FK506 Inhibits Antigen Receptor–mediated Induction of c-*rel* in B and T Lymphoid Cells

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Summary

Stimulation of B and T cells via the antigen receptor, by phorbol ester or by phorbol ester and ionomycin, leads to nuclear translocation of the inducible transcription factor NF-κB, comprising the p50 and p65 *rel*-related polypeptides. In this report we show that c-*rel* is a component of the antigen receptor-induced κB binding proteins in both B and T cells. Whereas NF-κB can be induced by phorbol ester alone, optimal induction of c-*rel* requires stimulation by both phorbol ester and ionomycin, the dual signal that is necessary for proliferation of untransformed lymphocytes. Furthermore, c-*rel* induction is blocked by the immunosuppressive drug FK506 that is known to inhibit B and T cell activation. c-*rel*-dependent transactivation of the interleukin-2 receptor α chain (IL-2Rα) promoter is augmented by coexpression of calcineurin, suggesting the involvement of a calcineurin-dependent intracellular pathway. Our results identify c-*rel* as a target of immunosuppressive agents and illustrate the similarity of activation pathways in both B and T cells.

Stimulation of B and T cells via the antigen receptor triggers a signaling cascade that results in clonal cell proliferation characteristic of the immune response. Alteration of the resting program of gene expression is brought about by inducible nuclear factors that transmit the signal generated at the plasma membrane to the cell nucleus. Several transcription factors have been identified that are induced in response to antigen receptor cross-linking, or its pharmacologic equivalent, the combined action of phorbol esters and calcium ionophore. We, and others, have shown that antigen-receptor cross-linking on both B and T cells induces the factors NF-κB, AP-1, and cAMP response element-binding protein (CREB) (1–4). However, these proteins can be induced by the action of phorbol ester alone on these cells, a signal that is not sufficient to induce proliferation. A notable exception is NF-AT, a nuclear factor present in activated T cells, whose induction requires the dual signal of phorbol ester and ionomycin (5). We have recently shown that both signals are also required to induce an indistinguishable DNA-binding protein in B cells, indicating that NF-AT should be regarded more generally as a factor of activated lymphoid cells (6). Because it is likely that proteins that are induced in response to the complete proliferative signal will play a critical role in normal and aberrant immune responses, it is essential to identify factors that satisfy this criterion.

The immunosuppressive drugs cyclosporin A and FK506 block B and T cell proliferation (7–9). The intracellular pathway of drug action in T cells has been recently elucidated. Both drugs bind to distinct cytosolic receptors and the drug receptor complexes inhibit the enzymatic activity of the calcium and calmodulin-dependent serine/threonine phosphatase, calcineurin (10–13). NF-$AT_{p/c}$, the preexisting cytoplasmic component of NF-AT (14–16), has been implicated as a direct target of calcineurin (17, 18). In this model, calcium-dependent translocation of NF-$AT_{p/c}$ to the nucleus is triggered by dephosphorylation of this protein by calcineurin. In contrast, the mechanism of immunosuppressive action in the B cell compartment is unknown. The observation that sIg, but not LPS, mediated B cell proliferation is blocked by these drugs (9, 19) suggests that they interfere with a calcium-dependent pathway, but no nuclear mediators of the effects of immunosuppressive drugs in B cells have been identified. We have recently demonstrated that NF-AT induction in splenic B cells is suppressed by cyclosporin A, indicating that this protein may be partly responsible for the effects of these drugs in B cells (6). In this report we show that the proto-oncogene c-*rel* is a component of antigen receptor-induced κB-binding proteins in both B and T cells. Whereas NF-κB (p50/p65 *rel* related heterodimer) can be induced by phorbol ester alone, optimal induction of c-*rel* requires stimulation by both phorbol ester and ionomycin. Furthermore, c-*rel* induction in both B and T cells is blocked by FK506. The effect of FK506 can be reversed by excess rapamycin, and c-*rel*–dependent transactivation of the IL-2Rα promoter is augmented by coexpression of calcineurin, suggesting the involvement of a calcineurin-dependent intracel-

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lular pathway. Our results identify c-*rel* as a target of immunosuppressive action and illustrate the fundamental similarity of activation pathways in B and T cells.

Materials and Methods

Cell Culture and Inductions. The murine B lymphoma cell line Bal 17 was maintained in RPM1 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum, 50 μM β-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were cultured at a density of 5–6 $\times$ 10^5 cells/ml during stimulation. Bal 17 B cells were stimulated for 1–2 h (37°C) with 20 ng/ml PMA (Sigma Chemical Co., St. Louis, MO), 20 ng/ml PMA, and 2 μM ionomycin (Calbiochem-Novabiochem Corp., La Jolla, CA) or affinity-purified goat anti-mouse $F(ab')_2$ fragment, μ chain specific, (anti-Ig; Jackson ImmunoResearch Labs., West Grove, PA) at 10–20 μg/ml. D5h3 is a hybridoma derived from Ar-5 cells by fusion with a TcR^- variant of BW 5147 thymoma cells. D5h3 cells were grown in DMEM medium supplemented with 10% FCS, 2 mM glutamine, 10 mM Hepes, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μM β-mercaptoethanol. These cells were stimulated for 4–5 h at a density of 5–6 $\times$ 10^5 cells/ml. Inducer concentrations were 40 ng/ml PMA, 2 μM ionomycin. D5h3 cells were also activated with cross-linked mAb to murine CD3ϵ. 2C11 (anti-CD3ϵ) antibody was used at 1:100 dilution, and was cross-linked by immobilized rabbit anti-hamster antibody (N. L. Cappel Laboratories, Cochranville, PA) as described previously (2). FK506, a generous gift from Dr. Stuart Schreiber, (Harvard University, Cambridge, MA) was used at a final concentration of 2 nM where indicated and was added 5–7 min before addition of other inducing agents.

Nuclear Extracts and Electrophoretic Mobility Shift Assays. Preparation of small scale nuclear extracts from untreated activated cells and labeling of oligonucleotide probes were done as described previously (2). The binding site (H2K) derived from the murine H2K MHC class I gene promoter was isolated as an EcoRI-HindIII fragment from the plasmid subclone (20) and used as a probe. In vitro binding reactions were carried out at room temperature with 6–10 μgm of nuclear extract for 15 min.

Antibody Shifts and Competitions. For antibody shifts, standard binding reactions were carried out, followed by addition of either rabbit anti c-*rel* antiserum 1050 gift of Dr. N. Rice (National Cancer Institute, Frederick, MD) (21) or normal rabbit serum (NRS) and overnight incubation on ice. 1–1.5 μl of a 1:5 dilution of these antisera were used in 20-μl binding reactions. For competition assays, where indicated, 1–2 μg of competitor peptides were included in the binding reaction followed by overnight incubation with antibodies as described above. Sequences of the peptides used for competitions are: (*a*) c-*rel*: NH_2-Asn-Pro-Asp-Asp-Leu-Ala-Arg-Met-Glu-Thr-Pro-Ser-Met-Ser-Pro-Thr-Asp-Leu-Cys; and (*b*) p50: NH_2-Met-Pro-His-Gly-Tyr-Gly-Gln-Glu-Gly-Pro-Ile-Glu.

Western Blots. Western blot analysis was done using the enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL). Nuclear extracts (10 μgm) derived from unstimulated and differently stimulated Bal 17/D5h3 cells were separated by electrophoresis through 8.8% SDS–polyacrylamide gels. Proteins were transferred to ECL-hybond nitrocellulose membranes (Amersham). Primary anti-c-*rel* antibody (SC71 (X); Santa Cruz Biotech, Inc., Santa Cruz, CA) or anti-*rel* B antibody (SC 226(X); Santa Cruz Biotech, Inc.) was used at 1:500 dilution for 3–4 h at room temperature, followed by peroxidase-conjugated anti-rabbit immunoglobulin (Amersham) at 1:1,000. Chemiluminescent detection was carried out according to the manufacturer's specifications.

Transfections and CAT Assays. 2 $\times$ 10^7 S194 plasma (B) cells or Jurkat T cells were transfected with 5 μgm of the reporter plasmid containing a fragment of the IL-2Rα promoter (residues -317 to -227) cloned upstream of the c-*fos* gene TATA box at -56 using the DEAE dextran procedure. The reporter plasmid was cotransfected with 5 μgm of either c-*rel* expression vector or the calcineurin expression vectors (12), or both together as indicated. In transfections containing only one transactivator, total DNA was made up to 15 μgm using pTZ 18R as carrier DNA. Transfected cells were left untreated, or treated with PMA (40 ng/ml) and ionomycin (500 nM), 24 h after transfection. For FK506 treatment, the drug was added at a concentration of 10 nM immediately after the transfection procedure. Whole cell extracts made 48–60 h after transfection were analyzed for CAT enzyme activity as previously described (22) using 100–150 μgm of heat-treated (65°C for 7 min) extract.

Results

Antigen Receptor Cross-linking, or the Dual Signal of Phorbol Ester and Calcium Ionophore, Is Required for Optimal c-rel Induction in B and T Cells. We have previously shown that in splenic B cells NF-κB is constitutively expressed, but is further induced following cross-linking of surface immunoglobulin (anti-Ig), or after stimulation with phorbol ester alone (PMA) or phorbol ester and ionomycin (P+I) (1). Because multiple *rel* family members can bind to the κB DNA probe (23), in this study we have characterized the induction of the c-*rel* component of inducible κB binding proteins. Bal 17 is a B cell line that has been extensively used to study signal transduction via surface immunoglobulin (24, 25), and it exhibits a pattern of NF-κB induction similar to that seen in splenic B cells. Nuclear extracts from Bal 17 cells were analyzed by electrophoretic mobility shift assays (EMSA)[1] in the presence or absence of anti–c-*rel* antibodies, using a DNA fragment containing a NF-κB–binding site. Whereas increased κB binding was evident in cells treated with PMA alone, P+I or anti-Ig, only nuclear extracts made from P+I-treated, or anti–Ig-treated Bal 17 cells, revealed an additional "super-shifted" nucleoprotein complex in the presence of anti–c-*rel* antibody (Fig. 1 *A*, lane *3*, *bold arrow*). Ionomycin treatment by itself did not induce κB binding proteins over the levels constitutively present in Bal 17 cells (data not shown). We conclude that P+I or anti-Ig signals, but not PMA alone, induce c-*rel* containing DNA-binding proteins in these cells. To confirm that the super-shifted complex contained c-*rel* determinants, competitor peptides were included during incubation of the extract and antiserum. The c-*rel* peptide used to generate the anti–c-*rel* antiserum abolished the super-shift (Fig. 1 *A*, lane *4*), whereas an equivalent amount of irrelevant peptide had no effect (lane *5*), supporting the idea that the super-shifted complex contains c-*rel* protein. The small amount of the super-shifted complex probably reflects both

[1] *Abbreviations used in this paper:* CN, calcineurin; CsA, cyclosporin A; EMSA, electrophoretic mobility shift assays; P+I, phorbol ester and ionomycin.

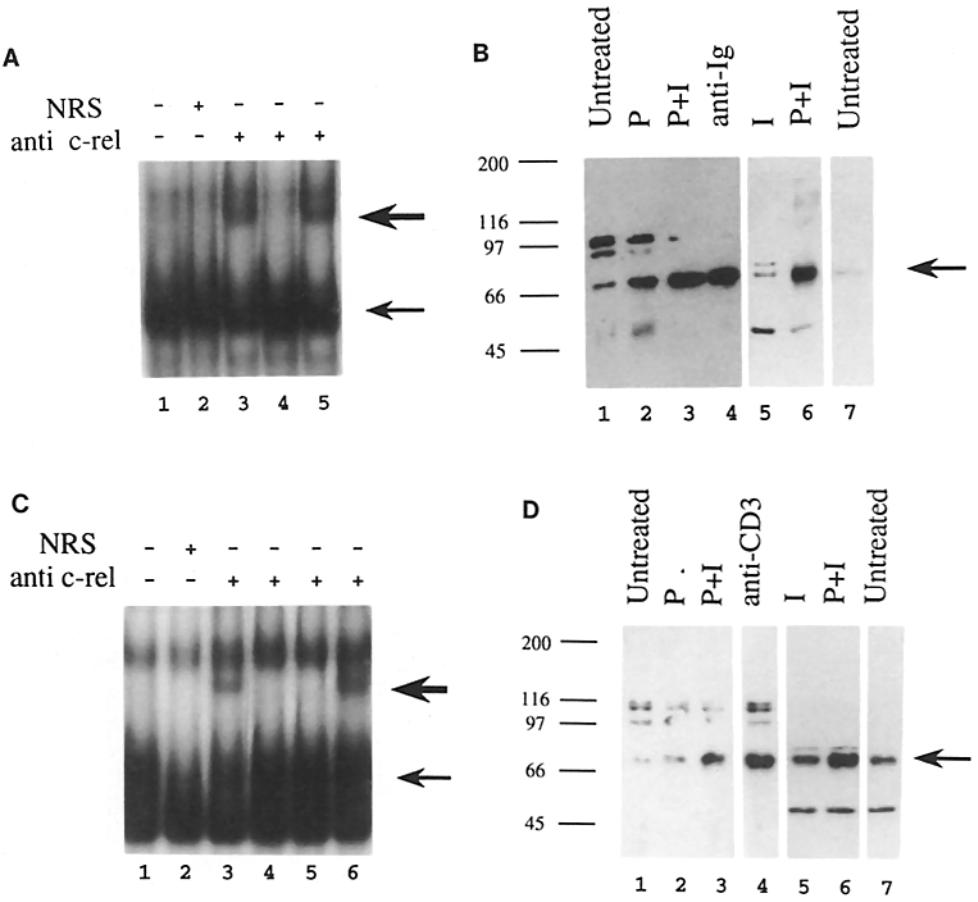

Figure 1. c-*rel* induction in B and T cells. (*A*) Electrophoretic mobility super-shift assays using the NF-κB–binding site from the H2K gene promoter and nuclear extracts derived from Bal 17 cells treated with PMA and ionomycin. Anti-c-*rel* antiserum or normal rabbit serum (*NRS*) were incubated as described, with in vitro binding reactions. (*Bold arrow*) Position of the anti c-*rel* specific super-shifted complex. (*Thin arrow*) Points to the position of NF-κB band. Specificity of super-shifted complex was determined by incubation of competitor peptides with the antiserum before binding analysis. Lane *1*, no added antibody; lane *2*, NRS; lane *3*, anti-c-*rel* antiserum with no added peptide; lane *4*, anti-c-*rel* antibody and 1 μg of c-*rel* peptide used to produce the anti-serum; lane *5*, anti-c-*rel* antibody and 2 μg of a 12-amino acid peptide from the COOH terminus of NF-κB, p50. (*B*) Western blot analysis of nuclear c-*rel* in Bal 17 cells. Nuclear proteins from cells treated as indicated were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with anti-c-*rel* anti-serum. *P*, PMA alone; *P+I*, PMA and ionomycin; anti-Ig, goat anti-mouse μ $F(ab')_2$ fragment; 5-7 represent a different set of extracts than those used in lanes 1-4. *I*, ionomycin alone. (*Arrow*) Position of 72-kD c-*rel* band. (*C*) Electrophoretic mobility super-shift assays using D5h3 (T) cells activated with PMA and ionomycin. Specificity of super-shifted complex was judged by in vitro peptide competition assays. Lane *1*, no added antibody; lane *2*, NRS; lane *3*, anti–c-*rel* anti-serum; lane *4*, anti–c-*rel* plus 2 μg c-*rel* peptide; lane *5*, anti–c-*rel* plus 1 μg c-*rel* peptide; lane *6*, anti–c-*rel* plus 2 μg p50 peptide. Super-shifted complex is indicated by the bold arrow, while the thin arrow points to the NF-κB band. The band below is likely to be the nucleoprotein complex generated by the p50 homodimer. (*D*) Western blot analysis of c-*rel* induction in D5h3 T cells. Nuclear proteins from cells treated as indicated were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti–c-*rel* anti-serum. –, PMA alone; *P+I*, PMA and ionomycin; *anti-CD3*, anti-CD3ε monoclonal antibody, 2C11 and secondary rabbit anti-hamster antibody; *I*, ionomycin alone. Lanes *5–7* represent a different set of extracts than those used in lanes *1–4*. Arrow indicates position of the 72-kD c-*rel* band.

the low abundance of nuclear c-*rel* and inefficient recognition of the DNA/protein complex by the antiserum.

Induction of c-*rel* was also directly visualized by Western blot analysis of nuclear extracts (Fig. 1 *B*). Nuclear extracts from untreated Bal 17 cells contain detectable levels of c-*rel* (Fig. 1 *B*, lane *1*), which were significantly increased by P+I or anti-Ig treatment (Fig. 1 *B*, lanes *3* and *4*). PMA treatment alone induced c-*rel*, albeit at much lower levels (Fig. 1 *B*, lane *2*) and ionomycin treatment alone had no effect (Fig. 1 *B*, compare lanes *5* and 7). We conclude that optimal induction of DNA-binding c-*rel* requires anti-Ig stimulation or the dual signals of P+I.

To extend these results, we examined c-*rel* induction in T cells. Nuclear extracts prepared from D5h3 hybridoma T cells (26) were used in electrophoretic mobility super-shift assays (Fig. 1 *C*). These T cells contain barely detectable levels of NF-κB in EMSA. Treatment with PMA alone for 4 h resulted in a modest increase of the NF-κB complex, whereas higher levels were seen in cells treated with P+I or anti-CD3 monoclonal antibody (data not shown). Similar to the observation in Bal 17 cells, an additional slower mobility nucleoprotein complex (*bold arrow*) was observed in the presence of anti–c-*rel* antibody only in extracts from anti-CD3 or P+I-treated cells (Fig. 1 *C*, lane *3*). Specificity of the super-shifted complex was assessed by peptide competition assays (Fig. 1 *C*, lanes *4–6*). Inclusion of 1 or 2 μg of peptide used to generate the anti-c-*rel* antiserum, abolished the super-shifted complex (Fig. 1 *C*, lanes *4* and *5*) whereas an irrelevant peptide had no effect (Fig. 1 *C*, lane *6*). Western blot analysis of nuclear extracts confirmed the c-*rel* induction pattern seen in EMSA (Fig. 1 *D*). Untreated, or PMA-treated, D5h3 cells contained low levels of nuclear c-*rel* (Fig. 1 *D*, lanes *1* and *2*), which were significantly increased by P+I or anti-CD3 treatment (Fig. 1 *D*, lanes *3* and *4*). In addition, treatment of D5h3 cells with ionomycin alone showed only a slight induction of c-*rel* (Fig. 1 *D*, compare lanes *5* and 7). We conclude that in T cells as well, nuclear DNA-binding c-*rel* is induced in response to T cell receptor or P+I signals. These results em-

phasize the similarity of the nuclear response to antigenic signals in both B and T lymphocytes.

c-rel Induction Is Suppressed by FK506. The immunosuppressive drugs cyclosporin A (CsA) and FK506 have been shown to inhibit T cell responses (7–9). Both drugs bind to distinct intracellular receptors and the drug/receptor complexes inactivate the calcium and calmodulin-dependent serine/threonine phosphatase and calcineurin (10–13, 27). Several studies in T cells have shown that both drugs affect the induction of several early activation-specific genes, including transcription factors such as NF-AT, NF-κB, NF-IL2A (5, 28–31), that presumably mediate the secondary steps of cellular activation leading to proliferation. In these earlier studies, NF-κB induction was seen to be only partially inhibited by FK506 and was therefore considered to be of little consequence for immunosuppression. In further exploring this effect, Frantz et al. have recently shown that calcineurin may participate in NF-κB (p50/p65) induction by indirectly stimulating I-κBα inactivation (32). Our observation that induction of the c-*rel* component of κB binding proteins required a Ca^{2+} signal in addition to a PMA signal, prompted us to explore the effects of immunosuppressive drugs on the induction of this protein. We find by Western blot analysis (Fig. 2), as well as in vitro mobility super shift assays (Fig. 3), that c-*rel* induction is abolished by treatment of Bal 17 and D5h3 cells with either 200 nM CsA (data not shown) or 2 nM FK506.

In Bal 17 cells, total nuclear c-*rel* assayed by Western blots was induced approximately twofold in cells treated with PMA alone, and approximately sixfold in cells treated with either P+I or anti-Ig (Fig. 2 *A*, lanes *3* and *5*). The levels of c-*rel* observed with P+I or anti-Ig were reduced to those seen with PMA alone in the presence of FK506 (Fig. 2 *A*, lanes *4* and *6*), and the drug had no effect on the low levels of c-*rel* induced by PMA alone (data not shown). Thus, induction of c-*rel* by P+I, or anti-Ig treatment of Bal 17 cells is sensitive to low concentrations of FK506, suggesting that some of the observed effects of the drug on B cell proliferation may be due to inhibition of c-*rel* function. Results from three independent sets of nuclear extracts were quantified by densitometry and the average induction is shown below each lane in Figs. 2, *A* and *B*.

Western blot analysis of nuclear c-*rel* expression in D5h3 (T) cells also showed dramatic reduction of this protein in extracts prepared from cells that were stimulated with P+I or anti-CD3, in the presence of FK506 (Fig. 2 *B*). Furthermore, optimal c-*rel* induction in primary adult thymocytes also required the combined action of P+I, and induction was diminished in the presence of FK506 (33). We conclude that in both B and T cell lines, signals transduced via the surface receptors are required for optimal induction of nuclear c-*rel* and that this process is markedly inhibited by CsA and FK506.

We further confirmed the inhibition of c-*rel* induction by FK506 using electrophoretic mobility super-shift assays. In extracts from P+I-treated Bal 17 cells, an additional super-shifted nucleoprotein complex was observed in the presence of anti c-*rel* antibodies (Fig. 3 *A*, lane *4*). Consistent with the immunoblotting analysis, this band was not observed when the extracts from P+I- and FK506-treated cells were used in this assay (Fig. 3 *A*, lane *6*). P+I-treated D5h3 cell extracts also showed a super-shifted nucleoprotein complex indicating the presence of DNA-binding c-*rel* protein (Fig. 3 *B*, lane *4*). Treatment of D5h3 cells with P+I in the presence of FK506 led to a marked reduction of the total induced κB-binding nucleoprotein complex (Fig. 3 *B*, lanes *5* and *6*), and loss of the anti c-*rel*–dependent super-shifted complex. These results suggest either that a large proportion of the

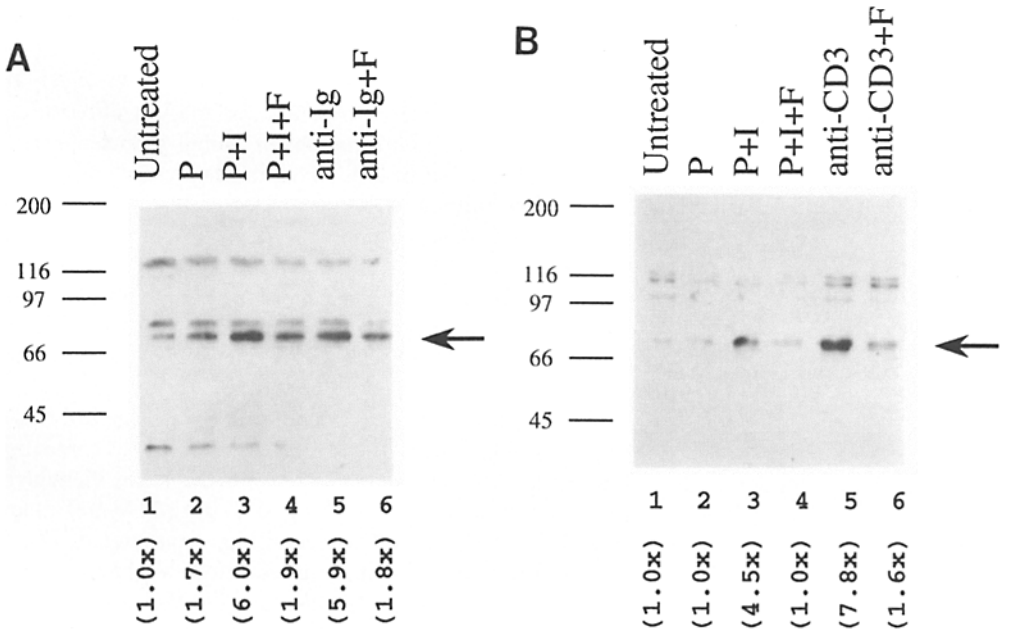

Figure 2. c-*rel* induction is suppressed by FK506 in Bal 17 and D5h3 cells. (*A*) Western blot analysis of Bal 17 cells stimulated as indicated in the presence of FK506. *P*, PMA alone; *P+I*, PMA and ionomycin; *P+I+F*, PMA and ionomycin in the presence of 2 nM FK506; *anti-Ig+F*, goat anti–mouse μ $F(ab')_2$ fragment in the presence of 2 nM FK506. 10 μgm of nuclear proteins were separated by SDS-PAGE, transferred to nitrocellulose paper and probed with anti–c-*rel* anti-serum (Santa Cruz Biotech). Arrow indicates the 72-kD c-*rel* band. Numbers below the lanes show the levels of c-*rel* expression after stimulation and FK506 treatment normalized to untreated Bal 17 nuclear extracts (lane *1*). These figures represent the average of 3 sets of independently generated extracts. (*B*) Western blot analysis of c-*rel* suppression by FK506 in D5h3 cells. 10 μgm of extracts derived from cells treated as indicated were analyzed. Arrow indicates position of c-*rel* protein. Numbers indicate the levels of c-*rel* induction and suppression relative to untreated cells (lane *1*), and represent the average of 3 sets of independently generated extracts.

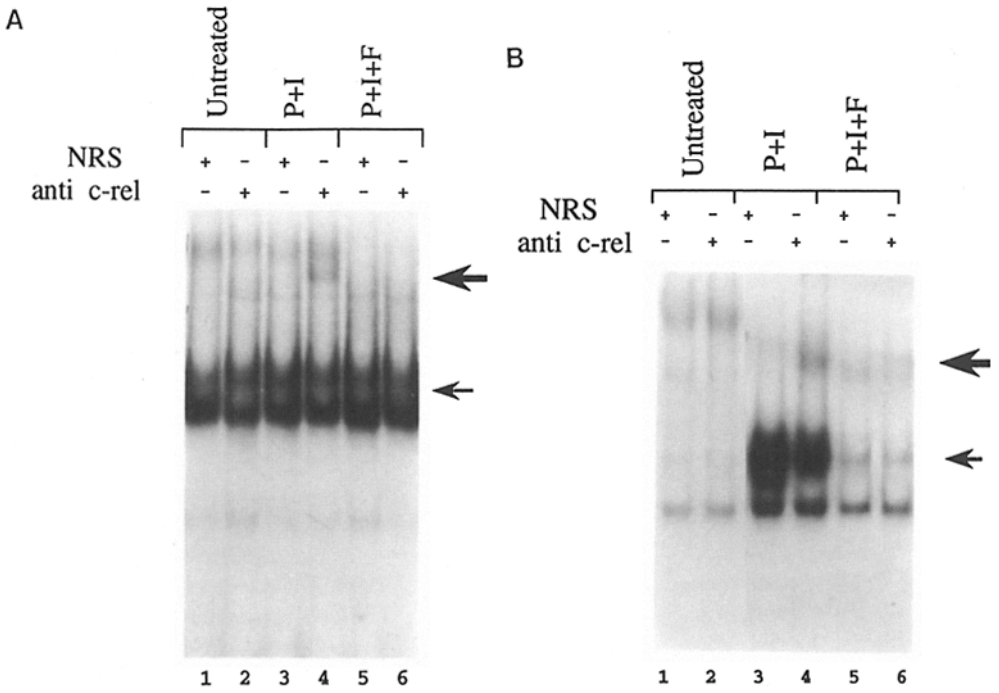

Figure 3. Electrophoretic mobility super-shift analysis of c-*rel* suppression by FK506 in Bal 17 and D5h3 cells. (*A*) Bal 17 cells were stimulated as shown above the lanes and nuclear extracts generated were used in in vitro binding assays in the presence of NRS or anti–c-*rel* serum (anti–c-*rel*) as described in Fig. 1 *A*. P+I+F cells treated with phorbol ester, ionomycin, and 2 nM FK506. Bold arrow indicates the c-*rel*-specific super-shift and the thin arrow the NF-κB complex. (*B*) Nuclear extracts from differently stimulated D5h3 cells were analyzed by EMSA as described above. Anti–c-*rel* specific super-shift is indicated by the bold arrow.

κB binding proteins contained c-*rel* or that induction of other *rel* family members was also sensitive to FK506 as observed in Jurkat cells (32).

The sensitivity of other *rel* family members was further studied by immunoblotting. In both Bal 17 and D5h3 cells, *rel* B protein was induced to similar levels by treatment with either PMA alone or P+I (Fig. 4, *A* and *B*, lanes *2* and *3*), suggesting that a calcium signal was not required for its induction. Consistent with this idea, inclusion of FK506 during P+I treatment did not affect *rel* B levels in either cell type (Fig. 4, *A* and *B*, lane *4*). PMA-treated HeLa cell extracts were included in these experiments as a positive control (Fig. 4, *A* and *B*, lane *5*). Analysis of p50 expression showed no alterations in the levels of this protein in this panel of nuclear extracts (data not shown). These results further indicate that *rel* proteins are differentially sensitive to the effects of FK506.

Rapamycin Reverses FK506 Inhibition of c-rel Induction. Although the major cytosolic receptor for FK506 (FKBP12) is different from that of cyclosporin A (cyclophilin A), both drugs when bound to their intracellular receptors are believed to affect cellular activation by a common mechanism, that is, by inhibiting calcineurin, a Ca^{2+}/calmodulin dependent, and serine/threonine phosphatase. The drug rapamycin competitively inhibits FK506 binding to FKBP12, but the resulting rapamycin/FKBP12 complex does not inhibit calcineurin activity (34, 35). Therefore, rapamycin reversal of FK506 inhibition has been considered to be an indication of the involvement of calcineurin in the process being studied. To determine whether suppression of c-*rel* induction by FK506 occurred via the calcineurin pathway, we carried out rapamycin competition experiments (Fig. 5). In Bal 17 B cells, 2 nM or 0.5 nM FK506 significantly reduced c-*rel* induction by P+I (Fig.

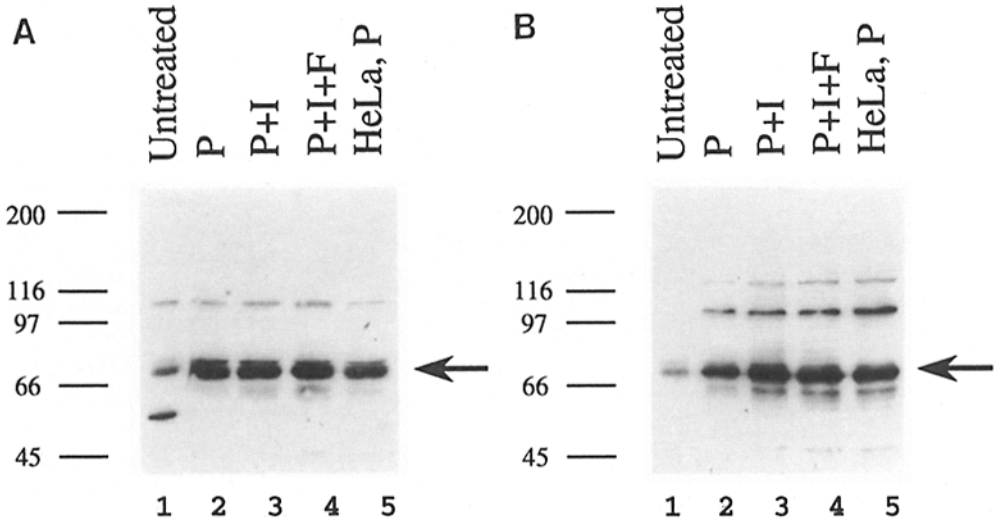

Figure 4. Western blot analysis of nuclear *rel* B in Bal 17 and D5h3 cells. (*A*) 10–20 μg of nuclear extracts from Bal 17 cells stimulated with various inducing agents were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-*rel* B antibody as described in the Materials and Methods section. HeLa P is nuclear extract from phorbol ester induced HeLa cells. Arrow indicates the position of *rel* B protein. (*B*) 10–20 μg of nuclear extracts derived from D5h3 cells treated as indicated were analyzed by immunoblotting. Arrow indicates the position of *rel* B protein.

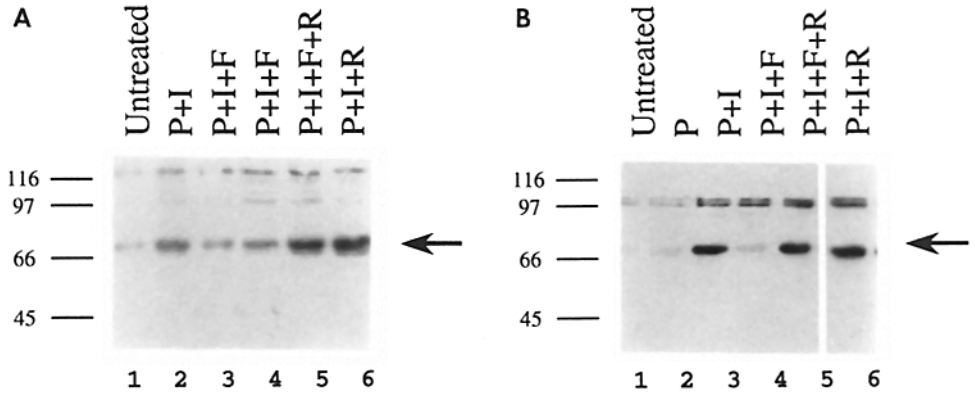

Figure 5. FK506 inhibition of c-*rel* induction is reversed by rapamycin. (*A*) Western blot analysis of Bal 17 nuclear extracts. 10 μgm of nuclear protein from cells treated as indicated above each lane were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-c-*rel* anti-serum (Santa Cruz Biotech); lane *3*, FK506 2 nM; lane *4*, FK506 0.5 nM; lane *5*, P+I+2 nM FK506 + 500 nM rapamycin; lane *6*, P+I+500 nM rapamycin. c-*rel* band is indicated by the arrow. Rapamycin was added first to the cell cultures, followed by FK506 10 min later and then inducers were added after a further 5 min. (*B*). Western blot analysis of D5h3 nuclear extracts derived from cells treated as indicated. c-*rel* band is indicated by the arrow. Drugs were added at the same concentrations as described above.

5 *A*, lanes *3* and *4*). A 250-fold molar excess of rapamycin did not affect c-*rel* induction by P+I (Fig. 5 *A*, lane *6*) but completely reversed FK506-dependent inhibition of c-*rel* induction (Fig. 5 *A*, lane *5*). The same pattern was true in D5h3 cells. FK506-mediated inhibition of c-*rel* induction (Fig. 5 *B*, lane *4*) was significantly diminished in the presence of excess rapamycin (Fig. 5 *B*, lane *5*). As expected, rapamycin had no effect on the induction of c-*rel* by P+I (Fig. 5 *B*, lane *6*). These results strongly suggest a role for calcineurin in c-*rel* induction in B and T lymphocytes, and identify c-*rel* as a nuclear target in the calcineurin pathway.

IL-2Rα Gene Induction Is Enhanced by Calcineurin Expression. c-*rel* has been implicated in the activation of the IL-2Rα gene, which is induced in response to mitogenic stimulation of both B and T lymphocytes. Tan et al. (36) showed that overexpression of c-*rel* in the Jurkat subline J.LEI transactivated a reporter plasmid containing the 5′ regulatory region of the IL-2Rα gene. We have shown that this IL-2Rα sequence is optimally activated in non-lymphoid cells by a combination of p50, c-*rel* and serum response factor (J. Pierce et al., manuscript submitted for publication). To assess whether calcineurin-dependent pathways participate in the generation of transcriptionally active c-*rel* protein, we assayed the effects of calcineurin expression on the transactivation of an IL-2Rα promoter containing reporter plasmid by c-*rel*. In S194 (plasma) cells, c-*rel* expression directed by the cytomegalovirus promoter and enhancer was insufficient to activate the reporter plasmid, in the presence or absence of PMA and ionomycin (Fig. 6 *A*). Lack of induction by cotransfected c-*rel* in the presence of PMA and ionomycin may be due to low c-*rel* expression or inefficient activation of intra-cellular calcium-dependent pathways in these terminally differentiated plasma cells. Coexpression of a calcineurin deletion mutant lacking the auto-inhibitory domain (ΔCaM-AI) resulted in threefold activation of transcription which was further increased by PMA and ionomycin treatment (Fig. 6 *A*). Consistent with the proposed role of the auto-inhibitory domain, a cotransfected calcineurin (CN) gene transactivated reporter plasmid expression only in the presence of PMA and ionomycin (Fig. 6 *A*). CN-mediated activation of the IL-2Rα promoter was diminished by treatment of the transfected cells with FK506 (Fig. 6 *A*). These results are consistent with a role for CN in generating transcriptionally active c-*rel*.

To extend the functional analysis, we examined the effect of CN expression on a cotransfected IL-2Rα promoter plasmid in Jurkat T cells. Expression of ΔCaM-AI alone, transactivated the IL-2Rα promoter 4.5-fold (Fig. 6 *B*) in these cells. We interpret this to indicate that the presence of the deregulated phosphatase in these cells activated endogenous κB-binding proteins that transactivate the IL-2Rα promoter. Coexpression of c-*rel* with ΔCaM-AI increased the expression level of the reporter plasmid, indicating that CN stimulated the transcription activity of transfected c-*rel*. Note that in the conditions of our assays, neither c-*rel* alone, nor CN alone, affected IL-2Rα expression (Fig. 6 *B*). Furthermore, inducible expression observed in the presence of ΔCaM-AI was suppressed by FK506. These experiments demonstrate that CN-dependent pathways increase the transcriptional activity of a cotransfected c-*rel* gene, as well as activate endogenous κB-binding proteins that are necessary for IL-2Rα expression.

Discussion

We have shown that the proto-oncogene c-*rel* is induced by PMA and ionomycin treatment of B and T lymphoid cells, or by stimulation of cells via the respective antigen receptors. The more sensitive Western blot assay revealed six- to eight-fold induction of nuclear c-*rel* in the B and T cell lines, and DNA-binding c-*rel* was only detected by antibody super-shift in extracts derived from optimally stimulated cells. The apparent low abundance of the super-shifted complex is likely to be an underestimate of the amount of DNA binding, and therefore presumably functional, nuclear c-*rel* protein. First, only a fraction of the nuclear c-*rel* may be bound the H2K-κB probe being used in these assays and second, only a subset of the DNA-bound protein may be super-shifted by the antiserum. In contrast to many other early response genes, such as c-*fos*, c-*jun*, c-*myc*, and *egr-1*, which are induced in response to phorbol ester alone, the dual signal requirement for c-*rel*

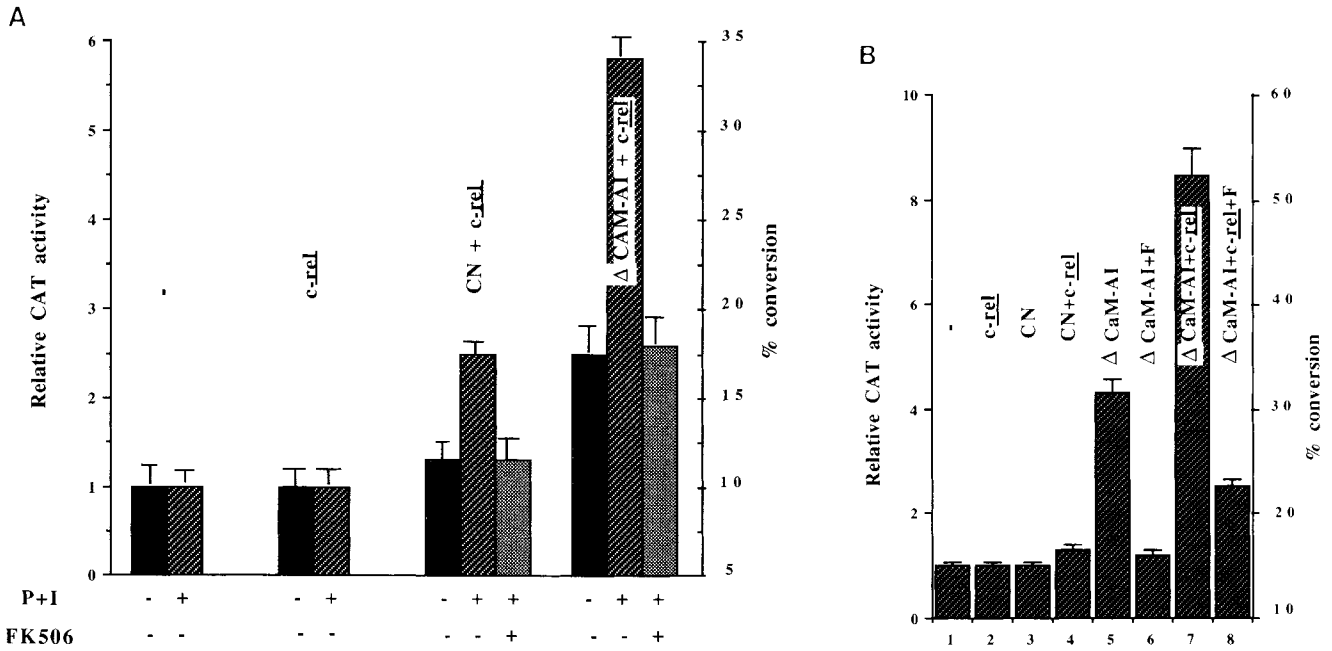

Figure 6. Coexpression of CN activates c-*rel*-mediated transcription in B and T cell lines. (*A*) Activation of the IL-2Rα promoter in S194 (plasma) cells by coexpression of c-*rel* and CN. S194 plasma (*B*) cells were transfected with a reporter plasmid containing a fragment of the IL-2Rα promoter (residues −317 to −227) cloned upstream of the c-*fos* gene TATA box at −56 and expression vectors for murine c-*rel* and CN as indicated above the bars. Transfected cells were left untreated, treated with PMA and ionomycin, or with PMA, ionomycin and FK506 as indicated below the graph. FK506 was used at a final concentration of 10 nM. Chloramphenicol acetyl transferase activity was assessed in whole cell extracts 48 h after transfection and is indicated as the percentage of substrate chloramphenicol converted to the acetylated form (*left y axis*). CAT enzyme activity was also normalized to the levels present in the absence of cotransfected transactivatory plasmids and is shown on the right y axis. The values shown are the average of three sets of transfection experiments carried out in duplicate. (*B*) Transactivation of the IL-2Rα promoter by CN in Jurkat cells. 5 μgm of IL-2Rα promoter containing reporter plasmid was transfected into 2 × 10^7 Jurkat cells together with expression vectors containing the murine c-*rel* gene; the complete CN gene indicated as CN or a deletion that removes the auto-inhibitory domain (indicated as Δ*CaM-AI*). In transfections where only one transactivator was used, total DNA was made up to 15 μgm using pTZ18R. FK506 was used at 10 nm final and added to the cells at the time of transfection. All experiments shown were performed in the absence of phorbol ester and ionomycin treatment to minimize the induction of endogenous κB-binding proteins. Results shown are the average of five experiments performed in duplicate.

induction implicates this proto-oncogene as a key participant in lymphocyte proliferation.

Cyclosporin A (CsA) and FK506 inhibit B and T cell proliferation. The effects of these drugs are best characterized in T cells where three different DNA elements have been shown to confer FK506 sensitive transcription activation (14, 35–37). However, the mechanism of drug action on the B cell compartment is less clear. We have recently shown that a factor indistinguishable from T cell NF-AT is induced in splenic B cells activated via sIg, and that the induction is sensitive to CsA treatment (6). In this report we show that c-*rel* induction in both B and T cells is blocked by FK506. Excess rapamycin competed away the FK506 effect, suggesting that c-*rel* induction requires a CN-dependent intracellular step. Furthermore, neither *rel* B induction nor p50 expression is sensitive to FK506 suggesting that *rel* proteins are differentially regulated in lymphocytes.

Unlike NF-AT or octamer-associated protein-1 (OAP-1), NF-κB can be induced by a PMA signal alone. However, higher levels of NF-κB are induced by the combined action of PMA and ionomycin, and CsA or FK506 reduce NF-κB levels to that observed in the presence of PMA alone, suggesting that the drugs affect the Ca^{2+}-dependent NF-κB induction. Increased NF-κB induction in the presence of ionomycin may occur by translation-dependent (28) or posttranslational mechanisms (32). Frantz et al. have proposed that FK506 inhibition of posttranslational NF-κB induction is caused by disruption of a CN-dependent pathway that normally synergizes with PMA to inactivate I-κBα (32). The signaling requirements for c-*rel* induction that we describe mimic the known properties of NF-AT and OAP-1, and the requirement for ongoing protein synthesis suggests that c-*rel* may be a part of the translation-dependent NF-κB induction in Jurkat cells (28).

To show that c-*rel* suppression by FK506 affects gene expression, we analyzed IL-2Rα gene induction by c-*rel*. The IL-2Rα gene is expressed in activated B and T lymphocytes. The promoter region of this gene contains binding sites for several factors, including a κB site that is essential for the induction of this gene by the HTLV-1 *tax* gene product and T cell receptor signals (37–39). We demonstrate that transactivation of the IL-2Rα gene promoter by c-*rel* is augmented by coexpression of active CN and suppressed by FK506. These results support a role for CN in generating transcriptionally active c-*rel* and suggest that c-*rel* suppression by immunosuppressive drugs may be the mechanism by which IL-2Rα in-

duction is abolished by these drugs. The characteristics of c-*rel* induction that we have found are consistent with this protein being a critical, immunosuppressive sensitive, component that is necessary for IL-2Rα expression. We have previously shown that in untransformed T cell clones, the IL-2Rα gene is induced in response to T cell receptor cross-linking and suppressed by CsA (40). Whereas the NF-κB–binding site in the IL-2Rα promoter (IL-2Rα-B) was necessary for inducible expression of the transfected gene, NF-κB levels detected by EMSA were not significantly affected by CsA treatment of the cells. These observations suggested that the CsA suppressible factor that activates the IL-2Rα promoter is not the bulk κB binding activity detected by EMSA, which is primarily p50/p65 NF-κB. The results presented in this paper suggest that the relatively small proportion of DNA-binding c-*rel* that is induced by TcR signals may regulate IL-2Rα gene transcription. Suppression of this factor by FK506 inhibits IL-2Rα expression without any apparent effect on the induced NF-κB seen in EMSA. Furthermore, induction of IL-2Rα mRNA required ongoing protein synthesis. This observation further supports a role for c-*rel* in activating the IL-2Rα promoter, as we have found that c-*rel* induction in response to PMA and ionomycin requires protein synthesis, whereas NF-κB induction is post-translational.

Sequence elements homologous to the κB element of the immunoglobulin κ gene enhancer are present in a variety of viral and cellular regulatory sequences. For example, several lymphokine genes, such as IL-2, IL-3, IL-4, GM-CSF, and TNF-α, whose induction is blocked by FK506, contain κB-related sequences in their promoters (41). Although the specific *rel*-family members that activate these promoters are not yet known, our results suggest that c-*rel* may play an important role in the FK506-sensitive expression of these genes. We propose that c-*rel* is a common target of immunosuppressive drugs in B and T lymphocytes.

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