Nuclear Factor-κB Dimer Exchange Promotes a p21$^{waf1/cip1}$ Superinduction Response in Human T Leukemic Cells

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Abstract

The nuclear factor-κB (NF-κB)/Rel transcription factors are recognized as critical apoptosis regulators. We reported previously that NF-κB contributes to chemoresistance of CEM human T leukemic cells in part through its ability to induce p21$^{waf1/cip1}$. Here, we provide evidence that sequential NF-κB-activating signals induce heightened NF-κB DNA binding and p21$^{waf1/cip1}$ induction in CEM and additional T leukemic cell lines. This response arises from exceedingly low basal expression of the p105/p50 NF-κB subunit encoded by the *NFKB1* gene in these cell lines. An initial NF-κB activation event enhances the recruitment of p65 and ELF1 to the *NFKB1* promoter, leading to p65- and ELF1-dependent synthesis of p105/p50, which promotes an exchange of NF-κB complexes to p50-containing complexes with an increased DNA-binding activity to certain NF-κB target elements. Subsequent stimulation of these cells with an anticancer agent, etoposide, results in augmented NF-κB-dependent p21$^{waf1/cip1}$ induction and increased chemoresistance of the leukemia cells. Thus, we propose that low basal *NFKB1* expression coupled with sequential NF-κB activation events can promote increased chemoresistance in certain T leukemic cells. (Mol Cancer Res 2006;4(2):101–12)

Introduction

The nuclear factor-κB (NF-κB)/Rel family of transcription factors regulates a wide range of cellular functions, including immune/inflammatory responses, proliferation, and apoptosis (1). In particular, recent studies identified NF-κB as a critical mediator that regulates transcription of survival genes implicated in resistance of cancer cells to chemotherapy and radiation therapy (reviewed in ref. 2). These NF-κB-induced survival genes include the antiapoptotic Bcl-2 family members and the cellular inhibitors of apoptosis family of caspase inhibitors (3-7). In addition, a NF-κB-dependent induction of p21$^{cip1/waf1}$, a cyclin-dependent kinase inhibitor that is normally under the control of the tumor suppressor p53 (8-10), also contributes to chemoresistance in certain p53-defective cancer cell types (11). Moreover, deregulation of NF-κB-regulatory pathways is a frequent occurrence in a variety of human cancer types (reviewed in ref. 12). Thus, pharmacologic inhibition of NF-κB pathways is considered to be a plausible anticancer strategy (reviewed in refs. 12, 13).

The NF-κB/Rel family consists of p50, p65, RelB, cRel, and p52 in mammals (reviewed in ref. 14). These five NF-κB proteins generate most combinations of homodimers or heterodimers to mediate NF-κB-dependent transcriptional regulation. The prototypical p50:p65 heterodimer is held inactive in the cytoplasm of most cell types due to association with the inhibitory proteins, such as IκBα (reviewed in ref. 14). Activation of NF-κB involves post-translational mechanisms involving the activation of the IκB kinase (IKK) complex and subsequent IKK-dependent phosphorylation and ubiquitin/proteasome-mediated degradation of IκBα to liberate free NF-κB that then translocates into the nucleus and activates transcription of target genes (reviewed in refs. 14, 15).

In addition to these post-translational mechanisms, the NF-κB/Rel dimer activation potential is also regulated at the transcriptional level. This regulation involves both basal and signal-inducible changes in the levels of each of the family members. For example, early studies suggested that p50 (encoded by the *NFKB1* gene) and p65 (encoded by the *RELA* gene) are ubiquitously expressed in most tissue types (16-18). The basal expression of other family members seems to greatly vary depending on specific cellular contexts (19, 20). Additionally, NF-κB can autoregulate the expression of most of its family members, except for p65 (21-26). In particular, the signal-dependent induction of *NFKB1* gene that encodes p105, the precursor of p50, was the first to be described as NF-κB dependent (25, 26). Although the involvement of the ETS family of proteins is implicated in this induction, the specific family member involved has not been revealed (27). Nevertheless, transcriptional autoregulation of NF-κB family members can modulate the cellular content of different NF-κB dimers, leading to differential activation potentials for different dimeric complexes. This type of regulation has been implicated in certain developmental processes, including B and dendritic cell development, where major NF-κB dimers seem to alter as the cell proceeds through maturation processes (28-30).

In T lymphocytes and T leukemic cell lines, differential activation of NF-κB dimers has also been described to impart important regulation on target gene transcription and cell functions. Studies have shown that T-cell receptor engagement

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of Th1 T-helper cells leads to rapid activation of a p50:p65 heterodimer, whereas similar stimulation of Th2 cells leads to delayed activation of p65 complexes devoid of the p50 subunit, thereby possibly contributing to differential cytokine gene regulation (31). A more recent study has shown that differential induction of cRel-containing NF-κB complexes regulates cytokine-mediated priming of naive T cells to overcome their intrinsic refractoriness for cytokine production in response to T-cell receptor engagement (32). Similarly, distinct NF-κB dimers have been shown to respond to different stimuli in various T leukemic cells (e.g., Jurkat, CEM, ACH-2, and MET-1), thereby contributing to differential target gene regulation (31-39). For example, CEM cells have been described to activate multiple ill-defined NF-κB complexes following stimulation with DNA-damaging agents, such as camptothecin, etoposide (VP16), and ionizing radiation (33, 40). Although NF-κB-dependent induction of $p21^{cip1/waf1}$ was found to promote chemoresistance of these cells (11), the contribution of different NF-κB dimers to such resistance gene induction remains to be determined.

In the present study, we were originally testing the hypothesis that activation of protein kinase C (PKC) is critical for NF-κB activation by genotoxic agents in CEM T leukemic cells. This hypothesis was based on previous observations that PKC activation can lead to NF-κB activation and PKC can be activated by different genotoxic agents (41-44). However, we did not find evidence for a critical role for PKC in this signaling pathway. Instead, we found an unexpected and highly enhanced NF-κB activation when CEM cells are first exposed to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and then to VP16. This enhanced NF-κB response resulted in superinduction (~1,800-fold over basal) of $p21^{cip1/waf1}$ RNA and heightened chemoresistance in CEM cells. Moreover, several other human T leukemic cell lines, but not many other cell lines examined, displayed a similarly augmented NF-κB and $p21^{cip1/waf1}$ responses. Mechanistic investigations revealed that this unusual NF-κB response in a select set of T leukemic cells derives from unusually low basal expression of p50 proteins. This leads to a reduced capacity of these T cells to activate the classic p50:p65 dimers and a lower $p21^{cip1/waf1}$-inducing potential. When these cells are preexposed to a NF-κB-activating stimulus, such as TPA, NF-κB-dependent induction of p50 occurs with the concomitant increase in the cellular potential to activate p50:p65 heterodimer and $p21^{cip1/waf1}$ gene in response to subsequent VP16 exposure. Our findings identify a previously unrecognized cellular setting in which a regulation of basal p50 expression has a profound effect on differential NF-κB dimer activation, NF-κB target gene induction, and cellular resistance to anticancer agents.

Results

Stimulation of CEM T Leukemic Cells with TPA Increases Subsequent NF-κB Activation Responsiveness

To determine the potential role of PKC in NF-κB activation by genotoxic agents in CEM T leukemic cells, we employed two different strategies: pharmacologic PKC inhibition and down-regulation of the expression of multiple PKC isoforms by a prolonged exposure to TPA. Although pretreatment of CEM cells with multiple PKC inhibitors displayed little inhibitory activity toward VP16-induced NF-κB activation (data not shown), cells pretreated with TPA for a prolonged period displayed greatly augmented, rather than reduced, VP16-dependent NF-κB activation in an electrophoretic mobility shift assay (EMSA; Fig. 1A). This NF-κB activity was greater than the sum of activations observed in cells treated with *TPA* or VP16 alone (Fig. 1B). There was no augmented Oct-1 activity seen (Fig. 1A). Thus, preexposure of these T leukemic cells to TPA modulated their NF-κB responsiveness to the genotoxic agent. Below, we describe the studies focusing on the mechanism and significance of this unusual NF-κB response.

Augmented NF-κB Response Is Associated with Superinduction of $p21^{cip1/waf1}$ and Increased Survival of CEM Cells

To gain insight into the potential significance of the above augmented NF-κB response, we examined the expression of $p21^{cip1/waf1}$ that we found previously to be NF-κB inducible in CEM cells (11). The expression of $p21^{cip1/waf1}$ as measured by quantitative real-time reverse transcription-PCR was induced ~35- and ~20-fold by VP16 and TPA treatment, respectively (Fig. 1C). Surprisingly, a sequential stimulation of CEM cells with TPA followed by VP16 led to ~1,800-fold induction of the $p21^{cip1/waf1}$ RNA. This $p21^{cip1/waf1}$ superinduction was largely NF-κB dependent, because its expression was markedly reduced in CEM cells stably expressing the superrepressor S32/36A-IκBα protein that prevents NF-κB activation (Fig. 1C). The TPA- and VP16-dependent $p21^{cip1/waf1}$ induction was also inhibited by the expression of the superrepressor by 70% and 50%, respectively, based on quantitative real-time analysis. Moreover, when induction of apoptosis was measured by the percentage of cells in sub-G_0-G_1 DNA content using propidium iodide staining of fixed cells, stable CEM $p21^{cip1/waf1}$ knockdown cells (generated and described in ref. 11; Fig. 1E) showed a significant ($P < 0.05$) increase in apoptosis under the $p21^{cip1/waf1}$ superinduction condition (72%) when compared with that induced in CEM cells expressing the control pSilencer vector under the same condition (33%; Fig. 1D).

TPA-Induced Increase in NF-κB Activation Responsiveness Involves an Exchange of NF-κB Dimers

Because NF-κB-dependent gene regulation was profoundly modulated in above experiments, we next to sought to determine the mechanism behind this augmentation of the NF-κB activation response. We examined whether VP16 dose response or kinetics of NF-κB activation was altered when CEM cells were preexposed to TPA. Results in Fig. 2A to D showed that TPA pretreatment did not alter the time course of VP16-dependent NF-κB activation or the half-maximal VP16 dose. The lack of changes in dose response and kinetics of VP16-dependent NF-κB activation suggested that the heightened NF-κB response might not involve an increased VP16-induced signaling capacity. To test this, we measured three key signaling events (IKK activation, IκBα degradation, and p65 nuclear translocation) in CEM cells. In line with the above hypothesis, none of these events correlated with the increased NF-κB response (data not shown). We also considered the possibility that previously described post-translational

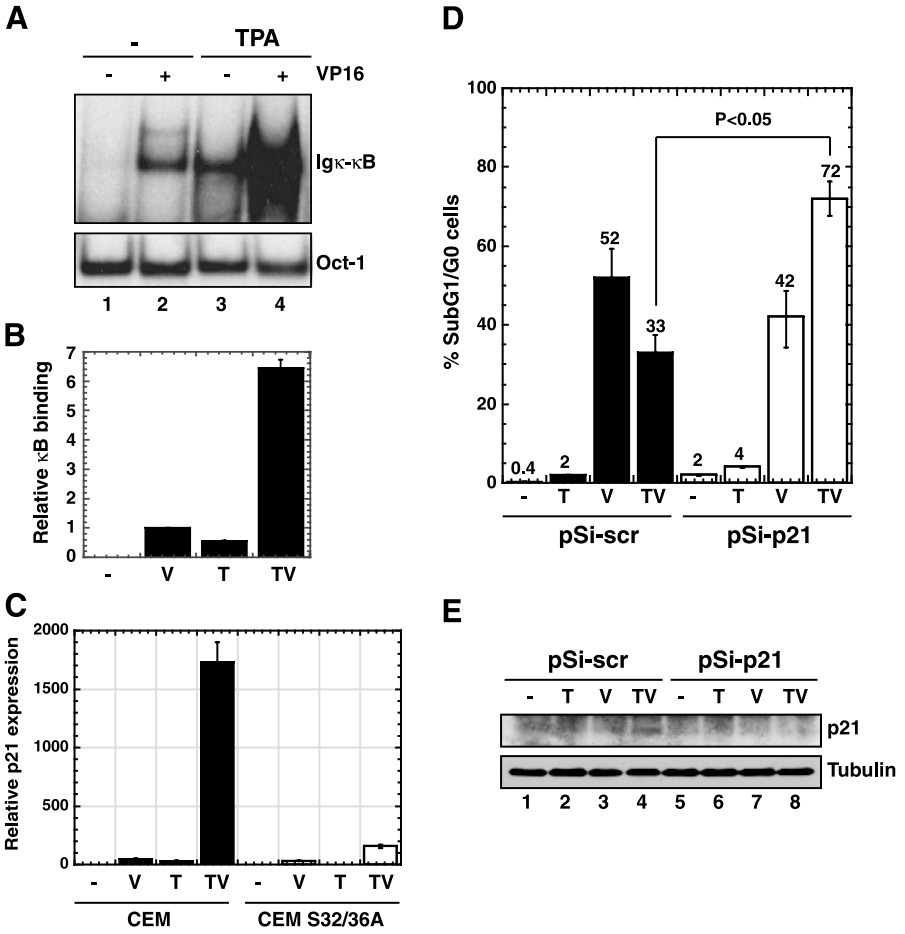

FIGURE 1. Stimulation of CEM T leukemic cells with TPA increases subsequent NF-κB activation responsiveness. **A.** CEM cells were left untreated or treated with TPA for 18 hours followed by VP16 (10 μmol/L) for 3 hours. Protein extracts (10 μg) were used for EMSA with Igκ-κB and Oct-1 site. **B.** Results from three experiments done as in (**A**) were quantified by phosphoimager. Columns, average; bars, SD. **C.** Parental CEM cells and CEM cells expressing S32/36A-IκBα cells were treated with TPA for 15 hours followed by a 6-hour VP16 treatment (*TV*). Untreated (−), TPA treatment for 21 hours (*T*), and exposure to VP16 for 6 hours (*V*) are included for comparison. RNA was purified with Qiagen RNeasy method and reverse transcribed, and cDNA levels of p21$^{cip1/waf1}$ were quantified by quantitative real-time PCR. Columns, average (as calculated by Microsoft Excel) of three experiments; bars, SD. Expression of p21$^{cip1/waf1}$ in untreated condition is defined as unity throughout the article. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. **D.** CEM cells stably expressing pSilencer-scramble (*pSi-scr*) or pSilencer-p21$^{cip1/waf1}$ (*pSi-p21*) were treated with TPA for 18 hours followed by a 96-hour VP16 treatment at 0.5 μmol/L. Cells were fixed and stained with propidium iodide. Propidium iodide incorporation was analyzed by fluorescence-activated cell sorting to identify the sub-G_0-G_1 fraction. Columns, average of three experiments; bars, SD. *, $P < 0.05$, ANOVA analysis; *, $P < 0.05$, Tukey's test (GraphPad Prism program). **E.** CEM cells stably expressing pSilencer-scramble or pSilencer-p21$^{cip1/waf1}$ were treated with TPA for 18 hours followed by a 6-hour VP16 treatment at 0.5 μmol/L. p21$^{cip1/waf1}$ expression was then analyzed by immunoprecipitation and Western blotting using antibody specific to p21$^{cip1/waf1}$ (11). Note that the induction of p21$^{cip1/waf1}$ protein is much lower than that reported previously (11) due to lower VP16 dose employed. Western blotting using an anti-tubulin antibody was employed as control.

modifications of p65, such as phosphorylation (45-48) and acetylation (49-54) events, contribute to the augmented DNA-binding response seen in TPA-exposed CEM cells. We employed a combination of pharmacologic inhibitors, site-directed mutagenesis, and stable reconstitution of CEM cells with different p65 mutant proteins; however, these studies yielded no positive association with the increased NF-κB DNA-binding activity seen in VP16-exposed cells after prolonged TPA treatment (data not shown).

Next, we explored the possibility that alterations in NF-κB complexes contributed to increased NF-κB DNA-binding activity. Different NF-κB dimers are known to possess differential affinities to various κB-binding elements (1). We did supershift experiments coupled with longer electrophoresis periods to enhance the detection of different NF-κB complexes in native gels. The VP16-induced NF-κB complexes in CEM cells migrated as two separable bands (denoted * and #; Fig. 3A) as found previously in these cells (33, 40). The slower migrating one (complex *) was supershifted by both anti-p65 and anti-cRel but not by anti-p50 antibody. The faster migrating band (complex #) reacted with anti-p65 and anti-p50 antibodies. These results showed that complex * consisted of p65 and cRel, possibly a p65:cRel heterodimer, whereas complex # consisted of the classic p50:p65 heterodimer. Under conditions where these cells are preexposed to TPA, an additional fast migrating band was detected (complex $) along with p65:p50 (#). It is supershifted by both anti-cRel and anti-p50 antibodies but not by anti-p65 antibody. These results suggested that the complex $ is composed of the p50:cRel heterodimer. Neither anti-RelB nor anti-p52 could supershift these complexes (data not shown).

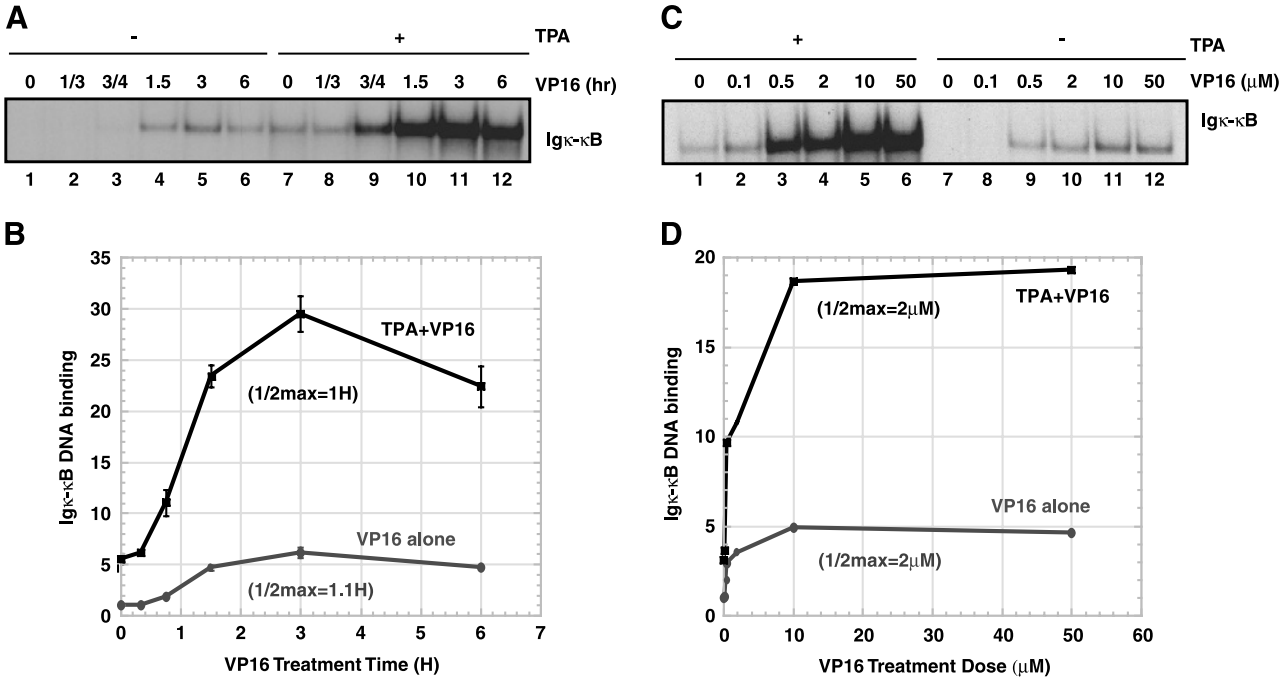

FIGURE 2. TPA preexposure alters the efficacy, but not the potency or kinetics, of a subsequent NF-κB activation response in CEM cells. **A.** CEM cells were treated with or without TPA for a total of 21 hours. Some of the cells were also treated with VP16 at the last 1/3 to 6 hours. **B.** Three experiments were done as in (**A**) and quantified and plotted. Points, average of three experiments; bars, SD. **C.** CEM cells were treated with or without TPA for 18 hours followed by VP16 for 3 hours at the indicated doses. EMSA was done with the Igκ-κB probe. **D.** EMSA in (**C**) was quantified by phosphoimager analysis and the levels of NF-κB binding were plotted against VP16 doses.

The NF-κB complexes seen under the TPA + VP16 condition (complexes # and $) were supershifted partially by anti-p65 and anti-cRel antibodies and completely reacted with anti-p50 antibody. Thus, the augmented NF-κB DNA binding was associated with increased p50-containing complexes, p50:p65 and p50:cRel, with the concomitant reduction in the p65:cRel binding. Coimmunoprecipitation experiments using anti-p65, anti-p50, or anti-cRel antibody also indicated that there were increases in the levels of p50 associated with both p65 and cRel following prolonged TPA treatment of CEM cells (Fig. 3B). These results suggested that TPA exposure of CEM cells altered the set point of NF-κB activation potential (as measured by EMSA) by altering the relative levels of NF-κB dimeric complexes.

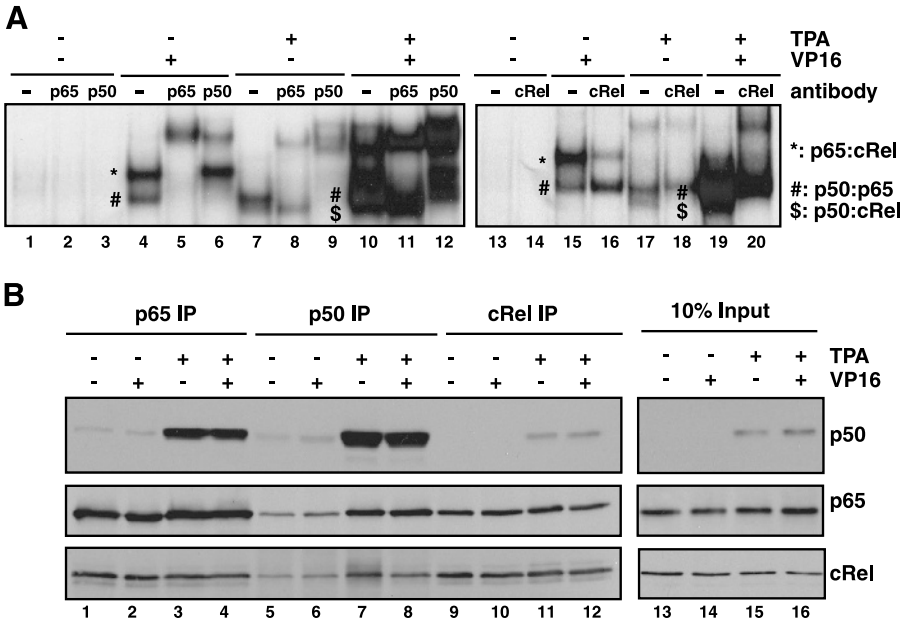

FIGURE 3. TPA-dependent increase in NF-κB responsiveness involves an exchange of NF-κB dimers. **A.** CEM cells were pretreated with TPA for 18 hours before the addition of VP16 for an additional 3 hours. Supershift analysis was done to determine the specific NF-κB dimers responsible for activation using the indicated antibodies. **B.** CEM cells were treated as in (**A**). Protein lysates were then used for immunoprecipitation by an anti-p65, anti-p50, or anti-cRel antibody followed by Western blot analysis to determine the levels of their associations. Input control (10%) was also analyzed for control. Note that p50 association with both p65 and cRel is increased following TPA treatment.

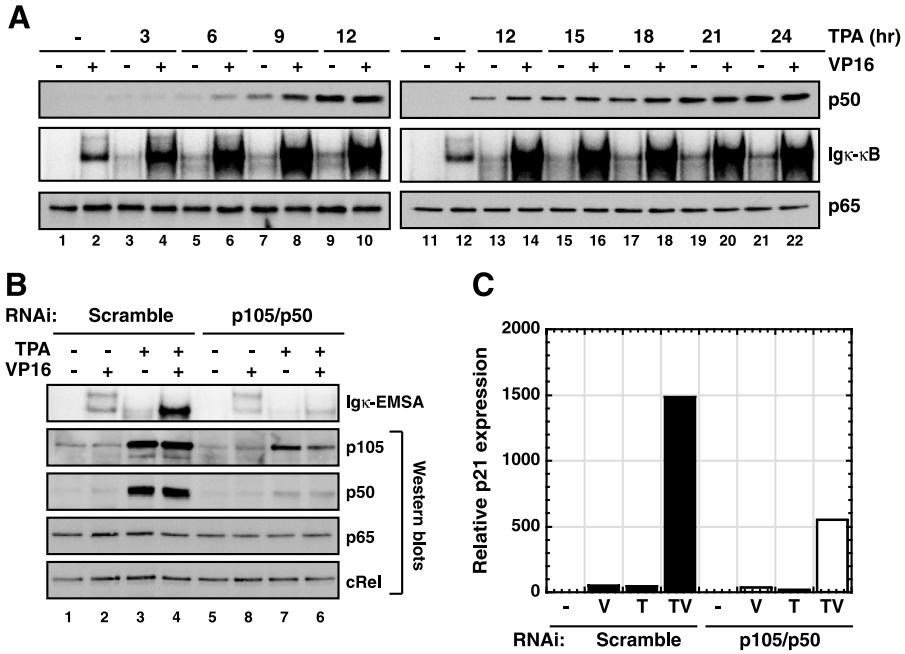

FIGURE 4. TPA-dependent induction of p50 is required for heightened NF-κB and p21$^{cip1/waf1}$ responses. **A.** CEM cells were treated with TPA for 3 to 24 hours followed by a 3-hour VP16 treatment in each case. EMSA was done to determine the DNA-binding activity, whereas Western blot was done to determine the levels of p50 and p65 proteins. **B.** CEM cells were transiently transfected with a siRNA Smart-pool selective to *NFKB1* or control siRNA by nucleofection and then treated as in Fig. 1A. Topmost row, EMSA using Igκ-κB site; last four rows, Western blots with indicated antibodies. **C.** *NFKB1* knockdown was done as in (**B**). The cells were then treated with TPA for 15 hours followed by a 6-hour VP16 treatment. Total RNA was analyzed for p21$^{cip1/waf1}$ expression by quantitative real-time PCR as described in Materials and Methods.

Exchange of NF-κB Dimers Requires p65- and ELF1-Dependent Induction of the NFKB1 Gene That Encodes p50 Protein

To determine whether the change of NF-κB dimeric complexes seen above is associated with TPA-dependent induction of p50, we next measured p50 protein levels and NF-κB-binding activity at different time points following TPA treatment. Induction of p50 could be evident as early as 6 hours following TPA treatment with the peak induction at ~18 to 24 hours (Fig. 4A). The degree of p50 induction directly correlated with that of the increase in the NF-κB DNA-binding activity.

To determine whether p50 expression was necessary for the increase in NF-κB DNA-binding activity, we silenced the expression of *NFKB1* gene that encodes p105/p50 by means of small interfering RNA (siRNA). Transient siRNA knockdown of the *NFKB1* gene expression greatly attenuated the NF-κB binding under the TPA+VP16 condition (Fig. 4B). Knockdown of *NFKB1* expression also attenuated the p21$^{cip1/waf1}$ superinduction by >60% (Fig. 4C). Thus, these results indicated that TPA-dependent induction of p105/p50 followed by exchange of NF-κB complexes to those containing the p50 subunit was critical for augmented NF-κB and p21$^{cip1/waf1}$ responses.

Previous studies reported that the *NFKB1* gene is a direct target of NF-κB (25, 26, 55). Accordingly, TPA increased not only p105/p50 protein levels but also *NFKB1* mRNA levels (Fig. 5A) and the increased p50 synthesis was blocked by expression of the S32/36A-IκBα superrepressor (Fig. 5B). Similarly, a prolonged exposure to tumor necrosis factor-α (TNF-α), another NF-κB inducer, also caused increased p50 levels and NF-κB DNA binding in response to subsequent VP16 stimulation (Fig. 5C and D). Furthermore, the augmented NF-κB DNA-binding response was also observed when the second inducer was a different DNA-damaging agent (camptothecin, doxorubicin, or ionizing radiation) or an extracellular ligand (TNF-α; Fig. 5E; others not shown). These findings suggested that the augmentation of the NF-κB response in CEM cells was a general phenomenon due to repetitive activation that was associated with the NF-κB-dependent *NFKB1* transcriptional induction.

Besides NF-κB, little is understood regarding other factors that also contribute to the induction of the *NFKB1* gene. A previous report suggested a role for the ETS family of proteins in the *NFKB1* gene regulation (27). The specific family member(s) that is involved in this regulation, however, is unknown. Supershift analysis using a *NFKB1* promoter DNA that contains both NF-κB and ETS binding sites showed that anti-ELF1 antibodies supershifted this complex *in vitro* (Fig. 6A). In addition, chromatin immunoprecipitation showed that ELF1 constitutively occupied the *NFKB1* promoter site in CEM cells and this occupancy was also increased in response to TPA exposure (Fig. 6B). In contrast, basal p65 occupancy of the *NFKB1* promoter site was not detected, but clear induction of binding was observed following TPA exposure (Fig. 6B). Transient siRNA knockdown of either p65 or ELF1 expression greatly attenuated p105 expression (Fig. 6C). Together, these findings indicated that both ELF1 and NF-κB are necessary for optimal *NFKB1* expression in response to TPA treatment of CEM cells.

p21$^{cip1/waf1}$ Superinduction Is Selective to a Subset of Human T Leukemic Cells

To determine whether the NF-κB and p21$^{cip1/waf1}$ superinduction responses observed in CEM cells could also be found in other cell systems, we next examined NF-κB responses in various human cell lines. The cell lines included several T leukemic cell lines (MOLT-4, RPMI-8402, PF382, ALL-SIL, HPB-ALL, Karpas-45, Loucy, and MOLT-13), acute lymphoblastic leukemia lines from the B-cell lineages (Sup-B15, Reh, and RS4;11), acute myeloid leukemia cell lines (GDM1 and Kasumi3), a Burkitt's lymphoma cell line (Ramos), an

erythroleukemia cell line (K562), a monocyte cell line (EL1), two prostate cancer lines (PC3 and DU145), an embryonic kidney cell line (HEK293), a cervical cancer cell line (HeLa), a lung cancer cell line (Hep2), a liver cancer cell line (HepG2), and a breast cancer cell line (MDA-MB-231). Our findings suggested that augmented NF-κB DNA-binding response (Fig. 7A-C) and $p21^{cip1/waf1}$ superinduction (Fig. 7D-F) under the TPA + VP16 condition were selectively seen in a subset of T leukemic lines. Intriguingly, and consistent with the finding in CEM cells, those T leukemic cell lines that displayed augmented NF-κB and $p21^{cip1/waf1}$ responses all showed very low basal p50 expression levels (summarized in Table 1). These findings indicated that, among the cell lines examined, only a subgroup of human T leukemic cell lines possessed this unique augmentation of the NF-κB and $p21^{cip1/waf1}$ induction potential due to the exceedingly low basal p50 expression levels.

Discussion

In this study, we have characterized an unusual NF-κB activation phenomenon in a subgroup of human T leukemic cells, which deviates from the classic NF-κB paradigm due to very low basal expression levels of *NFKB1* gene and its encoded p50 protein. Low p50 expression results in the formation of NF-κB complexes composed primarily of p65 and cRel (likely a p65:cRel heterodimer) as the major latent NF-κB complexes in these cells. When these cells are exposed to a NF-κB activation stimulus, such as TPA, *NFKB1* transcription is induced in an ELF1- and p65-dependent manner, leading to increased p50 (and its precursor p105) protein levels. Increased levels of p50 promotes a dimer exchange from the p65:cRel to the p50-containing dimers (p50:p65 and p50:cRel) as the major latent NF-κB complexes and a modified set point for the inducible NF-κB DNA-binding capacity in these stimulated cells. When these cells are subsequently exposed to another stimulus, activation of p50-containing dimers display increased DNA-binding activity to Igκ-κB site due to their inherent higher affinity to this κB site (56-59). This provides an explanation for why this augmented NF-κB response was only associated with the altered efficacy of DNA binding and not with altered VP16 activation kinetics, dose response, or increases of signaling potentials (e.g., IKK activation, IκBα degradation, and p65 nuclear translocation).

One outcome of this increased NF-κB response in T leukemic cells is heightened induction of $p21^{cip1/waf1}$ and increased resistance of these cells to VP16 treatment. The repertoire of NF-κB-dependent genes that behave in this manner is probably not limited to this gene alone. In *Drosophila* embryos, DNA-binding affinity of Dorsal (a *Drosophila* NF-κB family member) was shown to be a critical determinant for segregating Dorsal target gene expression (60). However, in mammalian cells, studies have shown that there is no simple correlation between NF-κB-binding affinity to a specific κB site *in vitro* and induction of genes regulated by such a site *in vivo*. For example, κB site bending changes due to intrinsic sequence differences, despite the similar binding affinity, greatly affected the outcomes of target gene expression, such as the IFN-β gene (61). Moreover, a recent study showed that a change of a single base pair within a κB element present in MCP-1 gene was sufficient to alter its induction property *in vivo* without altering the binding affinity (62). Such a complexity of NF-κB-dependent transcriptional regulation was also underscored by our observation of

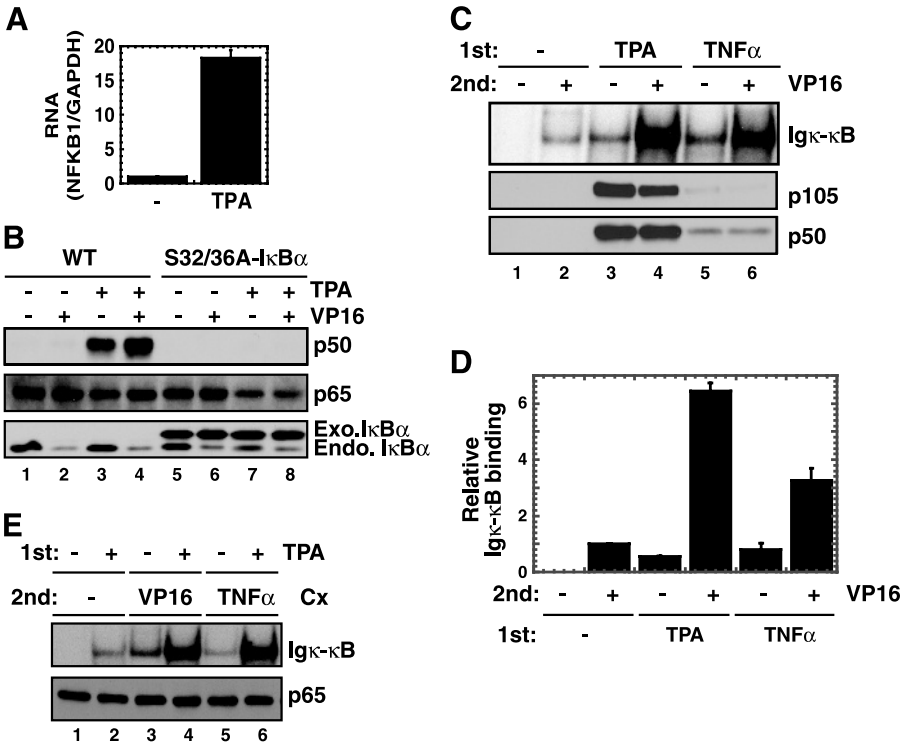

FIGURE 5. NF-κB-dependent induction of *NFKB1* is associated with heightened NF-κB responses irrespective of the nature of first or second NF-κB-activating stimuli. **A.** CEM cells were treated with TPA for 21 hours and total RNA was analyzed for *NFKB1* expression by quantitative reverse transcription-PCR. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was also analyzed as control. Columns, average *NFKB1*/glyceraldehyde-3-phosphate dehydrogenase radios of three experiments; bars, SD. **B.** CEM cells and those stably expressing S32/36A-IκBα were treated (or not) with TPA for 18 hours before the addition of VP16 for an additional 3 hours. Total protein extracts were analyzed by Western blotting for levels of p50, p65, and IκBα proteins (*bottom*). See also the position of the exogenous IκBα. **C.** CEM cells were treated (or not) with TPA or TNF-α for 18 hours followed by VP16 (10 μmol/L) treatment for 3 hours. Total protein extracts were used for EMSA with Igκ-κB and Oct-1 sites. **D.** Binding activities quantified by a phosphoimager from three experiments as done in (**C**). Columns, average; bars, SD. **E.** CEM cells were treated (or not) with TPA for 18 hours followed by treatment with VP16 (10 μmol/L) or TNF-α (10 ng/mL) for 3 hours and analyzed as in (*C*).

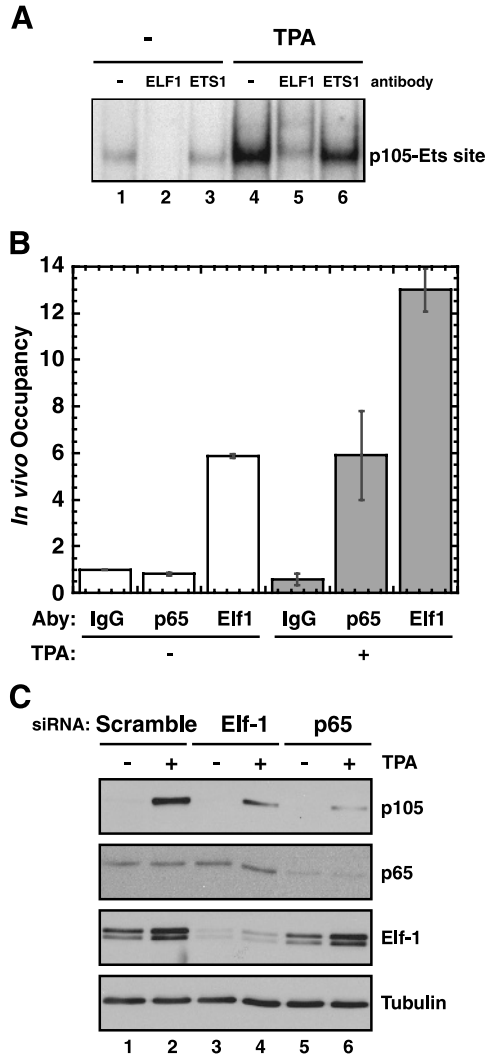

FIGURE 6. ELF1 and p65 are required for TPA-dependent *NFKB1* induction. **A.** Untreated and TPA (21 hours)–treated CEM cells were collected, and cellular lysates were prepared for EMSA. Supershift analysis was done with anti-ELF1 and anti-ETS1 antibodies to determine ETS1 and ELF1 association with the p105-Ets element. **B.** Chromatin immunoprecipitation assays were done with anti-p65 and anti-ELF1 antibodies as described in Materials and Methods. Results are presented using untreated CEM sample that is precipitated by control IgG as unity. Columns, average levels of *NFKB1* promoter DNA recovered in three experiments; bars, SD. **C.** CEM cells transfected with siRNAs specific to ELF1 or p65 were left untreated or treated with TPA for 21 hours. Control siRNA-transfected CEM cells (*Scramble*) were also analyzed in parallel. Western blotting was done to analyze the expression of p105, p65, ELF1, and tubulin (as loading control).

superinduction of the interleukin-8 gene under the TPA + VP16 condition, although there was no obvious increase of NF-κB binding to a κB site present in this gene promoter by EMSA under the TPA + VP16 condition.[2]

Interestingly, TPA-mediated increases in cell survival in response to chemotherapeutic agents, including several topoisomerase I/II inhibitors (e.g., camptothecin, VP16, VM-26, and 4-(9-acridinylamino) methanesulphon-*m*-anisidide), have been reported previously in several lymphoid cell lines analyzed in this study (e.g., CEM, MOLT-4, and RPMI-8402; refs. 63-68). These reports suggested that TPA-dependent survival is mediated through its ability to differentiate these cell lines. Additional studies implicated that NF-κB activation is a critical step in TPA-mediated differentiation (69-71). NF-κB can also regulate a wide range of target genes that alter apoptotic and proliferation responses. Our study identifies p21$^{cip1/waf1}$ as a critical player that promotes the TPA-dependent T leukemic cell survival response. However, because many NF-κB-regulated genes are known to retard apoptosis, such as the Bcl-2 and inhibitors of apoptosis families of proteins, there are likely other factors that contribute to TPA-dependent survival responses in T cells. Thus, the definition of the full transcriptional and functional effects of the augmented NF-κB response due to dimer exchange in T leukemic cells in response to TPA exposure requires additional genome-wide screening approaches.

The p21$^{cip1/waf1}$ expression has been widely implicated in the cell cycle regulation and some apoptotic functions. Although the p53-dependent induction of p21$^{cip1/waf1}$ gene is well established (8, 72), p21$^{cip1/waf1}$ gene regulation in p53-deficient cells is not fully understood. Recent studies also placed p21$^{cip1/waf1}$ under the regulation of several other transcription regulators, including NF-κB, Miz-1, Bcl-6, and Myc (73-76). We also reported previously that p21$^{cip1/waf1}$ induction can be induced in a NF-κB-dependent manner in p53-mutant CEM T leukemic and MDA-MB-231 breast cancer cells (11). We extended these observations by showing that p21$^{cip1/waf1}$ superinduction can be observed in several human T cell lines in response to sequential NF-κB activation events. Interestingly, all the T leukemic cells that display this p21$^{cip1/waf1}$ superinduction response (e.g., CEM, MOLT-4, RPMI-8402, and PF382) harbor mutant p53 proteins (77).[2] Although several p53-NF-κB cross-talk studies have been reported previously (78-81), these studies mostly addressed the functional relationships between NF-κB-dependent gene activation or activation of NF-κB in response to p53 modulation. Because many human cancer cell lines that are also defective for p53 do not display low basal *NFKB1* expression or augmented NF-κB DNA-binding responses under repetitive stimulation conditions (ref. 77; Table 1), the lack of p53 function does not seem to be the primary determinant for down-regulation of basal *NFKB1* gene expression in the above T cell lines.

Low basal expression of p105/p50 has been observed previously in certain other cellular contexts, including normal germinal center B cells (82), germinal center–derived diffuse large B-cell lymphoma cells (83), and transgenic murine Eμ-Myc lymphoma models (84). A recent study suggested that a proto-oncoprotein Bcl-6 is the direct transcriptional repressor responsible for the low basal p105/p50 expression level in these B-cell systems (85). Bcl-6 is not expressed in T cell lines that harbor very low basal levels of p105/p50 (86).[2] This observation suggests that other transcriptional repressors may negatively regulate p105/p50 expression in T leukemic cells. Aberrant expression and mutations of several transcription regulators (e.g., HOX11, HOX11L2, TAL1 + LMO1/2, LYL1 +

[2] P-Y. Chang, unpublished observations.

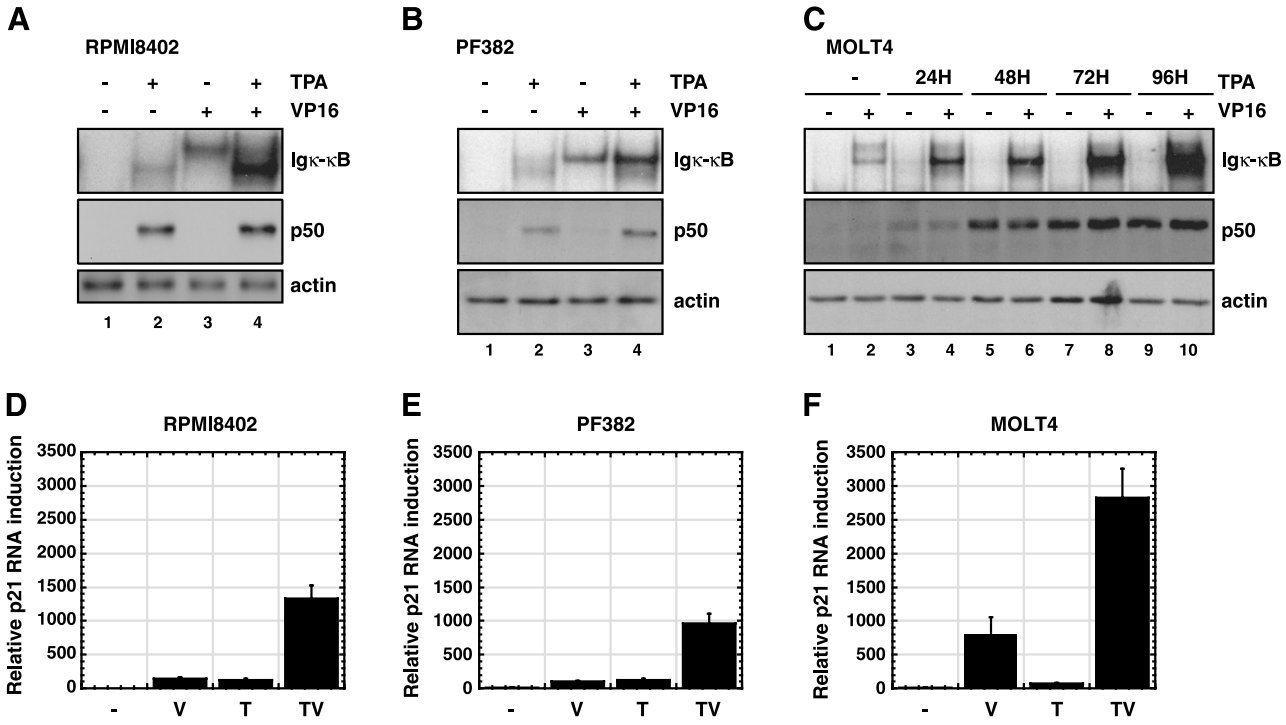

FIGURE 7. TPA-dependent increase in NF-κB responsiveness occurs in a subset of T leukemic cells. RPMI-8402 (**A**), PF382 (**B**), or MOLT-4 (**C**) cells were treated with or without TPA or TNF-α for 18 hours followed by treatment with VP16 (10 μmol/L) for 3 hours. Total protein extracts were used for EMSA analysis with Igκ-κB (*top*) or Western blot analysis with anti-p50 or tubulin antibodies (*bottom*). RPMI-8402 (**D**), PF382 (**E**), or MOLT-4 (**F**) cells were untreated or treated with TPA for 15 hours followed by VP16 (10 μmol/L) for 6 hours. Total RNA was analyzed by quantitative real-time PCR for $p21^{cip1/waf1}$ gene induction. Relative averages and ranges from two independent experiments for different conditions for different cell lines were plotted by setting the level of induction of $p21^{cip1/waf1}$ in CEM cells untreated condition (Fig. 1C) as unity.

LMO2, and MLL-ENL) have been reported to contribute to the T-cell leukemogenesis (87-89). It is possible that some of these oncoproteins is involved in repressing *NFKB1* gene in T leukemic cells.

Finally, our study identifies NF-κB as a potential therapeutic target for a subset of T-cell leukemias. Based on the microarray analysis published by Ferrando et al. (87), more than one third (14 of 39) of the T-acute lymphoblastic leukemia (T-ALL) patients displayed low expression levels of the *NFKB1* gene (<300 based on the published Affymetrix analysis). The leukemia cells in these patients could be predisposed for the augmented NF-κB response similar to what is described in our current study. Because inhibition of NF-κB activation and $p21^{cip1/waf1}$ induction sensitized CEM cells to VP16-induced apoptosis (this study and ref. 11), a combination of NF-κB and/or $p21^{cip1/waf1}$ inhibitors and chemotherapeutic agents might reduce a survival response of the leukemia cells. This approach could enhance the therapeutic success of a subset of the T-ALL patients.

Materials and Methods

Cell Culture and Chemicals

CEM, MOLT-4, Loucy, Sup-B15, Reh, RS4;11, GDM1, Kasumi3, Ramos, K562, EL1, Du145, PC3, HEK293, HeLa, Hep2, HepG2, and MDA-MB-231 were purchased from and maintained under conditions recommended by the American Type Cell Culture (Manassas, VA). Derivatives of the CEM (stably expressing S32/36A-IκBα; ref. 11) were cultured in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (HyClone Laboratory, Inc., Logan, UT), 100 units penicillin G, and 100 μg/mL streptomycin sulfate (Mediatech) with the addition of 500 μg/mL G418 (Mediatech). CEM cells stably expressing pSilencer-scramble and CEM pSilencer-p21 (generated and described in ref. 11) were maintained under the condition described above with the addition of 1 μg/mL puromycin. RPMI-8402, PF382, ALL-SIL, HPB-ALL, Karpas-45, and MOLT-13 were obtained from and cultured under conditions recommended by the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

Antibodies

IgG antibodies against IκBα (C-21), actin (C-11), c-Myc (9E10), p65 (C-20), and RelB (C-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cRel antibody (SA-172) was obtained from Biomol (Plymouth Meeting, PA). Anti-p52 (06-413) and anti-p50 (06-886) antibodies were obtained from Upstate Biotechnology (Charlottesville, VA). A monoclonal anti-tubulin antibody was purchased from EMD Biosciences (San Diego, CA) Horseradish peroxidase–conjugated protein A and horseradish peroxidase–conjugated anti-rabbit and anti-mouse antibodies were obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

Chemicals

VP16 and TPA were purchased from Sigma-Aldrich (Atlanta, GA). Human recombinant TNF-α was purchased from EMD Biosciences.

Transient siRNA Transfection

CEM cells (5×10^7) were used for nucleofection (Amaxa Biosystems, Gaithersburg, MD) under each treatment condition. Nucleofection was done under the manufacturer's recommended condition (O-017 with solution R). Smart-pool siRNA mixtures with four sets of oligonucleotides per target gene (specific to ELF1, p65, and p50) were purchased from Dharmacon (Lafayette, CO) and resuspended at 40 μmol/L concentrations. In each nucleofection reaction, 2.5 μL (100 pmol) siRNA was used.

EMSA and IKK Kinase Assay

EMSA (and the Igκ-κB oligonucleotide probe) and IKK kinase assays were done as described previously (90). The sequence for the plus strand of the p105-ETS probe used is 5′-TCGACAGTGGGAATTTCCAGCCAGGAAGTGAGA-GAGTGA-3′. The experiments were repeated at least thrice, and the results were quantified by exposing dried EMSA gels on a phosphoimager screen and analyzed by the IQMac1 program. The average and SD were calculated by the Microsoft Excel program and plotted by the KaleidaGraph software.

Immunoprecipitation and Western Blotting

Immunoprecipitation experiments were done with 10^7 cells. The cells were lysed in 20 μL PBS and 180 μL lysis buffer as described previously (11). Supernatants were diluted further in 300 μL lysis buffer, and 1 μg anti-p65, anti-p50, or anti-p21 antibody was added to each tube. Samples were rotated for 60 minutes at 4°C. Protein G-Sepharose beads (Amersham Pharmacia Biotech) were then added to each tube, and the samples were rotated for 90 minutes at 4°C. The precipitated protein was resolved in 10% SDS-PAGE gels (12.5% SDS-PAGE gels for p21 analysis) and analyzed by Western blotting using the appropriate antibody. Input controls were generated by taking 10 μL of the supernatant before lysis buffer dilution step. Quantified Western blot data were generated with the NIH ImageJ program. The average and SD were calculated by the Microsoft Excel program and plotted by the KaleidaGraph software.

Chromatin Immunoprecipitation Analysis

Chromatin immunoprecipitation solutions were purchased from Upstate Cell Signaling Solutions (Charlottesville, VA), and the assays were done according to the manufacturer's protocol with the minor modifications. Protein-DNA cross-linking was done by incubating 7.5×10^6 CEM cells with formaldehyde at a final concentration of 1% for 10 minutes at room temperature with gentle agitation. Glycine (0.125 mol/L) was added to quench the reaction. Cells were then collected by centrifugation at 3,000 rpm for 15 minutes and washed in PBS. The lysate was sonicated with 8 pulses of 40 seconds each at 50% to 60% of maximum power with a Heat Wave Systems W185F sonicator (Ultrasonics, Farmingdale, NY) equipped with a microtip to reduce the chromatin fragments to an average size of ~500 bp. Soluble chromatin was precleared by addition

Table 1. Summary of p50 Expression and NF-κB Responses in Different Human Cell Lines

Cell Line	Cell Type	Basal p50 Levels	TPA-Dependent p50 Induction	Igκ-κB Binding*	p21 Induction †
CEM	T-ALL	Very low	+++	6.5×	35×
MOLT-4	T-ALL	Very low	+++	5×	3.5×
RPMI-8402	T-ALL	Very low	++	2×	10×
PF382	T-ALL	Very low	++	2×	9×
ALL-SIL	T-ALL	Low	+	1×	<1×
HPB-ALL	T-ALL	Low	+	0.5×	ND
Karpas-45	T-ALL	Low	+	1×	<1×
Loucy	T-ALL	Medium	+	0.5×	ND
MOLT-13	T-ALL	ND	ND	1×	ND
Sup-B15	B-ALL	ND	ND	1×	ND
Reh	ALL	Medium	–	1×	ND
RS4;11	ALL bone marrow	Medium	+/–	1×	ND
GDM1	Acute myelogenous leukemia peripheral blood	Medium	–	0×	ND
Kasumi3	Acute myelogenous leukemia	ND	ND	1×	ND
Ramos	Burkitt's lymphoma	Medium	–	0.5×	ND
K562	Erythroleukemia	Medium	+/–	1×	ND
EL1	Monocytes	ND	ND	1×	ND
Du145	Prostate cancer	Medium	–	1×	ND
PC3	Prostate cancer	Medium	–	1×	ND
HEK293	Embryonic kidney	Medium	+/–	1×	ND
HeLa	Cervical cancer	Medium	+	1×	ND
Hep2	Lung cancer	Medium	–	1×	ND
HepG2	Liver cancer	Medium	–	1×	ND
MDA-MB-231	Breast cancer	ND	ND	1×	ND

NOTE: Quantification of Western blot results was done with the NIH ImageJ program. EMSA quantification was achieved by exposing dried EMSA gels to a phosphoimager screen. Quantitative results of p21$^{cip1/waf1}$ gene expression were generated by quantitative real-time PCR analysis. Results are average of three independent experiments.

Abbreviations: +/–, <2-fold; +, ~2-fold; ++, 2- to 4-fold; +++, >4-fold; ND, not determined.

*Igκ-κB EMSA binding of TPA + VP16 divided by the sum of TPA and VP16 alone.

†p21 induction of TPA + VP16 divided by the sum of TPA and VP16 alone.

of 50 μL preimmune serum followed by 100 μL salmon sperm DNA/protein A-Sepharose slurry. An aliquot of precleared chromatin was removed (input) and used in the subsequent PCR analysis. The remainder of the chromatin was diluted with immunoprecipitation dilution buffer and incubated with or without 10 μL antibody (anti-p65 from Biomol or anti-ELF1 from Santa Cruz Biotechnology) or rabbit preimmune serum in a final volume of 600 μL for 1 hour at 4°C. Immune complexes were collected by incubation with 30 μL protein A-Sepharose overnight at 4°C. Protein A-Sepharose pellets were washed according to the manufacturer's recommendations. RNase A (0.03 mg/mL) and NaCl (0.3 mol/L) were added, and cross-links were reversed by incubation for 4 hours at 65°C. Samples were digested with proteinase K (0.24 mg/mL) for 2 hours at 45°C. DNA was purified by one extraction with phenol/chloroform and one with chloroform followed by ethanol precipitation. Purified DNA was resuspended in 30 μL water. Aliquots of 2 μL were analyzed by real-time PCR with the appropriate primer pairs. In chromatin immunoprecipitation experiments, quantitative real-time data are presented by setting the untreated serum precipitated samples as unity. The average and SD were calculated by the Microsoft Excel program and plotted by the KaleidaGraph software. *NFKB1* promoter, forward GAATTCCATGGATGGCAGAAGATGATCCATAT and reverse GAATTCCTAGCTCATCAATGCTTCATCCC.

Quantitative Reverse Transcription-PCR Analysis

Total RNA from CEM cells or those stably expressing hemagglutinin-tagged S32/36A-IκBα was extracted with the Qiagen (Valencia, CA) RNeasy kit. cDNA was synthesized by annealing RNA (2 μg) with 250 ng of a 1:4 mixture of random and oligo(dT) primers by heating at 68°C for 10 minutes. After renaturation, the samples were incubated with Moloney murine leukemia virus reverse transcriptase (10 units/μL; Invitrogen, Carlsbad, CA) combined with 20 mmol/L DTT, 1 mmol/L deoxynucleotide triphosphates, and 2 units/μL RNasin (Promega, Madison, WI) at 42°C for 1 hour. The reaction mixture was heat inactivated at 95°C to 100°C for 5 minutes and diluted 1:10. Quantitative real-time reverse transcription-PCR (25 μL) contained 2 μL cDNA, 12.5 μL SYBR Green (Applied Biosystems, Warrington, United Kingdom), and the appropriate primers. Product accumulation was monitored by SYBR Green fluorescence with ABI Prism 7000 Sequence Detection Systems kindly provided by Dr. Bresnick (University of Wisconsin-Madison, Madison, WI). The relative expression levels were determined from a standard curve of serial dilutions of cDNA samples. CEM cells treated under VP16 alone condition is defined as unity in all experiments. Forward and reverse primers for real-time RT-PCR are described previously (11). The average and SD were calculated by the Microsoft Excel program and plotted by the KaleidaGraph software.

Fluorescence-Activated Cell Sorting and Cell Cycle Analysis

CEM cells and derivatives were exposed to 0.5 μmol/L VP16 for a total of 96 hours either with or without the pretreatment of TPA for 21 hours. For cell cycle analysis, cells were processed as described previously (11) and analyzed on a FACScan flow cytometer (BD PharMingen, San Jose, CA). Data were analyzed using the CellQuest (BD PharMingen) and ModFit (Verity Software House, Topsham, ME) software. The average and SD of three independent experiments were calculated by the Microsoft Excel program and plotted by the KaleidaGraph software. The statistical analysis was done by GraphPad Prism program with ANOVA analysis and Tukey's multiple comparison *t* test.

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