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Restoration of UV sensitivity in UV-resistant HeLa cells by antisense-mediated depletion of damaged DNA-binding protein 2 (DDB2)

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Abstract Damaged DNA-binding activity comprises two major protein components, DDB1 and DDB2, which are implicated in the repair of ultraviolet (UV) radiation-induced DNA damage. The possible role of DDB2 as a determinant of cellular sensitivity to UV was investigated. The abundance of DDB2 in UV-resistant HeLa cell lines was increased compared with that in the parental UV-sensitive cells. Stable transfection of the resistant cells with DDB2 antisense cDNA resulted in marked depletion of DDB2 protein and restored cellular sensitivity to UV-induced apoptosis. Whereas the extent of UV-induced activation of apoptosis executioners, including DNA fragmentation factor, and caspase-3 were reduced in the UV-resistant cells compared with those apparent in the sensitive cells, depletion of DDB2 from the resistant cells restored the normal activation patterns for these proteins. In contrast, overexpressing DDB2 in DDB2-depleted cells with recombinant adenovirus, which carries ddb2 cDNA, markedly inhibited the extent of UV-induced activation of DNA fragmentation factor, and caspase-3. Interestingly, a mutated form of DDB2, which is defective in interacting with DDB1 and binding to UV-damaged DNA, also markedly inhibited the activation of apoptosis executioners. These results indicate that DDB2 is a modulator of UV-induced apoptosis, and that UV resistance can be overcome by inhibition of DDB2. The findings also suggest that modulation of UV-induced apoptosis by DDB2 may be independent of DNA repair. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Antisense; Apoptosis; DDB2; UV resistance

1. Introduction

Ultraviolet (UV) radiation induces apoptosis by activation of the Fas signaling pathway in a variety of cell types [1]. Multimerization of Fas at the cell surface induces the recruitment of the signaling molecules FADD and caspase-8 to the activated receptor, resulting in the formation of the death-inducing signaling complex (DISC) [2,3]. Oligomerization of caspase-8 within the DISC results in its autoactivation by proteolysis [4]; the activated protease then mediates the activation of other caspases, including caspase-3 [5,6], by proteolytic cleavage. These downstream caspases cleave the death substrates, including DNA fragmentation factor (DFF) [7,8], that are central to apoptotic events such as changes in cell morphology and DNA fragmentation [9].

Nucleotide excision repair is a versatile mechanism for the repair of damaged DNA that operates in organisms ranging from mycoplasma to mammals. The ability of a cell to recognize damaged DNA is crucial in nucleotide excision repair and is a key determinant of cell sensitivity to genotoxic agents, including UV radiation. Damaged DNA-binding (DDB) activity has been purified to apparent homogeneity from human placenta and HeLa cells and characterized [10–12], and is identical to a similar activity originally identified in human placenta [13]. In vitro reconstitution studies have indicated that DDB proteins stimulate nucleotide excision repair but that they are not essential for this process [14]. Cells derived from individuals with the inherited disorder xeroderma pigmentosum (XP) of complementation groups A–G exhibit a reduced capacity for nucleotide excision repair of damaged DNA [15], and DDB proteins have been implicated in the primary defect of XP-E. Thus, whereas DDB activity, as measured by electrophoretic mobility shift assay, is increased in UV-resistant cells [16–19], it is absent from or reduced in several XP-E cell lines [20–23]. Furthermore, microinjection of purified DDB proteins into XP-E cells corrected the defect in DNA repair [24,25]. However, only indirect evidence suggests that DDB activity contributes to DNA repair [16,17,19,21].

DDB activity was originally purified as a single ~127-kDa protein from monkey cells [26] and as a complex of ~127-kDa (DDB1) and ~48-kDa (DDB2) subunits from human cells [11,27]. The gene encoding DDB1 has been isolated from monkey [28] and human [29,30], and the protein has been proposed to function in the recognition of DNA damage during UV-induced nucleotide excision repair [31]. However, several studies have shown that DDB1 is not essential for this step [32]. The detection of mutations in the DDB2 gene in a subset of XP-E cells that exhibit reduced or a lack of DDB activity [27] suggested that DDB2 might contribute to nucleotide excision repair and to cell sensitivity to genotoxic stress. The human DDB2 cDNA has been cloned [29] and the DDB2 protein was recently shown to require DDB1 in order to recognize DNA damaged by UV radiation [33]. However, the role of DDB2 in determination of cellular sensitivity to UV irradiation is unknown. We have now shown that antisense cDNA-induced down-regulation of DDB2 expression over-

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Abbreviations: UV, ultraviolet; DFF, DNA fragmentation factor; DDB2, damaged DNA-binding protein 2; XP, xeroderma pigmentosum; DAPI, 4′,6-diamidino-2-phenylindole; PCR, polymerase chain reaction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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comes the acquired resistance of cultured cells to UV radiation.

2. Materials and methods

2.1. Cells and reagents

Antibodies to β-actin, to DFF, and to caspase-3 were obtained from Santa Cruz Biotechnology. 4′,6-Diamidino-2-phenylindole (DAPI) and other reagents were from Sigma, unless indicated otherwise. Human cervical carcinoma HeLa cells were originally obtained from American Type Culture Collection. UV-resistant HeLa-CPR variants were maintained as previously described [34].

2.2. Cloning of DDB2 cDNA

An 1820-bp fragment of human DDB2 cDNA containing the entire reading frame was isolated from a placental cDNA library (QuickClone cDNA; Clontech) with the use of the polymerase chain reaction (PCR) [35] and primers based on the sequence deposited in the GenBank database (GenBank U18300) [29]. Recognition sequences for *Sma*I and *Hin*dIII were added to the 5′ end of the forward primer and the 3′ end of the reverse primer, respectively, to facilitate plasmid construction. The PCR product was cloned into the pGEM-T Easy vector (Promega) to yield pGTddb2. The cDNA insert was sequenced by the dideoxynucleotide method [36] with the use of a T7 or SP6 primer complementary to a vector region immediately adjacent to the insert. The continuity of the open reading frame in pGTddb2 was also confirmed by in vitro transcription and translation with the TNT reticulocyte lysate system (Promega), as described [37].

2.3. Preparation of antibodies to DDB1 and to DDB2

For preparation of antibodies to DDB2, we digested pGTddb2 with *Sma*I and *Hin*dIII, and inserted the released DNA fragment in frame into pET15bDH, a modified version of pET15b (Invitrogen), to form pETddb2. The recombinant protein produced from pETddb2 in bacteria was purified by chromatography on a nickel-NTA column (Qiagen). For preparation of antibodies to DDB1, a human DDB1 cDNA spanning nucleotides 2386–3723 (EMBL database accession number AJ002955) and encoding a 381-residue fragment of the protein was isolated by PCR from human placenta. Recognition sequences for *Sma*I and *Hin*dIII were added to the 5′ end of the forward primer and the 3′ end of the reverse primer, respectively. The DDB1 cDNA was inserted in frame into pET15bDH to yield pETddb1, which was then introduced into bacteria. The recombinant protein was purified as for DDB2. Antibodies to DDB1 and to DDB2 were generated in New Zealand White rabbits as described [38].

2.4. Generation of DDB2 transgenic cell lines

To generate cell lines that produce DDB2 antisense RNA, we transfected HeLa-CPR cells with pcDNA3ddb2as, a modified form of pcDNA3 (Invitrogen) that contains the full-length human DDB2 cDNA in the antisense orientation, a neomycin resistance gene, and a green fluorescent protein gene. The transfected cells were subjected to selection with medium containing G418 (300 μg/ml) for 2 weeks, and those expressing green fluorescent protein were identified by fluorescence microscopy [39].

2.5. Recombinant adenovirus and infection

Replication-deficient recombinant adenoviruses containing human ddb2 were generated as previously described [40] according to the method of He et al. [41]. Human ddb2 sequence that contains the full length of the open reading frame was amplified by PCR according to the reported sequence ([29], EMBL database accession number U18300). A 1.3-kb *Not*I/*Hin*dIII wild-type ddb2 fragment from pcDNA 3.1-ddb2 was ligated with pAdTrack-CMV. A ddb2 mutant (XP82TO) [27] fragment was released by *Stu*I and *Not*I and was also ligated with pAdTrack-CMV. After restriction enzyme mapping, linearized pAdTrack-CMV-ddb2 was cotransformed with pAdEasy1 into BJ5183 competent bacteria to generate recombinant pAdEasy1-GFP- ddb2. Recombinant viruses were produced by transfecting linearized pAdEasy1-GFP-ddb2 into 293 cells using lipofectamine reagent (Gibco). For large-scale virus production, the recombinant viruses were subjected to CsCl gradient centrifugation. The titer of viral stocks was determined by spectrophotometer at 260 nm, and one OD_{260} represents approximately 10^{12} pfu/ml.

Cells growing in log phase were infected with either empty or ddb2 adenovirus. After 30 h, cells were left untreated or treated with various doses of UV for 24 h. Cells were washed with phosphate-buffered saline and stained with DAPI to analyze the morphology and percentage of apoptotic cells. Samples were analyzed under a microscope. There were 50–60% cells infected by the virus (multiplicity of infection (MOI), 500) as shown by green fluorescence.

2.6. Preparation of cell extracts and immunoblot analysis

Cells were washed twice with phosphate-buffered saline and lysed by incubation on ice for 30 min in 1 ml of modified radioimmunoprecipitation buffer (50 mM Tris–HCl pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF, and aprotinin, leupeptin, and pepstatin each at 1 μg/ml). Insoluble material was removed by centrifugation using an Eppendorf centrifuge at 12 000 rpm for 10 min at 4°C, and the resulting supernatant was saved as cell extract. Protein concentration was measured by Bradford assay with the Bio-Rad dye reagent [42].

For immunoblot analysis, equivalent amounts of protein from each sample were fractionated by SDS–PAGE, and the separated proteins were transferred to a polyvinylidene difluoride membrane and exposed to antibodies. Immune complexes were detected with enhanced chemiluminescence reagents (Pierce).

2.7. Analysis of viability and apoptosis

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay [43]. In brief, cells were transferred to 96-well plates in a volume of 100 μl and, after 12 h, exposed to UV at 37°C. The number of surviving cells was determined after 72 h by their ability to convert the tetrazolium salt of MTT into a formazan product. For assessment of apoptosis, cells growing in six-well plates were exposed to UV, incubated for 24 h at 37°C, fixed with methanol, and incubated for 30 min in the dark with DAPI (2 μg/ml). Floating cells from each well were also fixed and added back to the respective wells. Cells were then examined with a fluorescence microscope at a wavelength of 420 nm, and those exhibiting morphological features of apoptosis, including chromatin condensation and nuclear fragmentation [44], were counted in six to eight randomly selected fields. Five hundred nuclei were examined for each sample, and the percentage of cells with apoptotic nuclei was determined in three independent experiments.

3. Results

3.1. Abundance of DDB2 in UV-resistant HeLa cells

UV-induced viability of sensitive HeLa, and its resistant cell lines (R1, R2, and R3), originally selected with increasing concentrations of cisplatin, was determined (Fig. 1A). Interestingly, cisplatin-selected cells are cross-resistant to UV. UV-induced apoptotic resistance in the resistant cell lines (R1, R2, and R3) was also determined (Fig. 1B). We previously showed that UV-resistant HeLa cells exhibited increased DDB activity and enhanced DNA repair compared with the parental cells [17,45]. Given the importance of DDB2 in DDB activity and DNA repair, we investigated the role of this protein in determination of UV sensitivity or resistance. With the use of immunoblot analysis, we examined the abundance of DDB1 and DDB2 in the crude extracts of these cells. Both DDB1 and DDB2 levels were increased in resistant cells, with most abundance in R3 cells (Fig. 1C).

3.2. Effect of DDB2 depletion on acquired UV resistance

Although both DDB1 and DDB2 are required for recognition of UV-induced DNA damage [46], mutation in DDB2 is associated with a defect in recognition and repair of UV-induced DNA damage [47]. DDB2 might be a determinant of UV sensitivity. This possibility was investigated further by stable transfection of R3 cells with a vector encoding DDB2

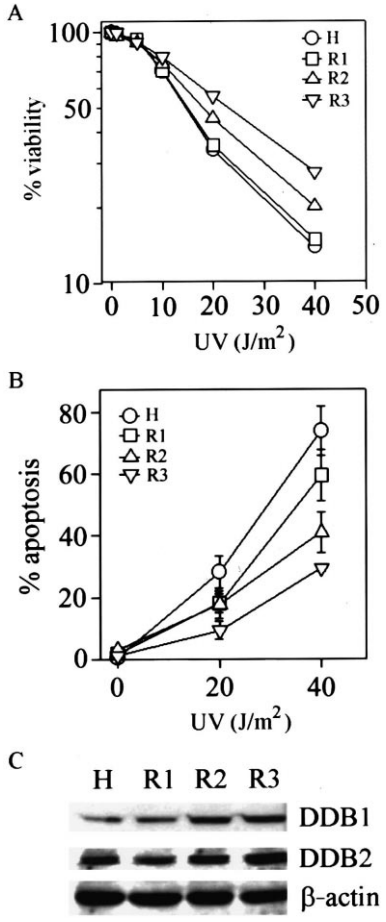

Fig. 1. Overexpression of DDB2 and apoptotic resistance in UV-resistant HeLa cell lines. A: Cell viability to UV treatment. HeLa (H), UV-resistant HeLa (R1, R2, R3) cells were exposed to the indicated doses of UV, and, after incubation for 72 h, cytotoxicity was assessed by the MTT assay. B: Apoptotic resistance in UV-resistant cells. Data are expressed as percentage survival relative to the survival of untreated cells, and are means ± S.D. of values from three independent experiments. C: Overexpression of DDB2 in UV-resistant cells. Cell extracts (50 μg of protein) of HeLa (H) cells, UV-resistant HeLa cells (R1, R2, and R3) were subjected to immunoblot analysis with antibodies to DDB1, to DDB2, or to β-actin.

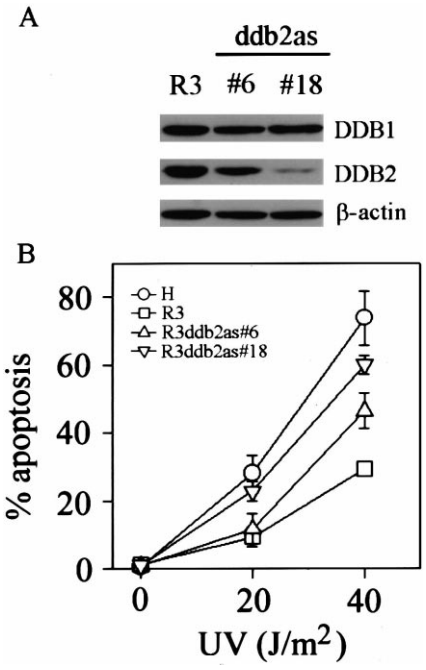

Fig. 2. Effect of DDB2 antisense cDNA expression on the abundance of DDB2 and on UV-induced apoptosis in UV-resistant HeLa cells. A: Effect of DDB2 antisense cDNA expression on the abundance of DDB2. Cell extracts (50 μg of protein) of UV-resistant HeLa (R3) cells, and R3 cells stably transfected with DDB2 antisense cDNA (HRddb2as #6, #18 cells) were subjected to immunoblot analysis with antibodies to DDB1, to DDB2, or to β-actin. B: Effect of DDB2 depletion on UV-induced apoptosis. The percentage of apoptotic cells was determined by DAPI staining 24 h after exposure to the indicated doses of UV. Data are means ± S.D. of values from three independent experiments.

antisense RNA. In typical resulting cell lines, R3ddb2as #6 and #18, the abundance of DDB2 was reduced (Fig. 2A). We next examined the effects of depletion of DDB2 on UV-induced apoptosis in the UV-resistant cells. Exposure of both sensitive and resistant HeLa cells to UV induced a dose-dependent increase in the percentage of apoptotic cells in both DDB2 knockdown cell lines (Fig. 2B). Depleting DDB2 by antisense cDNA expression restored the sensitivity of R3ddb2as #18 cells to apoptosis-inducing effects of UV to levels similar to those apparent for sensitive HeLa cells.

These observations were confirmed by immunoblot analysis of the activation of caspase-3 and DFF, which is required for fragmentation of chromosomal DNA during apoptosis. Activation of DFF is mediated by caspase-3 and caspase-7 in vitro [48]. Exposure of sensitive HeLa cells to UV at a dose of 40 J/m^2 induced a marked decrease in the abundance of the caspase-3 and DFF precursors (Fig. 3); this effect was dose-dependent (data not shown) and is indicative of the proteolytic cleavage of this protein to yield active caspase-3 and DFF. Whereas the extent of UV-induced activation of DFF was greatly reduced in R3 cells, depletion of DDB2 from these cells (R3ddb2as #6 and #18) restored the UV sensitivity of caspase-3 and DFF activation to a level similar to that of control HeLa cells. The average levels of corresponding apoptosis are also indicated. These results thus suggested that

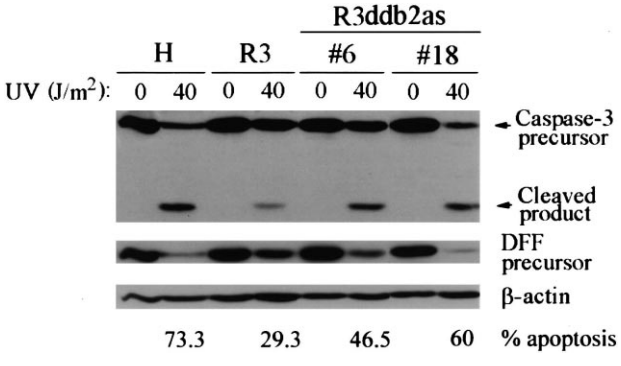

Fig. 3. Depleting DDB2 sensitizes UV-induced activation of caspase-3 and DFF. HeLa (H), UV-resistant HeLa (R3), and DDB2-depleted R3 (R3ddb2as #6, #18) cells were exposed or not to UV (40 J/m^2) and, after 24 h, were lysed. Cell extracts (50 μg of protein) were then subjected to immunoblot analysis with antibodies to caspase-3, to DFF, or to β-actin. The percentage of apoptotic cells, shown below, was determined by DAPI staining 24 h after exposure to the indicated doses of UV. Data are means ± S.D. of values from three independent experiments.

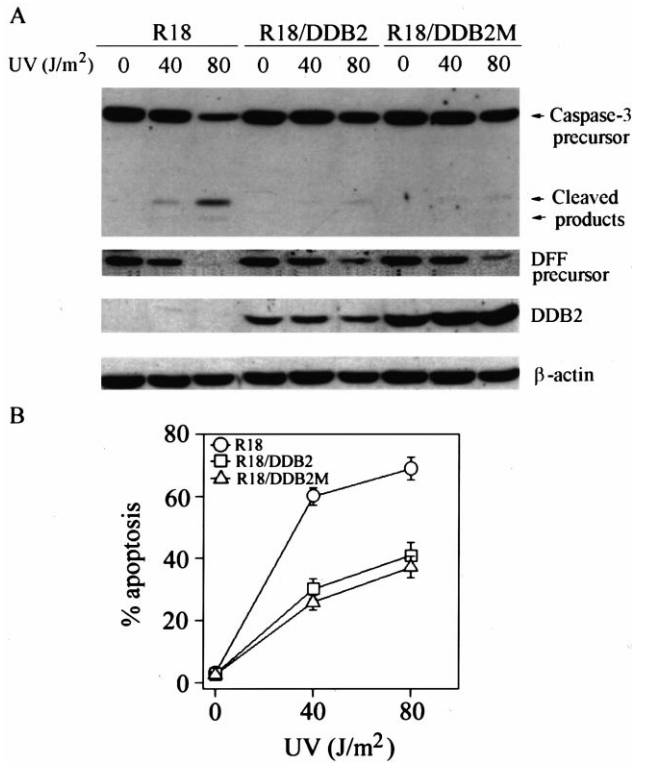

Fig. 4. Overexpressing DDB2 inhibits UV-induced activation of caspase-3 and apoptosis in DDB2-depleted cells. A: Inhibition of activation of caspase-3 and apoptosis by overexpressing DDB2. R3ddb2as #18 (R18) and R18/DDB2 or R18/DDB2M cells (which overexpress human DDB2 or DDB2 mutant) were exposed to the indicated doses of UV and then incubated for 24 h. Cell extracts (50 μg of protein) were subjected to immunoblot analysis with antibodies to caspase-3, to DFF, or to β-actin. B: Inhibition of UV-induced apoptosis by overexpressing DDB2. The percentage of apoptotic cells was determined by DAPI staining 24 h after exposure to the indicated doses of UV. Data are means ± S.D. of values from three independent experiments.

DDB2 overexpression might protect against UV-induced apoptosis.

3.3. Effects of DDB2 overexpression on UV-induced apoptosis

The potentiation of UV-induced apoptosis in R3 cells by depleting DDB2 suggested that DDB2 modulates these phenomena. To test this hypothesis further, we examined the effects of DDB2 overexpression in DDB2-depleted cells (R3ddb2as #18, or R18), in which DDB2 is nearly undetectable. We introduced recombinant adenoviruses, which carry the ddb2 gene, into these cells. Exposure of R18 cells to UV at a dose of 80 J/m^2 induced a marked decrease in the abundance of the caspase-3 and DFF precursors (Fig. 4A), whereas the extent of UV-induced activation of caspase-3 and DFF was greatly reduced in DDB2-overexpressing cells (R18/DDB2). Interestingly, overexpressing DDB2 mutant (R18/DDB2M) also greatly reduced UV-induced activation of caspases-3 and DFF. The extent of UV-induced apoptosis in these cells was reduced relative to that apparent in R18 cells (Fig. 4B).

4. Discussion

We have shown that the abundance of DDB2 is increased in UV-resistant HeLa cell lines compared with that in the parental cell line, and that depletion of DDB2 from resistant cells by expression of DDB2 antisense cDNA restored their sensitivities to UV-induced apoptosis to the original values. Our results therefore suggest that overexpression of DDB2 may contribute to the acquired UV resistance of HeLa cells. This conclusion was further supported by our observation that overexpression of recombinant DDB2 in DDB2-depleted cells protected these cells from UV-induced apoptosis. Depletion of DDB2 from R3 cells did not affect apoptosis induced by cisplatin or mitomycin C (data not shown), suggesting that modulation of apoptosis by DDB2 may be stimulus-specific.

DFF, a heterodimeric protein composed of 45-kDa catalytic (DFF45) and 40-kDa regulatory (DFF40) subunits, mediates fragmentation of nuclear DNA and chromatin condensation in apoptotic cells [8,48]. The extent of UV-induced activation of DFF in resistant R3 cells was reduced compared with that apparent in UV-sensitive HeLa cells. DFF is activated by caspases-3 and -7 in vitro [49], and activation of these caspases in the cytosol appears to be a critical step in apoptosis in mammalian cells [6,50]. The extent of UV-induced activation of caspase-3 in resistant cells was also reduced compared with that observed in UV-sensitive HeLa cells. Depletion of DDB2 from resistant R3 cells restored the extents of UV-induced activation of caspase-3 and DFF to levels similar to those apparent in UV-sensitive HeLa cells. Our results thus support the notion that various apoptotic stimuli, including UV, activate caspase-3 and thereby induce cleavage of apoptotic substrates such as DFF [6,48,51,52], and that impaired activation of caspase-3 contributes to the development of cellular resistance to UV in HeLa cells. These results also suggest that DDB2 may be a regulator for the activation of executioner caspases during UV-induced apoptosis.

Microinjection of purified DDB proteins into XP-E cells corrects the defect in DNA repair [24,25], suggesting that these proteins may play a specific role in the repair of chromosomal DNA. In the present study, depletion of DDB2 from UV-resistant HeLa cells, which exhibit enhanced DNA repair [17,46], increased the sensitivity of the cells to UV-induced apoptosis. Overexpression of DDB2 has also been shown to potentiate global genomic repair in hamster cells [47]. These observations further suggest that DDB2 functions in the repair of UV-induced DNA damage, and that the intracellular concentration of this protein is an important determinant of acquired UV resistance. The importance of DDB2 in the UV response is also supported by the observation that a subset of XP-E cells that expressed DDB1 but in which the DDB2 gene was mutated did not exhibit DDB activity [27]. Interestingly, overexpression of DDB2 mutant, which failed to interact with DDB1 and to induce DDB activity, in DDB2-depleted HeLa (R18) cells also inhibits UV-induced activation of caspase-3 and DFF. These results suggest that regulation of UV-induced apoptosis by DDB2 may be independent of DNA repair. Although a recent study also showed that overexpression of DDB2 by stable transfection in hamster V79 cells increased DDB activity [47], DDB2 overexpression did not protect the cells from UV cytotoxicity as measured by a colony formation assay. In contrast, we showed that overexpression of DDB2 protected HeLa cells from UV-induced apoptosis. Since apoptosis appears to be a major cell death pathway after UV, this apparent discrepancy is likely due to the difference between the cell lines.

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