

Down-regulation of Tumor Necrosis Factor α Expression by Activating Transcription Factor 2 Increases UVC-induced Apoptosis of Late-stage Melanoma Cells*

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To identify mechanisms whereby activating transcription factor 2 (ATF2) alters the radiation resistance of human melanoma cells, we examined the possible role of ATF2 in UVC-induced apoptosis. Forced expression of full-length or truncated (Δ 1–195 amino acids) forms of ATF2 in LU1205, a late-stage human melanoma cell line, elevated the levels of UVC-induced apoptosis. At the same time, either truncated or full-length forms of ATF2 reduced UVC-induced activation of the tumor necrosis factor- α (TNF α) promoter and decreased expression of TNF α . Forced expression of c-Jun in ATF2-expressing melanoma cells restored TNF α expression, suggesting that both forms of ATF2 sequestered transcription factors that positively regulate TNF α expression in response to UV irradiation. Antagonistic antibodies to Fas, but not to TNFR1, efficiently suppressed UVC-induced apoptosis, suggesting that the Fas pathway mediates the primary apoptotic signal in melanoma cells whereas the TNFR1 pathway elicits a survival signal. Indeed, treatment of melanoma cells with TNF α before UVC irradiation partially suppressed UVC-induced apoptosis, further supporting the protective role of TNF α in UVC-treated melanoma cells. Taken together, our findings suggest that ATF2 contributes to UVC-induced apoptosis through transcriptional silencing of TNF α , which balances Fas-mediated cell death in melanoma.

Programmed cell death (apoptosis) is a common cellular response to stress caused by environmental challenges (1). Altered expression of apoptosis-related proteins, which coincides with decreased or absent apoptosis, is commonly observed in various tumor types and is of fundamental importance in tumor resistance to host defenses as well as to clinical therapy (2). Indeed, reduced ability of tumors to undergo apoptosis is often associated with elevated drug resistance and poor clinical outcomes. Malignant melanoma is a primary example of a cancer that responds poorly to various treatments, including chemotherapy and γ -irradiation (3). Despite the alarming increase in the incidence of this tumor in the past decade, the molecular mechanisms of its progression as well as the regulation of apoptosis in human melanoma remain largely unknown (4).

The mechanisms underlying apoptotic signaling have been intensively studied in recent years. Two cell surface molecules,

Fas and TNFR1,¹ represent the main death signaling receptors (1). Receptor trimerization by ligands FasL and TNF α , respectively, initiate receptor interaction with the intermediate signaling molecules FADD and FLICE (caspase-8), followed by activation of the caspase cascade (1, 5, 6). In addition to its role in apoptosis, TNFR1-mediated signaling is also linked with the regulation of survival functions, including induction of cytokines, growth factors, and cell proliferation and differentiation (7–9). Furthermore, TNF α appears to be one of the primary growth regulators of metastatic cancer cells (10, 11), including late-stage melanoma cells (12–14). Hence, suppression of TNF signaling may enhance the apoptotic stimulus to tumor cells. Key to understanding the interplay between TNF α and Fas signaling is the nature of their upstream transcriptional regulators and downstream targets (*i.e.* inhibitors of apoptosis and survival proteins) in concordance with, or independent of, NF- κ B-dependent signaling (15–17). Expression of the human TNF α gene is controlled by the AP-1, ATF2, Erg-1, C/EBP β , and NF- κ B transcription factors (18–22). Transcriptional regulation of Fas and FasL genes was shown to be mediated by NF- κ B, AP-1, NF-AT, ATF2, and Egr3 (23–26). As a dynamic process, programmed cell death is dependent, in many cases, on new gene expression (27), and is tightly regulated at the transcriptional level (28–30).

Our interest in exploring mechanisms underlying key melanoma phenotypes led us to identify CREB/ATF transcription factors as regulatory proteins that play important roles in determining both melanoma's resistance to radiation and its metastatic potential (31, 32). ATF2, a member of the ATF/CREB family of basic region leucine zipper (bZIP) DNA-binding proteins (33–35), was shown to modulate melanoma's resistance to UVC irradiation but not to contribute to its metastatic potential (36). ATF2 is among several transcription factors, including AP-1, CREB, and Rel/NF- κ B, which have been found to be UV-inducible. ATF2 induction in response to stress occurs at the levels of transcription-translation and posttranslational modification, the latter of which is mediated by JNK/p38 kinases (37, 38). ATF2 has been implicated in the transcriptional control of various stress-responsive genes, including c-Jun (39), interferon- β (40), urokinase (41), TGF- β 2 (42), TNF α (18, 20), and DNA polymerase β (43). Differential splicing of ATF2 creates several isoforms which exhibit different transcriptional outputs (44). Full-length ATF2 is transcriptionally inactive in its native form as a result of intramolecular interaction of its

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¹ The abbreviations used are: TNFR, tumor necrosis factor receptor; AP-1, activator protein-1; Ab, antibody; mAb, monoclonal antibody; ATF2, activating transcription factor 2; CREB, cyclic AMP response element-binding protein; FasL, Fas ligand; NF- κ B, nuclear factor κ B; PI, propidium iodide; TNF, tumor necrosis factor; GFP, green fluorescence protein; PARP, poly(ADP-ribose) polymerase; X-gal, 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside; FITC, fluorescein isothiocyanate; IL, interleukin; bZIP, basic region leucine zipper.

DNA-binding domain with the amino-terminal transactivation domain (33). ATF2 as well as a related factor, ATF α , not only can form DNA-binding homodimers but also can efficiently heterodimerize with members of the ATF/CREB and Jun/Fos families, thus providing an important transcriptional control mechanism (41, 45–47). Duration and magnitude of ATF2 transcriptional output are also regulated at the level of phosphorylation and stability. While stable in its native non-active form, ATF2 is a short-lived protein after homo- or heterodimerization. ATF2 instability is further facilitated by heterodimerization with certain partners, which provide additional targeting molecules for ubiquitination through their own docking sites, as shown for c-Jun (48).

To identify mechanisms underlying ATF2's ability to modulate radiation resistance, we studied the possible role of ATF2 in apoptosis of human melanoma cells. We demonstrate that ATF2 expression increases UV-mediated apoptosis through suppression of TNF α expression, which elicits survival signals in tumors of this type.

EXPERIMENTAL PROCEDURES

Cell Lines—Human melanoma LU1205 cells were maintained in MCDB153/L15 medium (4:1) supplemented with 5% fetal bovine serum, L-glutamine, and antibiotics. LU1205/neo, LU1205/ATF2, LU1205/ATF2 Δ 1–195, and LU1205/I κ B Δ cell lines were maintained in the same medium supplemented with G418 (200 μ g/ml). Cells were grown at 37 °C with 5% CO $_2$.

Stable Transfection and Selection—The expression vectors pECE-ATF2 encoding full-size ATF2 and pECE-ATF2 Δ 1–195, a truncated form of ATF2 cDNA lacking the first 195 amino acids and fused with GAL4 (34, 35), were transfected by electroporation (230 V, 1050 microfarads) into LU1205 cells together with pBK-RSV-neo plasmid (Stratagene, La Jolla, CA) as described previously (36). In parallel, the pECE vector was cotransfected with pBK-RSV-neo to generate control LU1205/neo cells. The expression vector pCMV4-I κ B Δ (49) plus pcDNA3-neo (Invitrogen, Carlsbad, CA) was also cotransfected by electroporation into LU1205 cells. LU1205/neo, LU1205/ATF2, LU1205/ATF2 Δ 1–195, and LU1205/I κ B Δ cell lines were created as a mixed population of G418-resistant clones.

Transient Transfection and Luciferase Assay—Reporter constructs (20 μ g) were transiently cotransfected with the indicated expression vectors and pCMV- β -gal (5 μ g) into 10 7 LU1205 melanoma cells by electroporation (230 V, 1050 microfarads; Gene Pulser, IBI). The reporter constructs used were: 5xJun2tk-Luc and vector tk-Luc (50); -615 TNF-Luc, -615 TNF(mutCRE)-Luc, -615 TNF(mutAP-1)-Luc, -36 TNF-Luc (19), -453 FasL-Luc, -318 FasL-Luc, -237 FasL-Luc (23), -1.3kb FasL-Luc, and variants of this construct with mutations in the NF- κ B- and AP-1 sites (24). Expression constructs pECE-ATF2, pECE-ATF2 Δ 1–195 (34, 35), pCMV-c-Jun-HA (51), and TAM67, a dominant negative form of c-Jun (52, 53), were also used in these experiments. Luciferase activity was determined using the Promega luciferase assay system (Promega, Madison, WI). Luciferase activities were normalized on the basis of β -galactosidase levels in transfected cells.

Transient Transfection and GFP Assay—Melanoma cells (6×10^6) were transiently cotransfected with either ATF-2- or TAM-67 expression vectors together with marker plasmid encoding green fluorescent protein, pGFP (CLONTECH), 10 and 2.5 μ g, respectively, by electroporation. Twenty-four hours after transfection cells were irradiated by UVC (60 J/m 2) and 18 h after treatment were stained with PI and analyzed using flow cytometry. The ratio of GFP $^+$ PI $^+$ to total GFP $^+$ cells was used to measure frequencies of death in the transfected cells.

Transient Transfection and X-gal Staining—Melanoma cells (6×10^6) were transiently cotransfected with ATF2 expression vectors and pCMV- β -gal (10 and 2.5 μ g, respectively) by electroporation. Twenty-four hours after transfection, cells were irradiated with UVC (60 J/m 2) as described previously (32). To identify β -galactosidase activity, cells were fixed 18 h after UVC treatment and stained with X-gal (28). The ratio of blue cells with apoptotic morphology to the total number of blue cells served as the indicator of the level of apoptosis in transfected cells.

Cell Treatment and Apoptosis Studies of Stably Transfected Melanoma Cells—Cycloheximide (10 μ g/ml), actinomycin D (200 ng/ml) (Sigma), and the caspase inhibitor, zVAD-fmk (10–50 μ M) (Enzyme Systems, Dublin, CA) were added as indicated. The proteasome inhibitor lactacystin was purchased (Dr. E. J. Corey, Harvard University, MA) and used at 10 μ M. Recombinant TNF α (PharMingen, San Diego, CA)

was used at a final concentration of 1–4 ng/ml. Antagonistic monospecific antibodies against Fas (clone G254-274 from PharMingen) and against TNFR1 (clone 16803.1 from R&D Systems, Minneapolis, MN) were added as indicated at the final concentration of 1–5 μ g/ml.

Annexin-V-FITC staining in the presence of PI was performed for detection of early apoptosis levels using TACS Annexin-V-FITC kit (Trevigen, Gaithersburg, MD). Flow cytometric analysis was performed on an Epics-Profile II flow cytometer (Coulter, Hialeah, FL) with Elite software 4.01 by analyzing 10 4 cells/sample, using wide scatter gates to include late apoptotic cells.

DNA fragmentation analysis was performed as described previously (54). Cells were pelleted and resuspended in 0.5 ml of hypotonic buffer with 0.1% Triton X-100 containing propidium iodide (PI) (40 μ g/ml) and DNase-free RNase A (1 mg/ml). Cells were incubated at 37 °C for 30 min and analyzed on an Epics-Profile II flow cytometer (Coulter). The percentage of cells to the left of the diploid G $_{0/1}$ peak, diagnostic of hypodiploid cells that have lost DNA, was taken as the percentage of apoptotic cells. The analysis was performed without light scatter gating.

Western Blotting Analysis—Total cell extracts were resolved on 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and processed by standard methods. The polyclonal anti-PARP serum (Biomol, Plymouth Meeting, PA) was used at a 1:1000 dilution. Polyclonal Abs against ATF2 and phosphoATF2 (New England Biolabs) were used at a 1:1000 dilution. Monoclonal Abs against human TNF α (clone Mab1), Fas (clone G254–267) (PharMingen), and TNFR1(R&D System), and polyclonal Abs against FasL and I κ B α (Santa-Cruz Biotechnology) were used at the same dilution. The secondary Abs were goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase (dilution 1:5000). Signals were detected using the ECL system (Amersham Pharmacia Biotech).

RESULTS

Characterization of UVC-induced Apoptosis in Human Melanoma Cells—To study regulation of UVC-induced apoptosis in melanoma, we used the LU1205 cell line, which represents a late stage melanoma that harbors wild type p53. These cells exhibit higher resistance to irradiation than early stage melanoma cells (55). The finding that expression of a truncated ATF2 form (Δ 1–195) reduced radiation resistance of late-stage melanoma cells (36) prompted our interest in elucidating mechanisms by which ATF2 may elicit such changes. Since radiation resistance may be inversely correlated with the degree of programmed cell death, we studied the possible role of ATF2 in UV-induced apoptosis of these melanoma cells.

Several markers of apoptosis were used to monitor and characterize UVC-mediated programmed cell death in melanoma cells. To distinguish between early and late phases of apoptosis, annexin-V-FITC and PI staining, which detect early and late phases of apoptosis (56), respectively, were used. Eighteen hours after UVC irradiation, 20% of LU1205 melanoma cells were annexin-V $^+$, of which 11% were annexin-V $^+$ PI $^-$ and 9% annexin-V $^+$ PI $^+$, reflecting the early and late phases of apoptosis, respectively (Fig. 1A). In several independent experiments, we found that 25 \pm 6% LU1205 cells were annexin-V $^+$ (apoptotic) 18 h after UVC irradiation, compared with 5 \pm 3% for nontreated cells. DNA fragmentation analysis, performed by staining cell nuclei with PI and determining the percentage of apoptotic cells with hypodiploid DNA content (Fig. 1B), revealed that 36% of the total cell population had apoptotic nuclei with hypodiploid DNA content 40 h after UVC irradiation, compared with 2% for nontreated cells (Fig. 1B). Within 3 days after treatment the apoptosis level reached 70%; the vast majority of melanoma cells died 4 days after irradiation (data not shown). Cycloheximide (Fig. 1C) as well as actinomycin D (data not shown) partially suppressed UVC-induced apoptosis of melanoma cells, indicating that UVC-induced gene expression is required if maximal levels of apoptosis are to be achieved.

Since caspase-dependent proteolysis has been shown to play an important role in different types of programmed cell death (5), we determined the possible role of activation of the caspase

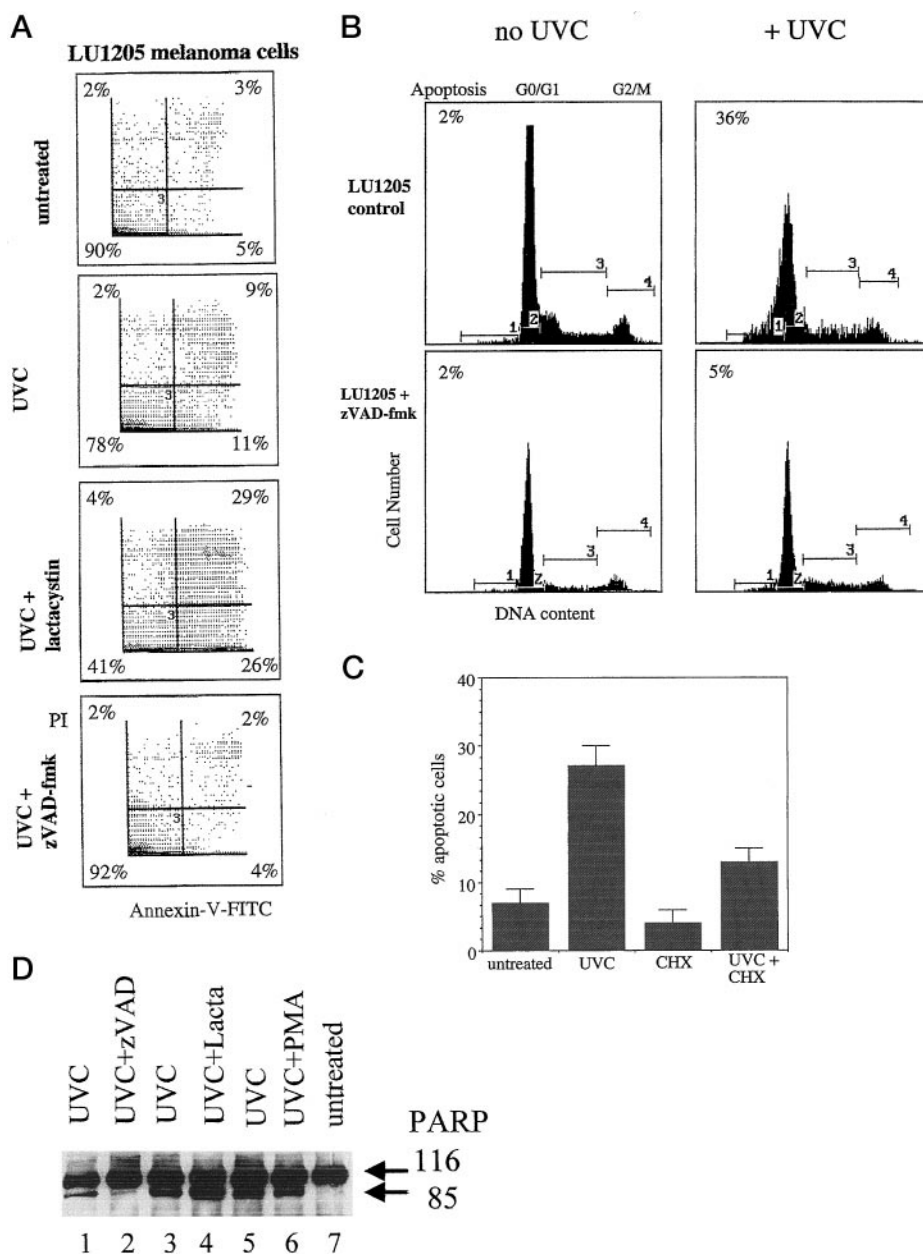


FIG. 1. UVC irradiation induces melanoma cell apoptosis. *A*, LU1205 cells were treated with UVC at 60 J/m² in the presence of zVAD-fmk (50 μ M) and lactacystin (10 μ M), added 1 h before irradiation and also in the absence of these materials. The percentage of early apoptosis was determined 18 h after UVC treatment using annexin-V-FITC plus PI staining and flow cytometry. The percentage of stained cells is shown near each quadrant. *Quadrant 3* contains Annexin-V⁻PI⁻ (live) cells. Annexin-V⁺PI⁻ and annexin-V⁺PI⁺ cells indicate early and late phases of apoptosis, respectively. *B*, DNA fragmentation analysis of LU1205 cells was performed 40 h after UVC treatment, as described under "Experimental Procedures." The percentage of cells in gate 1 indicates the level of apoptotic cells with hypodiploid DNA content. *C*, effect of cycloheximide (CHX) (10 μ g/ml) on UVC-induced apoptosis of LU1205 cells. The percentage of early apoptosis (annexin-V⁺PI⁻) is shown. *D*, UVC-induced cleavage of PARP. Western blot analysis of PARP was performed 18 h after UVC irradiation (40 J/m² for lane 1 and 60 J/m² for lanes 2–7) of LU1205 cells in absence or presence of zVAD-fmk (50 μ M), lactacystin (10 μ M) or PMA (10 ng/ml). The position of intact (116 kDa) and cleaved (85 kDa) forms of PARP is indicated by arrows.

cascade following UVC irradiation of melanoma cells. A 50 μ M quantity of zVAD-fmk, a caspase inhibitor, substantially suppressed UVC-induced apoptosis (Fig. 1, *A* and *B*). To monitor cleavage of poly(ADP-ribose) polymerase (PARP), a typical target of caspase-3 and an indicator of early apoptotic commitment (57), immunoblot analysis of nuclear proteins from UVC-treated melanoma cells was performed with the aid of anti-PARP Ab, revealing the presence of the 85-kDa cleavage product 18 h after UVC treatment, but not in the presence of zVAD-fmk (Fig. 1*D*).

The role of proteasome-dependent degradation in UVC-mediated apoptosis of melanoma cells was examined using the proteasome inhibitor lactacystin (58). Whereas 10 μ M lactacystin did not induce apoptosis in nonstressed melanoma cells (data not shown), a substantial increase in UVC-induced apoptosis was seen in the presence of 10 μ M lactacystin, indicating a protective role of proteasome-dependent protein degradation in UVC-induced apoptosis (Fig. 1*A*). Increased PARP cleavage was seen in lactacystin-treated cells following UVC irradiation (Fig. 1*D*). These observations suggest that all three major cell regulatory processes (new gene expression, proteasome-dependent

protein degradation, and caspase proteolysis) contribute to UVC-induced apoptosis of melanoma cells.

Role of ATF2 in UVC-induced Gene Response and Apoptosis of Human Melanoma Cells—To elucidate a probable function of ATF2 in the regulation of UVC-induced apoptosis of human melanoma cells, we utilized expression vectors encoding either GAL4 fused with truncated ATF2 lacking the transactivation domain (pECE-ATF2 Δ 1–195) or full-size ATF2 (pECE-ATF2) (34, 35). To monitor ATF2-mediated transactivation, a reporter construct containing five repeats of the ATF-2/AP-1 binding site from the Jun promoter (TRE-Jun2), which preferentially bind ATF2/Jun heterodimers (39, 50), was used. ATF2/AP-1-dependent luciferase activity increased 3-fold after UVC irradiation of control LU1205 cells. Transient transfection of truncated ATF2 (at a 2:1 ratio of expression vector to reporter construct) slightly increased the basal level of ATF2/AP-1-dependent transcription but decreased UVC-induced transactivation of ATF2/AP-1 (25–30%, compared with control cells; Fig. 2*A*). In contrast, expression of full-size ATF2 up-regulated the basal level of ATF2/AP-1-dependent luciferase activity 2.5-fold. Unexpectedly, the transfected full-size ATF2 also decreased

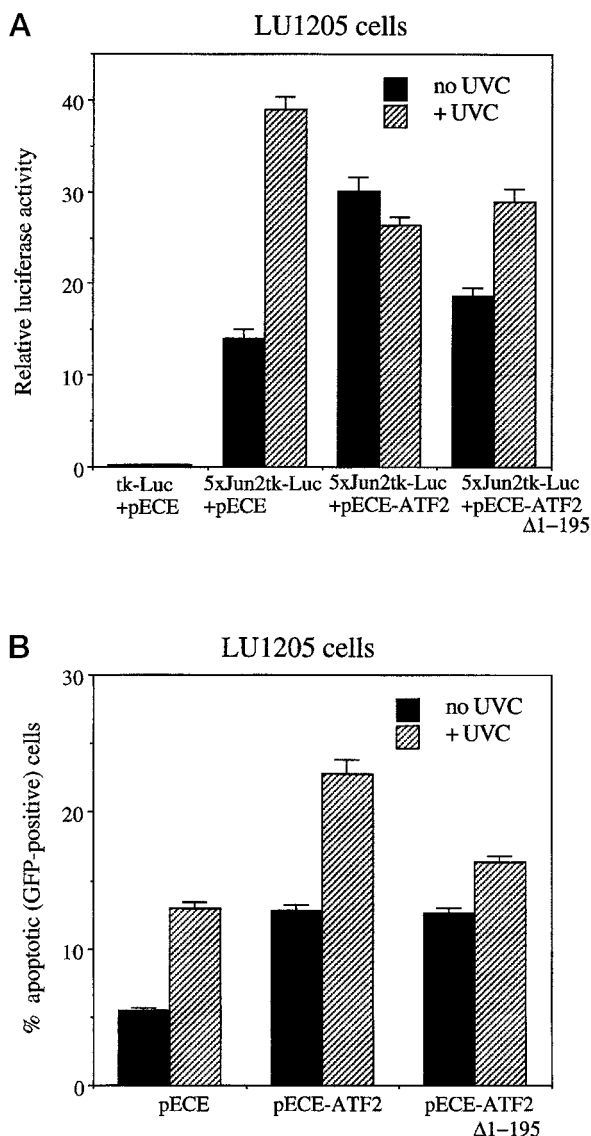


FIG. 2. Modulation of ATF2-dependent transcription affects UVC-induced apoptosis of melanoma cells. *A*, 8×10^6 LU1205 cells were transiently cotransfected with the reporter construct 5xJun2tk-Luc ($5 \mu\text{g}$) and expression vector pECE, pECE-ATF2, or pECE-ATF2 Δ 1-195 ($10 \mu\text{g}$) in the presence of pCMV- β -gal ($5 \mu\text{g}$). Cotransfection with tk-Luc ($5 \mu\text{g}$) was used as a control for basal luciferase activity. Transfected cells were divided into two plates. Twenty-four hours after transfection, half the plates were irradiated with UVC (60 J/m^2). After an additional 18 h, irradiated and nontreated cells were analyzed for luciferase and β -galactosidase activities. The normalized ratio of luciferase activity to β -galactosidase is shown. *B*, LU1205 cells were transiently transfected with pECE, pECE-ATF2, or pECE-ATF2 Δ 1-195 ($20 \mu\text{g}$) in the presence of pGFP ($5 \mu\text{g}$). Transfected cells were divided into two plates. Twenty-four hours after transfection, half the plates were irradiated with UVC (60 J/m^2). After an additional 18 h, irradiated and nontreated cells were stained with PI and analyzed by flow cytometry. The ratio of PI⁺ GFP⁺ cells to all GFP⁺ cells indicates the frequency of apoptotic cells in the population of transfected cells.

UVC-induced ATF2/AP-1-mediated transactivation (Fig. 2A). These observations suggest that both truncated and full-size ATF2 after transfection exert a certain silencing effect on UVC-induced ATF2/AP-1-dependent transcription in melanoma cells.

We next determined whether transcriptional suppression by ATF2 alters the degree to which melanoma cells undergo apoptosis in response to UV irradiation. Apoptosis studies were performed by cotransfection of ATF2 expression vectors with either pGFP or pCMV- β -gal encoding green fluorescent protein

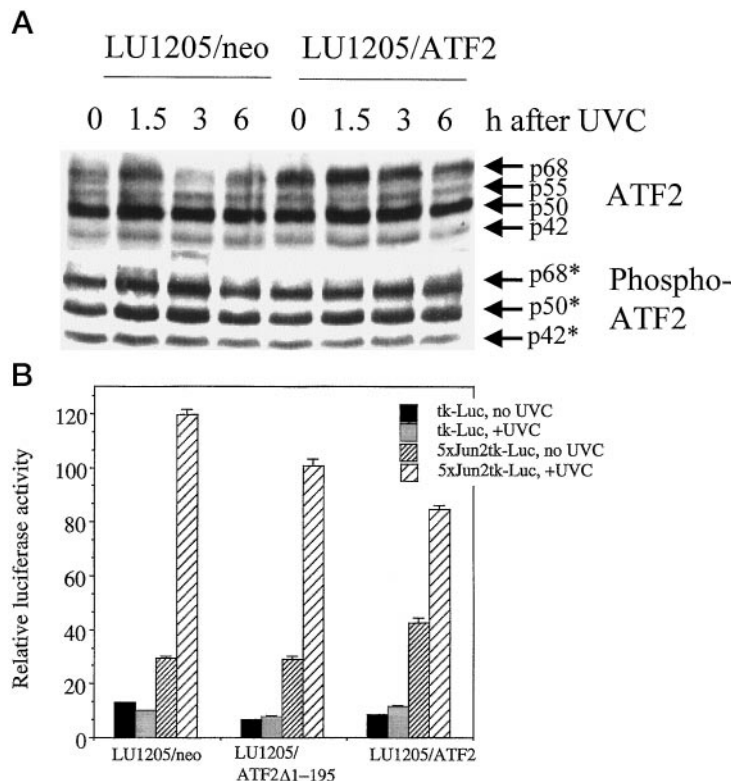


FIG. 3. A, total extracts of stably transfected cell lines LU1205/ATF2 and LU1205/neo were analyzed by Western blotting with anti-ATF2 and anti-phospho-ATF2 Abs. Positions of p68, p55, p50, and p42 forms of ATF2 and phosphorylated p68*, p50*, and p42* forms are indicated by arrows. *B*, effect of modulation of ATF2 levels in stably transfected melanoma cell lines (LU1205/ATF2, LU1205/ATF2 Δ 1-195, and LU1205/neo) on transcriptional activity of the ATF2-dependent reporter constructs. 10^7 LU1205/neo, LU1205/ATF2, and LU1205/ATF2 Δ 1-195 cells were transiently transfected with 5xJun2tk-Luc ($20 \mu\text{g}$) in the presence of pCMV- β -gal ($5 \mu\text{g}$). Twenty-four hours after transfection, cells were irradiated with UVC, and, after an additional 18 h, luciferase and β -galactosidase activity were determined. The normalized ratio of luciferase to β -galactosidase activity is indicated.

and β -galactosidase, respectively, as marker genes. To determine the frequency of death in ATF2-transfected cells, UVC-irradiated and nontreated cells were stained with PI and analyzed by flow cytometry, allowing quantification of GFP⁺PI⁺ cells in the overall population of GFP⁺ cells. Expression of either full-length or truncated forms of ATF2 notably increased (1.8- and 1.3-fold, respectively) the frequency of cell death following UVC irradiation of LU1205 cells (Fig. 2B). Parallel analysis using X-gal staining of β -galactosidase-expressing cells enabled visual detection of blue cells with apoptotic morphology in the total population of blue cells (28), revealing a similar increase in apoptosis levels after UVC irradiation in ATF2-transfected cells (data not shown).

To further investigate ATF2-dependent regulation of UVC-induced apoptosis of human melanoma cells, we established LU1205 cell lines stably transfected with either full-size ATF2 cDNA (LU1205/ATF2 cells) or the truncated form, GAL4-ATF2 Δ 1-195 (LU1205/ATF2 Δ 1-195), as well as the control cell line transfected with the empty vector and neo marker plasmid (LU1205/neo). Western blot analysis confirmed elevated expression of the respective forms of ATF2. Cells that constitutively express the full-size ATF2 revealed increased levels of 68-kDa ATF2 as well as spliced isoforms, before and after UVC irradiation (Fig. 3A). However, immunoblot with anti-phospho-ATF2 (Thr71) Ab revealed the same level of phosphorylated forms of ATF2 after UVC irradiation of LU1205/ATF2 cells, indicating a relative decrease in the ratio of phospho-ATF2 to

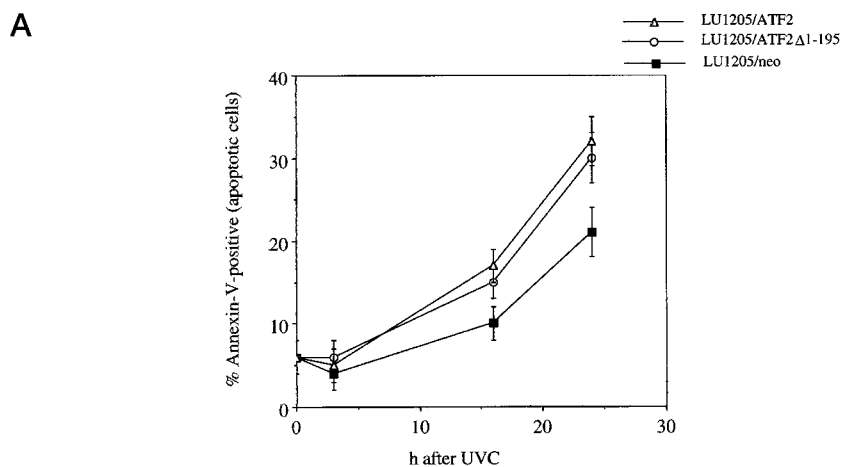
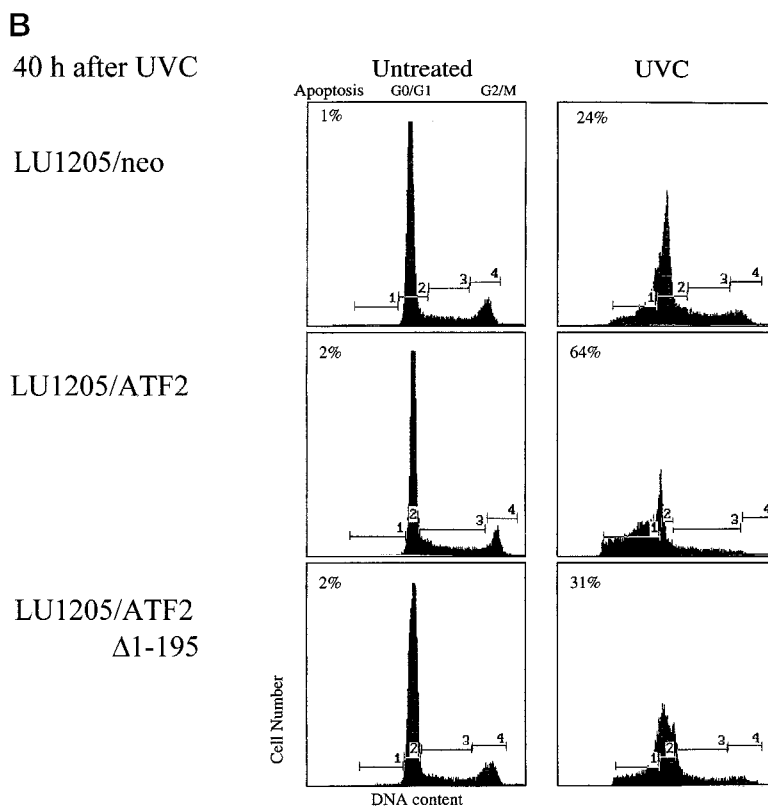


FIG. 4. UVC-induced apoptosis of LU1205 cells stably transfected with ATF2 constructs. LU1205/neo, LU1205/ATF2, and LU1205/ATF2Δ1-195 cells were irradiated with UVC (60 J/m²). *A*, apoptosis levels were determined using annexin-V-FITC plus PI staining 3–24 h after treatment. *B*, DNA fragmentation analysis was performed 40 h after treatment as described under “Experimental Procedures.”



total ATF2 (Fig. 3A). Immunoprecipitation with anti-GAL4 and subsequent Western blotting with anti-ATF2 Ab further confirmed the presence of the fused truncated form of ATF2 in LU1205/ATF2Δ1-195 cells (data not shown), as was observed previously with MeWo melanoma cells transfected with ATF2Δ1-195 (36).

We next examined transcriptional regulation of a reporter construct containing ATF2/AP-1 binding sites (5xJun2tk-Luc) in LU1205/ATF2 and LU1205/ATF2Δ1-195 cells. In agreement with the results of transient transfection studies, LU1205/ATF2 cells possessed increased basal levels of ATF2/AP-1-dependent luciferase activity and decreased UVC-induced levels of this activity (30–35%) compared with control cells. UVC-induced ATF2-dependent luciferase activity was also decreased (20–25%) in LU1205/ATF2Δ1-195 cells (Fig. 3B).

To monitor apoptosis in cell lines stably transfected with ATF2 constructs, we used Annexin-V staining and DNA fragmentation analysis. As found in the apoptosis study of transiently transfected cells, annexin-V plus PI staining (Fig. 4A)

showed increased levels of cell death in both LU1205/ATF2 and LU1205/ATF2Δ1-195 cells compared with the control LU1205/neo cells 16–24 h after UVC irradiation. UVC-induced apoptosis levels (annexin-V⁺) were enriched 21 ± 2%, 30 ± 3%, and 32 ± 3% relative to control LU1205/neo, LU1205/ATF2Δ1-195, and LU1205/ATF2 cells, respectively, 24 h after UVC irradiation (60 J/m²), based on the results of four independent experiments. DNA fragmentation analysis showed an additional increase of apoptosis levels 40 h after UVC treatment for LU1205/ATF2 (64 ± 10%), compared with LU1205/ATF2Δ1-195 (31%) and control (24 ± 4%) cells (Fig. 4B). These observations suggest that expression of either truncated or full-size ATF2 increase the level of UV-induced apoptosis in LU1205 melanoma cells. Interestingly, full-length ATF2 elicited a greater increase than the amino-terminal truncated form.

Effect of ATF2 on UVC-induced TNFα Expression—Metastatic melanomas actively produce a set of cytokines, including TNFα, that appear to provide autocrine growth conditions (13, 14, 59). Indeed, luciferase activity driven by TNFα promoter

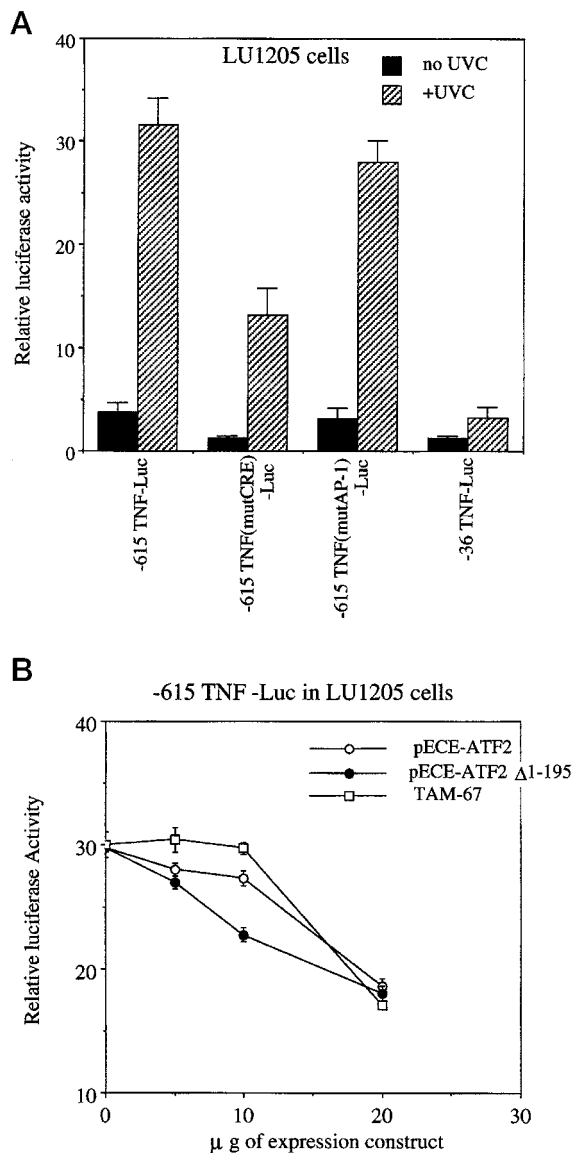


FIG. 5. *A*, effect of mutations of the CRE (−106) and AP-1 (−66) sites on TNF α promoter activity in LU1205 cells. LU1205 cells were transiently cotransfected with −615 TNF-Luc, −615 TNF(mutCRE)-Luc, or −615 TNF(mutAP-1)-Luc reporter constructs. *B*, effect of altered ATF2 levels on TNF α promoter activity. 10^7 LU1205 cells were transiently cotransfected with −615 TNF-Luc reporter construct (5 μ g) and with indicated amounts of the expression vectors pECE-ATF2, pECE-ATF2 Δ 1–195, or with pCMV-TAM67, a dominant negative mutant of c-Jun, in the presence of pCMV- β -gal (5 μ g). The total quantity of DNA for transfection was adjusted to 30 μ g with vector DNA. The normalized ratio of luciferase to β -galactosidase activity was determined 18 h after UVC treatment.

(−615 TNF-Luc) (19), was up-regulated (5–7-fold) after UVC irradiation of LU1205 cells (Fig. 5A). Mutation in the CREB/AP-1 site (at the −106 position) of this construct partially abrogated this increase (by 40–60%). In contrast, mutation within the AP-1 binding site (at the −66 position) was less pronounced (Fig. 5A), suggesting that the CREB/AP-1 site (at −106) is the primary regulator of TNF α expression in melanoma cells, possibly via ATF2, as was described previously for other cell systems (18–20).

To confirm the role of ATF2 in regulation of TNF α expression in the melanoma cells, LU1205 cells were transiently cotransfected with the −615 TNF-Luc reporter construct and either full-size or truncated ATF2 expression vectors. Both ATF2 constructs reduced the degree of UVC-mediated transactiva-

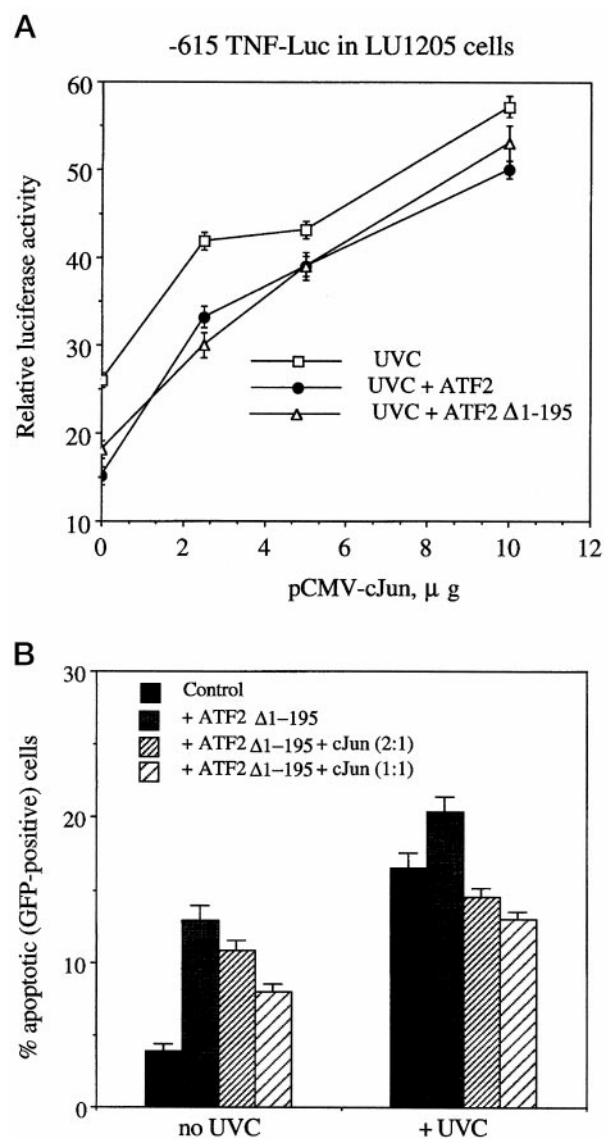


FIG. 6. *A*, c-Jun transactivates TNF α promoter activity in LU1205 cells. Effect of full-length and truncated forms of ATF2 on c-Jun-dependent transactivation of TNF α promoter activity. LU1205 cells were transiently cotransfected with −615 TNF-Luc (5 μ g) in the presence or absence of 10 μ g of pECE-ATF2 and pECE-ATF2 Δ 1–195 and indicated quantities of the pCMV-c-Jun expression construct and 5 μ g of β -galactosidase. Luciferase and β -galactosidase activities were determined after UVC treatment, as described in the legend to Fig. 2. *B*, c-Jun partially suppressed UVC-induced apoptosis, which was accelerated by ATF2 Δ 1–195 expression. LU1205 cells were cotransfected with either pECE-ATF2 Δ 1–195 (20 μ g) or with this vector and pCMV-c-Jun-HA (10 μ g or 20 μ g) in the presence of GFP marker plasmid. Two days after transfection, cells were UVC irradiated. Apoptosis levels of GFP-positive cells were detected using flow cytometry after PI staining.

tion by the TNF α promoter in a dose-dependent manner (Fig. 5B). That full-length (although hypophosphorylated upon UV irradiation; Fig. 3A) and truncated (without transactivator domain) forms of ATF2 elicit the same silencing effect on TNF α transcription could be attributed to squelching of a positive factor as a result of efficient heterodimerization with other bZIP family members. Among the primary transcription factors that associate with ATF2 is c-Jun (39, 47), which was also shown to increase TNF α transcription via the CRE/AP-1 site, following stimulation of monocytes by lipopolysaccharide (21). We therefore tested the possible effect of c-Jun on TNF α transcription in ATF2-expressing melanoma cells. To this end transcriptional activity of c-Jun was blocked by using a dominant

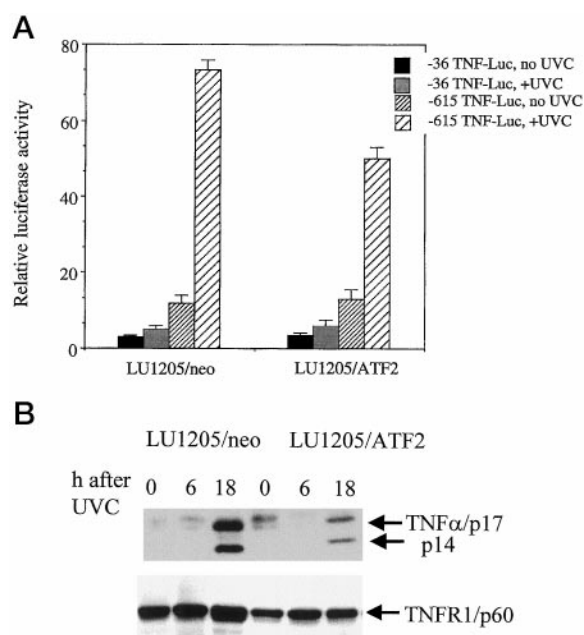


FIG. 7. *A*, determination of TNF α promoter activity in stably transfected melanoma cell lines (LU1205/ATF2, LU1205/ATF2 Δ 1–195, and LU1205/neo). These cells were additionally transiently cotransfected with –615 TNF-Luc or –36 TNF-Luc constructs (25 μ g). UVC treatment and determination of luciferase activity were performed as described above. *B*, Western blot analysis of TNF α and TNFR1/p60 expression in ATF2-transfected LU1205 cells.

negative c-Jun construct, TAM-67, which lacks the first 200 amino acids (52, 53), similar to the truncated ATF2 form used in the present study. Expression of TAM67 in LU1205 suppressed TNF promoter-dependent luciferase activity (Fig. 5B) as well as increased apoptosis (data not shown). Conversely, transfection of wild type c-Jun resulted in a dose-dependent increase of TNF α promoter activity in both non-treated and UVC-irradiated cells (Fig. 6A). Furthermore, forced expression of wild type c-Jun partially abrogated the negative effect of both full-length and truncated forms of ATF2 on TNF α promoter activity (Fig. 6A). c-Jun expression in LU1205 cells (co-transfected with the GFP marker plasmid) also blocked the increase in UVC-induced apoptosis caused either by ATF2 Δ 1–195 (Fig. 6B) or full-length ATF2 overexpression (data not shown). These observations suggest that the ability of ATF2 to increase the degree of UVC-induced apoptosis is mediated via suppression of positive transcription factors, such as c-Jun.

Analysis of TNF α promoter activity in stably transfected LU1205/ATF2 cells further confirmed the data obtained by transient transfection. LU1205/ATF2 cells exhibited reduced (35–40%) TNF α promoter activity after UVC treatment (without changes in basal levels) (Fig. 7A). Decrease in TNF α transcription coincided with down-regulation of TNF α protein levels. Western blot analysis revealed a UVC-dependent increase in the level of TNF α protein (p17) in LU1205/neo cells, whereas this increase was substantially suppressed in LU1205/ATF2 cells (Fig. 7B). Expression of TNFR1 (whose transcription is positively regulated by TNF α) was also decreased in LU1205/ATF2 cells prior to and after UV irradiation compared with control cells while TRAF2 (60) levels were relatively stable (data not shown).

Role of TNFR and Fas-mediated Signaling in UVC-induced Apoptosis of ATF2-expressing Melanoma Cells—To determine which death-signaling pathway mediates UVC-induced apoptosis in control and ATF2-expressing melanoma cells, antagonistic mAbs to either TNFR1 or Fas that block the relevant signaling cascade were added to cell cultures 1 h before UVC

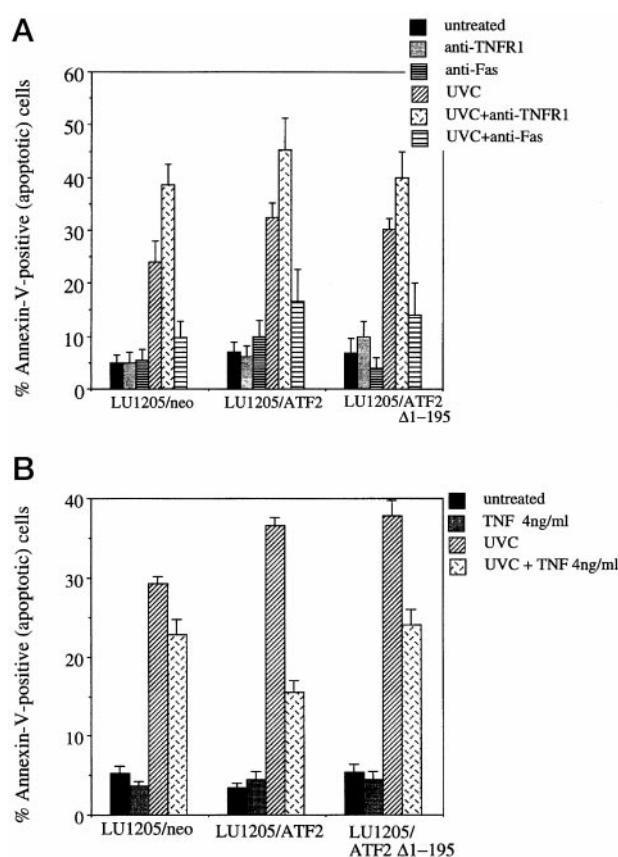


FIG. 8. *A*, effect of antagonistic anti-Fas and anti-TNFR1 mAbs on UVC-induced apoptosis of control LU1205/neo, LU1205/ATF2, and LU1205/ATF2 Δ 1–195 cells. Cells were pretreated with 5 μ g/ml of the indicated mAb 1 h before UVC irradiation (60 J/m²). Abs were maintained in the medium after UVC treatment. Cells were stained with annexin-V-FITC plus PI and analyzed using flow cytometry 18 h after UVC treatment. The percentage of annexin-V-positive (apoptotic) cells is shown. *B*, effect of TNF α on UVC-induced apoptosis of LU1205 cell lines. Cells were pretreated with TNF α (4 ng/ml) 1.5 h before UVC irradiation. TNF α was maintained in the medium after irradiation. The percentage of annexin-V-positive (apoptotic) cells is shown.

irradiation. Addition of anti-TNFR1 mAb further increased the degree of apoptosis after UVC irradiation of control and ATF2 (both full-length and truncated forms) transfected cells. Conversely, anti-Fas mAb substantially suppressed apoptosis in these melanoma cells (Fig. 8A). These results point to the pro-apoptotic role of Fas signaling and anti-apoptotic role of TNFR-mediated signaling for LU1205/neo, LU1205/ATF2, and LU1205/ATF2 Δ 1–195 cells following UVC irradiation. To confirm the protective role of TNFR1-mediated signaling, melanoma cells were pretreated with recombinant TNF α before UVC irradiation. The presence of TNF α (2–4 ng/ml) in the medium partially reduced the degree of UVC-mediated apoptosis of control and ATF2-transfected melanoma cells (Fig. 8B). These observations demonstrate that TNF α activates a survival signal in melanoma cells, which is suppressed upon ATF2 expression.

Suppression of UVC-induced TNF α promoter activity by both the truncated and the full-length forms of ATF2, in transient transfection as well as in LU1205/ATF2 cells (followed by down-regulation of TNF α expression), coincided with increased levels of UV-mediated cell death and pointed to a causal linkage between ATF2 and regulation of cell death in melanoma. It is important to note, however, that full-length ATF2 was more efficient in elevating the degree of UVC-induced apoptosis of melanoma cells than the truncated form of ATF2. Since the full-length form of ATF2 is also expected to bring about positive

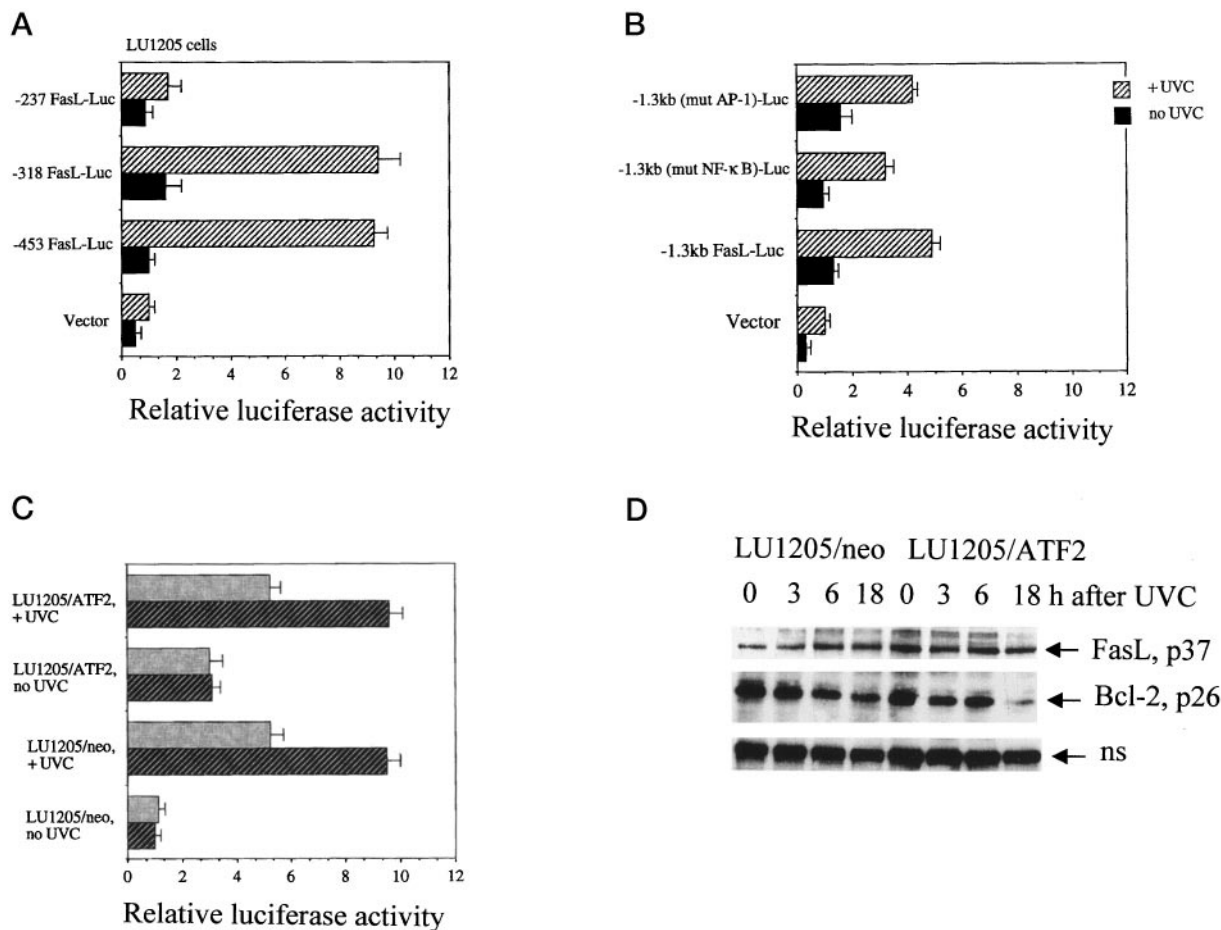


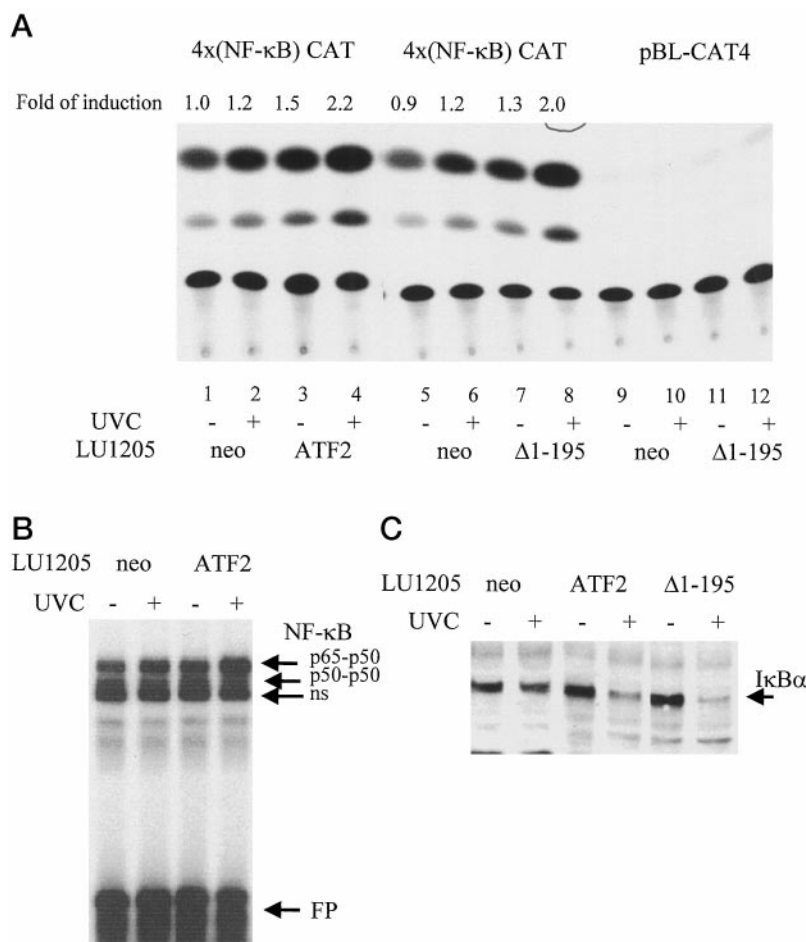
FIG. 9. A–C, FasL promoter activity in LU1205/neo and LU1205/ATF2 cells. The reporter constructs used are indicated. D, Western blot analysis of FasL and Bcl-2 expression in LU1205/neo and LU1205/ATF2 cells following UVC treatment; nonspecific band (*ns*) from Bcl-2 blot was used as a reference band for protein loading.

transcriptional output, we performed experiments to determine whether the apoptotic signal in melanoma cells, namely the Fas pathway, may be also affected by ATF2. Melanoma cell lines, including LU1205, express both the death receptor and its ligand, Fas and FasL (Ref. 61 and Fig. 9D). The human FasL promoter contains two NF-AT sites at positions –279 and –140 (23, 62), MEKK-RE (at –336), which binds ATF2-Jun heterodimers (63), and AP-1- and NF- κ B-binding sites at positions –1088 and –1050, respectively (23, 24). Transfection of nested deletions of 5' FasL promoter constructs (–1.3 kilobase pairs, and –453, –318, –237 base pairs) revealed activation of a UVC-dependent promoter. Increased FasL promoter activity after UV irradiation is likely to be mediated by target sequences located within the –453 and –318 region, as the –237 construct no longer exhibited strong activation by UV irradiation. Further support for the role in FasL activation of the MEKK-RE site located within this comes from analysis of 5' promoter sequences mutated within the NF- κ B or AP1 sites; neither was able to completely abrogate UV responsiveness (Fig. 9B). These results point to the role of MEKK-RL and its associated proteins in mediating increased FasL transcription. Indeed, cells expressing the ATF2 construct exhibited a higher basal level of FasL promoter activity. After UV irradiation, a further increase of 2–3-fold was noted in the LU1205/ATF2 cells (Fig. 9C). The overall level of FasL protein was also higher in LU1205/ATF2 cells before and after UVC treatment than in control cells (Fig. 9D). These observations suggest that ATF2 plays a positive role in the regulation of FasL transactivation/expression in melanoma (Fig. 9, A–D), as previously observed

in other cell systems (63). Because of blockage by different inhibitory proteins, including Bcl-2, the presence of both Fas-receptor and Fas ligand in melanoma cells is necessary but not sufficient to initiate death signaling. UVC-induced degradation of Bcl-2 (Fig. 9D) correlates with the progression of apoptosis seen in these cells. The elevated expression level of FasL in LU1205/ATF2 cells may explain the higher level of apoptosis seen after UV irradiation when compared with the apoptosis seen in cells expressing the truncated form of ATF2.

Melanoma cells are resistant to apoptotic signaling induced by TNF α , which, as noted, elicits a survival signal in these cells. Since NF- κ B has been implicated in protection against TNF-mediated apoptosis (64–66), we next examined the possible effects of ATF2 overexpression on NF- κ B DNA binding activity and transactivation before and after UVC treatment of melanoma cells. LU1205 cells contain relatively high basal NF- κ B activity (Fig. 10A). An additional increase in DNA binding activity of NF- κ B p65-p50 (Fig. 10B, the upper band of the specific NF- κ B DNA-binding complex) and transactivation of NF- κ B were observed in ATF2- and ATF2 Δ 1–195-transfected cells, both at the basal and at the UVC-induced levels (Fig. 10, A and B). Increased levels of NF- κ B activity after UVC irradiation of ATF2-overexpressing cells, when compared with the control cells, are due to down-regulation of I κ B α levels in these cells 18 h after UVC treatment (Fig. 10C), possibly as a result of ATF2-dependent regulation of I κ B expression. Since UVC treatment causes prolonged activation and nuclear translocation of NF- κ B (0.5–20 h) when compared with the short NF- κ B activation in response to TNF α treatment (10–90 min), it is not

FIG. 10. A, NF- κ B activity in ATF2-transfected LU1205 cells. LU1205/neo, LU1205/ATF2, and LU1205/ATF2 Δ 1-195 cells were transiently transfected with either 4xNF- κ B-CAT or with the empty vector pBL4-CAT in the presence of pCMV- β -gal. Two days after transfection, cells were irradiated with UVC and protein extracts were prepared 18 h later. The protein concentration of extracts was normalized based on β -gal activity. Relative CAT activity is indicated. B, electrophoretic mobility shift assay of nuclear extracts of control and LU1205/ATF2 cells 3 h after UVC irradiation was performed as described previously (29). The positions of NF- κ B DNA-binding complexes are indicated by arrows; the upper bands represent the p65-p50 heterodimer, the intermediate band reflects the p50-p50 complex, and the lower band, nonspecific binding (ns). C, Western blot analysis of I κ B α levels in the respective melanoma cell lines 18 h after UVC treatment.



possible to distinguish between primary and secondary TNF-mediated activation of NF- κ B in melanoma cells. However, the abundance of NF- κ B in these cells, especially in ATF2-overexpressing cells, is likely to represent an additional factor that promotes Fas- versus TNF-mediated apoptosis of late-stage melanoma cells.

DISCUSSION

The functional role of ATF2 in the regulation of cell death and survival in human melanoma was evaluated by overexpression of its full-size and truncated (without transactivation domain) forms. Here we provide evidence that both forms of ATF2 serve as potent accelerators of UVC-induced cell death in metastatic human melanoma LU1205 cells. Apoptosis following UVC treatment and its enhancement by overexpression of both forms of ATF2 were demonstrated by characteristic changes in cell morphology, redistribution of phosphatidylserine in the plasma membrane (annexin-V-FITC staining), activation of caspase cascade, PARP cleavage, and DNA fragmentation.

Among the important determinants influencing the degree of ATF2-mediated transactivation are: (i) alternative promoter usage of the ATF2 gene and differential splicing, which create several ATF2 isoforms that exhibit different transcriptional output (full-length ATF2 in its native form is transcriptionally inactive as a result of inhibitory intramolecular interaction, whereas a 42-kDa spliced form (Δ 150-248) is constitutively active; Refs. 33 and 44); (ii) regulation of the transcriptional activity of ATF2, like other members of the ATF/CREB and Jun/Fos families, by phosphorylation, including JNK- and p38-directed phosphorylation of threonine residues at positions 69 and 71 (39, 67-69); (iii) ATF2 heterodimerization with other proteins of the ATF/CREB and Jun/Fos families,

which results in varying degrees of transcriptional output (41, 46, 47) (ATF2 heterodimerization also plays an important role in its stability; Ref. 48); and (iv) interaction with the transcriptional co-activator p300 (dependent on phosphorylation of Ser¹²¹), which contributes to ATF2 transcriptional activity (70). This tight regulation suggests that any of these variables (hypophosphorylation, altered ratio between different full-length and spliced forms, a change in the nature of the heterodimers) may suppress ATF2-dependent transcription. Indeed, silencing effects were described for different ATF2/CREB family members. For example, binding of CREB and CREM to the AP-1 site inhibited activation by c-Jun (71), ATF2 down-regulated hepatitis B virus X promoter activity by competition for the AP-1 binding site and formation of ATF2-Jun heterodimers (72), and CREB and ATF1 were shown to be potent inhibitors of several transcription factors (73). The dominant negative form of CREB efficiently reduced radiation resistance (32), and inhibited tumor growth and metastatic potential of human melanoma through squelching of other CREB-associated proteins (31, 74). The relationship between changes in ATF2-dependent transactivation after UVC irradiation and the degree of apoptosis, as demonstrated in the present study, suggest that ATF2 tightly regulates expression of genes that control apoptosis.

One of the common causes of cell death is down-regulation of expression and decrease in the concentration within the medium of essential growth factors, for example, death of the IL-2-dependent CTLL-2 cell line upon deprivation of IL-2 (75). Late-stage metastatic melanoma cells are characterized by autocrine growth stimulation as a result of production of several cytokines, including bFGF, IL-6, IL-8, and TNF α (59). Numerous observations indicate that TNF α promotes the metastatic potential of melanoma cells (12-14) as well as of some other

tumors (10, 76–78). Down-regulation of c-KIT levels in melanoma cells has been implicated in the enhancement of melanoma tumorigenicity and metastasis (79). Although the effect of TNF α on negative regulation of c-KIT expression in melanoma has not been determined, it has been described for normal bone marrow progenitor cells and myeloid leukemia cells (80, 81). In general, TNF α plays a dual role in the regulation of cell death and survival. This cytokine is an essential growth factor not only for metastatic, but also for normal, cells in certain circumstances, *e.g.* in immature thymocytes (82). On the other hand, this cytokine induces apoptosis of some tumor cell lines and elicits an unusually wide range of biological responses (7–9). Expression of the TNF α gene is regulated by different transcription factors, including AP-1, ATF2, NFAT, Erg-1, C/EBP β , and NF- κ B (18–22). The present study demonstrated the ability to modulate TNF α transcription using both full-length and truncated forms of ATF2, resulting in increased susceptibility of UV-treated melanoma cells to programmed death. The dominant negative form of c-Jun (TAM 67) (53) also had a similar effect, further indicating that down-regulation of TNF α transcription could be due to heterodimerization of inactive ATF2 with c-Jun. In contrast, overexpression of functionally active c-Jun allowed the negative effect of ATF2 on TNF α expression to be overcome and partially rescued UV- and ATF2-mediated apoptosis, suggesting that ATF2 silences the c-Jun contribution to TNF α expression.

The present study also demonstrates that UVC-induced apoptosis of LU1205 as well as ATF2-transfected LU1205 cells is mediated by Fas death signaling. The role of Fas signaling in radiation-induced apoptosis was described in several normal and tumor cell systems (83–87). It was also demonstrated that UVC irradiation by itself may initiate trimerization of the Fas receptor and induction of signaling, substituting FasL (83, 87). However, it is still unresolved whether UVC-induced Fas trimerization *per se* is sufficient to mediate apoptosis, as it is difficult to exclude other cellular changes elicited by UV irradiation. Melanoma cells expressing full-length ATF2 were found to express higher levels of FasL protein, which may also contribute to Fas-mediated apoptosis, as was shown in UV-induced apoptosis of human lymphocytes (63, 84). Elevated FasL expression may explain the higher degree of apoptosis seen after UV irradiation of melanoma cells that express full-length ATF2 *versus* truncated form, as, in addition to silencing TNF, by both forms of ATF2, only the full-length form appears capable of mediating positive transcriptional signals. The ability of full-length ATF2 to elicit a negative signal in the case of TNF α and a positive signal in the case of FasL may be due to its heterodimerized partner, the overall contribution of neighboring transcription factors on a given promoter, and a possible requirement for the contribution of co-activators. Clearly, the effects of full-length ATF2 as a transcription factor are impaired due to its poor phosphorylation. We did not, however, complete analysis of all spliced forms of ATF2 expressed in these melanoma cells and thus cannot exclude further interplay between themselves and other bZIP partners.

The resistance of cells against TNF-mediated killing is linked to NF- κ B expression (9, 64–66). Metastatic melanoma LU1205 cells, used in the present study, are also characterized by moderate NF- κ B activity that may provide resistance against TNF α -mediated apoptosis. Indeed, inhibition of NF- κ B activity by I κ B Δ transformed TNF α from a protective to a death factor.²

Our data extend previous findings regarding down-regulation of radioresistance in human melanoma MeWo cells by

truncated forms of CREB and ATF2 (32, 36). We have shown in the present study that the truncated form of ATF2 increased UVC-induced apoptosis. Thus, an elevated level of apoptosis coincides with reduced resistance to UV irradiation. Surprisingly, comparison of ATF2 Δ 1–195 with full-length ATF2 overexpression revealed that while both elicit equally efficient silencing of TNF α expression, full-length ATF2 was more efficient in elevating UV-mediated apoptosis, possibly because of its ability to increase expression of FasL, which boosts death signaling in these melanoma cells. This is unlike the effect of full-length ATF2 in early stage melanoma cells DM3211, *i.e.* an increase radiation resistance (36), suggesting that the impact of ATF2 on cell survival depends on its proper activation and interaction with other factors that have been altered during melanoma progression. Indeed, the balance between various ATF2 forms, c-Jun, and other members of the CREB/ATF and AP-1 families is expected to change during melanoma development as was previously shown in the B16 mouse melanoma model (88).

In summary, we established an inverse correlation between TNF α expression as a survival factor and the consequences of Fas-mediated death signaling in melanoma cells. Our findings provide the foundation for design of ATF2-based reagents that could be useful in altering the rate of irradiation-mediated programmed death of melanoma cells.

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