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A cyclophilin-related protein involved in the function of natural killer cells

(natural killer tumor-recognition molecule/histone-related/arginine- and serine-rich domains)

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ABSTRACT **Natural killer cells are non-major histocompatibility complex-restricted large granular lymphocytes that can recognize and destroy tumor cells without prior stimulation. A 150-kDa molecule on the surface of human natural killer cells was identified as a component of a putative tumor-recognition complex. We report here the isolation of cDNAs coding for the 150-kDa tumor-recognition molecule from human and mouse cDNA libraries. The amino terminus of the predicted protein contains a large hydrophobic region followed by a domain that is highly homologous to cyclophilin/peptidylprolyl cis–trans isomerase. The remainder of the protein is extremely hydrophilic and contains three homologous positively charged clusters. There are also three regions that contain extensive arginine- and serine-rich repeats. Comparison of the human and mouse predicted amino acid sequences revealed >80% homology.**

Natural killer (NK) cells are defined as $CD3^-$, $CD16^+$, large granular lymphocytes (LGL) capable of killing tumor targets in a non-major histocompatibility complex-restricted fashion (1). The NK cell is thought to constitute a first line of defense against microorganisms and tumor cells (2). Although the NK phenomenon has been extensively studied, the molecule(s) responsible for the recognition of tumor cells by the NK cell has not been precisely defined. Numerous reports have implicated various NK cell and target-cell proteins in the recognition events that occur (3–6), but there is still no explanation of the process of specific recognition and lysis of tumors by the NK cell. Studies have shown that the T-cell receptor is not involved in target-cell recognition by NK cells and that $CD3^-$ LGL do not rearrange and express the α, β, or γ chains of the T-cell receptor (7–10).

To define the molecule(s) responsible for tumor-cell recognition by NK cells, a technique was used that involved the production of anti-idiotypic (ID) antisera to a monoclonal antibody that recognized a variety of NK-cell-susceptible tumor targets and blocked NK-cell-mediated lysis (11). The anti-ID antiserum was shown by immunofluorescence to bind specifically to $CD3^-$, $CD16^+$ NK cells, and it blocked the binding of LGL to NK-susceptible targets, thereby preventing lysis (12). Two proteins of 80 and 150 kDa were immunoprecipitated from ^{125}I surface-labeled NK cells but not from T cells or monocytes. The ability of NK cells to perform antibody-dependent cellular cytotoxicity was not affected by the anti-ID antiserum, indicating that the proteins recognized by the anti-ID antiserum were not associated with the CD16-mediated killing pathway. Prolonged pretreatment (18 hr) of effector cells with the anti-ID antiserum enhanced lytic activity and interferon γ production by NK cells, suggesting that these molecules also were linked to the triggering of NK cells. This hypothesis was supported by cross-linking anti-ID to anti-2,4-dinitrophenol, which redirected NK cytotoxicity against a NK-resistant target precoated with 2,4-dinitrophenol. On the basis of this evidence, the anti-ID antiserum was proposed to identify a putative NK receptor (pNKR; ref. 12) complex on the surface of NK cells that is involved in the recognition of tumor targets and is coupled to the cytolytic pathway. Therefore, we have named the 150-kDa member of the complex described herein as a NK-tumor recognition molecule (NK-TR).

The present study describes the isolation of a cDNA coding for a portion of the proposed NK-TR from a human NK-cell λgt11 expression library by screening with the anti-pNKR antiserum. Overlapping cDNAs containing the complete coding sequence were isolated from mouse and human cDNA libraries by DNA hybridization.§ The NK-TR gene is a single-copy nonrearranging gene specifically expressed in NK cells. The predicted amino acid sequence of the NK-TR indicates that it represents a discrete class of protein containing multiple domains with homology to cyclophilin and DNA/RNA-binding proteins.

MATERIALS AND METHODS

Synthesis of NK-Cell cDNA Library and Plaque Screening. Human NK cells were purified from human peripheral blood (donated by the Canadian Red Cross). Peripheral blood was depleted of monocytes by adherence to plastic tissue culture plates, and B cells were removed by passage of the cells over nylon wool columns. Two cycles of T-cell depletion with anti-CD3 and rabbit complement (Cedarlane Laboratories, Hornby, ON, Canada) were done. Activated NK cells were obtained by culture in the presence of interleukin 2 at 10 units/ml (Cetus) for 2 days. Total cellular RNA was isolated by using the method of Chirgwin *et al.* (13), and mRNA was purified by using two rounds of selection on oligo(dT)-cellulose. Double-stranded cDNA was synthesized by using a modification of the method of Gubler and Hoffman (14). After the addition of open-ended *Eco*RI linkers (Pharmacia), the cDNA was inserted into *Eco*RI-cut λgt11 (Promega). The resulting library contained 6×10^6 independent clones.

Antibody screening of the λgt11 library was conducted by using the procedure of Young and Davis (15). Additional

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Abbreviations: ID, idiotype; LGL, large granular lymphocytes; NK, natural killer; NK-TR, natural killer cell tumor-recognition molecule.

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§The sequences reported in this paper have been deposited in the GenBank data base [accession nos. L04288 (human cDNA) and L04289 (mouse cDNA)].

clones were isolated by plaque screening of the human NK library, a human bone-marrow λgt10 cDNA library (Clontech), a human thymus λgt10 cDNA library (from T. Mak, University of Toronto), or mouse peripheral blood lymphocyte λgt10 cDNA library (Clontech) with DNA probes following the procedure of Benton and Davis (16).

Radiolabeling of Proteins and Immunoprecipitation. Intact $CD3^-$ LGL, T cells, and monocytes were surface-labeled with ^{125}I by using lactoperoxidase that selectively labels only surface proteins (17). Proteins were immunoprecipitated from cell lysates with anti-peptide antiserum or normal rabbit serum, and protein A–Sepharose CL-4B. The complete coding region of the human cDNA was cloned into pBluescript KS(+) (Stratagene) and used as a template for a coupled *in vitro* transcription/translation system (Promega). Protein samples were analyzed by 7.5% SDS/PAGE (18).

DNA Sequencing. Direct sequencing of λgt11 clones was achieved through the use of forward and reverse sequencing primers (New England Biolabs). The *Eco*RI-cleaved cDNA inserts of individual λ clones were purified and digested with *Hae*III, *Alu* I, or *Mbo* I and cloned into the appropriate restriction site of M13mp18 or M13mp19 (19). Single-stranded template DNA was purified and sequenced with Sequenase (United States Biochemical). Both strands of all possible fragments were sequenced.

Nucleic Acid Hybridization. RNA and Southern blotting techniques were essentially as described (20). All radioactive DNA probes were synthesized by the random-primed method (BRL) using [α-^{32}P]dATP as label (Amersham).

RESULTS AND DISCUSSION

Molecular Cloning of the NK-TR. Affinity-purified anti-ID antiserum was used to screen a human NK-cell λgt11 cDNA library. Of ≈500,000 plaques screened, six positive clones were purified. These clones were used to produce λgt11 lysogens in *Escherichia coli* strain Y1089. The lysogens were then used to overproduce β-galactosidase fusion proteins by induction with isopropyl β-D-thiogalactoside at 42°C. Fusion proteins were purified on anti-β-galactosidase columns and tested for their ability to block immunoprecipitation of the 80- and 150-kDa proteins by the anti-ID serum. One clone (NK-2) produced a fusion protein that inhibited the immunoprecipitation of the 80- and 150-kDa molecules and therefore was chosen for further study. A synthetic peptide corresponding to a positively charged region of the predicted NK-2 fusion protein (peptide 104; residues 476–497 in Fig. 1) was injected into rabbits and produced the anti-p104 antiserum that reacted with the 150-kDa molecule (12). The antipeptide antiserum possessed properties similar to the original anti-ID antiserum.

These results indicate that clone NK-2 expresses an epitope contained in the 150-kDa molecule recognized by the anti-ID antiserum. The cDNA insert of clone NK-2 contained 250 bp that possessed no significant homology to any known sequence in the GenBank data base. The predicted amino acid sequence fused to β-galactosidase in NK-2 is underlined in Fig. 1. The NK-2 cDNA insert was used as a probe to screen the λgt11 NK cell cDNA library as well as a λgt10 human bone-marrow library and a human thymus cDNA library in λgt10. Overlapping cDNAs that spanned a total of 4272 bp were isolated.

Nucleotide and Deduced Amino Acid Sequence of the NK-TR. The nucleotide sequence and deduced amino acid sequence of the human NK-TR are shown in Fig. 1. The human NK-TR cDNA possesses a continuous open reading frame of 1403 amino acids (predicted M_r 157,713) that begins with an ATG initiation codon immediately preceded by an in-frame stop codon. The open reading frame is sufficient to code for the 150-kDa molecule observed by immunoprecipitation. Although there are several potential N-linked glycosylation sites, no appreciable N-linked glycosylation of the NK-TR was detected by treatment of protein lysates with N-glycosidase (data not shown). The NK-TR contains several distinct domains, as diagrammed in Fig. 2*A*. The first 58 amino acids of the NK-TR constitute a hydrophobic segment that resembles the multiple membrane-spanning region of the CD20 protein amino terminus (27). This result suggests that the NK-TR is attached to the membrane via its amino terminus, and most of the protein is expressed on the cell surface. The proposed orientation of the molecule in the membrane is supported by the ability of the anti-ID and antipeptide antibodies to bind to intact cells (12), indicating that the cyclophilin-related domain and the second positively charged domain are extracellular. There is a 24-amino acid hydrophobic region at position 641, and a 19-amino acid hydrophobic stretch at position 1286. Approximately half of the amino acids in these regions are polar, negatively charged, or proline residues, so they do not conform with typical transmembrane segments. However, we cannot exclude the possibility that they cross the membrane and result in the expression of residues 665–1285 in the cytoplasm.

Of particular interest is the presence of a domain that is homologous to cyclophilin/peptidylprolyl cis–trans isomerase (28, 29) immediately following the hydrophobic amino terminus of the protein. Cyclophilin is the name given to a cytoplasmic protein that represents the major cyclosporin A-binding protein isolated and protein sequenced from human and bovine thymus (30). Protein sequencing of the enzyme peptidylprolyl cis–trans isomerase (28, 29) revealed that it corresponded to the cyclophilin gene, and cyclosporin A was shown to inhibit the activity of this enzyme. Fig. 2*B* shows a comparison of the NK-TR cyclophilin-related domain with human thymus cyclophilin (23), the *Drosophila* ninaA protein (21), *N. crassa* cyclophilin (22), tomato cyclophilin (25), and *S. cerevisiae* cyclophilin (24). The NK-TR sequence possesses approximately the same degree of homology (from 47% to 53% amino acid homology) to each of the previously described cyclophilin-related sequences. Greater than 70% of the NK-TR amino acid residues in this region are homologous to one or more of the known cyclophilin proteins. The NK-TR is functionally more related to the ninaA protein because it contains a membrane-bound cyclophilin-like domain, and the ninaA gene product is expressed as a transmembrane protein with the cyclophilin domain contained in the endoplasmic reticulum (31).

The remainder of the predicted NK-TR protein is extremely hydrophilic, containing >20% serine residues and 37% charged residues. The positively charged amino acids are far more abundant, resulting in a net positive charge of 147 for the NK-TR protein. Three homologous positively charged clusters are present in the NK-TR (Fig. 2*C*). These positively charged regions of the molecule bear homology to several DNA-binding proteins, but the greatest homology was seen with sperm histone (ref. 26; Fig. 2*C*). Synthetic peptide p104 from the second positively charged region was able to induce an anti-NK-TR antiserum that reacted with the surface of freshly isolated NK cells and possessed functional properties similar to the original anti-ID antiserum (12). Because the anti-peptide antiserum could block killing of tumor targets, this result suggests that the positively charged domains are involved in triggering the NK lytic mechanism. The functional significance of the high density of positive charge in these regions is presently unclear. In addition, there are three large clusters (≈100 amino acids) containing numerous arginine-serine repeats. These regions have a general motif of alternating positive and polar amino acids. Arginine- and serine-rich domains have been reported in a number of proteins that regulate RNA splicing. The arginine-serine domains of the *Drosophila* suppressor of white apricot and transformer gene products target the proteins to a subcom-

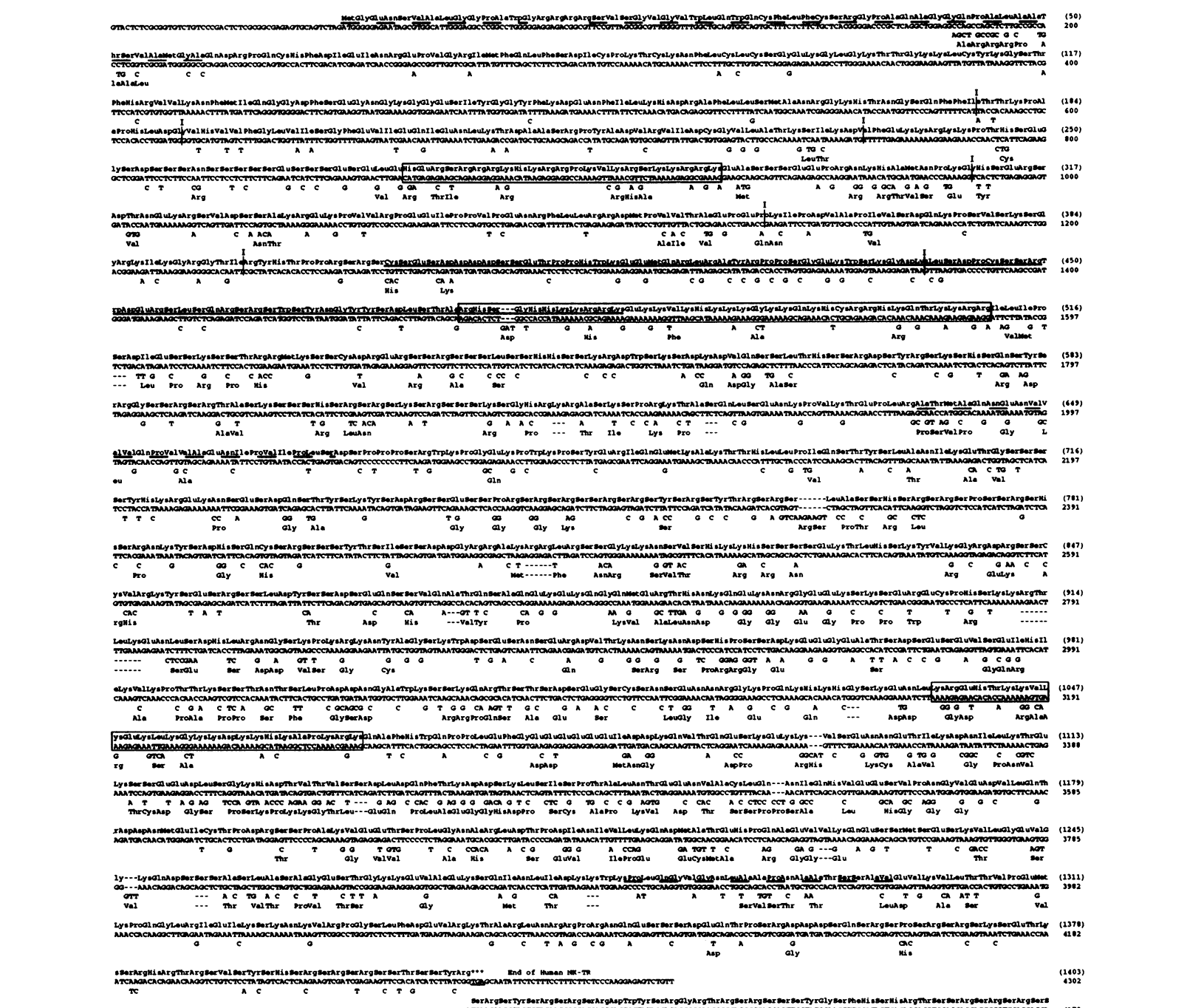

FIG. 1. NK-TR cDNA sequence. The sequence of the human NK-TR mRNA was determined from overlapping cDNAs. Nucleotides or amino acids of the mouse cDNA that differ from the human sequence are shown below the human sequence. Nucleotide numbers of human cDNA are shown at right; amino acid numbers are shown in parentheses. The symbol I above a vertical line indicates the position of an intron in the human NK-TR gene. The heavy underlining of amino acids 404–486 indicates the region expressed in λgt11 clone NK-2. The dashed underlinings indicate hydrophobic segments. Boxed regions represent clusters of positively charged amino acids.

partment of the nucleus thought to be the site of RNA splicing (32, 33).

The possible mechanism of signal transduction associated with the NK-TR is presently unclear because no significant homology to protein kinases or protein phosphatases was detected. Perhaps the NK-TR mediates signal transduction through other associated proteins. Also the cyclophilin/peptidylprolyl cis–trans isomerase region of the NK-TR is

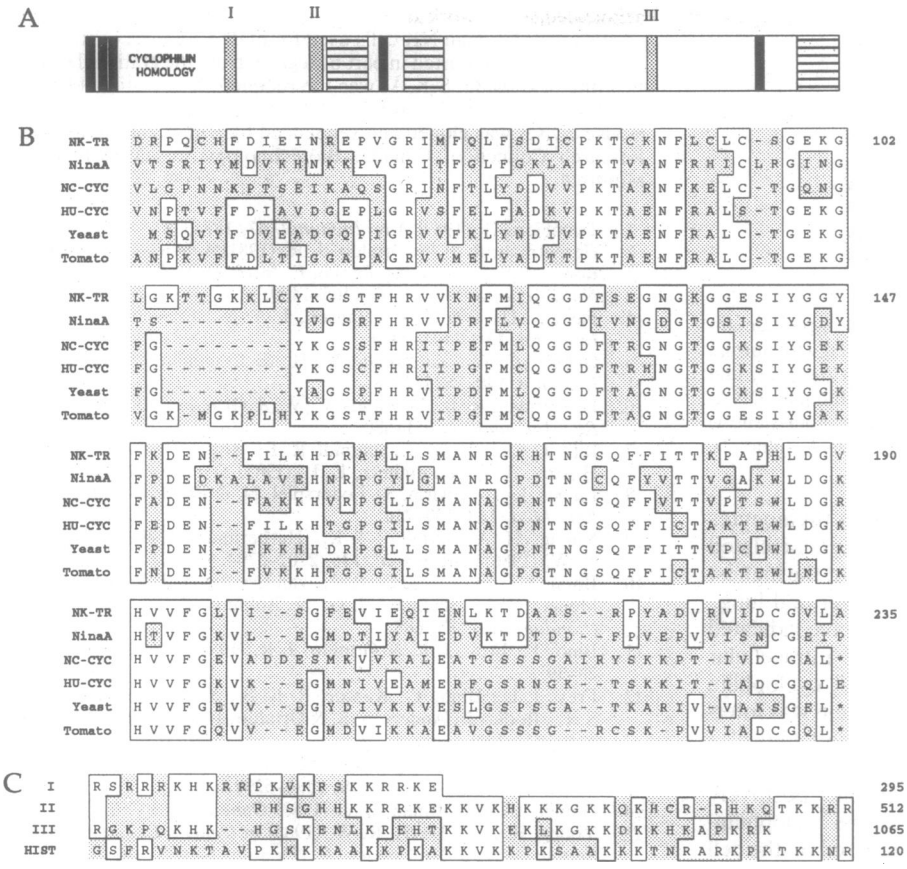

FIG. 2. Comparison of the NK-TR with other known proteins. (*A*) Schematic of NK-TR structure. A linear map of NK-TR protein structure is shown. Black rectangles indicate hydrophobic regions. The cyclophilin-related domain is labeled. Shaded rectangles indicate the three homologous positively charged clusters. Arginine- and serine-rich domains are indicated by boxes containing horizontal lines. (*B*) Alignment of NK-TR and known cyclophilin genes. The cyclophilin-related domain of NK-TR is aligned with amino acid sequences of *Drosophila* ninaA protein (NinaA; ref. 21), *Neurospora crassa* cyclophilin (NC-CYC; ref. 22), human thymus cyclophilin (HU-CYC; ref. 23), *Saccharomyces cerevisiae* cyclophilin (Yeast; ref. 24), and tomato cyclophilin (Tomato; ref. 25). Only the residues homologous to the corresponding NK-TR residues are boxed. (*C*) Alignment of positively charged regions with histone (HIST). The three positively charged domains of NK-TR are aligned with sperm histone (26). Identical residues are boxed. Amino acid numbers are shown at right.

involved in a signal-transduction process that occurs during NK-cell–target cell interaction.

Molecular Cloning of the Mouse Homologue of the NK-TR. Screening of 500,000 clones from a mouse peripheral blood lymphocyte λgt10 cDNA library (Clontech) with DNA probes derived from the 5′ and 3′ regions of the human NK-TR cDNA resulted in the isolation of 30 overlapping clones representing a 6.7-kb cDNA. The nucleotide sequence and proposed amino acid sequence of the mouse NK-TR that differs from the human NK-TR sequence are shown in Fig. 1. The 5′ initiation codon of the mouse gene was not contained within any of the clones sequenced; however, some sequence from the amino-terminal hydrophobic region was determined. The predicted open reading frame of the mouse NK-TR cDNA is 1460 amino acids with a predicted molecular mass of 160 kDa for the unmodified protein. The cyclophilin-related domain is 100% conserved between the human and mouse NK-TR. The remainder of the two proteins is highly conserved, except for several regions of elevated diversity. The mouse NK-TR possesses an additional 104 amino acids at the carboxyl terminus that contains numerous arginine-serine repeats.

Analysis of the NK-TR Gene and mRNA Expression. Southern blotting analysis of human and mouse genomic DNAs was conducted to investigate the nature of the loci coding for the NK-TR mRNA. Fig. 3*A* demonstrates that a single-copy cellular gene homologous to the NK-TR cDNA is present in both human and mouse genomic DNA. Analysis of various mouse tissues failed to reveal any gene rearrangements (data not shown). In addition, a human genomic DNA clone was isolated from a human fetal liver library (34). The DNA sequence of the 3′ region of the human NK-TR genomic locus has been determined, and the positions of introns in the gene are indicated in Fig. 1. The nucleotide sequence of the 3′ coding region of the cDNA, including the observed termination codon, has been confirmed by sequencing the genomic DNA. Several unspliced cDNAs were isolated from the mouse peripheral blood lymphocyte cDNA library, and sequence analysis of these clones demonstrated that the mouse and human genomic loci share an identical exon structure for all intron/exon junctions examined (data not shown).

The NK-TR cDNA was used to investigate the expression of NK-TR mRNA in both human and mouse. Fig. 3*B* shows the results of RNA blot analysis of human NK cells and T cells as well as several mouse tissues. A 7-kb mRNA was present in purified human NK cells but not in T cells. In humans, no significant expression was found in B cells purified by anti-immunoglobulin panning, in monocytes iso-

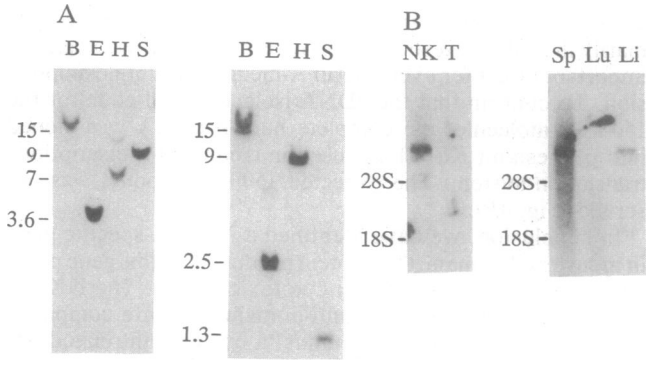

FIG. 3. Analysis of NK-TR gene. (*A*) Southern blot of human and mouse genomic DNA. *Bam*HI, *Eco*RI, *Hin*dIII, or *Sac* I-digested genomic DNA (10 μg per lane) was probed with [^{32}P]dATP-labeled cDNA insert from clone NK-2 for the human blot (*Left*) or a 1.1-kb mouse cDNA insert (PBL-21) for the mouse blot (*Right*). Lanes: B, *Bam*HI-digested human and mouse DNA; E, *Eco*RI digest; H, *Hin*dIII digest; S, *Sac* I digest. Molecular sizes in kbp are shown at left. (*B*) RNA blot of human and mouse NK-TR. Poly(A)$^+$ RNA (5 μg per lane) was electrophoresed on a 0.7% agarose gel containing formaldehyde. (*A*) Human mRNA. NK, purified NK cell mRNA; T, purified T-cell mRNA. (*B*) Mouse tissue mRNA. Sp, spleen mRNA; Lu, lung mRNA; Li, liver mRNA. Positions of 18S and 28S ribosomal RNAs are shown at left.

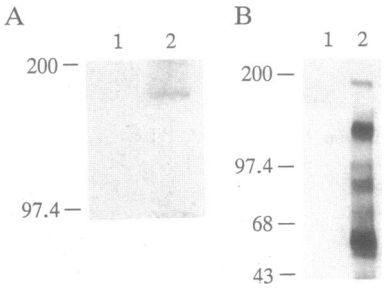

FIG. 4. (*A*) Immunoprecipitation of NK-TR by anti-peptide antiserum. ^{125}I-membrane-labeled LGLs were precipitated with normal rabbit serum (lane 1) or anti-peptide 106 antiserum (lane 2), and separated by SDS/PAGE. (*B*) *In vitro* translation of human cDNA. Coupled transcription/translation was done on vector alone (lane 1) or vector containing complete human NK-TR cDNA (lane 2). Positions of M_r standards ($\times 10^{-3}$) are shown at left.

lated by adherence to plastic, in platelets, or in thymocytes (data not shown). The expression in mouse tissues correlated approximately with the levels of NK cells present; the highest expression was found in spleen followed by liver and lung.

Analysis of NK-TR Protein. Previous studies have indicated that cyclophilins and other immunophilins are intracellular proteins (35). The observation that the NK-TR cyclophilin domain was part of a membrane-associated protein was interesting, particularly with regard to a recent report (36) of a cell-surface cyclosporin A-binding protein. Because anti-peptide antibodies directed against the second positively charged domain reacted with the cell surface, the cyclophilin-related domain of the NK-TR should be expressed on the cell surface. To confirm surface expression, several synthetic peptides from the cyclophilin-related region were used to immunize rabbits. One antipeptide antiserum (p106; amino acids 95–115 in Fig. 1) was found weakly reactive with the surface of CD3$^-$, CD56$^+$ NK cells by flow cytometry (data not shown) to a degree similar to that reported (12) with peptide p104. To further examine the surface expression of this cyclophilin-related region, NK cells were surface labeled with ^{125}I and immunoprecipitated with anti-p106. The resultant 150-kDa band is shown in Fig. 4*A*. These results support the contention that this cyclophilin-related region is on a NK cell-surface-associated protein. The presence of a putative cyclosporin A-binding domain on a molecule expressed primarily in LGL suggests that it may represent a physiologically important target for cyclosporin A-mediated immunosuppression. To confirm that the cDNA isolated could code for the 150-kDa molecule, the complete human cDNA was cloned into pBluescript KS(+) and used in a coupled transcription/translation system. The expected 150-kDa product was observed (Fig. 4*B*).

In conclusion, we have identified a NK-cell-specific gene in mouse and human. The protein produced by this gene plays an important role in NK-cell cytotoxicity (12). The NK-TR possesses a very unusual multi-domain structure composed of a hydrophobic amino terminus, a cyclophilin-related domain, three histone-like domains, and three arginine- and serine-rich domains. Although a direct role for the 150-kDa NK-TR protein in target-cell recognition has not yet been demonstrated, the NK-TR gene will be a useful tool for studying the ontology of NK cells as well as the molecular basis of tumor-cell recognition and lysis by the natural immune system.

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