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ORIGINAL PAPER

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AICL: a new activation-induced antigen encoded by the human *NK* gene complex

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Abstract The *NK* gene complex on mouse chromosome 6 and its human homologue on chromosome 12 encode type II transmembrane proteins with a C-type lectin domain which trigger or inhibit target cell lysis by NK cells (*NKR-P1*, *Ly49*, *NKG2*, *CD94*) or function as cellular activators of various hematopoietic cells (*CD69*). We herein report the cDNA cloning of a new molecule, designated activation-induced C-type lectin (*AICL*), whose gene maps to the human *NK* gene complex proximal to the *CD69* gene. *AICL* is a 149-amino acid (aa) polypeptide with a short cytoplasmic part of seven aa and a C-type lectin domain separated from the transmembrane region by only nine aa. The highest sequence similarity is found to the C-type lectin domains of *CD69* and the chicken lectin 17.5. The presence of *AICL* transcripts in different cell types of hematopoietic origin, a rapid increase of gene transcription during lymphocyte activation, and a short half-life of the mRNA characterize *AICL* as a new, broadly expressed activation antigen.

Introduction

NKR-P1 and *Ly49* gene families map to the same region on mouse chromosome 6 designated the *NK* gene complex (Yokoyama and Seaman 1993). The molecules encoded by

these gene families are homodimeric type II transmembrane proteins of 200–280 aa with a C-type lectin domain. Whereas *NKR-P1* family members trigger cellular activation, expression of *Ly49* receptors enable NK cells to recognize major histocompatibility complex (MHC) class I molecules on potential target cells and to kill only those cells that do not express critical MHC class I antigens (“missing self”). The latter finding was a fundamental contribution to the understanding of the molecular mechanisms employed by mouse NK cells to recognize their target cells (for a recent review see Gumperz and Parham 1995).

The observation that the distal region of mouse chromosome 6 is syntenic to the short arm of human chromosome 12 has led to the prediction that a human homologue of the *NK* gene complex would be localized there. Indeed, genes encoding four different type II proteins/protein families with C-type lectin domains have been located on the human chromosome 12. These are the *NKG2* family (Houchins et al. 1991), *NKR-PIA* (Lanier et al. 1994), *CD94* (Chang et al. 1995), and *CD69* (Hamann et al. 1993; Ziegler et al. 1993; Lopez-Cabrera et al. 1993). Localization of the *CD69* gene in the *NK* gene complex has been surprising, since function of this molecule is not restricted to NK cells. Cross-linking of the early activation antigen *CD69* triggers cell type-specific activation steps in T cells, B cells, NK cells, granulocytes, monocytes, and platelets (Testi et al. 1994). The variety of the induced processes indicates that *CD69* functions as a central molecule in cellular activation cascades.

The identification of additional genes within existing superfamilies has been facilitated by the growing number of expressed sequence tags (ESTs; Fields 1994). ESTs are partial 5′ or 3′ sequences from large-scale cDNA analyses of tissues or cell types and reflect specific gene expression patterns. Recently, the deposition of 87 983 distinct human sequences to nucleotide sequence databases has been reported (Adams et al. 1995). Based on the identification of an EST homologous to *CD69*, we cloned a new member of the human *NK* gene complex. Molecular structure and expression of the protein are described.

The nucleotide sequence data reported in this paper have been submitted to the EMBL/GenBank nucleotide sequence databases and have been assigned the accession number X96719

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A

CTGTGCTGTAACAAAGAGTAACATTTTATATTAAGTTAAATAAGTTACAACCTTG 60
 AAGAGAGTTTCGCAAGACATGACACAAAGCTGCTAGCAGAAATCAAACGCTGATTAAG 120
 AAGAAGCAGGTATGATGACCAACATAAAAGTGTTTATAATGTTGGTGTTTAATA 180
 M M T K H K K C F I I V G V L I
 ACAACTAATATTACTCTGATAGTTAACTAACTCGAGATTCTCAGAGTTTATGCCCC 240
 T T N I I T L I V K L T R D S Q S L C P 36
 TATGATTGGATTGGTTTCCAAAACAATGCTATTATTTCTCTAAAGAAGAAGGAGATTGG 300
 Y D W I G F G Q N K C Y Y F S K E E G D W 56
 AATTCAAGTAATACAAGTGTTCCTCACTCAACATGCCGACCTAACTATAATGACAACATA 360
 N S S K Y N C S T Q H A D L T I I D N I 76
 GAAGAAATGAATTTCTAGGCGGTATAAATGCAGTTCTGATCACTGGATTGGACTGAAG 420
 E E M N F L R R Y K C S S D H W I G L K 96
 ATGGCAAAAAATCGAACAGGACAATGGGTACATGGAGCTACATTTACCAATCGTTTGGC 480
 M A K N R T G Q W V H G A T F T K S F G 116
 ATGAGGGAGTGAAGGATGTGCCTCACTCAGCGATGATGGTGCAGCAACAGCTAGATGT 540
 M R G S E A Y L S D G A A T A R C 136
 TACACCGAAAGAAATGGATTTCGAGGAAAGAATCACTAAGTTAATGTCTAAGATAAT 600
 Y T E R K W I C R K R I H - 149
 GGGGAAATAGAAAATAACATTATTAAGTGTAAACCAGCAAGTACTTTTTTAAATAAA 660
 CAAAGTTCGAGTTTGTACCTGTCTGTTAATTTCTGCTACGTGTCAGGCTACACATAAA 720
 AGCCACTTCAAGATTGGCAAAAAAATAAAAAA 759

B

hs AICL	CRYDWIGFONKCYFFSKKECDWSSKYNCSQHADETIIDNIEEMNLRR	86
hs CD69	CSEDVVGYQRKCYFISTVKRSWTSAQNACSEHGATEAVIDSEKDMNFKR	134
gg 17.5	CRNAVVGFGKCYFFSDTESDWSREHCHRLGASEATLDTEEMEFLQ	178
	* * * * *	
hs AICL	YKSSDHWITGLKMAKNRTGQ-VVDGATFKS--FGMRGSECCAYLSDDGA	131
hs CD69	YAGREHHVGLKKEPGHPWK-WSNGKEFNW--ENVTSQDKVFLKNTVEV	184
gg 17.5	YQRPAQRWITGLHRAEGDEHWTWAGSFAFNRPVFEELRGGRCAYLNGGI	228
	* * * * *	
hs AICL	ATRCYTERKWTIERKRIH	149
hs CD69	SSMECEKNLYITCNPKYK	199
gg 17.5	SSALCHSEKFWVSRAADSYVRWRKGTNPQ	257
	* * * * *	

Fig. 1 **A** Nucleotide sequence and deduced amino acid sequence of the AICL cDNA. The hydrophobic membrane spanning region is shaded and potential N-glycosylation sites are boxed. Two polyadenylation signals which are alternatively used in clones 9.1 and 10.2 are underlined twice. **B** Amino acid sequence alignment of the C-type lectin domains of AICL with human CD69 (Hamann et al. 1993; Ziegler et al. 1993; Lopez-Cabrera et al. 1993) and chicken lectin 17.5 (Bernot et al. 1994). Amino acids conserved between AICL and other receptors are shaded. Asterisks indicate six cysteines which are conserved in all C-type lectin domains. Remarkably, the length of the C-type lectin domain matches exactly between AICL and CD69

Materials and methods

cDNA cloning and sequence analysis

A cDNA encoding AICL was isolated from a pCDM8 library from PMA-activated human PBMC (Hamann et al. 1993). Plasmid DNA, prepared from 12 pools of 3–4000 clones, was screened by polymerase chain reaction (PCR) (35 cycles, 30 s at 95 °C, 30 s at 53 °C, 1 min at 72 °C) with the specific primers 5'-ATTGACAACATAGAA-GAAATG-3' [(+) strand] and 5'-TGAGCTCCATCTACCCATTG-3' [(-) strand]. The primers were designed upon an EST of AICL (GenBank accession number T46996), identified in the Washington University-Merck EST project. In 10 of 12 pools a PCR product of 114 base pair (bp) was amplified. Repeated rounds of subdivision and PCR screening of one positive pool resulted in the isolation of a positive, but incomplete clone.

To identify a full-length cDNA, BA85 nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) were lifted from four positive pools of the cDNA library and hybridized with the ³²P-labeled incomplete clone for 20 h at 58 °C in a buffer consisting of 6 × standard sodium citrate (SSC), 5 × Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), 10 mM ethylenediaminetetraacetate (EDTA), and 0.1 mg/ml herring sperm DNA. The filters were washed with 2 × SSC/0.1% SDS at 50 °C and exposed to autoradiography. Positive clones were found on all four filters.

The isolated cDNA clones were sequenced on both strands with the fmol DNA-sequencing system (Promega, Madison, WI) and a series of 21mer synthetic oligonucleotide primers. Sequence analysis and multiple alignment of the predicted amino acid sequence with the CLUSTAL program were done in PC Gene (IntelliGenetics, Mountain View, CA).

RNA and DNA blot analysis

Total RNA was prepared with RNazol (Cinna/Biotech, Friendswood, TX) from human PBMC, either untreated or stimulated with 20 ng/ml PMA for 1, 3, 8, or 24 h. To investigate mRNA stability, cells were stimulated for 3 h with 20 ng/ml PMA and subsequently treated with 1 µg/ml actinomycin D for 30, 60, or 120 min. Of each sample, 10 µg RNA was separated on a 1.2% agarose/formaldehyde gel, transferred to a GeneScreen Plus nylon membrane (DuPont, Boston, MA), UV cross-linked and prehybridized with a buffer containing 1.5 × saline sodium phosphate-EDTA, 1% SDS, 0.5% non-fat dry milk, 0.5 mg/ml herring sperm DNA, and 10% dextran sulphate. Hybridization was performed in the same buffer with ³²P-labeled AICL cDNA at 60 °C for 20 h. Prior to autoradiography the blot was washed with 0.5 × SSC/0.1% SDS at 55 °C. Densitometry was performed by exposure of the blot on a phosphorimager BAS 2000 (Fuji, Tokyo, Japan). A human multi-tissue RNA blot (BIOS, New Haven, CT) was analyzed by the same procedure.

Genomic DNA was prepared from human PBMC. Aliquots of 15 µg were digested overnight with 100 units of restriction enzymes *Eco* RI, *Hin* dIII, *Pst* I, *Pvu* II, or *Xba* I and separated on a 0.8% agarose gel. Transfer to nylon membrane and hybridization with ³²P-labeled AICL cDNA at 65 °C were performed as described for RNA blot analysis. Subsequently, the same blot was hybridized with a ³²P-labeled PCR fragment from the C-type lectin domain of AICL (nucleotides 349–462).

Reverse transcription (RT)-PCR

Total RNA was prepared from human PBMC, T cells, B cells, monocytes, and granulocytes. Peripheral blood mononuclear cells (PBMC) and granulocytes were obtained from human venous blood by isolation on a Percoll density gradient and monocytes by subsequent counter-flow centrifugal elutriation. T and B cells of 90% purity were isolated by incubating PBMC with saturating amounts of CD19, CD14, and CD16 mAbs (to obtain T cells) or CD2, CD3, CD14, and CD16 mAbs (to obtain B cells) followed by negative selection with Dynabeads-M450 (Dyna, Oslo, Norway). Of each RNA sample, 1 µg was transcribed with Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD) into first-strand cDNA and used for PCR amplification (32 cycles, 30 s at 95 °C, 30 s at 52 °C, 1 min at 72 °C). Levels of hypoxanthine phosphoribosyltransferase (HPRT) cDNA were assessed as standard for the amounts of cDNA. PCRs specific for AICL, CD69, and CD94 were done with primer pairs flanking at least one intron to avoid false positive results due to amplification of genomic DNA. The specific primers were 5'-ATTGACAACATAGAA-GAAATG-3' [(+) strand] and 5'-TGAGCTCCATCTACCCATTG-3' [(-) strand] for AICL, 5'-CTCATTGCCTTATCAGTGGGC-3' [(+) strand] and 5'-ATTCCATGCTGCTGACCTCTG-3' [(-) strand] for CD69, 5'-CTAAACTGAGTATTGAGCCAG-3' [(+) strand] and 5'-GGAGAGTGCAGAGCCATTCTC-3' [(-) strand] for CD94, and 5'-TATGGACAGGACTGAACGCTTGC-3' [(+) strand] and 5'-GACACAAACATGATTCAAATCCCTGA-3' [(-) strand] for HPRT. Controls without cDNA were included in all experiments. PCR products were

Fig. 2 Analysis of transcription of the *AICL* gene in different human tissues. A multi-tissue RNA blot was hybridized with the ^{32}P -labeled *AICL* cDNA. Positions of RNA size markers are shown on the right

Fig. 3 RT-PCR detection of *AICL* transcripts in different cell types of hematopoietic origin. Total RNA of PBMC, purified T and B cells, monocytes, and granulocytes was transcribed into first-strand cDNA and analyzed for transcripts of *AICL*, *CD69*, *CD94*, and *HPRT* (control) by PCR amplification

separated on a 1.5% agarose gel containing ethidium bromide, visualized by UV illumination and photographed.

Chromosomal assignment of the gene

A sequence-tagged site (STS)-based yeast artificial chromosome (YAC) contig map has been generated using the Quickmap Based Pooling Strategy (QPS) as described previously (Krauter et al. 1995). YAC DNAs representing clones on the map were pooled according to their physical location. To place the *AICL* gene on the physical map, PCRs were performed with specific primers 5'-GGTATAAATG-CAGTTCTGATC-3' [(+) strand] and 5'-TGTAGCTCCATCTACC-CATTG-3' [(-) strand] and complex pools of YACs as templates, then less complex pools and finally individual YACs, until addresses were obtained for final localization.

Results and Discussion

Isolation and sequence analysis of *AICL* cDNA clones

To identify new members of the human *NK* gene complex, a search on ESTs in the EMBL/GenBank data base was

Fig. 4A, B Transcription kinetics of the *AICL* gene during lymphocyte activation. **A** RNA blot analysis of PBMC stimulated with 20 ng/ml PMA for 1, 3, 8, or 24 h prior to RNA isolation. Of each sample, 10 μg RNA was blotted and hybridized with ^{32}P -labeled *AICL* cDNA. The positions of the ribosomal RNAs are indicated. **B** Analysis of *AICL* mRNA stability. One $\mu\text{g}/\text{ml}$ actinomycin D was added to PBMC which were stimulated with 20 ng/ml PMA for 3 h. Equal amounts of RNA were prepared at the indicated time points and analyzed as above. Changes in the amount of *AICL* transcript were quantified by densitometry

performed. One EST (GenBank accession number T46996) with a high similarity to *CD69* (33%) identity in a stretch of 75 aa) was found. To characterize the protein, the cDNA was cloned from a cDNA library of human PBMC which had been activated for 6 h with PMA. By screening 12 pools of 3–4000 clones with a PCR-based strategy, 10 pools were found to be positive, indicating that the mRNA is highly expressed in short-term stimulated lymphocytes. One positive pool was successively subdivided and screened until a single clone was isolated. This clone was found to be incomplete as it lacked part of the 5' sequence. Subsequently, further cDNA clones were isolated by colony hybridization. Two clones with a length of 683 bp (clone 9.1) and 753 bp (clone 10.2) were sequenced.

The nucleotide sequence and the deduced aa sequence of clone 10.2 are shown in Figure 1A. The first seven nucleotides have been supplemented from the 5' end of clone 9.1, whose sequence was identical to the corresponding region of 10.2. The cDNA contains a single open reading frame of 447 bp encoding a polypeptide chain of 149 aa. Based on its expression characteristics (see below) the putative protein encoded by this cDNA was designated activation-induced C-type lectin (*AICL*). Its predicted molecular mass of 17300 M_r was confirmed by in vitro transcription and translation (data not shown).

AICL is a type II integral membrane protein with a C-type lectin domain

A typical feature of all molecules encoded by the *NK* gene complex is their type II membrane orientation (Yokoyama

Fig. 5 Partial physical map of chromosome 12 showing the location of *AICL*. The broad horizontal line at the top represents a segment of chromosome 12 containing *AICL*. STS marker names are shown above the line, and they are represented by cross-bars on the line. Individual YAC clones are shown below, with symbols indicating their STS content

and Seaman 1993). The *AICL* polypeptide contains a hydrophobic region between Cys⁸ and Val²⁵ but no cleavable signal sequence at the N-terminus. This indicates that *AICL* is also a type II membrane protein, with a C-terminal extracellular region of 124 aa, followed by a transmembrane region of 18 aa and an N-terminal cytoplasmic part of 7 aa. An equally short cytoplasmic region has recently been described for CD94 (Chang et al. 1995).

Sequence comparison revealed a significant similarity of the extracellular part of *AICL* to C-type lectin domains of various proteins. Highest similarity is found with the C-type lectin domains of CD69 (35% aa identity) and the chicken lectin 17.5 (45% aa identity). An alignment of the respective sequences is shown in Figure 1B. The 17.5 gene is located within the *Rfp-Y* system, a locus which encodes chicken *Mhc* genes of both class I and II. Expression of 17.5 was found in lymphoblastoid cell lines and a number of lymphoid tissues (Bernot et al. 1994).

C-type lectin domains consist of 115–130 aa (Drickamer 1993). With a size of only 124 aa, most of the extracellular part of *AICL* belongs to the C-type lectin domain which is separated by only nine aa from the transmembrane region. Remarkably, the short neck region contains no cysteines. Cysteines within this region are generally assumed to form disulfide bridges between the polypeptide chains of the homodimeric receptors encoded by the *NK* gene complex. It needs to be determined whether *AICL* indeed is expressed as a monomeric molecule.

Transcription of AICL is transiently upregulated during lymphocyte activation

To investigate transcription of the *AICL* gene, we analyzed a blot with RNA from different human tissues. As shown in Figure 2, only in spleen were small amounts of mRNA detected, implying that *AICL* is preferentially expressed in lymphoid tissues. RT-PCR was used to assess mRNA levels in different cell types of hematopoietic origin (Fig. 3). Transcription of *AICL* could be detected in lymphocytes, monocytes, and granulocytes, indicating that *AICL* is expressed by most hematopoietic cells and is not restricted to NK and T cells as previously shown for NKR-P1, Ly49, NKG2, and CD94 (Gumperz and Parham 1995).

The high frequency of *AICL* clones in a cDNA library from PMA-activated PBMC suggested that expression of *AICL* is upregulated during cellular activation. RNA was isolated from PBMC which had been stimulated with PMA for different periods of time (Fig. 4A). A rapid, but transient increase in *AICL* mRNA was found between 1 and 8 h after stimulation with a maximal induction at 3 h. Although the induction of *AICL* mRNA is probably less pronounced, transcription kinetics of *AICL* are similar to CD69 (Hamann et al. 1993; Lopez-Cabrera et al. 1993; Ziegler et al. 1994) and other rapidly upregulated antigens such as tissue factor, CD62E, and CD97 (Scarpatti and Sadler 1989; Bevilacqua et al. 1989; Hamann et al. 1995). The rapid mRNA turnover of these antigens is regulated by a fast degradation of the highly unstable mRNAs (Santis et al. 1995). *AICL* mRNA has a half-life

Fig. 6A, B DNA blot analysis of the *AICL* gene. **A** Genomic DNA from PBMC was digested with *Eco* RI, *Hin* dIII, *Pst* I, *Pvu* II, or *Xba* I and analyzed with 32 P-labeled *AICL* cDNA. The positions of DNA markers are shown on the right. **B** Analysis of the same blot with a 32 P-labeled 114 bp PCR fragment from the C-type lectin domain of *AICL*

of ≤ 1 h as examined by inhibiting transcription with actinomycin D after stimulation of PBMC with PMA for 3 h (Fig. 4B). Remarkably, no AU-rich elements, which are the most common determinants of mRNA instability (Chen and Shyu 1995), are found within the 3' untranslated (UT) region. However, the 3' UT region of *AICL* contains the motif TTTTGTA which is a *cis*-acting control element found in numerous immediate-early genes (Freter et al. 1992).

AICL is encoded by a single gene within the human NK gene complex

The first type II integral membrane protein with a C-type lectin whose gene was located to the predicted human NK gene complex on the short arm of chromosome 12 was CD69 (Lopez-Cabrera et al. 1993; Schnittger et al. 1993; Ziegler et al. 1994). Since the genes encoding NKG2, NKR-P1A, and CD94 were also mapped to chromosome 12 (Yabe et al. 1993; Lanier et al. 1994; Chang et al. 1995), we expected that the *AICL* gene would be localized there. Therefore, an STS-based YAC contig map of chromosome 12 (Krauter et al. 1995) was screened. As shown in Figure 5, the *AICL* gene was found on the short arm of chromosome 12 0.3 cM proximal to the *CD69* gene.

Within the NK gene complex, single genes as well as gene families have been described. To determine whether *AICL* is a member of a gene family, genomic DNA from human PBMC was analyzed (Fig. 6). Hybridization with the complete *AICL* cDNA revealed a diverse pattern of bands. When the same blot was subsequently hybridized

with a small PCR fragment covering a part of the C-type lectin domain (nucleotides 349–462), in most digests only one band was detectable, indicating that *AICL* is encoded by a single gene. From Figure 6A, the size of the gene can be estimated to be ~ 20 kb. The neck region and the first part of C-type lectin domain of CD69 are encoded by a common exon and not by separate exons as in NKR-P1, Ly49, and NKG2 (Ziegler et al. 1994; Santis et al. 1994; Giorda et al. 1992; Kubo et al. 1993; Plougastel et al. 1996). PCR analysis revealed that also the N-terminal part of the C-type lectin domain and the preceding neck region of *AICL* are encoded by a single exon (data not shown).

In conclusion, sequence analysis and chromosomal assignment identify *AICL* as a new protein encoded by the human NK gene complex whose expression kinetics resemble CD69 (Testi et al. 1994). These findings imply that the NK gene complex, next to NK cell receptors, encodes activation-induced molecules expressed by different types of hematopoietic cells. Further studies on *AICL* will contribute to a better understanding of the biological function of these molecules.

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