

Molecular Cloning, Expression, and Chromosomal Localization of the Human Earliest Lymphocyte Activation Antigen AIM/CD69, a New Member of the C-Type Animal Lectin Superfamily of Signal-transmitting Receptors

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Summary

The activation of T lymphocytes, both in vivo and in vitro, induces the expression of CD69. This molecule, which appears to be the earliest inducible cell surface glycoprotein acquired during lymphoid activation, is involved in lymphocyte proliferation and functions as a signal transmitting receptor in lymphocytes, natural killer (NK) cells, and platelets. To determine the structural basis for CD69 function, the cDNA coding for CD69 was isolated by a polymerase chain reaction-based strategy using oligonucleotides deduced from peptide sequences of the purified protein. The isolated cDNA exhibited a single open reading frame of 597 bp coding for CD69, and predicted a 199-amino acid protein of type II membrane topology, with extracellular (COOH-terminal), transmembrane, and intracellular domains. The CD69 clone hybridized to a 1.7-kb mRNA species, which was rapidly induced and degraded after lymphocyte stimulation, consistent with the presence of rapid degradation signals at the 3' untranslated region. Transient expression of the polypeptide encoded by CD69 cDNA in COS-7 cells demonstrated that it presented properties comparable to native CD69 protein. The CD69 gene was regionally mapped to chromosome 12 p13-p12 by both somatic cell hybrid DNA analysis and fluorescence in situ hybridization coupled with GTG banding (G bands by trypsin using Giemsa). Protein sequence homology search revealed that CD69 is a new member of the Ca²⁺-dependent (C-type) lectin superfamily of type II transmembrane receptors, which includes the human NKG2, the rat NKR-P1, and the mouse NKR-P1 families of NK cell-specific genes. CD69 also has structural homology with other type II lectin cell surface receptors, such as the T cell antigen Ly49, the low avidity immunoglobulin E receptor (CD23), and the hepatic asialoglycoprotein receptors. The CD69 protein also shares functional characteristics with most members of this superfamily, which act as transmembrane signaling receptors in early phases of cellular activation.

The activation of T lymphocytes by antigens or mitogens initiates a coordinate up- and downregulation of the expression of a wide number of known genes (1). The genes that are induced during the early phase of cell activation are also known as immediate-early genes. To this group belong several protooncogenes, such as *c-fos*, *c-jun*, or *c-myc*, and genes encoding molecules involved in signaling events, including triggering molecules, growth factors, and some cytokine receptors (1, 2).

The CD69 molecule (3), also designated as activation inducer molecule (AIM) (4), early activation antigen (EA-1) (5), Leu-23 (6), or MLR-3 (7) antigens, is a phosphorylated disulfide-linked 27/33-kD homodimeric protein (8). This an-

tigen seems to be the earliest inducible cell surface glycoprotein and is synthesized de novo and expressed upon T lymphocyte activation with a wide variety of stimuli, such as anti-CD3/TCR and anti-CD2 mAbs, activators of protein kinase C, or PHA (4, 5, 9, 10). The expression of CD69 by IL-2-activated NK cells parallels the acquisition of lytic activity by these cells (6). CD69 is undetectable on the plasma membrane of most circulating PBL; however, this molecule is expressed by a large fraction of T cells in the inflammatory infiltrates of several human diseases (11, 12), as well as by a small percentage of resident T and B cells in different normal lymphoid tissues (13). In this context, CD69 expression on thymocytes has been associated with the acquisition of im-

munocompetence during T cell maturation (14, 15). Other cell types, including platelets and epidermal Langerhans cells, also express the CD69 molecule (16, 17).

The CD69 glycoprotein functions as a signal-transmitting receptor in different cells. In T cells, the signals triggered by CD69 antibodies include rise of intracellular calcium concentration, expression of the IL-2R α subunit (CD25), synthesis of different cytokines, such as IL-2, TNF- α , and IFN- γ , and cell proliferation (4, 9, 18–20). Furthermore, the triggering of T cells through the CD69 activation pathway enhances the binding activity of transcription factor AP-1, which plays a key regulatory role in the initial steps of cell activation (21). Likewise, CD69 also acts as a triggering molecule on NK cells and platelets: CD69 antibodies are capable of inducing target cell lysis by IL-2-activated NK cells (22, 23), and of triggering platelet aggregation, Ca²⁺ influx, and hydrolysis of arachidonic acid (16). The possible coupling of CD69 with a GTP-binding protein has also been reported (24).

In an attempt to understand the molecular basis for the CD69 functions, the cDNA encoding this protein was cloned and its nucleotide sequence determined. We herewith report that CD69 protein is a type II membrane protein with a C-type animal lectin domain. Comparative protein sequence homology indicates that CD69 is a member of a gene superfamily of type II membrane proteins that includes a wide number of mouse, rat, and human NK cell-specific genes that, like CD69, function as signal-transmitting receptors.

Materials and Methods

mAbs. The different anti-CD69 TP1/8, TP1/55, and FAB/1 mAbs, directed to extracellular antigenic determinants of CD69, have been previously described (8). The CH/2 mAb is specific for an intracellular CD69 epitope (8). Monoclonal Ig (Ig1,K) from the myeloma line P3X63 was included as negative control in some assays.

Purification of CD69 Protein. The anti-CD69 FAB/1 mAb (IgG1,K) (8) was purified from ascites fluids by protein A affinity chromatography and coupled to CNBr-activated Sepharose CL-4B (Pharmacia, Uppsala, Sweden) at 2 mg/ml.

PBL were obtained from buffy coats by Ficoll-Hypaque (Pharmacia) centrifugation. Activation of PBL was carried out by culturing 2×10^6 cells/ml in the presence of PMA (10 ng/ml) and soluble anti-CD3 SPV-T3b mAb for 24 h (4). Activated PBL (2×10^8 cells/ml) were lysed in a buffer containing 20 mM Tris-HCl, pH 8.0, 1% Triton X-100, 5 mM iodoacetamide, 1 mM PMSF, 0.2 U/ml trypsin inhibitor of aprotinin, and 0.025% NaN₃. After 30 min at 4°C, the lysate was clarified by centrifugations at 1,000 g for 10 min followed by 50,000 g for 1 h. Preparative scale immunoaffinity isolation was performed by passing 50 ml of lysate over sequential glycine-quenched Sepharose CL-4B and anti-CD69 FAB/1-Sepharose CL-4B columns at a flow rate of 1 ml/min. The FAB/1 column was washed with a five-column bed volume of 25 mM Tris-HCl, pH 9.5, 1.5 M NaCl, 0.5% Triton X-100, 1 mM iodoacetamide, 0.5 mM PMSF, and 0.025% NaN₃. The column was then washed with 3 vol of 100 mM ammonium acetate, pH 6.0, 25 mM *N*-octyl- β -glucopyranoside (OG). The CD69 protein was eluted with 3 vol of 100 mM ammonium acetate, pH 2.5, 25 mM OG at a flow rate of 0.3 ml/min. Fractions (1 ml) were collected into tubes containing 50 μ l of 1 M ammonium bicarbonate. Homogeneity of immunoaffinity-purified protein was assessed by SDS-PAGE and silver staining.

Protein Digestion, Peptide Purification, and Sequencing. Approximately 200 pmol (1 ml) of purified protein was dissolved with 200 μ l of 1 M Na₂HPO₄ and denatured at 100°C for 5 min. After cooling, *N*-glycanase enzyme (Genzyme Corp., Cambridge, MA) was added and incubated for 16 h at 37°C. After incubation, the sample was concentrated, washed three times with 0.5 ml of 0.1% SDS, and reduced in volume to near dryness. The sample was then diluted up to 50 μ l with sample buffer containing 0.5% 2-ME without SDS and alkylated with the addition of 1 μ l of 4-vinylpyridine and run on SDS-11% PAGE.

In situ digestion of the protein in the polyacrylamide gel matrix was done by incubating the gel pieces with endoproteinase Lys-C (Promega Biotec, Madison, WI) for 20 h at 37°C.

The resulting peptides were then subjected to narrow-bore, reverse-phase HPLC. The first step of peptide purification was made on a Vydac C4 (2.1 \times 250-mm) column run in 0.1% TFA. Fractions of pure peptide were subjected to automated Edman sequencing on a protein sequencer (477A; Applied Biosystems, Inc., Foster City, CA) on line to the 120A Separation system.

cDNA Cloning. Degenerated sense and antisense oligonucleotides, deduced from the amino acid sequences of two Lys-C peptides (Fig. 1), were used as primers to achieve PCR (25) on a pool of reversely transcribed RNA from human PBL activated with 10 ng/ml of PMA at various time points (30 min to 48 h). Amplification was carried out for 35 cycles (1-min denaturation at 95°C, 1-min annealing at 50°C, and 1.5-min elongation at 72°C) using an RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT). Amplification of the 3' end of CD69 cDNA was achieved by PCR on reverse-transcribed RNA from PMA-activated PBL essentially as described (26), using the internal primer AIM 1 (5'-AGGAACCTGGTC-ACCCATGG-3', nucleotides [nt]' 521–540). To isolate the 5' end of CD69 cDNA, random-primed first-strand cDNA was synthesized from PMA-activated PBL poly(A)⁺ RNA and polyadenylated according to a protocol previously described (27), then the 5' cDNA end was amplified as described (26), using the internal primer AIM 3 (5'-TTCCATGCTGCTGACCTCTGT-3', nt 616–636 of the [–] strand). To verify the nucleotide sequence of the CD69 open reading frame (ORF), two independent PCR were performed on PMA-activated PBL RNA using the primers AIM 6 (5'-GAGCTCCAGCAAAGACTTTC-3' nt 12–31) and AIM 7 (5'-TCAATAAGTGAACATGTTTC-3', nt 686–705 of the [–] strand) from the 5'- and 3'-untranslated regions. Amplification was carried out for 35 cycles (1-min denaturation at 95°C, 1-min annealing at 58°C, and 1.5-min elongation at 72°C). The amplification products of PCRs were purified from agarose gels by using the Gene Clean kit (Bio 101 Inc., La Jolla, CA) and cloned into pCR II plasmid (Invitrogen Corp., San Diego, CA).

DNA sequencing was performed by the dideoxy termination method (28) either by subcloning restriction fragments into Bluescript vectors or by direct oligonucleotide-primed DNA sequencing with internal primers when no convenient restriction sites were available.

Northern Blot Hybridization Analysis. Total RNA was isolated from human PBL, either untreated or treated with 10 ng/ml of PMA at various time points (0.5, 1, 3, 6, 12, 24, and 48 h) by the guanidine isothiocyanate method (29). A total of 10–20 μ g of RNA of each sample was run on 1% formaldehyde-agarose gels and transferred onto nitrocellulose membranes. Hybridizations were performed using the cloned 197-bp fragment, obtained by PCR

¹ Abbreviations used in this paper: nt, nucleotide; ORF, open reading frame.

with oligonucleotides 3.1+ and 4.2- (Fig. 1), as a ³²P-labeled probe. To normalize by laser densitometry the CD69 mRNA expression, a ³²P-labeled β-actin cDNA probe was used as control.

Chromosome Mapping of the CD69 Gene. Chromosomal localization of the CD69 gene by PCR was performed on DNA from interspecies hamster-human somatic cell hybrids (BIOSMAP™ Somatic Cell Hybrids PCRable™ DNAs; BIOS Corp., New Haven, CT) using the AIM 1 and 3 primers. The specificity of PCR products was confirmed by hybridization with a genomic CD69 probe, which included the sequence between the AIM 1 and 3 oligonucleotides.

The regional localization of the CD69 gene was assessed by fluorescence in situ hybridization with an EMBL3 phage containing a 15-kb genomic CD69 DNA. The λ-h CD69-1 phage, containing a 15-kb genomic insert, was isolated from a human genomic library constructed in the EMBL3 phage vector, as will be described elsewhere. The genomic clone was labeled by nick translation with digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany) and diluted in hybridization buffer (50% formamide, 10% dextran sulphate, 2× SSC, and 50 mM phosphate, pH 7.0). After heat denaturation, 4 ng/μl of labeled probe was preannealed with a 500-fold excess of sonicated human placental DNA for 1 h at 37°C and hybridized overnight to denatured metaphase chromosomes from a normal male. Hybridization, washings, and detection with rhodamine-conjugated antibodies were performed as described (30). Chromosomes were counterstained with 75 ng/ml of 4'-6-diamino-2-phenylindole (DAPI) in antifade medium. After the fluorescent microscopy, GTG banding was performed as described (30).

FACS® Analysis. Fluorescence flow cytometry analysis was performed on a FACScan® cytofluorometer (Becton Dickinson & Co., Mountain View, CA). Cells were incubated at 4°C with 100 μl hybridoma culture supernatants, followed by washing and labeling with fluorescein isothiocyanate-labeled goat anti-mouse Ig (Pierce Chemical Co., Rockford, IL). Data were collected in a logarithmic scale, and percentage of positive cells was obtained by subtracting the background given by the mouse myeloma P3X63.

Transfection of COS Cells, Indirect Immunofluorescence, and Immunoprecipitation. To construct the CD69 expression vector (pAIMI-neo), a recombinant pCRII plasmid harboring the complete CD69 ORF and part of the 5' and 3' adjacent sequences (nt 12-705) was partially digested with BstXI and the resultant 700-bp fragment ligated to the plasmid pcDNAI-neo (Invitrogen Corp.) downstream of the cytomegalovirus promoter. The orientation of the insert was tested by restriction enzyme analysis.

For transient expression of CD69, monolayers of COS-7 cells were transfected with pAIMI-neo by the diethylaminoethyl-dextran method (31). Plasmid pcDNAI-neo was also included in the transfection as a control.

Transfected COS cells were grown on glass coverslips and cells were fixed in 3.7% formaldehyde in PBS for 10 min at room temperature. Cells were incubated with the primary antibody for 1 h at 37°C, rinsed in Tris-buffered saline, and stained with the appropriate dilution of the secondary antibody (FITC-labeled goat anti-mouse Ig; Pierce Chemical Co.). TP1/8 and TP1/55 mAbs were used as primary antibodies. Cells were observed using an Optiphot photomicroscope (Nikon Inc., Instr. Group, Melville, NY).

Activated PBL, and transfected COS-7 cells harvested 72 h after transfection, were radioiodinated in solution with chloroglycoluril (Iodogen; Pierce Chemical Co.). For immunoprecipitation, equal amounts of input radioactivity were mixed with 30 μl of different purified anti-CD69 mAb directly conjugated to Sepharose (2 mg/ml). Immunoprecipitates were processed as previously described

(4) and samples were subjected to SDS-PAGE on 12% gels either under reducing or nonreducing conditions.

Results

Isolation and Characterization of CD69 cDNA. The CD69 glycoprotein was purified from PMA-activated human PBL by mAb affinity chromatography. Homogeneous CD69 protein was obtained as determined by SDS-PAGE followed by silver staining (Fig. 1 A). The purified protein was treated with N-glycanase, digested with Lys-C endopeptidase, and the resulting peptides were separated by reverse-phase HPLC and subjected to amino acid micro-sequencing. A total of 38 residues were determined from four independent peptides (Fig. 1 B). Degenerated sense and antisense oligonucleotides (Fig. 1 C) based on two Lys-C peptides (Fig. 1 B, *underlined*) were designed and used as primers to carry out PCR on reversely transcribed poly(A)⁺ RNA from a pool of PBL activated with PMA at different times (30 min to 24 h) (see below, Fig. 5 A). A DNA fragment of 197 bp was amplified using the oligonucleotides 3.1+ and 4.2-, whereas no amplification product was obtained with the oligonucleotides 4.1+ and 3.3-. The specificity of the 197-bp product was verified by a second PCR performed on the first PCR products, using the oligonucleotide 3.2+, which overlaps oligonucleotide 3.1+, and the oligonucleotide 4.2- (Fig. 1 C). The specific products of both PCRs were cloned and sequenced. The full-length cDNA was obtained by PCR after the rapid amplification of cDNA 5' and 3' ends (RACE) protocols (Fig. 1 D). The complete nucleotide sequence of the 1.7-kb CD69 cDNA and the amino acid sequence of the translation product is shown in Fig. 2.

To confirm the predicted amino acid sequence of the CD69 translation product, two additional independent PCRs were performed using primers from the 5'- and 3'-untranslated sequences adjacent to the ORF. No polymerase-induced mutations were observed on these cloned PCR products. The ORF extended from nt 82 to 678, and contained the amino acid sequences of the four different Lys-C peptides. ORF was preceded by a 5'-untranslated region of 81 bp and followed by a 3'-untranslated region of 1,023 bp, which contained three poly(A) and multiple rapid degradation signals (Fig. 2).

Expression of CD69 Antigen in COS Cells. To confirm that the cDNA isolated was encoding CD69 antigen, COS-7 cells were transfected with the CD69 ORF, which was placed under the control of the cytomegalovirus promoter. Transfected cells reacted with several anti-CD69 mAbs as shown by immunofluorescence (Fig. 3 A). Furthermore, immunoprecipitation with mAb directed against extracellular and intracellular epitopes of the CD69 antigen (8) from ¹²⁵I externally labeled transfected COS-7 cells yielded two bands under reducing conditions, which corresponded to the 27- and 33-kD bands of the native CD69 precipitated from activated PBL (Fig. 3 B). Under nonreducing conditions, immunoprecipitation with anti-CD69 mAb yielded a single band of 60 kD, which is the expected molecular mass of the disulfide-linked CD69 homodimer (Fig. 3 B). These results demonstrate that

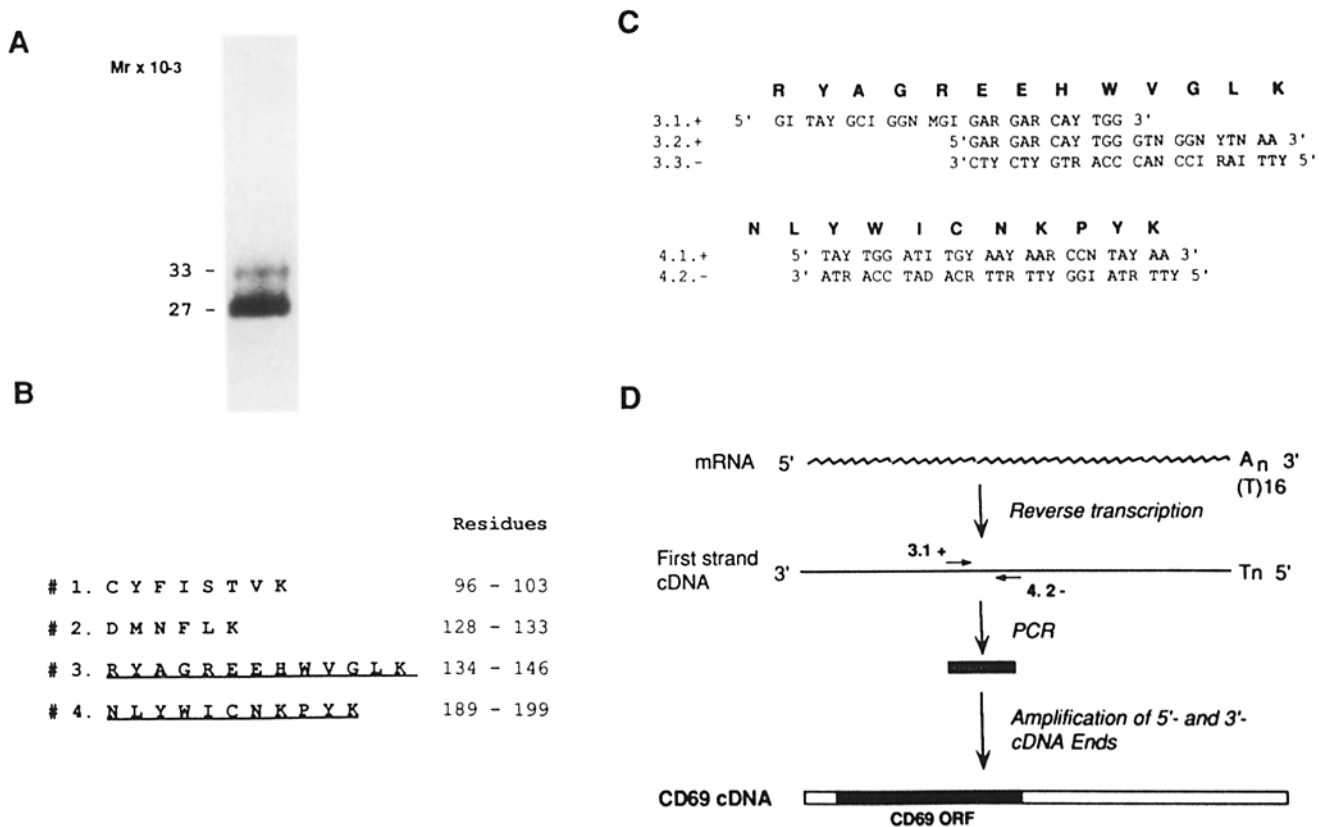


Figure 1. cDNA cloning procedure. (A) Homogeneity of immunopurified CD69 protein was analyzed by SDS-12% PAGE under reducing condition followed by silver staining. (B) CD69 Lys-C peptide sequences. The peptides and their position in the cDNA-derived sequence are shown. They have been represented in the same order as they are located in the CD69 ORF. The peptide sequences used for the design of degenerated PCR primers are underlined. (C) Degenerated oligonucleotides designed from CD69 Lys-C peptide sequences are shown. (N) G, A, T, or C; (Y) C or T; (R) G or A; (D) G, A, or T; and (M) A or C; + and - indicate that the oligonucleotide sequences correspond to the sense or antisense DNA strand, respectively. (D) Diagrammatic representation of the cDNA cloning procedure.

the cDNA-encoded CD69 protein possesses identical properties as native CD69 antigen.

Predicted Amino Acid Sequence of the CD69 Translation Product. The deduced amino acid sequence of the CD69 ORF corresponded to a polypeptide of 199 residues with a predicted molecular weight of 22,545, which is in agreement with the previously reported molecular weight of deglycosylated CD69 protein (22,000–24,000) (6, 8). This polypeptide was devoid of NH₂-terminal hydrophobic signal peptide but contained a transmembrane domain of 26 amino acids as predicted by hydrophobicity analysis (32). (Fig. 2). These features on the primary structure of CD69 indicated that it is a type II integral membrane protein and is therefore composed of a NH₂-terminal cytoplasmic domain of 38 residues, a single transmembrane region of 26 residues, and an extracellular COOH-terminal domain of 135 amino acids.

A putative site was found for N-linked glycosylation in the extracellular domain of CD69 (Fig. 2). The existence of this N-linked glycosylation site is in agreement with previous results demonstrating, by both glycosidase and tunicamycin treatments, the presence of N-linked glycosylation in the CD69 protein (6, 8). Several potential phosphorylation sites for

serine/threonine kinases, PKC, and casein kinase II were found within the cytoplasmic tail (Fig. 2), in accordance with the constitutive Ser/Thr phosphorylation of CD69 in both mature thymocytes and activated T lymphocytes (6, 14, 33).

CD69 Is a Member of the C-Type Animal Lectin Superfamily. Comparison of the predicted amino acid sequence with the SWISSPROT and EMBL databases using the search algorithm FASTA (34) indicated that CD69 has homology with proteins of the animal C-type lectin superfamily, which also possess the unusual type II membrane orientation (Fig. 4). Most markedly, computer-aided comparisons using the BESTFIT program revealed that CD69 has strong amino acid sequence similarity ranging from 44 to 48% with the human NKG2 (35), and the rat and mouse NKR-P1 protein families (36, 37). It was found that CD69 protein is also homologous to the human and mouse low affinity IgE receptor (CD23) (38–40), the hepatic asialoglycoprotein receptor (41), and the mouse T cell antigen Ly49 (42, 43). The highest sequence similarity was confined mainly to the C-type lectin domain, which covers a stretch of 116 amino acids of the CD69 polypeptide, the most striking feature being the presence of 12 invariant residues (Fig. 4). Particularly important is the

AGACTCAACAGAGCTCCAGCAAGAGCTTTCACCTGAGCTTGACTTGACCTGAGATTAACAGGGAATCTTGAGAATAAAG 81
 ATGAGCTCGAAAAATGGTTTCGTAGCAGAGAACAGCTCTTTCATCCGGAGAGTGGACAAGAAAATGATGCCACAGTCCC 162
M S S E N C F V A E N S S L E P E S G Q E N D A T S P 27
 CK2
 CATTTCCTCAACAGCTCATGAAGGGTCTCCAAAGTTCCTGTCTGTGTGTGAATGAATGTGGTCTTCATCACCATTTTA 243
H F S T R H E G S F Q V P V L C A V M N V V F I T I L 54
 PKC CK2
 ATCATAGCTCTCATTGCCTTATCAGTGGGCCAATACAATTGTCCAGGCCAATACACATTCTCAATGCCATCAGACAGCCAT 324
I I A L I A L S V G Q Y N C P G Q Y T F S M P S D S H 81
 GTTCTTCATGCTCTGAGGACTGGGTGGCTACCAGAGGAAATGCTACTTTATTCTACTGTGAAGAGAGCTGGACTTCA 405
V S S C S E D W V G Y Q R K C Y F I S T V K R S W T S 108
 GCCCAAAATGCTTGTCTGAACATGGTCTACTCTTCTGTCTGATTGATTCTGAAAAGGACATGAACTTTCTAAAACGATAC 486
A E N A C S E H G A T L A V I D S E K D M N F L K R Y 135
 GCAGGTAGAGAGGAACACTGGGTGGACTGAAAAGGAACCTGGTCACCCATGGAAGTGGTCAAATGGCAAAGAAATTTAAC 567
A G R E E H W V G L K K E P G E P W K W S N G K E F N 162
 AACTGGTTCAACGTTACAGGGTCTGACAAGTGTGTTTTCTGAAAAACACAGAGGTGAGCAGCATGGAATGTGAGAAGAAT 648
N W F N V T G S D K C V F L K N T E V S S M E C E K N 189
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 TTATACTGGATATGTAACAACTTACAATAATAGGAAACATGTTCACTTATTGACTATTATAGAACTGGAAGTCAAGGA 729
L Y W I C N K P Y K 199
 AATCTGTGTCAGTGGATGCTGCTGTGGTCCGAAGTCTCCATAGAGACTTGTGAAAAAAATTTTATAGTGTCTTGGG 810
 AATTTCTTCCAAACAGAACTATGGAAGAAAGGAAAGAAATCCAGGAAAATCTGCAGTGTGGCTTTTATGCCATGAGC 891
 TAGAAGCATCACAGGTTGACCAATAAACCATGCCAAGAATGAGAAGAATGACTATGCAACCTTTGGATGCACCTTATATTA 972
 TTTTGAATCCAGAAATAATGAAATAACTAGGCGTGGACTTACTATTATGCTGAATGACTACCAACAGTGAAGCCCTTC 1053
 ATGCATTGCACTACTGGAAGGAGTGTAGTGTGGTACTAGATACTGAATGTAACAAGGAATATGGCTGGTAACATAG 1134
 GTTTTGTAGTCTAATGAAATCCCTTAACTCAGGGAGCATTAAATGGACAAATGCTTATGAACTAAGATTGPAATAT 1215
 TTCTCTTTTTAGAGAAATTGCAATTGCTTTGTTATTTTTCCCAAAAAGAAATGGGATGATCGTGTATTATTTTTT 1296
TACTCTCAGCTGTAGACAGGCTCTTTTCGATGGTACATATTTCTTGGCTTTATAATCTTTTATACAGTGTCTTACAGA 1377
 GAAAAGACATAAGCAAAGACTATGAGGAATATTGCAAGACATAGAATAGTGTGGAAATGTGCAATATGTGATGTGGCAA 1458
 ATCTCTATTAGGAAATATTCTGTAATCTCAGAACCTAGAATAACTAGTCTTATAATAGTTTGTGACTTCTCTAAATCA 1539
 ATTCTATTACGTGCAACTACTCAACTCTGATTAAATATTTTATGTGCAATAAAAATGATTGTTGTTGATTTTGTGTT 1620
 CAGTACAATTATAAGCTGTTTTATATATGTGAAATAAAGTAGAATAAACACAAAAAATAAAAAAAAAAAAAAAAAAAAA 1701

Figure 2. CD69 cDNA nucleotide sequence and predicted amino acid sequence. The transmembrane domain is double underlined, and potential polyadenylation signals are single underlined. Potential rapid degradation signals are in boxes. Putative N-linked glycosylation sites are indicated by asterisks and phosphorylation sites are PKC and casein kinase II (CK2). These sequence data have been submitted to the EMBL Data Library under accession number Z22576.

conserved placement of six of the Cys residues (Fig. 4), which appear to be involved in disulfide bonds (41, 44). Although there are no long continuous stretches of invariant residues, almost all of the essential residues conforming the C-type carbohydrate recognition domain (41) are present at approximately the same spacing. CD69 also showed certain degree of similarity with the transmembrane and cytoplasmic regions of other C-type lectin cell surface receptors expressed by leukocytes, such as the NKG2 and NKR-P1 members.

CD69 mRNA Expression. The cell surface expression of CD69 is almost exclusively restricted to leukocytes and is transiently induced upon treatment of lymphocytes with several mitogenic reagents such as phorbol esters (4). Northern blot analysis of RNA from untreated and PMA-treated PBL using a specific CD69 cDNA probe showed a PMA-inducible RNA species of 1.7 kb, in agreement with the size of the isolated cDNA (Fig. 5 A).

The early expression of CD69 protein upon T cell activation together with the presence of rapid degradation signals at the 3' untranslated region of the CD69 cDNA led us to analyze the induction kinetics of the CD69 mRNA. Therefore, PBL were stimulated with PMA at various time points, and then the total RNA was subjected to Northern blot anal-

ysis with a CD69 cDNA probe (Fig. 5 B). In parallel, the surface expression of CD69 antigen was assessed by flow cytometry (Fig. 5 C). CD69 mRNA showed a transient increase after exposure of cells to PMA. It became detectable by 30 min to 1 h, with maximal expression by 6 h, reaching a 50-fold increase, and declined thereafter to undetectable levels (Fig. 5 B). The surface expression of CD69 antigen displayed a slower kinetics compared with that of the mRNA induction. It reached a peak by 12 h of PMA treatment, declined partially by 24 h, and then the induced level persisted for at least 48 h (Fig. 5 C). These results indicated that the level of CD69 mRNA regulates CD69 protein expression. Similarly, Northern blot analysis performed on RNA from different cell lines, which were either unstimulated or stimulated with PMA for 6 h, showed that the tissue-specific distribution of CD69 antigen is regulated at transcriptional level (data not shown).

Chromosomal Assignment of the CD69 Gene. The CD69 gene was located on chromosome 12 by PCR analysis of DNA from interspecies hamster-human somatic cell hybrids (Fig. 6, and data not shown). As observed in Fig. 6 A, an amplification product of 1.1 kb was obtained only in those hybrids containing chromosome 12 (lane 4). The presence

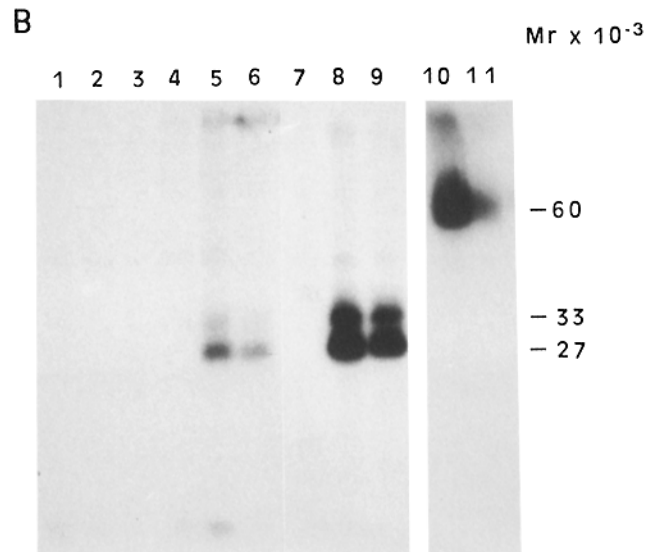
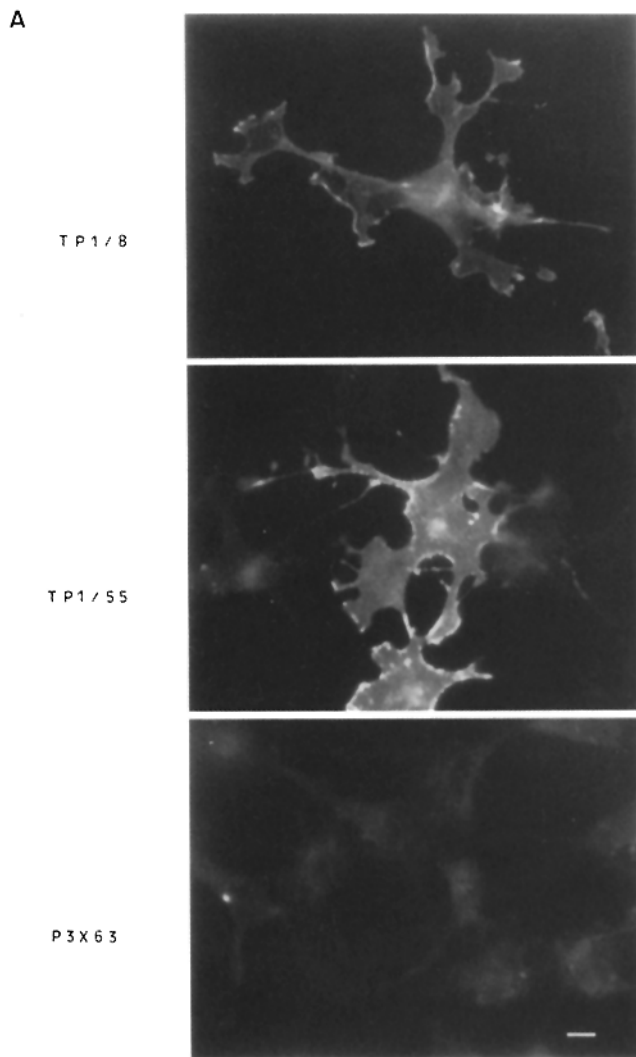


Figure 3. Transient expression of cDNA-encoded CD69 in COS-7 cells. (A) Immunofluorescence staining of CD69-transfected cells with anti-CD69 TP1/8 and TP1/55 mAbs. The monoclonal Ig from P3X63 myeloma was included as negative control. (B) Immunoprecipitation analysis of CD69 from transfected COS cells. Lysates from ^{125}I -labeled transfected cells with pcDNA1-Neo (vector without an insert) (lanes 1-3), transfected with pAIMI-Neo (containing the CD69 ORF) (lanes 4-6), and PMA-activated PBL (lanes 7-9) were precipitated with the following antibodies: P3X63 as negative control (lanes 1, 4, and 7), the anti-CD69 TP1/8 (lanes 2, 5, and 8), and CH/2 mAbs (lanes 3, 6 and 9) specific for extracellular and intracellular epitopes, respectively. The samples were analyzed by SDS-12% PAGE under reducing conditions. Precipitates with the anti-CD69 TP1/8 mAb from PMA-activated PBL (lane 10) and from pAIMI-Neo-transfected cells (lane 11) were also analyzed under nonreducing conditions.

of 1.0-kb intron between oligonucleotides AIM 1 and 3 (data not shown) is consistent with the size of the amplified fragment. The specificity of the PCR product was further confirmed by Southern blot hybridization (Fig. 6 B).

To determine the regional location of the human CD69 gene, we have performed fluorescence in situ hybridization on human metaphase chromosomes using a CD69 genomic probe. A total of 30 metaphase spreads were analyzed for the

h ASGR1	CCPVNVEHE	RSYWFSSRG	KAWADADNYC	RLEDAHLVVV	TSWEEQKFVQ	201
h CD23	TCPEKWINFO	RKCYFSGKGT	KQVHARYAC	DDMEGQLVSI	HSPEEQDFLT	211
h CD69	SCSEDWVGYO	RKCYFISTVK	RSWTSAENAC	SEHGATLAVI	DSEKDMNFLK	133
m NKR-P1	KCPKDNLSHR	DKCFHVSQTS	ITWKESLADC	GGKGATLLLV	QDQEELRFLR	142
m Ly49	GDKVYVFCYG	MKCYFVMDR	KTWSGCKQAC	QSSSLSLLKI	DDDELKFLQ	187
h NKG2a	HCPEEWITYS	NSCYIIGKER	RTWEESLLAC	TSKNSSLSLI	DNEEEMKFLS	167
h ASGR1	HHIGPVNT..	WMGLH..DQN	GPWKKVDCID	YETGFKNWRP	EQPDDWYGHG	247
h CD23	KHASHTGS..	WIGLRNLDLK	GEFIWVDGSH	VD..YSNWAP	GEPTSR....	253
h CD69	RYAGR..EEH	WVGLK.KEPG	HPWKKWSNGKE	FN.NWFN...VTC	169
m NKR-P1	NLTKRISSSF	WIGLSYTLSD	ENWKKWINGST	LNSDVLS...ITG	182
m Ly49	LVVP..SDSC	WVGLSYDNKK	KDWAVIDNRP	SKLALNT...GKY	225
h NKG2a	II....SPSS	WIGVFRNSSH	HPWVTMNGLA	PKHEIKD...SD	202
h ASGR1	LGGGEDCAHF	TDDGRWNDDV	GORPY.RWVC	E...T		278
h CD23	.SQGEDCVMM	RGSGRWNDAP	CDRKLGAWVC	DRLAT		287
h CD69	...SDKCVF.	LKNTFVSSME	CEKNLY.WIC	NKPYK		199
m NKR-P1	DTEKDSCAS.	VSQDKVLSSES	CDSDNI.WVC	QKELK		215
m Ly49	NIRDGGCML.	LSKTRLDNGN	CDQVFI.CIC	GKRLD		258
h NKG2a	NA.ELNCAV.	LQVNRLKSAQ	CGSSII.YHC	KHKL.		233

Figure 4. Sequence alignment of the lectin-like domains of CD69 protein and several other members of the supergene family. The amino acid positions within each sequence are shown. Filled circles indicate positions where the residue is highly conserved. The six invariant Cys residues are enclosed within black boxes. Identities between CD69 and other C-type lectins are shaded; (h) human, and (m) mouse.

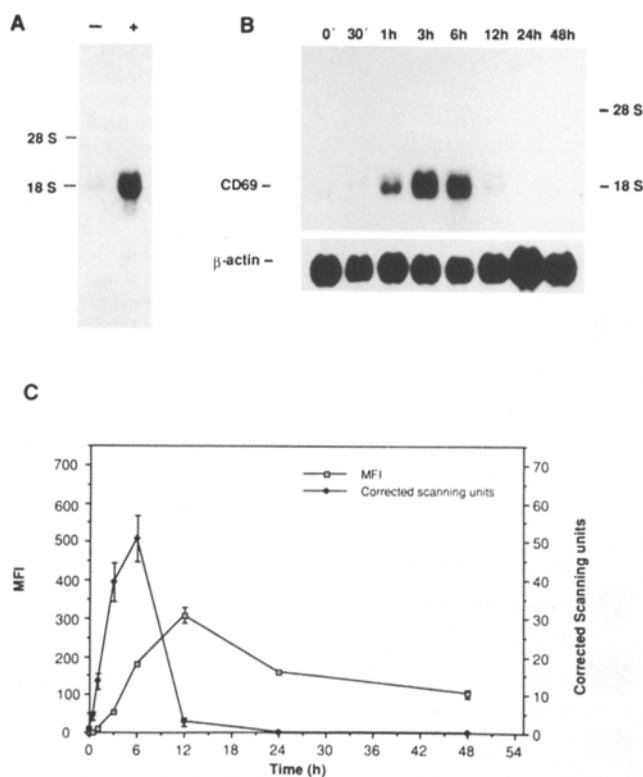


Figure 5. Analysis of human CD69 mRNA. (A) Northern blot analysis of RNA from untreated (lane -) and PMA-treated PBL (lane +) with a ^{32}P -labeled CD69-cDNA fragment spanning nt 482-678. Positions of the ribosomal 28S and 18S RNA are indicated on the left. (B) Induction kinetics of CD69 mRNA. PBL were stimulated with PMA (10 ng/ml) at various time points (30 min, and 1, 3, 6, 12, 24, and 48 h), and the total RNA of each sample was prepared and subjected to Northern blot analysis with the same CD69 cDNA probe described above. A β -actin cDNA probe was used as a control for loading amounts. (C) Diagrammatic representation of the induction kinetics of CD69 mRNA and protein. Laser densitometry giving corrected scanning units of the CD69 mRNA expression normalized to the β -actin control gene is represented by filled circles. Surface expression of CD69 protein analyzed by flow cytometry is represented by open squares. (MFI), mean fluorescence intensity.

presence of rhodamine fluorescent spots. As illustrated in Fig. 6 C, hybridization was specific and the signal was clearly detected on the short arm of chromosome 12, on bands p13-p12 based on GTG banding. As summarized in Fig. 6 D, 64% of the total fluorescent spots found on chromosome 12 were clustered on the p13-p12 bands.

Discussion

This study describes the cloning and sequencing of the cDNA encoding the human early lymphocyte activation antigen CD69. The molecular cloning of CD69 antigen was achieved by a combined strategy that included partial protein sequencing and PCR techniques. Confirmation that the cloned cDNA indeed represents CD69 is based on several lines of evidence. (a) The predicted amino acid sequence encoded by the ORF is a protein with a molecular weight of 22,545,

which is similar to the reported molecular weight (22,000-24,000) of the deglycosylated native CD69 protein (6, 8); (b) the four peptide sequences obtained from the CD69-purified protein are found in the translated sequence of the isolated gene; and (c) transfection of COS cells with the cloned CD69 cDNA shows that a disulfide-linked homodimer of the expected molecular weight under both reducing and non-reducing SDS-PAGE conditions can be precipitated by several anti-CD69 mAbs that are directed against extracellular and intracellular epitopes of the molecule (8).

The ORF of CD69 cDNA predicts a 199-amino acid polypeptide with a type II transmembrane topology. The extracellular COOH-terminal domain possesses an available site for N-linked glycosylation as proposed for the native protein (6, 8). Previous studies of cell protease treatments revealed the presence of a CD69 pronase-resistant peptide of 16,000 Mr that lost the external epitopes, suggesting that the native protein could be predominantly an intracellular protein (8). However, it is now evident that CD69 indeed possesses a large protease-resistant fragment in the extracellular region of the protein. This is in accordance with the observations that the purified glycosylated native protein was refractory to protease fragmentation, and that most of the currently used proteases failed to cleave the deglycosylated protein without previous SDS denaturation (8). Taken together, these findings show that the majority of the antigenic sites recognized by different CD69 antibodies, including those with agonistic properties, would be located in the terminal fragment of the external domain, likely into the lectin domain.

The regulation of the expression of CD69 in PBL appears to be of remarkable interest. Like other immediate early genes (45, 46), the CD69 gene displays a rapid and transient expression, followed by a rapid decay and degradation at the RNA level. The presence of reiterate rapid degradation signals (47) at the 3' end of CD69 mRNA may explain its rapid turnover. This tight regulation ensures the transient activation of this gene and contributes to prevent the uncontrolled expression of the CD69 receptor implicated in lymphocyte proliferation. The pattern of CD69 gene expression closely resembles that of proto-oncogenes and most cytokines in lymphocytes, and it is clearly distinct from that of other lymphocyte activation molecules such as the IL-2 and transferrin receptors, which exhibit a slower kinetics of both RNA induction and degradation (1, 48). However, the expression of the CD69 protein on lymphocyte cell surface persisted for longer periods of time. This may be attributed to the high stability of the CD69 glycoprotein, and its refractoriness to proteolytic mechanisms as discussed above. Whatever the mechanisms regulating the expression of the CD69 gene are, they remain unknown and deserve further investigation.

Comparison of CD69 cDNA with known sequences indicates that the extracellular region of CD69 is characterized by the presence of a C-type animal lectin domain with similar membrane orientation and protein sequence as those found in members of this lectin family. Hence, CD69 is related to a growing family of type II transmembrane receptors, which includes the gene families coding for human NKG2 (35), rat

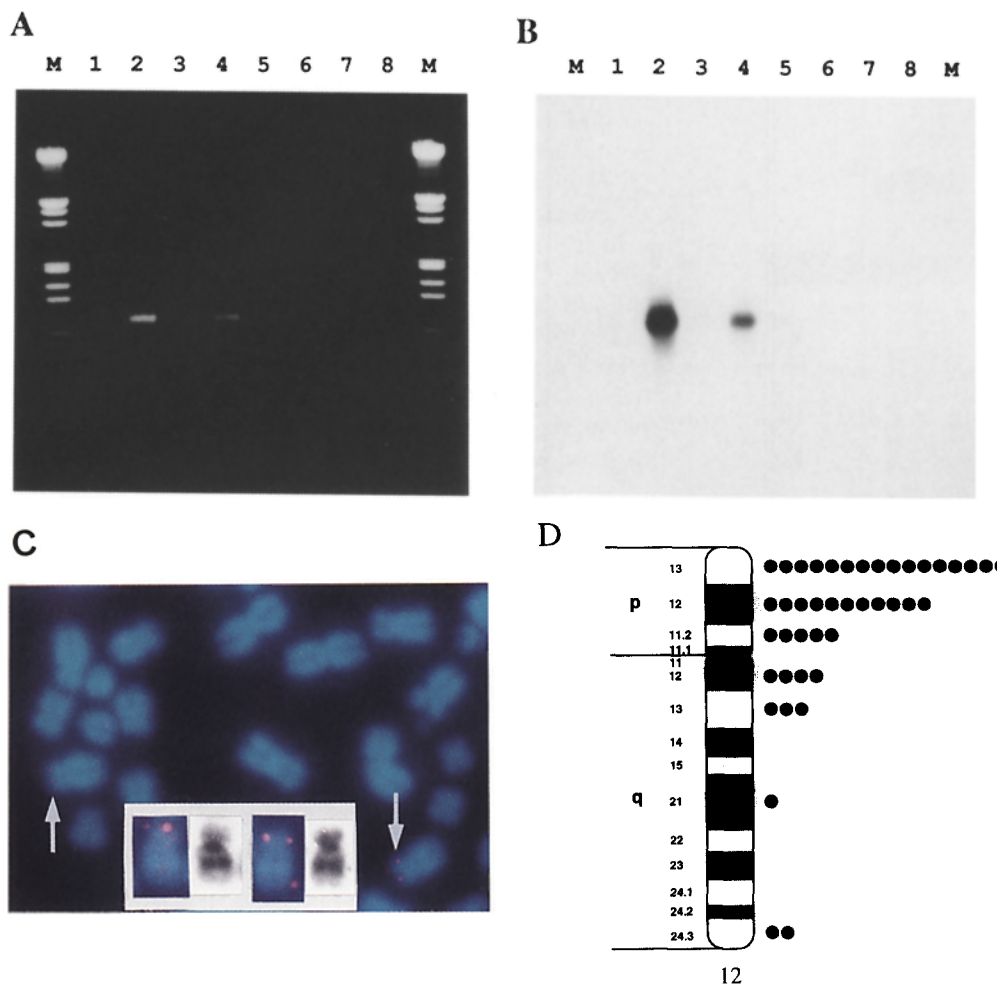


Figure 6. Chromosomal localization of the CD69 gene. (A) PCR analysis of DNA from human, hamster, and a set of human-hamster hybrids from the panel of BIOS-MAPTTM. PCR reactions were performed using AIM 1 and 3 primers. (Lane M) molecular weight markers; (lane 1) hamster; (lane 2) human; (lanes 3-8) hybrids containing the following human chromosomes: (lane 3) chromosome 3; (lane 4) chromosomes 3, 5, 12, 14, 20 (40%), and 22 (25%), Y; (lane 5) chromosomes 5, 11, 12 (45%), 14, 19, 21, and 22; (lane 6) chromosomes, 1 (60%), 5, 13, 14, 18, and 19; (lane 7) chromosomes 1, D5, 13, 19, 21, and 22; (lane 8) chromosomes 5 and 20. (B) Southern blot analysis of PCR products amplified in A. The corresponding genomic fragment of CD69 was used as a specific random primer probe labeled with α -[³²P]dCTP. A faint specific band can be detected after longer autoradiography exposure in lane 5. (C) Fluorescence in situ hybridization on human metaphases with a digoxigenin-labeled 15-kb genomic DNA probe for the human CD69 gene. Arrows indicate the specific site of hybridization of the CD69 probe detected by rhodamine-labeled antidigoxigenin antibodies on DAPI counterstained chromosomes. Fluorescent spots are located at 12 p13-p12. (Inset) Examples of DAPI and GTG-banding chromosome 12 from other cells. (D) Assignment of the CD69 gene to 12 p13-p12. The idiogram of chromosome 12 schematically indicates the distribution of fluorescent spots for the CD69 locus.

NKR-P1 (36), and mouse NKR-P1 (37, 49), which show preferential expression on LAK and NK cells. Other type II lectin cell surface receptors with structural similarities to CD69 are the mouse Ly 49 (42, 43) expressed by activated T cells, the CD23 low affinity Fc ϵ receptor, present on activated B cells and monocytes (38-40, 50), and the hepatic asialoglycoprotein receptors implicated in the clearance of plasma glycoproteins (41). Interestingly, the intracellular domain of CD69 maintains a certain degree of sequence homology only with members of the lectin superfamily expressed by leukocytes, but not with lectin receptors found in hepatocytes.

Our studies by PCR analysis on somatic cell hybrid DNA demonstrate that the human CD69 gene is located on chromosome 12, in accordance with previous serological analysis of human-mouse somatic cell hybrids (51). In addition, we have regionally mapped the CD69 gene to the distal region of the short arm of chromosome 12, bands p13-p12. Interest-

ingly, the genes coding for Ly 49 and NK1.1 antigens are closely linked within the distal portion of mouse chromosome 6, which is the homologous region with human chromosome 12p (52, 53). The possibility of a gene cluster encoding this family of C-lectin receptors expressed by activated T lymphocytes and NK cells raises important questions in terms of both the regulation of gene expression and their function in leukocyte activation.

The CD69 protein shares structural and functional features with NK-specific receptors encoded by human NKG2 and rat and mouse NKR-P1 (53). All are disulfide-linked dimers with a similar molecular size whose expression is either induced or enhanced during activation of T lymphocytes and NK cells. Although differentially expressed among leukocytes, all these molecules act as triggering structures. The activating effect of antibodies specific for the mouse and human CD69 antigen in stimulating redirected target cell lysis by IL-2-activated NK cells (22, 23) is similar to that described with

antibodies specific for other members of the family such as mouse NK1.1 and rat NKR-P1 antigens (22, 54). Furthermore, CD69 antibodies are capable of inducing intracellular signals, including mobilization of intracellular calcium and T cell proliferation in conjunction with suboptimal doses of phorbol esters (4, 19). Similarly, NKR-P1 also functions as an activating molecule on rat NK cells stimulating phosphoinositide turnover and rise of intracellular calcium (55). CD69 also displays common functional properties with the type II lectin Fc ϵ receptor CD23. The CD23 receptor, like CD69, is involved in lymphocyte proliferation and can be upregulated by cytokines (56). Moreover, a proteolytic soluble fragment of CD23 containing the lectin domain with mitogenic activity for B lymphocytes has been described (56). CD23 acts as a receptor for the Fc portion of IgE and, additionally, its interaction with the B cell antigen CD21 has recently been reported (57). The region involved in the interaction with IgE has been demonstrated to correspond almost exactly to the domain homologous with animal lectins (58). The integrity of the invariant cysteines is absolutely required for IgE binding (58).

The expression of a C-type lectin domain by the members of this superfamily of transmembrane receptors suggests their possible interaction with carbohydrates in a calcium-dependent manner. Evidence for such a role has been described for the CD23 member of the family (59). In addition, studies supporting the possibility that carbohydrate structures are in-

involved in recognition events mediated by NK cells have been reported (60). However, the identification of such putative carbohydrate ligands is required to elucidate the functional role of the lectin domain of CD69 and the other members of this family of signal transmembrane receptors.

It is important to remark that although CD69 is absent on circulating PBL, it is expressed at remarkably high levels in the majority of T cells in the inflammatory infiltrates of several human diseases. Thus, CD69 expression has been detected in T lymphocytes of synovial membrane and synovial fluid from rheumatoid arthritis patients (12), as well as on CD8⁺ T cells in infiltrates of virus-induced chronic inflammatory liver diseases (11). In addition, CD69 is also expressed by certain CD4⁺ T cells in the germinal centers of normal lymph nodes and by a small subpopulation of medullary thymocytes (13, 14). Therefore, it could be hypothesized that CD69 functions as an immediate early receptor that would be required for tissue positioning and localization, and/or for migration toward sites of inflammation. Conceivably, the expression of a C-type lectin domain externally exposed on CD69 may enable its interaction with carbohydrate moieties, allowing T cell migration across the endothelial cell layer and different tissue components. The molecular characterization of the CD69 protein and its adscription to this superfamily of C-lectin signaling molecules provide new insights regarding the possible functional role of these cell surface transmembrane receptors.

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