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Report

The NFAT1 Transcription Factor is a Repressor of Cyclin A2 Gene Expression

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KEY WORDS

cyclin, gene expression, lymphocytes, NFAT, transcription factor

ABBREVIATIONS

CHO	Chinese hamster ovary cells
CsA	cyclosporin A
Iono	ionomycin
NFAT	nuclear factor of activated T cell
OVA	ovalbumin
PMA	phorbol 12-myristate 13-acetate

ACKNOWLEDGEMENTS

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ABSTRACT

The NFAT (Nuclear Factor of Activated T cells) family of transcription factors plays a central role in the regulation of several genes related to the immune response. Recently, NFAT proteins have been implicated in the proliferation and differentiation of different cell types. Previous studies have shown that NFAT1-deficient mice display lymphocyte hyperproliferation, shortened cell cycle duration, and cyclin overexpression. Here, we demonstrate that cyclin A2 expression is upregulated in the absence of NFAT1 in lymphocytes. Ectopic expression of NFAT1 in CHO cells decreases cyclin A2 levels. Indeed, NFAT1 binds to a consensus binding site found at the mouse cyclin A2 promoter in vitro and in vivo. Luciferase reporter assays show that NFAT1 downregulates cyclin A2 expression by directly binding to the cyclin A2 promoter. Together, these results indicate that the NFAT1 transcription factor represses cyclin A2 expression in lymphocytes, and may act as a silencer of gene transcription during the cell cycle.

INTRODUCTION

Cell cycle progression is controlled by cyclin-dependent kinases (CDKs) and their partner cyclins. Cyclin-CDK activation is precisely coordinated throughout the cell cycle. Cyclins are regulated at transcriptional and post-transcriptional levels during cell cycle phases, and control multiple specific steps required for cellular division.[1,2] Cyclin A has been implicated in the control of S phase entry, as well as in the G_2/M phase transition, through binding to CDK2 and CDK1, respectively. Indeed, cyclin A levels are initially detected in late G_1, substantially increased through S phase, and start to decrease during early mitosis.[2,3] To date, two cyclin A members have been identified in mammals. In mice, cyclin A1 has been described to be exclusively expressed in male germ cells, while cyclin A2 is expressed in several adult as well as embryonic tissues.[4,5] Cyclin A2-deficient mice are not viable, suggesting a nonredundant role for this protein during embryonic development.[6]

Regulation of cyclin A2 expression has been widely investigated in different cell types and models.[7] Cyclin A2 transcription is repressed in quiescent (G_0) and early G_1 cells, and is rapidly derepressed/induced when cells approach the G_1/S boundary.[8-10] Several studies have identified at least four regulatory elements located within a 70-bp region near the transcriptional start site of cyclin A2 gene. Two of these elements, known as the CRE and CAAT box, are constitutively occupied throughout the different phases of the cell cycle by the CREB/ATF and NF-Y families of proteins, respectively.[10,11] These regions have been shown to be regulated by the TGF-β and cAMP signaling pathways in different cells.[12-15] The other two sites, named CCRE/CDE and CHR, are occupied in G_0/early G_1 cells, and are negative regulatory elements in the cyclin A2 promoter.[9,10,16] Binding proteins that regulate the activity of these elements may include E2F, Rb, CDF-1, and SWI/SNF chromatin remodeling factors.[16-19] Besides these elements, cyclin A2 promoter also seems to contain another negative regulatory region localized upstream of the CCRE/CDE core element.[8,10] However, the molecular mechanisms that dictate its activity have not been elucidated yet.

Increase of intracellular calcium levels is crucial for cellular proliferation. Several reports have shown that the calcium/calcineurin signaling pathway is able to regulate the expression of cell cycle-related genes, such as cyclin A and E.[20-22] Furthermore, the immunosuppressive drugs cyclosporin A and FK506, which inhibit the activity of calcineurin, also modulate the expression of cyclin genes and CDK inhibitors.[20,23] The NFAT family of transcription factors encodes four different proteins that are activated through the calcium/calcineurin

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signaling pathway.[24,25] Calcium influx induced by different stimuli activates calcineurin, which is able to dephosphorylate NFAT. NFAT dephosphorylation leads to nuclear translocation, and increased DNA binding affinity.[26,27] In fact, several reports have shown NFAT binding sites in the promoter/enhancer regions of many inducible genes, such as IL-2, IL-4, IFNγ, TNFα, and the cell surface molecules CD40L, Fas-L and CTLA-4.[24,25] Recently, the transcription factor NFAT1 has been demonstrated to directly repress the expression of CDK4 in lymphocytes.[28] Nevertheless, the involvement of NFAT proteins in the regulation of cell cycle-related genes is still poorly known.

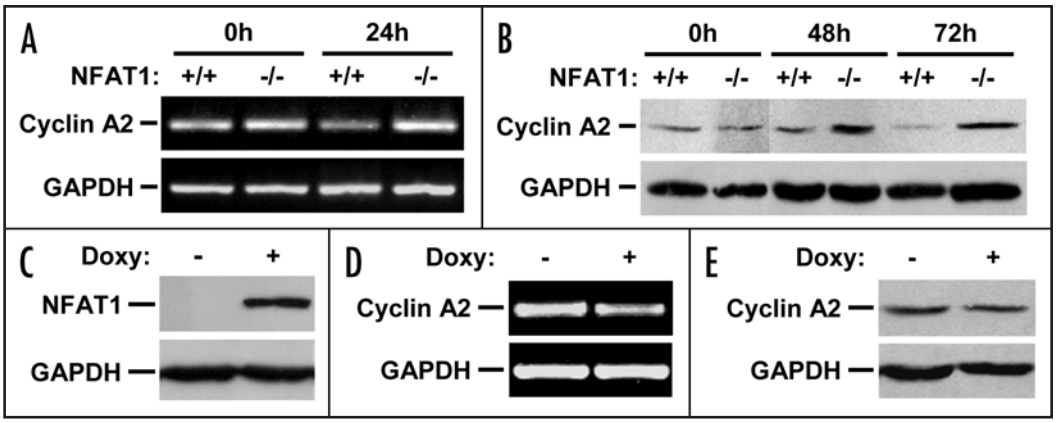

Figure 1. NFAT1 regulates cyclin A2 gene expression. (A and B) Animals (NFAT1$^{+/+}$ and NFAT1$^{-/-}$) were sensitized, and cells from the draining lymph nodes were collected and stimulated in vitro with ovalbumin (OVA) after 15 days of sensitization. At the indicated time points, total RNA or total protein lysates were obtained and cyclin A2 or GAPDH levels were analyzed either by RT-PCR (A) or western blot (B). (C–E) CHO-NFAT1 cells were cultivated either in the absence or presence of doxycycline (1 μg/mL) for 48 hours. Total protein lysates or total RNA was obtained and NFAT1, cyclin A2 or GAPDH levels were analyzed either by RT-PCR (D) or western blot (C and E). These results are representative of at least three independent experiments.

We have previously observed increased expression of cyclin genes in lymphocytes lacking the NFAT1 protein.[29] These results suggested to us that NFAT1 could play a negative regulatory role in cyclin expression in these cells. Here, we demonstrate that the NFAT1 transcription factor directly binds to the promoter region of the cyclin A2 gene, and regulates the expression of cyclin A2 in lymphocytes. NFAT1 acts as a repressor of cyclin A2 expression through the binding of a negative regulatory element identified at the cyclin A2 promoter, besides the canonical regions already described. These results indicate NFAT proteins as regulators of cyclin expression in lymphocytes, and central controllers of the mammalian cell cycle.

RESULTS

NFAT1 negatively regulates cyclin A2 expression. NFAT transcription factors regulate the expression of cell cycle-related proteins, such as CDK4, p21 and c-MYC.[23,28,35] We previously showed that NFAT1$^{-/-}$ lymphocytes display increased expression of certain cyclin genes upon antigen stimulation.[29] In this study, we investigate the mechanism underlying increased expression of cyclin A2 by NFAT1$^{-/-}$ lymphocytes. Compared to wild type cells, NFAT1$^{-/-}$ lymphocytes from OVA-sensitized mice expressed higher levels of cyclin A2 mRNA and protein after OVA challenge, as analyzed by RT-PCR and western blot (Fig. 1A, compare lanes 3 and 4; and Fig. 1B, compare lanes 3–6). To confirm this finding, we generated stably-transfected CHO cells in which NFAT1 can be conditionally expressed under the control of the tetracycline repressor. NFAT1 was expressed in these cells only upon doxycycline treatment (Fig. 1C), and NFAT1 induction was associated with decreased cyclin A2 mRNA and protein levels (Fig. 1D and E). Doxycycline treatment did not affect cyclin A2 expression in untransfected CHO cells (data not shown). Together, these results suggest that NFAT1 acts as a repressor of cyclin A2 expression.

The cyclin A2 promoter is repressed by NFAT1. We identified NFAT-responsive regions in the proximal promoter region of the mouse cyclin A2 gene by transfecting Jurkat cells with different promoter constructs (Fig. 2A). As previously described, a construct containing only 24 bp upstream of the transcription start site (CycA2-24-Luc) had no luciferase activity (Fig. 2A). On the other hand, a construct containing 302 bp of the proximal promoter (CycA2-302-Luc) was 3-fold more active than the original -862 bp promoter (CycA2-862-Luc) (Fig. 2A). Deletion of the -576 to -302 bp region in the context of the -862 bp promoter (CycA2Δ-576,-302-Luc) increased luciferase activity to the level observed with the partial construct CycA2-302-Luc, suggesting the presence of a negative regulatory element in the -576 to -302 bp region (Fig. 2A). Bioinformatic analysis indicated a consensus NFAT-binding site at position -456 bp of the mouse cyclin A2 promoter, which showed homology to the canonical NFAT binding site found at the mouse IL-2 promoter (Fig. 2B).

We showed that NFAT1 could act as a negative regulator of cyclin A2 expression in lymphocytes. PMA plus ionomycin treatment decreased expression of the -862 bp cyclin A2 promoter in Jurkat T cells, and this decrease was prevented by pretreatment with cyclosporin A (CsA) (Fig. 2C, left), suggesting that endogenous NFAT repressed cyclin A2 expression. Confirming this hypothesis, ectopic expression of NFAT1 resulted in decreased activity of the cyclin A2 promoter in unstimulated cells, with a further decrease observed after stimulation with PMA plus ionomycin; again, the stimulation-dependent decrease was prevented by CsA (Fig. 2C, right). As expected, cells ectopically expressing NFAT1 showed increased induction of a reporter construct containing tandem NFAT:AP-1 composite sites (3xNFAT-Luc) in response to PMA and ionomycin, and this was blocked by CsA (Fig. 2D). These results indicate that NFAT1 is a repressor of cyclin A2 expression in lymphocytes.

NFAT1 binds to the cyclin A2 promoter in lymphocytes. To confirm the role of NFAT1 in cyclin A2 gene repression in vivo, we used chromatin immunoprecipitation (ChIP) to monitor NFAT1 binding to the consensus NFAT binding site identified at position -456 bp of the cyclin A2 promoter. In Th1 cells derived from CD4^{+} T cells of wild type mice, NFAT1 bound to the cyclin A2 promoter, regardless of whether the cells were left unstimulated or were stimulated with PMA plus ionomycin, and stimulation-dependent binding was completely blocked by CsA (Fig. 3A and B). We also evaluated NFAT1 binding to the IFNγ and IL-4 promoters, as positive and negative controls respectively based on chromatin accessibility. After

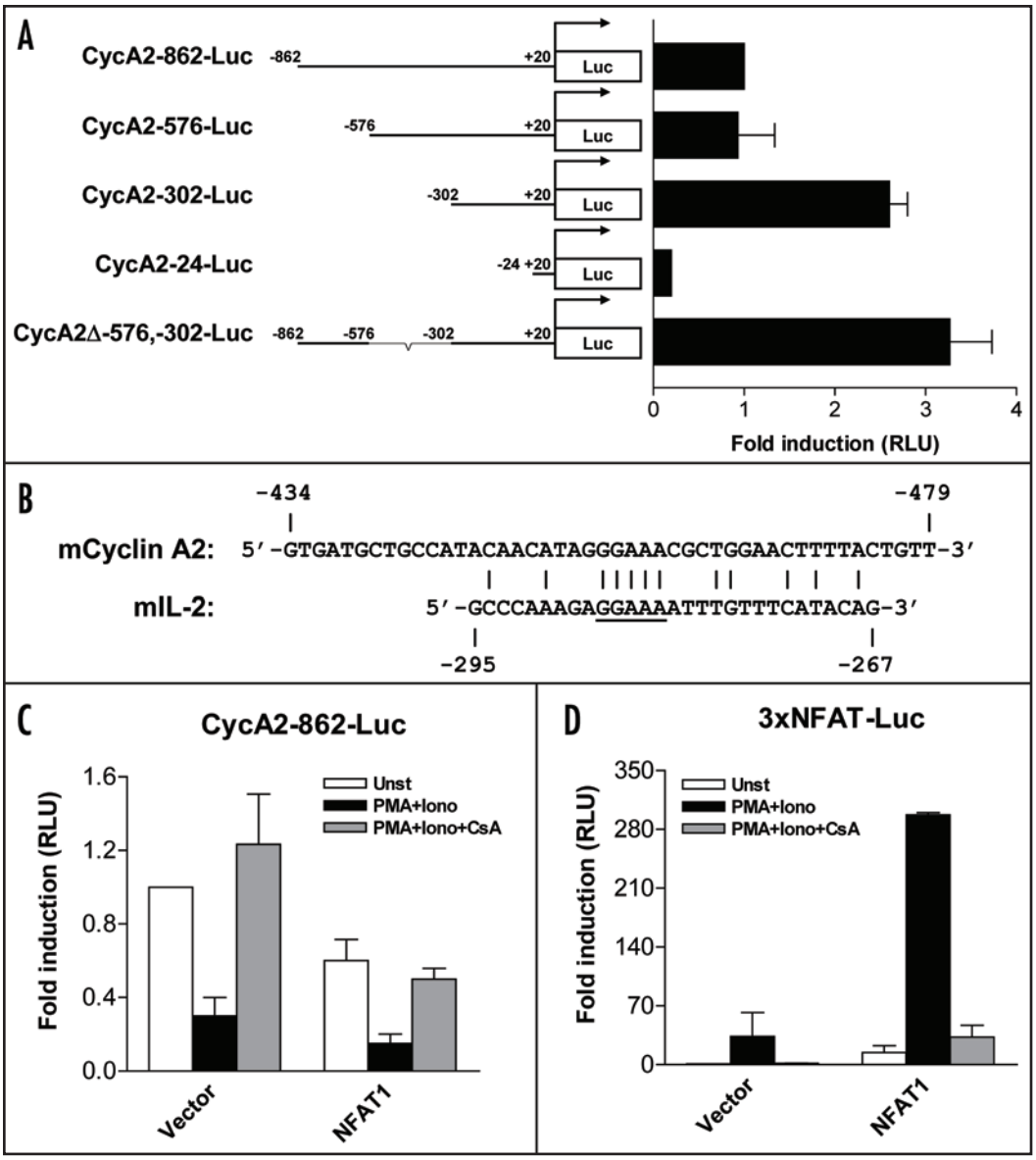

Figure 2. Negative regulatory region at cyclin A2 promoter contains an NFAT binding site. (A) Schematic representation of the luciferase reporter vectors used. The mouse cyclin A2 promoter region (between -862 and +20 bp) was cloned into the pGL3-basic vector (CycA2-862-Luc). The other plasmids were obtained by serial deletions of the full-length reporter plasmid as described: CycA2-576-Luc; -302-Luc; -24-Luc; Δ-576,-302-Luc. Jurkat cells were then transfected with each of the indicated vectors. After 24 hours, cells were stimulated for 16 hours with PMA (10 nM) plus ionomycin (1μM) and total cell lysates were obtained. Luciferase activity was measured as described, and expressed as relative light units (RLU) relative to CycA2-862-Luc vector. (B) Schematic representation of the mouse cyclin A2 promoter region (between -479 and -434 bp) aligned to the mouse interleukin-2 promoter region (mIL-2; between -295 and -267 bp). The putative NFAT binding site is underlined. (C and D) Jurkat cells were cotransfected with the expression vector (pcDNA5-empty or pcDNA5-NFAT1; Vector or NFAT1, respectively) and the luciferase reporter vector CycA2-862-Luc (C) or 3xNFAT-Luc (D). After 24 hours, cells were left unstimulated (Unst) or stimulated for 16 hours with PMA (10 nM) plus ionomycin (1 μM) either in the absence or presence of cyclosporin A (1 μM). Then, luciferase activity was measured, and expressed as relative light units (RLU) relative to unstimulated cells transfected with pcDNA5-empty (Vector). Results are expressed as mean ± SD of three independent experiments.

PMA plus ionomycin stimulation, NFAT1 bound by ChIP to the IFNγ promoter which is accessible in Th1 cells, but not to the IL-4 promoter which is inaccessible in these cells (Fig. 3C). As expected, NFAT1 did not bind the IFNγ or IL-4 promoters in unstimulated cells (Fig. 3C).

The cyclin A2 promoter contains a functional NFAT-responsive negative regulatory element. We then asked whether the NFAT-binding site identified at position -456 bp was a functional element that bound NFAT1. When tested in an electrophoretic mobility shift assay (EMSA), the recombinant DNA-binding domain of NFAT1 bound an oligonucleotide spanning the -456 bp element but was unable to bind a mutated oligonucleotide in which the GGAAA sequence which contacts NFAT1 was substituted with ACTCT (Fig. 4A; see Materials and Methods). NFAT1 binding was competed in a dose-dependent manner by increasing amounts of unlabelled oligonucleotides from the IL-2 or cyclin A2 promoters, with approximately 10-fold more cyclin A2 than IL-2 oligonucleotide required to produce the same level of competition (Fig. 4A). This result suggests that NFAT1 binds with lower affinity to the cyclin A2 promoter compared to the IL-2 promoter, consistent with the preference for T or A immediately 5' of the GGAAA sequence for high-affinity binding (A.R., unpublished observations).[24] The unlabelled mutant version of the cyclin A2 oligonucleotide did not interfere with NFAT1 binding, even at high concentrations (Fig. 4A).

To probe the function of the -456 bp NFAT-binding element, we mutated it in the context of the -862 bp cyclin A2 promoter (CycA2-862mut-Luc; Fig. 4B). The mutated promoter showed approximately 3-fold increase in luciferase activity compared to the wild type promoter (Fig. 4B). This result is in accordance with the increased luciferase activity observed with the -302 bp promoter construct (CycA2-302-Luc, Fig. 2A), which lacks the NFAT-binding site at position -456 bp. These results strongly support a negative regulatory role for NFAT1 during cyclin A2 expression in lymphocytes.

DISCUSSION

In this work, we demonstrate a previously unrecognized role for the transcription factor NFAT1 in cyclin A2 repression. NFAT1 directly binds to the cyclin A2 promoter, in a region demonstrated to have a negative regulatory function in both human and mouse promoters, suggesting an important evolutionary role.[8,10] This region is distinct from the CCRE/CDE and CHR negative regulatory regions which are involved in downregulating cyclin A2 expression through the binding of Rb, $p120^{E4F}$, and certain subunits of the SWI/SNF chromatin remodeling machinery.[18,19,36] Together, these results indicate that in addition to the repression driven by the CCRE/CDE and CHR elements, NFAT1 may account for the silencing of cyclin A2 during G_0/ G_1 cell cycle phase.[9,10,18]

NFAT1 has also been shown to bind to the promoter region and to repress the expression of CDK4 in lymphocytes, suggesting a general repressor role for this transcription factor during cellular proliferation.[28] As also observed in the *CDK4* and c-*MYC* promoter regions, the NFAT binding site found at the cyclin A2 promoter lies in close vicinity to a putative E2F-binding site. Recent data have indicated that E2F and NFAT proteins may compete for the binding and regulation of cdk4 and myc expression in different models.[28,35] Thus NFAT1 might be responsible for silencing cyclin A2 during the G_0/G_1 phase of the cell cycle, antagonizing the G_1/S phase transition mediated by E2F.

Notably, NFAT1 bound strongly to the cyclin A2 promoter even in unstimulated cells, although it could not be detected at the accessible promoter of the IFNγ gene in the same cell population (Fig. 3A and B). The binding was completely blocked by CsA, indicating that it reflected some basal activation of calcineurin as expected. The results suggest that NFAT1 occupies the cyclin A2 promoter with unusually high affinity in living cells. Given the relatively binding of NFAT1 to the -456 bp element in EMSA assays in vitro (10-fold lower affinity than binding to the IL-2 promoter element; Fig. 4A), one possibility is that the small amount of NFAT present in the nucleus of resting cells is preferentially recruited to the cyclin A2 promoter as a result of cooperation with a preexisting factor that tightly occupies a sequence adjacent to the -456 bp element even in the absence of stimulation. This hypothesis is currently being tested.

NFAT transcription factors have been associated with cellular proliferation and tumorigenesis in different cell types.[22,37] Deficiency of NFAT1 in vivo caused lymphocyte hyperproliferation, altered cell cycle control, and increased stage-specific cyclin expression.[29,30,38] Considering cell types other than immune cells, the absence of NFAT1 led to uncontrolled growth of connective tissue and skeletal muscle cells, suggesting a suppressor role for this protein in different tissues.[39,40] In contrast, the lymphocytes of NFAT2-deficient mice displayed impaired cellular proliferation as well as deficient thymus and lymphoid organ repopulation.[41,42] Moreover, NFAT2 has been implicated as an oncogenic factor, since it induces cells to acquire growth factor autonomy, and upregulate cyclin D and myc expression in adipocytes and pancreatic cells.[35,43] Together with these studies, our data suggest that NFAT proteins are critical regulators of cyclin gene expression in lymphocytes as well as other cell types.

To summarize, our evidence indicates that NFAT1 functions as a cell cycle repressor with a possible tumor suppressor role in vivo. Potentially, NFAT1-mediated cyclin A2 downregulation might be related to the control of cellular proliferation and tumor development in vivo. Preliminary results from our laboratory support this idea, since conditional expression of NFAT1 protein not only decreases the expression of cyclin A2 in CHO cells (Fig. 1C–E), but also inhibits tumor growth when these cells are subcutaneously injected in BALB/c nude/nude mice in a model of tumorigenesis (unpublished results). In fact, several studies have associated the dysregulation of cyclin A with different types of tumors, including hepatocellular carcinoma, soft tissue sarcoma, and leukemia.[44-47] A better understanding of the specific contribution of each NFAT family member to cell cycle progression will provide further insight into tumorigenic processes.

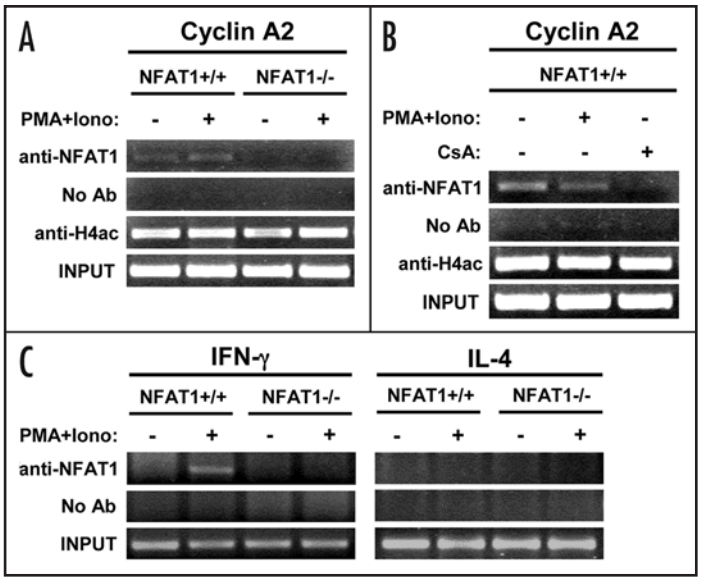

Figure 3. NFAT1 transcription factor directly binds to cyclin A2 promoter in vivo. $CD4^+$ T lymphocytes were isolated from naive $NFAT1^{+/+}$ and $NFAT1^{-/-}$ mice, and cells were stimulated in vitro with plate-bound anti-CD3 and anti-CD28 for 7 days in the presence of Th1 polarizing conditions as described. Cells were then left unstimulated or stimulated with PMA (10 nM) plus ionomycin (1 μM) for additional 4 hours either in the absence or presence of cyclosporin A (1 μM). Chromatin immunoprecipitation (ChIP) was carried out using nuclear extracts, and immunoprecipitation was performed with anti-NFAT1, anti-acetylated forms of histone H4 antibody (anti-H4ac), or no antibody as a negative control (No Ab). Then, DNA was purified and analyzed by PCR for cyclin A2 (A and B) or IFNγ and IL-4 (C) promoter regions containing the NFAT binding sites. As a control, PCR was also done directly on input DNA purified from chromatin before immunoprecipitation (INPUT). These results are representative of at least two independent experiments.

MATERIALS AND METHODS

Animals, sensitization and cell cultures. Control wild type ($NFAT1^{+/+}$) and NFAT1-deficient ($NFAT1^{-/-}$) 8- to 12-week old mice were used in all experiments. Animals were bred and maintained in the Brazilian National Cancer Institute (INCA) animal facility. Animals were treated according to the guide for the care and use of laboratory animals from the National Institutes of Health (NIH, USA). Mice were sensitized in the hind footpad with one subcutaneous injection of 0.1 mL of ovalbumin (OVA, 200 μg) emulsified in complete Freund's adjuvant as described before.[30,31] Fifteen days later, cells from the draining lymph nodes were harvested and restimulated in vitro with 0.5 mg/mL of OVA for different time points.[29] All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamine, streptomycin-penicillin, essential and nonessential amino acids, sodium pyruvate, vitamins, hepes, 2-mercaptoethanol (all from Invitrogen). Cells were cultivated

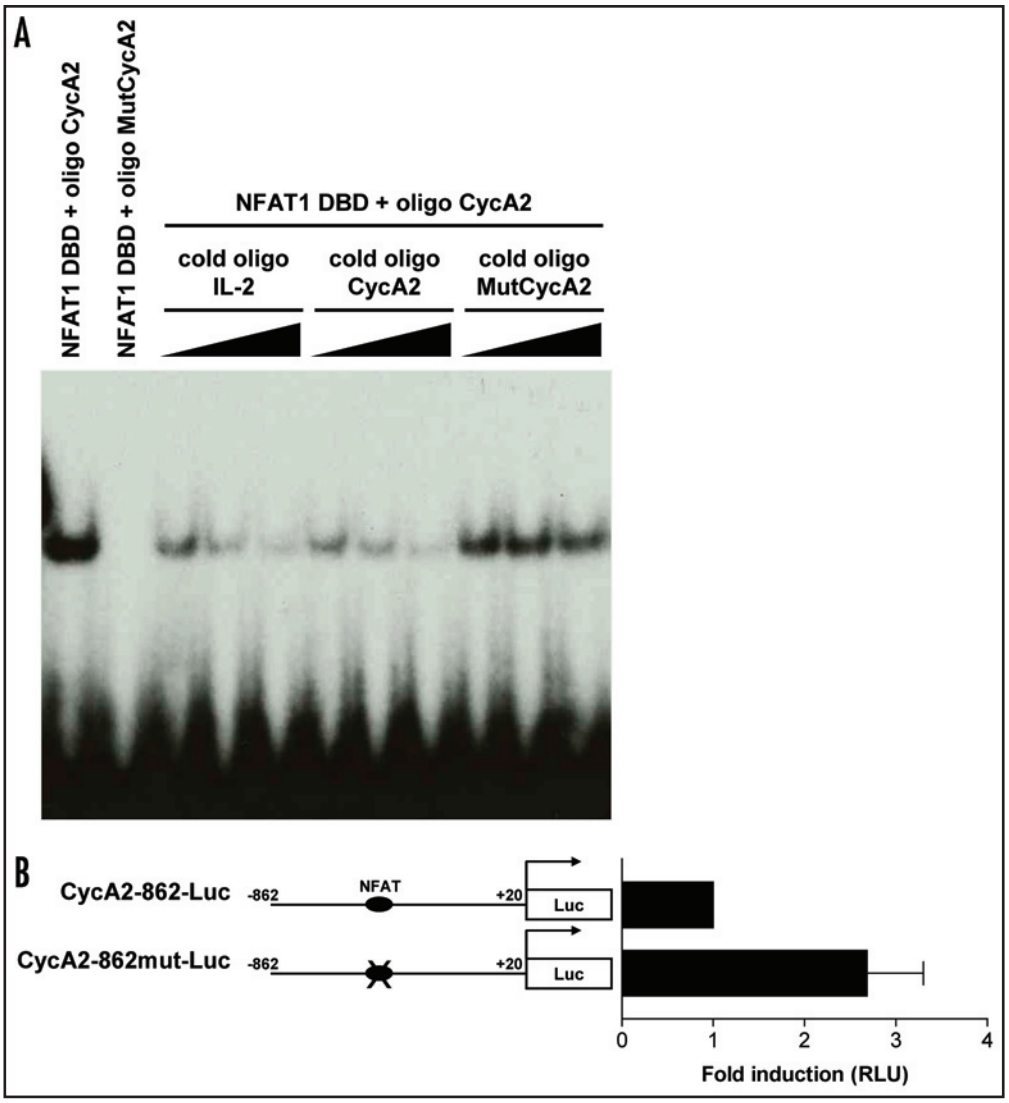

Figure 4. NFAT1 transcription factor inhibits cyclin A2 gene expression in lymphocytes. (A) NFAT1 peptide (NFAT1 DBD) was incubated with labeled oligos containing or not the NFAT binding site: wild type cyclin A2 promoter (CycA2) or the mutated oligo (MutCycA2), respectively. Increasing concentrations of cold oligos were included in the binding reaction before the addition of labeled probes when indicated: IL-2 (5, 10 and 25x), CycA2, and MutCycA2 (50, 100, and 250x). DNA-peptide complexes were analyzed by autoradiography. These results are representative of three independent experiments. (B) Schematic representation of cyclin A2 promoter constructs containing the wild-type NFAT binding site at position -456 (CycA2-862-Luc) or the specific mutation of this site (CycA2-862mut-Luc). Jurkat cells were then transfected as described before. After 24 hours, cells were stimulated for 16 hours with PMA (10 nM) plus ionomycin (1 μM). Luciferase activity was measured, and expressed as relative light units (RLU) relative to CycA2-862-Luc vector. Results are expressed as mean ± SD of three independent experiments.

in 5% CO_2 at 37°C. The phorbol 12-myristate 13-acetate (PMA), ionomycin (Iono), and the antibiotic doxycycline were obtained from Calbiochem®; the immunosuppressive drug cyclosporin A (CsA) was obtained from LC Laboratories; the complete Freund`s adjuvant (CFA), and the ovalbumin (OVA) were purchased from Sigma-Aldrich.

RT-PCR. Total RNA was extracted with Trizol Reagent (Invitrogen) and semi-quantitative RT-PCR for murine cyclin A2 and GAPDH expression were performed using the Ready-To-Go™ You-Prime First-Strand Beads (Amersham Biosciences). The primers were used as follows: cyclin A2, 5'-CCT CTC CTC CAT GTC TGT GTT AAG-3' and 5'-GTG CTC CAT TCT CAG AAC CTG CTT-3' (163-bp product); GAPDH, 5'-TGA AGG TCG GTG TGA ACG GAT TTG-3' and 5'-ACG ACA TAC TCA GCA CCA GCA TCA-3' (276-bp product). PCR conditions were as follows: 95°C 5 min; 28 cycles at 95°C 30s, 55°C 30s and 72°C 45s; and a final elongation at 72°C for 5 min. PCR products were resolved on 1.5% agarose gels and visualized with ethidium bromide.

Western blot. Total protein lysates were obtained as previously described.[27] Briefly, cells (2 x 10^6 cells) were lysed in buffer containing 40 mM Tris pH 7.5, 60 mM sodium pyrophosphate, 10 mM EDTA, and 5% SDS, followed by incubation at 100°C for 15 min. Total cell lysates were resolved in SDS-PAGE, and the separated proteins were transferred to nitrocellulose membrane. The antibodies were used as follows: NFAT1, polyclonal antibody anti-67.1,[27] Cyclin A, polyclonal antibody C-19 and GAPDH, monoclonal antibody 6C5 (Santa Cruz). The immunodetection was performed with ECL western blotting detection kit (Amersham Biosciences).

CHO-NFAT1 cell line construction. The CHO-NFAT1 cell line was constructed by using the Flp-In™ T-Rex™ System (Invitrogen). Briefly, Chinese Ovary Hamster (CHO) cells were transfected with the acceptor vector pFRT/lacZeo, and cultivated in the presence of zeocin (500 μg/mL). Recombinant clones were screened for unique insertion by Southern blotting, and high levels of β galactosidase activity. After clone selection, cells were transfected with the pcDNA5/FRT/TO-NFAT1 expression vector, and cultivated in the presence of hygromycin (500 μg/mL). The pcDNA5/FRT/TO-NFAT1 expression vector was generated by cloning an *Nr*uI/*Hi*ndIII fragment from NFAT1 cDNA (isoform C extracted from pFEBOS plasmid,[32]) into the pCDNA5/FRT/TO vector linearized with *Eco*RV and *Hi*ndIII (Flp-In™ T-Rex™ System, Invitrogen). Then, recombinant clones (CHO-NFAT1) were isolated and screened for low levels of β galactosidase activity, and the presence of NFAT1 protein by western blot. Finally, CHO-NFAT1 cells were transfected with the repressor vector pCDNA6, subsequently cultivated in the presence of blasticidin (5.0 μg/mL), and isolated as a clone once again. This system enables conditional expression of NFAT1 when derepressed upon tetracycline/doxycycline treatment.

Construction of cyclin A2-promoter reporters. The full-length luciferase reporter plasmid, named CycA2-862-Luc, was obtained by inserting an 882 bp fragment from the mouse cyclin A2 promoter (a generous gift from Dr. J.M. Blanchard,[10]) into the pGL3-basic vector (Promega). The CycA2-862-Luc vector contains the -862 to +20 bp region driving the expression of luciferase reporter gene. The *Bg*lII/*Pst*I cyclin A2 promoter fragment was cloned into the *Bg*lII/*Hin*dIII restriction sites from the pGL3-basic vector. The other plasmids were obtained by serial deletions of the full-length CycA2-862-Luc vector with different restriction enzymes, as follows: CycA2-576-Luc (*Eco*RV); CycA2-302-Luc (*Xho*I); CycA2-24-Luc (*Bss*HII). The CycA2Δ-576,-302-Luc vector was obtained by deletion of the -576 to -302 region from the full-lenght plasmid with *Eco*RV and *Xho*I enzymes. The CycA2-862mut-Luc mutant plasmid was generated through site-directed mutagenesis of the full-length plasmid (Gene Tailor™ Site-Directed Mutagenesis System; Invitrogen). For site-directed mutagenesis, forward primer was designed as follows: 5' AAACAATAAAAGTTCCAGCGAGAG TCTATGTTGTA 3' (IDT). The mutated NFAT consensus-binding site is underlined.

Transactivation Assay. Jurkat cells (3 x 10^6 cells/600 μL) were eletroporated (950 μF, 250 V) in a 0.4-cm GenePulser Cuvette with GenePulser II (Bio-Rad Laboratories). Cells were cotransfected with 3 different plasmids in serum-free media, as follows: (i) one of the indicated CycA2-promoter constructs (3.0 μg), (ii) the pcDNA5/FRT/TO-NFAT1 expression vector (30 μg), and (iii) the β galactosidase expression plasmid for normalization of transfection efficiency (1.0 μg; kindly provided by Dr. J.A. Garcia, Federal University of Rio de Janeiro, Brazil). After 24 hours, cells were washed, and stimulated in vitro for 16 hours at 37°C with PMA (10 nM) and ionomycin (1.0 μM). The next day, cells were harvested, and lysis was performed for 20 min at room temperature with 50 μL of 1X cell culture lysis reagent (Promega). Crude extracts (10 μL) were added to 100 μL of luciferase assay substrate (Promega), and luciferase activity was promptly measured in a Monolight 3010 Luminometer (Analytical Luminescence Laboratory). Luciferase activities were expressed as relative light units (RLU).

Electrophoretic mobility shift assay (EMSA). The NFAT1 DBD recombinant protein was expressed and purified as previously described.[33] Oligonucleotide duplexes (5.0 μg of each oligo) were generated by denaturation for 10 min at 95°C in hybridization buffer (10 mM Tris pH 7.4, 50 mM NaCl), and immediately followed by overnight hybridization at room temperature. Oligonucleotides (50 ng) were labeled for 1 hour at 37°C with 50 μCi [γ^{32}P] dATP (Amersham Biosciences) and T4 polynucleotide kinase (10 U; New England Biolabs). Probes were then purified with MicroSpin™ G-25 columns (Amersham Biosciences). Binding reaction was performed for 20 min at room temperature in a total volume of 20 μL of binding buffer (10 mM HEPES pH 7.0, 125 mM NaCl, 10% glycerol, 0.25 mM DTT, 0.8 mg/mL BSA). Purified NFAT1 DBD protein (10 nM) was incubated with 0.2 μg/reaction of poly (dI:dC) (Amersham Biosciences) and ~0.2–0.4 ng of labeled oligonucleotides (CycA2 or MutCycA2; 10.000 counts/min). When indicated, an excess of unlabeled oligonucleotides (cold oligos: IL-2, 5, 10, 25x; CycA2, 50, 100, 250x; and MutCycA2, 50, 100, 250x) was included in the binding reaction 10 min before the addition of the labeled probes. DNA-protein complexes were separated by electrophoresis under nondenaturating conditions on a 4% polyacrylamide gel in 0.5x TBE buffer. The gel was prerun for 1 hour at 100 V, and samples were run for an additional hour at 200 V. The gel was dried onto Whatman filter paper and analyzed by autoradiography. The oligonucleotides were used as follows: IL-2 5'-TGGAAAATTTGTTTCATAGT-3'; CycA2 5'-AGTTCCAGCG TTTCCCTATGTTGTA-3'; MutCycA2 5'-AGTTCCAGCGAGA GTCTATGTTGTA-3' (IDT). The NFAT consensus-binding site is depicted in bold, and the changed nucleotides are underlined.

Chromatin immunoprecipitation assay (ChIP). $CD4^+$ T cells were positively isolated by magnetic bead selection from total lymph nodes of $NFAT1^{+/+}$ and $NFAT1^{-/-}$ mice (Dynal). $CD4^+$ T lymphocytes (2 x 10^6 cells/well) were stimulated in vitro with plate-bound anti-CD3 antibody (2C11; 0.25 μg/mL) and anti-CD28 (37.51; 1.0 μg/mL) for seven days in the presence of Th1 polarizing conditions: IL-12 (10 ng/mL), anti-IL-4 (10 μg/mL), and IL-2 (20 U/mL). Then, cells were washed, rested for 2 hours, and left unstimulated or restimulated in vitro with PMA (10 nM) and ionomycin (1.0 μM) for additional four hours. When indicated, CsA (1 μM) was added to cells 15 min before stimulation. For ChIP assay, cells were treated as previously described.[34] Briefly, $CD4^+$ T cells (4 x 10^7) were fixed for 20 min at room temperature with 1:10 dilution of 11% formaldehyde solution (in 50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA), neutralized for 5 min on ice with 1:20 dilution of 2.5 M glycine, and rinsed twice with ice-cold 1x PBS. Nuclei were extracted in 10 mM HEPES pH 7.5, 10 mM EDTA, 0.5 mM EGTA, 0.75% Triton X-100 for 10 min at 4°C, followed by 10 min at 4°C in 10 mM HEPES pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA. Then, nuclei were sonicated 10–12 times of 20 sec each at maximal setting, with 1 min of cooling on ice in-between pulses in 25 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 1 mM PMSF, 25 μg/mL aprotinin, 12.5 μg/mL leupeptin (550 Sonic Dismembrator, Fisher Scientific). The size of sonicated DNA was monitored in 1.5% agarose gels, and was around 0.5 kb. Chromatin was cleared by centrifugation for 10 min at 20.000 g, and then precleared for 2 hours at 4°C with Protein A Sepharose CL-4B (Amersham Biosciences), previously blocked with sheared salmon sperm DNA (100 μg/mL). Immunoprecipitation was performed overnight at 4°C with a set of NFAT1-specific antibodies (anti-67.1 and anti-T2B1; 15 μg and 50 μg, respectively) or anti-acetyl-histone H4 (5 μg; Upstate Biotechnology). Immunocomplexes were captured with Protein A beads for 3 hours at 4°C, and precipitates were washed sequentially for 5 min at 4°C with different buffers, as previously described.[34] Protein A beads were treated for 30 min at 37°C with RNase A (50 μg/mL; Sigma-Aldrich) in TE buffer, followed by deproteination at 50°C for 4 hours with proteinase K (200 μg/mL; Roche) and 0.5% SDS. Formaldehyde crosslinks were reversed at 65°C by overnight incubation. Finally, DNA was purified by phenol-chloroform extraction, precipitated with ethanol in the presence of carrier glycogen, and resuspended in 100 μL of TE buffer. PCR was done with 5 μL of the immunoprecipitated DNA. PCR conditions were as follows: 3 min at 95°C, followed by 28 cycles of 15 sec at 95°C, 30 sec at 48°C or 60°C, and 30 sec at 72°C, completed by 5 min at 72°C. Primers were used as follows: cyclin A2 promoter, 5'-GTGAGTCACAGGACAATTGGGACA-3' and 5'-TAGGCAGGAGCGTATGGATCTGAA-3' (60°C; 209-bp product); IFNγ promoter, 5'-GCTCTGTGGATGAGAAAT-3' and 5'-AAGATGGTGACAGATAGG-3' (48°C; 250-bp product); and IL-4 promoter, 5'-AAGATTAGTCTGAAAGGCC-3' and 5'-TCA AGAGATGCTAACAATGC-3' (48°C; 290-bp product) (IDT). As loading control, the PCR was done directly on input DNA purified from chromatin before immunoprecipitation. PCR products were resolved on 1.5% agarose gels, and visualized with ethidium bromide.

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