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Differentially Induced Expression of C-Type Lectins in Activated Lymphocytes

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Abstract The human NK gene complex encodes for the leucocyte C-type lectins, CD69, AICL (activation-induced C-type lectin), LLT1 (lectin-like transcript), CD161/NKR-P1A, CD94, and for NKG-2 molecules. These gene products have been implicated in the regulation of the function of natural killer (NK) cells and other lymphocytes. In this study the expression of C-type lectins during the early activation of PMA-stimulated peripheral blood lymphocytes was examined. To investigate the influence of de novo protein synthesis on activation-dependent expression of C-type lectins, cells were cultured in presence of cycloheximide (CHX) and mRNA levels were analyzed by semi-quantitative reverse transcription-polymerase chain reaction. Upregulated levels of CD69, AICL, and LLT1, but less pronounced changes of CD161/NKR-P1A and CD94 mRNA were found at early time points of cellular activation. CD69 was superinduced by CHX at the nuclear precursor transcript and the mRNA level suggesting that regulation of transcriptional activity and mRNA stability contribute to extent of CD69 mRNA accumulation. CHX treatment resulted also in an overexpression of AICL, LLT1, and CD161/NKR-P1A mRNAs. Conversely, CHX blocked CD94 mRNA expression in PMA-stimulated cells, demonstrating that this process is dependent on new protein synthesis. Expression kinetics in context with susceptibility to CHX indicate that the mechanisms responsible for upregulated CD69, AICL, and LLT1 expression are distinct from those which control CD161/NKR-P1A or CD94 expression. J. Cell. Biochem. Suppl. 36:201–208, 2001.

Key words: CD69; AICL; LLT1; CD161/NKR-P1A; CD94; NK gene complex; superinduction; cycloheximide

The leucocyte type-II integral membrane glycoproteins, CD69, Ly-49, CD161/NKR-P1A, CD94, form together with AICL (activation-induced C-type lectin), LLT1 (lectin-like transcript 1), and NKG-2 gene products a separate group within a superfamily of lectin-like molecules. All these molecules possess a carboxy-terminal Ca^{2+}-dependent (C-type) carbohydrate recognition domain and are encoded by the NK gene complex on human chromosome 12, murine chromosome 6 or rat chromosome 4 [Santis et al., 1994; Brown et al., 1997; Renedo et al., 2000]. CD69 mediates numerous cellular responses such as proliferation, cytokine production of activated lymphocytes and elicits cytolytic activity of natural killer (NK) cells and γδ T lymphocytes [Testi et al., 1994]. Other gene products (CD161/NKR-P1A, Ly-49, CD94/NKG-2) have functions as receptors which initiate or inhibit target cell lysis by NK cells [Yokoyama and Seaman, 1993; López-Botet et al., 1997; Lanier, 1998]. Because of the extensive functional impact of C-type lectins in lymphocytes, regulation of their expression levels is of considerable interest. CD69, a phosphorylated disulfide-linked homodimer of differentially glycosylated 28- and 32-kDa subunits in humans, has characteristics of a lymphocyte surface activation antigen as it becomes immediately upregulated upon cellular activation [Hamann et al., 1993; López-Cabrera et al., 1993]. In contrast to its inducible expression on peripheral blood lymphocytes

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(PBL), CD69 is constitutively expressed on a small subset of medullar thymocytes, on monocytes, platelets, and epidermal Langerhans cells [Testi et al., 1994]. AICL and LLT1 are structurally closely related to CD69. The AICL transcript was identified in phorbol 12-myristate 13-acetate (PMA)-activated human peripheral blood mononuclear cells and shown to be expressed in various cell types of hematopoietic origin. The AICL mRNA has an open reading frame of 447 bp which encodes for a polypeptide chain of 149 amino acids with a predicted molecular weight of 17.3 kDa [Hamann et al., 1997]. Transcripts encoding for LLT1 were mainly detected in NK, T, and B cells. A LLT1 transcript described previously [Boles et al., 1999] encodes for a polypeptide of 191 amino acids. CD161/NKR-P1A, a disulfide-linked homodimer of 40–44 kDa subunits, is expressed on subpopulations of NK cells and T lymphocytes [Lanier et al., 1994; Poggi et al., 1998]. This molecule is involved in the modulation of the cytolytic activity of NK cells against tumor target cells [Lanier et al., 1994; Poggi et al., 1997a]. CD94 is a 70-kDa protein which covalently assembles with other C-type lectins of the NKG-2 family [Lazetic et al., 1996]. It is expressed in NK cells and subsets of $CD8^+$ T cells and was found to be involved in the inhibition or triggering of the NK cell function [Chang et al., 1995; López-Botet et al., 1997; Carretero et al., 2000].

The rapid upregulation of CD69 in lymphocytes prompted us to examine the expression behaviour of other C-type lectins during the early activation of lymphocytes. Moreover, the influence of ongoing protein synthesis on transcript levels of CD69 and of the evolutionarily related molecules, AICL, LLT1, CD161/NKR-P1A, and CD94 was investigated. The findings reported herein contribute to the further elucidation of regulatory mechanisms which control the expression of C-type lectins during lymphocyte activation.

MATERIALS AND METHODS

Culture and In-Vitro Stimulation of Lymphocytes

Peripheral blood mononuclear cells were separated from blood of healthy donors by standard density gradient centrifugation (density = 1.077). Fractions of PBL (purity 90–95%) were obtained by counter-flow elutriation. To induce expression of activation-dependent C-type lectins, PBL were stimulated using 20 ng/ml PMA (Calbiochem, Bad Soden, FRG) without or in presence of 50 μM cycloheximide (Sigma, Deisenhofen, FRG).

Immunofluorescence Analysis of CD69 Cell Surface Expression

The CD69 mAb BL-Ac/p26 (DiaMak, Leipzig, FRG) was used within this study. Expression studies were performed by staining aliquotes of 2×10^5 cells with BL-Ac/p26 followed by incubation with FITC-conjugated goat anti-mouse IgG (Sigma, Deisenhofen, FRG). Samples were analyzed on a flow cytometer (FACScan, Becton Dickinson, San José, CA) using the LYSYS version 1.1 software. Gates were set to discriminate between different cell populations and to exclude non-viable cells, and histograms were recorded to determine percentage and mean fluorescence intensity of labeled cells defined by scatter gates.

RNA Isolation and RT-PCR

Total RNA was prepared from whole cells using a commercially purchased RNA isolation kit (QIAGEN, Hilden, FRG). In several experiments, nuclei were prepared by lysis of PBL in RPMI medium/0.5% NP-40 for 10 min with subsequent centrifugation at 500*g*. The nuclei were washed and solubilized using 0.2% SDS, extracted with buffer-equilibrated (pH 4.7) phenol/chloroform followed by precipitation using ethanol. All RNA samples were dissolved in H_2O and subjected to a digestion with 1 U DNase I (Life Technologies, Eggenstein, FRG). No DNA was detected in the samples as examined by a glyceraldehyde-3-phosphate dehydrogenase (G3PDH)-specific polymerase chain reaction (PCR). Complemetary DNA (cDNA) was synthesized from 1 μg total RNA in a 20-μl reaction using 200 U of Superscript II reverse transcriptase (Life Technologies), 500 μM each of nucleotides, 5 mM DTT, and 0.5 μg of oligo$(dT)_{15}$ (InViTek, Berlin, FRG). In RNA samples obtained from isolated nuclei, oligo$(dT)_{15}$ was replaced by 1 μg of random primers (Roche Diagnostics, FRG). PCR was performed using a 20-μl volume with 0.5 U of InViTAQ DNA polymerase (InViTek), 1 μl of single-stranded cDNA, 100 μM dNTPs, 125 nM each of primers in 50 mM Tris-HCl, pH 8.8, 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.01% Triton X-100.

PCR products were separated by electrophoresis on a 1.8% agarose gel.

Quantitation of PCR Products

The levels of C-type lectin transcripts were analyzed by reverse transcription followed by PCR (RT-PCR). A cDNA of CD69 mRNA was generated using the primers 5′-TCTTCATGCTCTGAGGACTG-3′ (PR69F03) and 5′-CTATGGAAGACTTCGGACCA-3′ (PR69R03). The primer pairs 5′-ACGGTATGATGACCAAACAT-3′/ 5′-CTTTCGGTGTAACATCTAGC-3′ were used for AICL cDNA amplification, 5′-TTCCTATCCTGGGAGCAGGA-3′/ 5′-TAGTTGGGGCTTTGCTGTAA-3′ for LLT1 amplification, 5′-TATTGAGCCAGCATTTACTC-3′/ 5′-AGCTGTTGCTTACAGATAT-3′ for CD94 cDNA amplification, and 5′-CATGGACCAACAAGCAATAT-3′/ 5′-TACACAGATGTCTGTGAGAT-3′ for CD 161/NKR-P1A cDNA amplification, respectively. A cDNA derived from CD69 primary transcripts was generated using the primer 5′-TGGTTCTCTGGGATGACATT-3′ (PR69R4I), that binds to CD69 intron 3 [Santis et al., 1994], and primer PR69F03. All polymerase chain reactions were performed under conditions which permit linear accumulation of PCR products and using cDNA samples adjusted to equal glyceraldehyde-3-phosphate dehydrogenase (G3PDH) inputs. To estimate G3PDH levels, G3PDH cDNA was amplified in presence of a competitor and the primer pair, 5′-GCAGGGGGGAGCCAAAAGGG-3′/ 5′-TGCCAGCCCCAGCGTCAAAG-3′. Amplified region from the competitor (851 bp) was 285 bp longer than the amplicons derived from G3PDH cDNA samples. PCR cycle number determined to remain within the limit of the exponential relationship between PCR cycle number and amount of PCR product was chosen after amplification of cDNA derived from samples with the highest CD69, AICL, LLT1, CD161/NKR-P1A, or CD94 cDNA concentrations. Controls without cDNA were included in all experiments. Ethidiumbromide-stained agarose gels were scanned using a gel documentation system 1000 (BioRad, Hercules, CA). In order to facilitate comparison of the results obtained from different experiments, mRNA levels were expressed in relative units. For each gene product, one relative unit was arbitrarily related to the level of amplified cDNA from the cells which were stimulated for 2 h with PMA (a standard deviation cannot be given for this level).

RESULTS

Superinduction by Cycloheximide of CD69 Transcripts in Human Lymphocytes

The C-type lectin CD69 is known to be rapidly upregulated during the early activation of lymphocytes. Ongoing protein synthesis is a prerequisite for the CD69 cell surface expression [López-Cabrera et al., 1993]. Since the process of translation may play an important role in regulating mRNA levels [Atwater et al., 1990], we studied its influence on the expression of leucocyte C-type lectins in cultures of PMA-activated PBL. Therefore, mRNA and primary transcript levels of CD69 were examined in presence of the translational inhibitor, cycloheximide (CHX). The results of semi-quantitative RT-PCR analysis revealed that CD69 mRNA transiently increases within 20 h after the onset of PMA stimulation. CD69 transcripts were detectable after 45 min, exhibited maximal expression values at 2–4 h and declined thereafter rapidly (Fig. 1A). The experiments demonstrated also that the time course of CD69 mRNA expression is reflected at the cell surface by a retarded but persisting CD69 protein expression (Fig. 1B). When CD69 mRNA levels were analyzed in cells, which were cultured in presence of CHX, increasing intracellular concentration of activation-induced CD69 transcripts was detected (Fig. 1A). This superinduction was observed at 2 h and also at 6 h (Fig. 2A). Likewise, superinduction was detectable at later time points, e.g., 20 h, suggesting delayed CD69 mRNA degradation. Further experiments were carried out to determine whether transcriptional activation could account for the superinduction of CD69 mRNA. Compared to cells, which were stimulated with PMA alone, lymphocytes displayed elevated levels of nuclear CD69 precursor transcripts when CHX was additionally present (Fig. 2B). The transcripts were, in contrast to the corresponding mRNA, also markedly present in non-stimulated lymphocytes ($t = 0$) suggesting that resting lymphocytes possess low basal transcriptional activity of the *CD69* gene. Incubation with CHX only resulted in apparently unchanged CD69 precursor transcript levels at 2 h and reduced levels at 6 h. Thus it can be excluded that CHX activates pre-existing transcription factors. The results suggest rather that the PMA-mediated increase of transcriptional activity of the *CD69* gene might be

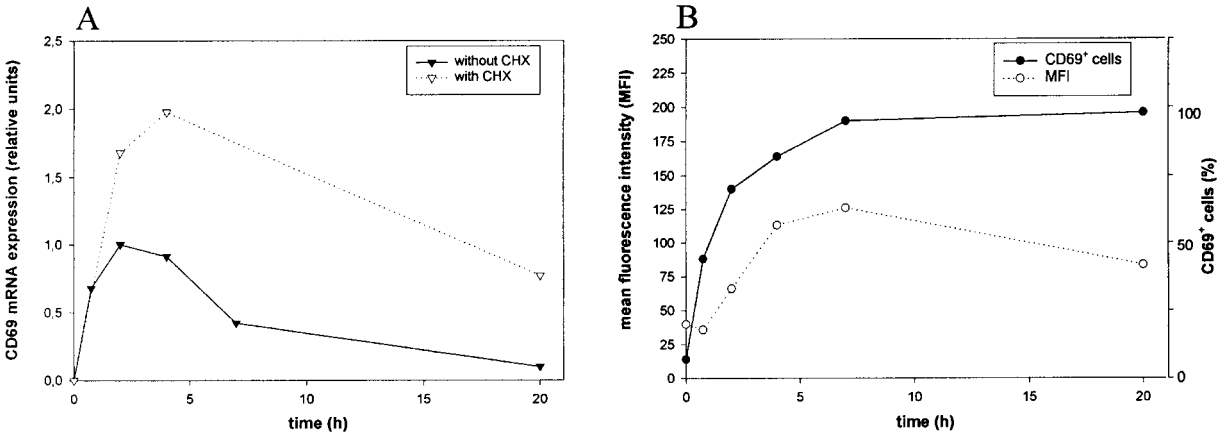

Fig. 1. Time course of early CD69 expression and superinduction of CD69 mRNA in activated lymphocytes. **A:** Kinetics of CD69 mRNA expression. Relative levels of CD69 mRNA are expressed as arbitrary relative units which were obtained by densitometric analysis of bands after RT-PCR. RNA was isolated at the indicated time points during PMA stimulation of PBL (cultured in presence or without CHX) and reverse-transcribed into cDNA. **B:** Cell surface expression, which was detected using immunofluorescence and flow cytometry analysis, is indicated as percentage of $CD69^+$ cells and mean fluorescence intensity (MFI) of cells. One experiment out of four with similar results is shown.

suppressed by newly synthesized protein factor(s).

mRNA Expression of AICL and LLT1 in Comparison to CD161/NKR-P1A and CD94 During the Early Activation of Human Lymphocytes

Further experiments revealed that the mRNAs of the C-type lectins, AICL and LLT1, are clearly upregulated during PMA stimulation of lymphocytes, at levels which exceeded at 2 h and 6 h those of non-stimulated cells. In contrast, less pronounced changes in transcript levels were found for CD161/NKR-P1A and CD94 (Fig. 3). This was in accordance with the cell surface expression of CD161/NKR-P1A and CD94 that varied only slightly (data not shown). AICL and LLT1 transcripts were relatively barely detectable in non-stimulated cells. As CD69 mRNA, AICL transcripts declined within the investigated temporal frame. In presence of CHX, the AICL mRNA was slightly elevated at 2 h whereas CHX did not affect significantly the increase of LLT1 mRNA at this time point. At 6 h, AICL and LLT1 mRNAs were superinduced suggesting that CHX attenuates mRNA decay (Fig. 3A). Although AICL and LLT1 have probably different expression kinetics these experiments demonstrated both C-type lectins as activation-dependent molecules of lymphocytes with a expression behavior similar to CD69.

In contrast to CD69, AICL, and LLT1 mRNAs, the CD161/NKR-P1A and CD94 mRNAs were readily detectable in non-stimulated PBL. A slight increase of CD161/NKR-P1A mRNA was observed 2 h after stimulation in cells from two donors; cells from another donor did not point to elevated mRNA levels (Fig. 3B, left panel). However, a hyperexpression in presence of CHX could be detected after 2 h suggesting that CD161/NKR-P1A mRNA levels are under control of protein synthesis during the early activation of lymphocytes. CD94 transcripts at 2 h exceeded slightly initial levels of non-stimulated cells and declined by 6 h. In contrast to the other C-type lectins investigated, CD94 mRNA levels were not amplified by CHX treatment in PMA-stimulated cells. Thus, CHX treatment blocked CD94 mRNA accumulation at 2 and 6 h (Fig. 3B, right panel) indicating that de novo protein synthesis contributes to the regulation of CD94 mRNA levels in PMA-stimulated lymphocytes.

DISCUSSION

CD69, AICL, LLT1, CD161/NKR-P1A, and CD94 are evolutionarily related members of the C-type lectin gene family. These surface receptors are encoded by the NK gene complex [Yokoyama and Seaman, 1993; Weis et al., 1998; Renedo et al., 2000]. Their carbohydrate recognition domains possess amino acid identity of 36% (CD69 with AICL), 41% (CD69 with LLT1), 25% (CD69 with CD161/NKR-P1A), and 26% (CD69 with CD94). In this report we demonstrated that the CD69, AICL, and LLT1 mRNAs are upregulated during the early

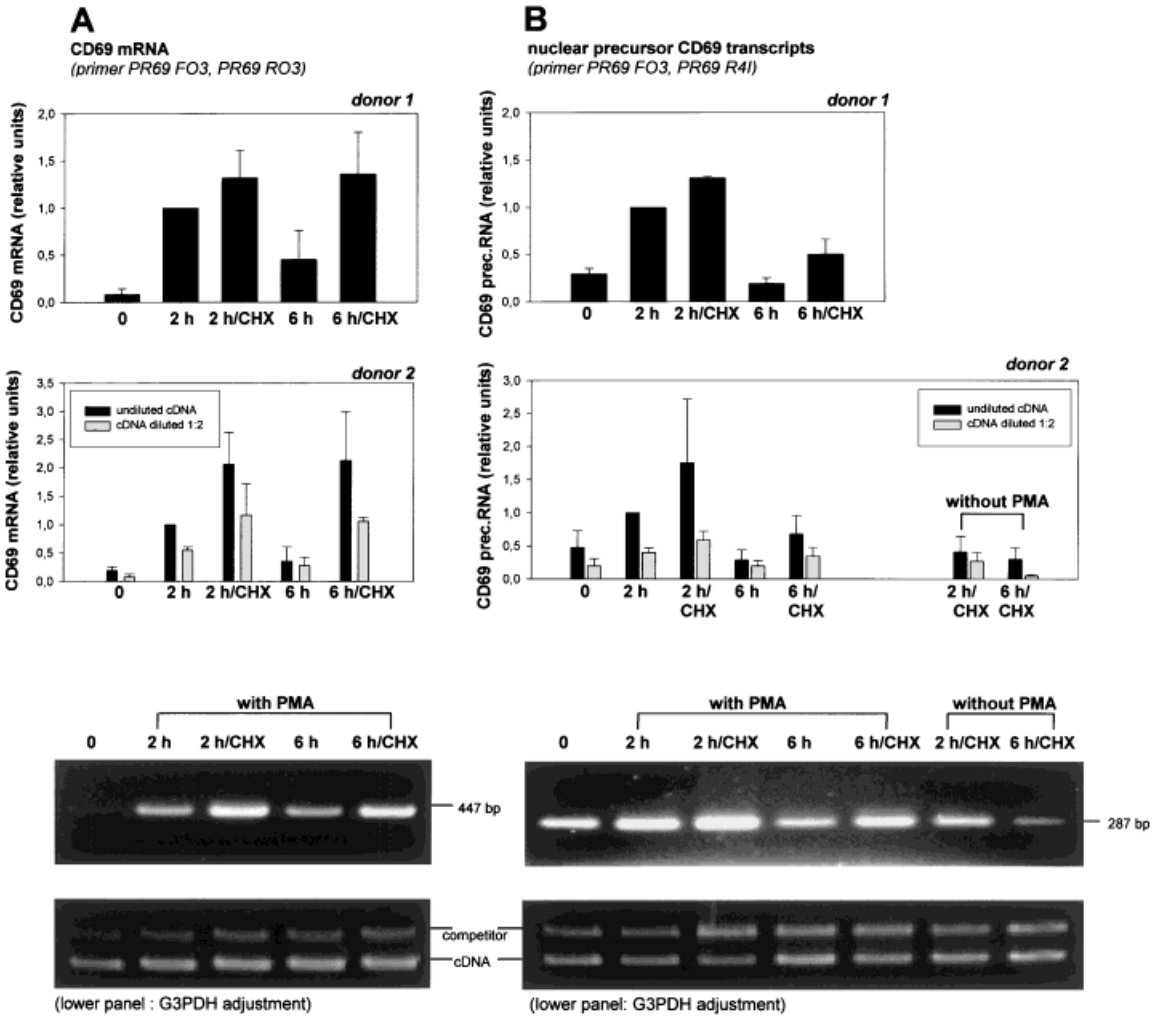

Fig. 2. **A:** Superinduction by CHX (50 μM) of CD69 mRNA in PMA-stimulated PBL. **B:** Superinduction of CD69 expression by CHX (50 μM) on the primary transcript level. Note that primer PR69R4I binds to CD69 intron 3 whereas PR69F03 and PR69R03 bind to exon 3 and the 3′ untranslated region, respectively. In addition to amplified cDNA samples from PMA-stimulated PBL, CD69 primary transcripts derived from cells after incubation with CHX but without PMA are shown. Adjustment of sample cDNA amounts to G3PDH housekeeping gene expression and analysis of two-fold diluted cDNA derived from one donor are also demonstrated. RNA was analyzed using RT-PCR and cDNA samples were amplified using the indicated primer combinations. Expression levels are indicated in relative units which were calculated as described in Materials and Methods. The average of three or more amplification reactions (the error bars represent standard deviation), performed on a single cDNA sample are presented. The results from two different donors are indicated, analysis of a third donor revealed comparable results (data not shown). The gels correspond to cDNA samples from donor 2.

activation of peripheral blood lymphocytes. Moreover, we showed that the levels of these transcripts are hyperinducible after translational inhibition whereas CD94 mRNA accumulation is blocked. Kinetics of C-type lectin expression in context with their susceptibility to CHX pointed to high similarity of mechanisms responsible for CD69, AICL, and LLT1 induction which may be distinct from those which control CD161/NKR-P1A or CD94 expression.

The mRNAs of CD69, AICL, and LLT1 were barely detectable in non-stimulated lymphocytes and increase early for a short time after stimulation. Upregulation of CD69 expression in PMA-stimulated lymphocytes proved to be dependent on the activation of the protein kinase C [Bjorndahl et al., 1988; Cebrián et al., 1989]. Increase of CD69 mRNA precedes transient expression of the CD69 protein, a scenario which is also likely for the AICL and LLT1 proteins. While structural and functional aspects of the activation-dependent CD69 expression in B and T lymphocytes have been extensively investigated [Cebrián et al., 1989;

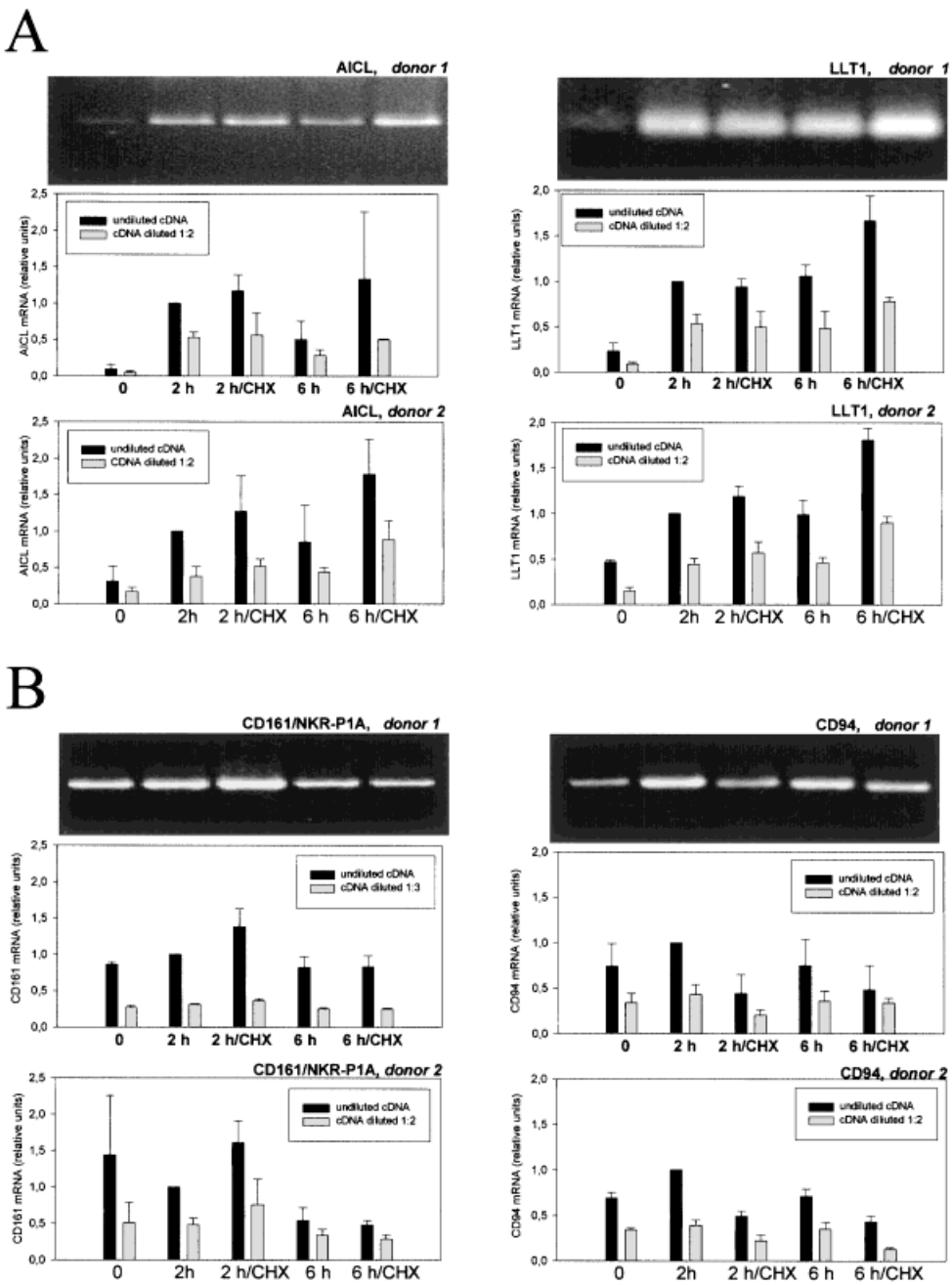

Fig. 3. **A:** Upregulation and superinduction of AICL mRNA (**left panel**) and LLT1 mRNA (**right panel**). **B:** Transiently hyperinducible mRNA expression of CD161/NKR-P1A mRNA (**left panel**) and inhibition of CD94 mRNA expression by CHX (**right panel**). PBL were stimulated with PMA and RNA was analyzed by RT-PCR. Analysis of diluted cDNA samples was additionally performed. Relative expression levels are presented as indicated in Figure 2. The results from the same donors as in Figure 2 are indicated, analysis of a third donor revealed similar results (data not shown). Gels show amplification of cDNA derived from donor 1.

Risso et al., 1989; Hamann et al., 1993; López-Cabrera et al., 1993], characteristics of the AICL and LLT1 proteins have not yet been elucidated. However, the early upregulation of both C-type lectins suggest a role of AICL and LLT1 as activation-dependent molecules which may resemble that of CD69 as signal-transmitting receptor.

The CD161/NKR-P1A protein can interact with appropriate carbohydrate ligands [Bezouška et al., 1994]. This molecule may deliver inhibitory signals in NK cells [Poggi et al.,

1998] and has also been shown to be involved in transendothelial migration of $CD4^+$ cells [Poggi et al., 1997b]. CD94/NKG-2 binds to HLA-E and operates as inhibitory receptor (CD94/NKG-2A) or triggers the cytolytic activity (CD94/NKG-2C) of NK cells [Carretero et al., 2000]. As opposed to CD69, AICL, and LLT1, transcripts encoding for CD161/NKR-P1A and CD94 are apparently more strongly expressed in non-stimulated PBL and are not greatly upregulated after PMA stimulation. Moreover, CHX-mediated decrease of mRNA levels in stimulated lymphocytes suggest that CD94 mRNA expression is dependent on the production of proteins which are induced, for example, as a response to protein kinase C activation.

The present study provides evidence that ongoing protein synthesis has influence on CD69 transcript levels in PBL. Both CD69 nuclear precursor transcript and cytoplasmic mRNA levels were found to increase following translational inhibition by CHX. These results indicate that CHX interferes with transcriptional activation of the *CD69* gene and post-transcriptional events which both influence the CD69 mRNA level. Transcriptional regulation of the *CD69* gene was previously demonstrated in experiments which indicated phorbol ester- and tumor necrosis factor (TNF)-α-inducible transcription [López-Cabrera et al., 1995]. Our findings point towards a CHX-sensitive process which may involve repressive protein factor(s) which additionally act(s) on transcription of the *CD69* gene. Posttranscriptional regulation is consistent with the previous demonstration that rapid degradation of the unstable cytoplasmic CD69 transcripts prevent their accumulation [Santis et al., 1995]. Thus, CD69 is similar to many unstable growth factor-inducible mRNAs, e.g., a variety of proto-oncogenes, transcription factors, and cytokine mRNAs, which contain pentameric AUUUA sequences within their 3′ untranslated regions. One important point is that these mRNAs are stabilized in the presence of inhibitors of protein synthesis. The resulting superinduction might be caused by suppression of the synthesis of a labile factor, which is responsible for mRNA degradation. Accordingly, regulation of mRNA stability, which is a crucial point in the regulation of CD69 gene expression [Santis et al., 1995], could be related to binding of cytoplasmic proteins to AU-rich elements at the 3′ untranslated region.

The mechanisms underlying amplification of AICL, LLT1, and CD161/NKR-P1A transcripts and suppressed CD94 mRNA expression in CHX presence remain to be defined. Whereas AUUUA sequences are present in the 3′ untranslated region of the LLT1 mRNA they are lacking in the AICL mRNA, and no information is available for CD161/NKR-P1A. Thus, it is uncertain whether expression of AICL and CD161/NKR-P1A is also a result of regulated mRNA stability.

Presumably, short-lived mRNAs and transient expression of CD69, AICL, and LLT1 are a prerequsite for their function within a close temporal frame of cellular activation. It is also conceivable that sensitivity to CHX of CD69, AICL, and LLT1 transcript levels reflects their accumulation as activation-induced molecules under stress conditions. Such situation, for example, viral infection or exposure to toxins and heat shock, may provoke down-regulated protein synthesis [de Haro et al., 1996]. Whether this is a mechanism which modulates C-type lectin expression remains to be resolved.

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