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Regulation of expression of the human lymphocyte activation gene-3 (*LAG-3*) molecule, a ligand for MHC class II

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Abstract The lymphocyte activation gene-3 (*LAG-3*), a major histocompatibility complex (MHC) class II ligand evolutionarily related to CD4, is expressed exclusively in activated T and NK lymphocytes and seems to play a role in regulating the evolving immune response. We first determined that surface LAG-3 expression on activated human T cells is upregulated by certain cytokines (IL-2, IL-7, IL-12) and not by others (IL-4, IL-6, IL-10, TNF-α, TNF-β, IFN-γ). Surface LAG-3 expression correlated with intracellular IFN-γ production in both CD4+ and CD8+ T-cell subsets. We then analyzed the 5' transcription control sequences of *LAG-3*. A DNase I hypersensitive site induced in T cells following cellular activation was found in the region including the transcriptional start site, showing that DNA accessibility is a mechanism which restricts LAG-3 expression to activated T cells. Transcription is initiated at three sites. A GC box, 80 base pairs (bp) upstream of the major transcription start site, forms a minimal promoter which is regulated by two upstream regions containing positive and negative regulatory elements with multiple protein binding sites as shown by footprinting analysis. In particular, a GATA/c-Ets motive was identified in a short segment homologous to the mouse *CD4* distal enhancer, suggesting that *LAG-3*, which is embedded in the *CD4* locus, may be controlled by some *CD4* regulatory elements. Finally, a 100 bp region downstream of the transcription start site was shown to be involved in the cell-specific control of LAG-3 expression. Understanding this highly regulated expression may help to determine the intriguing role of this activation-induced MHC class II ligand.

Key words T-cell activation · MHC class II · Promoter analysis · Cytokine

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Introduction

Following antigen exposure, T cells are activated and proliferate. Some activation antigens may function to actively down-regulate the evolving immune response, taking over the control of T-cell reactivity. Molecular interactions upregulated in activated lymphocytes and which have been proposed to play an important role in dampening the ongoing immune response include, for example, Fas/FasL (Brunner et al. 1995), CTLA-4/B7 (Walunas et al. 1994), 4-1BB/4-1BBL (Alderson et al. 1994), and LAG-3/MHC class II (Huard et al. 1994b, 1996).

T-cell interactions may control, in part, T-cell-mediated immune responses. In this regard, LAG-3-mediated cross-linking of T-cell MHC class II molecules is capable of negatively regulating activated T cells (Huard et al. 1996). In binding assays, soluble rLAG-3 molecules have a greater avidity for MHC class II (over two orders of magnitude) than CD4, an evolutionally related "first cousin" (Huard et al. 1995). This greater avidity may produce, in some circumstances, preferential binding of MHC class II molecules by LAG-3 over CD4, as observed, for instance, with homologous CTLA-4 and CD28 molecules and B7 counter-receptors (Linsley et al. 1991, 1994). Interestingly, both CD4 and LAG-3 are likely to oligomerize at the cell surface to interact more efficiently with MHC class II molecules (Huard et al. 1997; Sakihama et al. 1995; Wu et al. 1997). Following cell activation, the LAG-3 molecule is expressed on both CD4+ and CD8+ cell subsets, as well as on NK cells (Baixeras et al. 1992; Huard et al. 1994a). In CD4+ T cells, this expression is strongly associated with IFN-γ production (Annunziato et al. 1996), a potent MHC class II inducer. The expression of these two activation antigens (i.e., LAG-3 and MHC class II) could be coordinated in order to regulate the ongoing T-cell-mediated immune response in tissues. Taken together, these observations suggest that LAG-3 plays an important role in controlling T-cell responses as a physiological regulatory molecule. This assumption does not exclude a role for LAG-3 in NK-cell-mediated responses (Miyazaki et al. 1996).

LAG-3 expression is tightly regulated in vitro (activated T and NK cells) as well as in vivo (secondary lymphoid organs) (Baixeras et al. 1992; Huard et al. 1994a). In addition, *LAG-3* is nested 5' from the *CD4* locus between two enhancer regions shown to regulate CD4 expression in mice and the presence in this region of some boundary elements delineating a locus control region may not be excluded (Bruniquel et al. 1997). It is reasonable to assume that understanding the mechanisms controlling LAG-3 expression will help in understanding the mechanisms controlling certain T-cell responses which are influenced by MHC class II molecules. As for *CD4*, *LAG-3* has a TATA-less promoter (Triebel et al. 1990), and in the present study, we identified important regulatory regions that control inducible and T-cell-specific LAG-3 expression.

Materials and methods

Cell lines

Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood of healthy volunteer donors by Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) density gradient centrifugation. HeLa cells (human cervix epitheloid carcinoma) were grown at 37 °C and 10% CO_2 in DMEM supplemented with 10% fetal calf serum (FCS). Jurkat cells, K562 cells, and PBMC were grown in RPMI 1640 medium supplemented with 10% FCS, 4 mmol/l L-glutamine, 1 mmol/l pyruvate, 0.2 mmol/l NaOH, 50 000 IU/l penicillin and 50 mg/l streptomycin (Gibco BRL, Paisley, Scotland). For OKT3-activated PBMC, FCS was replaced by human AB serum. PHA-blasts were obtained by stimulating fresh PBMC with 2 µg/ml phytohemagglutinin (PHA), 100 IU/ml IL-2 (Roussel-Uclaf, Romainville, France).

Cytokine-induced LAG-3 upregulation

T lymphocytes from PBMC were first stimulated (day 0) by plating 5×10^5 cells/ml on 24-well flat-bottom plates in culture medium. Plates were previously coated overnight at 4 °C in borate buffer (pH = 8.4, 0.1 M) with 3 µg/ml protein-A Sepharose purified OKT3 [CD3-specific monoclonal antibody (mAb)]. Human recombinant cytokines tested for modulation of LAG-3 expression were: IL-2 (Roussel Uclaf), IL-4, IL-6, IL-10, IL-12, TNF-α, TNF-β obtained from R&D Systems (Abington, UK), IL-7 (Sanofi, Labège, France), and IFN-γ (Roussel Uclaf and R&D Systems). IL-2 (10 and 100 IU/ml), IL-4 (2 and 20 ng/ml), IL-6 (15 and 75 ng/ml), IL-7 (1.5 and 15 ng/ml), IL-10 (4 and 40 ng/ml), IL-12 (2.4 and 24 ng/ml), TNF-α (0.5 and 5 ng/ml), TNF-β (1.2 and 12 ng/ml), and IFN-γ (10 and 50 ng/ml) were added to culture at day 0 and day 5. In another set of experiments, T cells were stimulated by incubating 5×10^5 PBMC/ml with mouse fibroblasts (1.25×10^5 cells/ml) transfected with both human CD32 and CD80. (Demeure et al. 1995). Fibroblasts were first irradiated at 10 Gy and incubated with 10 ng/ml OKT3 for 30 min at 37 °C. For secondary response, cytokines were not added to culture during the first stimulation. Cytokine-induced LAG-3 modulation was also assessed on NK cells purified by negative selection and maintained in culture with 100 IU/ml IL-2.

The mAb used in immunostaining experiments were: 17B4-fluorescein isothiacyanate (FITC), LAG-3.1-specific mAb (Huard et al. 1994a) labeled in our laboratory, CD3-FITC (CD3) and NKH1-FITC (CD56) from Coulter (Hialeah, FC), Leu2a-PerCP (CD8), Anti-hu-IFN-γ-PE (IFN-γ), and γ1-FITC, γ1-PE, γ2a-PerCP used as negative controls were purchased from Becton Dickinson Immunocytometry Systems (San Jose, Calif.). LAG-3 expression was assessed by direct immunofluorescence. For intracytoplasmic staining, cells were activated 2 days after restimulation by phorbol myristyl acetate (PMA) (20 ng/ml) and ionomycin (0.8 µg/ml) for 4 h. Medium was supplemented with brefeldin A (10 µg/ml), a potent inhibitor of intracellular transport, for the last 3 h. Membrane staining of LAG-3 and CD8 was performed. Cells were washed twice and 0.5×10^6 cells were permeabilized with 500 l of 1/10 diluted FACS permeabilizing solution (Becton Dickinson, Mountain View, Calif.) for 10 min. Finally, intracytoplasmic staining was performed and cells were fixed in phosphate buffer saline (PBS) 1×1% formaldehyde. The mean fluorescence of 3×10^3 viable cells was measured by flow cytometry with an Elite cytometer (Coulter).

Oligonucleotides

The sequence of the synthetic DNA oligonucleotides used in this work was as follows: Den1: 5′-GAGAACCAGGAGAGGGCCTTGGAGA-3′, position –158 to –134 relative to the ATG translation start site, Den2: 5′-ATCTCTCCTATGGTCTGGGCAGCGC-3′, position –25 to –1 relative to the ATG translation start site and Den3: 5′-AGCTGGG-CAGATCAGGCAGCCTGA-3′, position –231 to –207 relative to the ATG translation start site on the non-coding strand.

DNase-I hypersensitive sites

DNase-I hypersensitivity assays were performed as previously described (Leroy-Viard et al. 1994). Briefly, 1×10^9 cells were lysed in buffer containing 0.05% NP40 for Jurkat cells, PBMC and PHA-blasts, 0.2% for K562 cells, and 0.6% for HeLa cell line. Nuclei were isolated by sucrose gradient centrifugation and subjected to DNase-I digestion (2–15 µg/ml) for 10 min at 37 °C. The DNA was then extracted, restricted with *Bam* HI, and analyzed by Southern hybridization. The ^{32}P-labeled genomic probes used were a 1 kilobase (kb) *Acc* I-*Bam* HI fragment, a 0.6 kb *Ava* I-*Bam* HI fragment, and a 0.8 kb *Bam* HI-*Eco* RI fragment.

S1 nuclease protection assay

Total RNA extraction was prepared by the guanidine isothiocyanate method (Chomczinsky and Sacchi 1987) and quantified by absorbance measurement at 260 nm. Ethidium bromide staining of ribosomal RNA after electrophoresis on agarose gel confirmed the integrity of total RNA. The hybridization probe was made using a single-stranded primer-extended genomic clone of the promoter region, with primers Den1, Den2, or Den3. The probe was uniformly labeled with [α-^{32}P] dCTP (3000 Ci/mmol, DuPont NEN Research Products, Boston, Mass.) restricted by *Bst* XI, purified on 6% denaturing polyacrylamide gel, and hybridized with 20 µg total RNA in 80% formamide at 55 °C for 16 h. The DNA-RNA hybrid was diluted into 250 µl of S1 Nuclease buffer (280 mM NaCl, 30 mM sodium acetate, pH 4.4, 4.5 mM zinc acetate with 250 µg/ml of denatured salmon sperm DNA) containing 400 units of nuclease S1 (Boehringer Mannheim, Mannheim, Germany), and incubated at 37 °C for 10 min. The digestion was terminated by adding 75 µl of 2.5 M ammonium acetate, 50 mM ethylenediaminetetraacetate (EDTA). The protected fragments were analyzed by electrophoresis on a 6% denaturing polyacrylamide sequencing gel. The same primers were used for the DNA sequence ladder.

Primer extension

Primer extension analysis was performed essentially as previously described (Townes et al. 1985). Briefly, the oligonucleotides Den1, Den2, or Den3 were radiolabeled with [γ-^{32}P] dATP (3000 Ci/mmol, DuPont NEN Research Products). Twenty micrograms of total RNA was annealed with 5 pmol of labeled antisense primer in buffer containing 400 mM NaCl, 10 mM Pipes (pH 6.5), and 1 mM EDTA for 6 h at 55 °C. The annealed RNA was concentrated by ethanol precipitation and re-suspended in a reaction mixture containing 10 mmol/l each of dGTP, dTTP, dATP and dCTP, 50 mM Tris-HCl

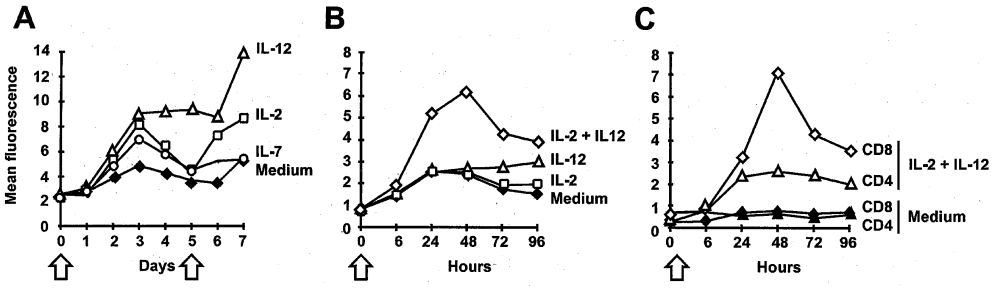

Fig. 1A–C Kinetics of LAG-3 expression by activated T cells: **A** PBMC of normal donors were stimulated with OKT3 (3 μg/ml) coated on plastic or in **B, C** a secondary response by OKT3 (10 ng/ml) added to CD32+ CD80+ fibroblasts. The mean fluorescence using LAG-3.1-specific (17B4) mAb was assessed at different time points of the **A** primary or **B, C** secondary response on **A, B** T cells or on the **C** CD4+ versus CD8+ subsets. IL-2 (10 IU/ml), IL-7 (15 μg/ml) or IL-12 (2.4 ng/ml) were added at **A** day 0 and 5 or **B** at the start of the secondary stimulation (day 10). **B, C** The combination of IL-2 (10 IU/ml) + IL-12 (1 ng/ml) was also tested

pH 7.5, 75 mM KCl, 3 mM $MgCl_2$, 20 mM DTT, 2 units/μl RNasin, and 1 μg/ml actinomycin *D*-mannitol. Primer-extended reverse transcription was performed using 100 units Moloney mouse leukemia virus reverse transcriptase (Boehringer Mannheim) at 37 °C for 40 min. The product was electrophoresed on a 6% denaturing polyacrylamide gel. Sequencing reactions were performed with each primer using the GD3 plasmid as a template (Triebel et al. 1990) and were run in parallel.

RNase protection assays

A 2.3 kb fragment of *LAG-3* (–2456 to –128 bp upstream of the ATG) was subcloned into a pBSSK+ vector and used as a template for in vitro transcription. The anti-sense RNA probe used to analyze the 5' end of *LAG-3* consisted of a 723 bp (*Sph* I to *Xba* I) fragment. This fragment was prepared from the linearized plasmid with a T7 polymerase RNA transcription kit (Stratagene, La Jolla, Calif.) and [α-^{32}P] dUTP (3000 Ci/mmol, DuPont NEN Research Products). The probe was hybridized with total RNA at 55 °C for 16 h and then digested by a combination of RNase T1 (20 units/ml) and RNase A (25 units/ml) (Boehringer Mannheim) for 20 min at 37 °C. The protected fragments were then electrophoresed on a 6% denaturing polyacrylamide gel.

Luciferase assay

Molecular cloning was performed using standard protocol (Sambrook et al. 1989). The promoterless pGL3-Basic vector (Promega, Madison, Wis.) containing the firefly luciferase gene was used to create all reporter gene constructs. Recombinant plasmids were analyzed by restriction enzyme digestion and partial DNA sequencing. All of the constructs were derived from a 2432 bp *Bam* HI-*Eco* 47-III DNA fragment containing the immediate upstream region of *LAG-3* from positions –2121 to +311 bp relative to the major transcription start site. This fragment was sub-cloned into the pGL3 Basic vector in the 5' to 3' orientation. A series of twenty 5' deletion mutants were prepared by exonuclease III/mung bean nuclease treatment (Stratagene, La Jolla, Calif.). The six constructs presented in the results section were termed Pr1 to Pr6. Removal of 100 additional nucleotides from +311 to +211 was done by digestion with *Xba* I, and ligation to the multiple cloning site (at the *Hin* dIII site) after filling the free ends.

Plasmids used for transfection were grown in *Escherechia coli* strain XL1-Blue, purified by Plasmid midi kit (Qiagen GmbH, Germany). Cells were re-suspended at 5×10^7 cells/ml in RPMI 1640 medium. Ten micrograms of uncut plasmid DNA was added to 300 μl of cell suspension in a 0.4 cm gap electroporation cuvette and incubated at room temperature for 5 min. Electroporation was performed in double pulse with the Celljet Electroporation System (Eurogentec S.A., Belgium) at 800 V, 74 Ω and 40 μF (Jurkat and K562 cells) or 1200 V, 132 Ω, and 40 μF (PHA-blasts) for the first pulse, and 150 V, 74 Ω, and 1500 μF for the second pulse. Electroporated cells were transferred to 90 mm Petri dishes containing 12 ml of culture medium.

The luciferase assay was performed using Promega's luciferase assay system (Promega) according to the manufacturer's instructions. Luminescence was measured for 30 s at room temperature in a luminometer (Top Count, Packard Instrument Co.). Each experiment was conducted at least 3 times. Data presented in the results section are representative of at least three separate experiments. In addition, all results reported were confirmed using several different plasmid preparations.

Cell transfection efficiencies were normalized by taking into account the quantity of plasmid DNA present in the transfected cells. To quantify the DNA, cells extracts were probed using an [γ^{32}P] dATP-labeled oligonucleotide termed pGL2 (Promega). The resulting autoradiogram was scanned and densitometry analysis was performed using Bioprofil software (Vilber Lourmat, France).

In vitro DNase footprinting analysis

Nuclear extracts were prepared from PHA-blasts according to the procedures of Dignam and co-workers (1983). DNase I footprinting was performed using Core Footprinting System (Promega) according to the manufacturer's instructions.

Three *LAG-3* promoter fragments (–1858/–1111, –1111/+309, and –158/+309) were purified and labeled with [γ-^{32}P] dATP (3000 Ci/mmol). The phosphorylated probes were then digested with various restriction enzymes to generate the footprint probe labeled at the 5′ or 3′ end. Binding assays were performed using 100 000 cpm of labeled DNA end-labeled fragments incubated with 0, 5, or 10 μg of PHA-blasts nuclear extract for 30 min. DNase I (0.02 units) was added for 1 min in all assays. The generated fragments were analyzed by electrophoresis on a 6% denaturing polyacrylamide gel.

Results

LAG-3 expression is upregulated by IL-2, IL-7, and IL-12 in T cells, and by IL-10 and IL-12 in NK cells

PBMC were stimulated with optimal (3 μg/ml) or suboptimal (0.3 μg/ml) concentrations of OKT3 coated on plastic plates or with OKT3 (10 ng/ml) added on CD32+ CD80+ fibroblasts. Under the latter conditions, the addition of a low concentration of OKT3 was sufficient to induce a strong proliferative response (>50000 cpm for 10^5 PBMC) due to the TCR cross-linking effect via OKT3 binding to CD32 receptors and co-stimulation via CD28, the receptor for CD80 (B7.1) molecules. In both culture conditions, a series of 9 cytokines, including IL-2, IL-4,

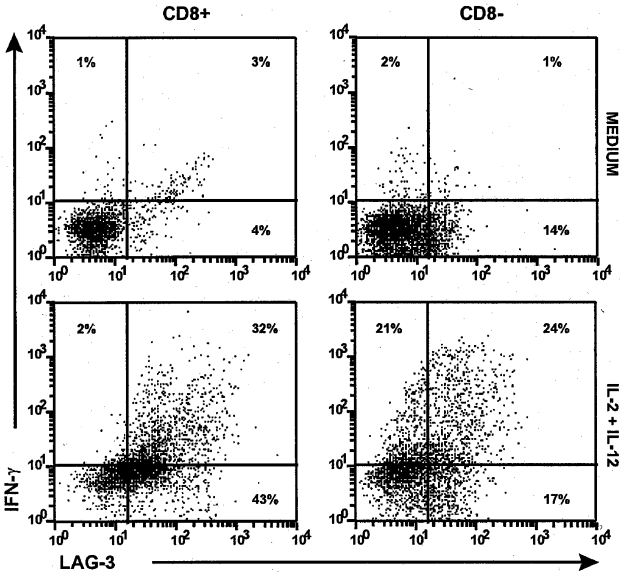

Fig. 2 LAG-3 expression by CD4+ (CD8-) and CD8+ cells and correlation with intracellular IFN-γ production: PBMC were stimulated with 10 ng/ml OKT3 added to CD32+ CD80+ fibroblasts. At day 10, activated T cells were restimulated with or without IL-2 + IL-12. Forty-eight hours later, cells were stimulated for 4 h with PMA + ionomycin in the presence of brefeldin A, and permeabilized for the detection of intracellular IFN-γ production. The CD8- subset corresponded to CD4+ cells (CD4+ could not be used due to its down-modulation by PMA)

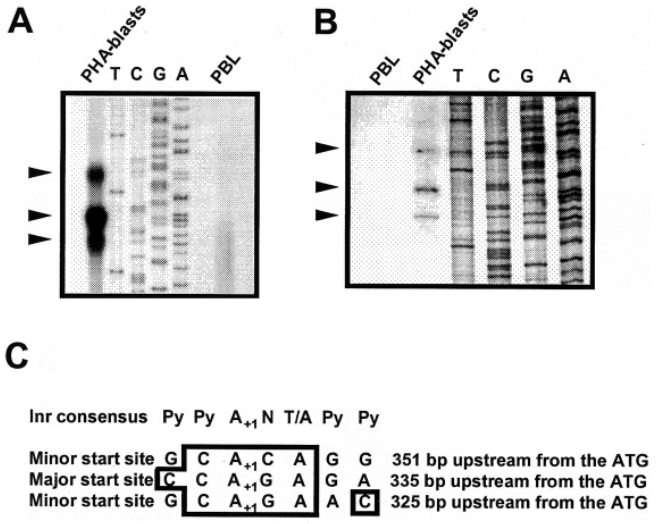

Fig. 3A–C Transcription initiation sites of *LAG-3*: **A** S1 nuclease protection mapping analysis. Twenty micrograms of total RNA from PHA-blasts or PBMC was hybridized with a uniformly ^{32}P-labeled *Bst* XI-*Den* 1 fragment. After S1 nuclease digestion, products were analyzed in a 6% denaturing polyacrylamide gel. *Arrows* indicate the position of the S1 products. **B** Primer extension analysis. Twenty micrograms of total RNA from PHA-blasts or PBMC was hybridized with a 5′ end ^{32}P-labeled *Den* 1 oligonucleotide primer. After incubation with reverse transcriptase, primer-extended products were analyzed in a 6% denaturing polyacrylamide sequencing gel. *Arrows* indicate the position of the extended products. **C** Comparison of three different putative *LAG-3* Inr motives with a consensus Inr sequence. The *boxed areas* underline similarity to the Inr consensus sequence

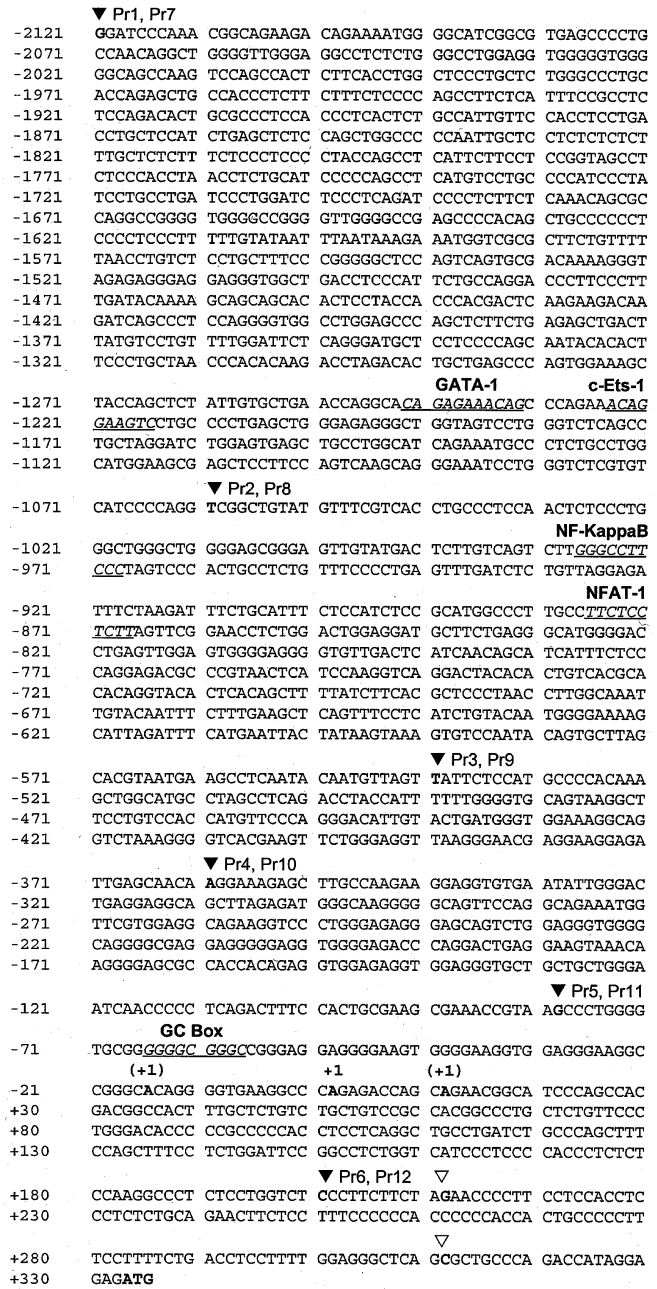

Fig. 4 The *LAG-3* 5' region. The major transcription start site is marked as +1 and the minor start sites as (+1). The consensus GC box, the GATA, c-Ets, NF-KappaB, and NFAT DNA binding motives are underlined. *Closed triangles* represent the 5' end of the various reporter constructs and *open triangles* the 3' ends of the DNA deletion in mutants

IL-6, IL-7, IL-10, IL-12, TNF-α, TNF-β, and IFN-γ, were tested at a concentration recommended by the supplier and also at a 5× or 10× concentration, for their capacity to modulate surface LAG-3 expression. None of these cytokines were able to down-modulate LAG-3 expression as determined in immunofluorescence assay. Only three (IL-2, IL-7, IL-12) were able to upregulate LAG-3 expression in all culture conditions tested (Fig. 1A, for 3 μg /ml of OKT3

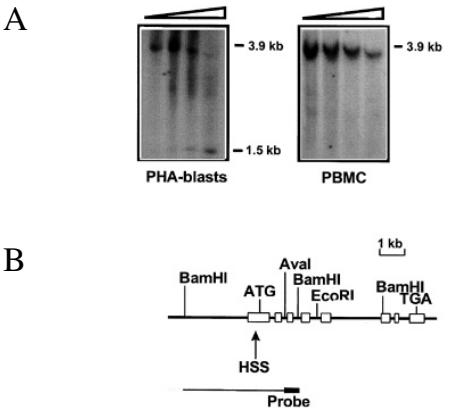

Fig. 5A, B Mapping of a Dnase-I HSS close to the *LAG-3* transcription initiation site. **A** Isolated nuclei from PHA-blasts or PBMC were treated with increasing amount of DNase I for 10 min at 37 °C. DNAs were purified from nuclei and digested with *Bam* HI. Fifty micrograms were electrophoresed on 0.7% agarose gel and blotted onto nitrocellulose filters. Blots were hybridized with a *Bam* HI/*Ava* I probe. **B** Shown is the location of the probe used to detect the HSS in LAG-3+ PHA-blasts

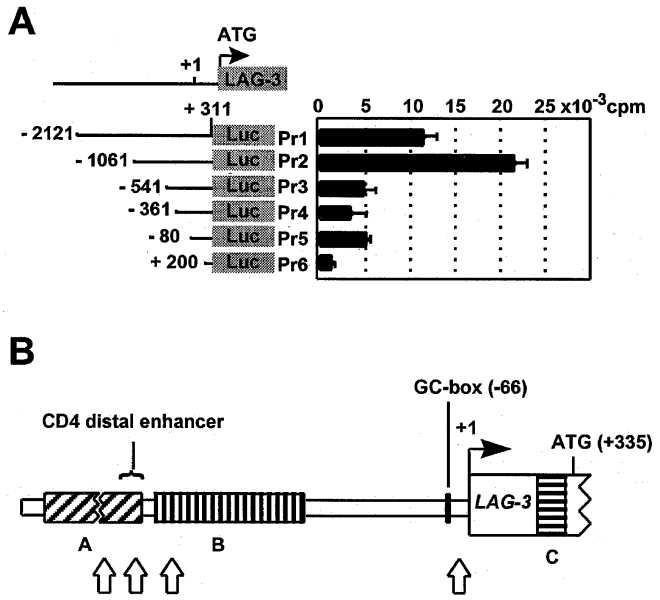

Fig. 6A–B Luciferase activity of *LAG-3* constructs in transiently transfected PHA-blasts (LAG-3+). **A** Day 2 PHA-blasts were transiently transfected with 5' deleted *LAG-3* constructs Pr1 to Pr6 (see Fig. 4 for description of the constructs). For each construct, results show the absolute cpm count obtained in one of six representative experiments. Average cpm values obtained with PHA-blasts were 10^6 cpm for a CMV construct and 200 cpm for a promoterless construct (pGL3 basic) used as transfection controls. **B** Diagrammatic representation of the elements in the *LAG-3* promoter shown to be involved in the regulation of gene expression. Positions are indicated relative to the major transcription start site (+1). Putative binding of the Sp1 transcription factor is shown (GC box). Enhancer element termed **B** and silencer elements **A** and **C** are indicated as *boxes*. The positions of DNase hypersensitive sites are indicated by *arrows*

coated on plastic). The most potent stimulus for LAG-3 expression was IL-12, which stably induced higher LAG-3 expression. The re-induction of LAG-3 expression following addition at day 5 of these three cytokines was also observed (Fig. 1A). Dose response experiments with these three cytokines indicated that the maximal effect was observed with 10 IU/ml IL-2, 10 ng/ml IL-7, and 1 ng/ml IL-12 (data not shown).

We also tested the capacity of these cytokines to upregulate LAG-3 expression following re-engagement of the TCR in a secondary response. Following a first stimulation with 10 ng/ml OKT3 added on CD32+ CD80+ fibroblasts, cells were collected at day 10 and re-stimulated in a similar assay. Due to the strong stimulatory response, effect of individual cytokines was not observed in the first 48 h of the culture (Fig. 1B). However, the IL-2 + IL-12 combination strongly increased LAG-3 expression. The combination of IL-7 + IL-12 did not increase LAG-3 expression compared with IL-12 alone (data not shown).

Paradoxically, LAG-3, a ligand for MHC class II, is expressed at higher levels on activated MHC class I-restricted CD8+ than on MHC class II-restricted CD4+ cells (Huard et al. 1994a). We compared the kinetics of LAG-3 induction by the most potent combination (IL-2 + IL-12) on CD4+- vs CD8+-activated cells (Fig. 1C). In the same secondary response shown in Fig. 1B, we observed that induction of LAG-3 expression was present on both CD4+ and CD8+ subsets with a greater increase on CD8+ cells following addition of IL-2 + IL-12 (Fig. 1C).

The same nine cytokines were tested on IL-2-dependent NK cell lines. IL-10 (maximal effect: 40 ng/ml) and mostly IL-12 (maximal effect: 1 ng/ml) were shown to upregulate LAG-3 expression (data not shown). IL-2 did not appear to upregulate LAG-3 expression but interpretation of this negative result is difficult, since these NK cells required high-dose IL-2 (100 IU/ml) to be maintained in culture.

Preferential LAG-3 expression by CD4+ and CD8+ cells producing IFN-γ

LAG-3 protein is expressed by most Th1 and Th0 clones, whereas most Th2 clones show either no or weak LAG-3 expression (Annunziato et al. 1996). In CD4+ T cells, expression of LAG-3 is associated with IFN-γ production (Annunziato et al. 1996). To confirm these results in our assay system where PBMC are stimulated by engagement of the TCR- and CD80-mediated signaling, and to putatively extend these results to the CD8+ compartment, we analyzed the induction of surface LAG-3 and intracellular IFN-γ in both subsets following a 4 h stimulation with PMA + ionomycin in the presence of brefeldin A (Fig. 2). The CD8- compartment corresponded to CD4+ cells (CD4 could not be used, since its expression is modulated by PMA). Strong induction of surface LAG-3 and intracellular IFN-γ was observed by stimulation with IL-2 + IL-12 in both subsets. Similar results were observed with either IL-2 or IL-12 used alone (data not shown).

The transcription initiation sites of LAG-3

Nuclease S1 protection, primer extension, and RNase protection assays were used to unequivocally identify the

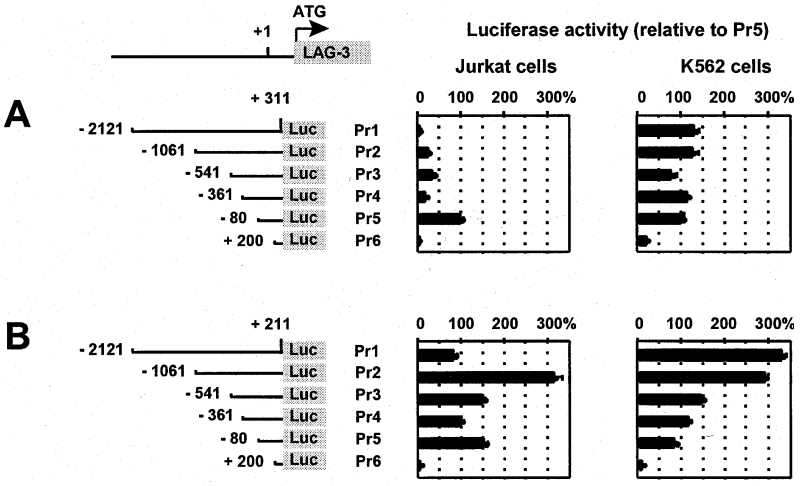

Fig. 7A, B Analysis of *LAG-3* promoter deletion constructs in transiently transfected Jurkat and K562 cells: A series of 5' to 3' deletions of *LAG-3* promoter were fused to a luciferase reporter gene **A** at position +311 and **B** at position +211 and transiently transfected into Jurkat or K562 cells by electroporation. Results are average values of luciferase activity (triplicates) normalized for transfection efficiency and expressed relatively to Pr5 activity. These results are representative of at least three separate experiments. Average absolute activities for the CMV promoter construct and for the promoterless construct (pGL3 basic) were ~ 250 times more and ~ 20 times less important than the Pr5 activity, respectively. Average cpm values obtained with the Pr5 construct in Jurkat and K562 cell lines were ~ 2×10^4 cpm and ~ 2×10^5 cpm, respectively

transcription initiation site(s). The 352 bp *Bst* XI-*Den* 1 fragment (–486 to –134) was uniformly labeled, hybridized to total RNA from either PHA-blasts, PBMC, Jurkat cells or K562 cells, and digested with S1 nuclease. Results revealed the presence of several transcription start sites in PHA-blasts. One major band at 335 and two minor bands at 351 and 325 bases upstream of the translation start site were identified with RNA from PHA-blasts (Fig. 3A). No transcription initiation site was identified with RNA from PBMC (Fig. 3A), Jurkat cells, or K562 cells (data not shown). Similar results were obtained with two additional *Bst* XI oligonucleotide fragments (data not shown) as described in Materials and methods. Hybridization for S1 mapping required a relatively high temperature (55 °C in 80% formamide), suggesting the existence of a significant secondary structure in this region, possibly related to the high G+C content of the region around transcription initiation sites.

To confirm the putative transcription initiation sites identified by S1 nuclease mapping, we used primer extension analysis. Extended products of 217, 201, and 191 nucleotides were identified with the Den1 primer (Fig. 3B). Three extended products of 350, 334, and 324 nucleotides and 144, 128, and 118 nucleotides were identified with Den2 and Den3, respectively (data not shown). These three fragments were consistent with the corresponding putative start sites at positions –351, –335, and –325 upstream of the ATG (see above). On the basis of the band intensity, we estimated position –335 to be the predominant start site for transcription. The degree of compliance of these sites with the Inr consensus sequence (Javahery et al. 1994), as shown in Fig. 3C, may be responsible, at least in part, for the predominance of position –335, which may, for example, have an increased affinity to an Inr binding protein compared with positions –351 and –325.

Finally, RNase protection assays were performed on RNA samples from PHA-blasts and PBMC to check whether any additional start sites could be detected upstream of the three described above. The synthetic RNA probe used in these assays extended 723 nucleotides from position –851 to –128 bp upstream of the ATG translation start codon. Three protected RNA probe fragments were found (223, 207, and 197 nucleotides) with RNA from PHA-blasts, corresponding to the three transcription initiation sites identified by S1 nuclease and primer extension analyses (data not shown). No protected fragment was detected with RNA derived from PBMC.

The *LAG-3* 5′ flanking sequence does not contain any sequence motive resembling a TATA box in the several hundred nucleotides upstream of the exon 1 coding sequence. Accordingly, like many genes lacking a TATA box, *LAG-3* has multiple transcription start sites.

Identification of regulatory regions upstream of the transcription start site

A genomic DNA fragment containing 311 bp of the first exon plus 2121 bp of the upstream region (accession number Y10211) was cloned into the pGL3-Basic vector. The corresponding DNA sequence is shown in Fig. 4.

To identify regions with regulatory activity, we constructed a series of expression plasmids containing various portions of this 2.4 kb fragment linked to the firefly luciferase gene in the appropriate 5' to 3' orientation. These constructs start between positions –2121 and +200 and end at position +311 relative to the major transcription start site (+1). They were tested in transient transfection assays using either T or non-T cells.

Pr1 to Pr6 were transfected in LAG-3$^+$-activated T cells (day 2 PHA-blasts), as described in Materials and methods (Fig. 6A). Transient expression in PHA-blasts was observed

with Pr5 that contained 80 bp 5' from position +1. Thus, the minimal *LAG-3* promoter region encompasses 80 bp. Indeed, a typical GC box (a putative Sp1-binding motive) (Kadonaga et al. 1987) is present at positions –66 to –58 (see Fig. 4). Pr3 to Pr5 exhibited low activity, barely above the background level. No significant variations were detected between these three clones in numerous experiments. The maximal luciferase activity was obtained with Pr2, showing the presence of a positive regulatory element (termed B, see Fig. 6B) in the region –1061/–541. Pr1 led to less activity than Pr2, suggesting the presence of a negative regulatory element (termed A) in the region –2121/–1061. Removal of the Inr sequences resulted in the complete loss of basal transcriptional activity (Pr6). In non-activated PBMC, these constructs led to no activity, comparable to the background level (300 cpm compared with 200 000 cpm for the CMV-positive control, data not shown).

We have previously examined the *LAG-3* locus for DNase I hypersensitive sites (HSS) in nuclei isolated from LAG-3$^+$ (PHA-blasts) cells and found seven HSS (Bruniquel et al. 1997). Two of them were found in regulatory region A, one in B, and one close to the transcription initiation site (Fig. 6B). These four HSS were not present in LAG-3$^-$ (PBMC, Jurkat, K562, and Hela) cells (Fig. 5 and data not shown). The presence of DNase I HSS in LAG-3$^+$ and not in LAG-3$^-$ cells suggests that the corresponding regions contribute to the specific regulation of LAG-3 expression.

We mapped the protein binding sites in the regions encompassing these four HSS by in vitro footprinting assays. The top strand of a *Bst* XI/*Eco* 47 III fragment encompassing the minimal promoter was protected from DNase cleavage at the Sp1 binding site (GC box) when PHA-blasts proteins were added (data not shown). The top strand of a *Sac* I/*Eco* 47 III fragment, encompassing the B-positive regulatory region, was analyzed. Several PHA-blast proteins bound to this fragment at the DNase I HSS (data not shown). Particularly, non-consensual NF-KappaB (–978/–968) and NFAT (–877/–866) motives were protected by DNA binding proteins (see Fig. 4). The top strand of a *Pvu* II/*Sac* I fragment showed protection by four PHA-blast proteins in the A negative regulatory region encompassing another HSS (–1600/–1350) (data not shown).

We have previously reported that *LAG-3* is embedded within the *CD4* locus between two *CD4*-enhancer-like regions, associated with DNase-I HSS, and shown to control CD4 expression in mice (Bruniquel et al. 1997). The bottom strand of a *Sac* I/*Sac* I fragment which contains a sequence homologous (80%) to the mouse *CD4* distal enhancer known to include a functional GATA/c-Ets site (Wurster et al. 1994) and encompasses an HSS was mapped in footprinting experiments. The pattern obtained (data not shown) is similar to that obtained with the mouse homologue (Bruniquel et al. 1997; Wurster et al. 1994). Thus, PHA-blast proteins also bind to the GATA/c-Ets site in humans.

A 100 bp region that inhibits LAG-3 expression in LAG-3$^-$ cells was found downstream of the transcription start site

The same series of six constructs joined at position +311 to the luciferase gene was transfected in Jurkat and K562 cell lines (Fig. 7A). The effects of the regulatory regions identified in PHA-blasts were not observed. To test whether an additional element downstream of the transcriptional start site may negatively regulate LAG-3 expression in LAG-3$^-$ cells, we deleted 100 bp upstream of the position +311. In this new series of constructs joined at position +211, the pattern of luciferase expression was quite similar to that obtained with PHA-blasts (Fig. 7B). In particular, the B region (–1061/–541) acts as a positive regulatory element by increasing the luciferase activity threefold. The putative negative regulatory element A (–2121 to –1061) was also found in Jurkat cells (difference between Pr1 and Pr2 in Fig. 7B). This element does not seem to be operational in a non-T-cell environment, as assessed with the transfections in K562. Altogether, the differences of pattern between the two series of constructs (joins at +311 vs +211) tested in Jurkat and K562 cell lines suggest that a regulatory element, termed C, is present in the region +211/+311, downstream of the transcription start site (see Fig. 6B). This C element was not found to be active in PBMC or PHA-blasts (data not shown).

Discussion

In the immunoglobulin superfamily (IgSF), the *CD4* and *LAG-3* genes have been grouped together, based on structural similarities and close proximity on human 12p13 (Triebel et al. 1990). Structural similarities have also been found across mouse/human species boundaries (Huard et al. 1997). The binding profile to MHC class II of these four extracellular IgSF domain molecules is broadly overlapping, although they can be functionally (Huard et al. 1994b) and structurally (Huard et al. 1997) distinguished from each other. This relatedness implies several primordial two-IgSF-domain gene duplication events, followed by the evolution and divergence of both structure and function. Indeed, we have proposed that both *LAG-3* and *CD4* have evolved by gene duplication from a pre-existing two-IgSF-domain structure (Huard et al. 1994a). As these two molecules diverge, it could be expected that promoter/regulatory elements responsible for genomic regulation of the MHC class II ligands will reflect this evolutionary divergence from a common ancestor, and that unique genetic features have become associated with distinct tissue-specific or activation-related expression and functional roles of these MHC ligands. Although it is difficult at this stage to pin-point the central physiological role of *LAG-3*, an analysis of its transcriptional regulation may provide some clues. Like *CD4* (Salmon et al. 1993), *LAG-3* has a TATA-less promoter and multiple transcription start sites. In contrast, regulatory regions are organized differ-

ently even if some regulatory elements such as the *CD4* enhancer region described here may be used as a locus boundary element regulating the coordinated expression of both genes in some cells.

In the present study, we isolated 2.1 kb from 5' flanking DNA of the human *LAG-3* and have delineated *cis*-acting sequences necessary for the expression of this gene. The promoter region contains several start sites characterized by an Inr-like sequence Py-Py-A_{+1}-N-T/A-Py-Py (Javahery et al. 1994). This feature has been shown for several TATA-less lymphoid-specific promoters (Smale and Baltimore 1989; Travis et al. 1991). The minimal promoter, as assessed by the luciferase assay, consists of 80 bp including a GC box. The latter motive is known to be the potential target of Sp1 (Pugh and Tjian 1990), which at that location has been shown to serve as a transcription initiation factor (Ernst and Smale 1995).

DNase I HSS within chromatin often correspond to sequences which play a role in activating transcription of the gene (e.g., promoter, enhancer, silencer, locus control region sequences) (Wolffe 1992). We found an HSS located around the transcription start sites. This HSS was only observed in LAG-3$^+$ PHA-blasts and not in resting PBMC cells or other LAG-3$^-$ cell lines (HeLa, Jurkat, K562). Therefore, DNA accessibility seems to be related to LAG-3 expression, as has been shown before for other genes (Felsenfeld 1992; Grunstein 1990). The lack of LAG-3 transcript expression in LAG-3$^-$ cells may derive, in part, from an inability to initiate or extend the synthesis of pre-mRNA due to the local chromatin configuration.

With regard to the modulation of LAG-3 expression with cytokines, activated T cells seem to respond specifically to only three of the nine cytokines tested, whereas NK cells respond to only two. LAG-3 is an activation antigen expressed in vivo primarily in lymphocyte-infiltrated tissues (results not published) or inflamed secondary lymphoid organs and not on in vivo-activated CD25$^+$, CD69$^+$ PBMC (e.g., in HIV$^+$ individuals) with the exception of cancer patients receiving high-dose IL-2 (Baixeras et al. 1992). This tissue-oriented expression in humans may be related to a need for some inflammatory cytokines such as IL-12 which could be met only at the inflamed site or in the afferent lymph node where the antigen is being processed by professional antigen presenting cells.

Our results show that IL-2 facilitates the upregulation of LAG-3 expression by IL-12 described for CD4$^+$ cells (Annunziato et al. 1996). LAG-3 may be associated with the IL-12-promoted development of both Th1- and Tc1-type cells, since our results indicate a correlation between LAG-3 and IFN-γ expression in both CD4$^+$ and CD8$^+$ subsets, as recently shown by Annunziato and co-workers (1997). It is known that IL-12 induces T and NK cells to produce IFN-γ while, on the other hand, IFN-γ upregulates the release of IL-12 by phagocyte cells. Therefore, these interactions appear to be involved in a positive feedback mechanism that results in the activation of phagocyte cells and the upregulation of immune functions pivotal for the protection against, for instance, infectious pathogens. In our hands, IFN-γ (from two sources) was unable to induce LAG-3 expression, suggesting that LAG-3 upregulation observed with IL-12 in the two experimental systems tested was associated with IFN-γ induction but independent of IFN-γ addition. On the other hand, the addition of a neutralizing anti-IFN-γ antibody to IL-12-primed CD4$^+$ T-cell cultures inhibits the development of LAG-3-expressing CD4$^+$ T cells, indicating that IFN-γ may nonetheless promote LAG-3 expression (Annunziato et al. 1997). It is therefore proposed that IL-12 induces T-cell Th1 and Tc1 differentiation associated with both LAG-3 expression and IFN-γ secretion, the latter being a known inducer of LAG-3 ligand (i.e., MHC class II molecules) expression and may be of LAG-3 itself.

In this paper, we show that the expression of *LAG-3* is regulated by complex interactions that involve numerous *cis*-acting regions that exert positive and negative effects on minimal promoter activity. We found that a 2.4 kb fragment could be subdivided into several regions as depicted by expression of a reporter gene in transient transfection assays in LAG-3$^+$ (PHA-blasts) or LAG-3$^-$ (Jurkat, K562) environments. In particular, a positive 5' regulatory element B was identified in PHA-blasts in the region –1061/–541. In LAG-3$^-$ cells, the 100 bp C region located in the first exon of *LAG-3* was found to hamper the activity of the regulatory elements upstream of the transcriptional start site. The inhibitory effect of the C region was not found in LAG-3$^+$ cells (i. e., PHA-blasts), suggesting that it plays a specific role in controlling the restricted expression of LAG-3. Multiple proteins including GATA, NF-KappaB and NFAT may bind the regions (A and B) regulating LAG-3 expression in PHA-blasts. In particular, we identified a GATA/c-Ets motive located in a region homologous to the mouse distal *CD4* enhancer (Bruniquel et al. 1997) and shown to actually bind proteins in mice (Wurster et al. 1994). We suggest that these two genes, *LAG-3* and *CD4*, may share some common regulatory elements. These could contribute to the discrepancy observed in CD8$^+$ vs CD4$^+$ cells in terms of LAG-3 expression intensity and upregulation by IL-12.

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