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Evidence that interleukin-1 and phorbol esters activate NF-κB by different pathways: role of protein kinase C

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Nuclear factor kappa B (NF-κB) is a ubiquitous transcription factor that affects expression of many genes, including immunoglobulin kappa (κ), the interleukin-2 receptor α chain, and two genes in HIV-1. NF-κB can be activated by a number of stimuli, including pharmacological stimulation of protein kinase C by phorbol 12-myristate 13-acetate (PMA) and treatment *in vitro* with either protein kinase C or protein kinase A. This has lead to the proposal that these kinases are key enzymes in the physiological activation of NF-κB as well. We have used a murine B cell line, 70Z/3, and T cell line, EL-4 6.1 C10, to study the activation of NF-κB by two physiological activators, interleukin-1α (IL-1) and lipopolysaccharide (LPS). There are four reasons to propose that these agents activate pathways that do not include protein kinase C as a major component in these cell lines. First, the protein kinase C inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) strongly inhibited PMA-induced activation of NF-κB in 70Z/3 cells but had no effect on NF-κB activated by IL-1 or LPS. Second, depletion of protein kinase C by prolonged growth of 70Z/3 in PMA abrogated the capacity of the cells to activate NF-κB in response to further PMA treatment. However, these same cells activated NF-κB normally after either IL-1 or LPS treatment. Third, IL-1 effectively activated NF-κB in EL-4 6.1 C10 cells, but PMA did not. Fourth, inteferon-γ is a potent activator of protein kinase C in 70Z/3 cells, but is completely inactive in the mobilization of NF-κB. These results suggest that the physiological inducers IL-1 and LPS activate NF-κB by pathways independent of protein kinase C in both 70Z/3 and EL-4 6.1 C10 cells.

Introduction

The control of transcription has been intensively studied, and it has become clear that a number of ubiquitous transcription factors are used in many different systems. In many of these situations, specifity is conferred by the unique combination of factors that affect the gene, not by the presence or absence of any one factor. For example, nuclear factor kappa B (NF-κB) has been identified in a wide variety of cell types and affects transcription of a number of genes. In B and T lymphocytes, the immunoglobulin kappa (κ) chain, interleukin-2 receptor-α chain, and several HIV-1 genes are all strongly activated by NF-κB (for review see Lenardo and Baltimore, 1989). Because this is a rather general mechanism, the specificity of the gene activations must depend on the constellation of other factors induced and on the mechanisms that activate the NF-κB rather than on the simple presence or absence of the factor itself. With this in mind, we have been studying the mode of activation of NF-κB by several different effectors in B and T cells.

NF-κB is located in the cytoplasmic fraction but is in an inactive state complexed with an inhibitor, IκB (Baeuerle and Baltimore, 1988; Lenardo and Baltimore, 1989; Ghosh and Baltimore, 1990; Zabel and Baeuerle, 1990). Treatment of cells with various activators can cause the dissociation of this complex, allowing NF-κB to translocate to the nucleus, where it binds to the EκB sequence (GGGACTTTCC) (Baeuerle and Baltimore, 1988; Bohnlein *et al.*, 1989; Lenardo and Baltimore, 1989; Ghosh and Baltimore, 1990; Zabel and Baeuerle, 1990). The mechanism(s) that activates NF-κB has not been defined, but phosphorylation of IκB has been identified as one step in the process (Ghosh and Baltimore, 1990; Zabel and Baeuerle, 1990). Two observations have focused attention on the family of enzymes called protein kinase C as a key part of this pathway. The majority of protein kinase C activity is found in the cytoplasmic fraction of the cell (Kikkawa *et al.*, 1988). However, treatment of cells with various agents can

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cause activation and relocation of most, if not all, of its isozymes to the membrane fraction (Nishizuka, 1984; Rosoff and Cantley, 1985; Ostrowski *et al.*, 1988). One very potent activator of protein kinase C is treatment of the cells with phorbol 12-myristate 13-acetate, (PMA) (Kikkawa *et al.*, 1988). This same treatment is very effective in dissociating NF-κB from its complex with IκB (Sen and Baltimore, 1986), suggesting that protein kinase C may be responsible for the phosphorylation of the IκB and subsequent liberation of NF-κB. Even more compelling, in vitro treatment of cytoplasmic extracts with protein kinase C can also liberate NF-κB from its inactive complex (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990).

We are interested in defining the pathways used in B and T cells to activate lymphoid-specific genes. We have used two murine cell lines as models for these activation events, the B lymphoid line 70Z/3 and the T lymphoma EL-4 6.1 C10. In 70Z/3 cells the κ light chain gene is transcribed at extremely low levels, but its expression can be greatly enhanced by treatment of the cells with a number of agents, including interleukin-1 (IL-1), (Calalb *et al.*, 1987; Shirakawa *et al.*, 1989), inteferon-γ (IFN) (Briskin *et al.*, 1988), and lipopolysaccharide (LPS) (Paige *et al.*, 1978). EL-4 6.1 C10 is a subline of the T lymphoma EL-4; IL-1α treatment of these cells induces expression of IL-2 (Lowenthal and MacDonald, 1986). In both cell lines, one early consequence of treatment with IL-1 is the activation of NF-κB (Shirakawa *et al.*, 1989; Bomsztyk *et al.*, 1990). Thus, we have used these lines to study the activation of NF-κB and its transcriptional sequelae.

We and others have shown previously that treatment of cells with LPS, IL-1, IFN, or PMA causes relocation of most of the protein kinase C activity from the cytoplasmic to the membrane fraction (Rosoff and Cantley, 1985; Fan *et al.*, 1988; Ostrowski *et al.*, 1988; Serfling *et al.*, 1989). However, in these same cells, NF-κB is activated only by treatment with IL-1, LPS, or PMA (Sen and Baltimore, 1986; Serfling *et al.*, 1989; Bomsztyk *et al.*, 1990). Treatment with IFN has no effect on NF-κB (Briskin *et al.*, 1988; Emery et al., 1989), suggesting that protein kinase C translocation may not be sufficient to activate NF-κB. Because of these apparent discrepancies, we set out to determine the role of protein kinase C in regulating the activity of NF-κB in 70Z/3 and EL-4 6.1 C10 cells.

Results and discussion

Our experiments were designed to test a straightforward hypothesis: if protein kinase C is a major component of the pathway of NF-κB activation by LPS and IL-1, treatments that inhibit the activity of protein kinase C should produce a comparable inhibition of the capacity of LPS and IL-1 to activate NF-κB.

We tested this assumption first by treating cells with 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine (H-7), an inhibitor that is effective on the α, β, and γ isozymes of protein kinase C (Kikkawa *et al.*, 1988) and on the cyclic AMP (cAMP) and cyclic GMP (cGMP)-dependent kinases (Hidaka *et al.*, 1984). Because H-7 can have nonspecific effects, it was carefully titrated and we used it only for short periods and at a concentration (100 μM) that effectively inhibits PMA-induced cytoplamsic alkalinization in 70Z/3 cells (Ostrowski *et al.*, 1988). The 70Z/3 cells were treated with various combinations of inducers and H-7; nuclear extracts were prepared and assayed by electrophoretic mobility shift assay (EMSA) for NF-κB activity. Gels from this experiment are shown in Figure 1. Treatment of the 70Z/3 cells with H-7 alone did not induce NF-κB (Lanes 2 and 8). More important, H-7 had no effect at all on the IL-1- or LPS-induced activation of NF-κB (compare lanes 3 and 4 and lanes 11 and 12). In sharp contrast, H-7 substantially inhibited the PMA-induced activation of NF-κB (compare lanes 5 and 6 or lanes 9 and 10). Two conclusions can be drawn. First, the equivalence of the NF-κB activation by IL-1 and LPS in the presence and absence of H-7 shows that the H-7 is not simply poisoning the cells: the inhibition of the PMA-induced activation of NF-κB is specific. Second, the physiological inducers IL-1 and LPS functioned without compromise even when the activity of protein kinase C was strongly inhibited by pharmacological treatment. We had previously observed that H-7 effectively inhibits another effect of PMA treatment in 70Z/3 cells, cytoplasmic alkalinization. In this case, as well, H-7 had no effect on the alkalinization caused by IL-1 treatment (Ostrowski *et al.*, 1988).

If activation of NF-κB does not require protein kinase C, depletion of the enzyme by prolonged treatment with PMA also should have no effect on the capacity of the cells to activate NF-κB in response to IL-1 or LPS. It has been shown previously that a 72-h incubation with 10^{-6} M PMA does greatly reduce the activity of protein kinase C (Nishizuka, 1984; Ostrowski *et al.*, 1988). To

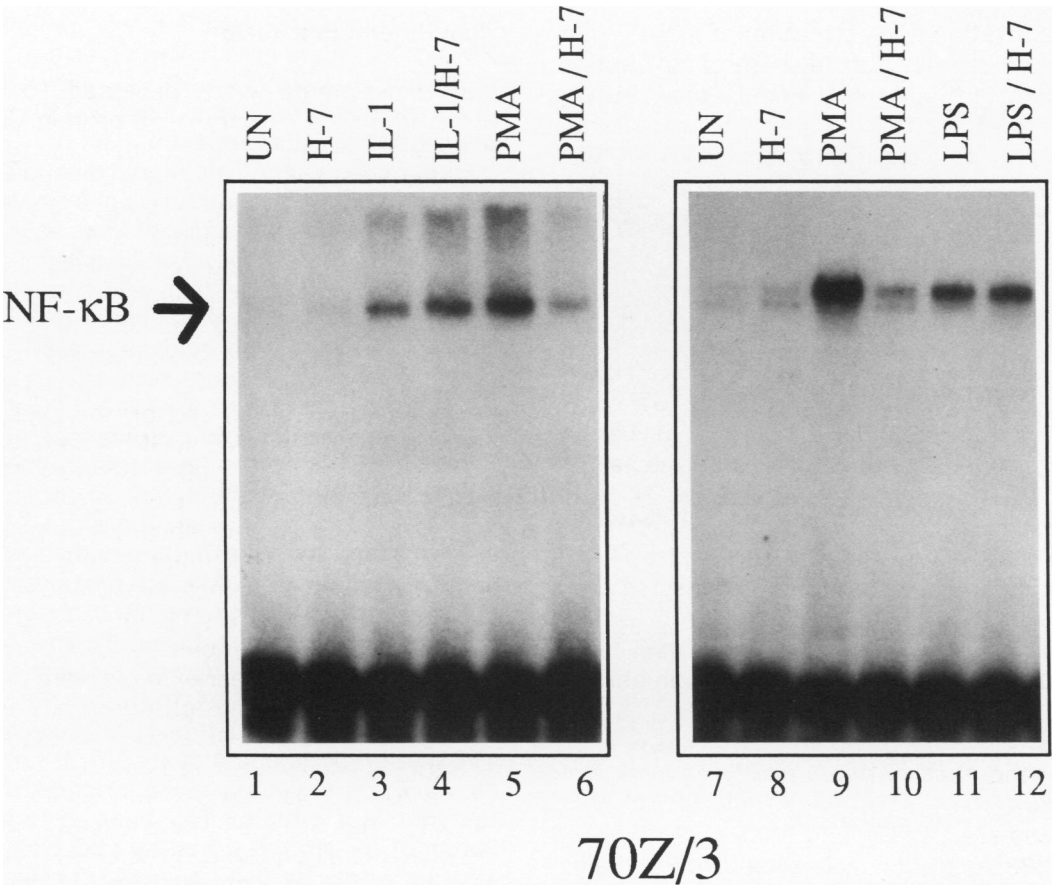

Figure 1. **Comparison of the effects of H-7 on IL-1, LPS, and PMA-induced activation NF-κB in 70Z/3 cells.** IL-1 (10^{-10} M), LPS (10 μg/ml), or PMA (10^{-6}M) were added to cells pretreated for 30 min with H-7 (100 μM) or medium alone. Nuclear proteins were extracted from 5–10 × 10^6 cells, and equal amounts of protein from these extracts were assayed by EMSA to determine the activity of NF-κB, all as described in Materials and methods.

test the effect of depletion, we treated 70Z/3 with PMA (10^{-6}M) or medium alone for 72 h and assayed by EMSA the level of NF-κB in nuclear extracts after a 30-min incubation with IL-1, LPS, or additional PMA. The results are displayed in Figure 2. The first four lanes show results from cells treated for 72 h with PMA; the last four are from those with no prior treatment. Comparison of Lanes 1, 5, and 8 demonstrates that the potent activation of NF-κB by PMA was transient; the cells treated for only 30 min showed high levels of NF-κB (lane 8), but after 72 h the level of NF-κB in nuclear extracts was barely above baseline (lane 4). Furthermore, restimulation of these PMA-treated cells with additional PMA was ineffective: lanes 1 and 4 are indistinguishable. However, the activation of NF-κB by IL-1 and LPS was equally effective in control cells and in those pretreated with PMA (compare lanes 2 and 6 and lanes 3 and7). Thus, the abrogation of protein kinase C activity by prolonged incubation in PMA also failed to diminish the ability of the cells to respond to either IL-1 or LPS. Treatment of 70Z/3 cells with either IL-1 or LPS does cause redistribution of protein kinase C (Rosoff and Cantley, 1985; Ostrowski *et al.,* 1988), so the independence of NF-κB activation from protein kinase C in these cells is even more striking.

Our third test of the role of protein kinase C in NF-κB activation employed a second cell line, the mouse T cell lymphoma EL-4 6.1 C10 (Lowenthal and MacDonald, 1986). We took advantage of the fact that, although IL-1 has many biological effects in T cells, it does not appear to activate protein kinase C as it does in B cells (Abraham *et al.,* 1988; Ostrowski *et al.,* 1988). IL-1 does induce surface expression of IL-2 receptors in these cells (Lowenthal and MacDonald, 1986), and in other T cell lines this tran-

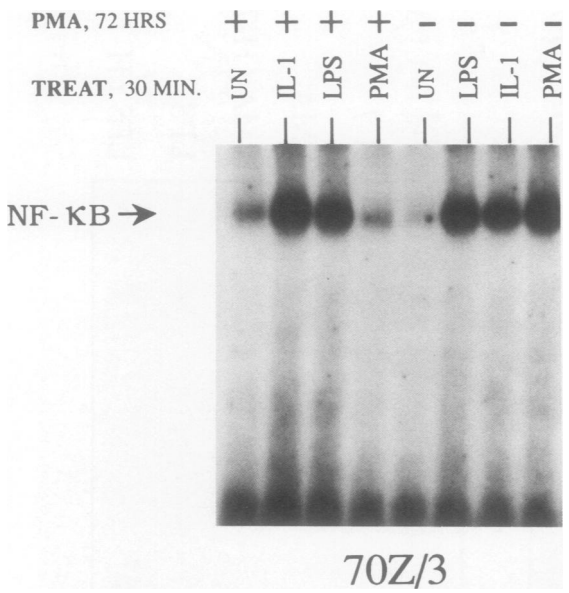

Figure 2. **Comparison of activation of NF-κB in cells treated for 72 h with medium alone or with PMA.** Equal numbers of 70Z/3 cells were grown for 72 h with 10^{-6} M PMA or with medium. These populations were then incubated with medium alone, additional PMA, IL-1, or LPS for 30 min. Nuclear proteins were extracted from 5–10 × 10^6 cells, and equal amounts of protein from these extracts were assayed by EMSA to determine the activity of NF-κB, all as described in Materials and methods.

scriptional activation of the IL-2 receptor α chain gene has been shown to depend on NF-κB (Bohnlein *et al.,* 1989). Therefore, we compared the capacity of 70Z/3 and EL-4 6.1 C10 to activate NF-κB in response to IL-1 or PMA treatment. Equal numbers of 70Z/3 and EL-4 6.1 C10 cells were treated with media alone, PMA, or IL-1; nuclear extracts were assayed for the presence of NF-κB by EMSA. Gels from this experiment are shown in Figure 3. Comparison of lanes 2 and 5 demonstrates that IL-1 was an equally effective inducer of NF-κB in both 70Z/3 and EL-4 6.1 C10 cells. As expected, PMA effectively induced NF-κB in 70Z/3 cells (lane 3). PMA did cause translocation of protein kinase C equally well in both cell lines (Ostrowski *et al.,* 1988; Bomsztyk *et al.,* 1989), but lane 6 shows that it failed to activate NF-κB in EL-4 6.1 C10 cells. If protein kinase C plays a major role in the activation of NF-κB by IL-1, this is a paradox. The paradox can be resolved by proposing that protein kinase C need not be the direct activator of NF-κB in these cells. The failure of PMA to activate NF-κB might be unique to the EL-4 6.1 C10 clone because, in another EL-4 subline (Serfling *et al.,* 1989), we found that NF-κB could be activated equally well by either PMA or IL-1 (data not shown). Even if the lesion is specific to this clone, it has allowed us to uncouple the effects of IL-1 and PMA and demonstrate their independence.

Both molecular and biochemical studies have shown that protein kinase C is not a single entity but rather a diverse family of kinases with different substrates and subcellular localizations (Kikkawa *et al.,* 1988). Thus, we cannot exclude the possibility that a minor isoform of protein kinase C is required for activation of NF-κB and that this kinase is neither sensitive to H-7 nor depleted by prolonged treatment with PMA. However, our data show a substantial reduction in NF-κB levels after H-7 treatment of PMA-induced cells and no effect at all on either the IL-1 or LPS-induced samples. Therefore, it seems reasonable to propose that the pathways of NF-κB activation used by PMA and the physiological activators are not the same. Four observations contributed to this conclusion. First, IFN treatment of 70Z/3 cells induces transient translocation of protein kinase C from the cytoplasmic to the membrane fraction (Ostrowski *et al.,* 1988) but it does not activate NF-κB (Briskin *et al.,* 1988; Emery et al., 1989). Second, the protein kinase C inhibitor H-7 blocked PMA activation of NF-κB, but it had no effect on the IL-1- or LPS-activated NF-κB in 70Z/3 cells. Third, depletion of protein kinase C by prolonged PMA treatment did not alter the capacity of 70Z/3 cells to activate NF-κB in response to IL-1 or LPS treatment. Fourth, in EL-4 6.1 C10 cells, IL-1 activated NF-κB but PMA treatment did not, even though PMA effectively activated protein kinase C. These observations do not support the assumption that protein kinase C is a major component in the activation of NF-κB by the physiological activators. Rather, they suggest that IL-1 and LPS may activate NF-κB by pathways different from that of the major isozymes of PKC.

Although the activation of NF-κB was initially thought to be unique to B cells (Sen and Baltimore, 1986), subsequent studies have demonstrated its ubiquity (Lenardo and Baltimore, 1989). Now it appears that the list of different pathways and ligands that can activate NF-κB is growing as well (Lenardo and Baltimore, 1989). It is clear that the pharmacological activation of protein kinase C by PMA can liberate NF-κB, but our observations suggest that neither of the physiological inducers, IL-1 and LPS, activates NF-κB using that pathway. This conclusion is supported by the recent observation

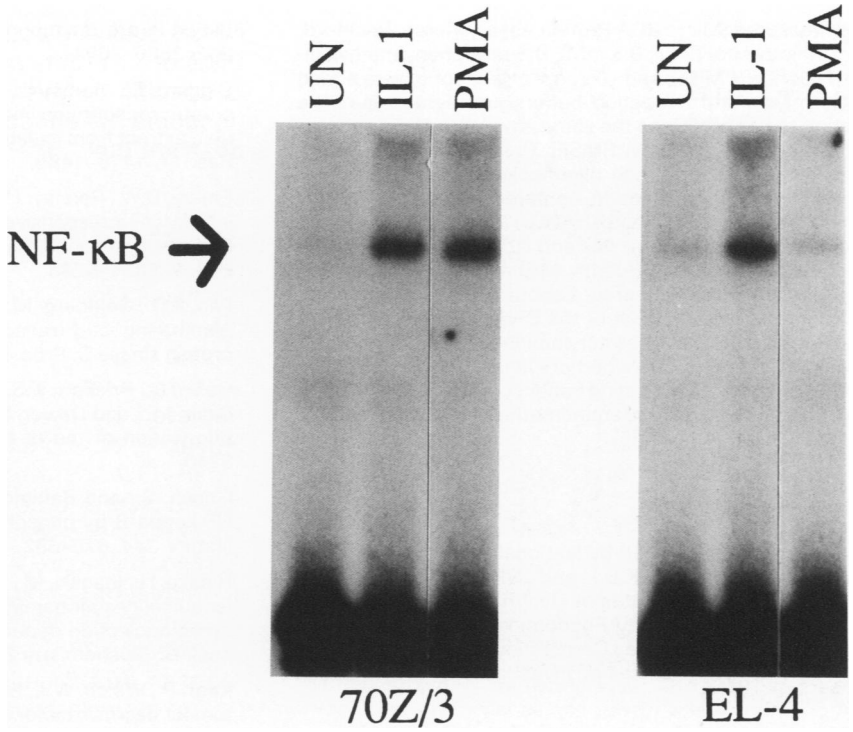

Figure 3. **Comparison of the effects of IL-1 and PMA on NF-κB activity in a B cell line 70Z/3 and T cell line EL-4 6.1 C10.** Equal numbers of the two cell lines were treated for 30 min with medium alone, IL-1 (10^{-10} M), or PMA (10^{-7} M). Nuclear proteins were extracted from 5–10 × 10^6 cells, and equal amounts of protein from these extracts were assayed by EMSA to determine the activity of NF-κB, all as described in Materials and methods. UN, untreated cells.

that tumor necrosis factor can also activate NF-κB in two human leukemic lines (K562 and Jurkat) without a requirement for protein kinase C activation (Meichle *et al.,* 1990).

Although there is a report that an increase in cAMP levels can activate NF-κB (Shirakawa *et al.,* 1989), we have recently shown that, in our clone of 70Z/3 cells, this is not the case (Bomsztyk *et al.,* 1990). This emphasizes again the disparity between phosphorylation in vitro and the normal physiological pathways. IL-1 has been shown to activate a protein serine/threonine kinase distinct from protein kinase C or protein kinase A (Matsushima *et al.,* 1988; Bird and Saklatvala, 1989; Gallis *et al.,* 1989; Kaur *et al.,* 1989). This enzyme phosphorylates the epidermal growth factor receptor (Bird and Saklatvala, 1989), a small heat shock protein (Kaur *et al.,* 1989), L-plastin (Matsushima *et al.,* 1988), and the IL-1 receptor itself (Gallis *et al.,* 1989). Because there is considerable evidence that NF-κB is activated by a phosphorylation event (Ghosh and Baltimore, 1990), we are currently examining the possibility that NF-κB or IκB can be a substrate for this other kinase.

Methods and materials

Cell lines

The murine lymphoid cell line 70Z/3 and murine thymoma EL-4 6.1 C10 were grown in complete RPMI medium supplemented with 5% fetal calf serum, 2 mM glutamine, 50 mM mercaptoethanol, penicillin (100 U/ml), and streptomycin (0.01%) at 37 ÂC in 5% CO_2/95% air atmosphere. Cells were treated with optimal concentrations of the various agents for 30 min: IL-1 (10^{-10} M), LPS (10 μg/ml), PMA (10^{-6}–10^{-7}M). When cells were grown in PMA for 72 h, the concentration was also 10^{-6} M.

Reagents

Recombinant human IL-1 was produced in *Escherichia coli* as described (Bomsztyk *et al.,* 1989). LPS was purchased from Difco, (Detroit, MI), PMA from Sigma (St. Louis, MO), and H-7 from Seikagaku America (St. Petersburg, FL).

Nuclear protein extraction and EMSA

Extracts were prepared with a modification of the method of Dignam *et al.,* (1983). After treatment, 5–10 × 10^6 cells were washed once with 1 ml phosphate-buffered saline and once with 1 ml of lysis buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid [HEPES] 10 mM KCl, and 1.5 mM $MgCl_2$, pH 7.9, 4°C). Cells were lysed by suspending the cell pellet in 20 μl of lysis buffer containing 0.1% NP-40 for 10 min on ice. To isolate nuclei, we vigorously mixed and centrifuged the lysis buffer suspensions (5 min at 12 000 x *g*, 4°C). Nucelar proteins were extracted by resuspending the nuclear pellet in 20 μl of protein extraction buffer (420 mM NaCl, 20 mM HEPES, 1.5 $MgCl_2$, 0.2 mM EDTA, and 25% glycerol, pH 7.9) for 10 min (4°C). After vigorous mixing the nuclear suspension was centrifuged (5 min at 12 000 x *g*, 4°C), the pellet was discarded, and the supernatant was centrifuged as before. The supernatant from the second centrifugation was diluted with 30 μl of diluting buffer (50 mM KCl, 20 mM HEPES, 0.2 mM EDTA, and 20% glycerol, pH 7.9), and the protein concentration

was measured (Micro BCA Protein Assay, Pierce, Rockford, IL). Dithiothreitol (DTT; 0.5 mM), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml of leupeptin were added to both lysis and extraction buffers just before use. The diluting buffer contained the same amounts of DTT and leupeptin, but only 0.2 mM PMSF. The NF-κB probe was a double-stranded synthetic oligonucleotide formed by annealing one oligonucleotide containing the sequence 5′ TGACAGA**GGGACTTTCC**GAGAGGA 3′ and its complement. The oligonucleotide probe was end-labeled using γ-[^{32}P]ATP (New England Nuclear, Boston, MA) and T4 polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, MD). The binding reaction of the DNA probe with nuclear protein extracts and polyacrylamide gel electrophoresis were performed as described previously (Bomsztyk *et al.*, 1990), except that the binding buffer contained 25 mM NaCl, 5 mM tris(hydroxymethyl) aminomethane-HCl, 1 mM EDTA, and 0.05% NP-40, pH 7.5.

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