Cell. Signal. Vol. 11, No. 5, pp. 371–383, 1999

ELSEVIER

ISSN 0898-6568/99 $–see front matter
PII S0898-6568(99)00022-4

Dual Activity of Pyrrolidine Dithiocarbamate on κB-Dependent Gene Expression in U937 Cells: II. Regulation by Tumour Necrosis Factor-α

*K. Watanabe, I. Kazakova, M. Furniss and S. C. Miller**

Signal Transduction Laboratory, Pharmaceutical Discovery Division,
SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025, USA

ABSTRACT. In the human promonocytic U937 cell line, pyrrolidine dithiocarbamate (PDTC) was a potent inhibitor of the nuclear factor-κB (NF-κB) signalling pathway induced by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). However, PDTC did not inhibit tumour necrosis factor-α (TNF-α)-induced NF-κB DNA binding activity but potentiated the effect of TNF-α on κB-dependent gene expression. The stimulatory effect of PDTC with TNF-α was not observed with an HIV-1 LTR reporter construct containing two mutated κB binding sites or with a construct with a mutation of the activating protein (AP)-2 binding site located between the two κB elements. Two distinct signalling pathways, one mediated by TPA and the other by TNF-α, were shown to interact, functionally defining a threshold important in the inhibitory or stimulatory effect of PDTC on κB-dependent gene expression. Evidence that PDTC induced AP-1 DNA binding and AP-1 reporter gene activity, raised the hypothesis that the effect of PDTC was mediated by an interaction between the AP-1 pathway and p65(RelA). Co-transfection with expression vectors for p65(RelA) and the AP-1 subunits c-Fos and c-Jun resulted in a decrease in the stimulatory effect of PDTC on HIV-1 LTR activity. Co-transfection of p65(RelA) with Tam67, a dominant negative mutant of c-Jun defective in transactivation, stimulated the effect of PDTC on HIV-1 LTR activity. Evidence that the stimulatory effect of Tam67 with PDTC was reduced with c-Jun is consistent with the hypothesis. CELL SIGNAL 11;5:371–383, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. Tumor necrosis factor-α, Dithiocarbamates, NF-κB, HIV-1 LTR, Gene regulation

INTRODUCTION

The regulation of the biological response of cells to tumour necrosis factor-α (TNF-α) is complex, resulting in divergent responses such as cell proliferation, differentiation and apoptosis. Binding of the trimeric TNF-α to the TNF receptor 1 results in receptor aggregation, which is thought to be a critical step in the activation of c-Jun amino-terminal kinase (JNK), the transcription factors NF-κB, activator protein 1 (AP-1) and apoptosis [1]. Recruitment of the signal transducer Fas associated death domain (FADD) mediates apoptosis, whereas JNK and NF-κB activation is mediated by receptor-interacting protein (RIP) and TNF receptor-associated factor 2 (TRAF2) [1]. Other signalling steps are important in apoptosis because the activation of NF-κB was shown to protect against TNF-α-induced apoptosis [2–4]. Recent work showed that TRAF2 defines a point of separate kinase cascades activating NF-κB and JNK, respectively [5]. The NF-κB-inducing kinase does not activate the JNK pathway but appears to be dedicated to the NF-κB pathway [5]. MEK kinase 1 (MEKK1) expression studies demonstrated that MEKK1 has a role in the activation of JNK [6]. MEKK1 expression was shown to stimulate NF-κB transcriptional activity [7–10]. However, it is not clear that MEKK1 is physiologically involved in NF-κB activation [6].

The biological response of cells to the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is mediated through the activation of protein kinase C (PKC). TPA and TNF-α were shown to phosphorylate IκB-α through parallel pathways, resulting in the translocation of NF-κB into the nuclear compartment [11]. Inhibitors of the TPA-induced signalling pathway that inhibit PKC by different mechanisms were used to demonstrate that TPA and TNF-α act through distinct signal transduction pathways leading to activation of NF-κB [12]. More recently, TNF-α and TPA were shown to activate the inhibitor of kappa B-α (IκB-α) kinase complex through distinct signals [13, 14].

Pyrrolidine dithiocarbamate (PDTC) was shown to inhibit the degradation of IκB-α induced by TNF-α and TPA and has been widely used as an inhibitor of the NF-κB signalling pathway [15, 16]. Although originally described as an antioxidant, PDTC was shown to possess metal chelator, antioxidant [17–19] and oxidant activity [16, 20]. Further, PDTC was shown to stimulate the transcription factor AP-1 through the JNK pathway [21]. The combined treat-

*Author to whom all correspondence should be addressed. Tel: +1-650-859-5788; Fax: +1-650-859-3153; E-mail: smiller@unix.sri.com
Received 20 January 1998; and accepted 30 November 1998.

ment of PDTC with TPA resulted in the persistent phosphorylation of extracellular signal-regulated kinase type 2.

Here we report on our examination of the interrelation of two distinct signalling pathways, one mediated by TPA and the other by TNF-α, and the effect of PDTC on κB-dependent genes in U937 cells. In this model system, TNF-α induced NF-κB, but, in contrast with TPA or PDTC treatment, TNF-α failed to stimulate AP-1-dependent DNA binding or reporter-gene activity. Although PDTC was a potent inhibitor of the NF-κB signalling pathway induced by TPA, PDTC did not inhibit TNF-α-induced NF-κB DNA binding activity, but it potentiated the effect of TNF-α on κB-dependent gene expression in U937 cells. Interestingly, other investigators demonstrated that PDTC inhibits TNF-α-induced NF-κB as well as AP-1-dependent gene expression [22]. Differences reported in the effects of PDTC on the TNF-α signalling pathway in different cell models in combination with our results suggested that another signalling pathway may be important. Our results are consistent with the hypothesis that a TPA-induced signalling pathway sets a threshold important for PDTC to potentiate the effect of TNF-α on κB-dependent gene expression.

MATERIALS AND METHODS

Cell Culture and Treatment

U937 cells were obtained from the ATCC and grown in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine (GIBCO BRL, Life Technology Inc., Grand Island, NY), 100 units/mL of penicillin and 100 μg/mL of streptomycin (Sigma Co., St. Louis, MO). TPA, PDTC, and *N*-acetyl-L-cysteine (NAC) were from Sigma Co. TPA was dissolved in DMSO to produce a 1-mg/mL stock. PDTC and NAC were dissolved in PBS, and pH was adjusted to 7.2 to produce a 1-M stock. TNF-α was from Cellular Products Inc. (Buffalo, NY).

Plasmids

To evaluate the effect of AP-1 on PDTC stimulated p65(RelA) HIV-1 long terminal repeat (LTR) activity, expression vectors for c-Fos, c-Jun and a dominant-negative mutant lacking the transactivation domain of c-Jun (Tam67) were used. The AP-1 subunits c-Jun and c-Fos were expressed from Rous sarcoma virus (RSV) expression plasmids; the construction of Tam67 in the pCMV vector was previously described [23]. The RSV expression plasmid containing human p65(RelA) also was previously described [24]. The plasmid pHIV chloramphenicol acetyltransferase (pHIVCAT) and expression vector of the HIV-1 transactivator protein (tat) pcDEBtat were obtained from P. Berg (Stanford University, Stanford, CA). These plasmids were previously described [25], and the pcDEBtat expression vector was previously shown to stimulate HIV-1 LTR activity through the Tat-responsive element (TAR). The effect of AP-1 subunits or Tam67 or Tat on HIV-1 LTR activity was measured. Briefly, 5 μg of the CAT reporter construct was co-transfected by electroporation with 5 μg of the test plasmid. Total DNA was kept constant by adding puc19 plasmid DNA. Similar studies were done with a 4 × AP-1CAT plasmid [41] in which CAT expression was shown to be dependent on four AP-1 recognition sequences.

To evaluate whether the effect of PDTC induced a stimulatory effect mediated through the κB elements in the HIV-1 LTR, a 4 × κBCAT construct containing four κB binding sites or HIV-1 LTR constructs with site-specific mutations in the κB or AP-2 sites [26] were used. The 4 × κBCAT, wild-type HIV-1CAT plasmid and mutant constructs were obtained from G. Nabel (University of Michigan, Ann Arbor, MI). HIV-1 CAT with wild-type κB elements (5′-GGGACTTTCCGCTGGGGACTTTCC-3′; κB elements underlined) or mut-κB (**TCT**ACTTTCCGC-TG**TCT**ACTTTCC) were described previously [27]. The site-directed mutagenesis of the HIV-1CAT plasmid was previously described for the mut-3′κB (5′-GGGACTTTC-CGCT**AGATCT**TTTCC-3′) or mut-3′κB + 5′Sp1 (5′-GGGACTTTCCGCT**AGATCT**TTTCC-3′; the 5′Sp1 site (underlined) was changed from GAGGCGTGGCC to GA**ACTCGAG**CC) [28] or mut-AP-2 (5′-GGGACTTT-CC**ATAT**GGGACTTTCC-3′) construct [26]; boldface type identifies the mutated sites. To determine the minimal κB sequence elements required for the stimulatory effect of PDTC, we evaluated BCAT reporter constructs (obtained from G. Nabel, University of Michigan, Ann Arbor, MI) regulated by two (κB/κB) elements [29] or one (κB/Sp1) κB element [28]. The sequences of the oligonucleotides used were: κB/κB, 5′-GATCCGCTGGGGACTTTCCAGGG-GGACTTTCCCTGA-3′ (the κB elements are underlined) and κB/Sp1, 5′-CGCTGGGGACTTCCAGGGAGGCG-TGGCCTGA-3′ (the transcription factor binding sites are underlined. Cells were electroporated with each construct, and 24 h post-electroporation duplicate wells were treated with TPA or with TNF-α alone or after a 1-h pre-incubation with PDTC. CAT protein was determined after 24 h by measuring CAT activity with radiolabelled ^{14}C-chloramphenicol (the CAT ELISA could not be used, because of the lower activity of the mutant constructs). In some experiments, triplicate samples of cells were treated, and changes in CAT activity were evaluated by Student's *t*-test for paired samples. Values of $P < 0.05$ were accepted as significantly different. CAT activity values were expressed as percent conversion of ^{14}C-chloramphenicol into its acetylated derivatives. The amount of TPA-induced CAT activity from each construct was normalised per 200 μg of cellular protein and an 8-h CAT enzyme reaction.

Electroporation and Treatment of U937 Cells

Cells (4×10^7) were harvested from stock cultures, pelleted and washed once in PBS and then once in serum-free RPMI 1640 ($RPMI_0$); the cell pellet was re-suspended in 0.4 mL of $RPMI_0$. The cell suspension (0.4 mL) was then transferred to the electroporation cuvette (Gene Pulser cuvette with 0.4-cm electrode; Bio-Rad, Hercules, CA). Cells were electroporated at 250 μF and 300 V with 10 μg of the indicated plasmid. Ten minutes after electroporation, cells were di-

luted into medium and aliquoted 1 mL per well (8×10^5 cells/mL) into 24-well multiwell plates. Twenty-four hours later, cells were pre-treated with PDTC or NAC (1 mL) for 1 h and then stimulated with TPA or TNF-α for 24 h. Cells were harvested, cell extracts were prepared by three cycles of freeze thawing and protein was determined by the method of Bradford (Bio-Rad). The amount of CAT protein in cellular extracts was determined by use of a CAT ELISA kit (Boehringer Mannheim, Indianapolis, IN) and a MAXline microplate reader (Molecular Devices, Menlo Park, CA). The amount of CAT protein was normalised to 100 μg of protein. In some experiments, the amount of CAT activity was determined with radiolabelled [^{14}C]chloramphenicol [30].

Nuclear Extracts and Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from U937 cells and electrophoretic mobility shift assays (EMSA) carried out as previously described [30]. For competition experiments, the unlabelled competitor oligonucleotides were added to the binding reactions before the addition of radiolabelled oligonucleotide. The oligonucleotides κB, HIV and AP-1 were previously described [41]. For measuring AP-2 DNA-binding activity, a previously described AP-2 oligonucleotide 5′-GATCCGAACTGACCGCCCGCGGCCCCGT-3′ (AP-2 binding site is underlined) was used [26]. For experiments using antibodies, 1 μL of p50, p52, p65, c-Fos, Fra-1, Fra-2, c-Jun, JunB or AP-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the nuclear extract, and a 15-min pre-incubation on ice was carried out before the addition of radiolabelled oligonucleotide.

RESULTS

Effect of PDTC on TNF-α-induced HIV-1 LTR and κB-dependent Gene Expression

A transient expression assay was used to investigate the effect of PDTC pre-treatment on TNF-α-induced HIV-1 LTR- or 4 × κB (containing four κB binding sites)-directed expression of the chloramphenicol acetyltransferase reporter gene with the use of the U937 pro-monocytic cell line. U937 cells were electroporated with the pHIVCAT plasmid, 24 h after electroporation cultures had been pre-treated with PDTC for 1 h, then various concentrations of TNF-α were added and CAT protein was determined after an additional 24-h incubation. Increasing concentrations of TNF-α induced HIV-1 LTR-directed CAT protein, which reached a maximum of >40-fold with ≥1 ng/mL of TNF (Table 1). The TNF-α-mediated increase in CAT protein was further stimulated maximally 2- to 3-fold by ≥50 μM PDTC.

We next evaluated the effect of PDTC on a 4 × κBCAT construct containing four binding sites for NF-κB. The 4 × κB-directed CAT protein was induced >100-fold with ≥1 ng/mL of TNF-α (Table 1). The TNF-α-mediated increase in CAT protein was further stimulated approximately 2- to 4-fold by PDTC. Notably, in contrast with the HIV-1 LTR construct, the stimulatory effect of PDTC with TNF-α on the 4 × κBCAT construct increased in a dose-dependent manner. This finding suggested that the HIV-1 LTR construct was more sensitive to lower concentrations of PDTC.

PDTC was shown to induce AP-1 DNA-binding activity [17–19], and AP-1 was shown to interact with the p65(RelA) subunit of NF-κB [31]. In U937 cells, TNF-α had no effect on AP-1 DNA-binding activity in nuclear extracts after a 1-h treatment by EMSA or AP-1-dependent reporter-gene expression after a 24-h treatment (data not shown). Therefore we evaluated the effect of a 1-h pre-treatment with PDTC on TPA-induced AP-1 DNA-binding activity in nuclear extracts after a 1-h treatment by EMSA. As shown in Figure 1, 50, 100 or 200 μM PDTC alone induced AP-1 DNA-binding activity in U937 cells. Of interest, PDTC in combination with TPA stimulated AP-1 DNA-binding activity more than TPA alone (Fig. 1) and potentiated the effect of TPA on AP-1-dependent (4 × AP-1CAT) reporter-gene expresion (data not shown). Notably, in the absence of TPA, 20 μM PDTC had little effect on AP-1 activity. However, the effect of 20 μM PDTC on AP-1 activity was markedly increased in a manner dependent on the concentration of TPA. Interestingly, as shown in Figure 1, the effect of PDTC on AP-1 activity was dependent on the concentration of TPA and PDTC. These data suggest that AP-1 activity became more sensitive to PDTC with increasing concentrations of TPA, from 0 to 2 to 4 ng/mL.

Effect of PDTC on the TNF-α-induced Signalling Pathway Depends on the Concentration of TPA

We next evaluated the interrelation between the TPA- and TNF-α-induced signalling pathways and the effect of PDTC on HIV-1 LTR activity. U937 cells were electroporated with the pHIVCAT plasmid and pre-treated with 200 μM PDTC for 1 h; increasing concentrations of TPA were added alone or with 10 ng/mL of TNF-α, and CAT protein was determined after a 24-h incubation. Figure 2 shows the following results: (1) PDTC increased the amount of CAT protein approximately 3-fold in the absence of TPA, and TNF-α alone increased the amount of CAT protein 31-fold over the untreated control; (2) TNF-α in combination with increasing concentrations of TPA (<1 ng/mL) resulted in a synergistic response that changed to a more additive response with concentrations of TPA > 1 ng/mL; (3) PDTC stimulated the effect of TPA at concentrations <1 ng/mL and inhibited it at >1 ng/mL (notably, PDTC had no effect on CAT protein with 1 ng/mL of TPA). Thus, the effect of PDTC with TNF-α changed from stimulation to inhibition in a manner that was dependent on the TPA concentration, functionally defining a threshold.

Effect of PDTC and the AP-1 Subunits c-Fos and c-Jun on p65(RelA)-stimulated HIV-1 LTR Activity

An expression vector encoding the p65(RelA) subunit of NF-κB was used to stimulate HIV-1 LTR activity and evaluate the effect of PDTC in the absence of TPA or TNF-α treatment. Initial studies demonstrated that CAT protein

TABLE 1. Effect of PDTC on TNF-α-induced HIV-1 LTR and 4 × κBCAT activity

Treatment[a]		HIV-1CAT activity[b]			4 × κBCAT activity		
PDTC (μM)	TNF-α (ng/mL)	Mean	S.D.	Fold[c]	Mean	S.D.	Fold
0	0.00	0.02	0.01	1.0	0.01	0.01	1.0
	0.05	0.18	0.01	9.0	0.08	0.01	8.0
	0.20	0.39	0.01	19.5			
	0.25				0.39	0.08	39.0
	0.50				0.72	0.03	72.0
	1.00	0.79	0.10	39.5	1.02	0.08	102.0
	2.00	0.89	0.06	44.5			
	10.00	0.90	0.06	45.0	1.26	0.07	126.0
50	0.00	0.05	0.01	2.5	0.02	0.01	2.0
	0.05	0.39	0.08	19.5	0.14	0.02	14.0
	0.20	0.82	0.06	41.0			
	0.25				0.54	0.01	54.0
	0.50				0.74	0.12	74.0
	1.00	1.55	0.25	77.5	0.96	0.03	96.0
	2.00	1.74	0.08	87.0			
	10.00	1.67	0.19	83.5	1.57	0.06	157.0
100	0.00	0.06	0.01	3.0	0.05	0.01	5.0
	0.05	0.51	0.03	25.5	0.24	0.01	24.0
	0.20	0.99	0.10	49.5			
	0.25				0.85	0.02	85.0
	0.50				1.26	0.16	126.0
	1.00	1.66	0.10	83.0	1.36	0.05	136.0
	2.00	1.62	0.09	81.0			
	10.00	1.89	0.16	94.5	1.64	0.09	164.0
200	0.00	0.06	0.01	3.0	0.03	0.01	3.0
	0.05	0.42	0.06	21.0	0.31	0.01	31.0
	0.20	0.84	0.05	42.0			
	0.25				0.98	0.01	98.0
	0.50				1.65	0.02	165.0
	1.00	1.47	0.11	73.5	1.96	0.06	196.0
	2.00	1.49	0.09	74.5			
	10.00	1.43	0.12	71.5	1.90	0.05	190.0

[a]U937 cells were electroporated with the pHIVCAT or p4 × κBCAT plasmid and duplicate wells were pretreated for 1 h with the indicated concentrations of PDTC and then stimulated for 24 h; CAT protein was determined after a 24-h incubation.

[b]The values represent the mean CAT protein (ng/mL) determined by CAT ELISA and normalized to 100 μg of cellular protein ± the sample standard deviations.

[c]The values represent the fold-induction with the data normalized to the untreated control.

increased in a manner dependent on the amount of the p65(RelA) plasmid electroporated and increased from a mean of 0.19 to 0.52 ng/mL with a 24-h treatment with 200 μM PDTC. To evaluate the potential role of AP-1 activity in mediating the effect of PDTC, U937 cells were electroporated with the pHIVCAT plasmid and expression vectors for the AP-1 subunits c-Fos and c-Jun, individually or together, in combination with an expression vector encoding the p65(RelA) subunit of NF-κB. In addition, some samples were electroporated with a Tat expression vector to stimulate HIV-1 LTR activity through the TAR element [25]. Twenty-four hours after electroporation, cells were treated with control or 200 μM PDTC, and CAT protein was determined after 24 h by CAT ELISA. Because of differences in the basal level of CAT protein between multiple electroporations, the CAT protein values shown in Figure 3 are also shown normalised to the untreated controls. PDTC treatment had a stimulatory effect on the basal (3.2-fold)-, p65(RelA) (1.6-fold)- or Tat (2.4-fold)-induced HIV-1 LTR activity.

Co-transfection of the AP-1 subunit c-Fos or c-Jun reduced the effect of PDTC on the basal level of CAT protein. Co-transfection of c-Fos and c-Jun reduced the stimulatory effect of PDTC observed with p65(RelA), but not Tat-induced HIV-1 LTR activity. These data were consistent with the effect of PDTC mediated through an interaction with p65(RelA). To further evaluate the effect of the AP-1 subunits c-Fos or c-Jun on the stimulatory effect of PDTC on p65-induced HIV-1 LTR activity, similar electroporation studies were carried out, but cells were stimulated with either control, 200 μM PDTC or 0.5 ng/mL of TPA. In addition, some samples were electroporated with an NH_2

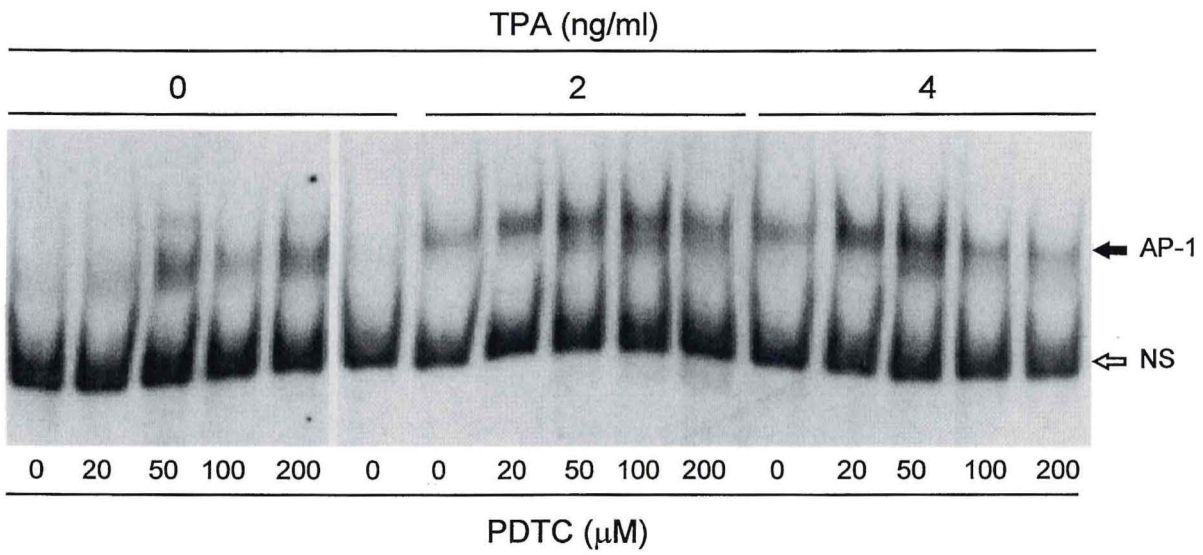

FIGURE 1. PDTC stimulates AP-1 DNA-binding activity and potentiates the effect of TPA. U937 cells were pre-treated for 1 h with the indicated concentrations of PDTC and then stimulated with 0, 2 or 4 ng/mL of TPA for 1 h, and nuclear extracts were prepared. The AP-1 DNA-binding complex was supershifted by the c-Fos antibody (data not shown). Non-specific binding (NS) indicated.

terminally truncated (deletion of amino acids 3–122) dominant negative mutant of c-Jun (Tam67). Tam67 is defective in transactivation, but the intact leucine zipper of Tam67 binds to leucine zippers of Jun and Fos family proteins, thus altering AP-1 complexes. As shown in Figure 4, in agreement with the results shown in Figure 3, the combination of c-Fos and c-Jun had the greatest effect in reducing the stimulatory effect of PDTC on p65(RelA)-mediated HIV-1 LTR activity. To control for differences in the basal level of CAT protein, the CAT protein values shown in Figure 4 are also shown normalised to the untreated controls for each electroporation. Notably, cells co-transfected with the c-Jun mutant Tam67 showed a greater PDTC-mediated stimulation (2.8-fold) of HIV-1 LTR activity than that of the control (1.9-fold), and the effect of Tam67 was reduced by wild-type c-Jun (1.9-fold). These data provide evidence that the Tam67 mutant increased the stimulatory effect of PDTC on p65(RelA)-induced HIV-1 LTR activity. This effect may be mediated by changes in AP-1 complexes. Interestingly, Tam67 stimulated the effect of PDTC (2.8-fold) or 0.5 ng/mL of TPA (4.1-fold) versus 1.9- and 2.2-fold for the control, respectively. Co-transfection of c-Jun with Tam67 inhibited the stimulatory effect of Tam67 with PDTC (1.9-fold) compared with that of the control (1.9-fold). However, the combination of c-Jun with Tam67 had marginal effect with 0.5 ng/mL of TPA (3.8-fold vs. the control of 4.1-fold).

Effect of Tam67 on TPA-induced AP-1 or HIV-1-Dependent Reporter-gene Expression and Effect of PDTC

The preceding results were obtained from studies comparing multiple electroporations and, because of obvious limitations, need to be interpreted with caution. Therefore, to further evaluate the effect of Tam67 and the effect of PDTC on HIV-1 LTR activity, U937 cells were electroporated with the pHIVCAT plasmid with or without Tam67 and pre-treated with 200 μM PDTC for 1 h; increasing concentrations of TPA were added and CAT protein was deter-

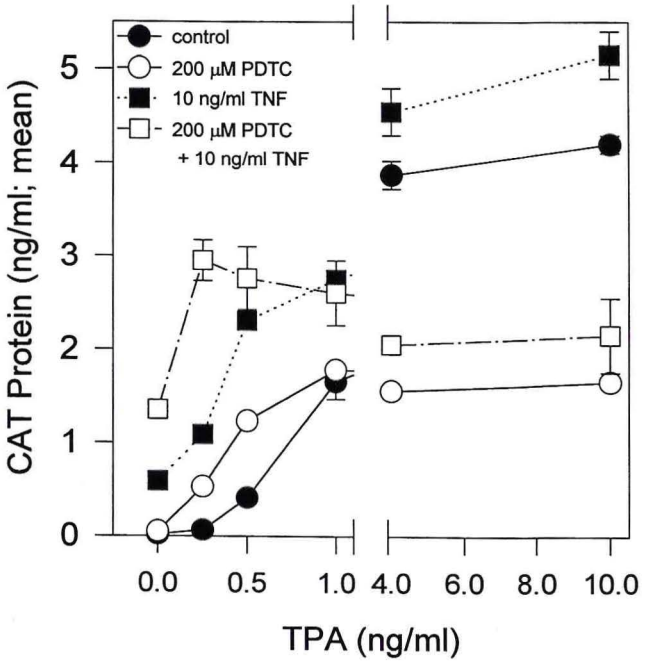

FIGURE 2. The effect of PDTC on the TNF-α-induced signalling pathway is dependent on the concentration of TPA. U937 cells were electroporated with the pHIVCAT plasmid and pre-treated with 200 μM PDTC for 1 h; increasing concentrations of TPA were added alone or with 10 ng/mL of TNF-α, and CAT protein was determined after a 24-h incubation. The results shown represent the mean CAT protein value (normalised to 100 μg of cellular protein) ± the sample deviation of duplicate cultures.

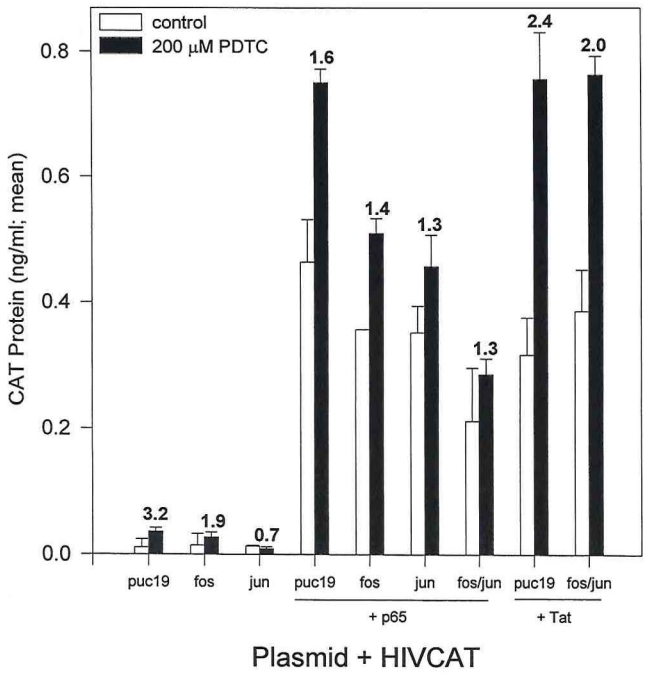

FIGURE 3. Effect of the AP-1 subunits c-Fos and c-Jun and PDTC on basal and p65(RelA)- or Tat-stimulated HIV-1 LTR activity. Cells were electroporated with 5 μg of the indicated plasmids (same amount of DNA was added using puc19), and cells were aliquoted into 4 wells of 24-multiwell dishes. Twenty-four hours post-electroporation, duplicate wells were treated with medium or 200 μM PDTC, and CAT protein was determined after 24 h. The results are from a representative experiment and represent the mean CAT protein value (normalised to 100 μg of cellular protein) ± the sample deviation of duplicate cultures. The fold-stimulation resulting from PDTC treatment is shown.

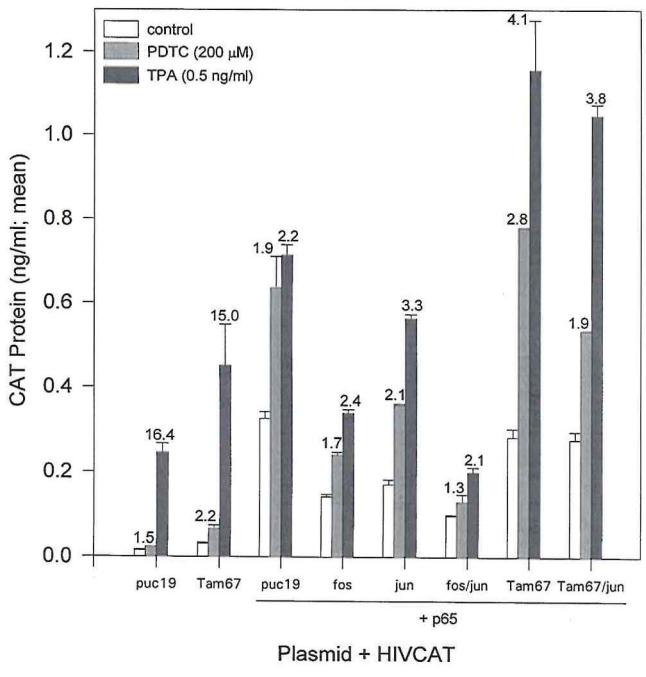

FIGURE 4. Effect of c-Fos, c-Jun or Tam67 on PDTC- or TPA-induced HIV-1 LTR activity. Cells were electroporated as described in Figure 3 but aliquoted into 8 wells, and duplicate wells were treated with medium, 200 μM PDTC or 0.5 ng/mL of TPA. The results are from a representative experiment and represent the mean CAT protein value (normalised to 100 μg of cellular protein) ± the sample deviation of duplicate cultures. The fold-stimulation resulting from PDTC or TPA treatment is shown over the respective bar. Notably, the combination of c-Fos and c-Jun produced a marked decrease in the effect of PDTC, a result consistent with the results shown in Figure 3, and Tam67 potentiated the effect of PDTC or TPA. However, co-expression of c-Jun with Tam67 reduced the stimulatory effect of Tam67 with PDTC but not with TPA.

mined after a 24-h incubation. An identical study was done with an AP-1-dependent (4 × AP-1CAT) reporter gene to define the effect of Tam67 on the AP-1 signalling pathway. As show in Figure 5A, Tam67 markedly stimulated the effect of TPA on HIV-1 LTR-dependent CAT protein. Notably, Tam67 also markedly stimulated the effect of PDTC. Moreover, and most significant, in the absence of Tam67, the effect of PDTC was inhibitory with TPA; but, with Tam67, PDTC stimulated with 1 ng/mL of TPA. The sample with 10 ng/mL of TPA with PDTC treatment showed a marked inhibition of CAT protein that was also inhibited with Tam67. Thus, these data suggest that Tam67 potentiated the effect of PDTC and shifted the functional threshold that was previously shown to be dependent on the TPA concentration. Tam67 also inhibited the effect of TPA on an AP-1-dependent (4 × AP-1CAT) reporter gene (Fig. 5B). Notably, the inhibitory effect of Tam67 was reduced in a manner dependent on the TPA concentration. Of interest, the stimulatory effect of PDTC on the basal level of AP-1-dependent gene expression was only slightly reduced by Tam67. In contrast, Tam67 increased the effect of PDTC on TPA-induced CAT protein. In addition, the results shown in Figure 5B also demonstrate that 10 ng/mL of TNF-α had no effect on AP-1-dependent CAT protein. Moreover, the effects of PDTC and Tam67 on unstimulated or TNF-α-treated cells were essentially identical. These data, taken together, demonstrate that Tam67 provided a means to strengthen the hypothesis that PDTC induces a change in the transcriptional activity of the HIV-1 LTR. Further, the findings suggest that PDTC altered the AP-1 composition of the transcription factor complex of p65(RelA) at the NF-κB DNA-binding site. Of note, Tam67 was previously shown to stimulate the activity of a κB-dependent luciferase reporter gene when stimulated with TPA [32].

An Intact AP-2 Binding Site between the Two κB Elements of the HIV-1 LTR Is Required for the Stimulatory Effect of PDTC with TNF-α

HIV-1 LTR constructs with site-specific mutations in the κB or AP-2 sites [26] were used to evaluate the effect of PDTC. The AP-2 site located between the two κB elements (Fig. 6) was shown to be important in the transcriptional activity of the HIV-1 LTR in Jurkat T cells [26]. Disruption of the AP-2 site prevented AP-2 protein binding, without

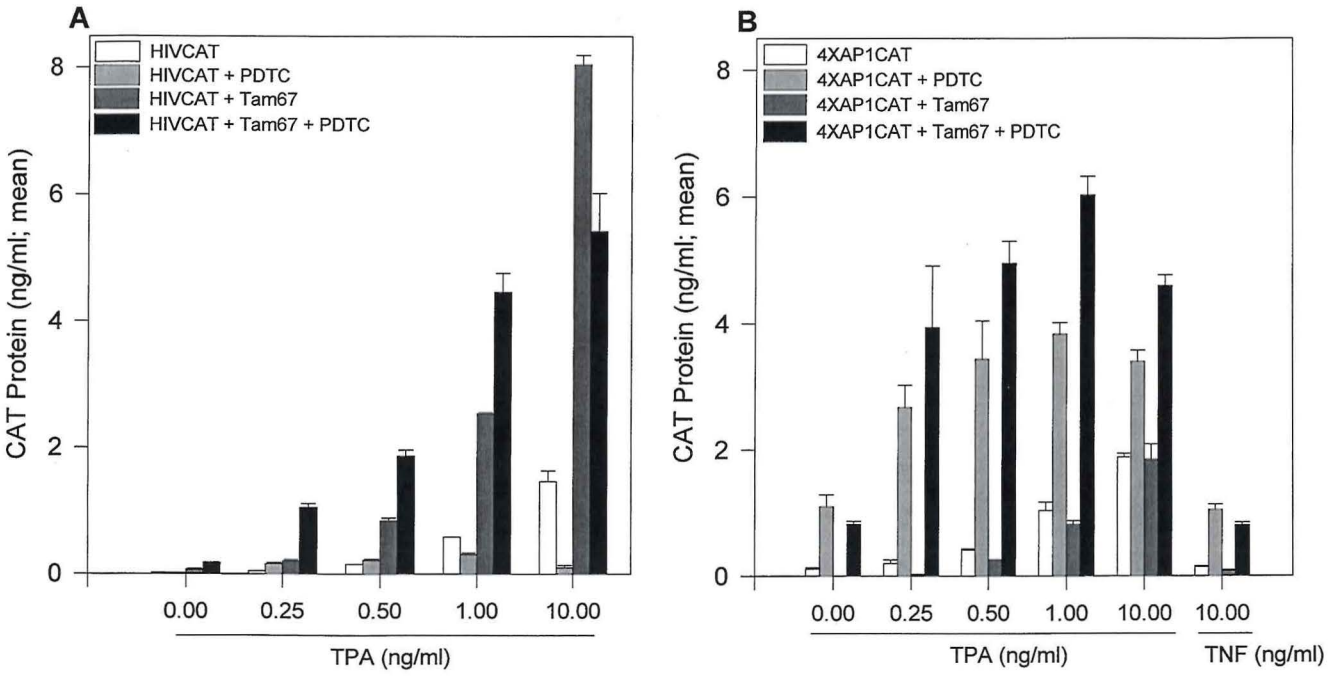

FIGURE 5. (A) Tam67 potentiates the effect of PDTC and prevents the inhibitory effect of PDTC on TPA-induced HIV-1 LTR activity. (B) PDTC stimulates and Tam67 inhibits the effect of TPA, but Tam67 potentiates the effect of PDTC on AP-1-dependent reporter-gene expression. Cells were electroporated with the (A) pHIVCAT or (B) 4 × AP-1CAT plasmid and Tam67, pre-treated 24 h later with 200 μM PDTC for 1 h and treated with the indicated concentrations of TPA or with 10 ng/mL of TNF-α; CAT protein was determined after a 24-h incubation. The results are from a representative experiment and represent the mean CAT protein value (normalised to 100 μg of cellular protein) ± the sample deviation of duplicate cultures. Treatment with TNF-α had no significant effect on AP-1 DNA-binding activity (data not shown) or AP-1-dependent reporter-gene activity.

affecting NF-κB binding or responsiveness to TPA, TNF-α or cotransfected NF-κB subunits [26]. Cells were electroporated with each construct, and 24-h pos-telectroporation triplicate wells were treated with 0, 0.25 or 10 ng/mL of TNF-α, alone or after a 1-h pre-incubation with PDTC (0, 50, 100 or 200 μM). CAT enzyme was determined after 24 h by measuring CAT activity with radiolabelled [^{14}C]chloramphenicol.

Compared with the wild type, the HIV-1 LTR construct containing mutations in both of the NF-κB binding sites was unresponsive to TNF-α or PDTC (data not shown). A construct with only the 3′ NF-κB-binding site mutated (mut-3′κB) remained responsive to TNF-α and the stimulatory effect of PDTC with TNF-α (Fig. 6 and Table 2). However, a construct with a mutation of the 3′ NF-κB-binding site and 5′ Sp1-binding site remained responsive to TNF-α but showed a marginal response to PDTC with TNF-α (Fig. 6 and Table 2). Notably, a construct with a mutation of the AP-2 site located between the two κB elements remained responsive to TNF-α but was unresponsive to the stimulatory effect of PDTC with TNF-α (Fig. 6 and Table 2).

To determine the minimal κB sequence elements required for the stimulatory effect of PDTC, we evaluated BCAT constructs (see the Materials and Methods section) regulated by two (κB/κB) or one (κB/Sp1) κB element. Only the κB/κB construct was responsive to TNF-α and the stimulatory effect of PDTC (Fig. 6; data not shown for the κB/Sp1 construct). Notably, an AP-2 element (CCN_3GG) is located between the κB elements of the κB/κB construct. The role of the AP-2 protein in the effect of PDTC is unclear because we failed to detect any AP-2 DNA-binding activity in U937 cells. This failure suggests that the AP-2 protein may not be the important requirement for the effect of PDTC (Miller, unpublished results). Thus, our overall data showed that the substitution of ATAT for the wild-type sequence in the spacer region between the two κB elements in the HIV-1 LTR had little effect on the response to TPA or TNF-α but decreased the stimulatory effect of PDTC with TNF-α. Evidence that a mutation of the AP-2 binding site in the HIV-1 LTR is associated with the loss of the PDTC-mediated potentiation of TNF-α-induced HIV-1 LTR activity is consistent with the possibility that PDTC induced AP-1 activity that participated in a transcription factor complex with p65(RelA).

No Effect of PDTC on TNF-α-induced NF-κB DNA-binding Activity

We characterised NF-κB DNA-binding activity to the κB (single site) versus the HIV (two κB sites) probe in nuclear extracts prepared from cells pre-treated for 1 h with 200 μM PDTC or treated for 1 h with 10 ng/mL of TNF-α. The NF-κB complex was shown to contain the NF-κB subunits p50 (NF-κB$_1$) and p65(RelA). As shown in Figure 7, the κB-specific binding complex was completely supershifted

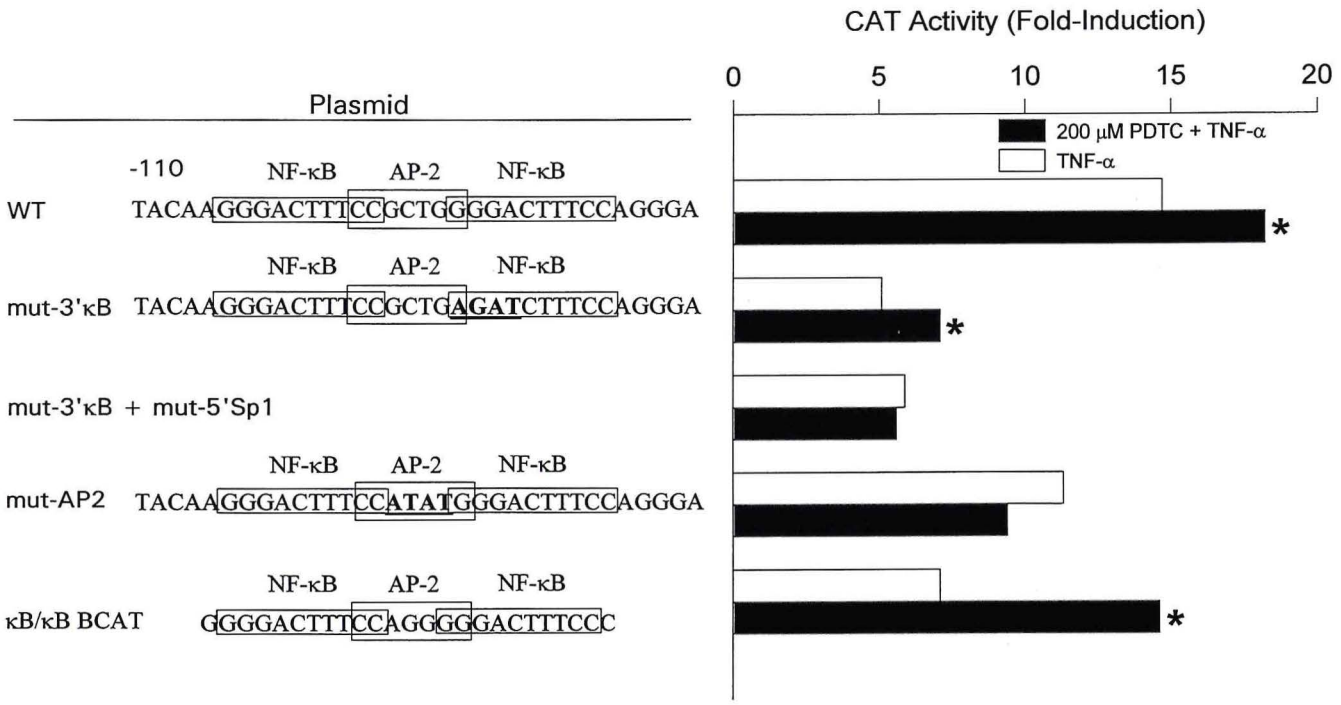

FIGURE 6. An AP-2 binding site located between the two κB elements is required for the stimulatory effect of PDTC with TNF-α. The AP-2 site defined as CCN_3GG (CCGCTGG sequence framed by box) located between the two κB elements (κB sites shown in boxed regions) was shown to be important in the transcriptional activity of the HIV-1 LTR in Jurkat T cells [26]. Cells were electroporated with 10 μg of the indicated construct, and 24-h post-electroporation duplicate wells were treated with 10 ng/mL of TNF-α, alone or after a 1-h pre-incubation with PDTC (200 μM). CAT protein was determined after 24 h by measuring CAT activity with radiolabelled [^{14}C]chloramphenicol. For each construct, the percent conversion of chloramphenicol into the acetylated form in the treated cells was divided by the percent conversion in untreated cells to obtain the values for fold-induction. The HIV-1 LTR construct with site-specific mutations (mutations shown in bold and underlined) in the 3′κB site (mut-3′κB) was responsive to TNF-α stimulation and PDTC (*samples that were significantly different at $P < 0.05$). Mutation of the AP-2 site (mut-AP2) resulted in a normal response to TNF-α, but the stimulatory effect of PDTC was reduced compared with the wild-type sequence. Two (κB/κB) elements in a BCAT construct (see the Materials and Methods section) define the minimal κB sequence elements required for the stimulatory effect of PDTC. A κB/Sp1 element was unresponsive to TNF-α or PDTC stimulation (data not shown).

into two bands by incubation with the p65 antibody. A slight smearing was observed with the p50 antibody, but no shift in mobility was detectable with the p52, c-Fos, Fra-1, Fra-2, c-Jun, JunB or AP-2 antibodies (data not shown). The EMSA studies showed no difference between NF-κB activity binding to the single κB probe versus the HIV probe. As shown in Figure 7, PDTC pre-treatment had no detectable effect on the non-specific binding complex or on the induction of NF-κB by TNF-α in nuclear extracts prepared from cells treated with 10 ng/mL of TNF-α alone or with PDTC at various times up to 25 h after treatment (data not shown).

The concentration of dI/dC used in the EMSA binding reaction was shown to be important in the detection of p65 homodimers [33]. As shown in Figure 8, a 10-fold decrease in the concentration of dI/dC used in the standard EMSA reaction resulted in the detection of two NF-κB binding complexes induced by TNF-α. The TNF-α-induced complexes were weakly decreased by a 1-h pre-treatment with 30 mM NAC but not with 200 μM PDTC. In addition, 200 μM PDTC had no detectable effect on the TNF-α-induced complexes throughout a 25-h incubation (data not shown). Thus, with the use of EMSA, we were unable to demonstrate that PDTC altered the AP-1 composition of the transcription factor complex of p65(RelA) at the NF-κB DNA-binding site.

Effect of PDTC Pre-incubation Time on TNF-α-induced HIV-1 LTR Activity

To further define the conditions required for the stimulatory effect of PDTC with TNF-α on HIV-1 LTR activity, we evaluated the effect of increasing the pre-incubation time with PDTC (200 μM) for 1, 4 or 17 h (0 h defined as 24 h post-electroporation). Samples treated with PDTC (200 μM) for 1 h in the absence of TNF-α showed an increase in CAT protein from a mean of 0.01 in the untreated control to 0.03 ng/mL. CAT protein further increased to 0.10 ng/mL with the 17-h PDTC pre-incubation. As shown in Figure 9, increasing the PDTC pre-incubation time to 17 h before the addition of TNF-α markedly stimulated the effect of 10 ng/mL of TNF-α by as much as 5-fold. In contrast, pre-treatment for 1 to 17 h with the antioxidant NAC only inhibited, in a concentration-dependent manner, the in-

TABLE 2. Effect of PDTC on TNF-α-induced CAT activity of wild-type and mutant HIV-1 LTR constructs

Treatment[b]		CAT activity[a]											
		HIV-1(wt)[c]			mut-3′κB			mut-3′κB + 5′Sp1			mut-AP-2		
PDTC (μM)	TNF-α (ng/mL)	Mean	S.D.	P[d]	Mean	S.D.	P	Mean	S.D.	P	Mean	S.D.	P
0	0.00	5.17	1.33		18.39	1.12		2.44	0.81		1.26	0.10	
0	0.25	42.45	2.47		43.20	8.22		5.12	0.77		7.65	0.29	
50		60.89	5.89		73.58	8.31		7.34	0.28	0.038	7.17	2.22	
100		54.24	6.19	0.036	87.48	17.91		9.82	3.13		7.16	0.77	
200		60.00	7.94		85.43	6.13	0.001	7.62	1.53		8.96	0.89	
0	10.0	75.84	7.72		94.24	13.01		14.33	0.72		14.18	0.95	
200		94.20	0.93	0.049	130.98	16.07	0.025	13.78	0.27		11.79	2.22	

[a]CAT activity values represent the mean ± the S.D. from triplicate samples. The percent conversion of [^{14}C]chloramphenicol into its acetylated derivatives was normalized to 200 μg of cellular protein and an 8-h CAT enzyme reaction.

[b]U937 cells were electroporated with 10 μg of the indicated plasmid, and triplicate wells were pre-treated for 1 h with the indicated concentrations of PDTC, then stimulated with various concentrations of TNF-α for 24-h and CAT activity was determined.

[c]HIV-1 CAT with wild-type κB elements (5′-GGGACTTTCCGCTGGGGACTTTCC-3′ (κB elements underlined) or mut-3′κB (GGGACTTTCCGC-TAGATCTTTTCC) or mut-3′κB + 5′ Sp1 (GGGACTTTCCGCTAGATCTTTTCC; the 5′ Sp1 site (underlined) was changed from GAGGCGTGGCC to GAACTCGAGCC) or mut-AP-2 (GGGACTTTCCATATGGGACTTTCC); boldface type identifies the mutated sites.

[d]Statistical analysis by Student's *t*-test for paired samples. Values of $P < 0.05$ are shown.

duction of HIV-1 LTR-directed CAT protein by TNF-α (data not shown). Therefore, it is unlikely that the antioxidant activity of NAC or PDTC is responsible for the potentiation of TNF-α-induced signalling.

DISCUSSION

As shown here, the U937 model system provides a cellular environment in which the TPA and TNF-α signalling pathways regulating NF-κB function are parallel pathways with selective sites of regulation by PDTC. It should be emphasised that the results described here were generated with U937 cells obtained directly from the ATCC. For these studies, these cells were expanded, and frozen stocks were prepared. Cells were thawed, grown and used for experiments for approximately 1 month before new cultures were initiated from frozen stocks. In these cells, AP-1 DNA binding and reporter-gene activity were induced by TPA or PDTC but not by TNF-α treatment. Notably, these cells are completely resistant to TNF-α-induced apoptosis, in contrast with U937 cells from other sources (Miller, unpublished results). Our efforts to understand the molecular mechanisms responsible for the resistance to TNF-α-induced apoptosis led us to the unexpected finding that PDTC po-

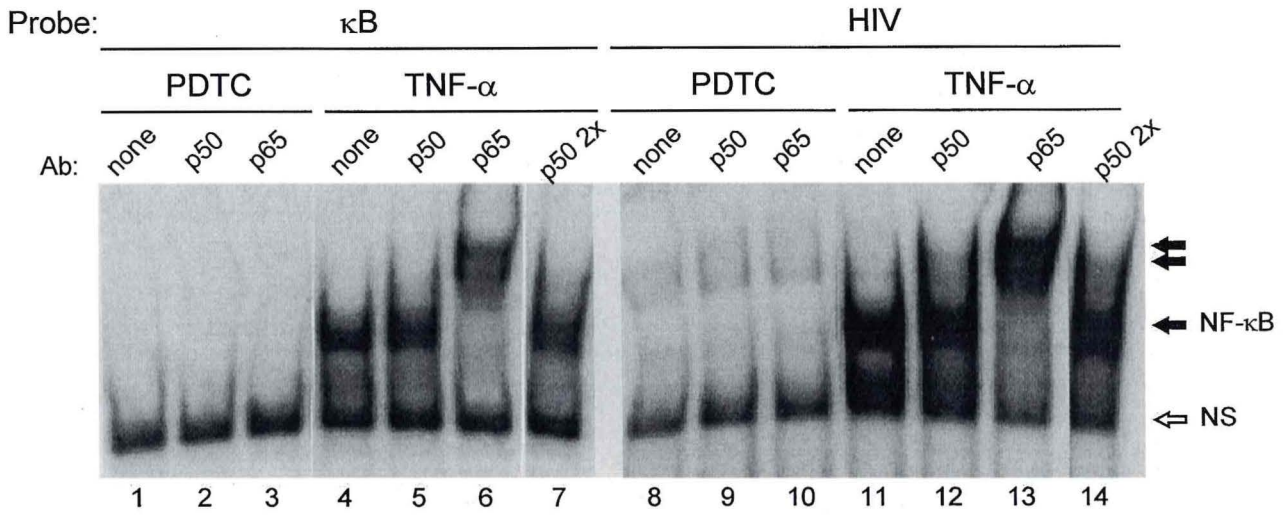

FIGURE 7. Characterisation of NF-κB binding activity in U937 cells induced by TNF-α to κB or HIV probe containing two κB binding sites. Cells were pre-treated for 1 h with 200 μM PDTC and treated for 1 h with 10 ng/mL of TNF-α, and nuclear extracts were prepared. PDTC treatment had no effect on NF-κB activity or on TNF-α-induced NF-κB mobility detected by antibodies (Ab). The TNF-α-induced NF-κB DNA-binding complex (solid arrow) was supershifted weakly by the p50 (lane 5) and strongly by the p65 (lane 6 for κB and lane 13 for HIV probe) antibodies (double arrows indicate DNA-binding activity with mobility shift by p65). Doubling the amount of p50 antibody (p50 2×) increased the detection of the p50 protein with the κB probe (lane 7). However, the supershift of the p50 antibody was markedly increased with the HIV probe (lanes 12 and 14). There was no detectable effect of the antibodies on the upper non-specific band shown with the HIV probe (lanes 8–10) or with any of the AP-1 subunit specific antibodies tested (see the Materials and Methods section; data not shown).

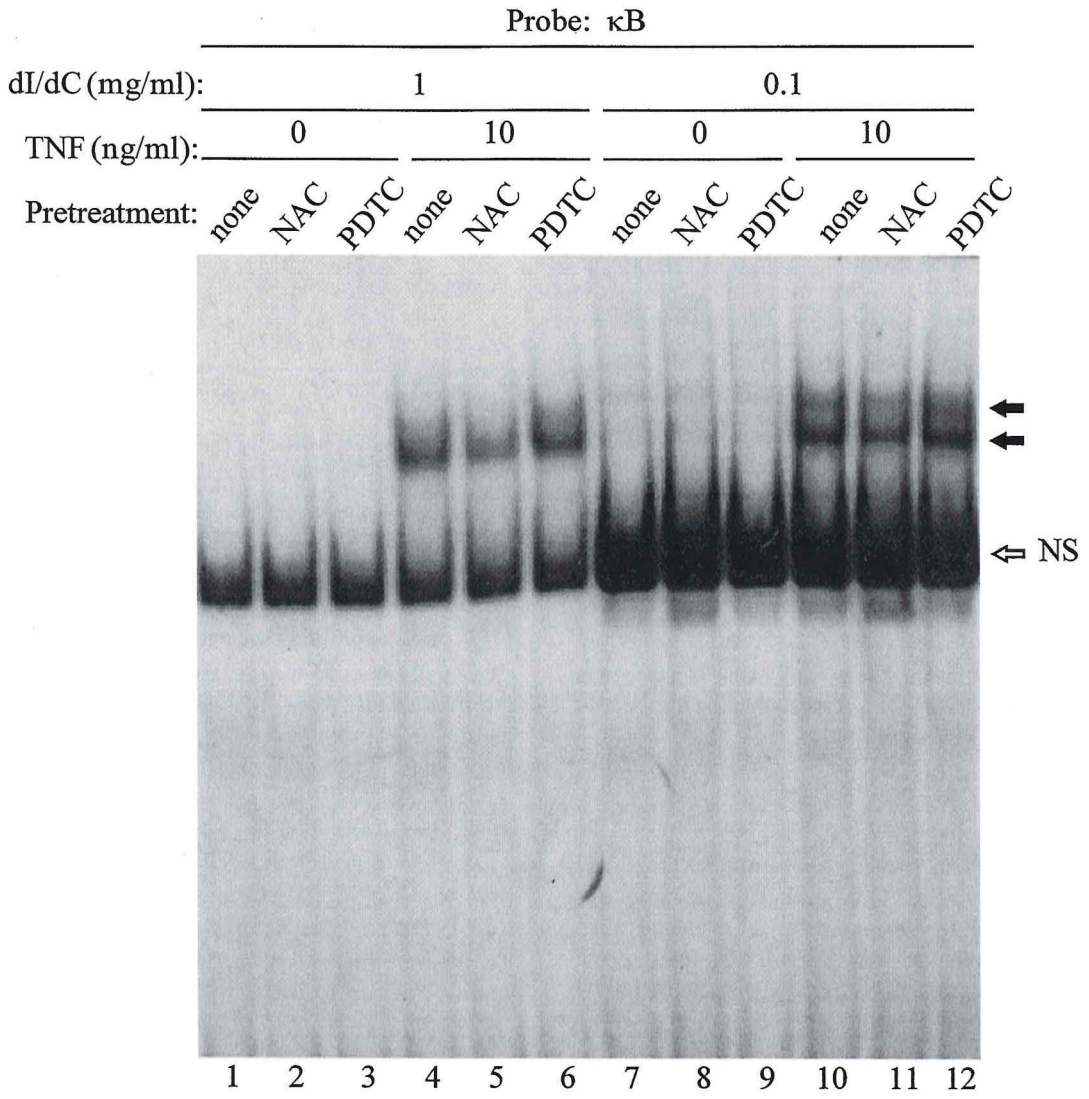

FIGURE 8. Effect of the antioxidant NAC versus PDTC on TNF-α-induced NF-κB DNA-binding activity. The concentration of dI/dC used in the EMSA binding reaction was shown to be important in the detection of p65 homodimers [33]. Increasing the time of electrophoresis and using our standard dI/dC (lanes 1–6) concentration resulted in the detection of two NF-κB binding complexes induced by TNF-α (lanes 4–6). The solid arrows indicate the NF-κB DNA-binding activity that was supershifted by the p65 antibody (data not shown). A 10-fold decrease in the concentration of dI/dC (lanes 7–12) resulted in an increased detection of two NF-κB binding complexes induced by TNF-α (lanes 10–12). Both TNF-α-induced complexes were weakly sensitive to pre-treatment with 30 mM NAC but not 200 μM PDTC.

tentiated the effect of TNF-α on the NF-κB signalling pathway.

Four lines of evidence are consistent with the hypothesis that the stimulatory effect of PDTC on κB-dependent gene expression is mediated through a PDTC-induced activity interacting with the NF-κB signalling pathway induced by TNF-α. First, PDTC potentiated the effect of TNF-α on the HIV-1 LTR-, 4 × κB- and κB/κB-directed CAT reporter constructs. Second, the stimulatory effect of PDTC in the absence or presence of TPA or TNF-α was not observed with an HIV-1 LTR reporter construct containing two mutated κB-binding sites. However, a construct with a single mutant 3′κB site was stimulated by PDTC with TNF-α. Notably, a construct with a mutant 3′κB and mutant 5′Sp1 site or a construct with a disruption of the AP-2 site located between the two κB elements resulted in HIV-1 LTR activity that remained responsive to TPA and TNF-α but reduced the stimulatory effect of PDTC with TNF-α. Evidence that the stimulatory effect of PDTC was dependent on an intact AP-2 site is consistent with the hypothesis that this intact site is required for a critical functional protein–protein interaction between NF-κB subunit p65(RelA) and unknown partners. Third, PDTC inhibited the activation of NF-κB DNA-binding activity by TPA but had little effect on the activation of NF-κB by TNF-α. Thus, two distinct signalling pathways, one mediated by TPA and the other by TNF-α, were shown to stimulate HIV-1 LTR activity in a manner consistent with the hypothesis that the

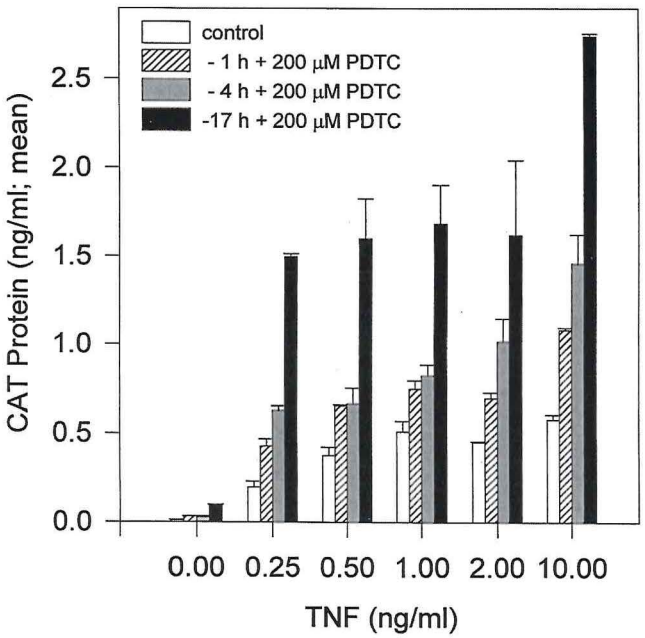

FIGURE 9. Effect of PDTC pre-incubation time on TNF-α-stimulated HIV-1 LTR activity. U937 cells were electroporated and treated as previously described, but the PDTC pre-incubation time was varied for 1, 4 or 17 h before TNF-α was added at 0 h (defined as 24 h post-electroporation) for a 24-h incubation. The results shown are from a representative experiment and represent the mean CAT protein value (normalised to 100 μg of cellular protein) ± the sample deviation of duplicate cultures.

concentration of TPA defines a threshold sensitive to regulation by PDTC. TPA-induced signals below this threshold were stimulated by PDTC. In contrast, TPA-induced signals above this threshold were inhibited by PDTC. Fourth, the p65(RelA) expression vector provided a means to activate HIV-1 LTR activity and evaluate the effect of PDTC without stimulating cells with TPA or TNF-α. Co-transfection of the expression vectors for the AP-1 subunits c-Fos and c-Jun resulted in a decrease in the stimulatory effect of PDTC on p65(RelA)-induced HIV-1 LTR activity. Of interest, Tam67 stimulated the effect of PDTC, an effect that was reduced by c-Jun.

The effect of PDTC in potentiating TNF-α-induced κB-dependent gene expression was unexpected. The experiments presented here and in a study by Watanabe *et al.* [41] define the conditions associated with demonstrating this effect of PDTC and show evidence for a threshold indicative of the activity of a TPA-induced signalling pathway. It is of interest that Tam67 increased the effect of PDTC on the basal level of HIV-1 LTR activity but that Tam67 had little effect on the basal level of AP-1-dependent reporter-gene activity. As expected for a mutant defective in transactivation, Tam67 inhibited the effect of TPA on the AP-1 reporter gene. Notably, increasing the concentration of TPA overcame the inhibitory effect of Tam67 on the TPA pathway. In contrast, Tam67 increased the effect of PDTC on the TPA-induced pathway. One interpretation of these data is that the results show evidence for an interaction of Tam67 with AP-1 complexes induced through the TPA pathway but for poor interaction with AP-1 complexes resulting from a PDTC-induced pathway. Tam67 has been shown to bind specifically to leucine zippers (bZip domains) of endogenous Jun and Fos family of proteins resulting in low activity AP-1 complexes. It remains to be seen whether the bZip domain of Tam67 or of p65(RelA) are important for the effect observed with PDTC. Of note, Tam67 was previously shown to stimulate the activity of a κB-dependent luciferase reporter gene when stimulated with TPA [32]. More studies are needed to address the mechanism responsible.

Although the nature of the threshold was not addressed in this study, it is significant that Tam67 affected the threshold, defined functionally by the ability of PDTC to make the transition from a stimulatory to an inhibitory effect on the TPA-induced HIV-1 LTR activity. We have evidence that this threshold correlated functionally with a Ca^{2+}-dependent co-signal required for PDTC to selectively inhibit IκB-α degradation and activation of NF-κB by the TPA but not the TNF-α-induced signalling pathway (Kazakova *et al.*, in preparation).

Our data are consistent with the hypothesis that an AP-1 family member may be responsible for the stimulatory effect of PDTC on TNF-α-induced κB-dependent gene expression. Other investigators showed that AP-1 DNA-binding activity and AP-1-dependent transcriptional activity were stimulated by PDTC [17–19]. This effect was independent of PKC activation, involved newly synthesised AP-1 components c-Jun and c-Fos [17, 18] and may be due to an oxidant effect of PDTC [16]. Interestingly, diethyldithiocarbamate (DDTC) was shown to stimulate the synthesis of TNF-α [34, 35]. Our results raise the possibility that this effect of DDTC on TNF-α gene expression may be mediated through NF-κB.

PDTC has been shown to stimulate the transcription factor AP-1 through the JNK pathway [21]. The combined treatment of PDTC with TPA resulted in the persistent phosphorylation of extracellular signal-regulated kinase type 2. In this context, the results presented here strongly suggest that the ultimate effect of PDTC is dependent on a TPA-induced pathway. We speculate that the strength of signals in the regulation of a PKC-dependent activity may be critical. Interestingly, in a JB6 mouse tumour-promotion-sensitive cell line, both NF-κB- and AP-1-dependent reporter-gene expression were inhibited by PDTC [22]. In this model system, TPA or TNF-α induced both NF-κB- and AP-1-dependent reporter-gene expression and cellular transformation, and both were inhibited by PDTC. Of interest, Tam67 also inhibited both NF-κB- and AP-1-dependent reporter-gene expression and cellular transformation. In contrast, in U937 cells, we have shown that Tam67 stimulated TPA-induced NF-κB-dependent reporter-gene expression and that Tam67 inhibited AP-1-dependent reporter-

gene expression with concentrations of TPA ≤ 1 ngmL; Tam67 had little effect with 10 ng/mL of TPA.

An important role of AP-1 in mediating the effect of PDTC on the regulation of the NF-κB signalling pathway is supported by data showing evidence of protein–protein interactions between AP-1 and NF-κB family members [31]. Functional protein–protein interactions with the HIV-1 LTR was shown for NF-κB family members [24] and Sp1 [28, 29], p53 [36], AP-2 [26], and C/EBP [37].

Perhaps one of the most unexpected findings of this study was obtained with the HIV-1 LTR construct with a mutation of the AP-2 site located between the two κB elements. In agreement with previous work [26], the mutant AP-2 construct remained responsive to TPA and TNF-α but, as described here, showed a decreased response to the stimulatory effect of PDTC with TNF-α. Interestingly, a construct with a mutant 3′κB element remained responsive to PDTC but a construct with a mutant 3′κB element and a mutant 5′Sp1 site was not. The minimal sequence element required for the response to PDTC was found to consist of an element composed of an AP-2 site between two κB sites (the κB/κB BCAT construct). It will be of interest to define the role of the AP-2 site and the effect of PDTC further. For example, mutational analysis of the AP-2 site in the κB/κB BCAT construct is needed to serve as a source of important insight into the assembly of the p65(RelA) transcription complex. Recent work showed that the cAMP response element binding protein (CREB), a CREB-binding protein and p300 are co-activators of the NF-κB signalling pathway through interactions with p65(RelA) [38]. PDTC may stimulate NF-κB activity through increasing the phosphorylation and transcriptional activity of p65(RelA) [39]. Other investigators showed that the combined treatment of PDTC with TPA resulted in the persistent phosphorylation of extracellular signal-regulated kinase type 2 and stimulated the transcription factor AP-1 through the JNK pathway [21]. This finding raises the possibility that PDTC may be acting through the CREB pathway, because the interaction between the transcriptional activator and the co-activator is phosphorylation dependent for CREB. Alternatively, a change in the redox state of the cell may be important, as shown for AP-1 [40]. In this context, in the U937 cell model, PDTC is a potent inhibitor of the generation of intracellular reactive oxygen intermediates (ROIs), and PDTC treatment results in a rapid increase in intracellular glutathione levels (Miller, unpublished results).

In summary, an understanding of the molecular mechanisms responsible for the stimulatory effect of PDTC with p65(RelA) may help to define additional regulatory pathways controlling signal integration important in the NF-κB signalling pathway.

We would like to thank the laboratories of M. Birrer, G. Nabel and P. Berg for plasmids. This research was partly supported by National Institutes of Health Grant No. GM48229 to S. C. M.

References

1. Liu Z. G., Hsu H. L., Goeddel D. V. and Karin M. (1996) *Cell* **87**(3), 565–576.
2. Wang C. Y., Mayo M. W. and Baldwin A. S., Jr. (1996) *Science* **274**, 784–787.
3. Beg A. A. and Baltimore D. (1996) *Science* **274**, 782–784.
4. Van Antwerp D. J., Martin S. J., Kafri T., Green D. R. and Verma I. M. (1996) *Science* **274**, 787–789.
5. Song H. Y., Regnier C. H., Kirschning C. J., Goeddel D. V. and Rothe M. (1997) *Proc. Natl. Acad. Sci. USA* **94**(18), 9792–9796.
6. Karin M. and Delhase M. (1998) *Proc. Natl. Acad. Sci. USA* **95**(16), 9067–9069.
7. Hirano M., Osada S., Aoki T., Hirai S., Hosaka M., Inoue J. and Ohno S. (1996) *J. Biol. Chem.* **271**(22), 13234–13238.
8. Yin M. J., Christerson L. B., Yamamoto Y., Kwak Y. T., Xu S., Mercurio F., Barbosa M., Cobb M. H. and Gaynor R. B. (1998) *Cell* **93**(5), 875–884.
9. Lee F. S., Hagler J., Chen Z. J. and Maniatis T. (1997) *Cell* **88**(2), 213–222.
10. Lee F. S., Peters R. T., Dang L. C. and Maniatis T. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 9319–9324.
11. Baldwin A. S., Jr. (1996) *Annu. Rev. Immunol.* **14**, 649–683.
12. Daniel L. W., Civoli F., Rogers M. A., Smitherman P. K., Raju P. A. and Roederer M. (1995) *Cancer Res.* **55**, 4844–4849.
13. Regnier C. H., Song H. Y., Gao X., Goeddel D. V., Cao Z. and Rothe M. (1997) *Cell* **90**(2), 373–383.
14. Schouten G. J., Vertegaal A. C., Whiteside S. T., Israel A., Toebes M., Dorsman J. C., van der Eb A. J. and Zantema A. (1997) *EMBO J.* **16**, 3133–3144.
15. Schreck R., Meier B., Mannel D. N., Droge W. and Baeuerle P. A. (1992) *J. Exp. Med.* **175**, 1181–1194.
16. Pinkus R., Weiner L. M. and Daniel V. (1996) *J. Biol. Chem.* **271**, 13422–13429.
17. Meyer M., Schreck R. and Baeuerle P. A. (1993) *EMBO J.* **12**, 2005–2015.
18. Meyer M., Pahl H. L. and Baeuerle P. A. (1994) *Chem. Biol. Interact.* **91**(2–3), 91–100.
19. Schulze-Osthoff K., Los M. and Baeuerle P. A. (1995) *Biochem. Pharmacol.* **50**, 735–741.
20. Nobel C. I., KimLand M., Lind B., Orrenius S. and Slater A. F. (1995) *J. Biol. Chem.* **270**, 26202–26208.
21. Gomez del Arco P., Martinez-Martinez S., Calvo V., Armesilla A. L. and Redondo J. M. (1996) *J. Biol. Chem.* **271**, 26335–26340.
22. Li J. J., Westergaard C., Ghosh P. and Colburn N. H. (1997) *Cancer Res.* **57**, 3569–3576.
23. Brown P. H., Chen T. K. and Birrer M. J. (1994) *Oncogene* **9**, 791–799.
24. Schmid R. M., Perkins N. D., Duckett C. S., Andrews P. C. and Nabel G. J. (1991) *Nature* **352**, 733–736.
25. Feng S. and Holland E. C. (1988) *Nature* **334**, 165–167.
26. Perkins N. D., Agranoff A. B., Duckett C. S. and Nabel G. J. (1994) *J. Virol.* **68**, 6820–6823.
27. Nabel G. and Baltimore D. (1987) *Nature* **326**, 711–713.
28. Perkins N. D., Edwards N. L., Duckett C. S., Agranoff A. B. Schmid R. M. and Nabel G. J. (1993) *EMBO J.* **12**, 3551–3558.
29. Perkins N. D., Agranoff A. B., Pascal E. and Nabel G. J. (1994) *Mol. Cell. Biol.* **14**, 6570–6583.
30. Miller S. C., Taylor A., Watanabe K., Mok K. and Torti F. M. (1997) *Exp. Cell Res.* **230**, 9–21.
31. Stein B., Baldwin A. S., Jr., Ballard D. W., Greene W. C., Angel P. and Herrlich P. (1993) *EMBO J.* **12**, 3879–3891.
32. Petrak D., Memon S. A., Birrer M. J., Ashwell J. D. and Zacharchuk C. M. (1994) *J. Immunol.* **153**, 2046–2051.

33. Ganchi P. A., Sun S. C., Greene W. C. and Ballard D. W. (1993) *Mol. Cell. Biol.* **13**, 7826–7835.
34. East C. J., Abboud C. N. and Borch R. F. (1992) *Blood* **80**, 1172–1177.
35. Schmalbach T. K., Datta R., Kufe D. W. and Sherman M. L. (1992) *Biochem. Pharmacol.* **44**, 365–371.
36. Gualberto A., Hixon M. L., Finco T. S., Perkins N. D., Nabel G. J. and Baldwin A. S., Jr. (1995) *Mol. Cell. Biol.* **15**, 3450–3459.
37. Ruocco M. R., Chen X., Ambrosino C., Dragonetti E., Liu W., Mallardo M., De Falco G., Palmieri C., Franzoso G., Quinto I., Venuta S. and Scala G. (1996) *J. Biol. Chem.* **271**, 22479–22486.
38. Gerritsen M. E., Williams A. J., Neish A. S., Moore S., Shi Y. and Collins T. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 2927–2932.
39. Schmitz M. L., dos Santos Silva M. A. and Baeuerle P. A. (1995) *J. Biol. Chem.* **270**, 15576–15584.
40. Hirota K., Matsui M., Iwata S., Nishiyama A., Mori K. and Yodoi J. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 3633–3638.
41. Watanabe K., Kazakova I., Furniss M. and Miller S. C. (1999) *Cell Signal.* **11**, In press.