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*J Immunol* 2003; 170:2259-2263; doi: 10.4049/jimmunol.170.5.2259

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The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Cutting Edge: Molecular Cloning of a Killer Cell Ig-Like Receptor in the Mouse and Rat

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We report the molecular cloning of a KIR3DL1 receptor in the mouse and the rat, between 37.4 and 45.4% identical with primate killer cell Ig-like receptors (KIRs/CD158). Both mouse and rat molecules contain a pair of immunoreceptor tyrosine-based inhibition motifs in their cytoplasmic regions, suggesting an inhibitory function. Southern blot analysis indicated a single KIR gene in the rat, whereas the mouse genome contains more than one KIR-related element. The rat Kir3dl1 locus was mapped to the leukocyte receptor gene complex on chromosome 1, whereas mouse Kir3dl1 was localized to the X chromosome. RT-PCR demonstrated that KIR3DL1 was selectively expressed by NK cells in both rat and mouse. An epitope-tagged expression construct of mouse KIR3DL1 transfected into 293T cells induced expression of a ~55-kDa protein. Our data indicate that KIR receptors may contribute to the NK cell receptor repertoire in rodents, alongside the Ly-49 family. The Journal of Immunology, 2003, 170: 2259–2263.

Natural killer cells have the capacity to spontaneously kill tumor cells, virally infected cells, and MHC class I disparate allogeneic target cells (1). Recognition and discrimination between different allelic variants of MHC class I are mediated by surface receptors belonging either to the killer cell lectin-like receptor (KLR) family (2, 3) or to the family of killer cell Ig-like receptors (KIR) or closely related receptors (4–6). Multigene families of receptors exist, comprising both members with an inhibitory function and members that activate NK cell cytotoxicity. Inhibitory NK cell receptors, including inhibitory KIR (KIR2DL or KIR3DL variants), contain immunoreceptor tyrosine-based inhibition motifs (ITIM) in their cytoplasmic region, that on phosphorylation may recruit and activate the protein tyrosine phosphatase Src homology protein-1 (5). Whereas mouse and rat NK cells express a repertoire of many different Ly-49 receptors that specifically bind subsets of allelic MHC class I variants (7), only a single Ly-49 appears to exist in the human (as a pseudogene) (8) as well as in cattle (9). Although ~13 KIR family members exist in the human (4), numerous attempts to identify KIR genes in rodents have failed, leading to the current view that rodent NK cells do not express KIR.

We here describe the molecular cloning and characterization of the first KIR family members in the mouse and rat.

Materials and Methods

cDNA isolation and sequence analysis

A full-length cDNA was obtained from a C57BL/6 mouse IL-2-activated NK cell cDNA library (10) by PCR using a primer (5′-CCCTCGAGATGAGGTCCATCGTATGGGCCC-3′/5′-CTGGAAATTCTCATCGGTGCTAGATTCACTGCATC-3′) derived from a mouse EST (GenBank accession number BB314335) against a vector-specific forward primer. cDNA library hybridization screening was performed as described (11), using medium stringency conditions (final two washes in 0.5× SSC, 0.1% SDS at 50°C). The rat D0 exon was amplified from genomic liver DNA by PCR (5′-TGCCTCAGCAAGTTATATTGTTCCTC-3′/5′-TCATATGCGCTGAAATTTCATTAGGCTAAGTT-3′). A cDNA library generated from KLRH1* IL-2-activated NK cells from PVG rats (12) was probed with the resulting 230-bp fragment under the same conditions as above.

Radiation hybrid (RH) mapping

The T31 mouse/hamster RH panel (Research Genetics, Huntsville, AL) was analyzed by PCR with mKIR3DL1-specific primers (5′-TGATGGGGCCCTGTGCCTCATGTGATGAGTTTCAGGGGGTTGCTCGACTT-3′/5′-CCCTCGAGATGAGGTCCATCGTATGGGCCC-3′) for the presence of a 150-bp mouse-specific product and with primers specific for mouse NKp46 (GenBank accession number AJ223765; 5′-CCAAACCCAGCATCATGTCACAATAAGTTTCAGGGGTTGCTCGACTT-3′). The PCR data were submitted to The Jackson Laboratory Mouse RH Database (http://www.jax.org/resources/documents/cmdata/rhmap/RHIntro.html). The T35 rat/hamster RH panel (Research Genetics) was analyzed using the D0 exon-specific primers described above.

Transfection, immunoprecipitation, electrophoresis, and blotting

293T cells were transiently transfected with an expression construct of mouse KIR3DL1 with an N-terminal Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (FLAG) tag (pCMV-FLAG-1; Sigma-Aldrich, St. Louis, MO) using Lipofectamine/Opti-MEM (Invitrogen, San Diego, CA) (12). Forty-eight hours after transfection, cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 5 mM EDTA, 0.02% NaN3, 1 mM PMSF, 1 mM Na3VO3, 10 mM NaF, protease inhibitor mixture; Sigma-Aldrich) containing 1% Igepal CA-630 (Sigma-Aldrich). Cleared lysate from 3 × 106 cells was immunoprecipitated with 1

1 This study was supported by the Norwegian Cancer Society, the Norwegian Research Council, and Bergljot and Sigurd Skauen’s Fund (to S.E.H., O.N., P.C.S., I.O., S.F., and E.D.), National Institutes of Health Grant RO1 AI 44126 (to J.C.R.), and the U.S. Veterans Administration (to J.C.R.).

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3 Abbreviations used in this paper: KLR, killer cell lectin-like receptor; KIR, killer cell Ig-like receptor; ITIM, immunoreceptor tyrosine-based inhibition motif; RH, radiation hybrid; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; IgSF, Ig superfamily; LRC, leukocyte receptor complex.
µg of anti-FLAG mAb (M2; Sigma-Aldrich) followed by 30 µl of protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA). Western blot detection (SuperSignal, Pierce, Rockford, IL/BioMaxMR film; Eastman Kodak, Rochester, NY) was either with 1 µg/ml rabbit anti-FLAG Ab (Sigma-Aldrich) or 0.2 µg/ml 4G10 anti-phosphotyrosine mAb (Santa Cruz Biotechnology) followed by HRP-conjugated secondary Abs (Jackson ImmunoResearch Laboratories, West Grove, PA).

Southern blot analysis and semiquantitative RT-PCR
Genomic DNA was isolated from mouse liver and subjected to Southern blot analysis as previously described (11) using a full-length mouse KIR3DL1 cDNA probe. Total cellular RNA isolation, first-strand cDNA synthesis, and analysis of rat CD45 transcription was performed as described previously (13). A primer pair specific for the first 5’-CATGCGCTGCGCAAGGAATTATGTTATATTGTCT-3’ and second 5’-AATGACACTCCAGGGTCACATTCTC-3’ Ig superfamily (IgSF) exons of rat KIR3DL1 was used to analyze transcription by PCR (25 cycles). Mouse KIR3DL1 expression was analyzed by PCR (35 cycles; primers 5’-CCTATCTGCTCTGCGCAAGGAATTATGTTATATTGTCT-3’/5’-TCAATCCCTCCTGATTCACC-3’) on first-strand cDNA (13) from C57BL/6 lymph node T cells (Pan T cell isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany) or IL-2-activated spleen NK cells cultured in rat IL-2 as described (13).

Results
cDNA cloning and sequence analysis
Searching GenBank for rodent EST sequences with homology to primate KIRs, a mouse EST was retrieved. Primers were generated from the 3’-untranslated region of this EST, and a full-length cDNA was obtained by PCR. The open reading frame fragment of this cDNA was used as a probe to screen a cDNA library from IL-2-activated C57BL/6 NK cells by hybridization. Six clones were isolated and sequenced, all apparently representing transcripts from the same gene.

In parallel, the rat Trace archive of short genomic sequences was searched for exons with homology to the mouse cDNA sequence. An exon homologous to the first IgSF domain of the mouse cDNA was retrieved and amplified from rat genomic DNA by PCR, using specific primers. This fragment was used to screen a cDNA library from IL-2-activated PVG rat NK cells. Fifty-one primary clones were detected, 10 of which were isolated and sequenced. All clones appeared to be transcribed from a single gene but represented several different splice variants. Four different polyadenylation sites were observed. The mouse and rat cDNA molecules contain ORFs encoding putative type I transmembrane proteins of 432 and 422 amino acids, respectively. They consist of three extracellular IgSF domains, a stem region (very short in the rat cDNAs), a transmembrane region lacking charged amino acids, and cytoplasmic regions containing one pair of ITIM-like motifs. Their predicted amino acid sequences are 68.6% identical with each other, 41.9% identical with <i>Bos taurus</i> Kir2DL1, and between 45.4% (mouse to <i>Maca</i> <i>c</i> <i>a</i> <i>c</i> <i>a</i> <i>m</i> <i>v</i> <i>u</i> <i>a</i> <i>t</i> <i>t</i> <i>a</i> <i>r</i> <i>i</i>s <i>K</i> <i>i</i>r2D<i>DL</i>6) identical with members of the primate KIR family. Furthermore, the mouse and rat predicted proteins were more similar to the KIR multigene family than to other related IgSF-containing receptors (Fig. 1).

Accordingly, they have been named mouse and rat Kir3DL1 (GenBank accession numbers AF130461 and AF527797, respectively) to indicate three extracellular IgSF domains in combination with long cytoplasmic tails with ITIMs. The predicted mouse and rat Kir3DL1 mature polypeptides have relative molecular masses of 46.2/45.6 kDa and 3/5 potential sites for N-linked glycosylation, respectively. The extracellular IgSF domains of mouse and rat Kir3DL1 share the D0-D1-D2 organization (4) of primate KIRs (Fig. 1A). Sequence comparison with human Kir2DL1 and -DL2 does not provide particular clues to suggest ligands for rodent Kir3DL1. The stem region of mouse Kir3DL1 is somewhat shorter than that of primate KIRs and contains a cysteine residue, suggesting the ability to form disulfide-linked Kir3DL1 dimers. None of the sequenced rat cDNA clones contained a stem region, present in most KIR variants in primates as well as in cattle (9).

Southern blot analysis of rat genomic DNA under low stringency hybridization conditions using different rat Kir3DL1 probes yielded a simple pattern compatible with a single rat KIR gene (data not shown). Southern blot analysis of mouse (C57BL/6 and BALB/c) genomic DNA (Fig. 2A) yielded more bands than in the rat, and the EST and genomic sequence information available through GenBank and ENSEMBL provides evidence for at least one additional KIR locus. Two ESTs corresponding to this second gene were retrieved from GenBank, indicating that it can be transcribed. A hypothetical amino acid sequence of this second mouse KIR was predicted from the two ESTs in addition to genomic sequence information. A single base deletion in the cytoplasmic tail of this second KIR gene results in a frame shift and a premature stop codon, yielding a short cytoplasmic tail without ITIMs. We have therefore named this hypothetical protein Kir3DS1. In contrast to the activating human Kir2DS and Kir3DS, no positively charged amino acid is present in the transmembrane domain of mouse Kir3DS1 (Fig. 1A).

Chromosomal localization
A rat/hamster RH panel was screened by PCR with Kir3DL1-specific primers, localizing rat Kir3dl1 to the leukocyte receptor gene complex (LRC) on chromosome 1 (14), between DXMit174 and DXMit66 (not shown). In contrast, screening a mouse/hamster RH panel with mouse-specific primers reproducibly localized the mouse locus to the X chromosome, between DXMit174 and DXMit66. To investigate the possibility of a local error in the mouse RH marker framework, the panel was screened with primers specific for mouse Nkp46, which is closely linked to the KIR genes in the human (15) as well as in the rat (14). The Nkp46 RH distribution pattern was not related to that obtained for Kir3DL1 and localized Nkp46 to the mouse LRC on chromosome 7, next to Piral6 (not shown). Human homologues of the genes that flank mouse Kir3dl1 are also localized on the X chromosome, and there is no evidence of shared synteny between the mouse Kir3dl1 region and the human LRC region on 19q13.4.

Transcription and surface expression of Kir3DL1
Semiquantitative RT-PCR analysis demonstrated that Kir3DL1 is selectively transcribed by NK cells both in the mouse and the rat (Fig. 2, B and C). No transcription was detected in rat T cells (CD4+, CD8+ or ConA blasts). A weak band was detected with mouse lymph node T cells after additional cycles of PCR (not shown), possibly due to a small number of NK cells in the lymph node T cell preparation.

A mouse Kir3DL1-FLAG construct induced surface expression of Kir3DL1 when transiently transfected in 293T cells. This was demonstrated by flow cytometry (Fig. 2D) as well as by Western blot analysis of surface biotinylated cells immunoprecipitated with anti-FLAG (data not shown). Anti-FLAG Western blots of whole cell lysates as well as immunoprecipitates (not shown) yielded a major band of ~55 kDa under non-reducing conditions, migrating slightly slower in the gel under...
FIGURE 1. A. Amino acid sequence alignment of mouse and rat KIR3DL1 together with selected members of the human KIR family. A hypothetical peptide sequence of mouse KIR3DS1 is also shown, based on EST together with genomic trace sequence information. Uncertain residues are marked X. B. Phylogram displaying similarity between mouse and rat KIR3DL1 and selected KIR-related IgSF-containing leukocyte receptors. H, Homo sapiens; M, Mus musculus; R, Rattus norvegicus; Bt, Bos taurus (cattle); Ma, Macaca mulatta (rhesus monkey); Pt, Pan troglodytes (chimpanzee). Mouse KIR3DS1 is based on EST (BB644731 and BB314335) and genomic (ENSEMBL ENSMUSG00000031424) sequences.
reducing conditions (Fig. 2, E and F). An additional weaker ~110 kDa band could in some experiments be observed under nonreducing conditions but was absent under reducing conditions. Although this band may represent an artifact, the latter observation suggests that mouse KIR3DL1 may partially exist in a disulfide-linked dimer form in 293T cells. The 9-kDa discrepancy between the observed molecular mass and the molecular mass predicted from the polypeptide backbone likely reflects glycosylation.

Discussion
We have identified in the mouse and the rat cDNA molecules belonging to the KIR family, as demonstrated by sequence analysis and comparison. A mouse KIR3DL1 cDNA expression construct induced surface expression in 293T cells, suggesting it is also expressed at the surface of NK cells. It contains two bona fide ITIM motifs, suggesting an inhibitory role, and the primary sequence does not appear to contain abnormal elements to suggest loss of function.

Similar to human KIRs, rat KIR3DL1 was selectively expressed by NK cells and localized within the leukocyte receptor gene complex. However, some of the features of the rat Kir3dl1 gene suggest that it may be a pseudogene. First, the transmembrane region exon contains a trinucleotide repeat yielding an abnormally long transmembrane region. Second, the stem exon appears not to be present or not to contain the proper splicing signals, leading to its loss during mRNA splicing. Third, among the 10 clones sequenced, several different splice variants were observed, revealing cryptic splice sites in the second signal peptide exon (exon 2); the transmembrane exon (exon 7) and importantly in exon 9, leading to the loss of both ITIMs. Among the 51 primary rat cDNA clones obtained, 28 contained the correct version with ITIMs; the rest represented splice variants lacking ITIMs, as demonstrated by PCR (data not shown). Finally, four different polyadenylation sites were observed. In contrast, the large number of cDNA clones detected in the library screening, together with the RT-PCR analysis demonstrating readily detectable levels of mRNA, indicate that the gene is transcribed at a high level in NK cells. Additional experiments are necessary to explore the functional nature of rat KIR3DL1.

The identification of a bovine KIR (9) demonstrated that the KIR family did not originate at the level of primates, but instead that one or more ancestral KIR genes existed before the split between cattle and primates. This is further supported by our identification of KIR genes in the rat and the mouse. The typical primate KIR gene organization, with three exons encoding C2 set-like IgSF domains, and with the same D0-D1-D2 organization (4), indicates that an ancestral KIR gene was of the KIR3DL type, with a D0-D1-D2 organization and with a long cytoplasmic tail containing two ITIMs. In contrast to the human KIR family, that consists of ~13 members, the mouse and rat KIR families seem to be small, possibly restricted to 2 genes in the mouse and a single gene in the rat. Like primate KIR, the rodent Ly-49 family contains several members with either inhibitory or activating function, capable of discriminating...
between allelic variants of MHC class I molecules (4, 7). Conversely, only a single Ly-49 pseudogene is present in the human (8). It would therefore seem likely that the Ly-49 receptor repertoire in rodents may perform functions parallel to those of the KIR family in primates. The functional role played by a single or a very limited number of KIR receptors in rodents remains to be investigated. Depending on the outcome of such investigations, the identification of rodent KIRs might lead to further understanding of the role of KIR receptors through the availability of an experimental animal model.

Acknowledgments
We thank M. Lauritzen and W. Jensen for technical assistance and J. T. Vaage, L. L. Spruyt, and J. G. M. C. Damoiseaux for sharing reagents.

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