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Cloning of a mouse homolog of CD94 extends the family of C-type lectins on murine natural killer cells

Two families of major histocompatibility complex (MHC) class I-specific receptors are found on natural killer (NK) cells: immunoglobulin-like receptors and C-type lectin receptors. In mice, the latter category is represented by the Ly49 family of receptors, whereas in humans, NK cells express the distantly related CD94, which forms MHC class I-specific heterodimers with NKG2 family members. Humans also express the MHC class I-specific p50/p58/p70 family of immunoglobulin-like receptors, but these have not been identified in mice. Hence, there is no known instance of an MHC class I-specific receptor that is expressed by both human and murine NK cells. Here we report the cloning of CD94 from the CB.17 and C57BL/6 strains of mice. Mouse CD94 is 54 % identical and 66 % similar to human CD94, and is also a member of the C-type lectin superfamily. Mouse CD94 is expressed efficiently on the cell surface of cells transiently transfected with the corresponding cDNA, but surface CD94 was unable to mediate detectable binding to MHC class I-expressing ConA blasts. Notably, mouse CD94, like human CD94, has a very short cytoplasmic tail, suggesting the existence of partner chains that may play a role in ligand binding and signaling. Like many other C-type lectins expressed by NK cells, mouse CD94 maps to the NK complex on distal chromosome 6, synteneic to human CD94. We also demonstrate that mouse CD94 is highly expressed specifically by mouse NK cells, raising the possibility that mice, like humans, express multiple families of MHC class I-specific receptors on their NK cells. Murine homologs of human NKG2 family members have not yet been identified, but we report here the existence of a murine NKG2D-like sequence that also maps to the murine NK complex near CD94 and Ly49 family members.

1 Introduction

An "enigma" [1] of recent NK cell biology has been the phylogenetic relationship between MHC class I-specific receptors on human and murine natural killer cells. Human NK cells express two main types of MHC class I-specific receptors: the Ig-like p50/p58/p70 family of receptors, and the lectin-like CD94/NKG2 family of receptors (for recent reviews, see [2–4]). Murine NK cells, on the other hand, express a distinct family of MHC class I-specific lectins, the Ly49 family, which bears only about 25 % homology to the human CD94/NKG2 receptors [5–8]. Murine MHC class I-specific, Ig-like receptors homologous to the human p50/p58/p70 receptors have not been identified, although the distantly related mouse gp49 receptor may be a candidate [9, 10]. Thus, we are left with a situation, puzzling from an evolutionary standpoint, in which human and murine NK cells appear to be regulated by unique families of MHC class I-specific receptors.

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Abbreviations: **A-LAK:** Adherent lymphokine-activated killer cell **EST:** Expressed sequence tag **YAC:** Yeast artificial chromosome **HA:** Hemagglutinin

Key words: CD94 / Natural killer cell / Mouse / Major histocompatibility complex class I

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Human CD94 was first identified by monoclonal antibodies developed by López-Botet and colleagues [11]. A considerable body of evidence has since accumulated to implicate CD94 in HLA class I recognition [12–14], but the consequences of CD94 ligation were complex [15, 16]: anti-CD94 mAb activated some NK clones, whereas other clones from the same donor were inhibited or unaffected. The ability of CD94 to transmit signals was also puzzling, given the absence of a significant cytoplasmic domain [17].

Some recent work has added to our understanding of MHC class I-specific lectins on human NK cells. NKG2 family members, first identified in a differential hybridization screen [18], are believed to form signaling heterodimers with CD94 [19–21]. NKG2A/CD94 heterodimers appear to transmit inhibitory signals, whereas NKG2C/CD94 heterodimers transmit activating signals [22]. Little is known about NKG2D, except that it is only distantly related to other NKG2 receptors ([18] and Fig. 2B), and may, therefore, not be a *bona fide* NKG2 family member. The structural heterogeneity in CD94/NKG2 complexes appears to underlie the previously observed functional heterogeneity. The MHC class I specificity of the NKG2/CD94 heterodimers seems to be relatively broad and reportedly includes recognition of HLA-A, -B and -C alleles [12, 14, 23]. The relative contributions of CD94 and NKG2 to MHC class I binding have not yet been determined. Diverse biological roles for MHC class I recognition by the CD94/NKG2 complex on human NK cells have been suggested by recent studies [24, 25].

Here we report the cloning of murine CD94 from two inbred strains of mice. Mouse CD94 is 54 % identical and

66% similar to human CD94 at the amino acid level, a degree of cross-species similarity in line with other members of the C-type lectin family. Like human CD94, mouse CD94 has a very short cytoplasmic tail, suggesting the existence of partner chains (possibly mouse NKG2 molecules) which may play a role in signaling. Southern blot analysis suggests that mice possess only one CD94-like sequence, which we have physically linked to the Ly49 locus within the NK complex [26–28] on mouse chromosome 6, syntenic to human CD94. We further report the existence of a mouse NKG2D-like sequence that also maps to the NK complex. The mouse CD94 message is abundantly expressed in NK cells and not elsewhere, suggesting that it may indeed be a functional counterpart of human CD94. The identification of mouse CD94 will allow CD94 function to be examined in the murine system.

2 Materials and methods

2.1 Cloning of murine CD94

The human CD94 protein sequence (GenBank accession number U30610) was used to search the expressed sequence tag (EST) database for homologous sequences, using the XREFdb website (http://www.ncbi.nlm.nih.gov/XREFdb). A mouse EST (GenBank accession number AA161895) was identified which contains a stretch of sequence with high homology ($p = 2.2 \times 10^{-21}$) to human CD94. The entire clone (I.M.A.G.E. Consortium Clone ID 598616; [29]) from which the EST was derived was sequenced, but was not found to contain a complete open reading frame exhibiting homology to the full length of human CD94. However, the 3′ end of the clone did contain a small open reading frame that exhibited homology to the 3′ end of human CD94. Thus, it was hypothesized that the clone represented an incompletely spliced transcript that contained the 3′ exons of CD94 cDNA fused to an intronic sequence. To obtain a full-length cDNA, 100 ng of a CB.17 SCID lymphocyte plasmid cDNA library [30], the kind gift of P. Mathew and V. Kumar (Houston, TX), was used as a template for a 100-μl PCR reaction containing Taq polymerase (Promega), a 3′ primer specific for the 3′ end of the cDNA (primer AA161Xho/Not = 5′-tactcgagcggccgcATTTTTGCTTCATCTGAAC-3′) and a 5′ primer specific for the plasmid cloning site (primer pME18S-3′Cla = 5′-ccatcgatGAATTCCGGAATTTCC-TCG-3′). A somewhat diffuse band about 1 kb in length was amplified by this reaction, and was subsequently cloned by digestion with NotI and ClaI (New England Biolabs) into pBluescript SKII(−) (Stratagene). Six independent clones from two independent PCR reactions were sequenced using an automated sequencer (Applied Biosystems, Foster City, CA) to arrive at a consensus sequence. Three clones (clones 1, 10 and 21) exhibited a structure similar to the original I.M.A.G.E. clone and contained a 5′ intronic sequence fused to 3′ CD94 exons. Three other cDNA clones (clones 5, 24 and 28) appeared to be correctly spliced, full-length homologs of human CD94.

To clone the B6 cDNA of CD94, 3 μg of total B6 A-LAK RNA (gift from J. Dorfmann) was reverse transcribed in a 20 μl volume using an oligo-$(dT)_{15}$ primer (Boehringer Mannheim) and M-MLV reverse transcriptase (Gibco BRL) and then amplified in ten parallel reactions using PCR and two mouse CD94-specific primers [5′ primer mCD94#3Xho = 5′-atctcgagACACATCTCTGCACCAT-CCTTGG-3′ and 3′ primer AA161Xho/Not (above)]. The PCR product was sequenced directly to compensate for errors introduced by Taq polymerase and was cloned into the XhoI and NotI sites of pBluescript SKII(−).

2.2 Southern, Northern and PCR analysis

For Southern blotting, tail DNA was prepared from various strains of inbred mice (Jackson Labs, Bar Harbor, ME) by overnight digestion in proteinase K (Boehringer Mannheim) followed by phenol:chloroform extractions and ethanol precipitation. Total genomic DNA from *Mus spretus* (SPRET/Ei) mice was obtained from Jackson Labs. Ten micrograms of each sample was digested electrophoresed in 1% agarose (Boehringer Mannheim), exposed to UV light to fragment the DNA and then transferred in 0.4 M NaOH by capillary action onto Hybond Nylon membrane (Amersham). Membranes were hybridized overnight with a random primed ^{32}P-labeled probe generated by PCR from the 3′ end of mouse CD94 (bp 313–618), washed three times at 65°C in 2 × SSC/1% SDS and once at 65°C in 0.5 × SSC/1% SDS, and then exposed to film for 1–3 days at −70°C.

For Northern blotting, total RNA was isolated according to manufacturer's instructions with the Ultraspec RNA isolation kit (Biotecx Labs, Houston, TX). Approximately 10 μg of RNA was electrophoresed in a 1% agarose/formaldehyde gel, essentially as described by Maniatis [31]. RNA was transferred to Hybond Nylon membrane (Amersham) in 20 × SSC, and then cross-linked by exposure to UV light. RNA blots were then treated essentially as described above for the DNA blots, except that stripping was performed by incubating the blot in 94% formamide/1% SDS at 68°C for 2 h, followed by a 30-min wash in 0.1 × SSC/1% SDS, also at 68°C.

PCR was generally conducted using Taq polymerase (Promega) according to the manufacturer's instructions. Annealing was performed at 55°C for 1 min, extension was at 72°C for 30 s and denaturation was at 95°C for 1 min, for a total of 35 cycles using a Perkin Elmer thermal cycler. PCR from genomic templates was performed using a "hot start" protocol, which involved addition of Mg^{2+} after heating the samples to 99°C for 2 min, just prior to the commencement of normal thermal cycling. Primers used for the analysis of the predicted intron 3/exon 4 boundary of CD94 (see text) were as follows: 5′ primer AA161-5′#1 = 5′-TTCTCAAGTTGTTCATGCCC-3′ and 5′ primer AA161-3′#2 = 5′-GGATTCCTTT-TAAATATGTTG-3′ recognize putative intronic sequences in intron 3 of mouse CD94. 3′ primer mCD94-3′#1 = 5′-atggatccGCATTCCAATCCAGAAAAAGG-3′ recognizes bp 338–358 within exon 4 of mouse CD94. Primer AA161-3′#1 = 5′-GAACATGCTCTGAGGGCAAC-3′ was used in mapping CD94 (see below).

2.3 Mapping of CD94 to distal mouse chromosome 6

A panel of B6 yeast artificial chromosomes (YAC) [32] shown to hybridize to Ly49-specific oligo probes (D. M. T.

and D. H. R., unpublished results) were screened by PCR (using primers AA161-5′#1 and AA161-3′#1) for the presence of CD94 intronic sequence. Two overlapping YAC of approximately 360 kb and 630 kb produced PCR products of the predicted size. The PCR product was sequenced to confirm its identity. To confirm the presence of CD94 exons, the two positive YAC were digested with a panel of restriction enzymes, Southern blotted and hybridized to the CD94 cDNA probe.

2.4 Expression of epitope-tagged CD94 in COS cells

A hemagglutinin (HA) epitope-tagged version of mouse CD94 was generated by PCR using a 5′ primer containing a XhoI site and a Kozak sequence fused to the first 23 bp of the CD94 open reading frame (primer mCD94-Xho-Koz = 5′-atctcgagccaccATGGCAGTTTCTAGGATCAC-TCG-3′) and a 3′ primer (primer mCD94-3′HA-Bam) which replaces the presumed stop codon of CD94 with the HA epitope (YPYDVPDYA), followed by a stop codon and a BamHI site: 5′-atggatcctcaggcgtagtcgggca-cgtcgtaggggtaAATAGGCAGTTTCTTACAGATG-3′. A CB.17 cDNA clone (clone 28) was used as the template for the PCR reaction, and the resulting PCR product was cloned by XhoI/BamHI digestion into pBluescriptSKII(−) and then subcloned (using XhoI and NotI) into a pME18S expression vector that had been modified to contain a XhoI site. The final construct (called pME-CD94HA) was confirmed by automated sequencing on both strands.

COS-7 cells were transfected with supercoiled DNA (Qiagen) using the Lipofectamine reagent (Gibco BRL) according to the manufacturer's instructions. After 2 days, expression of CD94HA was assessed by staining of transfectants and controls with anti-HA (Babco, Berkeley, CA) or PK136 (mouse isotype control) followed by PE-conjugated donkey anti-mouse IgG (Jackson). To assess adhesion of CD94 to different MHC class I molecules, a semi-quantitative adhesion assay was developed (T. H. and D. H. R., unpublished results). Briefly, ConA blasts from splenocytes of various mouse strains expressing different H-2 antigens were [^{3}H]thymidine labeled and incubated with adherent COS-7 cells that had been transfected with pME-CD94HA or other pME-Ly49 constructs as controls. After 2 h incubation at 37 °C degrees, nonadherent cells were removed by five washes with prewarmed medium. The remaining cells in the well were lifted with trypsin/EDTA, lysed, and their radioactivity determined with a β-counter.

3 Results and discussion

3.1 EST-based cloning of mouse CD94 from CB.17 SCID

By making use of a growing EST database, a mouse cDNA clone (I.M.A.G.E. Consortium Clone ID 598616) was identified which exhibited homology to the 3′ end of human CD94. This clone did not, however, appear to encode an open reading frame for a full-length mouse homolog of CD94 and in particular did not contain a putative transmembrane segment or a signal sequence. The possibility that the clone was derived from an incompletely spliced pre-mRNA was investigated by using a PCR strategy designed to amplify and clone the 5′ ends of additional CD94-like sequences from a mouse CB.17 SCID plasmid cDNA library (see Sect. 2.1). A total of six clones were obtained and sequenced, three of which appeared to encode a full-length mouse homolog of human CD94. The consensus CB.17 cDNA sequence of CD94 was obtained by sequencing both strands of these three clones (derived from two independent PCR reactions) and is shown in Fig. 1A. The cDNA appears to encode a protein of 179 amino acids that contains motifs conserved by all members of the C-type lectin family. Like human CD94 [17], mouse CD94 has only a short cytoplasmic tail (10 amino acids) which is probably not sufficient for mediating signal transduction.

The mouse CD94 sequence was aligned to the human CD94 sequence and to other NK lectin sequences from both mice and humans (see Fig. 2A, B). The results indicated that the sequence we had obtained was indeed most homologous to human CD94 (54% identical and 66% similar), and exhibited a degree of inter-species similarity in line with other members of the C-type lectin family (for example CD69).

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-49                TTAAAAAGC ACAAACACAT CTCTGCACCA TCCTTGGAAC ATCACTTCTC

1    ATG GCA GTT TCT AGG ATC ACT CGG TGG AGA CTG ATG TCT GTC ATC TTT
1    Met Ala Val Ser Arg Ile Thr Arg Trp Arg Leu Met Ser Val Ile Phe

49   GGA ATA AAA TGT CTT TTC TTG ATG GTT ACT TTG GGA GTT TTG CTG ATA
17   Gly Ile Lys Cys Leu Phe Leu Met Val Thr Leu Gly Val Leu Leu Ile

97   AAT TCA TTT ACT ATA CAG AAC ATT CAA TCA ACA CCT TCT CCA ACC ACC
33   Asn Ser Phe Thr Ile Gln Asn Ile Gln Ser Thr Pro Ser Pro Thr Thr

145  ACT GTA GAA TTC CAG GAA GTT TCT GAA TGC TGT GTT TGC CTG GAC AAG
49   Thr Val Glu Phe Gln Glu Val Ser Glu Cys Cys Val Cys Leu Asp Lys

193  TGG GTT GGG CAT CAG TGC AAT TGT TAC TTT ATT TCC AAA GAA GAA AAG
65   Trp Val Gly His Gln Cys Asn Cys Tyr Phe Ile Ser Lys Glu Glu Lys

                A
241  TCT TGG GAA AGA AGC AGA GAT TTC TGT GCT TCG CAG AAT TCC AGC CTT
81   Ser Trp Glu Arg Ser Arg Asp Phe Cys Ala Ser Gln Asn Ser Ser Leu
           Lys
                                        ▽
289  CTT CAG CCC CAA TCC AGA AAT GAA TTG AGT TTT ATG AAC TTC AGT CAA
97   Leu Gln Pro Gln Ser Arg Asn Glu Leu Ser Phe Met Asn Phe Ser Gln

337  ACC TTT TTC TGG ATT GGA ATG CAT TAT AGT GAA AAA AGA AAT GCC TGG
113  Thr Phe Phe Trp Ile Gly Met His Tyr Ser Glu Lys Arg Asn Ala Trp

                                        ▽
385  CTA TGG GAG GAT GGC ACA GTT CCC TCA AAA GAT TTA TTT CCA GAA TTC
129  Leu Trp Glu Asp Gly Thr Val Pro Ser Lys Asp Leu Phe Pro Glu Phe

          T
433  TCA GTC ATC AGG CCA GAG CAC TGC ATT GTT TAC AGT CCA AGC AAA AGC
145  Ser Val Ile Arg Pro Glu His Cys Ile Val Tyr Ser Pro Ser Lys Ser

481  GTT TCT GCT GAA TCC TGT GAA AAT AAA AAC CGT TAC ATC TGT AAG AAA
161  Val Ser Ala Glu Ser Cys Glu Asn Lys Asn Arg Tyr Ile Cys Lys Lys

529  CTG CCT ATT TAA ATGTTTCTTA AGGCAAAGGG TATAGACAAG GAAGGTCCAC
177  Leu Pro Ile ***

581  GGTTACTAAA AATAGTAACA GCTCTCAATA TTACTACCTT TCCATCTTGT GTCTGTAGAA

                              C   T
641  TGTTTTTGTT TGGTAGTATA TAATTTTCAC ACATTTGTAC CCAAGTAATT TATACTTGAT

                   A
701  GGCTTCTTCA CGCGTGTGCA TAAA
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Figure 1. Nucleotide and predicted protein sequence of the CB.17 SCID allele of CD94. The numbering of nucleotides begins with the ATG start codon. The putative transmembrane segment is boxed, and potential exon boundaries are indicated by inverted triangles. The C57BL/6 allele of CD94 is identical except for five substitutions, as described in the text. The B6 nucleotide and amino acid substitutions are shown in bold above and below (respectively) the CB.17 sequence. Because of the primers that were used to amplify the B6 sequence, B6 polymorphisms in the 5′ untranslated region could not be discerned.

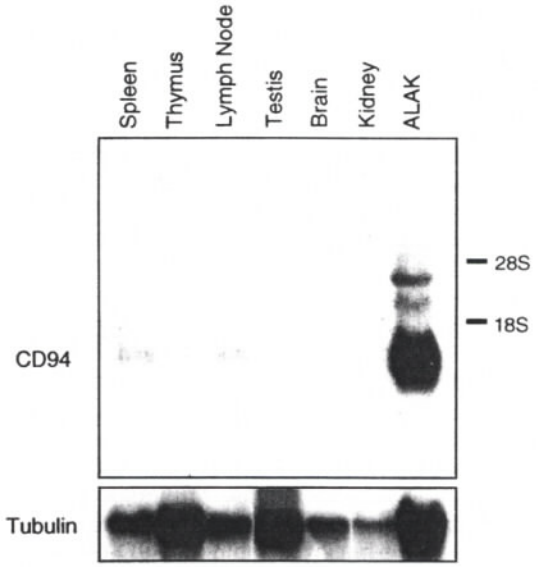

Figure 4. Northern blot of total RNA isolated from the indicated tissues of a male C57BL/6 mouse. The blot was probed with a 305-bp ^{32}P-labeled probe derived from the 3′ end of mouse CD94, or with a tubulin probe as a control. After washing, the labeled blot was exposed to film overnight at −70 °C.

the B6 CD94 sequence reported here. However, none of these EST appear to contain a completely spliced CD94 cDNA. Instead, the identity breaks down at one of two defined positions, corresponding to the location of predicted exon-intron boundaries, independently determined by aligning CD94 with Ly49A and CD69, two C-type lectins for which the exon-intron structure is known and apparently conserved (see Fig. 1 and [33, 34]). EST AA161895, AA177592 and AA267100 (GenBank accession numbers) appear to have failed to properly splice together the predicted exons 3 and 4 of CD94, whereas EST AA122795 and AA177664 appear to have resulted from a failed splicing of the predicted exons 4 and 5 of CD94. When these splice site sequences are compared to a "consensus" splice site sequence [35], it appears that the faulty splicing of exons 3 and 4 may be due to an imperfect splice acceptor sequence. In contrast, the faulty splicing of exons 4 and 5 may be the result of a potentially poor donor sequence; however, none of the clones extended far enough into the intron to identify the full donor sequence.

In order to confirm that the non-homologous sequence seen in the 5′ portions of these EST originates from CD94 intronic sequence, we attempted to amplify the putative intron 3/exon 4 boundary from B6 genomic DNA using two different primer sets (see Sect. 2.2). Products of the predicted size were obtained in each case (data not shown), confirming that the putative intronic sequence seen in the EST is contiguous in the genome with the CD94 exon sequence we had independently obtained.

3.4 Northern Blotting of RNA from several tissues

The presence of a number of improperly spliced CD94 transcripts in the EST database raised the possibility that CD94 is not properly expressed in the mouse. We therefore probed a Northern blot of total RNA from several tissues with a probe derived from the 3′ end of CD94 (Fig. 4). A prominent band of about 1 kb was detected in RNA derived from NK cells that had been expanded with IL-2 (A-LAK cells). Weaker bands of the same size were detected in spleen and lymph node RNA, and are possibly due to the presence of a small number of NK cells in these tissues. The prominent 1-kb band is approximately the size of the mature CD94 transcripts we isolated previously, and strongly argues that CD94 transcripts in NK cells are predominantly mature and properly spliced. No expression of CD94 was detected in thymus, brain, testis or kidney, arguing that the role of CD94 may be specific to NK cells. In addition to the prominent 1-kb transcript, additional minor bands were observed in A-LAK RNA that were about 3 kb and 5 kb in length. Bands of a similar size are also seen in human CD94$^+$ NK cells [17]. Given our analysis of EST, these minor bands are hypothesized to be unspliced pre-mRNA sequences.

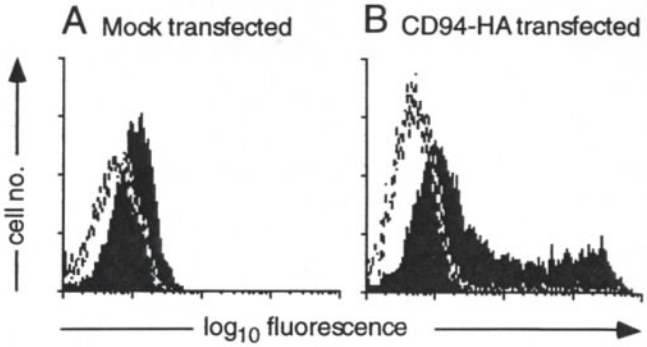

Figure 5. Staining of CD94-HA and mock transfected COS-7 cells with anti-HA (filled histograms) or isotype control (dotted histograms).

3.5 Expression of CD94 in COS-7 cells and an analysis of its MHC class I specificity

A nine amino acid HA tag was recombinantly added to the C terminus of mouse CD94 and the resulting CD94-HA protein was transiently expressed in COS-7 cells (Fig. 5). We achieved readily detectable surface expression of CD94, as assessed by staining of transfectants with an anti-HA antibody, demonstrating that proper signals for plasma membrane insertion and targeting are present in mouse CD94. Since human CD94 has been implicated in the recognition of MHC class I molecules, we were interested in determining whether mouse CD94 exhibited any binding capacity for MHC class I. We assessed the ability of CD94-HA transfectants to bind to ConA blasts from mice of the H-2^{b}, ^{d}, ^{k}, ^{q}, ^{f}, ^{r} and ^{s} haplotypes, but did not observe any binding of the blasts to CD94 transfectants (data not shown). Binding was observed, however, for COS-7 cells transfected with several different Ly49 cDNA, demonstrating the efficacy of the assay (T. H. and D. H. R., unpublished results). The failure of CD94 to bind MHC class I-expressing cells is consistent with the hypothesis that CD94 requires heterodimerization with NKG2 family members in order to recognize MHC class I. However, we cannot rule out the possibility that CD94 binding to MHC class I is of insufficient affinity for detection with this assay.

3.6 Murine NKG2 family members

It is anticipated that NKG2 family members will exist in the mouse, since the mouse CD94 gene, like its human counterpart, appears to contain only a short cytoplasmic tail which is probably insufficient to mediate signaling. We have discovered an NKG2D homolog in mice, again by searching the EST database (GenBank accession number AA178606; $p = 1.5 \times 10^{-69}$; see Fig. 2B). In contrast to the CD94 EST, this EST clone (I.M.A.G.E. Consortium Clone ID 621324; [29]) appears to encode a full-length protein. The coding region of the mouse NKG2D cDNA was used to probe the panel of Ly49-containing YAC and was found to hybridize specifically to the same two YAC that contained CD94 (data not shown), placing mouse NKG2D in the mouse NK complex. However, NKG2D is relatively distant from other NKG2 family members (see Fig. 2) and there is no evidence that NKG2D forms heterodimers with CD94 in humans. NKG2D may therefore serve a distinct type of function from the other NKG2 proteins.

Since they are found in diverse phyla, NK cells are usually considered an ancient or primitive arm of the immune system. However, recent molecular analyses of the MHC class I-specific receptors on the surface of NK cells suggest rapid evolution of the corresponding genes. Humans appear to express at least two different families of such receptors (the p50/p58/p70 family and the CD94/NKG2 family) whereas mice appear to express a distinct family of genes (the Ly49 family). In this report we partly bridge the evolutionary gap by providing the first evidence of an NK receptor, implicated in MHC class I recognition, that is shared between mice and humans. Our finding raises important questions as to how multiple families of NK receptors are coordinately expressed to generate a functional NK repertoire.

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Note added in proof: The following sequences have been deposited in Genbank: B6-CD94 (AF030311), CB.17-CD94 (AF030312), (AF030312), B6-NKG2D homolog (AF030313). The sequence of rat CD94 has also recently been described (*Eur. J. Immunol.* 1997. 27: 2080), as has a YAC contig of the NK complex (*Genomics* 1997. 42: 16).