

Characterization of a Novel Receptor That Maps Near the Natural Killer Gene Complex: Demonstration of Carbohydrate Binding and Expression in Hematopoietic Cells¹

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ABSTRACT

A novel type II integral membrane protein has been identified in the course of screening for genes overexpressed in a mouse model of chronic myelogenous leukemia blast crisis. This new protein, designated NKCL, consists of a 210-amino acid polypeptide with a short, NH₂-terminal cytoplasmic tail of 17 amino acids preceding a transmembrane domain and a COOH-terminal extracellular region. The COOH-terminal 132 amino acids bear typical features of the C-type animal lectin carbohydrate-recognition domain. The *Nkcl* gene is unique in that it maps just proximal to the region of the genome that encodes group V members of the C-type animal lectin family near the natural killer gene complex on mouse chromosome 6, but its protein product also has features of several group II C-type animal lectins. Most notably, it has a complete Ca²⁺-binding site 2, which forms part of the sugar-binding site in other members of the family, and binds mannose in a Ca²⁺-dependent manner. Moreover, its expression is not restricted to natural killer cells, as reported for the majority of group V lectins. *Nkcl* is expressed in pluripotent myeloid precursors, precursor and mature macrophages, and neutrophils.

INTRODUCTION

CML³ (1) is a hematopoietic malignancy arising from the neoplastic transformation of a primitive stem cell by the oncoprotein BCR/ABL (1–3). The initial chronic phase is characterized by an accumulation of myeloid precursors in all stages of maturation in the bone marrow and peripheral blood. The chronic phase is unstable and ultimately progresses to a terminal stage called blast crisis characterized by the expansion of undifferentiated myeloid or lymphoid blast cells (4). Although the Philadelphia chromosome is the only cytogenetic abnormality in CML chronic phase, progression from chronic to blast crisis is accompanied by the acquisition of additional genetic aberrations (5). The most common of these secondary abnormalities is the loss of function of the p53 tumor suppressor in ~25% of cases (6). To characterize further the blastic phase at the molecular level, we sought to identify genes expressed differentially in a mouse model of CML blastic transformation.

The mouse model was developed by injecting p53-deficient bone marrow cells, expressing BCR/ABL, into SCID mice (7). These primary recipients succumb to an acute myeloid leukemia within 6–9 weeks. Transplantation of the spleen cell suspension from these terminally ill animals to secondary recipients decreases the survival to

3–4 weeks. Tertiary recipients injected with the leukemic spleen cell suspension from secondary recipients succumb to a very aggressive acute leukemia and have to be sacrificed within 2 weeks. One of the mRNAs expressed differentially in tertiary stage splenocytes encodes a protein with similarity to several members of the C-type animal lectin family and is characterized in the present study.

Animal lectins are carbohydrate-binding proteins that are devoid of enzymatic activity (8). They may be intracellular, membrane-bound, or secreted proteins. Membrane-bound lectins participate in a variety of biological processes including homing of lymphocytes, neutrophil extravasation, fertilization, and serum glycoprotein turnover (8).

The C-type lectins, which are characterized by dependence on Ca²⁺ for carbohydrate-binding activity, are diverse in overall organization but share a common sequence motif indicative of similarly folded CRDs. The C-type lectin family can be subdivided into groups based on the domain context within which the CRDs are found and the degree of divergence of the amino acid sequences of the CRDs (8, 9). Two of the groups are of particular interest in the present context. Both groups II and V comprise type II transmembrane proteins with extracellular, COOH-terminal C-type CRDs. Proteins in group II are generally endocytic receptors, often forming trimers through coiled-coils of α -helices in a stalk region that projects the CRD from the membrane. These receptors are involved in a variety of biological phenomena, from glycoprotein turnover to IgE synthesis and cell-cell adhesion (10).

All of the group V proteins are encoded in a single region of the distal arm of mouse chromosome 6, which is designated the NKC (11, 12). The majority of these proteins are expressed solely on natural killer or T lymphocytes and are either involved in NK cell activation or in specifically inhibiting natural killer cell responses (12). The absence of key binding site residues suggests that these proteins would be unlikely to interact with either Ca²⁺ or sugar in the same way as most C-type animal lectins. Indeed, binding of anionic polysaccharides to *Ly49* is Ca²⁺ independent (13). The designation of NK cell domain has been suggested for these divergent domains (14).

We report the characterization of a novel gene, named *Nkcl*, that was identified as part of a screen for genes overexpressed in a mouse model of CML blast crisis. The *Nkcl* gene maps in close proximity to the NKC and is expressed in different cell types of hematopoietic origin. The protein product, NKCL, is a novel receptor that binds mannose in a Ca²⁺-dependent manner.

MATERIALS AND METHODS

Cells. COS-7 cells, established from CV-1 simian cells transformed by an origin-defective mutant of SV40 (American Type Culture Collection number CRL1651), were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum and 1 mM-glutamine. Cells were tested routinely for the presence of *Mycoplasma* using the enzyme immunoassay detection kit from Boehringer Mannheim.

RNA Isolation. Total RNA was isolated from cells or tissue with Tri-Reagent (Molecular Research Center) according to the manufacturer's instructions.

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³ The abbreviations used are: CML, chronic myelogenous leukemia; SCID, severe combined immunodeficiency; CRD, carbohydrate-recognition domain; RACE, rapid amplification of cDNA ends; HA, hemagglutinin; NKC, natural killer gene complex; ITIM, immunoreceptor tyrosine-based inhibitory motif.

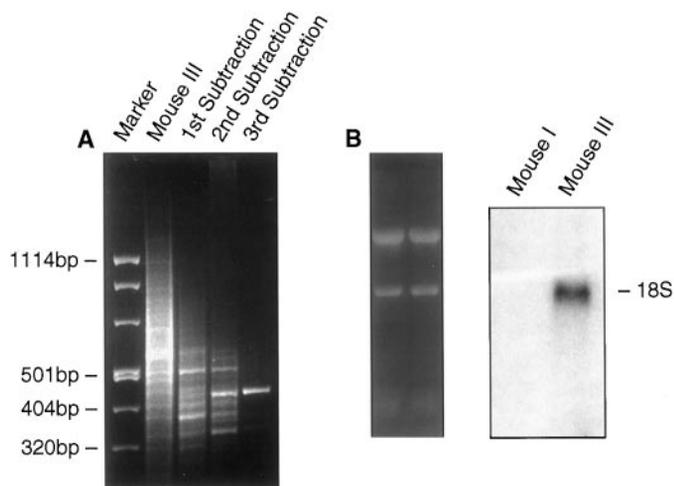


Fig. 1. Representational difference analysis of splenocyte cDNAs from mice I and III. A, cDNA was prepared from poly(A)⁺ RNA isolated from the spleen suspension of mice I and III. The products of the first, second, and third subtractions were loaded onto an ethidium bromide-agarose gel. For comparison, mouse III cDNA, prior to subtraction, is shown. B, to confirm the differential expression of *Nkcl*, 10 µg of mouse I and III total RNA were fractionated electrophoretically on a 1% agarose/formaldehyde gel and blotted onto a nylon membrane. The membrane was visualized under UV light to verify the equal loading of RNA (shown to the left of the blot) and hybridized with a ³²P-labeled probe of *Nkcl* corresponding to the 3' half of the cDNA (right).

cDNA Synthesis. Polyadenylated RNA was purified from total RNA with oligotex spin columns (Qiagen, Inc.), and cDNA was synthesized with the cDNA synthesis kit from Boehringer Mannheim.

Representational Difference Analysis. Total RNA was isolated from spleen suspensions of SCID mice in the blastic phase of CML. The leukemia in these mice was induced as described previously (7). Mouse bone marrow cells deficient in p53 and expressing BCR/ABL were injected i.v. into SCID mice, who succumbed to an acute leukemia within 6–9 weeks. The serial transplantation of splenocytes from these primary recipients to secondary recipients decreased the survival to 4 weeks. Tertiary recipients had to be sacrificed within 2 weeks. cDNA prepared from RNA of a primary (mouse I) and tertiary (mouse III) recipient was digested with *DpnII*, and representational difference analysis was performed according to the method of Hubank and Schatz (15). The digested cDNA populations were ligated to adaptors and amplified prior to subtractive-hybridization as described (15). Three successive subtractive-hybridizations were performed with ratios of mouse III to mouse I cDNA of 1:100, 1:800, and 1:50,000, respectively. The products of the second and third subtractions were subcloned into the *BamHI* site of pBluescript (SK+) using standard subcloning techniques (16).

RACE. The complete coding sequence of *Nkcl* was obtained by performing adaptor-ligated 5' and 3' RACE with the Marathon cDNA amplification kit (Clontech) on an adaptor-ligated library created from mouse III cDNA. The 5' RACE product was obtained by amplifying the library, by PCR, with the Marathon adaptor primer and a *Nkcl* antisense primer (5'-CTC TGT CAG ATG TGC AGG CCA AGA AG-3'). Similarly, the 3' RACE product was obtained by amplifying with the Marathon adaptor primer and a *Nkcl* sense primer (5'-GGG GCT GGA ATG ATG TTT TCT GTG-3'). RACE products were subcloned and sequenced with the PRISM Ready Reaction Dye-deoxy Terminator Cycle Sequencing kit (Applied Biosystems).

Northern Blot Analysis. Ten µg of total RNA were heated at 65°C for 10 min in loading buffer (8% formaldehyde, 17 M formamide, 1 M 4-morpholinopropanesulfonic acid, and 0.1 mg/ml ethidium bromide), fractionated electrophoretically on a 1% agarose/formaldehyde gel and blotted onto a nylon membrane. The membrane was then probed with a nick-translated ³²P-labeled fragment of *Nkcl*. Filters were routinely hybridized in 5× SSC, 5× Denhardt's and 0.5% SDS at 65°C for 16 h and washed in 2× SSC/0.1% SDS for 10 min at room temperature and for 15 min at 65°C. If required, a final wash was carried out in 0.2× SSC/0.1% SDS at 65°C for 15 min. Filters were visualized under UV light to monitor possible differences in the quantity of RNA loaded per lane.

Plasmids. To study NKCL *in vitro*, its coding sequence was inserted into the expression vector pRc/RSV (Invitrogen) using standard subcloning tech-

niques (16). Briefly, the cDNA of *Nkcl* was amplified with two primers flanking the coding sequence, the amplification product subcloned into the TA-cloning vector (Invitrogen), and then inserted into the *EcoRI* site of pRc/RSV. To permit the immunodetection of NKCL, a 27-bp sequence that encodes the HA epitope of influenza virus (YPYDVPDYA) was included in the 3' primer at a position that places the epitope between the last amino acid and the stop codon of the open reading frame (17). A model for NKCL based on the crystal structure of the C-type CRD of lithostatine (18) was used to predict that the tag at this position would be unlikely to affect the structure of the protein. The final construct, pNKCL-HA, was sequenced with the PRISM

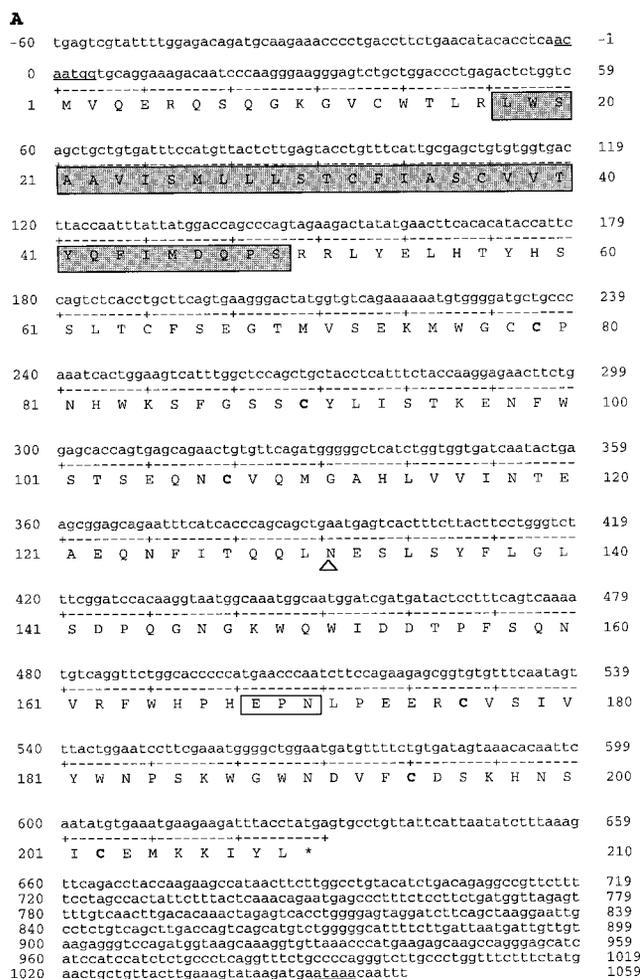


Fig. 2. cDNA, deduced amino acid sequence, and domain structure of NKCL. A, cDNA and deduced amino acid sequence: the Kozak-like translational start site is underlined once, and the polyadenylation signal is underlined twice. The transmembrane domain is shaded, the EPN motif in Ca²⁺-binding site 2 is boxed, the conserved cysteines are shown in bold, and the N-linked glycosylation site is indicated by the arrow. Asterisk, all stop codon. B, domain structure. The dashed lines represent bonds, and the circle corresponds to the N-glycosylation site. The 3-amino acid motif, EPN, of Ca²⁺-binding site 2 that has direct contact with the ligand is shown with asterisks.

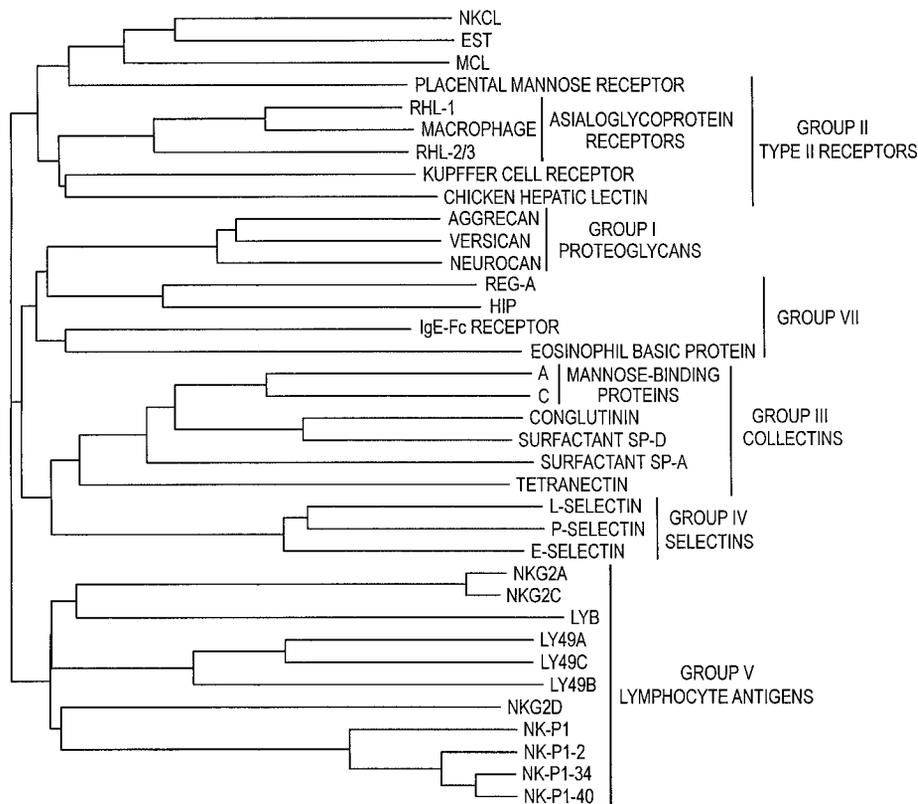


Fig. 3. Cluster analysis of NKCL and other C-type CRDs. The dendrogram was created based on the amino acid sequence similarity between the C-type lectin-like domains of C-type lectins. *EST*, EST AA868502; *RHL*, rat hepatic lectin.

Ready Reaction Dyedeoxy Terminator Cycle Sequencing kit (Applied Biosystems).

Transfection. COS cells were transiently transfected with pNKCL-HA by the Ca^{2+} -phosphate coprecipitation method (Profection kit; Promega Corp.). Forty-eight h after transfection, cells were harvested by washing once in PBS and lysing in 400 μ l of lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA (pH 7.5), 10% glycerol, 1% Triton X-100, 5 mM pepstatin A, 1 mM PMSF, 5 μ l/ml aprotinin, and 5 μ l/ml leupeptin] per 100-mm plate for 2 min on ice. Lysates were collected after centrifugation at $12,000 \times g$ for 10 min, and the protein concentration was determined by the Bradford method (19).

Western Blot Analysis. COS cells transiently transfected with pNKCL-HA were harvested as described in "Transfection." Ten μ g of total protein were fractionated on a 4–15% gradient gel and transferred to a nitrocellulose membrane. The membrane was probed with a 1:1000 dilution of a monoclonal anti-HA antibody (BAbCo), followed by an incubation in a 1:2000 dilution of a sheep anti-mouse IgG antibody (Amersham) coupled to horseradish peroxidase. Immunoblots were developed by standard enhanced chemiluminescence procedures according to the manufacturer's instructions (Amersham).

Analysis of Glycosylation. COS cells transiently transfected with pNKCL-HA were harvested as described in "Transfection." Fifteen μ g of total protein were deglycosylated with 2.5 units of *N*-glycosidase F (Boehringer Mannheim), fractionated on a 4–15% gradient gel, and transferred to a nitrocellulose membrane. The membrane was probed as for Western blots.

Analysis of Ligand Binding. COS cells transfected with pNKCL-HA were harvested 48 h after transfection by washing twice in PBS and lysing in 1 ml of low salt loading buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.8), 25 mM $CaCl_2$, and 0.05% Triton X-100] supplemented with Triton X-100 to 0.5%. The lysate was sonicated twice for 2 s and centrifuged 5 min at $18,000 \times g$. The supernatant was loaded onto 1-ml columns of mannose- or galactose-Sepharose (20). The columns were washed with 5 aliquots of 1 ml of low salt loading buffer, and bound protein was eluted with three aliquots of 1 ml of low salt eluting buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.8), 2.5 mM EDTA, and 0.05% Triton X-100]. Fractions were supplemented with 2 μ g of BSA and precipitated by addition of 0.5 ml of 30% trichloroacetic acid, followed by incubation for 10 min on ice. The precipitate was collected by centrifugation

for 5 min at $18,000 \times g$ and washed twice with 0.5 ml of ethanol:ether (1:1). Dried fractions were suspended in lysis buffer, fractionated by SDS-PAGE, and electroblotted onto a nitrocellulose membrane. The membrane was probed as for Western blots.

Southern Blot Analysis. Southern blotting was performed as described (16). Membranes were hybridized in $5 \times$ SSC, $5 \times$ Denhardt's and 0.5% SDS for 16 h. They were then washed in $2 \times$ SSC/0.1% SDS for 20 min at room temperature and 15 min at $65^\circ C$. The final wash at $65^\circ C$ for 10 min was carried out in $0.2 \times$ SSC/0.1% SDS.

Chromosomal Localization. An interspecific backcross mapping panel of (AEJ/Gn \times *Mus spretus*) $F_1 \times$ AEJ/Gn progeny, established from the AEJ/Gn and *M. spretus* mouse strains (21), was used for genetic linkage analysis to determine the map location of *Nkcl* in the mouse genome. Informative RFLPs that could be used to follow the inheritance of the *Nkcl* allele from the *M. spretus* parent were identified by digestion of AEJ/Gn and *M. spretus* genomic DNA and subsequent Southern blotting with a *Nkcl* probe. An informative polymorphism obtained with the *MspI* enzyme was used to follow the N2 progeny by Southern blot analysis to determine the allelic pattern of inheritance of *Nkcl*. The segregation pattern of *Nkcl* among the N2 progeny was then compared with that of markers that scan the entire mouse genome.⁴ The segregation analysis used to determine the chromosomal location of *Nkcl*, as well as the calculation of recombination frequencies of loci mapped in the interspecific backcross, was carried out using the computer program SPRETUS MADNESS: PART DEUX.⁵

Sequence Comparisons. Trees were constructed for comparison of the CRDs of various C-type lectins using the ClustalW program (22).

Immunofluorescence Microscopy. COS cells transiently transfected with pNKCL-HA were trypsinized 12 h after transfection, plated onto 18-mm round glass coverslips, and grown overnight. The cells were washed three times in PBS and fixed for 10 min in 3.7% formaldehyde at room temperature. After a second wash in PBS, the cells were blocked for 20 min in 4% goat serum

⁴ A. M. Buchberg and L. D. Siracusa, unpublished data.

⁵ The computer program, SPRETUS MADNESS: PART DEUX, was developed by K. Smalley, J. Averbach, L. D. Siracusa, and A. M. Buchberg at the Kimmel Cancer Center, Philadelphia, PA.

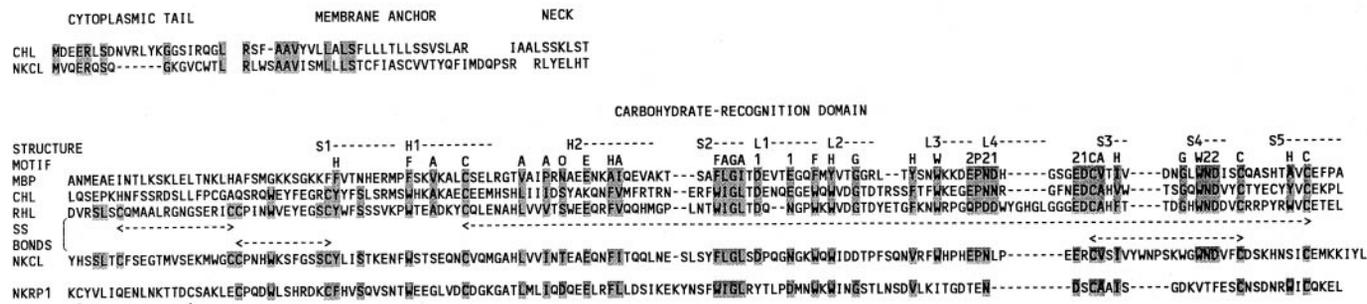


Fig. 4. Alignment of NKCL with members of the C-type lectin family. Alignment of the different domains of NKCL to those of rat serum mannose binding protein (MBP-A), chicken hepatic lectin (CHL), rat hepatic lectin (RHL), and the NKC-encoded protein NKRPI is shown. Regions of secondary structure (*H*, α -helix; *S*, β -strand; and *L*, loop) and residues that define the C-type CRD (*H*, hydrophobic; *F*, aromatic; *A*, aliphatic; *O*, oxygen-containing; *C*, cysteine; *E*, glutamic acid; *G*, glycine; *W*, tryptophan; and *P*, proline) are shown above the sequence. Residues that ligate Ca^{2+} are designated 1 and 2. Dashed lines, disulfide bonds; asterisks, cysteines involved in interchain disulfide bonding in NKRPI.

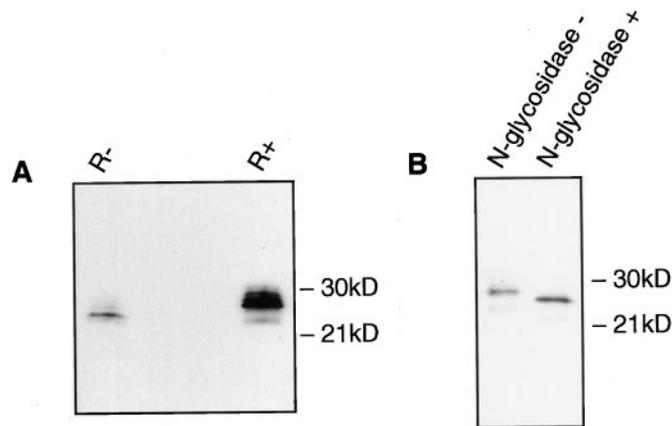


Fig. 5. Electrophoretic mobility and glycosylation of HA-tagged NKCL. Total lysate of COS cells transiently transfected with pNKCL-HA was fractionated by SDS-PAGE under nonreducing (R⁻) or reducing (R⁺) conditions (A) or incubated with *N*-glycosidase F and fractionated under reducing conditions (B) before immunoblotting with an anti-HA antibody and detection by enhanced chemiluminescence.

diluted in Triton X-100 solution (0.05% Triton X-100 in PBS). The cells were then incubated in a 1:250 dilution of a monoclonal anti-HA antibody (BAbCo) for 30 min, rinsed three times, and washed for 30 min in the Triton X-100 solution. The incubation with the secondary antibody (1:20 dilution of a FITC-conjugated, goat anti-mouse IgG antibody from Life Technologies, Inc.) was carried out as described for the first antibody. Prior to mounting the cells onto slides, they were rinsed three times with PBS, once with water for 2 min, and dried. For experiments with nonpermeabilized cells, the above procedure was carried out by replacing the Triton X-100 solution with PBS. Confocal microscopy was performed on a Bio-Rad MRC 600 laser scanning confocal microscope (Hemmelhlostadt, United Kingdom) attached to a Zeiss Axiovert 100 microscope, using a Zeiss Plan-Apo 63 \times 1.40 NA oil-immersion lens.

Expression in Hematopoietic Cells. A slot blot was prepared with cDNA amplified globally from single hematopoietic progenitor precursor cells or from populations of terminally maturing cells in various lineages (23). Each slot was loaded with cDNA pooled from multiple samples of individual cells at the same stage of differentiation. A probe corresponding to the 3' untranslated region of *Nkcl* was used for hybridization to the slot blot. Hybridization was performed following the same protocol as for "Southern Blot Analysis."

RESULTS

Identification of Genes Overexpressed in a Mouse Model of CML Blast Crisis. A mouse model of CML blastic transformation in which p53-deficient bone marrow cells were infected with a BCR/ABL-containing retrovirus and injected into primary recipient mice was developed recently (7). These mice succumb to an acute leukemia within 6–9 weeks. After serial transplantation, the acute leukemia is

much more aggressive, and tertiary recipients have to be sacrificed within 2 weeks.

The subtractive hybridization technique, cDNA representational difference analysis, was used to identify gene(s) that may be responsible for the more aggressive leukemia observed in a tertiary (mouse III) versus a primary (mouse I) recipient. To enrich for mRNAs overexpressed in mouse III, three successive subtractive hybridizations were performed with mouse III to mouse I cDNA ratios of 1:100, 1:800, and 1:50,000, respectively (Fig. 1). The products from the second and third subtractions were subcloned and sequenced. Several differentially expressed transcripts were identified, one of which encodes the novel protein NKCL (Fig. 1).

Cloning of the Full-Length cDNA of *Nkcl*. Adaptor-ligated RACE was performed on cDNA from mouse III to obtain the complete coding sequence of *Nkcl* (Fig. 2). Because the *Nkcl*-specific primer used for 5' RACE maps 80 bp downstream from the stop codon, the complete coding sequence for *Nkcl* is contained in the 5' RACE product. Four independent clones from the 5' RACE reaction were analyzed. To verify the sequence and to confirm that the open-reading frame could be amplified in one fragment, primers flanking the coding region were used to amplify *Nkcl* from cDNA obtained by reverse transcribing mouse III total RNA. Several additional, independent amplifications with these primers have been performed, and no variations in the sequence have been observed. The open-reading frame was initially determined by searching the available nucleotide and protein databases for sequences similar to *Nkcl*. *Nkcl* shares a significant degree of similarity with the CRD of C-type lectins. The translational initiation codon of the *Nkcl* sequence was assigned to an

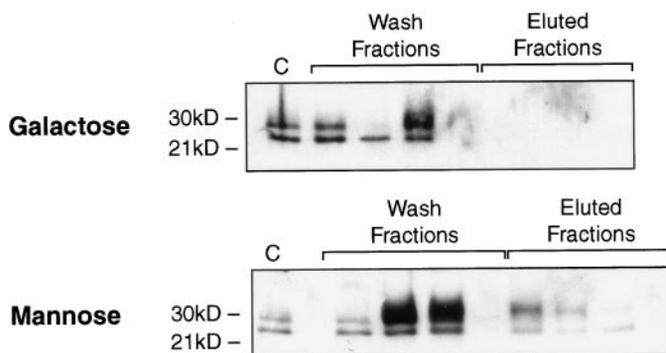


Fig. 6. Affinity chromatography of NKCL on carbohydrate-containing columns. Lysate from COS-7 cells transiently transfected with pNKCL-HA was loaded onto a 1-ml galactose-Sepharose or mannose-Sepharose column. Fractions collected during washes with Ca^{2+} -containing buffer and eluted with EDTA-containing buffer were analyzed by SDS-PAGE, and the protein was visualized as in Fig. 5. A fraction of the total lysate was also loaded as a control (Lane C).

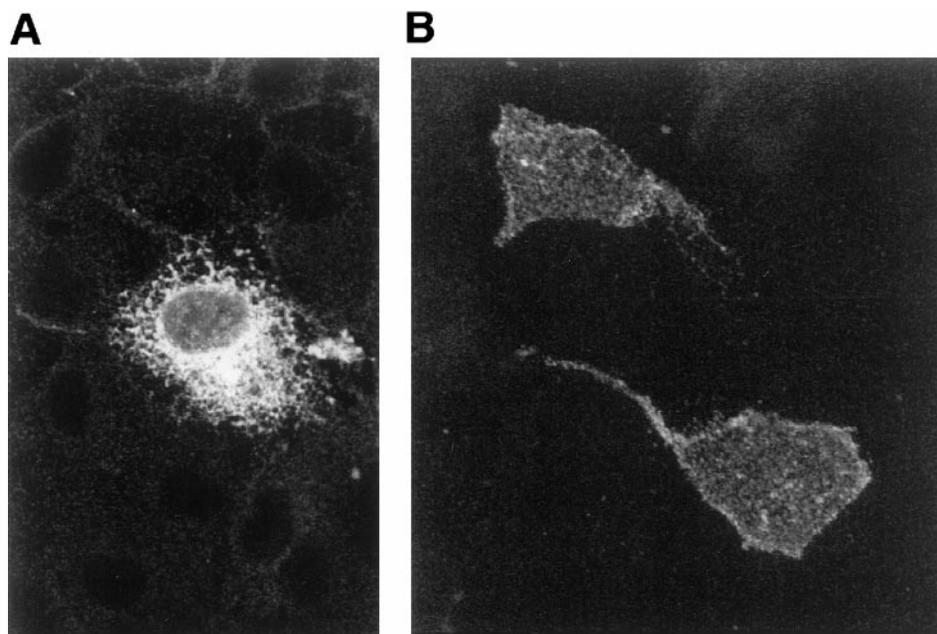


Fig. 7. Immunocytochemistry of transiently transfected COS cells with pNKCL-HA. Transiently transfected COS cells were grown on coverslips and stained with a monoclonal anti-HA antibody and a FITC-conjugated anti-mouse antibody and visualized. Immunostaining of permeabilized (A) and non-permeabilized (B) cells is shown.

ATG located 234 bp upstream of its CRD based on: (a) an upstream termination codon that prevents translation from other potential start sites in the same reading frame; and (b) the similarity of the translational start site to the Kozak motif (PuNNATGG; Ref. 24). The polyadenylation signal maps 417 bp downstream of the translational stop codon, as determined by 3'RACE. *Nkcl* encodes a 210-amino acid polypeptide, NKCL, with a predicted molecular mass of 23 kDa.

NKCL Is a Member of the C-Type Animal Lectin Superfamily. Analysis of the amino acid sequence of NKCL reveals features of a type II integral membrane protein, including the absence of a cleavable signal sequence at the NH₂ terminus and a characteristic hydrophobic transmembrane domain between Arg17 and Arg50 (Fig. 2). It is composed of a cytoplasmic tail of 17 amino acids preceding the transmembrane domain and a neck region of 29 amino acids at the extracellular side of the membrane. The COOH-terminal 132 amino acids bear the hallmarks of the C-type CRD.

Attempts to classify NKCL into one of the two groups of C-type animal lectins that share the type II transmembrane organization, groups II and V, are complicated by the distinct characteristics that it shares with each group. A global comparison of the CRD of *Nkcl* with other members of the C-type lectin family indicates, however, that it is most closely related to the group II lectins and may define a new subgroup, together with the recently cloned *mpcl* (formerly *mcl* gene; Ref. 25) and the human EST AA868502 (Fig. 3).⁶ The NH₂-terminal cytoplasmic domain and the first portion of the membrane anchor of NKCL are particularly similar to the chicken hepatic lectin (Fig. 4), which belongs to group II. The presence of a relatively short neck region on the extracellular side of the membrane is also reminiscent of the chicken hepatic lectin.

A key feature of the sequence that helps to define the overall organization of the remainder of the extracellular domain is the arrangement of cysteine residues and disulfide bonds. In the COOH-terminal 110 amino acids, the four cysteine residues that form the two disulfide bonds characteristic of the C-type lectin fold are perfectly conserved in NKCL (Fig. 4). The likely arrangement of the remaining four cysteine residues in two disulfide bonds can be predicted by

analogy with additional disulfide bonds in the asialoglycoprotein receptors. Western blot analysis of transiently expressed, HA-tagged NKCL in COS cells revealed that these four cysteine residues are most likely to be involved in intrachain disulfide bonding. NKCL-HA migrates faster on SDS-PAGE under nonreducing than under reducing conditions (Fig. 5A), which is consistent with the presence of extensive intrachain disulfide bonding preventing the unreduced protein from unfolding completely in SDS. There is no evidence for a disulfide-linked dimer under nonreducing conditions.

Within the CRD, the residues that define the C-type lectin fold are largely conserved in NKCL, with the exception of glycine residues in loop 2 and in the turn between β -strands 3 and 4 (Fig. 4). In the latter case, the turn is probably replaced by a more extended loop because of the insertion of four residues. A similar insertion is seen in the selectins, although the character of the inserted sequence is quite different. Among the amino acid residues that define the two Ca²⁺-binding sites in mannose-binding protein, those for Ca²⁺-binding site 2 are completely conserved. In contrast, three of the four site 1 amino acids are not conserved, making it unlikely that this Ca²⁺-binding site is present in NKCL. Although Ca²⁺-binding site 2 is conserved among sugar-binding CRDs, site 1 is not. C-type CRDs with single Ca²⁺-binding sites, as well as examples in which there is an alternative site 1 have been described (26–29).

One additional protein motif noted in the extracellular domain of NKCL is a potential *N*-linked glycosylation site at Asn131. To demonstrate that this residue forms a functional glycosylation site, extracts of COS cells expressing the HA-tagged NKCL were digested with *N*-glycosidase F and resolved by SDS-PAGE, followed by immunoblotting with an anti-HA antibody (Fig. 5B). A shift in the mobility of NKCL-HA was observed after *N*-glycosidase F treatment corresponding to ~3 kDa, which is consistent with the presence of a single *N*-glycosylation site at Asn131.

Ligand Binding Specificity. C-type CRDs can be broadly divided into two groups based on ligand specificity, those that bind mannose and those that bind galactose (30). The primary determinant of ligand-binding specificity, in most cases, is a three amino acid motif that forms part of Ca²⁺-binding site 2. C-type lectins containing the QPD motif bind galactosides and their derivatives

⁶ M. Fernandes, personal observation.

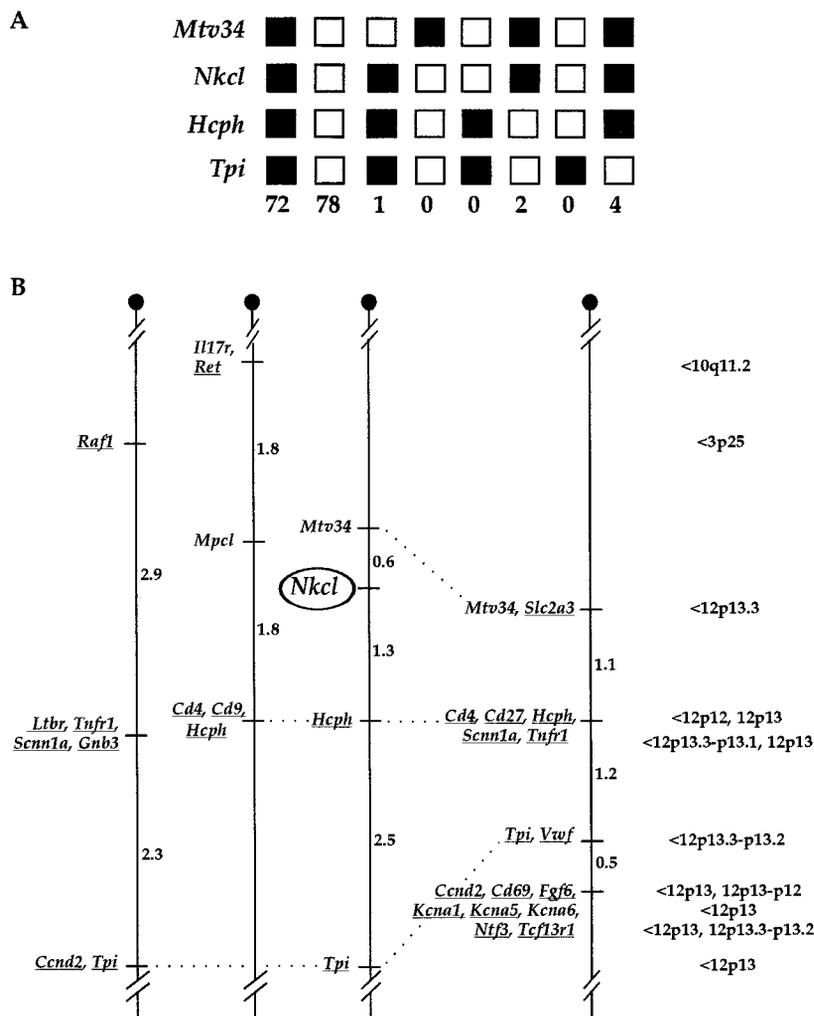


Fig. 8. Mapping of *Nkcl*. **A**, haplotype analysis of the N2 progeny from the interspecific backcross. Each column represents the chromosome identified in the N2 offspring that was inherited from the (AEJ/Gn \times *M. spretus*) F₁ parent. The number of N2 progeny carrying each type of chromosome is listed at the bottom, and the loci followed in the backcross are listed at the left. ■, AEJ/Gn allele; □, *M. spretus* allele. **B**, genetic linkage maps showing the chromosomal localization of *Nkcl* on mouse chromosome 6. The third map from the left represents loci typed in our interspecific mouse backcross (described in "Materials and Methods"). Genetic distances between loci are given in CM. The map to the right shows data from the Copeland-Jenkins mapping panel, the first map on the left shows the consensus data of three crosses from the Kozak mapping panel, and the second map from the left shows data from the Seldin mapping panel for the same region.⁷ This comparison shows that the *Nkcl* gene resides proximal to genes within the NKC, such as *Hcph*. Genes that have been mapped in the human genome are underlined, and their corresponding position in the human genome is listed to the far right. This comparison also shows that the human homologue of the *Nkcl* gene most likely resides on human chromosome 12p13. The dotted lines between the chromosomes show the alignment of the *Mtv34*, *Hcph*, and *Tpi* loci.

and those with an EPN motif bind mannose, *N*-acetylglucosamine, and related sugars. The presence of an EPN motif in Ca²⁺-binding site 2 of NKCL is suggestive that it binds mannose and not galactose. The ligand-binding specificity of NKCL was determined by passing Triton X-100 solubilized lysate of COS cells transiently expressing NKCL-HA through mannose and galactose-containing columns. As shown in Fig. 6, NKCL is retarded on the mannose but not the galactose column. Although some of the protein elutes from the mannose column in the Ca²⁺-containing wash fractions, a portion is retained and eluted with EDTA. This behavior is characteristic of C-type CRDs with relatively weak affinity for monosaccharides (31, 32).

Subcellular Localization of NKCL. To determine whether NKCL is expressed on the cell surface as predicted from its domain organization and the surface-expression of groups II and V lectins, immunocytochemistry using an anti-HA antibody was performed on COS cells transfected with pNKCL-HA. Staining of permeabilized cells with an anti-HA antibody, followed by a FITC-conjugated goat anti-mouse IgG antibody, revealed that NKCL-HA is expressed in transfected cells (Fig. 7A). Nonpermeabilized cells showed a membrane-specific staining pattern indicative that

NKCL is targeted to the plasma membrane and expressed on the cell surface (Fig. 7B).

***Nkcl* Maps to the Distal Arm of Mouse Chromosome 6.** The interspecific backcross mapping panel of (AEJ/Gn \times *M. spretus*) F₁ \times AEJ/Gn progeny, for which markers throughout the mouse genome have been characterized, was used to determine the chromosomal localization of *Nkcl*. This interspecific backcross was established from the AEJ/Gn and *M. spretus* mouse strains (21). Informative RFLPs for *Nkcl* in mouse strains AEJ and *M. spretus* were identified by Southern blot analysis. The RFLP obtained with *Msp*I was used to determine the segregation pattern of the *M. spretus*-specific *Nkcl* allele in the N2 progeny of a (AEJ/Gn \times *M. spretus*) F₁ \times AEJ/Gn mating. The *Nkcl* probe detected *M. spretus*-specific *Msp*I fragments of 7.4 and 14 kb, which were distinguishable from the AEJ/Gn-specific *Msp*I fragments of 2.6, 2.9, 3.2, 5.0, 6.2, and 8.7 kb. The chromosomal location of *Nkcl* was then determined by comparing its segregation pattern to that of well-characterized markers in the N2 progeny (33). *Nkcl* has a pattern of segregation very similar to that of marker *Mtv34*, which resides on chromosome 6 (Fig. 8).

Because *Mtv34* maps near but not within the NKC, *Nkcl* was mapped with respect to a NKC-encoded locus, *Hcph* (34). The *Hcph* probe (kindly provided by Dr. T. Yi) detected a *M. spretus*-specific, *Pst*I fragment of 3.3 kb and a AEJ/Gn-specific fragment of 3.8 kb. The data positions *Nkcl* in the distal region of mouse chromosome 6 between the *Mtv34* and *Hcph* loci, where it is closely linked to the NKC. The order of loci and the ratio of the number of recombinants

⁷ Mouse Genome Informatics (MGI) Resource, Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, Maine. The *Nkcl* was approved by the International Committee for Standardized Genetic Nomenclature in Mice. World Wide Web (URL: <http://www.informatics.jax.org/>) November, 1998. Copeland-Jenkins: (C57BL/6J \times *M. spretus*) F₁ \times C57BL/6J. Kozak FvC58: (NFS/N \times *M. spretus*) F₁ \times C58/J. Kozak FvSpr: (NFS/N \times *M. spretus*) F₁ \times *M. spretus*. Kozak Skive: (NFS/N or C58/J \times *M.m. musculus*) F₁ \times *M.m. musculus*.

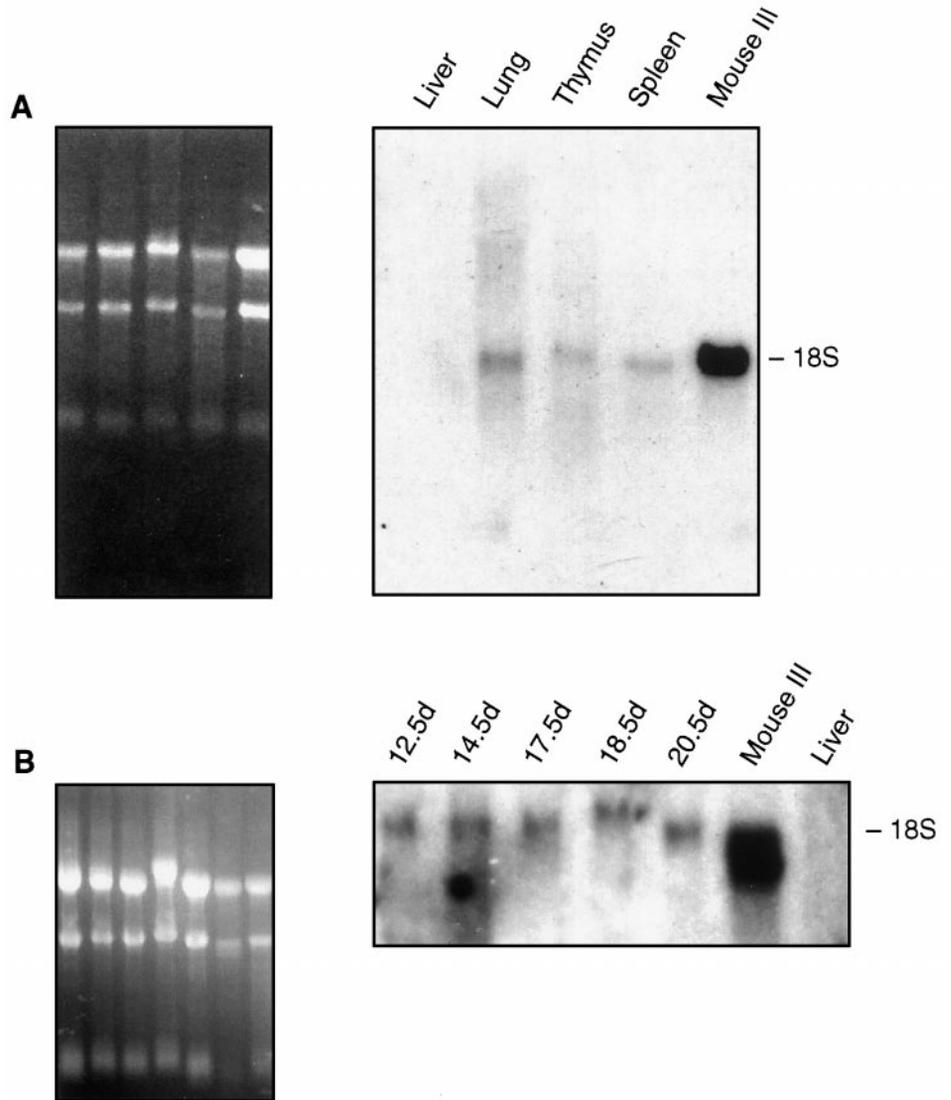
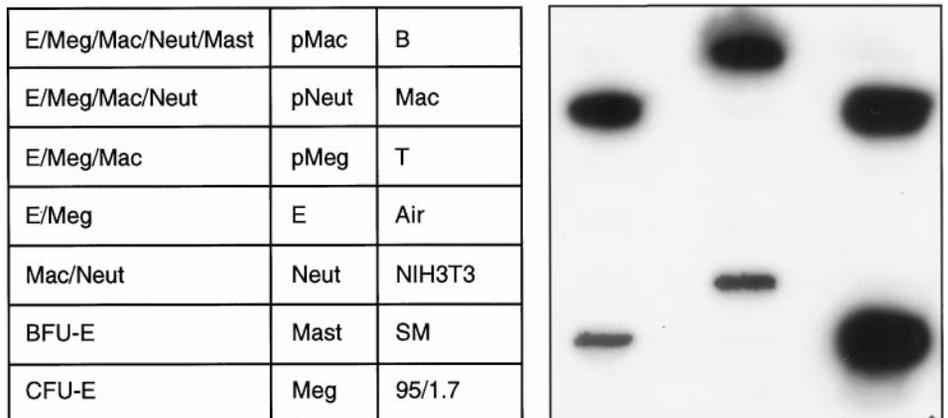


Fig. 9. Expression of *Nkcl* in adult mouse tissues and whole embryos at different stages of development. Total RNA from liver, lung, thymus, spleen, and mouse III (A) or from whole embryos corresponding to days 12.5, 14.5, 17.5, 18.5, and 20.5 (B) was hybridized with a ³²P-labeled *Nkcl* probe. The nylon membranes were visualized under UV light to verify the equal loading of RNA (shown to the left of each blot).

to the total number of N2 offspring examined are: centromere - *Mtv34* - 1/157 - *Nkcl* - 2/157 - *Hcph* - 4/157 - *Tpi* - telomere. The genetic distances between loci in cM (± SE) are: centromere - *Mtv34* - 0.6 cM (± 0.6) - *Nkcl* - 1.3 cM (± 0.9) - *Hcph* - 2.5 cM (± 1.3) - *Tpi* - telomere. The results show that *Nkcl* resides 1-2 cM proximal to the NKC-linked locus *Hcph* (10) and in a region of synteny with human chromosome 12p13-p12 (Fig. 8).

Expression of *Nkcl* in Normal Mouse Tissues and Hematopoietic Cells. Northern blot analysis of a variety of adult mouse tissues reveals that *Nkcl* is expressed in the spleen, thymus, and lung but not in liver (Fig. 9A). It is also expressed in the bone marrow (data not shown) and throughout mouse development (Fig. 9B). *Nkcl* mRNA was not detected in the brain, eye, heart, or kidney (data not shown). Because *Nkcl* has a hematopoietic-like tissue distribution, its presence

Fig. 10. Slot blot analysis of *Nkcl* expression in normal mouse hematopoietic cells representing various stages of the developmental hierarchy. cDNA from hematopoietic cells indicated in the schematic diagram (left) was loaded onto each slot of a nitrocellulose membrane and hybridized with a ³²P-labeled *Nkcl* probe (right). The following abbreviations were used to designate the different hematopoietic cell types: *E*, erythroid; *Meg*, megakaryocyte; *Mac*, macrophage; *Neut*, neutrophil; and *Mast*, mast cell. *B*, B lymphocytes; *T*, concanavalin A-stimulated T lymphocytes. *SM*, cells of macrophage origin; *95/1.7*, a fibroblast line derived from adult marrow cells (45).



in hematopoietic cells representing various stages of the developmental hierarchy was also determined (Fig. 10). A fragment of *Nkcl* cDNA was used to probe a slot blot containing pooled cDNA populations of progenitor precursor, differentiating, and mature hematopoietic cells. The *Nkcl* gene is expressed in the erythroid/megakaryocyte/macrophage/neutrophil myeloid precursor, burst-forming units erythroid, both precursor and mature macrophages, and mature neutrophils.

DISCUSSION

We report the identification and characterization of a novel gene that maps close to the NKC, *Nkcl*. The NKC is a region of the genome rich in loci implicated in regulating NK cell function (12, 35). The NKC, which spans at least 2.1 megabases (36), was originally defined by two families of group V C-type animal lectin-like genes, the *Nkrp1* and *Ly49* gene clusters, that encode dimeric type II integral membrane proteins expressed predominantly on NK cells (37). Recently, the boundaries of the NKC have been expanded to include additional linked genes encoding structurally diverse proteins that play a role in natural killer cell activity (12).

Like the *Nkrp1* and *Ly49* gene products, *Nkcl* is a member of the C-type lectin superfamily (Fig. 4) and maps to the distal arm of mouse chromosome 6 (Fig. 8). It is a type II integral membrane protein with a COOH-terminal C-type lectin-like domain. Similar to most group V lectins, NKCL has a small neck domain connecting the CRD to the cell surface and lacks Ca²⁺-binding site 1.

NKCL, however, exhibits a significant degree of divergence at the amino acid level from the group V lectins. Within the CRD, Ca²⁺-binding site 2 is conserved, conferring upon NKCL the ability to bind carbohydrate (Fig. 6) in a Ca²⁺-dependent manner. This observation further supports the hypothesis that the inability of other group V lectins to bind carbohydrate ligands in a Ca²⁺-dependent manner can be explained by the absence of a conserved Ca²⁺-binding site 2. At the NH₂-terminal end of its CRD and in the neck region, NKCL lacks cysteine residues that could engage in interchain disulfide bonds, which is typical of the group V lectins. Thus, oligomers of NKCL would have to form noncovalently. The two pairs of cysteine residues in this region probably form intrachain disulfide bonds similar to the asialoglycoprotein receptors. Because the ligand-binding specificity of many C-type lectins is attributable to the specific geometric arrangement of their CRDs achieved by oligomerization, it will be of interest to determine the oligomeric state of NKCL (38–40).

NKCL is also devoid of protein motifs within its cytoplasmic and transmembrane domains that are characteristic of the group V lectins. NKC receptors that inhibit NK cell function mediate their effects via an ITIM in their cytoplasmic domain (35). NKC receptors that lack ITIMs activate NK cell function by oligomerizing with DAP12 through a charged residue in their transmembrane domain (41). NKCL lacks both an ITIM and a charged residue. It is conceivable, however, that signaling through NKCL, if it occurs, involves a noncovalent association with an as yet unidentified receptor polypeptide that contains signaling motifs.

In some respects, NKCL resembles the group II C-type lectins (Fig. 4). The cytoplasmic and transmembrane domains are particularly similar to those of the chicken hepatic lectin, and Ca²⁺-binding site 2 is conserved.

Despite its similarity to the groups II and V lectins, cluster analysis suggests that *Nkcl* is most closely related to the group II lectins and may be a member of a new subgroup of C-type lectins comprised of *Nkcl*, the recently cloned *Mppl* gene (25), and the human expressed sequence tag AA868502 (Fig. 3). Interestingly, *Nkcl* and *Mppl* map very close to each other and at a similar distance of ~2 cMs from the NKC-encoded locus *Hcph* (Fig. 8). These two loci may possibly

represent a new cluster of C-type lectin genes that are more closely related to each other than to the group V lectins. Analogous to the NKC, this novel cluster may encode loci that are involved in biological processes of a particular branch of the immune system.

The *Mppl* gene product is macrophage specific and is structurally similar to *Nkcl*. mMPCL is composed of a short cytoplasmic domain devoid of known protein motifs, followed by a transmembrane, neck, and C-type lectin-like domain. The most significant difference between *Nkcl* and *Mppl* at the amino acid level is a proline to serine substitution in the "EPN motif" of Ca²⁺-binding site 2, making it unlikely that mMPCL binds carbohydrates in a Ca²⁺-dependent manner.

Nkcl is preferentially expressed in the spleen, thymus, bone marrow, and lung (Fig. 9A). These data suggest that *Nkcl* is hematopoietic in origin. Indeed, it is expressed in pluripotent myeloid precursors and mature neutrophils and macrophages. The expression of *Nkcl* in hematopoietic cells is in agreement with its pattern of expression in normal tissues. Its presence in the spleen, thymus, and lung may be explained, in part, by its expression in macrophages and neutrophils. Likewise, the expression of *Nkcl* in cells of myeloid origin is consistent with its presence in the bone marrow. The functional significance of the differential expression of *Nkcl* in hematopoietic cells is unknown. However, it is suggestive that *Nkcl* is turned on and off during hematopoiesis, depending on the requirement for its expression at different stages of differentiation. The detection of *Nkcl* mRNA in a population of SCID spleen cells enriched to 95% in NK 1.1⁺-NK cells is suggestive that *Nkcl* may also be expressed in NK cells (data not shown).

Nkcl was initially identified in a screen for genes overexpressed in a mouse model of CML blast crisis. CML is a hematopoietic malignancy arising from the transformation of the hematopoietic stem cell, resulting in an abnormal expansion and tissue distribution of cells of the myeloid lineage. Because *Nkcl* is expressed in hematopoietic cells of myeloid origin (Fig. 10) and may be required for cell-cell or cell-matrix interaction, it is conceivable that NKCL is involved in the altered trafficking of myeloid cells in CML.

In this regard, several lines of evidence strongly suggest that endogenous tumor cell lectins play a role in the pathogenesis of cancer (42, 43). Recently, L-selectin, an adhesion molecule that is involved in leukocyte trafficking and possibly in the homing and proliferation of stem cells and progenitors in the bone marrow, was implicated in the chronic phase of CML (44). Analysis of CD34⁺ cells from patients in the chronic phase of CML revealed a significant decrease in L-selectin expression in comparison to the normal cellular counterparts, raising the possibility that the premature release of progenitors in CML may be due in part to decreased expression of L-selectin.

Biochemical and biological approaches to assess the role of NKCL in carbohydrate-mediated target recognition will be required to determine whether its expression correlates with the ability of CML-blast crisis cells to infiltrate hematopoietic and nonhematopoietic tissues. In conclusion, the identification of a novel gene that is closely linked to the NKC, *Nkcl*, with its broad pattern of expression and potential role in leukemogenesis, adds to the repertoire of proteins that may be involved in the immune response and to the diversity of type II integral membrane receptors that map to this region of the genome.

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