Rat and Mouse CD94 Associate Directly with the Activating Transmembrane Adaptor Proteins DAP12 and DAP10 and Activate NK Cell Cytotoxicity

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Signaling by the CD94/NKG2 heterodimeric NK cell receptor family has been well characterized in the human but has remained unclear in the mouse and rat. In the human, the activating receptor CD94/NKG2C associates with DAP12 by an ionic bond between oppositely charged residues within the transmembrane regions of NKG2C and DAP12. The lysine residue responsible for DAP12 association is absent in rat and mouse NKG2C and -E, raising questions about signaling mechanisms in these species. As a possible substitute, rat and mouse NKG2C and -E contain an arginine residue in the transition between the transmembrane and stalk regions. In this article, we demonstrate that, similar to their human orthologs, NKG2A inhibits, whereas NKG2C activates, rat NK cells. Redirected lysis assays using NK cells transfected with a mutated NKG2C construct indicated that the activating function of CD94/NKG2C did not depend on the transmembrane/stalk region arginine residue. Flow cytometry and biochemical analysis demonstrated that both DAP12 and DAP10 can associate with rat CD94/NKG2C. Surprisingly, DAP12 and DAP10 did not associate with NKG2C but instead with CD94. These associations depended on a transmembrane lysine residue in CD94 that is unique to rodents. Thus, in the mouse and rat, the ability to bind activating adaptor proteins has been transferred from NKG2C/E to the CD94 chain as a result of mutation events in both chains. Remarkable from a phylogenetic perspective, this sheds new light on the evolution and function of the CD94/NKG2 receptor family.

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NK cells recognize and kill tumor cells, infected cells, and MHC class I disparate hematopoietic cells (1–4). Unlike B and T cells, NK cell specificity is provided by an array of inhibitory and activating receptors coexpressed on the same cell. It is believed that the balance of activating and inhibitory signals, as well as the nature of the signals, determines the NK cell response (5). Inhibitory receptors contain ITIMs that recruit and activate protein tyrosine phosphatases upon phosphorylation. Activating receptors signal by binding to activating transmembrane adaptor proteins. Four such adaptor proteins have been found to bind NK cell receptors: DAP12, DAP10, FcεRIγ, and CD3ζ (6). The cytoplasmic regions of DAP12, FcεRIγ, and CD3ζ contain ITAMs necessary for recruitment and activation of Syk (and ZAP70) tyrosine kinases. The cytoplasmic region of DAP10 contains a tyrosine-based motif (YINM) that recruits the p85 subunit of PI3K, as well as Grb2 (7).

Stable associations between activating receptors and transmembrane adaptor proteins typically depend on ionic bonds between a positively charged residue (lysine or arginine) present in the transmembrane region of the receptor and a negatively charged residue (aspartate) within the transmembrane region of the adaptor protein (6). Although these adaptor proteins may associate with several different receptors, individual receptors usually bind selectively to one (or, in some cases, two) adaptor proteins. The oppositely charged residues are typically juxtaposed within their respective transmembrane regions. The aspartate residue is centrally located within the transmembrane regions of DAP12 and DAP10 but closer to the extracellular surface in FcεRIγ and CD3ζ. Consequently, DAP12 and DAP10 associate with receptors that have a positively charged residue that is more centrally located, whereas FcεRIγ and CD3ζ bind receptors harboring the charged residue closer to the cell surface. There are also other features within the transmembrane region that influence the specificity of activating receptors and transmembrane adaptor proteins (8, 9), but these features are poorly understood.

The killer cell lectin-like receptors (KLRs) are type II transmembrane receptors with extracellular C-type lectin-like domains, encoded by the NK cell gene complex. KLRs are found in all mammalian species and include several families. In rodents, the Ly49 (KLRA), NKR-P1 (KLRB), NKG2 (KLRC), and KLRI families have multiple members, whereas NKG2D (KLRK), CD94 (KLRD), KLRH, and KLRE are single-member families (10). Most of the KLRs are expressed as homodimers. The exceptions are CD94, which forms disulphide-linked heterodimers with members of the NKG2 family (11), as well as the more recently identified KLRE/I1 and -2 heterodimeric receptors (12). The inhibitory CD94/NKG2A receptor, expressed on a majority of NK cells and subsets of T cells, maintains tolerance toward cells expressing HLA-E, its nonclassical MHC class I ligand (13–16). Increasing evidence supports a role for the activating CD94/NKG2C and -E receptors in virus infections (17–19). In the human, CD94 does not have any known signaling motifs. Human NKG2A has two ITIM motifs in the cytoplasmic region, and CD94/NKG2A heterodimers function as inhibitory receptors. In contrast, NKG2C and NKG2E both have a positively charged lysine residue within the transmembrane region allowing the formation of an ionic bond with the negatively charged aspartate

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Abbreviation used in this article: KLR, killer cell lectin-like receptor.

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residue of DAP12. The formation of this bond is necessary for stable surface expression of activating CD94/NKG2C receptor complexes (20, 21). Similar to human, mouse CD94 associates with NKG2A, NKG2C, or NKG2E to form functional heterodimeric NK cell receptor complexes (14, 22). However, little is known about CD94/NKG2C receptor signaling in rodents.

In this study, by comparing the transmembrane region sequences of CD94 and NKG2C, we find that mouse and rat CD94 differ markedly from other mammalian orthologs by the presence of a lysine residue in the transmembrane region. Moreover, rat and mouse NKG2C and -E lack the transmembrane lysine residue necessary for DAP12 interaction in the human. In transfection experiments, we show that rat CD94 directly associates with DAP10, as well as DAP10, and that these interactions depend critically on the presence of the lysine residue within the transmembrane region of CD94. Finally, cytoxicity assays provided evidence that the resulting rat CD94/NKG2C receptor activates NK cell cytotoxicity and that the activation is mediated through the CD94 chain.

Materials and Methods

Sequence analysis

Genomic or EST sequence information of mammalian CD94 and NKG2 family members was obtained using search algorithms and genome browser applications at the Ensembl (http://www.ensembl.org) and National Center for Biotechnology Information/GenBank (http://www.ncbi.nlm.nih.gov) Web sites. Sequence analysis was performed using the GCG program package (Accelrys) and ClustalX. Transmembrane region topology prediction was performed with TMpred (23), TMHMM (24, 25), and TopPred (25).

Expression constructs and transfections

Kozak sequence and open reading frame of rat NKG2A, rat NKG2C, and mouse and rat CD94 (all excluding stop codons) were amplified by PCR and inserted into pECHA in frame with a C-terminal HA tag sequence (26). For 293T cell transfections, double-expression constructs were generated in pBudCE4.1 (Invitrogen) by inserting a rat CD94-HA fragment (with a C-terminal HA tag) under the EF-1α promoter and NKG2A or NKG2C fragments (untagged) under the CMV promoter. Single-expression constructs carrying CD94-HA, NKG2A, or NKG2C were also generated. Mutated versions of mouse and rat CD94-HA (replacing lysine residue 19 with isoleucine), as well as rat NKG2C and NKG2C-HA (replacing arginine residue 86 with isoleucine), were generated by site-directed mutagenesis (Quick Change; Stratagene). Open reading frame minus leader sequence of rat CDAP10, DAP12, FcɛRIγ, and CD3ε were inserted into pCMV-FLAG (Sigma-Aldrich) in frame with the N-terminal leader and FLAG epitope sequence.

Transient transfection of 293T cell transfections, 7 µg DNA was mixed with 32 µg polyethylenimine (Polyscience) in 0.5× PBS (pH 7.4). After a 25-min incubation at room temperature, DNA/polyethylenimine complexes were added directly to a 25-cm² flask culture of 293T cells growing in 7 ml complete medium as a 50-80% confluent monolayer. Cells were harvested for flow cytometry and biochemical analyses 36–48 h after transfection.

For stable transfections, 5 × 10⁶ RNK-16 cells were mixed with 25 µg linearized plasmid at 4°C in complete medium (RPMI 1640 with 1 mM sodium pyruvate, 1% antibiotic/antimycotic solution, 10% FCS [all from Invitrogen], and 5 × 10⁻⁵ M 2-ME) and electroporated in a 2-mm cuvette at 120 V, 960 µF (GenePulsor; Bio-Rad Laboratories). Stably transfected cells were selected by cultivation in complete medium supplemented with 2 µg/ml Geneticin (G-418 disulfate; Duchaef Biochemie) and tested for gene expression by flow cytometry.

Flow cytometry

A total of 0.5 × 10⁶ cells was incubated with anti-HA (10 µg/ml HA.11; Covance Research Products), anti-FLAG (2 µg/ml M2; Sigma-Aldrich), anti-rat NKG2A/C/E (2 µg/ml, WEN28, P.C. Saether, S. Fossum, and E. Dissen, unpublished observations), anti-rat NKp46 (2 µg/ml, WEN23 (27)), or anti-rat CD48 (2 µg/ml, OX45 (28)) primary mAb in a total volume of 50 µl at 4°C for 30 min, washed three times, and incubated with FITC-conjugated Fab’ (1 µg/ml), fragments of polyclonal sheep anti-mouse IgG serum (Sigma-Aldrich). Samples were analyzed with FACSCalibur or FACSCanto II flow cytometers using CellQuest Pro, FACSDiva (BD Biosciences), and FlowJo v7.5 (Tree Star) software.

Biochemistry

Transiently transfected 293T cells were lysed in 1% Digitonin (Calbiochem), 0.1% Igepal CA-630 (Sigma-Aldrich), 20 mM Tris-HCl, 150 mM NaCl, and protease/phosphatase inhibitors (1:100, Halt Protease and Phosphatase Inhibitor Cocktail; Thermo Scientific) at 4°C for 30 min. For immunoprecipitations, lysates were either incubated with anti-FLAG mAb-conjugated agarose beads (EZview anti-FLAG M2 affinity gel; Sigma-Aldrich) for 2 h or 3 µg anti-rat NKG2A/C/E mAb (WEN28) for 25 min, followed by incubation with protein G-coupled magnetic beads (Dynabeads; Invitrogen) for 45 min. All incubations were performed with constant rotation at 4°C. Protein samples were subjected to SDS-PAGE under reducing conditions and transferred onto polyvinylidene difluoride membranes (Immobilon P; Millipore). Membranes were probed with either 1 µg/ml rabbit anti-FLAG Ab (Sigma-Aldrich) or 0.2 µg/ml rabbit anti-HA Ab (Santa Cruz Biotechnology), followed by incubation with HRP-conjugated sheep anti-rabbit Ig (Jackson ImmunoResearch Laboratories).

Cytotoxicity assays

Standard 4-h redirected cytoxicity assays were set up as follows: 5 × 10⁶ P388D1, P815, or YAC-1 target cells were labeled with 100 µCi Na₂¹⁷⁷Cl for 2 h at 37°C, washed three times in PBS, resuspended to 10⁸ cells/ml in complete medium, and added to 96-well plates at 100 µl/well. Untransfected or stably transfected effector RNK-16 cell lines were preincubated in complete medium with 2 µg/ml anti-HA (HA.11; Covance), 2 µg/ml anti-rat NKG2A/C/E (WEN28), 2 µg/ml anti-rat NKp46 (WEN23), 2 µg/ml anti-rat CD48 (OX45), all mouse IgG1 mAbs, or no Ab for 15 min at room temperature and mixed with target cells in different ratios, as indicated. After a 4-h incubation at 37°C, the supernatants were harvested and counted in a gamma counter (Minaxi Auto Gamma 5000 Series; Packard). Spontaneous release was between 5 and 10% for all experiments. Specific lysis was calculated from median values, as previously described (29).

Results

Sequence comparison of the human and rodent CD94 and NKG2 transmembrane regions suggests different signaling mechanisms

The assembly and signaling properties of the human CD94/NKG2A and CD94/NKG2C receptors have been well characterized. However, little is known about how rodent CD94/NKG2 receptors regulate NK cells. Peptide sequence analysis of mouse and rat CD94 and NKG2A, -C, and -E suggests that these receptors may associate differently with adaptor proteins compared with their human orthologs. Both mouse and rat CD94 have a positively charged lysine residue located in the center of the transmembrane region (30), a feature not seen in other species investigated (Fig. 1A). Moreover, the transmembrane lysine residue critical for association of human NKG2C and -E with DAP12 is absent in the mouse and rat (14, 31) (Fig. 1B). Instead, the mouse and rat NKG2C and -E transmembrane regions contain a positively charged arginine residue located close to the extracellular surface, suggesting that these receptors do not associate with DAP12 (Fig. 1B).

Requirements for mouse and rat CD94 cell surface expression

To investigate the requirements for mouse and rat CD94 cell surface expression, we generated expression constructs encoding mouse or rat CD94 with extracellular HA epitope tags (CD94-HA). When stably transfected into the rat NK cell line RNKDA1, rat CD94-HA was readily detected on the cell surface using an anti-HA mAb in flow cytometry analysis (Fig. 2A). In contrast, neither mouse nor rat CD94-HA was detected on the cell surface of transiently transfected 293T cells (Fig. 2B). In this respect, mouse and rat CD94 differ from human CD94, which is readily expressed on the cell surface of transiently transfected 293T cells (11). Together, these observations suggested that the surface expression of mouse and rat CD94 depends on association with molecules present in NK cells but absent in 293T cells. Because surface expression of many
activating NK cell receptors requires association with adaptor protein (probably due to masking of the positively charged residue in the transmembrane region of the receptor), we tested whether the presence of the charged transmembrane lysine residue hindered cell surface expression of rodent CD94. Indeed, both mouse and rat CD94 were expressed on the cell surface of transfected 293T cells if the transmembrane lysine was mutated to the electrostatically neutral isoleucine (CD94K19I-HA) (Fig. 2B). This suggests that both mouse and rat CD94 associate directly with one or several transmembrane proteins expressed in NK cells but absent in 293T cells. The arginine residues of mouse and rat NKG2C and -E reside in a region (light gray background) that most likely is external to the plasma membrane (different transmembrane topology-prediction algorithms give different results).

To search for transmembrane adaptor proteins that would induce surface expression of rat CD94, we transfected 293T cells with expression constructs encoding rat CD94-HA together with different NK cell adaptor proteins: DAP10, DAP12, FcεRIg, and CD3ζ (all with extracellular FLAG epitope tags). Flow cytometry analysis demonstrated cell surface expression of CD94 in the presence of DAP10 or DAP12 (Fig. 3). In contrast, cotransfecting with FcεRIg-FLAG, CD3ζ-FLAG, or a combination of FcεRIg-FLAG plus CD3ζ-FLAG did not induce rat CD94 cell surface expression. All of the adaptor proteins were expressed on the cell surface (Fig. 3). These results indicated that rat CD94 may associate directly with both DAP10 and with DAP12.
Surface expression of rat CD94 is influenced by coexpression of rat NKG2A or NKG2C. NKG2A expression was detected using a newly generated mAb specific for rat NKG2A,-C, and -E (P.C. Saether, S. Fossum, and E. Dissen, unpublished observations). In these experiments, NKG2A induced bright cell surface expression of wild-type CD94 (Fig. 4). Notably, the K19I-mutated CD94 was expressed at a still brighter level than wild-type CD94 in double transfections with NKG2A (Fig. 4). Similar to NKG2A, NKG2C induced surface expression of CD94, albeit at a lower level compared with NKG2A. Surface expression of CD94 and NKG2C was markedly enhanced when using the K19I-mutated CD94 construct (Fig. 4). Similar results were obtained for NKG2E (data not shown). In single transfections, rat NKG2A was readily expressed on the cell surface alone, whereas NKG2C displayed modest cell surface expression (Fig. 4). To investigate the role of the transmembrane arginine residue on cell surface expression of rat and mouse NKG2C, we generated an expression construct in which this residue had been mutated to isoleucine (NKG2CR86I). The mutated protein was not expressed at a higher level than NKG2C in single transfections nor in double transfections with CD94-HA or CD94K19I-HA. Together, these data suggested that surface expression of rat CD94/NKG2 receptors requires a transmembrane arginine residue to juxtapose to mask the positively charged transmembrane lysine residue of rat and mouse CD94. Moreover, these observations demonstrated that the transmembrane/stalk arginine residue of NKG2C does not appear to affect the efficiency of surface expression.

**Rat CD94 coinmunoprecipitates with both DAP12 and DAP10**

To assess association between rat CD94 and adaptor proteins more directly, we performed immunoprecipitation experiments with 293T cells double transfected with CD94-HA and selected FLAG-tagged adaptor proteins. Using an anti-FLAG Ab, CD94 precipitated together with DAP12 or DAP10 but not with FcεRIγ. Although CD94 appeared to bind DAP12 more efficiently than DAP10 in these experiments, the results demonstrated a direct association between rat CD94 and DAP12, as well as between CD94 and DAP10 (Fig. 5). This is consistent with the flow cytometry analysis showing that DAP12 and DAP10 both can induce surface expression of CD94 (Fig. 3).

To clarify whether association with NKG2 molecules influenced the association between CD94 and adaptor proteins, 293T cells were triple transfected with combinations of expression constructs encoding CD94-HA, NKG2A, or NKG2C together with FLAG-tagged adaptor proteins. Transfected cells were then lysed and subjected to immunoprecipitation. In parallel, efficient transfection and surface expression of all proteins were verified in flow cytometry analyses (data not shown). Immunoprecipitation experiments using an anti-FLAG mAb demonstrated that wild-type CD94 efficiently precipitated together with DAP12 but not together with FcεRIγ (Fig. 6A). In contrast, K19I-mutated CD94 could not be immunoprecipitated together with DAP12. Similarly, wild-type CD94, but not the mutated CD94K19I variant, was detected in DAP10 immunoprecipitates (Fig. 6B). To confirm that the precipitated receptor complexes were indeed trimeric complexes of CD94, NKG2C, and adapter protein (and not merely dimers of CD94 plus adaptor protein), immunoprecipitation experiments with triple-transfected 293T cells were performed using the anti-rat NKG2A/C/E-specific mAb. In these experiments, CD94, in addition to either DAP12 or DAP10, precipitated together with NKG2C (Fig. 7). Importantly, wild-type CD94 and K19I-mutated CD94 were coprecipitated with NKG2C at seemingly equal levels, whereas coprecipitation of DAP12 or DAP10 was not observed with K19I-mutated CD94. To investigate the possible influence of the NKG2C transmembrane/stalk arginine residue in the assembly of DAP12 or DAP10 together with CD94/ NKG2C, we performed anti-FLAG immunoprecipitation experiments with NKG2CR86I. Efficient precipitation of CD94 was observed in the absence of the NKG2C R86 residue, both in DAP12 and DAP10 cotransfections (Fig. 6). Together, these data demonstrated that the direct associations between rat CD94 and the adaptor proteins DAP12 and DAP10 both critically depend on...
the positively charged lysine residue within the CD94 transmembrane region. Furthermore, both DAP12 and DAP10 associated with CD94 in the presence of NKG2A, as well as NKG2C. These observations suggested that mouse and rat CD94/NKG2 receptors form trimeric receptor complexes consisting of either DAP12 or DAP10, together with CD94/NKG2A or CD94/NKG2C heterodimers. Furthermore, the association between CD94 and adaptor protein did not rely on the positively charged arginine residue within the NKG2C transmembrane/stalk region.

**FIGURE 6.** The rat CD94 transmembrane lysine residue is critical for association with DAP12 and DAP10. Western blot analyses under reducing conditions. 293T cells were mock transfected (EV) or triple transfected with expression constructs encoding DAP12-FLAG (A) or DAP10-FLAG (B), together with combinations of CD94-HA or CD94K19I-HA and NKG2A, NKG2C, or NKG2CR86I. FcεRIg-FLAG was used as a negative control for DAP12-FLAG and DAP10-FLAG. Transfected cells were lysed and subjected to immunoprecipitations with anti-FLAG mAb. Anti-HA and anti-FLAG Abs were used for Western blot detection of CD94-HA and FLAG-tagged adaptor proteins, respectively. Relative masses (kDa) are indicated.

**FIGURE 7.** Rat DAP12-CD94 and DAP10-CD94 are expressed as multimeric complexes including NKG2 receptor chains. Western blot analyses under reducing conditions. 293T cells were mock transfected (EV) or triple transfected with CD94-HA or CD94K19I-HA, together with NKG2C and DAP12-FLAG or DAP10-FLAG, lysed, and subjected to anti-NKG2A/C/E immunoprecipitations. Precipitated CD94-HA and FLAG-tagged adaptor proteins were detected by anti-HA and anti-FLAG Abs, respectively. Relative molecular mass is indicated.

**FIGURE 8.** The rat CD94/NKG2C heterodimer displays a slight, but nondominant, intrinsic preference for association with DAP12 over DAP10. 293T cells were mock transfected (EV) or transfected with CD94-HA and NKG2C, together with combinations of DAP12-FLAG and DAP10-FLAG expression constructs in different molar ratios, as indicated, and then subjected to immunoprecipitation with anti-NKG2A/C/E mAb, followed by Western blot analysis under reducing conditions. DAP12 and DAP10 were detected with anti-FLAG Ab. Whole-cell lysate (WCL) was used as control of the overall DAP10-FLAG and DAP12-FLAG expression in the transfected cells. The migration of DAP12-FLAG and DAP10-FLAG is indicated with arrows.
To examine whether CD94/NKG2 receptors preferentially associate with either DAP12 or DAP10, we transfected 293T cells with CD94 and NKG2C, as well as five molar ratios of DAP12 to DAP10 (w/w DAP12 and DAP10 expression plasmids). Both DAP12 and DAP10 were found to coimmunoprecipitate together with CD94/NKG2C at all ratios tested (Fig. 8). Although the experimental results displayed a tendency toward greater amounts of coimmunoprecipitated DAP12 compared with DAP10, they did not support a dominant intrinsic preference for DAP12 over DAP10.

Rat NKG2A inhibits, whereas NKG2C activates, NK cell cytotoxicity

To evaluate the role of CD94/NKG2 receptor complexes in regulating NK cell cytotoxicity, we stably transfected the rat NK cell line RNK-16 with expression constructs encoding rat NKG2A or NKG2C containing extracellular HA epitope tags. Individual clones were tested for NKG2A-HA and NKG2C-HA cell surface expression in flow cytometry analyses, as well as their ability to kill standard target cells (Fig. 9A, data not shown). NKG2A-HA⁺, NKG2C-HA⁺, and untransfected RNK-16 cells were incubated with anti-HA or anti-NKG2A/C/E mAbs before incubation with the FcR⁺ target cell line P388D1 in redirected cytotoxicity assays. Both anti-HA and anti-NKG2A/C/E mAbs reduced cytotoxicity toward P388D1 targets when using NKG2A-HA⁺ transfectants as effector cells (Fig. 9B). In contrast, incubating NKG2C-HA⁺ effector cells with the same mAbs enhanced killing of P388D1 cells (Fig. 9C). These results are in agreement with previous observations describing the function of human NKG2C and NKG2A (20) and demonstrated that rat NKG2C enhances NK cell cytotoxicity, whereas rat NKG2A induces inhibition of target cell killing.

**FIGURE 9.** Rat NKG2C activates NK cell cytotoxicity independent of the transmembrane/stalk region arginine residue. A and D, Flow cytometry analyses of RNK-16 cells (rat NK cell line) stably transfected (Tx) with expression constructs encoding rat NKG2A-HA or NKG2C-HA (A) or a mutated expression construct in which the NKG2C transmembrane/stalk region arginine residue has been replaced by isoleucine (NKG2CR86I-HA) (D). Surface expression of transfected receptors was detected using an anti-HA mAb (solid lines); RNK-16 cells endogenously express CD94/NKG2 receptors, as detected by the anti-rat NKG2A/C/E mAb (dotted lines). Surface expression of CD48 (dashed lines; A) and Nkp46 (long dashed lines; D) was detected using anti-rat CD48 mAb or anti-rat Nkp46 mAbs, respectively (used as isotype-matched negative and positive control mAbs in the redirected cytotoxicity experiments, see below). B and C, Redirected cytotoxicity assay using transfected or untransfected RNK-16 cells. The effector cells were preincubated with anti-HA (○) or anti-rat NKG2A/C/E (□) mAbs before incubation with P388D1 (FcR⁺) target cells. Preincubation with no mAb (●) and anti-rat CD48 mAb (■) served as negative controls. The FcR⁻ cell line YAC-1 was used as negative control target cells. E, Redirected cytotoxicity assay against P815 (FcR⁺) target cells using RNK-16 cells transfected with mutated NKG2CR86I-HA or untransfected RNK-16 cells, as indicated. Preincubation with Abs indicated as in B and C. Effector cell preincubation with mAb against the activating NK cell receptor Nkp46 served as positive control (▲). E:T ratios are presented on the horizontal axis, and specific lysis is presented as the percentage of specific [⁵¹Cr] release on the vertical axis. Median values from one representative experiment (out of at least two) are shown. Similar observations were made with at least two additional independent transfected clones for NKG2A-HA, NKG2C-HA, and NKG2CR86I-HA.
Rat NKG2C-mediated activation does not depend on the transmembrane/stalk region arginine residue

The functional role of the positively charged arginine residue located between the transmembrane and stalk region of rat and mouse NKG2C and -E has not been investigated. Therefore, we generated RNK-16 clones stably transfected with a mutated construct encoding rat NKG2CR86I-HA (Fig. 9D). In redirected cytotoxicity assays, engaging this receptor with anti-HA or anti-NKG2A/C/E mAbs induced killing of the FcR+ target cell line P815 (Fig. 9E). This demonstrated that the ability of rat CD94/NKG2C to induce cytotoxicity does not depend on the NKG2C transmembrane/stalk region arginine residue. Instead, these data supported that the activating function of rat CD94/NKG2C is mediated by activating adaptor proteins linked directly to the CD94 chain (Fig. 10).

Discussion

In this study, we set out to investigate the functional role of the charged lysine residue in the transmembrane regions of rat and mouse CD94, not seen in any other species investigated thus far. Unfavorable in the hydrophobic lipid environment of the plasma membrane interior, as a rule, charged residues are neutralized by salt bridge formation with oppositely charged residues situated in other transmembrane regions, stabilizing the complex formation between immunoreceptors and activating adaptor proteins (9). Indeed, the experimental data gathered in our study demonstrated that the CD94 lysine residue hinders surface expression of CD94, unless it is paired with the oppositely charged aspartic acid residues of the activating adaptor proteins DAP12 and DAP10. Flow cytometry and coimmunoprecipitation data indicated that rat and mouse CD94/NKG2C heterodimers bind efficiently to both DAP12 and DAP10 and that this binding is critically dependent on the transmembrane lysine residue of CD94. Thus, two alternative trimeric receptor complexes can exist side by side: CD94/NKG2C/DAP12 and CD94/NKG2C/DAP10. In the same manner, our observations indicated the parallel existence of CD94/NKG2A/DAP12 and CD94/NKG2A/DAP10 receptor complexes (Fig. 10). These findings were unexpected. First, our data indicated that the transmembrane regions of rat and mouse CD94 bind directly to adaptor protein, in remarkable contrast to human CD94/NKG2C, which instead binds DAP12 via the transmembrane region of NKG2C (21). Second, trimeric CD94/NKG2A/DAP10 or CD94/NKG2A/DAP12 receptors have not been described in the human, where charged residues to support such interactions are lacking.

These differences suggest that CD94/NKG2 receptors may regulate NK cells differently in mice and rats compared with humans. The signaling capacities of rat and mouse NKG2C and -E had not previously been investigated. Our experiments demonstrated that rat NKG2C is an activating receptor, triggering NK cell cytotoxicity in redirected lysis experiments. In these experiments, the activation signals were most likely transmitted via CD94 (endogenously expressed CD94 in complex with transfected NKG2C-HA) bound to DAP12 or DAP10 or a combination of these.

The transmembrane regions of rat and mouse NKG2C and -E all lack the centrally located lysine residue seen in most other species, including humans. In contrast, these four rodent NKG2 chains all contain a surface-proximal arginine residue, previously suggested to substitute for the centrally located lysine in binding to an activating adaptor protein (14, 31). Our data demonstrated that the surface-proximal arginine residue of CD94/NKG2C did not influence binding to DAP12 or DAP10, as demonstrated by mutation analysis. Similarly, redirected cytotoxicity assays with a mutated NKG2C chain (R86I) demonstrated that the activating role of the rat CD94/NKG2C heterodimer is not dependent on the arginine residue. Still, this residue is conserved between rat and mouse NKG2C and -E, suggesting some functional significance.

**FIGURE 10.** Schematic model of CD94/NKG2 receptor assembly in the human compared with the mouse and rat. Mouse and rat CD94 harbor a positively charged (lysine) residue centrally located in the transmembrane region that is absent in humans. Moreover, mouse and rat NKG2C lack the transmembrane lysine residue responsible for DAP12 association in human NKG2C. In this model, based on our observations in this study, rat and mouse CD94 directly associate with DAP12 and DAP10 instead of indirectly via NKG2C, as is the case in humans. Notably, mouse and rat CD94/NKG2A differ from the corresponding human receptor in simultaneously holding the structural motifs capable of engaging both activation (ITAM) and inhibition (ITIM) pathways.
As demonstrated above, mutating the arginine did not have any effect on surface expression. Although it is commonly observed that activating receptors with surface-proximal arginine residues that pair with FcεR1γ or CD3ζ can be readily expressed in the absence of a partner adaptor molecule, rat NKG2C did not bind to FcεR1γ in these experiments (Fig. 6), and NKG2C surface expression was not enhanced in cotransfections with transmembrane adaptor proteins (data not shown). We cannot completely rule out the possibility that the surface-proximal arginine residue of rat and mouse NKG2C and -E stabilizes the binding to an as-yet-unidentified fourth partner in the receptor complex. As suggested by transmembrane topology prediction algorithms, it seems more likely that this arginine residue is not embedded in the membrane but instead marks the boundary between the extracellular and transmembrane regions (Fig. 1B).

Biochemical analyses from transfected 293T cells demonstrated that rat CD94/NKG2A receptors associate with either DAP12 or DAP10 (Fig. 10). The resulting receptor complexes would have the potential both to inhibit and activate NK cell effector functions. This is not without precedence: cattle NKG2A7 contains two ITIM and a positively charged transmembrane residue (32); the same is true for several rat Ly49 receptors (33) and KIR2DL4 in primates (34). A priori, ligand engagement of a CD94/NKG2A/ DAP12 receptor could lead to recruitment and activation of tyrosine phosphatases, resulting in inhibition, but also to phosphorylation of the DAP12 ITAM motifs, leading to recruitment and activation of Syk and finally resulting in activation of effector functions. Similarly, a CD94/NKG2A/DAP10 receptor complex would have the potential both to recruit phosphatases and to engage the p85 subunit of PI3K and Grb2. In our cytotoxicity assays, using NKG2A-HA–transfected RNK-16 clones as effector cells, engagement of rat NKG2A led to inhibition of NK cell killing of target cells. This indicated that rat NKG2A is able to recruit and activate protein tyrosine phosphatases, although the sequence (IVYADF) of the membrane-proximal of its two ITIM diverges from the core definition of the canonical ITIM motif (V/ILxYxxLxV). In support of this observation, mouse NK cells displayed reduced cytotoxicity toward target cells expressing QA-1, the ligand for mouse CD94/NKG2A (14). The observed inhibitory effect of engaging NKG2A may reflect that inhibition dominates and blocks activation in the possible bifunctional rat/mouse CD94/NKG2A receptor. Alternatively, the signaling outcome may depend on cell type, activation status of the cell or local conditions in the immune synapse, such as ligand concentration, avidity, coengagement of other receptors, and associated kinases and phosphatases.

The inhibition observed in the NKG2A-HA–redirected cytotoxicity assays could have been mediated by NKG2A-HA in the form of homodimers. Biochemical analyses demonstrated that NKG2A was expressed as a disulfide-linked homodimer in 293T cells transfected with NKG2A-HA alone (data not shown). Both mouse and rat NKG2A-HA were readily surface expressed in single transfections of 293T cells, as well as CHO cells (Fig. 4, data not shown). Moreover, mouse NKG2A-HA could be expressed on the cell surface of transfected COS-7 cells (14). Thus, the NKG2A-transfected NK cells used in the cytotoxicity experiments may have expressed a combination of NKG2A in complex with endogenous CD94, as well as NKG2A in homodimeric form. However, surface expression of NKG2A was not detectable on NK cells from CD94-deficient mice (35, 36), suggesting that expression of NKG2A homodimers are a result of overexpression in transfected systems. To investigate the signaling role of rat CD94/NKG2A further, IL-2–activated splenic NK cells were incubated with the anti-NKG2A/C/E mAb in redirected lysis assays. No significant effect was observed (data not shown). Further experiments are warranted to explore, in more detail, the possibly bifunctional potential of CD94/NKG2A in mouse and rat, but they await Abs that discriminate between CD94/NKG2A and CD94/NKG2C or targeted gene disruptions of Nkg2a or Nkg2c together with Nkg2e.

Until recently, the short isoform of mouse NKG2D was the only NK cell receptor known to associate with both DAP12 and DAP10 (37, 38). Domain-mapping studies demonstrated that structures in the transmembrane region of mouse NKG2D allow association with both DAP12 and DAP10, whereas human NKG2D is incapable of associating with DAP12 (39). More recently, studies using DAP12−, DAP10−, and DAP12/DAP10 double-deficient mice demonstrated that Ly49H and Ly49D can signal through DAP10, in addition to DAP12 (40, 41). In this article, we showed that yet another KLR family, rat CD94/NKG2, associates with both DAP12 and DAP10 in transfected cells. CD94/NKG2C demonstrated no dominant intrinsic preference for either adaptor protein. The observations that several activating NK cell receptors may signal through both adaptor proteins has led to speculation of additive or synergistic effects. Signal pathways from DAP12 and DAP10 display both distinct and overlapping characteristics (6). DAP12 has an ITAM motif in the cytoplasmic region that binds the tyrosine kinases Syk and ZAP-70, whereas the YINM motif in the DAP10 cytoplasmic region binds either the p85 subunit of PI3K or Grb2 upon phosphorylation. In functional studies, triggering DAP12-associated receptors induced both NK cell cytotoxicity and cytokine secretion (42). In contrast, engagement of mouse NKG2D/DAP10 complexes induced cytotoxic responses but inefficient cytokine production (43). It is possible that engagement of both DAP12− and DAP10-signaling pathways in some instances is necessary for optimal effector responses and that this “two-signal” requirement can be fulfilled by CD94/NKG2 alone, as a single receptor. Further studies are required to understand the significance of signaling through both DAP12 and DAP10 by the same NK cell receptor, including the mouse and rat CD94/NKG2 receptors.

The arrangement in mouse and rat differs from other mammals and has resulted from at least two independent genetic events: the DAP12-binding capacity of NKG2C and -E has been lost by the loss of a centrally located transmembrane lysine residue. Reciprocally, CD94 has gained the ability to bind DAP12 (and DAP10) by the introduction of a transmembrane lysine residue. One can only speculate about the selective forces that brought about this rare example of evolutionary reshuffling of functions between heterodimeric partner chains. In any respect, the findings presented in this article should inspire a closer comparison of the human and rat/mouse CD94/NKG2 receptor families and their functional roles in regulating the effector functions of a majority of NK cells, as well as subsets of T cells.

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Disclosures
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