TUMOR NECROSIS FACTOR-α, INTERLEUKIN 1, AND PHORBOL MYRISTATE ACETATE ARE INDEPENDENT ACTIVATORS OF NF-κB WHICH DIFFERENTIALLY ACTIVATE T CELLS

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Gene expression in eukaryotic cells can be altered in different ways by extracellular agents, including mitogens and cytokines. Such differential gene expression is mediated in part through the effects of these stimuli on distinct sets of cellular transcription factors. In this report, the effects of phorbol myristate acetate, tumor necrosis factor-α (TNF-α), and interleukin 1 (IL-1) on differential gene expression in the LBRM mouse T-lymphoma cell line are examined. Although these three different stimuli produce similar levels of induction of the NF-κB transcription factor, it is reported that they cause differential expression of other cellular activation genes, including c-*fos* and IL-2. The roles of IL-1 and TNF-α were also analyzed in EL-4 cells in the presence of a second activator, ionomycin. IL-1, but not TNF-α, was found to stimulate the IL-2 enhancer in the presence of this costimulator. These findings suggest that one transcription factor can be the target of cellular activators that exert otherwise different effects on gene expression. Cellular activation pathways can therefore be defined by the set of transcription factors stimulated within a cell. This approach may allow a more precise definition of the requirements for differential gene activation in different cell types and thereby provide a basis for the selective manipulation of gene expression in cytokine-responsive cells.

Cytokines, including tumor necrosis factor-α (TNF-α) and interleukin 1 (IL-1), act on a variety of immune and nonimmune cells where they induce changes in cell proliferation and differentiation. These cytokines can alter gene expression by modulating transcription factors that regulate specific genes. Despite their divergent effects, some cytokines and mitogens have common functions. For example, TNF-α, IL-1, and phorbol myristate acetate (PMA) all stimulate the DNA binding activity of NF-κB.[1-3] NF-κB is a transcription factor that stimulates the human immunodeficiency virus (HIV) enhancer in activated T cells[2] and regulates several cellular gene products in different cell types including immunoglobulin light chain, IL-2 receptor alpha, beta interferon, serum amyloid A, histocompatibility antigens, and cytokines.[4-15]

Although the effects of these agents had been examined in different cell types, it was unknown whether these independent activators caused similar changes in gene expression in one cell. In this study, we have analyzed the mouse T-cell line LBRM[16] whose NF-κB binding activity can be activated comparably by PMA, TNF-α, and IL-1. Our data suggest that these three agents, which activate NF-κB binding, act disparately within these cells, having different effects on c-*fos* and AP-1–dependent gene activation, as well as differentially regulating IL-2 secretion. These findings suggest that cytokines and mitogens that activate a common transcription factor can also stimulate distinct sets of transcription factors and activation genes in one T-cell type. Because these agents probably act through different second messengers, it is likely that convergent pathways result in the activation of common transcription factors, such as NF-κB. By defining the unique and overlapping sets of transcriptional regulatory proteins activated by each agent, cellular activation pathways can be defined specifically, the mechanisms of gene

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activation can be further elucidated, and control of specific cellular genes can be achieved.

RESULTS

NF-κB Binding Activity and Stimulation of the Human Immunodeficiency Virus Enhancer

The mouse LBRM T-cell lymphoma synthesizes IL-2 in response to IL-1.[16-18] Incubation of LBRM cells with IL-1 also induces NF-κB binding activity and stimulates the HIV enhancer through the κB regulatory element.[1] We first examined whether PMA, TNF-α, and IL-1 stimulated comparable NF-κB binding activity within LBRM cells. LBRM cells were incubated with PMA, TNF-α, or IL-1, and an electrophoretic mobility shift assay was performed to determine NF-κB binding activity.[2,12] All agents induced comparable NF-κB binding activity, and all inducible complexes were competed specifically by unlabeled κB site, but not by an unrelated IL-2 fragment (Fig. 1A, lanes 1 through 12). The time course of stimulation with all three agents was similar, and NF-κB binding activity was maximal between 15 and 240 min after activation (data not shown). Another DNA binding protein that recognizes the immunoglobulin octamer motif[19,20] showed no change in binding following stimulation with any of these stimulants (Fig. 1A, lanes 13 through 16).

To determine whether the binding activity induced by each agent correlated with transcriptional activation, the HIV enhancer linked to the chloramphenicol acetyltransferase (CAT) gene[2] was transfected into LBRM cells. Twenty-four hours after transfection, cells were incubated for an additional 20 h in the presence of recombinant murine TNF-α, recombinant murine IL-1, or PMA. CAT activity was comparably stimulated using 150 U/mL of TNF-α or 200 U/mL of IL-1 (Fig. 1B). Similar results have been obtained with plasmids containing multiple copies of κB linked to a heterologous promoter, and mutations of the κB sites abolished this stimulation (data not shown) as in other PMA, TNF-α, or IL-1 responsive cells.[1]

Differential Activation of AP-1 Transcription

NF-κB is one of several transcription factors stimulated by PMA. Among the other known PMA-

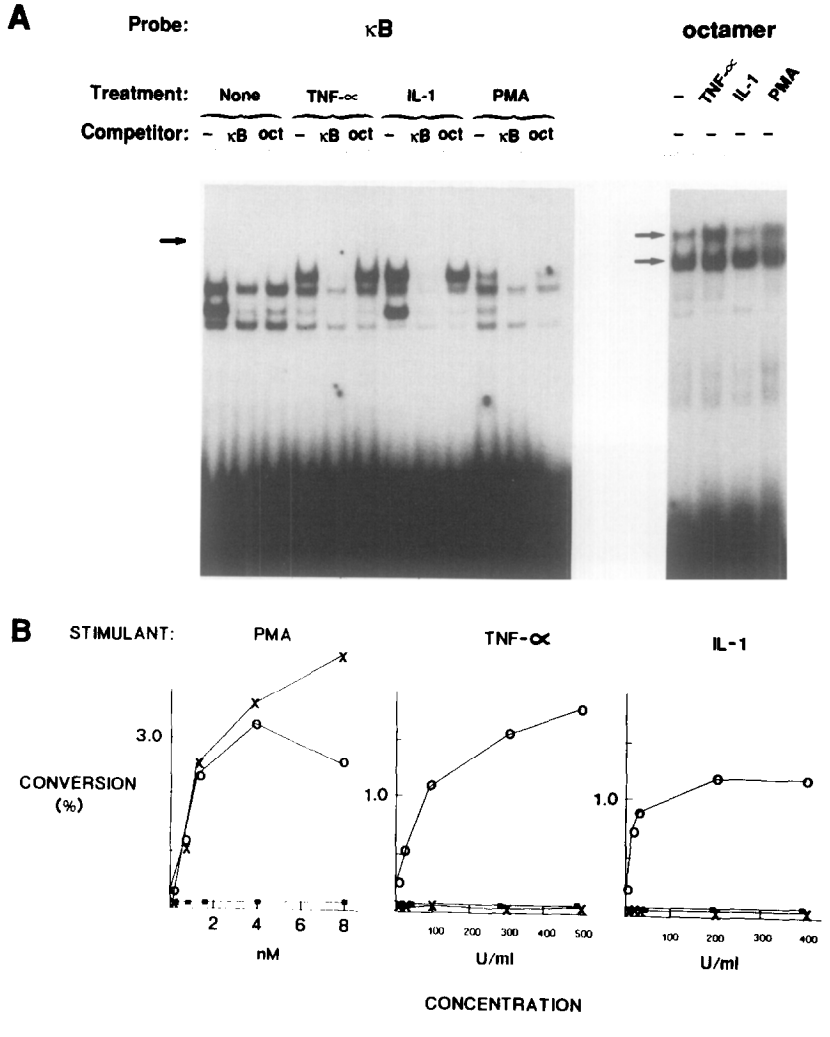

Figure 1. NF-κB binding activity and function in LBRM cells.

(A) Analysis of PMA and cytokine inducible complexes in LBRM cells using the electrophoretic mobility shift assay. Nuclear extracts were prepared from cells treated as indicated and incubated with a radiolabeled κB probe (lanes 1 through 12) or an immunoglobulin octamer probe (lanes 13 through 16) alone or with the unlabeled competitors (κB) from the HIV enhancer or octamer from IL-2.[12] Arrows denote specifically competed complexes. (B) 10^7 LBRM cells were transfected using DEAE-dextran with 20 μg of HIV-CAT plasmid (○), AP-1–CAT plasmid (X), or a mutant AP-1–CAT (■) plasmid altered in the AP-1 sites.

responsive elements is the AP-1 motif,[21,22] which binds complexes of Jun and Fos transcription factors. We compared activation of κB and AP-1 sites by PMA, TNF-α, and IL-1. Because the mechanism that regulates AP-1 activation is controversial,[21-25] we examined the ability of LBRM cells to functionally activate an AP-1 reporter plasmid. Although PMA stimulated the AP-1 enhancer element in LBRM cells, TNF-α and IL-1 had no detectable effect on this element at concentrations that activated NF-κB binding and the HIV enhancer (Fig. 1B). So despite their ability to stimulate NF-κB, TNF-α and IL-1 do not affect another PMA-responsive element, AP-1, in T cells.

To determine whether AP-1 activation was mediated by an increase in c-*jun* RNA, total RNA, prepared from LBRM cells stimulated with PMA, TNF-α, or IL-1 for 1 h, was evaluated by Northern blot analysis. At least two major RNA species, as described in other cells,[25-28] hybridized to a c-*jun* probe; however, the steady-state levels of any of these mRNAs changed by less than twofold (Fig. 2A). In contrast, a 2.2-kilobase (kb) RNA species that hybridized to a c-*fos* probe was induced by PMA but not by cytokines (Fig. 2B). Similar results were observed with RNA prepared after stimulation for 30 min (data not shown). Although other explanations are possible, this result is consistent with the notion that expression of the AP-1 reporter is regulated by the induction of c-*fos*, which subsequently interacts with a c-*jun*–like product that facilitates binding to the AP-1 site, perhaps through formation of a heterodimer.[29-37]

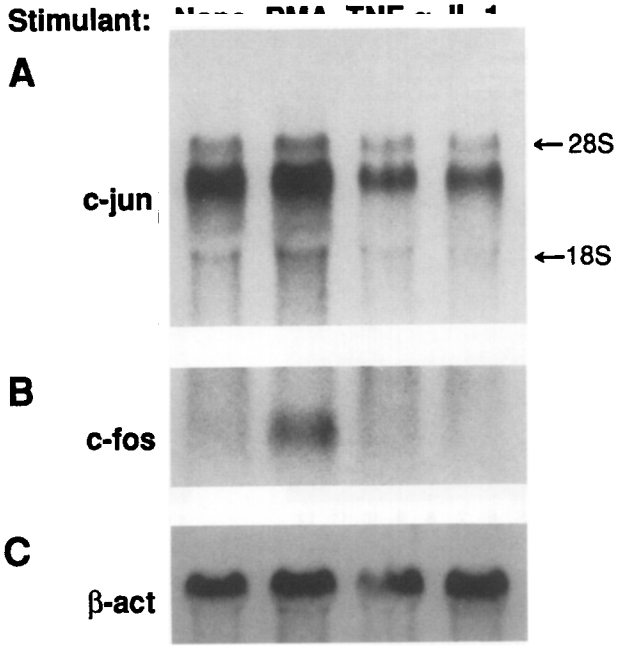

Figure 2. The effect of PMA and cytokines on c-*jun* and c-*fos* gene expression in LBRM cells.

10^7 LBRM cells were incubated for 1 h with 16 nM PMA, 100 U/mL recombinant murine TNF-α, or 10 U/mL recombinant murine IL-1. Total RNA (20 μg) was isolated from these cells and transferred to Gene Screen Plus (Du Pont, NEN, Boston, MA). (A) Hybridization of Northern blot with a radiolabeled murine c-*jun* cDNA probe.[27] (B) A second Northern blot of the same preparations of total cellular RNA as in (A) hybridized with radiolabeled murine c-*fos*.[69] (C) Northern blot shown above (A) was rehybridized with a radiolabeled β-actin probe.[70]

Selective Activation of Interleukin 2 Secretion by Cytokines

To compare the effects of TNF-α, IL-1, and PMA on a different activation pathway,[38] their effects on IL-2 secretion were analyzed. Supernatants from LBRM cells incubated with TNF-α, IL-1, or PMA for 24 h were assayed for IL-2 activity. IL-1, similar to PMA, stimulated significant levels of IL-2 production (Fig. 3A). In contrast, TNF-α levels even as high as 1,000 U/mL (data not shown), had no effect (Fig. 3A). Thus, although TNF-α and IL-1 are similar in their ability to stimulate NF-κB binding and activate the HIV enhancer, these two agents have markedly different effects on IL-2 production in LBRM cells. These results are also observed in the presence of a costimulant. In EL-4 cells, activation of the IL-2 enhancer was induced in the presence of ionomycin, a costimulant. As in LBRM cells, PMA or IL-1 stimulation caused marked enhancer activation, although TNF-α did not. This effect was mediated in part through κB (Fig. 3C). With a collagenase AP-1 reporter in these cells or in LBRM cells, IL-1 does not stimulate CAT activity (Fig. 1) in contrast to its effect on the IL-2 AP-1 site[39] (Fig. 3B), suggesting that Fos or Jun proteins differ in their ability to regulate the collagenase or IL-2 AP-1 sites.

Previous studies have shown that multiple gene products bind to κB-like sequences,[11,40-47] although it is not known whether all can activate transcription. Because it remained possible that PMA and cytokines could activate potentially distinct κB binding proteins, UV-crosslinking studies were performed. Several complexes were detected, including proteins of M_r ~160 kDa, ~90 kDa, and ~50 kDa (Fig. 4 a, b, and c). (The precise size of the proteins may vary by up to 5 kDa due to covalently bound DNA). However, no differences were noted between κB binding proteins from cells stimulated with PMA, TNF-α, or IL-1. These complexes were specific and not found in the presence of specific competitor or in unstimulated nuclear extracts (data not shown). The complex of ~50 kDa is consistent with previous reports of NF-κB[11,45] and KBF1.[47] A κB binding protein of 86 kDa, HIVen86A, has also been identified by two-dimensional gel electrophoresis[44] and is consistent with the ~90 kDa protein. The 160 kDa complex has not been previously described.

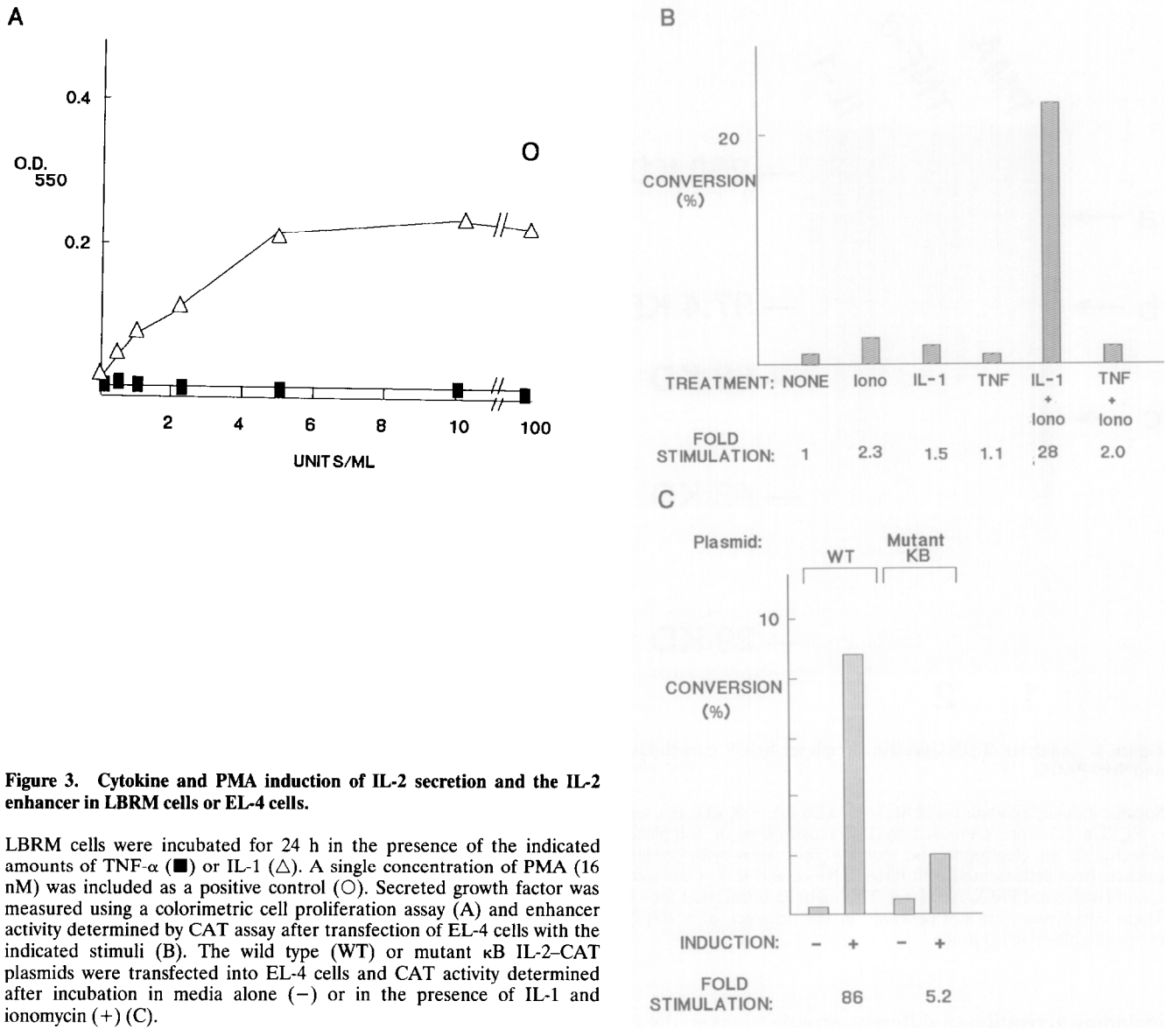

Figure 3. Cytokine and PMA induction of IL-2 secretion and the IL-2 enhancer in LBRM cells or EL-4 cells.

LBRM cells were incubated for 24 h in the presence of the indicated amounts of TNF-α (■) or IL-1 (△). A single concentration of PMA (16 nM) was included as a positive control (○). Secreted growth factor was measured using a colorimetric cell proliferation assay (A) and enhancer activity determined by CAT assay after transfection of EL-4 cells with the indicated stimuli (B). The wild type (WT) or mutant κB IL-2–CAT plasmids were transfected into EL-4 cells and CAT activity determined after incubation in media alone (−) or in the presence of IL-1 and ionomycin (+) (C).

DISCUSSION

PMA, TNF-α, and IL-1 are alike in their ability to activate NF-κB binding and stimulate the HIV enhancer. In this study, we have used a target cell, LBRM, which is responsive to these agents and can be induced to secrete IL-2. Therefore, this cell line allows analysis of the relationship between cellular activators of NF-κB and those that stimulate IL-2 secretion. Our results suggest that different modes of NF-κB activation, e.g., TNF-α or IL-1 stimulation, differentially affect IL-2 secretion. A third inducer of NF-κB binding, PMA, acts differently from TNF-α or IL-1, stimulating IL-2 secretion and another PMA-responsive element, AP-1, which is not responsive to TNF-α or IL-1 in this cell line. Among its many effects, PMA is known to stimulate protein kinase C.[48-50] Although controversial,[51,52] there is no conclusive evidence that IL-1–induced gene expression is mediated by protein kinase C.[53-58] With the exception of one study using a different cell type,[59] it does not appear that TNF-α activates protein kinase C.[56,58] Moreover, TNF-α stimulates NF-κB binding in cells unresponsive to PMA.[1] It therefore appears that NF-κB is activated in association with different signal transduction pathways, some probably independent of protein kinase C.[10,14,15]

Although the mechanism is not fully defined, it is likely that multiple proteins regulate NF-κB binding. For example, a cytoplasmic inhibitor of NF-κB, IκB, complexes to NF-κB and inhibits DNA binding activity.[40,60] This complex can be dissociated by treatment with protein kinase C or with cyclic AMP-responsive protein kinases in vitro, facilitating dissociation and translocation of NF-κB to the nucleus.[57] Binding of IκB to NF-κB can also be inactivated in vitro by incubation with multiple protein kinases.[61] Thus, it is possible that TNF-α and IL-1 affect different steps in this pathway,

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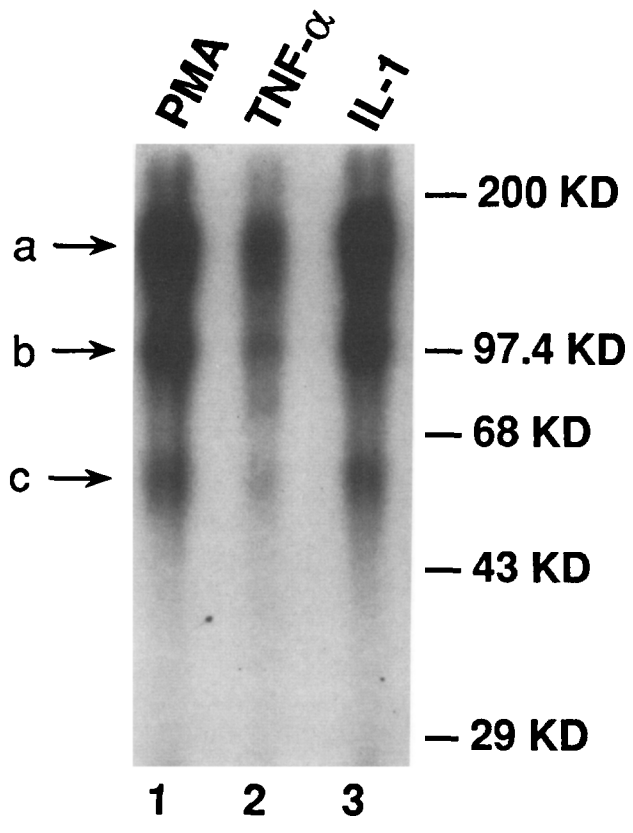

Figure 4. Analysis of DNA-protein complexes by UV crosslinking and SDS-PAGE.

Specific inducible complexes of M_r ~160 kDa (a), ~90 kDa (b), and ~50 kDa (c) were identified by UV crosslinking of complexes detected in an electrophoretic mobility shift assay with nuclear extracts from cells treated with PMA, TNF-α, and IL-1. Cells were treated with 8 nM PMA, 100 U/mL TNF-α, or 25 U/mL IL-1 for 4 h. These complexes were not observed in the presence of a 10-fold excess of unlabeled κB probe.

including activation of different protein kinases, dissociation from the inhibitor, transport to the nucleus, or binding to DNA. Although it is possible that different κB binding proteins[12,15,41,42,46] could be stimulated by different activators, no differences in binding complexes were detected in this study using UV crosslinking. Furthermore, NF-κB complexes induced by all agents showed similar patterns of competition, suggesting that NF-κB is a set of transcription factors common to several activation pathways.

These studies also address the role of κB binding proteins in the IL-2 enhancer. Previous studies have implicated a κB-like site in the stimulation of the IL-2 enhancer.[62] Using point mutations in this site, we also found a modest contribution of the κB site in the IL-2 enhancer in EL4 cells (Fig. 3C). Similarly, this site was not required for induction by PMA, suggesting alternative responsive elements in this cell line. Previous studies have implicated activation of Fos and Jun in such T cells.[39] Our findings also suggest that Fos induction can be stimulated by PMA, although Jun was detected without deliberate activation, perhaps due to differences in basal transcription induced by serum. This finding may explain why IL-2 can be induced by PMA alone in this cell line.

Although the precise mechanism by which these transcription factors become activated in T cells is not yet known, several different models can be postulated to explain our findings (Fig. 5). For example, it is possible that TNF-α, IL-1, and PMA each stimulate separate signal transduction pathways which independently activate NF-κB in association with different activation genes (Fig. 5A). A second possibility is that alternative second messengers converge to activate gene products through common mechanisms (Fig. 5B). Yet another possibility is that the same transcription factor (NF-κB) can become activated through separate signal transduction pathways by different mechanisms, some of which are shared among activators (Fig. 5C). Considerable complexity would be generated by the first model, and because activation genes such as IL-2 are induced by both IL-1 and PMA, we would suggest that one of the latter models is relevant to our findings. Because we have shown previously that TNF-α can induce NF-κB binding in cells unresponsive to PMA,[1] our data would support the third model, whereby second messenger pathways converge on common transcription factors by shared and unique mechanisms.

Although we cannot yet define the precise mechanism, this study suggests that a major determinant of gene activation in different cell types is the substrate of each second messenger pathway. Because distinct cellular activators stimulate some transcription factors in common, the ability to understand the molecular basis of their activation and to delineate this network of interactions will help to achieve selective gene expression using cytokines. This approach may also help to clarify confusion regarding disparate effects of cytokines in different cell types. For example, TNF-α increases c-*jun* and c-*fos* expression in fibroblasts[59] but has little effect on T cells (Fig. 2B). Taken together, the definition of the transcription factors stimulated by various extracellular agents provides a first step in the analysis of these signal transduction pathways. Further studies of the activation of these transcription factors will allow these common and unique pathways to be fully defined. In addition, because NF-κB induction is associated with increases in HIV transcription,[2,63-65] these findings also suggest potential strategies to decrease HIV gene expression while minimizing effects on other T-cell activation genes that mediate immunologic function.

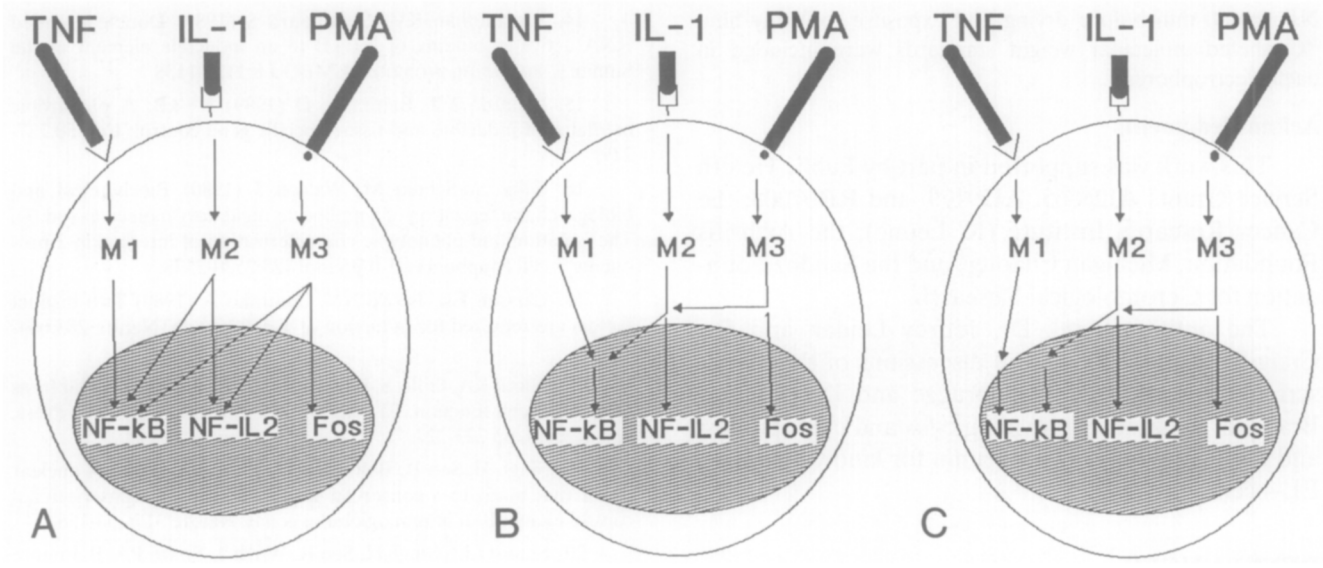

Figure 5. Alternative models of cellular activation.

Schematic representation of potential activation pathways that might lead to activation of NF-κB through (A) independent, (B) totally convergent, or (C) partially convergent mechanisms. M1, M2, and M3 indicate putative second messengers for TNF-α, IL-1, and PMA, respectively. NF–IL-2 refers to the set of transcription factors, other than Fos or NF-κB, which lead to IL-2 secretion.

MATERIALS AND METHODS

Cells and Nuclear Extracts

Nuclear extracts were prepared from cells incubated for 2 h at 37°C in medium alone, 500 U/mL recombinant TNF-α (Genzyme, Boston, MA), 100 U/mL (15 ng/mL) recombinant IL-1 (Genzyme), or in 16 nM PMA (Sigma, St. Louis, MO) as indicated. 10 μg of nuclear extract, prepared as previously described,[1] was used in the presence of 1 μg dIdC. EL4-6.1 cells were kindly provided by Dr. Christopher D. Benjamin (Biogen).

Plasmids

The AP-1–CAT plasmid was prepared by inserting an oligonucleotide consisting of four tandem copies of the AP-1 sequence from the collagen I gene, ATGAGTCAG, into the *Sac*I and *Sma*I sites of an SV-40 promoter expression vector pSP-CAT.[12] The AP-1–CAT mutant plasmid was prepared identically; the mutated sequence inserted in four copies was ATGATCACG. The IL-2–CAT plasmid was used as described,[66] or the κB mutant by modification of positions −205 to −195 to TTCTAGATCGA by site-specific mutagenesis.[2]

Transfections and Chloramphenicol Acetyltransferase Assays

Cells were transfected using DEAE-dextran as previously described.[12] Twenty-four hours after transfection, cells were incubated for an additional 20 h in the presence of the indicated amounts of PMA, recombinant murine TNF-α, or recombinant murine IL-1. In Fig. 3, ionomycin (1 μM), 25 U/mL recombinant human IL-1α (Genzyme), and 200 U/mL of mouse recombinant TNF-α (Genzyme) were used as indicated. Cell extracts were prepared 44 h after transfection, transfection efficiencies normalized, and conversion of chloramphenicol to its acetylated forms assayed by standard methods.[2,67] Results are representative of at least three independent transfections, and standard deviation for each CAT determination was ≤10%. Percent conversion of [^{14}C]chloramphenicol to its acetylated forms is indicated.

Cell Proliferation Assays

Quantitation of secreted growth factor was performed by using an HT-2 cell indicator cell line, which was not responsive to IL-1 or IL-4, and a colorimetric assay was used to determine proliferation.[68] Standard deviations were <25% for each data point.

UV Crosslinking and SDS-PAGE Analyses

Radiolabeled probe for the κB site was prepared by incubation of the 25 base long sense strand of single copy κB site with a complementary 10-base primer, 25 μM dATP, dGTP, 5-bromo-dUTP and 10 μCi ^{32}P-α-dCTP in the presence of the Klenow fragment of *Escherichia coli* DNA polymerase for 30 min at room temperature. At the completion of electrophoresis of the mobility shift gel, the polyacrylamide gel was exposed to UV light (305 nm) generated from an inverted UV light source at a distance of 5 cm from the light source for 60 min on ice. The gel was then exposed to Kodak X-Omat AR film (Rochester, NY) with an intensifying screen at 4°C overnight. Gel slices containing specific DNA-protein complexes were excised, soaked in 0.1% SDS for 10 min at room temperature, and transferred to a 7% SDS-polyacrylamide gel. After electrophoresis, gels were incubated with Autofluor (National Diagnostics, Manville,

NJ) for 60 min before drying and exposure to X-ray film. ^{14}C-labeled molecular weight standards were included in each electrophoresis.

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