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A Structural Basis for the Association of DAP12 with Mouse, but Not Human, NKG2D[1]

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Prior studies have revealed that alternative mRNA splicing of the mouse *NKG2D* gene generates receptors that associate with either the DAP10 or DAP12 transmembrane adapter signaling proteins. We report that NKG2D function is normal in human patients lacking functional DAP12, indicating that DAP10 is sufficient for human NKG2D signal transduction. Further, we show that human NKG2D is incapable of associating with DAP12 and provide evidence that structural differences in the transmembrane of mouse and human NKG2D account for the species-specific difference for this immune receptor. *The Journal of Immunology,* 2004, 173: 2470–2478.

The activity of NK cells is regulated by a balance of positive and negative signals transduced, respectively, via activating and inhibitory cell surface receptors (1, 2). The activating receptor NKG2D, a C type-like lectin and type II transmembrane (TM)[4] protein (3), is expressed on all NK cells, $\gamma\delta$ TCR$^+$ T cells, and human CD8$^+$ T cells and is induced on activated mouse CD8$^+$ T cells (4). NKG2D recognizes several MHC-related ligands, including the UL16-binding protein and MHC class I-related chain family of proteins in humans (5, 6) and H60, RAE-1, and MULT-1 in mice (7–10). These NKG2D ligands, though usually absent from or expressed at low levels by normal adult tissues, are often induced on stressed, infected, or tumor cells in adult life (reviewed in Ref. 11). In this fashion, leukocytes expressing NKG2D can directly recognize transformed or infected cells.

Activation through NKG2D can have multiple outcomes including the production of IFN-γ and the triggering of cell-mediated cytotoxicity (5, 12). In $\alpha\beta$ TCR$^+$ T cells, NKG2D has been suggested to provide a costimulatory role similar to CD28, enhancing TCR-mediated signaling events (13, 14). In vivo NKG2D is involved in antitumor as well as antiviral immunity (reviewed in Ref. 15).

NKG2D itself lacks intrinsic signaling capabilities. Like the TCR, NKG2D contains a positively charged amino acid within its TM region and requires association with adapter signaling proteins for cell surface expression. These adapter proteins contain complementary negatively charged amino acids within their TM regions that provide a salt bridge with NKG2D. Both in mice and humans, NKG2D homodimers associate with and signal through homodimers of the TM adapter protein DAP10 (12, 16). Signaling through DAP10 involves phosphorylation of its cytoplasmic YXXM motif, recruitment of the p85 subunit of phosphatidylinositol-3 kinase, and downstream signaling through AKT (16–18).

In mice, alternative mRNA splicing generates two functionally distinct isoforms of the NKG2D protein (12). The mouse NKG2D long (mNKG2D-L) protein comprises 232 amino acids, whereas the mouse NKG2D short (mNKG2D-S) protein lacks the first 13 N-terminal amino acids and initiates translation at a second methionine in the cytoplasmic domain of this type II protein. This shorter isoform is expressed in activated, but not resting, mouse NK cells and is capable of pairing with homodimers of either DAP10 or DAP12, an ITAM-containing TM adapter protein. Like other ITAM sequences, DAP12 intracellular YXXL$_{6–8X}$ YXXL/I motif recruits Syk family kinases (19). Mouse NKG2D-L pairs exclusively with DAP10 (12). Both DAP10 and DAP12 signaling contribute to NK cell-mediated cytotoxicity, whereas DAP12 signaling also definitively stimulates cytokine production, such as IFN-γ (12, 16, 20, 21).

The ability of the mouse NKG2D to generate identical receptors with distinct signaling properties by virtue of association with different adapter proteins prompted the question of whether human NKG2D also demonstrates this property. In this study, we have examined NKG2D expression and functional activity in human PBMCs of patients lacking a functional *DAP12* gene and have explored the structural basis for association of human and mouse NKG2D with the DAP10 and DAP12 adapter proteins.

Materials and Methods

Characterization of patients with Nasu-Hakola

The *DAP12* (*tyrobp*) gene in a patient with Nasu-Hakola, designated NH1, has a single base mutation in the start codon of exon 1 that has recently been identified in Japanese patients (22). Patients NH2 and NH3 have a single base deletion in exon 3 (22, 23). Loss of DAP12 protein expression in these patients was confirmed by Western blotting as previously described (22). Studies of all these subjects were conducted according to the institutional guideline.

Cytotoxicity assays

PBMC were prepared from peripheral blood samples of healthy individuals and three patients with Nasu-Hakola disease using density gradient centrifugation by using Ficoll-Hypaque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden). PBMC were resuspended at 2×10^6/ml in RPMI 1640

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[4] Abbreviations used in this paper: TM, transmembrane; EC, extracellular; mTM hEC, mouse-human NKG2D chimeras; IRES, internal ribosomal entry site; mCJS, murine cytoplasmic juxtamembrane sequence.

medium supplemented with 10% FCS, 2 mM L-glutamine, HEPES, penicillin/streptomycin, and 2-ME. PBMC were plated into 24-well plates in the presence of 100 U/ml rIL-2, for mAb-induced redirected cytolytic assays, or 1000 U/ml rIL-2, for BaF/3 cytotoxicity assays rIL-2 (Shionogi, Osaka, Japan). A lower concentration of IL-2 was used for assays with P815 target cells as high doses of IL-2 cause considerable background cytotoxicity against P815, likely due to lymphokine-activated killer activity. Activated PBMC were harvested after 48-h culture, then used in 4-h ^{51}Cr release cytotoxicity assays as described (24). BaF/3, BaF/3 cells expressing MICA*0019, and FcR$^+$ P815 cells were labeled with 100 μCi of ^{51}Cr (PerkinElmer, Boston, MA) for 2 h at 37°C, washed three times, and used as target cells in cell-mediated cytotoxicity assays. For mAb-induced redirected cytotoxicity assays using P815 target cells, PBMC were cultured in the presence of medium only, control mAb anti-CD56 mAb (Leu19; BD Immunocytometry Systems, San Jose, CA) or anti-NKG2D mAb (made in collaboration with Dr. J. Houchins; R&D Systems, Minneapolis, MN). Monoclonal Abs were used at a final concentration of 2.5 μg/ml.

cDNAs, chimeras, and plasmids

Human "short" NKG2D, human truncated NKG2D, mouse truncated NKG2D, mouse-human NKG2D chimeras (mTM hEC), and NKG2D-CD69 chimeras were created by standard PCR mutagenesis. TM regions were determined by using the following TM prediction programs and a consensus sequence was obtained by comparison of the analyses: Tmap (http://srs.ebi.ac.uk/srsbin/cgi-bin/wgetz?-page+Launch+-id+1dvsK1MjgHZ+-appl+tmap+-launchFrom+top); DAS (http://www.sbc.su.se/~miklos/DAS/); Tmpred (http://www.ch.embnet.org/software/TMPRED_form.html); HMMTOP (http://www.enzim.hu/hmmtop/html/submit.html); SOSUI (http://sosui.proteome.bio.tuat.ac.jp/cgi-bin/sosui.cgi?/sosui_submit.html); and TMHMMM (http://www.cbs.dtu.dk/services/TMHMM/). Protein chimeras and truncations were created using the following amino acids: mΔNKG2D begins at K48, hΔNKG2D at R47, and ΔCD69 at V39 (with numbering beginning at the first amino acid in the mNKG2D-L isoform and the human NKG2D and CD69 sequences). Mouse NKG2D TM construct consists of amino acids K48-V84 and the human NKG2D TM constructs span R47-N80. Human NKG2D extracellular (EC) construct begins at L82, and the human CD69 EC construct at G65. The human NKG2D short intracellular region construct consists of M15-E48, the human long intracellular construct of M1-E48 and the mouse NKG2D tail construct spans M1-E13. CD69 cDNA was synthesized from mRNA isolated from Jurkat T cells stimulated 24 h with 25 ng/ml PMA (Calbiochem, Darmstadt, Germany) by reverse transcription with Superscript II (Invitrogen Life Technologies, Carlsbad, CA). Site-directed mutagenesis was performed using a Quick Change kit (Invitrogen Life Technologies), according to the manufacturer's instructions. All constructs were confirmed by DNA sequencing. cDNAs were subcloned into pMx-pie (containing a puromycin resistance gene, an internal ribosomal entry site (IRES) element, and the enhanced GFP gene) or pMX-puro retroviral vectors (25).

Cells and transfectants

Plasmid constructs were transfected with Lipofectamine 2000 (Invitrogen Life Technologies) into the Phoenix packaging cell lines (generous gifts from Dr. G. Nolan, Stanford University, Palo Alto, CA) (26) to produce retroviruses. Retroviruses in medium containing 8 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO) were used to infect BaF/3 reporter cells. Briefly, polybrene was added to retroviral supernatants and the mixture was used to resuspend BaF/3 reporter cells. Following centrifugation of the cells in the retrovirus-containing medium (1300 × g for 2.5 h), transfected reporter cells were incubated for 48 h at 37°C and then assayed for transgene expression. BaF/3 reporter cells were created from the mouse pro-B BaF/3 cell line. Dr. S. Tangye (Centenary Institute, Sydney, Australia) generously provided BaF/3 cells transfected with a mouse IL-3 cDNA to permit autocrine production of this requisite growth factor. To create Myc-DAP10 reporter cells, IL-3$^+$ BaF/3 cells were infected with retroviruses (pMX-puro vector) containing a cDNA including the human CD8 leader segment, followed by the Myc epitope (EQKLISEEDL) joined to the EC N-terminal domain of human DAP10 (27). Similarly, to create Flag-DAP12 reporter cells, IL-3$^+$ BaF/3 cells were infected with retroviruses (pMX-puro vector) containing cDNA including the human CD8 leader segment, followed by the Flag epitope (DYKDDDDK) joined to the EC N-terminal domain of human DAP12, as described (19, 28). Infected cells were then selected in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, and 1 μg/ml puromycin.

Flow cytometry and Abs

For immunofluorescence analysis of transfected *myc*-DAP10 BaF/3 reporter cells, 1 × 10^6 cells were stained with anti-*myc* mAb 9E11 (generously provided by Dr. G. Evan, University of California, San Francisco,

CA), followed by a donkey anti-mouse IgG secondary Ab conjugated to PE (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were then preincubated in 10% normal mouse serum before being stained with either biotin-conjugated mouse anti-human NKG2D mAb (clone 149810, R&D Systems), biotin-conjugated rat anti-mouse NKG2D mAb CX5, or allophycocyanin-conjugated mouse anti-human CD69 mAb Leu23 (BD Pharmingen, San Diego, CA). Biotin-conjugated Abs were detected with CyChrome-conjugated or allophycocyanin-conjugated streptavidin (BD Pharmingen). For immunofluorescence analysis of transfected Flag-DAP12 BaF/3 reporter cells, 1 × 10^6 cells were stained with biotin-conjugated anti-Flag mAb M2 (Sigma-Aldrich), followed by CyChrome-conjugated streptavidin (BD Pharmingen). Cells were also stained prior with PE-conjugated mAb to mouse NKG2D (CX5), anti-human NKG2D, or anti-CD69 (Leu23; BD Pharmingen) followed by donkey anti-mouse IgG PE. Live cells were gated based on forward and side scatter profiles. Retrovirus-infected cells were gated based on GFP fluorescence. Cells were analyzed by using a FACSCaliber (BD Biosciences, San Jose, CA) or a small desktop Guava Personal Cytometer with Guava ViaCount and Guava Express software (Hayward, CA).

Immunoprecipitations and Western blots

A total of 50 × 10^6 transfected BaF/3 cells were solubilized in 1 ml Brij-Nonidet P-40 lysis buffer (0.875% Brij 97, 0.125% Nonidet P-40, 10 mM Tris base, 150 mM NaCl (pH 8.0), and protease inhibitors; all Sigma-Aldrich). Cell lysates were precleared with 60 μl of protein G-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4°C. Anti-human NKG2D (clone 149810; R&D Systems) or isotype-matched control mAb were cross-linked to protein G beads by incubation in PBS for 30 min, followed by incubation in 10 mM dimethyl pimelimidate dihydrochloride (DMP; Pierce, Rockford, IL), 200 mM triethanolamine (Sigma-Aldrich) pH 8.2 for 45 min and extensive washing. Monoclonal Ab-coated beads were used for immunoprecipitation of precleared lysates for 3 h at 4°C. After washing, immunoprecipitates were eluted by adding nonreducing sample buffer and incubating for 30 min at room temperature. 2-ME (Sigma-Aldrich) was added, samples were boiled, and analyzed by 15% SDS-PAGE. Samples were transferred to Immobilon P membrane (Millipore, Bedford, MA), blocked and probed with goat anti-human DAP12 Ab N-17 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-human DAP12 mAb DX37 (27), followed by HRP-conjugated donkey anti-goat IgG (Amersham Pharmacia Biotech) or goat anti-mouse IgG (Amersham Pharmacia Biotech), respectively, and visualized with chemiluminescent substrate (Pierce).

Results

PBMCs from DAP12$^{-/-}$ patients display normal NKG2D function

Only the counterpart of the mNKG2D-L isoform has been described in humans and has been shown to coimmunoprecipitate with DAP10, but not DAP12 (16, 27). Nonetheless, this does not exclude a weak or indirect association between human NKG2D and DAP12 that may contribute to NKG2D receptor function in human lymphocytes. Therefore, studies were undertaken to address formally a potential role for DAP12 in human NKG2D receptor-dependent NK cell activation. Nasu-Hakola disease, also called polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy, is a globally distributed recessively inherited disease caused by loss of function mutations in the *DAP12* (also called *tyrobp*) gene (23). In this report, we analyzed PBMC of three Japanese patients with Nasu-Hakola disease for their NKG2D-mediated cytolytic function. Each of these patients has a single point mutation in their *DAP12* gene that causes premature stop codons and results in a complete lack of detectable DAP12 protein (see *Materials and Methods*). We examined the peripheral blood CD3$^-$CD56$^+$ NK cells of these patients and found that the expression of NKG2D on the cell surface of their NK cells was indistinguishable from normal, healthy individuals (Fig. 1a). Previously, we reported that loss of function mutations in human *DAP12* did not affect expression of the closely linked *DAP10* gene (23); however, in this prior study NKG2D receptor-dependent functions were not directly analyzed.

To formally address the activity of the NKG2D receptor in patients lacking DAP12, we tested the ability of PBMCs from these
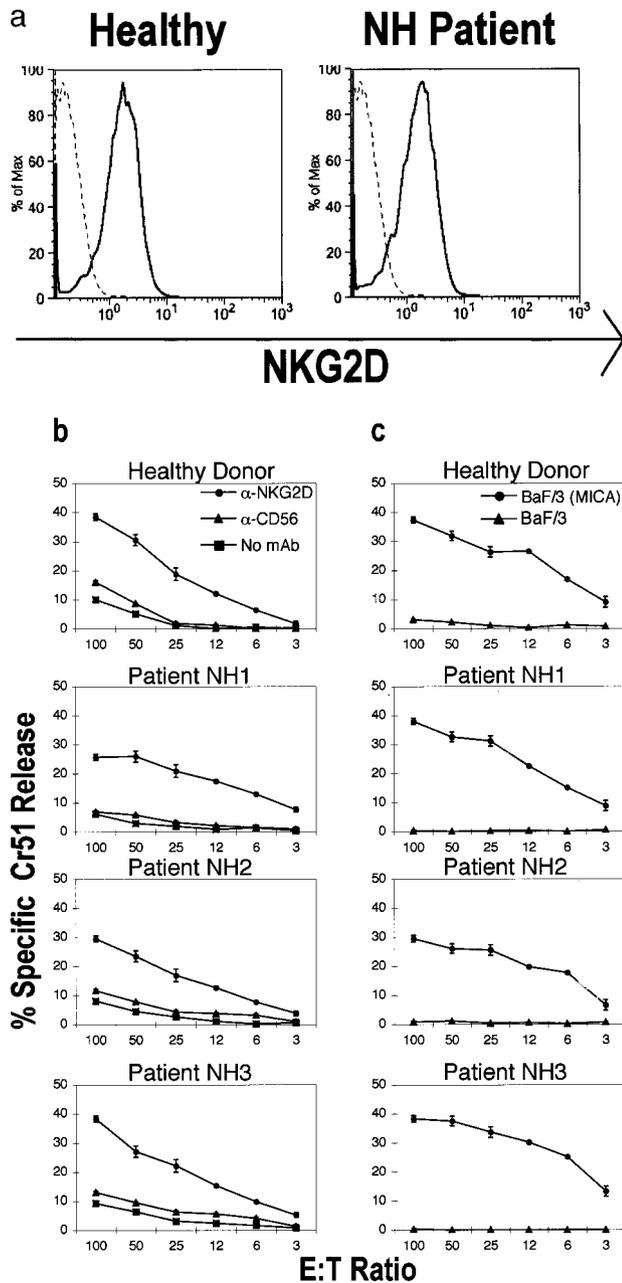
FIGURE 1. NKG2D expression and function in Nasu-Hakola patients. *a*, NKG2D levels on NK cells are identical in normal healthy humans and patients with Nasu-Hakola disease. CD3⁻CD56⁺ NK cells from PBMC of a normal, healthy individual, and Nasu-Hakola patients (NH1, NH2, and NH3) were stained with mAb against CD3, CD56, and NKG2D or appropriate control Ig and were analyzed by flow cytometry. Representative data from one Nasu-Hakola patient are shown. Isotype-matched Ig control (dashed line) and anti-NKG2D (bold lines) are shown. *b*, PBMC from a normal, healthy individual and three Nasu-Hakola patients (NH1, NH2, and NH3) were cultured for 48 h in IL-2 and assayed for mAb-induced redirected cytotoxicity against FcR⁺ P815 target cells in the absence or presence of anti-NKG2D mAb or anti-CD56 mAb (used as a negative control). *c*, IL-2-activated PBMC from a normal, healthy individual, and three Nasu-Hakola patients (NH1, NH2, and NH3) were assayed for cytolytic activity against mouse Ba/F3 target cells or Ba/F3 cells stably transfected with MICA*0019, a human NKG2D ligand.

patients to kill target cells by the NKG2D-dependent pathway. This was achieved by using an anti-NKG2D mAb-induced redirected cytotoxicity assay and by using mouse target cells trans-

fected with a human NKG2D ligand, MICA*0019. PBMCs from the three patients with Nasu-Hakola disease, as well as a normal, healthy individual, were cultured for 48 h in IL-2 and then used as effector cells in these cytotoxicity assays. Using ^{51}Cr-labeled FcR⁺ P815 target cells, anti-NKG2D mAb induced cytotoxicity mediated by PBMCs of healthy individuals and patients with Nasu-Hakola disease, whereas the anti-CD56 mAb used as a negative control failed to augment lytic activity (Fig. 1*b*). No significant difference was seen in NKG2D-mediated cytotoxicity levels between the patient and control cells. To further characterize NKG2D function in patients with Nasu-Hakola disease, we performed cytotoxicity assays against BaF/3 mouse pro-B cells stably transfected with MICA*0019, a physiological ligand of human NKG2D. Although minimal cytotoxicity was seen against untransfected, parental BaF/3 cells, BaF/3 cells stably expressing MICA stimulated elevated cytotoxicity from activated PBMCs of both normal individuals and patients with Nasu-Hakola disease. No significant difference was observed in cytotoxicity levels mediated by PBMCs of healthy individuals and Nasu-Hakola patients against MICA-bearing target cells. Thus, NKG2D expression on NK cells and NKG2D-dependent functions were indistinguishable among healthy individuals and the patients with Nasu-Hakola disease. These experiments demonstrate normal human NKG2D function in the absence of DAP12.

Unlike mouse NKG2D-S, human NKG2D does not associate with DAP12

Many NK receptors, including NKG2D, KIR2DS, Ly49D, NKR-P1C, NKp30, NKp44, NKp46, and CD94/NKG2C, are multisubunit receptor complexes that convey signals via the TM adapters FcεRIγ, CD3ζ, DAP10, or DAP12 (reviewed in Ref. 29). All of these adapters have a negatively charged aspartic acid residue in their hydrophobic TM domain, which is critical for interaction with an oppositely charged basic residue in their associated ligand-binding receptors. For NKG2D, this basic residue is a conserved arginine in the TM region (Fig. 2*a*, starred residue). The human and mNKG2D-L receptors have been shown to pair and signal through DAP10, but not DAP12 (12, 16).

Recently, a second isoform of mouse NKG2D was discovered that lacks the first 13 N-terminal cytoplasmic amino acids and uses an alternative methionine start site due to alternative splicing of the transcript (12, 30). This shorter murine NKG2D isoform is capable of pairing and signaling with both DAP10 and DAP12 adapters (12, 30). Examination of the predicted amino acid sequence of human NKG2D reveals that it also contains a second N-terminal methionine residue that could potentially act as an alternative start site (Fig. 2*a*). Although to date no alternatively spliced human transcript involving the NKG2D cytoplasmic domain has been identified (31), it is impossible to exclude the existence of such isoforms at a low abundance or that they are only expressed in certain conditions of activation or in selected cell types. Therefore, we have addressed the issue using a different approach. In this model, we ask whether a theoretical short human NKG2D isoform lacking the first 14 amino acids could be able to pair with DAP12.

To address this experimentally, we artificially created a short human NKG2D using the second methionine residue as the start site and tested its ability to pair with DAP10 and DAP12. For these assays, we used mouse BaF/3 reporter cells stably transfected with Myc epitope-tagged human DAP10 or with Flag epitope-tagged human DAP12. In the absence of an associated receptor, Myc-DAP10 and FLAG-DAP12 were expressed at only low levels on the cell surface of the reporter cells. These reporter cells were infected with retroviruses encoding the wild-type human NKG2D (in our study designated as hNKG2D-L), the artificially created

FIGURE 2. Human and mouse NKG2D comparisons and receptor constructs. *a,* Alignment of predicted mouse and human NKG2D amino acid sequences. Identical residues (shaded) in the mouse and human proteins are shown. An arrow indicates the methionine (M) beginning of the mouse NKG2D-short isoform and the artificial human "NKG2D-Short" protein. The mCJS (brackets) present in mouse, but not human, NKG2D is shown. The putative TM region (overlined) is starred to indicate the conserved arginine (R) residue required for association with DAP10 (16). Putative TM regions were defined as the consensus from six TM prediction programs (see *Materials and Methods*). Regions and residues (boxed) of interest are shown. *b,* Schematic representation is shown of the truncations and chimeric proteins generated, with the boundaries as defined in *Materials and Methods. c,* Phylogenetic comparison of the TM regions of NKG2D in the indicated species, as determined by Clustal W analysis of the proteins using MegAlign (DNASTAR Software, Madison, WI).
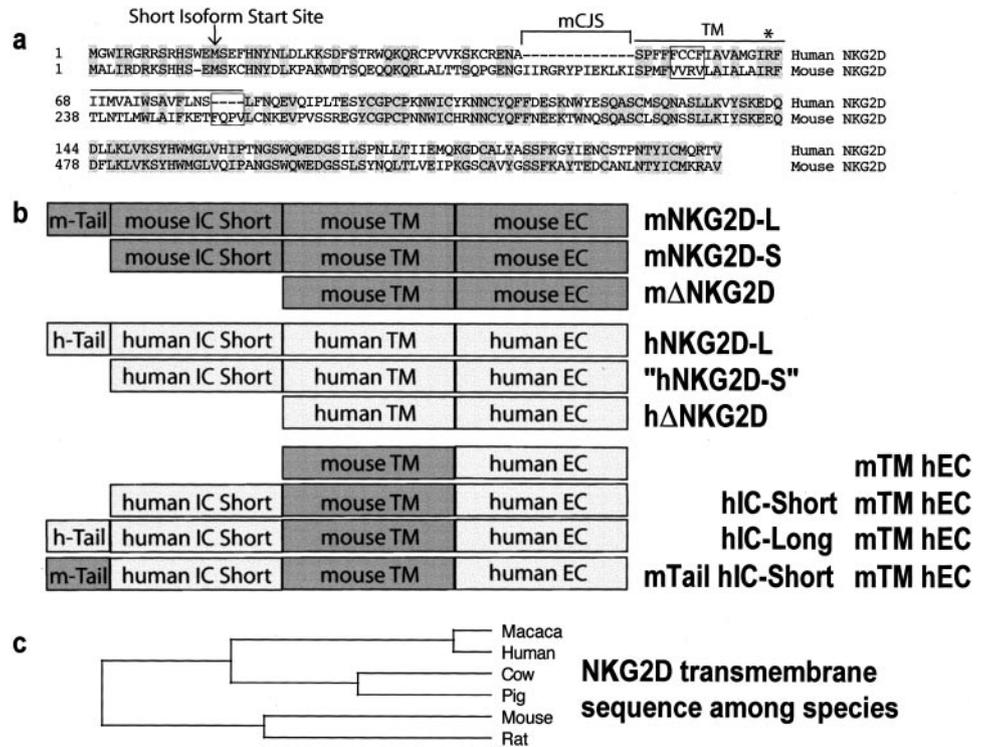
human "short" NKG2D (or hNKG2D-S), mNKG2D-L, and mNKG2D-S or an empty retroviral vector. The pMX-pie retroviral vector used in these experiments harbors an IRES-GFP element downstream of the inserted cDNA, allowing for infected cells to be readily detected by the expression of green fluorescence.

Cells were stained with mAbs against the appropriate epitope tags and either human or mouse NKG2D. Infected cells, detected by gating on GFP-positive cells using flow cytometry, were then analyzed for coexpression of NKG2D and the epitope-tagged adapter protein of interest. Coordinate expression of NKG2D and its associated adapter protein on the surface of transfected cells is indicated by the "diagonal" relationship observed in the bivariate dot plots. The long NKG2D proteins of both species and the mNKG2D-S protein paired with DAP10, as would be expected
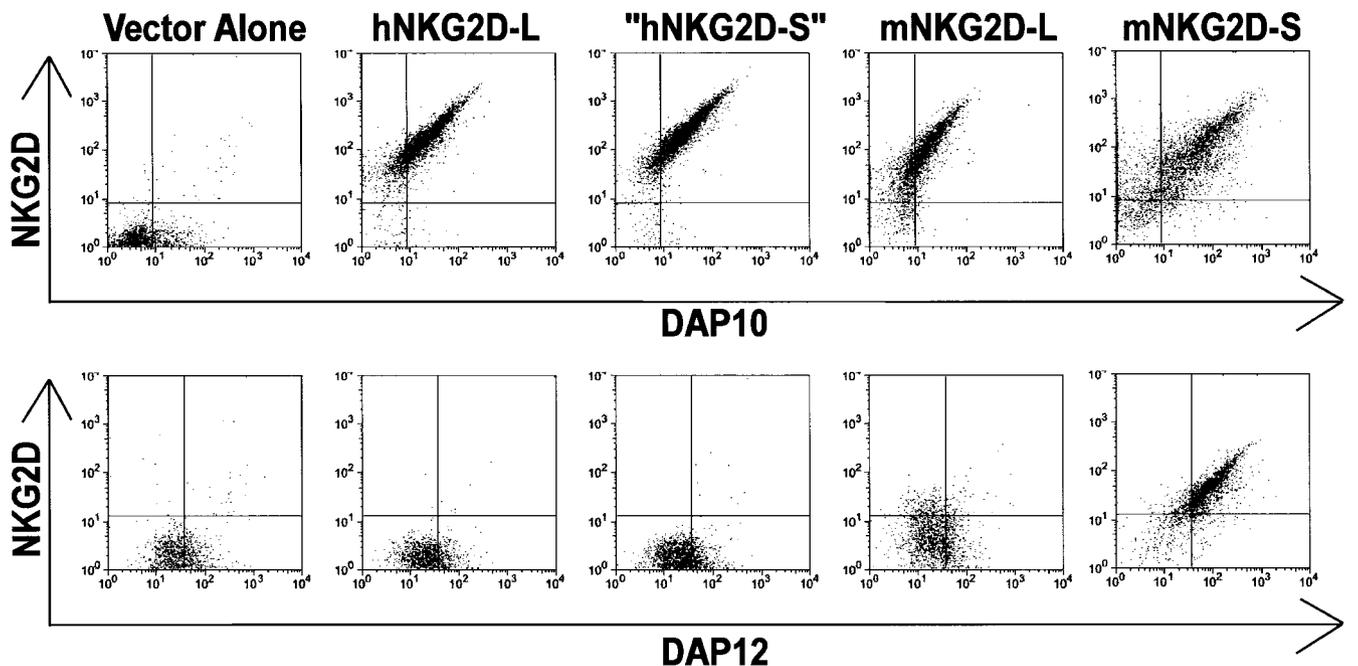
FIGURE 3. Unlike mNKG2D-S, human short NKG2D does not pair with DAP12. Ba/F3 reporter cells stably expressing Myc-DAP10 (*upper panels*) or Flag-DAP12 (*lower panels*) were infected with retroviruses with the indicated NKG2D constructs in a vector containing an IRES-GFP element. Samples were stained with the relevant anti-NKG2D and anti-epitope tag mAbs and analyzed by flow cytometry. Data shown are gated on GFP$^+$ cells, which confirmed expression of the construct in the infected cells. Results shown are representative of at least three independent experiments.
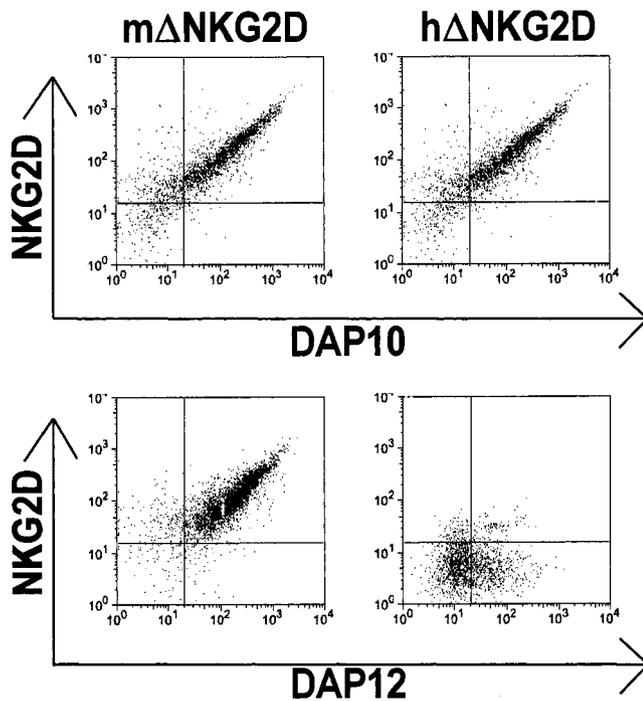
FIGURE 4. Mouse NKG2D TM and EC domains are sufficient for DAP12 association. Ba/F3 reporter cells stably expressing Myc-DAP10 or Flag-DAP12 were infected with retroviruses with the indicated NKG2D constructs, as shown in Fig. 2b, in a vector containing an IRES-GFP element. Samples were stained with the relevant anti-NKG2D and anti-epitope tag mAbs and analyzed by flow cytometry. Results shown are representative of at least three independent experiments.

based on prior reports (12, 16) (Fig. 3). In accordance with prior findings (12, 30), mNKG2D-S efficiently paired with DAP12. By contrast, although the human short NKG2D associated with DAP10, it was completely unable to pair with DAP12. Human and mouse NKG2D-L were able to pair interchangeably with either human or mouse DAP10, without species preference (data not shown). Similarly, mNKG2D-S associated equally with mouse or human DAP12, whereas human short NKG2D failed to assemble with either mouse or human DAP12. Collectively, these results demonstrate a fundamental difference between the human and mouse NKG2D proteins, rather than species-specific differences in the conserved adapter proteins.

Mouse NKG2D cytoplasmic domain is not required for DAP12 association

In mouse NKG2D, the 13 amino acid N-terminal cytoplasmic tail present in mNKG2D-L, absent in mNKG2D-S, abrogates DAP12 binding (12). Similar to mouse NKG2D, the first 14 amino acids of human NKG2D preceding the second methionine in the cytoplasmic domain is a highly charged region (isoelectric point pH 12). As our artificial human short NKG2D also lacks this potentially inhibitory sequence, and is still unable to pair with DAP12, other structural elements must explain the difference between human and mouse NKG2D with respect to association with DAP12. By comparison of mouse and human NKG2D (Fig. 2), one explanation lies in a 13 amino acid stretch present in mouse, but not human NKG2D, in the cytoplasmic juxtamembrane region. This unique murine cytoplasmic juxtamembrane sequence (mCJS), designated in Fig. 2a, might provide a positive signal for DAP12 association, as this sequence is present in mouse but not human NKG2D. To address this possibility, we created two N-terminal truncations of

mouse NKG2D: one that contained the mCJS and another that only contained the TM and EC domains (mΔNKG2D). Similarly, we created a truncated human NKG2D, which contained only the TM and EC domains (hΔNKG2D) (Fig. 2b). Retroviruses encoding mΔNKG2D and hΔNKG2D were used to infect Myc-DAP10 or Flag-DAP12 reporter BaF/3 cells, as previously described.

Ab staining for the appropriate epitope tags and NKG2D revealed that mΔNKG2D was capable of pairing with both DAP10 and DAP12 (Fig. 4). This result suggests that the mCJS region is not required for DAP12 association and that the mouse NKG2D TM and EC domains are sufficient for DAP12 association. The same experiment with a truncated mouse NKG2D still containing the mCJS did not significantly improve DAP12 association, suggesting that this sequence plays no major role in DAP12 association (data not shown). Like human short NKG2D, hΔNKG2D was still able to pair with DAP10, but was incapable of pairing with DAP12. This excluded the possibility that the inability of human NKG2D to pair with DAP12 was due to an inhibitory sequence present in the cytoplasmic region. Furthermore, these results suggest that the difference between mouse and human NKG2D, with respect to DAP12 association, must lie within the TM and/or EC domains.

*The mouse NKG2D TM sequence allows human NKG2D to pair with DAP12*

Previous studies with a chimeric protein composed of the DAP10 EC-DAP12 TM-DAP10 cytoplasmic domains suggested a nonpermissive interaction between the TM regions of DAP12 and human NKG2D (27). Furthermore, examination of the protein sequences of human and mouse NKG2D reveals significant sequence divergence within the TM region (Fig, 2a, underlined). These observations spurred the question of whether the difference in DAP12 pairing ability between mouse and human NKG2D could be attributed to their TM regions. To test the hypothesis that the mouse NKG2D TM is permissive for DAP12 association whereas the human NKG2D TM is not, we created a chimeric NKG2D construct containing the mTM hEC domain (Fig. 2b) and tested its ability to pair with DAP10 and DAP12 in BaF/3 reporter cells, as previously described. We found that replacing the human TM with the mouse TM region allowed the chimeric protein to stabilize expression of human DAP12 on the cell surface of the transfectants (Fig. 5a). In other words, the mouse NKG2D TM permitted the chimeric receptor to associate with DAP12. This conclusion was further supported by the ability to coimmunoprecipitate both DAP10 and DAP12 with the chimeric receptor containing the mouse NKG2D TM region (Fig. 5c). By contrast, as reported previously, human NKG2D coimmunoprecipitates with DAP10, but not with DAP12 (27, 31). These results suggest a critical difference between the TM domains of human and mouse NKG2D such that human NKG2D is not permissive for DAP12 pairing whereas mouse NKG2D is permissive.

In these experiments, we also tested whether the FQPV motif (Fig. 2a, boxed) on the EC membrane-proximal side of the mouse TM was necessary for DAP12 interaction, as this motif is absent from human NKG2D. Our experiments suggest this motif is unnecessary as chimeric mTM hEC proteins with or without this sequence associate equivalently with DAP12 (data not shown).

The human NKG2D cytoplasmic domain when associated with the mouse NKG2D TM region is permissive for DAP12 association

Like the mouse NKG2D 13 amino acid tail, the N-terminal portion of human NKG2D-L contains multiple charged amino acid residues (Fig. 2a). Because of this similarity, it is possible that the

FIGURE 5. The mouse NKG2D TM region conveys DAP12 specificity to human NKG2D. *a* and *b*, Ba/F3 reporter cells stably expressing Myc-DAP10 or Flag-DAP12 were infected with retroviruses with the indicated NKG2D constructs, as shown in Fig. 2*b*, in a vector containing an IRES-GFP element. Samples were stained with the relevant anti-NKG2D and anti-epitope tag mAbs and analyzed by flow cytometry. *c*, mTM hEC chimeric NKG2D receptor coimmunoprecipitates with DAP10 and DAP12. Transfected mTM hEC reporter cells (*a*) were lysed in Brij-Nonidet P-40 lysis buffer. The receptor complexes were immunoprecipitated from lysates with anti-human NKG2D or isotype-matched control mAb. Samples were analyzed by SDS-PAGE and transferred to Immobilon P membrane and probed with goat anti-DAP10 antisera N-17 or anti-DAP12 mAb DX37, followed by HRP-conjugated donkey anti-goat IgG or goat anti-mouse IgG, respectively, and visualized with chemiluminescent substrate. As described previously (16), the heterogeneous migration pattern of DAP10 is likely due to *O*-linked glycosylation of its EC domain.
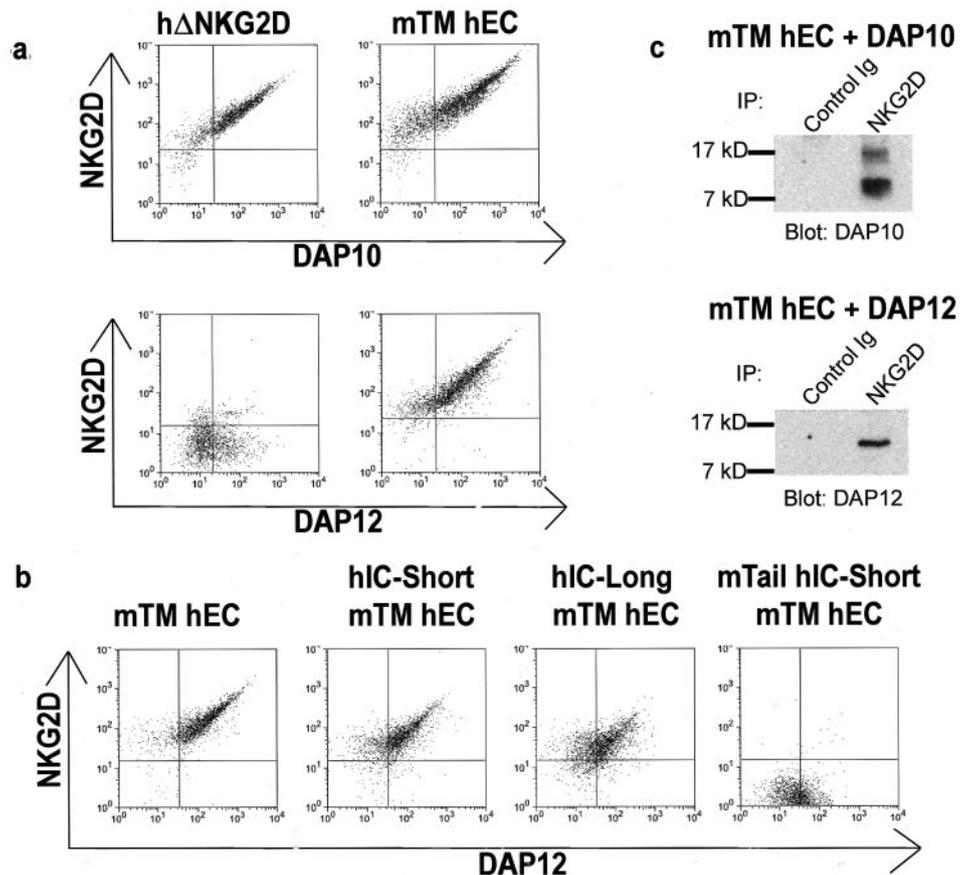
human tail behaves like the mouse tail and may also be repulsive to DAP12 association. Thus, in addition to having a nonpermissive TM region in human NKG2D, the repulsive segment present in mNKG2D-L cytoplasmic domain might have been conserved in humans to prevent potential association with DAP12. To answer the question of whether the long cytoplasmic domains of human NKG2D or the artificial "short" hNKG2D are permissive for DAP12 binding, we created NKG2D chimeras from the mTM-hEC construct adding back the human "short" intracellular domain (hIC-"Short" mTM hEC), the human long intracellular domain (hIC-Long mTM hEC), or the human "short" intracellular domain with the mouse N-terminal 13-aa tail (mTail hIC-"Short" mTM hEC), as portrayed graphically in Fig. 2*B*. As expected, all of these chimeric constructs paired with DAP10 (data not shown).

Using retroviral infection of the Flag-DAP12 reporter BaF/3 cells, we found that the entire human intracellular domain, in both the artificial short and natural long isoforms, failed to prevent DAP12 association when the TM region of mNKG2D is present in the chimeric receptors (Fig. 5*b*). In contrast, addition of the mouse tail to the human short intracellular domain abrogated DAP12 association. These results demonstrate that the N-terminal tail sequence of mouse NKG2D is necessary to prevent association of DAP12 with the mNKG2D-L isoform. This repulsion cannot be due to charge alone because the N-terminal segments of both mouse and human NKG2D are rich in acidic and basic amino acid residues. From an evolutionary perspective, mouse NKG2D may have evolved a repulsive tail and alternative splicing of NKG2D as mechanisms to regulate DAP12 adapter signaling. In contrast, as the TM of human NKG2D is not permissive for association with DAP12 it requires no such regulatory region in the cytoplasmic domain and hence this structural feature has not been conserved.

NKG2D TM domains are necessary and sufficient to confer adapter specificity

As the mouse NKG2D truncation (mΔNKG2D) and mTM hEC chimeric NKG2D proteins paired with DAP12, it was possible that the EC portions of human or mouse NKG2D could be necessary for association with DAP12. To address this question and further to ask whether the TM regions of mouse and human NKG2D are sufficient to confer adapter specificity, we created chimeric proteins consisting of the TM domain of either mouse or human NKG2D fused to the EC domain of human CD69 (Fig. 6*a*). CD69 has some structural similarity to NKG2D, as it is also a type II TM-anchored homodimer with an EC region consisting of a single C type-like lectin domain (32–34). However, in contrast to NKG2D, CD69 does not pair with signaling adapters, such as DAP10 or DAP12, and does not possess charged amino acids in its TM region. A truncated form of CD69 consisting of only the TM and EC domains was also constructed as a control (in our study designated as ΔCD69) (Fig. 6*a*).

We tested the CD69 chimeras in the DAP10 or DAP12 reporter cells, as previously described, and found that the TM domain of NKG2D was necessary and sufficient to convey adapter specificity to the chimeric proteins (Fig. 6*b*). Although the control protein ΔCD69 did not pair with either adapter, the human TM NKG2D-CD69 protein was able to pair with DAP10, but not DAP12. In contrast, the mouse TM NKG2D-CD69 chimeric protein was able to associate equivalently with both DAP10 and DAP12.

We further wished to define requirements for DAP12 association. Closer examination of the mouse TM region revealed a second basic residue present in the mouse but not human TM region. We tested whether this second arginine residue (Fig. 2*a*, boxed)
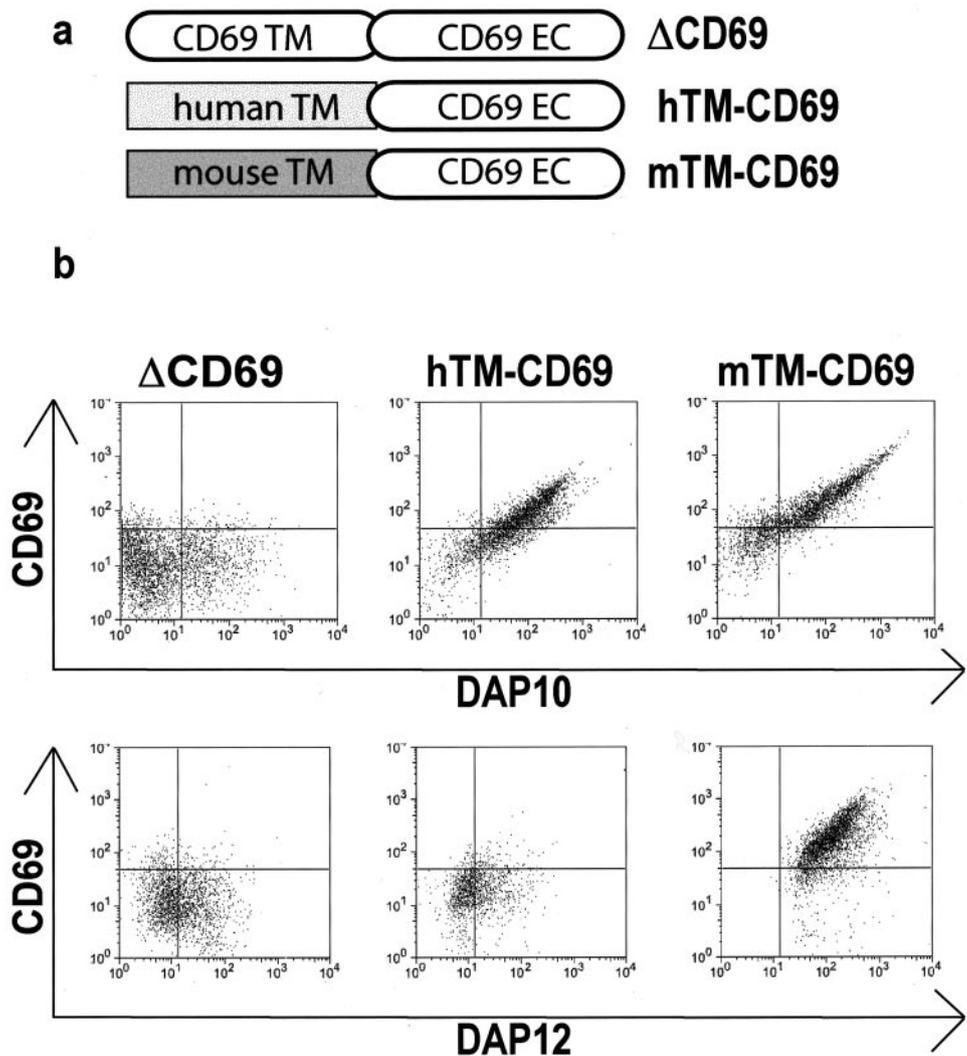
FIGURE 6. NKG2D TM regions are necessary and sufficient to confer DAP10 and DAP12 specificity. *a*, Schematic representation is shown of chimeric receptors containing the EC domain of human CD69 and the TM region of human or mouse NKG2D (as described in *Materials and Methods*). *b*, Ba/F3 reporter cells stably expressing Myc-DAP10 or Flag-DAP12 were infected with retroviruses with the indicated NKG2D TM-CD69 EC constructs in a vector containing an IRES-GFP element. Samples were stained with anti-human CD69 mAb and anti-epitope tag mAbs and analyzed by flow cytometry. Results shown are representative of at least three independent experiments.

was necessary for DAP12 association by mutating it to an alanine residue via site-directed mutagenesis. We found that an R→A mouse TM CD69 mutant still paired with DAP12, indicating that this putative second TM basic residue does not contribute to DAP12 association (data not shown).

These results formally demonstrate that the adapter specificity for human and mouse NKG2D lies exclusively in the TM domain, and this domain is necessary and sufficient to allow adapter pairing. A similar phenomenon has been observed for the receptor specificity of the human DAP10 and DAP12 signaling adapters. Chimeras between DAP10 and DAP12 have illustrated that the receptor specificity of these two adaptors also lies in their TM domains (27). Thus, for NKG2D, DAP10, and DAP12 all of the critical and necessary pairing interactions occur within the TM regions.

Overall, there is little conservation of the TM sequence of human and mouse NKG2D. Only a few amino acids have been conserved, conspicuously the arginine residue in the center of the TM. This extensive diversity in the sequence of the mouse and human NKG2D TM region precludes easy predictions about the critical residues in mouse NKG2D that permit or in human NKG2D prevent association with DAP12.

Discussion

Our results indicate a fundamental structural difference between mouse and human NKG2D. Although mouse NKG2D is capable

of associating with both DAP10 and DAP12 signaling adapters, human NKG2D is only able to partner with DAP10. We show that this species difference can be mapped to the TM regions of mouse and human NKG2D. As DAP10 signals through a p85 phosphatidylinositol-3 kinase-mediated AKT pathway (16, 17), and as DAP12 signals through a Syk/ZAP70-mediated ITAM pathway (19), the ability of NKG2D to pair with various combinations of adapters represents its ability to initiate discreet signaling effects in mice, but not humans. Moreover, direct examination of NKG2D-dependent NK cell activation in DAP12-deficient humans suffering from the Nasu-Hakola disorder demonstrated that the NKG2D receptor functions normally with respect to its ability to induce cytolytic activity in the complete absence of DAP12.

Our findings are consistent with the studies of Billadeau et al. (21), which demonstrate that a chimeric protein with the cytoplasmic domain of human DAP10 was capable of triggering NK cell-mediated cytotoxicity. However, in these experiments the chimeric DAP10 was introduced into human NK cells expressing a functional DAP12 protein, leaving open the possibility of indirect interactions between the cytoplasmic domains of the chimeric DAP10 and endogenous DAP12 proteins. Our findings provide conclusive evidence for a DAP12-independent role of DAP10 in human NK cell NKG2D-mediated cytotoxicity.

Activated mouse NK cells expressing mNKG2D-S can thus signal through DAP10 and DAP12, whereas resting mouse NK cells and all human NK cells express long NKG2D and only signal

through DAP10. In many cases, DAP10 alone suffices to initiate cytotoxicity. This is evident from the fact that human NKG2D, which only uses DAP10, stimulates cytotoxicity independent of Syk kinases (21), as well as the evidence that NKG2D triggers cytotoxicity in mouse NK cells lacking DAP12 or Syk family kinases (20). In mice, DAP12 is also independently capable of initiating cytotoxicity through NKG2D-S, as demonstrated by experiments in DAP10-deficient mice (30). Furthermore, mouse NKG2D-S initiated DAP12 signaling contributes to proliferative responses and IFN-γ production (12). A role for DAP10 in IFN-γ production has been suggested using human NK cells and recombinant human NKG2D ligands (17, 31, 35).

It is not completely surprising that the mouse NKG2D TM region can associate with both DAP10 and DAP12 as these two adaptor proteins share significant homology. DAP10 and DAP12 share 20% sequence homology (16) and moreover their TM domains are very homologous in both sequence (45% homology) and structure (27). In the genomes of both mice and humans, the *DAP10* and *DAP12* genes are adjacent to one another, but in opposite transcriptional orientation (36), a circumstance that likely arose through gene duplication.

Our results illustrate that the TM domain of the immunoreceptor NKG2D provides signaling adapter specificity and accounts for the species difference. Although the human NKG2D TM associates only with DAP10, the mouse NKG2D TM associates with both DAP10 and DAP12. A comparison of NKG2D TM domain sequences across species demonstrates a close relationship between mouse and rat TM sequences (Fig. 2c). Furthermore, the only reported sequence of rat NKG2D lacks an N-terminal "tail" region and is thus structurally similar to mNKG2D-S. Based on the sequence similarity of mouse and rat TM regions and the absence on a potentially inhibitory tail sequence in rat NKG2D that could block DAP12 association, rat NKG2D likely pairs with DAP12. In contrast, other species such as macaque, cow, and pig, display TM sequences more homologous to human NKG2D and may solely signal through the DAP10 adapter protein, although this requires experimental verification.

Although many genes are highly conserved in sequence, expression and function between mice and humans, *NKG2D* represents a gene that has undergone evolutionary divergence between the two species. Although it acts as an innate immune receptor in both mice and humans, NKG2D demonstrates distinct signaling mechanisms in the two species and none of the human and mouse NKG2D ligands are highly conserved in primary sequence. For example, mice do not possess structural homologues of the human *MICA* and *MICB* genes that are present in the human MHC (37). Furthermore, although the human ULBP genes clearly are functional orthologs of the mouse *RAE-1*, *H60* and *MULT-1* genes they have diverged significantly and only demonstrate 15–20% sequence similarity (14). Expression of NKG2D also differs in humans and mice. Whereas all human CD8$^+$ T cells constitutively express NKG2D (4, 5), in mice only activated CD8$^+$ T cells possess NKG2D. Nonetheless, there is evidence that NKG2D is important in immune defense in mice and humans. Viruses and tumors have evolved immune evasion mechanisms to counter the effects of NKG2D (38–41). Thus, perhaps an evolutionary pressure from the host to fight rapidly evolving viral infections and tumors helps explain some of the divergence and distinctions of NKG2D between mice and humans.

Acknowledgments

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