, ULBP2, and ULBP3 molecules, respectively, published after submission of our study (Cosman et al. 2001). ULBPs were identified by virtue of interactions of ULBP1 and ULBP2 with the CMV UL16 protein. They can activate NK cells in a manner similar to MICA and MICB, via engagement of NKG2D (Cosman et al. 2001). To investigate interactions between NKG2D and the N2DL molecules, we expressed *N2DL-1*, -2, and -3 constructs fused to a MICA leader peptide in C1R cells (see Materials and methods). The stable transfectants obtained after drug selection bound sNKG2D strongly as shown by flow cytometry after staining with mAb M2 and secondary antibody reagent (Fig. 3). These results show that N2DL-1, -2, and -3 are physical ligands for human NKG2D.

Binding of NKG2D correlates with MIC expression on cell lines

The specificity and high affinity of sNKG2D binding enabled us to scan a panel of cell lines for the presence of NKG2D ligands other than MICA and MICB, which are mainly expressed on epithelial cell lines (Groh et al. 1996). There was no knowledge of whether N2DL (or ULBP) are surface expressed at readily detectable levels and/or whether their expression is limited to certain cell types. As expected, application of sNKG2D and flow cytometry showed significant staining of fibroblast and epithelial cell lines (MRC-5, DLD-1, SW480, Lovo, HCT116, HT-29, Caco-2, AGS, SNB19, U-373, HeLa) that express variable amounts of MIC (Fig. 3; data not shown). However, no staining was observed with any of a number of other cell lines, including T-cell lines (Peer, HPB-ALL), B-cell lines (C1R, Raji, JY, Daudi), a gastric carcinoma (KATO III), a cervix carcinoma (C4I), and a mammary carcinoma (BT474), which are all negative for cell surface MIC (Fig. 3; data not shown). Moreover, there was no cell population among peripheral blood lymphocytes (PBLs) that detectably bound sNKG2D. Thus, with the caveat that this screening included a limited number of cell lines, these results suggested that there may be no ligands other than MIC that are expressed at relatively high surface levels on major populations of cells. These results also suggest that expression of N2DL (or ULBP) may be similar to MIC or, alternatively, highly cell type restricted or developmentally regulated, and/or at lev-

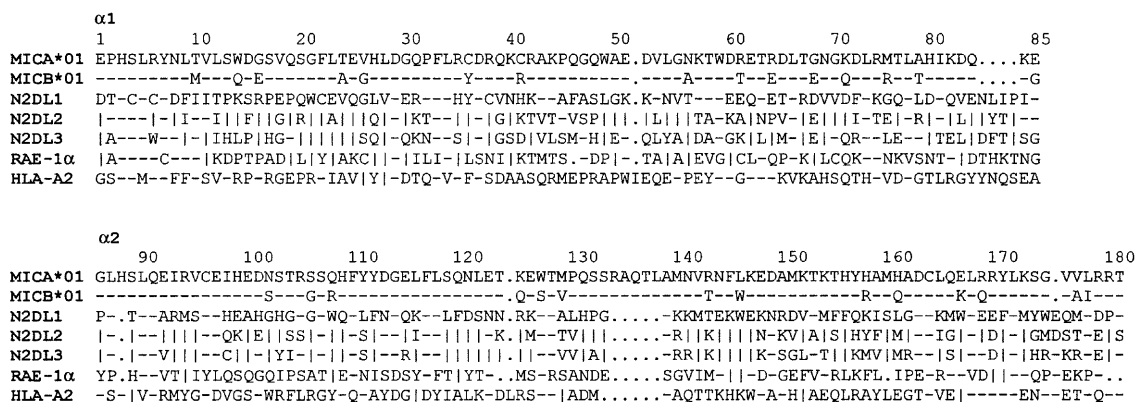


Fig. 4 Diversity of NKG2D ligands. Alignment of the $\alpha 1\alpha 2$ domain amino acid sequences of MICA*01, MICB*01, N2DL-1, -2, and -3, mouse RAE-1 α , and HLA-A2. Dashes indicate residues that are identical with the top MICA*01 sequence. Dots indicate gaps or unaligned amino acid positions. Vertical lines identify identical positions shared by the bottom four sequences with N2DL-1

els insufficient for unambiguous detection with the sNKG2D-binding reagent. Future studies with specific antibody reagents will be necessary to conclusively address these issues.

Allelic variants of MICA differ in their affinity for NKG2D

Several studies have investigated associations between MICA polymorphism and susceptibility to MHC-associated diseases (Ghaderi et al. 1999; Goto et al. 1998; Kawabata et al. 2000; Mizuki et al. 1997; Ricci-Vitani et al. 2000). However, a functional correlate for this polymorphism has not yet been found. We analyzed binding of sNKG2D to five MICA alleles expressed on transfected C1R cells. These included MICA*01, *04, *07, *08, and *16, which are among the most frequent and disparate variants (Fodil et al. 1996; Visser et al. 1999). By staining with anti-MICA mAbs, all transfectants expressed similarly high levels of surface MICA, including MICA*08, which has a transmembrane region truncation. By contrast, sNKG2D binding varied substantially, with MICA*01 and *07 representing strong binding alleles, whereas MICA*04, *08, and *16 were weak binding alleles (Fig. 5A). Compared to the mAb stainings, the sNKG2D-binding differences were in the range of 10- to 50-fold. To substantiate this observation, soluble MICA*04 (sMICA*04) was produced in *E. coli*, refolded and tested for binding to sNKG2D in solution. In contrast to sMICA*01, sMICA*04 failed to form a stable complex with sNKG2D as shown by SEC (Fig. 2D). This indicated that the reduced binding affinity of NKG2D for MICA*04 versus MICA*01 was not due to posttranslational modifications but could be attributed to amino acid differences between these variants.

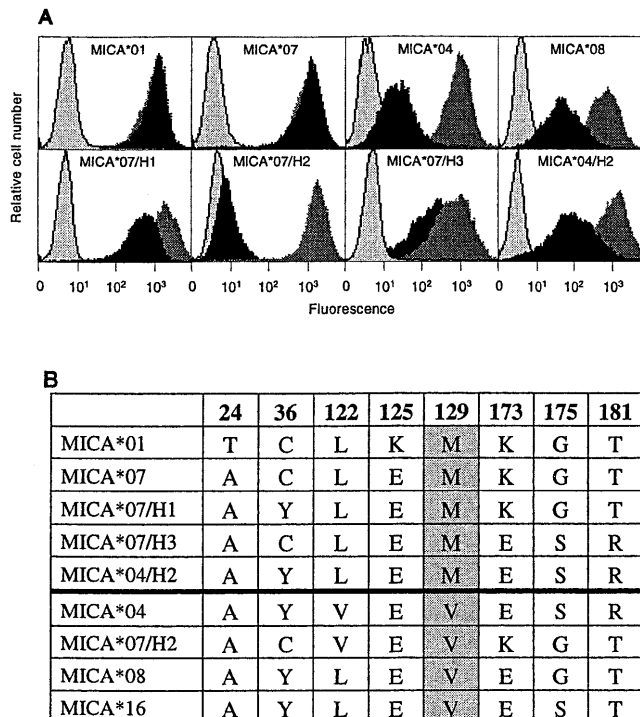


Fig. 5A,B Differential binding of NKG2D by allelic variants of MICA. **A** Stainings of MICA alleles (top panels) and of sequence exchange hybrid alleles (bottom panels) with sNKG2D/mAb M2 (black profiles), anti-MICA (mAb 6D4; dark-gray profiles), and mAb M2 alone (light-gray profiles). See text for further explanations. **B** Allelic amino acid positions in the $\alpha 1\alpha 2$ domains of the MICA*01, *07 (strong NKG2D binding), *04, *08, and *016 (weak NKG2D binding) alleles. Of these, positions 36, 129, and 173 segregated with strong or weak binding of NKG2D. Exchange of sequences in the MICA*07 and MICA*04 hybrid alleles (H1, H2, and H3; see Materials and methods) showed the association of methionine and valine at position 129 with strong and weak binding, respectively

MICA valine 129 is associated with reduced affinity for NKG2D

MICA*04 and *07 differ in six amino acid residues in the $\alpha 1\alpha 2$ domain (Fig. 5B). To investigate which of these positions affected NKG2D binding, we constructed four hybrid sequences by mutually exchanging

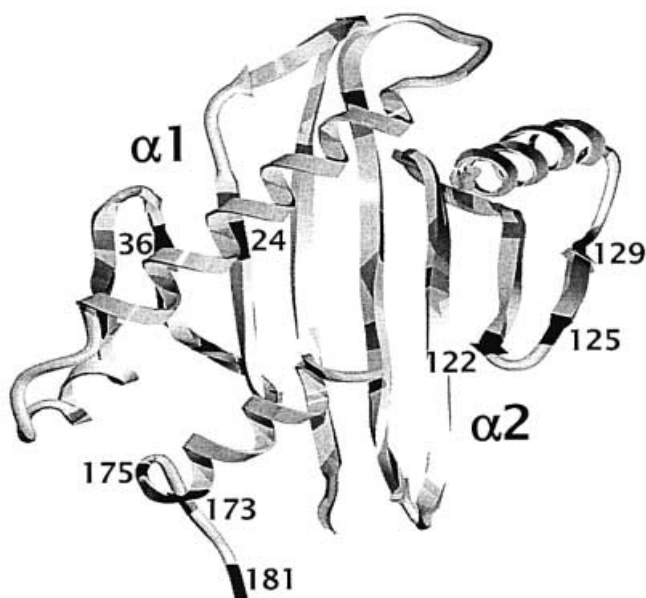


Fig. 6 Position of methionine/valine 129 on a ribbon diagram of the crystal structure of MICA (Li et al. 1999). See text for further explanations

three segments of *MICA*04* and *MICA*07* cDNAs. All of the encoded hybrid molecules were expressed at high levels on transfected C1R cells as confirmed by flow cytometry with anti-MICA mAb (Fig. 5A). Stainings with sNKG2D showed that substitution of valine 129 by methionine conferred high NKG2D binding affinity to *MICA*04* (hybrid *MICA*04/H2*). Conversely, substitution of methionine 129 by valine drastically reduced binding of NKG2D to *MICA*07* (hybrid *MICA*07/H2*). Exchange of the remainder biallelic positions had no effect on NKG2D binding (Fig. 5A, B). According to the *MICA*01* crystal structure, amino acid position 129 is located in the $\beta 4$ strand of the β -pleated sheet in the $\alpha 2$ domain (Fig. 6) (Li et al. 1999). Because the side chain of methionine 129 is partially buried and forms hydrophobic interactions with glutamine 136, alanine 139, and methionine 140, its replacement by valine likely affects NKG2D binding indirectly by a conformational change.

Discussion

Functional evidence together with the crystal structure of MICA has indicated that MIC molecules, unlike the structurally related MHC class I and CD1, are not associated with peptide or other ligands. This is corroborated by our present results showing complex formation of purified MICA with NKG2D in solution. Complexes with unglycosylated MICA appeared a little less stable than those incorporating glycosylated MICA. Carbohydrates enhance binding of mouse

H2-D^b to the Ly49A NK-cell receptor (Lian et al. 1998). However, of the three N-linked glycosylation sites in the MICA $\alpha 1\alpha 2$ domain (Asn8, Asn56, and Asn102), none is present in MICB which also bound strongly to NKG2D, suggesting that they may have an accessory but not a pivotal role in the MIC-NKG2D interaction. Likewise, the glycosylation state of NKG2D, which was purified from insect cell supernatant or refolded from bacterial inclusion bodies, had no substantial effect on complex formation. Despite the dual symmetry of NKG2D homodimers, MICA was bound as a monomer, which is distinct from Ly49A-D^b complexes in which Ly49A homodimers are in contact with two D^b molecules (Tormo et al. 1999).

NKG2D interacts similarly with a heterogeneous set of ligands, represented by MIC in most mammals and by the RAE-1 protein family in the mouse. To identify human homologues of the mouse molecules, we searched sequence databases and retrieved three orphan sequences that are distantly related among each other and to RAE-1. Common features shared by all of these molecules are an MHC class I-like $\alpha 1\alpha 2$ platform domain, absence of an $\alpha 3$ -like domain, and a putative GPI-membrane anchor. Further support for a direct homology relationship is provided by the location of *RAE-1* and of *N2DL-1*, -2, and -3 on the syntenic mouse and human Chromosomes 10 and 6, respectively (Nomura et al. 1996; BLAST search of NCBI human genome contig database; see Materials and methods). As with RAE-1, the N2DL molecules were expressed on the cell surface and tightly bound NKG2D. Comparison of the N2DL amino acid sequences with those of the ULBPs, published soon after submission of our study (Cosmann et al. 2001), showed that these are identical, with N2DL-1, -2 and -3 matching ULBP1, ULBP2, and ULBP3, respectively. The aligned nucleotide sequence pairs differ in only three silent substitutions in N2DL2/ULBP2.

Engagement of ULBPs by NKG2D triggers NK cells (Cosmann et al. 2001), hence confirming that the interactions of N2DL molecules with NKG2D are biologically significant. This raises the question as to how NKG2D can specifically interact with five diverse ligands (N2DLs/ULBPs, MICA, MICB), which share about 25% identical amino acids in their $\alpha 1\alpha 2$ domains that are variably scattered throughout the aligned sequences without discernible patterns of sequence conservation. Moreover, 18 of the 27 amino acid residues that are preserved among all of the five MIC and N2DL polypeptides are buried in the MICA crystal structure (Li et al. 1999). These profound differences cannot be accommodated in a binding model without information derived from complex structures.

Because NKG2D is a dominant activator of NK cells and may positively modulate T cells (Bauer et al. 1999), tight regulation of the expression of all of its ligands seems imperative. In the mouse, abundant *RAE-1* mRNA transcripts have only been detected in

early stage embryos and small amounts have been found in adult liver and spleen (Zou et al. 1996). As inferred from binding of soluble NKG2D, RAE-1 is expressed on some mouse tumor cell lines (Cerwenka et al. 2000; Diefenbach et al. 2000). In our screening of a panel of human cell lines and PBLs, binding of sNKG2D correlated exclusively with MIC expression on fibroblast and epithelial tumor cells. Thus, N2DL molecules may not be present in significant amounts on major populations of cells or may be expressed scarcely or similarly to MIC. The regulation and immunological relevance of N2DL expression remain to be fully explored in studies which will require suitable antibody reagents.

MICA and, to a lesser extent, MICB, are polymorphic, the former being represented by 47 alleles that variably include 18 biallelic amino acid substitutions with fairly random distributions in the $\alpha 1\alpha 2$ domains (Fodil et al. 1996; Pellet et al. 1997; Visser et al. 1999). However, there has been no evidence for functional significance of this allelic variation. Our results show that the methionine/valine bimorphism at position 129 in MICA determines substantial differences in NKG2D binding. According to the crystal structure of MICA*001, this amino acid position is located in the $\beta 4$ strand of the β -pleated sheet in the $\alpha 2$ domain (Fig. 6) (Li et al. 1999). The side chain of methionine 129 is partially buried and forms hydrophobic interactions with glutamine 136, alanine 139, and methionine 140 in the first $\alpha 2$ helical stretch, which may thus become differently positioned by the valine 129 substitution. This indicates that instead of a direct participation in the MICA-NKG2D interface, the biallelic position 129 is likely to affect NKG2D binding indirectly, perhaps by association with a conformational change. This implies some involvement of the α helices on the upper side of the $\alpha 1\alpha 2$ domain of MICA in its interaction with NKG2D. However, this finding cannot be extrapolated to MICB*01, which has valine at position 129 and showed strong binding of sNKG2D (Fig. 3).

The pronounced differences in binding affinities of MICA alleles for NKG2D could have significant effects in NK-cell activation and in the modulation of T-cell responses, in particular under conditions of sub-optimal MIC expression. In addition, since the MIC-NKG2D system has the ability to costimulate CD8 $\alpha\beta$ T-cell responses (Groh et al. 2001), interactions of high-affinity MICA alleles with NKG2D could play a role in precipitating or exacerbating autoimmune reactions.

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