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# New insights on the transcriptional regulation of CD69 gene through a potent enhancer located in the conserved non-coding sequence 2

Teresa Laguna[a], Laura Notario[a], Raffaella Pippa[b], Miguel G. Fontela[a], Berta N. Vázquez[a,1], Miren Maicas[b,2], Noemí Aguilera-Montilla[c], Ángel L. Corbí[c], María D. Odero[b], Pilar Lauzurica[a,*]

[a] Activación Immunológica Group, Carlos III National Health Institute (ISCIII), Majadahonda, Spain
[b] Laboratory of Genetics, Division of Oncology, CIMA, University of Navarra, Pamplona, Spain
[c] Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain

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ABSTRACT

The CD69 type II C-type lectin is one of the earliest indicators of leukocyte activation acting in lymphocyte migration and cytokine secretion. CD69 expression in hematopoietic lineage undergoes rapid changes depending on the cell-lineage, the activation state or the localization of the cell where it is expressed, suggesting a complex and tightly controlled regulation. Here we provide new insights on the transcriptional regulation of CD69 gene in mammal species. Through *in silico* studies, we analyzed several regulatory features of the 4 upstream conserved non-coding sequences (CNS 1–4) previously described, confirming a major function of CNS2 in the transcriptional regulation of CD69. In addition, multiple transcription binding sites are identified in the CNS2 region by DNA cross-species conservation analysis. By functional approaches we defined a core region of 226 bp located within CNS2 as the main enhancer element of CD69 transcription in the hematopoietic cells analyzed. By chromatin immunoprecipitation, binding of RUNX1 to the core-CNS2 was shown in a T cell line. In addition, we found an activating but not essential role of RUNX1 in CD69 gene transcription by site-directed mutagenesis and RNA silencing, probably through the interaction with this potent enhancer specifically in the hematopoietic lineage. In summary, in this study we contribute with new evidences to the landscape of the transcriptional regulation of the CD69 gene.



## 1. Introduction

CD69 is an inducible receptor expressed in leukocytes. It is rapidly upregulated on the membrane of lymphocytes upon stimulation, as it is observed in T cells after 1 h of treatment with PMA (Hara et al., 1986), while it reaches its maximum expression in myeloid populations in about 24 h (Yoshimura et al., 2002; Urasaki et al., 2000; Marzio et al., 1997; Ochiai et al., 2000). This time-specific regulation of CD69 expression is suggested to be in part due to distinct transcriptional regulation mechanisms, since several *cis*-acting elements have previously been found in CD69 locus with lineage-specific effects on transcription (Vazquez et al., 2009).

In the human and mouse CD69 promoters, regulatory elements binding NF-κB, AP-1, OCT, CREB and the Early Growth Response (EGR) proteins have been identified and proposed as responsible for inducible expression (Ziegler et al., 1994; López-Cabrera et al., 1995; Castellanos et al., 1997; del Carmen Castellanos et al., 2002). Apart from these, other *cis*-regulatory regions have been identified previously in the CD69 locus (Vazquez et al., 2009, 2012): four upstream conserved non-coding sequences (CNS 1–4) and a non-conserved hypersensitivity site (HS) located within the first intron of the CD69 gene. It has been previously shown that the four CNS are regulatory regions being in open conformation and possessing marks of active transcription on histones in mouse lymphocytes (Vazquez et al., 2009). It was also observed a differential regulation between T and B cells in transgenic mice bearing the hCD2 reporter under the control of the CD69 promoter and different combinations of the CNSs (Vazquez et al., 2009). Although transcriptional studies

* Corresponding author. Tel.: +34 918223718.
*E-mail address:* lauzurica@isciii.es (P. Lauzurica).
[1] Current address: Department of Genetics, Human Genetics Institute of New Jersey, Rutgers University, Piscataway, NJ 08854, USA.
[2] Current address: Biomedicine Institute of Valencia (IBV), Valencia, Spain.

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confirmed CNS2 as a potent transcriptional enhancer, in transgenic mouse lines, the construct formed by CNS2 plus CNS1 plus promoter showed an inhibition of the transgene expression (Vazquez et al., 2009).

Here we further analyzed the role of CNS2 in CD69 gene transcription, defining specific regulatory elements within this region and identifying transcription factors which probably intervene in the enhancer mechanism. For that purposes, we employed both *in silico* and experimental procedures.

We performed data mining of predicted conserved Transcription Factor Binding Sites (TFBS) in CNS2, which permitted the finding of *cis*-acting elements on their basis of conservation during evolution (Wang et al., 2006). This method has been successfully applied to find regulatory elements in other immune inducible genes, such as γ interferon (Hatton et al., 2006). After comparing these results with data from ENCODE consortium, we further analyzed the *cis*- and *trans*-acting elements of CNS2 by experimental means. These approaches allowed us to obtain new insights on the transcriptional regulation of CD69, such as the identification of a minimal enhancer sequence within CNS2 and the role of different transcription factors in this function. The attempt to delineate the function of RUNX1 in CD69 transcription regulation and the discussion of the results founded is presented.

## 2. Materials and methods

### 2.1. Data from ENCODE consortium

Human open chromatin regions, histone H3K27Ac marks and transcription factor binding by Chromatin Immunoprecipitation followed by sequencing (ChIP-seq) in different cell lines were obtained from the *ENCODE Consortium* (Anon, 2012) and displayed on the *University of California-Santa Cruz (UCSC) Genome Browser* (https://genome.ucsc.edu/ENCODE/). Input sequences were employed from *UCSC* (https://genome-euro.ucsc.edu/cgi-bin/hgGateway/) (Figs. 1–3).

### 2.2. Identification of predicted conserved transcription factor binding sites (TFBSs) within CNS2

Sequences of CNS2 for human (*Homo sapiens*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), rhesus (*Macaca mulatta*), dog (*Canis familiaris*) and horse (*Equus caballus*) species were downloaded from the online platform *Vista-Point* from the portal *VISTA tools from comparative genomics* (http://genome.lbl.gov/vista/index.shtml) using as base genome the human genome version March 2006 from the *UCSC Genomic Browser* website. These sequences were introduced into the application *Genomatix DiAlign* on the *Genomatix* website (http://www.genomatix.de/), and the output data were depicted as arrows indicating the binding sites over a plot of sequence conservation in mammals obtained from the *UCSC Genome Browser* (human March 2006: chr12: 9,808,600–9,809,300).

### 2.3. Plasmids

Mouse CD69 promoter (−1 to −609, BAC clone RP24-188C4) was cloned into BglII and HindIII restriction enzyme (RE) cloning sites of the commercial luciferase vector pGL3 basic (Promega). After that, CNS2 region (mouse 2010 chr6: 129,234,359–129,235,318) was cloned into KpnI and XhoI RE sites, introducing an EcoRI site by KpnI for further cloning. Modified CNS2 constructs containing single and double deletions were generated by overlap PCR (Higuchi et al., 1988) employing custom primers (Supplementary Table 1) and cloned into EcoRI and XhoI RE sites in the plasmid containing the CD69 promoter.

### 2.4. Site-directed mutagenesis

The kit *QuikChange Lightning Site-Directed Mutagenesis kit* (Agilent) was employed following manufacturer instructions using primers to perform the mutations shown in Supplementary Table 2. Every PCR product and *DpnI* digestion was checked by agarose gel electrophoresis before transformation in bacteria.

### 2.5. Luciferase assays

Jurkat T cells ($5–7 \times 10^5$), K562, U937 and C1R cells ($2–3 \times 10^5$) were transfected with 1 μg of modified firefly luciferase plasmid (purified with *Plasmid Maxi Kit* from Qiagen) plus 20 ng of pRL-TK (Renilla luciferase plasmid from Promega, to standardize the luciferase activity independently of the efficiency of transfection between samples) using *Superfect* (Qiagen) following manufacturer's protocol. RAJI cells ($5–7 \times 10^5$) were transfected with 2 μg of firefly luciferase plasmid and 20 ng of renilla plasmid per condition employing 6 μl of *X-tremeGENE 9* reagent from Roche. After transfection, cells were cultured at 37 °C with 5% $CO_2$ for 24 h. Next, they were stimulated or not with 10 ng/ml of PMA and 500 ng/ml of Ionomycin, PMA alone or plate-bound anti-CD3 (clone OKT3; eBioscience) and anti-CD28 (clone CD28.2; eBioscience) mouse antibodies (plated at 5 μg/ml) or were mock incubated, for other 24 h. 48 h after transfection, cells were lysed using *Passive Lysis Buffer* (Promega) and luciferase activity (firefly/renilla) was measured with the *Dual Luciferase Kit* from Promega.

### 2.6. Nucleofection

RUNX1 RNA silencing experiments were performed using *Cell Line Nucleofector® Kit V* from Amaxa and siRNAs *siRUNX1-59* (ref: s2459) and *siNeg* were from Ambion. $10^6$ Jurkat cells were used per transfection. Cells were washed 3 times in 1× PBS and resuspended in 100 μl of *Cell Line Nucleofector Solution V*. Then 600 ng of *siRUNX1* or *siNeg* were mixed with the cell suspension in an Amaxa certified cuvette and nucleofected applying the program X-05 in the Amaxa Nucleofector. After 10 min at room temperature, cells were harvested with 500 μl of pre-warmed complete medium rinsing the cuvette, transferred to a 6-well culture dish and incubated at 37 °C and 5% $CO_2$ for 24 h in a final volume of 1 ml of complete medium. Next, cells were harvested or stimulated with 10 ng/ml of PMA plus 500 ng/ml of Ionomycin for 24 extra hours. Effective RUNX1 silencing at 24 h was confirmed by Western blot.

### 2.7. RNA extraction and real-time PCR

Cells nucleofected for 24 h (unstimulated) or nucleofected for 24 h and then stimulated for 24 extra hours were washed in cold 1× PBS and resuspended in 350 μl of lysis buffer RP1 (Macherey-Nagel). RNA extraction was performed employing *NucleoSpin® RNA/Protein* kit from Macherey-Nagel following manufacturer directions. cDNA was synthesized using *AMV Reverse Transcriptase* from Promega according to manufacturer's instructions. Real-time PCR was performed using *LightCycler® FastStart DNA Master$^{PLUS}$ SYBR Green I* from Roche. Relative quantification was carried out amplifying hCD69 and 18s RNA (housekeeping control gene). Primers for hCD69 amplify a 50nt-amplicon located between exons 1 and 2. The primers used were: hCD69_F: 5′-CAGTCCAACCCAGTGTTCCT-3′; hCD69_R: 5′-CGTGTTGAGAAATGGGGACT-3′; RNA18S_F: 5′-CTCAACACGGGAAACCTCAC-3′; RNA18S_R: 5′-CGCTCCACCAACTAAGAACG-3′.