We examined the effect of inhibiting p38 MAPK on UVA-irradiated HaCaT cells, a spontaneously immortalized human keratinocyte cell line. Recent work from our laboratory has shown that UVA (250 kJ/m²) induces a rapid phosphorylation of p38 MAPK in the HaCaT cell line. Inhibition of p38 MAPK activity through the use of a specific inhibitor, SB202190, in combination with UVA treatment induced a rapid cleavage of caspase-9, caspase-8, and caspase-3, whereas UVA irradiation alone had no effect. Similarly, cleavage of the caspase substrate poly(ADP-ribose) polymerase was observed in UVA-irradiated HaCaT cells treated with SB202190 or in cells expressing a dominant-negative p38 MAPK. No effect of p38 MAPK inhibition upon caspase cleavage was observed in mock-irradiated HaCaT cells. In addition, increases in apoptosis were observed in UVA-irradiated cells treated with SB202190 by morphological analysis with no significant apoptosis occurring from UVA irradiation alone. Similar results were obtained by using normal human epidermal keratinocytes. UVA induced expression of the anti-apoptotic Bcl-2 family member, Bcl-XL, with abrogation of expression by using the p38 MAPK inhibitor SB202190. Overexpression of Bcl-XL prevented poly(ADP-ribose) polymerase cleavage induced by the combination of UVA and p38 MAPK inhibition. UVA enhanced the stability of Bcl-XL mRNA through increases in p38 MAPK activity. We determined that increases in UVA-induced expression of Bcl-XL occur through a post-transcriptional mechanism mediated by the 3′-untranslated region (UTR). We used Bcl-XL 3′-UTR luciferase constructs to determine the mechanism by which UVA increased Bcl-XL mRNA stability. Additionally, RNA binding studies indicate that UVA increases the binding of RNA-binding proteins to Bcl-XL 3′-UTR mRNA, which can be decreased by using SB202190. In conclusion, p38 MAPK and Bcl-XL expression play critical roles in the survival of UVA-irradiated HaCaT cells.

Programmed cell death, commonly referred to as apoptosis, is an essential process in the development of an organism, tissue remodeling, and immune system development (1). An imbalance between the processes of cell proliferation and death has been proposed to be a critical step in the pathogenesis of human tumors (2, 3). The process of apoptosis has also been described to play an important role in human epidermis, including controlling the size of its major cell population, the epidermal keratinocytes, maintaining epidermal barrier function, and aging (1, 4). Ultraviolet radiation has been shown to trigger apoptosis in epidermal cells. With excessive UVB exposure, “sunburn cells” can be observed within the epidermis (5, 6). These cells are thought to be keratinocytes that have undergone apoptosis, selectively removing those cells that have sustained irreversible damage and that may pose a risk for malignant transformation (7).

UV light has been described to affect directly the apoptotic response of keratinocytes; however, UVA-induced signaling leading to apoptotic susceptibility remains to be fully elucidated. In this report, we focus upon the effects of UVA (320–400 nm), which comprises the largest portion of solar radiation reaching the surface of the earth. It is estimated that 90–99% of solar radiation reaching surface of the earth is comprised of UVA, with the remaining portion consisting of portions of UVB not filtered by the ozone layer (8, 9).

The p38 mitogen-activated protein kinases (MAPKs) and c-Jun N-terminal kinases are stress-regulated protein kinases belonging to the superfamily of MAPKs in addition to extracellular signal-regulated kinases (10). Induction of p38 MAPK activity has a variety of effects, including changes in transcription, protein synthesis, cell surface receptor expression, and cytoskeletal structure with the result ultimately leading to either cell survival or programmed cell death (10). Various extracellular stimuli have been shown to activate stress-regulated kinase cascades, including p38 MAPK. Our laboratory has shown that both UVA and UVB can activate p38 MAPK in the HaCaT cell line (11, 12). The role of p38 MAPK in apoptosis is controversial, appearing to be dependent upon cell type and stimuli. In some systems, activation of p38 MAPK results in apoptosis. Overexpression of MEKKs, specific for p38 MAPK activation, results in apoptosis in T cells and fibroblasts (13). In contrast, inhibition of p38 MAPK in Jurkat cells inhibited Fas induction, and UV-induced apoptosis (14). Another complication of the role of p38 MAPK as it relates to the induction of apoptosis is that the induction or inhibition of apoptosis may be sensitive to the nature of p38 MAPK activation, i.e. the duration of activation.

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tion. For example, a transient early activation of p38 MAPK in tumor necrosis factor-α-treated neutrophils prevents apoptosis, whereas late phase activation contributes to it (15). The opposite effect has also been reported in mouse erythroblastosis cell lines (16). UVA-induced p38 MAPK activity and its role in UVA-induced apoptosis has not been addressed in previous studies. In the studies described here, we demonstrate that UVA-induced p38 MAPK activity plays a dramatic role in the survival of keratinocytes. We observed that inhibition of p38 MAPK decreases expression of Bcl-XL and results in the mitochondrial permeabilization through release of cytochrome c, cleavage of initiator caspases (caspase 8 and caspase 9), the effector caspase (caspase-3), as well as the apoptotic substrate poly-(ADP-ribose) polymerase (PARP). Morphological analysis revealed typical features of apoptosis in UVA-irradiated keratinocytes with inhibited p38 MAPK activity including nuclear condensation and formation of apoptotic bodies. Apoptosis observed in these cells was determined to be caspase-dependent. We further demonstrate that UVA results in increases in the anti-apoptotic Bcl-2 family member, Bcl-XL, through a post-transcriptional mechanism involving the 3′-untranslated region (3′-UTR). Overexpression of Bcl-XL demonstrates its functional role in preventing PARP cleavage in UVA-irradiated HaCaTs where p38 MAPK activity has been inhibited. UVA-induced p38 MAPK activity and the subsequent increase in Bcl-XL resulted in a resistance to UVA-induced apoptosis.

MATERIALS AND METHODS

Cells and UVA Treatment—HaCaT cells, a spontaneously immortalized human keratinocyte cell line, were cultured in DMEM supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. They were grown to 90% confluence and placed in serum-free DMEM for 24 h prior to irradiation. During irradiation, cells were placed in PBS supplemented with 0.01% MgCl2 and 0.01% CaCl2. Control cells were mock-irradiated in supplemented PBS in a separate tissue culture hood. Four F20T12/BL/HO PUVA bulbs (National Bio-Products, Upland, CA) were used for each treatment. After exposure to light, cells were treated for 1 h prior to irradiation with tacrine (10 μmol/L) (Calbiochem) for 30 min prior to UVA irradiation and were placed in serum-free medium containing Z-VAD-fmk following irradiation until the time of harvest. For message half-life studies, cells were pretreated with 1 μmol/L of PMA for 1 h prior to irradiation withycin D (1 μg/ml) (Sigma) and again immediately following UVA irradiation. HaCaT cells stably transfected with a tetracycline controlled Bcl-XL construct were a kind gift from Dr. Ulrich Rodeck (Thomas Jefferson University, Philadelphia) (18). Mock-transfected HaCaTs were used as controls in these experiments. Cells grown in the presence of tetracycline (1 μg/ml) expressed control levels of Bcl-XL. For experiments described here, cells were seeded at 4 × 105 cells/cm2, grown for 48 h, and serum-starved for an additional 24 h prior to irradiation.

Normal human epidermal keratinocytes were obtained from individual adult donors (Cambrex, Walkersville, MD) and used between passages 2 and 4. Cells were maintained according to manufacturer's protocols. Briefly, normal human epidermal keratinocytes (NHEKs) were grown in KGM®-2 Bullet Kit® (containing bovine pituitary extract, human epidermal growth factor, insulin, hydrocortisone, GA-1000 (gentamicin and amphotericin-B), epinephrine, and transferrin) to 80% confluency and subcultured at 3500 cells/cm2.

Toxic Cellular Protein Extraction and Western Analysis—Cells were lysed in 1 ml T-PER Tissue Protein Extraction Reagent (Pierce) containing 1% Triton X-100, 2.5 mm Na3VO4, 1 mm β-glycerophosphate, 1 mm Na3PO4, and 1 μg/ml leupeptin. Cells were scraped and centrifuged at 14,000 rpm for 5 min at 4 °C. Bio-Rad D1 reagent (Bio-Rad) was used to determine protein concentration. For Western analysis, 40 μg of protein were resolved on a 12.5% SDS-polyacrylamide gel. The protein was then transferred to a polyvinylidene difluoride membrane overnight at 4 °C. The membrane was then blocked with 5% milk in TBST at room temperature for 1 h. Primary antibodies for caspase-8, caspase-9, caspase-3, PARP, Bcl-XL, MAPKAPK-2 (Thr-222) (Cell Signaling, Beverly, MA), and cytochrome c (Pharmingen) were used at 1:1000 dilution and incubated at 4 °C overnight. The primary antibody for human GAPDH (Oncogene, La Jolla, CA) was used at a dilution of 1:5000 and incubated at room temperature for 2 h. Membranes were incubated with the appropriate horseradish peroxidase secondary antibody in 5% milk/ TBST for 1 h at room temperature. Goat anti-mouse secondary antibody, antimouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was used for cytochrome c and α-tubulin Western blots at 1:5000, and anti-rabbit (Cell Signaling, Beverly, MA) was used at 1:2500 dilution for all other antibodies mentioned previously. Membranes were washed three times for 10 min each in TBST between antibody incubations and were detected by using ECL Western blotting detection reagents (Amersham Biosciences).

Isolation of Cytoplasmic and Mitochondrial Fractions—Cells were treated as indicated, harvested, and resuspended in cytosolic extraction buffer (250 mm sucrose, 10 mm KCl, 1 mm EDTA, 20 mm Tris-HCl (pH 7.2), 1 mm dithiothreitol, 1.5 mm MgCl2, 100 μm phenylmethylsulfonfyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin). Cells were incubated on ice for 5 min and centrifuged for 10 min at 4 °C at 14,000 rpm. The supernatant was saved as a cytosolic fraction. The remaining pellet was resuspended in mitochondrial extraction buffer (50 mm Tris-HCl (pH 7.4), 150 mm NaCl, 2 mm EDTA, 0.5% Nonidet-P-40, 100 μm phenylmethylsulfonfyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin), placed on ice for 5 min, and centrifuged at 12,500 rpm for 10 min at 4 °C. The supernatant was saved as the mitochondrial extract.

Real Time PCR—Total RNA was isolated from HaCaT cells by using the RNeasy mini kit (Qiagen, Carlsbad, CA). 1 μg of total RNA was used to generate random primed hexamer cDNA by using Omniscript reverse transcriptase kit (Qiagen). Primers and Taqman® probes for Bcl-XL and human GAPDH were purchased from Assay-on-Demand (Applied Biosystems, Foster City, CA). The real time PCR was performed using the Taqman® Universal PCR Master Mix and the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR conditions are as follows: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative Cm value was used to calculate mRNA levels of Bcl-XL normalized to GAPDH in each sample as described in User Bulletin 2, ABI PRISM® 7700 Sequence Detection System.

Flow Cytometry—Apoptosis was measured by determining the DNA content of cells by propidium iodide staining and flow cytometry. Cells were trypsinized and stained with Modified Krishan Buffer (0.1% sodium citrate, 0.2 mg/ml RNase, 0.5% Nonidet P-40, and 0.05 mg/ml propidium iodide (pH 7.4)) before being analyzed. Cells with DNA content less than the G1 amount of untreated cells were considered apoptotic and were recorded by using the ModFit LT software program (Beverly, MA) was used at a 1:2000 dilution for all other antibodies and appropriate horseradish peroxidase secondary antibody in 5% milk/TBST at 1:2000. Membranes were washed three times for 10 min each in TBST between antibody incubations and were detected by using ECL Western blotting detection reagents (Amersham Biosciences).

Generation of p38 MAPK Dominant-negative HaCaT Cell Line—A p38 DNK construct was obtained from Dr. Zigang Dong (Hornet Institute, University of Minnesota, Austin). The plasmid expresses a dominant negative form of p38 MAPK (p38 SN). p38 SN-p38DNK was transfected into THG-Cell, a human keratinocyte cell line stably transfected with a tetracycline-controlled Bcl-XL construct by using the DNeasy tissue kit (Qiagen, Valencia, CA). An EagI site was inserted into the PCR primers to facilitate cloning downstream of the luciferase reporter. 

Luciferase Reporter Construction—The 5′-untranslated region of Bcl-XL was cloned from genomic DNA isolated from HaCaT cells using the DNeasy tissue kit (Qiagen, Valencia, CA). An EagI site was inserted into the PCR primers to facilitate cloning downstream of the luciferase gene in the pRL-TK vector (Promega, Madison, WI). A nested PCR was performed by using the following primers in the first amplification:
5′-TGGCCGCGCCGAGACTGACATCCATCCAT-3′ and 5′-GAAGCTG-CACTTTGACACTTCAACA-3′. A second round of PCR was performed by using the following primer (EagI restriction site underlined): 5′-GGGGCGGGCGGATCAGGCCGTCCAATCT-3′. The 3′-UTR of Bcl-XL was inserted into the EagI site of the expression vector pRL-TK Renilla luciferase (Promega, Madison, WI), the region adjacent to the coding sequence. The portion of the Bcl-XL 3′-UTR cloned into the pRL-TK vector corresponds to base pairs 1130–2396 of Bcl-XL (GenBank™ accession number NM_138578). The DNA construct was analyzed by restriction mapping and DNA sequencing.

**Transient Transfections—**HaCaT cells were transiently transfected by using LipofectAMINE PLUS (Invitrogen) according to the manufacturer’s protocol. Briefly, cells were plated in 6-well plates 1 day prior to transfection and grown to 95% confluence. Plasmid DNA was prepared in 100 µl of serum-free DMEM and incubated with 3 µl of PLUS reagent at room temperature for 15 min, followed by 2.5 µl of LipofectAMINE in an additional 100 µl of DMEM. The mixture was incubated for another 15 min and then added to the well containing 1 ml of serum-free DMEM. Transfections were allowed to proceed for 6 h. A pGL2 firefly luciferase construct was co-transfected along with the Renilla luciferase reporter to control for transfection efficiency. The cells were then washed two times with serum-free DMEM and incubated for an additional 18 h in DMEM before UV irradiation.

**Luciferase Assay—**After treatments, cells were washed two times with PBS and lysed in passive lysis buffer (dual luciferase assay, Promega). All treatments were done in triplicate for each experiment. Luciferase activity was measured by using the dual luciferase reporter assay system (Promega, Madison, WI) and a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Luciferase activity is expressed as the ratio of Renilla/firefly.

**RNA-EMSA—**The region of the Bcl-XL 3′-UTR corresponding to base pairs 1258–1419 were cloned into pBluescript and used in *in vitro* transcription incorporating [32P]ATP (50 µCi) into sense RNAs using Riboprobe® system-T7 (Promega, Madison, WI). Unlabeled RNA transcripts were used for cold competition controls. Following mock or UV irradiation with or without SB202190 treatment, cells were washed twice with cold phosphate-buffered saline and lysed (25 mM Tris-HCl (pH 7.5), 0.5% Triton X-100). Cells were scraped and centrifuged at 14,000 × g for 10 min at 4 °C. Protein concentration was determined using Bio-Rad D, reagent (Bio-Rad). RNA-EMSAs were performed as reported previously by Dixon et al. (19). Briefly, 10 µg of protein was incubated with radiolabeled RNA in binding buffer (20 mM HEPES (pH 7.5), 3 mM MgCl₂, 30 mM KCl, 1 mM dithiothreitol, 5% glycerol) in a total volume of 20 µl for 20 min. Heparin was added at 5 mg/ml and incubated for an additional 20 min. The samples were resolved on 4% polyacrylamide gels (60:1 acrylamide/bisacrylamide) in 1× TBE. The gel was dried and visualized using a PhosphorImager (Amersham Biosciences).

**RESULTS**

**UVA in Combination with SB202190 Induces Caspase Cleavage and PARP Cleavage**—HaCaT cells were UVA-irradiated (250 kJ/m²) or mock-irradiated with or without treatment with SB202190, a specific inhibitor of p38α and p38β1, and collected at various time points. Our laboratory has previously shown SB202190 to inhibit p38 MAPK activity at 0.5 and 2.0 µM by demonstrating inhibition of phosphorylation of a downstream target, MAPKAP-K2 (12, 20). Caspase activation resulting from a cleavage of the inactive pro-form to the active cleaved form was analyzed by Western analysis. Beginning at 1 h post-irradiation, increases in the cleaved forms of caspase-9 and caspase-8 were observed in UVA-irradiated HaCaTs.
The cytosol was analyzed by Western blot analysis. Lower doses of the p38 MAPK inhibitor (0.5 μM) converted to the active cleaved forms. Increases in caspase-3 and PARP were also observed under these conditions. Lower doses of p38 MAPK were determined by examining phosphorylation of a downstream target of p38 MAPK, MAPKAPK-2 in UVA-irradiated cells. HaCaT-empty vector and HaCaT + p38 DNM were grown to 95% confluence and serum-starved for 24 h prior to irradiation. Cells were UVA-irradiated (250 kJ/m²) and collected at 30 min post-UVA irradiation. Proteins were resolved by SDS-PAGE, and Western analysis was performed for active MAPKAPK-2 (Thr-222) (Cell Signaling, Beverly, MA). B, HaCaT-empty vector and HaCaT + p38 DNM cells were grown and treated and described above. Cells were collected at 1 and 2 h post-UVA irradiation, and PARP cleavage was analyzed by Western blot.

A.

B.

30’

1hr

2hr

UVA

+ + + + + + +

SB (2μM)

+ + + + + + +

cytochrome c

α-tubulin

UVA in combination with SB202190 induced release of cytochrome c from the mitochondria. HaCaT cells were grown to 95% confluence, serum-starved for 24 h, and UVA-irradiated. Cells were treated for 1 h prior to and immediately following irradiation with SB202190 (SB, 2 μM). Cyttoplasmic and mitochondrial fractions were isolated as described under “Materials and Methods.” Release of cytochrome c into the cytosol was analyzed by Western blot analysis. α-Tubulin (Oncogene, La Jolla, CA) is shown as a loading control.

Regulation of UVA-induced Bcl-X₇ Expression

Our laboratory has published data previously (20) showing that inhibition of UVA-induced p38 MAPK by SB202190 is specific and does not alter extracellular signal-regulated kinase or c-Jun N-terminal kinase phosphorylation. To confirm further the specificity of the apoptotic effects we observed with the combination of UVA and p38 MAPK inhibition using SB202190, we examined effects of PARP cleavage using an inactive analogue of SB202190, SB202474. As shown in Fig. 2, PARP cleavage was only observed in combination with UVA in cells treated with the active p38 MAPK inhibitor, SB202190. Treatment with UVA alone or in combination with SB202474 had no effect on PARP cleavage indicating the effects on apoptosis observed with UVA irradiation and p38 MAPK inhibition are specific.

By using a retroviral vector, we established a HaCaT cell line that expressed a dominant-negative p38 MAPK to confirm the results obtained from pharmacological inhibition of p38 MAPK by using SB202190. The dominant-negative p38 MAPK construct used contains double point mutations at the activating sites of phosphorylation, Thr-Gly-Tyr to Ala-Gly-Phe. To establish that the expression of the dominant-negative p38 MAPK was decreasing endogenous p38 MAPK activity, we examined the phosphorylation of one of its downstream targets, MAPKAPK-2 (Thr-222). In cells containing the vector control, UVA induced phosphorylation of MAPKAPK-2 as expected from previously published work from our laboratory (12, 20). A significant decrease in MAPKAPK-2 phosphorylation was observed in cells containing the dominant-negative p38 MAPK, indicating that cells containing the dominant-negative p38 MAPK have lower levels of p38 MAPK activity when exposed to UVA (Fig. 3A). Significant increases in PARP cleavage were observed in UVA-irradiated HaCaT cells expressing a dominant-negative p38 MAPK, whereas UVA irradiation alone had no effect on PARP cleavage in vector control HaCaT cells (Fig. 3B). These data further confirm our findings that inhibition of p38 MAPK leads to increases in PARP cleavage and subsequently apoptosis.

UVA Plus SB202190 Induces Release of Cytochrome c—Apoptosis mediated through mitochondrial permeabilization results in the release of cytochrome c from the mitochondria where it is able to form the apoptosome in combination with APAF-1, dATP, and procaspase-9. Cells that were UVA-irradiated in combination with the p38 MAPK inhibitor showed an increase in release of cytochrome c into the cytosol (Fig. 4).
These changes were only observed in cells that were UVA-irradiated in combination with SB202190 and did not result from UVA irradiation alone.

**UVA Plus SB202190 Induces Morphological Changes Typical of Apoptosis and Increases in Cells with Sub-G₀ DNA Content**—The morphology of cells was examined under 100× oil immersion lens and evaluated for apoptotic features (condensed chromatin, nuclear fragmentation, cell shrinkage, and apoptotic body formation) (Fig. 5A). These hallmarks of apoptosis were observed only in UVA-irradiated cells treated with SB202190. The effects were again shown to be dose-dependent with increases in the number of cells undergoing apoptosis with...
increasing amounts of SB202190. In Fig. 5A, the arrows indicate the appearance of apoptotic bodies. We analyzed sub-G0 DNA content in cells to quantitate the levels of apoptosis that occurred. Approximately 4.2% of cells showed increases in sub-G0 DNA content at 6 h post-irradiation, whereas higher doses of SB202190 in UVA-irradiated cells showed 70.3% sub-G0 DNA content (Fig. 5C). It is important to note that no morphological change or increase in sub-G0 content was observed in cells receiving UVA irradiation alone. These observed increases in sub-G0 DNA are consistent with the pattern of caspase activation and morphological analysis shown in Figs. 1 and 5A.

Apoptosis Induced by UVA and SB202190 Treatment Is Caspase-dependent—Both caspase-dependent and -independent pathways leading to apoptosis have been described. We investigated whether the apoptotic effects induced by UVA in combination with SB202190 were dependent on caspase activity. By using the general caspase inhibitor Z-VAD-fmk, we observed cleavage of PARP induced by UVA in combination with p38 MAPK inhibition was caspase-dependent by using the general-caspase inhibitor Z-VAD-fmk (Fig. 6B). Treatment with Z-VAD-fmk completely reversed the observed cleavage of PARP induced by UVA and SB202190 treatment. Both morphological analysis and sub-G0 DNA content were performed concomitantly. The results shown with PARP cleavage were in accordance with both the morphological status of the cell as well as any change in sub-G0 DNA content (data not shown). The experiment was performed with NHEKs from three individual donors. Fig. 6, A and B, is representative of the observations made from three independent experiments.

Bcl-XL Expression Modulates the Apoptotic Effects of UVA in Combination with p38 MAPK Inhibition—In Fig. 7A, we demonstrate that UVA (250 kJ/m2) increases expression of Bcl-XL at the protein level. Most interestingly, inhibition of p38 MAPK resulted in decreased Bcl-XL expression in UVA-irradiated HaCaTs. We also confirmed UVA induced steady-state levels by using real time PCR for Bcl-XL. Statistically significant increases in Bcl-XL were observed at both 30 min (p = 0.04) and 2 h (p = 0.02) post-UVA irradiation (Fig. 7B). To establish that modulations in Bcl-XL expression played a functional role in the observed apoptosis, we used HaCaT cells that overexpressed Bcl-XL in a Tet-Off System (HaCaT-Bcl-XL) (18). Cells were plated 72 h prior to irradiation at 40,000 cells/cm2 in tetracycline-free medium. After 48 h, the medium was changed to serum-free medium and was cultured for an additional 24 h. Under these conditions, Bcl-XL was significantly overexpressed as observed by Western analysis (Fig. 7C). HaCaT-MOCK cells were treated in a similar manner and used as the control cell line for these experiments. Overexpression of Bcl-XL inhibited PARP cleavage induced by UVA and SB202190 treatment indicating a functional role of Bcl-XL expression in the observed apoptotic response (Fig. 7C).

**UVA-induced Bcl-XL Expression Is Post-transcriptionally Regulated**—To investigate the possibility that Bcl-XL was post-transcriptionally regulated, we performed mRNA half-life
studies. UVA-irradiated or mock-irradiated HaCaT cells were treated with actinomycin D. Analysis by real time PCR indicated a significant increase in message half-life in UVA-irradiated cells (t_{1/2}/H11005 8.3 h) (Fig. 8). Those cells treated with UVA in combination with SB202190 had a significant decrease in message half-life (t_{1/2}/H11005 4.6 h), close to that of mock-irradiated controls (t_{1/2}/H11005 3.7 h). These data indicate that UVA acts to stabilize Bcl-X\textsubscript{L} mRNA through increases in p38 MAPK activity.

Regulation of UVA-induced Bcl-X\textsubscript{L} Expression through the 3'-Untranslated Region—We investigated the post-transcriptional regulation of Bcl-X\textsubscript{L} mediated through the 3'-UTR by cloning the 3'-UTR of Bcl-X\textsubscript{L} downstream of the coding region of Renilla luciferase. In Fig. 9A, we demonstrate that UVA irradiation increases luciferase activity in cells transiently transfected with the pRL-TK Bcl-X\textsubscript{L} 3'-UTR construct (p < 0.05 at all time points). To implicate p38 MAPK signaling in the post-transcriptional regulation of UVA-induced Bcl-X\textsubscript{L} expres-

FIG. 7. UVA-induced Bcl-X\textsubscript{L} mediates apoptosis induced by UVA and p38 MAPK inhibition. A. HaCaT cells were cultured as described previously. Following UVA irradiation, cytosolic and mitochondrial fractions were collected at 30 min post-irradiation as described under "Materials and Methods." Protein was resolved by SDS-PAGE, and Western blot analysis was performed for Bcl-X\textsubscript{L} (Cell Signaling, Beverly, MA). B. HaCaT cells were cultured as described previously. Following UVA irradiation, total RNA was harvested at 30 min and 2 h post-UVA irradiation by using RNeasy (Qiagen). cDNA was generated using Omniscript reverse transcription kit (Qiagen) using random primers. Real time PCR was performed using Bcl-X\textsubscript{L} and GAPDH primers and Taqman® probes (Applied Biosystems, Foster City, CA). The relative \textit{C}_\text{t} method was used to calculate mRNA levels of Bcl-X\textsubscript{L} normalized to GAPDH. \textit{p} values were calculated by using the Student’s \textit{t} test (30 min post-UVA irradiation, \textit{p} = 0.04; 2 h post-UVA irradiation, \textit{p} = 0.02). C. HaCaT cells were pretreated with SB202190 (SB) (0.5 or 2.0 \textmu M) or vehicle control (Me\textsubscript{2}SO) for 1 h prior to irradiation. Cells were UVA-irradiated (250 kJ/m\textsuperscript{2}), placed in medium containing vehicle or SB202190, and harvested at 1 h post-irradiation. Protein was resolved by SDS-PAGE and analyzed for PARP cleavage by Western blot analysis.
Regulation of UVA-induced Bcl-X<sub>L</sub> Expression

FIG. 8. UVA induces stabilization of Bcl-X<sub>L</sub> mRNA. HaCaT cells were cultured as described previously. Cells were pretreated with SB202190 and actinomycin D (1 μg/ml) for 1 h prior to irradiation. Following UVA irradiation, cells were placed in medium containing SB202190 or vehicle with actinomycin D. Total RNA was collected at various time points following irradiation. Total RNA was reverse-transcribed using Omniscript reverse transcription kit (Qiagen) using random primers. Real time PCR was performed using Bcl-X<sub>L</sub> and GAPDH primers and Taqman® probes and expression was analyzed by relative C<sub>T</sub> method. + UVA; ▲, + UVA/− SB202190).
of promoter regions leading to increased expression of Bcl-XL and Bcl-XS are attributed to cell type and differentiation status of the cell (24). However, the functions of the other mRNA splice variants, bclΔTM and bcl-β, have not been thoroughly investigated. We investigated the potential transcriptional regulation of bcl-xL by using an ~900-kb human promoter construct. Although we were able to demonstrate increases in promoter activity by using other tumor promoters such as 12-O-tetradecanoylphorbol-13-acetate, UVA did not increase promoter activity (data not shown).
Several genes have been reported to be regulated at the post-transcriptional level through the 3′-untranslated region, including c-fos, cyclooxygenase-2, granulocyte-macrophage colony-stimulating factor, interleukin-3, β-interferon, c-jun, junB, and c-myc (19, 33–41). One mechanism by which stabilization of mRNA can occur is through adenylyl/uridylyl-rich elements (AREs) within the 3′-untranslated region, consisting of the pentamer core AUUUA (42). We identified three previously unidentified AU-rich elements within the 3′-UTR of Bcl-XL. RNA-binding proteins belonging to the embryonic lethal abnormal vision family of proteins have remained of interest as trans-acting factors regulating mRNA turnover. Other RNA-binding protein families shown to bind to AREs include AUF1 (ARE/poly-(U)-binding/degradation factor 1), TIA-1 (T cell restricted intracellular antigen and TIAR (TIA-related protein), FBP (far upstream sequence element binding protein), TTP (tristetraprolin), hnRNP (heterogeneous nuclear ribonucleoprotein) A0, and poly(A)-binding protein. In our investigation, we report increases in binding of proteins to the 3′-UTR in UVA-irradiated cells with a subsequent decrease through inhibition of p38 MAPK activity. We observed a 20% inhibition of overall binding to the Bcl-XL 3′-UTR in cells treated with UVA and SB202190 compared with UVA-irradiated cells alone. RNA-binding proteins have been reported to act as both positive and negative regulators of message stability (43). The observed change could be the result of a change in the composition of the complex itself or a change in its ability to regulate message stability through post-translational modifications as a result of decreased p38 signaling as demonstrated in previous investigations (44, 45). It will be of interest to identify those proteins that bind to the 3′-UTR of Bcl-XL in response to UVA irradiation.

Inhibition of apoptosis is of interest because of the connection between this process and skin carcinogenesis (24, 46). UVB has been shown to induce apoptosis in keratinocytes, whereas the role of UVA in modulating expression of mitochondrial proteins has remained largely unexplored. Decreases in Bcl-XL through antisense treatment has been shown to sensitize cells to UVB-induced apoptosis (2). These studies, however, did not demonstrate that UVB induced Bcl-XL expression but more directly implicated Bcl-XL in mediating resistance to UVB-induced apoptosis. p53 also plays a role in UVB-induced apoptosis through phosphorylation on Ser-15 by p38 MAPK (47). Phosphorylation of p53 by p38 MAPK stabilizes cytoplasmic p53 in normal keratinocytes (47). UVB irradiation and p38 MAPK activity did not alter distribution or expression of p53 in HaCaT cells, presumably due to the fact that it contains two mutant p53 alleles. Although these experiments suggest a role for p38 in UVB-induced apoptosis, our data suggest that apoptosis induced by UVA and p38 MAPK inhibition is p53-independent as our findings of UVA-induced protection through increased p38 MAPK activity were consistent in both normal keratinocytes and HaCaT cells.

Most interestingly, UVA irradiation alone did not induce apoptosis in HaCaT cells or NHEKs as determined biochemically through caspase and PARP cleavage, sub-G0 analysis, and morphological status. Contradictory reports (48–50) have been published regarding UVA-induced apoptosis, described as both inducing apoptosis and having no significant effect in human keratinocytes by using various UV sources. In our experiments, any contaminating UVB emission was removed through filtering. UVA spectra with and without filtering have been published previously (17) by our laboratory. It is of interest to note that UVA is of lower energy than UVB, thus penetrating into deeper layers of skin. Pourzand et al. (51) reported UVA-induced apoptosis through antioxidant properties of Bcl-2 in Rat 6 fibroblasts. Studies by Bernerd et al. (52) demonstrate that UVA induces apoptosis of fibroblasts in the dermis without significant effects on keratinocytes residing in the epidermis by using an in vitro reconstructed skin model. These data suggest a differential effect of UVA on sensitivity to apoptosis depending upon cell type. Our data confirm the findings that UVA does not induce apoptosis of keratinocytes at a physiologically relevant dose and further extend these observations to suggest a mechanism by which these cells become resistant to apoptosis through modulation of Bcl-XL.

In this study, we report several novel findings. First, UVA-induced p38 MAPK activity regulates Bcl-XL expression and susceptibility to apoptosis in UVB-irradiated keratinocytes. Previous findings have not demonstrated a role for p38 MAPK in Bcl-XL expression. Second, p38 MAPK regulates Bcl-XL expression through an interplay with p53.
pression at the post-transcriptional level. Although previous investigations have elucidated transcriptional regulation of Bcl-X<sub>L</sub>, this is the first report, to our knowledge, that demonstrates post-transcriptional regulation of Bcl-X<sub>L</sub>. Finally, the post-transcriptional regulation of Bcl-X<sub>L</sub> is mediated through the 3′-UTR and is dependent upon p38 MAPK activity. As Bcl-X<sub>L</sub> is overexpressed in squamous cell carcinoma (27) and potentially other cancer types, new insights into mechanisms regulating its expression are of great interest. In summary, we demonstrate modulation of Bcl-X<sub>L</sub> expression by UVA-induced p38 MAPK activity providing protection from apoptosis.

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