CD4$^+$ T Cells from Simian Immunodeficiency Virus Disease-Resistant Sooty Mangabeys Produce More IL-2 Than Cells from Disease-Susceptible Species: Involvement of p300 and CREB at the Proximal IL-2 Promoter in IL-2 Up-Regulation[1]

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IL-2 is an important cytokine required for the physiological function of CD4$^+$ T cells. Immunological unresponsiveness—anergy—of CD4$^+$ T cells is characterized by the inability of these cells to synthesize IL-2. Both progressive HIV infection leading to AIDS in humans and SIV infection in rhesus macaques (RM) are associated with dysregulation of IL-2 synthesis. In certain nonhuman primate species, such as sooty mangabeys (SM), SIV infection does not lead to AIDS. We have shown that this is associated with the resistance of the CD4$^+$ T cells from SM to undergo anergy in vitro. In this study, we show that CD4$^+$ T cells from SM spontaneously synthesize 2- to 3-fold higher levels of IL-2 than corresponding cells from RM. Proximal IL-2 promoter constructs derived from SM show significantly higher activity than the RM-derived constructs in primary CD4$^+$ T cells, which is associated with an element at approximately nt −200. Activity of both constructs was up-regulated by p300 and down-regulated by CREB to a similar degree. Chromatin immunoprecipitation analysis showed significantly higher binding of p300 and lower binding of CREB to the SM promoter in vivo. Two single nucleotide substitutions present in the SM sequence around position −200 and −180 seem to increase the affinity of these sites for the binding of transcription factors, one of which was identified as Oct-1. These unique characteristics of the proximal IL-2 promoter in SM therefore can represent one of the mechanisms contributing to the resistance of these cells to undergo anergy. *The Journal of Immunology,* **2007, 178: 7720–7729.**

Gradual loss of Ag-specific T cell responses, immunological anergy, in both HIV-1-infected humans and SIV-infected rhesus macaques (RM)[3] (1–5) is associated with an impairment of the immune system and is characteristic for progressive HIV infection of humans and pathogenic SIV infection in RM. In SIV-infected RM, immunological anergy is detectable in both the CD4$^+$ and CD8$^+$ cells before any detectable signs of CD4$^+$ T cell depletion (6). Interestingly, such loss of CD4$^+$ T cell responses with subsequent AIDS-like pathogenesis does not occur in naturally or experimentally SIV-infected sooty mangabeys (SM) despite significant plasma and cellular viral loads associated with disease progression in Asian macaques (5, 7). IL-2 is a key factor in multiple processes involved in the survival of T lymphocytes and in the regulation of T cell responses. It plays an important role in the regulation of anergy because the anergic CD4$^+$ T cells are characterized by their inability to proliferate and express IL-2 following TCR-specific stimulation in the presence of adequate costimulation (8, 9). It has been proposed that anergy results from TCR activation in the absence of IL-2 production (10). Conversely, IL-2-induced cell cycle progression from G_1 to S phase, even without proliferation, is sufficient to prevent anergy (11). In addition, it was shown that CD4$^+$ T cells from HIV-infected patients exhibit deficient IL-2 autocrine function, and the addition of exogenous IL-2 is sufficient to correct cell cycle abnormalities characteristic for these cells (12). In anergic T cells, the down-regulation of IL-2 gene expression is achieved by an active process (13). This process involves regulatory elements located within the proximal IL-2 promoter, and one of the sites, the −180 AP1-like site in mice, was shown to be essential for anergy induction (14, 15).

Interestingly, the CD4$^+$ T cells from the SIV disease-resistant SM are resistant to the induction of immunological anergy; they show an anergy-resistant phenotype, which was shown to predominantly reside in the central memory CD4$^+$ T cell population (5, 16). This phenotype is characterized by their ability to produce significant levels of IL-2 even when stimulated via TCR alone. Increased IL-2 production may be beneficial also to the regulatory T cell subset, which was recently described to express the high-affinity receptor for IL-2, and subsequently contributes to the low apparent immune activation seen in SIV-positive SM (7, 17). Previous sequence analysis of the −180 AP-1-like site from macaques and mangabeys revealed a single nucleotide substitution within this region only in SM that target this regulatory site (5).

In efforts to further understand the molecular mechanisms that underlie these functional differences present in CD4$^+$ T cells from SM that may have important implications in the immune response and resistance to AIDS in these monkeys, we conducted a more

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Received for publication August 3, 2006. Accepted for publication March 27, 2007.

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[1] This work was supported by National Institutes of Health Grants R01 AI65362 (to P.B.) and R01 AI51994 (to A.A.A.).

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[3] Abbreviations used in this paper: RM, rhesus macaque; ChIP, chromatin immunoprecipitation; SM, sooty mangabey; NHP, nonhuman primate.

detailed study of the IL-2 promoter in SM. In this study, we show that T cells from SM show significantly higher baseline production of IL-2, which correlates with higher baseline activity of the IL-2 promoter proximal fragment. A regulatory site immediately upstream of the −180 AP-1-like site seems to directly contribute to this higher activity. The higher baseline production of IL-2 is accompanied by increased p300 and decreased CREB binding in vivo to the proximal promoter region in $CD4^+$ T cells from SM. These results suggest that these unique characteristics of the IL-2 promoter contribute to the anergy-resistant phenotype of $CD4^+$ T cells from this species.

Materials and Methods

Cells

The peripheral blood samples were obtained from normal healthy adult RM (*Macaca mulatta*) and adult healthy SM (*Cercocebus atys*) housed at the Yerkes Regional Primate Research Center of Emory University. All animals were maintained according to the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, and the Health and Human Services guidelines "Guide for the Care and Use of Laboratory Animals." PBMC were isolated using Lymphocyte Separation Medium (Mediatech). The human PBMC were obtained from healthy human adult volunteers. $CD4^+$ T cells were isolated from PBMC using Dynabeads M450 CD4 (Dynal Biotech). The purity of the cell population was always >95%, as determined by FACS analysis.

Cloning of promoter sequences

DNA was isolated from PBMC samples using the Wizard Genomic DNA purification kit (Promega). IL-2 promoter sequences were amplified from 10 animals from each species using primer pairs as follows: 5′-CTG GCAATAAGGGCTGAGTG-3′ and 5′-GAGGTTACTGTGAGTAGT GAT-3′. Amplification was performed using the Advantage2 PCR kit (BD Clontech) in 25 cycles (95°C for 30 s; 58°C for 90 s). PCR products were directly cloned into the pGEM-T vector (Promega) and sequenced using SP6, T7, and internal primers 5′-GCTTCAGGTACTGTTTAACGC-3′ and 5′-GAGTATTTAACAATCGCACCCC-3′. Sequences were analyzed using the DNASTAR analysis package and submitted to the GenBank (accession numbers EF457240 and EF457241). Sequences were analyzed for putative transcription factor binding sites using the TFSEARCH software (Y. Akiyama; Searching Transcription Factor Binding Sites; http://www.rwcp.or.jp/papial/) and the TRANSFAC database (18).

Analysis of IL-2 production

The ICC analysis for IL-2 was performed, as previously described (19), using allophycocyanin-conjugated anti-IL-2 mAb (clone MQ1-17H12; BD Biosciences).

The IL-2-dependent HT-2 cell line (HT-2; American Type Culture Collection) was IL-2 starved for 24 h before assay and then cultured in 96-well microtiter plates (10^5 cells/well). The cells were then cultured alone or cocultured with PBMC from RM or SM (5×10^4/well) in triplicate wells. PBMC from SM or RM were cultured alone in parallel as a control for background proliferation. In select experiments, IL-2R-blocking Ab (clone M-A251; BD Biosciences) was added to the well at concentration 1 μg/ml. The microculture plates were then incubated for 72 h at 37°C, 7% CO_2-humidified atmosphere, and to each well was added 20 μl of medium containing 1 μCi of [^{3}H]thymidine (NEN). The cultures were harvested 16 h later using a Wallac harvester and a betaplate reader. The mean uptake of [^{3}H]thymidine by triplicate cultures was calculated, and the SD was derived. Addition of 1 μg/well anti-IL-4 to the supernatant from the sooty mangabey $CD4^+$ T cells did not appreciably alter the results denoting that the proliferation of the HT-2 cell line was not likely due to the presence of IL-4.

IL-2 promoter constructs and transfections

The proximal IL-2 promoter fragments from SM and RM were PCR amplified from the pGEM clones using primers IL2PU 5′-GCGAGATCTGT GAGTAGTGATTAAAGAG-3′ and IL2PD 5′-CGCGGTACCTCTTGTC CACCACAATATG-3′; amplified fragments were digested with *Kpn*I and *Bgl*II and cloned into pGFPemd-b reporter plasmids (Packard Instrument) to generate pIL2pSM and pIL2pRM. Hybrid reporter constructs (Fig. 3*A*) containing the SM-proximal IL-2 promoter sequences upstream of the −200 site spliced with the RM IL-2 promoter sequences downstream of the −200 site and vice versa, pIL2pSM/RM and pIL2pRM/SM, were obtained by PCR mutagenesis using primers 5′-AATGGATGTAGGTGAAATC CCTC-3′ and 5′-GAGGGATTTCACCTACATCCATT-3′ with either IL2PD or IL2PU to generate upstream and downstream fragments, respectively. These products were subsequently spliced together by a second round of PCR with IL2PU and IL2PD primers and cloned into pGFPemd-b, as described above. The construct pIL2PSMDBL was obtained similarly by first generating 5′ and 3′ parts of the SM-proximal promoter using primers 5′-CTGACAGAATGGATGACCCCCAAAGACTGACA-3′ and 5′-CATCCATTCTGTCAGTCTTTGGGGGT-3′ together with IL2PU or IL2PD and subsequent PCR splicing, as above, leading to a duplication of the −180 site from SM. The plasmids expressing CREB and dominant-negative A-CREB were gifts from M. Green (University of Massachusetts, Worcester, MA) and C. Vinson (National Cancer Institute, Bethesda, MD) (20, 21). Plasmids expressing p300 were a gift from K. Gardner (22). Transfections of primary enriched human $CD4^+$ T cells were performed using the Amaxa transfection technology and the Amaxa human CD4 T cell transfection kit (Amaxa Biosystems), according to the manufacturer's instructions. Transfection efficiencies for all of the experiments were between 30 and 40%. Cells were cultured for 48 h after transfection in RPMI 1640 (Life Sciences) medium supplemented with 10% FCS, L-glutamine, and gentamicin (Life Sciences). The mean fluorescence intensity (MFI) was quantitated by flow cytometry. Experiments were always performed in triplicates and mean values ± SD derived.

Nuclear extracts and EMSA

Nuclear extracts were prepared from enriched $CD4^+$ T cells from SM and RM or from the A301 lymphocytic cell line by first incubating the cells in 10 mM HEPES (pH 8.0), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and protease inhibitors Complete tablet (Roche), 3 μg/ml pepstatin, and 1.5 mM DTT for 15 min on ice. Cells were then lysed by adding an equal volume of the same buffer containing 2% Triton X-100, and nuclei were collected by centrifugation at 10,000 rpm for 2 min. The nuclear pellet was then incubated on ice in a buffer containing 20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, protease inhibitors, and DTT for 30 min at a concentration of 10^5 cell equivalents/μl. The nuclear lysate was spun down at 20,000 × *g* for 20 min, and the supernatant (nuclear extract) was stored at −70°C until use. EMSA were performed using 6% acrylamide gels in 0.5× TAE buffer (0.04 M Tris acetate, 0.001 EDTA). Typically, 2-μl aliquots of nuclear extracts were incubated with 20,000 cpm of ^{32}P-end-labeled double-stranded oligonucleotide probe (~10–20 fmol) with 60 ng of poly(dG-dC) in a buffer containing 10 mM Tris-Cl (pH 8.0), 50 mM KCl, 1 mM EDTA, 5% glycerol, 4 mM DTT, and 200 μg/ml BSA for 30 min at 22°C and then electrophoresed at 4°C. The oligonucleotides used as probes were as follows (sense strand): SM180, 5′-CATCCATTCTGTCA GTCTTTGGGGGT-3′ and RM180, 5′-CATCCATTCAGTCAGTCTTTG GGGGT-3′ (corresponding to the −180 IL-2 promoter site in SM and RM, respectively); SM210, 5′-CTAATGTAATCATGAGGGATTC-3′ and RM210, 5′-CTAATGTAACCATGAGGGATTC-3′ (corresponding to the −210 IL-2 promoter site in SM and RM, respectively). Oligonucleotides containing the Oct-1 and Sp1 consensus binding sites were obtained from Promega. In some experiments, in which oligonucleotides were used as competitors, the unlabeled oligonucleotides were added to the binding reaction with appropriate nuclear extracts and preincubated on ice for 10 min before the addition of a probe. Anti-Oct-1 and anti-Sp-1 Abs were obtained from Upstate Biotechnology and added to select experiments at 1 μl/reaction 10 min after the beginning of the incubation step for supershift analysis.

Chromatin immunoprecipitation (ChIP) assay

Cells were cultured in medium or activated with anti-CD3/CD28 immunobeads (Dynal Biotech) for 8 h, and chromatin-DNA complexes were then cross-linked by 1% formaldehyde for 10 min. Cells were then washed twice with cold PBS and lysed on ice for 5 min in a buffer containing 5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40, and protease inhibitors (as above). After centrifugation (3000 × *g* for 5 min), the nuclear pellet was lysed in a buffer containing 50 mM Tris-Cl (pH 8.1), 10 mM EDTA, 1% SDS, and protease inhibitors on ice at a concentration of 10^7 cell equivalents/ml. The chromatin lysate was sonicated to obtain fragments ~500 bp long. For each ChIP reaction, typically 80 μl of the lysate was diluted with 1.2 ml of ChIP dilution buffer containing 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16 mM Tris-Cl (pH 8.1), and 160 mM NaCl. The chromatin was then precleared with a mixture of protein A-Sepharose, 0.2 mg/ml tRNA (both Roche), and 0.2 mg/ml sheared herring sperm DNA (Promega) for 30 min at 4°C. After centrifugation to remove the Sepharose, aliquots of the chromatin were incubated with 5 μl of the appropriate Ab overnight at 4°C. No Ab and anti-*fnk* mAb (clone 53; BD Biosciences)

were used as negative and background controls, respectively. A 50-μl aliquot of the chromatin was removed before the addition of the Ab to be used as a control allowing for standardization for equivalent chromatin loading. Rabbit polyclonal anti-acetyl H3, anti-acetyl H4, anti-p300, and anti-CREB Abs were all purchased from Upstate Biotechnology. Protein A/tRNA/ssDNA mix was then added for 1 h to pull down the ChIP complexes. Protein A beads were then washed sequentially with (two washes in each buffer for 10 min): ChIP dilution buffer; subsequently with buffer containing 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl (pH 8.1), and 500 mM NaCl; then with buffer containing 100 mM Tris-Cl (pH 8.1), 500 mM LiCl, 1% Nonidet P-40, and 1% deoxycholic acid; and final wash was performed with 1×TE. Protein/DNA complexes were then eluted by incubating the beads twice for 15 min at room temperature in a buffer containing 50 mM $NaHCO_3$ and 1% SDS.

Protein/DNA cross-links in the ChIP samples and chromatin-loading controls were reversed by incubating the complexes with 0.2 M NaCl at 65°C overnight, and DNA was purified by standard phenol:chloroform extraction and ethanol precipitation. Real-time PCR was performed to quantify the immunoprecipitated DNA in an iCycler using SYBR Green fluorescence quantification (both Bio-Rad). Primers 5′-AGAAGGCGTTAATTGCAT GAATT-3′ and 5′-TCCTCTTCTGATGACTCTTTGGA-3′ were used for cycles of 95°C for 15 s and 60°C for 1 min. Serial dilutions of standards containing a known number of IL-2 promoter copies were analyzed with each PCR run to quantify the number of copies in each sample, which was subsequently adjusted relative to the chromatin-loading controls to normalize all of the samples to the standard chromatin input from the cultures. Each PCR analysis was performed in triplicate, and mean copy numbers were derived. Melt-curve analysis was performed in all of the assays to monitor the specificity of results in each sample.

Real-time PCR quantification of cDNA

cDNA samples from RM and SM (8–10 animals in each group) were prepared from RNA isolated from $CD4^+$ T cells from each animal ex vivo and subjected to real-time PCR in an iCycler (Bio-Rad) and SYBR Green fluorescence quantification using primers for IL2 cDNA: 5′-TGTCA CAAACAGTGCACCTACTTC-3′ and 5′-AATGTGAGCATCCTGGTG AGTTT-3′. As a control, an amplification of the GAPDH fragment was performed using the primers 5′-ACCACCATGGAGAAGGCTGG-3′ and 5′-CAGTTGGTGGTGCAGGAGGC-3′. Parameters of the cycle were 95°C for 15 s and 60°C for 1 min. The target cDNA quantitation in duplicate samples was then performed by first normalizing the threshold cycle number of the target gene to the GAPDH. The copy numbers of the target gene were then expressed relative to the calibrator samples assayed in each run. Each target sequence and GAPDH control was quantitated from two independent cDNA preparations from each sample/animal, and the resulting relative quantitation is expressed as an average of two measurements.

Results

Increased basal IL-2 production of SM $CD4^+$ T cells

Our laboratory has previously documented the proliferation and IL-2-secreting patterns in in vitro stimulated subpopulations of $CD4^+$ T cells from SM that were significantly different from those in RM and humans. These differential patterns were not secondary to differences in the frequency of naive, central memory, or effector memory $CD4^+$ T cells from the two species (5, 16). To further investigate whether at least some of these characteristics could be ascribed to the ability of the $CD4^+$ T cells from SM to produce higher baseline levels of IL-2, which may subsequently contribute to increased survival and well being of cells in the SIV-infected animals, we analyzed baseline IL-2 production in PBMC from RM and SM (Fig. 1). The cells were cocultured with the IL-2-dependent HT2 cell line (which allowed us to quantitate the relatively low levels of IL-2 produced by the PBMC at baseline) and the relative levels of proliferation of the cell cultures determined. As can be seen, the background proliferation of the IL-2-starved HT2 cells was very low (916 ± 18 cpm), and the background proliferation of the RM and SM PBMC cultured in medium alone was relatively comparable in both species (1640 ± 654 cpm and 2119 ± 529 cpm, respectively). However, whereas the levels of proliferation seen in the RM coculture with the HT2 cells remained low (1456 ± 542 cpm), the proliferation of SM cells cocultured

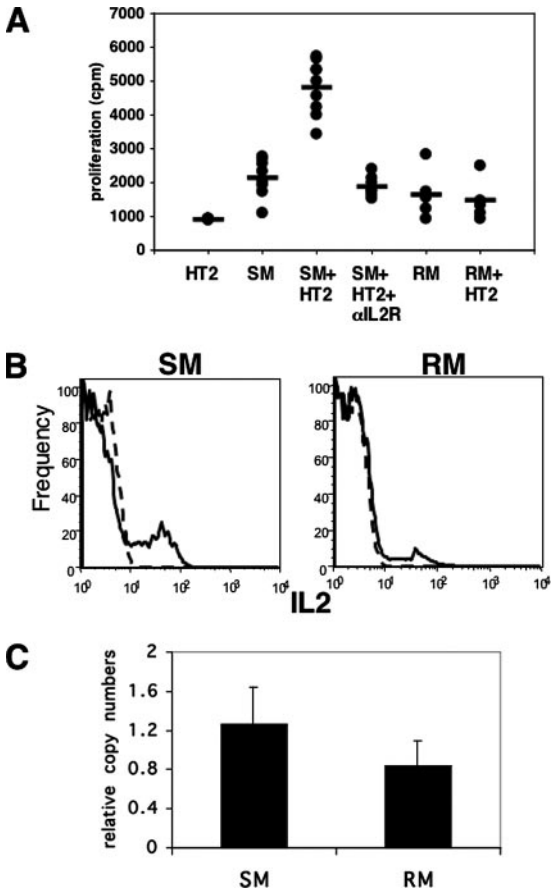

FIGURE 1. Expression of IL-2 by PBMC from nonhuman primates. *A*, PBMC from RM and SM were cultured alone or cocultured with the IL-2-starved IL-2-dependent HT-2 cell line. Proliferation was measured by the uptake of [^{3}H]thymidine in the HT2 cells alone (HT2), PBMC from RM and SM alone, and mixed cultures of HT2 and PBMC (SM + HT2, RM + HT2). As a control, PBMC from SM were cocultured with HT2 cells in the presence of anti-IL-2R mAbs (SM + HT2 + α-IL2R). Each data point represents mean value of triplicate assays performed with the cells from one monkey, and means for each experimental group are indicated by bars. *B*, Intracellular cytokine analysis for IL-2 expression in the representative $CD4^+$ T cell samples from RM ($n = 6$) and SM ($n = 6$). Dashed line: isotype/background control. *C*, $CD4^+$ T cells from SM ($n = 8$) and RM ($n = 10$) ex vivo were analyzed for the transcription of IL-2 by real-time PCR, normalized to GAPDH, and expressed as copy numbers relative to the calibrator sample. Error bars represent SD of values obtained from samples from different animals.

with the HT2 cells showed significantly increased proliferation ($p < 10^{-6}$) ~2.5 times (4812 ± 796 cpm). This was clearly due to the IL-2 production and subsequent stimulation via IL-2R because addition of the anti-IL-2R Abs completely inhibited this proliferation increase to the background levels (1880 ± 280 cpm). Intracellular cytokine staining confirmed that higher frequency of $CD4^+$ T cells from SM expressed increased levels of IL-2 (Fig. 1*B*). To investigate whether the increase in IL2 expression in SM-derived cells was at least in part due to the increase in transcription of IL2 mRNA, we performed real-time PCR quantitation of IL-2 message in $CD4^+$ T cells from both species. The data show (Fig. 1*C*) moderately higher number (~1.5-fold) of IL-2 copies in SM-derived cells, although this difference was not significant. This indicated that the $CD4^+$ T cells from SM produce higher levels of IL-2 even without any stimulation than the similar cultures from RM, and that these increased levels of IL-2 can have significant biological effects.

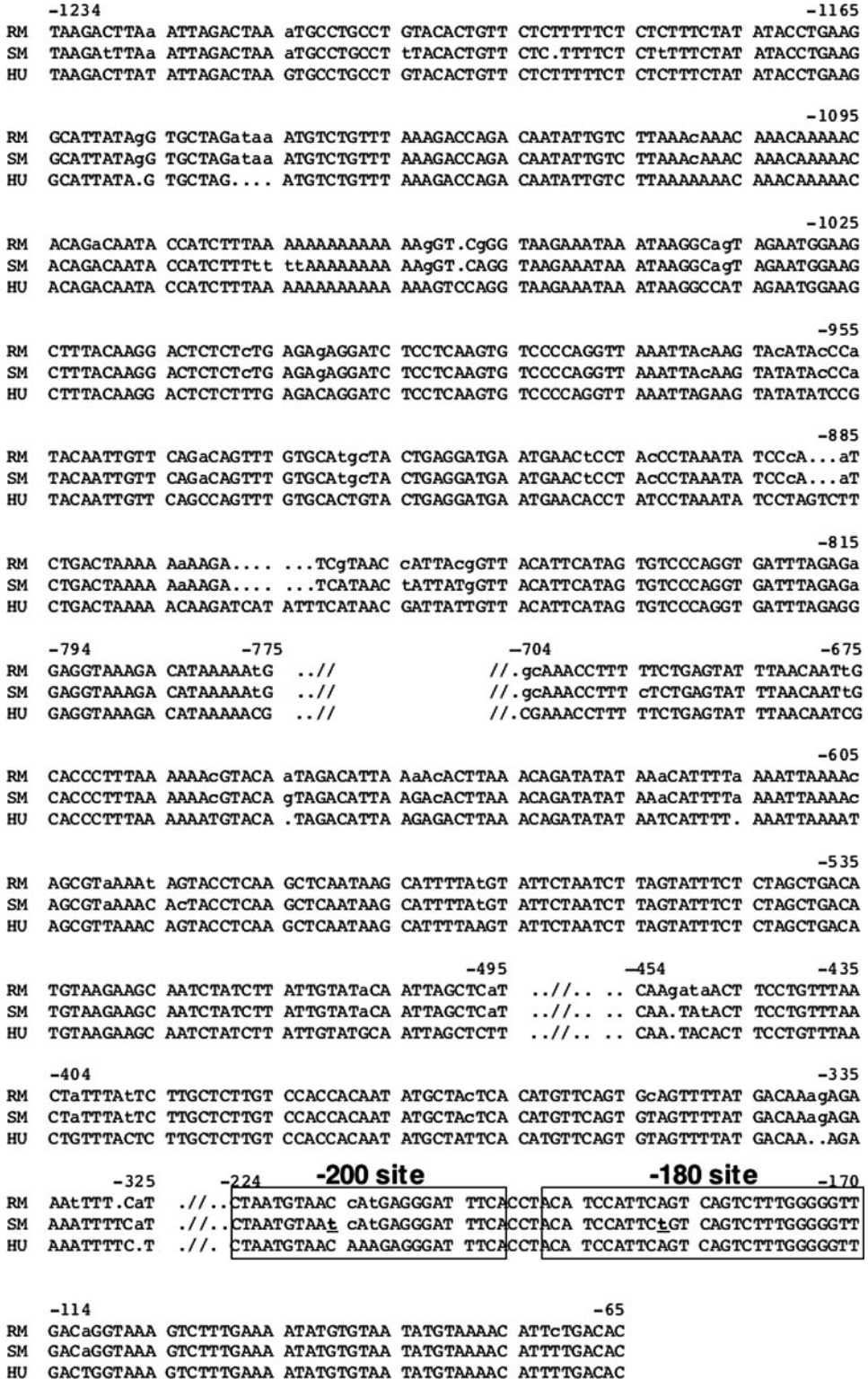

FIGURE 2. Sequence analysis of the IL-2 promoter. IL-2 promoter sequences from SM and RM were aligned to the corresponding human sequence (HU). The numbering reflects positions of the human sequence. Only regions showing variability of the NHP sequences are shown, and regions corresponding to the −180 and −210 sites are indicated by boxes.

Structure of the IL-2 promoter in RM and SM

These initial findings prompted us to perform detailed studies of the IL-2 promoter sequences from the two species with the hypothesis that such low levels of baseline IL-2 synthesis are sufficient to lead to the anergy resistance of $CD4^+$ T cells from SM. In this regard, several studies have reported that IL-2 secretion is regulated in anergic cells at least in part at the transcription level, and that the proximal promoter sequences (i.e., ~300 bp upstream of start site), specifically the −180 site, are critical for this regulation (14, 23). Our laboratory has also previously suggested potential correlation between differences in the sequences of a number of cytokine promoters with several phenotypic characteristics of $CD4^+$ T cells from the two nonhuman primate (NHP) species (24). Therefore, as a first step to gain insight into whether the differences in IL-2 production and associated phenotypic characteristics of $CD4^+$ T cells from SM could be based on the differential transcriptional regulation of IL-2, we cloned and sequenced ~1.3-kb fragments corresponding to the IL-2 promoter sequences

from RM and SM. As seen in Fig. 2, the majority of the sequence variability is contained within the upstream/distal part of the promoter. Thus, the most distal part between −1300 and −700 bp contains several relatively extensive deletions and insertions, as well as numerous 1- to 2-bp substitutions compared with the human sequence. Most of these are, however, present in both NHP species and therefore unlikely to be the basis for the differences in the outcome of SIV infection observed between these two species. The mid-part of the analyzed promoter sequence, i.e., −700 to −300 bp, shows less variability between the two species. Most of the differences noted were single base pair substitutions or insertions that were present either in both NHP species, or the SM sequence corresponded to the human sequence and therefore also unlikely to be the basis for a differential regulation in SM. The proximal promoter region of −300 to −1 bp shows the least variability of the whole analyzed fragment. It contains, compared with the human sequence, six substitutions in RM and only five substitutions in SM, three of which are concordant in both NHP species. However, the two SM-specific variable sites, which include the −187 T/A and the −215 T/C substitutions in SM, directly affect or lie in the close proximity to the −180 site previously shown to play a role in anergy (14, 23) and, therefore, reasoned to be potential candidates that may play a role in the observed SM-specific IL-2 synthesis and anergy phenotype.

Activity of the IL-2-proximal promoter and the role of −180 and −210 sites

It has been previously shown that an experimental 3-bp substitution spanning the area of the T/A substitution at position −187 in SM contributed significantly to a change in the regulation of the IL-2 promoter during anti-CD3-induced stimulation/anergy induction (23). In addition, whereas $CD4^+$ T cells from both humans and RM have been previously shown to manifest similar phenotypes with regard to IL-2 production and anergy resistance and because the −187 and −215 sites are the only two sequence variations in the proximal IL-2 promoter fragment that are discordant with both the human and RM sequences, it was hypothesized that these sequence variations had the highest potential to play a role in the observed phenotype of $CD4^+$ T cells from SM. As a next step, it was therefore important to further characterize what effect the two proximal promoter substitutions in SM may have on the promoter activity. To test the activity of the promoter, we prepared reporter constructs in which the GFP expression is driven by the proximal IL-2 promoter fragment from RM or SM (Fig. 3*A*) and transfected these constructs into primary human $CD4^+$ T cells. Primary human $CD4^+$ T cells as opposed to primary $CD4^+$ T cells from RM or SM were used because of our repeated failure to obtain consistent enough efficiency of transfection of nonhuman primate $CD4^+$ T cells (data not shown). Fig. 3 shows that the activity of the proximal IL-2 promoter from SM was consistently ~2-fold higher than the activity of the corresponding RM-derived promoter sequence (Fig. 3, *B–E*). This activity was clearly dependent on the methylation status of the promoter, because the hypermethylation of these constructs led to a decrease of the signal to background levels (Fig. 3*B*). To further characterize which of the two discordant variable sites may underlie this increased activity, we prepared hybrid promoter constructs by splicing together the upstream promoter part containing the −210 site from RM with the downstream part containing the −180 site from SM and vice versa. In addition, we made a construct in which we duplicated the −180 site in the SM promoter, reasoning that it would further potentiate and highlight any effects this site might have on the promoter activity (IL-2p DBL). Transient transfections of these constructs into primary $CD4^+$ T cells showed that the −180 site from SM by itself was not sufficient to induce the higher promoter activity because the hybrid containing the downstream part of the promoter from SM showed activity corresponding to the RM construct. However, the −210 site from SM was found sufficient to at least partially increase the activity of the SM promoter, because the activity of the SM/RM hybrid was higher than the activity of the native RM construct, although it was lower than the activity of the native SM construct, suggesting potential coordinate contribution of the two sites to the construct activity in SM. The –180 site alone, if anything, exerts probably a negative effect because the SM construct with the duplication at the −180 site showed levels of activity similar to the RM construct. The −180 site is in close proximity to the CD28-TRE element, in which p300 exhibits a strong up-regulatory effect during T cell activation and spans the distal AP-1 site shown to bind CREB as a negative factor during T cell anergy (22, 23). In efforts to investigate whether the sequence variations detected in SM affected p300- and CREB-mediated regulation of the IL-2 promoter, we performed cotransfection experiments of p300 and/or CREB expression constructs with the IL-2p constructs as reporters. Fig. 3*C* shows that overexpression of the p300 led to the up-regulation of activity of all tested constructs ~2-fold. This up-regulation was dose dependent (data not shown). However, the p300-induced activity of low expressing IL2p RM, IL2p RM/SM, or IL2p DBL constructs never reached the levels of stimulated activities of the high expressing IL-2p SM construct or IL-2p SM/RM hybrid. Overexpression of CREB (Fig. 3*D*), on the contrary, had a consistent negative effect, but only on the high expressing constructs IL2p SM or IL2p SM/RM, where it led to the down-regulation of their activity to the levels of other, low expressing IL-2p constructs. These data suggest that p300 has a positive regulatory function that is independent of the −187 sequence variability and that may be, at least in part, responsible for higher IL-2 promoter activity in SM. CREB, in contrast, has a clear negative effect on the activity of the high expressing constructs, and to further investigate whether it can function as a dominant-negative factor for the p300-mediated up-regulation, we cotransfected the IL-2p SM and RM constructs simultaneously with both CREB- and p300-expressing constructs (Fig. 3*E*). In addition to wt CREB, we also used a dominant-negative mutant A-CREB that dimerizes with wt CREB and prevents its binding to the DNA. The overexpression of CREB consistently inhibited p300-mediated up-regulation of IL-2p activity from both RM and SM reporters in a dose-dependent manner. Interestingly, however, so did A-CREB, and to even greater extent, suggesting that this effect is most likely mediated by CREB functioning in a polyprotein complex rather than binding directly to DNA.

Analysis of histone status and in vivo protein binding at the proximal IL-2 promoter

It was previously shown that chromatin modifications, such as histone acetylation and methylation, constitute important epigenetic mechanisms that regulate IL-2 transcription in T cells (25–28). To investigate whether these regulatory mechanisms could play a role in the differences related to the level of IL-2 expression between the two NHP species, we performed ChIP analysis of the proximal promoter fragments of the IL-2 promoters in primary $CD4^+$ T cells from RM and SM ($n = 5–6$ for each species). Cells were analyzed ex vivo (baseline) and after an 8-h stimulation in vitro to study potential differences in activated cells. Kinetic analysis was first performed in activated cells to identify the optimal time interval for the assessment (data not shown). The anti-acetyl histone 3 and

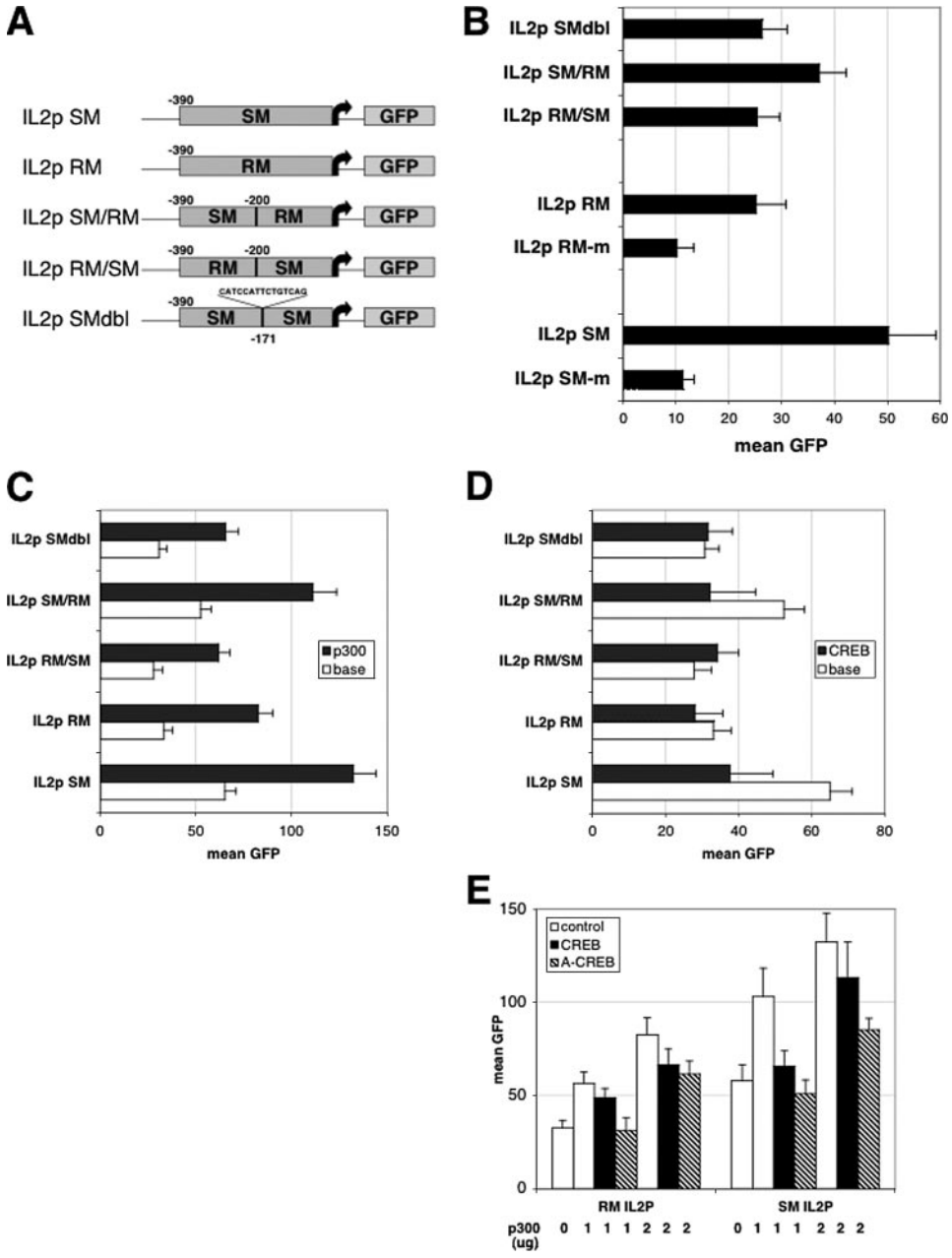

FIGURE 3. Transcriptional activity of the IL-2 promoters from RM and SM. *A*, Structures of GFP reporter vectors. *B*, Basal activity of the IL-2 promoter constructs. GFP constructs described in *A* were transfected into human primary $CD4^+$ T cells, and mean fluorescence intensity (mean GFP) was measured 48 h later by flow cytometry. IL-2p SM-m and IL-2p RM-m correspond to in vitro methylated IL-2p SM and IL-2p RM constructs, respectively. *C* and *D*, GFP promoter constructs were cotransfected with the p300 expression vector (*C*) or CREB expression vector (*D*), and mean fluorescence intensity was measured by flow cytometry 48 h later. *E*, GFP promoter constructs were cotransfected with the indicated amounts of the p300 expression construct and one of the following CREB expression constructs: wt CREB (CREB), dominant-negative CREB mutant (A-CREB), or empty vector (control) into $CD4^+$ T cells, and mean GFP intensity was determined by flow cytometry 48 h later.

anti-acetyl histone 4 Abs were used to assess acetylation status of both histones, and anti-dimethyl-lysin-K9 histone 3 Ab was used to assess methylation of H3 reported to play an important role in IL-2 regulation during T cell activation (29). Fig. 4 shows that there were no major significant differences between the histone acetylation and methylation of the IL-2 promoters assayed, except for a ~2-fold higher baseline level of H4 acetylation in RM. Otherwise, interestingly, the $CD4^+$ T cells from RM also showed a trend of higher baseline acetylation levels and lower methylation levels, which were, however, not statistically significant. Furthermore, we did not detect any significant differences in the acetylation characteristics within the cells at baseline and following anti-CD3/28 stimulation. The in vitro stimulation led only to a modest and nonsignificant decrease in the K9 methylation of H3 in SM and, surprisingly, to an increase in the H3 K9 methylation in RM. These results therefore did not suggest any significant and clear-cut differential effect of these epigenetic mechanisms in the promoter regulation in the two species. Because our data above indicated that p300 and CREB regulation may play an important role in the higher activity of the SM IL-2-proximal promoter, we decided to test whether there are any differences in the p300 and CREB occupancy of the proximal IL-2 promoter in vivo directly in primary cells from both species by ChIP (Fig. 4). ChIP analysis of p300 binding in cells from SM showed that in both baseline and stimulation induced p300 binding was reproducibly and significantly higher (5–6-fold) than the levels detected in RM. Conversely, levels of CREB binding to the IL-2 promoter, detected by ChIP were significantly lower (3- to 4-fold) in the $CD4^+$ T cells from SM than from RM. Stimulation of the cells did not lead to any significant changes in $CD4^+$ T cells from either species. These data were therefore predictive of the higher baseline IL-2 synthesis in SM. In addition to the higher activity of the IL-2 promoter in SM, higher levels of p300 binding would lead to further stimulation of the promoter, whereas low levels of CREB did not inhibit the activity of the SM promoter, which is otherwise more sensitive to CREB-mediated inhibition.

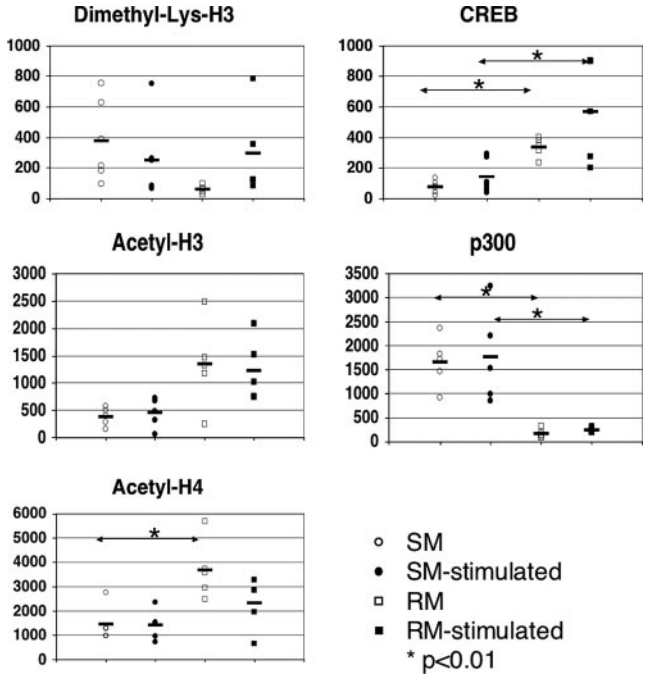

FIGURE 4. Histone status and binding of transcription factors to the IL-2 promoter in vivo. Protein/DNA complexes were isolated from $CD4^+$ T cells from SM (circles) and RM (squares) incubated in medium (open symbols) or with anti-CD3/CD28-coated beads (closed symbols) for 8 h, and chromatin was immunoprecipitated using anti-dimethyl-lysine-histone3, anti-acetyl-histone3, anti-acetyl-histone4, anti-CREB, or anti-p300 polyclonal Abs (indicated). The quantity of coprecipitated DNA sequences corresponding to the proximal IL-2 promoter fragment was determined by real-time PCR. Results for individual monkeys ($n < 5$) are presented with horizontal bars representing means for each group and asterisks indicating statistically significant differences ($p < 0.01$).

Binding of the transcription factors to the −180 and −200 sites

We have shown above that the differences in IL-2 production between $CD4^+$ T cells from SM and RM correlate with the differential binding of p300 and CREB to their respective IL-2 promoter sequences in the two species, and that the differences in the proximal promoter sequences, albeit very low, can account for the higher baseline activity in SM. We have putatively identified the −180 and −200 sites in the promoter sequence as those whose sequence variation between the species affects the baseline promoter activity in primary $CD4^+$ T cells in line with the levels of IL2 synthesized (Fig. 1). To further identify whether the two single nucleotide substitutions at the −180 and −200 IL-2 promoter sites in SM affect binding of regulatory factors, we performed comparative EMSA analysis with nuclear extracts generated from $CD4^+$ T cells from each of the two species. When the probe was derived from the sequence corresponding to the −180 site from SM (Fig. 5), we could see similar complexes forming with extracts from both species (*left panel*) and identical complexes formed when RM sequence was used as a probe (data not shown). Interestingly, however, when we tested the affinity of these two sequences to form these complexes by performing competition experiments, in which unlabeled competitor oligonucleotide was added together with the SM derived probe, we could observe that the SM-derived sequence competed out the protein complexes at ~5 times lower concentrations than the RM-derived competitor. This effect was highly reproducible specifically for the central complex (no. 2) and could be observed for both SM and RM nuclear proteins. Subsequently, we attempted to identify the proteins in these complexes by supershift assays using anti-CREM and anti-Oct-1 Abs. The complex at the top (no. 1) clearly contained CREM (as previously published (23)) because it was almost entirely supershifted by CREM-specific Ab. The Oct-1 Ab was chosen on the basis of our comparative sequence analysis using TFSEARCH, which indicated that a single nucleotide change in the sequence (present in SM) increases the homology to the consensus binding site for the Oct-1. This is also supported by the fact that the addition of the cold competitor oligo representing Oct-1 consensus binding site clearly competed out the complex no. 2 at very low concentrations (Fig. 5). However, the anti-Oct-1 Ab did not supershift any of the complexes. Identical data were obtained when RM-derived nuclear extracts were used for both the cold competition and supershift analyses (data not shown). Taken together, the −180 IL-2 promoter sites from both NHP species seem to form identical protein complexes, but the SM-derived sequence shows higher affinity for at least one of these complexes. This complex binds with high affinity to the octamer-binding sequence, but was not confirmed to contain Oct-1 by Ab supershift analysis.

Subsequently, we applied a similar comparative EMSA approach to analyze the binding properties of the −200 IL-2 promoter element. This element was shown to be responsible, at least in part, for the higher baseline activity of the SM-proximal IL-2 promoter. EMSA of nuclear extracts from both species with labeled probes corresponding to the −200 site from RM or SM showed that in any combination extract/probe a similar pattern of one dominant complex with a faint upper band is formed (Fig. 6, *left panel*). Once again, however, when we performed competition experiments, the SM sequence-derived competitor oligo competed the main complex at ~5-fold lower concentration, indicating that the SM oligo has a relatively higher affinity for this complex than

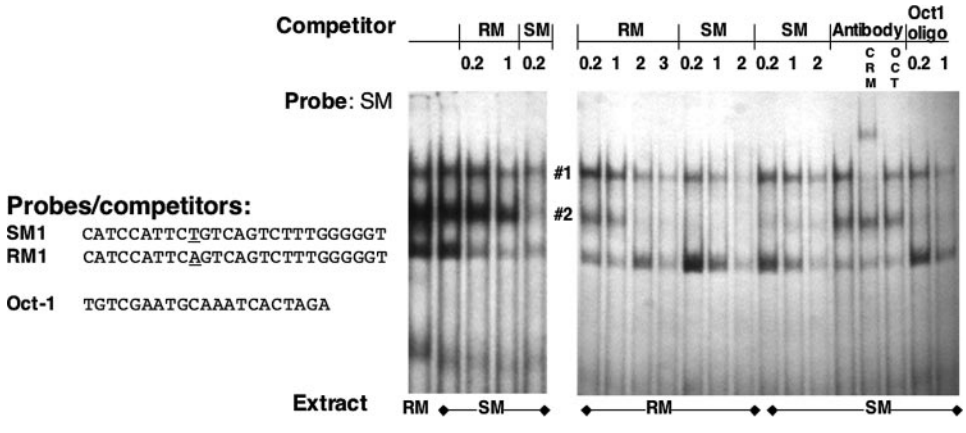

FIGURE 5. EMSA analysis of the −180 promoter segment. Nuclear extracts from $CD4^+$ T cells from RM or SM were incubated with a ^{32}P-labeled probe corresponding to the SM −180 IL-2 promoter sequence (SM1). EMSA was performed without (*two left lanes*) or with unlabeled competitor oligonucleotides RM1, SM1, or Oct-1 in concentrations indicated (in μM). Supershift analysis (Ab) was performed by incubation of probe-protein complexes with anti-CREM (CRM) and anti-Oct-1 (OCT) Abs. Complexes no. 1 and no. 2 are labeled.

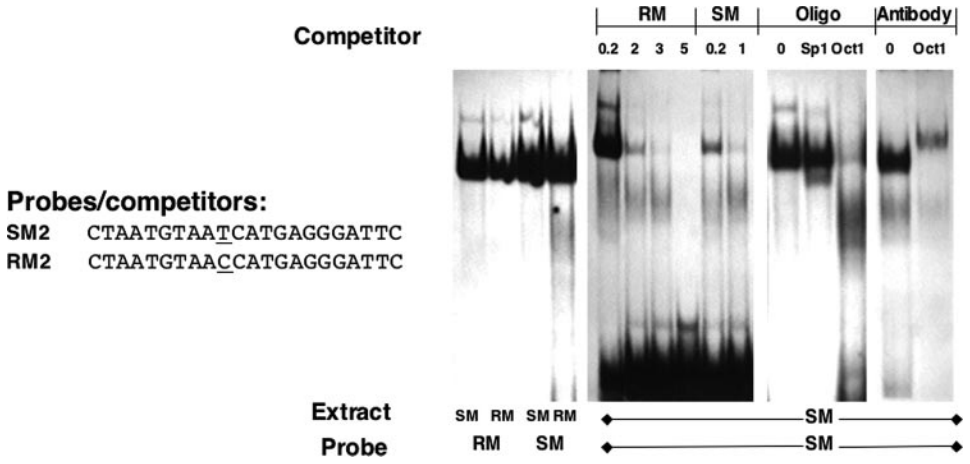

FIGURE 6. EMSA analysis of the −200 promoter segment. Nuclear extracts from $CD4^+$ T cells from RM or SM were incubated with a ^{32}P-labeled probe corresponding to either the RM or SM −200 IL-2 promoter sequence (RM2, SM2). EMSA was performed without (*left panel*) or with unlabeled competitor oligonucleotides RM2 or SM2 in concentrations indicated (μM). Competition analysis (Oligos) was performed by adding unlabeled Oct-1 or Sp1 consensus oligonucleotides in 1 μM concentrations. Supershift analysis (Abs) was performed by incubation of probe-protein complexes with anti-Oct-1 Abs.

RM-derived sequences (Fig. 6, *middle panel*). We subsequently attempted to identify the protein involved in this complex by both competition experiments and Ab supershift assays. The Oct-1 consensus oligo competed the main complex entirely at low concentrations (1 μM), whereas the addition of the Sp1 consensus oligo did not have any effect on the complex formation. In addition, the anti-Oct-1 Ab shifted the main complex in the supershift analysis (Fig. 6, *right panel*). These data therefore suggest that the −200 element binds to a single complex containing Oct-1 in both SM and RM, which, however binds to the SM sequence with significantly higher affinity, and this may present an additional mechanism that leads to higher baseline activity of the IL-2-proximal promoter in SM.

Discussion

Gradual impairment of immune responses characterized by T cell anergy with a deficient IL-2 autocrine function, followed later on by a decrease in $CD4^+$ T cell numbers, is one of the hallmarks of the development of AIDS (30). It has clearly been shown that T cell anergy is present in HIV-infected patients with opportunistic infections (31) and that HIV infection-induced defective IL-2 production in T cells can be partially restored by highly active antiretroviral therapy (32). Other studies showed that anergy in T cells can be induced by HIV-1 proteins gp120 (33) and Env (34) and can be reversed by IL-2 (35, 36).

It is clear, however, that whereas only a small fraction of $CD4^+$ T cells is infected by the virus, the decrease of T cell responses is broad and general, suggesting that the underlying mechanisms of this dysregulation are, at least in part, indirect, i.e., dysregulation affecting the whole system rather than only infected cells. Keeping that in mind, it is therefore reasonable to postulate that in the models of lentivirus infection, which exhibit resistance to the development of AIDS despite significant virus replication, such as SM, at least some of the mechanisms underlying this phenomenon of disease resistance represent general characteristics of the immune system and not specific responses to virus infection. One such characteristic that we described previously is the relative resistance of $CD4^+$ T cells from SM to the experimentally induced anergy (5, 16).

Previous studies have shown ample evidence that it is the IL-2 production and autocrine function/signaling that regulate subsequent Ag responsiveness (37), and not only that the signaling through the common γ-chain of the IL-2R together with Ag stimulation prevents anergy (38), but also that the IL-2 can reverse existing anergy (39, 40). At the same time, dysregulation of IL-2 expression in $CD4^+$ T cells with accompanying defects in their function has been shown to be characteristic for $CD4^+$ T cells from HIV-infected patients (12). It should be noted that although anergy is characterized by the presence or absence of downstream events that lead to IL-2 production (41), the induction of anergy is an active process associated with differences in downstream TCR signaling (42) rather than loss-of-function, as was determined by cell fusion experiments, in which anergic phenotype was dominant in the fused cells (13). It was therefore interesting to see, as we have shown previously, that SM $CD4^+$ T cells exhibit significant IL-2 production with TCR stimulation only associated with positive up-regulation of ERK signaling (5). Because these cells also routinely exhibit higher levels of spontaneous proliferation and viability in vitro, we were further interested in IL-2 regulation, and in this study we show that this phenotype is associated with significantly higher spontaneous IL-2 production compared with similar cell cultures from RM. Although there could be other reasons for this finding, such as increased intercellular signaling between cells in SM cultures leading to increased activation and IL-2 production, the higher baseline activity of IL-2 promoter observed in the reporter assays suggests that an increased spontaneous transcriptional activity of the IL-2 promoter in SM is at least one of the underlying mechanisms. Although the increase of IL-2-specific transcripts was only modest, it should be noted that such quantitative differences observed in the bioassay or subsequent GFP reporter assays, i.e., 2- to 3-fold, are difficult to quantitate using PCR-based methodology. Our sequence analysis showed only minor nucleotide variations within the proximal SM IL-2 promoter fragment as compared with RM. However, one such variation was within the −180 *cis*-acting negative regulatory site shown to be essential for anergy (15) with an additional variation in an adjacent sequence around position −200. Although one study suggested that the −180 site target of CREB mediated activation as a consequence of PKC-θ leading (43), the majority of the evidence suggests that −180 site is a target of negative regulation by CREB/CREM complexes, which represses IL-2 transcription and is essential for the induction of anergy in T cells (23, 44). In addition, this site was shown to bind Jun-Jun/Oct complexes, which were, however, dispensable for the negative regulation in anergy. Our mutational analysis suggests that the −180 site still functions as a negative site in SM despite the 1-bp variation, and that one of the complexes binding to it contains CREM. Interestingly, the nucleotide variation seemed to increase the affinity of this site for the other complex, presumably containing Jun/Oct1, which we, however, were not able to confirm by the supershift analysis. Regardless of these data, this complex has been reported previously to not having any essential effect in the development of anergy (23). In

contrast, we have demonstrated that the −200 site from SM is a positive regulatory site that is responsible for the increased spontaneous promoter activity in the SM. The supershift analysis identified Oct-1 binding to this site in both RM and SM, but that the sequence variation found in SM seemingly increases the affinity of this site to bind the Oct-1-containing complex.

CREB and p300 are factors important for the regulation of the IL-2 transcription. Both p300 and the related CBP protein are nuclear factors that have a capacity to bind various specific transactivators, such as CREB, AP-1, NF-AT, and others. They function through their histone acetyl transferase activity, increasing the accessibility of chromatin for other transcription factors as well as functioning as scaffolds that facilitate assembly of complexes and their interaction with Pol II (reviewed in Ref. 45). It has been shown that CD28 stimulation-responsive element (CD28RE) located at nt −150 within IL-2 promoter is *trans*-activated by CREB after appropriate T cell costimulation, and this effect is even more dramatic with p300/CBP binding after CD28 activation (21). This complex, which assembles around p300, is essential for the induction of IL-2 expression (22). We found that both RM- and SM-derived promoter constructs were inducible by overexpression of p300 to a similar extent. However, simultaneous expression of CREB down-regulated the promoter activity in all of our constructs. Interestingly, both the wt and a dominant-negative CREB mutant inhibited the IL-2 promoter activity, suggesting that CREB may be acting and inhibiting as a part of a protein complex, rather than directly binding to the target sequence. It was indeed reported previously that CREB can serve as a repressor of various promoters and this function is not associated with CRE element binding or activator binding (20). One possibility is that binding of CREB or CREB-containing complexes prevents binding or assembly of other activating complexes, which exhibit higher affinity for the SM-derived sequence; however, the inhibitory effect of CREB per se is independent of the differences in sequence between the species. Furthermore, we found by ChIP analysis that in vivo, i.e., in cells, the T cells from SM contain significantly higher level of IL-2 promoter-associated p300 and lower level of CREB. At the same time, the overall levels of CREB were similar in $CD4^+$ T cells from both species (data not shown). This suggests that although the promoters from both species can be equally up-regulated by overexpressed p300 and down-regulated by overexpressed CREB, at physiological levels of p300 and CREB in cells, the higher IL-2 spontaneous synthesis observed in SM may be due in part to a more favorable p300/CREB ratio or differences in CREB activation and binding.

The previously reported regulation of IL-2-proximal promoter by remodeling of nucleosome present in resting cells following activation (46) that includes histone H3 and H4 acetylation and demethylation (47), did not seem to greatly differ in SM vs RM. Rather the opposite, we found that it is actually the IL-2 promoter in the $CD4^+$ T cells from RM that exhibits higher levels of histone acetylation. This apparent discrepancy could be reconciled by a previous report that states that the actual decrease of acetylated H3 and H4 at the proximal IL-2 promoter corresponds not to a decreased acetylation of histones, but to a temporary loss of histones from the proximal IL-2 promoter fragment after T cell activation (27). From this perspective, it would seem that the IL-2 promoter in $CD4^+$ T cells from SM, which exhibits a loss of acetylated histones, is in a somewhat activated state even without a requirement for additional external stimulation and costimulation.

Taken together, the higher activity of IL-2 promoter in $CD4^+$ T cells from SM, which may subsequently play an important role in anergy resistance together with the SIV disease resistance in this species, seems to be a result of complex regulatory events. Furthermore, the higher spontaneous production of IL-2 could appear to be in contrast with the recent report of lower T cell activation in SIV-infected SM when compared with RM (7). One potential explanation to reconcile these seemingly contradictory findings might be that low levels of IL-2 primarily benefit T cells expressing the high-affinity receptor for IL-2 (reviewed in Ref. 48). One subset characteristically expressing this receptor are regulatory $CD4^+$ T cells that control T cell activation and prevent autoimmune responses (49). Our recent data do not suggest the presence of higher frequencies of regulatory T cells ($CD25^{high}/FOXP3^+$) in mangabeys (52). Low, but consistent IL-2 levels detected in SM may, however, potentiate their function (17, 50, 51) and limit the activation of effector T cells, therefore limiting immunopathology associated with chronic high viremia infection such as HIV or SIV. This mechanism is being addressed in ongoing studies.

The SM model represents an excellent model not only for further studies for the SIV disease resistance, but also for other important immunological mechanisms, such as anergy, regulation of IL-2, or regulatory T cell-induced homeostasis.

Acknowledgments

We are sincerely grateful to Drs. M. Green and C. Vinson for plasmids expressing CREB and dominant-negative A-CREB, and Dr. K. Gardner for plasmids expressing p300.

Disclosures

The authors have no financial conflict of interest.

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