Xeroderma Pigmentosum p48 Gene Enhances Global Genomic Repair and Suppresses UV-Induced Mutagenesis

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Summary
UV-damaged DNA-binding activity (UV-DDB) is deficient in some xeroderma pigmentosum group E individuals due to mutation of the p48 gene, but its role in DNA repair has been obscure. We found that UV-DDB is also deficient in cell lines and primary tissues from rodents. Transfection of p48 conferred UV-DDB to hamster cells, and enhanced removal of cyclobutane pyrimidine dimers (CPDs) from genomic DNA and from the nontranscribed strand of an expressed gene. Expression of p48 suppressed UV-induced mutations arising from the nontranscribed strand, but had no effect on cellular UV sensitivity. These results define the role of p48 in DNA repair, demonstrate the importance of CPDs in mutagenesis, and suggest how rodent models can be improved to better reflect cancer susceptibility in humans.

Introduction
Nucleotide excision repair (NER) removes a broad spectrum of lesions. Many of the lesions are medically important, including cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts induced by ultraviolet radiation (UV) from the sun, intrasstrand cross-links generated by the anticancer drug cisplatin, and benzo[a]pyrene adducts derived from tobacco smoke (Friedberg et al., 1995). NER recognizes these lesions, excises the damaged oligonucleotide, and restores the DNA by replication of the complementary undamaged DNA strand.

Recognition of lesions for NER involves two mechanisms: transcription-coupled repair (TCR) and global genomic repair (GGR). TCR repairs lesions on the transcribed strand of expressed genes (Hanawalt, 1994), while GGR repairs lesions from both nontranscribed genomic DNA and the nontranscribed strand of expressed genes. TCR appears to be initiated by the arrest of RNA polymerase II at lesions (Hanawalt, 1994), but recognition of lesions for GGR has been poorly understood (Wood, 1999).

Inherited defects in NER occur in xeroderma pigmentosum (XP), an autosomal recessive disease associated with UV sensitivity and skin cancer susceptibility (Bootsma et al., 1998). This association has contributed to the clinical impression that sun sensitivity is a marker for skin cancer risk in the general population (Naylor, 1997). XP includes seven genetic complementation groups, corresponding to seven gene products involved in NER. Groups A, B, D, F, and G are defective in both TCR and GGR. Group C is defective only in GGR (Venema et al., 1991). XP variant defines individuals with the clinical symptoms of XP despite normal NER.

XP group E is biochemically heterogeneous, with the absence of a highly specific UV-damaged DNA-binding activity (UV-DDB) in some individuals (Chu and Chang, 1988) but not others (Keeney et al., 1992). UV-DDB requires the expression of two subunits, p125 (or DDB1) and p48 (or DDB2) (Takao et al., 1993; Dualan et al., 1995; Hwang et al., 1996). The p48 gene is inactivated by missense mutations in those XP group E cells lacking UV-DDB (Nichols et al., 1996; Hwang et al., 1998). In normal cells, p48 expression is rate limiting for UV-DDB (Hwang et al., 1998), and p48 transcription is induced by the p53-dependent response to DNA damage (Hwang et al., 1999).

Several observations have challenged the role of UV-DDB in NER. Among XP cells, group E cells have the mildest defect in NER as measured by UV-induced unscheduled DNA synthesis and are the least sensitive to UV (Bootsma et al., 1998). Furthermore, UV-DDB is not required for NER in cell-free extracts (Aboussekhra et al., 1995; Kazantsev et al., 1996).

Balanced against these observations is the recent discovery that XP group E cells either lacking or expressing UV-DDB are deficient in the GGR of CPDs (Hwang et al., 1999). Despite the imperfect correlation between the loss of UV-DDB and loss of GGR, we proposed that UV-DDB is involved in GGR. A direct test of this proposal was not possible because available XP group E cells were refractory to transfection.

Wild-type hamster cell lines also fail to express UV-DDB (Hwang et al., 1998) and are deficient in GGR of CPDs (Bohr et al., 1985). Here, we establish that several primary tissues from hamsters and mice likewise express very low levels of UV-DDB, defining a specific biochemical difference between rodents and humans in DNA repair. Transfection of hamster cells with human p48 enhanced GGR of CPDs, suppressing UV-induced mutagenesis without affecting survival. These results establish the role of p48 in DNA repair, illustrate the possible dissociation of sun sensitivity from cancer risk, and question the validity of rodent models for assessing cancer risk in humans.

Results
Hamster Cells Are Deficient in UV-DDB
UV-DDB is absent or expressed at very low levels in 10 Chinese hamster cell lines (Hwang et al., 1998). To
determine whether the low level of UV-DDB was an artifact of cell culture or a property shared by primary hamster tissues, we measured UV-DDB in extracts of peripheral blood lymphocytes isolated directly from Syrian golden hamsters.

Hamster lymphocytes contained barely detectable levels of UV-DDB (Figure 1A, lanes 5 and 6), while extracts from human peripheral blood lymphocytes contained at least 30-fold higher levels of UV-DDB (lanes 2 and 3). Similarly, UV-DDB was expressed at barely detectable levels in hamster V79 cells (lanes 9 and 10), but expressed at high levels in human HeLa cells (lanes 7 and 8). Low levels of UV-DDB were also seen in primary mouse tissues, including peripheral blood lymphocytes, spleen, lung, and kidney (data not shown). The lack of UV-DDB in rodent cells is not due to absence of the p48 or p125 gene, since treatment of hamster cells with the demethylating agent azacytidine induces expression of UV-DDB (Hwang et al., 1998). Thus, UV-DDB may be transcriptionally suppressed in many rodent tissues.

Hamster Cell Lines Transfected with p48 Express UV-DDB

The failure of hamster cells to express UV-DDB presented an opportunity to define its role in DNA repair, since hamster cell lines are highly receptive to DNA transfection. An expression vector lacking a cDNA insert or containing either p48 or FLAG-p48 was transfected into V79 hamster cells, and clones were screened for UV-DDB by EMSA (Figure 1B). Little UV-DDB was detected in the parental V79 cell line or in V79 cells stably transfected with the control vector. However, clones with p48 message or FLAG-p48 protein have significant levels of UV-DDB (Figure 1B). Thus, expression of p48 conferred UV-DDB to the hamster cells.

Three clones were selected for further analysis: 1A, 3B4, and 5E. Expression of p48 had no significant effect on the division times of these clones: 16 hr for 1A (vec), 15 hr for 3B4 (p48), and 16 hr for 5E (FLAG-p48).

p48 Is Required for Global Genomic Repair of CPDs but Not 6-4 Photoproducts

To test the effect of p48 on GGR of 6-4 photoproducts, unreplicated DNA from UV-irradiated cells was probed with a monoclonal antibody against 6-4 photoproducts. Expression of p48 in hamster cells had no effect on the already rapid repair of 6-4 photoproducts (Figure 2A).

To test the effect of p48 on GGR of CPDs, unreplicated DNA from UV-irradiated cells was probed with a monoclonal antibody against CPDs. GGR of CPDs was undetectable in the hamster clone transfected with control vector, consistent with previous reports that hamster cell lines are defective in GGR of CPDs (Bohr et al., 1985), but occurred at significant levels in hamster clones expressing FLAG-p48 or p48 (Figure 2B).

p48 Is Not Required for Transcription Coupled Repair of CPDs

To determine whether p48 has a role in TCR, we measured CPD repair in the transcribed and nontranscribed strands of the DHFR gene. Wild-type hamster cells (wt) and hamster cells transfected with control vector showed negligible levels of CPD repair in the nontranscribed strand (Figure 2C) as previously reported (Mellon et al., 1987). Hamster cells expressing p48 showed a significantly higher level of CPD repair on the nontranscribed strand, consistent with the role of p48 in GGR. Expression of p48 had no effect on the proficient repair of CPDs from the transcribed strand.

p48 Expression Does Not Affect UV Survival

To determine the physiological significance of GGR of CPDs, we measured the effect of p48 on UV survival. Colony formation after UV was indistinguishable among
p48 Enhances DNA Repair and Suppresses Mutagenesis

Figure 2. p48 Enhances Global Genomic Repair of CPDs
(A) GGR of 6-4 photoproducts. (B) GGR of CPDs. Repair was measured after exposure to UV in hamster cells expressing p48, FLAG-p48, or vector. Each point represents the mean of three independent experiments. (C) TCR of CPDs. Removal of CPDs from the nontranscribed and transcribed strands of the DHFR gene was measured after exposure to UV.

Figure 3. p48 Expression Does Not Affect UV Survival but Suppresses Mutagenesis
(A) UV survival. Wild-type hamster cells (V79 or AA8), V79 hamster cells transfected with p48 or vector, and hamster cells with mutations in the Cockayne syndrome group B gene (CSB) or the XP group D gene (XPD) were exposed to UV from a Westinghouse IL782-30 germicidal lamp at an incident dose of 0.33 J/m²/s. Surviving colonies were counted after 2 weeks. (B) UV-induced mutagenesis. Mutations in the HPRT gene were measured in hamster cells stably transfected with p48, FLAG-p48, or vector. Cells were grown synchronously (asyn) or synchronized in G1 (syn) by serum starvation, exposed to different doses of UV, and selected for HPRT mutations with 6-thioguanine.

p48 Suppresses Mutations Only from Nontranscribed DNA
Hamster cells were exposed to UV (2 J/m²) and grown in divided populations to select for independent HPRT mutant clones. Table 1 lists independent mutations arising in the hamster cells transfected with vector or p48. Most mutations were single base pair substitutions, 29% of which were C-to-T transitions. Significantly, 3 tandem base pair mutations at dipyrimidines were observed: a CT-to-TC transition, a CC-to-TT transition, and a TT-to-105 CT transversion. Such mutations have been reported previously (Friedberg et al., 1995). The damaged DNA strand that led to each mutation could be inferred in most cases from sequence context. For hamster cells expressing p48, the vast majority of UV-induced lesions (94%) occurred at sites containing adjacent pyrimidines, where CPDs and 6-4 photoproducts could have formed. Only 22% (7 of 32) of the mutations were attributable to dipyrimidine lesions on the transcribed strand, while 72% (23 of 32) were from the nontranscribed strand (Table 1), consistent with the poor GGR of CPDs in hamster cells. By contrast, for hamster cells expressing p48, 36% (13 of
lesion was undetermined. Deletion (Del) in the cDNA led to a truncated (Trunc) open reading frame (ORF), but the mutation in genomic DNA dipyrimidine sequence at the site of the mutation. NP, no pyrimidine dimer was associated with the mutation. UD, strand with the mutagenic transcribed DNA, the percentage of mutations found on statistically significant (p < 0.05) was attributable to the transcribed strand. The effect of p48 on the strand specificity ever, on the nontranscribed strand, the mutations from TT dipyrimidines (Table 1). This bias was seen on the transcribed strand (1 mutation from TT, 6 from non-TT) and the nontranscribed strand (7 from TT, 12 from non-TT). When p48 was expressed in hamster cells, there was no significant effect on mutations from the transcribed strand (3 from TT, 10 from non-TT). However, on the nontranscribed strand, the mutations from non-TT dipyrimidines declined significantly (9 from TT, 3 from non-TT). The difference in the effect of p48 on mutations arising from TT and non-TT dipyrimidines was statistically significant (p = 0.037 by Fisher’s exact test), suggesting that UV-DDB targets non-TT CPDs for repair more efficiently than TT CPDs (Figure 4B).

**Discussion**

The Role of UV-DDB in Intact Cells

Expression of p48 conferred UV-DDB to hamster cells and enhanced removal of CPDs from genomic DNA and from the nontranscribed strand of a transcribed gene. Thus, UV-DDB is required for the GGR of CPDs, presumably by binding to CPDs on nontranscribed DNA and recruiting the core NER complex.

GGR of 6-4 photoproducts was not affected by p48

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* The strand of the mutagenic lesion was either the transcribed (−) or nontranscribed (−) strand depending on which strand contained a dipyrimidine sequence at the site of the mutation. NP, no pyrimidine dimer was associated with the mutation. UD, strand with the mutagenic lesion was undetermined. Deletion (Del) in the cDNA led to a truncated (Trunc) open reading frame (ORF), but the mutation in genomic DNA was not defined.
was damaged with a UV dose of 100 J/m² to produce results suggest that the UV sensitivity in XP group E cells (Reardon et al., 1993). Consistent with this, when DNA but unlike hamster cells, have a mild UV sensitivity. Our cis-syn for TT CPDs in the predominant than on TT CPDs (Figure 4). UV-DDB has a weak affinity as they do for DNA replication. transcribed DNA, with a larger effect on non-TT CPDs bypass polymerases do not exist for RNA transcription in human XP group E cells. sensitive. Failure to repair CPDs on the transcribed DNA in the absence of UV-DDB in hamster cells but only partially of the Cockayne syndrome B gene, are extremely UV-sensitive. Failure to repair CPDs due to mutation (perhaps XPC/HR23B), which substitutes fully for the have a specific defect in TCR of CPDs due to mutation et al., 1999). By contrast, UV61 hamster cells, which targeted for repair by both UV-DDB and a second protein in intact cells. Aboussekhra et al. utilized naked DNA substrates containing both CPDs and 6-4 photoproducts after exposure to a high dose of UV. Addition of UV-DDB resulted in only a 2-fold stimulation of UV survival. Cells deficient in GGR of CPDs show a delay in the repair of 6-4 photoproducts (Hwang and Chu, 1993; Reardon et al., 1993), cisplatin may survive by replicating unrepaired DNA with transle-

expression in hamster cells. However, XP group E cells show a delay in the repair of 6-4 photoproducts (Hwang et al., 1999; Itoh et al., 1999). UV-DDB binds to 6-4 photoproducts (Treiber et al., 1992) in addition to CPDs (Hwang and Chu, 1993; Reardon et al., 1993), cisplatin adducts, and many other lesions (Payne and Chu, 1994). These observations suggest that 6-4 photoproducts are targeted for repair by both UV-DDB and a second protein (perhaps XPC/HR23B), which substitutes fully for the absence of UV-DDB in hamster cells but only partially in human XP group E cells.

Expression of p48 suppressed mutagenesis from non-transcribed DNA, with a larger effect on non-TT CPDs than on TT CPDs (Figure 4). UV-DDB has a weak affinity for TT CPDs in the predominant cis-syn conformation (Reardon et al., 1993). Consistent with this, when DNA was damaged with a UV dose of 100 J/m² to produce predominantly cis-syn TT CPDs, binding by UV-DDB was largely unaffected when the CPDs were removed by pretreatment of the DNA with photolyase (Hwang and Chu, 1993). On the other hand, when the DNA was damaged with higher UV doses to produce a significant number of non-TT CPDs, binding by UV-DDB decreased after pretreatment of the DNA with photolyase. Thus, both binding and mutagenesis data suggest that UV-DDB recognizes non-TT CPDs more efficiently than TT CPDs.

Three damage-specific DNA-binding proteins have now been implicated in NER: the XPA protein (Jones and Wood, 1993), the XPC/HR23B heterodimer (Sugasawa et al., 1998), and UV-DDB. During NER in cell-free extracts, binding of XPC/HR23B precedes XPA protein in one report (Sugasawa et al., 1998), but not in another (Wakasugi and Sancar, 1999). Additional experiments are required to determine the order in which UV-DDB, XPC/HR23B, and XPA protein direct the GGR of CPDs.

Failure of Cell-Free Systems to Reveal the Role of UV-DDB in NER

Although UV-DDB appears to be dispensable for the reconstitution of NER in cell-free systems (Aboussekhra et al., 1995; Kazantsev et al., 1996), our experiments with intact cells reveal a critical role for UV-DDB in GGR. Indeed, the results of the cell-free experiments can now be better understood. The failure of UV-DDB to stimulate repair in the system of Kazantsev et al., who utilized an oligonucleotide substrate containing a 6-4 photoproduct, is consistent with our conclusion that 6-4 photoproducts are targeted for repair by either UV-DDB or a second protein in intact cells. Aboussekhra et al. utilized naked DNA substrates containing both CPDs and 6-4 photoproducts after exposure to a high dose of UV. Addition of UV-DDB resulted in only a 2-fold stimulation of NER. Furthermore, extracts from XP group E cells failed to reveal a repair defect (Otrin et al., 1998). Perhaps the high dose of UV (450 J/m²) required to generate the substrate for this assay does not fully test the very high specificity of UV-DDB for UV-damaged DNA (Hwang and Chu, 1993). Alternatively, the naked DNA substrate does not test possible interactions between chromatin and p48, which contains a WD domain conserved among several chromatin remodeling proteins (Hwang et al., 1998).

CPDs on Nontranscribed DNA

Do Not Affect Survival

When hamster cells acquired GGR of CPDs, there was no effect on UV survival. Cells deficient in GGR of CPDs may survive by replicating unrepaird DNA with translesion DNA polymerases, such as polymerase δ, which is mutated in XP variant (Johnson et al., 1999; Masutani et al., 1999). By contrast, UV61 hamster cells, which have a specific defect in TCR of CPDs due to mutation of the Cockayne syndrome B gene, are extremely UV-sensitive. Failure to repair CPDs on the transcribed DNA strand is associated with cell death, perhaps because bypass polymerases do not exist for RNA transcription as they do for DNA replication.

XP group E cells are deficient in the GGR of CPDs, but unlike hamster cells, have a mild UV sensitivity. Our results suggest that the UV sensitivity in XP group E cells can be attributed to their mild deficiency in repairing 6-4 photoproducts. Consistent with this, XP group C cells have a severe defect in the GGR of 6-4 photoproducts and are much more sensitive to UV (van Hoffen et al., 1995).

CPDs Are a Major Source of UV-Induced Mutations

Previous reports have disagreed about the role of CPDs in mammalian mutagenesis (Zdzienicka et al., 1992; Vreeswijk et al., 1998). In these studies, unrecognized genetic differences may have affected the mutagenesis assay in comparisons between nonisogenic cell lines. This problem was circumvented by transfection of mouse fibroblasts with bacteriophage T4 endonuclease V (Kusewitt et al., 1998) or transfection of human XP group A cells with photolyase (Asahina et al., 1999), which specifically target CPDs for repair. UV-induced mutagenesis was suppressed, but the experiments involved ectopic repair systems not endogenous to mouse.
or human cells. We utilized transfected hamster cells isogenic except for UV-DDB expression, which can be induced by demethylation with azacytidine and is therefore indigenous to hamster cells (Hwang et al., 1998). In our experiments, GGR of CPDs led to a 3.7-fold suppression of mutagenesis from the nontranscribed DNA strand (Figure 4A). Thus, CPDs are indeed a major source of UV-induced mutations.

Implications for Rodent and Human Carcinogenesis

The discovery that UV-DDB was suppressed in many rodent tissues suggests that models of human carcinogenesis in rodents may have serious shortcomings. In the hairless mouse, which has been used as a model for UV-induced skin cancer, nearly all of the p53 mutations in the tumors arise from the nontranscribed strand (Dumaz et al., 1997), while in humans, only 54% of p53 mutations arise from the nontranscribed strand (Giglia et al., 1998). Inactivation of the Cockayne syndrome group B gene increases the risk for UV-induced skin cancer in mice (van der Horst et al., 1997) but not in humans (Nance and Berry, 1992). Our findings indicate that mice will reflect the skin cancer susceptibility of humans more accurately if they are engineered to express similar levels of p48.

A dissociation between UV sensitivity and mutagenesis occurs in human diseases of DNA repair. Defective GGR of CPDs in XP group E leads to relatively mild UV sensitivity that is nevertheless associated with skin cancer. Conversely, defective TCR of CPDs in Cockayne syndrome causes severe UV sensitivity without affecting skin cancer risk, presumably because GGR of CPDs is sufficient to suppress mutagenesis. Our observation of a dissociation between UV sensitivity and cancer susceptibility has clinical implications. Sensitivity to sunburn has been accepted as a clinical indicator of skin cancer risk (Naylor, 1997). Our data suggest that this is not always true and that variations in GGR should be considered in assessing skin cancer risk.

Experimental Procedures

Cell Lines and Primary Lymphocytes

Cell lines were grown in DMEM medium supplemented with 10% fetal bovine serum. Rodent blood was collected by cardiac exanguination of Syrian golden hamsters (Charles River, Kingston, NY) or Balb/c mice generously provided by I. Weissman. Lymphocytes were isolated from the rodent blood using Lympholyte-M density separation medium (Cedarlane, Hornby, Canada). Lymphocytes were isolated from human peripheral blood by Ficoll-Paque PLUS density centrifugation (Pharmacia Biotech, Uppsala, Sweden).

DNA Transfection

To generate cell lines expressing FLAG-p48, cells were transfected by calcium phosphate with 2 μg of pRSVneo and either 23 μg of the pB8 J expression vector lacking a cDNA insert (vec) or containing the human p48 open reading frame fused at its N terminus to the FLAG epitope (FLAG-p48) (Hwang et al., 1998). To generate cell lines expressing unmodified p48, cells were transfected by Lipofectamine-PLUS (Gibco-BRL, Gaithersburg, MD) with 1.3 μg of pRSVneo and 2.7 μg of the pB8 J expression vector containing p48. Two days after transfection, cells were plated in medium containing 500 μg/ml of the antibiotic G418 (Gibco-BRL, Gaithersburg, MD) to select for stable transfectants expressing the neo gene.

DNA Repair Assays

For GGR, the relative number of UV-induced photoproducts in unreplicated genomic DNA was determined by an immunoblot assay with mouse monoclonal antibodies specific for either CPDs or 6-4 photoproducts (Mori et al., 1991; Ford and Hanawalt, 1997). In brief, after exposure to UV, hamster cells were incubated in growth medium containing bromodeoxyuridine (BrdUrd). Incorporation of BrdUrd in newly replicated DNA allowed isolation of unreplicated DNA by cesium chloride density gradient sedimentation. Unreplicated DNA was adsorbed to a nylon membrane in triplicate using a slot-blot apparatus, and incubated with mouse monoclonal antibody directed against either CPDs or 6-4 photoproducts and horseshadish peroxidase-conjugated goat anti-mouse antibody.

For TCR, repair of CPDs was examined in the dihydrofolate reductase (DHFR) gene, as previously described (Mellon et al., 1987). In brief, unreplicated DNA from UV-irradiated cells was cleaved with KpnI, and treated or mock treated with T4 endonuclease V, which specifically nicks DNA at CPD sites. The DNA was resolved by electrophoresis under denaturing conditions, transferred to a nylon membrane, and hybridized with RNA probes specific for either the transcribed or nontranscribed strand of the DHFR gene. The ratio of intact restriction fragments in the endonuclease-treated and -untreated DNA was used to calculate the number of CPDs per fragment from Poisson statistics.

HPRT Mutants

UV-induced mutants in the hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene were generated as previously reported (Zdzienicka et al., 1988), except that before UV irradiation, hamster cells were either grown asynchronously or arrested in G1 phase by starvation in medium containing low serum (0.2%) for 48 hr. G1-arrested cells were exposed to UV and then switched to standard medium containing 10% fetal bovine serum. The cells were passaged for 7 days to allow loss of HPRT protein in the newly induced UV sensitivity that is nevertheless associated with skin cancer. Converse-ly, cells were exposed to a UV dose of 2 J/m² and then divided into 36 separate populations to ensure that mice will reflect the skin cancer susceptibility of humans more accurately if they are engineered to express similar levels of p48.

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Acknowledgments

The authors thank A. van Zeeland, M. Zdzienicka, L. DeFazio, V. Goss, and T. Tan for helpful suggestions. The research was supported by the Medical Scientist Training Program and a Paul and Goss, and T. Tan for helpful suggestions. The research was sup-

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To define HPRT mutations, total cytoplasmic RNA was isolated from each HPRT mutant clone using the RNAsesy Mini Kit (Qiagen, Chatsworth, CA). Synthesis of cDNA with reverse transcriptase was initiated with the primer (5'-TAATTTCAGGAACAT-3') and the reverse primer (5'-ATGGGAC TCTCGTGTGTC-3'). The DNA was purified and sequenced on both strands.

References


