Sequential binding of UV DNA damage binding factor and degradation of the p48 subunit as early events after UV irradiation

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ABSTRACT

The UV-damaged DNA binding protein complex (UV-DDB) is implicated in global genomic nucleotide excision repair (NER) in mammalian cells. The complex consists of a heterodimer of p127 and p48. UV-DDB is defective in one complementation group (XP-E) of the heritable, skin cancer-prone disorder xeroderma pigmentosum. Upon UV irradiation of primate cells, UV-DDB associates tightly with chromatin, concomitant with the loss of extractable binding activity. We report here that an early event after UV, but not ionizing, radiation is the transient dose-dependent degradation of the small subunit, p48. Treatment of human cells with the proteasomal inhibitor NIP-L3VS blocks this UV-induced degradation of p48. In XP-E cell lines with impaired UV-DDB binding, p48 is resistant to degradation. UV-mediated degradation of p48 occurs independently of the expression of p53 and the cell’s proficiency for NER, but recovery of p48 levels at later times (12 h and thereafter) is dependent upon the capacity of the cell to repair non-transcribed DNA. In addition, we find that the p127 subunit of UV-DDB binds in vivo to p300, a histone acetyltransferase. The data support a functional connection between UV-DDB binding activity, proteasomal degradation of p48 and chromatin remodeling during early steps of NER.

INTRODUCTION

Nucleotide excision repair (NER) is the major process responsible for the removal of structurally diverse lesions from DNA. In human cells, a major function of NER is the removal of UV-induced DNA photoproducts caused by sunlight. In recent years, the general mechanism of NER in humans has been uncovered, primarily based upon analyses of NER-defective individuals with the inherited skin cancer-prone disorder xeroderma pigmentosum (XP). Cell fusion studies have revealed seven complementation groups in XP, XP-A–XP-G, implicating all of the corresponding genes in NER (1–3). The genes and associated proteins for each of the XP groups have been isolated and their roles assigned to the recognition or incision phases of NER (4,5). However, the molecular function remains unknown for the UV DNA damage binding protein complex (UV-DDB) which, when defective, yields the XP-E phenotype (3,6). XP-E is the mildest and one of the least common forms of the disorder. The cells from XP-E patients have a partial deficiency in NER (7–10), but only a subset of putative XP-E cell lines lack UV-DDB binding activity (8,11). UV-DDB is the only readily detectable damage binding activity in crude extracts of mammalian cells due to its much greater molar affinity for UV-damaged DNA than two other damage recognition proteins, XPC-HR23B and XPA (12,13). The UV-DDB protein complex comprises two subunits of 127 and 48 kDa (p127 and p48, also known as DDB1 and DDB2) (3,14,15), and it appears that the DNA binding activity resides only in the heterodimeric complex (15–17).

Despite a particular affinity for UV-induced photoproducts (15,18,19) and some DNA lesions caused by other agents (20), UV-DDB seems to have only an accessory role in NER in vivo. The repair of UV-damaged, chromatin-free DNA has been reconstituted in vitro in the absence of UV-DDB (21,22) and the addition of the purified protein stimulates repair synthesis only 2-fold (21). However, microinjection of UV-DDB protein into XP-E cells lacking the DNA binding activity does complement NER activity in vivo (8,23), indicating an important function for this factor in vivo and suggesting a key role for UV-DDB when DNA damage is present in a chromatin context. In support of this notion, UV irradiation induces a tight association of UV-DDB with chromatin in mammalian cells. A similar pattern was observed for RPA, a protein involved in the formation of the NER incision complex (8). Recent investigations have found interactions between UV-DDB (as a complex or as p127 alone) and human or viral proteins likely to be involved in transcriptional regulation. This implies an association of UV-DDB with the transcriptional machinery as well as DNA repair, perhaps facilitating
chromatin remodeling at the site of the specific DNA damage (24–27).

Sequence changes have been found in the p48 gene of XP-E patients, but no mutations have been found in the p127 gene of any XP-E cell lines examined to date (16,28,29). Several lines of evidence suggest that p48 is the regulatory factor of the UV-DDB complex. Mutations in the p48 subunit are linked with the loss of UV-DDB binding activity and appear to cause the repair-deficient phenotype in humans with XP-E (6,8,16).

A similar phenotype is detected in hamster cells, which normally express p127 but have no detectable p48. Transfection of hamster cells with human wild-type, but not mutated, p48 cDNA restores the binding activity in hamster cells to a level comparable with that in normal human cells (30). Furthermore, the XP2RO cell line carries an amino acid change, R273H, in the p48 subunit, which impairs the formation of a stable complex with p127 (31). Finally, three XP-E cell lines mutant for p48 are deficient in carrying out the nuclear localization of its complex with p127 (31). Recently it has been shown that p53 can regulate the UV-mediated induction of DDB2 mRNA, suggesting that p53 can promote NER via UV-DDB activity (10). However, in normal fibroblasts the maximum increases in DDB2 transcript, p48 protein and UV-DDB activity were detected 48 h after UV irradiation, after NER is almost completed (16). In our earlier work (33,34), we reported that mammalian cells show an early dose-dependent inhibition of extractable UV-DDB activity after treatment with UV light. Although all p127 could be recovered through fractionation of the nuclei of irradiated cells, UV-DDB activity was inhibited (9). We found, moreover, that after initial inhibition, UV-DDB activity in primate cells was UV-inducible and dependent on de novo protein synthesis (35). Thus, in view of this apparently complex regulation, our goal was to investigate the fate of the p48 subunit and how it is regulated in normal and repair-deficient cells following UV irradiation. Specific anti-p48 antibodies were generated to measure protein levels. We also examined the interaction of UV-DDB with the histone acetyltransferase (HAT) p300 in normal human cells compared with XP-E cells, to gain clues as to the true function of UV-DDB during NER in the context of chromatinized DNA.

MATERIALS AND METHODS

Cell culture

Monolayer cultures of TC-7 cells, a clone of the African green monkey kidney cell line CV-1, were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). The same medium with 15% FCS and MEM amino acids was used for cultivation of primary and SV40-transformed human fibroblasts from normal and DNA repair-deficient individuals. The lymphoblastoid cell lines GM01953, GM01646 and XP23PV were maintained in RPMI 1640 medium supplemented with 15% FCS. TR9-7 cells were derived from spontaneously immortalized p53−/− human fibroblasts (MDAHO41/041) and contain a stably transfected tetracycline-regulated system for the expression of wild-type p53. This cell line was continuously grown in DMEM containing 10% FCS, G148 (600 µg/ml), hygromycin B (50 µg/ml) and, when needed, the indicated amount of tetracycline. All cells were kept at 37°C in a humidified atmosphere with 5% CO2. The cell lines and strains used in this study were obtained from Coriell Cell Repositories and as indicated previously (8,9). A proteasome inhibitor, NIP-L3VS (carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone) (Calbiochem), was reconstituted in dimethyl sulfoxide and control cells were treated with an equal volume of solvent.

UV and ionizing irradiation (IR) treatment

All cell lines, except the lymphoblastoid cells, were grown in 150 mm Petri dishes to a subconfluent level, prior to UVC irradiation (254 nm) at the indicated dose. The dose intensity, emitted from a 15 W germicidal lamp, was measured using a Spectrolite model DR UV meter (Spectronics, Westbury, NY). With the exception of the samples marked as 0 time after UV or mock treatment, which were harvested within 10 min after irradiation, all other cells were incubated further for the indicated times before harvesting. The cells from two to four plates were harvested at each time point. The lymphoblastoid cell line was exposed to IR from a 137Cs source with total doses of 2, 5 and 15 Gy (fluxes of 1 or 5 Gy/min) and harvested at the same time points as cells irradiated with UV.

Preparation of antibodies

Two types of p48 antibodies were produced. One antibody (GPP48) was made by immunization of guinea pigs with the Escherichia coli expressed, his-tagged, full-length p48 purified with a Ni–NTA protein purification system (Qiagen) and by SDS–PAGE. GPP48 antiserum was further affinity purified (13) and concentrated with a Microset 10 apparatus (Filtron Technology Corp.). The other antibody (p48N) was prepared against a synthetic peptide encompassing residues 5–24 of human p48 (NH2-KRPETKSEIVLRPRNKR-COOH) in rabbits by Covance Research Products (VA). p48N antibody was affinity purified using a column of synthetic peptide coupled to AminoLink Plus Gel (Pierce) and concentrated as described above.

Immunoblotting and immunoprecipitation

Whole cell extracts as well as cytosol and nuclear extracts, from UV-, IR- or mock-irradiated cells harvested at the indicated times, were prepared as described (8,34). Briefly, whole cell extract was made by resuspending the cells, harvested by scraping, in lysis buffer [20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM DTT and a protease inhibitor cocktail (Complete; Boehringer)], freeze-thawed five times and incubated on ice with a magnetic stirrer for 30 min. The supernatant, obtained after centrifugation, was used as the whole cell extract (wce).

For nuclear fractionation, GM01953 cells were harvested 10 min and 4 h after UV irradiation or mock treatment. Nuclei were prepared from 3–5 × 10⁶ cells and a sequential extraction procedure applied according to the method of Humphrey et al. (36), with the indicated modifications. For preparation of nuclei, cells were washed twice in phosphate-buffered saline (PBS), resuspended in hypotonic buffer (20 mM MES, pH 6.5, 10 mM NaCl, 1.5 mM MgCl2 and 1 mM DTT) and lysed by Dounce homogenization. The nuclear pellet was washed in isotonic sucrose buffer (STM) (250 mM sucrose, 50 mM Tris pH 7.4, 5 mM MgCl2 and 1 mM DTT), yielding the STM wash, and the nuclear envelope was removed by addition of 1%...
Triton X-100, yielding the Triton wash (TW). The nuclear pellet was further extracted with low salt buffer (10 mM Tris–HCl, pH 7.4, 0.2 mM MgCl₂ and 1 mM DTT) and consecutively with increasing concentrations of NaCl in the same buffer. The resulting supernatants are designated low salt (LS), 0.3, 0.5 and 2.0 M. All buffers contained a protease inhibitor cocktail (Complete; Boehringer).

The levels of p127, p48, p53, actin and lamin B were analyzed by immunoblotting with chemiluminescent detection (Tropix, Bedford, MA) using anti-p127 (37), GPP48, anti-p53, anti-actin and anti-lamin B (Oncogen Science). Proteins were separated on 10% SDS–PAGE gels and transferred to Immobilon-P membranes. The membranes were incubated overnight with primary antibodies (diluted 1:1000) and for 30 min with the corresponding alkaline phosphatase-conjugated secondary antibodies (diluted 1:10 000). The chemiluminescent signal was captured by BioMax-light film (Kodak) and a Chemi-Doc System (Bio-Rad).

For immunoprecipitation, TC-7 cells were lysed directly on the plates in NP-40 buffer (50 mM Tris–HCl, pH 8, 100 mM NaCl, 1% Nonident P-40, 5 mM MgCl₂, 1 mM Na₂VO₄, 1 mM NaF and protease inhibitor cocktail) for 1 h on ice and centrifuged at 16 000 g for 10 min at 4°C. The supernatants were precleared with protein A–Sepharose beads (Pharmacia Biotech) and subjected to immunoprecipitation with GPP48 as described (9).

For co-immunoprecipitation of UV-DDB and p300, the lymphoblastoid cells were lysed in buffer consisting of PBS containing 10 mM CHAPS, 1 mM Na₂VO₄, 1 mM NaF and protease inhibitor cocktail for 1 h on ice and centrifuged at 16 000 g for 10 min at 4°C (38). The supernatants were incubated with anti-p300 antibody (Pharminogen) for 1 h at 4°C, before adding 40 μl of protein G–agarose (Gibco BRL). After 1 h at 4°C, the immunoprecipitated complexes were washed and bound proteins were analyzed by western blotting, probing for both subunits of UV-DDB.

### DNA binding assay

The band shift assay of UV-DDB activity was carried out with a UV-irradiated double-stranded 60mer DNA oligonucleotide (60/54), and free and bound complexes were separated on a 6% non-denaturing polyacrylamide gel as described previously (35,39).

### Northern blotting

mRNA was prepared from ∼1 × 10⁷ mock- or UV-irradiated TC-7 cells following the protocol of the QuickPrep mRNA purification kit (Pharmacia Biotech). mRNAs were separated on a denaturing gel and transferred to a GeneScreen Plus membrane (NEN Research Products). Northern blots were hybridized with random primed probes containing the whole cDNA for DDB2 and the coding region of the first 1630 nt for DDB1. A probe for actin was purchased from Clontech (Palo Alto, CA). After overnight hybridization at 42°C in Hybrisol I buffer (Oncor), membranes were washed twice in 2× SSC at room temperature for 15 min and subsequently twice in 2× SSC, 2% SDS at 65°C for 45 min. More stringent conditions were obtained by washing the blots in 0.1× SSC at 65°C for 15–30 min.

### RESULTS

**Tight association with chromatin and a dose-dependent reduction in p48 protein as early events after UV irradiation**

Soon after exposure to UV light, monkey kidney TC-7 cells and human fibroblasts show a reduction in extractable UV-DDB binding activity, concomitant with translocation of the p127 protein to a tight chromatin association (9). We found in a previous study that all p127 could be recovered through fractionation of the nuclei of irradiated cells, but that extractable UV-DDB activity remained inhibited. The total UV-DDB binding activity in irradiated cells was only ~20% of that found in unirradiated cells, and that was retained in high salt fractions. The majority of binding activity (~80%), which in control cells was associated with the low and 0.3 M salt fractions, was somehow lost. Thus, translocation and complexing of p48 with chromatin after irradiation could not solely account for the disappearance of binding activity.

To address the role of the p48 subunit in this phenomenon and to clarify the issue further, here we have used lymphoblastoid cells, grown in suspension and irradiated collectively, instead of a large number of plates with monkey kidney cells or human fibroblasts. We also modified the method for fractionation, replacing overnight DNase I treatment of isolated nuclei with 1% Triton X-100 detergent extraction (36), which enabled faster processing of the samples. We assayed the distribution of p48 in the nuclei from human GM01953 cells collected 10 min and 4 h post-UV (12 J/m²), utilizing specific anti-p48 antibodies that we generated. Ten minutes after treatment, p48 associated more tightly with chromatin (the 2 M salt fraction of irradiated cells contained 58% of total nuclear p48 versus 17% in mock-treated cells) and was almost completely depleted from fractions loosely associated with chromatin (TW and LS) (Fig. 1A and B). The total amounts of p48 detected in nuclear fractions 10 min and 4 h after UV were 52 and 25% of the level in mock-treated cells, respectively, strongly suggesting UV-induced loss of p48. As shown for TC-7 cells, there was only a small increase in p127 associated with the 2 M salt fraction immediately after irradiation (23% in irradiated versus 14% in control nuclei). After 4 h, when p48 levels were low, 33% of total p127 protein was extracted in the detergent wash. A progressive loss of immunodetectable p48 within 4 h after UV (Fig. 1A and B) correlated with the UV-DDB binding activity detected in the same fractions (not shown). Thus, a reduction in the amount of p48, and not the formation of an unextractable complex with the damaged DNA, appeared to be causing the disappearance of binding activity.

To examine whether UV-induced reduction of p48 was dose dependent, as was shown for UV-DDB binding activity (33), we sought to detect p48 in TC-7 cells irradiated with different doses of UV immediately after treatment. We utilized immunoprecipitation as a method of extraction of p48 from irradiated cells. Even a low dose (6 J/m²) triggered a strong response, decreasing the amount of p48 to 61% of the level in unirradiated cells, and with a higher dose (18 J/m²) to 19% (Fig. 1C).
UV but not ionizing irradiation transiently decreases the level of p48

It has been well established by Nichols et al. (16) that in normal fibroblasts the level of p48 is transcriptionally induced 38 h after UV treatment. This previous work focused on the late effect of UV irradiation on UV-DDB. Considering the result above, we chose to further explore the early response of UV-DDB to UVC in a whole cell extract from the TC-7 cell line, which was used extensively in our initial study. We were particularly interested in how long after irradiation depletion of p48 lasts and how this correlates with UV-DDB binding activity and with the level of p53, which has been implicated in UV-mediated induction of DDB2 mRNA (30). A whole cell extract, obtained with 0.42 M salt lysis buffer, was used for this analysis, because the majority of the p48 and binding activity, which in control cells was associated with the low and 0.3 M salt nuclear fractions, was missing in corresponding fractions of irradiated cells (Fig. 1A and B) due to a tight association with chromatin and a concomitant reduction in the amount of p48.

Cells exposed to UV and corresponding mock controls were harvested at the indicated time points within the course of 48 h and the levels of UV-DDB subunits in whole cell extracts were measured by immunodetection (Fig. 2A). In the samples collected during the first 6 h after irradiation, p48 was undetectable. There followed a period of recovery, and by 24 h the level of p48 in irradiated cells reached the basal level of mock controls. At 48 h this level had increased 10–12-fold over the level in unirradiated cells, marking the induction phase. In contrast to the dramatic changes which occurred in expression of p48, the level of the p127 subunit remained constant.

As found in human fibroblasts (30), p53 levels begin increasing 3 h after UV in monkey TC-7 cells (Fig. 2A). The basal level of p53 was almost undetectable in mock-treated cells. The highest level of p53 accumulation was reached between 18 and 24 h, and was substantially unchanged in later hours.

To see further if the dramatic UV-induced changes in p48 (Fig. 2A) were associated with changes in cellular localization of both proteins, we examined the distribution of p127 and p48 between cytosol and nucleus following irradiation. As shown for human cells (32), we found that in unirradiated TC-7 cells, p127 was predominantly localized in the cytosol fraction, whereas all p48 was in the nuclear fraction. The profile of p48 immunodetected in the nuclear fractions collected after irradiation (Fig. 2B) was similar to the profile in wce (Fig. 2A). UV-induced depletion of p48 was seen in the first 6 h, with a decrease in nuclear content of p127. Later, induction of p48 took place, and p127 translocated to the nucleus. Moreover, at 48 h after UV, the amount of p127 detected in the nuclear fraction was significantly elevated (Fig. 2B).

The same cytosol and nuclear fractions that had been examined for UV-DDB protein expression following irradiation were further tested for UV-DDB binding activity in a band shift assay. As observed earlier (7), only nuclear fractions harbored UV-DDB binding activity (Fig. 2C). The UV-DDB binding activity correlated with the p48 level in the same nuclear fractions, confirming that the loss of UV-DDB activity was the result of UV-mediated depletion of p48.

The results above (Figs 1 and 2A and B) demonstrated that UV irradiation transiently decreased the level of p48 and binding activity (Fig. 2C). We wondered if this was unique to UV or if it was a general early response to any damaging agent. Recent information suggests that even a very low level of IR induced DDB2 mRNA in a p53-dependent manner (10,40). To assess how IR affects the protein level of p48, the lymphoblastoid cell line was exposed to doses of 2, 5 and 15 Gy and harvested at the same time points as cells irradiated with UV. The levels of p48, p127 and p53 in extracts were analyzed by immunoblotting. In contrast to UV treatment, the exposure of the lymphoblastoid cells to IR did not change the protein level of p48 for the first 3–6 h post-irradiation (15 Gy is shown in Fig. 2D). At later times (24 h and thereafter), only a modest induction of p48 was detected (2, 5 and 15 Gy induced 1.2-, 1.4- and 2.3-fold higher levels of protein than in unirradiated samples, respectively; not shown and Fig. 2D). The IR induction
of p48 was accompanied by an ∼2-fold increase in binding activity (not shown). The level of p53 was dose dependent, with the peak between 6 and 12 h after IR, while the level of p127 was nearly constant throughout the entire time course (Fig. 2D).

**Early suppression of p48 protein is not transcriptionally controlled**

The very prompt, dose-dependent decrease in p48 suggested UV-induced degradation of this UV-DDB subunit (Figs 1 and 2), as shown for several other proteins (41,42), rather than a transcriptionally controlled event. To define the mechanism by which UV induces down-regulation of p48, we measured DDB2 mRNA as a function of post-exposure incubation time in TC-7 cells. DDB2 mRNA is low in abundance in human cells and in cells of other species (30) and isolation of mRNA for northern detection is necessary. In contrast to the protein level, a dose of 10 J/m² did not cause a decrease in DDB2 mRNA during the first 6 h post-irradiation (Fig. 3), as also seen in human cells (10,16). DDB2 mRNA induction at
later times (after 24 h) correlated with the increase in p48 protein and binding activity (Fig. 2A and C). DDB1 mRNA, as was true for p127 protein, was substantially unaffected by UV.

The effect of a proteasome inhibitor on p48 degradation
We next considered the possibility that the UV-induced decrease in p48 might be regulated through ubiquitin–proteasome degradation (43). In support of this possibility, it has been shown recently that overexpression of cullin 4A (Cul-4A), a ubiquitin ligase component, results in ubiquitination of p48 (44). We studied the effect of the proteasome inhibitor NIP-L3VS (45) on p48 degradation in normal human lymphoblastoid cells (GM01953). These cells had a higher content of p48 per mg cell extract protein than normal human fibroblasts (NHF) (GM05757). The intensity of the p48 signals in the cell extracts from cells treated with NIP-L3VS was 90% of the level in unirradiated samples, suggesting that the mutated protein is resistant to proteasomal degradation (Fig. 5A). The above result came as a surprise, because the K244E mutation does not affect interaction of the overexpressed XP82TO p48 with Cul-4A and presumably ubiquitination, as shown for another XP-E mutant, XP2RO (48). Our results suggest that the binding of UV-DDB to UV-damaged DNA has an important role in mediating the UV-induced degradation of p48.

Figure 4. A proteasomal inhibitor blocks UV-induced degradation of p48. Normal human lymphoblastoid cells (GM01953) were pretreated with the specific proteasomal inhibitor NIP-L3VS at the indicated concentrations for 1 h and then subjected to UV irradiation. Fresh medium with NIP-L3VS was used for further post-UV incubation for 3 h. The levels of p127, p48, p53 and total ubiquitinated proteins were detected in whole cell extracts (75 µg) using the specific antibodies. The membrane was probed with anti-actin antibodies, as the internal control.

Degradation and recovery of p48 in repair-deficient cells
We have shown previously that after the initial inhibition, the recovery of UV-DDB binding activity after UV damage occurs in a NER-dependent manner (9). We speculated that degradation of p48 might be a general early post-UV event in cell lines with normal p48, with the reappearance of p48 at later times of UV and/or NIP-L3VS treatment (not shown). This result suggests that ubiquitinated forms of p48, like endogenous p48, are localized in nuclei and not in cytosol.

In a co-immunoprecipitation assay using anti-p48 and probing with anti-ubiquitin, we could not detect a convincing signal for ubiquitinated p48 induced by UV and NIP-L3VS treatment under the conditions employed to detect endogenous levels of p48, Cul-4A and ubiquitin in lymphoblastoid cells (not shown). However, to confirm that the proteasomal inhibitor (NIP-L3VS) induces accumulation of proteins though ubiquitination, we tested for the appearance of total ubiquitinated proteins in the extracts from these cells. The anti-ubiquitin antibody clearly detected a dose-dependent smear and the signal ranged from ~300 to 50 kDa, with the intensity decreasing with the lower molecular weight proteins (Fig. 4).

As shown with other proteasome inhibitors (46), treatment with 25 µM NIP-L3VS stabilized the level of p53 in unirradiated lymphoblastoid cells, and the amount of p53 increased further in irradiated cells. The level of stabilized p53 correlates with the immunodetected signal for ubiquitinated proteins in whole cell extracts from cells treated with NIP-L3VS. It is unlikely that stabilized p53 directly affected the level of p48 because degradation of p48 is not mediated through p53 (see Fig. 7).
dependent on the cell’s proficiency for global genome repair (GGR). To test this hypothesis, and considering that UV regulation of p48 occurs in a p53-dependent manner, we compared the levels of UV-DDB and p53 in NER-deficient XP and Cockayne’s syndrome (CS) cell lines at various time after irradiation. We chose to irradiate repair-deficient cells with the dose that yielded at least 85% survival of the respective cells 48 h after treatment (with the exception of XP12RO, an exquisitely sensitive SV40-transformed line which had 40% survival at 3 J/m²; a lower dose would have produced too few lesions to permit interpretation of p48 kinetics).

Recovery and induction of p48, comparable with that found in NHF 48 h post-UV, was seen only in XP-V and CS-A cell lines, which are deficient in DNA translesion synthesis and transcription-coupled repair (TCR), respectively (49,50) (Fig. 6B). In cells from other XP groups (XP-A, XP-B, XP-C, XP-D and XP-F), which are primarily deficient in NER, p48 was significantly degraded after presumably binding to UV-damaged DNA (1 h after irradiation; Fig. 6A), and recovery of p48 was not seen after 24 h post-treatment. Moreover, the level of p48 decreased further and was almost undetectable in the wce of these cells prepared 24 h after irradiation. The recovery of p48 to a varying extent, presumably due to p53-mediated induction, was detected after 48 h (Fig. 6A).

Except for XP-A, the highest level of accumulated p53, similarly to that in NHF, was observed 24 h after UV in other XP cells. A prolonged p53 accumulation was still present at 48 h post-UV only in XP-F and CS-A cell strains (Fig. 6A and B). Interestingly, in another binding-deficient XP-E cell line (XP2RO), with an R273H amino acid substitution in p48 (28), the basal level of p48 was almost undetectable in 60 µg wce (Fig. 6B), and no post-UV accumulation was detected.

Thus, up to 24 h post-UV the recovery of p48 seems to occur in a GGR-dependent manner, and we wondered if it was further dependent on the repair of a specific lesion, such as (6–4) photoproducts or cyclobutane pyrimidine dimers (CPD). The same type of experiment as in Figure 6A was carried out with XP129, a UV-resistant revertant of the SV40-immortalized XP-A cell line XP12RO (51). The XP12RO cells contain a termination codon, resulting in no detectable level of XPA protein and a deficiency in GGR of CPD and (6–4) photoproducts. Reversion of the parental mutation in XP129 allows the cells to make a reduced amount of full-length XPA protein, and might also contribute to its altered substrate specificity; XP129 is proficient in GGR of (6–4) photoproducts, but deficient in GGR of CPD (52,53). Revertant and parental cells displayed a constitutively expressed level of p48 similar to that of the corresponding SV40-transformed normal cell line (Fig. 6C). The kinetics of post-UV degradation of p48 in the SV40-transformed XP-A cell line (XP12RO) were similar to those found in primary XP-A fibroblasts; the lowest amount of p48 was detected 24 h post-UV (Fig. 6C). The same dose of UV (3 J/m²) induced a lower level of degradation of p48, 1 h after treatment, in XP129 than in XP12RO cells, and the amount of p48 did not change significantly through the course of 48 h. Whether the higher level of p48 at 24 h post-UV in XP129 than in XP12RO cells is the result of proficient repair of (6–4) photoproducts in this cell line is difficult to conclude from these experiments. In all three cell lines, as a consequence of SV40-transformation and the presence of T antigen (54), the constitutive levels of p53 were greatly enhanced (Fig. 6C), although this form of p53 is transcriptionally inactive. The post-UV induction of p48 was not detected in a repair-proficient immortalized cell line (GM00637), most likely as a consequence of compromised p53 function.

**UV-induced degradation of p48 is not mediated through p53**

Because the results above indicate that p53-mediated induction of p48 with the time after NER has already been completed, we wanted to address the role of p53 in the degradation of p48 more directly. We chose the TR9-7 cell line (55,56) to examine the fate of p48 in unirradiated cells and in the early hours after irradiation. To ensure full expression or suppression of p53 protein, TR9-7 cells were grown without or with tetracycline, respectively, for 3 days prior to irradiation. Independent of the p53 status controlled by the tetracycline-regulated system, p48 was constitutively expressed in TR9-7 cells to a significant level (Fig. 7) and was subject to UV-induced degradation. The kinetics of p48 degradation in samples with and without p53 expression were similar for the first 3 h post-UV. In TR9-7 cell extracts, GPp48 antibody detected a band that was probably a degraded form of p48 because it appeared as intact p48 was lost (Fig. 7). A similar band was detected by GPp48 antibody in several other cell types.

**Interaction of UV-DDB with p300 in vivo occurs through the p127 subunit**

In earlier work, we proposed that UV-DDB has a role in the repair of DNA in the context of chromatin (8). The recent finding that UV-DDB can be found in multiprotein complexes which harbor HAT activity (27,57) strengthens the notion that the role of UV-DDB might be to target the chromatin remodeling machinery to DNA lesions, facilitating access by DNA repair.
enzymes. In the light of our finding that UV induces proteasomal degradation of p48, it was important to define the role of each UV-DDB subunit in the interaction with a chromatin remodeling complex.

Lymphoblastoid cells were irradiated with 15 J/m² and lysed with buffer containing detergent. The supernatants were incubated with anti-p300 antibody and the presence of p127 and p48 in co-immunoprecipitated complexes was detected by immunoblotting. Both subunits were detected in the complex with p300 in amounts that corresponded to the signals in input lysates (Fig. 8A). This result suggested that the constitutive interaction with p300 occurs through the p127 subunit, and UV-induced degradation of p48 had no effect on the amount of p127–p300. Co-immunoprecipitation was also tested in two binding-deficient XP-E cell lines (GM01646 and XP23PV) were immunoprecipitated in the same way as in (A).

**Figure 6.** Defective global genome repair affects the post-UV recovery of p48. (A and B) Normal human primary fibroblasts and various XP and CS repair-deficient primary fibroblasts were irradiated with the following UV doses: 10 J/m² (normal fibroblasts, GM05757; CS-A, GM01865; XP-E, GM02415; XP-V, XP7Br) or 6 J/m² (XP-A, GM05509; XP-B, GM13025; XP-C, MG00676; XP-D, GM03248; XP-F, GM04313). (C) A normal SV40 transformed cell line (GM00637), XP-A revertant (XP129) and its parental cell (XP12RO) were irradiated at 6, 3 and 3 J/m², respectively. The wce from unirradiated and irradiated cells were prepared at the indicated time points after irradiation and 60 µg were used for immunoblot analysis.

**Figure 7.** UV-induced degradation of p48 is not mediated through p53. TR9-7 cells, with a stably integrated Tet-regulated wild-type p53 gene, were grown without (–Tet) or with 2 µg/ml tetracycline (+Tet) for 3 days and then subjected to UV irradiation. The cells were treated with or without tetracycline for the indicated times after irradiation, after which wce were prepared and 75 µg used for western blot analysis.

**Figure 8.** Interaction of UV-DDB with p300 in vivo occurs through the p127 subunit. (A) GM01953 cells were irradiated with 15 J/m² and cell lysates prepared 10 min and 2 h after treatment were immunoprecipitated with anti-p300 antibody. The co-immunoprecipitates were analyzed by immunoblotting using anti-p127 and anti-p48 antibodies. (B) The lysates from normal and two UV-DDB binding-deficient XP-E cell lines (GM01646 and XP23PV) were immunoprecipitated in the same way as in (A).

**DISCUSSION**

**UV irradiation and proteasomal-mediated degradation of p48**

In this study we have demonstrated that the earliest detectable effect of UV irradiation (10 min after treatment) in mammalian
cells is the tight association of the UV-DDB complex with chromatin, presumably binding to damaged DNA, followed by the transient dose-dependent degradation of the p48 subunit (Figs 1 and 2). This degradation is induced by UV and does not occur in human cells after IR (Fig. 2D). We analyzed a diversity of cell lines with respect to their proficiency for NER and found that UV-induced degradation of p48 occurred in all lines except the cell lines harboring a mutated p48, which impairs binding activity (Figs 5 and 6). This finding suggests that the binding step is necessary for the degradation of p48, perhaps resulting in a conformational change that would favor a post-transcriptional modification necessary for degradation. However, we cannot exclude the possibility that in addition to recognition of UV-induced damage as the prelude to the degradation of p48, the mutation in p48 could alter the ubiquitin acceptor site and affect the activity of the proteasome.

The prompt decrease in cellular levels of p48 is not a transcriptionally controlled event, but is due to selective degradation mediated most likely by the proteasome. While this manuscript was in preparation, Nag et al. demonstrated that overexpressed Cul-4A was able to target co-expressed p48 for ubiquitination and subsequent degradation (44). It is believed that members of the cullin family function as E3 ligases by facilitating ubiquitin-mediated degradation at the proteasome (43).

The phase of p48 degradation (up to 12 h post-UV; Figs 2A and 5B) is concomitant with the period of intensive NER in vivo, particularly the period required for the repair of (6–4) photoproducts (58,59). A period of recovery followed, and by 24 h, when NER was completed, the level of p48 protein increased to that of p48 in untreated cells. While degradation of p48 occurs in a NER-independent manner, recovery of the protein, as previously shown for UV-DDB activity (9), is dependent on the cell’s capacity for GGR. These findings imply that the accumulation of un repaired DNA lesions in GGR-deficient cells leads to prolonged binding of UV-DDB to damaged DNA, and subsequently to prolonged degradation. In turn, this prolongation results in postponed recovery of endogenous p48, compared with the situation in GGR-proficient cells (Fig. 6A and B).

The repair defect of XP-E cells lacking the DNA binding activity is corrected by microinjection of UV-DDB (8,23), but the complex is only marginally required for in vitro repair (21). This discrepancy suggests the importance of a chromatin context for the role of UV-DDB during in vivo repair. However, Araki et al. utilized an NER assay with a UV-irradiated SV40 minichromosome substrate and the same purified proteins essential for NER of naked DNA; addition of purified UV-DDB did not enhance the efficiency of repair synthesis on this chromosomal DNA (60). The results presented here strongly support the notion that UV-induced degradation of p48 is a pertinent event for UV-DDB function in NER. Therefore, the failure of an in vitro system to reveal the involvement of UV-DDB in NER might be due to the failure of such a system to catalyze proteolysis of p48.

**p53 and the regulation of p48**

p53 itself is a target of ubiquitin-mediated proteasomal degradation, which efficiently controls the basal level of this protein. DNA damage regulates the level of p53 and its transactivation capacity (47). Among its other roles, in UV-exposed cells p53 influences the removal of DNA photoproducts during GGR (56,61). It has been suggested by Hwang et al. (10) that p53 affects NER through transcriptional induction of DDB2 mRNA.

Our results, where the levels of p48 protein were measured after UV treatment in a variety of normal or NER-deficient cells, suggest a more complex mechanism by which p53 is involved in the regulation of p48 than that proposed (10). In UV-irradiated cells, the accumulation of p53 at 3 h takes place at a time when p48 is being degraded and UV-DDB binding activity is lost. Moreover, in the TR9-7 cell line, p48 is constitutively expressed and degraded after UV irradiation in a p53-independent manner. Based upon our results obtained with NER-deficient cells, we speculate that the level of p53-mediated induction of p48 (48 h post-UV) is not only on the function of p53, but also on the competency of the cells for GGR (Fig. 6) (9). Therefore, the involvement of p53 in the UV-induced regulation of p48 occurs at late times post-UV (24 h and thereafter), after NER has been completed to a varying extent, and therefore the amount of p48 partially or completely recovered depends on the cell type. Consequently, a delay in repairing CPD in p53+/− cells in the first 3–4 h after UV cannot be attributed to compromised induction of p48 (62).

**A role for UV-DDB in NER on chromatin**

Recognition of the DNA damage sites within the densely packed chromosome structure requires that DNA repair be coupled with chromatin disassembly and reassembly (63). The human NER machinery requires a space that is larger than 80 bp to excise (6–4) photoproducts efficiently (64). The stalled polymerase II complex on damaged DNA appears to function as a recognition signal in TCR and helps to recruit the core repair apparatus to damaged sites (65). In addition, a model for polymerase II involvement in TCR has been proposed which assumes that the complex must be dissociated from the lesion for repair to take place (41,65). The full complement of proteins involved in damage recognition and recruitment of the repair apparatus in GGR is as yet unresolved. The XPC–HR23 complex is indispensable for initiating the cell-free repair reaction, binding to sites with a DNA helix distortion (66).

Considering that (i) UV-DDB has a high affinity for photoproducts in vitro and forms a tight complex with chromatin after UV-induction in vivo, (ii) p127 constitutively interacts with RPA in vivo (9) and (iii) p48 contains a WD motif characteristic of proteins involved in recognition of chromatin proteins (30), we have speculated that the role of UV-DDB is to permit the access of repair enzymes to photolesions within chromatin-obsured DNA (8). This notion is further supported by the recent observation that UV-DDB can be detected within multi-protein complexes having HAT activity (27,57), implicated in the remodeling of chromatin (67). Our demonstration that the constitutive interaction of the UV-DDB complex with p300 in vivo occurs through the p127 subunit (Fig. 8) supports a functional connection between UV-DDB binding activity and chromatin remodeling. We propose that the p48 subunit targets UV-DDB, in a complex with HAT (either p127 binds to p300 directly or indirectly through intermediate proteins), to the specific DNA lesion, which enables local chromatin remodeling through HAT activity. In a step that follows, p48 is degraded and the catalytic enzymes of the repair machinery are loaded.
onto the damaged DNA. The function of this degradation step remains to be determined. Removal of p48 and potentially p127 might create unencumbered space for other repair enzymes. Alternatively, removal of p48 might allow direct interaction of p127 with other repair factors, facilitating their recruitment.

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