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Transcription Factor NF-κB Is Activated by Photosensitization Generating Oxidative DNA Damages

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The abbreviations used are: HIV, human immunodeficiency virus; PF, proflavine; LTR, long terminal repeat; IL, interleukin; TNF, tumor necrosis factor; ROI, reactive oxygen intermediate; EMSA, electrophoretic mobility shift assay; PMA, phorbol 12-myristate 13-acetate; CHX, cycloheximide; NAC, N-acetyl-L-cysteine.

Abstract : Reactive oxygen intermediates like hydrogen peroxide (H_2O_2) have been shown to serve as messengers in the induction of NF-κB and, then, in the activation and replication of human immunodeficiency virus (HIV)-1 in human cells. Because H_20_2 can be converted into the highly reactive OH' at various locations inside the cells, we started to investigate the generation of Reactive oxygen intermediates by photosensitization. This technique is based on the use of a photosensitizer which is a molecule absorbing visible light and which can be located at various sites inside the cell depending on its physicochemical properties. In this work, we used proflavine (PF), a cati-onic molecule having a high affinity for DNA, capable of intercalating between DNA base pairs. Upon visible light irradiation, intercalated PF molecules oxidize guanine residues and generate DNA single-strand breaks. In lymphocytes or monocytes latently infected with HIV-1 (ACH-2 or U1, respectively), this photosensitizing treatment induced a cytotoxicity, an induction of NF-κB, and a reactivation of HIV-1 in cells surviving the treatment. NF-κB induction by PF-mediated photosensitization was not affected by the presence of N-acetyl-L-cysteine while strong inhibition was recorded when the induction was triggered by H_20_2 or by phorbol 12-myristate 13-acetate. Another transcription factor like AP-1 is less activated by this photosensitizing treatment. In comparison with other inducing treatments, such as phorbol 12-myristate 13-acetate or tumor necrosis factor *α*, the activation of NF-κB is slow, being optimal 120 min after treatment. These kinetic data were obtained by following, on the same samples, both the appearance of NF-κB in the nucleus and the disappearance of IκB-α in cytoplasmic extracts. These data allow us to postulate that signaling events, initiated by DNA oxidative damages, are transmitted into the cytoplasm where the inactive NF-κB factor is resident and allow the translocation of p50/p65 subunits of NF-κB to the nucleus leading to HIV-1 gene expression.

Considerable interest has been focused recently on the role that some host transcriptional factors may play in the initial activation of human immunodeficiency virus (HIV-1)[1] gene expression by interacting with the long terminal repeat (LTR) of the integrated provirus (1). Transcriptional activation of the LTR depends largely on a major enhancer made of two directly repeated sequences able to respond to the transcription factor NF-κB (2, 3). The consensus recognition site for this factor is a decamer with two pentameric half-sites, each of which participates in the recognition and stabilization of binding of the NF-κB dimer (for a review, see Refs. 4 to 6). For this DNA binding to occur and HIV-1 transcription to be initiated, NF-κB usually associating p50 and p65 subunits must be translocated into the nucleus from the cytoplasm where it is normally retained by interaction with its inhibitory subunit named IκB (7, 8). Related κB enhancers are present in the regulatory region of various cellular genes like interleukin-2 (IL-2), IL-2 receptor-α, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and have further been shown to be functionally involved in the transcriptional induction by various T-cell stimulants (9). Functionally active NF-κB complexes are induced after cellular activation in one of the following ways: through the CD3-T-cell receptor complex in T-lymphocytes, in response to antigen recognition (10,11) or to anti-CD3 antibodies (12), or following stimulation with other inducers such as phorbol esters, selected cytokines, and lipopolysaccharide in both lymphocytes and monocytes-macrophages (13-15).

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Treatment of T-lymphocytes with hydrogen peroxide (H_20_2) induces NF-κB **DNA** binding activity and nuclear appearance of this factor (16) followed by a transcriptional activation of the proviral DNA in cells latently infected with HIV-1 (17). The activation of NF-κB by treatment of T-cells with H_20_2 appears to be a specific event because it occurs at low extracellular concentrations, and other DNA-binding proteins do not seem to be affected (16). Presumably, the mechanism involves a passive diffusion of H_20_2 through the cell membrane where it would trigger indirectly IκB phosphorylation and its controlled proteolytic degradation, through a cytoplasmic chymotrypsin-like protease, providing therefore an irreversible NF-κB activation (18).
Eukaryotic and prokaryotic cells produce reactive oxygen intermediates (ROIs) continuously as side products of the mitochondrial electron transfer chain reaction but also upon exposition to solar radiations (UV and visible light) or γ-rays (reviewed in Refs. 19 and 20). Most inducers of NF-κB seem to rely on the production of ROIs as evidenced by the inhibitory effect of antioxidants such as cysteine derivatives (16, 21), metal chelators and dithiocarbamates (16, 22, 23), vitamin E and α-lipoic acid (24). It has then been proposed that ROIs serve as common messengers in the activation of NF-κB (16) and that NF-κB is primarily an oxidative stress-responsive transcription factor.
The molecular pathways leading to the critical dissociation of NF-κB heterodimers from IκB are not yet fully elucidated, especially the nature of the biochemical events capable of initiating the redox controlled pathway. With UV, several authors have postulated that DNA damages can trigger the events leading to the activation of NF-κB and then to HIV-1 gene expression. The demonstration that the initial event in UV activation of HIV-1 could well be lesions induced to DNA has been obtained by (i) determining the action spectrum of UV-induced HIV-1 gene expression (25), (ii) demonstrating that the HIV-1 LTR is activated by much lower doses of UV when it is resident in a cell from the repair-deficient disease, xeroderma pigmentosum, than when it is in a repair-proficient cell (25), and (iii) by abrogation of the viral gene activation when cells are fused with liposomes encapsulating T4 endonuclease V (26). Thus, a signal which leads to HIV-1 gene expression could be initiated in the nucleus by UV. It would migrate to the cytoplasm where it activates NF-κB by releasing its inhibitory subunit IκB and allowing p50/p65 subunits to translocate to the nucleus where they bind to their DNA responsive elements situated in the LTR of the HIV-1 provirus. However, it has also been shown (27, 28) that the mammalian UV response is triggered by the activation of tyrosine kinases situated at the plasma membrane level, and, also, cells anucleated by cytocha-lasin B treatment are still fully responsive to UV in terms of NF-κB induction. Thus, HIV-1 activation by UV-C could well involve two distinct pathways, one being initiated by UV-prod-ucts in DNA such as pyrimidine dimers, the other through oxidative damages induced in membranes. In this paper, we show that oxidative DNA modifications like 7,8-dihydro-oxyo-guanine and single-stranded breaks, generated by a photosen-sitizer which intercalates between DNA base pairs (29, 30), can activate NF-κB and HIV-1 reactivation, supporting the idea that oxidative DNA damages would be molecular intermediates able to trigger a signaling pathway to the cytoplasm.

Materials and methods

Cell Culture

The lymphocytic ACH-2 and the promonocytic U1 cell lines are both cultivated at 37 °C in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 100 units of penicillin/ml and 100 mg of streptomycin/ml.

Exposure of the U1 and ACH-2 Cell Lines to Photosensitization

Exponentially growing cells were washed with PBSG (140 mM NaCl, 3 mM KCl, 8 mM Na_2HP0_4, 1 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% glucose, pH 7.4) and resuspended in this buffer at a concentration of 2.5 x 10^6 cells/ml. PF was added at different concentrations (between 1 and 0.1 mM) to cells suspended (in PBSG) for 15 min in the dark before irradiation to allow its penetration inside the cells. Before irradiation, cells were washed in PBSG to eliminate extracellular and noninterca-lated PF. At various times of irradiation with filtered light (Osram XBO-150, Germany, $\lambda > 360$ nm, 40 watts/m^2), cells were collected and replaced in culture. Between 30 and 240 min after the treatment, aliquots were removed and used to prepare nuclear and cytoplasmic extracts. After 24 and 48 h, cells were counted and aliquots of supernatant fluid were removed for the determination of the virus

associated-reverse transcriptase activity. PF localization inside the cell was determined by fluorescence using either a conventional (Zeiss) or a confocal microscope (Meridian).

Reverse Transcriptase Assay

The reverse transcriptase assay was done as described (29). Briefly, the virus was purified and concentrated by ultracentrifugation of supernatant fluids (131,000 X *g* at 4 °C for 2 h). Pellets were resuspended in 25 µl of TNE (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA) containing 0.1% Triton X-100 and left on ice for 30 min. Then, 10 µl of the virus preparations were incubated at 37 °C for 60 to 90 min with 40 µl of a solution containing 62.5 mM Tris-HCl, pH 7.8, 6.25 mM dithiothreitol, 6.25 mM $MgCl_2$, 180 mM KCl, 0.06% Triton X-100, 0.375 mM glutathione, 0.325 mM EGTA, 31 mg/ml bovine serum albumin, 2.5% ethylene glycol, 250 milliunits/ml poly(rA)-oligo(dT), and 125 µCi/ml [^{3}H]deoxythymidine. The DNA resulting from the extension reaction carried out by the reverse transcriptase was precipitated with 10% trichloroacetic acid and filtered through 2.4-mm Whatman GF-A filters. Filters were rinsed with 0.01 M sodium pyrophosphate, dried with 95% ethanol, and then counted by liquid scintillation.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were isolated as described by a rapid micropreparation technique based on the use of a hypotonic lysis followed by high salt extraction of nuclei (30) and which is derived from the large scale procedure of Dignam *et al.* (31). Binding reactions were performed for 25 min at room temperature with 3-5 µg of total protein in 10 to 20 µl of 20 mM Hepes-KOH, pH 7.9, 75 mM NaCl, 1 mM EDTA, 5% glycerol, 0.5 mM $MgCl_2$,1 *µg* of aeetylated bovine serum albumin, 1.5 µg of poly(dI-dC)-poly(dI-dC) (Pharmacia Biotech Inc.), 1 mM dithiothreitol, and 0.2 ng of ^{32}P-labeled oligonucleotides (Eurogentech, Belgium). Oligonucleotides were labeled by end-filling with the Klenow fragment of *Escherichia coli* DNA polymerase (Boehringer Mannheim) with [^{32}P]dATP, [^{32}P]dCTP (DuPont NEN), and cold dTTP + dGTP. Labeled probes were purified by spin chromatography on Sephadex G-25 columns. DNA-protein complexes were separated from unbound probe on native 6% polyacrylamide gels at 150 V in 0.25 M Tris, 0.25 M sodium borate, 0.5 mM EDTA, pH 8.0. Gels were vacuum-dried and exposed to Fuji x-ray films at -80 °C for 16 to 24 h. The amount of specific complexes were determined either by counting the radioactivity with a Phosphorlmager (Molecular Dynamics) or by photodensitometry (LKB) of the autoradiography. Supershift experiments were carried out as described (32) and following the same EMSA protocol as described above except that the gel concentration is 4%, The sequences of the probes used in this work are:

Wild-type NF-κB probe:
5′-GATCAGGGACTTTCCGCTGGGGACTTTCCAG
TCCCTGAAAGGCGACCCCTGAAAGGTCCTAG-5′

Mutated NF-κB probe:
5′-GATCACTCACTTTCCGCTGCTCACTTTCCAG
TGAGTGAAAGGCGACGAGTGAAAGGTCCTAG-5′

IκB-α Detection

IκB-α subunit was detected by Western blot analysis using specific antibodies. Cytoplasmic extracts were prepared at various times after the PF-mediated photosensitization by hypotonic lysis, pelleting of the nuclei, and collecting the supernatant fraction (32). Cytoplasmic proteins were added to a loading buffer (10 mM Tris-HCl pH 6.8, 1% SDS, 25% glycerol, 0.1 mM β-mercaptoethanol, 0.03% bromphenol blue), boiled, and electrophoresed on a 10% poly-acrylamide-SDS gel and electrotransferred to Immobilon-P membranes (Millipore). Filters were incubated in primary antibody for 60 min at 37 °C (1:2000 dilution), then in peroxidase-conjugated goat anti-rabbit IgG (1:250 dilution) for 60 min at 37 °C, and finally analyzed using Amersham's enhanced chemiluminescence system (ECL) (Amersham) using Fuji x-ray films.

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DNA *Modifications*

Promonocytic and lymphocytic cell lines were cultivated as described above. Irradiation in the presence of PF (0.25 and 1.0 μM) or rose bengal (1.0 and 2.5 μM) was carried out in PBSG on ice in a shallow dish (10^6 cells/ml). Subsequent damage analysis by alkaline elution analysis is described elsewhere (33, 34); it follows essentially the protocol of Kohn *et al.* (35) with an additional incubation with a repair endonuclease solution (3 μg/ml formamidopyrimidine-DNA glycosylase protein) which was applied after cell lysis and prior to treatment with proteinase K. To quantify strand breaks, this incubation was carried out without endonuclease.

Results

An oxidative stress mediated by H_2O_2 can lead to the HIV-1 reactivation (17) through the activation of the cellular factor NF-κB (16, 22, 36). Several authors speculated that H_20_2 can passively cross the plasma membrane and, within the cell, is either converted by catalases into H_20 and O_2, or, by the Fenton reaction into hydroxyl radicals (OH'). Because H_20_2 can be converted into the highly reactive OH radical at various cellular localizations, it was unclear from these data what type of cellular oxidative damage triggers NF-κB activation and then HIV-1 reactivation. To try to clarify whether or not DNA oxidative damages can participate in the NF-κB activation pathway, we have used photosensitization reactions to generate ROIs in the DNA of lymphocytic (ACH-2) or monocytic (U1) cell lines latently infected by HIV-l. The photosensitizer used in this work is proflavine (PF) (Fig. 1A) which is a water-soluble cationic chromophore having an important affinity for DNA (37, see Ref. 38 for review).

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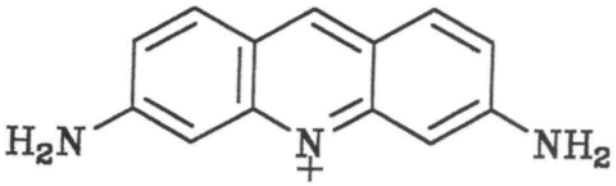
A



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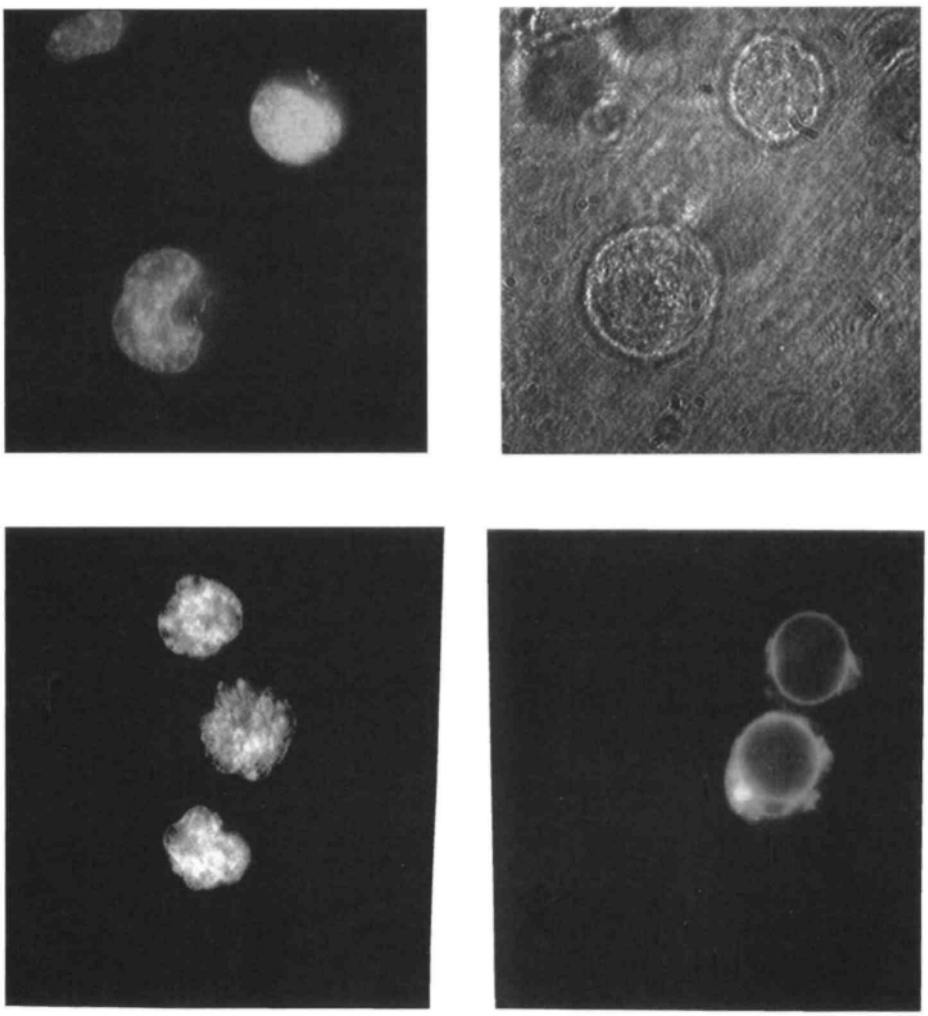

FIG. 1. A, chemical structure of proflavine (PF). β, localization of proflavine in ACH-2 cells. Cells were mixed with 2 μM PF in the dark and mounted on slides before being observed by confocal fluorescence microscopy (λ_{exc} = 450 nm). The two upper panels show cells mixed with 2 μM PF observed under fluorescence (left panels) or by phase contrast (right panels). The two lower panels show cells mixed with PF at 2 μM (left panels) or with mero-cyanine 540 at 5 μM (right panels).

PF intercalates between DNA base pairs, and, when mixed with ACH-2 or U1, it binds to DNA and is localized in the cell nucleus (Fig. IS). No chro-mophore fluorescence was detected outside the cell nucleus either by classical fluorescence microscopy or by confocal microscopy using a highly sensitive camera even in the presence of a large molar excess of PF compared to the concentrations used in the following experiments (Fig. 1*1*3). No PF fluorescence was detected in the cytoplasm or in membrane and the nuclear restriction of PF is particularly striking when compared with a membrane-associated

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chromophore such as merocyanine 540 (Fig. 1B).

Photosensitization by PF Produces DNA Oxidative Damages

Promonocyte U937 and lymphocytic CEM T-cells, which are the parental cell lines used to generate U1 and ACH-2 cell lines, respectively, were mixed with PF to characterize and quantify DNA damages induced by PF-mediated photosensitization. The irradiation of PF with visible light is known to generate singlet oxygen ($^1O_2$) by an energy transfer mechanism (type II reaction) and a so-called type I reaction causing electron transfer from guanine to PF (see Refs. 39 and 40 for reviews). These two photochemical pathways are known to generate guanine oxidation products and single-strand breaks (41). In order to clarify the specificity of DNA oxidation products induced by PF-mediated photosensitization, the amount of DNA lesions induced by this treatment was compared to those obtained by photosensitization with rose bengal which is an anionic photosensitizer known to enter inside cells and to localize predominantly in the cell cytoplasm (42). Rose bengal photosensitization generates singlet oxygen but also superoxide anion which can be dismutated into H_20_2 (43).

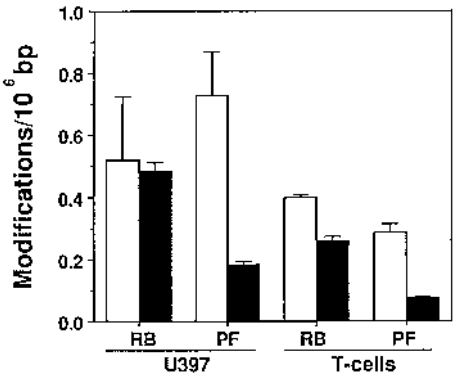

FIG. 2. Damage profiles induced either by proflavine (PF) or rose bengal (RB) in promonocytic cells (U397 cells) treated with 1.0 μw PF and 2.5 μM rose bengal, respectively, and lymphocytic cells (T-cells) treated with 0.25 μM PF and 1.0 μM rose bengal, respectively. These cells were irradiated for 20 s with a 1000-watt halogen lamp emitting visible light. Columns indicate the number of DNA single strand breaks (open bars) and formamidopyrimidine-DNA glycosylase-sensitive sites (filled bars) as determined by alkaline elution. Data are means of two independent experiments.

DNA single-stranded breaks and DNA modifications sensitive to the repair endonuclease formamidopyrimidine-DNA glycosylase protein were quantified by means of an alkaline elution technique. Formamidopyrimidine-DNA glycosylase protein is known to recognize 7,8-dihydro-8-oxoguanine residues in DNA, in addition to sites of base loss and formamidopyrimidines (44, 45). Results obtained are shown in Fig. 2 for both cell lines. In the reactions photosensitized by PF, formamidopyrimidine-DNA glycosylase-sensitive sites (0.75 and 0.3 modifications per 106 base pairs for U937 and CEM cells, respectively) are induced in high excess of single-stranded breaks (0.18 and 0.08 modifications per 10^β base pairs for U937 and CEM cells, respectively). The predominant formation of formamidopyrimi-dine-DNA glycosylase-sensitive base modifications (7,8-dihy-dro-8-oxoguanine) is characteristic for the DNA damage induced by singlet oxygen and type I photosensitization and was described for L1210 cells exposed to acridine orange plus light, a photosensitizer closely related to PF (33). In the case of rose bengal, single-stranded breaks and formamidopyrimidine-DNA glycosylase-sensitive modifications are induced in almost the same amount (Fig. 2). This confirms that DNA damages by rose bengal were generated indirectly, *e.g.* via hydroxyl radicals formed in a Fenton reaction or via nucleases, which would be activated by damage to membranes or calcium stores. These indirect mechanisms are consistent with the prominent localization of rose bengal in the cytoplasm.

PF Photosensitization Is Cytotoxic and Reactivates HIV-1 from Latently Infected Cells

Cell survival is strongly affected by the PF-mediated photosensitization reaction. Fig. *3A* shows that

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this treatment induces an important lethal effect which increases with the irradiation time. ACH-2 cells turns out to be somewhat more sensitive than the U1 cell line. For each cell line, the level of cytotoxicity is also proportional to PF concentration up to 1.5 μM (Fig. 3*13*). Cell irradiation with visible light only does not lead to any cytotoxicity demonstrating that a chromophore is required to induce a lethal damage.

Among the cells surviving PF photosensitization, HIV-1 reactivation can be detected by measuring reverse transcriptase activity in the cell supernatant 24 and 48 h after the pho-totreatment (Fig. 4). After 24 h, the level of reverse transcriptase activity is still relatively low (data not shown), but becomes maximal 48 h after photosensitization. Two important results can be deduced from reverse transcriptase measurements: (i) reverse transcriptase values increase as a function of stress intensity, and (ii) for similar survival fractions, reverse transcriptase activities are somewhat higher for ACH-2 than for U1 cells.

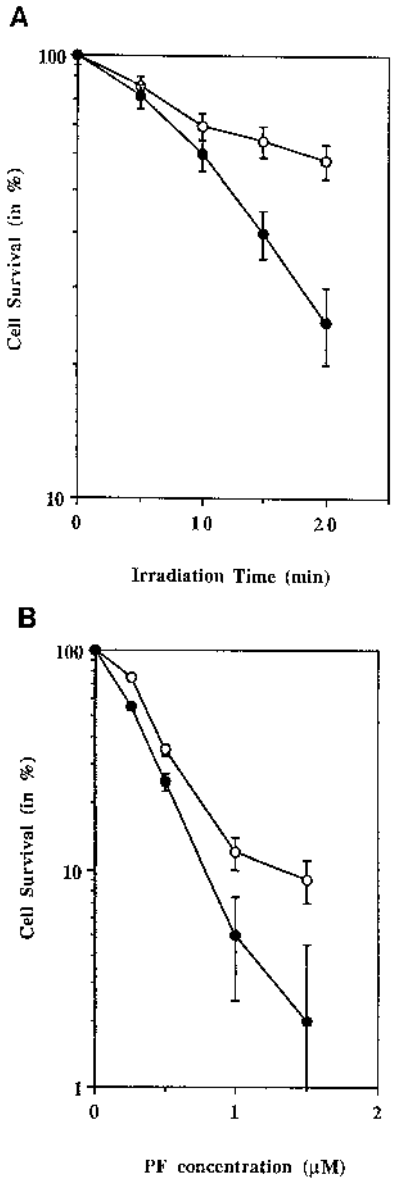

FIG. 3. A, cytotoxic effect induced 48 h after the oxidative stress induced in ACH-2 cells (filled symbols) or in U1 cells (open symbols) photoreacted with proflavine during various irradiation times (PF, 1.0 mM for U1 and 0.25 μM for ACH-2 cells, respectively). Percentage of cell survival (log_{10}) is plotted versus irradiation time (min). B, cytotoxic effect induced 48 h after the oxidative stress induced in ACH-2 cells (filled symbols) or in U1 cells (open symbols) photoreacted with increasing PF concentrations and irradiated with visible light for 10 min. Percentage of cell survival (log_{10}) is plotted versus PF concentration (μM).

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These results indicate also that the cells have to be cultured for at least 48 h after the stress to release virus particles in the supernatant. 30% survival seems to correspond to a stress intensity leading to the optimal virus reactivation. In other words, when the survival fraction is too low, there are not enough viable cells in culture to proliferate and to reactivate HIV-1.

Photosensitization Mediated by PF Activates NF-κB

ACH-2 cells are widely used not only for studying HIV-1 reactivation (14) but also because they provide an excellent model for investigating the T-cell activation processes (46). This cell line is known to respond to treatments with phorbol ester (PMA), lectins, and TNF-α producing the activation of NF-κB but also of NF-κB-controlled genes (46, 47).

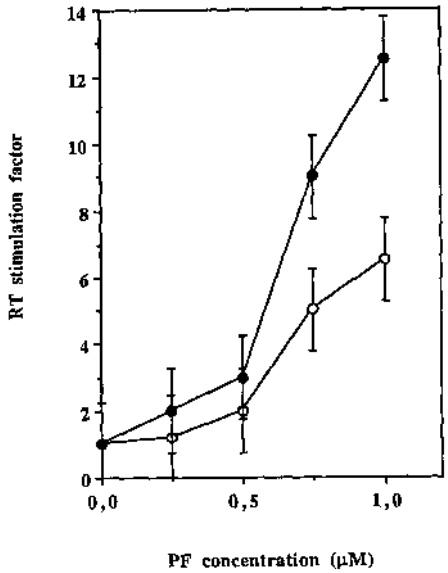

FIG. 4. Induction of HIV-1 reactivation in ACH-2 cells (filled symbols) or in U1 cells (open symbols) mediated by PF (concentrations are the same than in Fig. 3A). Supernatant fluids, corresponding to an identical cell number, were taken 48 h after the photo-sensitization to determine reverse transcriptase activities as described by Ref. 29. The stimulation of the reverse transcriptase activity is the ratio between the activities measured for various PF concentrations and the initial value. Stimulation of reverse transcriptase is plotted versus PF concentration (μM).

Because an oxidative stress mediated by H_2O_2 is known to strongly activate NF-κB (16, 22, 36), we have investigated whether or not the PF-mediated photosensitization can activate this transcriptional factor. ACH-2 cells were incubated in PBSG in the presence of various concentrations of PF before being irradiated with visible light for 10 min. After the photosensitization, the cells were replaced in culture for at least 120 min before preparing nuclear extracts and analyzing DNA binding to a NF-κB probe using EMSA. As shown in Fig. 5A, PF-mediated photosensitization induced an activity that retarded, in native gels, a ^{32}P-labeled DNA probe encompassing the decameric NF-κB motif from the HIV-1 enhancer. The NF-κB activity was already significantly increased at a very low PF concentration (0.25 μM) corresponding to one PF molecule intercalated per several hundreds of DNA base pairs to be maximum at 1.0 μM (Fig. 5B). Above this concentration, the stimulation of the NF-κB activity starts to decrease probably because of the shielding effect of PF molecules stacked along the sugar-phosphate backbone of the DNA (37) or due to a too important cytotoxic effect observed at these PF concentrations (Fig. *5B).* No NF-κB DNA binding activity could be detected during cell irradiation with visible light only (in the fluence range used in this work) or with PF alone. On the other hand, the NF-κB activity could also be detected at a low PF concentration (0.2 μM) and at very low fluences (Fig. 5C). Photosensitization reactions carried out during increased times (from 0 to 15 min) lead to a linear accumulation of NF-κB activities. Because NF-κB activities were determined in HIV-1-infected cells (ACH-2), it was possible to evaluate both the degree of NF-κB stimulation and the level of reactivation by reverse transcriptase measurements (Fig. 5C). From these experiments, it turned out

that the oxidative stress mediated by PF leads to higher stimulation of NF-κB activities than of HIV-1 reactivation, demonstrating that the induction of this factor is very sensitive to PF-mediated photosensitization.

The level of NF-κB activation by the PF-mediated photosen-sitization was compared to those induced by well-characterized agents such as H_2O_2 and PMA. EMSA shows that NF-κB DNA binding activity is stimulated at comparable levels by PF and H_2O_2 (data not shown). Indeed, the stimulation level reached by the PF-mediated treatment at 1.0 μM is similar to the one obtained after treatment of ACH-2 cells with 250 μM H_20_2, demonstrating that PF-mediated photosensitization is very efficient in inducing NF-κB activity. PMA also induces comparable levels of stimulation (data not shown).

The identity of the PF-activated DNA-protein complex with a NF-κB probe was further investigated by several different ways. First, by competition analysis (Fig. 6A) and by the use of antisera specific for the DNA-binding p50 and p65 subunits of NF-κB (Fig. 6B). Nuclear extracts were prepared 240 min after PF-mediated photosensitization and were incubated with a ^{32}P-labeled NF-κB probe alone or in the presence of increasing amounts of two different unlabeled competitor oligonucleotides. Competition with either a 25- or 250-fold molar excess of an oligonucleotide encompassing the same NF-κB elements of HIV-1 eliminated the formation of the radioactive protein-DNA complex induced by PF-mediated photosensitization. Competition experiments carried out with this competitor mutated at the NF-κB motifs did not abolish the binding, demonstrating unambiguously the κB-specific DNA binding of the PF-activated factor. The binding of one minor activity to the DNA probe was only weakly influenced at a high molar excess of both competitors demonstrating that this DNA binding activity was not sequence-specific (Fig. 6A).

Next, we have examined whether the PF-activated protein-DNA complex could react with antisera raised against the DNA-binding p50 and p65 subunits of NF-κB. These two sera clearly gave rise to a characteristic supershift of the retarded complex demonstrating unambiguously that the PF-activated factor is p50- and p65-containing NF-κB (Fig. 6*B).*

Another characteristic of NF-κB is its activation by a post-translational mechanism involving the release of the inhibitory subunit IκB from a cytoplasmic inactive form (7). In order to investigate whether the activation of NF-κB by PF-mediated photosensitization involved a post-translational mechanism, the photosensitization reaction was performed in the presence of cycloheximide (CHX), a protein synthesis inhibitor (Fig. 6C). When ACH-2 cells alone were treated with 50 μg/ml CHX, only a very weak activation of NF-κB was seen. On the other hand, NF-κB activity induced by PF photosensitization was not affected by the presence of CHX indicating that, in these conditions, NF-κB is activated by a post-translational mechanism. Because several cytokines such as IL-1 and TNF-α are efficiently processed from precursors only under inflammatory conditions (48, 49), an autocrine mechanism involving IL-1 or TNF-α release could be rather unlikely in this case.

NF-κB Activation by PF Photosensitization Is Not Affected by NAC

In order to ensure that NF-κB induction by PF-mediated photosensitization is triggered by DNA oxidative damages and not by ROIs leaking from the nucleus to the cytoplasm, ACH-2 cells were cultivated in the presence of 30 mM N-acetyl-L-cysteine (NAC) for 1 h. NAC possesses two main characteristics: (i) it is an antioxidant compound which can directly react with ROIs (16) and (ii) it functions as a precursor of reduced glutathione (GSH) capable of replenishing intracellular GSH concentration, thereby protecting cells from the effects of ROIs (50). Due to their physicochemical properties, neither NAC nor GSH can interact with DNA or can modify DNA-PF complexes, but they both seem to be localized mainly in the cell cytoplasm or in mitochondria. ACH-2 cells were preincubated with NAC before being stressed by PF-mediated photosensitization or H_20_2 or PMA.

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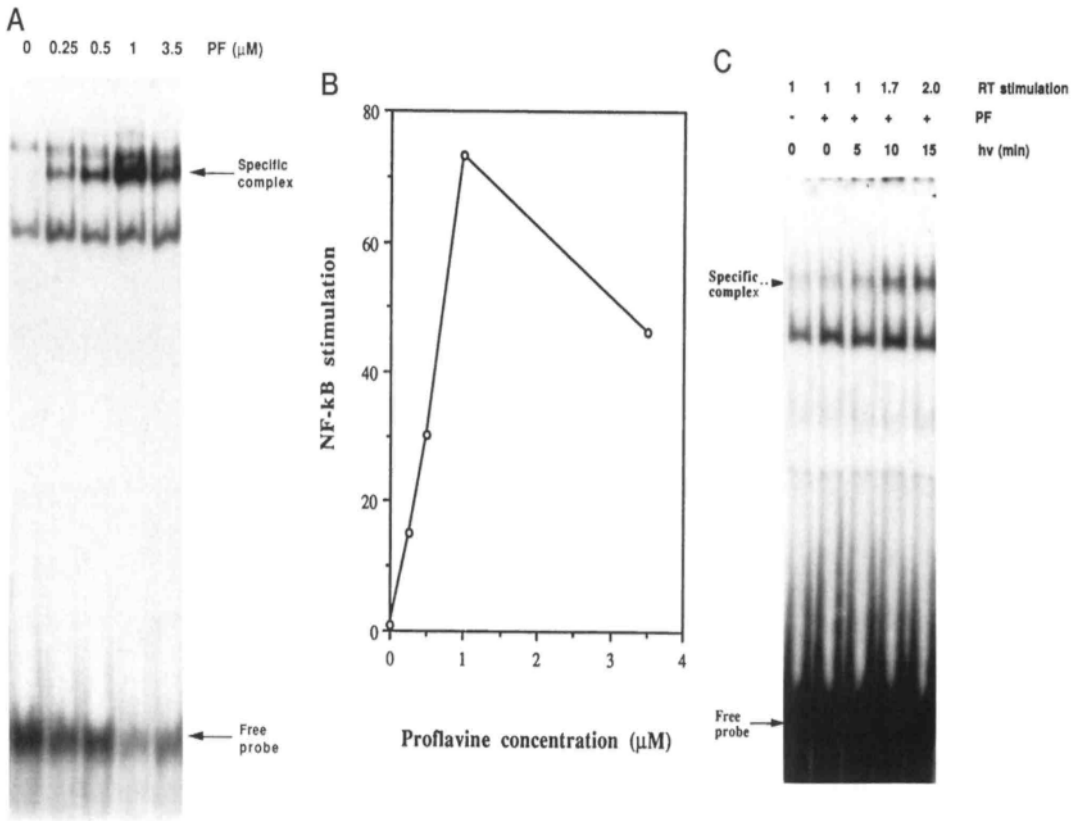

FIG. 5. The effect of a photoreaction mediated by PF on κB-DNA binding activities in ACH-2 cells. A, rapid induction of a nuclear κB enhancer DNA-binding protein by treatment of ACH-2 cells with increasing concentrations of PF and irradiated for 10 min with visible light. Nuclear extracts were prepared 240 min after the reaction with equal amounts of protein and mixed with a ^{32}P-labeled probe encompassing the κB elements of the HIV-1 enhancer. Samples were loaded on 6% native polyacrylamide gels and electrophoresed at 150 V. An autoradiogram of the gel is shown, and the arrows indicate the position of the specific complex and of the free probe. B, amount of κB DNA binding activity detected in the nucleus of ACH-2 cells is plotted as a function of the PF concentrations. C, appearance of a nuclear κB enhancer DNA-binding protein by treatment of ACH-2 cells with PF (0.2 μM) and irradiated with visible light between 0 and 15 min. The sample noted - corresponds to cells treated with PF and nonirradiated. Nuclear extracts were prepared in equal protein amounts 240 min after the reaction and mixed with a ^{32}P-labeled probe encompassing the κB elements of the enhancer of the HIV-1 LTR. Samples were loaded on 6% native polyacrylamide gels and electrophoresed at 150 V. Autoradiogram of the gel is shown, and the arrows indicate the position of the specific complex and of the free probe.

The addition of NAC to the culture media effectively protects ACH-2 cells against NF-κB activation induced by either H_20_2 or PMA (Fig. 7); confirming in these cases that cytoplasmic ROIs are involved in the pathway leading to IκB phosphorylation and to NF-κB activation (16, 22). In the case of PF-mediated photosensitization, the induction of NF-κB turns out to be almost unaffected by NAC (Fig. 7). At all the PF concentrations and the irradiation times tested, NF-κB is induced at the same level in the presence or in absence of NAC; demonstrating that (i) the generation of oxidative DNA damages by intercalated PF molecules was not affected by NAC or GSH which did not interact with DNA and were localized in the cytoplasm and (ii) ROIs generated by photoexcited PF reacted almost completely with DNA without any leakage in the cytoplasm.

NF-κB Activation by PF Photosensitization Is Slow

We have also followed the appearance of NF-κB in the nucleus (Fig. 8A) in connection with the disappearance of IκB-α (formerly MAD3, Ref. 51) in the cytoplasmic extract (Fig. *8B).* ACH-2 cells

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were photosensitized with PF in the same conditions as mentioned above, then replaced in culture. Aliquots were prepared at various times after the stress to follow both the induction of NF-κB in connection with the decrease of the total amount of IκB-α. In both untreated cells or at very short times after the stress, IκB-α-specific IgG detected a single 38K band on Western blots. Between 60 and 90 min after the stress, the amount of IκB-α strongly decreased from cytoplasm (Fig. *SB),* coinciding with the appearance of NF-κB activity in the nucleus (Fig. 8A). These results confirmed the causal relationship between the two biochemical events. However, the kinetic data presented here are quite different from those reported by several authors (52-54) who followed both IκB and NF-κB after stimulation of either T-cells or 70Z/3 pre-B cells by various inducers such as PMA, TNF-α, IL-lβ, and lipopolysaccharide. These authors reported that IκB-α started to disappear very quickly (5 min) after the induction with PMA, TNF-α, and IL-lβ, the reaction being somewhat slower in the case of lipopolysaccharide (between 30 and 60 min). In the case of the stress mediated by PF, the reaction is much slower (90 min), but, despite these kinetic differences, the depletion of IκB-α is coincident with the appearance of NF-κB in the nucleus. These data strongly support the idea that the mechanism of NF-κB activation after a stress mediated by PF involves intermediates different from those implicated in the NF-κB activation by PMA, TNF-α, or ILl-β, the action of which being located at the cytoplasmic membrane.

AP-1 Is Very Weakly Activated by PF

To determine whether or not the activation of NF-κB by the PF-mediated oxidative stress is characteristic of this type of treatment, we have examined the induction of AP-1, another DNA binding activity with responsive elements in the HIV-1 LTR. ACH-2 cells were treated with PF as described above and replaced directly in culture for various times. Nuclear extracts from control and PF-treated cells were incubated with a ^{32}P-labeled probe encompassing the TRE of the type I collagenase gene promoter.

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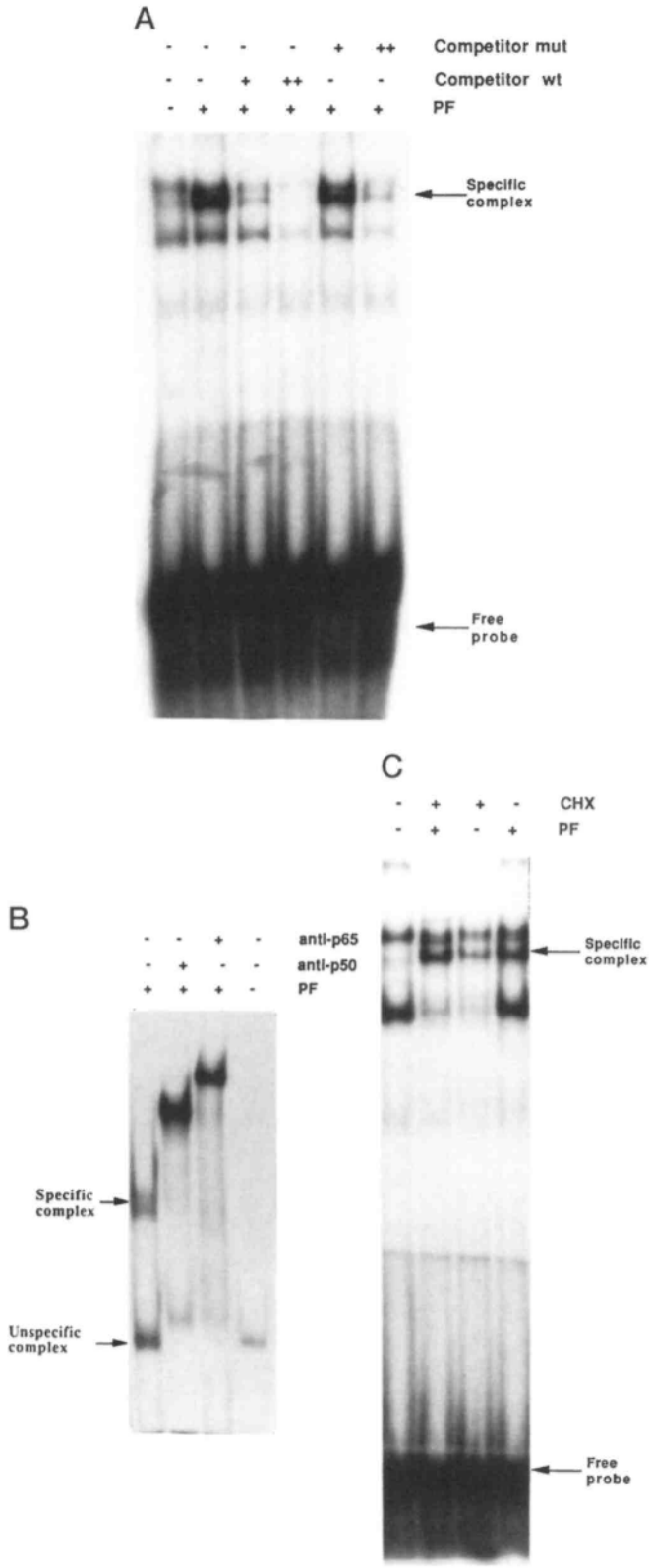

FIG. 6. Characterization of the PF-induced DNA binding activity as being NF-κB. A, competition analysis: nuclear extracts from ACH-2 cells photoreacted for 10 min with 3.5 μM PF were prepared 240 min after the stress and analyzed by EMSA as described in Fig. 5. Nuclear extracts were either mixed directly with the ^{32}P-labeled probe or with the labeled probe in the presence of 25 (+) or 250 (+ +) molar excess of wild type or mutated κB site of the HIV-1 enhancer (77). B, immunoreactivity of the PF-inducible protein-κB enhancer complex. Nuclear extracts from ACH-2 cells photoreacted with PF (1 μM) for 10 min were either mixed directly with the ^{32}P-labeled κB probe or incubated with

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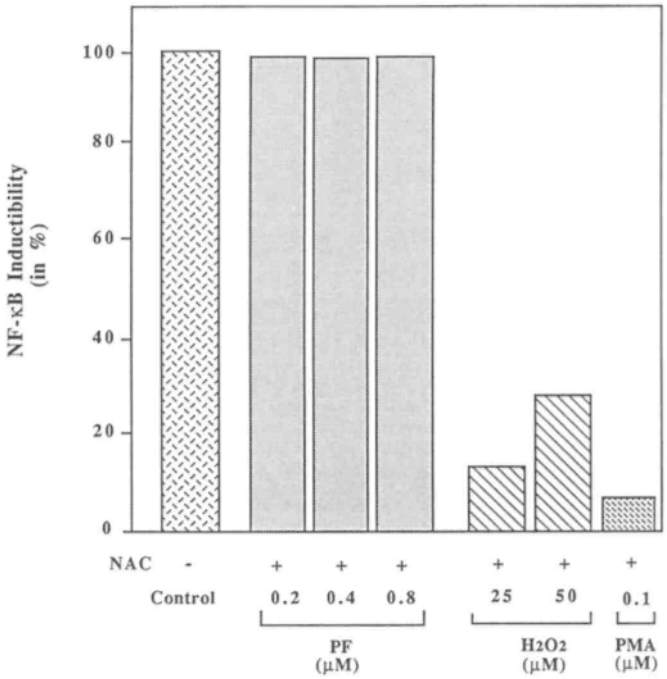
antisera specific for p50 or p65 before being mixed with the ^{32}P-labeled κB probe (34). Samples were then loaded on a 4% poly-acrylamide native gel and electrophoresed as in Fig. 5. C, the effect of cycloheximide {CHX) on the induction of κB DNA-binding proteins. ACH-2 cells were left untreated, were phototreated for 10 min with PF (0.25 μM), were incubated with 50 μg/ml CHX alone, or phototreated 10 min with PF (0.5 μM) and treated with 50 μg/ml CHX.



Fκ;. 7. NF-κB inducibility in ACH-2 cells grown in the absence (control) or in the presence of 30 mM NAC. NF-κB activation was performed by PF (0.2, 0.4, or 0.8 μM) photosensitization as described above or by H_20_2 (25 or 50 μM) or by PMA (0.1 μM). NF-κB DNA binding activities were determined by EMSA as reported in Fig. 5 and the intensities of the κB bands were quantitated by phosphorimaging. The amount of NF-κB activities observed in the presence of NAC were compared directly to those obtained in the absence of NAC (determined as being equal to 100%).

The specificity of protein-DNA complexes was tested by competition with the unlabeled oligonucleotide. Fig. 9 shows that a very low AP-1 DNA binding activity can be detected in nuclear extracts prepared 60 min after the stress with PF, no clear induction being observed for longer periods of time. However, AP-1 activity is stimulated only 5-fold which is far less than observed in the case of NF-κB. The same low induction can be observed when using a larger range of PF concentrations (data not shown). In conclusion, oxidative DNA damages induced in the nucleus of HIV-1-infected lymphocytes appear to activate specifically NF-κB, and the kinetics of this activation differ totally from those reported in the case of other inducers.

Discussion

Treatment of T-lymphocytes latently infected by HIV-1 with a photosensitizer having a high affinity for DNA induces, upon visible light irradiation, DNA oxidative damage, cytotoxicity, HIV-1 reactivation in cells surviving the treatment as well as the binding to DNA, and nuclear appearance of a cellular transcription factor. Evidence that this factor is NF-κB is based on its κB-specific DNA binding, its immunoreactivity with an-tisera raised against the p50 and p65 subunits of NF-κB, and the post-translational induction of the DNA binding activity. The stimulation of the NF-κB DNA binding activity can be detected at very low treatment intensity and it is very high in comparison with the induction of another transcription factor like AP-1. Moreover, NF-κB induction with ROIs generated in DNA appears to be rather slow when compared with other inducing events like phorbol ester treatment (18).

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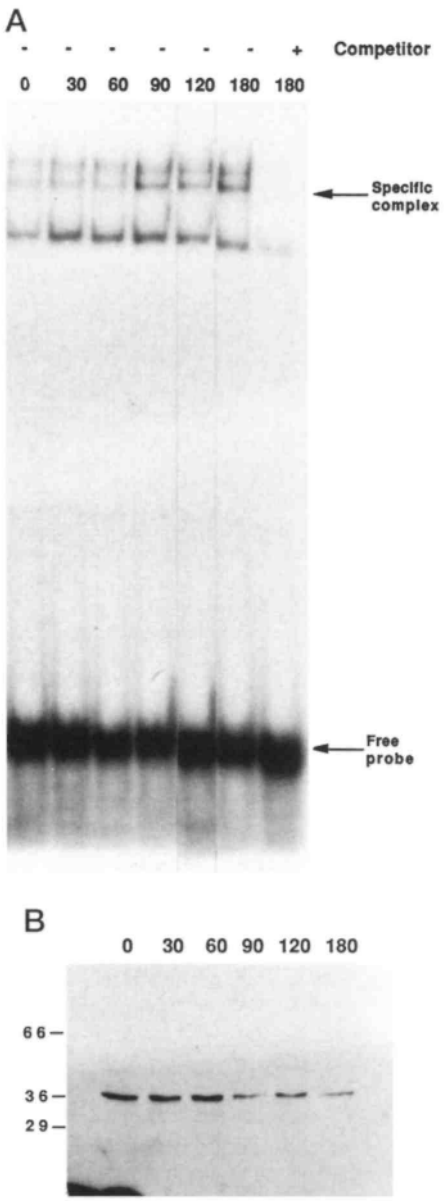

FIG. 8. Disappearance of IκB and appearance of NF-κB after the oxidative stress mediated by PF. A, evolution of the NF-κB complex induced in ACH-2 cells by phototreatment with PF (3.5 μM) for 10 min. Cells were taken at various times after the photoreaction (from 0 to 240 min) and used to prepare nuclear extracts to be analyzed by EMSA as described in Fig. 4. B, fate of IκB-α in ACH-2 cells photore-acted exactly as described in A. Cytoplasmic proteins (15 μg) from the same extracts as above were analyzed by SDS-polyacrylamide gel electrophoresis and transferred on nylon membrane followed by Western blot analysis using an anti-IκB-α IgG.

All these features allow us to conclude that a photosensitization reaction capable of generating DNA oxidative damages can induce specifically NF-κB.
Similar induction of NF-κB has already been described by several authors in the case of H_2O_2 (16, 22, 23). These authors have postulated that H_2O_2 penetrates the cell before being converted into highly reactive OH radicals through a Fenton-like reaction. Since this conversion can take place at various cellular locations where iron is available to catalyze this reaction, it is not possible from these works to determine what are the primary cellular targets responsible for the initiation of the signaling pathway leading to NF-κB activation and then to HIV-1 reactivation.

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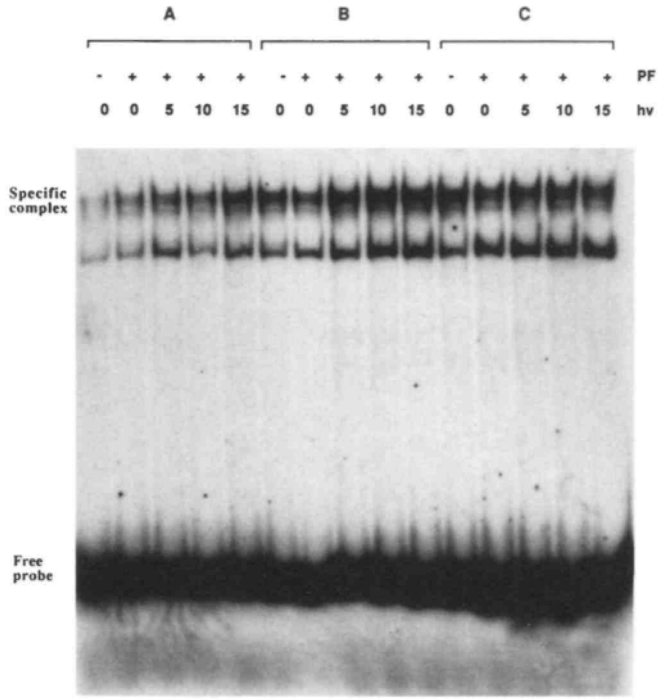

FIG. 9. Kinetics of induction of AP-1 DNA binding activities in ACH-2 cells phototreated during increasing times (0 to 15 min) with 0.25 μM PF. Nuclear extracts were prepared 30 min (A), 60 min (B), and 90 min (C) after the photoreaction. The nuclear extracts were mixed with a ^{32}P-labeled probe encompassing the TRE elements of the type I collagenase gene promoter.

To provide insights to this question, we have used photosensitization to generate ROIs. Photosensitization is based on the use of a chromophore absorbing visible light which in turn transfers the absorbed energy either to molecular oxygen or directly to a substrate (for review, see Refs. 40 and 55). In this work, PF has been used as photosen-sitizer because it is an acridine-derived molecule having a very high affinity for DNA (29, 56). The interaction of small planar acridine molecules with DNA has been studied in detail (for review, see Refs. 39 and 57) to show that these molecules can draw DNA base pairs apart from their normal 3.4-Å spacing to 6.8 A causing the DNA to become longer and stiffer. At high molar ratios between acridine and DNA, when all the intercalation sites are filled, acridines can stack along the phosphate-sugar backbone (37, 38, 57). With chromatin, the analysis of the binding isotherms shows that high affinity sites are clustered on the nucleosomal DNA and at a very high intercalating drug concentration it could cause dissociation of the nucleosome core particle as observed with ethidium bromide (58, 59). The confirmation that acridines localize in cell nuclei has been brought by numerous techniques such as fluorescence microscopy (60), electron miscrocopy (61), and electron spin resonance combined with the use of a spin-labeled acridine derivative (62). All these data confirm our observation made using fluorescence microscopy to localize PF inside lymphocytes or monocytes. Although no PF fluorescence can be detected in the cytoplasm of these cells, intercalation into mitochondrial DNA cannot be totally ruled out. However, the absence of PF fluorescence in the cytoplasm would lead us to conclude that the vast majority of PF molecules are localized in the cell nucleus. It should also be pointed out that the lack of NF-κB inhibition by cell preincubation with NAC can be used as an argument to exclude mitochondria as an intermediate in the activation pathway initiated by PF-mediated photosensitization.

Photosensitization reactions mediated by PF in solution or bound to DNA have also been studied in detail (see Refs. 63 and 55 for review). Both type I (electron transfer to DNA base) and type II ($^1O_2$ generation) reactions have been shown to take place. With isolated DNA bases, these reactions occurred with a yield of 0.55 and 0.45 for types I and II, respectively (63). The first mechanism (type I) occurred only when PF was in contact with a substrate like DNA, but the second pathway (type II) generated $^1O_2$ with PF either free in solution or bound to DNA. Both photochemical reactions have been shown to produce 7,8-dihydro-8-oxoguanine in DNA efficiently (64, 65). $^1O_2$ is a rather long-lived species in aqueous solution (2 μs in water); however, in cells, its lifetime is very short (between 5 and 100 ns for L1210 and erythrocyte ghosts, respectively) because the intracellular concentration of substrates which are readily oxidizable by this species is very high (66). Then, generated by PF inside

the DNA of ACH-2 cells, $^1O_2$ could not diffuse easily outside DNA because its lifetime is too short and the amount of oxidizable substrates too high. This interpretation is in accordance with the observation that pretreatment of cells with NAC does not affect the level of NF-κB induction by PF-mediated photosensitization. Indeed, NAC itself or GSH does not interact with DNA, then cannot interfere with a process generating ROIs inside DNA explaining why no protection can be conferred by NAC against NF-κB induction by PF photosensitiza-tion. On the other hand, NAC was shown to be very effective when NF-κB induction was performed by H_2O_2 or PMA because NAC, in that case, localizes in cell compartments where it can efficiently scavenge ROIs (16, 22, 23, 36).

Oxidative stress mediated by PF induces DNA damage at purines, single-strand breaks, and cytotoxicity in ACH-2 cells. Among the induced DNA modifications, it has been shown that 7,8-dihydro-8-oxoguanine is not capable of blocking DNA replication (67) while other modifications, if not repaired, can inhibit DNA replication (68) and transcription; therefore, they are possibly responsible for the lethal effects observed with this photosensitizer. Thus, unrepaired purine oxidation products can well be suspected to play an important role in the lethal effect and in the HIV-1 reactivation which is induced among cells surviving the treatment. This hypothesis is supported by the observation that the cytotoxic effect induced by the photo-sensitization mediated by PF is always more intense 48 h after the reaction than at earlier times.

A central event in the HIV-1 reactivation from ACH-2 cells photosensitized with PF is the induction of NF-κB. This activation occurs through a post-translational mechanism leading to the release of the inhibitory subunit IκB from its complex with p65 and p50 in the cytoplasm (for review, see Refs. 4—6). Release of IκB allows translocation to the nucleus of NF-κB and its DNA binding. The reactions that abolish binding of IκB to p65 have been elucidated. When the stimulation is due to phorbol esters, the degradation seems to occur in two steps (18, 51, 52, 53, 69), (i) transfer of phosphoryl groups onto IκB by a protein kinase which causes its release and (ii) degradation of IκB through a cytoplasmic chymotrypsin-like protease (18) or proteasome (70, 71). With PMA, TNF-α, or IL-1β, these events appear soon after the induction (less than 5 min), but turn out to be much slower with the reaction mediated by PF. These results allow us to postulate that the IκB release from p65 and p50 after the oxidative stress induced by PF involves other additional intermediates. The signaling pathway from the nucleus to the cytoplasm would then be more complex and slower than the one initiated at the plasma membrane level by PMA, TNF-α, or IL-1β. A similar signaling pathway has been postulated by several authors in the case of the UV-mediated activation of AP-1 (25, 72, 73) and NF-κB (25, 26). These authors postulate that DNA modifications, *i.e.* pyrimidine dimers, induced by UV would be responsible for the initiation of a signaling pathway from the nucleus to the cytoplasm. However, Devary *et al.* (28) have shown that UV activation of NF-κB can also occur without cell nucleus implying that another pathway is likely to be initiated from other cellular targets, *e.g.* membranes, growth factor receptors (74). The existence of a pathway initiated at the membrane level has found a support in the demonstration that the breakdown of phosphatidylcholine by the addition of exogenous phospholipase C activates NF-κB and increases HIV-1 replication in human monocytes and T-lym-phocytes (75). On the other hand, DNA modifications have been shown to be directly responsible for the transcriptional activation *of gadd.153* gene (76). A large variety of DNA modifications can lead to this activation like those induced by UV, UV-mimetic, DNA cross-linking, and alkylating agents but also by intercalators and inhibitors of topoisomerases. These authors postulate that the induction of *gadd153* gene does not occur by chromatin decondensation as proposed by Valerie and Rosenberg (77) and by Verdin *et al.* (78) but through the activation of cellular factors like AP-1 (79). Moreover, the activation of AP-1 binding activity and *gadd153* promoter transcription activation after treatment of WI-38 human fibroblasts with either UV or alkylating agents is diminished in late passaged cells compared to early passages (79).

In the case of the NF-κB activation by the photosensitization mediated by PF, the pathway is very likely to be initiated by oxidative damage induced in DNA because (i) the photosensi-tizer exhibits a high DNA affinity and can be visualized in the cell nucleus and (ii) the ROIs generated by the photoreaction induce DNA damages. Looking at the level of NF-κB stimulation which is quite similar to the one induced by H_2O_2 for identical survival fractions, we think that it is rather unlikely that NF-κB induced in PF-treated cells would be due to a very small fraction of PF which would not be intercalated into DNA. We postulate thus that oxidative base damage, probably in genomic DNA, is the event initiating the signaling pathway leading to the cytoplasmic release of IκB and in turn to NF-κB translocation to the nucleus. Experiments are now in progress to try to identify the nature of the intermediates involved in this pathway paying special attention to the activation of DNA-activated protein kinase (80) or to kinase homologs of a yeast protein kinase encoded by the *DUN1* gene which is known to control the DNA damage response in yeast (81).

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