Negative Transcriptional Regulation of Human Interleukin 2 (IL-2) Gene by Glucocorticoids through Interference with Nuclear Transcription Factors AP-1 and NF-AT

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Abstract

IL-2 gene transcription is affected by several nuclear proteins. We asked whether dexamethasone (Dex) and cyclosporin A (CsA) inhibit IL-2 gene transcription by interfering with the activity of nuclear proteins that bind to the IL-2 promoter. Nuclear extracts from primary human T lymphocytes were analyzed by electrophoretic DNA mobility shift assays. Both Dex and CsA inhibited the binding of transcription factors AP-1 and NF-AT, but not of NF-kB and OCT-1/OAF, to their corresponding sites on the IL-2 gene promoter. To correlate changes in nuclear factor binding in vitro with transcriptional activity in vivo and define the structural requirements for IL-2 promoter repression, we used transient DNA transfections. Jurkat cells were transfected with plasmids containing either the intact IL-2 promoter or its AP-1, NF-AT, and NF-kB motifs. Dex inhibited the IL-2 promoter and the AP-1, but not the NF-AT and NF-kB plasmids. In contrast, CsA inhibited the IL-2 promoter and the NF-AT, but not the AP-1 and NF-kB plasmids. These results suggest that in human T lymphocytes both Dex and CsA inhibited IL-2 gene transcription through interference with transcription factors AP-1 and NF-AT. We propose that, while maximum inhibition may involve interaction with both transcription factors, AP-1 is the primary target of Dex. (*J. Clin. Invest.* 1993. 91:1481–1489.) Key words: glucocorticoids • interleukin 2 • gene transcription • nuclear factor AP-1 • nuclear factor AT

Introduction

Glucocorticoids (GC)[1] have assumed a major role in the treatment of allograft rejection, autoimmune, allergic, and malignant diseases. Their widespread use is based on their profound effects on inflammatory and immune responses. GC affect the growth, differentiation, and function of monocytes and lymphocytes, the distribution of cellular subsets, and cytokine production (1). In spite of their widespread medical use, the mechanism of their inhibitory effects has not been completely defined.

We have recently reported that dexamethasone (Dex), a synthetic GC hormone, inhibits the nuclear transcription of IL-2 gene in primary human T lymphocytes (2). IL-2 gene transcription is mediated by a region extending ~ 326 bp upstream of the transcription start site. This promoter region contains binding sites for at least seven nuclear proteins (Fig. 1; references 3–9). Analysis of the IL-2 promoter has not revealed any *cis*-reacting sequences (glucocorticoid response elements, GRE) for binding of the activated GC receptor and negative transcriptional regulation (10). Thus, delineation of the mechanism of negative transcriptional regulation of IL-2 promoter has been the subject of active investigation over the last two years. Even though several recent publications have explored this area in transformed human or mouse lymphocytes (11, 12), the mechanisms of GC-mediated inhibition of IL-2 gene transcription in primary human T lymphocytes remain unknown.

Using electrophoretic DNA mobility shift assays (EMSA) and transient DNA transfection assays, we have examined whether interference with activity of nuclear proteins binding to IL-2 promoter may explain the inhibitory effects of GC on IL-2 nuclear transcription. To better understand the effect of GC on primary human T lymphocytes, we have also examined their effect on transiently transfected transformed T lymphocytes with reporter constructs containing specific IL-2 promoter motifs and have compared it with the effect of another unrelated inhibitor of IL-2 gene transcription, cyclosporin A (CsA) (13–17). Our data suggest that in primary human T lymphocytes GC and CsA inhibit IL-2 gene transcription by interfering with transcription factors AP-1 and NF-AT. Our data also suggest that while AP-1 is the primary target for GC-mediated repression of IL-2 gene transcription, NF-AT appears to be the main target of CsA action.

Methods

Cell cultures. Human PBMC were obtained and purified by gradient centrifugation over lymphocyte separation medium (Organon Teknica, Durham, NC) from leukapheresis of normal volunteers by the Department of Transfusion Medicine, Clinical Center, National Institutes of Health (NIH). Cells were aspirated from the resulting interface and washed three times in PBS without Ca^{2+} or Mg^{2+}. PBMC were depleted of macrophages by fractionation in Sepracell-MN (Sepratech, Oklahoma City, OK), according to the instructions of the manufacturer. Monocyte-depleted cells were collected, washed in PBS, and rosetted with 2-aminoethyliso-thiouronium bromide pretreated sheep red blood cells (Sigma Chemical Co., St. Louis, MO) and separated over lymphocyte separation medium. E(+) cells were resuspended at a density of 5×10^6/ml in medium consisting of RPMI 1640 (Gibco,

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1. *Abbreviations used in this paper:* CsA, cyclosporin A; Dex, dexamethasone; EMSA, electrophoretic mobility shift assay; GC, glucocorticoids; OAP, OCT-1-associated protein; PKC, protein kinase C; TRE, TPA-responsive element.

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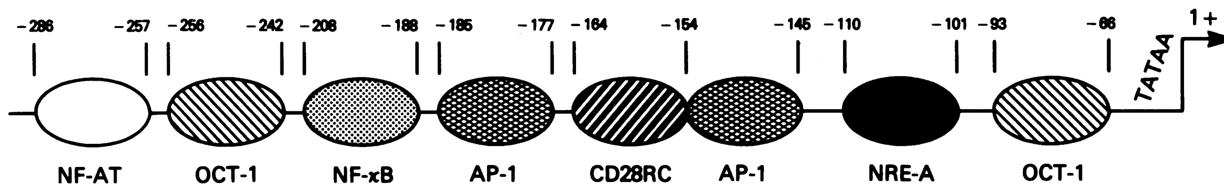


Figure 1. IL-2 promoter with known protein binding sites. Numbers at the top represent the position in base pairs relative to the initiation of transcription site. In contrast to the other motifs, which upregulate IL-2 expression, the NRE-A motif binds a zinc finger protein that inhibits IL-2 gene expression (9). (Modified from reference 8 with the permission of the American Association for the Advancement of Science.)

Grand Island, NY) supplemented with 10% heat-inactivated FCS and 25 μg/ml gentamicin, and were cultured in 5% CO_2/95% air at 37°C for various times with 1 μg/ml ionomycin (Sigma Chemical Co.), 10–30 ng/ml of phorbol myristate acetate (PMA, Sigma Chemical Co.), 10^{-5}–10^{-7} M Dex (Sigma Chemical Co.) and 100 to 1 ng/ml of CsA (Sandoz, Basel, Switzerland). Cells were preincubated with Dex for ≥ 8 h and with CsA for ≥ 0.5 h before stimulation. Human Jurkat cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 25 μg/ml gentamicin, and 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co.).

Nuclear extracts. Nuclear extracts were prepared from 2×10^8 T-lymphocytes by a modification of the method of Dignam et al. (18). Briefly, cells were washed once with PBS and twice with buffer A (10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF). The cell pellet was resuspended in 20 μl of buffer A/0.1% Nonidet P-40, incubated 10 min on ice, mixed briefly, and spun in a microcentrifuge (full speed) for 10 min at 4°C. The supernatant was removed and the nuclear pellet was suspended in 20 μl of buffer C (20 mM Hepes, 25% glycerol, 0.42 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF), incubated 15 min at 4°C, mixed briefly, and microcentrifuged for 10 min at 4°C. The supernatant was diluted with equal volume of modified buffer D (20 mM Hepes, 20% glycerol, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) (18). The protein concentration in the nuclear extracts was determined by using the Bradford assay (19).

DNA-protein binding assay. End-labeled DNA fragments (0.2–0.5 ng, ~ 2×10^4) cpm were incubated at room temperature for 30 min with 5 to 10 μg of nuclear protein in the presence of 4 μg poly-dI-dC in 15 μl of buffer. Two buffers were used: Buffer I (10 mM Tris HCl, pH 7.5; 50 mM NaCl; 1 mM DDT; and 5% glycerol) was used for NF-kB and NF-AT EMSA. Buffer II (20 mM Hepes, pH 7.9; 2.5 mM $MgCl_2$; 40 mM KCl; 1 mM DTT; and 4% Ficoll) was used for AP-1 and OCT-1. Protein–DNA complexes were separated from free probe on a 5% polyacrylamide gel in 0.25× Tris borate buffer at 150 V for 1.5 h at room temperature. The gels were dried and exposed to x-ray film. Specificity of binding was determined by using 20-fold excess of specific cold oligonucleotide, which competed the binding of the proteins to radiolabeled probe, whereas a similar excess of unrelated oligonucleotide did not compete. To determine whether the binding of proteins to the AP-1 site contained c-*jun* or c-*fos* protein, nuclear extracts from stimulated cells were incubated with 0.1 μg of purified c-*jun* rabbit polyclonal IgG antibody against the DNA binding site of the protein (Oncogene Science, Inc., Uniondale, NY), 1 μl of anti-*jun* antibody 636/5 provided by Dr. R. Bravo (Bristol-Myers, Squibb Institute, Princeton, NJ) (20), 0.1 μg of purified anti-*fos* rabbit polyclonal antibody against the NH_2-terminal domain of c-*fos* (Oncogene Science), or 0.1 μg normal rabbit immunoglobulin before addition of the labeled AP-1 probe.

DNA probes. The oligonucleotides were synthesized with a DNA synthesizer (Applied Biosystems; Foster City, CA). Complementary sequences were annealed at 68°C for 10 min and then cooled to room temperature, labeled with [^{32}P]ATP, and used for EMSA after gel purification. The following probes were used, all derived from sequences present in the IL-2 promoter region: For AP-1 binding site from −157 to −140 ^{5}TTCCAAAGAGTCATCAG^3; NF-AT binding site sequences −285 to −254, ^{5}GGAGGAAAAACTGTTTCATACAGAAGGCGT^3; OCT-1 binding site from −82 to −67, ^{5}TAATATGTAAAACATT^3; and NF-kB binding site from −206 to −195, ^{5}GGGATTTCACCT^3 (21).

Plasmids. The plasmid pIL-2 CAT (a gift from Dr. U. Siebenlist, National Institutes of Health, Bethesda, MD) contains 632 bp of the 5-flanking sequences of the human IL-2 gene, including 575 bp upstream of the transcription start site through position +57 bp linked to the CAT gene (22). The plasmid AP-1 CAT (TRE-tk CAT, a gift from Dr. M. Karin, University of California, San Diego, La Jolla, CA) contains a synthetic oligonucleotide from −71 to −65 of the human collagenase gene flanked by HindIII/Bam linker sequences cloned into HindIII/BamHI cut of pBLCAT2, which contains the herpes simplex virus thymidine kinase (HSV-tk) promoter from position −109 to +51 in front of the CAT structural gene (23). The NF-AT CAT plasmid (a gift from Dr. G. Crabtree, Stanford University, Stanford, CA) has three copies of NF-AT binding site containing 5′ IL-2 sequences −255 to −285 linked to the IL-2 promoter sequences from −72 to +47 linked to the CAT gene (7). The plasmid NF-Kb (provided by Dr. U. Siebenlist) contains the kB site from the IL-2 promoter linked to minimal c-*fos* promoter inserted at the Sal site at −56, and drives the expression of the CAT gene (24). The plasmid pCH110 (Pharmacia Inc., Piscataway, NJ) contains a functional lacZ gene, coding for β-galactosidase, under the transcriptional control of the SV40 early promoter.

DNA transfections. Transfections of lymphoid cells were carried out by the DEAE–dextran method (4). To decrease variations in transfection efficiency, cells were transfected in single batches, which were then separated into different drug treatment groups. As an internal marker for monitoring and normalizing transient expression, cells were transfected with pCH110. For each treatment, 1.5–2×10^7 cells harvested in log phase of growth (3–5×10^5 cells/ml) were incubated with 20 μg DNA and 100 μg/ml DEAE–dextran in serum-free RPMI-1640 medium for 50 min at room temperature. After two washes with serum-free RPMI-1640 medium they were replated in complete medium. 24 h after transfection, cells were treated with various concentrations of Dex ≥ 8 h and for 0.5 h with various concentrations of CsA. Then they were stimulated for 8 h with ionomycin 1 μg/ml and 30 ng/ml of PMA. After stimulation, cells were harvested and extracts were prepared by rapid freeze-thawing. Protein concentration was determined by the Bradford assay (19) and equivalent amounts of protein extracts were assayed for enzyme activity.

CAT assay. CAT assay was carried out as described previously (25) by incubation of 50 μg of cell lysate protein with 0.1 μCi of [^{14}C]-chloramphenicol (specific activity 57.3 mg/mmol, NEN Research Products, Boston, MA) in the presence of 9 mM acetyl-coenzyme A (Pharmacia Inc.) for 3 h at 37°C. Acetylated and unacetylated chloramphenicol were separated by thin layer chromatography, and acetylation quantified by autoradiography.

β-galactosidase assay. Equal amounts of the same cell lysate used for CAT assays were diluted in 100 mM $NaPO_4$, 10 mM KCl, 1 mM $MgSo_4$, 50 mM 2-mercaptoethanol, pH 7.0, and β-galactosidase activity was determined spectrophotometrically at 420 nm by the hydrolysis of *o*-nitrophenol-β-D-galactoside.

Results

Dex and CsA inhibit the protein kinase C– and calcium-mediated trans-activation of the IL-2 promoter. Both Dex and CsA inhibit the nuclear transcription of the human IL-2 gene (2,

13–17). To determine whether their inhibition is mediated through interference with the transcriptional activity of the human IL-2 promoter, Jurkat cells were transiently transfected with plasmid (p) IL-2 CAT, which contains the IL-2 promoter region driving the expression of CAT gene (see Methods). After transfection, cells were incubated with Dex (10^{-6}–10^{-8} M) or CsA (1,000 to 0.1 ng/ml) and stimulated with ionomycin (1 μg/ml) and PMA (30 ng/ml). 18 h later, cell extracts were prepared and assayed for CAT activity. pIL-2 CAT was not expressed in unstimulated Jurkat cells or in cells incubated with Dex or CsA (Fig. 2). Stimulation with ionomycin and PMA significantly increased CAT activity. Both Dex and CsA inhibited the expression of IL-2 CAT after stimulation with ionomycin and PMA in a dose-dependent fashion (minimum effective dose 10^{-8} M and 0.1 ng/ml respectively, not shown). Dex at the dose of 10^{-6} M inhibited by a mean of 60% (range 54–67%) the CAT activity (three different experiments), whereas at 10^{-7} M inhibition was ~ 20% (range 15–28%). Inhibition by CsA was as follows: 1,000–10 ng/ml, 100%; 1 ng, ~ 80%, 0.5 ng, ~ 40% (data not shown). These results suggest that both Dex and CsA inhibit IL-2 gene transcription by interfering with the activity of IL-2 promoter, and that CsA is more potent than Dex (on a molar to molar basis) in mediating this inhibition.

Stimulation through ionomycin and PMA induces the activity of nuclear proteins AT, NF-kB, AP-1 and OCT/OAP, all of which are thought to contribute to the transcriptional activation of IL-2 promoter in various degrees (7, 9, 26, 27). Recent data have suggested a functional antagonism between the AP-1 transcriptional factor for the collagenase gene and the activated GR (28–30). We therefore first examined the effect of Dex on the activity of the AP-1 site of the human IL-2 promoter.

Binding of nuclear proteins from stimulated primary human T lymphocytes to the AP-1 site of the IL-2 promoter is inhibited by Dex or CsA. Common to many genes in which transcription is induced by the phorbol ester TPA is a conserved sequence TGAGTCA functioning as a TPA responsive element (TRE). This sequence is recognized by the transcriptional factor AP-1, which is encoded by the cellular proto-oncogene c-*jun* (31–34). c-*fos* protein interacts with AP-1 through their "leucine zipper." This complex binds to the TRE with an affinity 300-fold higher than AP-1 alone, leading to transactivation of AP-1-dependent transcription (34). The human IL-2 promoter contains two TREs, one proximal and one distal. Footprint and deletion analysis has shown that only proximal TRE binds strongly AP-1 and has revealed its importance in IL-2 enhancer activity (7–9). Human peripheral blood T lymphocytes were therefore stimulated with ionomycin and PMA after preincubation with Dex ($\geq$ 8 h) or CsA (0.5 h). Nuclear extracts were isolated 16 h later and analyzed in an EMSA using end-labeled DNA fragment corresponding to the proximal AP-1 site of the IL-2 enhancer. In agreement with previous observations (21), unstimulated T lymphocytes contain little or no AP-1 activity (Fig. 3 *A*). Stimulation of these cells effectively induced AP-1 activity. The sequence specificity of the binding was assessed by using molar excess of homologous and heterologous oligonucleotide as competitors. Protein binding to the ^{32}P-labeled IL-2 AP-1 site was inhibited by a cold homologous, but not with a heterologous (NF-kB) oligonucleotide (see Methods). An antibody raised against a peptide common to the DNA-binding region of *Jun* proteins, but not an equivalent concentration of normal rabbit immunoglobulin, specifically blocked protein binding, suggesting that it contains *Jun* protein and identifying it as the AP-1 factor (Fig. 3 *B*). An antibody raised against c-*fos* decreased the mobility of this complex, suggesting that it contains c-*fos* protein. Both Dex and CsA inhibited the formation of the AP-1 complex. Lower concentration of Dex (10^{-7} M) had little effect (Fig. 3 *A*), whereas 10^{-8} M had no effect (not shown). These results suggest that both Dex and CsA inhibit the binding of the AP-1 transcription factor to the AP-1 site of the human IL-2 promoter.

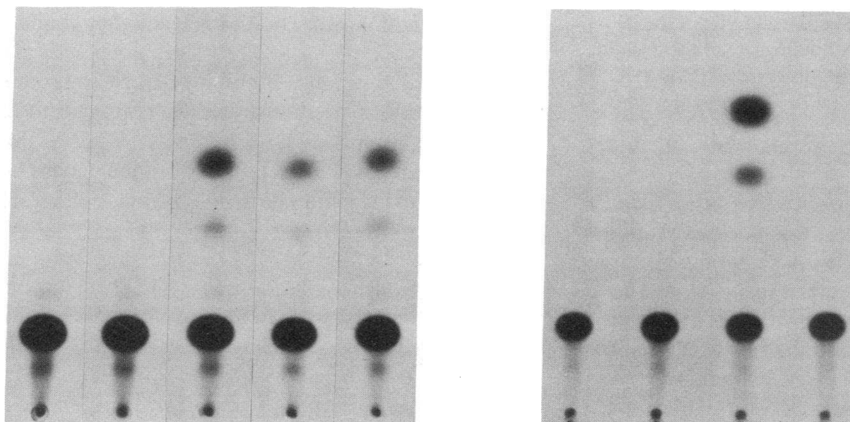


Figure 2. Dex and CsA inhibit the Ca^{2+} and PKC-mediated trans-activation of the IL-2 promoter. Jurkat cells were transiently transfected with plasmid IL-2 CAT, which contains the IL-2 promoter driving that expression of CAT gene (see Methods). After transfection, cells were incubated with Dex (10^{-6} or 10^{-7} M) or CsA (100 ng/ml) and stimulated with ionomycin and PMA. 18 h later, cell extracts were prepared and assayed for CAT activity. To decrease variations in transfection efficiency, cells were transfected in single batches, which were then separated into different drug treatment groups. pCH110 (which contains functional lacZ gene, coding for β-galactosidase) was used as an internal marker for monitoring and normalizing transient transfections. Acetylated and unacetylated chloramphenicol were separated by thin layer chromatography and quantified by autoradiography. Results are representative of three independent experiments. During this experiment inhibition of IL-2 CAT activity by CsA was 100%, whereas for Dex 10^{-6} M and 10^{-7} M it was 67 and 28%, respectively.

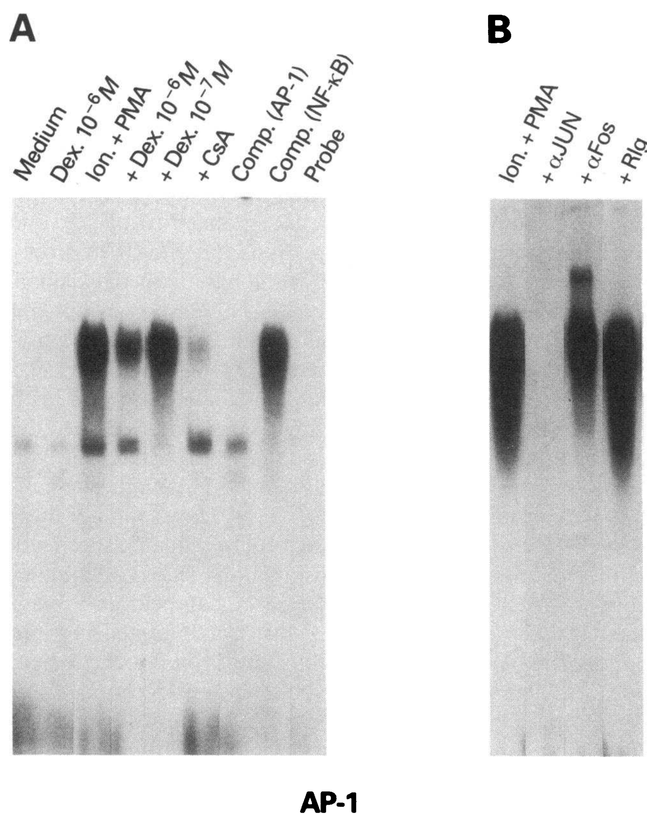


Figure 3. (*A*) Dex and CsA inhibit the binding of nuclear proteins from stimulated primary human T lymphocytes to the AP-1 site of the IL-2 promoter. In EMSA a ^{32}P–end-labeled 17-bp DNA probe that included the AP-1 binding domain of the human IL-2 promoter was incubated with nuclear extracts (10 μg/lane) prepared from primary human T lymphocytes resting or stimulated, in the presence or absence of Dex or CsA for 16 h, in buffer II (see Methods). Protein-DNA complexes were separated from free probe on a 5% polyacrylamide gel in 0.25 × Tris borate buffer. Gels were dried and exposed to x-ray film. Specificity of binding was determined by the use of a 20-fold excess of specific or unrelated oligonucleotide. (*B*) To determine whether the binding proteins contain c-*jun* and c-*fos* protein, nuclear extracts from stimulated cells were incubated with 0.1 μg of affinity purified c-*jun* (α*Jun*) or c-*fos* (α*fos*) antibodies, or 0.1 μg of normal rabbit immunoglobulin (*RI*) before addition of the labeled AP-1 probe. α*Jun* binds to the DNA binding site of the protein, thus inhibiting its binding. The "supershift" observed with α*Fos* is consistent with this antibody binding to the *Jun-Fos* complex, thus decreasing its mobility.

Dex but not CsA inhibits the in vivo transcriptional activity of a construct containing the AP-1 site. Jurkat cells were transfected with plasmid AP-1 CAT (TRE-CAT), which contains the AP-1 site of the human collagenase gene cloned into pBLCAT2 (23). Cells were preincubated with Dex (10^{-6}–10^{-8} M) or CsA (10–1,000 ng/ml) as described in Methods and stimulated by ionomycin and PMA. Cell extracts were prepared and assayed 8, 18, and 36 h later. Unstimulated Jurkat cells expressed low but detectable AP-1 CAT activity, which was suppressed by Dex (Fig. 4). In contrast to Dex, CsA failed to suppress the activity of this construct. As expected, when cells were stimulated with ionomycin and PMA, activity of AP-1 CAT was induced by severalfold. Dex inhibited the inducible activity of AP-1 CAT by a mean of ~ 70% (range 60–80%) at 10^{-6} M and ~ 30% (range 35–25%) at 10^{-7} M, when cells were harvested at 18 h. Similar results were obtained when cells were harvested at 8- and 36-h time points (not shown). Lower doses of Dex (10^{-8} M) did not have a significant effect on AP-1 CAT activity (not shown). In contrast to Dex, CsA failed to inhibit the inducible activity of AP-1 CAT (Fig. 5) at concentrations that were previously found to be effective for downregulation of IL-2 promoter activity. Concentrations of CsA as high as 1 μg/ml were not effective (not shown). Taken together, these results confirm the in vitro data for inhibition of AP-1 by Dex and suggest that this inhibition does not require the presence of other motifs of the IL-2 promoter. The discrepancy between the in vitro and the in vivo data observed when CsA was used may be due to the difference in cell types (primary vs. transformed). These data are in agreement with previous reports suggesting that in Jurkat cells AP-1 activity by EMSA or transfection assays is not inhibited by CsA (14, 15, 17). Inhibition of AP-1 activity in vitro by CsA in primary human T lymphocytes (by EMSA) has also been reported by Granelli-Piperno et al. (16).

NF-AT activity in primary human T lymphocytes is inhibited by Dex or CsA. In contrast to nuclear factor AP-1, which is ubiquitous, NF-AT has been found only in lymphoid cells (27). Unstimulated primary human T lymphocytes contain no detectable activity for NF-AT as determined by EMSA. In stimulated T lymphocytes NF-AT activity appears within 6 h after stimulation and continues to increase until it reaches a peak within ~ 36 h (21). Induction of NF-AT in transformed human T lymphocytes requires two activation-dependent effects: a CsA-sensitive translocation of a preexisting cytoplasmic component to the nucleus and a CsA-resistant synthesis of a nuclear component (35). Recent data in murine T lymphocytes suggest that the CsA-insensitive nuclear component is AP-1 (which is added to the preexisting NF-AT complex) and that there is close physical and functional interaction (crosstalk) between AP-1 and NF-AT (36). We therefore examined the effect of Dex on NF-AT activity. Primary human T lymphocytes were stimulated by ionomycin and PMA in the presence of Dex or CsA. Nuclear extracts were isolated 18 and 36 h later and analyzed by EMSA using a radiolabeled oligonucleotide probe for the AT site of the human IL-2 promoter. Unstimulated cells did not exhibit any AT activity (Fig. 5). Stimulation of cells resulted in induction of AT activity, which was inhibited by both Dex or CsA at 36 h (Fig. 5). Analysis at 8 and 18 h revealed similar results. Binding of nuclear proteins was sequence specific, as demonstrated by competition assays with related and unrelated (NF-kB) DNA (Fig. 5). Inhibition of NF-AT activity by CsA in primary human T lymphocytes corroborates previous findings in both primary and transformed T lymphocytes (14–16).

CsA but not Dex inhibits the transcriptional activity of a transfected plasmid containing the AT site in Jurkat cells. NF-AT activity is inhibited by Dex in vitro. We examined these phenomena in vivo by transfection assays. Jurkat cells were transiently transfected with pNF-AT CAT, which contains three copies of the NF-AT site of the human IL-2 promoter linked to the CAT gene (5). Plasmids containing a single copy of the NF-AT site are not inducible (5, 11, 12). As shown in Fig. 6, unstimulated Jurkat cells did not exhibit any detectable CAT activity. Neither Dex nor CsA affected the baseline activity of this plasmid (Fig. 6). Activation of Jurkat cells led to a dramatic induction of pNF-AT CAT. Dex at 10^{-6} or 10^{-7} M did not affect the activity of this plasmid (Fig. 6). Harvesting of

AP-1 CAT

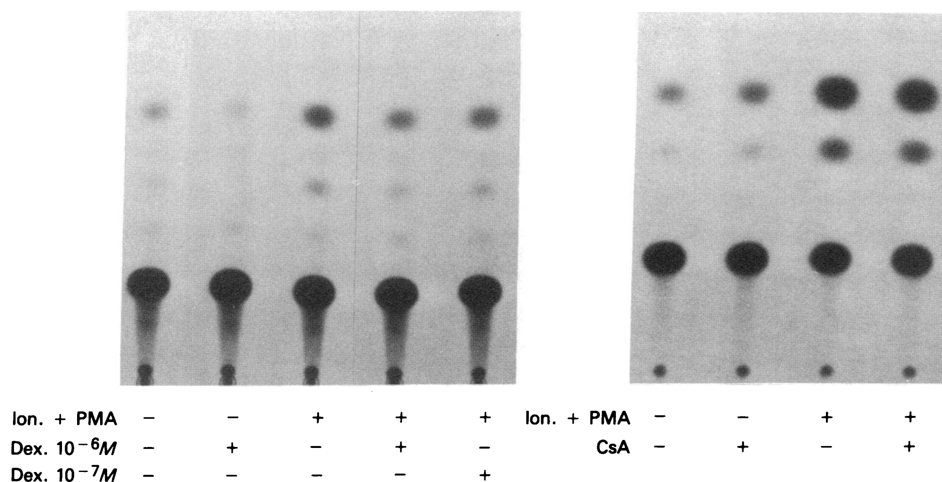


Figure 4. Dex but not CsA inhibits the activity of an AP-1 reporter construct. Jurkat cells were transiently transfected with plasmid AP-1 CAT, which contains a single copy of the AP-1 site of the human collagenase gene cloned in pBLCAT2 (23). After transfection, cells were stimulated with ionomycin and PMA in the presence of Dex (10^{-6} or 10^{-7} M) or CsA (100 ng/ml) (separate experiment) for 18 h and analyzed for CAT activity as described in the legend to Fig. 1. Results are representative of three independent experiments. Dex inhibited the CAT activity in this experiment by ~ 70 (10^{-6} M) and ~ 30% (10^{-7} M). No inhibition was observed with CsA.

cells at various time points (8, 16, or 36 h) also did not affect the results (not shown). In contrast to Dex, CsA (100 ng/ml) completely inhibited the activity of pNF-AT CAT. Doses of CsA as low as 1 ng/ml inhibited the activity of this plasmid (not shown). Taken together, these results suggest that CsA is a potent inhibitor of NF-AT activity and that its inhibitory effect does not require the presence of other sites of the IL-2 promoter.

Lack of inhibition of pNF-AT activity in vivo by Dex in Jurkat cells could be due to (*a*) resistance of transformed T lymphocytes (compared with primary T lymphocytes); (*b*) the presence of three NF-AT sites in the plasmid used for these experiments instead of a single site in the IL-2 promoter; or (*c*) to Dex requiring the presence of other sites of the IL-2 promoter in order that it may inhibit NF-AT activity. Analysis of Jurkat cells by EMSA demonstrated that both Dex (10^{-6} M) and CsA inhibit the binding of nuclear proteins to the human NF-AT site of the human IL-2 promoter (Fig. 7). When higher concentrations of Dex were used (10^{-5} M) in DNA transfection assays with pNF-AT, CAT results were identical (not shown). Taken together, these results suggest that the NF-AT site, by itself, is not sufficient for GC mediated inhibition. Given the highly cooperative interaction among the various transcription factors for the IL-2 gene promoter (27), it is possible that additional sites are required in order that NF-AT may confer susceptibility to GC (see Discussion).

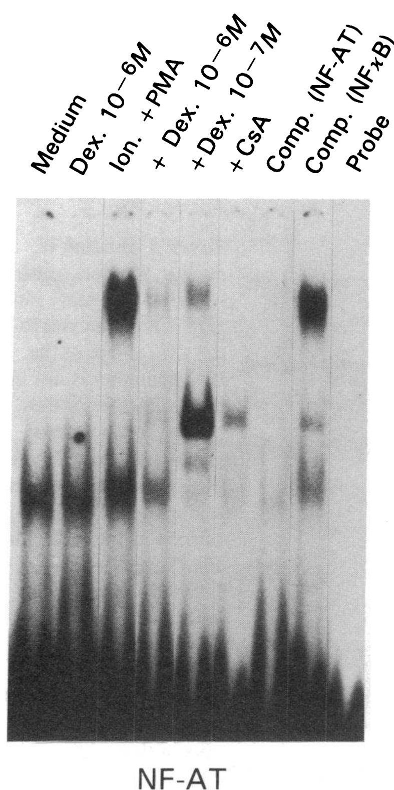
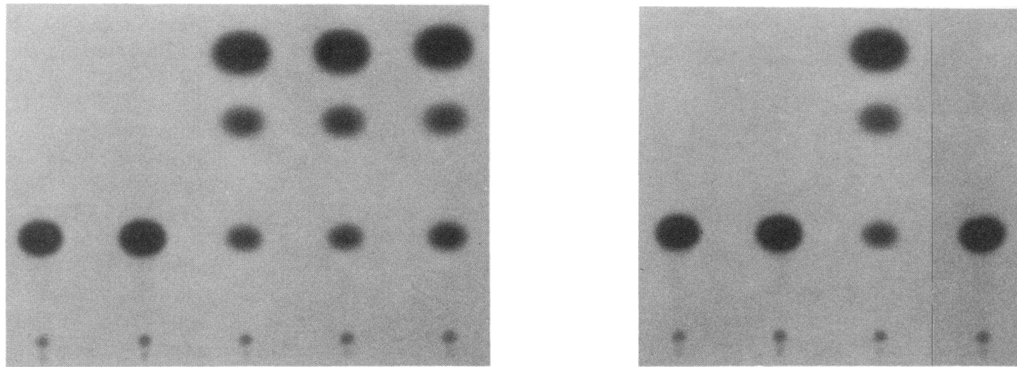


Figure 5. NF-AT activity in primary human T lymphocytes is inhibited by DEX or CsA. Nuclear extracts from resting or stimulated T lymphocytes were analyzed by EMSA as described in the legend to Fig. 3. A ^{32}P–end-labeled 30 bp DNA probe that included the NF-AT binding domain of the human IL-2 promoter was used in this assay. Results are representative of four different experiments.

Neither Dex nor CsA inhibits the activity of nuclear proteins binding to the OCT-1 site of the human IL-2 promoter. OCT-1 is a ubiquitous octamer binding nuclear protein that participates in the induction of IL-2 promoter activity by interacting with both DNA and a 40-kD protein called OCT-1–associated protein (OAP) (37). Two octamer-binding sites are identified in the human IL-2 promoter, one proximal and one distal (Fig. 1). Protein extracts of stimulated primary T lymphocytes bind very poorly to the OCT-1 distal site (21). In contrast, binding to the proximal OCT-1 is strong. Levels of OCT-1 activity are low in unstimulated T cells and are induced after stimulation reaching a peak at ~ 6 h (21). As shown in Fig. 8, unstimulated primary T lymphocytes exhibited low levels of OCT-1 binding activity that were not affected by pretreatment by Dex or CsA (not shown). Two bands are apparent in this figure, one upper and one lower. Competition with related cold oligonucleotide (OCT-1) completely inhibited the binding of the label in the upper band, whereas it competed less effectively for binding in the lower band. Competition with a heterologous (NF-kB) oligonucleotide did not exhibit any demonstrable effect on binding of these proteins. This suggests that upper band is specific for OCT-1, whereas binding to the lower band represents nonspecific binding. Both Dex or CsA failed to inhibit the activity of OCT-1/OAP nuclear proteins.

NF-kB activity is not inhibited by Dex or CsA. NF-kB is involved in the inducible transcription of several genes coding for cytokines or their receptors (27, 38–41). In contrast to IL-

NF-AT CAT



Ion. + PMA	−	−	+	+	+
Dex. $10^{-6}M$	−	+	−	+	−
Dex. $10^{-7}M$	−	−	−	−	+

Ion. + PMA	−	−	+	+
CsA	−	+	−	+

Figure 6. CsA but not Dex inhibits the activity of a transfected plasmid containing the NF-AT site. Jurkat cells were transiently transfected with plasmid NF-AT CAT, which contains three copies of the AT site of the human IL-2 promoter linked to the CAT gene, as described in the legend to Fig. 2. Cells were exposed to Dex or CsA (100 ng/ml) before stimulation. After stimulation for 16 h, cells were analyzed for CAT activity. Densitometric analysis revealed no inhibition for Dex and 100% inhibition for CsA. Results are representative of three different experiments.

2Rα gene, where NF-kB is essential for transcription, its relative importance for IL-2 promoter activity is not well established (5, 7, 27). In unstimulated human T lymphocytes low to undetectable levels of NF-kB activity are present by EMSA. Stimulation of T lymphocytes leads to a rapid induction of NF-kB activity, which peaks within 6 h and persists at similar levels for ≥ 36 hours (21). As depicted in Fig. 9, unstimulated T lymphocytes expressed low levels of activity for the kB site of the IL-2 promoter by EMSA. Dex increased approximately twofold the baseline NF-kB activity (Fig. 9) but not CsA (not shown). Stimulation with ionomycin and PMA led to a marked induction of NF-kB activity, which was not affected by Dex or CsA. Specificity of binding was again confirmed by

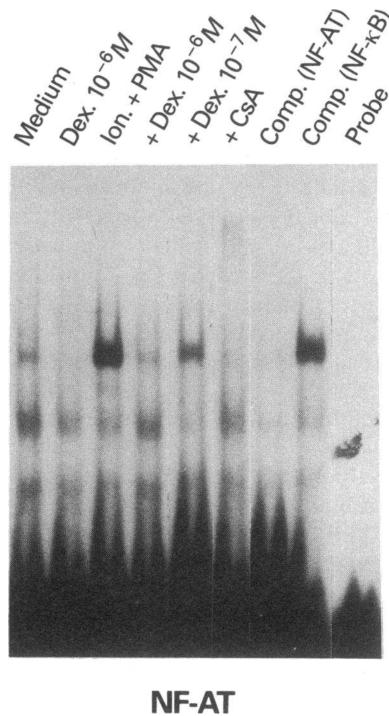


Figure 7. Both Dex and CsA inhibit the binding of nuclear proteins to the NF-AT site of the human IL-2 promoter. Jurkat cells were stimulated with ionomycin and PMA in the presence or absence of Dex (10^{-6} or 10^{-7} M) or CsA (100 ng/ml) and analyzed by EMSA as described in the legend to Fig. 6. Results are representative of two different experiments.

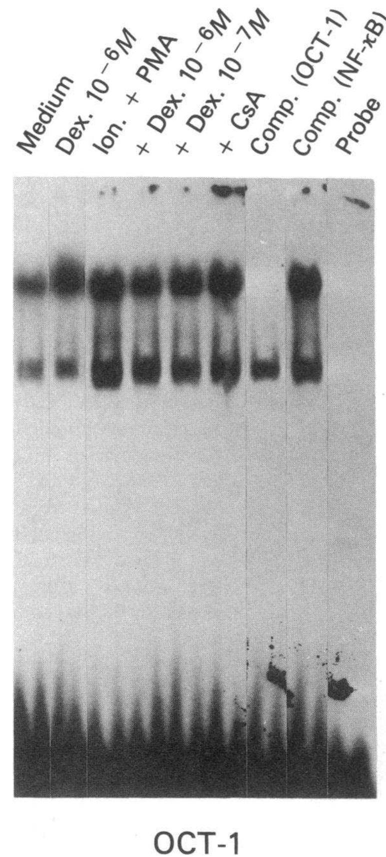


Figure 8. Binding of nuclear proteins from stimulated primary human T lymphocytes to the OCT-1 site of the IL-2 promoter is not inhibited by Dex or CsA. Nuclear extracts from primary human T lymphocytes were prepared and analyzed as described in the legend to Fig. 3. A ^{32}P–end-labeled DNA probe (16 bp) that includes the DNA binding domain of OCT-1/OAF was used in this experiment. Results are representative of three different experiments.

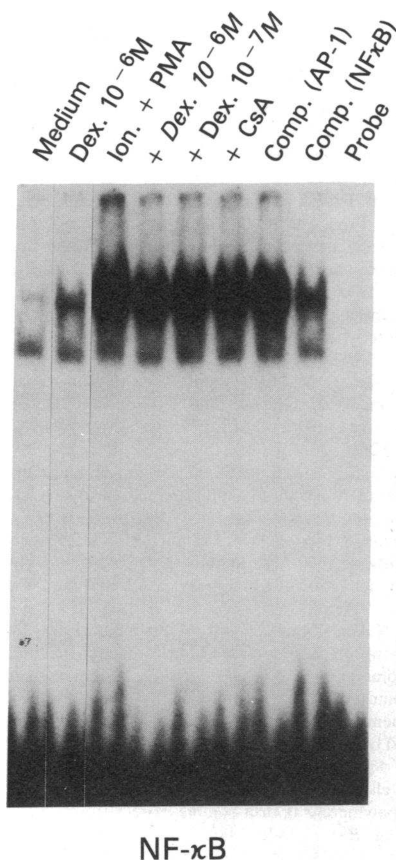


Figure 9. Neither Dex nor CsA inhibits the binding of nuclear proteins to kB site of the IL-2 promoter. Nuclear extracts from primary human T lymphocytes were analyzed by EMSA as described in Fig. 3. A DNA probe (−206 to −195 to the human IL-2 promoter) that includes to the DNA binding site of NF-kB on the IL-2 promoter was used. Results are representative of two different experiments.

competition assays with cold related (NF-kB) and unrelated (AP-1) oligonucleotide (Fig. 8).

We next determined whether Dex or CsA will inhibit NF-kB activity in Jurkat cells. Plasmid NF-kB CAT, which contains a single copy of the human IL-2 kB site, was transfected into Jurkat cells. Neither Dex or CsA inhibited the induction of NF-kB activity by ionomycin and PMA (not shown). Taken together, these results suggest that NF-kB is not likely to mediate inhibition of IL-2 promoter activity by Dex. Lack of inhibition of NF-kB activity by CsA has been reported also by Granelli-Piperno et al. (16), whereas other groups have reported partial inhibition (14, 17).

Discussion

We have presented a series of studies aimed at defining the mechanism(s) of GC-mediated inhibition of IL-2 gene nuclear transcription in primary human T lymphocytes. To better understand this inhibition and put the effect of GC in perspective, we have also studied transformed human T lymphocytes (Jurkat cells) and have compared the effects of GC with those of CsA. Our results suggest that inhibition of IL-2 transcription by GC, both in primary and transformed human T lymphocytes, is mediated by interference with nuclear factors AP-1 and AT. Furthermore, our data suggest that while maximum inhibition of IL-2 promoter transcription may involve interaction with both transcription factors, AP-1 seems to be the primary target of GC because, in contrast to NF-AT, its inhibition does not require the presence of other motifs of the IL-2 promoter. Finally, we have shown that inhibition of IL-2 transcription by CsA in primary T lymphocytes involves interference with both NF-AT and AP-1, whereas in Jurkat cells inhibition is mediated mainly through NF-AT.

Our results are reminiscent of findings recently reported by Vacca et al. (11). By mutational analysis of human IL-2 promoter in a system using transfection in Jurkat cells, these investigators found that mutants carrying deletions disrupting the NF-AT and the proximal AP-1–like motif impaired their ability to be inhibited by GC. A homooligomer of NF-AT was found to be inducible by ionomycin and PMA. Dex did not inhibit the activity of the construct. When an AP-1 motif concatemer was used, calcium ionophores and PMA failed to induce it significantly. Based on these results, they concluded that the AP-1 motif confers GC sensitivity to a flanking region containing an NF-AT element and suggested that, in order that GC may inhibit IL-2 gene transcription, a synergistic cooperativity is required between the NF-AT and the AP-1 sites.

The experiments presented herein, while not necessarily excluding cooperation between AP-1 and NF-AT in mediating the inhibitory effect of Dex, also suggest that the AP-1 motif (but not the NF-AT) of IL-2 promoter may be sufficient for inhibition by Dex. Inability to demonstrate inhibition of the AP-1 noncatemer used by Vacca et al. (11) by Dex is due to its failure to be induced after stimulation. A multimerized AP-1 construct derived from the AP-1 site of the IL-2 promoter (AP-CAT/NFIL2B2H), when used by Northrop et al. (12), was inducible after stimulation. Dex inhibited the activity of this construct by 78% (compared with 100% inhibition of the intact IL-2 promoter). Taken together, these results raise the intriguing possibility that, similar to activation of IL-2 promoter, where multiple transcriptional factors are required for optimal activity, maximal repression by GC may involve interaction with more than a single factor and/or binding site. Recent work in murine cells has suggested that AP-1 is important for binding and activity of NF-AT and has also suggested a close physical and functional interaction between these two factors (36). Our work raises the possibility that the primary target of GC in mediating inhibition of IL-2 promoter activity is transcriptional factor AP-1, whereas its effect on NF-AT activity may be, at least in part, indirect, through decreasing AP-1 activity.

A functional antagonism that involves direct protein to protein interactions between AP-1 and GR has been reported by several investigators (28–30). We have shown that the proximal AP-1 site of the human IL-2 promoter binds an inducible protein in primary human T lymphocytes, a protein that contains both c-*jun* and c-*fos* proteins and mediates repression by GC. Vacca et al. (11) have also shown that the proximal AP-1 motif of the human IL-2 promoter binds an inducible complex containing c-*fos* protein in Jurkat cells. Our results differ from those recently reported by Northrop et al. (12), who suggest that inhibition of IL-2 transcription by GC is mediated by direct interaction between GR and a factor that binds to two sequences located in the proximal half of the IL-2 promoter (NKIL2B/AP-1 and NFIL2A/OCT-1/OAP). These sequences bind a similar, if not identical, inducible nuclear factor that has biological characteristics that distinguish it from AP-1 (likely OAP) (12). The discrepancy of these results may be due to differences in cell types used (primary or transformed human vs. transformed human or murine) or to differences in experimental protocols.

To our knowledge this is the first demonstration of GC inhibiting IL-2 transcription by interfering with nuclear factors

for IL-2 promoter in primary human T lymphocytes. Granelli-Piperno et al. (16) have also studied the effects of GC on the induction of nuclear factors binding in the IL-2 promoter in primary human T lymphocytes. Dex at 10^{-7} M, while blocking the increase in IL-2 mRNA in stimulated primary human T lymphocytes, did not inhibit binding of nuclear factors AP-1, AP-3, NF-kB, OCT-1, and NF-AT. We have previously shown that relatively high doses of Dex are required for inhibition of IL-2 gene transcription as determined by nuclear run-off assays (minimum effective dose 10^{-6} M), whereas lower concentrations (10^{-8} M) are required for inhibition of IL-2 mRNA and protein product. This discrepancy is due to posttranscriptional effects of GC on IL-2 gene expression (2). Thus, the difference between our results and those reported by Granelli-Piperno et al. (16) is due to lower doses of Dex used by these investigators (10^{-6} M vs. 10^{-7} M). Concentrations of GC comparable to 10^{-6} M of Dex used in these experiments are certainly achievable in vivo by doses of prednisone of ≥ 1 mg/kg or methylprednisolone similar to those used for pulse therapy (≥ 15 mg/kg). An infusion of 40 mg of cortisol (equivalent to 10 mg of prednisone or 1.3 mg of Dex) will result in plasma cortisol levels of $\sim 1\text{-}4 \times 10^{-6}$ M (42). These facts are important to bear in mind when using data obtained in vitro to interpret the immunosuppressive effects of GC in vivo.

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