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Cyclic Adenosine 5′-Monophosphate Response Element Modulator Is Responsible for the Decreased Expression of c-fos and Activator Protein-1 Binding in T Cells from Patients with Systemic Lupus Erythematosus

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T cells from patients with systemic lupus erythematosus express increased levels of the cAMP response element modulator (CREM) that has been shown to bind to the IL-2 promoter and suppress its activity. In this study, we demonstrate that CREM binds to the proximal promoter of the c-fos proto-oncogene in live systemic lupus erythematosus T cells and represses its expression following stimulation in vitro. Decreased levels of c-fos protein result in decreased AP-1 activity, as determined in shift assays. Blockade of the translation of CREM mRNA with an antisense CREM vector increases the expression of c-fos and the AP-1 activity. The levels of c-fos mRNA vary with disease activity. We conclude that CREM represses the expression of c-fos and limits the activity of the enhancer AP-1. Thus, CREM is involved indirectly in the modulation of transcriptional regulation of multiple genes including IL-2. The Journal of Immunology, 2004, 173: 3557–3563.

Abbreviations used in this paper: SLE, systemic lupus erythematosus; AICD, activation-induced cell death (AICD) (3, 4). Accordingly, limited production of IL-2 by SLE T cells has been linked to increased susceptibility to infections (5) and persistence of autoreactive T and B cells (6) in SLE patients.

Decreased transcription of the IL-2 gene upon activation of the T cell has been shown to be responsible for the decreased production of IL-2 by SLE T cells (7). Multiple transcription factors including NF-κB, Oct, and CREB have been shown to bind to the proximal 300 bp of the IL-2 promoter (8). Initial studies from our group in SLE T cells showed a decrease of the activating transcription factor NF-κB p65 subunit (9), and gene transfer of the p65 subunit into SLE T cells increased the activity of the IL-2 promoter (10). Furthermore, we have shown that the transcription repressor cAMP response element (CRE) modulator (CREM) binds the −180 site of the IL-2 promoter in SLE T cells and directly inhibits IL-2 production in these cells (7). Transfection of SLE T cells with a plasmid encoding antisense CREM limited the expression of CREM and resulted in increased production of IL-2 (11). These experiments lead us to propose that CREM is a key repressor of the IL-2 transcription in SLE T cells.

CREM is suspected to bind to the promoters of at least 3000 genes, including the proto-oncogene c-fos. Indeed, CREM binds to the −57 CRE site of the c-fos promoter and blocks the cAMP-induced expression of the c-fos proto-oncogene (12), a prototype of the early response genes. c-fos belongs to the AP-1 family of transcription factors that consists of a mixture of heterodimers and homodimers of jun (v-jun, c-jun, junB, junD) and fos (v-fos, c-fos, fosB, fra1, fra2) proteins (13, 14). Each protein contains a leucine zipper region that enables its dimerization with other members of the fos/jun family. The jun proteins can form homodimers among themselves, while fos proteins form only heterodimers with jun proteins. Both jun-jun and jun-fos dimers bind to the 12-O-tetradecanoyl-acetate response element (TRE) that contains the TGACTCCTA motif. The binding though of the jun-jun homodimers to DNA is weak compared with jun-fos heterodimers (15, 16). Soon after antigenic stimulation, jun and fos proteins are expressed, and subsequently, AP-1 (particularly the c-jun/c-fos dimer) binds to the IL-2 promoter. This in turn induces the expression of the IL-2 gene (8, 17).

Because CREM is transcriptionally up-regulated in SLE T cells (11), we conducted experiments to investigate the effect of CREM on the expression of the c-fos gene and the binding of AP-1 in SLE T cells. We demonstrate that CREM indeed binds to the c-fos promoter in SLE T cells, and it is directly responsible for the decreased production of c-fos and decreased AP-1 binding.

Materials and Methods

Study subjects

Twenty-five female patients diagnosed with SLE according to the American College of Rheumatology criteria (18) donated blood for these studies. The mean age of the patients was 38 (21–80) years old, and the mean SLE disease activity index (SLEDAI) was 3.7 (0–12) (19). Eighty-four percent of the patients were taking oral prednisone at a mean dose of 8.3 mg

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Chromatin immunoprecipitation analysis (ChIP)

Five million T cells were used per investigated Ab. The cells were treated with Formalin (1% final concentration) for 10 min, washed, lysed, and sonicated. The DNA-protein complexes were immunoprecipitated with a desired Ab and extracted by protein A/G-Sepharose beads (Santa Cruz Biotechnology). After several washing steps, the cross-link between DNA and protein was reversed at 65°C, followed by protein digestion with proteinase K, and the DNA was extracted (QiAmp DNA extraction kit; Qiagen, Valencia, CA). The DNA was amplified with primers flanking the c-fos promoter, including the −57 site. The sequences are: forward, 5'-ATGCTCAGGATAGTACAGAC-3'; reverse, 5'-GCTGCGATGCGTTGGAGT-3'. DNA from 1 million cells was used for each PCR. PCR products were separated on a 1.0% agarose gel. Using QuantityOne software (Bio-Rad), the OD of each band was quantitated after background subtraction.

Preparation of mRNA, RT-PCR, and real-time PCR

One million T cells were used for extracting RNA (RNA Easy Mini kit; Qiagen). The RNA was treated with DNases I (Qiagen) and quantitated; 250 ng of total RNA was used for cDNA synthesis by reverse transcription (RT-PCR kit; Promega, Madison, WI). Sigma Genosys synthesized the PCR primers. PCR beads were used for amplification (Pharmacia, Piscataway, NJ). PCR was conducted in a conventional thermocycler. Real-time PCR was conducted with a Cepheid Smart Thermocycler (Cepheid, Sunnyvale, CA) by adding SYBR green to the reaction mixture. Primers used for PCR were as follows: GAPDH forward, 5'-CAACTCATGTTTAGACATGCC-3' reverse, 5'-GGATCTTGTTGTCATGCTCT-3'; c-fos forward, 5'-CGGAAAGGAAAGGAAATAG-3'; reverse, 5'-CGGGTACCCAGGAAAATG-3'; PCRs products were separated on a 1.0% agarose gel, and the OD was quantitated with the QuantityOne software (Bio-Rad) after background subtraction from each band. The real-time PCR products were quantitated, as previously described (21). Briefly, a cDNA specimen was used to create serially diluted samples that were subsequently amplified using different primers. A standard curve was generated for each set of primers, by plotting the threshold cycles of the real-time PCR against the logarithm of the relative concentrations of the diluted samples. Thereafter, the relative mRNA copies of the experimental samples were calculated after real-time amplification, using the linear regression of the standard curve.

Transfection of Jurkat cells

Jurkat cells were cultured in RPMI 1640 and 10% FBS without stimulation. Plasmids encoding CREM antisense (a kind gift from P. Sassone-Corsi, Laboratoire de Génétique Moléculaire des Eucaryotes, Institute National de la Santé et de la Recherche Médicale, Strasbourg, France) and correspond- ing empty vector plasmid were used for transfection. Five micrograms of each plasmid were used per transfection. Approximately 5–10 × 10^6 T cells were transfected by electroporation with an AMAXA electroporator (AMAXA, Cologne, Germany).

Statistical analysis

The paired two-tailed Student’s t test and the Pearson product moment correlation coefficient were used for statistical analysis. Statistical significance was defined as p < 0.05.

Results

CREM binding to the c-fos promoter is increased in SLE T cells

Antigenic stimulation of T cells leads to CAMP-dependent protein kinase A-mediated phosphorylation of the CREB that in turn induces the expression of the c-fos gene. The site that CREB binds to the c-fos promoter is a CRE located on the −57 site. The same CRE is the target of CREM binding to the promoter, as shown in JEG-3 cells (12).

To determine whether CREM that is overexpressed in unstimulated SLE T cells binds to the c-fos promoter in live SLE T cells, we performed ChIP assays using anti-CREM Ab (Fig. 1). Eight of 10 patients with SLE had increased CREM binding to the c-fos promoter (mean relative density of immunoprecipitated DNA levels as assessed by PCR: SLE, 2046 ± 564; control, 801.8 ± 207.2; p = 0.05). As control, ChIP using an unrelated anti-E47 Ab showed no binding to the c-fos promoter. These results demonstrate that CREM binding to the c-fos promoter is significantly
increased in live unstimulated SLE compared with control T cells. Furthermore, we performed ChIP with anti-CREB Ab and found no specific pattern difference between SLE and control T cells in CREB binding to the c-fos promoter (Fig. 1d).

To ascertain that CREM indeed binds to the −57 site of the c-fos promoter in T cells, we performed EMSA using a 32P-labeled oligonucleotide containing the −57 CRE sequence and nuclear protein extracted from unstimulated SLE T cells (Fig. 2a). The EMSA indeed showed a band that can be shifted with anti-CREM Ab, but not an unrelated anti-CBP Ab. Supershift was also seen with anti-CREB Ab, indicating that CREB and CREM can bind to the c-fos −57 site as a heterodimer. Similar results were seen with extracts from control T cells (Fig. 2b).

c-fos up-regulation after stimulation in T cells is impaired in SLE patients

To determine whether the increased CREM binding to the c-fos promoter is associated with impaired c-fos production after stimulation, we incubated T cells from SLE patients and control individuals with PMA and calcium ionophore. Real-time RT-PCR was used to assess c-fos mRNA levels, and Western blot to assess c-fos protein levels. Kinetic studies (Fig. 3a) show that c-fos mRNA in both SLE and control T cells is up-regulated within 10 min, reaching its peak at 30 min, and returns to baseline levels at 1 h after stimulation. Similarly, Fig. 3b shows that following cell stimulation c-fos protein production is quickly up-regulated, reaching a plateau at 1 h. These experiments show that the kinetics of c-fos production upon stimulation in our system is similar to previously published data.

Using RT-PCR, we then calculated the peak levels of c-fos mRNA in SLE and control T cells 30 min after stimulation with PMA and calcium ionophore. GAPDH mRNA levels were determined also, and the ratio of c-fos to GAPDH mRNA was compared between SLE and control T cells. As shown in Fig. 4, c-fos mRNA production in SLE T cells is markedly decreased when compared with controls (mean ratio of c-fos-GAPDH mRNA: SLE, 0.30 ± 0.09; control, 0.54 ± 0.11; p = 0.02). We also analyzed mRNA levels in unstimulated T cells from SLE patients and controls (data not shown). Because the mRNA levels were very low, it was impossible to reach a conclusion regarding true differences between SLE T cells and controls, although there was a trend for higher basal c-fos mRNA levels in controls (basal c-fos mRNA production in SLE T cells is markedly decreased when compared with controls (mean ratio of c-fos-GAPDH mRNA: SLE, 0.30 ± 0.09; control, 0.54 ± 0.11; p = 0.02). We also analyzed mRNA levels in unstimulated T cells from SLE patients and controls (data not shown). Because the mRNA levels were very low, it was impossible to reach a conclusion regarding true differences between SLE T cells and controls, although there was a trend for higher basal c-fos mRNA levels in controls (basal c-fos mRNA production in SLE T cells is markedly decreased when compared with controls (mean ratio of c-fos-GAPDH mRNA: SLE, 0.30 ± 0.09; control, 0.54 ± 0.11; p = 0.02). We also analyzed mRNA levels in unstimulated T cells from SLE patients and controls (data not shown). Because the mRNA levels were very low, it was impossible to reach a conclusion regarding true differences between SLE T cells and controls, although there was a trend for higher basal c-fos mRNA levels in controls (basal c-fos mRNA production in SLE T cells is markedly decreased when compared with controls (mean ratio of c-fos-GAPDH mRNA: SLE, 0.30 ± 0.09; control, 0.54 ± 0.11; p = 0.02).
As mentioned above, early after T cell stimulation, the c-jun/c-fos AP-1 binding in SLE T cells is lower than controls. The mRNA was isolated, reverse transcribed, and subjected to PCR using specific primers for the c-fos and the GAPDH genes, as described in Materials and Methods. a, Representative experiments showing the c-fos and GAPDH mRNA levels as measured by RT-PCR in SLE and control T cells. The density of the bands was measured by densitometry, and the background was subtracted; subsequently, the ratio of c-fos-GAPDH mRNA was calculated. b, Means and SEM of c-fos-GAPDH mRNA from 13 patients and controls are shown here. The results are statistically significant (p = 0.02).

mRNA copies as assessed by real-time PCR: SLE, 17.9 ± 15.5; control, 48.7 ± 35.7; p = 0.16).

Next, we determined the levels of c-fos protein in nuclear extracts from SLE and control T cells stimulated with PMA and calcium ionophore for 60 min using Western blotting. hnRNP nuclear protein levels were used as control, and the ratio of c-fos to hnRNP was compared between SLE and control T cells. As shown in Fig. 5a, SLE T cells had significantly decreased c-fos protein levels in their nucleus (ratio of c-fos-hnRNP nuclear protein levels: SLE, 1.37 ± 0.25; control, 1.87 ± 0.35; p = 0.04). Along the same lines, when we used anti-CD3/CD28 and anti-mouse cross-linking Ab to stimulate the cells, T cells from three SLE patients had decreased c-fos protein levels in their nucleus relative to controls (one of three representative experiments in T cells from the three SLE/control pairs is shown in Fig. 5a). Similar results were observed when we compared the cytoplasmic levels of c-fos protein in SLE and control T cells (Fig. 5c). β actin protein levels were used as control, and the c-fos to β actin ratio was calculated. Fig. 5d depicts the significant decrease in the ratio of c-fos to β actin protein levels in the cytoplasm of SLE when compared with control T cells (mean ratio of c-fos-actin cytoplasmic protein levels: SLE, 3.52 ± 0.44; control, 5.94 ± 0.47; p = 0.01). Taken together, these results demonstrate that following activation of the T cell, the expected early up-regulation of c-fos expression is significantly impaired in SLE. On the contrary, c-fos protein levels in T cells from four RA patients did not significantly differ from the controls, as shown in Fig. 5e (mean ratio of c-fos-actin cytoplasmic protein levels: RA, 2.31 ± 0.39; control, 1.68 ± 0.34; p = 0.13; mean ratio of c-fos-actin nuclear protein levels: RA, 12.08 ± 3.93; control, 12.79 ± 5.09; p = 0.92).

**AP-1 binding in SLE T cells is lower than controls**

As mentioned above, early after T cell stimulation, the c-jun/c-fos dimer forms and binds strongly to the TRE motif containing AP-1 binding sites. Because c-fos expression upon stimulation was found to be deficient in SLE T cells, we hypothesized that this would result in decreased overall AP-1 transcription factor binding. Accordingly, we constructed an oligonucleotide containing the consensus AP-1-binding TRE motif. EMSA using this oligonucleotide (Fig. 6a) and nuclear protein extracts showed a band that was shifted with both anti-c-jun and anti-c-fos Abs, but not an unrelated anti-GKLF Ab. This experiment demonstrates that our construct binds specifically to the c-fos/c-jun heterodimer, the main AP-1 complex. Fig. 6, b and c, shows the results of EMSA using this AP-1 consensus oligonucleotide labeled with 32P and nuclear extracts from SLE and control T cells that have been stimulated for 1 h with PMA and calcium ionophore. The SLE T cells had a significantly lower poststimulation AP-1 binding when compared with controls (mean AP-1 binding as assessed by the EMSA
band density: SLE, 2168 ± 430.7; control, 3590 ± 421; p = 0.03). AP-1 binding in T cells from patients with RA showed no clear difference from controls (mean AP-1 binding as assessed by the EMSA band density: RA, 1570 ± 644; control, 1721 ± 262; p = 0.8). Thus, the decreased expression of c-fos in stimulated SLE T cells results in decreased AP-1 binding.

Antisense CREM up-regulates c-fos mRNA and AP-1 binding
To determine whether the increased binding of CREM to the c-fos promoter and the decreased production of c-fos and AP-1 binding upon stimulation are directly related, we transfected Jurkat cells with either a control plasmid or a plasmid encoding antisense CREMα. Fig. 7 shows an increase in the c-fos mRNA level and the

FIGURE 6. AP-1 binding is decreased in stimulated SLE compared with control T cells. a, T cell nuclear protein extracts from an SLE patient were incubated with a radiolabeled oligonucleotide encoding two TRE sites (AP-1 binding sites) in the absence or presence of anti-c-fos, anti-c-jun, or anti-GKLF Abs, as described in Materials and Methods. The same was performed in the presence of the same oligonucleotide in excess as well as the presence of an unrelated elf-1-binding oligonucleotide in excess. b, T cell nuclear protein extracts from SLE patients (L), and controls (N) were incubated with the same radiolabeled oligonucleotide encoding two TRE sites, as described in Materials and Methods. A representative experiment is shown. The density of the bands was measured by densitometry, and the background was subtracted. c, Means and SEM of AP-1 binding from 10 SLE patients and 10 controls are shown. The results are statistically significant (p = 0.03).

FIGURE 7. Jurkat cells were transfected with plasmids encoding antisense CREM or empty vector. The cells were cultured for 24 h and then subjected to stimulation with PMA and calcium ionophore, as described in Materials and Methods. a, Jurkat cells transfected with control plasmid or antisense CREMα were stimulated with PMA and calcium ionophore for 30 min. The mRNA was isolated, reverse transcribed, and subjected to PCR using specific primers for the c-fos and the GAPDH genes, as described in Materials and Methods. The ratios of c-fos-GAPDH mRNA are shown under each specimen’s blot. b, T cell nuclear protein extracts from Jurkat cells transfected with either control plasmid or antisense CREMα and stimulated with PMA and calcium ionophore for 60 min were incubated with a radiolabeled oligonucleotide encoding two TRE sites (AP-1 binding sites), as described in Materials and Methods. The density of the bands was measured by densitometry, and the background was subtracted.

AP-1 binding in cells that were transfected with the antisense CREMα-encoding plasmid compared with cells transfected with a control vector and stimulated with PMA and calcium ionophore (ratio of c-fos-GAPDH mRNA: transfected with antisense CREM, 0.70; control, 0.51; AP-1 binding as assessed by the EMSA band density: transfected with antisense CREM, 3212; control, 1149). Because antisense CREMα causes a decrease in the levels of CREMα protein (11), these data indicate that CREMα is involved directly in the transcriptional repression of c-fos gene that results in decreased c-fos protein and AP-1 binding.

c-fos mRNA is decreased in active SLE patients
Because SLE patients in this cohort were at different stages of their disease, we evaluated the effect of disease activity on the expression of c-fos. Fig. 8 shows that the poststimulation c-fos mRNA levels are significantly decreased in active SLE patients (patients with SLEDAI of 4 or more) compared with both inactive patients (SLEDAI <4) and controls (mean ratio of c-fos-GAPDH mRNA levels: control, 55.39 ± 12.99 (n = 13); inactive SLE patients, 44.16 ± 15.50 (n = 7); active SLE patients, 13.35 ± 4.98 (n = 6); p = 0.03). Furthermore, a decrease in the SLEDAI was observed in two patients who were studied twice over time, and this was associated with an increase in the levels of c-fos mRNA. The first patient (L13) had a decrease of SLEDAI from 8 to 4 with a concomitant increase in c-fos-GAPDH mRNA ratio from 3.95 to 35.85, and the second patient (L10) had a decrease of SLEDAI from 2 to 0 with a concomitant increase in c-fos-GAPDH mRNA ratio from 17.43 to 89.16.

On the contrary, there was no association between the levels of c-fos mRNA and the type and dose of medications that the patients were receiving at the time of the study. Specifically, the prednisone dose did not correlate with the ratio of c-fos-GAPDH mRNA levels (Pearson product moment correlation coefficient: −0.271, p = 0.37) or AP-1 binding activity (Pearson product moment correlation coefficient: −0.373, p = 0.288).

Discussion
The present study shows that CREM binding to the c-fos promoter, in unstimulated live T cells from SLE patients, is significantly increased. Increased binding results in decreased transcription of c-fos gene, decreased production of c-fos protein, and AP-1 binding in the nuclei of SLE T cells. Of importance is the finding that
interference with the translation of the c-fos mRNA using antisense CREM vector resulted in increased c-fos protein levels and AP-1 binding. Besides reaffirming that CREM can suppress the transcription of c-fos gene in human T cells, as is the case with JEG-3 cells (12), our experiments demonstrate a mechanism whereby CREM contributes to decreased binding of an important transcriptional enhancer AP-1.

This decreased induction of c-fos was mainly observed in active SLE patients, underscoring the fact that this is probably a secondary phenomenon directly related to the activity of the disease. Kliman et al. (22) have shown decreased c-fos mRNA in PBMC from SLE patients 90 min after mitogenic stimulation. Other investigators have looked also at the levels of c-fos mRNA in unstimulated T cells from patients with SLE. Although the spontaneous expression of c-fos was lower in bone marrow extracts from patients with SLE when compared with controls (23), there was no difference in the levels of c-fos mRNA in unstimulated peripheral blood mononuclear cells (24). In contrast to SLE patients, patients with RA have significantly higher expression of c-fos (25, 26) and AP-1 (27) than normal individuals.

Multiple signaling abnormalities have been recognized in SLE. Upon Ag binding, the TCR/Ca2+ complex triggers a rapid rise in intracellular calcium (2). This rise in calcium is significantly higher and accelerated in SLE T cells as compared with controls (28). The substitution of TCR ζ-chain by the FcRγ chain in SLE T cells (29) contributes to the increased calcium response in SLE T cells. In T cells, calcium acts as a second messenger, leading to the activation of NF-AT-responsive genes, including the IL-2 gene. But, despite the fact that the SLE T cells show this particularly robust calcium response, they have a phenotype similar to anergic cells with deficient in vivo response to new and recall Ags as well as in vitro responses to recall Ags and mitogens (28) and deficient production of IL-2 upon activation (4). It is well recognized now that a second stimulus initiated by the CD28-B7 interaction and resulting in the activation of the transcription factors AP-1, CREB, and NF-κB is essential for the induction of IL-2 and the propagation of the immune response. In SLE T cells, the decrease of NF-κB and the increase of CREM are responsible for the decreased expression of IL-2. CREM further affects a second stimulatory pathway by blocking the expression of c-fos and the activation of AP-1, as shown in this study.

The imbalance between an overactive Ca2+-mediated signaling and impaired AP-1 activation has broader consequences. As mentioned earlier, Ca2+ influx in the T cell results in the activation of NF-AT. AP-1 interacts with NF-AT, forming complexes that up-regulate several cytokine gene promoters, including IL-2, IL-3, IL-4, GM-CSF, Fas ligand, and MIP1α (30). The up-regulation of such genes is important for the productive immune response. In the absence or decrease of AP-1 (particularly the inducible c-fos-jun dimer), the NF-AT:AP-1 complexes are not formed. Thus, NF-AT can only up-regulate genes such as TNF-α and IL-13 (30, 31) that do not require the NF-AT:AP-1 complex to form. Along the same lines, T cells that have activated NF-AT while lacking sufficient AP-1 do not undergo AICD as readily (30). Thus, the activation of NF-AT with impaired AP-1 activation results in a nonproductive immune response.

In the SLE T cell paradigm, a relative imbalance between NF-AT and AP-1 activation can explain the dichotomy between high calcium responses and deficient production of IL-2. It can also help explain the in vivo and in vitro abnormal T cell responses to Ags (28) and the deficient AICD (3, 32). In the same context, Th2 cell cytokine production would be skewed toward IL-10, a cytokine that has been found to be elevated in SLE patients (33). Interestingly, IL-10 is associated with in vivo spontaneous apoptosis of T cells (34), a mechanism that can explain the lymphopenia seen in SLE patients. It has also been proposed that this increased lymphocytic apoptosis can flood the circulation with nuclear and cytoplasmic autoantigens (28, 34), leading to perpetuation of the abnormal immune response.

The study of the molecular aberrations in SLE T cells has suggested approaches to correct the production of IL-2. We have shown that replenishing the TCR ζ-chain of the TCR (35) in SLE T cells results not only in normalization of the calcium responses, but also in the restoration of IL-2 production. Also, replenishment of the missing NF-κB p65 subunit in SLE T cells (10) results in increased IL-2 production. Along the same lines, the elimination of CREM by introducing an antisense CREM plasmid in SLE T cells (11) resulted in the increase of IL-2 production.

In conclusion, this study has shown that the increased CREM expression in SLE T cells can limit the expression of c-fos that is a key component of the transcriptional enhancer AP-1. Therefore, CREM contributes to the decreased expression of IL-2 by both binding directly to the IL-2 promoter (7) and limiting the AP-1 binding by the c-fos promoter.

References


