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Inducible Nuclear Factor Binding to the κB Elements of the Human Immunodeficiency Virus Enhancer in T Cells Can Be Blocked by Cyclosporin A in a Signal-Dependent Manner

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Cyclosporin A (CsA) is thought to exert its immunosuppressive effects by inhibiting the expression of a distinct set of lymphokine genes which are induced upon T-cell activation, among them the gene coding for interleukin-2. In addition, the activation of the human immunodeficiency virus (HIV) is partially suppressed. To better understand the molecular mechanisms underlying suppression by CsA, we have investigated the effects of this drug on transcription factors in T cells. Here we report that the formation of two distinct mitogen-inducible DNA-binding complexes, the κB complex within the HIV enhancer and the NFAT-1 complex within the interleukin-2 enhancer, is inhibited in the presence of CsA. The κB-binding activity with the HIV enhancer is inhibited only if it is activated via the mitogen phytohemagglutinin, whereas phorbol myristate acetate-mediated activation is completely insensitive to the drug. This suggests a model in which functionally indistinguishable κB complexes can be activated via two separate pathways of signal transduction distinguishable by CsA.

Cyclosporin A (CsA) is a clinically important immunosuppressive drug which suppresses induction of a number of genes, including that coding for interleukin-2 (IL-2), in T cells (9, 25), and to some extent expression of human immunodeficiency virus (HIV) (26, 28). The molecular mechanisms of this suppression are unknown, but it has been shown that CsA inhibits the induced expression of several genes at the transcriptional level (10, 14). Two tandemly repeated κB sequence elements are essential to the activation of the HIV genome, and these elements confer enhancer function (19). This enhancer element binds inducible nuclear factors in T cells, the NF-κB factor, and additional, possibly related factors including HIVEN-86 (2, 3, 8, 19). κB nuclear factor-binding elements also play important roles in the activation of the gene coding for IL-2 and other genes in T cells (11, 15, 16, 22). In addition to κB, other IL-2 enhancer elements appear to mediate signal transduction, and some of these elements show inducible factor binding after activation of Jurkat T cells (4, 6, 11, 16, 22, 24). Among the elements that show inducible factor binding is the site termed IL-2-IIA by us (4) and NFAT-1-binding site by others (24), which has been implicated directly in playing a major role during transcriptional activation of IL-2 (6, 24). We show here that the activation of the κB and NFAT-1 complexes in T cells is sensitive to CsA, whereas activation of an inducible AP-1 complex is not. This suggests selective action of the drug. We further show that the HIV-κB-binding complex as well as the function of the HIV enhancer in transfection experiments is completely inhibited when mediated through a phytohemagglutinin (PHA)-induced signal but is insensitive to a phorbol myristate acetate (PMA)-induced signal.

We investigated the effect of CsA on the activation of the κB-binding complex of the HIV long terminal repeat in T cells with the electrophoretic mobility shift assay. A radioactively labeled oligonucleotide fragment representing the κB-binding site (nucleotides −104 to −79, 5′-GGGACTTTCCGCTGGGGACTTTCCAG-3′ [19]) was incubated with nuclear extracts (10 μg of protein) in a total volume of 20 μl {40 mM NaCl, 40 mM KCl, 0.2 mM EDTA, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], approximately 7% glycerol [vol/vol], 0.5 mM dithiothreitol, 25 μM NPGB, 1 mM phenylmethylsulfonyl fluoride, 2 μg of poly[dI-dC)] [Pharmacia]} for 30 min at room temperature. Resulting complexes were separated on a 4% low-ionic-strength polyacrylamide gel (27) in a cold room. Activation of T cells by PHA-PMA induced the binding of a major complex (termed B) to the κB enhancer elements (Fig. 1, lane 3). An additional inducible complex (termed A), closely spaced and slightly more slowly migrating, could also be discriminated (Fig. 1, lane 3). Although detected repeatedly, the A shift was weak and variable in strength; it was most clearly seen when cells were stimulated with PHA alone (Fig. 1, lane 5) but never seen when cells were stimulated with PMA alone (Fig. 1, lane 7). The nature of the two constitutively present complexes is unknown; they were essentially unaffected by the activation protocols. The major inducible shift (B complex) appears identical to the previously reported (2, 3, 19) inducible shift obtained with similar HIV κB oligonucleotide probes because (i) the shift was not detected with a mutant enhancer, nor was the B shift competed for by this mutant (data not shown; the mutation κB/M [19] is 5′-CTCACTTTCCGCTGCTCACTTTCCAG-3′), and (ii) this induced shift comigrated with a shift formed with nuclear extracts from Namalwa cells, which contain a constitutively activated κB complex (13) (data not shown). The activable binding activity giving rise to this shift was ascribed to the nuclear factor NF-κB and possibly other closely related factors (2, 3, 8, 19). Addition of CsA during stimulation with both PHA and PMA significantly reduced the major shift (B shift) and completely abrogated the A shift (Fig. 1, lane 4).

Since the induction of the major κB shift was only partially

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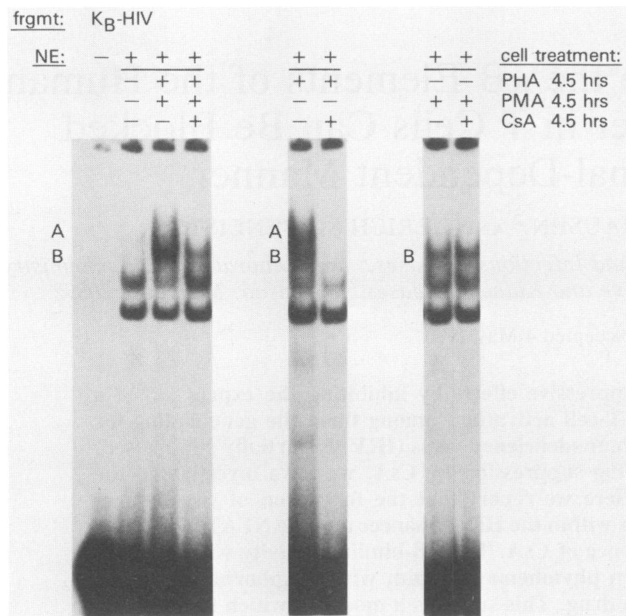

FIG. 1. Dependence of CsA inhibition of HIV-κB binding activity on the inducing signal. Complementary single-stranded oligonucleotide fragments representing the HIV enhancer (frgmt: κB-HIV; nucleotides −104 to −79, with directly abutting 3′-end *Sst*I overhangs) were radioactively labeled with polynucleotide kinase, annealed, and then incubated with nuclear extracts. Nuclear extracts (NE) were prepared from untreated (−) Jurkat cells or cells treated for 4.5 h with various agents (PHA, 1 μg/ml; PMA, 20 ng/ml; CsA, 1 μg/ml) (+) by hypotonic swelling of cells, Dounce homogenization, salt extraction of the resulting nuclei, and precipitation of the extracted nuclear proteins with ammonium sulfate, as described by Shapiro et al. (23). Lane −, Free labeled oligonucleotides without nuclear extracts added; lanes +, nuclear extracts added. B and A, Positions of shifts B and A, respectively. In addition, two constitutively present complexes are seen.

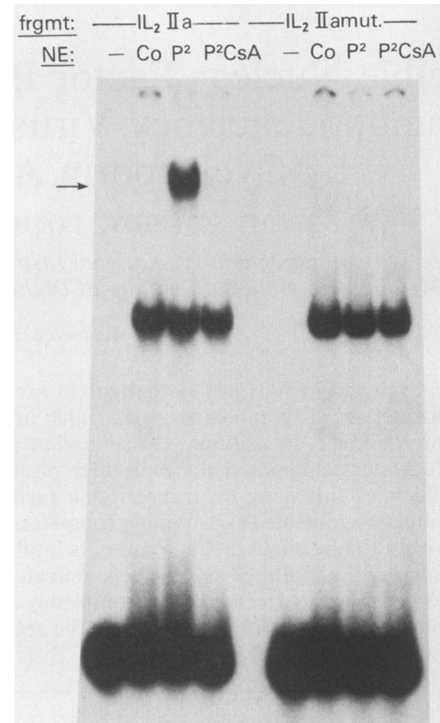

FIG. 2. Sensitivity of inducible NFAT-1 nuclear factor complex formed with IL-2 promoter region IIA to CsA. Double-stranded oligonucleotide fragments (frgmt) representing the NFAT-1-binding site IL-2-IIa (4, 24) (IL-2 nucleotides −288 to −266; 5′-AAGGAG GAAAAACTGTTTCATAC-3′ [left]) or a mutant of this binding site (IL-2-IIamut; AAGGAGGCAAAACTGTTGCATAC [right]) (both sets of oligonucleotides have directly abutting 5′ *Bgl*II [upper strand] or *Bam*HI [lower strand] overhangs) were radioactively labeled by filling in the ends with Klenow enzyme. The oligonucleotides were then incubated with nuclear extracts, and the resulting complexes were separated as described in the legend to Fig. 1. Nuclear extracts (NE) were prepared from Jurkat cells treated with various agents, as described in the legend to Fig. 1. Lanes Co, Untreated cells; lanes P^2, PHA-plus-PMA treatment; lanes P^2CsA, PHA plus PMA plus CsA; lanes −, free labeled oligonucleotides without nuclear extracts added. →, Position of the inducible shift. The additional nonspecific shift was largely unaffected by the various types of cell treatment, and the nature of the shift is unknown.

suppressed when the broadly acting, mitogenic stimuli PHA and PMA were used, we investigated the activity of CsA with each agent alone. PHA mimics induction via the T-cell receptor, while PMA stimulation is thought to be mediated at least in part via direct activation of protein kinase C. While PHA-mediated induction of complexes binding to the κB enhancer was completely abrogated by CsA (Fig. 1, lane 6; no B or A shifts), the PMA-induced shift was not at all affected (Fig. 1, lane 8). These data were confirmed with multiple different independent extracts. We conclude that very similar or identical κB complexes were activated via different pathways during stimulation with PHA versus PMA and that these pathways were distinguishable by their sensitivities to CsA.

As shown by electrophoretic mobility shift assay, an oligonucleotide encompassing the IL-2 enhancer site IIA (NFAT-1-binding site) bound a complex inducible upon activation of Jurkat T cells with PHA and PMA (Fig. 2, left panel, arrow). However, the addition of CsA during the cellular activation phase completely abolished this binding. When a mutant of this element was used, no inducible binding activity could be detected in any of the variously treated cellular extracts (Fig. 2, right panel). This shows the sequence specificity of the induced factor, as do previous data (6). In addition to the findings reported here, we have observed good activation of NFAT-1 with PHA alone but not with PMA alone (data not shown). Therefore, the complete suppression of NFAT-1 in the presence of both PHA and PMA is consistent with the notion that CsA interferes with some necessary action(s) initiated by PHA but not with those initiated by PMA (10). Direct addition of CsA to a prepared nuclear extract from activated cells had no effect on the factor binding, including binding of κB-binding factors (data not shown), which suggests that inhibition occurs during the activation phase of NFAT-1 or the κB complex.

The AP-1 site is known to confer inducibility on many genes. To test whether the inducible binding to this element (18) is sensitive to CsA as well, we performed electrophoretic mobility shift assays with the identical CsA-treated and untreated nuclear extracts described above. Inducible binding activity was completely insensitive to CsA (Fig. 3), even when PHA was used alone as a stimulus. This experiment shows that some activable binding complexes are not inhibited by CsA, and therefore AP-1 represents a control for the inhibition of the NFAT-1 and κB factors. This experiment

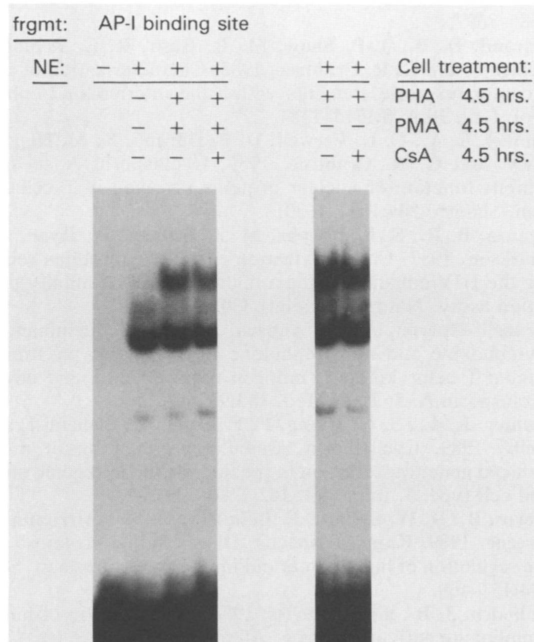

FIG. 3. Inducible binding to an AP-1 site is insensitive to CsA. The double-stranded oligonucleotide fragment (frgmt) bearing an AP-1 consensus core sequence (5'-AGAAATAGATGAGTCAAC AG-3', with CG dinucleotide 5' overhangs on both strands [21]) was radioactively labeled by filling in the ends with Klenow enzyme and was subjected to electrophoretic mobility shift assay with nuclear extracts (NE) from cells treated (+) or not treated (−) as indicated and as described in the legend to Fig. 1. The inducible band (upper band) is essentially insensitive to CsA. The nature of the lower constitutively present band shift is unknown. Lane −, Free labeled oligonucleotides without nuclear extracts added; lanes +, nuclear extracts added.

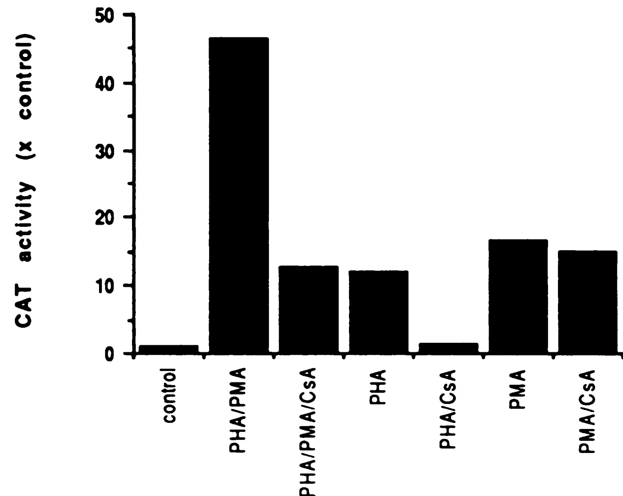

FIG. 4. Effects of CsA upon CAT inducibility conferred by HIV enhancer (κB-binding sites) in Jurkat cells. CAT activities determined after the transfection with a construct in which the HIV κB enhancer is driving the expression of the CAT gene are shown (κB-WAP-CAT). To make this construct, a 112-base-pair fragment from the mouse WAP promoter, extending from −88 to +24 (*Xba*I to *Kpn*I) (5), was cloned into a CAT vector containing no promoter or enhancer sequences (pA10 CAT 3M), resulting in the minimal promoter construct WAP-CAT. Two copies of the wild-type κB-binding region (nucleotides −104 to −79) were integrated in the same orientation into an *Sst*I site at −88 of the WAP-CAT construct via *Sst*I 3' overhangs, creating κB-WAP-CAT. We also integrated two copies of the mutant κB/M (see above) to create κB/M-WAP-CAT. The activities under various types of cell treatment (indicated below each bar) are expressed relative to the total CAT activity found with untreated, transfected cells, which was arbitrarily given a value of 1.0. Results of a single representative experiment are shown. In repeated experiments with the same construct, qualitatively identical results were obtained.

also suggests that only some activation paths initiated by PHA are sensitive to CsA in T cells (10).

To correlate the signal-dependent changes in factor binding in vitro with transcriptional regulatory function in vivo, HIV wild-type or mutant enhancer-driven chloramphenicol acetyltransferase (CAT) constructs containing a minimal whey acidic protein (WAP) promoter were transfected into Jurkat cells and tested for CAT activity after various cellular stimulations (Fig. 4). Several independent transient transfections of the constructs were carried out by using a modified DEAE method in which cells are slightly swollen with hypotonic medium before transfection (17). Following a 36- to 48-h incubation period after transfection, cells were split for the individual treatments, eliminating the need for internal transfection efficiency controls. These cells were then treated for 4.5 h with PHA, PMA, and CsA, and CAT assays were performed as previously described (20). Regulation of transcription conferred by the HIV enhancer correlated directly with the mobility shift binding studies of the κB complex. Specifically, CsA inhibited the PHA-derived activation signal but not the PMA signal. Thus, CsA caused no general inhibition of cellular function. The activation mediated by PHA and PMA exceeded that of either agent alone, and as expected, costimulation with PHA and PMA was only partially inhibited by CsA. The transfection experiments were also done with a 10-fold-lower level of CsA (0.1 μg/ml), and we observed qualitatively identical results (data not shown). In additional controls, the HIV enhancer mutation κB/M, which prevented binding of κB-like complexes (19), also completely abrogated the inducibility of CAT activity by PHA or PMA (or both), as did a construct with the minimal WAP promoter only (WAP-CAT) (data not shown). Our transfection data are consistent also with earlier transfection reports which demonstrated that parts or all of the HIV long terminal repeat confers inducibility by signals originating from the antigen receptor or originating with PMA-mediated activation but that only the signal from the antigen receptor is sensitive to CsA (26, 28).

CsA suppressed the induction-dependent binding activity in T cells of two apparently unrelated DNA-binding complexes, the NF-κB-like complex formed with the HIV enhancer and the NFAT-1 complex formed with the IL-2-IIA site. Additional inducible DNA-binding activities may be affected, which would be consistent with a broad action of this drug as documented by the relatively large number of genes in T cells which are suppressible (10, 29). On the other hand, not all inducible complexes were suppressed, as exemplified by the induction of the AP-1 complex in the presence of the drug. Surprisingly, suppression of the κB complex of the HIV enhancer in T cells occurred only when the cells were activated by PHA (which is likely to act through the antigen receptor) but not when activated by PMA. Apparently, identical κB complexes were formed after the delivery of either signal, but the paths for transducing the signal must have been different. When PHA plus PMA was used to activate cells, inhibition of the κB complex was only partial, which suggests that the PMA-initiated

signals remained unaffected by CsA. Since the NFAT-1 factor required stimulation by PHA as opposed to PMA, it was very sensitive to CsA even in the presence of both PHA and PMA.

After completion of this work, a related report appeared which demonstrated CsA inhibition of the NFAT-1- and the κB-binding activities in PHA-PMA-stimulated T cells (7). The partial inhibition of the κB-binding activity in the presence of CsA after induction with these combined signals was not analyzed further, however.

The results of the transfection experiments with HIV enhancer-driven CAT constructs demonstrated that the in vivo effects closely corresponded to the results of κB-binding studies in vitro. The PHA-stimulated but not the PMA-stimulated cells were sensitive to CsA. Thus, CsA appears to inhibit a step(s) in cellular activation which follows PHA or antigen receptor stimulation but does not inhibit the PMA signal. Since it is known that stimulation via the antigen receptor leads to production of inositol phosphate derivatives and diacylglycerol even in the presence of CsA (12), the suppression must occur downstream of these second messengers or involve an as yet unidentified second messenger. Also, activation of AP-1 with PHA was not affected by CsA, which suggests that only some PHA-derived signals are suppressible.

It is not known what links the activation of the κB and NFAT-1 factors. NFAT-1 appears to involve the synthesis of an induced gene, since cycloheximide interferes with NFAT-1 activation (4, 24). On the other hand, the κB factor(s) is already present in the cell and requires only release from a cytoplasmic inhibitor (1). Considering that several similar but possibly distinct nuclear factors may bind to the HIV κB region, we cannot rule out that such factors are separately activated depending on the inducing signal. This could be the cause of the observed differential sensitivity to CsA. Whether the same or a distinct factor(s) binds as a result of the two activation signals, these signal-transducing paths converge functionally at the DNA level, as shown by the transfection experiments. It is possible that different kinases (or other modifications) effect the release of NF-κB from its inhibitor (1) and that some of these kinases in turn are regulated by distinct signaling pathways.

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