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Evidence of a Functional Role for p21$^{WAF1/CIP1}$ Down-Regulation in Synergistic Antileukemic Interactions between the Histone Deacetylase Inhibitor Sodium Butyrate and Flavopiridol

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ABSTRACT

The functional significance of disruption of p21$^{WAF1/CIP1}$ induction by flavopiridol (FP) in human leukemia cells (Jurkat) exposed to the histone deacetylase (HDAC) inhibitor sodium butyrate (SB) was investigated. Coexposure of leukemic cells to FP blocked SB-mediated induction of p21$^{WAF1/CIP1}$ and resulted in a marked increase in mitochondrial injury, activation of procaspases-3 and -8, Bid cleavage, and PARP degradation. Enforced expression of p21$^{WAF1/CIP1}$ (i.e., in Jurkat cells inducibly expressing p21$^{WAF1/CIP1}$ under the control of a doxycycline-responsive promoter) partially but significantly reduced cytochrome *c* and apoptosis-inducing factor release, loss of mitochondrial membrane potential, caspase-3 and -8 activation, Bid cleavage, poly(ADP-ribose)polymerase (PARP) degradation, and apoptosis in response to SB/FP. Furthermore, increasing expression of p21$^{WAF1/CIP1}$ (i.e., by culturing cells in the presence of higher concentrations of doxycycline) rendered cells more resistant to SB/FP-mediated lethality. Enforced expression of p21$^{WAF1/CIP1}$ did not modify SB/FP-mediated JNK activation or generation of reactive oxygen species. Consistent with these results, Jurkat cells stably expressing a p21$^{WAF1/CIP1}$ nuclear localization mutant (p21ΔNLS) were also resistant to SB/FP-mediated mitochondrial injury, activation of procaspases-3 and -8, PARP cleavage, and apoptosis. Finally, enforced expression of full-length or ectopic expression of ΔNLS p21$^{WAF1/CIP1}$ increased the amount of p21$^{WAF1/CIP1}$ coimmunoprecipitating with procaspase-3. Together, these findings suggest that interruption of HDAC-mediated p21$^{WAF1/CIP1}$ induction by FP plays a significant functional role in potentiating apoptosis, possibly by preventing the formation of a procaspase-3/ p21$^{WAF1/CIP1}$ complex.

Histone deacetylase (HDAC) inhibitors represent a novel class of agents that has recently been developed as potential anticancer agents for the treatment of solid tumors and hematological malignancies. These agents promote the acetylation of histones, chromatin relaxation, and the transcription of diverse genes involved in cellular differentiation, among other functions (Melnick and Licht, 2002; Rosato and Grant, 2003). HDAC inhibitors such as sodium butyrate (SB), suberoylanilide hydroxamic acid (SAHA), apicidin, and trichostatin A represent structurally diverse compounds that share the capacity to interrupt cell cycle progression in G_1 and G_2M phase, resulting in growth arrest, differentiation, and/or cell death (Marks et al., 2001; Rosato and Grant, 2003). The latter represent alternative and, under some circumstances, mutually exclusive cell fates (Selvakumaran et al., 1994; Rosato et al., 2001). The degree to which each of these processes occurs is highly dependent on cell type, agents employed, and drug dose and treatment interval (Melnick and Licht, 2002). Notably, exposure of different cell lines to HDAC inhibitors results in up-regulation, at the transcriptional level, of the endogenous cyclin-dependent kinase (CDK) inhibitor p21$^{WAF1/CIP1}$, which inhibits multiple cyclin/CDK complexes and may also exert direct antiapoptotic actions (El Deiry et al., 1993; Rosato et al., 2002), possibly by

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ABBREVIATIONS: HDAC, histone deacetylase; SB, sodium butyrate; SAHA, suberoylanilide hydroxamic acid; CDK, cyclin-dependent kinase; FP, flavopiridol; PMA, phorbol 12-myristate 13-acetate; ΔNLS, p21$^{WAF1/CIP1}$ lacking the nuclear localization signal; PBS, phosphate-buffered saline; BOC-D-fmk, benzyloxycarbonyl-Asp(Ome)-fluoromethyl ketone; PBS-T, phosphate-buffered saline/Tween 20; PARP, poly(ADP-ribose)polymerase; AIF, apoptosis inducing factor; CDKI, cyclin-dependent kinase inhibitor; Dox, doxycycline; $\Delta\psi_m$, mitochondrial membrane potential; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; ASK, apoptosis signal-regulating kinase; ROS, reactive oxygen species; PAGE, polyacrylamide gel electrophoresis; PI, propidium iodide; $DiOC_6$, 3,3-dihexlyoxacarbocyanine.

directly blocking activation of caspases (e.g., caspase-3) (Asada et al., 1999). CDK inactivation leads in turn to dephosphorylation of retinoblastoma protein, which then binds to and inactivates E2F, resulting in transcriptional repression of cell cycle progression genes, growth arrest, and cell differentiation (Rosato et al., 2002). We and others have demonstrated the critical functional role of p21$^{WAF1/CIP1}$ in HDAC inhibitor-induced cell growth arrest, differentiation, and apoptosis in malignant cells (Han et al., 2000; Rosato et al., 2001).

Flavopiridol [FP (L86-8275; NSC 649890)] represents a family of polyhydroxylated flavones that includes quercetin and genistein and that has demonstrated promising preclinical activity (Wang, 2000). FP binds to the CDK ATP binding pocket and potently inhibits cdk2, cdc2, and cdk4 at submicromolar concentrations, although at higher concentrations it inhibits other kinases, including protein kinase C, epidermal growth factor receptor, and extracellular regulated kinase 1 (Sedlacek et al., 2002). In preclinical studies, FP has been shown to induce apoptosis in lung cancer cells (Shapiro et al., 1999) and, at nanomolar concentrations, in malignant hematopoietic cells (Parker et al., 1998; Semenov et al., 2002). Administration of FP has been associated with G_2M and G_1S arrest, depending upon the model system (Carlson et al., 1996; Motwani et al., 1999), as well as down-regulation of cyclin D1 (Carlson et al., 1999). In addition, FP, by inhibiting the positive transcription elongation factor-b complex, may act as a transcriptional repressor (Chao and Price, 2001). FP also interacts synergistically with more established chemotherapeutic agents, including cytarabine (Bible and Kaufmann, 1997) and paclitaxel (Bible and Kaufmann, 1997; Motwani et al., 1999). More recently, we demonstrated that FP synergistically induces apoptosis in human leukemia cells when combined with certain differentiation-inducing agents, including phorbol 12-myristate 13-acetate (PMA) and HDAC inhibitors such as SAHA and SB, events associated with abrogation of p21$^{WAF1/CIP1}$ expression (Cartee et al., 2001; Almenara et al., 2002; Rosato et al., 2002). In view of evidence that disruption of p21$^{WAF1/CIP1}$ induction promotes HDAC inhibitor-mediated lethality (Rosato et al., 2001), it is tempting to speculate that FP-mediated repression of this CDKI contributes to lethality.

Currently, direct evidence that interference with p21$^{WAF1/CIP1}$ expression by FP contributes functionally to synergistic antileukemic interactions with HDAC inhibitors is lacking. To address this issue, we have employed human leukemia cells inducibly expressing p21$^{WAF1/CIP1}$ under the control of a tetracycline-responsive promoter, as well as leukemia cells stably expressing a nuclear localization signal p21$^{WAF1/CIP1}$ mutant (ΔNLS). The latter accumulates in the cytoplasm, where it blocks caspase activation (Asada et al., 1999). Our results support the notion that FP-mediated attenuation of p21$^{WAF1/CIP1}$ induction plays a key functional role in promoting HDAC inhibitor-induced apoptosis in human leukemia cells, possibly through a mechanism involving blockade of the apoptotic caspase cascade.

Materials and Methods

Cells. Jurkat cells were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in a 37°C, 5% CO_2, fully humidified incubator and cultured in RPMI 1640 medium supplemented with sodium pyruvate, minimal essential medium, essential vitamins, L-glutamate, penicillin, streptomycin, and 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Jurkat cells stably expressing cytoplasmic p21$^{WAF1/CIP1}$ (i.e., lacking the nuclear localization signal; aa 1–140) (p21$^{WAF1/CIP1}$-ΔNLS) were obtained by polymerase chain reaction amplification as described by Asada et al. (Asada et al., 1999). Briefly, a full-length p21$^{WAF1/CIP1}$ cDNA (kindly provided by Dr. Steven Elledge, Baylor College of Medicine, Houston, TX) was used as template in conjunction with the following set of primers: forward: 5′-ATGTCAGAACCGGCTGGGGAT-3′; reverse: 5′-CCTGGAGACTCTCAGGGTCGATAA-3′. The corresponding polymerase chain reaction fragment was subcloned using the TOPO-TA cloning kit (Invitrogen), removed as an EcoRI fragment, and cloned into pcDNA3.1 (Invitrogen), sequenced and transfected into Jurkat cells. Neomycin-resistant clones (designated p21ΔNLS 8 and 35) were isolated and expanded in medium containing G418 (0.4 mg/ml) for 3 weeks and then tested by Western immunoblot analysis for the ectopic expression of a truncated protein using an antibody against p21$^{WAF1/CIP1}$. All experiments were performed using cells in logarithmic phase growth suspended at 2.5×10^5 cells/ml.

Tet-On Inducible p21$^{WAF1/CIP1}$-Jurkat Cell Lines. A stable Jurkat lymphoblastic leukemia cell line inducibly expressing p21$^{WAF1/CIP1}$ was generated as follows. A full-length p21$^{WAF1/CIP1}$ cDNA (kindly provided by Dr. Steven Elledge, Baylor College of Medicine, Houston, TX) was subcloned into the pTRE2-hygro2-Myc expression vector (BD Biosciences Clontech, Palo Alto, CA) according to standard techniques. Jurkat "Tet-On" cells that stably express a reverse tet transactivator regulator protein (BD Biosciences Clontech) were transfected with Myc-tagged p21$^{WAF1/CIP1}$-pTRE2-hygro2 by electroporation (600 V, 60 ms) using 0.4-μm cuvettes. Stable clones derived from single cells were selected by limiting dilution in RPMI 1640 medium supplemented with 10% of Tet-System-approved fetal bovine serum (BD Biosciences Clontech) in the presence of 0.4 mg/ml hygromycin. To test for induced expression of the myc-p21$^{WAF1/CIP1}$, stable clones were left untreated or treated for 24 h with 2 μg/ml doxycycline, after which they were harvested and analyzed for myc-p21$^{WAF1/CIP1}$ by Western blot analysis as described below.

Drugs and Chemicals. Sodium butyrate was supplied as a powder (Calbiochem, La Jolla, CA) and dissolved in PBS before use. Flavopiridol FP (L86 8275; NSC 649890) was kindly provided by Dr. Edward Sausville (Cancer Treatment and Evaluation Program, National Cancer Institute, Bethesda, MD). FP was formulated in dimethyl sulfoxide (Sigma-Aldrich, St. Luis, MO) and 10^{-2} M stock solution was stored at −20°C. The pan-caspase inhibitor BOC-D-fmk was purchased from Enzyme System Products (Livermore, CA) and dissolved in dimethyl sulfoxide.

Assessment of Apoptosis. Apoptotic cells were evaluated by both morphological assessment of Wright-Giemsa–stained cytospin preparations and by annexin V/PI staining (BD Biosciences PharMingen, San Diego, CA) as per the manufacturer's instructions, as described previously (Almenara et al., 2002). The extent of apoptosis was determined using a Becton Dickinson FACScan flow cytometer.

Assessment of Mitochondrial Membrane Potential. At the indicated intervals, cells were harvested and 2×10^5 cells were incubated with 40 nM $DiOC_6$ for 15 min at 37°C. Analysis was then carried out on Becton-Dickinson FACScan cytofluorometer. The percentage of cells exhibiting low levels of $DiOC_6$, reflecting loss of mitochondrial membrane potential, was determined as described previously (Rosato et al., 2002)

Analysis of Cytosolic Cytochrome *c* and AIF. A technique described previously was employed (Almenara et al., 2002). The S-100, or cytosolic fraction, was subjected to Western analysis as described above. For each condition, 30 μg of the S-100 fraction was loaded on the gel and probed with the corresponding antibody.

Western Blot Analysis. Whole cell-pellets were washed twice in PBS, resuspended in PBS, and lysed by the addition of 1 volume of loading buffer. Total proteins (30 μg) per point were separated by 4 to 12% Bis-Tris Nu-Page precast gel system (Invitrogen) and electroblotted to nitrocellulose. The blots were blocked in 5% nonfat milk in PBS-T and probed for 1 h with the appropriate dilution of primary antibody. Blots were washed 3 × 10 min in PBS-T and then incubated with a 1:2000 dilution of peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were again washed 3 × 10 min in PBS-T and then developed by enhanced chemiluminesence (PerkinElmer Life and Analytical Sciences, Boston, MA). Where indicated, blots were stripped and reprobed with antibodies directed against actin.

Antibodies for Western Blot Analysis. Primary antibodies for the following proteins were used at the designated dilutions: procaspase 3, caspase 9, p21$^{WAF1/CIP1}$ (1:1000; BD Transduction Laboratories, Lexington, KY); cleaved caspase-3 and -9 (1:1000; Cell Signaling Technology, Beverly, MA); PARP (1:1000; BioMol, Plymouth Meeting, PA); cytochrome *c* (1:1000; BD Biosciences PharMingen); apoptosis-inducing factor (AIF), Bid, pJNK, JNK2 (1:2000; Santa Cruz, Santa Cruz, CA); caspase 8 (1:2000; Alexia Corporations, San Diego, CA); actin (1:2000; Sigma-Aldrich Chemicals). Secondary antibodies conjugated to horseradish peroxidase were obtained from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD).

Measurement of ROS Production. Cells were treated with 20 μM 2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR) for 30′ at 37°C, fluorescence was measured by flow cytometry on a FACS scan, and analyzed with CELLQuest software. H_2O_2 was used as a positive control.

Immunoprecipitation Assay. Cells were harvested (2×10^7), washed in ice-cold PBS, suspended in immunoprecipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) containing protease inhibitors, and quickly sonicated on ice. After centrifugation, the supernatant was incubated with antibody against pro-caspase 3 (BD Transduction Laboratories) and rocked overnight at 4°C. Immune complexes were immunoprecipitated with immunomagnetic microspheres, Dynabeads M-450 precoated with sheep anti-Mouse IgG (Dynal Biotech, Lake Success, NY). The beads were washed with radioimmunoprecipitation assay buffer, treated with 1× sample buffer, boiled, and loaded in precast gels for Western blot analysis.

Immunohistochemistry. After cytospin, the cells were fixed for 10 min in a mix of ethanol/acetic acid (95/5%) and washed with water. The cytospin preparations were permeabilized for 15 min with 0.1% Triton and washed again with water. All the slides were placed in endogenous blocking solution (methanol + 10% hydrogen peroxide) for 20 min at room temperature. The slides were washed three times with water and once with PBS. All sections were blocked with PBS/1% bovine serum albumin for 1 h at room temperature and then incubated overnight at 4°C in a humidified chamber with the appropriate dilutions of mouse monoclonal anti-human p21$^{WAF1/CIP1}$ antibody (BD Transduction Laboratories). The slides were washed in PBS and detected as per manufacturer's instructions using a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA). Positive cells were revealed by the use of diaminobenzidine as a substrate (BioGenex, San Ramon, CA), washed, and counterstained with modified Harris hematoxylin for 30 s. No positive cells were identified when the specific antibodies were replaced with isotype-matched control antibodies.

Statistical Analysis. The significance of differences between experimental conditions was determined using the student's *t* test for unpaired observations.

Results

Cotreatment with SB/FP Blocks p21$^{WAF1/CIP1}$ Induction and Promotes Apoptosis in Jurkat Lymphoblastic Leukemia Cells. Previously, we demonstrated that coadministration of HDAC inhibitors (e.g., SAHA, SB) with FP resulted in a striking increase in apoptosis in human myelomonocytic U937 leukemia cells (Almenara et al., 2002; Rosato et al., 2002). In Jurkat lymphoblastic leukemia cells, 1 mM SB or 200 nM FP administered alone induced apoptosis only modestly, reflected by the percentage of AnexinV/PI$^+$ cells (Fig. 1A). However, the combination of SB and FP resulted in a pronounced apoptotic response (i.e., ≥60% within the first 24 h). Furthermore, exposure of Jurkat cells to FP resulted in abrogation of both the basal expression of p21$^{WAF1/CIP1}$ as well as that induced by exposure to SB (Fig. 1B). The mechanism by which FP blocked p21$^{WAF1/CIP1}$ expression was caspase-independent (Fig. 1B), in that the caspase inhibitor Boc-D-fmk (20 μM) failed to block down-regulation of this CDKI (Fig. 1B, BF+I). Thus, analogous to results obtained in U937 cells exposed to PMA (Cartee et al., 2001) or SAHA (Almenara et al., 2002), interference with induction of p21$^{WAF1/CIP1}$ by FP dramatically increased apoptosis in Jurkat cells.

Enforced Expression of p21$^{WAF1/CIP1}$ Protects Cells against HDAC Inhibitors/FP-Induced Cell Death. To investigate the functional role of dysregulated p21$^{WAF1/CIP1}$ expression in SB/FP-induced apoptosis, two separate Jurkat cell lines transfected with a p21$^{WAF1/CIP1}$ cDNA (J-p21-5 and J-p21-15) under the control of a Tetracycline-responsive promoter were employed. In these lines, expression of the gene of

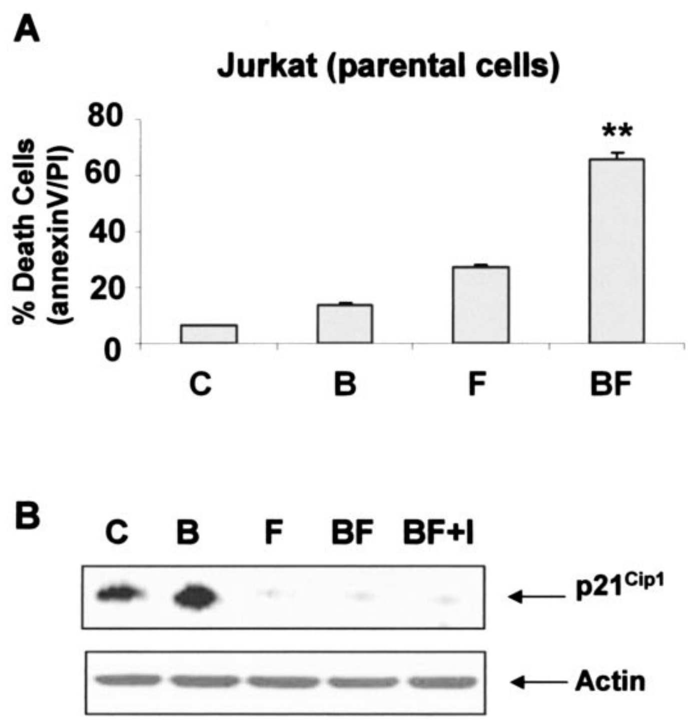

Fig. 1. Exposure of Jurkat cells to SB/FP induces apoptosis and p21$^{WAF1/CIP1}$-down-regulation. A, Jurkat cells were exposed to 1 mM SB, 200 nM FP, or both for 24 h, after which the percentage of apoptotic cells was monitored by AnexinV/PI staining. B, Western blot analysis of lysates from Jurkat cells treated for 24 h with 1 mM SB (B), 200 nM FP (F), or the combination SB/FP (BF) with or without the pan-caspase inhibitor Boc-D-fmk (20 μM; BF+I). After treatment, cells were pelleted and lysed, and 30 μg of protein was separated by SDS-PAGE as described under *Materials and Methods*. Blots were then probed with an anti-p21$^{WAF1/CIP1}$ antibody, after which they were stripped and reprobed with an antibody to actin to ensure equivalent loading and transfer. The results of a representative study are shown; two additional experiments yielded similar results. **, $p < 0.01$; values significantly greater than those obtained from untreated cells (C) or cells treated with either drug alone (B, F). Results represent the means for three separate experiments ± S.D.

interest (in this case, $p21^{WAF1/CIP1}$) can be induced by culturing cells in the presence of either tetracycline or doxycycline (Dox). When cells were exposed individually to 1 mM SB, 300 nM FP (data not shown), or 400 nM FP for 24 h, minimal apoptosis occurred in the presence or absence of Dox. In contrast, a marked increase in apoptosis was observed in cells exposed to SB and either 300 or 400 nM FP in the absence of Dox. Moreover, the extent of cell death was significantly reduced (i.e., approximately 40 to 50%; $p < 0.001$) in both of the clones (J-p21-5 and J-p21-15) when Dox was added to the medium (Fig. 2A). In separate studies, treatment of untransfected cells or cells transfected with an empty vector with Dox did not modify SB/FP-mediated cell death (data not shown). Apoptosis was also essentially abrogated by addition of the pan-caspase inhibitor Boc-D-fmk (+I; 25 μM). Western blot analysis of lysates collected after treatment demonstrated that for both inducible clones, addition of Dox resulted in a very pronounced increase in $p21^{WAF1/CIP1}$ expression in control cells (Fig. 2B). Levels of Dox-induced $p21^{WAF1/CIP1}$ expression (C^+) were between three and five times higher than those achieved by exposing uninduced (−Dox) to SB (B) (Fig. 2A, insets). Although levels of $p21^{WAF1/CIP1}$ in cells exposed to 300 nM SB/FP, and particularly to 400 nM SB/FP, in the presence of Dox were less than those of controls, they were nevertheless substantially greater than those observed in drug-treated cells cultured in the absence of Dox, and comparable with, if not quite as great as, those observed in uninduced cells exposed to SB alone. Addition of Boc-D-fmk partially restored $p21^{WAF1/CIP1}$ levels to those of controls, suggesting that reductions in Dox-induced $p21^{WAF1/CIP1}$ expression in drug-treated cells reflected caspase-mediated degradation of ectopic protein.

Western blot analysis of lysates from J-p21-15 cells exposed to SB (1 mM) + FP (400 nM) in the presence or absence of Dox revealed that enforced expression of $p21^{WAF1/CIP1}$ resulted in a pronounced decrease in the activation of the

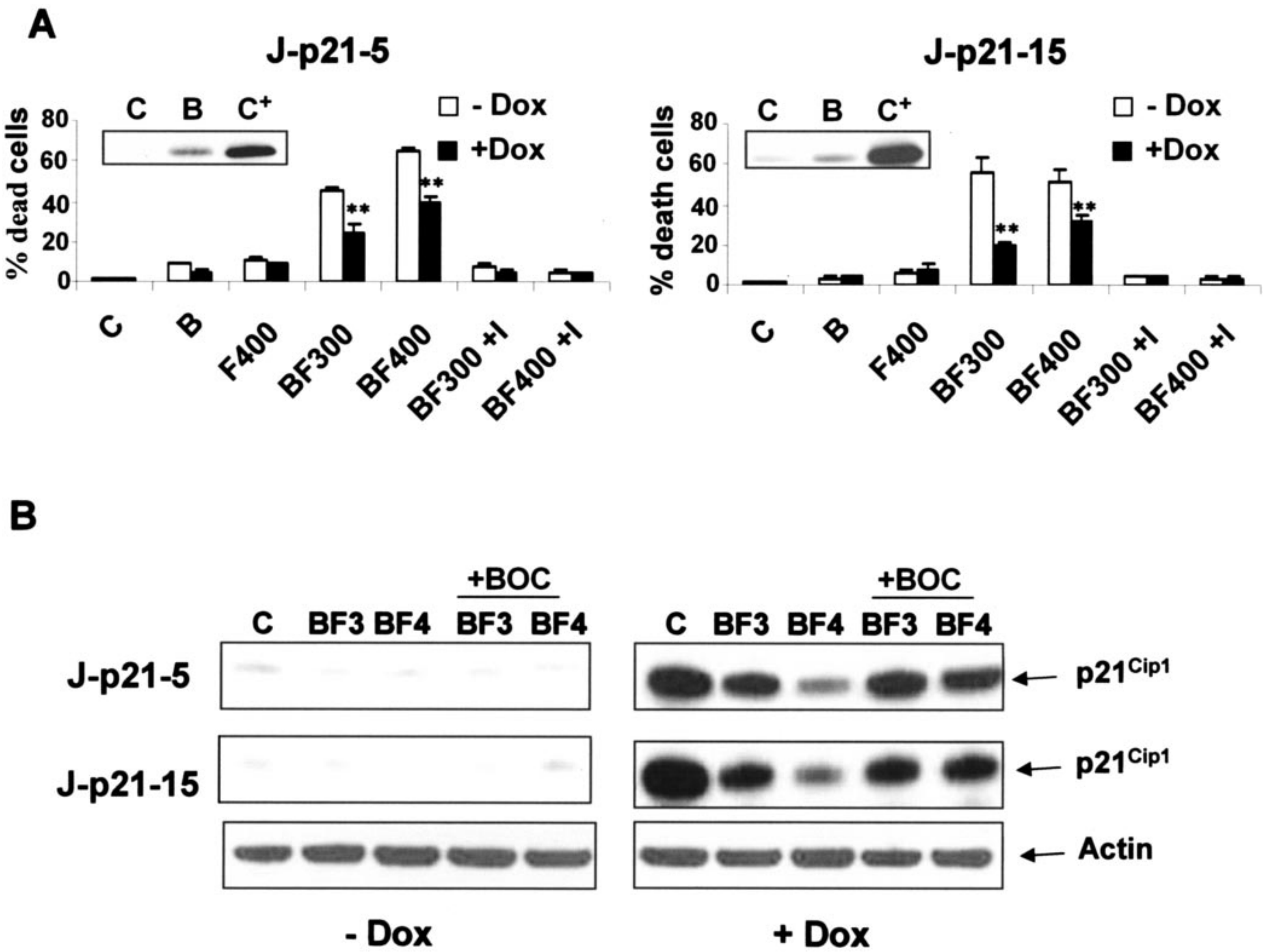

Fig. 2. Enforced expression of $p21^{WAF1/CIP1}$ protects cells against HDAC inhibitor/FP-induced cell death. A, Jurkat cells inducibly expressing $p21^{WAF1/CIP1}$ cells (J-p21, clones 5 and 15) were pretreated with or without Dox (□, −Dox; ■, +Dox) for 24 h, after which they were exposed to 1 mM SB (B), 300 or 400 nM FP (F), or the combination SB/FP (BF) in the presence (+I) or absence of the caspase inhibitor Boc-D-fmk (20 μM) for 24 h. The apoptotic response was then evaluated by flow cytometric analysis of AnnexinV/PI staining; results correspond to the means for three separate determinations ± S.D. **, $p < 0.01$; values significantly lower than those obtained in cells cultured in the absence of Dox (−Dox). A, inset, WB analysis of $p21^{WAF1/CIP1}$ expression in lysates from J-p21-5 and J-p21-15 clones cultured in the absence (C) or presence of 1 mM SB (B). For comparison, $p21^{WAF1/CIP1}$ expression in cells induced with Dox for 24 h (C^+) is shown. B, after treatment as indicated, cell lysates were collected and 30 μg of protein was separated by SDS-PAGE as described under *Materials and Methods*. Blots were then probed with an anti-myc tag antibody, after which they were stripped and reprobed with an antibody to actin to ensure equivalent loading and transfer. The results of a representative study are shown; two additional experiments yielded similar results.

caspase cascade, manifested by diminished cleavage/activation of caspases-9, -3, and-8, and Bid, as well as PARP degradation and the appearance of a caspase-9 cleavage fragment (Fig. 3). Together, these findings indicate that enforced expression of $p21^{WAF1/CIP1}$ in SB/FP-treated cells significantly attenuates the lethal effects of this regimen. They are also consistent with the notion that $p21^{WAF1/CIP1}$ down-regulation plays a significant functional role in synergistic antileukemic interactions between these agents.

SB/FP-Induced Cell Lethality Is Dependent Upon the Level of $p21^{WAF1/CIP1}$ Expression. Results from the previous studies suggested that enforced expression of $p21^{WAF1/CIP1}$ blocked SB/FP-induced apoptosis. Attempts were therefore undertaken to determine whether $p21^{WAF1/CIP1}$-inhibitory effects could be related to the level of Dox-induced protein expression. To address this issue, J-p21-5 cells were incubated for 24 h in the presence of 0, 0.1, or 2.0 μg/ml Dox followed by exposure to 1 mM SB/300 nM FP for an additional 24 h, after which the extent of apoptosis was evaluated. Western blot analysis of lysates from J-p21 cells after 24 h of Dox revealed that 0.1 μg/ml Dox induced a modest increase in $p21^{WAF1/CIP1}$ expression, whereas 2.0 mg/ml resulted in a more pronounced increase in protein levels. As shown in Fig. 4B, administration of 0.1 mg/ml Dox modestly but significantly ($P < 0.05$) reduced apoptosis induced by SB/FP compared with cells cultured in the absence of Dox. Moreover, addition of 2.0 μg/ml Dox to the medium, which robustly induced $p21^{WAF1/CIP1}$, resulted in a further reduction in SB/FP-mediated lethality ($P < 0.05$ versus cells exposed to 0.1 μg/ml Dox). Together, these findings suggest that

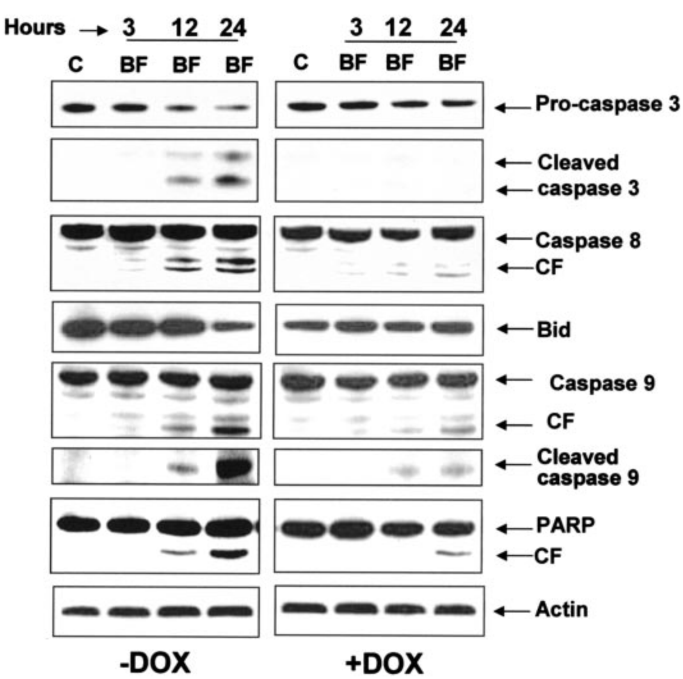

Fig. 3. Enforced expression of $p21^{WAF1/CIP1}$ diminishes activation of the caspase cascade by SB/FP. J-p21-15 cells were pretreated with or without Dox for 24 h, after which they were coexposed to 1 mM SB/300 nM FP (BF) for an additional 24 h. Cell lysates were collected, and 30 μg of protein was separated by SDS-PAGE as described under *Materials and Methods*. Blots were then probed with the antibodies against pro-caspase-3, cleaved/activated caspase-3, caspase-8, Bid, caspase-9, cleaved/activated caspase-9, and PARP; they were then stripped and reprobed with an antibody to actin to ensure equivalent loading and transfer. The results of a representative study are shown; two additional experiments yielded similar results. CF, cleavage fragment.

the level of $p21^{WAF1/CIP1}$ induction plays a role in determining the extent to which SB/FP-mediated lethality is attenuated.

Enforced $p21^{WAF1/CIP1}$ Expression Diminishes SB/FP-Mediated Mitochondrial Injury. Previous studies have demonstrated that coadministration of HDAC inhibitors with FP results in a marked increase in mitochondrial injury including a loss of mitochondrial membrane potential ($\Delta\psi_m$) and release of cytochrome *c* and AIF into the cytosol (Almenara et al., 2002; Rosato et al., 2002). Therefore, mitochondrial events were evaluated in J-p21-15 cells treated for 24 h ± 1 μg/ml Dox followed by exposure to 1 mM SB + 300 nM FP for an additional 24 h. Treatment of uninduced cells (−Dox) with SB or FP alone had no effect on either $\Delta\psi_m$ or release of cytochrome *c* and AIF into the cytosolic S-100 fraction (data not shown). However, coadministration of SB/FP resulted in a marked increase in the percentage of cells displaying loss of $\Delta\psi_m$ (Fig. 5A) as well as increased cytosolic release of cytochrome *c* and AIF (Fig. 5B). When parallel studies were performed in cells cultured in the presence of Dox, a significant reduction in loss of $\Delta\psi_m$ was noted ($P < 0.02$ versus cells cultured in the absence of Dox). Similarly, SB/FP-mediated release of cytochrome *c* and AIF was modestly but discernibly diminished in cells exposed to Dox. These findings indicate that enforced expression of $p21^{WAF1/CIP1}$ attenuates mitochondrial damage induced by simultaneous exposure of cells to SB in conjunction with FP.

Protection against SB/FP-Induced Cell Death by $p21^{WAF1/CIP1}$ Does Not Involve Activation of the Pro-Apoptotic SAPK/JNK Pathway or ROS Generation. There is evidence that the apoptosis inhibitory activity of $p21^{WAF1/CIP1}$ is related to the inhibition of the stress-activated mitogen-activated protein kinase cascade through binding to the apoptosis signal-regulating kinase-1 (ASK) (Asada et al., 1999), which in turn is activated by oxidative stress. Attempts were therefore made to determine the effects, if any, of enforced expression of $p21^{WAF1/CIP1}$ on SB/FP-mediated activation of the stress-related JNK pathway or on generation of reactive oxygen species (ROS). Western blot analysis of lysates from J-p21-15 cells cultured in medium or Dox for 24 h followed by exposure to SB/FP for 3, 12, and 24 h revealed no changes in expression of phospho-JNK (Fig. 6A). In addition, flow cytometric analysis revealed no significant effect of Dox exposure on ROS generation after SB/FP treatment [Fig. 6B; results obtained after 3-h treatment are shown; similar observations were made at 12 and 24 h (data not shown)]. These findings argue against the possibility that $p21^{WAF1/CIP1}$-mediated cytoprotective effects involve perturbations in the oxidative stress-SAPK/JNK axis.

Cytoplasmic Localization of $p21^{WAF1/CIP1}$ Mediates its Protective Role against SB/FP-Induced Cell Death. Recent reports have attributed the apoptosis-inhibitory activity of $p21^{WAF1/CIP1}$ to its translocation into the cytoplasm (Asada et al., 1999; Suzuki et al., 2000). Based on these observations, we investigated the cellular localization of ectopically expressed $p21^{WAF1/CIP1}$ to identify possible mechanisms responsible for the cytoprotective role of $p21^{WAF1/CIP1}$. In situ immunocytochemical analysis demonstrated very low expression of $p21^{WAF1/CIP1}$ in noninduced (−Dox) J-p21-15 cells (Fig. 7A). After 24-h exposure to Dox, robust expression of $p21^{WAF1/CIP1}$ was detected that localized in both the nucleus, where the strongest signals were observed, and in the cytoplasm (Fig. 7B). Because cytoplasmic accumulation of

$p21^{WAF1/CIP1}$ has been proposed to account for its antiapoptotic effects, Jurkat cells were stably transfected with a $p21^{WAF1/CIP1}$-cDNA lacking the C-terminal domain coding for the nuclear localization signal (p21-ΔNLS; aa 1–140) (Asada et al., 1999). These cells displayed clear cytoplasmic expression of the ΔNLS $p21^{WAF1/CIP1}$-deletant mutant, detected by immunohistochemical analysis (Fig. 7D), whereas no signal was observed in the corresponding J/EV (empty vector) cells (Fig. 7C). The specificity of the immunohistochemical staining was verified by the use of irrelevant control primary antibodies (data not shown) as well as by immunostaining with secondary antibody alone (Fig. 7D, inset). Expression of $p21^{WAF1/CIP1}$-ΔNLS was further confirmed by Western blot analysis of lysates obtained from two separate clones (8 and 36) demonstrating robust expression of a rapidly migrating species compared with full-length (aa 1–164) $p21^{WAF1/CIP1}$, as shown in lysates from Jurkat cells treated for 24 h with either 10 nM PMA or 1 mM SB (Fig. 7E). In contrast to the effects of full-length $p21^{WAF1/CIP1}$ expression on leukemia cells (Cartee et al., 2001; Rosato et al., 2001), ectopic expression of p21-ΔNLS affected neither cell cycle traverse nor differentiation (data not shown).

Exposure of two separate p21-ΔNLS clones (designated 8 and 36) to SB/FP resulted in a significant decrease in apoptosis monitored by AnnexinV/PI ($P < 0.01$ in each case; Fig. 8A). Inhibition of drug-induced apoptosis by cytoplasmic $p21^{WAF1/CIP1}$ correlated closely with diminished mitochondrial damage, reflected by a decrease in the percentage of cells exhibiting a reduction in $\Delta\Psi_m$ ($P < 0.02$ in each case; Fig. 8B). In accord with these findings, reductions in caspase-8 and -3 activation, the appearance of a caspase-3 cleavage product, and PARP degradation were observed in both p21-ΔNLS clones (Fig. 8C). Together, these findings are consistent with the notion that cytoplasmic localization of $p21^{WAF1/CIP1}$ plays a key role in the antiapoptotic actions of this CDKI in cells exposed to the SB/FP regimen.

Enforced Expression of $p21^{WAF1/CIP1}$ Leads to Increased Formation of a Complex with Caspase 3. Accumulating evidence suggests that the antiapoptotic activity of cytoplasmic $p21^{WAF1/CIP1}$ stems from formation of a complex with caspase 3, thereby inhibiting activation of the caspase cascade (Suzuki et al., 1999, 2000). In view of the association between cytoplasmic accumulation of $p21^{WAF1/CIP1}$ and inhibition of SB/FP-induced cell death (Figs. 7 and 8), the forma-

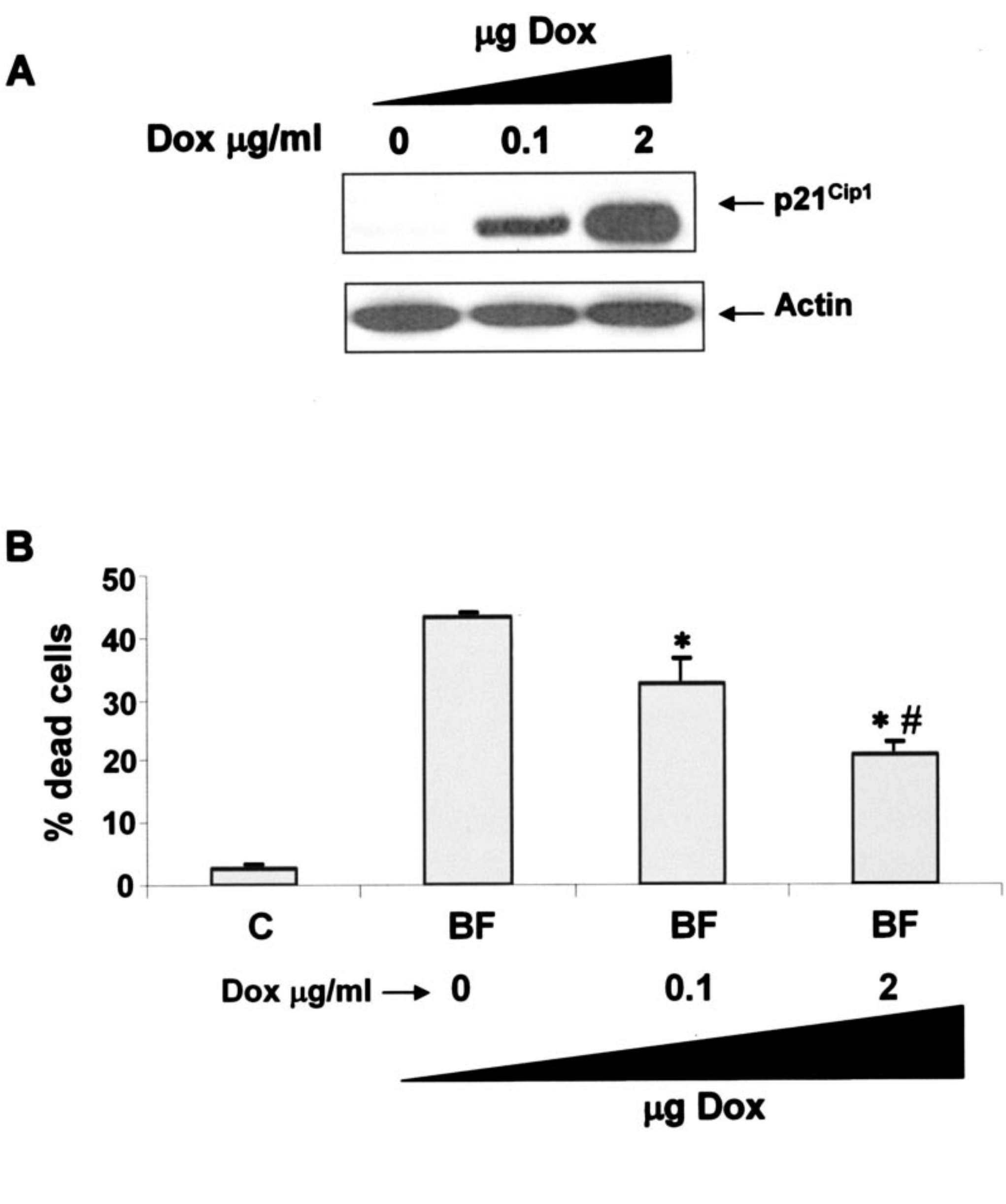

Fig. 4. Relationship between $p21^{WAF1/CIP1}$ induction and SB/FP-induced cell death. A, J-p21-15 cells were pretreated with 0, 0.1, or 2.0 μg/ml Dox for 24 h, after which they were coexposed to 1 mM SB/300 nM FP (BF) as above. Cell lysates were prepared, and 30 μg of was protein separated by SDS-PAGE as described under *Materials and Methods*. Blots were then probed with an anti-myc tag antibody that recognizes inducible $p21^{WAF1/CIP1}$, after which they were stripped and reprobed with an antibody to actin to ensure equivalent loading and transfer. The results of a representative study are shown; two additional experiments yielded similar results. B, J-p21-15 cells pretreated for 24 h with the indicated concentrations of Dox were exposed for an additional 24 h to 1 mM SB/300 nM FP (BF) and the extent of cell death was determined by flow cytometry (AnnexinV/PI, 7-amino-actinomycin D uptake) as described under *Materials and Methods*. Values represent the means for three separate experiments performed in triplicate ± S.D. *, $p < 0.01$; values significantly lower than those obtained with Dox-untreated (−Dox) cells. #, $p < 0.01$; values significantly lower than those obtained with cells pretreated with 0.1 μg Dox.

tion of caspase 3-$p21^{WAF1/CIP1}$ complexes was examined in cells ectopically expressing full-length and ΔNLS $p21^{WAF1/CIP1}$. Whole-cell lysates from ±Dox J-p21 and p21-ΔNLS cells were immunoprecipitated with an anti-pro-caspase-3 monoclonal antibody and the composition of the pro-caspase-3/$p21^{WAF1/CIP1}$ complex was analyzed by Western blot. Figure 9 demonstrates that $p21^{WAF1/CIP1}$ was undetectable in immunoprecipitates from either noninduced J-p21 (−Dox) cells or empty-vector control 3.1-Jurkat cell lysates (Fig. 9, A and B, respectively). In contrast, $p21^{WAF1/CIP1}$ was readily detected in pro-caspase 3 immunoprecipitates in lysates obtained from Dox-induced J-p21 and p21-ΔNLS cells. Reverse immunoprecipitates (i.e., IP: $p21^{WAF1/CIP1}$; Western blot: caspase-3) yielded similar results with respect to detection of complex formation (data not shown). Finally, as shown in Fig. 9C, for both ±Dox-treated J-p21 cells, exposure to SB increased the amount of $p21^{WAF1/CIP1}$ in complex with pro-caspase 3. Furthermore, these levels seemed significantly diminished after SB/FP treatment of Dox-induced J-p21 cells (Fig. 9C). These findings indicate that cytoplasmic $p21^{WAF1/CIP1}$ associates with pro-caspase 3 and raise the possibility that this event may inhibit activation of the caspase cascade, thereby blocking SB/FP-induced apoptosis. Conversely, interference with $p21^{WAF1/CIP1}$ induction e.g., by FP may attenuate this process and, in so doing, promote SB-induced cell death.

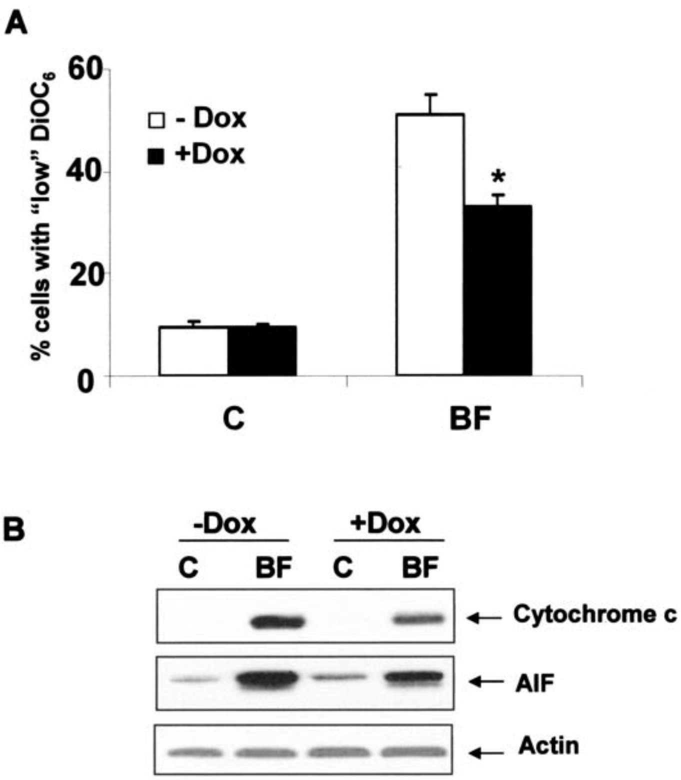

Fig. 5. Enforced expression of $p21^{WAF1/CIP1}$ attenuates SB/FP-mediated mitochondrial damage. A, J-p21-15 cells were pretreated with or without Dox for 24 h followed by exposure to 1 mM SB/300nMFP (BF) for an additional 24 h, after which the percentage of cells exhibiting loss of $\Delta\psi_m$ was evaluated by flow cytometric analysis of $DiOC_6$ uptake as described under *Materials and Methods*. Results represent the means for three separate experiments performed in triplicate ± S.D. *, $p < 0.01$; values significantly lower than those obtained with Dox-untreated cells (−[Dox). B, after pretreatment for 24 h with or without Dox, J-p21-15 cells were exposed to SB + FP as indicated above and pelleted; protein lysates were extracted from the cytosolic S-100 fraction as described under *Materials and Methods*. Protein (30 μg) were separated by SDS-PAGE, and blots were then probed with the indicated antibodies, after which they were stripped and reprobed with an antibody to actin to ensure equivalent loading and transfer. The results of a representative study are shown; two additional experiments yielded similar results.

Discussion

The CDKI $p21^{WAF1/CIP1}$ is critically involved in cell cycle arrest after DNA damage (Bartek and Lukas, 2001) as well as that accompanying differentiation induction by various agents including HDAC inhibitors (Freemerman et al., 1997; Rosato et al., 2001). However, there is accumulating evidence that this CDKI plays a key role in regulating the apoptotic response of leukemia and other neoplastic cells to such agents. For example, stable expression of a $p21^{WAF1/CIP1}$ antisense construct has been shown to increase the sensitivity of human leukemia cells to SB, SAHA, and, most recently, the benzamide HDAC inhibitor MS-275 (Vrana et al., 1999; Rosato et al., 2001, 2003). Conversely, up-regulation of p21 correlated with reduced sensitivity and interference with activation of the apoptotic cascade induced by the HDAC inhibitor azelaic bishydroxamic acid (Burgess et al., 2001). Recently, we reported that the pharmacologic CDK inhibitor FP interacts synergistically with SAHA and SB to induce mitochondrial injury and apoptosis in human leukemia cells (Almenara et al., 2002; Rosato et al., 2002). The rationale for these studies stemmed from the premise that because cell cycle arrest is required for leukemic cell maturation (Freytag, 1988), a pharmacologic CDK inhibitor might be expected to enhance maturation by HDAC inhibitors and other differentiation-inducing agents. However, contrary to expectations, FP opposed HDAC inhibitor-mediated maturation and instead promoted leukemic cell apoptosis. Significantly, this phenomenon was accompanied by a marked attenuation of the $p21^{WAF1/CIP1}$ response (Almenara et al., 2002; Rosato et al., 2002). Based upon earlier results involving leukemia cells displaying an impaired $p21^{WAF1/CIP1}$ response (Vrana et al., 1999; Rosato et al., 2001), it was tempting to postulate that FP-mediated antagonism of $p21^{WAF1/CIP1}$ induction contributed to the lethality of this drug combination. However, direct support for this concept has been lacking. The results described herein provide evidence that FP-mediated abrogation of the $p21^{WAF1/CIP1}$ response in human leukemia cells plays a significant functional role in promoting HDAC inhibitor-mediated lethality.

The mechanism by which FP blocks $p21^{WAF1/CIP1}$ induction is not known with certainty but may be related to the ability of this agent to inhibit transcription. For example, FP has been shown to form duplexes with DNA (Bible et al., 2000) and to mimic the actions of the transcriptional inhibitor actinomycin D (Lam et al., 2001). More recently, FP has been found to be a potent inhibitor of the positive-transcription elongation factor-b by virtue of its ability to block phosphorylation of the carboxyl-terminal domain (Chao et al., 2000). In this regard, the ability of FP to down-regulate expression of the antiapoptotic protein Mcl-1 at the transcriptional level has been implicated in induction of cell death in multiple myeloma cells (Gojo et al., 2002). Analogously, FP has been shown to interfere with transcription of $p21^{WAF1/CIP1}$ in human leukemia cells exposed to the phorbol ester PMA (Cartee et al., 2001). Although $p21^{WAF1/CIP1}$ is a known target of apoptotic caspases (Gervais et al., 1998), the inability of the broad caspase inhibitor Boc-D-fmk to block FP actions argues against this as a mechanism of p21$^{WAF1/CIP1}$ down-regulation. Taken together, these findings suggest that FP interferes with expression, at the transcriptional level, of a number of proteins required to oppose apoptosis in malignant hematopoietic cells, particularly those undergoing maturation.

There is considerable evidence, primarily indirect, supporting the notion that interference with p21$^{WAF1/CIP1}$ induction by FP contributes, at least in part, to potentiation of HDAC inhibitor-mediated apoptosis. For example, expression of p21$^{WAF1/CIP1}$ has been shown to protect tumor cells from the lethal effects of ionizing radiation and doxorubicin (Wang et al., 1999a). Conversely, loss of p21$^{WAF1/CIP1}$ has been found to sensitize cells to cytotoxic drugs (Wang et al., 1998), low-dose cytarabine (Wang et al., 1999b), and various differentiation-inducing agents, including phorbol esters (Wang et al., 1998) and HDAC inhibitors (Vrana et al., 1999; Rosato et al., 2001). In view of these findings, it is tempting to propose that interruption of p21$^{WAF1/CIP1}$ induction in HDAC inhibitor-treated cells contributes to enhanced lethality. However, direct evidence that this is in fact the case has been lacking. It is therefore noteworthy that enforced expression of p21$^{WAF1/CIP1}$ significantly, although partially, blocked HDAC inhibitor/FP-mediated apoptosis, as well as activation of the caspase cascade (e.g., cleavage of caspases-3 and -8, Bid, and PARP). The observation that enforced expression of p21$^{WAF1/CIP1}$ reduced the extent of FP/HDAC inhibitor-mediated apoptosis by only 50% could reflect multiple factors, including caspase-mediated protein degradation, which may prevent levels from reaching those achieved in uninduced cells exposed to an HDAC inhibitor alone. In support of this notion, cells displaying greater (enforced) expression of p21$^{WAF1/CIP1}$ were more resistant to HDAC inhibitor-mediated lethality than those exhibiting a more modest induction (Fig. 4B). Alternatively, heterogeneous expression of the Δ-NLS mutant (Fig. 7) could be responsible for incomplete protection from FP/HDAC inhibitor lethality. Finally, other factors (e.g., Mcl-1 down-regulation) (Rosato et al., 2002) could contribute to synergistic interactions between these agents. Nevertheless, taken together, such findings argue that interference

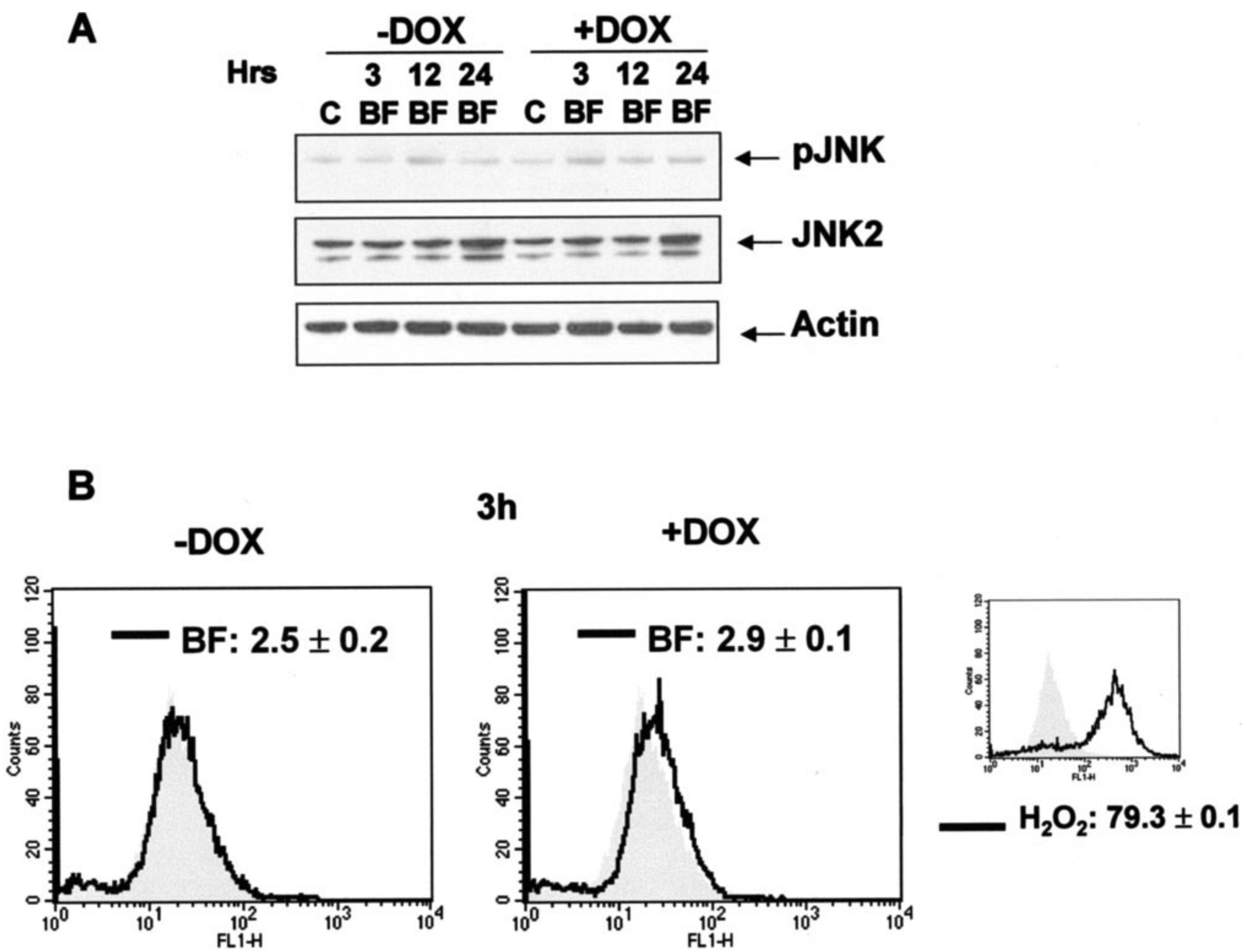

Fig. 6. Analysis of the SAPK/JNK pathway and ROS production. A, J-p21-15 cells were pretreated with or without Dox for 24 h followed by exposure to 1 mM SB/300 nM FP (BF) for an additional 3, 12, or 24 h, after which cell lysates were prepared and 30 μg of protein was separated by SDS-PAGE as described under *Materials and Methods*. Blots were then probed with the corresponding phospho-JNK and JNK antibodies. The blots were stripped and reprobed with an antibody to actin to ensure equivalent loading and transfer. The results of a representative study are shown; two additional experiments yielded similar results. B, after induction with Dox as described above, J-p21 cells were exposed to 1 mM SB/300 nM FP (BF) for additional 3 h, after which they were labeled with an oxidative-sensitive dye (2′,7′-dichlorodihydrofluorescein diacetate) and analyzed by flow cytometry to quantify the percentage of cells displaying an increase in ROS production (reflected by a rightward shift of the histogram). As controls, cells were treated with 10 μM H_2O_2 for 15 min and analyzed in parallel (far right). Values represent the means for three separate experiments ± S.D.

with HDAC inhibitor-mediated p21$^{WAF1/CIP1}$ induction by FP plays a significant, albeit partial functional role in synergistic antileukemic interactions between these agents.

The mechanism by which p21$^{WAF1/CIP1}$ inhibits apoptosis is not known with certainty but may involve, at least in part, cell cycle arrest permitting repair of DNA damage (McDonald et al., 1996). Alternatively, p21$^{WAF1/CIP1}$ is known to associate with ASK-1, an upstream activator of the stress-activated MAP kinase cascade, resulting in inactivation of the proapoptotic SAPK/JNK pathway (Asada et al., 1999). However, the failure of enforced expression of p21$^{WAF1/CIP1}$ to modify JNK activation in SB/FP-treated cells would argue against this mechanism in the present case. Recently, p21$^{WAF1/CIP1}$ has been shown to form a complex with procaspase-3, thereby protecting cells against Fas-mediated apoptosis (Suzuki et al., 1998). The binding region responsible for p21$^{WAF1/CIP1}$procaspase 3 interactions was later localized to the N-terminal region of the p21$^{WAF1/CIP1}$ protein and independent of the Cdk2 or proliferating cell nuclear antigen-binding domains (Suzuki et al., 1999). Furthermore, it has

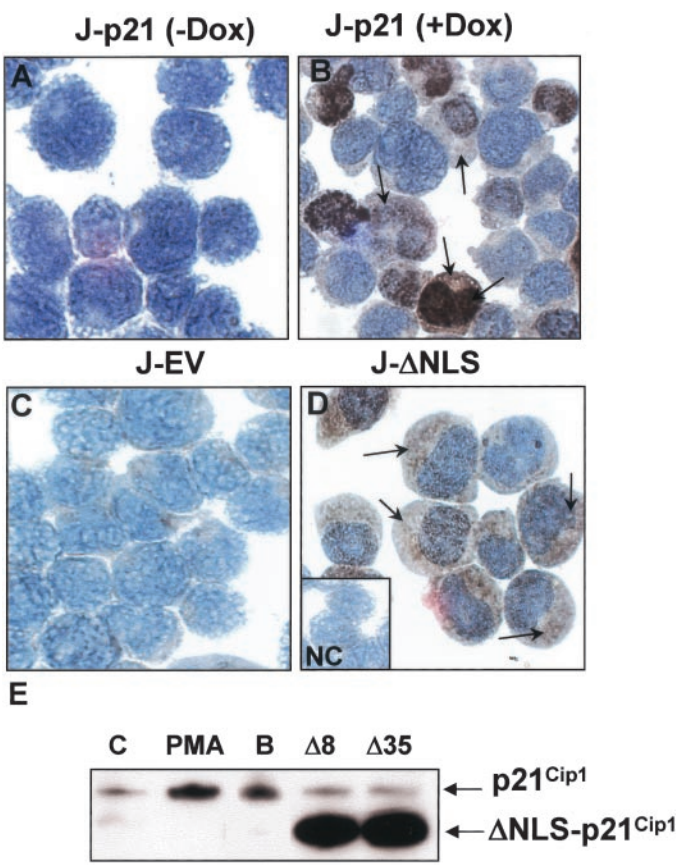

Fig. 7. Subcellular localization of enforced ectopically-expressed full-length p21$^{WAF1/CIP1}$ and ΔNLS p21$^{WAF1/CIP1}$. Immunohistochemical analysis of non-induced J-p21 cells (A), 24-h Dox-induced J-p21 cells (B), Jurkat cells transfected with either the pcDNA3.1 empty vector (C), or pcDNA3.1-p21$^{WAF1/CIP1}$-ΔNLS (D). Cells were treated as described under *Materials and Methods*. Arrows indicate cells in which enforced p21$^{WAF1/CIP1}$-expression is detected in both the nucleus and the cytoplasm (A, B) or in the cytoplasm (p21$^{WAF1/CIP1}$ -ΔNLS) (D), as identified with a specific p21$^{WAF1/CIP1}$ antibody. D, inset, negative control (NC) with secondary antibody alone. E, Western blot analysis of lysates from p21$^{WAF1/CIP1}$-ΔNLS clones (Δ8 and Δ35). PMA and B indicate lysates from Jurkat cells treated for 24 h with either 10 nM cPMA) or 1 mM SB as positive controls for induced endogenous full-length p21$^{WAF1/CIP1}$. Two additional studies yielded equivalent results.

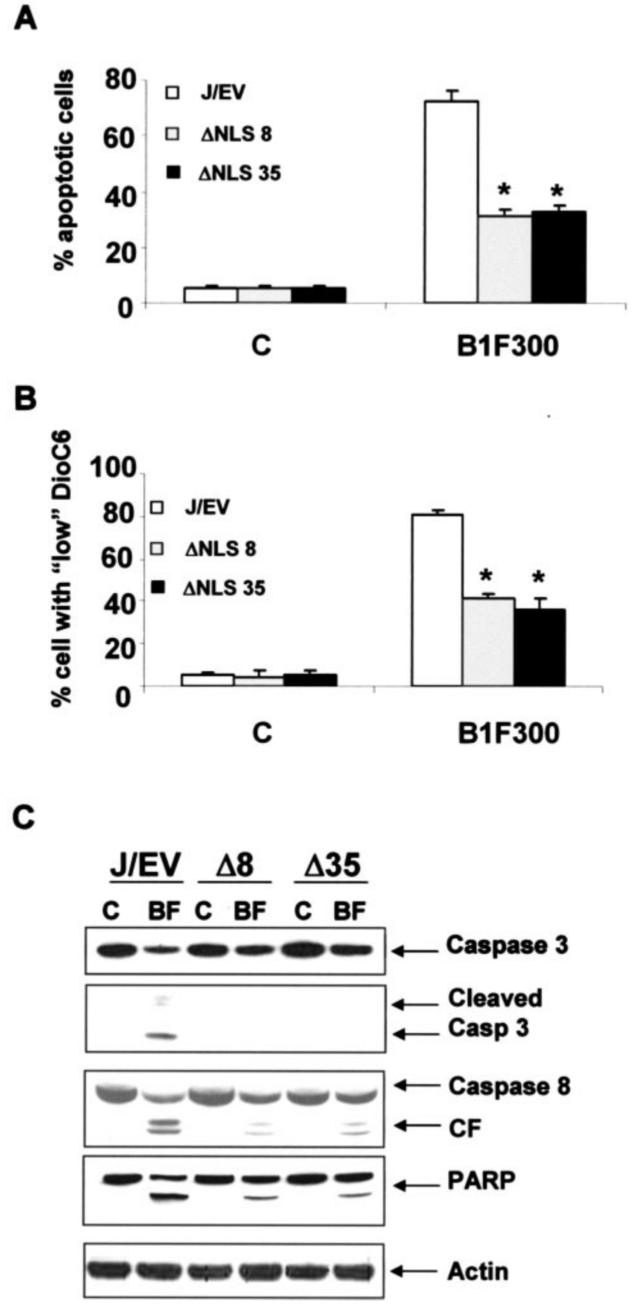

Fig. 8. Jurkat cells stably expressing p21$^{WAF1/CIP1}$-ΔNLS are resistant to FB-induced cell death. A, Jurkat cells transfected with either empty vector (J/EV) or two clones expressing p21$^{WAF1/CIP1}$-ΔNLS (Δ8 and Δ35) were treated with 1 mM SB/300 nM FP for 24 h, after which the extent of apoptosis was determined by flow cytometry analysis of Annexin V/PI and 7-amino-actinomycin D uptake. Values represent the means for three separate experiments performed in triplicate ± S.D. *, $p < 0.01$; values significantly lower than those obtained with J/EV control cells. B, Jurkat control EV and p21$^{WAF1/CIP1}$-ΔNLS cells were treated as above and the percentage of cells exhibiting loss of $\Delta\psi_m$ was evaluated by flow cytometry analysis as described under *Materials and Methods*. Values represent the means for three separate experiments performed in triplicate ± S.D. *, $p < 0.01$; values significantly lower than those obtained with J/EV control cells. C, Western blot analysis of lysates from the same samples tested in A and B. After treatment, cell lysates were prepared and 30 μg of protein was separated by SDS-PAGE as described under *Materials and Methods*. Blots were then probed with the corresponding antibodies against procaspase-3, cleaved/activated caspase-3, caspase-8, and PARP. The blots were stripped and reprobed with an antibody to actin to ensure equivalent loading and transfer. The results of a representative study are shown; two additional experiments yielded similar results. CF, cleavage fragment.

been suggested that the function of p21$^{WAF1/CIP1}$ as an inhibitor of cell cycle progression versus apoptosis may be determined by its subcellular localization (Asada et al., 1999). Specifically, in human leukemia cells, p21$^{WAF1/CIP1}$ nuclear localization correlates with growth arrest and induction of differentiation, whereas in the cytoplasm this CDKI may primarily act by forming inhibitory complexes with procaspase-3 and ASK-1 (Suzuki et al., 1998; Asada et al., 1999). Thus, FP, by blocking p21$^{WAF1/CIP1}$ induction and thereby preventing its cytosolic accumulation, may promote HDAC inhibitor-mediated activation of the apoptotic caspase cascade.

The results of the present study, in which two genetically engineered cell types were employed [i.e., one expressing inducible full-length p21$^{WAF1/CIP1}$ and the other stably expressing a C-terminal-deleted form lacking the nuclear localization signal (cytoplasmic p21$^{WAF1/CIP1}$)], support this concept. For example, cells inducibly expressing full-length p21$^{WAF1/CIP1}$ or stably expressing a nuclear localization signal mutant (ΔNLS) displayed diminished caspase activation, PARP cleavage, and apoptosis in response to combined treatment with FP and an HDAC inhibitor. Furthermore, enforced expression of full-length or ΔNLS-p21$^{WAF1/CIP1}$ resulted in a marked increase in the amount of p21$^{WAF1/CIP1}$ associating with procaspase-3. The possibility that such an event might antagonize activation of a key effector caspase such as procaspase-3 seems plausible (Asada et al., 1999). However, why inactivation of caspase-3 would diminish mitochondrial injury in cells exposed to the HDAC inhibitor/FP regimen is unknown (Fig. 5). In this context, studies by other groups (Sun et al., 2002) as well as our own (Almenara et al., 2002; Rosato et al., 2002) have suggested the presence of an amplification loop in which activation of the intrinsic and receptor-related extrinsic pathways cooperate to promote mitochondrial injury. Such a loop has been postulated to involve activation of procaspase-8 by activated procaspase-3, resulting in cleavage of Bid, which translocates to the mitochondria and promotes release of pro-apoptotic mitochondrial proteins (Sun et al., 2002). Consistent with this view, enforced expression of p21$^{WAF1/CIP1}$ diminished HDAC inhibitor/FP-mediated caspase-8 activation and cleavage of Bid. It is therefore possible that by antagonizing procaspase-3 activation, p21$^{WAF1/CIP1}$ may interfere with an amplification loop that promotes mitochondrial injury; conversely, interference with p21$^{WAF1/CIP1}$ induction (i.e., by FP) may promote HDAC inhibitor-mediated release of cytochrome *c* and related proteins.

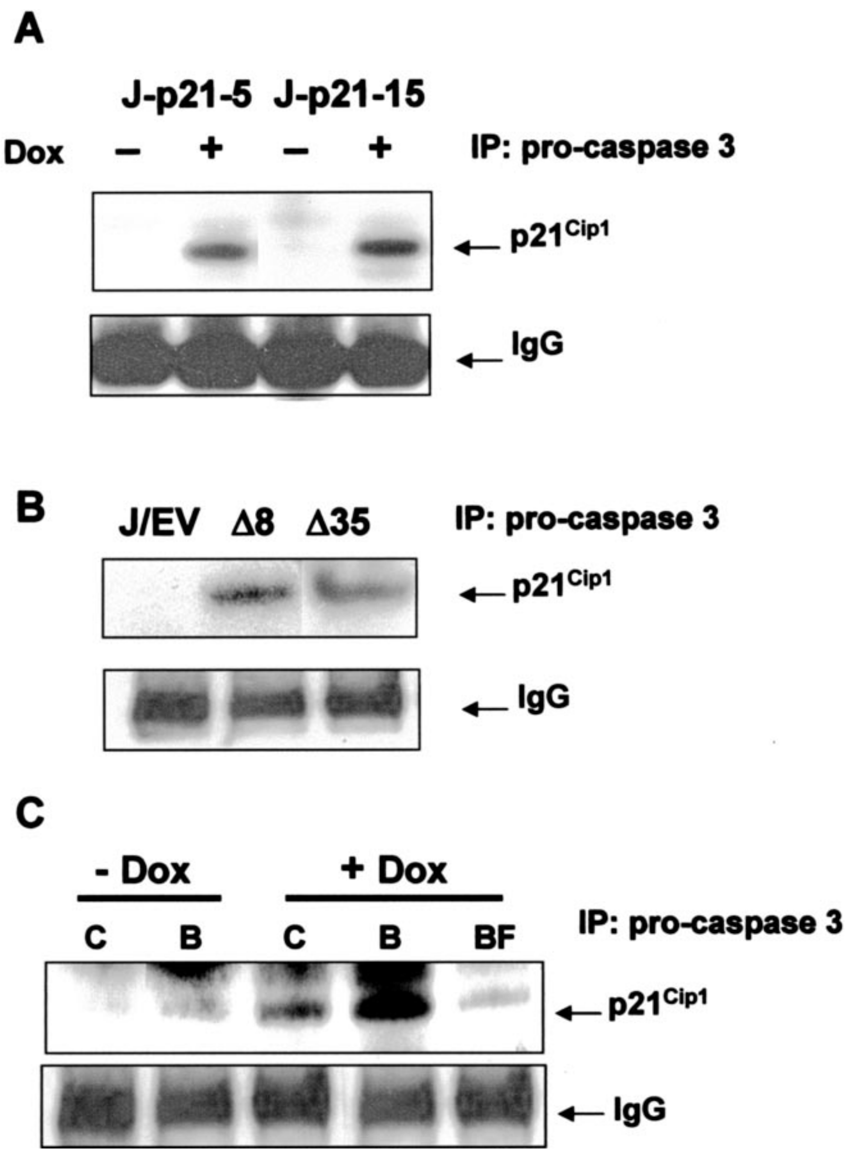

Fig. 9. Ectopically expressed p21$^{WAF1/CIP1}$ coimmunoprecipites with pro-caspase 3. Both inducible J-p21 cell lines (clones 5 and 15) as well as p21$^{WAF1/CIP1}$-ΔNLS cell lines (Δ8 and Δ35) were monitored for coimmunoprecipitation of pro-caspase 3 and p21$^{WAF1/CIP1}$. Lysates from J-p21 cells preincubated with or without Dox for 24 h (A) and from J/EV control and p21-ΔNLS cells (Δ8 and Δ35) (B) were immunoprecipitated with an anti-procaspase-3 antibody as described under *Materials and Methods*, after which they were subjected to Western blot analysis with p21$^{WAF1/CIP1}$ antibody. C, J-p21 cells (clones 5 and 15) were pretreated with or without Dox for 24 h followed by exposure to 1 mM SB alone (B) or in combination with 300 nM FP (BF) for an additional 24 h. The cell lysates were then immunoprecipitated with an anti-procaspase-3 antibody followed by Western blot analysis using an anti-p21$^{WAF1/CIP1}$ antibody. The band corresponding to IgG represents a loading control. The results of a representative study are shown; two additional experiments yielded similar results.

In summary, the present studies provide, for the first time, evidence that interference with p21$^{WAF1/CIP1}$ induction by FP plays a significant functional role in promoting leukemic cell mitochondrial injury, caspase activation, and apoptosis after exposure to HDAC inhibitors. They also suggest that attenuation of cytoplasmic p21$^{WAF1/CIP1}$ accumulation, as well as activation of a procaspase-3, procaspase-8, and Bid amplification loop, may contribute to this phenomenon. Given accumulating support for the notion that p21$^{WAF1/CIP1}$ plays a major role in regulating the apoptotic response of leukemic as well as other neoplastic cells to HDAC inhibitors (Burgess et al., 2001; Rosato et al., 2001), the concept of combining these agents with FP, which transcriptionally represses the expression of p21$^{WAF1/CIP1}$ (Cartee et al., 2001) and other antiapoptotic proteins (e.g., Mcl-1) (Gojo et al., 2002) seems to be a rational one. Finally, it will be of interest to determine whether other CDK inhibitors can mimic the actions of FP in opposing p21$^{WAF1/CIP1}$ induction and/or in promoting HDAC inhibitor-mediated lethality. Accordingly, studies designed to answer these questions are currently in progress.

Acknowledgments

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