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# Regulation of the Interleukin-2 CD28-Responsive Element by NF-ATp and Various NF-κB/Rel Transcription Factors

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**The CD28 costimulatory signal enhances antigen-mediated induction of interleukin-2 (IL-2) gene transcription through activation of an enhancer termed the CD28-responsive element (CD28RE). Although various nuclear proteins have been shown to bind to CD28RE, their in vivo functions in the regulation of this enhancer remain elusive. In this report, we show that CD28RE binds distinct transcription factors in cells treated with different mitogenic stimuli. Stimulation of the T-cell receptor (TCR) complex in the absence of a CD28 costimulatory signal induces a member of the nuclear factor of the activated T cells, NF-ATp; however, this treatment fails to activate the CD28RE enhancer activity. Significant activation of CD28RE was detected when the cells were treated with both the TCR stimulators and an anti-CD28 monoclonal antibody (anti-CD28), which induces the NF-κB/Rel enhancer binding proteins in addition to NF-ATp. The costimulatory activity of anti-CD28 can be further enhanced by a phorbol ester. Kinetic analyses demonstrate that activation of endogenous IL-2 gene transcription is correlated with the binding of CD28RE by NF-ATp and different NF-κB/Rel species. Transient-transfection studies reveal that expression of either NF-ATp or the p50-RelA NF-κB heterodimer leads to the potent transactivation of both the CD28RE enhancer and the intact IL-2 promoter in mitogen-stimulated cells. Remarkably, coexpression of these two families of enhancer-binding proteins in Jurkat T cells results in the transactivation of the CD28RE enhancer even in the absence of any cellular stimuli. Together, these results suggest that activation of IL-2 gene transcription by the TCR- and CD28-mediated signals involves the interaction of CD28RE with NF-ATp and various NF-κB/Rel transcription factors.**

Optimal activation of T cells requires both the engagement of the T-cell receptor (TCR) complex and the interactions of other surface molecules, the latter of which provide the so-called costimulatory signals (36). One major costimulatory signal is mediated by the interaction between the CD28 accessory molecule on the surface of T cells and its cognate ligand B7 on antigen-presenting cells or an anti-CD28 monoclonal antibody (22, 26, 37). Stimulation of the TCR complex in the absence of this costimulatory signal drives the T cells to enter a state of anergy or undergo programmed cell death (15, 42, 45). Conversely, induction of T-cell anergy can be prevented by stimulation of the CD28 molecule with B7 or an anti-CD28 monoclonal antibody (20).

Stimulation of CD28 alone has no detectable effect on the activation of T cells. However, in T cells treated with agents that stimulate the TCR complex, such as monoclonal antibodies against the CD3 chains (anti-CD3) and the mitogenic lectin phytohemagglutinin (PHA), the anti-CD28 antibody markedly enhances T-cell proliferation and production of cytokines, including interleukin-2 (IL-2) (43). The synergy between the primary and costimulatory signals in induction of the IL-2 gene is seen at both transcriptional and posttranscriptional (RNA stability) levels (9, 25, 47). The mitogen phorbol 12-myristic 13-acetate (PMA) has also been shown to synergize with anti-CD28 in enhancing the steady-state level of IL-2 mRNA (25), although this combination has only little effect on the promoter activity of IL-2 (47).

The intracellular signaling and biochemical events triggered by the CD28 costimulatory signal remain unclear. It has been shown that the moderate induction of IL-2 mRNA by PMA and anti-CD28 is not inhibited by cyclosporin A (CSA) (25), an immunosuppressive drug specifically interfering with the function of a calcium/calmodulin-regulated phosphatase, calcineurin (35). However, the synergistic action of the TCR-mediated primary and CD28-mediated costimulatory signals in the induction of IL-2 mRNA and IL-2 promoter activity is either markedly or completely inhibited by CSA (25, 47). These findings may suggest the involvement of both calcium-dependent and calcium-independent signaling pathways in the activation of the IL-2 gene by the two signals. Regarding the downstream nuclear events, a CD28-responsive element (CD28RE) has been identified in the promoter of the IL-2 gene and shown to be essential for the transcriptional induction of this gene through the CD28 signaling pathway (9). Recent studies have shown that the CD28RE enhancer can be bound by NF-κB/Rel (5, 13, 44), a family of enhancer-binding proteins involved in many parts of the immune response (2, 3, 18, 30, 40). However, some other studies have demonstrated that in activated T cells, the CD28RE is primarily occupied by a member of the nuclear factor of activated T cells (NF-AT [8, 31, 32]), NF-ATp, but not by the NF-κB/Rel factors (33). Here we demonstrate that in response to different cellular stimuli, CD28RE is bound by distinct transcription factors, and these factors are induced with different kinetics and appear to play important roles in regulation of the CD28RE enhancer.

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## MATERIALS AND METHODS

**Cells, reagents, and antibodies.** Jurkat T cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. These human leukemia T cells mimic many of the early events of

T-cell activation (14, 46) and have been extensively used as a model system to study the transcriptional activation of the human IL-2 gene (7–9, 38). The 293 kidney cell lines were maintained in Iscove's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. Human peripheral blood T cells were prepared from partially purified human blood (Biological Specialty Corporation, Colmar, Pa.) with a Ficoll-Hypaque gradient (Pharmacia Biotech) followed by negative selection using human T-cell enrichment immunocolumns (Biotex Laboratories Inc., Edmonton, Alberta, Canada). The murine T-cell clone 16B.2 (kindly provided by Daniel Mueller) was cultivated as previously described (29). PHA and PMA were purchased from Sigma and used at concentrations indicated in the figure legends. CSA was provided by Sandoz Pharmaceuticals Co. (East Hanover, N.J.). The monoclonal antibody for human CD28 (clone 9.3) was provided by the Bristol-Myers Squibb Pharmaceutical Research Institute and used at 1:10,000 dilution (or 0.3 μg/ml). Anti-human CD3 (OKT3) was purchased from Ortho Diagnostic Systems, Inc. (Raritan, N.J.) and was immobilized onto tissue culture plates (1:250 dilution) at 4°C overnight before use. The monoclonal antibodies for murine CD28 (clone 37.51) and CD3 (clone 145-2C11) were purchased from PharMingen (San Diego, Calif.). The anti-murine CD3 was immobilized onto tissue culture plates (2 μg/ml), and the anti-murine CD28 was used at a concentration of 1 μg/ml in solution. The antibodies for c-Fos (4-10G) and c-Jun (c-Jun/AP-1 [D]) were obtained from Santa Cruz Biotechnology, Inc. These two antibodies recognize different members of the Fos and Jun families, respectively. The antibody against NF-ATp was purchased from Upstate Biotechnology, Inc. The peptide-specific antisera for various NF-κB/Rel components were gifts from Warner C. Greene (The Gladstone Institute of Virology and Immunology, San Francisco, Calif.).

**Plasmid constructs.** The full-length NF-ATp expression vector (NF-ATpFL) was generated by PCR amplification of a human NF-ATp cDNA (a kind gift from Timothy Hoey, Tularik, Inc., South San Francisco, Calif.) and subsequent cloning of the PCR product into the pCMV4 mammalian expression plasmid (1). NF-ATpXS encoded a truncated murine NF-ATp similar to the NF-ATpXS previously described (28) but lacked the N-terminal hexahistidine tag. In brief, a 2-kb *Msc*I-*Cla*I restriction fragment from the murine NF-ATp cDNA (kindly provided by Anjana Rao, Dana-Farber Cancer Institute, Boston, Mass.) was inserted into the *Eco*RV and *Cla*I sites of a modified pCMV4 mammalian expression vector, pCMV4HA (41). This intermediate construct was digested with *Kas*I and *Xba*I to remove the C-terminal untranslated sequence as well as part of the coding sequence. After the ends were filled in with the Klenow fragment of DNA polymerase I, the vector was religated. When transfected into human kidney 293 cells, the encoded NF-ATpXS protein (containing 412 amino acids) was primarily expressed in the nucleus (data not shown). The cDNAs encoding RelA, p50, and c-Rel have been described previously (6, 10). To construct the reporter plasmid CD28RE-luc, a *Hin*dIII-*Xho*I insert (containing four copies of the human IL-2 CD28RE motif linked in front of a thymidine kinase promoter) of plasmid 4xCD28RE-CAT (gift from Paritosh Ghosh and Howard Young, National Cancer Institute, Frederick, Md.) (13) was transferred into the pGL2-basic luciferase plasmid (Promega). The luciferase reporter driven by the IL-2 distal NF-AT enhancer (NF-AT-luc) was constructed by transferring the insert (containing three copies of NF-AT sites and the thymidine kinase promoter) of NF-AT-TK-βgal (12) to pGL2. The construction of the luciferase reporter plasmid driven by the wild-type IL-2 promoter (IL-2-luc) or IL-2 promoter lacking the CD28RE site (IL-2-lucΔCD28RE) has been described previously (16).

**Transient-transfection and luciferase assays.** Jurkat cells ($5 \times 10^6$) were transfected by using DEAE-dextran (21) with the indicated amounts of reporter plasmid and effector cDNA expression vectors. At 40 h after transfection, the recipient cells were either left untreated or incubated with the indicated inducers for the indicated time periods and then subjected to extract preparation by using a reporter lysis buffer (luciferase reagent; Promega) at about 40 μl per $10^6$ cells. Luciferase activity was detected by mixing 5 μl of extract with 25 μl of luciferase substrate (Promega) and measured with a single photon channel of a scintillation counter (Beckman).

**Nuclear extract preparation and EMSA.** Human peripheral blood T cells, Jurkat cells, or murine 16B.2 T cells were treated with the indicated inducers for the indicated time periods, collected by centrifugation at $800 \times g$ for 5 min, and then subjected to nuclear extract preparation as previously described (34). For the experiments involving CSA, the CSA was added to the cell culture 5 min before cellular stimulation. Human 293 cells were transfected in six-well plates by using DEAE-dextran and collected for nuclear extract preparation 40 h after transfection. Electrophoresis mobility shift assay (EMSA) was performed by incubating the nuclear extracts (5 μg) with $^{32}$P-radiolabeled probes at room temperature for 10 min and then resolving the DNA-protein complexes on native 4% polyacrylamide gels (10). For antibody supershift assays, 1 μl of diluted (fivefold for anti-NF-ATp and threefold for the various anti-NF-κB antisera) or undiluted (anti-Jun and anti-Fos) antibodies was added to the EMSA reaction 5 min before electrophoresis.

Oligonucleotide probes used in EMSA were as follows: CD28RE, AAAGAA ATTCCAAAGAGT; NF-AT, GGAGGAAAAACTGTTTCATA; and κB, CAA CGGCAGGGGAATTCCCCTCTCCTT.

**Nuclear run-on assays.** Nuclei were isolated from Jurkat T cells by using a standard protocol (19). For nuclear transcription, the nuclei were incubated in a reaction buffer (10 mM Tris [pH 8.0], 5 mM $MgCl_2$, 0.3 M KCl, 5 mM dithiothreitol, 1 mM nucleoside triphosphates, 50 μCi of $^{32}$P-labeled UTP) at 30°C for 30 min followed by isolation of the $^{32}$P-labeled RNA as previously described (19). The radiolabeled RNA samples were hybridized at 65°C for 36 h to cDNA for either human IL-2 or glyceraldehyde-3-phosphate dehydrogenase blotted onto nitrocellulose membranes (25 μg per blot). The free RNA molecules were removed by RNase A digestion followed by extensive washing in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (17). The membranes were then subjected to autoradiography.

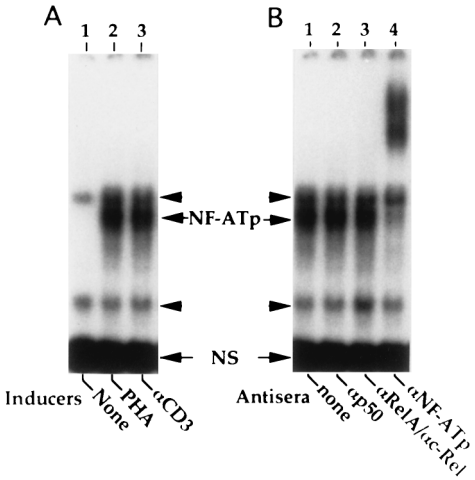


FIG. 1. PHA and anti-CD3 induce NF-ATp but not NF-κB/Rel. (A) Jurkat T cells were either untreated (None) or incubated for 2 h with PHA (1 μg/ml) or anti-human CD3 (4 μl/ml). Nuclear extracts isolated from these cells were subjected to EMSA using a $^{32}$P-labeled human IL-2 CD28RE probe. (B) The nuclear extract isolated from PHA-induced cells was subjected to EMSA in the absence (none) or presence of the indicated antisera. The arrowheads indicate two constitutive CD28RE-binding protein complexes. NS indicates a protein complex that nonspecifically binds to different oligonucleotide probes (data not shown).

## RESULTS

**Stimulation of human Jurkat T cells with anti-CD3 or PHA induces the binding of NF-ATp to CD28RE but fails to activate the CD28RE-luc reporter.** To investigate the molecular mechanism by which CD28RE modulates the primary and costimulatory T-cell activation signals, we first examined whether stimulation of the TCR complex in the absence of a CD28 costimulatory signal could induce the CD28RE-binding proteins. For these studies, human Jurkat T cells were stimulated for 2 h with either the lectin PHA or anti-human CD3 (OKT3), and the nuclear CD28RE-binding proteins were analyzed by EMSA using a $^{32}$P-labeled oligonucleotide probe covering the CD28RE from the human IL-2 promoter. Two constitutive protein complexes were detected from the nuclear extract of unstimulated cells (Fig. 1A, arrowheads). The intensity of these complexes varied among different experiments (data not shown). More importantly, stimulation of the cells with either PHA (Fig. 1A, lane 2) or anti-CD3 (lane 3) led to the induction of a novel protein complex. Antibody supershift assays (Fig. 1B) revealed that this inducible protein complex exhibited no immunoreactivity with antisera for p50 (lane 2), RelA and c-Rel (lane 3), or other NF-κB/Rel subunits (data not shown). However, it was readily supershifted by the anti-NF-ATp antibody (lane 4). Thus, stimulation of the TCR complex appears to induce an NF-ATp-containing CD28RE-binding complex but not NF-κB/Rel.

To examine the potential biological function of NF-ATp in the activation of the CD28RE enhancer, functional reporter gene assays were performed with a CD28RE-driven luciferase reporter (CD28RE-luc). As shown in Fig. 2A, stimulation of

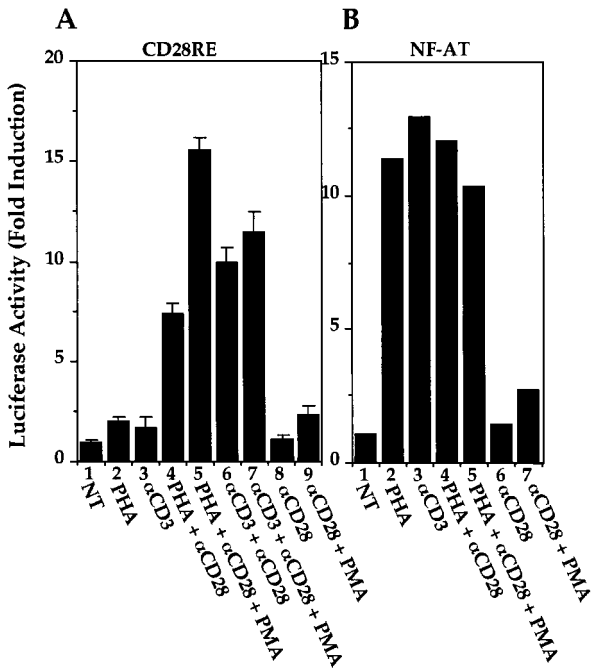


FIG. 2. Activation of CD28RE-luc, but not NF-AT-luc, requires multiple cellular stimulation signals. (A) Human Jurkat T cells ($5 \times 10^6$) were transfected with 10 μg of CD28RE-luc. At 40 h after transfection, the recipient cells were either untreated (NT) or treated for 8 h with PHA (1 μg/ml), anti-human CD3 (4 μl/ml), anti-human CD28 (0.3 μg/ml), or the indicated combinations (PMA was used at a concentration of 10 ng/ml). The cells were then collected for luciferase assay (Promega). Luciferase activity is presented as fold induction relative to the basal level measured in untreated cells (about $8 \times 10^4$ cpm). The values represent the means of fold induction of three independent experiments. (B) Human Jurkat T cells were transfected with 2 μg of NF-AT-luc. The transfectants were stimulated with the indicated inducers and then subjected to luciferase assays as described for panel A. The values shown are representative of two independent experiments.

the CD28RE-luc transfectants with either PHA (column 2) or anti-CD3 (column 3) only slightly elevated the level of luciferase expression. The inability of these stimuli to activate the CD28RE enhancer was not due to the lack of NF-AT in these cells, since both inducers potently activated a reporter driven by the IL-2 distal NF-AT enhancer (NF-AT-luc [Fig. 2B]). Significant activation of the CD28RE-luc expression was detected in cells treated with both the TCR stimuli (PHA or anti-CD3) and anti-CD28 (Fig. 2A, columns 4 and 6), thus confirming the notion that activation of this enhancer requires the CD28 costimulatory signal (9). The CD28 costimulatory effect was further enhanced by the protein kinase C (PKC) activator PMA (columns 5 and 7). On the other hand, in the absence of anti-CD3 or PHA, anti-CD28 failed to activate the reporter gene expression (column 8), and even in the presence of PMA, only moderate enhancer activity was detected (column 9). Together, these biochemical and functional studies suggest that induction of NF-ATp is not sufficient to activate the CD28RE enhancer. Additional factors may be induced in the costimulated cells and participate in the enhancer activation.

**Activation of the CD28RE enhancer is correlated with the rapid induction of both NF-ATp and NF-κB/Rel transcription factors.** The transcription factors mediating activation of CD28RE in the costimulated T cells were then examined by EMSA. As expected, stimulation of the cells for 2 h with PHA in the absence of anti-CD28 led to the induction of the NF-ATp-containing complex (Fig. 3A, lane 2). However, when the cells were costimulated with PHA and anti-CD28, a more rapidly migrating band was induced in addition to NF-ATp (lane 3). Supershift assays revealed that formation of this lower band was abrogated by antibodies against p50 (lane 4) and RelA (lane 5). After a longer time of autoradiography, supershifted complexes could be visualized (data not shown). Furthermore, the anti-NF-ATp antibody did not influence the formation of the more rapidly migrating band although this antibody efficiently supershifted the NF-ATp-containing upper complex (lane 6). A similar result was obtained with the anti-CD3–anti-CD28-costimulated cells (data not shown). Thus, costimulation of Jurkat T cells with a TCR stimulus (PHA or anti-CD3) and anti-CD28 induces both NF-ATp and the p50-RelA NF-κB/Rel heterodimer. Stimulation of the cells in the presence of PMA further enhanced the induction of NF-κB/Rel (Fig. 3A, lane 7). In these PMA-costimulated cells, the NF-κB/Rel complex was again primarily composed of p50 and RelA since the formation of this complex was abrogated by either anti-p50 (lane 9) or anti-RelA (lane 10) but not influenced by antibodies for c-Rel (lane 11) and other NF-κB subunits (data not shown). A faint band that migrated more rapidly than the p50-RelA heterodimer was consistently detected when the p50-RelA heterodimer was cleared by the anti-RelA antibody (Fig. 3A, lane 10). This complex was probably the p50–c-Rel heterodimer since its formation was prevented in the presence of antibodies for either c-Rel (lane 12) or p50 (lane 9). As expected, when antibodies for NF-ATp, RelA, and c-Rel were added together to the EMSA reaction, both the NF-ATp and NF-κB/Rel complexes were completely supershifted (lane 14). The supershift studies also revealed that in contrast to findings for NF-κB/Rel- and NF-ATp-specific antibodies, none of the CD28RE-binding protein complexes immunoreacted with antibodies recognizing various members of the Jun (anti-c-Jun/AP-1 [D]; Santa Cruz Biotechnology) and Fos (anti-c-Fos [4-10G]; Santa Cruz Biotechnology) families of proteins (lanes 15 and 16). These antibodies could supershift the NF-AT–AP-1 complex formed on the IL-2 distal NF-AT site (data not shown). A parallel EMSA revealed that while anti-CD28 alone failed to induce either NF-ATp or NF-κB/Rel (Fig. 3A, lane 17), together with PMA, this CD28 stimulator induced the p50-RelA NF-κB heterodimer but not NF-ATp, as demonstrated by supershift assays (Fig. 3A, lanes 18 to 23). Of note, the immunoreactivities of the NF-ATp and NF-κB antibodies with the CD28RE-binding complexes were specific since the effects of these antibodies could be blocked by corresponding antigenic peptides (for the NF-κB subunits) or a recombinant protein (for NF-ATp [data not shown]).

To compare the relative binding affinities of the NF-κB and NF-ATp factors to different target sequences, EMSA was performed with $^{32}$P-radiolabeled probes covering CD28RE, the IL-2 distal NF-AT, and a palindromic κB site (Fig. 3B). In cells stimulated with PHA, the NF-ATp band was readily detected with the CD28RE probe (lane 1). With the NF-AT probe, two complexes were detected (lane 3); the more rapidly migrating band was NF-ATp, while the more slowly migrating complex was composed of both NF-ATp and Fos and Jun, as determined by supershift assays (data not shown). No κB binding complexes were detected in the PHA-stimulated cells (lane 5), suggesting that NF-ATp did not appreciably bind to the κB element. As expected, both the NF-ATp and NF-κB complexes were detected with CD28RE probe in cells stimulated with PHA together with anti-CD28 and PMA (lane 2). However, under the same conditions, the NF-AT probe failed to bind NF-κB, although this probe formed the NF-ATp-containing complexes, predominantly the more slowly migrating NF-

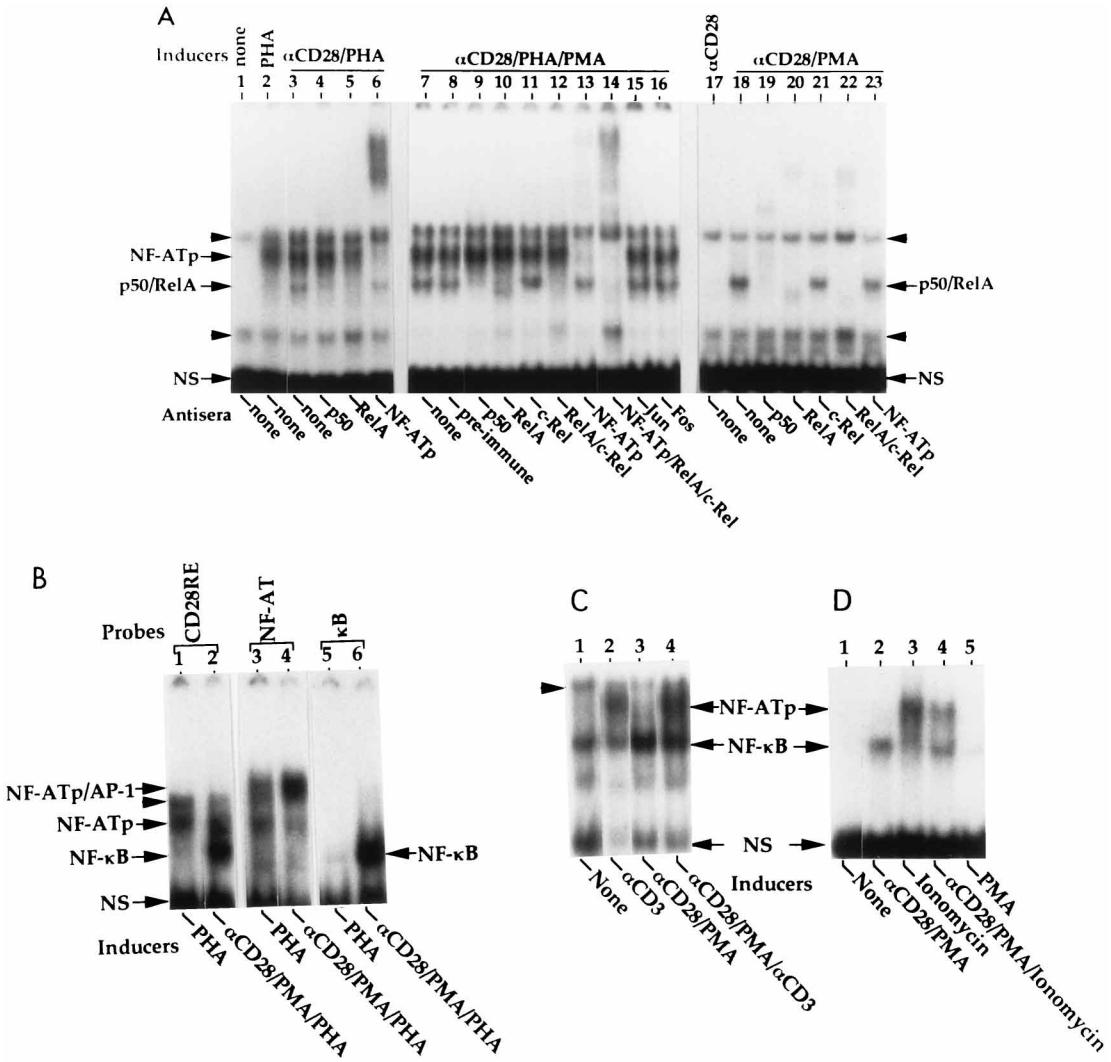


FIG. 3. NF-κB/Rel and NF-ATp are induced by different cellular stimuli. (A) Jurkat T cells were either untreated (lane 1) or incubated for 2 h with the inducers indicated at the top. The concentrations for the inducers were the same as those indicated in the legend to Fig. 2. Nuclear extracts were subjected to EMSA in the absence (none) or presence of various antibodies specific for the proteins indicated at the bottom. The DNA-protein complexes are marked according to the results of the antibody supershift assays. (B) The CD28RE probe, but not the κB and NF-AT probes, binds both NF-ATp and NF-κB. Jurkat cells were stimulated for 2 h with the indicated inducers and collected for nuclear extract preparation. The nuclear extracts were then subjected to EMSA using the $^{32}$P-radiolabeled probes indicated at the top. (C) EMSA of nuclear extracts isolated from murine 16B.2 cells stimulated for 2 h with the indicated inducers. The anti-murine CD3 antibody was coated onto plates at 2 μg/ml; anti-murine CD28 was used in solution (1 μg/ml); PMA was used at a concentration of 10 ng/ml. (D) EMSA of nuclear extracts isolated from human peripheral blood T cells stimulated for 2 h with the indicated inducers. Ionomycin was used at a concentration of 2 μM; the rest of the inducers were as described in the legend to Fig. 2A. The protein bands marked with arrowheads or NS are as described in the legend to Fig. 1.

ATp–Fos–Jun complex (lane 4, NF-ATp/AP-1). On the other hand, the κB probe formed a strong complex with NF-κB but failed to associate with NF-ATp (lane 6). Thus, while CD28RE is able to bind both NF-κB and NF-ATp, the NF-AT and κB enhancers selectively associate with NF-ATp and NF-κB, respectively.

To examine whether induction of the CD28RE-binding complexes could be recapitulated in nontransformed T cells, studies were performed with a murine T-cell clone (16B.2) and human peripheral blood T cells. In untreated 16B.2 cells, a basal level of nuclear NF-κB binding activity was detected (Fig. 3C, lane 1), which was likely due to the presence of IL-2 in the medium. When the cells were stimulated with anti-CD3, the NF-ATp DNA binding activity was induced (lane 2). Furthermore, as observed in Jurkat cells, stimulation of the cells with anti-CD28 plus PMA failed to induce NF-ATp although this treatment significantly enhanced the binding of NF-κB (lane 3). Induction of both NF-κB and NF-ATp was detected in cells incubated with both anti-CD3 and PMA–anti-CD28 (lane 4). The NF-κB complex was composed of primarily p50 and RelA, as demonstrated by a parallel supershift assay (data not shown). In untreated human T cells, neither NF-ATp nor NF-κB could be detected with the CD28RE probe (Fig. 3D, lane 1). Stimulation of the cells with PMA led to only a slight induction of the NF-κB band (lane 5). As expected, NF-κB was potently induced by anti-CD28-PMA (lane 2), whereas NF-ATp was induced by the calcium ionophore ionomycin (lane 3). The binding of both NF-ATp and NF-κB to the CD28RE probe was detected in cells stimulated with both the calcium and CD28-PMA signals (lane 4). The identities of the NF-κB and NF-ATp complexes in both Fig. 3C and Fig. 3D were confirmed by antibody supershift assays (data not shown).

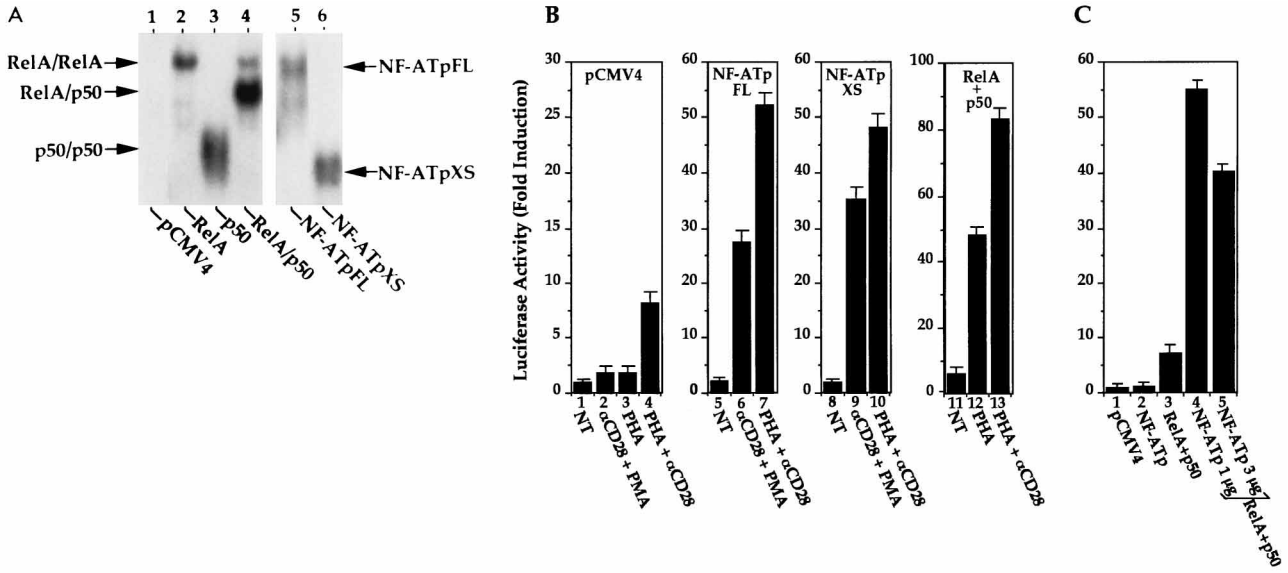


FIG. 4. Transfected NF-ATp and p50-RelA NF-κB proteins bind to the CD28RE probe and transactivate the CD28RE-luc reporter in stimulated cells. (A) EMSA of the nuclear extracts isolated from 293 cells transfected with either the parental vector pCMV4 lacking a cDNA insert or the indicated NF-κB and NF-ATp cDNA expression vectors. The identity of each of the DNA-protein complexes was confirmed by antibody supershift assays (data not shown). (B) Luciferase reporter gene assays showing the function of NF-κB and NF-ATp in the activation of the CD28RE enhancer. Jurkat cells ($5 \times 10^6$) were not transfected (NT) or transfected with either 2 μg of the parental pCMV4 vector, 2 μg of the cDNA expression vectors encoding the full-length (NF-ATpFL) or truncated form (NF-ATpXS) of NF-ATp, or the p50 and RelA cDNA expression vectors together (1 μg of each), along with the CD28RE-luc reporter plasmid (8 μg). After 40 h posttransfection, the recipient cells were incubated for 8 h with the indicated inducers and then subjected to luciferase assay as described for Fig. 2A. (C) Synergy of NF-ATp and NF-κB/Rel in the activation of CD28RE. Jurkat cells were transfected with either pCMV4 (column 1), the cDNA for NF-ATpXS (1 μg; column 2), or the cDNA for NF-κB (1 μg of RelA plus 0.5 μg of p50) in the absence (column 3) or presence (columns 4 and 5) of the indicated amounts of the NF-ATpXS cDNA. The cells were also cotransfected with the CD28RE-luc reporter plasmid (2 μg). The recipient cells were subjected to luciferase assay 40 h posttransfection.

Thus, activation of NF-κB/Rel appears to be mediated by the CD28 costimulatory signal in synergy with the PKC stimulator PMA, whereas a calcium signal mediated by stimulation of the TCR complex may be required for the activation of NF-ATp. Furthermore, together with results of the reporter gene assays (Fig. 2), these results suggest that functional activation of the CD28RE enhancer by the multiple stimulation signals is correlated with the rapid induction of both NF-ATp and the p50-RelA NF-κB heterodimer.

**Ectopic NF-ATp and p50-RelA heterodimer participate in the transactivation of the CD28RE-luc in Jurkat T cells.** To investigate the functional importance of NF-ATp and p50-RelA in the transcriptional control of CD28RE, transient-transfection studies were performed to examine the ability of each of these proteins to bind to and transactivate CD28RE. For the DNA binding studies, cDNA expression vectors encoding NF-ATp or the p50-RelA NF-κB proteins were transfected into human 293 cells, and the nuclear extracts were subjected to EMSA using a $^{32}$P-labeled CD28RE as probe (Fig. 4A). As expected, the transfected RelA (lane 2) and p50 (lane 3) both bound to CD28RE, and when expressed together in the cells, these two NF-κB subunits formed a heterodimer on the CD28RE probe (lane 4). Furthermore, under these overexpression conditions, a portion of the full-length NF-ATp was expressed in the nucleus of the cells and able to bind to the CD28RE probe (lane 5). An NF-ATp truncation mutant, NF-ATpXS, previously shown to be functional on the transactivation of the NF-AT enhancer (28), was more readily expressed in the nucleus (data not shown) and thus exhibited stronger CD28RE binding activity (lane 6). Functional reporter gene assays performed in Jurkat T cells revealed that overexpression of the full-length NF-ATp or the truncation mutant, NF-ATpXS, failed to transactivate the CD28RE-luc reporter (Fig. 4B, columns 5 and 8). However, both types of NF-ATp molecules markedly enhanced the activation of CD28RE-luc by PHA plus anti-CD28 (compare columns 4, 7, and 10). More importantly, expression of the NF-ATp constructs rendered the cells responsive to stimulation by anti-CD28 plus PMA in the absence of PHA (columns 6 and 9). The latter function was seen better with the NF-ATp truncation mutant than with its full-length form, which was probably because that the truncated form was more readily expressed in the nucleus in the absence of a calcium signal. No significant induction of luciferase activity was detected when these NF-ATp-transfected cells were stimulated with the calcium ionophore ionomycin although the reporter gene activity was moderately induced by PHA (data not shown). Since ionomycin and PHA were both able to induce the nuclear expression of NF-ATp (Fig. 3A and D), the weak stimulatory effect of PHA (also shown in Fig. 2A) might be due to the induction of some factors other than the elevation of NF-ATp nuclear translocation. Transfection of the cells with cDNAs encoding p50 and RelA only slightly activated the CD28RE-luc reporter gene (Fig. 4B, column 11) although this NF-κB heterodimer could translocate to the nucleus and readily bound to the CD28RE probe in EMSA (Fig. 4A). However, this exogenously transfected NF-κB heterodimer potently transactivated the CD28RE-luc in synergy with PHA (column 12) and markedly enhanced the transactivation activity triggered by PHA plus anti-CD28 (column 13). These results strongly suggest that both NF-ATp and the NF-κB heterodimer participate in activation of the CD28RE enhancer although neither of them appears to be sufficient for this transactivation event.

To directly test whether NF-ATp and NF-κB functionally synergize in the activation of CD28RE, luciferase studies were performed with cDNA expression vectors encoding NF-

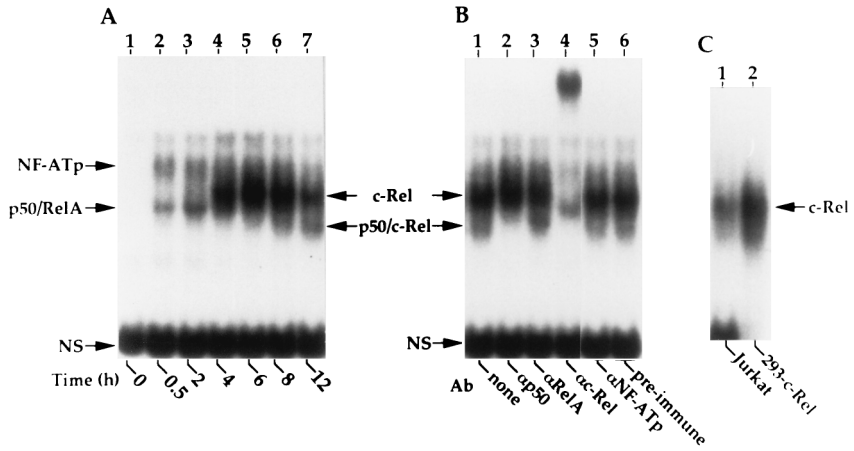


FIG. 5. CD28RE is bound by different factors at different phases of induction. (A) Induction kinetics of the CD28RE-binding proteins. Jurkat cells were stimulated for the indicated time periods with anti-human CD28 together with PMA and PHA (concentrations were as indicated in the legend to Fig. 2). Nuclear extracts were subjected to EMSA using the $^{32}$P-labeled CD28RE probe. (B) Antibody supershift assays that identify the CD28RE-binding proteins. A nuclear extract isolated from Jurkat cells that were stimulated for 8 h (as described for panel A) was subjected to EMSA in the absence (none) or presence of either a preimmune serum (pre-immune) or the indicated specific immune antisera. Similar results were obtained in assays using extracts from cells stimulated for 4 and 6 h, which revealed c-Rel as the predominant CD28RE-binding protein (data not shown). The identities of NF-ATp and p50-RelA heterodimer were also confirmed by supershift assays (Fig. 3A and data not shown). (C) The c-Rel-containing Jurkat CD28RE-binding complex comigrates with the transfected c-Rel homodimer. EMSA was performed with nuclear extracts isolated from either Jurkat cells stimulated for 6 h or 293 cells transfected with a c-Rel cDNA expression vector. The c-Rel–DNA complex is indicated.

ATpXS and the NF-κB p50-RelA heterodimer (Fig. 4C). As expected, when expressed alone, these factors either failed to activate or very weakly activated CD28RE-luc (columns 2 and 3). However, when these two families of transcription factors were cotransfected into the cells, luciferase activity was markedly induced (columns 4 and 5). Of note, higher concentrations of NF-ATp tended to lower the level of synergy (column 5 and data not shown), suggesting that the ratio between these factors played an important role in the synergy. We also observed that when these cotransfected cells were stimulated with either PHA or anti-CD28-PMA, markedly higher luciferase activity could be detected (data not shown). Together, these results suggest that optimal activation of CD28RE may require the induction of both NF-ATp and NF-κB as well as certain additional factors.

**CD28RE is bound by different factors at different phases of induction.** The foregoing studies suggest that NF-ATp and the NF-κB heterodimer are the predominant CD28RE-binding proteins during the early time (2 h) of cellular stimulation and that these factors appear to participate in the activation of the CD28RE enhancer. To investigate whether different CD28RE-binding proteins are induced during different phases of cellular stimulation, we performed a kinetic study using anti-CD28 together with PHA and PMA as inducers. As shown in Fig. 5, stimulation of the cells for up to 2 h induces primarily NF-ATp and the p50-RelA NF-κB heterodimer (Fig. 5A, lanes 2 and 3), as confirmed by antibody supershift assays (data not shown and Fig. 3). However, further cellular stimulation led to the potent induction of a more diffused protein band that migrated slightly more slowly than the p50-RelA heterodimer (Fig. 5A, lanes 4 to 7). This novel protein complex was readily supershifted by the anti-c-Rel antibody (Fig. 5B, lane 4) but did not appreciably react with antibodies against p50 (lane 2), p65 (lane 3), or other NF-κB/Rel subunits, including p52 and RelB (data not shown), or the anti-NF-ATp antibody (lane 5). Furthermore, this c-Rel-containing complex comigrated with the c-Rel homodimer generated with a c-Rel-transfected 293 cell extract (Fig. 5C). Thus, although a definitive answer requires further biochemical analyses, these results indicate that the c-Rel-containing CD28RE-binding complex is likely a c-Rel homodimer. This potential c-Rel homodimer was clearly detected at 4 h of cellular stimulation and declined after 12 h. Interestingly, another protein complex that migrated more rapidly than p50-RelA was detected at 8 h (Fig. 5A, lane 6); it became more apparent at 12 h (lane 7) and was sustained until at least 16 h after cellular stimulation (data not shown). This late-induced complex appeared to be the p50–c-Rel heterodimer since its formation was abrogated in the presence of anti-p50 or anti-c-Rel (Fig. 5B, lanes 2 and 4, and data not shown). Together, these results suggest that CD28RE binds to different transcription factors at different phases of cellular stimulation.

To correlate the induction of the early and late CD28RE-binding proteins with the activation of IL-2 gene transcription, the kinetics of transcriptional activation of the IL-2 gene was analyzed by using nuclear run-on assays. As shown in Fig. 6, transcription of the IL-2 gene was clearly detected as early as 2 h following cellular stimulation and remained active at least until 6 h. Transcriptional activity reached the peak level between 6 and 8 h (data not shown) and declined at 12 h (Fig. 6). Therefore, the kinetics of IL-2 gene transcription correlated with the early induction of NF-ATp and p50-RelA as well as the subsequent induction of the c-Rel homodimer.

**Inhibition of the induction of either NF-ATp or NF-κB/Rel is correlated with the blockade of CD28RE activation.** To further correlate the induction of CD28RE-binding proteins and the activation of CD28RE, the effect of the pharmacological agent CSA on these biochemical and functional events was investigated. CSA is an immunosuppressive drug known to specifically inhibit the calcium-dependent phosphatase calcineurin and influence NF-AT induction (8). As expected, the early-phase induction of NF-ATp, but not the NF-κB p50-RelA heterodimer, was blocked by CSA (Fig. 7A; compare lanes 1 and 4). Interestingly, the subsequent induction of the c-Rel complex (lanes 2 and 3) was also markedly inhibited by CSA (lanes 5 and 6), suggesting that induction of the potential c-Rel homodimer was dependent on the calcium/calcineurin signaling pathway. In support of this notion, stimulation of the cells with anti-CD28-PMA in the absence of PHA failed to induce the c-Rel complex, although this treatment was suffi-

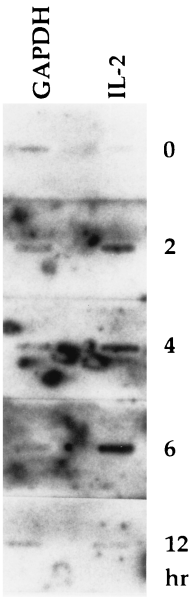


FIG. 6. Nuclear run-on analysis to determine the induction kinetics of IL-2 gene transcription. Jurkat T cells were stimulated for the indicated time periods with anti-human CD28 together with PMA and PHA (at the concentrations indicated in the legend to Fig. 2A). The nuclei isolated from these cells were subjected to nuclear run-on reactions, and the generated $^{32}$P-radiolabeled RNAs were used to detect the IL-2 transcript by hybridization to a human IL-2 cDNA immobilized on nitrocellulose membranes. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as a control.

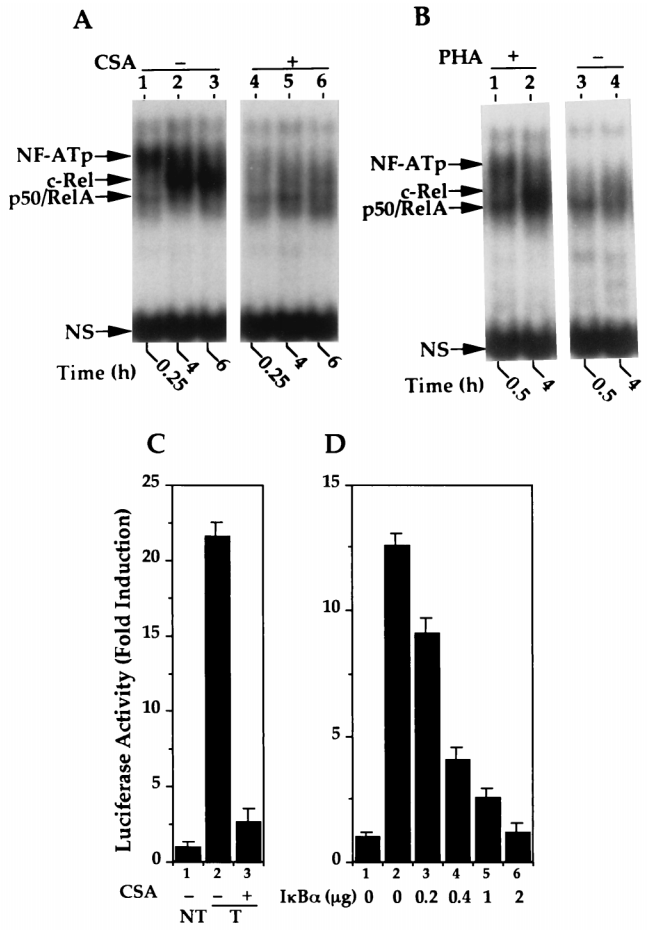


FIG. 7. Inhibition of either NF-ATp or NF-κB/Rel blocks activation of CD28RE-luc. (A) Induction of NF-ATp and the c-Rel homodimer is inhibited by CSA. Jurkat cells were either not treated (−) or incubated for 5 min with 1 μg of CSA per ml (+) and then stimulated for the indicated time periods with anti-human CD28 together with PMA and PHA. Nuclear extracts were subjected to EMSA. The DNA-protein complexes are indicated. NS, nonspecific band. (B) Induction of NF-ATp and c-Rel requires PHA. Jurkat cells were stimulated for the indicated time periods with anti-human CD28 plus PMA in either the presence (+) or absence (−) of PHA and then collected for nuclear extract preparation. The CD28RE-binding proteins were analyzed by EMSA. (C) Induction of CD28RE-luc is inhibited by CSA. Jurkat cells were transfected with CD28RE-luc (10 μg), and the recipient cells were either not treated (NT) or stimulated for 8 h with PHA together with anti-human CD28 and PMA (as described for Fig. 2A) in the presence (+) or absence (−) of CSA (T). The luciferase assay was performed as described in the legend to Fig. 2A. (D) Jurkat cells were transfected with CD28RE-luc (5 μg) along with the indicated amounts of cDNA expression vector encoding an IκBα mutant, IκBα(37-317). The recipient cells were either not treated (column 1) or stimulated (columns 2 to 6) and then subjected to luciferase assay as described for panel C.

cient to induce the p50-RelA heterodimer (Fig. 7B; also see Fig. 3). Parallel luciferase assays revealed that CSA potently inhibited the activation of the CD28RE-luc reporter gene (Fig. 7C). A similar negative result was obtained when the cells were stimulated with anti-CD28-PMA in the absence of PHA (Fig. 2). Thus, inhibition or failure of induction of the NF-ATp and c-Rel complexes is correlated with the inhibition of CD28RE activation.

The role of NF-κB/Rel in the activation of CD28RE was further examined by analyzing the effect of an NF-κB/Rel dominant-negative inhibitor, IκBα(37-317), on the signal-mediated induction of CD28RE-luc. This inhibitory molecule, which lacked the sites for inducible phosphorylation and degradation, had previously been shown to be able to block the activation of the κB enhancer by various NF-κB/Rel inducers (4, 27, 41). As expected, when the CD28RE-luc gene was cotransfected with increasing amounts of the IκBα cDNA, induction of luciferase expression was markedly inhibited (Fig. 7D).

**Activation of CD28RE by NF-ATp and NF-κB/Rel plays an important role in induction of the IL-2 promoter.** To confirm that the functional results obtained with CD28RE-luc reflect the regulatory features of the intact IL-2 promoter, studies were performed to examine the functional importance of the NF-ATp and NF-κB factors in the activation of a luciferase reporter gene driven by the full-length IL-2 promoter (IL-2-luc, 575 bp [39]). For these studies, IL-2-luc was transfected into human Jurkat T cells followed by various stimulations. As shown in Fig. 8A, significant activation of the IL-2 promoter occurred only when the cells were stimulated with both PHA and anti-CD28 (Fig. 8A, columns 1 to 4). However, as seen with activation of the CD28RE enhancer (Fig. 4B), expression of NF-ATp rendered the cells responsive to stimulation by anti-CD28 and PMA in the absence of PHA (Fig. 8A, columns 5 and 6). Parallel studies with the RelA-p50 heterodimer revealed that, as previously demonstrated (24), expression of this NF-κB heterodimer induced low levels of IL-2 promoter activity (Fig. 8A, column 7). More importantly, the RelA-p50 heterodimer markedly induced luciferase expression in synergy with PHA (column 8). To prove that the synergy between the transfected proteins and the CD28 and TCR signals was mediated through CD28RE, an IL-2-luc construct lacking the CD28RE site (ΔCD28RE) was used in the luciferase assay. As shown in Fig. 8B, deletion of the CD28RE enhancer markedly diminished the response of the IL-2 promoter to the stimulation signals. Thus, activation of CD28RE by NF-ATp and

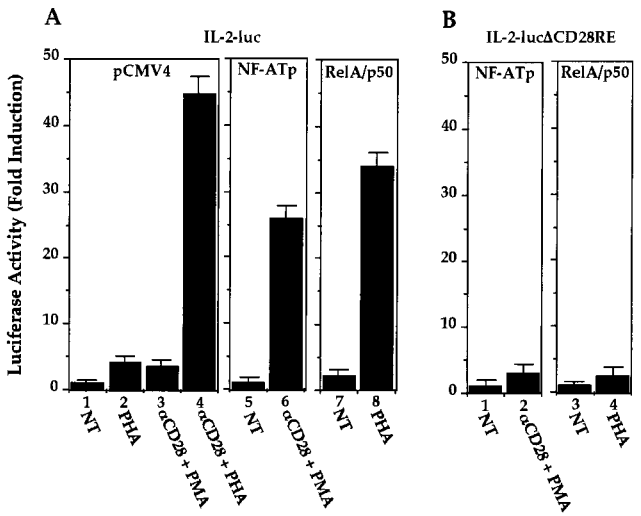


FIG. 8. CD28RE is required for NF-ATp- and NF-κB-mediated activation of the IL-2 promoter. (A) Both NF-ATp and NF-κB transactivate the IL-2 promoter in synergy with cellular stimuli. Jurkat cells were transfected with either 2 μg of the parental pCMV4 vector (columns 1 to 4) or the same amount of cDNA expression vector encoding NF-ATpXS (columns 5 and 6) or the RelA-p50 NF-κB heterodimer (columns 7 and 8), along with a luciferase reporter driven by the human IL-2 promoter (IL-2-luc; 2 μg). The cells were stimulated with the indicated inducers and then subjected to luciferase assay as described for Fig. 2A. (B) Effect of CD28RE deletion on the inducible activity of the IL-2 promoter in NF-ATp- and NF-κB-transfected cells. Jurkat cells were transfected with 2 μg of an IL-2-luc construct lacking the CD28RE site (IL-2-lucΔCD28RE), along with 2 μg of the NF-ATpXS (columns 1 and 2) or NF-κB (columns 3 and 4) cDNA expression vector. The recipient cells were either left untreated (NT) or stimulated with the indicated inducers and then subjected to luciferase assay.

NF-κB/Rel appears to be critical for the promoter activity of the IL-2 gene.

## DISCUSSION

Transcriptional activation of the IL-2 gene requires both the TCR-mediated primary and the CD28-mediated costimulatory signals. The costimulatory effect of the CD28 signal is modulated by the CD28RE enhancer located in the promoter region of the IL-2 gene. Deletion of CD28RE markedly diminishes the activation of the IL-2 promoter by anti-CD28 together with mitogens that mimic TCR signaling (9). The identities of the transcription factors involved in the regulation of CD28RE have remained elusive. While some studies have shown that the NF-κB/Rel proteins bind to CD28RE in activated Jurkat and human peripheral T cells (5, 13), other studies have suggested that NF-ATp, not NF-κB/Rel, is responsible for the activation of the CD28RE. In the present study, we analyzed the CD28RE-binding proteins in cells stimulated with different inducers. Our studies demonstrate that CD28RE is bound by different factors in response to different cellular stimuli. Consistent with a prior study (33), stimulation of the TCR complex with anti-CD3 or PHA led to the induction of an NF-ATp-containing CD28RE-binding complex but not NF-κB/Rel. On the other hand, treatment of the cells with anti-CD28 and PMA in the absence of TCR stimulation induces only NF-κB/Rel but not NF-ATp, which is in agreement with some (5, 13) but not all (33) of the previous studies. Interestingly, although anti-CD28 alone fails to induce any detectable CD28RE-binding proteins, together with PHA or anti-CD3, it induces both NF-ATp and the p50-RelA NF-κB heterodimer, and this costimulatory effect is further potentiated by the PKC activator PMA. We have also shown that the CD28RE enhancer is bound by different factors at different phases of induction in the costimulated cells. During the early phase of cellular stimulation (15 min to 2 h), NF-ATp and the p50-RelA NF-κB heterodimer are the predominant CD28RE-binding factors. However, further cellular stimulation leads to the potent induction of a potential c-Rel homodimer that remains predominant until 8 h following cellular stimulation. Induction of the c-Rel complex, but not that of NF-ATp and the p50-RelA heterodimer, is sensitive to the protein synthesis inhibitor cycloheximide (data not shown). Thus, while activation of NF-ATp and the p50-RelA NF-κB heterodimer likely results from the nuclear translocation of these proteins, the activation of c-Rel appears to be mediated through de novo protein synthesis. Interestingly, like the early induction of NF-ATp, the late-phase induction of c-Rel is sensitive to CSA (Fig. 7A). These results suggest that activation of c-Rel may require a calcium signal. It remains an interesting question whether the calcium-dependent NF-AT transcription factors are involved in the transcriptional activation of the c-*rel* gene.

A recent study has shown that anti-CD28 and PMA are able to induce the binding of NF-AT to the IL-2 distal NF-AT site in the absence of a calcium signal (12). However, under these conditions, we were unable to detect any NF-ATp DNA binding activity in assays using the CD28RE probe (Fig. 3A, lane 23). This apparent discrepancy is most likely due to the different probes used in the EMSA. For example, the NF-AT and CD28RE sequences may preferentially bind to different NF-AT members. In support of this notion, the NF-AT-binding complex detected in the prior study only weakly immunoreacted with the anti-NF-ATp antibody (12), suggesting that NF-ATp is not the major NF-AT species in this calcium-independent NF-AT complex. In contrast, the CD28RE-binding NF-AT complex contains primarily NF-ATp since the entire complex can be supershifted by the anti-NF-ATp antibody (Fig. 3A).

Our functional reporter gene studies have suggested that the CD28RE enhancer activity is tightly controlled by multiple transcription factors. Cellular stimuli that induce either NF-ATp or NF-κB/Rel fail to significantly activate the CD28RE. Optional activation of this enhancer is correlated with the induction of both NF-ATp and the NF-κB/Rel factors. Consistently, expression of neither NF-ATp nor the p50-RelA NF-κB heterodimer in Jurkat cells is sufficient for activation of the CD28RE-luc reporter; however, both ectopic factors are able to synergize with cellular stimuli in the activation of CD28RE as well as the intact IL-2 promoter (Fig. 4 and 8). These findings may suggest that NF-ATp and NF-κB/Rel activate CD28RE cooperatively. However, our in vitro studies have shown that NF-ATp and NF-κB/Rel do not seem to cooperate in binding to the CD28RE probe since both factors can readily bind to CD28RE alone (Fig. 4A). Thus, it is likely that NF-ATp and NF-κB/Rel function independently, both requiring additional factors for the transactivation of the CD28RE enhancer. On the other hand, it is also possible that NF-ATp and NF-κB/Rel cooperate indirectly by, for example, cooperatively inducing other transcriptional activators such as c-Rel. A functional cooperation between NF-ATp and NF-κB/Rel is suggested by the finding that coexpression of NF-ATp and the RelA-p50 NF-κB heterodimer in Jurkat T cells leads to a marked induction of the CD28RE-luc reporter gene (Fig. 4C). While further studies will be required to test these notions, our present study strongly suggests that both NF-ATp and the p50-RelA heterodimer participate in the activation of the CD28RE enhancer.

Our studies have revealed a potential c-Rel homodimer that

represents the predominant form of a CD28RE-binding factor during the late phase of cellular stimulation. This c-Rel complex appears 4 h following cellular stimulation and remains predominant until 8 h, while the DNA binding activity of NF-ATp and the p50-RelA heterodimer becomes undetectable at this later time point (Fig. 5B). A role of c-Rel in the activation of CD28RE has been demonstrated by a previous study, which shows that when transiently expressed in Jurkat T cells, c-Rel alone is able to moderately activate the CD28RE enhancer (13). We obtained similar results in the present study (data not shown). These results suggest that the c-Rel homodimer may serve as a positive factor in regulation of the CD28RE enhancer. The role of c-Rel in regulation of the endogenous IL-2 gene is suggested by our finding that the appearance of the c-Rel homodimer in the nucleus is correlated with a marked increase in IL-2 gene transcription (Fig. 5 and 6). Furthermore, recent gene knockout studies have revealed that inactivation of the c-*rel* gene in mice markedly inhibits the induction of the IL-2 gene by anti-CD3 and anti-CD28 (11, 23). Thus, c-Rel appears to be important for activation of the IL-2 gene through the TCR and CD28 signals. Together, our studies strongly suggest that activation of the IL-2 gene through the CD28 costimulatory signal involves both NF-κB/Rel and NF-ATp and that these factors functionally interact with CD28RE.

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