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# Cloning of a new lectin-like receptor expressed on human NK cells

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**Abstract** Natural killer (NK) cells constitute the third major population of lymphocytes. They possess the inherent capacity to kill various tumor and virally infected cells and mediate the rejection of bone-marrow grafts in lethally irradiated animals. A large family of NK cell receptors belong to the C-type lectin superfamily and are localized to the NK gene complex on Chromosome (Chr) 6 in the mouse and Chr 12 in the human. Genes in the NK gene complex encode type II receptors and examples include the families of NKR-P1, Ly-49, and NKG2 receptors. Examples of other C-type lectin-like NK cell receptors that occur as individual genes are *CD94*, *CD69*, and *AICL*. Here we report the molecular characterization and chromosomal mapping of a human lectin-like transcript (LLT1) expressed on NK, T, and B cells and localized to the NK gene complex within 100 kilobases of *CD69*. The cDNA encodes a predicted protein of 191 amino acid residues with a transmembrane domain near the N-terminus and an extracellular domain of 132 amino acid residues with similarity to the carbohydrate recognition domain of C-type lectins. The predicted protein of LLT1 shows 59 and 56% similarity to AICL and CD69, respectively. The predicted protein does not contain any intracellular ITIM motifs, suggesting that LLT1 may be involved in mediating activation signals.

**Key words** NK cells · Human · Surface molecule · Lectin superfamily · NK gene complex

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## Introduction

The C-type carbohydrate recognition domain (CRD) is the common feature among $Ca^{2+}$-dependent animal lectins and structurally related proteins. A subset of the C-type lectin family found on natural killer (NK) cells contains domains homologous to other C-type lectin domains, but whether they mediate interactions through carbohydrate or protein binding remains unresolved (Weis et al. 1998). NK cell receptors with lectin-like domains are encoded in the NK gene complex on Chromosome (Chr) 6 in the mouse and Chr 12 in the human. (Brown et al. 1997; Yabe et al. 1993; Yokoyama and Seaman 1993).

The majority of NK cell receptors encoded by the NK gene complex belong to groups of highly related genes such as the *NKR-P1, Ly-49*, and *NKG2* families. The *Ly-49* and *NKG2* families contain members that are mostly inhibitory, but have a few members that transduce activation signals (Lanier 1998; Long and Wagtmann 1997; Yokoyama and Seaman 1993). The NKR-P1 receptors have been observed to act as activating receptors in rodents. Cross-linking of the human NKR-P1 homologue with antibody leads to inconsistent results (Lanier et al. 1994; Poggi et al. 1996), suggesting the possibility of isoforms that have not yet been isolated (Lanier 1998). CD94 is a type II receptor expressed on most NK cells and was originally implicated as an inhibitory receptor (Chang et al. 1995; Long and Wagtmann 1997). Subsequently, it was discovered to form a heterodimer with members of the NKG2 family (Lazetic et al. 1996).

CD69 and AICL (activation-induced C-type lectin) are two structurally similar receptors localized to the NK gene complex, but have interesting differences from the other genes located there. As opposed to the other type II receptors in the NK gene complex, which are restricted to NK cells and a subset of T cells, CD69 and AICL are expressed in most cells of hematopoietic origin (Hamann et al. 1997; Lanier 1998; Long and

Wagtmann 1997; Testi et al. 1994). The function of AICL is not known, but CD69 cross-linking leads to the activation of NK cells, T cells, B cells, monocytes, granulocytes, and platelets (Testi et al. 1994). In addition, these genes appear to have single rather than multiple isoforms.

In the present study, we report the molecular cloning, characterization, and expression pattern of a new lectin-like transcript predominantly expressed on human NK cells. Chromosomal mapping indicated that *LLT1* is localized to the human NK gene complex on Chr 12, close to *CD69*.

## Materials and methods

*Expressed sequence tag database search and cDNA library screening*

The expressed sequence tag (EST) database at GenBank (http://www.ncbi.nlm.nih.gov) was searched with the TblastN program vs. a consensus sequence of human (CD69, CD94, and NKG2's) and mouse (Ly-49's) C-type lectin receptors (Boguski et al. 1993, 1995). Several overlapping clones were identified and polymerase chain reaction (PCR) primers were designed to amplify a 350 base pair (bp) fragment within the C-type lectin CRD. cDNA from a NK cell library constructed in $\lambda$ phage by J. Houchins (R & D Systems, Minneapolis, MN, and kindly provided by A. Brooks, NIH, Bethesda, MD) was successfully used as template. PCR cycle conditions were 94 °C for 30 s, 50 °C annealing temperature for 30 s, and a 72 °C extension for 45 s repeated for 30 cycles using *Taq* DNA polymerase from GIBCO BRL (Grand Island, NY) at 2 mM $MgCl_2$. The same library was then screened with the resulting PCR fragment labeled with $\alpha^{32}$P dCTP (Feinberg and Vogelstein 1983; Sambrook et al. 1989). Approximately $5 \times 10^5$ clones were screened. After three rounds of screening, phage DNA was isolated from positive clones by the method of Lee and Clark (1997). All positively selected clones were sequenced (Automated sequencing facility, Department of Pathology, UT Southwestern Medical Center, Dallas) and analyzed (Genetics Computer Group, Wisconsin package). One clone (Y9A2) which contained an open reading frame was identified for further study. The transcript was named LLT1 (lectin-like transcript 1) due to sequence similarity to other C-type lectin-like receptors found on NK cells.

*Cell culture*

Human tumor cell lines Jurkat (T cell), YT (NK cell), HL-60 (monocytic), and DB (B cell), in addition to a murine lymphoma cell line (YAC-1), were cultured in RPMI 1640 supplemented with 10% fetal calf serum (Hyclone, Logan, Utah), 2 mM L-glutamine, 100 Units/ml of penicillin and streptomycin, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids (Gibco BRL). A lymphokine-activated killer cell (LAK) culture was obtained by isolating peripheral blood mononuclear cells (PBMC) from 60 ml of venous blood from a healthy donor by Ficol-Paque centrifugation (Pharmacia, Piscataway, N.J.). The cells were grown in the above media supplemented with 1000 Units/ml of human rIL-2 for three days. The non-adherent cells were removed and the culture was continued in 500 Units/ml of human rIL-2 and conditioned media until day 10, when RNA was extracted. All cell lines were grown to one million per ml and split 1:2 24 h before RNA isolation.

*RNA and DNA blot analysis*

Total RNA was isolated with the RNAstat 60 reagent according to the manufacturer's protocol (Teltest Inc., Friendswood, Tex.), divided into 20 μg aliquots, and stored in 70% EtOH at −80 °C until used. One percent agarose gels for northern analysis were stained with ethidium bromide after electrophoresis to insure equal loading by comparison of rRNA. Northern blots were probed with 25 ng of the full-length cDNA labeled with $\alpha^{32}$P dCTP (Feinberg and Vogelstein 1983; Sambrook et al. 1989). The first blot consisted of 20 μg of total RNA from human monocytic, T, B, and NK cell lines (HL-60, Jurkat, DB, and YT, respectively), a mouse cell line (YAC-1), and LAK and PBMC cells from a healthy donor immobilized on Hybond nylon (Amersham, Arlington Heights, Ill.). Prehybridization and hybridizations were performed according to the instructions of Amersham for the Hybond nylon membrane at 65 °C. The second membrane was purchased from Clontech (Palo Alto, Calif.) and contained mRNA samples from human spleen, lymph node, thymus, peripheral blood leukocytes, bone marrow, and fetal liver (human immune system multiple tissue northern blot II). It was hybridized according to the manufacturer's instructions with the included ExpressHyb Hybridization solution at 65 °C. Blots were exposed to Hyperfilm (Amersham). The membrane was subsequently stripped and reprobed for $\beta$ actin in ensure equal loading.

Genomic DNA was isolated from human liver according to standard protocol (Sambrook et al. 1989). For DNA blot analysis, human genomic DNA samples (20 μg each) were digested with various restriction enzymes (*Bam*HI, *Eco*RI, *Hin*dIII, and *Xba*I) and separated on 0.8% agarose gel by electrophoresis. The DNA was transferred to Hybond nylon membrane under alkaline condition (0.4 N NaOH) and fixed by UV cross linking. The membrane was prehybridized for 2 h at 65 °C in hybridization buffer (1 mM ethylenediaminetetraacetate (EDTA), 0.5 M sodium phosphate, pH 7.2, 7% sodium dodecyl sulfate (SDS), 100 μg/ml ssDNA). The probe (50 ng of the full-length cDNA labeled with $\alpha^{32}$P dCTP) was added to the same buffer and hybridization was continued for 18 h at 65 °C (Feinberg and Vogelstein 1983; Sambrook et al. 1989). The membrane was washed with a buffer containing 40 mM sodium phosphate, pH 7.2, 1% SDS at 65 °C for 1 h. The membrane was exposed to Hyperfilm for one day (Amersham) and developed.

*PAC identification*

A gridded human PAC library (RPCI1) was hybridized for 20 h with 40 ng of LLT1 (full-length cDNA labeled with $\alpha^{32}$P) (Feinberg and Vogelstein 1983; Ioannou et al. 1994; Sambrook et al. 1989). The membranes were washed twice with 2× standard sodium citrate, 0.1% SDS at 65 °C for 20 min and exposed to X-ray film at −70 °C for 24 h. Positive clones were provided by the Human Genome Mapping Project resource center. DNA was extracted using a Qiagen kit (Crawley, UK) according to standard protocols. PAC DNA (200 ng) was digested to excise the insert and separated on a pulse field gel with ramped switch times from 1 to 13 s at 200 V for 16 h.

*PAC polymerase chain reaction and sequencing*

PCR was performed in the presence of 2.5 mM $MgCl_2$ using primers designed to the cDNA sequence (RBC151, RBC130, RBC136, and RBC141; Table 1). PAC DNA was treated for 10 s at 94 °C, followed by 30 cycles at 94 °C for 10 s, 55 °C for 10 s, and 72 °C for 2 min with a 10 min final extension at 72 °C. The PCR products were purified using a PCR purification kit from Qiagen and sequenced on an ABI377 automated sequencer as described previously (Wilson et al. 1997).

## Results

### *Isolation and sequence analysis of the LLT1 cDNA clone*

The molecular basis of target cell recognition by NK cells is poorly understood. Unlike T and B cells, NK cells do not rearrange DNA to generate diversity. Therefore, one could predict that NK cells might express several receptors to recognize various targets or utilize some other mechanism to generate diversity. In fact, over the last few years a number of receptors have been identified on NK cells (Lanier 1998). However, all the functions of NK cells could not be accounted by the known receptors. In order to understand the mechanism by which NK cells recognize and kill target cells, we searched the EST database with a consensus sequence of human (CD69, CD94, and NKG2 s) and mouse (Ly-49 s) C-type lectin receptors (Boguski et al. 1993; Boguski 1995). Several overlapping clones were identified and PCR primers were designed and used in PCR to yield a 350 bp fragment within the C-type lectin CRD. The primers used for PCR amplifications are given in Table 1. We screened a human NK cell cDNA library with the PCR fragment and a positive clone (Y9A2) was selected for further analysis. The clone contained a cDNA insert of 850 bp with an open reading frame predicting a polypeptide of 191 amino acid residues with a type II receptor structure (Genbank accession number AF133299). The predicted protein sequence had a single transmembrane domain of 29 amino acid residues (Fig. 1A, B) and an intracellular domain of 30 amino acid residues. Additionally, it had an extracellular lectin-like domain of 132 amino acid residues which contained two putative N-linked glycosylation sites (Fig. 1A).

The predicted protein sequence of LLT1 has an extracellular domain with similarity to the C-type lectin-like domains shared with other NK cell receptors (Fig. 2; Weis et al. 1998). It has the highest similarities to AICL and CD69 of 59 and 56%, respectively (Hamann et al. 1993, 1997; Lopez-Cabrera et al. 1993; Ziegler et al. 1993). In addition, representative similarities to other NK cell receptors belonging to the C-type lectin superfamily are 53, 51, and 41% to NKG2-D, CD94, and Ly-49D, respectively (Chang et al. 1995; Houchins et al. 1991; Weis et al. 1998; Wong et al. 1991).

Analysis of the sequences of LLT1 clones from another NK cell cDNA library made from pooled NK cells (NKTRP, kindly provided by B. Passer, NIH, Bethesda, Md.) revealed no differences, indicating that the gene is not highly polymorphic. Furthermore, sequence data available in the EST database did not show variation beyond what was expected for single-pass sequences.

### *Expression of LLT1 in different tissues and cells*

The expression of LLT1 transcripts in various cell lines and different human tissues was analyzed by northern blotting of total RNA or poly(A)$^+$ RNA. The full-length cDNA hybridized to transcripts of approximately 5, 3.5, 2, and 0.9 kilobases (kb) in total RNA from a human NK cell line (YT) and hybridized weakly to transcripts of similar sizes from human T cell (Jurkat), B cell (DB), or monocytic (HL-60) tumor cell lines. Hybridization signals for the same size transcripts were strong in donor samples from a LAK culture and PBMC except for the 900 bp transcript (Fig. 3A). Tissue distribution of LLT1 showed that human peripheral blood leukocytes, lymph node, thymus, and spleen expressed transcripts of the same relative sizes as the YT cell line with the exception of the 900 bp transcript (Fig. 3B). No hybridizing transcripts were detected in mRNA from fetal liver or bone marrow. LLT1 may be expressed only in the later stages of NK cell differentiation, similar to Ly49 expression.

### *Southern analysis of human genomic DNA*

Several lectin-like receptors expressed on NK cells belong to multigene families (Lanier 1998; Long and Wagtmann 1997; Weis et al. 1998). Southern blot analysis of human genomic DNA was carried out to explore this possibility for LLT1. Genomic DNA was isolated from human liver and digested with four different restriction enzymes (*Bam*HI, *Eco*RI, *Hin*dIII, and *Xba*I), separated on an agarose gel and transferred to a nylon membrane. The full-length LLT1 cDNA hybridized to several restriction fragments (Fig. 4). The strongly hybridizing restriction fragments identified in Fig. 4 ranged from 22 to 35 kb for the different digestions in addition to several weakly hybridizing bands.

**Table 1.** PCR primers used to amplify LLT1 and CD69 sequences

| Name | Gene | Sequence (5′-3′) | Sense |
|---|---|---|---|
| YF1 | LLT1 | GAGCTAACTGCCATCAAGAGCCATCAGTATGTC | + |
| YR1 | LLT1 | AGAACCTAAGTAAGCTCCTTGAACACTTTGGAA | − |
| RBC141 | LLT1 | TTCCTGTTGAGATATAAAGGC | + |
| RBC136 | LLT1 | GGAACAAATCCACTTCCTCT | − |
| RBC151 | CD69 | GGGTCTGACAAGTGTGTTTT | + |
| RBC130 | CD69 | TGCCACATCACATATTGCAC | − |

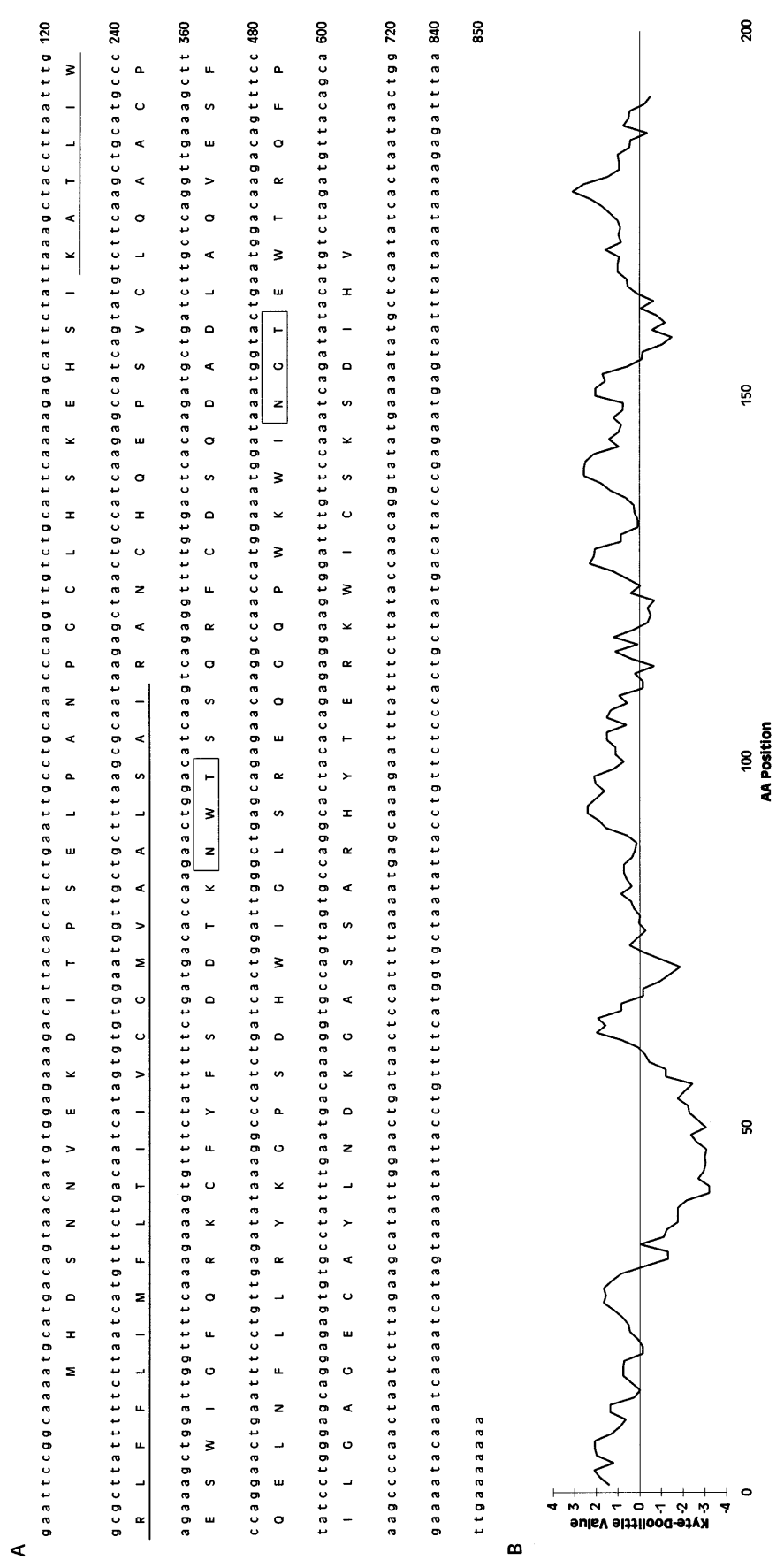


**Fig. 1A,B** Analysis of the cDNA sequence of LLT1. **A** The nucleotide sequence and predicted translation of LLT1. The transmembrane domain is *underlined*. Glycosylation sites in the extracellular domain are *boxed*. **B** Hydrophilicity plot of the LLT1 putative peptide sequence determined by the Kyte-Doolittle method

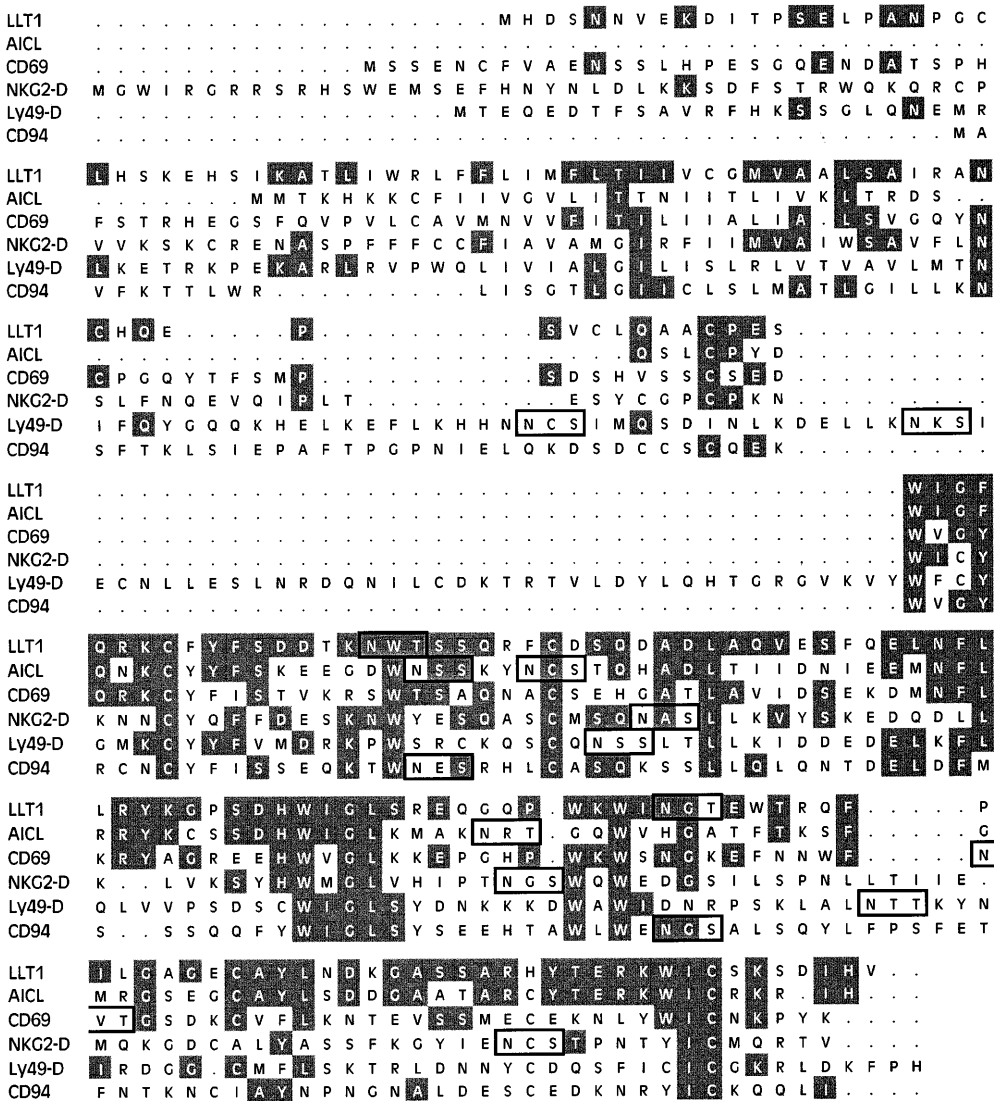


**Fig. 2** Pileup of LLT1 and other NK cell C-type lectin superfamily receptors. Conserved residues are *shaded* and putative glycosylation sites in the extracellular domains are *boxed*

*Chromosomal localization*

Due to the sequence similarity of the LLT1 clone to AICL and CD69, we expected that *LLT1* might be localized in the NK gene complex on Chr 12. Therefore, a PAC library containing the NK gene complex was screened. Two PACs were isolated from the human RCP1 library using a probe for LLT1. The inserts of PAC NKCP4 and NKCP5 were sized on a pulsed field gel to 110 and 160 kb, respectively.

LLT1 is located in the human NK gene complex within 100 kb of the *CD69* gene. PCR products of approximately 0.9 kb (RBC150/RBC130) as expected for the *CD69* gene and 1.8 kb (RBC136/RBC141) for the *LLT1* gene were obtained with both PAC DNAs as templates. The PCR products were sequenced. The *LLT1*-specific PCR product sequence revealed amplification of an intron. The exon sequence showed 100% identity to the cDNA of LLT1. The presence of intron sequence is consistent with this being the authentic *LLT1* gene and not a processed pseudogene. Consistently, we observed in all our *CD69* sequences three nucleotide exchanges (out of 796 bp) in comparison with the published *CD69* sequence (GenBank accession number Z30428). This is most likely due to polymorphism in the untranslated 3′ end of the gene.

## Discussion

This study describes the cloning and molecular characterization of a new member of the human NK gene complex. The conserved C-type CRD found in NK cells receptors localized to the NK gene complex allowed identification of related sequences in the EST database. The predicted peptide of LLT1 (Fig. 1A) bears strong similarity to CD69 and AICL (Hamann et al. 1993, 1997; Lopez-Cabrera et al. 1993; Ziegler et al. 1993). The function of CD69 has been extensively characterized. Cross-linking with anti-CD69 Ab activates the cell-specific functions of lymphocytes, granulocytes, monocytes, and platelets (Testi et al. 1994). Further-

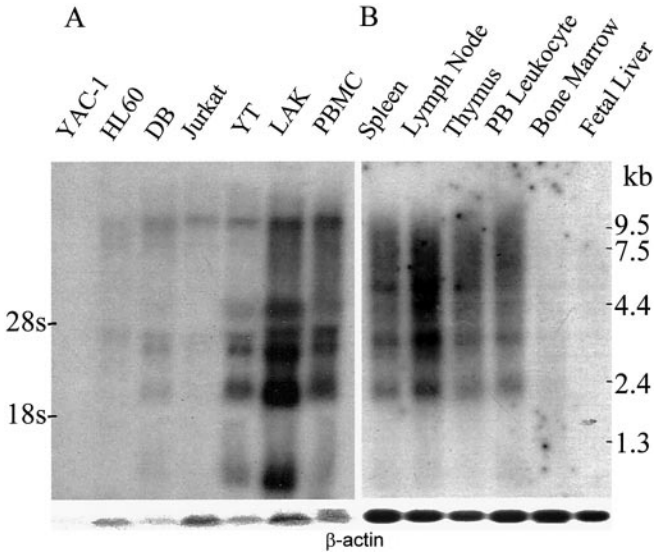


**Fig. 3A,B** RNA blot analysis of LLT1 transcripts hybridized with $^{32}$P-labeled, full-length LLT1 cDNA. **A** Total RNA (20 μg) isolated from the YAC-1, HL-60, DB, Jurkat, and YT tumor cell lines was electrophoresed in a formaldehyde agarose gel, blotted, and probed. In addition, samples were included from the PBMC and a LAK culture from a healthy donor. **B** Northern blot of poly $(A)^+$ RNA from spleen, lymph node, thymus, peripheral blood leukocytes, bone marrow, and fetal liver tissues. Both membranes were stripped and hybridized with a *β*-actin probe. The position of 28 S and 18 S rRNA and the sizes of RNA molecular standards are shown at the *left* and *right* of panels *A* and *B*, respectively

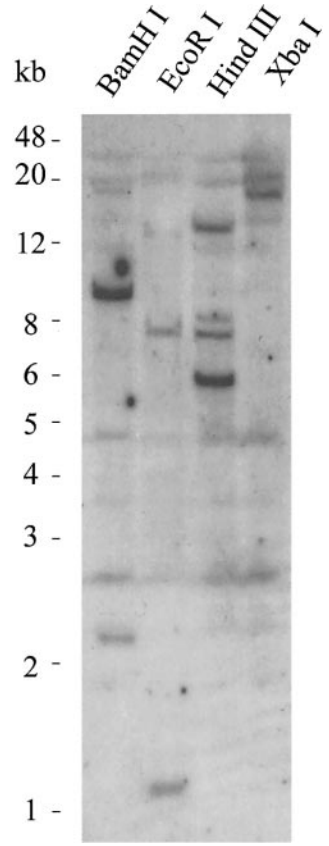


**Fig. 4** Genomic DNA blot analysis. Aliquots of human genomic DNA (20 μg) from liver were digested with the restriction enzymes *Bam*HI, *Eco*RI, *Hin*dIII, and *Xba*I, electrophoresed in 0.8% agarose, blotted, and hybridized with a $^{32}$P-labeled, full-length LLT1 cDNA. Sizes of DNA standards are shown at the left

more, CD69 is a useful indicator of cell activation and immune arousal. The signaling of neither AICL nor LLT1 has been studied yet. In light of the structural similarity of CD69 to AICL and LLT1, it is likely these new transcripts will demonstrate novel functions.

The observation of multiple bands in northern analysis implies the existence of highly related transcripts or splice variants of LLT1 (Fig. 3). Both LLT1 and CD69 are expressed in lymphocytes, at a high level in NK cells and less in T and B cells (Fig. 3; Hamann et al. 1993). We do not know whether LLT1 may be inducible on T and B cells. The restricted expression of LLT1 to tissues representing the later stages of NK cell differentiation implicates it as a receptor involved in immune response rather than development (Fig. 3B). The Southern blot showed a simple pattern indicating a single gene or a small number of genes. A similar pattern has been reported for *CD69* (Santis et al. 1994) and *AICL* (Hamann et al. 1997). The *CD69* gene is localized to approximately 15 kb and the *AICL* gene is approximately 20 kb. Taken together, this supports the notion that *LLT1* may be a single gene.

We co-localized the *LLT1* gene to within 100 kb of the *CD69* gene. The *AICL* gene has been previously localized to 0.3 cM proximal to the *CD69* gene (Hamann et al. 1997). Consequently, the proximity of the genes and the sequence similarities between the AICL, CD69, and LLT1 cDNAs suggests that the genes might be derived from the duplication of a common ancestral gene.

In conclusion, sequence analysis and chromosomal localization classify LLT1 as a new member of the NK cell receptors located in the human NK gene complex. Sequence similarity to CD69 and AICL suggest that LLT1 may have a comparable role in the immune system. Antibodies are being generated which will enable us to decipher the functional role of LLT1 in human lymphocytes.

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