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Molecular Determinants Regulating the Pairing of NKG2 Molecules with CD94 for Cell Surface Heterodimer Expression

Michelle L. LaBonte, Elisa I. Choi, and Norman L. Letvin

The lytic capacity of a NK cell is regulated, in part, by the balance in cell surface expression between inhibitory CD94/NKG2A and activating CD94/NKG2C heterodimers. We demonstrate that, in the absence of DAP12, rhesus monkey NKG2A is preferentially expressed at the cell surface with CD94 due to a single amino acid difference in the transmembrane of NKG2A and NKG2C. Furthermore, in the context of an NKG2A transmembrane, the stalk domain of NKG2C was found to enhance heterodimer formation with CD94 compared with the stalk domain of NKG2A. In the presence of DAP12, the ability of NKG2C to compete for cell surface CD94 heterodimerization is enhanced and approaches that of NKG2A. Finally, allelic differences that affect the ability of rhesus NKG2A to reach the cell surface with CD94 could also be mapped to the transmembrane. These differences in the ability of inhibitory and activating NKG2 molecules to reach the cell surface provide a mechanism for the regulation of NK cell activity.

Although the importance of NK cells in controlling microbial infections and tumors is well appreciated, the regulation of the effector function of these cells remains poorly understood. Novel families of NK cell-associated molecules have recently been defined, including the CD94/NKG2, killer cell Ig-like receptors, and Ly49 molecules (1, 2). In general, each of these families of molecules includes some members that can initiate and other members that can inhibit cellular activation. The mechanisms that regulate the differential cell surface expression of these activating and inhibitory molecules are likely to prove critical in the immunopathogenesis of infections and neoplastic diseases.

The CD94/NKG2 family of NK cell receptors has been identified in humans, chimpanzees, orangutans, rhesus monkeys, and rodents (3–9). These receptors are heterodimers composed of the CD94 molecule covalently bound to activating or inhibitory NKG2 molecules (10–12). The inhibitory NKG2A molecules contain two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails. Upon receptor ligation, the tyrosines within the ITIMs of NKG2A molecules become phosphorylated, initiating a downstream signaling cascade that inhibits NK cell lytic activity (13–15). The activating NKG2C molecules lack ITIMs, and instead contain a charged lysine in their transmembrane domains that is essential for interaction with the immunoreceptor tyrosine-based activation motif-containing DAP12 molecule (16, 17). Upon CD94/NKG2C cross-linking, a signaling cascade is initiated that leads to activation of NK cell-mediated lysis (16, 17).

Both the inhibitory and activating CD94/NKG2 receptor complexes interact with the nonclassical MHC class I b molecule HLA-E on the surface of target cells. HLA-E must be complexed to a signal peptide derived from HLA-A, -B, -C, or -G to be expressed at the cell surface (18–20). Many viruses down-regulate the expression of classical MHC class I molecules on the surface of cells in which they replicate, allowing these cells to evade the host CTL response (21). The CD94/NKG2 family is thought to indirectly measure the level of classical MHC class I molecules expressed on the surface of cells by monitoring the level of HLA-E expressed by these infected cells. Whether or not a lytic response is triggered by these binding events is thought to reflect the balance of signals to NK cells from inhibitory and activating molecules at their surface. Thus, the ratio of inhibitory and activating molecules expressed on its surface is likely to be an important determinant of the functional status of a NK cell.

Interestingly, NKG2 family members can be transported to the cell surface only in the presence of CD94 (11, 12). Competition for binding of activating and inhibitory CD94/NKG2 molecules to CD94 may therefore regulate the balance between receptors expressed on the cell surface. Moreover, the presence of DAP12 is known to enhance the cell surface expression of NKG2C, suggesting that the level of DAP12 expression may also be important in regulating cell surface NKG2 expression (16). Rhesus monkeys have provided powerful animal models for exploring the pathogenesis of a number of human diseases (22–24). Therefore, we have begun to characterize the NK cell receptors in this species, hoping to develop model systems to study the role of NK cells in disease immunopathogenesis. In the course of these studies, we have identified multiple NKG2A and NKG2C alleles in this species (7).

In the present study, we evaluated the ability of rhesus monkey NKG2A and NKG2C molecules to compete for heterodimerization and cell surface expression with CD94. In pursuing this work, we also evaluated whether NKG2 allelic differences can alter the balance of receptors at the cell surface through differential abilities to compete for CD94 heterodimerization. In addition, we explored the effect of the adaptor molecule DAP12 on the ability of NKG2C...
Transformations were incubated overnight at 30 or 37°C. Epitope tag (GKPIPNPLLGLDST). Purified addition of a C-terminal linker (PPPELLGGP) followed by a V5 tag. Sequencing was performed using the QuickChange Site-Directed Mutagenesis kit (Stratagene) and 5 pmol of each primer. Following mutagenesis, PCR products were digested with DpnI for 1 h at 37°C. Fifteen microliters of each digest was transformed into DH5α Max Efficiency (Invitrogen) or Stbl2 (Invitrogen) cells. Transformations were incubated overnight at 30 or 37°C.

DNA constructs

Full-length rhesus NKG2A*01, NKG2A*02, NKG2A*04, NKG2C*02, and NKG2C*04 were cloned into pcDNA3.1A (Invitrogen). Full-length rhesus monkey NKG2A*01 and NKG2C*04 molecules were generated by PCR-mediated addition of a C-terminal linker (PPPELLGGP) followed by a V5 tag (Invitrogen) and EcoRI restriction sites. Rhesus mouse CD94 was cloned into pcDNA/zeo+ (Invitrogen) using the XhoI and BamHI restriction sites. The V5-tagged NKG2 molecules were generated by PCR-mediated addition of a C-terminal linker (PPPELLGGP) followed by a V5 tag (Invitrogen) and EcoRI restriction sites. The V5-tagged NKG2 molecules were generated by PCR-mediated addition of a C-terminal linker (PPPELLGGP) followed by a V5 tag (Invitrogen) and EcoRI restriction sites. The V5-tagged NKG2 molecules were generated by PCR-mediated addition of a C-terminal linker (PPPELLGGP) followed by a V5 tag (Invitrogen) and EcoRI restriction sites. The V5-tagged NKG2 molecules were generated by PCR-mediated addition of a C-terminal linker (PPPELLGGP) followed by a V5 tag (Invitrogen) and EcoRI restriction sites.

Materials and Methods

PCR and cloning

Standard PCR was conducted with 50 pmol of each primer and 10 ng of plasmid DNA using AmpliTaq Gold polymerase (PerkinElmer, Foster City, CA). Recursive PCR was conducted using Pfu Turbo Hot Start DNA Polymerase (Stratagene, La Jolla, CA), and DNA was isolated using the QiAprep Spin Miniprep kit (Qiagen, Valencia, CA) and sequenced. Site-directed mutagenesis was conducted using the QuickChange Site-Directed Mutagenesis kit (Stratagene) and 5–10 pmol of each primer. Following mutagenesis, PCR products were digested with DpnI for 1 h at 37°C. Fifteen microliters of each digest was transformed into DH5α Max Efficiency (Invitrogen) or Stbl2 (Invitrogen) cells. Transformations were incubated overnight at 30 or 37°C.

Results

For the competition experiments, 1 × 10⁵ 293T cells were transfected by the calcium phosphate method (Invitrogen) with a combination of 1 μg of plasmid DNA encoding NKG2A*02, a limiting amount (0.1 μg) of CD94 plasmid, and increasing amounts (0–12 μg) of untagged NKG2 competitor plasmid in the presence of 0–0.5 μg of a plasmid encoding FLAG-DAP12 DNA. Twenty-four hours posttransfection, cells were harvested and stained with 1 μl of the anti-V5-FTIC Ab. After the addition of Ab, cells were washed with 1% PBS, and then resuspended in 2% formaldehyde. Stained cells were analyzed by flow cytometry. The staining was expressed as mean fluorescence intensity (MFI). Values were calculated as a percentage of the baseline MFI (MFI with no untagged NKG2 competitor). All competition experiments were done in triplicate. In addition, both the anti-NKG2-PE and anti-V5-FTIC mAbs were used to stain cell surface of transfected with plasmid DNA encoding NKG2 and CD94.

For intracellular staining, 250 μl of Cytoperm/Cytofix (BD Pharmingen, San Jose, CA) was added to 0.3–1 × 10⁶ cells. After vortexing, cells were incubated at room temperature for 45 min. Cells were washed twice with Cytoperm/Cytowash (BD Pharmingen) and resuspended in the remaining supernatant. Cells were then incubated with either the anti-NKG2-PE or anti-V5-FTIC Ab for 1 h in the dark. Cells were washed once with Cytoperm/Cytowash (BD Pharmingen), once with 1× PBS, and then resuspended in 2% formaldehyde for analysis by flow cytometry.

Surface NK2GA and NK2GC expression

In view of the importance of the NKG2A and NKG2C molecules in transducing inhibitory and activating signals to NK cells, studies were initiated to explore the regulation of the cell surface expression of the rhesus monkey NKG2 molecules. NKG2 molecules can only be expressed at the cell surface in association with CD94, and DAP12 is necessary for optimal cell surface expression of the CD94/NKG2C heterodimer. We sought to determine the molecular mechanisms that control cell surface CD94/NKG2 expression. To this end, we generated plasmid DNAs encoding C-terminal V5-tagged rhesus monkey NK2GA*01 and NK2GC*04 molecules.
(pNKG2A*01-V5 and pNKG2C*04-V5). 293T cells were transfected with either pNKG2A*01-V5 or pNKG2C*04-V5 alone, or each of these in combination with a plasmid encoding CD94 (pCD94). The transfected cells were stained with an anti-V5 mAb and then evaluated by flow cytometric analysis. In the absence of CD94, NKG2A*01-V5 and NKG2C*04-V5 cell surface expression was not detected. When cotransfected with pCD94, the MFI of cells stained with the anti-V5 Ab was greater for the cells expressing NKG2A*01-V5 than for those expressing NKG2C*04-V5 (Fig. 1A).

We sought to elucidate the mechanism accounting for the difference in expression of these CD94/NKG2 heterodimers. Therefore, a study was done to determine whether the higher surface density of NKG2A was due to higher levels of expression of NKG2A intracellularly or whether both NKG2 proteins were equivalently expressed intracellularly but differentially expressed as heterodimers on the cell surface. 293T cells were transfected as in the previous experiment with pNKG2A*01-V5 or pNKG2C*04-V5, permeabilized, stained with the monoclonal anti-V5 Ab, and analyzed by flow cytometry. Intracellular staining was comparable in the transfected cell populations, suggesting that NKG2A*01-V5 and NKG2C*04-V5 were expressed at equivalent levels in the transfected 293T cells (Fig. 1B). These data demonstrated that the NKG2A*01 and NKG2C*04 proteins were comparably expressed following DNA transfection, yet NKG2A*01 was better able to reach the cell surface as a heterodimer with CD94.

The ability of NKG2A and NKG2C to compete for heterodimerization and cell surface expression with CD94

To explore why NKG2A was found at the cell surface as a heterodimer with CD94 at higher levels than NKG2C, we developed a competition assay to evaluate the relative efficiency of individual NKG2 molecules to heterodimerize and be expressed at the cell surface with CD94. 293T cells were transfected with pNKG2A*01-V5, limiting amounts of pCD94 as determined by titration, and untagged pNKG2A*01 or pNKG2C*04 competitor. The total amount of DNA in each transfection was kept constant by the addition of empty vector DNA. Twenty-four hours following transfection, cells were stained with an anti-V5-FITC Ab to measure the amount of NKG2A*01-V5 that reached the cell surface. The rationale underlying this assay was that an unlabeled NKG2A or NKG2C protein should compete with the NKG2A*01-V5 protein for heterodimerization with CD94. Because CD94 is expressed in limiting quantities in the transfected cells, the better an untagged NKG2 molecule binds to CD94, the less CD94 is available to heterodimerize with NKG2A*01-V5 and reach the cell surface. Thus, the better the untagged NKG2A or NKG2C molecules bind to CD94, the lower the NKG2A*01-V5 expression on the cell surface. As shown in Fig. 1, C and D, the addition of untagged NKG2C*04 competitor to NKG2A*01-V5 and CD94 resulted in only a modest decrease in the MFI of the anti-V5 Ab staining of NKG2A*01-V5 expression at the cell surface of the transfected cells. However, the addition of untagged NKG2A*01 to NKG2A*01-V5 and CD94 resulted in a substantial reduction of the MFI of the anti-V5 Ab staining of the NKG2A*01-V5 expressed on the surface of the cells. Thus, this NKG2A molecule was better able than the NKG2C molecule to compete with NKG2A*01-V5 for CD94 heterodimerization and cell surface expression. This finding suggests that CD94 may pair with NKG2A in preference to NKG2C for cell surface expression. To confirm that the measurement of NKG2A-V5 surface expression reflects the ability of untagged NKG2 molecules to compete

FIGURE 1. Expression of NKG2A*01-V5 or NKG2C*04-V5 by 293T cells following transient transfection. A, 293T cells were transfected with DNA encoding CD94 and either NKG2A*01-V5 or NKG2C*04-V5. Twenty-four hours posttransfection, cells were isolated, stained with an anti-V5-FITC Ab, and analyzed by flow cytometry. B, 293T cells were transfected with pNKG2A*01-V5 or pNKG2C*04-V5. Twenty-four hours posttransfection, cells were isolated, permeabilized, stained with an anti-V5-FITC Ab, and analyzed by flow cytometry. C, Flow cytometric analysis of the expression of CD94/NKG2A*01-V5 in the presence of NKG2A*01 or NKG2C*04 competitor. 293T cells were cotransfected with 0.1 μg of pCD94, 1 μg of pNKG2A*01-V5, and 2 μg of untagged pNKG2A*01 or pNKG2C*04 competitor. Twenty-four hours posttransfection, cells were isolated, stained with an anti-V5-FITC Ab, and analyzed by flow cytometry. MFI is indicated. D, Histograms of transfected cells described in C.
for heterodimerization with CD94, we measured total NKG2A expression and NKG2A-V5 expression in the presence of increasing amounts of NKG2A competitor. In fact, the total NKG2A (untagged NKG2A competitor plus NKG2A-V5) on the cell surface remained relatively constant, whereas the cell surface NKG2A-V5 expression decreased in the presence of competitor NKG2A (data not shown). In addition, although the preferential ability of NKG2A to pair with CD94 for cell surface expression is seen most clearly in the setting of limiting quantities of CD94 (Fig. 2A), the preferential heterodimerization and subsequent cell surface expression of NKG2A can also be seen with nonlimiting concentrations of CD94 (Fig. 2B). At very high concentrations of CD94, the preferential heterodimerization and cell surface expression of NKG2A was not seen (data not shown). Therefore, the results of the competition experiments described above reflect CD94/NKG2 heterodimerization. It is also important to note that, due to variation between experiments, it is only appropriate to compare results between groups within a single experiment.

We next sought to determine whether the findings in this study for selected NKG2A and NKG2C alleles could be extended to other NKG2A and NKG2C alleles of the rhesus monkey. Cells were co-transfected with pNKG2A*01-V5, limiting amounts of pCD94, and increasing amounts of DNA encoding each of three rhesus monkey NKG2A alleles. Twenty-four hours posttransfection, cells were stained with a FITC-conjugated anti-V5 Ab and assessed by flow cytometry. Two of the alleles, NKG2A*01 and NKG2A*02, were comparably efficient in competing with NKG2A*01-V5 for heterodimerization and cell surface expression with CD94. The third allele, NKG2A*04, competed more efficiently with NKG2A*01-V5 for surface expression with CD94 than NKG2A*01 or NKG2A*02 (Fig. 3A).

In parallel experiments, we assessed the ability of three NKG2C alleles to compete with NKG2A*01-V5 for binding and surface expression.

**FIGURE 2.** Effect of varying the concentration of CD94 on the competition of NKG2A and NKG2C for cell surface expression. A, Competition of CD94/NKG2A*01-V5 expression with either NKG2A*01 or NKG2C*04 competitor, at a limiting concentration of CD94. 293T cells were cotransfected with 0.1 μg of pCD94, 1 μg of pNKG2A*01-V5, and 2 μg of untagged pNKG2A*01 or pNKG2C*04 competitor, or 4 μg of untagged pNKG2A*01 or pNKG2C*04 competitor. Twenty-four hours posttransfection, cells were isolated, stained with an anti-V5-FITC Ab, and analyzed by flow cytometry. Results are plotted as a percentage of the baseline MFI. Baseline is defined as the MFI of cells transfected with no untagged NKG2A*01 and is set at 100%. B, Competition of CD94/NKG2A*01-V5 expression with either NKG2A*01 or NKG2C*04 competitor, at a nonlimiting concentration of CD94. 293T cells were cotransfected with 0.25 μg of pCD94, 1 μg of pNKG2A*01-V5, and 2 μg of untagged pNKG2A*01 or pNKG2C*04 competitor. Twenty-four hours posttransfection, cells were isolated, stained with an anti-V5-FITC Ab, and analyzed by flow cytometry. MFI is indicated.

**FIGURE 3.** Effect of various NKG2A and NKG2C alleles on CD94/ NKG2A*01-V5 expression. A, Competition of CD94/NKG2A*01-V5 expression with three NKG2A alleles. 293T cells were cotransfected with 0.1 μg of pCD94, 1 μg of pNKG2A*01-V5, and increasing amounts of DNA encoding three untagged NKG2A alleles. Twenty-four hours posttransfection, cells were isolated, stained with an anti-V5-FITC Ab, and analyzed by flow cytometry. Baseline is defined as the MFI of cells transfected with no untagged NKG2A*01 and is set at 100%. B, Competition of CD94/NKG2A*01-V5 expression with three untagged NKG2C alleles. Experiments were conducted as described in A. C, Competition of CD94/NKG2A*01-V5 expression with NKG2A*02, NKG2A*04, and NKG2C*02. CD4 was included as a negative control. Experiments were performed as described in A.
expression with CD94. Cells were transfected with pNKG2A*01-V5, limiting amounts of pCD94, and increasing amounts of DNA encoding each of three rhesus monkey NKG2C alleles. As shown in Fig. 3B, rhesus monkey NKG2C*02, NKG2C*03, and NKG2C*04 were comparably inefficient in competing with NKG2A*01-V5 for heterodimerization and cell surface expression with CD94.

These studies suggested that the biologic properties of NKG2A*01 and NKG2C*04 required for heterodimerization and cell surface expression with CD94 are, in fact, representative of the properties of the other NKG2A and NKG2C alleles. To confirm the differences between the NKG2A and NKG2C alleles in their heterodimerization with CD94 and cell surface expression, 293T cells were cotransfected in the same experiment with pNKG2A*01-V5, limiting amounts of pCD94, and pNKG2A*04, or pNKG2C*02 competitor. A plasmid encoding untagged CD4 was similarly assessed in this experiment as a negative control molecule. Both NKG2A*02 and NKG2C*04 efficiently competed with NKG2A*01-V5 for cell surface expression (Fig. 3C). As shown previously, untagged NKG2A*04 was better able to compete for cell surface expression with NKG2A*01-V5 than NKG2A*02. Importantly, both NKG2A alleles were better able to compete for NKG2A*01-V5 surface expression than NKG2C*02. The negative control molecule CD4 did not compete with NKG2A*01-V5 for surface expression when added to the transfections in small quantities, but equaled NKG2C*02 in its efficiency for competition at high concentrations. This finding suggested that, at high concentrations, the ability of NKG2C to compete for surface expression with CD94 might be nonspecific. Taken together, these data demonstrated that the three NKG2C alleles tested were better able to compete for cell surface expression with CD94 than the three NKG2C alleles tested.

The effect of DAP12 on NKG2A and NKG2C surface expression with CD94

Because it has previously been shown that NKG2C cell surface expression is increased in the presence of DAP12 through interactions between charged residues in the transmembrane domains of these molecules, we determined whether DAP12 expression would improve the ability of NKG2C to compete with NKG2A for surface expression with CD94. To assess this possibility, we generated a FLAG-tagged rhesus monkey DAP12 construct and confirmed its expression following transfection into 293T cells by cell surface staining (data not shown). To determine the amount of DAP12 DNA to use in the planned experiments, we examined the ability of DAP12 to regulate the cell surface expression of NKG2A and NKG2C. 293T cells were cotransfected with plasmids expressing CD94, either NKG2A*01-V5 or NKG2C*04-V5, and increasing amounts of DAP12 DNA. Twenty-four hours posttransfection, cells were isolated and stained with an anti-V5-FITC Ab, and analyzed by flow cytometry. As shown in Fig. 4A, NKG2A*01-V5 cell surface expression remained constant in the presence of increasing quantities of DAP12. However, the expression of NKG2C*04-V5 dramatically increased in the presence of even small quantities of DAP12.

Having thus confirmed that DAP12 specifically up-regulates NKG2C surface expression without nonspecifically altering NKG2A surface expression in this assay, we evaluated the relative ability of NKG2A and NKG2C to compete for heterodimerization and cell surface expression with CD94 in the presence of DAP12. 293T cells were cotransfected with pNKG2A*01-V5, limiting quantities of pCD94, a fixed, excess quantity of untagged pNKG2A*01 or pNKG2C*04 competitor, and increasing quantities of pDAP12. The NKG2C*04 plasmid cotransfected with increasing quantities of CD4 DNA served as a negative control for the effects of DAP12. The total quantity of DNA in each transfection was kept constant by the addition of empty vector DNA. As shown in Fig. 4B, the addition of increasing quantities of DAP12 dramatically enhanced the ability of NKG2C*04 to compete with NKG2A*01-V5 for cell surface expression. In fact, this NKG2C molecule competed with NKG2A*01-V5 for expression as efficiently in the presence of DAP12 as the NKG2A molecule did in the absence of DAP12. This effect of DAP12 was specific, because the addition of increasing quantities of the control CD4 did not significantly enhance the ability of NKG2C*04 to compete with NKG2A*01-V5 for surface expression. As expected, the addition of increasing quantities of DAP12 had no effect on the ability of NKG2A*01 to compete for cell surface expression with NKG2A*.
01-V5. Thus, although NKG2A molecules are intrinsically better able than NKG2C molecules to reach the cell surface as heterodimers with CD94, the presence of DAP12 enhances the ability of NKG2C molecules to compete for heterodimerization and cell surface expression with CD94 to levels equal to or greater than NKG2A molecules. The molecular interaction with DAP12 may therefore provide an additional level of regulation of NKG2C competition for cell surface heterodimerization with CD94.

**Chimeric NKG2A/NKG2C molecules**

Rhesus monkey NKG2A and NKG2C molecules can be distinguished by amino acid residues in their cytoplasmic, transmembrane, and stalk domains. We reasoned that one or more of these residues must be responsible for the differential abilities of NKG2A and NKG2C to bind to CD94 and reach the cell surface. To explore this possibility, we generated chimeric rhesus monkey NKG2A*02/NKG2C*04 molecules to localize these regions.

We first generated NKG2A/NKG2C (A/C) and NKG2C/NKG2A (C/A) chimeras at the transmembrane/stalk junction and assessed these chimeric molecules for their ability to compete with NKG2A*01-V5 for cell surface expression with CD94 (Fig. 5A). Cells were cotransfected with pNKG2A*01-V5, a limiting quantity of pCD94, and two quantities of plasmid encoding each untagged NKG2 molecule. Both NKG2C*04 and the C/A TM/Stalk chimera were relatively inefficient in competing with NKG2A*01-V5 for cell surface expression. NKG2A*02 was more effective than these other two molecules in competing for cell surface expression and heterodimerization with CD94. However, the A/C TM/Stalk chimera was better able than NKG2A*02 to compete with NKG2A*01-V5 for surface expression. This observation indicated that the NKG2A*04 stalk and/or lectin domain may enhance the ability of the NKG2A*02 molecule to compete for cell surface CD94 heterodimerization. Furthermore, these data suggested that the differential ability of NKG2A and NKG2C to compete for cell surface expression as a heterodimer with CD94 maps to the cytoplasmic and/or transmembrane portions of the molecules.

To define further the critical domains of the NKG2 molecules, we generated two additional chimeras, pA/C Cyt/TM and pA/C Stalk/Lectin, and assessed their ability to compete with NKG2A*01-V5 for cell surface expression. As shown in Fig. 5B, NKG2C*04 and A/C Cyt/TM were relatively inefficient in their ability to compete with NKG2A*01-V5 for cell surface expression with CD94. NKG2A*02 and A/C Stalk/Lectin were more efficient than both NKG2C*04 and A/C Cyt/TM in competing for cell surface expression with CD94. This experiment demonstrated that the transmembrane and/or stalk regions are important in determining the cell surface expression of the NKG2 molecule. Taken together, the data shown in Fig. 5, A and B, suggest that the transmembrane region determines the differential ability of NKG2A or NKG2C to compete with NKG2A-V5 for CD94 binding and cell surface expression. Furthermore, the stalk and/or lectin domains of NKG2C may enhance the ability of NKG2A to heterodimerize with CD94.

Eleven amino acid residues differentiate the transmembrane regions of rhesus monkey NKG2A*02 and NKG2C*04 (Fig. 5C). These amino acid changes are clustered into four different regions, one of which includes a threonine-to-lysine change. Because this lysine residue in the NKG2C transmembrane is essential for the molecule’s interaction with the aspartic acid residue in the transmembrane domain of DAP12 (25), we postulated that this cluster of amino acids might also be important in determining the differential ability of rhesus monkey NKG2A and NKG2C molecules to compete with NKG2A*01-V5 for CD94 heterodimerization and cell surface expression. To assess this possibility, we generated gene constructs with point mutations in this region of the transmembrane domains of NKG2A*02 and NKG2C*04. The four amino acids in NKG2A were changed to the corresponding residues in NKG2C and vice versa. As shown in Fig. 5D, NKG2A*02 TLKT (S86T, V88L, T89K, I90T) behaved comparably to NKG2C*04 in a competition experiment. Thus, the four amino acid mutations S86T, V88L, T89K, and I90T were sufficient to change the phenotype of NKG2A*02 to that of NKG2C*04 in this assay. In addition, NKG2A*02 and NKG2C*04 SVTI (T86S, L88V, K89T, T90I) were both better able than NKG2C*04 to compete with NKG2A*01-V5 for CD94 heterodimerization and cell surface expression. As seen with other chimeras containing an NKG2A transmembrane domain, NKG2C*04 SVTI was able to compete better than NKG2A*02 for surface expression with CD94.

Because the lysine residue in the transmembrane region of NKG2C is essential for the interaction with DAP12, we were interested in determining whether the lysine/threonine residue in the NKG2 transmembrane domain was responsible for differentiating the phenotypes of NKG2A and NKG2C. To this end, we generated gene constructs with single mutations in the transmembrane (T/K) region through site-directed mutagenesis and tested their ability to compete with NKG2A*01-V5 for cell surface heterodimer formation. As shown in Fig. 5E, NKG2A*02 K TM behaved similarly to NKG2C*04 in competition experiments. Thus, the single threonine-to-lysine change in the transmembrane region of NKG2A was sufficient to change the phenotype of NKG2A*02 to that of NKG2C*04. In addition, the NKG2C*04 T TM mutant had an increased ability to compete with NKG2A*01-V5 for cell surface expression with CD94. As seen with other NKG2C*04 transmembrane mutants, NKG2C*04 T TM was actually better able to compete for cell surface heterodimer expression than the wild-type NKG2A*02.

Although the lysine/threonine in the transmembrane region is an important determinant of the ability of the NKG2 molecules to compete for cell surface expression with CD94, the data generated in these studies suggested that other regions of these molecules also play an important role in this function. The data shown in Fig. 5 indicate that any chimeric molecule with an NKG2C transmembrane region behaves like NKG2C. However, some chimeric molecules with an NKG2C transmembrane region appeared to be better able to compete with NKG2A*01-V5 for cell surface expression than the wild-type NKG2A*02. To evaluate this phenomenon in greater detail, we performed a competition experiment with a panel of chimeric molecules, each of which contained the four NKG2A-specific amino acids (SVTI) in their transmembrane regions. As suggested from the data shown in Fig. 5, replacing the NKG2A*02 lectin domain with that of the NKG2C*04 molecule resulted in a subtle enhancement of the ability of NKG2A*02 to compete for heterodimerization and cell surface expression with CD94 (Fig. 5B and data not shown). A much more striking enhancement was observed with the A/C TM/Stalk chimera, a molecule in which the NKG2A*02 stalk and lectin domains were replaced with those of NKG2C*04 (Fig. 5A and data not shown). No additional enhancement of this phenotype was seen by further replacement of NKG2A*02 domains with regions of NKG2C*04. These data suggest that, if an NKG2 molecule contains the NKG2A*02-specific SVTI residues in its transmembrane domain, the ability of the molecule to compete for surface expression with CD94 can be substantially enhanced by the addition of an NKG2C*04 stalk region.

Interestingly, as shown in Fig. 5A, the addition of the NKG2A*02 stalk domain to an NKG2C*04 transmembrane domain in a chimeric molecule (NKG2C/A TM/Stalk) did not decrease the ability of that molecule to compete for cell surface expression with CD94. We reasoned that a molecule containing the transmembrane...
The domain of NKG2C*04 may compete so poorly for CD94 heterodimerization and cell surface expression that an inhibitory effect of the NKG2A*02 stalk cannot be detected. There are a number of amino acid residues that differentiate the stalk of NKG2A*02 from that of NKG2C*04, including a 2-aa deletion/insertion. Furthermore, the cysteine required for disulfide bond formation is present at the stalk junction.

**FIGURE 5.** Competition of CD94/NKG2A*01-V5 surface expression by NKG2A*02/NKG2C*04 chimeric molecules. A, Competition of CD94/NKG2A*01-V5 expression by NKG2A*02, NKG2C*04, and NKG2A*02/C*04 transmembrane/stalk junction chimeras. 293T cells were cotransfected with 0.1 μg of pCD94, 1 μg of pNKG2A*01-V5, and 2 or 4 μg of pNKG2A*02, pNKG2C*04, pNKG2A*02/C*04 TM/Stalk, or pNKG2C*04/A*02 TM/Stalk. Twenty-four hours posttransfection, cells were isolated, stained with an anti-V5-FITC Ab, and analyzed by flow cytometry. Baseline is defined as the MFI with no untagged NKG2 competitor and is set at 100%. B, Competition of CD94/NKG2A*01-V5 expression with NKG2A*02, NKG2C*04, and NKG2A*02/NKG2C*04 cytoplasmic/transmembrane and stalk/lectin junction chimeras. Experiments were conducted as described in A. C, Amino acid alignment of the transmembrane domains of NKG2A*02 and NKG2C*04. Identical amino acids are indicated by a period. Amino acid residues mutated in the generation of the chimeric A*02 TLKT or C*04 SVTI molecules are indicated by an asterisk. The charged lysine in the transmembrane domain of NKG2C*04 that is essential for the interaction with DAP12 is shown in bold.

D, Competition of CD94/NKG2A*01-V5 expression with NKG2A*02, NKG2C*04, and the point mutants NKG2A*02 S86T V88L T89K I90T and NKG2C*04 T86S L88V K89T T90I. Experiments were conducted as described in A. E, Competition of NKG2A*01-V5 expression with NKG2A*02, NKG2C*04, and the single point mutants NKG2A*02 K TM, and NKG2C*04 T TM. Experiments were conducted as described in A.
formation between an NKG2 molecule and CD94 is located in the stalk region of the NKG2 molecule. Although the transmembrane domains of the NKG2A alleles have features that are more favorable than those of the NKG2C alleles for association with CD94, the stalk regions of the NKG2C molecules may have a structure that allows for more efficient disulfide bond formation with CD94.

**Chimeric NKG2A*04/NKG2A*02 molecules**

Having characterized these expression level differences between the NKG2A and NKG2C molecules, we were also interested in evaluating possible differences in cell surface expression between the various rhesus monkey NKG2A alleles (Fig. 3A). NKG2A*02 can be distinguished from NKG2A*04 by 9 aa in the cytoplasmic, 2 aa in the transmembrane, 2 aa in the stalk, and 8 aa in the lectin domains. To determine which amino acid residues account for the cell surface expression level differences between NKG2A*02 and NKG2A*04, we generated and evaluated a series of NKG2A/A chimeric DNA constructs. As shown in Fig. 6A, we created NKG2A/A transmembrane/stalk junction chimeras and tested their ability to compete with NKG2A*01-V5 for cell surface expression with CD94. NKG2A*02 and the A*02/A*04 TM/Stalk chimera showed a comparable ability to compete with NKG2A*01-V5 for surface expression. The other chimera, A*04/A*02 TM/Stalk, was better able to compete for surface expression with CD94 and behaved like the NKG2A*04 allele. Therefore, we can conclude that important determinants of the NKG2A allele differences are located in the cytoplasmic and/or transmembrane domains of the molecules.

We next generated NKG2A/A chimeras at the junction between the cytoplasmic and transmembrane domains of the alleles. The untagged NKG2A*02 and the A*04/A*02 Cry/TM chimera were comparable in their ability to compete for heterodimerization and cell surface expression with CD94. Furthermore, both NKG2A*04 and the A*02/A*04 Cry/TM chimera were better able to compete with NKG2A*01-V5 for cell surface expression with CD94 than NKG2A*02 (Fig. 6B). Taken together, the data shown in Fig. 6A and B, suggest that the amino acid residues that distinguish NKG2A*02 and NKG2A*04 in their ability to compete with NKG2A*01-V5 for surface expression in CD94 are located in the transmembrane domains of the molecules.

To demonstrate formally that the transmembrane domain is the essential determinant of the functional difference between the NKG2A alleles, we generated chimeric molecules that contain the transmembrane domain of one allele and the cytoplasmic, stalk, and lectin domains of the other allele. Because the transmembrane domains of NKG2A*02 and NKG2A*04 differ at only two amino acid residues, we performed double mutagenesis to change both residues in the transmembrane domains. As shown in Fig. 6C, NKG2A*02 and the A*04 w/A*02 TM chimera both competed equally well with NKG2A*01-V5 for surface expression. In addition, NKG2A*04 and the A*02 w/A*04 TM chimera were both better able to compete with NKG2A*01-V5 for CD94 heterodimerization and surface expression than NKG2A*02. Therefore, the data shown in Fig. 6, A–C, demonstrate that the transmembrane domains of NKG2A*02 and NKG2A*04 determine their different abilities to bind to CD94 and reach the cell surface.

As stated previously, the transmembrane domains of NKG2A*02 and NKG2A*04 differ at only two amino acid residues. To determine whether one or both of these residues are important in determining the phenotypic differences between NKG2A*02 and NKG2A*04, we generated point mutations in the transmembrane domains of each allele. As illustrated in Fig. 6D, we used site-directed mutagenesis to generate molecules with an NKG2A*02 backbone and the corresponding NKG2A*04 amino acid at either residue 82 or 87 of the transmembrane domain. These constructs were then tested for their ability to be expressed on the cell surface in association with CD94. As expected, NKG2A*04 was better able than NKG2A*02 to compete with NKG2A*01-V5 for cell surface expression. Interestingly, constructs containing either the single point mutation A*02 R82V or A*02 V87L showed an intermediate phenotype in this assay, suggesting that both amino acid residues in the transmembrane domain confer the increased ability of NKG2A*04 to compete with NKG2A*01-V5 for cell surface expression.

Conversely, we generated and tested mutants that contained the NKG2A*04 backbone and the corresponding NKG2A*02 amino acid at either residue 82 or 87 of the transmembrane domain. These two constructs containing the mutations A*04 V82I or A*04 L87V were able to compete with NKG2A*01-V5 for CD94 surface expression as well as the wild-type NKG2A*04 allele (Fig. 6E). These data also suggest that the amino acid residues at both positions 82 and 87 of the transmembrane domains of the molecules are required to change the functional properties of NKG2A*04 to those of NKG2A*02. Thus, as seen in studies of the NKG2A/C chimeras, the determinants of the capability of the NKG2A alleles to heterodimerize with CD94 and be expressed on the cell surface map to the transmembrane regions of the molecules.

**Discussion**

Many multimeric proteins with distinct functions consist of a single common polypeptide subunit that can oligomerize with members of a diverse group of polypeptides. For example, some cytokine receptors, such as those for IL-2 and IL-15, share a common γ-chain but have distinct functions determined by their α- and β-chains (26). The human DNA mismatch repair system, important in maintaining genomic stability, includes functionally distinct heterodimeric complexes composed of three different chains, each of which binds to a single conserved chain (27). The pairing of the members of the CD94/NKG2 family of molecules to form functionally distinct heterodimers therefore has many biologic precedents.

There is also precedent for the findings in the present study that the regulation of heterodimeric complex formation can determine important functional characteristics of a cell. For instance, apoptosis is regulated by homo- and heterodimeric pairing of members of the Bcl-2 family of proteins (28, 29). Furthermore, nuclear import is regulated by the formation of two different heterodimeric protein complexes that share a common subunit, the karyopherin αβ heterodimer and the karyopherin β/Ran-GTP heterodimer (30). Analogous to these systems, the regulation of cell surface expression of the CD94/NKG2A and CD94/NKG2C molecules likely contribute to determining the activation state of an NK cell.

The transmembrane domains of protein chains have been shown to be important in the formation of many heterodimeric complexes. The transmembrane domains of the hepatitis C virus E1 and E2 envelope glycoproteins are necessary for their heterodimerization (31, 32). In addition, transmembrane domains are necessary for the interactions of chains of the MHC class II molecules, the TCR, and glycoporphin A molecules (33–36). In fact, charged residues in the transmembrane domains of the TCR/CD3 receptor complex are important for their interactions (34, 37). In the present studies, we did not formally examine the contributions of various domains of NKG2 molecules to their ability to form heterodimers. Rather, we evaluated the importance of specific domains of NKG2A and NKG2C in their ability to differentially reach the cell surface in association with CD94. In testing chimeric molecules generated by swapping corresponding regions of NKG2A and NKG2C, we
demonstrated that a single residue in the transmembrane domains of NKG2 proteins is important for the preferential ability of NKG2A to reach the cell surface with CD94. Interestingly, the particular amino acid residue within the transmembrane domains of NKG2A (T) and NKG2C (K) is fully conserved between rhesus monkeys, great apes, and humans, suggesting that these domains are critically important in the regulation of this receptor family in primates.

We have also shown in the present studies that the NKG2 stalk region can modify the contribution of the transmembrane domain to the cell surface expression of NKG2 molecules. These findings are consistent with those of Boyington et al. (38) demonstrating the

**FIGURE 6.** Analysis of NKG2A*02/NKG2A*04 chimeras. A, Competition of CD94/NKG2A*01-V5 expression by NKG2A*02, NKG2A*04, NKG2A*02/A*04 TM/Stalk, and NKG2A*04/A*02 TM/Stalk. 293T cells were transfected with 0.1 μg of pCD94, 1 μg of pNKG2A*01-V5, and 2 or 4 μg of DNA encoding untagged NKG2 molecules. Twenty-four hours posttransfection, cells were isolated, stained with an anti-V5-FITC Ab, and analyzed by flow cytometry. Baseline is defined as the MFI with no untagged NKG2A and is set at 100%. B, Competition of CD94/NKG2A*01-V5 expression by NKG2A*02, NKG2A*04, NKG2A*02/A*04 Cyt/TM, and NKG2A*04/A*02 Cyt/TM. C, Competition of CD94/NKG2A*01-V5 expression by NKG2A*02, NKG2A*04, NKG2A*02 w/A*04 TM, and NKG2A*04 w/A*02 TM. D, Competition of CD94/NKG2A*01-V5 expression by NKG2A*02, NKG2A*04, NKG2A*02 I82V, and NKG2A*02 V87L. E, Competition of CD94/NKG2A*01-V5 expression by NKG2A*02, NKG2A*04, NKG2A*04 V82I, and NKG2A*04 L87V.
importance of the stalk domain in dimer formation by bacterially expressed extracellular CD94. In addition, CD94 crystal structure data suggest that residues in the lectin domains of the chains are important for CD94/NKG2 dimerization, implying that the transmembrane domains may not be necessary for heterodimerization per se (39). However, the transmembrane domains are likely important in stabilizing the cell surface expression of CD94 and NKG2.

Because the presence of DAP12 has been shown to enhance CD94/NKG2C cell surface expression, we also analyzed the regulation of CD94/NKG2 pair formation and cell surface expression in the presence of DAP12. We demonstrated that, in the presence of excess DAP12, the ability of NKG2A and NKG2A to preferentially reach the cell surface shifted slightly in favor of NKG2C. We do not know the steady-state level of cell surface DAP12 protein expression in NK cells and CD8+ T lymphocytes in a normal monkey or human. Therefore, we cannot predict whether in normal physiologic conditions we expect high levels of DAP12 expression to drive increased expression of CD94/NKG2C on the surface of lymphocyte populations. Moreover, because mAbs have not yet been generated that can differentiate NKG2A and NKG2C molecules in the monkey, we do not know whether the default state of an NK or CD8+ T cell is one that favors NKG2A expression or one in which NKG2A and NKG2C expression are comparable. Nevertheless, because it has been shown that stimulation of murine myeloblastic leukemia (M1) cells by LPS resulted in a dramatic increase in DAP12 expression per cell, we expected high levels of DAP12 expression to result in increased expression of CD94/NKG2C on the surface of cells of an animal expressing an alternate NKG2A allele such as NKG2A*04 that reaches the cell surface with an especially high efficiency may in turn affect the ability of NKG2C molecules to reach the cell surface, ultimately affecting the capacity of these cells to be triggered to mediate lytic activity. It will therefore be important to determine whether the expression of particular NKG2A alleles by cells of an individual affect that individual’s ability to contain tumors or infection by particular microbes.

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