Molecular Cloning of NKp46: A Novel Member of the Immunoglobulin Superfamily Involved in Triggering of Natural Cytotoxicity

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Summary

NKp46 has been shown to represent a novel, natural killer (NK) cell–specific surface molecule, involved in human NK cell activation. In this study, we further analyzed the role of NKp46 in natural cytotoxicity against different tumor target cells. We provide direct evidence that NKp46 represents a major activating receptor involved in the recognition and lysis of both human and murine tumor cells. Although NKp46 may cooperate with other activating receptors (including the recently identified NKp44 molecule) in the induction of NK-mediated lysis of human tumor cells, it may represent the only human NK receptor involved in recognition of murine target cells. Molecular cloning of the cDNA encoding the NKp46 molecule revealed a novel member of the immunoglobulin (Ig) superfamily, characterized by two C2-type Ig-like domains in the extracellular portion. The transmembrane region contains the positively charged amino acid Arg, which is possibly involved in stabilizing the association with CD3ζ chain. The cytoplasmic portion, spanning 30 amino acids, does not contain immunoreceptor tyrosine-based activating motifs.

Analysis of a panel of human/hamster somatic cell hybrids revealed segregation of the NKp46 gene on human chromosome 19. Assessment of the NKp46 mRNA expression in different tissues and cell types unambiguously confirmed the strict NK cell specificity of the NKp46 molecule. Remarkably, in line with the ability of NKp46 to recognize ligand(s) on murine target cells, the cDNA encoding NKp46 was found to be homologous to a cDNA expressed in murine spleen. In conclusion, this study reports the first characterization of the molecular structure of a NK-specific receptor involved in the mechanism of NK cell activation during natural cytotoxicity.

Key words: natural killer cells • cell-mediated cytotoxicity • molecular cloning • activating receptors • tumor target cells

The molecular mechanism by which NK cells can discriminate and kill autologous cells that do not express sufficient amounts of MHC class I molecules has been identified recently (1, 2). NK cells express a number of MHC class I–specific receptors that, upon interaction with their ligands, inhibit NK cell cytotoxicity (3–5). In humans, two distinct families of inhibitory receptors for HLA–class I molecules have been identified. The first one is represented by members of the Ig superfamily that display specificity for different groups of HLA–class I alleles encoded by the HLA-C, -B, or -A loci (5–7). The second group is represented by heterodimers formed by members of the C-type lectin superfamily. They are composed by the covalent association of CD94 with members of the NKG2 family (8–10). The CD94–NKG2A heterodimer has recently been shown to represent the receptor for HLA-E molecules (11–13). On the other hand, thus far only limited information has been available on the surface molecules involved in triggering of NK cells during natural cytotoxicity. In this context, we showed that the activating forms of the HLA-C–specific receptors (p50) may play a role in cytotoxicity against HLA–class I-positive target cells (14–16). However, it is evident that this activating pathway is not involved in cytotoxicity against HLA–class I-negative target cells. In the search for receptors responsible for NK cell triggering in the process of non-MHC-restricted cyto-

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toxicity, we recently identified two novel NK-specific triggering surface molecules. The first one is a 46-kD molecule (termed NKp46 in this study) that is selectively expressed by all resting and activated human NK cells (17), and associates with the CD3ζ chain (18). The second one is a 44-kD molecule (NKp44) that is only expressed by activated human NK cells, and associates with the recently described killer cell activating protein (KAR)[1]-associated polypeptide (KARAP)/DNAX activation protein (DAP)12 signal transducing molecule (18–20). Clues that these molecules may represent triggering receptors involved in natural cytotoxicity have been provided by their ability to activate NK cell function after cross-linking mediated by anti-NKp46 or anti-NKp44 mAb (17, 18). In addition, anti-NKp46 or anti-NKp44 mAb could partially inhibit the NK cytotoxicity against FcγR-negative tumor target cells (18). Remarkably, this inhibitory effect varied depending upon the target cells analyzed. Moreover, a synergistic effect could be demonstrated when the two mAbs were used in combination (18). A likely interpretation of these data is that the inhibitory effect on NK cytotoxicity is consequent to mAb-mediated masking of relevant receptors involved in recognition of target cell ligands. In this study we show that the NKp46 molecule initiates a pathway of NK cell activation involved not only in the recognition of human but also of murine target cells. Molecular cloning of NKp46 revealed a novel member of the Ig superfamily characterized by two Ig-like extracellular domains, a transmembrane region containing the charged amino acid Arg, and a short cytoplasmic tail that does not contain immunoreceptor tyrosine-based activating motifs (ITAMs).

Materials and Methods

Cells Lines, Antibodies, Generation of NK Cell Clones, and Cytolytic Assays. Human NK cells were isolated from peripheral blood lymphocytes of healthy donors as previously described (21). The purity of the cell population was evaluated by flow cytometric analysis after staining with anti-CD3, anti-CD56 and anti-NKp46 mAbs. NK cell clones were generated as previously described (21). Polyclonal NK cell cultures and clones were maintained in RPMI 1640 medium supplemented with 10% FCS and 100 U/ml recombinant IL-2. PHA-activated human T lymphocytes and T cell clones were obtained and maintained as previously described (22). The Raji, Daudi, 721.221 B, Jurkat T, HL60 and U937 myelomonocytic, M14 melanoma, and IGROV ovarian carcinoma cell lines (all of human origin), and mouse thymoma BW1502 were cultured in RPMI 1640 medium supplemented with 10% FCS. Monkey kidney COS-7 cells were maintained in DMEM supplemented with 10% FCS.

mAbs BAB281 (IgG1, anti-NKp46), Z231 (IgG1, anti-NKp44), C218 (IgG1, anti-CD56), C227 (IgG1, anti-CD69), and EB6 (IgG1, anti-p58.1) were obtained in our lab (17, 18, 23). NK cell clones were assessed for cytolytic activity in a 4-h ^{51}Cr-release assay as previously described (23).

cDNA Library Construction. Total RNA was extracted from IL-2–activated polyclonal NK cells obtained from two healthy donors by guanidinium thiocyanate lysis followed by centrifugation on a cesium chloride cushion (24). Poly A^+ RNA was purified by magnetic separation with Dynabeads Oligo(dT)25 (Dynal A.S., Oslo, Norway). 5 μg of polyA^+ RNA was used to construct a directional cDNA library by using the SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (GIBCO BRL, Gaithersburg, MD). In brief, first strand cDNA synthesis was catalyzed by SuperScript II reverse transcriptase by using an oligo-dT-NotI primer-adapter. After second strand synthesis, cDNA was ligated to SalI adapters and subsequently digested with NotI. Digested cDNA was size-fractionated by column chromatography and the larger inserts were ligated into SalI-NotI–digested VR1012 plasmid (Vical Inc., San Diego, CA), a mammalian expression vector containing CMV IE promoter/enhancer regions. The ligation products were transformed into XL1-Blue MRF′ bacterial cells (Stratagene, La Jolla, CA) by electroporation. The library (~10^6 independent recombinant clones) was divided into 10 fractions (10^5 clones each) and grown for several hours, and then frozen glycerol cultures were prepared and DNA was isolated by the alkaline lysis method (24).

Library Screening by cDNA Expression in COS-7 Cells and Sib-Selection. The library screening procedure was adapted from the method reported by Brakenhoff et al. (25). In brief, 10 μg of plasmid DNA from each library fraction was transfected into COS-7 cells by electroporation (3×10^6 cells each electroporation) and cells were seeded into 100-mm petri dishes. A control transfection with p58.1 cDNA in VR1012 plasmid was performed in each experiment. On day 3 after transfection, cell monolayers were immunocytochemically stained as described below. The library fraction containing the highest number of positive cells and the lowest number of bacterial colonies was chosen for further analysis. The corresponding glycerol stock was titered and divided into 10 smaller pools (~2×10^5 colonies each), and then plasmid DNA was isolated from bacterial overnight cultures of each library subpool and transfected into COS-7 cells. Subsequent rounds of screening of progressively smaller pools of bacterial colonies were performed until a single positive colony (clone 2F) was identified.

Immunocytochemical Staining of Transfected COS-7 Cells. Immunocytochemistry was essentially performed according to Brakenhoff et al. (25). In brief, cells in 100-mm petri dishes were air dried, fixed with methanol, washed once with Tris buffer saline (TBS: 25 mM Tris-HCl, pH 7.4, 2 mM KCl, 150 mM NaCl), and incubated with 2% normal rabbit serum/1% BSA in TBS for 15 min at room temperature. Incubation with either BAB 281 (library transfected cells) or EB6 (control cells) mAb, each diluted 1:2 in TBS/1% BSA, was conducted for 1 h at room temperature. After extensive washes in TBS, cells were incubated with rabbit anti–mouse immunoglobulins (DAKO, Glostrup, Denmark) diluted 1:25 in TBS/1% BSA for 30 min at room temperature. The dishes were washed again in TBS and subsequently incubated with alkaline phosphatase (AP) anti-AP mAb (DAKO) diluted 1:200 in TBS/1% BSA for 30 min at room temperature. Cells were washed again in TBS, rapidly equilibrated in AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 10 mM $MgCl_2$), and then incubated for 10 min at room temperature with nitro blue tetrazolium (100 μg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (50 μg/ml) in AP buffer. Cell monolayers were then rinsed with deionized water and visually analyzed under an inverted phase

[1]*Abbreviations used in this paper:* AP, alkaline phosphatase; EST, expressed sequence tags; ILT, immunoglobulin-like transcript; ITAM, immunoreceptor tyrosine-based activating motif; KAR, killer cell activating receptor; LIR, leukocyte immunoglobulin-like receptor; ORF, open reading frame; RT-PCR, reverse transcriptase PCR; TBS, Tris-buffered saline.

microscope. Positive cells stained purple and the membrane localization of the immunoreactive protein was detectable as a well delineated dark cell profile.

DNA Sequencing. DNA sequencing was performed using d-Rhodamine Terminator Cycle Sequencing Kit and an automatic sequencer (Automatic Sequencer 377; PE Applied Biosystems, Foster City, CA).

Transient Transfections. COS-7 cells (5×10^5/plate) were transfected with clone 2F cDNA by the DEAE-dextran method, as previously described (5). After 48 h, transfected cells were detached with 2% EDTA in PBS, stained with mAb BAB281 followed by a PE-conjugated goat antibody to mouse IgG1, and analyzed by flow cytometry using a FACSort® (Becton Dickinson, San Jose, CA).

Analysis of NKp46 Transcript Expression by Northern Blotting. Northern blot analysis of RNA extracted from multiple human tissues was performed using Northern Territory™ Human Normal Tissue Blots I, II, and III from Invitrogen (Carlsbad, CA). The Northern blots contained total RNA from heart, brain, kidney, liver, lung, pancreas, spleen, and skeletal muscle (Blot I); esophagus, stomach, intestine, colon, uterus, placenta, bladder, and adipose tissue (Blot II); and tonsil, thymus, appendix, lymph node, gall bladder, prostate, testis and ovary (Blot III). To analyze NKp46 gene expression in leukocytes, total RNA was purified from the indicated cells, size-separated by electrophoresis in agarose gels containing formaldehyde, and transferred onto a nylon membrane (DuPont-NEN, Boston, MA). Northern blots were hybridized under high stringency conditions as previously described (26). The NKp46 cDNA probe (a 1.3-kb SalI–NotI fragment excised from the VR1012 recombinant vector) was ^{32}P-labeled by random priming (27).

Reverse Transcriptase PCR Analysis. cDNA was obtained by reverse transcription of mRNA from various leukocytic cells using oligo dT priming. Primers used for cDNA amplification of complete NKp46 open reading frame (ORF; 984 bp) were as follows: 5′CTGAGCGATGTCTTCCACAC (46FOR) and 5′CCGCCCAGGCTCAACACC (46REV). Amplification was performed with 25 pmol of each primer 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 products were subcloned in pCR2.1 vector by TOPO-TA Cloning kit (Invitrogen), and subsequently were sequenced.

Biochemical Characterization of the NKp46 Molecule. Cyanogen bromide Sepharose-coupled (Amersham Pharmacia Biotech, Uppsala, Sweden) BAB281 mAb was used to immunoprecipitate NKp46 molecules from 1% NP-40 lysates of ^{125}I surface-labeled cells (DuPont-NEN), as previously described (17). Immunoprecipitates were analyzed by discontinuous SDS-PAGE under reducing conditions (5% 2-ME).

NKp46 molecules, purified from dried gel, were treated with neuraminidase (2 mU), α-l-fucosidase (2 μg), *N*-acetyl-β-d-glucosaminidase (1 mU), *O*-glycosidase (2.5 mU), and *N*-glycosidase F (0.4 U) (all from Boehringer Mannheim, Mannheim GmbH, Germany), for 18 h at 37°C in 30 μl of 20-mM phosphate buffer, pH 7, 0.1% SDS, 1% NP-40, and 10 mM 2-ME. Digestion was stopped by adding 10 μl of 4× SDS sample buffer. Samples were then analyzed by discontinuous SDS-PAGE.

Chromosomal Localization of NKp46 Gene and Southern Blotting. A panel of genomic DNA samples from human/hamster cell hybrids (BIOS Laboratories, New Haven, CT) was used to assign NKp46 gene to a specific chromosome by Southern blotting. The somatic cell hybrid blot contained 20 multichromosomal somatic cell hybrids plus 3 control genomic DNAs from human, hamster, and mouse tissues. High stringency hybridization and washes were performed according to the manufacturer's instructions. The NKp46 labeled probe was the same as that used for Northern blotting.

Analysis of cross-species conservation of the NKp46 gene was performed using Zoo-Blot from Clontech (Palo Alto, CA). The Southern blot contained genomic DNA from human, Rhesus monkey, Sprague-Dawley rat, BALB/c mouse, dog, cow, rabbit, chicken, and *Saccharomyces cerevisiae* yeast. The probe used was a cDNA segment corresponding to nucleotides 310–715 of the NKp46 ORF obtained by PCR. Washes were carried out at low stringency conditions as previously described (26).

Results and Discussion

NKp46 Represents an NK cell Triggering Receptor Involved in the Recognition of both Human and Murine Tumor Cells. A series of human NK cell clones derived from different donors were selected for their ability to lyse a panel of human or murine FcγR-negative tumor target cells. In agreement with previous data, all NK clones expressed NKp46 surface molecules (17). To assess the possible role of NKp46 in the recognition of different target cells, we evaluated the cytolytic activity in either the absence or presence of anti-NKp46 mAb. Based on the results of these experiments, three different groups of target cells could be identified. In the first group, addition of anti-NKp46 mAb led to an almost complete (>70%) inhibition of target cell lysis. Remarkably, target cells belonging to this group (including Bw1502 and YAC cells) were of murine origin. In the second group, addition of anti-NKp46 mAb resulted in a partial (30–60%) inhibition of cytolysis. This group included various human tumor cell lines including lung, liver or mammary gland carcinomas, melanomas, and EBV-transformed cell lines. Lysis of the third group (which included only a few human tumor cell lines) was not affected by anti-NKp46 mAb. Fig. 1 shows the cytolytic activity of NK clones against target cells representative of each group. It can be seen that the murine thymoma BW1502 (representative of the first group of target cells) is lysed in the absence of added mAb, whereas lysis was sharply inhibited in the presence of anti-NKp46 mAb. A similar inhibitory effect was consistently detectable in all analyzed NK clones. Addition of isotype-matched anti-NKp44 (Fig. 1) or anti-CD69 or anti-CD56 mAb (data not shown) had no effect. In the case of the M14 melanoma cell line (representative of the second group), anti-NKp46 mAb partially (~50%) inhibited target cell lysis, whereas anti-NKp44 mAb (Fig. 1), as well as anti-CD69 and anti-CD56 mAbs (data not shown), had no effect. Finally, killing of IGROV ovarian carcinoma cell line (representative of the third group) was affected by neither anti-NKp46 nor anti-NKp44 mAb. Taken together, these data provide further evidence that NKp46 functions as a triggering receptor involved in natural cytotoxicity. It is conceivable that the inhibitory effect of anti-NKp46 mAb may reflect masking of the NKp46 receptor. This would prevent NKp46 interaction with ligand(s) expressed on target cells. Remarkably, the mAb-

mediated abrogation of cytolytic activity against murine target cells suggests that NKp46 may represent the only triggering receptor expressed by human NK cells capable of recognizing a ligand on the surface of murine cells. If this interpretation is correct, one may speculate that the ligand(s) for NKp46 may be conserved in human and mouse. The finding that lysis of M14 melanoma could be inhibited only partially by anti-NKp46 mAb suggests that, in this case, NKp46 may cooperate with other, still undefined, NK receptors for optimal induction of NK cell triggering. In this context, it is of note that NK-mediated cytotoxicity against M14 is not affected by masking of NKp44 molecules. It should be also noted that NKp44 has been shown to cooperate with NKp46 in the NK-mediated cytolytic activity against other tumor targets such as the lung carcinoma A549 and the EBV-transformed B cell lines LCL721.221 and RPMI 8866 (18). Taken together, these data would suggest that different triggering receptors expressed by a given NK cell may differentially contribute to activation of cytotoxicity, depending on the ligands expressed by the analyzed target cells.

The inability of anti-NKp46 mAb to inhibit cytolytic activity against IGROV cells may reflect the absence (or inadequate expression) of the NKp46 ligand(s). Alternatively, several additional still unknown receptor–ligand interactions may be responsible for NK cell activation against IGROV cells, and blocking of the NKp46 receptor may not significantly impair the overall triggering of cytolysis. It is of note that although lysis of most melanoma cell lines was inhibited by anti-NKp46 mAb (indeed, most melanomas belong to the above-defined "group 2" of cell targets), lysis of a melanoma cell line (from melanoma patient no. 15392) was not affected by masking of NKp46 (data not shown). This suggests that the ligand(s) for the NKp46 receptor may be differentially expressed even by human tumor cells of the same histotype.

Cloning of cDNA that Encodes NKp46. To isolate the cDNA that encodes the NKp46 molecule, we applied an expression cloning strategy. An expression library was prepared from human NK cell cDNA derived from two healthy donors, and was divided into 10 fractions of $\sim 10^5$ independent recombinant clones. Individual fractions were transiently transfected into COS-7 and analyzed by immunocytochemical staining with anti-NKp46 mAb. The first screening revealed two positive library fractions. Screening of progressively smaller pools of recombinants allowed us to isolate a single clone (2F) that directed cell surface expression of the NKp46 protein, as demonstrated by staining with anti-NKp46 mAb and by flow cytometric analysis (Fig. 2 *A*).

The cDNA comprises a 915-bp ORF encoding a novel protein of 304 amino acids (Fig. 2 *B*). The predicted amino acid sequence is consistent with a type I transmembrane protein belonging to the Ig superfamily. The extracellular region is preceded by a 21-residue signal peptide, and consists of two cysteine-bridged C2-type Ig-like domains followed by a stretch of amino acids that probably form a stem

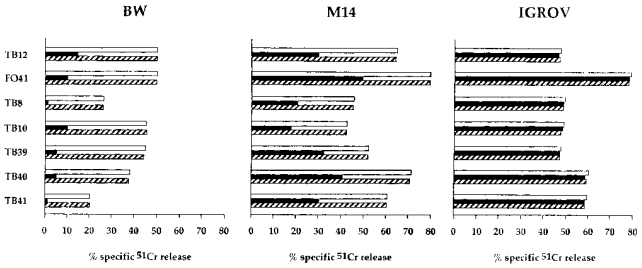
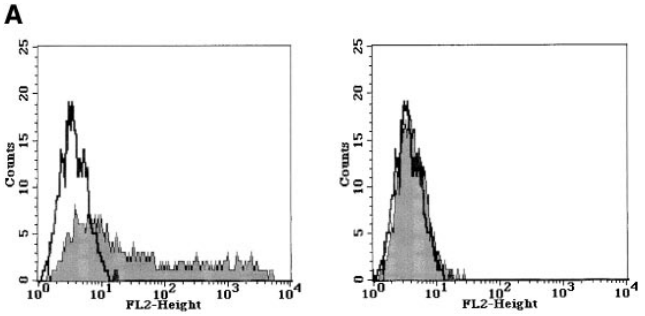


Figure 1. Inhibition of natural cytotoxicity by mAb-mediated masking of NKp46 molecules. Seven representative NK cell clones, derived from two donors, were analyzed for cytotoxic activity in a ^{51}Cr-release assay against the following FcγR-negative target cell lines: BW1502 (murine thymoma), M14 (human melanoma), and IGROV (human ovarian carcinoma), in either the absence (□) or presence of BAB281 (anti-NKp46, ■), or in Z231 (anti-NKp44, ◪) mAb. Both mAbs are IgG1. The E/T ratio was 5:1 for human target cells and 8:1 for murine target cells. Each bar represents the mean of triplicate experiments.

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TCC CCA CTG CTC AGC ACT TAG GCC GGC AGA ATC TGA GCG ATG TCT TCC ACA CTC CCT GCC CTG CTC TGC GTC GGG                    36
CTG TGT CTG AGT CAG AGG ATC AGC GCC CAG CAG CAG ACT CTC CCA AAA CCG TTC ATC TGG GCC GAG CCC CAT TTC                   111
l  c  l  s  q  r  i  s  a  Q  Q  Q  T  L  P  K  P  F  I  W  A  E  P  H  F
ATG GTT CCA AAG GAA AAG CAA GTG ACC ATC TGT TGC CAG GGA AAT TAT GGG GCT GTT GAA TAC CAG CTG CAC TTT                   186
M  V  P  K  E  K  Q  V  T  I  C  Ⓒ  Q  G  N  Y  G  A  V  E  Y  Q  L  H  F
GAA GGA AGC CTT TTT GCC CTG GAC AGA CCA AAA CCC CCT GAG CGG ATT AAC AAA GTC AAA TTC TAC ATC CCG GAC                   261
E  G  S  L  F  A  V  D  R  P  K  P  P  E  R  I  N  K  V  K  F  Y  I  P  D
ATG AAC TCC CGC ATG GCA GGG CAA TAC AGC TGC ATC TAT CGG GTT GGG GAG CTC TGG TCA GAG CCC AGC AAC TTG                   336
M  N  S  R  M  A  G  Q  Y  S  Ⓒ  I  Y  R  V  G  E  L  W  S  E  P  S  N  L
CTG GAT CTG GTG GTA ACA GAA ATG TAT GAC ACA CCC ACC CTC TCG GTT CAT CCT GGA CCC GAA GTG ATC TCG GGA                   411
L  D  L  V  V  T  E  M  Y  D  T  P  T  L  S  V  H  P  G  P  E  V  I  S  G
GAG AAG GTG ACC TTC TAC TGC CGT CTA GAC ACT GCA ACA AGC ATG TTC TTA CTG CTC AAG GAG GGA AGA TCC AGC                   486
E  K  V  T  F  Y  Ⓒ  R  L  D  T  A  T  S  M  F  L  L  L  K  E  G  R  S  S
CAC GTA CAG CGC GGA TAC GGG AAG GTC CAG GCG GAG TTC CCC CTG GGC CCT GTG ACC ACA GCC CAC CGA GGG ACA                   561
H  V  Q  R  G  Y  G  K  V  Q  A  E  F  P  L  G  P  V  T  T  A  H  R  G  T
TAC CGA TGT TTT GGC TCC TAT AAC AAC CAT GCC TGG TCT TTC CCC AGT GAG CCA GTG AAG CTC CTG GTC ACA GGC                   636
Y  R  Ⓒ  F  G  S  Y  N  N  H  A  W  S  F  P  S  E  P  V  K  L  L  V  T  G
GAC ATT GAG AAC ACC AGC CTT GCA CCT GAA GAC CCC ACC TTT CCT GCA GAC ACT TGG GGC ACC TAC CTT TTA ACC                   711
D  T  E  [N]  T  S  L  A  P  E  D  P  [T]  F  P  A  D  T  W  G  T  Y  L  L  T
ACA GAG ACG GGA CTC CAG AAA GAC CAT GCC CTC TGG GAT CAC ACT GCC CAG AAT CTC CTT CGG ATG GGC CTG GCC                   786
T  E  T  G  L  Q  K  D  H  A  L  W  D  H  T  A  Q  N  L  L  Ⓡ  M  G  L  A
TTT CTA GTC CTG GTG GCT CTA GTG TGG TTC CTG GTT GAA GAC TGG CTC AGC AGG AAG AGG ACT AGA GAG CGA GCC                   861
F  L  V  L  V  A  L  V  W  F  L  V  E  D  W  L  S  R  K  R  T  R  E  R  A
AGC AGA GCT TCC ACT TGG GAA GGC AGG AGA AGG CTG AAC ACA CAG ACT CTT TGA AGA ATG ACC ATG AGA CAC AGT                   936
S  R  A  S  T  W  E  G  R  R  R  L  N  T  Q  T  L  •
GGC CAT GGG TGG ATC TGA AAG CTG GTG TTG AGC CTG GGC GGC GTG AGC TCT GTG TTG GAC CCA CGG AGG AGG GAG                  1011
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CAT GCT AAA TTT CTT TTT TTT TTT TGA GCC TGG GCG ACA GAG CGA GAC TCC ATC TCA AAA AAA AAA AAA AAA                  1161
AAA AAA ACC ACA TAG GCT CAG TCT TTT CAG TAT CTG CTT TAC TGG TTC AGT AAA AGC CAG GAA ACA CAA CTT TGT                  1236
GGT AAT CTG AAT GTT ATT GAA CTG TAT TTT GTT CAC TTT ATT GTA AAT ACT AGT GAA CAG TGA ATA AAT GGT TGT                  1311
ATA TTC CTA ATA AGA AAA AAA AAA AA                                                                                   1337
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Figure 2. (*A*) Cell surface expression of NKp46 protein in COS-7 transfected cells. COS-7 cells, transfected with clone 2F cDNA (*left*) or vector alone (*right*), were stained with anti-NKp46 mAb, followed by PE-conjugated goat anti–mouse IgG1 and were analyzed by flow cytometry. White profiles represent cells incubated with second reagent alone (i.e., negative controls). (*B*) Nucleotide and predicted amino acid sequences of NKp46. The beginning of translation is marked by an arrow. The putative signal peptide is indicated in lowercase letters, the minimal predicted transmembrane region is underlined, and the charged amino acid Arg is circled and shaded in gray. Cysteines involved in the Ig-like fold are circled and putative *N*- and *O*-glycosylation sites are boxed. DNA and protein sequence analysis were performed using GeneWorks, MacVector suites (Oxford Molecular Group Inc., Oxford, UK), NetOGlyc 2.0 (http://www.cbs.dtu.dk/services/NetOGlyc/), and PSORT (http://psort.nibb.ac.jp/) Prediction Servers. These sequence data are available from EMBL/GenBank/DDBJ under accession number AJ001383.

connecting the ectodomain with the transmembrane region. The putative transmembrane segment may be unusually short, comprising 19 amino acids that include a positively charged residue (Arg). Alternatively, inclusion of the two acidic amino acids Glu and Asp would result in a longer transmembrane region characterized by the atypical presence of several charged residues. The intracellular portion is 30 amino acids in length, is rich in basic residues, and does not contain consensus sequences for any known ITAM. Remarkably, recent biochemical analysis revealed an association of the NKp46 receptor with the ITAM-containing CD3ζ subunit (18). However, unlike CD16 cDNA, the NKp46 cDNA does not require cotransfection with CD3ζ cDNA to induce surface expression of the NKp46 protein. In addition, cotransfection of NKp46 and CD3ζ cDNAs did not result in any significant increase of NKp46 surface expression in COS-7 cells (data not shown). It is of note that the NKp46 molecule, unlike other activating NK receptors (including KAR and NKp44), does not appear to be associated with the KAR-associated polypeptide (KARAP)/DNAX-associated protein (DAP)12 signal transducing subunit (18–20).

Next, COS-7 cells untransfected or transfected with the NKp46 cDNA were surface labeled with ^{125}I, lysed in 1% NP-40, and then immunoprecipitated with the anti-NKp46 mAb. As shown in Fig. 3 *a*, the anti-NKp46 mAb immunoprecipitated a surface molecule of ~46 kD from NKp46-transfected COS-7 cells, corresponding to the surface molecule immunoprecipitated from a polyclonal NK cell population used as a control. A second faint band displaying a molecular mass of ~33 kD could be visualized in immunoprecipitates derived from NKp46-transfected COS-7 cells. The NKp46 molecule was originally described as a poorly glycosylated protein characterized by a slight decrease in molecular mass after treatment with *O*-glycosidase (17).

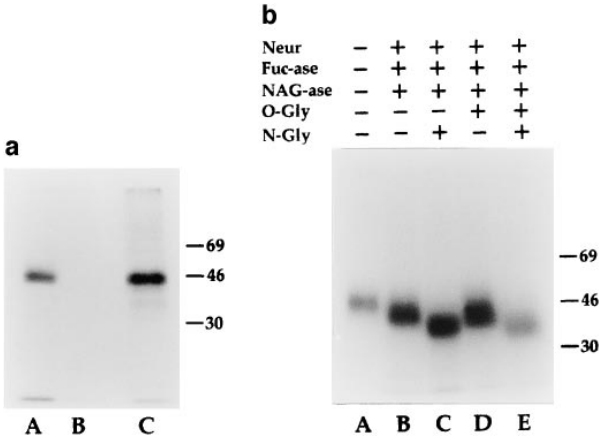

Figure 3. Biochemical analysis of NKp46 glycoprotein. (*a*) A polyclonal NK cell population (*A*) and COS-7 cells, untransfected (*B*) or transfected with NKp46 cDNA (*C*), were surface-labeled with ^{125}I and immunoprecipitated with BAB281 (anti-NKp46) mAb. Samples were analyzed in an 11% SDS-PAGE under reducing conditions. (*b*) NKp46 molecules, purified from a ^{125}I surface-labeled NK cell population, were treated with various enzymes as indicated. Samples were run in a 9% SDS-PAGE under reducing conditions.

On the other hand, the difference between the molecular size of the protein predicted from the cDNA sequence (~33 kD) and the molecular mass of the NKp46 molecule derived from normal NK cells or transfected COS-7 cells (46 kD), suggested the presence of previously undetected glycosylations. Moreover, computer search for putative glycosylation sites indicated one potential N-linked glycosylation site at Asn216 and two potential O-linked glycosylation sites at Thr125 and Thr225, respectively (28). This was confirmed by experiments in which NKp46 molecules were treated with *O*- and/or *N*-glycosidase in the presence of fucosidase and *N*-acetyl-glucosaminidase. As shown in Fig. 3 *b*, under these conditions we could demonstrate a decrease in molecular mass after treatment with either N- or O-glycosidase (lanes *C* and *D*, respectively). Moreover, when the NKp46 molecules were digested with both *N*- and *O*-glycosidase (lane *E*), a band of ~33 kD, corresponding to the predicted NKp46 protein backbone, could be visualized. Thus, the NKp46 molecule presumably displays both N- and O-glycosylations, and the faint band of 33 kD visualized in immunoprecipitates derived from COS-7 transfected cells may represent a nonglycosylated form of the NKp46 molecule.

Tissue Distribution and Sequence Analysis of the NKp46 Transcript. NKp46 cDNA and amino acid sequences were compared with the EMBL/GenBank/DDBJ databases. This analysis revealed a partial homology with members of the immunoglobulin-like transcript (ILT)/leukocyte immunoglobulin-like receptor (LIR) receptor family that are mainly expressed on human lymphoid and myelomonocytic cells (29–31). In particular, the greatest amino acid identity (39%) was observed between the extracellular domain of NKp46 and that of the inhibitory ILT3 receptor (29), also known as HM18 (30) or LIR-5 (31) (Fig. 4).

Northern blot experiments performed using RNA from multiple human tissues showed that the NKp46 gene is weakly expressed only in spleen (data not shown). More detailed Northern blot analysis, using RNA isolated from purified NK cell populations or clones, polyclonal T cells, and cell lines including the T, B, and myelomonocytic lines, revealed that NKp46 expression is confined to NK

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Consensus   Q...LPKP.. WAEP..... ...VTI.CQG ...A.EY.L. .E.S....DR    50
ecto NKp46  .QQT....FI ....HFMVPK EKQ...C... NYG.V..Q.H F.G.LFAV..   50
ecto ILT3   .AGP....TL ....GSVISW GNS...W... TLE.R..R.D K.E.PAPW..   50

Consensus   ..P.E..NK. .F.IP.M... .AG.Y.C.YR ....WS.PS. .L.LV.T..Y  100
ecto NKp46  PK.P.RI..V K.Y..D.NSR M..Q.S.I.. VGEL..E..N L.D..V.EM.  100
ecto ILT3   QN.L.PK..A R.S..S.TED Y..R.R.Y.. SPVG..Q..D P.E..M.GA.  100

Consensus   ..PTLS..P. P.V.SG..VT ..C..... .FLL.KE... ....H.....   150
ecto NKp46  DT....VH.G .E.I..EK.. FY.RLDTATS M...L..--G RS-S.VQRGY  147
ecto ILT3   SK....AL.S .L.T..KS.. LL.QSRSPMD T...I..RAA HPLL.LRSEH  150

Consensus   G....QAEFP ..PVT..H.G TYRCF.S... .....S.PS. P..L.V.G..  200
ecto NKp46  .--KV..... LG...TA.R. .....G.--Y NNHAW.F..E .VK.L.T.DI  193
ecto ILT3   .AQQH..... MS...SV.G. .....S.HGF SHYLL.H..D .LE.I.S.SL  200

Consensus   E.....P... ...A..... .L........ ...L..H... .           241
ecto NKp46  .NTSLA.EDP TFP.DTWGTY L.TTETGLQK DHA.WD.TAQ N           234
ecto ILT3   .GPRPS.TRS VST.AGPEDQ P.MPTGSVP- HSG.RR.--W E           238
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Figure 4. Alignment of amino acid sequences corresponding to the extracellular regions of NKp46 and ILT3 proteins. The putative signal peptides were deleted from both sequences. Consensus sequence is indicated on top, dashes were introduced to maximize homologies, and amino acids identical to the consensus are indicated by dots.

cells, whereas other lymphoid or myelomonocytic cells do not express NKp46 mRNA (Fig. 5). The NK-specific expression pattern of NKp46 transcript was subsequently confirmed by reverse transcriptase (RT)-PCR analysis (data not shown). These findings are in agreement with previous data demonstrating NK-specific cell surface expression of NKp46 protein, as determined by flow cytometric analysis using anti-NKp46 specific mAb (17). Remarkably, the NK cell–restricted expression of NKp46 differentiates this molecule from ILT/LIR molecules that generally display a broader cellular distribution.

As shown in Fig. 5, the NKp46 main transcript is ~3.4 kb long, and an additional faint band of ~0.9 kb is also detectable. Subsequent RT-PCR experiments, using specific primers and RNA isolated from NK cell clones derived from different donors, revealed the existence of shorter cDNA sequences, probably representing alternative splice variants of NKp46 (data not shown). It is possible that the 0.9-kb RNA transcript that weakly hybridizes with the NKp46 full length cDNA probe might correspond to a transcript encoding a lower molecular weight isoform of NKp46 molecule. At present, it is not known whether these alternative splice variants of NKp46 (nucleotide sequence data are available from EMBL/GenBank/DDBJ under accession numbers AJ006121, AJ006122, and AJ006123) represent functional molecules.

The NKp46 cDNA sequences obtained by RT-PCR from clonal and polyclonal NK cells isolated from several unrelated donors were identical (data not shown). This finding indicates that the NKp46 gene does not display significant allelic variations among different individuals.

Chromosomal Localization and Southern Blot Analysis of the NKp46 Gene. We performed analysis of chromosomal localization of the NKp46 gene by Southern blotting. To this aim, we used genomic DNA, extracted from a panel of human/hamster somatic cell hybrids, hybridized with the NKp46 cDNA probe at high stringency conditions. This analysis revealed segregation of the NKp46 gene on chromosome 19 (Fig. 6). Since the genes encoding killer cell inhibitory protein (KIR)/KAR, FcαR (1), ILT/LIR (29–31), and LAIR-1/p40 (32) molecules are also located on chromosome 19 (in particular, they are linked within a short segment of 19q13.14; reference 33), this suggests the existence of a common ancestral gene for all these molecules. Southern blot analysis of human genomic DNA (Fig. 6) also revealed a relatively simple hybridization pattern for the NKp46 gene, suggesting that the NKp46 probe may hybridize with a single gene or a few genes.

We next investigated the species-specificity of the NKp46 gene by Southern blot analysis under lower stringency conditions, using genomic DNA from different species. Interestingly, the NKp46 probe displays cross-hybridization with genomic DNA from monkey, dog, rabbit, cow, mouse, and rat DNA (data not shown). These data suggest a significant cross-species conservation of the NKp46-encoding gene. In addition, by searching the National Center for Biotechnology Information (NCBI) database of expressed sequence tags (EST), we found an EST clone from a C57BL/6J mouse spleen cell library (accession number AA170207) that shared high degree of sequence identity with the human NKp46 cDNA. Based on the sequence information obtained from this EST clone, we could isolate a murine homologue of the NKp46 molecule that shares 69% homology with the human NKp46 cDNA sequence (Biassoni, R., A. Pessino, C. Bottius, A. Morette, and L. Morette, manuscript in preparation).

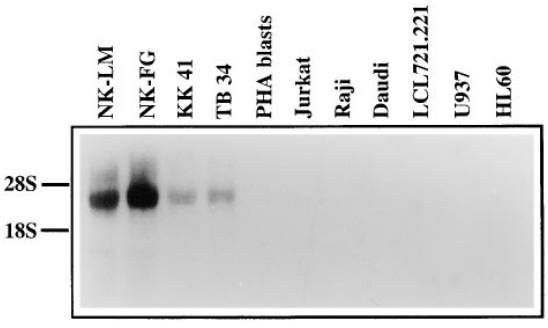

Figure 5. Northern blot analysis of NKp46 transcript expression. Total RNA was isolated from cells of different origins as follows: polyclonal NK cell populations (NK-LM and NK-FG); NK cell clones (KK41 and TB34); a polyclonal $CD3^+$ T cell population (PHA blasts); a T lymphoma cell line (Jurkat); Burkitt's lymphoma B cell lines (Raji and Daudi); an EBV-transformed B cell line (LCL721.221); a histiocytic lymphoma cell line (U937); and an acute promyelocytic leukemia cell line (HL60). 10 μg of each RNA preparation (2 μg of RNA from NK cell clones KK41 and TB34) were hybridized with the 1.3-kb NKp46 probe. The positions of 28S and 18S ribosomal RNA subunits are indicated on the left.

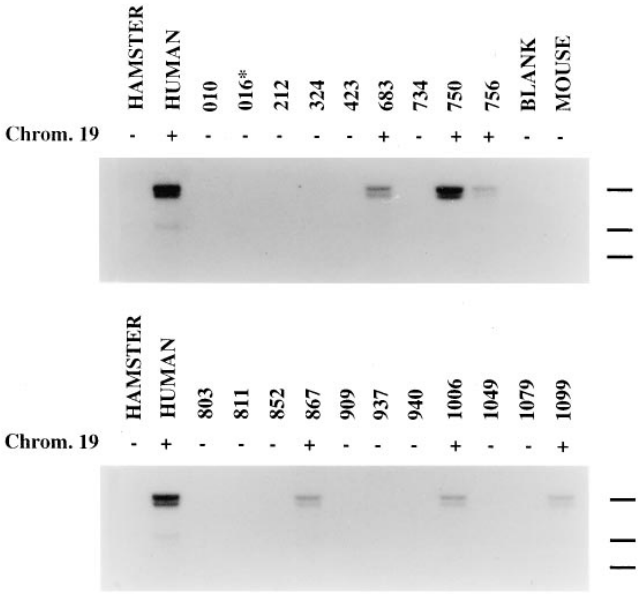

Figure 6. Chromosomal localization of NKp46 gene. Genomic DNA derived from a panel of hamster/human somatic cell hybrids, or from human, hamster, or mouse tissues, and digested with EcoRI, was hybridized with the 1.3-kb NKp46 probe. The hybrid cell lines containing chromosome 19 are indicated on top. The positions of the 23.1-, 9.4-, and 6.6-kb fragments of the λ HindIII-digested molecular weight marker are indicated on the right side of each autoradiograph.

Conclusion. This study reports the molecular cloning of the first human NK-specific receptor involved in triggering of non MHC-restricted natural cytotoxicity. Sequence analysis revealed a new member of the Ig superfamily, characterized by two C2-type Ig-like domains, a transmembrane region containing at least one charged amino acid (Arg), and a short cytoplasmic tail, that does not contain ITAM. The presence of a positively charged amino acid within the transmembrane domain is a typical feature of many triggering receptors, including KAR, NKG2-C, and TCRs. Since the prediction of the transmembrane region of NKp46 is not unequivocal from its amino acid sequence, the possibility exists that the complete transmembrane segment may include also the two negatively charged residues Glu and Asp. If this is the case, NKp46 would possess an unusual transmembrane domain with several charged amino acids potentially mediating interactions with other molecules. In this context, previous data demonstrated that NKp46 associates with CD3ζ subunit (18), which is characterized by a negatively charged amino acid (Asp) in its transmembrane region and by the presence of three ITAM sequences in its cytoplasmic portion. Although this association is likely to be essential for signal transduction via NKp46, CD3ζ is not required for NKp46 surface expression in cell transfectants. It is of note that CD16, which also binds to CD3ζ, displays an Asp residue in its transmembrane region.

The specificity of the NKp46 molecule for NK cells was shown previously by selective reactivity of NK populations and clones with anti-NKp46 mAb (17). In this study, this finding was further substantiated by the analysis of the expression of the Nkp46-specific transcript. Therefore, the NKp46 molecule represents the only NK-specific marker for human NK cells identified thus far.

The anti-NKp46 mAb has also been used to analyze the regulatory function of the NKp46 molecule. Mab-mediated cross-linking of NKp46 molecule triggered different NK cell functions (17). On the other hand, mAb-mediated masking (i.e., under non–cross-linking conditions) of NKp46 molecule inhibited NK cytotoxicity against different tumor target cells (17, 18). Here we show that the degree of this inhibition depends upon the target cell analyzed, and that NKp46 is involved not only in cytotoxicity against human tumor cells of different histotype and origin, but also against murine target cells. It is likely that additional triggering receptors (including NKp44 and other as yet undefined molecules) are involved in natural cytotoxicity against human target cells. However, NKp46 receptor would appear to play a predominant role in triggering of cytotoxicity against most tumor target cells analyzed. It is possible that this is due to the expression of putative Nkp46 ligands on most NK-susceptible tumor cell lines analyzed. It will be important to identify these ligands to clarify their tissue distribution, their expression on tumor versus normal tissues, and their expression in different species.

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