NKp44, A Triggering Receptor Involved in Tumor Cell Lysis by Activated Human Natural Killer Cells, Is a Novel Member of the Immunoglobulin Superfamily

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Summary

Surface receptors involved in natural killer (NK) cell triggering during the process of tumor cell lysis have recently been identified. Of these receptors, NKp44 is selectively expressed by IL-2–activated NK cells and may contribute to the increased efficiency of activated NK cells to mediate tumor cell lysis. Here we describe the molecular cloning of NKp44. Analysis of the cloned cDNA indicated that NKp44 is a novel transmembrane glycoprotein belonging to the Immunoglobulin superfamily characterized by a single extracellular V-type domain. The charged amino acid lysine in the transmembrane region may be involved in the association of NKp44 with the signal transducing molecule killer activating receptor–associated polypeptide (KARAP)/DAP12. These molecules were found to be crucial for the surface expression of NKp44. In agreement with data of NKp44 surface expression, the NKp44 transcripts were strictly confined to activated NK cells and to a minor subset of TCR-γ/δ^+ T lymphocytes. Unlike genes coding for other receptors involved in NK cell triggering or inhibition, the NKp44 gene is on human chromosome 6.

Key words: natural killer cells • activating receptor • natural cytotoxicity • immunoglobulin superfamily • cDNA

The mechanism by which NK cells lyse certain virally infected or tumor cells while sparing normal cells has recently been elucidated. They express MHC class I–specific inhibitory receptors (killer inhibitory receptors [KIRs])[1] that block NK cell function when interacting with their ligand(s) (1). Thus, the expression of inadequate amounts of MHC molecules at the cell surface renders cells susceptible to NK cell lysis. On the other hand, the molecular mechanism(s) responsible for NK cell triggering remains largely unknown. Although the activating counterparts of KIRs have been identified (killer activating receptors [KAR]), their contribution to NK cell triggering is clearly limited to the case of MHC class I^+ target cells. However, a major function of NK cells is to kill cells that do not express MHC class I molecules, thus implying a major role in NK cell triggering of receptors recognizing non-MHC ligands. In this context, we have recently described two novel, highly NK-specific, receptor molecules (termed NKp46 and NKp44) that are involved in NK cell triggering during the process of natural cytotoxicity. Whereas NKp46 is expressed by all resting and activated NK cells (2), NKp44 is selectively expressed by NK cells only upon culture in IL-2 (3). In this respect, the de novo expression of NKp44 may play a relevant role in the marked increase of cytolytic activity displayed by the so-called LAK cells (4) that have been used in the adoptive immunotherapy of cancer (5, 6). Molecular cloning of NKp46 (7) has recently revealed a novel member of the Ig superfamily (Ig-SF) associated to CD3ζ, a signal transducing molecule containing immunoreceptor tyrosine-based activation motifs (ITAM). In this study, we report the molecular cloning of NKp44. NKp44 also represents a novel member of the Ig-SF which differs from NKp46 in that it is associated with the recently identified signal transducing molecule killer activating receptor–associated polypeptide (KARAP)/DAP12 (3). Moreover, the gene encoding NKp44 is located on human

[1] *Abbreviations used in this paper:* ITAM, immunoreceptor tyrosine-based activating motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; KARAP, killer activating receptor–associated polypeptide; KIR, killer inhibitory receptor; ORF, open reading frame; RT-PCR, reverse transcriptase-polymerase chain reaction; SF, superfamily.

chromosome 6 whereas NKp46 and most of the NK receptors belonging to the Ig-SF are encoded by genes on chromosome 19 (1, 7).

Materials and Methods

cDNA Library Construction. The expression cDNA library was prepared in VR1012 plasmid (Vical Inc.) using RNA extracted from IL-2–activated polyclonal NK cells as described (7, 8), and was divided into 10 fractions of $\sim 10^5$ clones each.

Library Screening by cDNA Expression in COS-7 Cells. The library screening procedure was performed as described (7, 9). Briefly, cDNA library was transiently transfected in COS-7 cells; selection of positive pools was performed by immunocytochemical staining using the specific anti-NKp44 mAb Z231 (3) and sib selection.

DNA Sequencing. DNA sequencing was performed using d-Rhodamine Terminator Cycle Sequencing Kit and a 377 Applied Biosystems Automatic Sequencer (Perkin Elmer-Applied Biosystems).

Reverse Transcriptase PCR (RT-PCR) Amplification of cDNAs Encoding for NKp44 and DAP12. Total RNA extracted using RNAzol (Cinna/Biotecx) from polyclonal NK and T cell populations and clones and from different hemopoietic cell lines (Table I) was reverse transcribed using oligo dT priming. Primers used for cDNA amplification of complete NKp44 open reading frame (ORF) (857 bp) were the following: 5′ CCA CGA GCG CAC AGG AAA AGG (NKp44 ORF UP) and 5′ TCA CAA AGT GTG TTC ATC ATC ATC ATC GCT TAT CTT AGT CC (NKp44 ORF DOWN). Amplification was performed for 30 cycles (30 s at 94°C, 30 s at 65°C, and 30 s at 72°C), followed by a 7-min incubation at 72°C, utilizing TAQ-GOLD (Perkin Elmer-Applied Biosystems) after preactivation for 15 min at 95°C. The cDNA obtained from a polyclonal NK cell population (NKp44 ORF) was subcloned in pcDNA3.1/V5/His$^-$TOPO vector (Invitrogen) and sequenced. Primers used for cDNA amplification of complete DAP12 ORF (10) (353 bp) were the following: 5′ TCA TGG GGG GAC TTG AAC C (DAP12 UP) and 5′ GAT TCG GGC TCA TTT GTA ATA C (DAP12 DOWN). Amplification was performed for 30 cycles (30 s at 94°C, 30 s at 60°C, and 30 s at 72°C), followed by a 7-min incubation at 72°C. The amplification product obtained from the NK cell clone K17 was subcloned in pCR3.1 vector (Invitrogen) and sequenced. Polyclonal or clonal NK cell populations and clones were obtained as previously described (2, 3, 11).

RT-PCR analysis of NKp44 and DAP12 mRNA on a panel of different cell populations of lymphoid origin and hemopoietic human cell lines was performed utilizing a semiquantitative PCR technique (12, 13).

Transient Transfections. COS-7 cells (5×10^5 per plate) were cotransfected with VR1012-15C and pCR3.1-DAP12 or with pcDNA3.1/V5/His$^-$TOPO NKp44 ORF (pcDNA3.1–NKp44 ORF) and pCR3.1–DAP12 constructs by the DEAE-dextran method as described (11). Cells were stained with different anti-NKp44 mAbs (Z231, IgG1; AZ140, IgG1; KS38, IgM) followed by a phycoerythrin-conjugated goat antibody to mouse IgG1 or IgM and analyzed by flow cytometry using a FACSort® (Becton Dickinson).

Expression of NKp44 and DAP12 mRNA in Transfected COS-7 Cells by RT-PCR and by Dot Blot Analysis. Total RNA was extracted from COS-7 cells (untransfected, transfected with pcDNA3.1–NKp44 ORF, or co-transfected with pcDNA3.1–NKp44 ORF and pCR3.1–DAP12) utilizing CsCl gradient. Poly A^+ fraction was purified from total RNA using oligo dT Dynabeads (Dynal, Norway) following the manufacturer's instructions. cDNA was obtained by RT reaction using oligo dT priming starting from 200 ng poly A^+. PCR amplification of NKp44 ORF and DAP12 ORF was carried out with the primers described above for 25 cycles, each consisting of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. A 228-bp β-actin fragment was amplified as control using the following primers: 5′ ACT CCA TCA TGA AGT GTG ACG (β-actin UP) and 5′ CAT ACT CCT GCT TGC TGA TCC (β-actin DOWN). PCR amplification was carried out for 25 cycles, each consisting of 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C. As a negative control we included for each set of primers poly A^+ without RT reaction step. PCR products were run on a 1.5% agarose gel and stained with ethidium bromide.

For the dot blot analysis, 200 ng poly A^+ were denatured in 60 μl 20× SSC and 40 μl 37% formaldehyde at 60°C for 15 min and spotted onto a positively charged nylon membrane (GeneScreen plus; Dupont-NEN) in 15× SSC using a microfiltration apparatus (Bio-dot; BioRad). The dot blot was hybridized with the following probes: 857-bp NKp44 ORF fragment, 353-bp DAP12 fragment, and 228-bp β-actin fragment. cDNA probes were ^{32}P labeled by random priming (14). Blot was washed at high stringency conditions as described (8).

Biochemical Characterization of the NKp44 Molecule. Cyanogen bromide Sepharose (Pharmacia, Sweden) –coupled Z231 mAb was used to immunoprecipitate NKp44 molecules from 1% NP-40 lysates of ^{125}I surface labeled cells (DuPont-NEN) as previously described (2, 3). Immunoprecipitates were analyzed by discontinuous SDS-PAGE under reducing conditions (5% 2-mercaptoethanol).

Biochemical Characterization of the NKp44-associated KARAP/DAP12 Molecules. NK cells were treated with sodium pervanadate (3) or with KS38 (IgM, anti-NKp44) mAb (3×10^6, 5 μg, 30 min, 4°C) followed by affinity purified F(ab′)$_2$ rabbit anti–mouse IgM (1.5 μg, 1 min, 37°C, ZYMED). Untreated and treated cells were lysed in 1% digitonin and immunoprecipitated with various mAbs as previously described (3). Samples were analyzed in SDS-PAGE, transferred to Immobilon P (Millipore Corp.) and the membranes probed with PY20-HRPO (anti-phosphotyrosine mAb; Transduction Laboratories) or with anti-FcεRIγ rabbit antiserum (provided by E. Vivier, INSERM, Marseille, France) or anti-DAP12 rabbit antiserum (SI-28) followed by donkey anti–rabbit HRPO (Amersham, UK). The Renaissance Chemiluminescence Kit (Dupont-NEN) was used for detection. SI-28 antiserum was obtained as previously described (11), using the COOH-terminal peptide (SDVYSDLNTQRPYYK) of DAP12 molecule (10).

Analysis of NKp44 Transcript Expression by Northern Blotting. 5 μg of total RNA prepared from LCL721.221 and Jurkat cell lines, NK cell clones and polyclonal NK cell populations using CsCl gradient was size-fractionated by denaturing agarose gel electrophoresis and transferred onto a positively charged nylon membrane (GeneScreen plus; DuPont-NEN). Northern blot was performed under high stringency conditions as described (8). The NKp44 cDNA probe (857-bp fragment NKp44 ORF) and the 228-bp β-actin probe were ^{32}P labeled by random priming (14).

Southern Blotting and Chromosomal Localization of NKp44 Gene. 10 μg of genomic DNA extracted from human, monkey, and mouse was digested with EcoRI, HindIII, or SacI restriction enzymes. DNA was size-fractionated by electrophoresis on a 0.8% agarose gel, transferred onto a positively charged nylon membrane (DuPont-NEN), hybridized as described (8, 14), and washed at low stringency conditions (0.5× SSC at 65°C).

The Somatic Cell Hybrid blot (BIOS Laboratories) was used to assign NKp44 gene to a specific chromosome by Southern blotting. The NKp44 ORF probe was used for high stringency hybridization; washes were performed as previously described (8). Chromosomal assignment was further confirmed by PCR on DNA from human–hamster monochromosomal hybrids (BIOS Laboratories) utilizing the following primers: 5′ CCA CGA GCG CAC AGG AAA AGG (NKp44 ORF UP) and 5′ GTA GAT TCT ACA CCA GTA ATG (15C-REV2). These primers allowed us to discriminate, by the size of the amplified products, between genomic and cDNA amplified sequences.

Results

Molecular Cloning of the cDNA Encoding the NKp44 Receptor. The availability of different mAbs specific for NKp44 molecules allowed us to attempt the isolation of the cDNA

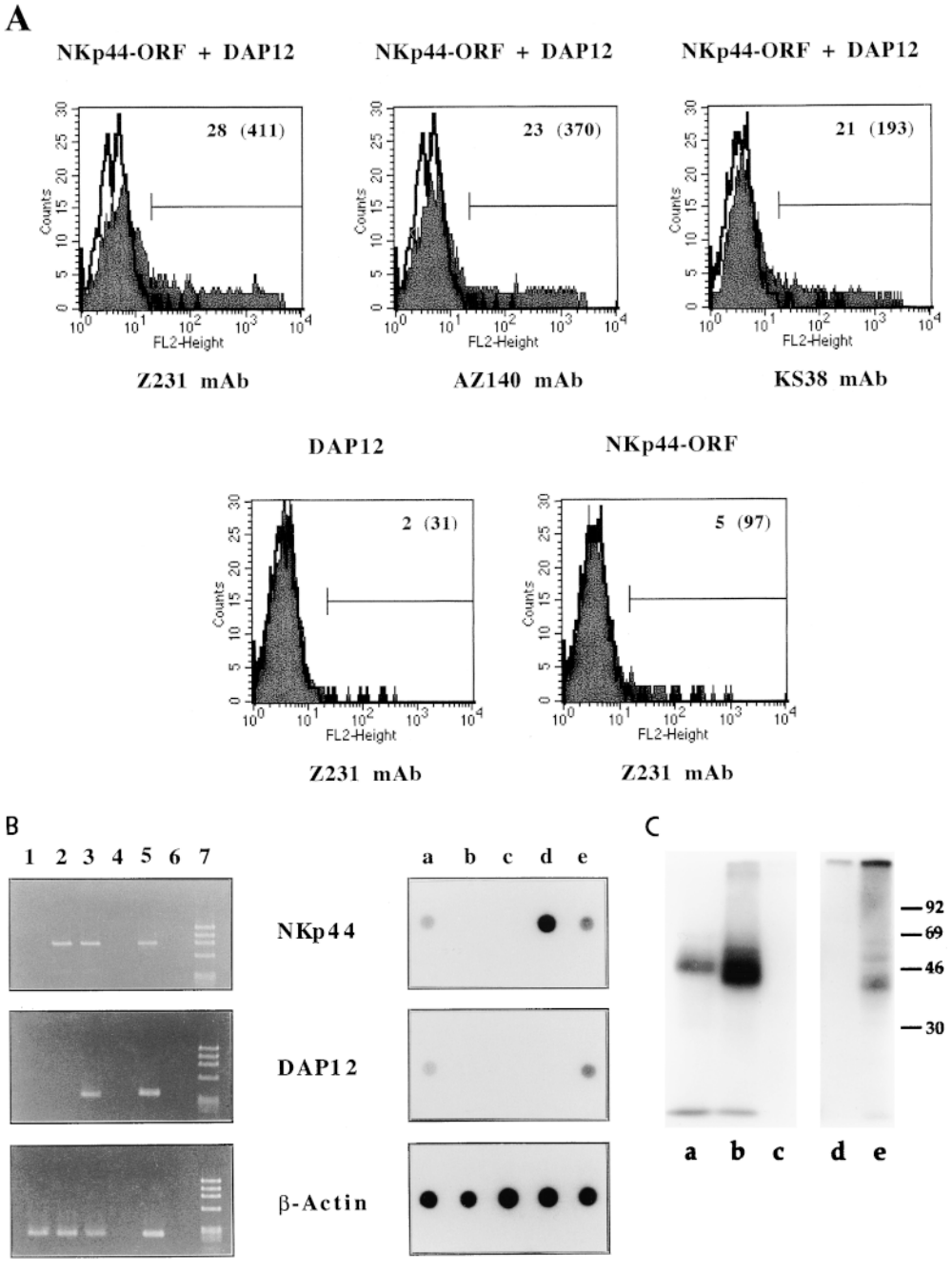

Figure 1. NKp44 surface expression in COS-7 cell transfectants. (A) Cell surface expression of NKp44 protein in COS-7 cells requires cotransfection with DAP12 cDNA. COS-7 cells were cotransfected with NKp44 ORF and DAP12 cDNAs (upper panels) and stained with three different anti-NKp44 mAbs: Z231, AZ140, and KS38. Lower panels represent COS-7 cells transfected with DAP12 or NKp44 ORF cDNAs alone and stained with the prototype Z231 mAb. Cells were then stained with phycoerythrin–conjugated goat anti–mouse IgG1 or IgM and analyzed by flow cytometry. White profiles represent cells incubated with the second reagent only. Although not shown, the anti-NKp44 mAbs AZ140 and KS38 failed to stain COS-7 cells transfected with NKp44 ORF or DAP12 cDNAs alone. In each panel, the percent of positive cells is indicated; numbers in parentheses indicate the mean fluorescence intensity. (B) Expression of NKp44 and DAP12 mRNA in COS-7 cell transfectants. NKp44 and DAP12 mRNA expression was analyzed by RT-PCR and dot blot using poly A^+ RNA (see Materials and Methods). On the left, RT-PCR was performed using primers specific for NKp44, DAP12, and β-actin. (Lane 1) Untransfected COS-7 cells; (lane 2) NKp44 transfected COS-7 cells; (lane 3) NKp44/DAP12 cotransfected COS-7 cells; (lane 4) nonretrotranscribed poly A^+ RNA; (lane 5) NK cell clone LOS64; (lane 6) negative control (PCR reaction mix without cDNA); (lane 7) HinfI digested ϕX 174 molecular weight markers. On the right, a dot blot prepared using poly A^+ RNA was hybridized with NKp44, DAP12, and β-actin probes. (lane a) NK cell clone C-11; (lane b) untransfected COS-7 cells; (lane c) mock transfected COS-7 cells; (lane d) NKp44 transfected COS-7 cells; (lane e) NKp44/DAP12 cotransfected COS-7 cells. (C) Biochemical analysis of NKp44 glycoproteins. A polyclonal NK cell population (lanes a and b), and COS-7 cells, untransfected (lane c) or cotransfected with NKp44 and DAP12 cDNAs (lanes d and e), were surface labeled with ^{125}I and immunoprecipitated with BAB281 (anti-NKp46) (lanes a and d) or Z231 (anti-NKp44) (lanes b, c, and e) mAbs. Samples were analyzed in a 11% SDS-PAGE under reducing conditions. Molecular mass markers (kD) are indicated on the right.

encoding NKp44 by an expression cloning strategy. To this end, a cDNA library was prepared from human NK cells and divided into 10 fractions of $\sim 10^5$ independent recombinant clones. Individual fractions were transiently transfected in COS-7 cells and analyzed by immunocytochemical staining using the prototype NKp44-specific mAb Z231 (3). The first screening yielded 2 (out of 10) positive fractions. By subsequent screening of progressively smaller cDNA library pools, we obtained positive fractions, each containing $\sim 3 \times 10^3$ recombinants. Further attempts aimed at obtaining positive pools of smaller size (e.g., 500 recombinants/pool) were unsuccessful. However, cotransfection of pairs of these small pools allowed us to identify two pools (termed C67 and C68) yielding $Z231^+$ COS-7 cells. Since individual transfection of C67 and C68 pools gave negative results, a possible explanation was that NKp44 expression at the surface of COS-7 cells required cotransfection with other molecule(s). On the basis of this assumption, the C67 fraction was transfected with progressively smaller pools of C68. A single clone (clone 15C) was isolated that could direct surface expression of the NKp44 protein when cotransfected with the C67 fraction. Since we recently demonstrated that NKp44 is associated with KARAP/DAP12 ITAM-bearing molecules (3), we analyzed whether these molecules were required for NKp44 expression at the cell surface. Indeed, cotransfection of clone 15C and DAP12 cDNAs resulted in surface expression of NKp44 (not shown). This clearly indicated that clone 15C encoded the NKp44 molecule. Importantly, in agreement with these results, DAP12 cDNA could be amplified by PCR from the C67 library fraction, thus confirming that, at least in COS-7 cells, DAP12 is required for the surface expression of NKp44.

Next, COS-7 cells were either cotransfected with NKp44 ORF and DAP12 cDNAs or transfected separately with the individual constructs and stained with different anti-NKp44 mAbs (including Z231, AZ140, and KS38). Fig. 1 A shows that COS-7 cells were efficiently stained by the anti-NKp44 mAbs only when cotransfected with both NKp44 ORF and DAP12 cDNAs. On the contrary, COS-7 cells transfected with NKp44 ORF cDNA alone either failed to express NKp44 or (in some experiments) displayed a low level of NKp44 surface expression. As illustrated in Fig. 1 B, the expression of NKp44 and DAP12 mRNA in COS-7 cell transfectants was checked both by RT-PCR and by dot blot analysis. Surface molecules reacting with anti-NKp44 mAb were immunoprecipitated from ^{125}I surface labeled COS-7 cells transfected with NKp44 ORF and DAP12 cDNAs. As shown in Fig. 1 C, the surface molecules immunoprecipitated from COS-7 cell transfectants (lane e) displayed a molecular size similar to that of molecules isolated from activated NK cells (lane b).

Notably, cotransfection of COS-7 cells with NKp44 ORF and cDNAs encoding for other ITAM-bearing molecules, such as CD3ζ or FcεRIγ (15), did not result in NKp44 surface expression (not shown). Thus, we can conclude that neither CD3ζ nor FcεRIγ can substitute KARAP/DAP12 to allow surface expression of NKp44. In this context, we previously showed that, in activated NK cells, NKp44 molecules do not associate with CD3ζ molecules (3). In Fig. 2 A, we show that NKp44 do not associate with FcεRIγ chains either. On the other hand, FcεRIγ associated with NKp46. Remarkably, the use of an antiserum specific for the recently cloned DAP12 molecules formally demonstrates that DAP12 is identical to the previously described NKp44-associated KARAP molecules (3, 16). As shown in Fig. 2 B, in NKp44 immunoprecipitates derived from activated NK cells treated with sodium pervanadate, the DAP12-specific antiserum recognized both nonphosphorylated and tyrosine-phosphorylated KARAP molecules. Moreover, as shown in Fig. 2 C, tyrosine phosphorylation of the NKp44-associated KARAP/DAP12 molecules also occurred when cross-linking of NKp44 molecules was induced by a specific mAb. Taken together, these data may suggest a direct involvement of KARAP/DAP12 molecules in the signal transduction leading to the NKp44-dependent NK cell activation.

Nucleotide sequence analysis showed that the 1,192-bp isolated cDNA (clone 15C) contained a 828-bp ORF preceded by a 338-bp 5′ untranslated region. The ORF encoded a 276–amino acid (aa) type I transmembrane protein belonging to the Ig-SF. This protein is characterized by a

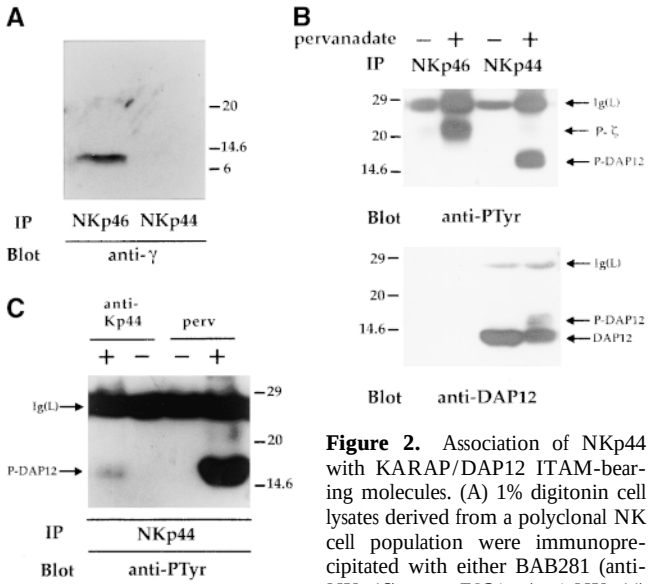

Figure 2. Association of NKp44 with KARAP/DAP12 ITAM-bearing molecules. (A) 1% digitonin cell lysates derived from a polyclonal NK cell population were immunoprecipitated with either BAB281 (anti-NKp46) or Z231 (anti-NKp44) mAbs. Samples were analyzed in a 15% SDS-PAGE under reducing conditions, transferred to PVDF membranes, and probed with the anti-FcεRIγ antiserum. (B) 1% digitonin cell lysates derived from a polyclonal NK cell population, untreated or treated with sodium pervanadate, were immunoprecipitated with mAbs to the indicated molecules. Samples were analyzed in a 15% SDS-PAGE under reducing conditions, transferred to PVDF membranes, and probed with either the antiphosphotyrosine mAb (anti-PTyr) or the anti-DAP12 antiserum. (C) A polyclonal NK cell population was treated or not treated with KS38 (anti-NKp44) mAb or sodium pervanadate. Cell lysates were immunoprecipitated with Z231 (anti-NKp44) mAb. Samples were analyzed in a 15% SDS-PAGE under reducing conditions, transferred to PVDF membranes, and probed with the antiphosphotyrosine mAb (anti-PTyr). Ig(L), Ig light chains; P-ζ, Tyr-phosphorylated CD3ζ; P-DAP12, Tyr-phosphorylated KARAP/DAP12, and DAP12, unphosphorylated form of KARAP/DAP12 are indicated by arrows. Molecular mass markers (kD) are indicated on the right.

21-aa leader sequence, a 169-aa extracellular region, a 23-aa transmembrane portion, and a 63-aa cytoplasmic tail (Fig. 3). Two cysteines in the extracellular region (positions 19 and 88 of the mature protein) determine the formation of a single Ig-related domain of the V type. Thus, the NKp44 V domain displays the typical long spacing (68 aa) between the two conserved cysteines as well as an arginine at position 59 and an aspartic acid at position 82 characteristic of Ig V domains (17). It also displays an additional pair of cysteines at positions 34 and 42, possibly involved in the formation of a second disulphide bond similar to that described in the NH_2-terminal V domain of the poly Ig receptor (18). The membrane-proximal region of the NKp44 receptor contains a high proportion of proline (20%), serine (16%), and threonine (13%). By analogy with other Ig-SF members, this region is predicted to display an extended open conformation typical of hinge-like sequences (19). Computer search (20) revealed seven serine and six threonine residues in the hinge-like region as putative O-glycosylation sites and an asparagine at position 159 as a potential N-linked glycosylation site. The predicted molecular mass of the NKp44 polypeptide is ~29 kD. Thus, glycosylation of the molecule may account for the apparent molecular mass (44 kD) of the glycoprotein isolated from normal NK cells (3). The transmembrane portion contains the positively charged amino acid lysine. This may be involved in the association with the negatively charged residue aspartic acid contained in the transmembrane region of KARAP/DAP12 protein (10). The cytoplasmic tail of NKp44 contains the amino acid sequence ILYHTV, which fits the consensus sequence for immunoreceptor tyrosine-based inhibitory motif (ITIM) (i.e., I/LxYxxL/V) (21). Remarkably, this motif is typical of all the HLA class I–specific inhibitory receptors, while none of the activating NK receptors identified so far (including CD16, p50, and NKp46) display ITIM in their cytoplasmic region (1, 21). On the other hand, it should be stressed that other surface receptors that are involved in cell activation, such as the erythropoietin receptor, the stem cell factor receptor, and the IL-3 receptor β chain, are characterized by a cytoplasmic tail containing ITIM sequences (22–24). The homology of NKp44 with known proteins was confined to the Ig-like V domain and limited to few members of the Ig superfamily. In particular, a low degree of homology was observed between the Ig-like V domain of NKp44 and that of the human poly-Ig receptor (18) (25% identity) and of the human CMRF35 protein (25) (29% identity).

Cellular Distribution of NKp44 Transcripts. Northern blot analysis was performed on RNA isolated from clonal and polyclonal NK cells as well as from LCL721.221 and Jurkat cell lines. As shown in Fig. 4, hybridization with the NKp44 ORF probe yielded two transcripts of ~3.7 and 1.2 kb. Since the cloned 1.2-kb cDNA encodes for a 44-kD protein that is specifically recognized and immunoprecipitated by anti-NKp44 mAbs, it is likely that the long 3.7-kb transcript may use a different polyadenylation site located 3′ with respect to the 1.2-kb transcript. Moreover, analysis of the cDNA sequence 5′ to the ATG initiation codon showed that there are four in-frame stop codons, making it unlikely that the 3.7-kb transcript may encode for a longer protein (not shown). Whereas β-actin hybridization showed that similar amounts of RNA were loaded in each lane, the amount of NKp44 mRNA varied considerably among different NK cell clones or populations ana-

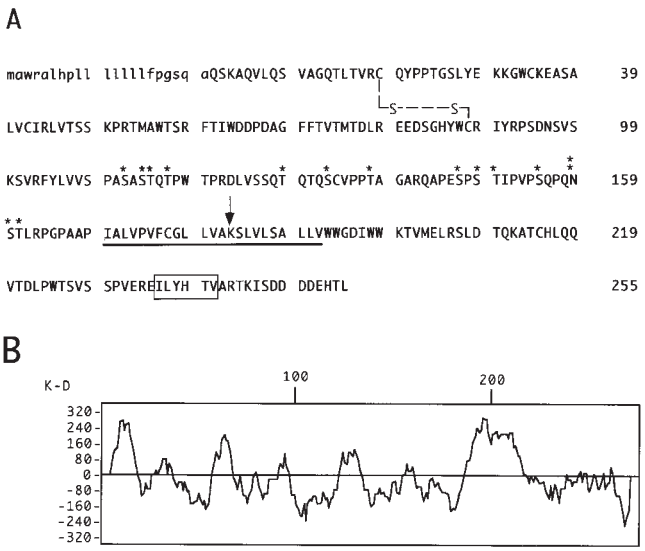

Figure 3. Predicted amino acid sequence and hydrophobicity plot of the NKp44 receptor. (A) Amino acids included in the signal peptide are indicated in small letters. The disulphide bond of the Ig-like V domain is indicated. The potential O-glycosylation sites are marked by single asterisks, and the potential N-glycosylation site by double asterisks. The predicted transmembrane portion is underlined and the charged residue is marked by an arrow. The ITIM sequence is boxed. These sequence data are available from EMBL/GenBank/DDBJ under accession number AJ225109. (B) Kyte-Doolittle hydrophobicity plot of the NKp44 protein.

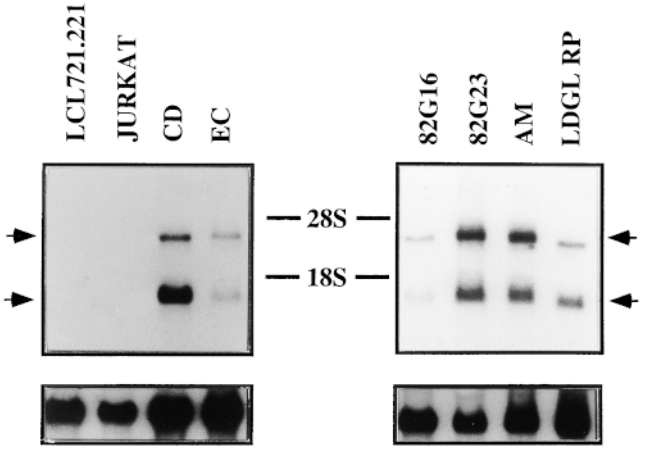

Figure 4. NKp44 transcript expression in polyclonal and clonal NK cells. Total RNA (5 μg/lane) isolated from the B-EBV cell line LCL721.221, the leukemic T cell line Jurkat, cultured polyclonal NK cell populations (CD, EC, and AM), two NK cell clones (82G16, 82G23), and a $CD3^-$ NK cell expansion (LDGLRP) were hybridized with the NKp44 ORF probe. The positions of 28S and 18S ribosomal subunits are indicated. Arrows indicate the two NKp44-specific transcripts. The two bottom insets represent the control hybridization with a β-actin cDNA probe.

lyzed. This is consistent with previous data indicating that the amount of NKp44 expressed at the cell surface differs among NK cell clones (3).

Further analysis of the expression of NKp44 and DAP12 mRNA in different cell populations of lymphoid origin and in hemopoietic human cell lines was performed by a semiquantitative RT-PCR technique. Table I shows that all IL-2–cultured NK cell populations and clones analyzed (all reactive with anti-NKp44 mAbs) expressed both NKp44 and DAP12 transcripts. On the contrary, fresh PBL expressed DAP12 but not NKp44 transcript, in agreement with the lack of surface reactivity with anti-NKp44 mAb. This is consistent with previous data indicating that only activated NK cells express surface NKp44 (3). Likewise, all

Table I. *NKp44 Surface Expression and RT-PCR Analysis of NKp44 and DAP12 mRNA Expression in Different Lymphocyte Cell Populations and Clones or in Human Cell Lines of Hemopoietic Origin*

Cells		NKp44 surface reactivity	NKp44 transcript	DAP-12 transcript
$CD3^-$ LM	(polyclonal NK cells)	+	+	+
$CD3^-$ CD	(polyclonal NK cells)	+	+	+
$CD3^-$ EC	(polyclonal NK cells)	+	+	+
LDGL RP	(NK expansions)	+	+	+
LDGL DF	(NK expansions)	+	+	+
SA92	(NK clone)	+	+	+
KK52	(NK clone)	+	+	+
LOR402	(NK clone)	+	+	+
LP72	(NK clone)	+	+	+
C11	(NK clone)	+	+	+
29	(TCR α/β^+ clone)	−	−	−
201	(TCR α/β^+ clone)	−	−	−
13	(TCR α/β^+ clone)	−	−	−
6	(TCR α/β^+ clone)	−	−	−
33	(TCR α/β^+ clone)	−	−	−
R3.2	(TCR α/β^+ clone)	−	−	+
X50B	(TCR γ/δ^+ clone)	−	−	+
X50F	(TCR γ/δ^+ clone)	−	−	+
M50F	(TCR γ/δ^+ clone)	−	−	−
DG16	(TCR γ/δ^+ clone)	−	−	−
DG29	(TCR γ/δ^+ clone)	−	−	−
17.12	(TCR γ/δ^+ clone)	+	+	+
17.31	(TCR γ/δ^+ clone)	+	+	+
PBL		−	−	+
PHA blasts		−	−	+
Total thymus		−	−	+
Monocytes		−	−	+
NK3.3	(NK cell line)	−	−	−
NKL	(NK cell line)	−	−	+
Jurkat	(T cell line)	−	−	−
HSB-2	(T cell line)	−	−	−
CEM-B	(T cell line)	−	−	−
MOLT-4	(T cell line)	−	−	−
PEER	(T cell line)	−	−	−
LCL721.221	(B cell line)	−	−	−
U937	(histiocytic lymphoma)	−	−	+
HL60	(promyelocytic leukemia)	−	−	−
THP-1	(monocytic cell line)	−	−	+
Eo/A3	(Eosinophilic cell line)	−	−	+

TCR-α/β^+ T cell clones were not stained by anti-NKp44 mAb and did not express the NKp44 transcript. The DAP12 transcript was only expressed in one of the clones analyzed; notably, this clone (R3.2) expressed the p50.2 activating receptor, known to associate with KARAP/DAP12 molecules (16). Among TCR-γ/δ^+ T cell clones, most did not express surface NKp44 molecules. However, two of them expressed DAP12 transcripts. Two TCR-γ/δ^+ T cell clones (17.12 and 17.31), isolated from a single donor, displayed low levels of surface NKp44 and expressed both NKp44 and DAP12 transcripts. In both clones, the NKp44 receptor was functional, as revealed by redirected killing assays in the presence of anti-NKp44 mAbs (not shown). We also analyzed several human cell lines of different histotype. All these lines lacked surface expression of NKp44 and did not display NKp44 transcripts; on the other hand, the DAP12 transcript was also expressed in cells of nonlymphoid origin. Unlike normal activated NK cells, the two NK cell lines analyzed (NK3.3 and NKL) displayed neither surface NKp44 molecules nor NKp44 transcripts.

Conservation Across Species and Chromosomal Localization of the NKp44 Gene. Southern blot analysis of the NKp44 gene was performed on genomic DNA extracted from human, monkey, and mouse cells and digested with three different restriction enzymes (Fig. 5). Hybridization performed with the NKp44 ORF probe under low stringency conditions revealed a relatively simple hybridization pattern for the NKp44 gene. Interestingly, cross-species hybridization could be observed with both monkey and mouse genomic DNA. These data suggest that the NKp44 ORF probe hybridizes with a single or relatively few human genes and that the NKp44 gene may be conserved between humans and rodents.

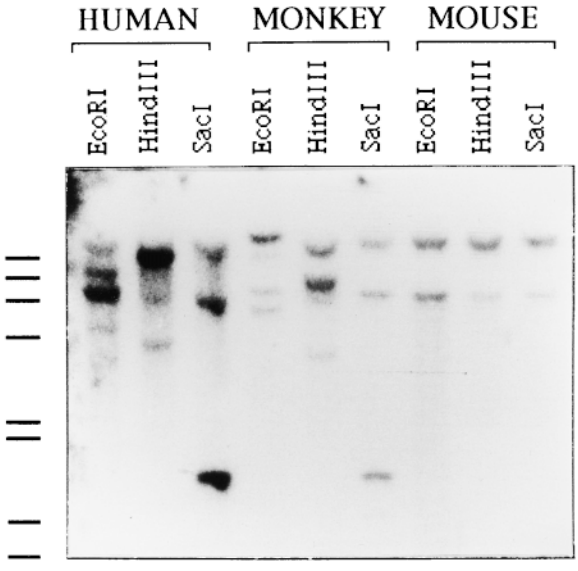

Figure 5. Southern blot analysis of NKp44 gene. Genomic DNA (10 μg/lane) extracted from human, monkey, and mouse cells was digested with the indicated restriction enzymes and hybridized with the NKp44 ORF probe. Molecular weight markers (23,130; 9,416; 6,557; 4,361; 2,322; 2,027; 1,353; and 1,078 bp [top to bottom]) are shown on the left.

Chromosomal localization of the NKp44-encoding gene was determined by two different approaches. First, we performed Southern blot analysis, using genomic DNA derived from a panel of polychromosomal human–hamster cell hybrids. Hybridization with the NKp44 ORF cDNA probe showed segregation of the NKp44 gene on human chromosome 6. The same conclusion was achieved by PCR analysis of a panel of genomic DNA extracted from monochromosomal human–hamster cell hybrids by using NKp44 specific primers (not shown). Taken together, these results indicate that the NKp44 gene is localized on human chromosome 6.

Discussion

The present study reports the molecular cloning of NKp44, a NK-specific triggering receptor involved in non-MHC–restricted natural cytotoxicity (3). Different from NKp46 (2, 7), which is expressed by all resting and activated NK cells, NKp44 is selectively expressed by activated human NK cells. In this context, it is well known that culture in IL-2 greatly enhances the NK-mediated anti-tumor cytotoxicity both in vitro (4) and in vivo (5, 6). It is conceivable that the increased cytolytic activity mediated by activated NK cells may be consequent, at least in part, to the de novo expression of triggering receptors. NKp44 may well represent one of these receptors since it is involved in triggering of activated NK cells in the process of tumor cell lysis (3).

Molecular cloning revealed that NKp44 is a type I glycoprotein belonging to the Ig-SF, which does not display any major amino acid sequence homology with known proteins. NKp44 is characterized by an extracellular region containing an Ig-like domain of the V type. The transmembrane portion contains the charged amino acid lysine, possibly involved in the association with KARAP/DAP12 molecules. Indeed, biochemical data confirmed that NKp44 associates with KARAP/DAP12 molecules (3). Moreover, molecular cloning of NKp44 demonstrated that the association with KARAP/DAP12 molecules is required for the surface expression of NKp44.

Interestingly, the cytoplasmic tail of NKp44 contains a classical ITIM (21). In this context, it is of note that, under the experimental conditions resulting in a strong tyrosine phosphorylation of the KARAP/DAP12 molecules associated to the NKp44 receptor (3), no tyrosine phosphorylation of the NKp44 molecule itself could be detected. Moreover, under the same experimental conditions, no evidence has been obtained so far that NKp44 may associate with phosphatases such as SHP-1, SHP-2, or SHIP, which have been reported to associate with ITIM-bearing receptors (21). The functional role of this ITIM is presently under investigation.

Remarkably, unlike most of the genes encoding Ig-SF receptors involved in the regulation of NK cell–mediated cytolytic activity (including KIR [1], KAR [1], NKp46 [7], and ILT/LIR [26, 27]), which are on human chromosome 19, the NKp44 gene is on chromosome 6. Southern blot

analysis of human genomic DNA revealed a restriction enzyme digestion pattern that is compatible with the existence of a single NKp44 gene. Moreover, the NKp44 gene appears to be conserved across species since the human NKp44 ORF cDNA probe cross-hybridized with genomic DNA from monkey and mouse. It will be of interest to analyze whether the murine counterpart of NKp44 exists and whether its expression is restricted to NK cells and to a subset of TCR-γ/δ^+ T lymphocytes as it occurs in humans.

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