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Intracellular thiols regulate activation of nuclear factor κB and transcription of human immunodeficiency virus

(transcription factor/glutathione/signal transduction/AIDS/N-acetyl-L-cysteine)

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ABSTRACT The activation of nuclear factor κB (NF-κB) has been implicated in the regulation of transcription of a variety of genes and has been shown to be essential for the expression of genes controlled by the long terminal repeat of human immunodeficiency virus (HIV LTR). We show here that intracellular thiol levels play a key role in regulating this process. That is, stimulation with tumor necrosis factor α and/or phorbol 12-myristate 13-acetate activates NF-κB and markedly decreases intracellular thiols; N-acetyl-L-cysteine, an efficient thiol source, prevents this thiol decrease and blocks the activation of NF-κB; and the lack of activated NF-κB prevents the activation of the HIV LTR and the transcription of genes under its control. These findings reveal a previously unrecognized genetic regulatory mechanism in which cytokine-induced shifts in intracellular thiol levels are crucial in the control of NF-κB activity and thereby influence the spectrum of genes expressed by cytokine-stimulated cells.

Fauci and colleagues (1) have demonstrated that tumor necrosis factor α (TNF-α) stimulates human immunodeficiency virus (HIV) transcription via activation of the transcription factor nuclear factor κB (NF-κB). We have shown (2) that this stimulation is inhibited by N-acetyl-L-cysteine (NAC), a nontoxic drug that enters cells readily and serves both as a scavenger for reactive oxidative intermediates (ROI) and as a precursor for glutathione (γ-glutamylcysteinylglycine, GSH), the major intracellular thiol and ROI scavenger present in all eukaryotic forms of life (3–5).

GSH is a cofactor for many glycolytic enzymes (6) and for enzymes involved in amino acid catabolism and conversion (7). It plays a key role in the accurate formation of protein disulfide bonds. In addition, by maintaining the redox potential within cells, it protects against oxidative damage that would otherwise be caused by free radicals and ROI produced during cell metabolism or as the result of drug overdose (e.g., acetaminophen) (8). NAC, which replenishes the intracellular cysteine required to produce GSH, also reacts directly with ROI and thereby provides further protection against cell damage caused by conditions that induce oxidative stress.

In our previous studies, we showed that NAC blocks the TNF-α- or phorbol 12-myristate 13-acetate (PMA)-stimulated HIV expression (as measured by the production of the viral glycoprotein p24) in normal peripheral blood lymphocytes and T lymphocyte cell lines that are acutely infected with HIV *in vitro* (2). In addition, we showed that NAC blocks HIV long terminal repeat (LTR)-directed gene expression in an HIV model system, which we also use in studies presented here. In this system, NAC blocks the cytokine-stimulated β-galactosidase expression by blocking transcription of a *lacZ* gene that is stably transfected under the control of the HIV promoter and enhancer sequences. Since NF-κB has been shown to play a key role in HIV-controlled transcription, these findings suggest that NAC regulates HIV expression by regulating the supply of activated NF-κB.

NF-κB is present in the cytoplasm of unstimulated cells in a complex with inhibitor of NF-κB (IκB) (9, 10). When the cells are stimulated with phorbol esters or with cytokines such as TNF-α and interleukin 1, IκB is phosphorylated through the action of protein kinase C or other kinases (acting directly or indirectly) (11). This phosphorylation dissociates NF-κB from IκB and allows NF-κB to migrate to the nucleus, where it activates its target genes.

In this publication, we show that intracellular thiols play a key role in regulating NF-κB activation, in that low thiol levels are required for activation and high thiol levels inhibit activation. Thus we introduce a previously unrecognized mechanism for regulation of a transcription factor and, through that mechanism, a method for regulating the replication and expression of HIV.

MATERIAL AND METHODS

Cell Lines and Cell Culture. The 293.27.2 cell line contains a construct (pNAZ) consisting of the HIV LTR fused to bacterial β-galactosidase gene as described previously (2). Construction of the Jurkat tri-κB gene fusion (3κB 5.2) will be described elsewhere (P. S. Matilla, G. R. Crabtree, and L.A.H., personal communication). The 293 cells were grown in Dulbecco's modified Eagle's medium (GIBCO), supplemented with 5% (vol/vol) fetal calf serum (FCS) and 5% horse serum, L-glutamine at 290 mg/ml, penicillin at 100 units/ml, and streptomycin at 70 mg/ml and kept under G418 (1 mg/ml) selection. The Jurkat cells were grown in complete RPMI-1640 medium (GIBCO), supplemented with 10% FCS penicillin at 100 units/ml and streptomycin at 70 mg/ml.

Stimulation of Cells. Cells were plated out in individual wells of 96- or 24-well Falcon plates at a density of 2000 cells per well in 0.2 ml (96-well plate) or 10,000 cells per well in 1.0 ml (24-well plate). The next day PMA (Sigma), TNF-α (recombinant human TNF-α, Cetus), and NAC (Aldrich) were added to the desired concentrations from stock solutions. Incubations were for 6 or 8 hr in an atmosphere of 5% CO_2 in air humidified at 37°C. In experiments with diamide [diazenedicarboxylic acid bis(N,N-dimethylamide), Calbiochem, 1 M stock solution in Dulbecco's phosphate-buffered saline (PBS)], medium was aspirated prior to stimulation and fresh, prewarmed medium with diamide or with the same volume of PBS (negative control) was added. After 10 min,

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Abbreviations: EMSA, electrophoretic mobility shift assay; FACS, fluorescence-activated cell sorter; GSH, glutathione; HIV, human immunodeficiency virus; IκB, inhibitor of nuclear factor κB; LTR, long terminal repeat; MCB, monochlorobimane; MUG, 4-methylumbelliferyl β-D-galactoside; NAC, N-acetyl-L-cysteine; NF-κB, nuclear factor κB; PMA, phorbol 12-myristate 13-acetate; ROI, reactive oxidative intermediates; TNF-α, tumor necrosis factor α.

the diamide was washed away and cells were stimulated with TNF-α.

Measurement of β-Galactosidase. The fluorescence-activated cell sorter (FACS) and 4-methylumbelliferyl β-D-galactoside (MUG) assays of β-galactosidase were carried out as described (2, 12).

Measurement of GSH Concentration with Monochlorobimane (MCB). FACS measurements of GSH and internal thiols were performed by slight modification of methods described (13). Cells (1×10^6 per ml) were stained at room temperature for 15 min with MCB (Molecular Probes) at a final concentration of 40 μM. The reaction was stopped by addition of 3 ml of ice-cold staining medium and centrifuging through 0.5 ml of underlaid serum to remove unreacted MCB. Samples were washed and analyzed by FACS (Facstar Plus, Becton Dickinson). In Fig. 2, data (means of duplicates) are plotted from the scaled means of MCB fluorescence.

Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA). Nuclear protein extracts were made by two methods. First, extracts were made as described previously (14). Results with this standard method (not shown) were equivalent to results presented here, which were obtained by using a method adapted from Schaffner and colleagues (15), which allows extracts to be made from 10^6 cells. The conditions were optimized for our cells as described below. Cells from the appropriate stimulations (PMA, TNF-α, NAC) were harvested and centrifuged (10 min, 1200 rpm, 4°C), washed in 1 ml of ice-cold Tris-buffered saline (TBS), and spun for 15 sec at 14,000 rpm, 4°C, in an Eppendorf Brinkman-5412 centrifuge. All subsequent steps were done in the cold room and on ice. Cells were pelleted and washed once in 0.4 ml of buffer A (10 mM Hepes, pH 7.8/10 mM KCl/2 mM $MgCl_2$/1 mM dithiothreitol/0.1 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride), supplemented with the protease inhibitors antipain (1 mg/ml, Sigma) and leupeptin (0.3 mg/ml, Sigma) and incubated on ice for 15 min. Then 25 μl of a 10% Nonidet P-40 solution (Sigma) was added, and cells were vigorously mixed for 15 sec and then centrifuged (30 sec, 14,000 rpm). Pelleted nuclei were resuspended in 50 μl of buffer C [50 mM Hepes, pH 7.8/50 mM KCl/300 mM NaCl/0.1 mM EDTA/1 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride/10% (vol/vol) glycerol]. Following gentle mixing for 20 min, the tubes were spun for 5 min at 14,000 rpm, after which the supernatant containing the nuclear proteins was transferred to another tube. Protein samples were stored at −70°C. EMSAs were done essentially as described (16, 17). The binding reactions were carried out with 10 μg of protein for NF-κB and 5 μg for the Oct-1 transcription factor. Oligonucleotides used in these studies are monomers of the binding site for NF-κB (GGGACTTTCC) or Oct-1 (NFII-2A, nucleotides −93 to −63 from human interleukin-2 promoter).

RESULTS

NAC Inhibits HIV LTR Expression Stimulated by PMA and TNF-α. Using a reporter cell system in which the HIV LTR directs expression of β-galactosidase, the clone 293.27.2, we previously showed that TNF-α and PMA activate HIV transcription (2). This activation results in the production of *Escherichia coli* β-galactosidase, which can be detected in individual viable cells by the FACS assay (12) or in cell lysates by the biochemical (MUG) assay used in our previous studies. Fig. 1 shows FACS β-galactosidase analyses after 6-hr stimulations with TNF-α, PMA, or both. TNF-α and PMA both give good activation of HIV LTR-dependent expression in this cell line. Synergy between PMA and TNF-α can be noted as a stronger β-galactosidase activation than by optimal levels of either alone. These activations, measured either by the MUG assay (2) or by the FACS assay (Fig. 1), are effectively blocked by NAC.

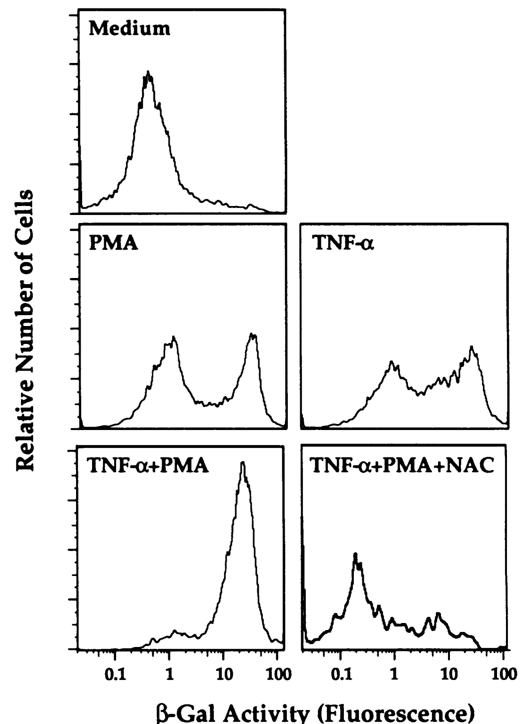

FIG. 1. Activation of HIV LTR by TNF-α and PMA can be inhibited by NAC. FACS β-galactosidase plots of 293.27.2 cells [in which HIV LTR drives the transcription of the β-galactosidase reporter gene (*lacZ*)] after a 6-hr stimulation period. (*Top Left*) Basal activity of the clone, without stimulators. (*Middle Right*) Stimulation of HIV LTR with TNF-α (10 ng/ml). (*Middle Left*) Stimulation with PMA (20 ng/ml). (*Bottom Left*) Synergistic stimulation by TNF-α (10 ng/ml) and PMA (20 ng/ml). (*Bottom Right*) Inhibition of TNF-α- and PMA-induced stimulation by 20 mM NAC, with NAC, PMA, and TNF-α added simultaneously. NAC slightly reduced basal activity of the clone and effectively inhibited the PMA activation (>95%); the TNF-α stimulation was inhibited ≈70% by this concentration of NAC (data not shown, but see ref. 2).

In this system, as in the gel retardation analyses presented below, NAC inhibits stimulation with PMA considerably more effectively than it inhibits stimulation with TNF-α. A possible explanation is that TNF-α stimulates *lacZ* expression via two separate pathways, one of which is NAC-insensitive; the other is NAC-sensitive and also used by PMA. This model is consistent with the observed synergy of activation between PMA and TNF-α (Fig. 1 *Bottom Left*) and with recent evidence demonstrating that TNF-α and PMA synergize in the stimulation of HIV transcription (2, 18) and NF-κB activation (18). Alternatively, the TNF-α pathway may simply be less sensitive to thiols than is the PMA pathway.

TNF-α and PMA Decrease Intracellular Thiols; NAC Blocks This Decrease. To measure intracellular thiol levels, we use a FACS assay in which we stain cells with MCB, a membrane-permeant non-fluorescent substrate that becomes fluorescent after reaction with sulfhydryl groups (13). Inside cells, it is almost exclusively coupled to GSH by glutathione-*S*-transferase.

Stimulating cells with TNF-α, PMA, or both results in an immediate decrease in intracellular thiols (Fig. 2); however, the stimulated cells regain their normal intracellular thiol levels after 4 hr (data not shown). Thus cytokine stimulation apparently induces rapid, short-term production of oxidants which transiently deplete GSH in the stimulated cells.

Intracellular thiol levels do not decrease, however, when cells are stimulated with cytokines in the presence of NAC. In fact, thiol levels (detected by MCB staining) in these cells

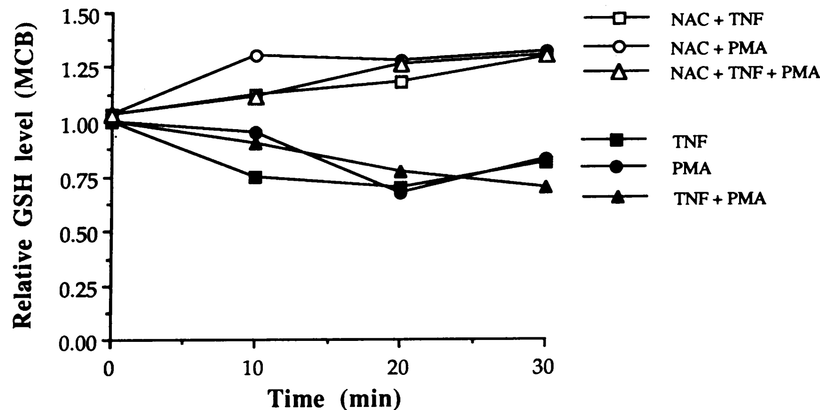

FIG. 2. Decrease in internal thiol concentration after TNF-α and PMA stimulation is prevented by NAC. 293.27.2 cells were stimulated in six-well trays with TNF-α (10 ng/ml), PMA (20 ng/ml), or both in the absence or presence of 20 mM NAC. Measurements were done in duplicate. Cells were harvested and stained with MCB. Relative thiol (mainly GSH) levels were determined from the scaled mean of MCB fluorescence. Incubations were for 0, 10, 20, and 30 min. The internal thiol level without stimulation is given as 1.0.

rise to substantially above normal (Fig. 2) and remain elevated for 2–3 hr (data not shown). Thiol levels in unstimulated cells cultured in NAC for the same length of time do not rise, indicating that the rise is due to a cytokine-stimulated increase in the rate of GSH synthesis and not to reactivity of MCB with NAC.

Activation of HIV LTR by TNF-α Is Greatly Enhanced in Cells with Lowered GSH. The stimulation of HIV expression by cytokines is greatly enhanced when cells are preincubated with diamide, which rapidly consumes intracellular GSH by nonenzymatically converting it to the oxidized dimer GSSG (19). Culturing cells (293.27.2) for 10 min in the presence of 3 mM diamide decreases intracellular thiols by 50%. This treatment does not affect viability, and the intracellular GSH levels return to normal within 45–60 min when the diamide is washed away (data not shown).

Preincubation with diamide greatly enhances the stimulation of HIV expression by TNF-α, which gives an 8-fold increase in β-galactosidase activity. This enhancement increases roughly linearly with increasing concentrations of diamide, which result in progressively lower intracellular GSH levels (Fig. 3). Because diamide reacts with GSH in a 1:2 stoichiometry, the activation of the HIV LTR is inversely related to the intracellular GSH concentration.

Thus the low GSH and/or high intracellular oxidant (ROI) levels induced by certain cytokines and diamide facilitate activation, while the high GSH and/or low ROI induced or maintained by the addition of NAC inhibit activation. Taken together, these findings demonstrate that the intracellular levels of GSH and/or ROI regulate the extent to which the HIV LTR is activated and genes under its control are expressed.

Tri-κB-Directed *lacZ* Expression Is Activated by TNF-α and PMA; NAC Inhibits This Activation. The HIV LTR contains two well-defined NF-κB sites that are essential for the induction of HIV expression (20). TNF-α and PMA induce HIV expression at least partly through the activation of NF-κB. To study the role of thiols in NF-κB activation, we used a construct composed of three κB sites linked to bacterial *lacZ* stably introduced into Jurkat cells. The tri-κB construct in this clone (3κB 5.2) behaves similarly to the HIV LTR construct in that TNF-α and PMA independently stimulate *lacZ* expression and synergize to give very strong stimulation readily detectable in MUG assays (Fig. 4). NAC at 20 mM was highly effective in inhibiting these stimulations. As with the HIV LTR, the PMA stimulation of the tri-κB construct was blocked more effectively than the TNF-α stimulation. This is consistent with the hypothesis that NF-κB is the major transcription factor in the regulation of HIV LTR-directed transcription (1).

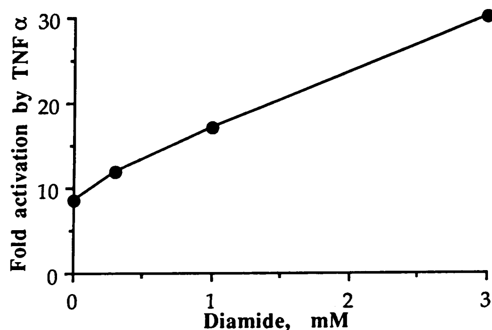

FIG. 3. TNF-α stimulation of HIV LTR is enhanced when GSH concentrations are lowered. 293.27.2 cells were treated with diamide to lower GSH concentrations. After washings, cells were stimulated with TNF-α (10 ng/ml) for 6 hr and stained with fluorescein digalactoside for FACS analysis. Stimulation of HIV LTR is determined by β-galactosidase activity. The basal activity of the cells without stimulation is set as 1.0. Data are plotted as the fold activation over basal activity and were calculated as mean of β-galactosidase activity for 30,000 cells.

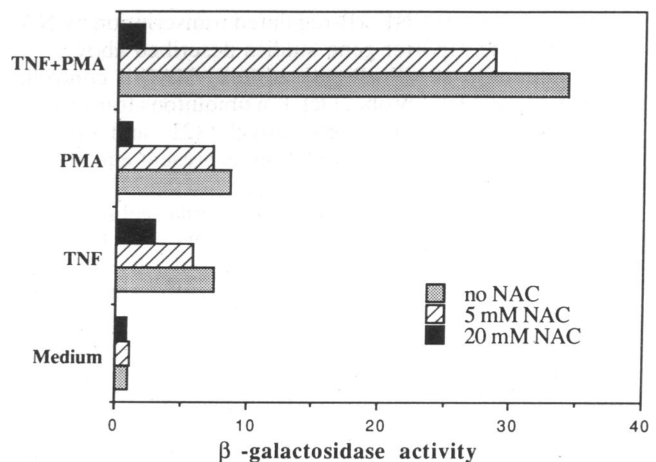

FIG. 4. NAC inhibition of PMA and TNF-α stimulation of Jurkat tri-κB cells. Jurkat tri-κB cells were stimulated in 24-well microtiter plates with TNF-α (10 ng/ml), PMA (20 ng/ml), or both, and with 0, 5, or 20 mM NAC. After 8 hr, β-galactosidase activity was determined with the MUG assay. Relative β-galactosidase activity (mean of duplicate samples) is plotted on the horizontal axis, with the activity without stimulation or NAC added given as 1.0.

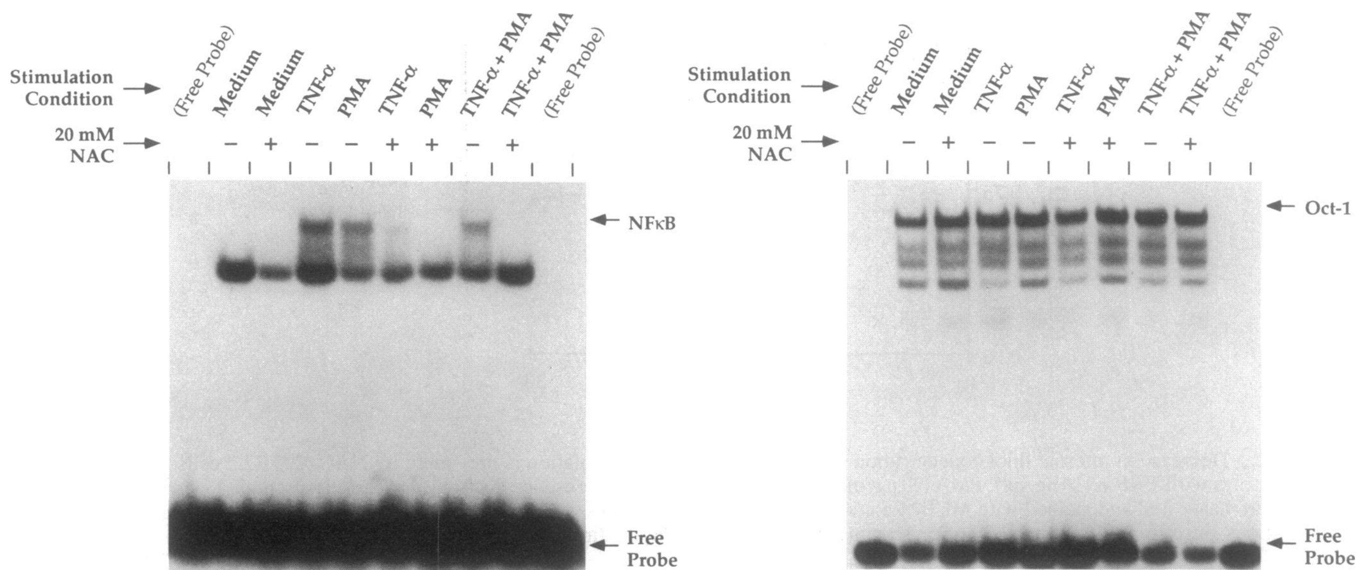

FIG. 5. Inhibition of TNF-α- and PMA-induced NF-κB activity by NAC. 293.27.2 cells were stimulated with TNF-α, PMA, or both together in the presence or absence of NAC (20 mM) for 4 hr. Nuclear protein extracts were made and EMSAs were done with an NF-κB probe (*Left*) or Oct-1 probe (*Right*). The first and last lanes in each gel contain free probe without nuclear protein added. In *Left*, the upper arrow indicates the induced NF-κB band. In *Right*, the upper arrow indicates the position of the Oct-1 band. The lower arrow in both figures indicates the unbound probe.

NAC Selectively Inhibits the Activation of NF-κB. Activated NF-κB is detectable as a uniquely positioned band in EMSA of nuclear extracts from 293 or Jurkat cells stimulated with TNF-α and/or PMA (Fig. 5 *Left*). On the basis of the results from Ghosh and Baltimore (11), we interpret the upper band as the activated form of NF-κB—i.e., the $(p65)_2(p50)_2$ tetramer. The lower band corresponds to the $(p50)_2$ dimer, which provides the DNA-binding domain of NF-κB but is known to be transcriptionally inactive.

Gel retardation analyses of extracts from cells stimulated with TNF-α and/or PMA in the presence of NAC demonstrate that NAC selectively inhibits the activation of NF-κB. The activated NF-κB band $[(p65)_2(p50)_2]$ is only barely detectable in the extracts from NAC-treated cells. In contrast, the $(p50)_2$ dimer band is clearly present and may even be stronger than in extracts from cells in which activated NF-κB is detectable. Since the p50 subunit itself is not transcriptionally active, these results from the EMSA are consistent with the inhibition of NF-κB-regulated transcription by NAC detected in the reporter gene studies described above.

The inhibition of NF-κB activation by NAC is controlled for by using an Oct-1 probe. Oct-1, a ubiquitous transcription factor that is produced constitutively (21) and does not increase in amount upon stimulation, is present equally in all extracts (Fig. 5 *Right*). These findings with the 293 cells were confirmed and extended by data from similar gel retardation and reporter gene expression experiments with the Jurkat human T-cell line (data not shown). In these cells stimulation with TNF-α, PMA, or both resulted in an efficient activation of NF-κB. The combined stimulation was synergistic in Jurkat cells, resulting in a much stronger NF-κB band than found with either agent alone. These stimulations were also effectively blocked by NAC, as is the case with the 293 cells (Fig. 5 *Left*).

There are subtle differences in the extent to which NAC inhibits NF-κB activation, as detected in EMSAs. A faint NF-κB band is visible (Fig. 5 *Left*) in the extracts from cells stimulated with TNF-α in the presence of NAC, whereas no band can be detected in extracts from cells stimulated with PMA or PMA plus TNF-α in the presence of NAC. These differences mirror the known differences in the effectiveness of NAC inhibition of transcription in reporter gene assays. That is, NAC is more effective in inhibiting PMA-stimulated NF-κB activation and less effective against TNF-α.

DISCUSSION

Studies presented here define a previously unsuspected role for intracellular thiols in the control of gene expression. In essence, we have shown that intracellular thiol levels regulate the activation of NF-κB. Since GSH is the most abundant intracellular thiol, these findings suggest that the mechanisms that control GSH levels also regulate those genes whose expression is dependent on the activation of NF-κB and possibly other transcription factors.

We discuss these mechanisms in terms of intracellular thiol levels, since we have directly measured these levels in our studies. However, intracellular thiol levels actually reflect a dynamic balance between the amount of GSH available and the amount of recently generated oxidants in the cells, since oxidants react with (consume) GSH and vice versa. Thus, the data we interpret as demonstrating that intracellular thiols regulate the activation of NF-κB could equally well be interpreted as indicating that NF-κB activation is regulated by intracellular oxidant levels.

Intracellular thiols (or oxidants) could regulate NF-κB activation at one or more points in the signal transduction pathway. For example, high intracellular thiol levels could influence protein folding or enzyme activation (8) and thus block the activation of the protein kinases (e.g., protein kinase C) that phosphorylate the IκB/NF-κB complex and liberate activated NF-κB. Alternatively, high thiols could interfere directly with IκB phosphorylation or with the transport of activated NF-κB into the nucleus. Finally, if intracellular oxidants can directly activate any of the enzymes involved in NF-κB activation, high thiol levels could prevent NF-κB activation simply by scavenging oxidants.

The maintenance of the thiol/oxidant balance appears to be crucial for protection against the toxic effects of overstimulation with TNF-α or other inflammatory cytokines. Lieberman and Baltimore (22) recently suggested that activation of NF-κB might be one of the body's earliest responses to infection and inflammation. This transcription factor is known to control the expression of a number of genes that

code for cytokines and other proteins involved in inflammatory processes. It is present in most cell types as an inactive complex (NF-κB/IκB) that can be activated very rapidly by phosphorylation of the inhibitory IκB subunit. Thus, under normal circumstances, the animal can respond rapidly to an invading pathogen by generating an inflammatory response.

This defensive process is subverted by HIV and other viruses whose replication is regulated by NF-κB (produced by the host). These viruses exploit the host responses to inflammatory agents and tend to replicate whenever inflammation occurs because TNF-α and other inflammatory cytokines which activate NF-κB are produced. Thus, HIV replication would be expected to proceed more rapidly in immunosuppressed individuals [e.g., with AIDS-related complex (ARC) and AIDS] who have infections which induce inflammatory responses. Indeed, TNF-α levels are known to become progressively elevated (23) and GSH levels are known to become progressively lower (23, 24) as the HIV infection develops from its asymptomatic stage to AIDS. Thus, the existing data are consistent with the idea that inflammatory responses in HIV patients shift the thiol/oxidant balance so that NF-κB activation and HIV transcription are favored.

NAC, which we have used here to block NF-κB activation and HIV transcription *in vitro*, is a well-known, nontoxic drug that is commonly used to replenish GSH and other thiols. We have shown elsewhere that NAC blocks HIV replication and HIV-induced syncytia formation and therefore have suggested that this drug may be useful for slowing the progression of AIDS (2). In addition, findings presented here demonstrating that NAC blocks cytokine-stimulated NF-κB activation suggest that this drug might also be effective for dampening overshoot reactions in inflammation that cause tissue injury and for other pathologies where oxidative stress and/or free radical formation plays an important role. Such diseases include acute respiratory distress syndrome (ARDS), toxic and septic shock, porphyria, and cerebral malaria.

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