Journal of Cellular Biochemistry 103:1657–1669 (2008)

Ultraviolet Radiation (UVR) Activates p38 MAP Kinase and Induces Post-Transcriptional Stabilization of the C/EBPδ mRNA in G_0 Growth Arrested Mammary Epithelial Cells

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Abstract The G_0 growth arrest (quiescent) state is highly conserved in evolution to promote survival under adverse environmental conditions. To maintain viability, G_0 growth arrested cells limit gene expression to essential growth control and pro-survival genes. CCAAT enhancer binding proteinδ (C/EBPδ), a member of the C/EBP family of nuclear proteins, is highly expressed in G_0 growth arrested mammary epithelial cells (MECs). Although C/EBPδ gene transcription is elevated during G_0 growth arrest, C/EBPδ mRNA and protein are relatively short lived, suggesting tight control of the cellular C/EBPδ content in unstressed, quiescent cells. Treatment of G_0 growth arrested MECs with ultraviolet radiation (UVR) dramatically increases the C/EBPδ mRNA half-life (∼4-fold) and protein content (∼3-fold). The mRNA stabilizing effects of UVR treatment are mediated by the C/EBPδ mRNA 3′untranslated region, which contains an AU rich element. UVR increased p38 MAP kinase (MAPK) activation and SB203580, a p38 MAPK inhibitor, blocked UVR-induced C/EBPδ mRNA stabilization. UVR increased the nuclear to cytoplasmic translocation of HuR, an ARE-binding protein that functions in mRNA stabilization. Finally, HuR siRNA treatment blocked UVR-induced stabilization of the C/EBPδ and C/EBPβ mRNAs but had no effect on C/EBPζ (CHOP) mRNA stability. In summary, G_0 growth arrested MECs respond to UVR treatment by activating p38 MAPK, increasing HuR translocation and HuR/C/EBPδ mRNA binding and stabilizing the C/EBPδ mRNA. These results identify post-transcriptional stabilization of the C/EBPδ mRNA as a mechanism to increase C/EBPδ levels in the stress response of quiescent cells to UVR. J. Cell. Biochem. 103: 1657–1669, 2008.

Key words: ultraviolet radiation (UVR); CCAAT/enhancer binding proteinδ; C/EBPδ; mRNA stability; p38 mitogen activated kinase (MAPK); post-transcriptional regulation; G_0 growth arrest

CCAAT-enhancer binding proteinδ (C/EBPδ) is a member of the highly conserved C/EBP family of nuclear proteins [Ramji and Foka, 2002; Vinson et al., 2002]. C/EBPδ gene expression is rapidly and persistently induced in human and mouse mammary epithelial cells (MECs) in response to G_0 growth arrest induction by serum and growth factor withdrawal or contact inhibition or treatment with IL-6 family cytokines [O'Rourke et al., 1997; Hutt et al., 2000; Sivko and DeWille, 2004]. C/EBPδ functions in the regulation of G_0 growth arrest, acute phase response, apoptosis, differentiation and the maintenance of genomic stability [Cao et al., 1991; Cantwell et al., 1998; O'Rourke et al., 1999b; Huang et al., 2004]. Reduced C/EBPδ expression has been reported in primary human breast tumors, suggesting that loss of function alterations in C/EBPδ plays a role in breast cancer [Porter et al., 2001; Tang et al., 2006]. Using nuclear run-on assays we demonstrated that C/EBPδ gene transcription is induced ∼6-fold in G_0 growth arrested MECs, however, the C/EBPδ mRNA and protein half-lives are relatively short (∼45 and ∼120 min) [O'Rourke et al., 1999a; Dearth and DeWille, 2003b]. These results indicate that C/EBPδ plays a key role in

Grant sponsor: NCI; Grant numbers: CA57607, P30 CA16058.

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Received 12 June 2007; Accepted 26 July 2007

DOI 10.1002/jcb.21554

the regulation of a number of cell fate determining programs, but the cellular content of C/EBPδ is tightly controlled.

G_0 growth arrest is highly conserved in evolution to promote survival in response to adverse environmental conditions [Gray et al., 2004]. In multi-cellular organisms, G_0 growth arrest plays a major role in the maintenance of tissue and organ homeostasis [Nelson and Daniel, 2002]. Gaining a better understanding of G_0-specific intracellular signaling and gene regulatory mechanisms is important as most cells in adult animals have exited the cell cycle and exist in a quiescent, G_0-like state [Ford and Pardee, 1999]. The conserved, pro-survival mechanisms inherent in the G_0 growth arrest state may contribute to the increased resistance of G_0 growth arrested cells to oxidative stress compared to actively dividing cells [Naderi et al., 2003]. However, the aberrant preservation of G_0 growth arrest induced pro-survival mechanisms may also contribute to the increased resistance of cancer cells to chemotherapy induced cell death [Pinski et al., 2001; Naderi et al., 2003].

Exposure of G_0 growth arrested cells to exogenous stresses and DNA damage treatments, such as ultraviolet radiation (UVR), activates specific intracellular signaling pathways, including p38 MAP kinase, and alters the expression of downstream genes at multiple levels, including post-transcriptional stabilization of selected mRNAs [Cheadle et al., 2005; Fan et al., 2005]. The mechanism underlying p38 MAPK regulation of mRNA stabilization involves sequence specific RNA binding proteins that bind to, and stabilize mRNAs by binding to mRNA AU rich elements (AREs) [Dean et al., 2004]. Two ARE binding proteins, HuR and hnRNP D, increase mRNA stability by binding to mRNA AREs [Dean et al., 2004]. AREs are present in mRNA 3′UTRs of wide variety of mRNAs including mRNAs that encode stress or "early response" genes [Dean et al., 2004]. We previously reported that the 3′UTR of the C/EBPδ mRNA contains two consensus AREs, but the potential regulatory role of the C/EBPδ mRNA 3′UTR AREs under normal growth arrest or in response to extracellular stresses has not been investigated.

The goal of the present study was to investigate the influence of UVR treatment on intracellular signaling pathways and the regulation of C/EBPδ, a transcription factor with a documented functional role in the control of G_0 growth arrest, genomic stability and programmed cell death [O'Rourke et al., 1999a,b; Hutt et al., 2000; Huang et al., 2004; Thangaraju et al., 2005]. The results demonstrated that UVR treatment of G_0 growth arrested MECs activated p38 MAP kinase, increased cytosolic localization of HuR and increased C/EBPδ mRNA stability. Importantly, UVR treatment increased phosphorylated p38 MAP kinase levels but did not significantly increase C/EBPδ levels in growing (actively cycling) MECs, demonstrating that post-transcriptional stabilization of C/EBPδ mRNA is G_0-specific. Post-transcriptional regulation provides an efficient mechanism to regulate the content of gene products that may be required to respond to an extracellular stress in quiescent cells when gene transcription rates are generally repressed [Johnson et al., 1975, 1976] and global gene repair mechanisms are inactive [Bielas and Heddle, 2000, 2004; Bielas, 2006].

EXPERIMENTAL

Cell Culture

Mouse MECs (HC11) were cultured in RPMI 1640 medium plus 10% fetal bovine serum (FBS), 100 U/ml penicillin: 100 μg/ml streptomycin, 500 ng/ml Fungizone, 10 μg/ml bovine insulin and 10 ng/ml epidermal growth factor. To induce G_0 growth arrest (quiescence) HC11 cells were grown to ~80% confluence and then switched to growth arrest media (RPMI 1640 + 0.1% FBS) for 24–48 h. MCF-12A cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in 1:1 Dulbecco's modified Eagle's medium (DMEM): F-12 phenol red free media supplemented with 5% horse serum, 20 ng/ml human recombinant EGF, 100 ng/ml cholera toxin, 10 μg/ml bovine insulin, 500 ng/ml hydrocortisone, 100 U/ml pencillin:100 μg/ml streptomycin and 500 ng/ml Fungizone. To induce G_0 growth arrest (quiescence) MCF-12A cells were grown to ~80% confluence and the media were switched to 1:1 DMEM/F-12 with 0.1% horse serum.

UV Radiation (UVR), RNA Isolation and Northern Blotting

G_0 growth arrested HC11 and MCF-12A cells were washed with PBS and exposed to UVR

(60 J/m^2 UVC, 254 nm) using a UV Stratalinker 2400 (Stragene, La Jolla, CA). Following UVR exposure, cells were cultured in cell line specific growth arrest media. Actinomycin D (10 μg/ml) was added to the cell cultures to block transcription and total RNA was isolated at the indicated time points. Total RNA was harvested using RNABee (TelTest, Inc.). Total RNA was separated by electrophoresis on 1.2% agarose gels, transferred to DuralonUV membranes and probed with (α-^{32}P)dCTP labeled cDNA probes. Cyclophilin (CP) was used as loading control.

Construction, Transfection mRNA Stability Analysis of the pBBB/C/EBPδ 3′UTR Construct

The mouse C/EBPδ mRNA 3′UTR was inserted into the *Bgl*II site of the pBBB β-globin encoding plasmid (generously provided by Dr. Ann-Bin, Shyu, University of Texas, Houston TX). The 3′UTR of the existing pBBB β-globin construct was then deleted by site-specific mutagenesis. The orientation and nucleotide sequence of the resulting pBBB/C/EBPδ 3′UTR plasmid construct was validated by sequencing. The pBBB/C/EBPδ 3′UTR was transfected into HC11 cells using Lipofectamine (Invitrogen) according to the manufacturer's recommendations. Briefly, ~80% confluent HC11 cells were co-transfected with 1 μg pBBB/C/EBPδ 3′UTR construct and 1 ng phRL (Renilla expressing transfection efficiency control). Following transfection cells were switched to growth arrest media for 24 h, treated with actinomycin D, and exposed to UVR. Total RNA was isolated at the designated time points and Northern blots performed as described with detection of the β-globin mRNA using an α-^{32}P dCTP labeled β-globin cDNA probe. Cyclophylin (CP) was used as loading control. The $t_{1/2}$ values were calculated by scanning Northern blots using a Molecular Dynamics Densitometer with ImageQuant software. ImageQuant software quantitation was performed by the volume integration method with manual background correction. The quantitated data was exported to an Excel spreadsheet file where the data was normalized to the loading control (cyclophilin (CP)). The normalized data for the $t = 0$ time point was set at 100% and data for individual time points was plotted as a % of the $t = 0$ data in Excel. Data time points were graphed and a best fit line was plotted.

Whole Cell, Nuclear and Cytoplasmic Protein Isolation and Western Blot Analysis

Following experimental treatments HC11 cells were harvested and lysed in RIPA buffer containing 50 mM Tris–HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1× Complete Protease Inhibitors (Roche Molecular Biochemicals) and Kinase inhibitors (1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and 1 mg/ml Aprotinin). Protein concentration was determined by the BCA Microprotein assay kit (Pierce) according to the manufacturer's instruction. Proteins were separated by SDS–PAGE and transferred to a PVDF membrane. After membrane blocking in 7.5% milk, specific antibodies (anti-P-p38 MAP kinase, anti-p38 MAP kinase, anti-pSTAT3, anti-STAT3, anti-β-actin (Cell Signaling), and anti-C/EBPδ, anti-HuR (Santa Cruz Biotechnology)) were used to detect cellular steady state protein levels. Nuclear and cytoplasmic lysates were prepared by washing cells 2× in ice-cold phosphate buffered saline (PBS) and gentle suspension in 10 mM HEPES (pH7.9) lysis buffer containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.2% NP-40, 0.5 mM DTT, 1× Complete Protease Inhibitors (Roche Molecular Biochemicals) and Kinase inhibitors (1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and 1 mg/ml Aprotinin). After a 20 min incubation the disrupted cells were centrifuged (5 min, 5,000 rpm) and the supernatant was aliquoted and stored at −80°C as the cytoplasmic fraction. The pellet was washed once with ice-cold PBS and resuspended in 20 mM HEPES (pH 7.9) lysis buffer containing 20 mM $MgCl_2$, 0.42 M NaCl, 25% Glycerol, 0.2 mM EDTA, 0.5 mM DTT, 1× Complete Protease Inhibitors (Roche Molecular Biochemicals) and Kinase inhibitors. Samples were vortexed, centrifuged (10 min, 13,000 rpm) and the supernatant stored at −80°C as the nuclear fraction.

In Vivo RNA–Protein Binding Assay

The RNA–protein binding assay was performed by a modification of the protocol described by Niranjanakumari et al. [2002]. HC11 cells were growth arrested for 48 h, UV treated (60 J/m^2), cultured in growth arrest media for the indicated times, washed with cold PBS, crosslinked using 37% formaldehyde (final concentration of 1%) for 10 min. The crosslinking reaction was then

stopped by adding 2 M Glycine (pH 7.0), final concentration of 0.25 M. The cells were resuspended in RIPA buffer and lysed by sonication. The resulted cell lysate was precleared by mixing with protein A-agarose beads and nonspecific competitor tRNA (final concentration of 100 μg/ml). Following incubation with rotation for 60 min at 4°C the beads were collected by centrifugation at 4,000 rpm for 4 min and the supernatants were stored for immunoprecipitation. HuR antibody coated protein A-agarose beads were prepared by incubation of HuR antibody (or mouse IgG isotype controls) with rotation in NT2 buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$ and 0.05% NP-40] overnight at 4°C. After washing 3× with RIPA buffer, the antibody coated protein A-agarose beads were incubated with precleared cell lysates for 90 min at 4°C. RNase out (Ambion) was added to this mixture. The beads containing the immunoprecipitated complexes were collected by centrifugation, resuspended in Resuspension buffer [50 mM Tris–HCl (pH 7.0), 5 mM EDTA, 10 mM DTT and 1% SDS] and incubated in a 70°C waterbath for 40 min to reverse the protein–RNA crosslinking. Total RNA was harvested using RNABee (TelTest, Inc.), resuspended in DEPC water, denatured for 5 min at 70°C and chilled on ice before reverse transcription (RT) with a random 6-mer primer in 20 μl using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen Life-Technologies). The RT reaction (5 μl) was used as template for PCR reaction (45 s at 94°C, 45 s at 58°C and 1 min at 72°C for 30 cycles) with specific primers for C/EBP δ 3′UTR as following: 5′-GCGTGTCGGGGCCAAATCCAG-3′ (forward) and 5′-TTTTCTAGCCCCAGCTG-ACGC-3′ (reverse).

SiRNA Analysis

Specific SMARTpool SiRNA oligos for HuR were purchased from a commercial supplier (Dharmacon). Transfection of cells with SiRNA oligos was performed by nucleofection using the Amaxa Nucleofector. Briefly, 80% confluent HC11 cells (about 2×10^6 cells, 10 cm plate) were harvested in PBS and transferred to a 15-ml tube. After centrifugation, the cell pellet was suspended in 100 μl Nucleofector Solution and mixed with 300 nmol SiRNA. Nucleofection was performed using the Amaxa Nucleofector program T20. Following nucleofection cells were cultured in growth arrest media for 24 h.

RESULTS

C/EBPδ mRNA is Stabilized by UVR Treatment of G_0 Growth Arrested (Quiescent) Mouse and Human Mammary Epithelial Cells

The C/EBPδ gene transcription rate is highly induced in G_0 growth arrested mouse and human MECs [O'Rourke et al., 1999a; Dearth and DeWille, 2003b; Sivko et al., 2004]. A large body of evidence has accumulated from a number of laboratories demonstrating that C/EBPδ gene transcription is induced by activated STAT3 (pSTAT3) [Cantwell et al., 1998; Hutt et al., 2000; Sivko et al., 2004; Dauer et al., 2005; Zhang et al., 2007]. The influence of UV treatment on STAT3 is controversial, with reports indicating that UV both increases and decreases STAT3 levels [Ahsan et al., 2005; Sano et al., 2005]. To investigate the role of the STAT3/C/EBPδ signaling pathway in the response of G_0 growth arrested cells to UVR we exposed HC11 mouse MECs to UVR and assessed pSTAT3, STAT3 and C/EBPδ protein levels by western blot. The results demonstrated that UVR induced a rapid and persistent decline in pSTAT3 levels; however, UVR significantly induced C/EBPδ protein levels (Fig. 1A,B). This result was unexpected due to the documented role of pSTAT3 as a major transcriptional activator of C/EBPδ gene expression [Cantwell et al., 1998; Hutt et al., 2000; Dauer et al., 2005]. This suggested that UVR treatment activated an alternate regulatory mechanism to increase C/EBPδ levels.

Since UVR treatment has been shown to stabilize ARE containing mRNAs [Blattner et al., 2000], we hypothesized that the C/EBPδ mRNA, which contains an ARE in the 3′UTR, may be stabilized by UVR treatment [Dearth and DeWille, 2003a]. UVR treatment dramatically increased the C/EBPδ mRNA $t_{1/2}$ in G_0 growth arrested HC11 mouse and MCF-12A human MECs (Fig. 1C–F). These results indicate that UVR treatment stabilizes the short lived C/EBPδ mRNA transcript in G_0 growth arrested mouse and human nontransformed MEC lines. To further investigate the role of the C/EBPδ mRNA 3′UTR in the UVR-induced stabilization of the C/EBPδ mRNA an expression construct composed of the β-globin coding sequence plus the C/EBPδ 3′UTR was trans-

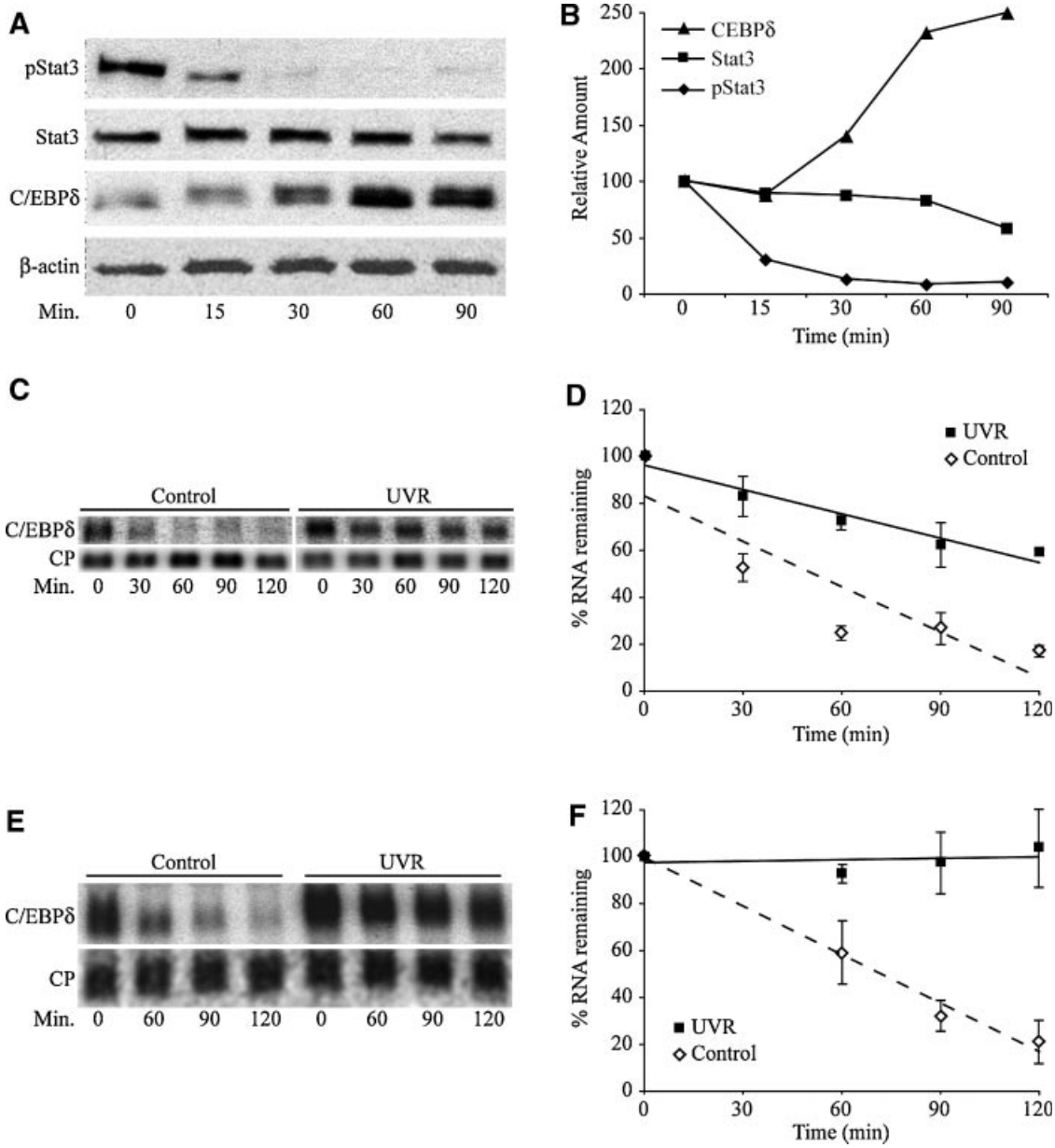

Fig. 1. C/EBPδ RNA stability is increased in G_0 growth arrested mouse and human mammary epithelial cells treated with ultraviolet radiation (UVR). **A**: Western blot analysis of pSTAT3, STAT3, C/EBPδ and β-actin (loading control) levels in whole cell lysates from G_0 growth arrested HC11 mouse mammary epithelial cells irradiated with UVC (60 J/m^2) and harvested at the designated time points. **B**: Western blots were scanned, normalized to β-actin (loading control) and plotted. Results were plotted relative to protein levels in control, non-UVR treated G_0 growth arrested HC11 cells. **C**: G_0 growth arrested HC11 cells were exposed to UVR, treated with Actinomycin D (10 μg/ml) and harvested at the designated time points. Total RNA was isolated and the Northern blot was probed with a ^{32}P labeled C/EBPδ cDNA probe. Cyclophilin (CP) was used as the loading control. **D**: C/EBPδ mRNA levels were quantified (ImageQuant®) and normalized to CP. The normalized C/EBPδ/CP level at T = 0 was set at 100% and the relative amount of mRNA expressed as a % of this value and plotted. **E**: G_0 growth arrested human MCF-12A mammary epithelial cells were irradiated, treated with Actinomycin D (10 μg/ml) and harvested at the designated time points. Total RNA was isolated and Northern blot performed and probed with a ^{32}P labeled C/EBPδ cDNA probe. Cyclophilin (CP) was used as the loading control. **F**: C/EBPδ mRNA levels were quantified, normalized and plotted as a % of the C/EBPδ/CP level at T = 0. The results shown are representative of two independent experiments (Western blot) and three independent experiments (Northern blots).

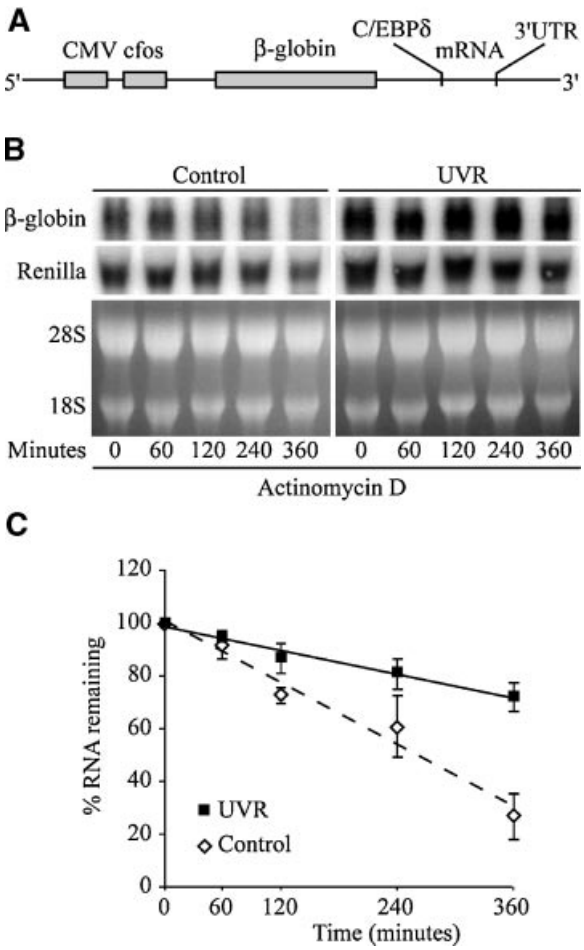

Fig. 2. The C/EBPδ 3′-UTR functions in UVR-mediated mRNA stabilization. **A**: The structure of the pBBB reporter construct with the C/EBPδ 3′UTR. **B**: HC11 cells were cotransfected with the pBBB/C/EBPδ 3′UTR construct plus phRL (Renilla, control). Cells were growth arrested, actinomycin D treated, exposed to UVR and total RNA was isolated at the designated time points. Northern blots performed and blots were probed with a ^{32}P labeled β-globin cDNA plus a Renilla cDNA (control). 18S and 28S RNA bands are shown as evidence of even loading. **C**: β-Globin mRNA levels were quantified, normalized and plotted as a % of the Renilla controls at T = 0. The results shown are representative of three independent experiments.

fected into HC11 cells (Fig. 2A). β-Globin mRNA levels were assessed by northern blot at selected times following induction of G_0 growth arrest and UVR treatment. The intact β-globin mRNA transcript is extremely stable, with a $t_{1/2}$ of ~20 h [Jiang et al., 2006]. Fusing the β-globin coding region to the C/EBPδ 3′UTR produced a β-globin fusion mRNA with a $t_{1/2}$ ~4 h (240 min; Fig. 2B,C). UVR treatment induced an approximately 2.5-fold increase in the β-globin fusion mRNA half life from a $t_{1/2} \sim 4$ to $t_{1/2} \sim 10$ h (Fig. 2B,C). These results indicate that UVR increases the stability of the β-globin/C/EBPδ 3′UTR fusion construct mRNA.

UVR and Anisomycin (Translation Inhibitor) Stabilize C/EBPδ mRNA by Activation of p38 MAPK in G_0 Growth Arrested Mouse Mammary Epithelial Cells

A variety of exogenous cell stresses, including UVR exposure, activate the p38 MAPK pathway and increase the stability ARE-containing mRNAs [Frevel et al., 2003; Dean et al., 2004]. To investigate the influence of p38 MAPK on C/EBPδ mRNA stability G_0 growth arrested HC11 cells were treated with UVR and Anisomycin, a potent activator of stress-inducible MAPKs [Hazzalin et al., 1998]. Anisomycin and UVR treatments both stabilized the C/EBPδ mRNA compared to controls (Fig. 3A). Anisomycin and UVR treatments increased p38 MAPK activation (phosphorylation) and increased C/EBPδ protein content in G_0 growth arrested HC11 cells (Fig. 3B). Phosphorylated p38 MAPK (P-p38) levels are highly induced by UVR and anisomycin treatments at 30 min, declining by 60 min (Fig. 3B). This result suggests that UVR transiently induces p38 MAPK activation. Transient MAP kinase pathway activation plays a key role in cell biology as transient activation of the ERK signaling pathway has been shown to regulate critical cell fate decisions, such as cell cycle control and differentiation [Murphy and Blenis, 2006].

We previously reported that C/EBPδ expression is G_0 growth arrest specific and that C/EBPδ is not induced in actively cycling MECs arrested in other phases of the cell cycle by hydroxyurea (G1/S arrest) or nocodozole (G2/M arrest) [O'Rourke et al., 1999b]. To determine the effects of UVR treatment on p38 MAPK activation and C/EBPδ mRNA levels in actively cycling cells we treated exponentially growing HC11 cells with UVR. The results indicated that UVR increased p38 MAPK activation but little or no induction of C/EBPδ (Fig. 3C). These results are consistent with UVR induced stabilization of existing C/EBPδ mRNA transcripts present in G_0 growth arrest cells, with little or no effect in cycling cells where C/EBPδ mRNA levels are minimal. Collectively, these results demonstrate that cellular stress treatments such as anisomycin and UVR activate p38 MAPK activity and stabilize existing C/EBPδ mRNA transcripts in G_0 growth arrested MECs.

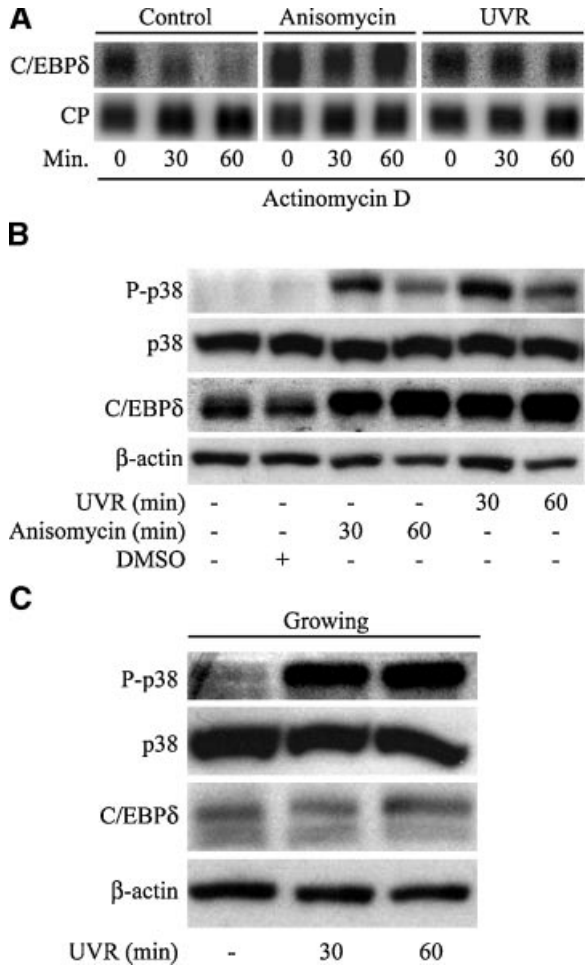

Fig. 3. UVR and Anisomycin (translation inhibitor) activate p38 MAPK in G_0 growth arrested mouse mammary epithelial cells. **A**: G_0 growth arrested, actinomycin D treated HC11 cells were treated with DMSO (control), exposed to UVR (60 J/m^2), or Anisomycin (20 μM). Cells were harvested at 0, 30, and 60 min, total RNA was isolated and northern blots performed. **B**: G_0 growth arrested HC11 cells were treated with DMSO (control), exposed to UVR or Anisomycin. Whole cell lysates were isolated at 0, 30, and 60 min and western blots performed. β-Actin was used as the loading control. **C**: Exponentially growing HC11 cells were exposed to UVR (60 J/m^2). Whole cell lysates were isolated at 0, 30, and 60 min and Western blots performed. β-Actin was used as the loading control. The results shown are representative of three independent experiments.

SB203580 Blocks UVR Induced C/EBPδ mRNA Stabilization by Blocking p38 MAPK Activation, HuR Nuclear → Cytoplasmic Translocation and HuR/C/EBPδ mRNA Binding

To investigate the role of p38 MAPK on UVR induced C/EBPδ mRNA stabilization G_0 growth arrested HC11 cells were pretreated with SB203580, a p38 MAPK inhibitor, and exposed to UVR. SB203580 pretreatment reduced C/EBPδ mRNA to a $t_{1/2}$ of ~60 min, a reduction of ~60%, from the $t_{1/2}$ ~150 min in the vehicle treated controls (Fig. 4A,B). This result demonstrates a link between UVR induced p38 MAPK activation and increased C/EBPδ mRNA stability. To further investigate the mechanism by which UVR treatment increases C/EBPδ mRNA stability we assessed the cellular localization of HuR, an Hu/ELAV family RNA binding protein [Chen and Shyu, 1995; Peng et al., 1998]. HuR functions in mRNA stability by translocating from the nucleus to the cytoplasm and binding to 3′UTR AREs [Chen and Shyu, 1995; Peng et al., 1998]. UVR treatment induces HuR nuclear → cytoplasmic translocation (Fig. 4C, top row, lane 1 vs. lanes 3 and 5). HuR nuclear → cytoplasmic translocation is blocked by SB202580 pretreatment, indicating that p38 MAPK activation influences HuR translocation (Fig. 4C, second row, lane 1 vs. lanes 3 and 5). To more directly assess the interaction between HuR and the C/EBPδ mRNA transcript we assessed HuR/C/EBPδ mRNA binding using immunoprecipitation coupled with RT-PCR analysis. The results demonstrated that UVR significantly induces HuR/C/EBPδ mRNA binding compared to non-UVR treated G_0 growth arrested HC11 cells (Fig. 4D, UVR vs. GA). These results are consistent with UVR induced p38 MAPK activation, p38 MAPK mediated HuR nuclear → translocation and increased HuR/C/EBPδ mRNA binding.

HuR Is Required for UVR Induced Stabilization of C/EBPδ mRNA

To investigate the functional role of HuR in UVR-mediated C/EBPδ mRNA stabilization HC11 HuR protein levels were reduced to ~10% of scrambled siRNA-treated controls by HuR specific siRNA transfection (Fig. 5A). The northern blot results demonstrated that reducing HuR levels was associated with loss of UVR induced C/EBPδ mRNA stabilization (Fig. 5B,C). We next investigated the influence of UVR and HuR on the post-transcriptional regulation of additional C/EBP family members expressed in MECs, C/EBPβ and C/EBPζ. HuR has been shown to bind to the C/EBPβ 3′UTR ARE, but the effects of HuR on C/EBPβ mRNA stability are poorly understood [Jones et al., 2007]. C/EBPζ (C/EBP Homologous Protein (CHOP, GADD153)) is a well-described stress inducible C/EBP family member [Ubeda et al.,

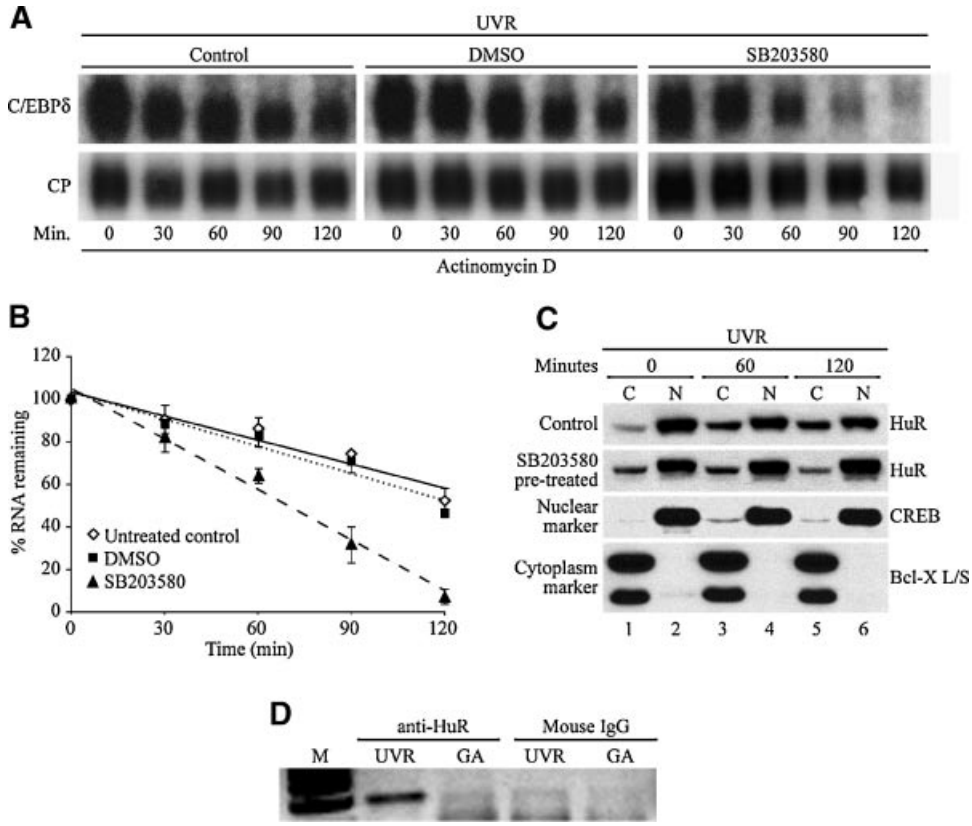

Fig. 4. UVR stabilization of the C/EBPδ mRNA requires p38 MAPK activation and is associated with HuR nuclear→cytoplasm translocation and HuR/C/EBPδ mRNA binding. **A**: G_0 growth arrested, actinomycin D treated HC11 cells were exposed to UVR (60 J/m^2) and untreated (control), or pretreated with DMSO (vehicle control), or SB203580 (20 μM) a p38 MAP kinase inhibitor for 120 min. After UVR exposure cells were harvested at 0, 30, 60, 90, and 120 min, total RNA was isolated and northern blots performed. CP was used as the loading control. **B**: C/EBPδ mRNA levels were quantified and normalized to CP. The normalized C/EBPδ/CP level at T = 0 was set at 100% and the relative amount of mRNA expressed as a % of this value and plotted. **C**: G_0 growth arrested HC11 cells were exposed to UVR (60 J/m^2) and pretreated with DMSO (control) or SB203580 (20 μM) for 120 min. After UVR treatment cells were harvested at 0, 60, and 120 min and fractionated into cytoplasmic (C) and nuclear (N) lysates. Western blots were performed to detect HuR cytoplasmic and nuclear localization. BCL-$X_{L/S}$ was detected as a cytoplasmic marker and CREB was detected as a nuclear marker to assess fractionation protocol. **D**: G_0 growth arrested HC11s were untreated (growth arrest (GA)) or exposed to UVR. Cell lysates were isolated 30 min after UVR treatment and immunoprecipitated with an HuR monoclonal antibody or mouse IgG. Total RNA was isolated from the immunoprecipitates, RT-PCR amplified and the RT-PCR product was re-amplified using primers specific for the C/EBPδ mRNA 3′UTR. The C/EBPδ specific PCR product (300 bp) was detected by ethidium bromide staining following agarose gel electrophoresis. The results shown are representative of two to three independent experiments.

1996; Ramji and Foka, 2002]. Previous reports have demonstrated that CHOP is regulated at the post-translational level by p38 MAPK phosphorylation at serine 78 and serine 81 but the potential role of p38 MAPK on C/EBPζ post-transcriptional control is unknown [Wang and Ron, 1996]. The CHOP 3′UTR contains an ARE, suggesting a possible role for HuR in the regulation of mRNA stability [Ubeda et al., 1996]. UVR treatment of G_0 growth arrest HC11 increased C/EBPβ mRNA stability ~50% (from ~90 to ~140 min) and this effect was abrogated by HuR siRNA treatment (Fig. 5B,C). However, C/EBPζ (CHOP) mRNA stability was unaffected by UVR or HuR siRNA treatments (Fig. 5B,C). These results demonstrate that HuR plays an important role in UVR induced post-transcriptional regulation of C/EBPδ and C/EBPβ mRNA stability, but HuR does not appear to influence C/EBPζ (CHOP) mRNA stability.

DISCUSSION

The major finding of this study is the characterization of a post- transcriptional regulatory mechanism, that is, increased mRNA stability; to increase C/EBPδ levels in UVR treated, G_0 growth arrested MECs. UVR induced DNA

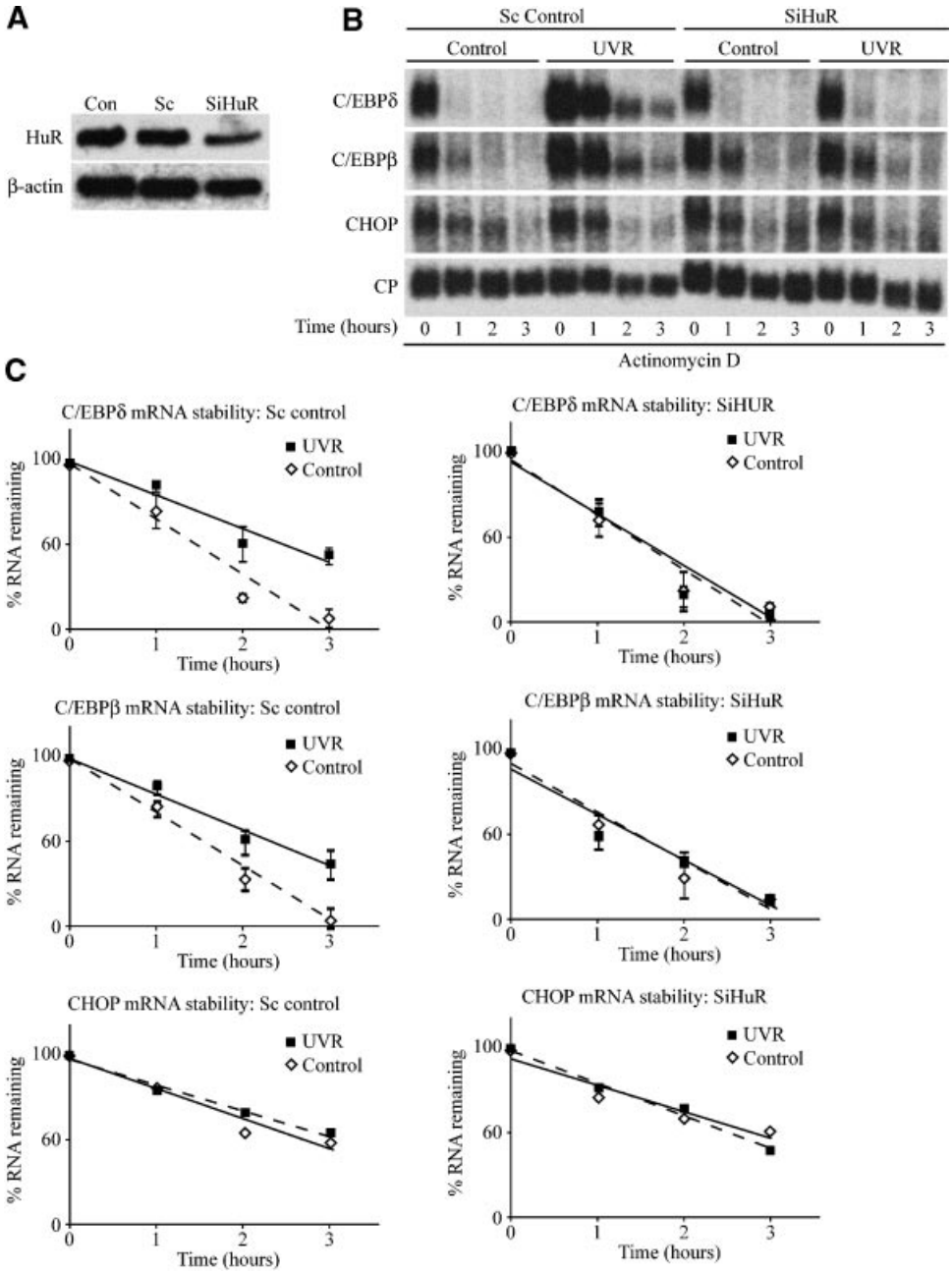

Fig. 5. HuR is required for UVR mediated C/EBPδ and C/EBPβ mRNA stabilization. **A**: HC11 cells were subjected to ''mock'' nucleofection (nucleofection without siRNA) (Con, control), nucleofection with scrambled siRNAs (Dharmacon non-targeting four-oligo pool, Sc (SiRNA control)) and nucleofection with HuR siRNAs (SiHuR). Nucleofected cells were growth arrested, whole cell lysates isolated and analyzed by western blot. β-Actin was used as a loading control. **B**: HC11 cells were nucleofected with scrambled siRNAs (Sc control) and HuR siRNAs (SiHuR), growth arrested and treated with Actinomycin D (10 μg/ml). Cells either received no UV (Control) or were exposed to UVR and harvested at 0, 1, 2, and 3 h post-UV treatment. Total RNA was isolated and Northern blot performed and probed with ^{32}P labeled C/EBPδ, C/EBPβ and C/EBPζ (CHOP) probes. Cyclophilin (CP) was used as the loading control. A representative northern blot result is shown. **C**: C/EBPδ, C/EBPβ and CHOP mRNA levels were quantified (ImageQuant®) and normalized to CP. The normalized levels at T = 0 was set at 100% and the relative amount of each mRNA expressed as a % of this value and plotted. The data shown for C/EBPδ and C/EBPβ are plotted from three independent experiments. The data for C/EBPζ (CHOP) are plotted from one experiment.

damage produces DNA lesions that stall or block the progression of RNA polymerase II and, as a result, UVR induced DNA damage is a potent inhibitor of transcription [Mone et al., 2001; Laine and Egly, 2006]. C/EBPδ gene transcription is persistently induced during G_0 growth arrest, suggesting that C/EBPδ plays a key role in the maintenance of important cell functions during G_0 growth arrest state [O'Rourke et al., 1999a]. Recent evidence indicates that C/EBPδ knockout mouse embryo fibroblasts exhibit chromosome instability, suggesting a connection between "loss of function" alterations in C/EBPδ and the recognition and repair of DNA lesions [Huang et al., 2004]. Using "ChIP-chip" assays we recently identified a number of C/EBPδ target genes including two, TATA binding protein (TBP) and the xeroderma pigmentosum group C (XPC), that function in the DNA damage response (Zhang et al., unpublished). UVR induced DNA damage sites are enriched in DNA binding proteins including TBP and XPC plays a key role in DNA damage recognition as a component of global genome nucleotide excision repair [Vichi et al., 1997; Laine and Egly, 2006]. The present results suggest that DNA damage activates post-transcriptional mechanisms that stabilize the C/EBPδ mRNA and increase C/EBPδ levels in quiescent cells. C/EBPδ may function in the transcriptional activation of genes that function in cell survival, DNA repair and/or the maintenance of a stable genome.

Activation of the p38 MAPK pathway is well established as an important regulator of mRNA stability in response to environmental stress, such as UVR treatment, and inflammatory cytokines [Dean et al., 2004]. Intrinsically unstable mRNAs that are stabilized as a result of p38 MAPK activation are characterized by the presence of AU rich elements (AREs) in the mRNA 3′UTR [Dean et al., 2004]. For example, the early response gene c-fos encodes the prototype, unstable, mRNA that contains a 3′UTR ARE and exhibits UVR mediated stabilization [Chen and Shyu, 1995]. AREs play a complex role in the biology of eukaryotic mRNAs, functioning in both mRNA de-stabilization and mRNA stabilization depending on the complement of RNA binding proteins associated with the mRNA ARE [Stoecklin et al., 2003; Dean et al., 2004]. Emerging evidence indicates that cytoplasmic processing bodies (P bodies) play a major role in the rapid decay of ARE containing mRNAs [Eulalio et al., 2007; Stoecklin and Anderson, 2007]. Cellular stress conditions induce the mobilization of mRNAs from P-bodies to polysomes by a mechanism that involves binding of ARE containing mRNAs to HuR [Bhattacharyya et al., 2006; Eulalio et al., 2007; Stoecklin and Anderson, 2007]. HuR has also been shown to stabilize p38 MAPK regulated mRNAs by binding to AREs and blocking the degradation of deadenylated mRNAs [Peng et al., 1998; Dean et al., 2004]. In the present report we show that UVR induces a sequence of events involving activation of p38 MAPK, translocation of HuR from the nucleus to the cytoplasm, increased HuR binding to the C/EBPδ mRNA and increased C/EBPδ mRNA stabilization. These results are consistent with previous reports in which UVR treatment resulted in HuR mediated stabilization of $p21^{waf1/cip1}$, RhoB and prothymosinα ARE-containing mRNAs by inducing a similar sequence of events [Wang et al., 2000; Lal et al., 2005; Westmark et al., 2005]. These results suggest that UVR treatment induces the stabilization of mRNAs that encode proteins with functional roles in the cellular DNA damage response. For example, the universal cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$ is well established as a cell cycle inhibitor that is expressed in response to DNA damage [Wang et al., 2000]. RhoB encodes a small GTPase that is involved in a number of cell signaling pathways that may facilitate apoptosis in cells exposed to significant levels of DNA damage [Liu et al., 2001]. Prothymosinα appears to function as a pro-survival effector gene in UVR treated cells [Lal et al., 2005]. Whether C/EBPδ mRNA stabilization is associated with a pro-survival or pro-apoptotic cellular response to UVR is currently under investigation. Taken together this evidence supports the general hypothesis that HuR stabilizes ARE containing mRNAs that play critical roles in determining the fate of cells exposed to UVR or DNA damage. The utilization of post-transcriptional regulation to stabilize existing mRNAs may be particularly important in G_0 growth arrested cells in which gene transcription is reduced.

The initial reports identifying C/EBP gene family members determined that C/EBPs are expressed in a coordinated, sequential pattern in the adipocyte differentiation model and that C/EBPs contain highly conserved, interacting structural domains (i.e., leucine zippers) [Cao

et al., 1991; Williams et al., 1991]. The results presented in this study demonstrate a common post-transcriptional regulation by mRNA stabilization for both C/EBPδ and C/EBPβ. This shared regulation may result in increased C/EBPδ and C/EBPβ interactions in the cellular response to UVR. In contrast, C/EBPζ (CHOP) was not stabilized by HuR, suggesting that C/EBPζ may not function in the UVR response or that C/EBPζ may be regulated by alternate mechanisms such as post-translational modification. Further studies are now needed to identify which C/EBPs interact and how these interactions affect downstream gene expression and cellular responses to treatments such as UVR.

The cellular pathways and gene regulatory mechanisms that are activated in G_0 growth arrested cells in response to DNA damage treatments have important implications in cell biology and clinical medicine. With regards to DNA repair, G_0 growth arrested cells utilize primarily transcription coupled DNA repair mechanisms which results in repair of DNA damage in transcribed genes but may be ineffective in repairing DNA damage in nontranscribed genes [Bielas and Heddle, 2000, 2004; Bielas, 2006]. This could result in reduced levels of mutations in genes that are expressed in quiescent cells. Interestingly, C/EBPδ, which is highly expressed in G_0 growth arrested cells, is rarely mutated in human cancers [Vegesna et al., 2002]. In the context of cancer, LnCAP prostate cancer cells that are arrested in a G_0, or G_0-like state exhibit increased resistance to DNA damage inducing chemotherapy, suggesting that cancer cells capable of cell cycle arrest may survive chemotherapy and contribute to cancer recurrences after the cessation of cancer treatment [Pinski et al., 2001]. It is of interest that growth arrested LnCAP cells express elevated levels of C/EBPδ [Sanford and DeWille, 2005]. A better understanding of the intracellular signaling, alterations in gene expression and the resulting DNA repair or apoptotic responses of cancer cells may help direct decisions regarding the type of chemotherapy provided to cancer patients. It is of interest that UVR mediated mRNA stabilization is p53 independent, suggesting that UVR mediated p38 MAP kinase activation and mRNA stabilization parallels, but is not dependent on, the p53 mediated DNA damage response [Blattner et al., 2000].

In conclusion, the C/EBPδ mRNA is unstable in G_0 growth arrested MECs. UVR activates a coordinated cellular response that results in the post-transcriptional stabilization of C/EBPδ mRNA and an increase in C/EBPδ protein levels. The functional significance of UVR induced C/EBPδ post-transcriptional regulation and its role in the cellular response to UVR and other DNA damage treatments is currently under investigation.

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