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Mechanism of Ultraviolet B-Induced Cell Cycle Arrest in G2/M Phase in Immortalized Skin Keratinocytes with Defective p53

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The ultraviolet B (UVB) portion (280–320 nm) of solar radiation is considered to be a major etiologic factor in human skin cancer and is a known cause of extensive DNA damage. In this study, we observed that UVB exposure of immortalized epidermal keratinocytes (HaCat cells) harboring mutant p53 leads to G_2/M cell cycle arrest in both asynchronously growing and synchronized cells in a dose dependent manner. Following UVB exposure (200 mJ/cm^2), we observed a threefold increase in G_2/M population at 6 h, which increased to sixfold. The observed G_2/M arrest was associated with an increase in cyclin B level whereas cdc2 protein remained unchanged. However, we observed an accumulation of tyrosine 15 hyperphosphorylated cyclin B-cdc2 complex. In addition, we observed an increase in chk1 kinase and a decrease in cdc25C protein levels. Chk1 phosphorylates cdc25C on serine 216 and inactivates it whereas cdc25C dephosphorylates tyrosine 15 phosphate of cdc2 and activates the cdc2-cyclin B complex. Therefore, the increase in chk1 and the decrease in cdc25C both participate in inhibiting the G2/M transition. Our data identifies two upstream targets leading to inhibition of cyclin B-cdc2 complexes, which explain the inhibition in cyclin B-associated cdc2 kinase following UVB exposure. The inactive phosphorylated cdc2-cyclin B complex remains sequestered in cytoplasm and may migrate to the nucleus following activation. Our data also indicate that UVB exerts unique effects in different types of skin keratinocytes having nonfunctional or mutant p53. **© 2000 Academic Press**

Solar radiation and in particular its UVB component is a ubiquitous environmental carcinogen and a known risk factor for nonmelanoma skin cancers (NMSC) (basal cell and squamous cell carcinomas) in humans (1). More than one million patients are diagnosed annually with NMSC in the U.S.A. (2). In addition to the induction of skin cancers both in humans and experimental animals, UVB causes many other skin disorders including sunburn, photoaging, actinic keratoses, etc. (3).

It is known that UVB induces signature mutations in cutaneous DNA (4). DNA damage evokes a wide range of acute cellular responses that ultimately lead to delay of cell cycle progression, stimulation of DNA repair and alterations in the expression of genes necessary for cell survival. p53 mutations have been identified in more than 50% of NMSC (5, 6). p53 is a critical sensor of genotoxic insult that can mediate G1 cell cycle arrest or apoptosis (7). The basic purpose of cell cycle regulation is to ensure that DNA is faithfully replicated only once during S phase and that identical copies of chromosomes are formed and distributed equally to the progeny cells during M phase (8). Cell cycle progression is regulated by surveillance mechanisms that operate through checkpoints located before entry into S phase and M phase (9). Progression through G1/S involves activation of cyclin D/cdk4, cyclin D/cdk6 and cyclin E/cdk2 complexes. D-type cyclins (D1, D2 and D3) complexed with cdk4/6 phosphorylates serine and threonine residues on Rb which untethers it from E2Fs enabling activation of a series of target genes essential for S phase entry (10) whereas cyclin E/cdk2 is essential for DNA replication (11). G2/M transition is mediated by a signaling cascade leading to the regulation of cyclin B/cdc2 complex through phosphorylation (12). Cyclin B/cdc2 is phosphorylated on inhibitory sites at residues threonine 14 and tyrosine 15 by Wee1/Mik1 protein kinases (13). As a consequence, it remains inactive in cytosol (14). Ac-

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tivation of cyclin B/cdc2 complex occurs by cdc25C phosphatase-mediated dephosphorylation of inhibitory phosphates, which regulates the activation and nuclear translocation of the cyclin B/cdc2 complex (14, 15). Recently, atr, which is induced both by UV and ionizing radiation (IR), has been shown to be the upstream target for regulating chk1 kinase activity by phosphorylating at its 345 serine residue (16–18). Both atm and atr can activate Chk1 protein kinase, which in turn phosphorylates cdc25 leading to the inhibition of its activity by creating a 14-3-3 binding site (16–18), and promoting its sequestration in cytoplasm leading to the accumulation of inactive forms of CDKs phosphorylated at tyrosine 15 (19). In addition, p53-dependent induction of 14-3-3σ and GADD45 genes mediates G2 arrest (20). It has been demonstrated that the 14-3-3σ gene product sequesters phosphorylated cdc25C needed to dephosphorylate cyclin B/cdc2 essential for G2/M transition (20). Finally, GADD45 can directly interact with cdc2 to disrupt the cyclin B/cdc2 complex leading to G2 arrest (21).

Recently, we observed that UVB induces G1 arrest in transformed keratinocytes (A431cells) lacking functional p53 by down regulating cyclin D and cdk4 levels and associated kinase activity (22). It has been shown that UVB exposure of normal human skin keratinocytes carrying wild type p53 produces differential effect on cell cycle progression and causes G1 arrest by inducing the cyclin kinase inhibitory proteins, p21 and p27 (23). To further investigate the effect of UVB in nonmalignant skin cells with nonfunctional p53, we studied the effect of UVB exposure on immortalized skin keratinocytes (HaCat cells) carrying two copies of mutant p53 (24). UVB exposure to HaCat cells induces G2/M arrest by increasing cyclin B1 protein without altering cdc2. The induction of cyclin B1 is concomitant with the accumulation of the hyperphosphorylated form of cdc2. UVB exposure results in increased chk1 and decreased cdc25C proteins but does not alter 14-3-3σ levels. Our results indicate that UVB induces G2/M arrest in immortalized skin keratinocytes (HaCat cells) by inactivating the cyclin B-cdc2 complex as a result of chk1-mediated inactivation of cdc25C and its trapping by 14-3-3σ protein.

MATERIALS AND METHODS

Cell culture and UVB irradiation. HaCat keratinocytes were grown in DMEM supplemented with 10% fetal bovine serum. For UVB treatment cells were washed with PBS, irradiated while under a thin film of PBS and replenished with the media thereafter. Trypan blue dye exclusion test indicated greater than 80% cell survival at 100 mJ/cm^2 of UVB dose.

Flow cytometry. Cells were harvested by trypsinization and low speed centrifugation. The resulting pellet was then fixed in ice cold 70% ethanol and stored at −20°C until flow cytometric analysis. Fixed cells were then centrifuged, washed and resuspended in PBS containing RNAse A (1 mg/ml), and incubated at 37°C for 30 min. Propidium iodide was added to a final concentration of 10 μg/ml. Propidium iodide-stained cells (1–2 $\times$ 10^6 cells/ml) were analysed by a fluorescence-activated cell sorter (FACSCaliber, Becton Dickenson) followed by determination of the percentage of the cells in sub-G1, G1, S, and G2/M.

UV light source. We used a UV Irradiation Unit (Daavlin Co., Bryan, OH) equipped with an electronic controller to regulate dosage. The UVB source consisted of eight FS72T12–UVB-HO lamps that emit UVB (290–320 nm, 75–80% of total energy) and UVA (320–380 nm, 20–25% of total energy). The dose of UVB was quantified with a UVB Spectra 305 Dosimeter obtained from the Daavlin Co. (Bryan, OH). The radiation was further calibrated with an IL1700 Research Radiometer/Photometer from International Light Inc. (Newburyport, MA). The radiation source to target distance was maintained to 30 cm. No measurable increase in temperature was noticed during the irradiation.

Preparation of cell lysate. Cells were harvested at different time intervals as mentioned in figure legends by incubating them with 0.25% trypsin. The cells were washed with ice cold PBS (10 mM, pH 7.4) and treated with ice cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 1% Triton-X 100, 0.5% NP-40, and protease inhibitor) for 30 min. Clear cell lysate was prepared by centrifugation at 10,000*g* for 10 min. It was aliquoted in small volumes and stored at −80°C before it was used.

Immunoblotting. Aliquots of total cell lysate were mixed with equal volume of SDS sample buffer (85 mM Tris–HCl, pH 6.8 containing 1.4% (W/V) SDS, 14% (V/V) glycerol, 5% (V/V) mercaptoethanol and traces of bromophenol blue), boiled for 5 min and subjected to SDS–PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes. After transfer, nonspecific sites were blocked with 5% (W/V) nonfat, dry milk in TTBS (0.1% Tween 20, 20 mM Tris base, 137 mM NaCl, 3.8 mM HCl, pH 7.6) for two h at ambient temperature followed by overnight probing with primary antibody at 4°C. After washing the blot three times in TTBS for 10 min each, the membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (dilution-1:10,000, Jackson Labs, Maine). The blot was washed three times in TTBS for 10 min each and was developed with ECL according to the manufacturer's instructions (Amersham, Arlington Heights, IL).

Immunoprecipitation. Cells were lysed for 30 min in cold lysis buffer consisting of 1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM PMSF, 0.5% NP-40. A clear lysate was obtained by sonicating for 15 s on ice followed by centrifugation at 10,000*g* for 10 min. The protein concentration was determined by DC Bio-Rad assay using manufacturer's protocol. The cell lysate (500 μg protein) was precleared with 15 μl of 50% protein A sepharose. The mixture was incubated for 1 h at 4°C and supernatant was collected, which was again incubated with 15 μl of 50% protein A sepharose and appropriate antibody overnight at 4°C. Beads were collected by centrifugation at 3000 rpm for 10 min at 4°C. Immune complexes were washed three times in lysis buffer, and the pellets were either suspended in SDS sample buffer for SDS–PAGE electrophoresis or washed further for kinase activity assay.

RESULTS

UVB irradiation of immortalized keratinocytes induces G2/M arrest. To investigate the effect of UVB on cell cycle distribution of immortalized human skin keratinocytes carrying mutated p53 both alleles on (HaCat cells) (24), we exposed subconfluent and asynchronously growing cells to various doses of UVB. As shown in Fig. 1, UVB induces a dose-dependent accumulation of cells in G2/M. 100 mJ/cm^2 of UVB en-

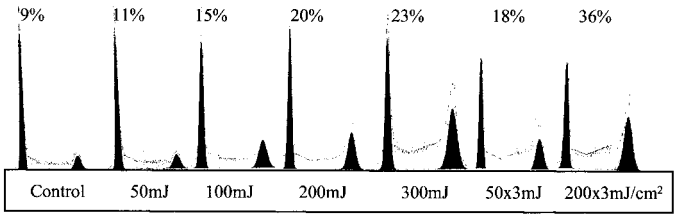

FIG. 1. DNA content flow cytometric histograms of control and UVB-irradiated HaCat cells. Cells were irradiated and harvested 24 h later. First peak represents cells in G1, second peak represents cells in G2/M and area between the peaks represents cells in S phase of cell cycle.

hanced G2/M population from 9% in untreated controls to 20%. Three consecutive exposures of 200 mJ/cm^2 further increased this number to 36% (Fig. 1).

UVB induces cyclin B1 accumulation but does not alter cdc2 protein levels. Since the cyclin B-cdc2 complex is a key regulator of entry into mitosis, we analyzed the effect of UVB on cyclin B1 and cdc2 proteins in these cells. As shown in Fig. 2, exposure to UVB results in modest accumulation of cyclinB1 protein in a dose-dependent fashion. The dose- and time-dependent effects of UVB on cdc2 levels are shown in Fig. 3. No significant alterations in cdc2 levels occurred at any dose or time point following UVB exposure.

UVB induces accumulation of hyperphosphorylated cdc2. The activity of the cyclinB1-cdc2 complex is regulated positively by phosphorylation at the threonine-161 residue of cdc2 and inactivating phosphorylation at threonine-14 and tyrosine-15 residues of the cdc2 subunit (15). As shown in Fig. 4 using specific antibodies recognizing exclusively cdc2 phosphorylated at tyrosine 15, UVB (200 mJ/cm^2) induces accumulation of tyrosine-15 hyperphosphorylated cdc2 complex in HaCat cells. Maximum accumulation occurs 6 h following exposure and returns to base line by 24 h.

UVB induces chk1 kinase and reduces cdc25C phosphatase levels. Cdc25C phosphatase cleaves inhibitory phosphates from the cyclinB1-cdc2 complex. This enzyme is inactivated by chk1 kinase-mediated phosphorylation at residue serine-216 (19). The effect of exposure of HaCat cells to UVB on chk1 kinase and cdc25C phosphatase levels is shown in Fig. 5. UVB dose-dependently enhances chk1 protein levels, while concomitantly reducing cdc25C phosphatase level by 9 h, which completely disappears by 15 h. Phosphorylation at serine-216 creates a recognition motif for

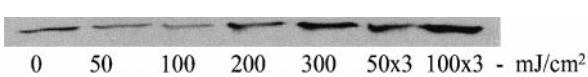

FIG. 2. UVB induces cyclin B1 in HaCat cells. Cells were exposed to various doses of UVB (50–300 mJ/cm^2) and harvested 24 h later.

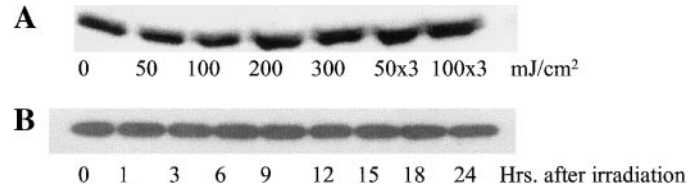

FIG. 3. UVB does not alter cdc2 protein levels in HaCat cells. (A) Cells were exposed to various doses of UVB (50–300 mJ/cm^2) and harvested 24 h later. (B) Cells were exposed to 50 mJ/cm^2 of UVB and harvested at different time points.

binding of 14-3-3σ, which was found to be virtually stable up to 24 h following UVB exposure (data not shown).

DISCUSSION

G2/M transition is regulated by cdc2 kinase, which is a heterodimeric complex of cdc2 and cyclin B proteins activated by threonine 161 phosphorylation (12). However, this complex is inactivated by threonine 14 and tyrosine 15 inhibitory phosphorylations (13). At G2/M transition, these inhibitory phosphates are removed by cdc25C phosphatase (15). G2 arrest is considered a period of repair and recovery following DNA damage. It has been shown that inhibitory phosphorylations are maintained during this period resulting in the accumulation of hyperphosphorylated cdc2/cyclin B complexes (13, 15). Our results show accumulation of hyperphosphorylated cdc2/cyclin B1 complex and consequent G2 arrest following UVB irradiation. Herzinger *et al.* observed a decrease in cdc2/cyclin B kinase activity in UVB exposed HaCat cells (25). In addition, we observed a modest increase in cyclin B1 but cdc2 remained constant suggesting that UVB might have a down regulating effect on its activating kinase or phosphatase. In contrast to our results with UVB in HaCat cells, exposure of HeLa cells which also lack functional p53 to IR, induces instability in cyclin B1 mRNA, decreases cyclin B1 protein level (12) and cdc2/cyclin B kinase activity leading to G2 arrest (12).

The kinase activity of the cyclin B-cdc2 complex is controlled by cdc25C phosphatase, which removes in-

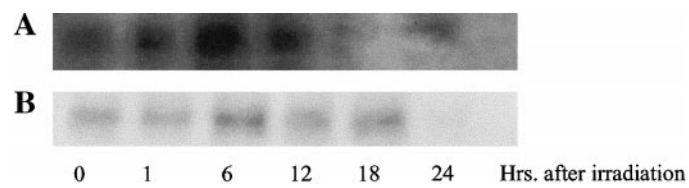

FIG. 4. UVB induces accumulation of hyperphosphorylated (tyr-15) cdc2 in HaCat cells. Cells were exposed to 200 mJ/cm^2 of UVB and harvested at different time points (0–24 h). (A) Cell lysate was immunoprecipitated with tyrosine-15 antibody and blotted with the cdc2 antibody. (B) Cell lysate was immunoprecipitated with cdc2 antibody and blotted with tyrosine-15 antibody.

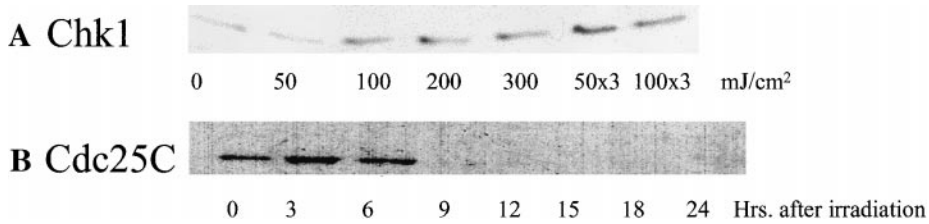

FIG. 5. UVB-induces chk1 and decreases cdc25C in HaCat cells. (A) Cells were exposed to various doses of UVB and harvested 24 h later. (B) Cells were exposed to 200 mJ/cm^2 of UVB and harvested at different time points.

hibitory phosphatases to activate the complex (15). We observed a substantial reduction in cdc25C level after UVB, which correlates with and may be responsible for the observed accumulation of tyrosine 15 phosphorylated cyclin B-cdc2 complex in these cells. Chk1 kinase phosphorylates cdc25C and inhibits its phosphatase activity (19). Concomitantly, we observed an increase in chk1 protein levels following UVB, which may further preserve cdc25C in a dormant state before its protein level is reduced. *Chk1* is an essential gene (26), which is induced by DNA damage as a result of the induction of its activating upstream kinase, ATR in both yeast and humans (27). Chk1 protein inactivates cdc25C, blocking the progression of cells through mitosis (28). Human chk1 can phosphorylate cdc25C, creating a site on this protein for binding with 14-3-3σ, which helps to sequester cdc25C in the cytosol (15, 27). Recently, it has been shown that UV and IR induce ubiquitin- and proteasome-dependent degradation of cdc25A, which also involves activation of chk1 protein kinase leading to the maintenance of inhibitory tyrosine-15 phosphorylation of cdk2 (28). The observed disappearance of cdc25c in the present study may also involve the ubiquitin- and proteasome-dependent degradation mechanism but it remains to be investigated.

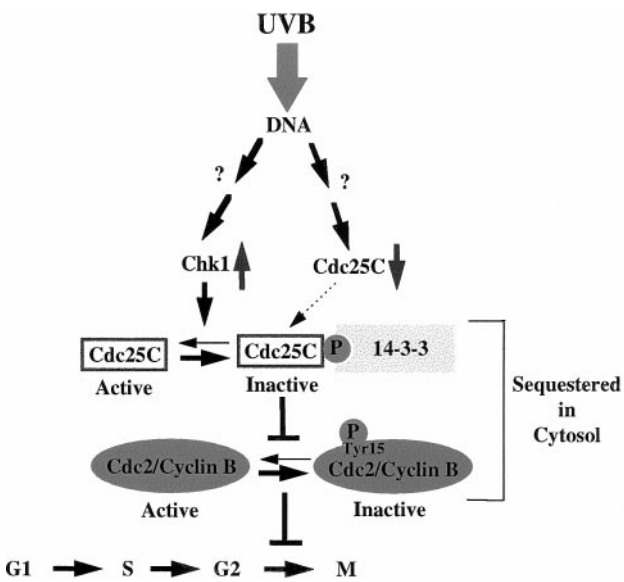

FIG. 6. Proposed mechanism for UVB-mediated G2/M arrest in HaCat cells.

In summary our results indicate that UVB induces G2/M arrest in HaCat cells by inactivating the cyclin B-cdc2 complex, which remains sequestered in cytoplasm as a result of chk1-mediated inactivation of cdc25C and its trapping by 14-3-3σ protein (Fig. 6). Since pharmacological interference with G2 arrest is known to increase the fraction of cells undergoing apoptosis following irradiation, the understanding of the mechanism of G2/M checkpoint may be helpful in designing more effective cancer preventive and therapeutic strategies.

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