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Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1

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Hydrogen peroxide and oxygen radicals are agents commonly produced during inflammatory processes. In this study, we show that micromolar concentrations of H_2O_2 can induce the expression and replication of HIV-1 in a human T cell line. The effect is mediated by the NF-κB transcription factor which is potently and rapidly activated by an H_2O_2 treatment of cells from its inactive cytoplasmic form. *N*-acetyl-L-cysteine (NAC), a well characterized antioxidant which counteracts the effects of reactive oxygen intermediates (ROI) in living cells, prevented the activation of NF-κB by H_2O_2. NAC and other thiol compounds also blocked the activation of NF-κB by cycloheximide, double-stranded RNA, calcium ionophore, TNF-α, active phorbol ester, interleukin-1, lipopolysaccharide and lectin. This suggests that diverse agents thought to activate NF-κB by distinct intracellular pathways might all act through a common mechanism involving the synthesis of ROI. ROI appear to serve as messengers mediating directly or indirectly the release of the inhibitory subunit IκB from NF-κB.

Key words: *N*-acetyl-L-cysteine/activation of NF-κB/HIV-1/hydrogen peroxide/NF-κB

Introduction

NF-κB is a multisubunit transcription factor that can rapidly activate the expression of genes involved in inflammatory, immune and acute phase responses (for reviews see Baeuerle and Baltimore, 1991; Baeuerle, 1991). The protein is found in many different cell types and tissues but has been characterized best in cells of the immune system such as pre-B, B and T lymphocytes, macrophages and monocytes. Many target genes of the ubiquitous NF-κB show a tissue- or cell type-specific expression which might come from a synergistic action of NF-κB with cell type-specific factors within enhancers and promoters. Most of the target genes fall into three classes: genes encoding (i) immunomodulatory cytokines such as TNF-α, IL-6, β-interferon and GM-CSF, (ii) immunoregulatory cell surface receptors such as MHC class I antigens, non-polymorphic subunits of MHC genes and the IL-2 cytokine receptor, and (iii) acute phase proteins such as serum amyloid A precursor and angiotensinogen.

In most cells, NF-κB is present in a non-DNA-binding form in the cytoplasm (Baeuerle and Baltimore, 1988a,b). This complex is composed of three subunits: a DNA-binding 48-55 kd protein (p50) (Kawakami *et al.*, 1988; Kieran *et al.*, 1990; Ghosh *et al.*, 1990), a DNA-binding 65-68 kd protein (p65) (Baeuerle and Baltimore, 1989; Ruben *et al.*, 1991; Urban *et al.*, 1991) and a third inhibitory subunit, called IκB, which is bound to p65 (Baeuerle and Baltimore, 1988b; Urban and Baeuerle, 1990; Urban *et al.*, 1991). IκB inhibits DNA-binding of NF-κB and appears to be responsible for the cytoplasmic localization of the complex (Baeuerle and Baltimore, 1988b). It is apparently the release of IκB which triggers the activation of the NF-κB transcription factor.

Recent molecular cloning of the p50 (Bours *et al.*, 1990; Ghosh *et al.*, 1990; Kieran *et al.*, 1990) and p65 subunits (Ruben *et al.*, 1990) revealed that their DNA-binding/dimerization domains share high homology with that of the *rel* proto-oncogene proteins. There is at least one more c-rel/NF-κB-like protein with a molecular size of 75 kd (p75) (Ballard *et al.*, 1990). p50 can associate with c-rel and p75 after a combined renaturation *in vitro* but it is unclear yet whether this can occur *in vivo* as well.

A characteristic of NF-κB is that many different agents can induce its DNA-binding activity (reviewed in Baeuerle and Baltimore, 1991; Baeuerle, 1991). Among them are viruses (which can activate NF-κB either by the action of viral transactivator proteins or double-stranded RNA intermediates), bacterial lipopolysaccharide, protein synthesis inhibitors, the cytokines TNF-α, TNF-β and interleukin-1, and T cell mitogens, such as phorbol 12-myristate 13-acetate (PMA), lectins, calcium ionophores and antibodies directed against T cell receptors. Very little is known of how such diverse agents can all cause the same reaction, i.e., the release of IκB from p50-p65 in the cytoplasm.

Some progress was recently made in understanding the activation of NF-κB by PMA, an activator of protein kinase C (PKC). The reaction of PKC with cytoplasm (Shirakawa and Mizel, 1989) or partially purified NF-κB-IκB complex (Ghosh and Baltimore, 1990) under kinasing conditions caused an activation of the DNA-binding of NF-κB. Furthermore, treatment of purified IκB with PKC inactivated the inhibitor and the incorporation of radioactive phosphate into a protein of the molecular size of IκB was observed (Ghosh and Baltimore, 1990). This suggested that a direct phosphorylation of IκB through PKC released the inhibitor and thereby activated NF-κB. On the other hand, activation of NF-κB by TNF-α appears to be independent of PKC (Meichle *et al.*, 1990). Although TNF induces a rapid and transient activation of PKC (Schütze *et al.*, 1990), depletion of the kinase by chronic PMA treatment and the use of PKC inhibitors did not affect NF-κB activation by TNF. Also the NF-κB activation by protein synthesis inhibitors (Sen and Baltimore, 1986) and double-stranded RNA (Visvanathan and Goodbourn, 1989; Lenardo *et al.*, 1989) are unlikely to be mediated by PKC.

Very recently, Roederer *et al.* (1990) reported that *N*-acetyl-L-cysteine (NAC) is a potent inhibitor of the PMA-

and TNF-α-induced activation of the HIV-1 LTR. While our study was in progress, a second study from the Herzenberg laboratory (Staal *et al.*, 1990) showed that NAC blocked specifically the activation of NF-κB. The authors suggested that the intracellular glutathione (GSH) level, which is increased by NAC, is an important regulator of the activity of NF-κB. Because GSH levels control the concentration of ROI within cells via the GSH peroxidase (for review, see Halliwell and Gutteridge, 1990), we were prompted to test the possibility that oxygen radicals are involved in the activation of NF-κB in the cytoplasm. We used H_2O_2 as a membrane-permeable reagent which allows studies of the effects of oxygen radicals in living cells. Moreover, H_2O_2 is physiologically produced in large amounts by granulocytes and macrophages during inflammatory processes and is implicated, together with oxygen radicals, in many pathological situations (for reviews see Cerutti, 1985; Blake *et al.*, 1987; Halliwell and Gutteridge, 1989, 1990).

In this study, we report on the activation of NF-κB by treatment of Jurkat T cells with micromolar amounts of hydrogen peroxide. The same treatment could also potently transactivate the enhancer/promoter in the HIV-1 LTR depending on intact binding sites for NF-κB, and increase the production of new HIV-1 virus in latently infected T cells. The antioxidant and radical scavenger NAC inhibited the activation of NF-κB by H_2O_2, strongly supporting the idea that oxygen radicals were involved in the activation process. We also found that the activation of NF-κB by cycloheximide, double-stranded RNA, interleukin-1 (IL-1), bacterial lipopolysaccharide (LPS), calcium ionophore, lectin and, as reported earlier (Staal *et al.*, 1990), by TNF-α and PMA, was strongly inhibited by NAC. Also other thiol compounds blocked the activation of NF-κB and the effect was not restricted to T cells. These findings could provide a unifying concept of how many different agents can induce the DNA-binding of the cytoplasmic form of NF-κB: ROI, which can transiently increase in cells by different mechanisms, could serve as messengers that directly or indirectly cause the release of IκB from the p50–p65–IκB complex.

Results

Treatment of T cells with H_2O_2 activates NF-κB

Jurkat T cells are widely used in the study of T cell activation processes and provide a model system for studying the induction of HIV-1 gene expression in latently infected cells (Nabel and Baltimore, 1987; Osborn *et al.*, 1989). The human T lymphoma cell line responds to treatments with the active phorbol ester PMA, lectins and TNF with the activation of NF-κB and NF-κB-controlled genes. Here we have tested hydrogen peroxide, an agent produced during inflammatory processes (for review see Halliwell and Gutteridge, 1989), for its capability to activate NF-κB. Because H_2O_2 can permeate the plasma membrane and can be converted intracellularly into more reactive oxygen intermediates, it allows investigation of the effects of H_2O_2 and of oxygen radicals in living cells.

Jurkat T cells were incubated in the presence of 150 μM H_2O_2 (Figure 1A). After various times, aliquots of the cell culture were harvested and cells fractionated into cytosol and nuclei. Nuclear salt extracts and cytosol were then prepared and analyzed for the specific DNA-binding of NF-κB using

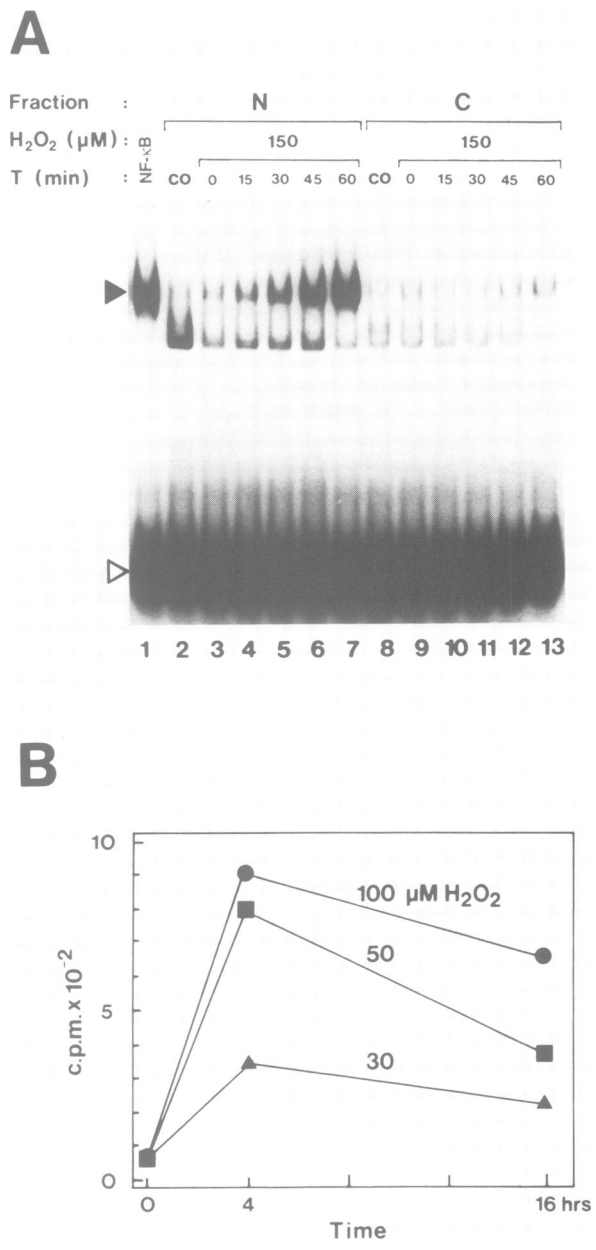

Fig. 1. The effect of an H_2O_2 treatment on DNA-binding activities in Jurkat T cells. (**A**) Rapid induction of a κ enhancer-binding protein by treatment of T cells with H_2O_2. Jurkat T cells were left untreated (Co, lanes 2 and 8) or incubated for various times with 150 μM H_2O_2. Nuclear extracts (lanes 2–7) and cytosolic fractions (lanes 8–13) were prepared and equal proportions (2–8 μg of protein) reacted with a ^{32}P-labeled DNA probe encompassing the κB motif of the mouse κ light chain enhancer (Sen and Baltimore, 1986). A protein–DNA complex with purified NF-κB composed of p50 and p65 subunits (Baeuerle and Baltimore, 1989) was electrophoresed in lane 1. Samples were analyzed on a native 4% polyacrylamide gel. A fluorogram of the gel is shown. The filled arrowhead indicates the position of a NF-κB–DNA complex and the open arrowhead the position of unbound DNA. (**B**) Dose dependence and the effect of long-term incubation by H_2O_2. Jurkat T cells were incubated for 4 and 16 h with either 30, 50 or 100 μM H_2O_2. The radioactivity in the induced protein–DNA complex co-migrating with that of NF-κB was determined by Cerenkov counting and the numbers corrected for the same amount of protein.

electrophoretic mobility shift assays (EMSAs). As shown in Figure 1A (lanes 2–7), H_2O_2 rapidly activated an activity that retarded in native gels a ^{32}P-labeled DNA

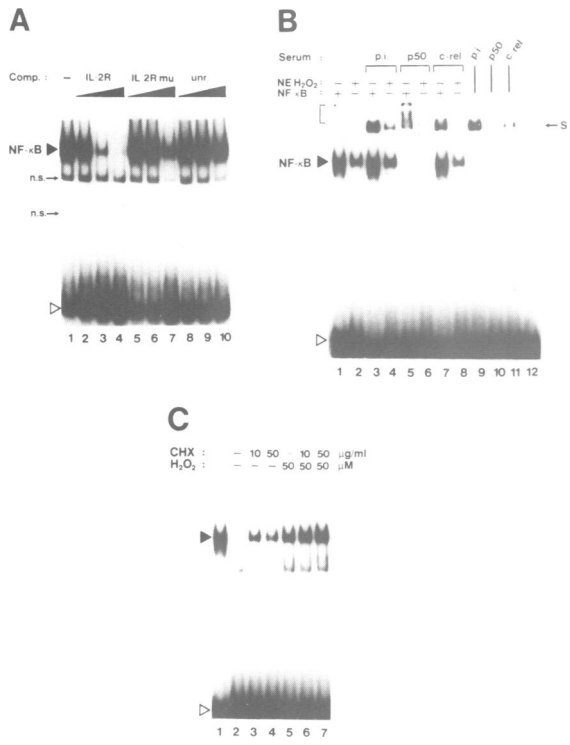

Fig. 2. Characterization of the H_2O_2-induced DNA-binding activity. **(A)** Binding competition analysis. A nuclear extract from H_2O_2-treated (100 μM; 3 h) Jurkat cells was used. 2.5-, 25- and 250-fold molar excesses of unlabeled competitor oligonucleotides (described in Zabel *et al.*, 1991) were mixed with the ^{32}P-labeled κ enhancer probe and the binding reaction started by the addition of the nuclear extract. The competitor oligonucleotide 'IL-2R' encompassed the NF-κB binding site from the interleukin 2 receptor α chain promoter, 'IL-2R mu' is the same oligonucleotide with two point mutations (see text), and 'unr' is an unrelated DNA fragment described in Urban and Baeuerle (1990). A fluorogram of a native gel is shown. The filled arrowhead indicates the position of the κB-specific DNA-binding activity and arrows the positions of two non-specific (n.s.) activities. The open arrowhead shows the position of unbound DNA. **(B)** Immunoreactivity of the H_2O_2-inducible protein−DNA complex. Purified human NF-κB (Zabel *et al.*, 1991) (lanes 1, 3, 5 and 7) and a nuclear extract from Jurkat cells treated with 50 μM H_2O_2 for 1 h (lanes 2, 4, 6 and 8) were reacted with a mix of pre-immune sera (lanes 3 and 4), an antiserum specific for the p50 subunit of NF-κB (no. 2; Kieran *et al.*, 1990) (lanes 5 and 6) or an antiserum reacting with the unique C-terminus of the human c-rel protein (Brownell *et al.*, 1988) (lanes 7 and 8). After immunoreaction, the DNA probe was added together with a DNA-binding mix and samples were electrophoresed on a native gel. A fluorogram of a native gel is shown. Lanes 9−11 show a slowly migrating binding activity from the serum labeled with an arrow (S). Lane 12 shows the DNA-binding mix without additions. A bracket on the left indicates the position of immune-complexed NF-κB. **(C)** The effect of cycloheximide (CHX) on the induction of NF-κB by H_2O_2. Jurkat T cells were left untreated (lane 2) or incubated with 10 μg/ml (lane 3), 50 μg/ml cycloheximide (lane 4) and 50 μM H_2O_2 (lane 5). Lanes 6 and 7 show a combined treatment of 50 μM H_2O_2 with 10 and 50 μg/ml CHX, respectively. The protein−DNA complex of purifed NF-κB was electrophoresed in lane 1. A fluorogram of a native gel is shown.

probe encompassing the decameric NF-κB motif from the mouse κ light chain enhancer. The newly activated protein−DNA complex co-migrated with that formed by purified human NF-κB composed of p50 and p65 protein subunits (Figure 1A, lane 1). More conclusive evidence for the H_2O_2-activated factor being NF-κB came from a binding competition analysis and an immunoreactivity experiment shown below (see Figure 2).

In cytosolic fractions, no accumulation of the H_2O_2-activated protein−DNA complex was seen (Figure 1B, lanes 9−13) indicating that the newly activated NF-κB was rapidly translocated from the cytoplasm into the nucleus. None of the faster migrating factors binding to the κ enhancer probe was affected by the H_2O_2-treatment of cells indicating that the activation was specific for NF-κB and did not reduce the DNA-binding of other proteins (see also Figure 3).

Also concentrations <150 μM H_2O_2 could efficiently activate NF-κB (Figure 1B). A kinetic analysis showed that a treatment of cells with 30 μM H_2O_2 accumulated significant amounts of active NF-κB in nuclei. With 25 μM H_2O_2, no significant activation was seen after 4 h (data not shown). However, we observed some variation between experiments with respect to the minimal concentration of H_2O_2 that induced NF-κB activity. This could be due to different extents of decomposition of H_2O_2 in the cell culture medium catalyzed by either serum components (Link and Riley, 1988) or enzymes released from cells. In the presence of 50 and 100 μM H_2O_2, more NF-κB was activated after 4 h than with 30 μM. After 16 h of incubation, a slight reduction in the amount of active nuclear NF-κB was seen (Figure 1B). There was only a small difference between the activation potential of 50 and 100 μM H_2O_2, suggesting that a maximal stimulation of NF-κB was reached between 50 and 100 μM. Prolonged treatment of cells with H_2O_2 concentrations >150 μM significantly decreased the survival of Jurkat T cells. This was observed with other cell lines too (Link and Riley, 1988). H_2O_2 could also activate NF-κB in various other cell lines tested, among them the mouse fibroblast line Ltk$^-$ (see Table I) and the pre-B cell line 70Z/3 (data not shown).

The possible identity of the H_2O_2-activated DNA-binding protein with NF-κB was further investigated by a binding competition analysis (Figure 2A) and by the use of antisera specific for the DNA-binding p50 subunit of NF-κB (Figure 2B). Nuclear extracts from cells treated with 100 μM H_2O_2 were reacted with a ^{32}P-labeled κ enhancer probe in the absence (Figure 2A, lane 1) or presence of increasing amounts of various unlabeled competitor oligonucleotides (lanes 2−10). Competition with a 250-fold molar excess of an oligonucleotide encompassing the NF-κB binding motif 5′-GGGAATCTCC-3′ from the IL-2R promoter completely eliminated the formation of the radioactive protein−DNA complex induced by H_2O_2 treatment (Figure 2A, lane 4). At a 250-fold molar excess, the competition with increasing amounts of a mutant κB motif from the interleukin 2 receptor gene promoter (5′-GGGAATCTAA-3′) showed only a weak effect on binding (Figure 2A, lane 7). A DNA fragment which does not contain sequences similar to κB motifs showed no competition within the concentration range tested (lanes 8−10). These results demonstrate the κB-specific DNA-binding of the H_2O_2-activated factor. The binding of the two minor activities to the radioactive DNA probe was not strongly influenced by any of the competitor oligonucleotides demonstrating that their DNA-binding was not sequence-specific. These activities must have been endogenous because they were not detectable in a reaction without nuclear extract (Figure 2B, lane 12).

Next, we tested whether the H_2O_2-activated protein−DNA complex could react with an antiserum raised against the DNA-binding p50 subunit of NF-κB (Kieran *et al.*, 1990). The serum did not cross-react with the related

c-rel protein in EMSAs (U.Zabel and P.Baeuerle, unpublished). The presence of anti-p50 serum during the DNA-binding reaction abolished the protein–DNA complex of purified NF-κB (Figure 2B, compare lanes 1 and 5). Also, the co-migrating inducible complex from a nuclear extract of H_2O_2-treated Jurkat cells was abolished by the antiserum (Figure 2B, compare lanes 2 and 6). The pre-immune serum had no effect on the protein–DNA complex of NF-κB (Figure 2B, lanes 3 and 4). c-rel is another protein which can recognize κB sequence motifs (Kieran *et al.*, 1990; Ballard *et al.*, 1990) and shares high homology with p50 NF-κB within a 300 amino acid long DNA-binding and dimerization domain (Bours *et al.*, 1990; Kieran *et al.*, 1990; Ghosh *et al.*, 1990). An antiserum raised against the unique C-terminus of the human c-rel protein (Brownell *et al.*, 1988) did not react with the purified NF-κB and H_2O_2-activated factor (Figure 2B, lanes 7 and 8). All three sera contained a factor which gave rise to a very slowly migrating protein–DNA complex in EMSAs (Figure 2B, lanes 3–11) and was present in different amounts in the sera (lanes 9–11).

A characteristic of NF-κB is its activation by a post-translational mechanism involving the release of the inhibitory subunit IκB from a latent cytoplasmic form (Baeuerle and Baltimore, 1988a,b; for a review see Baeuerle, 1991). In order to investigate whether the activation of NF-κB by H_2O_2 was a post-translational event, we performed the treatment with H_2O_2 in the presence of the protein synthesis inhibitor cycloheximide (Figure 2C). If Jurkat T cells were treated with 10 or 50 μg/ml cycloheximide alone, a weak activation of NF-κB was seen (Figure 2C, compare lane 2 with lanes 3 and 4). A treatment with 50 μM H_2O_2 for 2 h was chosen to obtain only a sub-optimal activation of NF-κB (Figure 2C, lane 5). In the presence of 10 μg/ml cycloheximide, the H_2O_2 treatment could further increase the amount of nuclear NF-κB (Figure 2C, lane 6). As determined by Cerenkov counting of the protein–DNA complexes, the effects of the protein synthesis inhibitor and H_2O_2 were additive. In a combined treatment with 50 μg/ml cycloheximide and 50 μM H_2O_2, a slight superinduction was observed (Figure 2C, lane 7). These results show that the activation of NF-κB by H_2O_2 occurred post-translationally.

The activation of NF-κB by oxidant stress is specific

We tested whether treatment of cells with H_2O_2 influences the DNA-binding activity of other inducible and constitutive transcription factors (Figure 3A). The nuclear extracts from control and H_2O_2-treated cells were incubated with ^{32}P-labeled DNA probes which allow detection of the DNA-binding activities of NF-κB, AP-1/c-fos proteins, glucocorticoid receptor and various octamer-binding proteins (for a review, see Johnson and McKnight, 1989). The specificity of protein–DNA complexes was tested by competition with the respective unlabeled oligonucleotide. An induction of a DNA-binding activity was only seen with the κB probe (Figure 3A, first panel). H_2O_2 did not induce activities binding to the AP-1 probe or a glucocorticoid response element (Figure 3A, panels 2 and 3). The constitutive DNA-binding activities of the ubiquitous oct-1 and faster-migrating lymphoid-specific oct-2 proteins were unchanged after treatment of cells with H_2O_2 (Figure 3A, last panel). In conclusion, the treatment of Jurkat cells with

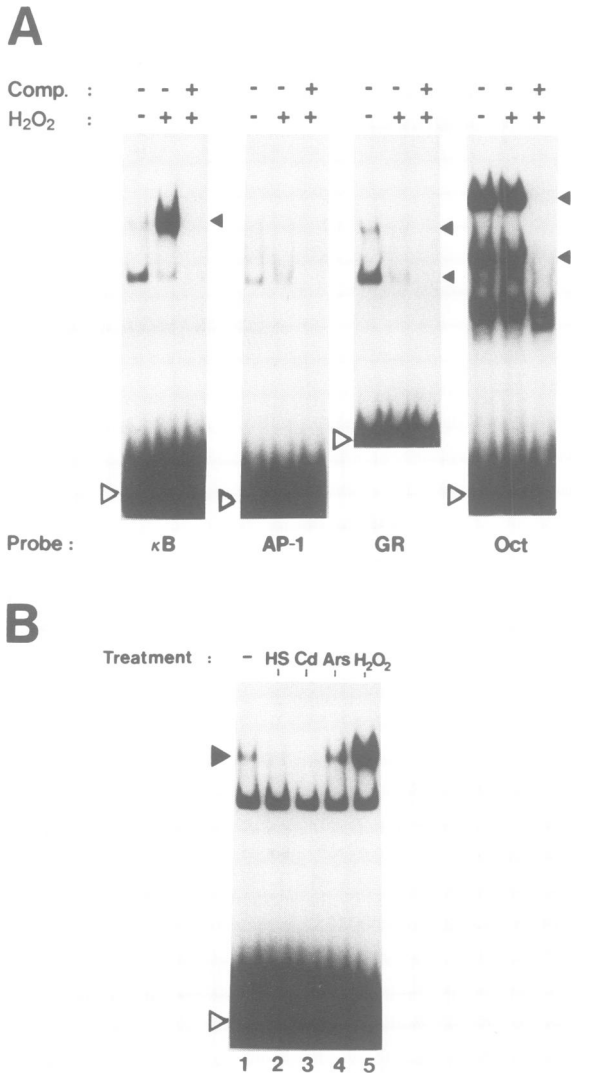

Fig. 3. The specificity of the H_2O_2 effect. (**A**) The effect of an H_2O_2 treatment on the DNA-binding activity of other inducible and constitutive transcription factors. Nuclear extracts from control (lanes 1, 4, 7 and 10) and H_2O_2 treated Jurkat cells (100 μM; 3 h) were reacted with ^{32}P-labeled DNA probes detecting NF-κB (κB; lanes 1–3), *jun*/c-fos proteins (AP-1; lanes 4–5), the glucocorticoid receptor (GR; lanes 7–9) and octamer-binding proteins (oct; lanes 10–12). In lanes 3, 6, 9 and 12, a 100-fold molar excess of the respective unlabeled specific oligonucleotide was added as competitor. Samples were analyzed by EMSA. Fluorograms of native gels are shown. Filled arrowheads indicate the positions of presumably specific protein–DNA complexes. The open arrowhead shows the position of the unbound DNA probes. (**B**) The effects of heat shock and chemical stress factors on the activity of NF-κB. Jurkat cells were treated for 1 h at 42°C (HS; lane 2) or for 4 h with 50 μM cadmium sulfate (Cd; lane 3), 50 μM sodium arsenite (Ars; lane 4) and 50 μM H_2O_2 (lane 5). Lane 1 shows control cells. Nuclear extracts were analyzed by EMSA using a labeled κ enhancer probe. A fluorogram of a native gel is shown. The filled arrowhead indicates the position of the NF-κB–DNA complex and the open arrowhead the position of unbound DNA probe.

H_2O_2 appears to activate specifically the NF-κB transcription factor. Various DNA-binding activities detected with other DNA probes are either unchanged or show a slight decrease in activity which might be indicative of some oxidative damage.

Besides oxidant stress, also heat shock and chemical

inducers of the cellular stress response can induce the expression of genes (Ashburner and Bonner, 1979; Ananthan *et al.*, 1986; Zimarino and Wu, 1987; Ciavarra and Simeone, 1990). We therefore investigated whether exposure of Jurkat cells to heat shock (Figure 3B, lane 2), cadmium sulfate (lane 3) and sodium arsenite (lane 4) under previously reported conditions (Geelen *et al.*, 1988) and for the same duration as the H_2O_2 treatment (1−4 h) can activate NF-κB. None of the treatments caused a rapid appearance of detectable amounts of NF-κB binding activity in nuclear extracts from Jurkat T cells (Figure 3B). This suggests that NF-κB is a transcription factor which is specifically activated if T cells are exposed to oxidant stress.

Low concentrations of H_2O_2 induce expression of HIV-1

In Jurkat T cells, NF-κB binding sites in promoters and enhancers of genes serve as response elements that confer activation of genes following treatment with TNF-α (Osborn *et al.*, 1989; Lowenthal *et al.*, 1989), TNF-β (Messer *et al.*, 1990; Paul *et al.*, 1990), PMA and lectins (Tong-Starksen *et al.*, 1989; Nabel and Baltimore, 1987). In the following experiments, we have tested whether the NF-κB binding sites in the HIV-1 LTR can also serve as response elements for H_2O_2, an agent produced during inflammatory processes by granulocytes and macrophages (Figure 4A and B). A reporter gene construct with the chloramphenicol acetyltransferase (CAT) gene under the control of the HIV-1 enhancer/promoter was transfected into Jurkat T cells. Cells were then treated for 20 h with either a combination of PMA and the lectin phytohemagglutinin (PHA), 10−50 μM H_2O_2, or with human TNF-α. Thereafter, cells were lysed and extracts assayed for the activity of the reporter gene product, the CAT enzyme.

H_2O_2 caused a strong induction of CAT enzyme activity as was evident from the increased acetylation of chloramphenicol compared with control cells (Figure 4A and B). While 10 μM H_2O_2 were ineffective, 30 μM gave a maximal induction which was not further augmented by treatment with 50 μM H_2O_2. The 11-fold increase in CAT activity after treatment with 30 μM H_2O_2 was as strong as that obtained with PMA/PHA treatment and even higher than that seen after treatment with TNF-α (seven-fold induction) (Figure 4B). In order to test whether the NF-κB-binding sites in the HIV-1 LTR are responsible for the H_2O_2-inducible CAT gene expression, a construct was used in which the two κB motifs were mutated such that binding of NF-κB is abolished (Nabel and Baltimore, 1987, 1990). This κB mutant construct of the HIV-1 LTR showed no induction of CAT activity upon treatment of cells with H_2O_2, PMA/PHA or TNF-α. The dependence of the gene inducibility of the HIV-1 LTR by a TNF-α and PMA/PHA treatment on intact NF-κB-binding sites is consistent with earlier data (Nabel and Baltimore, 1987; Osborn *et al.*, 1989; Duh *et al.*, 1989). Since the increase in CAT enzyme activity after H_2O_2 treatment of cells was completely dependent on the NF-κB elements in the HIV-1 enhancer, it is very unlikely that H_2O_2 increased the stability of the CAT mRNA, induced an endogenous acetylation activity or stimulated the basal CAT enzyme activity. Rather, the data show that μM-concentrations of H_2O_2 can potently activate HIV-1 LTR-controlled gene expression depending on the two NF-κB binding motifs in the LTR.

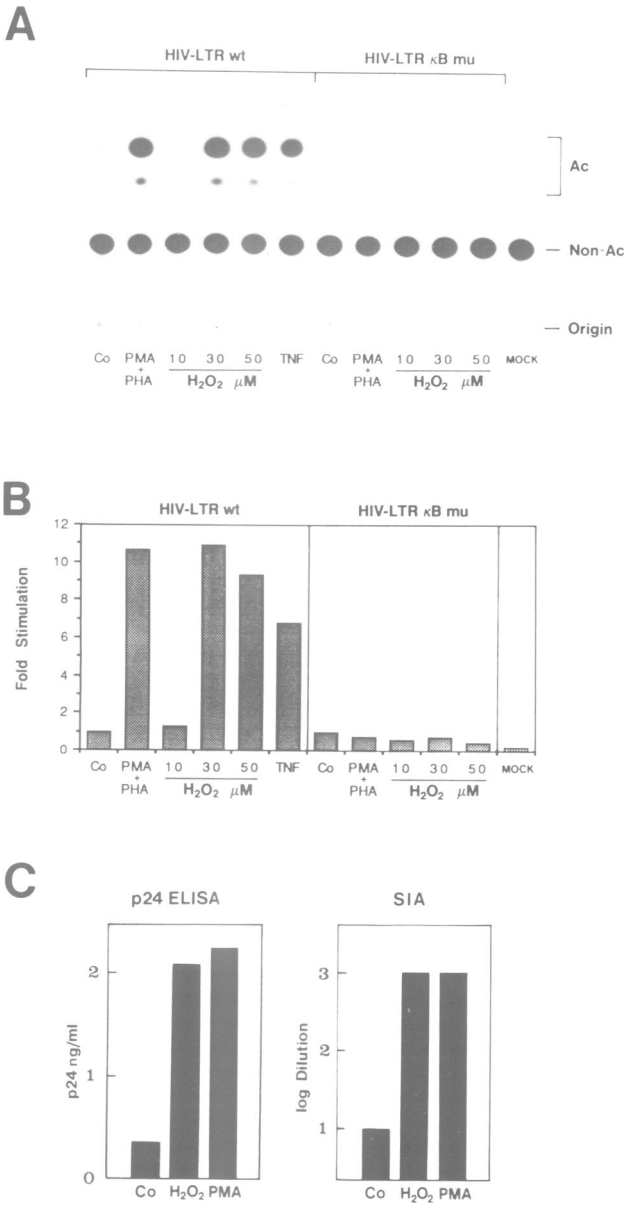

Fig. 4. The effect of an H_2O_2 treatment in Jurkat cells on the expression of HIV-1 T cells. (**A**) The effect of H_2O_2 on the HIV-1 LTR-controlled expression of a CAT reporter gene in Jurkat cells. Cells were transfected with a CAT reporter gene construct controlled by the promoter/enhancer of HIV-1 (HIV-LTR wt) or a construct with mutations altering the two NF-κB-binding sites in the LTR (HIV-LTR κB mu) (Nabel and Baltimore, 1987, 1990). In the last position, the effect of a mock transfection is shown. Cells transfected by the DEAE−dextran method were left untreated (Co) or incubated for 20 h with the phorbol ester PMA in combination with the lectin phytohemagluttinin (PHA), 10, 30 or 50 μM H_2O_2 or recombinant human TNF-α. The acetylated forms of [^{14}C]chloramphenicol (Ac) were separated from unreacted reagent (Non-Ac) by ascending thin layer chromatography. An autoradiogram is shown. (**B**) Quantification of the CAT activity by liquid scintillation counting. Bars indicate the -fold stimulation of CAT activity in comparison with untreated cells (Co; set to 1.0). Results from two independent experiments are shown as average values. (**C**) The effect of H_2O_2 on the production of HIV-1 in latently infected Jurkat cells. Jurkat T cells were inoculated with an HIV-1 isolate and cultured for 8 days. Washed cells were then incubated for 24 h with 50 μM H_2O_2, 50 ng/ml PMA or left untreated (Co) followed by determination of the viral p24 protein in culture supernatants by ELISA and the induction of syncytia by a titration assay (SIA).

We next tested whether treatment with H_2O_2 can activate HIV-1 replication in latently HIV-1 infected Jurkat T cells. Infected cells were treated with either 50 μM H_2O_2, 50 ng/ml PMA or left untreated. Twenty-four hours later, the production of the p24 protein was determined by ELISA and the formation of syncytia was determined in a titration assay using C8166 cells. Untreated cells exhibited a basal level of p24 production and their cell culture supernatants caused induction of syncytia only at a low dilution (Figure 4C). Treatment of cells with 50 μM H_2O_2 caused a 6.4-fold increase in the production of the viral p24 protein and the cell culture supernatants from treated cells could induce the formation of syncytia at a 100-fold lower dilution than culture supernatants from control cells (Figure 4C). Treatment of cells with PMA showed effects very similar to those of H_2O_2. The activation of the HIV-1 LTR and viral replication in T cells by H_2O_2 might be of great significance in the onset of HIV-1 production in AIDS patients that suffer from secondary infections and inflammatory processes.

NAC blocks the activation of NF-κB by H_2O_2

NAC can raise intracellular GSH levels and thereby protect cells from the effects of ROI (Aruoma *et al.*, 1989, and references therein). In addition, the SH group of the agent can directly react with radicals. Here, we have tested whether the activation of NF-κB by H_2O_2 in Jurkat T cells was sensitive to NAC which would indicate an involvement of oxygen radicals. Cells were pre-incubated in the absence (Figure 5A, lanes 1, 3 and 4) or presence of 20 mM NAC (lanes 2, 5 and 6) followed by a treatment with 50 or 100 μM H_2O_2 (lanes 3–6). As is seen in a fluorogram (Figure 5A) and after Cerenkov counting of the protein–DNA complexes (not shown), 20 mM NAC reduced the induction of NF-κB binding after H_2O_2 treatment by ~70%. NAC at a concentration of 30 mM showed an inhibition of 90% (see Figure 6). NAC did not have an inhibitory effect on two endogenous non-specific DNA-binding activities. To test whether the millimolar amounts of NAC (sodium form) simply blocked the DNA-binding of NF-κB in the assay system, we incubated purified NF-κB with increasing amounts of NAC (Figure 5B, lanes 3 to 6). All samples were adjusted to the same Na^+ concentration because sodium ions had previously been shown to influence the DNA-binding activity of NF-κB (Zabel *et al.*, 1991). Even at a concentration of 100 mM NAC, no significant reduction in the amount of NF-κB–DNA complex was found (Figure 5B, lane 6). Also, when NAC was added to cells at the end of an H_2O_2 treatment, no inhibition was observed (Figure 5C, compare lanes 1 and 2). Since NAC can only react very slowly, if at all, with H_2O_2 (Aruoma *et al.*, 1989), our results suggest that NAC exerts its inhibitory effect on the activation of NF-κB primarily within intact cells by elevating GSH levels and/or by directly reacting with a metabolite of H_2O_2. Also other thiol compounds, such as 2-mercaptoethanol, dithiocarbamate, glutathione or disulfiram at concentrations in the micromolar to millimolar range potently blocked the activation of NF-κB (R.Schreck and P.Baeuerle, in preparation).

We tested whether NF-κB is activated by H_2O_2 in a cell free system (Figure 5D). NF-κB, NF-κB–IκB complex and a cytosolic fraction and nuclear extract from unstimulated Jurkat cells were treated for 30 min with 100 μM H_2O_2. In contrast to intact cells, no induction of the binding activity of NF-κB was observed, suggesting that H_2O_2 cannot directly inactivate and release IκB from NF-κB (Figure 5D, lanes 4 and 6). The DNA-binding of active NF-κB was also

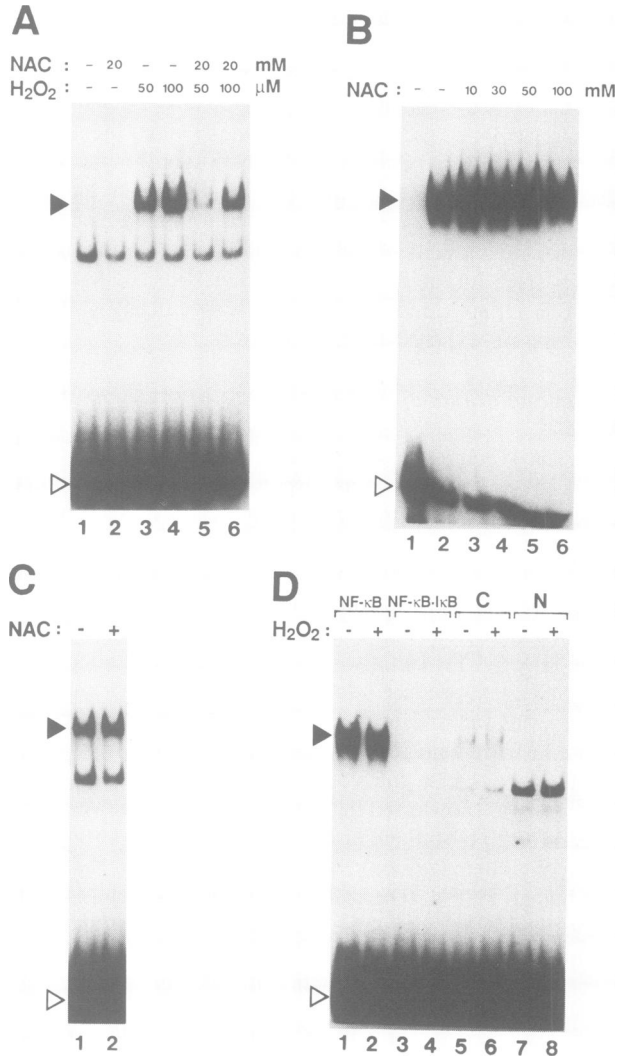

Fig. 5. The effect of NAC on the activation of NF-κB by H_2O_2 *in vivo* and *in vitro*. **(A)** The *in vivo* effect of NAC on the activation of NF-κB by H_2O_2. Jurkat cells were incubated for 1 h in the absence (lane 1) or presence of 20 mM NAC (sodium form) followed by a one hour treatment in the absence (lane 2) or presence of 50 μM (lane 5) or 100 μM H_2O_2 (lane 6). Lanes 3 and 4 show a treatment with 50 and 100 μM H_2O_2 alone. Nuclear extracts were analyzed by EMSA using a κ enhancer probe. A fluorogram of a native gel is shown. The filled arrowhead indicates the position of the NF-κB–DNA complex, the open arrowhead the position of the unbound DNA probe. **(B)** The effect of NAC on the DNA-binding activity of NF-κB. Purified NF-κB was incubated for 30 min with the indicated amounts of the sodium form of NAC. By the addition of NaCl, all samples were adjusted to a final concentration of 100 mM sodium. A binding reaction in the absence of NaCl or NAC is shown in lane 1. **(C)** The effect of addition of NAC at the end of a H_2O_2 treatment. After a 1 h incubation with 100 μM H_2O_2 (lane 1), 20 mM NAC was added to the culture (lane 2) and nuclear extracts prepared and analyzed by EMSA. **(D)** The effect of H_2O_2 *in vitro* on the binding activity of active NF-κB and its latent cytoplasmic form. Purified NF-κB (lanes 1 and 2), purified NF-κB–IκB complex (lanes 3 and 4), and cytosol (lanes 5 and 6) or nuclear extract (lanes 7 and 8) from control Jurkat cells were incubated with 100 μM H_2O_2 for 30 min at room temperature followed by a DNA-binding reaction for 30 min. Samples were analyzed on native gels. A fluorogram is shown.

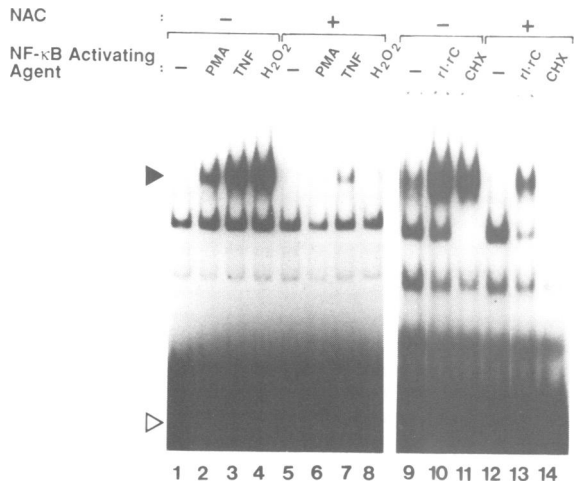

Fig. 6. The effect of NAC on the activation of NF-κB by five different agents. Jurkat T cells were treated with PMA (50 ng/ml; lanes 2 and 6), human TNF-α (13 ng/ml; lanes 3 and 7), H_2O_2 (100 μM; lanes 4 and 8), poly(rI)−poly(rC) (rI·rC, 0.1 mg/ml; lanes 10 and 13) or cycloheximide (CHX, 50 μg/ml; lanes 11 and 14) for 3 h in the absence (lanes 1−4 and 9−11) or presence of 30 mM NAC added 1 h prior to the activating treatment (lanes 5−8 and 12−14). Nuclear extracts were prepared and analyzed by EMSA using a labeled κ enhancer probe. Fluorograms from two native gels are shown. The filled arrowhead indicates the position of the NF-κB−DNA complex and the open arrowhead the position of unbound DNA probe.

not influenced by H_2O_2 (Figure 5D, lane 2). This finding lends further support to the idea that a metabolite of H_2O_2 is involved in the activation of NF-κB.

In contrast to NF-κB, the activation of the AP-1 factor by PMA appears not to depend on ROI because the presence of NAC did not effect the induction of AP-1 DNA-binding following a PMA treatment of cells (R.Schreck and P.Baeuerle, in preparation). This also suggests that NAC did not interfere with the activity of PKC.

Thiol compounds block the activation of NF-κB by many different agents

Different agents are known to induce the DNA-binding activity of NF-κB. Among them are TNF-α (Osborn *et al.*, 1989; Lowenthal *et al.*, 1989), PMA (Sen and Baltimore, 1986), double-stranded RNA (Lenardo *et al.*, 1989; Visvanathan and Goodbourn, 1989), cycloheximide (Sen and Baltimore, 1986) and H_2O_2. These agents are thought to act intracellularly by distinct signalling pathways. TNF-α was shown to activate NF-κB independently of PKC (Meichle *et al.*, 1990), while PMA is thought to inactivate NF-κB by PKC which can phosphorylate and subsequently release IκB (Ghosh and Baltimore, 1990). Double-stranded RNA could act via the dI kinase (for review see London *et al.*, 1987) and cycloheximide by preventing the synthesis of a labile inhibitor of NF-κB activation. Finally, H_2O_2 seems to act via ROI as supported by the sensitivity of the induction towards NAC. However, NAC was recently shown to also inhibit HIV-1 replication and transactivation of the HIV-1 LTR following TNF-α or PMA treatment of T cells (Roederer *et al.*, 1990). The effect of NAC was mediated by the NF-κB binding sites in the HIV-1 enhancer and the induction of the DNA-binding activity of NF-κB by TNF-α and PMA is decreased by the compound (Staal *et al.*, 1990). In the following, we have investigated whether the activation of NF-κB in Jurkat T cells by five distinct agents is inhibited in each case by NAC.

Table I. The effect of two different thiol compounds on the activation of NF-κB by different agents in different cell lines.

NF-κ-activating agent	Cell lines tested	Inhibition by 30 mM NAC	Inhibition by 0.1 mM PDTC
H_2O_2	Jurkat	++++	n.d.
	Ltk⁻	++++	n.d.
Tumor necrosis factor α	Jurkat	++++[a]	++++
	70Z/3	n.d.	++++
	Ltk⁻	n.d.	++++
Interleukin-1	70Z/3	+++	+++
	Ltk⁻	+++	n.d.
Double-stranded RNA	Jurkat	++++	n.d.
Lipopolysaccharide	70Z/3	+++	++++
PMA	Jurkat	++++[a]	++++
	70Z/3	+++	++++
	Ltk⁻	n.d.	++++
PMA + lectin	Jurkat	++++	++++
Cycloheximide	Jurkat	+++	n.d.
Calcium ionophore	Jurkat	++++	n.d.

Jurkat T cells, mouse fibroblasts (Ltk⁻) and mouse pre-B cells (70Z/3) were treated as indicated with H_2O_2 (100 μM; 2 h), human recombinant TNF-α (13 ng/ml; 1−3 h), human recombinant IL-1β (10 U/ml; 1−2 h), double-stranded RNA [poly(rI)−poly(rC); 4 h], PMA (50 ng/ml; 1−3 h), PMA plus lectin (5 μg/ml PHA; 2 h), cycloheximide (10 μg/ml; 3 h) or the calcium ionophore A23187 (1 μM; 3 h). Cells were incubated in the absence or presence of 30 mM NAC or 0.1 mM PDTC added 1 h prior to the activating agents. Nuclear extracts were analyzed by EMSA and the protein−DNA complex of NF-κB quantified by Cerenkov counting. +++, 50−75% inhibition; ++++, 75−100% inhibition
[a]Also reported by Staal *et al.* (1990); n.d., not determined

Cells were incubated in the presence or absence of 30 mM NAC with the following compounds: the phorbol ester PMA (Figure 6, lane 2), human TNF-α, (lane 3) H_2O_2 (lane 4), poly(rI)−poly(rC) (lane 10) and cycloheximide (lane 11). In all cases, a significant accumulation of active NF-κB in nuclear extracts from Jurkat T cells was seen. PMA and cycloheximide gave the weakest induction of DNA-binding. In the presence of NAC, the induction of NF-κB by all five agents was efficiently blocked (Figure 6, lanes 6−8, 13 and 14). The extent of inhibition by NAC was very similar with the five inducers and was in the range 80−90% as determined by Cerenkov counting of the radioactivity in the protein−DNA complexes.

In search of a more potent inhibitor of NF-κB, we tested a variety of other agents mainly encompassing thiol compounds and metal chelators (R.Schreck and P.Baeuerle, to be published elsewhere). Most of the agents showed inhibitory effects but at very different concentrations. One of the most potent and specific agents was a pyrrolidone derivative of dithiocarbamate (PDTC). In Table I, we compare the effects of 30 mM NAC and 0.1 mM PDTC on the inhibition of NF-κB induction by the five agents shown in Figure 6 and by four other agents known to activate NF-κB. These other agents were LPS, which is a strong inducer of NF-κB in the pre-B cell line 70Z/3 (Sen and Baltimore, 1986), IL-1, which can activate NF-κB in 70Z/3 but not in Jurkat cells (Osborn *et al.*, 1989) and the calcium ionophore A23187 and the lectin phytohemagglutinin (PHA) which activate NF-κB in T cells strongly when in

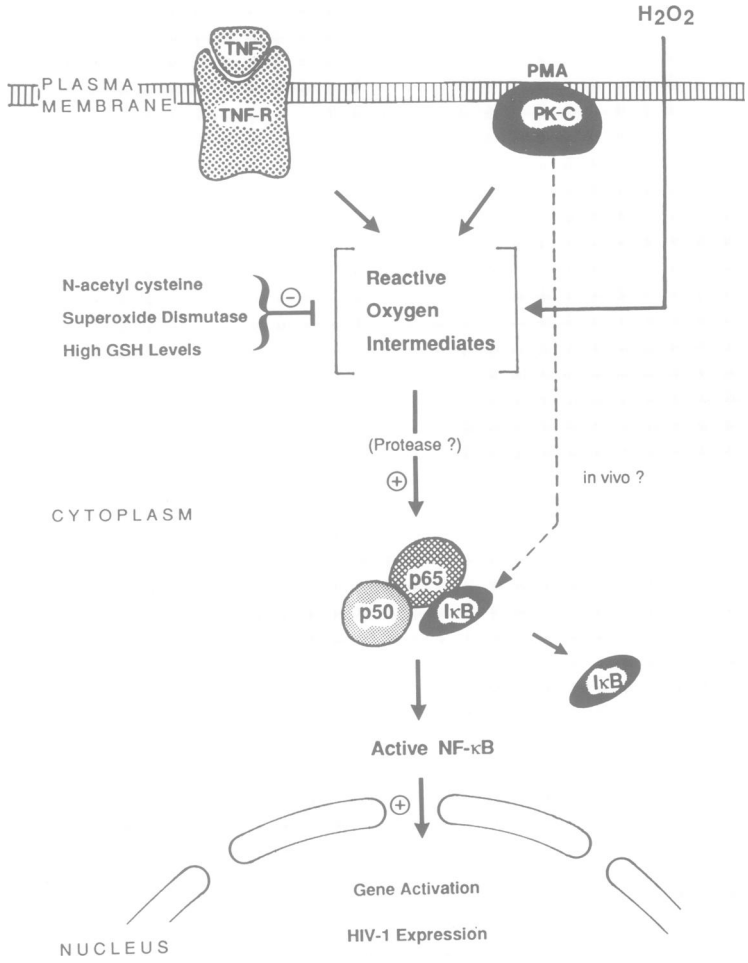

Fig. 7. A model showing the presumed involvement of reactive oxygen intermediates in the activation of NF-$\varkappa$B.

combination with PMA (Nabel and Baltimore, 1987) but only weakly if applied on their own (unpublished observation). First, the inhibition of NF-$\varkappa$B by NAC and PDTC was not restricted to Jurkat cells but was also observed in a mouse fibroblast line (Ltk$^-$) and a mouse pre-B cell line (70Z/3) (Table I). Second, both thiol agents, though chemically distinct, inhibited the induction of NF-$\varkappa$B binding activity regardless of the activating agent or cell line tested. Third, the induction of NF-$\varkappa$B by LPS, IL-1, calcium ionophore and PHA/PMA was also efficiently blocked by NAC and/or PDTC. These result suggests that PMA, TNF-α, IL-1, LPS, double-stranded RNA, cycloheximide, calcium ionophore, lectin and H_2O_2 might all activate NF-$\varkappa$B by the same mechanism involving a NAC/PDTC-sensitive intracellular signalling step. The common messenger which is sensitive to radical-scavenging thiol agents appears to be a reactive oxygen intermediate. A model summarizing this idea is shown in Figure 7.

Discussion

Treatment of T cells with hydrogen peroxide induces the DNA-binding and nuclear appearance of a factor. Evidence that this factor is NF-$\varkappa$B includes the $\varkappa$B-specific DNA-binding, the co-migration of its protein–DNA complex with that of purified NF-$\varkappa$B, its immunoreactivity with antisera raised against the DNA-binding subunit of NF-$\varkappa$B and the post-translational induction of its DNA-binding. The activation of NF-$\varkappa$B by treatment of cells with H_2O_2 appears to be a specific event because (i) it occurs at low extracellular concentrations of H_2O_2; (ii) other DNA-binding proteins appear to be unaffected by the treatment and (iii) because other kinds of cellular stress do not induce the activity of NF-$\varkappa$B under the conditions tested.

Addition of only 30 μM H_2O_2 to the culture medium was sufficient to transactivate the HIV-LTR to an extent seen with TNF-α or a PMA/PHA treatment of cells. It should however, be considered that H_2O_2 can decompose in serum-containing medium; within one hour the initial H_2O_2 concentration was found to decrease by 60% (Link and Riley, 1988). Enzymes released from cells, such as catalases, could further deplete the initial amount of H_2O_2 added to the cell cultures. Presumably, the actual concentration of H_2O_2 that induced the activation of NF-$\varkappa$B and HIV-1 in cell cultures was therefore much lower than the theoretical one. Another important criterion for the specificity of the H_2O_2 effect is that other cellular stress reactions such as heat shock and chemicals did not activate NF-$\varkappa$B. Thus, $\varkappa$B elements in regulatory domains of genes appear to serve specifically as response elements for oxidant stress but not for other types of cellular stress. It has recently been proposed that NF-$\varkappa$B is a transcription factor which has specialized in the organism to induce the synthesis of defense and signalling proteins rapidly upon exposure of cells to a wide variety of mostly pathogenic agents (Baeuerle and

Baltimore, 1991; Baeuerle, 1991). The present findings are fully consistent with this idea and add H_2O_2 to the list of inducers produced under pathogenic conditions.

In liver cells, measurements have shown that the normal intracellular concentration of H_2O_2 is in the sub-micromolar range whereas other tissues such as the eye lens reach up to 25 μM (for review see Halliwell and Gutteridge, 1990). In blood plasma of healthy subjects, values between 0.25 and 5 μM were determined (Frei *et al.*, 1988). These low basal levels make H_2O_2 a suitable signal for the activation of NF-κB when there is an increase in the extracellular H_2O_2 concentration during an inflammatory process. It is well possible that the local H_2O_2 concentrations in extracellular fluids during inflammatory processes are sufficient to activate NF-κB in tissue and blood cells thereby allowing induction of the synthesis of cytokines, immunologically active cell surface receptors and also of viruses such as HIV-1. The efficient propagation of HIV-1 in macrophages (for review see Meltzer *et al.*, 1990) might be related to the presence of an effective oxidative burst machinery in this cell type. There is an overwhelmingly large number of reports dealing with the effects of oxygen radicals in biological and pathobiological systems. Future studies can now address in these systems the role of NF-κB as a transcription factor which is specifically activated under conditions that increase the intracellular concentration of ROI.

Possible mechansims of H_2O_2-mediated NF-κB activation

A central event in the post-translational activation of NF-κB is the release of the inhibitory subunit IκB from its complex with p65 and p50 in the cytoplasm (for reviews see Baeuerle and Baltimore, 1991; Baeuerle 1991). Release of IκB allows DNA-binding of NF-κB and its translocation to the nucleus. Any reaction that abolishes binding of IκB to p65 or damages IκB should thus enable the activation of NF-κB. As shown in this study, H_2O_2 on its own is unable to activate the purified NF-κB−IκB complex or that contained in a cytosolic fraction. It therefore appears that a metabolite of H_2O_2 or an intracellular reaction induced by H_2O_2 caused the release of IκB.

After its passive diffusion through the cell membrane, H_2O_2 can be converted into more reactive oxygen compounds such as the superoxide anion, O_2^-, and the hydroxyl radical OH· (for review, see Halliwell and Gutteridge, 1989). While OH· might react instantanously with any macromolecule, O_2^- is less reactive and can diffuse further prior to a reaction. An involvement of radicals in the H_2O_2-induced activation of NF-κB is supported by the inhibitory effect of NAC and that of various other thiol compounds tested (R.Schreck and P.Baeuerle, in preparation). NAC raises intracellular GSH levels and thereby provides GSH peroxidase with the co-substrate required to eliminate ROI (for review, see Halliwell and Gutteridge, 1989). In addition, NAC can directly scavenge radicals (Aruoma *et al.*, 1989). The metal chelators desferroxamine, diethyldithiocarbamate and *o*-phenanthroline also blocked the induction of NF-κB by H_2O_2 (R.Schreck and P.Baeuerle, in preparation). Metal ions such as Fe^{2+} are required for the interconversion of oxygen radicals. Within cells, oxygen radicals can generate other reactive substances, for instance, by oxidizing membrane lipids (for review see Wolff *et al.*, 1986). However, it is unclear whether this can occur to a relevant extent at the low concentrations of H_2O_2 used here to activate NF-κB.

ROI could directly activate NF-κB by degrading or modifying IκB in the cytoplasmic p50−p65−IκB complex. The oxidation of a single cysteine residue in the DNA-binding domains of jun and fos proteins was recently found to allow regulation of the DNA-binding of the two proteins *in vitro* (Abate *et al.*, 1990). A similar reaction could selectively inactivate and release IκB from p50−p65. A frequently observed effect of oxidant stress is also the induction of proteolysis (for review see Pacifici and Davies, 1990). Oxidative damage or a controlled proteolytic degradation of IκB would both provide an irreversible mechansim of NF-κB activation. This idea would be consistent with the preliminary observation that IκB released *in vivo* during a PMA treatment of pre-B cells can apparently not be reused to inhibit the activated NF-κB (Baeuerle *et al.*, 1988). *In vitro* studies are in progress to understand the precise role of ROI in the release of IκB from the cytoplasmic p50−p65−IκB complex.

Does protein kinase C directly activate NF-κB in vivo?

The activation of NF-κB by PMA is inhibited by the radical scavengers NAC and PDTC. We therefore discuss here the possibility that NF-κB is activated by ROI produced in response to the activation of PKC rather than directly by the kinase. *In vitro* studies provided evidence for a direct phosphorylation of IκB by PKC (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990). Using highly purified components, we obtained the same results (E.Link, L.Kerr, R.Schreck, U.Zabel, I.Vetma and P.Bauerle, submitted). There is, however, also a series of observations that does not argue in favor of a direct mechanism. Ghosh and Baltimore (1990) only observed activation of NF-κB by PKC with fractions highly enriched for p50-p65−IκB but not with crude cytosol. Also the heme-regulated kinase and the cAMP-dependent kinase A can activate NF-κB *in vitro* although there is no conclusive evidence for an *in vivo* involvement of the two kinases in the activation of NF-κB. This raises doubts about the specificity of the PKC reaction. In HL-60 cells, TNF-α can rapidly induce PKC but the NF-κB activation by TNF-α is unchanged when PKC was depleted by chronic PMA treatment or inactivated by the inhibitors staurosporine and H-7 (Schütze *et al.*, 1990; Meichle *et al.*, 1990). Moreover, the PMA-induced activation of NF-κB in this cell line is very slow compared with that induced by TNF-α (Hohmann *et al.*, 1990).

PKC could activate NF-κB by directly enhancing the activity of an NADPH oxidase-like enzyme. These enzymes produce superoxide anions and H_2O_2 from O_2 and NADPH. In granulocytes and macrophages, PKC can activate the plasma-membrane bound NADPH oxidase within seconds after PMA stimulation causing the oxidative burst reaction (Gennaro *et al.*, 1986; Christiansen, 1988; Tauber *et al.*, 1989, and references therein). PMA induces the production of superoxide anions not only in granulocytes and macrophages but also in many other cell types (Rosen and Freeman, 1984; Matsubara and Ziff, 1986; Meier *et al.*, 1989, 1990). Staal *et al.* (1990) observed that treatment of Jurkat cells with PMA rapidly decreased intracellular thiol levels, supporting the idea that there is a depletion of GSH by oxidants. Moreover, numerous studies have shown that the tumor-promoting effects of phorbol esters are inhibited by antioxidants (for review see Cerutti, 1985). The inhibitory

effect of the antioxidant NAC and PDTC on the activation by PMA of NF-κB but not of AP-1 strongly suggest that this effect of the phorbol ester treatment also relies on the production of ROI.

Reactive oxygen intermediates as widely used second messengers

While there is good evidence that PMA can exert its effects on gene activity by interaction with its receptor PKC, the signal transduction pathway used by TNF is not known and apparently does not require PKC for the activation of NF-κB (Meichle *et al.*, 1990). Nevertheless, PMA and TNF both activate NF-κB from its latent cytoplasmic form and share κB elements as enhancers of gene expression. The possibility is now raised that the two agents might activate NF-κB by a common pathway diverging upstream of IκB and involving oxygen radicals. Support for this idea includes the observations that, (i) both PMA and TNF-α stimulate the production of superoxide anions and H_2O_2 in granulocytes, fibroblasts and other cell types (Rosen and Freeman, 1984; Matsubara and Ziff, 1986; Klebanoff *et al.*, 1986; Meier *et al.*, 1989, 1990); (ii) the activation of NF-κB by both agents is inhibited by the radical scavenger NAC and other thiol compounds; and (iii) PMA and TNF cause a rapid depletion of the GSH levels in Jurkat T cells (Staal *et al.*, 1990). More evidence for an involvement of oxygen radicals in TNF-α effects comes from experiments using oxygen depletion, thiol reagents and superoxide dismutase (SOD), an enzyme eliminating the superoxide anion. Anaerobic conditions (Matthews *et al.*, 1987), agents elevating GSH levels (Zimmerman *et al.*, 1989) or the overexpression of SOD (Wong *et al.*, 1989) desensitized cells for the cytotoxic effects of TNF-α. At present, it is not clear to what extent the cytotoxicity of TNF relies on the activation of NF-κB (for a recent review see Larrick and Wright, 1990).

In this study we tested nine conditions that were reported to induce NF-κB and, without any exception, the induction of NF-κB appeared to be dependent on oxygen radicals. Future studies have to address the generality of this requirement by testing other conditions under which NF-κB is induced. It is of great interest to see whether NAC and PDTC inhibit the effect of UV light on the induction of HIV-1 and NF-κB (Valerie *et al.*, 1988; Stein *et al.*, 1989) and the activity of NF-κB-inducing viral transactivator proteins such as tax (Leung and Nabel, 1988; Ballard *et al.*, 1988), X (Twu *et al.*, 1989) and ie1 (Sambucetti *et al.*, 1989).

There are many ways in which intracellular levels of ROI could be increased. One is to inhibit mechanisms for their elimination. An alternative way is the induction of enzymes actively producing oxygen radicals. Further experiments have to address in detail the origin of ROI produced in response to inflammatory cytokines and various other NF-κB activating agents and the putative role of ROI in signal transduction processes. The activation of the cytoplasmic form of the NF-κB transcription factor will provide a valuable monitoring reaction for such studies. A role of radicals as second messengers is not without precedent. In the case of the nitric oxide radical (NO) such a role is now becoming widely accepted (for review, see Crossin, 1991). NO stimulates guanylate cyclase and is known to relax vascular smooth muscle and to modulate messenger pathways in the developing and adult brain.

Does it make sense for the cell to engage highly toxic compounds as messenger molecules? First of all, ROI are not only undesired side products of cellular electron transfer reactions but there are ubiquitous enzyme systems which have specialized during evolution in the production of oxygen radicals and H_2O_2. Second, ROI are available in every cell type, either from intracellular reactions or, as H_2O_2, from other cells. Third, eukaryotic cells contain multiple enzymes allowing a precise and rapid regulation of intracellular levels of reactive oxygen intermediates, among them superoxide dismutase, the GSH peroxidase/GSH system, catalase and peroxidases. Thus, ROI fulfil important prerequisites for second messenger molecules: they are small, diffusible and ubiquitous molecules that can be synthesized and destroyed rapidly. However, there might be only a narrow concentration range in which they can function exclusively as second messengers. At elevated concentrations, ROI serve as physiologically important cytotoxic agents.

Materials and methods

Electrophoretic mobility shift assays

Binding conditions for NF-κB were characterized and EMSAs performed as described in detail elsewhere (Zabel *et al.*, 1991). Briefly, binding reactions (20 μl) contained 2 μg poly(dI-dC) (Pharmacia), 5-10 000 c.p.m. (Cerenkov) ^{32}P-labeled DNA probe, 2 μl buffer D (Dignam *et al.*, 1983) containing 1% (v/v) Nonidet P-40, 20 μg bovine serum albumin and binding buffer. Binding reactions were started by the addition of cell extracts or purified protein and allowed for 30 min. Samples were analyzed on native 4% polyacrylamide gels.

Cells were fractionated into cytoplasm and nuclei, and cytosol and nuclear extracts were prepared as described (Baeuerle and Baltimore, 1988a). Equal proportions of cell fractions with 2-8 μg of protein were used in the assays. Approximately 50 pg of purified human NF-κB was used per assay. The NF-κB was purified from cytosol of human placenta as described in detail elsewhere (Zabel *et al.*, 1991). It consisted of the p50 and p65 subunits.

For the competition experiments, 0.1 ng of the labeled oligonucleotide was mixed with 0.25, 2.5 and 25 ng of unlabeled competitor oligonucleotides prior to the addition of proteins. The p50 antiserum was kindly given by Dr A.Israel (Pasteur Institute, Paris) and the c-rel antiserum by Dr Nancy Rice (NCI, Frederick). The antisera and a mix of pre-immune sera (1.5 μl) were diluted with a DNA-binding mix (see above) devoid of dithiothreitol and the labeled DNA probe. After addition of nuclear extracts or purified NF-κB, reactions were allowed for 15 min at room temperature. Thereafter the ^{32}P-labeled DNA probe was added and the incubation continued for 30 min. Samples were then subjected to electrophoresis. NAC (Sigma) was adjusted to a neutral pH value by NaOH.

Oligonucleotides and plasmid constructs

Oligonucleotides were synthesized on an Applied Biosystems synthesizer A380 by the phosphoroamidate method and purified on OPC cartridges (Applied Biosystems) according to the instructions provided by the manufacturer. The sequence of the double-stranded oligonucleotide encompassing the κB motif from the mouse κ light chain enhancer is shown in Zabel *et al.* (1991). The sequences of the oligonucleotides used to detect the DNA-binding activities of octamer binding proteins (oct), AP-1/c-fos, and glucocorticoid receptor were the following (the binding site is underlined). oct: 5′-AGCTTTGGGTAATTTGCATTTCTA-AG-3′; AP-1/c-fos: 5′-AGCTTAAAAAAGCATGAGTCAGACACC-TG-3′; glucocorticoid receptor: 5′-AGCTTGAGAACACAGTGTTCTGA-TCATGAGAACACAGTGTTCTCG-3′. The complementary strands created 5′-overhanging ends (*SalI* sites) which allowed labeling by the Klenow polymerase (Boehringer) using one [α-^{32}P]dNTP (Amersham, 3000 Ci/mmol) and the other three dNTPs in unlabeled form. The labeled DNA probe was purified on push columns (Stratagene).

The plasmids called HIV-LTR wt and HIV-LTR mu contain HIV-1 sequences from −453 to +80 from the transcription start site of the viral genome in front of a CAT reporter gene (Nabel and Baltimore, 1987). In the HIV-LTR mu construct the two binding sites for NF-κB were altered by mutations as described (Nabel and Baltimore, 1990).

Cell culture, transfections and CAT assays

Jurkat T cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 1% (w/v) penicillin/streptomycin (all purchased from Gibco Laboratories). The medium did not contain iron salts which

are known to promote the decomposition of H_2O_2 into hydroxyl radicals. IL-1β was purchased from Genzyme (Boston), LPS, PHA, PDTC, NAC, A23187, cycloheximide and PMA from Sigma and poly(rI)−poly(rC) from Pharmacia.

Transfections were performed by the DEAE−dextran method according to Pomerantz *et al.* (1990). Briefly, 1×10^7 cells were washed with PBS and resuspended in Tris-buffered saline containing DEAE−dextran (Pharmacia) at 200 μg/ml and 15 μg/ml of plasmid DNA in a total volume of 1 ml. Incubations were continued for 90 min at 37°C with frequent agitation. After a shock with 10% (v/v) DMSO for 2 min at room temperature, cells were washed with PBS and resuspended in 20 ml of RPMI 1640 medium supplemented with 10% FCS. Twenty-four hours after transfection, cells were stimulated for 20 h with recombinant human TNF-α (30 ng/ml, a kind gift from Hofmann LaRoche, Basel), a combination of PMA (50 ng/ml) and PHA (5 μg/ml; both Sigma) or H_2O_2 (Merck). Cell extracts were prepared by three freeze−thaw cycles and protein concentrations determined by the method of Bradford (Biorad).

CAT activity was determined essentially as described (Gorman *et al.*, 1983) using samples of the same protein content. In a reaction mix of 150 μl containing 20 mM acetyl CoA (Sigma) and 0.3 μCi [^{14}C]chloramphenicol (Amersham), 100 μg of protein was incubated for 4 h at 37°C. Reaction products were analyzed by thin-layer chromatography followed by autoradiography and liquid scintillation counting. Transfections were performed in duplicate. Mock transfections showed a chloramphenicol acetylation of 0.3%.

p24 ELISA and syncytia induction assay

Jurkat cells (3×10^5 cells/ml) were infected with 10 IE of the HIV-1 isolate M899 (kindly provided by Prof. Dr Gürtler, Pettenkofer Institut, Munich). On day 8 post-infection, cells were washed. On day eleven, cells were treated with 50 ng/ml PMA, 50 μM H_2O_2 or left untreated. The next day, cell culture supernatants were harvested. The amount of p24 protein in the supernatants was determined by ELISA (HIVAG-1 test from Abott GmbH, Wiesbaden-Delkenheim) and the amount of newly produced virus quantified by a syncytia induction assay using C1866 cells (Weiss *et al.*, 1986).

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References

Abate,C., Patel,L., Rauscher,F.J. III and Curran,T. (1990) *Science*, **249**, 1157−1161.

Ananthan,J., Goldberg,A.L. and Voellmy,G.R. (1986) *Science*, **232**, 522−524.

Aruoma,O.I., Halliwell,B., Hoey,B.M. and Butler,J. (1989) *Free Radical Biol. Med.*, **6**, 593−597.

Ashburner,M., and Bonner,J.J. (1979) *Cell*, **17**, 241−254.

Baeuerle,P.A. (1991) *Biochim. Biophys. Acta*, in press.

Baeuerle,P.A. and Baltimore,D. (1988a) *Cell*, **53**, 211−217.

Baeuerle,P.A. and Baltimore,D. (1988b) *Science*, **242**, 540−546.

Baeuerle,P.A. and Baltimore,D. (1989) *Genes Dev.*, **3**, 1689−1698.

Baeuerle,P.A. and Baltimore,D. (1991) In Cohen,P. and Foulkes,J.G. (eds), *Molecular Aspects of Cellular Regulation, Hormonal Control Regulation of Gene Transcription*. Elsevier/North Holland Biomedical Press, Amsterdam pp. 409−432.

Baeuerle,P.A. Lenardo,M., Pierce,J.W. and Baltimore,D. (1988) *Cold Spring Harbor Symp. Quant. Biol.*, **53**, 789−798.

Ballard,D.W., Böhnlein,E., Lowenthal,J.W., Wano,Y., Franza,B.R. and Greene,W.C. (1988) *Science*, **241**, 1652−1655.

Ballard,D.W., Walker,W.H., Doerre,S., Sista,P., Molitor,J.A., Dixon,E.P., Peffer,N.J., Hannink,M. and Greene,W.C. (1990) *Cell*, **63**, 803−814.

Blake,D.R., Allen,R.E. and Lunec,J. (1987) *Brit. Med. Bull.*, **43**, 371−385.

Bours,V., Villalobos,J., Burd,P.R., Kelly,K. and Siebenlist,U. (1990) *Nature*, **348**, 76−80.

Bradford,M. (1976) *Anal. Biochem.*, **72**, 248.

Brownell,E., Ruscetti,F.W., Smith,R.G. and Rice,N.R. (1988) *Oncogene*, **3**, 93−98.

Cerutti,P.A. (1985) *Science*, **227**, 375−381.

Christiansen,N.O. (1988) *FEBS Lett.*, **239**, 195−198.

Ciavarra,R.P. and Simeone,A. (1990) *Cellular Immunol.*, **131**, 11−26.

Crossin,K.L. (1991) *Trends Biochem. Sci.*, **16**, 81−82.

Dignam,J.P., Lebovitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475−1489.

Duh,E.J., Maury,W.J., Folks,T.M., Fauci,A.S. and Rabson,A.B. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5974−5978.

Frei,B., Yamamoto,Y., Niclas,D. and Ames,B.N. (1988) *Anal. Biochem.*, **175**, 120−130.

Geelen,J.L.M.C., Minnaar,R.P., Boom,R., van der Nordaa,J. and Goudsmit,J. (1988) *J. Gen. Virol.*, **69**, 2913−2917.

Gennaro,R., Florio,C. and Romeo,D. (1986) *Biochem. Biophys. Res. Commun.*, **134**, 305−312.

Ghosh,S. and Baltimore,D. (1990) *Nature*, **344**, 678−682.

Ghosh,S., Gifford,A.M., Riviere,L.R., Tempst,P., Nolan,G.P. and Baltimore,D. (1990) *Cell*, **62**, 1019−1029.

Gorman,C.M., Merlino,G.T., Willingham,M.C., Pastan,I. and Howard,B.H. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 6777−6781.

Halliwell,B. and Gutteridge,J.M.C. (1989). *Free Radicals in Biology and Medicine*. Second Edition. Clarendon Press, Oxford.

Halliwell,B. and Gutteridge,J.M.C. (1990) *Methods Enzymol.*, **186**, 1−85.

Hohmann,H.-P., Brockhaus,M., Baeuerle,P.A., Remy,R., Kolbeck,R. and van Loon,A.P.G.M. (1990) *J. Biol. Chem.*, **265**, 22409−22417.

Johnson,P.F. and McKnight,S.L. (1989) *Annu. Rev. Biochem.*, **58**, 799−839.

Kawakami,K., Scheidereit,C. and Roeder,R.G. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4700−4704.

Kieran,M., Blank,V., Logeat,F., Vandekerckhove,J., Lottspeich,F., LeBail,O., Urban, M.B., Kourilsky,P., Baeuerle,P.A. and Israel,A. (1990) *Cell*, **62**, 1007−1018.

Klebanoff,S.J., Vadas,M.A., Harlan,J.M., Sparks,L.H., Gamble,J.R., Agosti,J.M. and Waltersdorph,A.M. (1986) *J. Immunol.*, **136**, 4220−4225.

Larrick,J.W. and Wright,S.C. (1990) *FASEB J.*, **4**, 3215−3223.

Lenardo,M.J., Fan,C.-M., Maniatis,T. and Baltimore,D. (1989) *Cell*, **57**, 287−294.

Leung,K. and Nabel,G.J. (1988) *Nature*, **333**, 776−778.

Link,E.M. and Riley,P.A. (1988) *Biochem. J.*, **249**, 391−199.

London,I.M., Levin,D.H., Matts,R.L., Thomas,N.S.B., Pteryshyn,R., and Chen,J.-J. (1987) In Boyer,P.D. and Krebs,E.G. (eds), *The Enzymes*. Academic Press, New York. Vol. 18, pp. 359−380.

Lowenthal,J.W., Ballard,D.W., Böhnlein,W. and Greene,W.C. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 2331−2335.

Matthews,N., Neale,M.L., Jackson,S.K. and Stark,J.M. (1987) *Immunology*, **62**, 153−155.

Matsubara,T. and Ziff,M. (1986) *J. Cell. Physiol.*, **127**, 207−210.

Meichle,A., Schütze,S., Hensel,G., Brunsing,D. and Krönke,M. (1990) *J. Biol. Chem.*, **265**, 8339−8343.

Meier,B., Radeke,H.H., Selle,S., Younes,M., Sies,H., Resch,K. and Habermehl, G.G. (1989) *Biochem. J.*, **263**, 539−545.

Meier,B., Radeke,H.H., Selle,S., Habermehl,G.G., Resch,K. and Sies,H. (1990) *Biol. Chem. Hoppe Seyler*, **371**, 1021−1025.

Meltzer,M.S., Skillman,D.R., Hoover,D.L., Hanson,B.D., Turpin,J.A., Kalter,D.C. and Gendelman,H.E. (1990) *Immunology Today*, **11**, 217−223.

Messer,G., Weiss,E.H. and Baeuerle,P.A. (1990) *Cytokine*, **2**, 389−397.

Nabel,G. and Baltimore,D. (1987) *Nature*, **326**, 711−713.

Nabel,G.J. and Baltimore,D. (1990) *Nature*, **344**, 178.

Osborn,L., Kunkel,S. and Nabel,G.J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 2336−2340.

Pacifici,R.E. and Davies,K.J.A. (1990) *Methods Enzymol.*, **186**, 485−502.

Paul,N.L., Lenardo,M.J., Novak,K.D., Sarr,T., Tang,W.-L. and Ruddle,N.H. (1990) *J. Virol.*, **64**, 5412−5419.

Pomerantz,R.J., Feinberg,M.B., Trono,D. and Baltimore,D. (1990) *J. Exp. Med.*, **172**, 253−261.

Roederer,M., Staal,F.J.T., Raju,P.A., Ela,S.W., Herzenberg,L.A. and Herzenberg,L.A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 4884−4888.

Rosen,G.M. and Freeman,B.A. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7269−7273.

Ruben,S., Dillon,P.J., Schreck,R., Henkel,T., Chen,C.-H., Maher,M., Baeuerle,P.A. and Rosen,C. (1991) *Science*, **251**, 1490−1493.

Sambucetti,L.C., Cherrington,J.M., Wilkinson,G.W.G. and Mocarski,E.S. (1989) *EMBO J.*, **8**, 4251−4258.

Schütze,S., Nottrott,S., Pfizenmaier,K. and Krönke,M. (1990) *J. Immunol.*, **144**, 2604−2608.

Sen,R. and Baltimore,D. (1986) *Cell*, **47**, 921−928.

Shirakawa,F. and Mizel,S.B. (1989) *Mol. Cell. Biol.*, **9**, 2424−2430.

Staal,F.J.T., Roederer,M., Herzenberg,L.A. and Herzenberg,L.A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9943–9947.
Stein,B., Rahmsdorf,H.J., Steffen,A., Litfin,M. and Herrlich, P. (1989) *Mol. Cell. Biol.*, **9**, 5169–5181.
Tauber,A.I., Cox,J.A., Curnutte,J.T., Carrol,P.M., Nakakuma,H., Warren,B., Gilbert, H. and Blumberg,P.M. (1989) *Biochem. Biophys. Res. Commun.*, **158**, 884–890.
Tong-Starksen,S.E., Luciw,P.A. and Peterlin,B.M. (1989) *J. Immunol.*, **142**, 702–707.
Twu,J.-S., Chu,K. and Robinson,W.S. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5168–5172.
Urban,M.B. and Baeuerle,P.A. (1990) *Genes Dev.*, **4**, 1975–1984.
Urban,M.B., Schreck,R. and Baeuerle,P.A. (1991) *EMBO J.*, in press.
Valerie,K., Delers,A., Bruck,C., Thiriart,C., Rosenberg,H., Debouck,C. and Rosenberg, M. (1988), **333**, 78–81.
Visvanathan,K.V. and Goodbourn,S. (1989) *EMBO J.*, **8**, 1129–1138.
Weiss,R.A., Clapham,P.R., Weber,J.N., Dalgleish,A.G., Lasky,L.A. and Berman.,P.W. (1986) *Nature*, **324**, 572–575.
Wong,G.H.W., Elwell,J.H., Oberley,L.W. and Goeddel,D.V. (1989) *Cell*, **58**, 923–931.
Wolff,S.P., Garner,A. and Dean,R.T. (1986) *Trends Biochem. Sci.*, **11**, 27–31.
Zabel,U. and Baeuerle,P.A. (1990) *Cell*, **61**, 255–265.
Zabel,U., Schreck,R. and Baeuerle,P.A. (1991) *J. Biol. Chem.*, **266**, 252–260.
Zimarino,V. and Wu,C. (1987) Nature, **327**, 727–730.
Zimmerman,R.J., Marafino,B.J.Jr., Chan,A., Landre,P. and Winkelhake,J.L. (1989) *J. Immunol.*, **142**, 1405–1409.

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Note added in proof

While this manuscript was in press, Devary *et al.* (*Mol. Cell. Biol.*, **11** (1991), 2804–2811) also reported that the DNA binding of AP-1 transcription factor is induced with H_2O_2 treatment. In the study, 250 μM H_2O_2 was used for stimulation which might be the reason why we have not detected AP-1 DNA binding with H_2O_2 treatment at 100 μM (see Figure 3).