NF-κB Activation by Camptothecin

A LINKAGE BETWEEN NUCLEAR DNA DAMAGE AND CYTOPLASMIC SIGNALING EVENTS*

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Activation of the transcription factor NF-κB by extracellular signals involves its release from the inhibitor protein IκBα in the cytoplasm and subsequent nuclear translocation. NF-κB can also be activated by the anticancer agent camptothecin (CPT), which inhibits DNA topoisomerase (Topo I) activity and causes DNA double-strand breaks during DNA replication to induce S phase-dependent cytotoxicity. Here we show that CPT activates NF-κB by a mechanism that is dependent on initial nuclear DNA damage followed by cytoplasmic signaling events. NF-κB activation by CPT is dramatically diminished in cytoplasts and in CEM/C2 cells expressing a mutant Topo I protein that fails to bind CPT. This response is intensified in S phase cell populations and is prevented by the DNA polymerase inhibitor aphidicolin. In addition, CPT activation of NF-κB involves degradation of cytoplasmic IκBα by the ubiquitin-proteasome pathway in a manner that depends on the IκB kinase complex. Finally, inhibition of NF-κB activation augments CPT-induced apoptosis. These findings elucidate the progression of signaling events that initiates in the nucleus with CPT-Topo I interaction and continues in the cytoplasm resulting in degradation of IκBα and nuclear translocation of NF-κB to attenuate the apoptotic response.

The NF-κB/Rel family of transcription factors regulates expression of genes critical for multiple biological processes, including immune responses, inflammatory reactions, and apoptosis (1–3). In mammalian cells, NF-κB exists as dimeric complexes composed of p50, p65 (RelA), c-Rel, RelB, or p52. These proteins share a conserved Rel homology domain that encodes dimerization, DNA binding, and nuclear localization functions. NF-κB associates with members of the IκB family of proteins, most notably IκBα, which masks the nuclear localization sequence of NF-κB and retains it in the cytoplasm (4, 5). Dissociation from IκBα is essential for NF-κB to enter the nucleus and to activate gene expression. Several signaling cascades that control NF-κB activation converge at an IκB kinase (IKK) complex, responsible for site-specific phosphorylation of IκBα at serines 32 and 36 (6–10). Phosphorylation of IκBα induces mult ubiquitination of IκBα and its subsequent degradation by the ubiquitin-dependent 26 S proteasome (11, 12). This sequence of events can be induced without de novo protein synthesis by multiple extracellular stimuli, including tumor necrosis factor α (TNFα), interleukin-1, phorbol ester (PMA), bacterial lipopolysaccharide (LPS), and others. However, NF-κB activation can also be achieved through mechanisms that are distinct from the above IKK-dependent model. These include phosphorylation-independent yet proteasome-mediated IκBα degradation induced by ultraviolet irradiation (13, 14), calpain-dependent degradation of IκBα by silica and TNFα (15, 16), and tyrosine phosphorylation-induced dissociation of IκBα from NF-κB following hypoxia and reoxygenation (17). Thus, depending on the stimuli, NF-κB can be activated through multiple distinct regulatory pathways.

Activation pathways of NF-κB typically originate from ligand-receptor interactions on the cell membrane. However, NF-κB can also be activated by a group of agents that damage DNA in the nucleus. A paradox confounding our current understanding of the mechanism of NF-κB activation by agents that damage DNA is that the major source of damaged DNA is in the nucleus, whereas latent NF-κB complex is in the cytoplasm. It was previously hypothesized that a signal may transfer from the nucleus to the cytoplasm (18). In support of this model, a recent study by Piette and Piret (19) provides evidence that NF-κB activation by DNA-damaging agents correlates with their capacity to induce DNA breaks. However, the requirement of damaged DNA in the nucleus has not been directly demonstrated. In contrast, Devary et al. (20) showed that nucleated cells (i.e. cytoplasts) retained full capacity to activate NF-κB following UV irradiation, indicating that nuclear DNA damage is not necessary for NF-κB activation by UV irradiation. There is now substantial evidence to support the notion that UV activation of NF-κB involves activation of cell surface receptors by ligand-independent oligomerization (14, 21). The abbreviations used are: IKK, IκB kinase; TNFα, tumor necrosis factor α; PMA, phorbol myristyl acetate; LPS, lipopolysaccharide; CPT, camptothecin; Topo I, DNA topoisomerase I; SSB, DNA single-strand break; DSB, DNA double-strand break; TPT, topotecan; ALLN, acetyl-leucin-leucin-norleucin; EMSA, electrophoretic mobility shift assay; HA, hemagglutinin; GFP, green fluorescent protein; WT, wild type; FACS, fluorescence-activated cell sorter; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.
NF-κB Activation by DNA-Topo I Lesions

21–24) and/or oxidative stress-mediated inactivation of receptor tyrosine phosphatases, which ultimately leads to ligand-independent activation of receptor tyrosine kinases (25). Whether nuclear DNA damage can directly activate an intracellular NF-κB signaling pathway without involving cell surface receptors remains an important question yet to be resolved.

We and others have observed that an anti-cancer agent, camptothecin (CPT), can activate NF-κB in pre-B or T cell lines (19, 26). CPT inhibits the activity of DNA topoisomerase (Topo) I (27–29). Topo I changes the supercoiling of DNA and therefore plays critical roles in DNA replication, in RNA transcription, and, indirectly, in DNA damage repair (30). CPT selectively binds to and stabilizes a covalent Topo I-DNA reaction intermediate, referred to as the cleavable complex, which contains a single-strand DNA break (SSB) (31, 32). DNA double-strand breaks (DSBs) are then generated during DNA replication when the replication fork collides with the cleavable complex (33). In the present study, our objective was to determine whether or not nuclear events associated with the DNA-damaging action of CPT and a clinically utilized derivative of CPT, topotecan (TPT) (34, 35) were required for activation of cytoplasmically localized NF-κB complexes. We also examined whether CPT activation of NF-κB modulated an apoptotic response. Our findings elucidate a series of nuclear events induced by CPT/TPT that converge with cytoplasmic signaling events responsible for the activation of NF-κB, which can provide anti-apoptotic function.

EXPERIMENTAL PROCEDURES

Cell Culture—Culture conditions for 70Z/3 and 70Z/3-CD14 murine pre-B cells have been described (36). CEMp and CEM/C2 human T cell lines were kindly provided by Dr. Y. Pommier (National Institutes of Health) and maintained in RPMI 1640 medium (Cellgro, Mediatech) supplemented with 10% fetal bovine serum (Sigma), 2 mM glutamine, 100 units of penicillin G (Sigma), and 0.5 mg/ml streptomycin sulfate (Sigma) in a humidified 5% CO2–95% air incubator (Forma Scientific). HeLa human cervical carcinoma cells and PC-3 human prostate carcinoma cells were maintained in Dulbecco’s modified Eagle’s medium (Cellgro) supplemented with 10% fetal bovine serum and antibiotics as above in a 10% CO2–90% air incubator. The human kidney embryonic fibroblast 293 (HEK293) was maintained in the latter medium on 0.1% gelatin-coated plastic culture dishes.

Retrovirus Construction and Infection—For infection, a human kidney cDNA library in AZAPII with a polyclonal chain reaction-amplified DNA fragment using a HeLa cDNA library (CLONTECH) and TO297 (5’-CTGACAGTCCTTGTCCGCG-3’) and TO240 (5’-CCAGAGCTCCTTGTCCGCG-3’) primers. IKKa and IKKβ with a Lyo-to-Ata substitution at the conserved ATP binding site were generated by polymerase chain reaction mutagenesis and confirmed by DNA sequencing. The mutant genes were placed under the control of the cytomegalovirus promoter in the pDNA3.1(+) expression vector (Promega). HEK293 cells were transfected with these constructs by calcium phosphate precipitation and then treated with either TNFα (10 ng/ml) or TPT (30 μm) for 2 h. Nuclear extracts were analyzed by EMSA as described above. Cytoplasmic extracts were analyzed by Western blotting using the anti-FLAG monoclonal antibody (Kodak) to determine expression levels of respective dominant-negative mutants in each condition. An horseradish peroxidase-conjugated donkey anti-mouse antibody (Amersham Pharmacia Biotech) was used for secondary antibody followed by ECL development.

Retrovirus Construction and Infection—Production and infection of HA-tagged wild-type and HA-tagged S32A/S36A mutant IκBα expression constructs were described (36). Other IκBα deletion mutants were generated by polymerase chain reaction-mediated mutagenesis and confirmed by sequencing. Stable pools were selected with hygromycin (1 mg/ml, Roche Molecular Biochemicals), and the expression levels of the corresponding proteins were examined by either anti-IκBα (C21, a C-terminal epitope) or anti-HA antibodies. For experiments shown in Figs. 7B and 9, HA-S32A/S36A clone 5 that expressed a relatively high level of the mutant protein was used. Similar but less pronounced effects were also seen with pooled cultures and in five isolated clones expressing varying levels of mutant protein (not shown).

Generation of a Green Fluorescent Protein-IκBα Fusion Construct—N-terminally fused GFP-IκBα was generated by subcloning polymerase chain reaction amplified human IκBα (MAD3) into HindIII-BamHI sites of the pEGFP vector (CLONTECH), such that the entire MAD3 coding sequence was in-frame with the GFP coding sequence. Stable IκBα cell clones and G4 were generated by G418 selection and subsequent FACS sorting. Cells were photographed using a Zeiss Axioskop microscope equipped for fluorescence with the aid of a fluorescein-specific filter.

FACS Analyses—For FACS sorting of G1, S, and total cell fractions for EMSA analyses, 70Z/3 cells untreated or treated with CPT, TPT, or LPS for appropriate durations were stained with Hoechst 33342 (stock at 10 mg/ml in water) at the final concentration of 10 μg/ml for 15 min before the AP-1 site used for control EMSA reactions was obtained from Promega.

Enucleation Procedure—Enucleation was performed as described (37) with the following modifications. PC3 or HeLa cells were seeded in 30-mm dishes, grown overnight, and exposed to cycloheximide B (10 μg/ml) for a total of 30 min at 37 °C. Plates were placed upside down in 400-ml centrifuge bottles and bathed in growth medium containing cycloheximide B at the same concentration. Plates were secured at the bottom of the centrifuge bottles by appropriately sized styrofoam. Samples were then centrifuged at 10,000 rpm for 15 min at 37 °C using a Beckman JA-14 rotor. The rotor and centrifuge chamber were prewarmed to 37 °C by centrifugation without samples. Plates with enucleated cells (i.e. cytoplasts) were then removed from the centrifuge bottle, gently rinsed with phosphate-buffered saline, and incubated with prewarmed growth medium for 30 min at 37 °C. Samples of enucleated cells were fixed in 3:1 methanol/acetic acid, stained with Hoechst dye 33258, and photographed under a fluorescence microscope equipped with a 4’6-diamidino-2-phenylindole-specific filter. The enucleation efficiency varied from ~75 to 95% for PC-3 and HeLa cells.

Transient Transfection Assay—Cells (HEK293, HeLa, or PC-3) were transiently transfected using a similar cloning strategy starting with the 3xM-B-CAT (40). 24 h following transfection, cells were treated with TPT (30 μm) or CPT (10 μm) for 2 h, rinsed twice with growth medium, and further incubated without drugs for 6 h before termination of the cultures. Positive control samples were treated with TNFα (10 ng/ml) for a total of 8 h. Control samples were transfected with the LacZ cDNA under the control of the cytomegalovirus promoter in the pCMX vector (CMV-LacZ). For CEMp and CEM/C2 cells, total proteins were used for normalization.

Full-length human IKKα cDNA was provided by Dr. I. M. Verma (Salk Institute). Full-length human IKKβ cDNA was cloned by screening a human kidney cDNA library in AZAPII with a polyclonal chain reaction-amplified DNA fragment using a HeLa cDNA library (CLONTECH) and TO297 (5’-CTGACAGTCCTTGTCCGCG-3’) and TO240 (5’-CCAGAGCTCCTTGTCCGCG-3’) primers. IKKα and IKKβ with a Lys-to-Ala substitution at the conserved ATP binding site were generated by polymerase chain reaction mutagenesis and confirmed by DNA sequencing. The mutant genes were placed under the control of the cytomegalovirus promoter in the pDNA3.1(+) expression vector (Promega). HEK293 cells were transfected with these constructs by calcium phosphate precipitation and then treated with either TNFα (10 ng/ml) or TPT (30 μm) for 2 h. Nuclear extracts were analyzed by EMSA as described above. Cytoplasmic extracts were analyzed by Western blotting using the anti-FLAG monoclonal antibody (Kodak) to determine expression levels of respective dominant-negative mutants in each condition. An horseradish peroxidase-conjugated donkey anti-mouse antibody (Amersham Pharmacia Biotech) was used for secondary antibody followed by ECL development.

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RESULTS

CPT Induces Transient NF-κB Activation in the Absence of de Novo Protein Synthesis—NF-κB activity is dictated by its ability to bind cognate κB sites present in responsive genes. We utilized a κB site from the immunoglobulin κ intronic enhancer in EMSA analyses to evaluate NF-κB activation by CPT or TPT treatments. CPT induces dose-dependent (Fig. 1A, saturating at 10 μM) and transient (Fig. 1B, peaking at 1–2 h) NF-κB binding activity in 70Z/3-CD14 pre-B cells. Addition of 50-fold excess specific and nonspecific oligonucleotides (Fig. 1C, lanes 1–3) shows that the binding activity is specific to NF-κB. Specificity is further demonstrated by the interaction of binding complex with antibodies to p50, RelA, and c-Rel (Fig. 1C). Antibodies to other NF-κB family members, p52 and RelB, did not alter binding, indicating that these proteins are not components of the NF-κB complex induced by CPT in 70Z/3-CD14 cells. Pretreatment with cycloheximide (Fig. 1D, lanes 3 and 4) did not interfere with this pathway, which shows that CPT action does not require de novo protein synthesis. This activation is not limited to lymphoid cells because it was also induced at 37 °C in RPMI growth medium, followed by cell isolation using FACStar® plus (Becton Dickinson) at 4 °C. 10⁶ cells each were purified, and total cell extracts were prepared for EMSA analyses. The status of the cell cycle of purified fractions was confirmed by propidium iodide, and analyzed using FACSCalibur (Becton Dickinson). Detailed protocols for apoptosis analyses using FACS have been published (41). Briefly, cells were fixed in ethanol, treated with a citric acid buffer to release fragmented DNA out of the cells, stained with propidium iodide, and analyzed using FACSCalibur.

by both CPT and TPT in CEM T leukemic, PC-3 prostate cancer, HEK293 embryonic kidney fibroblast, and HeLa cervical cancer cell lines (see below, others not shown). Induction of
NF-κB DNA binding activity by CPT or TPT treatment resulted in increased NF-κB-dependent transcription of a luciferase reporter gene (see below). Thus, CPT or TPT activation of NF-κB occurs without de novo protein synthesis and may utilize pre-existing regulatory component(s).

Interaction of CPT with Nuclear Topo I Is Necessary for NF-κB Activation—The primary molecular target of CPT or TPT is nuclear Topo I enzyme (27, 32). However, mitochondria also contain CPT-sensitive Topo I (42). It is also possible that CPT activation of NF-κB may involve molecular target(s) other than nuclear Topo I. To evaluate the requirement of a nuclear event in NF-κB activation by CPT, we enucleated PC-3 and HeLa cells by the cytochalasin B-mediated enucleation procedure (37). This protocol produced enucleated cells with approximately 90% efficiency as determined by nuclear staining with Hoechst dye (Fig. 2A). Consistent with a previous report (43), EMSA of total cell extracts prepared from intact and enucleated cells demonstrated that NF-κB activation by activators, such as PMA (Fig. 2B) or TNF (80), does not require an intact nucleus. By contrast, the NF-κB response by CPT and TPT was dramatically diminished in the enucleated cells (Fig. 2B). Most activation by TPT in enucleated cells is likely due to low numbers of intact cells present in the enucleated cell population (Fig. 2B). NF-κB (p65), and upstream kinases in the signaling pathway, IKKα and IKKβ, are still present in the cytoplasts (Fig. 2B, lanes 2, 4, and 6), demonstrating that the lack of NF-κB activation response in certain enucleated samples (lane 4) is not due to potential leakage of these signaling components. Thus, these results are consistent with the hypothesis that a nuclear event is necessary for NF-κB activation by CPT-related compounds.

Although the above data are consistent with the notion that an intact nucleus is required for NF-κB activation by CPT, it is unknown whether Topo I-induced DNA damage is also required for this process. Its DNA-damaging function requires CPT to interact with Topo I-DNA cleavable complexes (33). To address whether direct interaction of CPT and a Topo I-DNA complex is necessary for activation of NF-κB, we examined human CEM/C2 cells, which exclusively express a mutant Topo I enzyme (44). This mutant Topo I enzyme contains two amino acid substitutions, Met370 to Thr and Asp722 to Ser. The latter substitution is critical for CPT-mediated DNA cleavage (33). It has been suggested that aphidicolin-induced inhibition of DNA polymerase activity prevents DSB liberation (34). Aphidicolin prevents S phase-specific toxicity of CPT (46). To evaluate whether a SSB or DSB is critical for NF-κB activation by CPT, we examined the influence of aphidicolin on CPT induction of NF-κB. FACS analysis confirmed that ~50% of 70Z/3-CD14 cells were in S phase of the cell cycle at the time of CPT treatment (see below). The EMSA demonstrated that CPT activation of NF-κB was efficiently blocked by this treatment (Fig. 4A, lanes 9–5). Aphidicolin, however, did not block NF-κB activation by bacterial LPS (lanes 10–12). Aphidicolin also did not block NF-κB DNA binding activity (lanes 6–8). These results are consistent with the hypothesis that the generation of DSB, not SSB, is necessary for efficient NF-κB activation by CPT (19). These data also imply that this activation pathway may occur only in the S phase of the cell cycle. We therefore enriched 70Z/3-CD14 cells in the S phase by FACS sorting after cells were stimulated with CPT or LPS for 2 h (Fig. 4B). Compared with a similarly obtained G1 cell population, NF-κB activation was 2.8-fold higher in the S phase.
population when equivalent amounts of cell extracts were analyzed by EMSA (Fig. 4C). LPS stimulation did not show any significant differences between S and G1 cells. These findings demonstrate that CPT activation of NF-κB is cell cycle coupled and predominantly takes place during the S phase of the cell cycle in a DNA-polymerase-dependent fashion. This also can be explained why NF-κB activation by CPT or TPT is relatively lower in virtually all cell types examined when compared with LPS or TNFα. NF-κB activation by CPT or TPT is dependent on the percentage of replicating cells in S phase, whereas activation by either LPS or TNFα is not cell cycle coupled.

CPT Induces Degradation of IκBα by a Ubiquitin-Proteasome Pathway—The regulatory events governing NF-κB activity induced by cytokines and LPS are well characterized and involve activation of cytoplasmic signaling cascades (2). The primary regulator is the inhibitory protein, IκBα, which maintains NF-κB in the cytoplasm. Release of NF-κB to the nucleus depends on degradation of IκBα. To determine whether CPT activation of NF-κB is solely dependent on nuclear events or whether cytoplasmic events are also required, IκBα protein levels were monitored following treatment with CPT by Western immunoblot analyses. CPT treatment caused a reduction in IκBα protein levels (Fig. 5A, compare lanes 6 and 7), consistent with induction of IκBα degradation. This degradation was prevented by the proteasome inhibitors, ALLN and lactacystin (Fig. 5A, lanes 8 and 9). A longer exposure of the film (Fig. 5B, lanes 8 and 9) revealed an accumulation of characteristic high molecular mass multiubiquitinated IκBα ladders (11, 12). Proteasome inhibitors not only prevented IκBα degradation but also NF-κB activation by CPT treatment (Fig. 5C, compare lanes 2 and 3). TPT induced similar IκBα degradation (Fig. 6A). Inhibition of IκBα degradation by ALLN resulted in accumulation of IκBα in the cytoplasm, as visualized by an IκBα protein N-terminally tagged with the green fluorescent protein (GFP-IκBα) (Fig. 6B, right panel). Control coimmunoprecipitation experiments with RelA-specific antibodies confirmed that the addition of the GFP tag did not interfere with its association with NF-κB (80). The GFP tag also did not affect TPT-induced proteolysis (Fig. 6A, lanes 2–5). Thus, induction of IκBα degradation by CPT or TPT is similar to that induced by LPS in 70Z/3 cells or TNFα in multiple cell types (1, 2), which utilizes a ubiquitin-proteasome pathway. Moreover, we also found that IκBα degradation by TPT was markedly reduced in enucleated PC3 cells (not shown). These data demonstrate that the progression of events initiated in the nucleus by TPT or CPT treatment is continued in the cytoplasm.

IκBα Degradation Induced by CPT or TPT Is Ser32/36-dependent—Although cytokines and LPS cause IκBα degradation by a ubiquitin-proteasome pathway that requires intact Ser32 and Ser36 residues, UV irradiation causes IκBα degradation by a ubiquitin-proteasome pathway independent of these Ser residues (13, 14). To evaluate whether CPT or TPT-induced IκBα degradation requires intact Ser32/36 residues, the S32A/S36A mutant protein was N-terminally tagged with an HA epitope (HA-S32A/S36A) (36), stably introduced in 70Z/3 cells, and analyzed for sensitivity to degradation by TPT treatment. The S32A/S36A mutant protein was completely resistant to degradation induced by TPT treatment (Fig. 7A). This was not due to the presence of the HA tag because the control HA-WT IκBα protein was efficiently degraded. Moreover, a Ser22/36 deletion mutant without the HA tag also failed to be efficiently degraded (Fig. 7A, ΔS20–40). Stable expression of the HA-S32A/S36A mutant, but not HA-WT, selectively eliminated the appearance of NF-κB DNA binding in the nucleus after treatment with CPT or TPT (Fig. 7B, lanes 9 and 10). Consistent with the formation of multiubiquitinated IκBα ladders (Fig. 5B, lanes 8 and 9), substitution of the primary ubiquitination sites Lys21 and Lys22 (11, 50), with Arg resulted in retardation of degradation following TPT treatments (Fig. 7A, HA-K21/22R). These results are similar to those obtained with LPS (Fig. 7A, lane 2).
The use of EMSA analysis to investigate potential inhibitory epitope (not shown). B, degradation of GFP-IKKα protein is largely cytoplasmic. Left panel, HEK293 stably expressing the GFP-IKKα protein was left untreated and visualized under fluorescein-aided fluorescence microscopy. Middle panel, parallel cultures as in the left panel were treated with TPT (30 μM) for 2 h as in A and visualized as above. There were decline of GFP signals in ~50% of the cell population (those showing reduced fluorescence are shown), potentially corresponding to replicating cells as suggested from results (Fig. 4). Right panel, parallel cultures were treated with 100 μM ALLN and 30 μM TPT and visualized as above. The exposure settings for all three panels were identical. The above data are representative of two independent experiments.

The IKK Complex Is Essential for NF-κB Activation by CPT—To further elucidate the events upstream of IκBα degradation that are involved in CPT activation of NF-κB, we evaluated the NF-κB response by EMSA in HEK293 cells transiently expressing dominant-negative IKKα and β proteins. The use of EMSA analysis to investigate potential inhibitory responses was possible because transfection efficiency was consistently >95% in this cell type (Fig. 5A), and thus almost all cells in the transfected population expressed the IKK mutant proteins. Both IKK mutants (N-terminally tagged with a FLAG epitope) reduced the level of NF-κB activation by TPT in a dose-dependent manner (Fig. 5B). By contrast, these mutants did not appreciably alter the DNA binding levels of AP-1 complex (Fig. 5B, upper panel). Dose-dependent expression of IKK mutant proteins is shown by Western blot analysis of cell extracts using monoclonal anti-FLAG antibody (Fig. 8C).

To independently determine the requirement of IKKα and IKKβ in NF-κB activation by CPT, embryonic fibroblast lines derived from IKKα and IKKβ knockout mice (51, 52) were treated with TNFα or CPT. Consistent with published observations (51–55), TNF activation of NF-κB was much weaker in IKKβ knockout cells than in IKKα knockout cells (Fig. 8D). However, NF-κB activation by CPT treatment was undetectable in both IKKα- and IKKβ-deficient cells (Fig. 8D, lanes 12 and 8, respectively). In addition, CPT activation of NF-κB was also undetectable in IKKγ-deficient 1.3E2 cells (56) (Fig. 8E). Thus, key components of the IKK complex (IKKα/β/γ) are essential for NF-κB activation by CPT.
population in S phase of the cell cycle (Fig. 9, OT, WT). Expression of S32A/S36A mutant protein did not significantly affect the cell cycle status of untreated cells (OT, S32A/S36A). After treatment with 1 μM CPT for 24 h, however, most of the cells appeared in either a G2/M or sub-G0/G1 apoptotic peak (Fig. 9, 24 h, WT). The fraction of apoptotic peak was approximately twice as great in S32A/S36A expressing cells as WT expressing cells (24 h, S32A/S36A). Similar results were obtained with higher CPT doses or TPT treatments (not shown). Thus, these observations indicate that activation of NF-κB retards some cancer cells from undergoing apoptosis. These findings demonstrate that CPT activation of NF-κB can provide an anti-apoptotic activity.

**DISCUSSION**

The activity of NF-κB depends on a series of reactions that release it from an inhibitory complex in the cytoplasm and allows it to migrate to the nucleus. The elucidation of the individual steps within NF-κB signaling cascades induced by a variety of structurally and functionally unrelated stimuli has revealed the use of both shared and unique components that may contribute to the diverse functions of this ubiquitous transcription factor (38, 60–68). DNA-damaging agents represent a unique group of NF-κB activators because their primary site of action is in the nucleus. In this study, we demonstrate that nuclear events arising from the DNA-damaging function of CPT and TPT are components of a NF-κB signaling pathway that converges in the cytoplasm with events associated with signaling pathways induced by cytokines or LPS stimulation.

The DNA-damaging function of CPT in replicating cells is a multi-step process that initiates with intercalation of CPT into a covalent Topo I-DNA reaction intermediate. CPT stabilizes this transient intermediate, forming the cleavable complex with a SSB. The cleavable complexes and associated SSBs are mostly reversible until the cell undergoes replication, during which the replication fork collides with the cleavable complex and yields a DSB. Our results obtained by utilizing mutant CEM/C2 cells, pharmacological agents, and FACS enrichment of S phase cells indicate that S phase-dependent generation of DSB is essential for NF-κB activation. Of note, however, is that...
CPT or TPT activation of NF-κB is transient in all cell lines tested thus far. Similar dose-dependent and time course responses in lymphoid, fibroblastic, and epithelial cell lines suggest that a conserved activation mechanism may be involved. It has been demonstrated that CPT can induce degradation of Topo I enzyme by the ubiquitin-proteasome pathway causing marked reduction of Topo I enzyme within 2–4 h (69). Although Topo I enzyme by the ubiquitin-proteasome pathway causing has been demonstrated that CPT can induce degradation of gest that a conserved activation mechanism may be involved. It responses in lymphoid, fibroblastic, and epithelial cell lines sug-
tested thus far. Similar dose-dependent and time course re-
idative stress-responsive transcription factor (72), determina-
ted UV-C treatment of Xeroderma pigmentosa group A fibro-
Because NF-
port chain, indicate that NF-
activation induced by CPT or TPT requires an intact nucleus. It is plausible that event(s) downstream of DSbs or those coupled to cell cycle may be responsible for transient NF-κB activation by CPT-related compounds. A recent study has implicated the involvement of the ataxia telangiectasia mutant protein in sustained activation of NF-κB by CPT (81). Enucleation studies demonstrated that the process of NF-κB activation induced by CPT or TPT requires an intact nucleus. To our knowledge, this is the first demonstration of the lack of NF-κB activation in enucleated cells. Although this may be implied from the demonstration that events associated with DNA damage are required for NF-κB activation, mitochondria also contain DNA and CPT-sensitive Topo I enzyme (42). Studies utilizing L929 fibrosarcoma cells deficient for mitochondrial (DNA) and antimycin A, which increases the generation of reactive oxygen intermediates by inhibiting the electron transport chain, indicate that NF-κB activation by TNFα requires reactive oxygen intermediates derived from mitochondria (71). Because NF-κB is implicated as an important mammalian ox-
itive stress-responsive transcription factor (72), deter-
mination of the contribution of nuclear versus potential mitochondrial events was crucial for elucidating the NF-κB activation mechanism induced by CPT and TPT. Our findings provide direct evidence that CPT- or TPT-induced DNA damage in the nucleus is a primary component of the signaling events required for NF-κB activation. Although recent studies that utilized UV-C treatment of Xeroderma pigmentosa group A fibroblasts suggested the involvement of DNA damage in late stage NF-κB activation (14), whether or not an intact nucleus is required for this late activation was not investigated. Previous studies demonstrated that UV activation of NF-κB could efficiently take place in enucleated cells (20).

CPT induction of DNA damage translates into activation of a cytoplasmic signaling cascade that liberates active NF-κB from the inhibitor protein, IκBα. We utilized well-characterized mu-
tants of signaling components within the cytokine-inducible NF-κB signaling pathway to dissect the signaling cascade acti-
vated by CPT and TPT. Ser-to-Ala mutations at positions 32 and 36 of IκBα disrupt inducible phosphorylation and prevent subsequent ubiquitination and degradation by the proteasome pathway (47–49). We showed that these mutants also prevent IκBα degradation and activation of NF-κB induced by CPT and TPT. We additionally demonstrated that dominant-negative IKK mutants that inhibit phosphorylation of IκBα at these sites also prevent NF-κB activation by TPT. IKKα/β, or γ-deficient cells fail to activate NF-κB by CPT treatment. Mu-
tation of Lys residues critical for the attachment of ubiquitin moieties further disrupts CPT-inducible IκBα degradation. To-
gether with pharmacological evidence using proteasome inhib-
itors, our findings show that CPT and TPT induction of IκBα degradation is similar to that induced by cytokines, LPS, and several other signals (7, 8, 10, 11, 47–50, 73, 74). Our findings therefore demonstrate that nuclear DNA damage causes IKK-depen-
dent degradation of IκBα in the cytoplasm. This activation may involve signal transfer from the nucleus to the cyto-
plasm. This type of nuclear-to-cytoplasmic signaling has also been suggested for the late stage NF-κB activation induced by UV irradiation, which involves the production of an autocrine/paracrine factor, interleukin-1α (14). A recent study has also implicated the involvement of the DNA-dependent protein kinase in NF-κB activation by certain DNA damaging agents (82). Further definition of signaling components and reactions will help to determine whether NF-κB activation by CPT indeed involves a nuclear-to-cytoplasmic signal transduction pathway.

CPT derivatives, including TPT, are utilized clinically as part of cancer therapy regimes (34, 35). Recently, several studies have reported that NF-κB may control expression of genes involved in the regulation of apoptosis (57–59, 75–78). In particular, Wang et al. (58) have shown that NF-κB activation by ionizing radiation and daunorubicin may have anti-apoptotic effects in HT1080 human fibrosarcoma cells. The same group recently showed that NF-κB activation by CPT-11 can display similar anti-apoptotic effects in the above cell line (79). We have also shown that CPT activation of NF-κB provides an anti-apoptotic function. NF-κB-dependent survival of even a fraction of cancer cells after treatment with DNA-damaging agents, such as TPT or ionizing radiation, will likely lead to increased mutation rates and accelerated manifestation of ma-
lignancy. Moreover, it may also contribute to transformation of normal cells during the therapy. Understanding the mechanism(s) of NF-κB activation, therefore, may help improve the current methods of cancer therapy by defining a resistance mechanism to Topo I inhibitors and potentially other clinically important DNA-damaging and NF-κB-activating agents, such as ionizing radiation and Topo II inhibitors.

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REFERENCES
1. Baeuerle, P. A., and Baltimore, D. (1996) Cell 87, 13–20
2. Verma, I. M., Stevenson, J. K., Schwartz, E. M., Van Antwerp, D., and Miyamoto, S. (1995) Genes Dev. 9, 2723–2735
3. Sonenshein, G. E. (1997) Semin. Cancer Biol. 8, 113–119
4. Berg, A. A., Ruben, S. M., Muller, J. F., Miller, J. S., Rosen, C. A., and Baldwin, A. S. J. (1992) Genes Dev. 6, 1899–1913
5. Ganchi, P. G., Sun, S. C., Greene, W. C., and Ballard, D. W. (1992) Mol. Biol. Cell 3, 1539–1552
6. Chen, Z. J., Parent, L., and Maniatis, T. (1996) Cell 84, 853–862
7. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) Nature 388, 548–554
8. Mercier, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J. W., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) Science 278, 860–866
9. Woronicz, J. D., Guo, X., Cao, Z., Roche, M., and Goeddel, D. V. (1997) Science 278, 866–869
10. Zandi, E., Rothwarf, D. M., Delhaye, M., Hayakawa, M., and Karin, M. (1997) Cell 91, 243–252
11. Alkalai, I., Yaron, A., Hatzubai, A., Orian, A., Chechanover, A., and Ben-Neriah, Y. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10599–10603
12. Chen, Z., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995) Genes Dev. 9, 1566–1577.
13. Li, N., and Karin, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13012–13017
14. Bender, K., Gottlicher, M., Whiteside, S., Rahmsdorf, H. J., and Herrlich, P. (1998) EMBO J. 17, 5170–5181
15. Han, Y., Weimann, S., Boldog, I., Walker, R. K., and Brasier, A. R. (1999) J. Biol. Chem. 274, 787–794
16. Chen, P., Lu, Y., Kuhn, D. C., Mak, M., Shi, X., Sun, S. C., and Demers, L. M. (1997) Arch. Biochem. Biophys. 342, 383–388
17. Imbert, V., Rupe, R. A., Livosi, A., Pahl, H. L., Traenckner, E. B. M., Mueller-Deckmann, C., Faralduf, D., Roost, B., Auberge, P., Baerueer, P. A., Cell, S., and Peyron, J. F. (1996) EMBO J. 15, 3860–3866
18. Stein, B., Kramer, M., Rahmsdorf, H. J., Ponta, H., and Herrlich, P. (1989) J. Virol. 63, 4540–4544
19. Piret, B., and Piette, J. (1996) Nucleic Acids Res. 24, 4242–4248
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