Mutations Specific to the Xeroderma Pigmentosum Group E Ddb− Phenotype*

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The activity of a damage-specific DNA-binding protein (DDB) is absent from a subset, Ddb−, of cell strains from patients with xeroderma pigmentosum group E (XP-E). DDB is a heterodimer of 127-kDa and 48-kDa subunits. We have now identified single-base mutations in the gene of the 48-kDa subunit in cells from three known Ddb− individuals, but not in XP-E strains that have the activity. An A → G transition causes a K244E change in XP82TO and a G → A transition causes an R273H change in XP2RO and XP3RO. No mutations were found in the cDNA of the 127-kDa subunit. Overexpression of p48 in insect cells greatly increases DDB activity in the cells, especially if p127 is jointly overexpressed. These results demonstrate that p48 is required for DNA binding activity, but at the same time necessitate further definition of the genetic basis of XP group E.

Xeroderma pigmentosum is a rare human hereditary disease, characterized by a high incidence of skin cancer upon exposure to ultraviolet (UV) light and by defective nucleotide excision repair, characterized by a high incidence of skin cancer upon exposure to ultraviolet (UV) light and by defective nucleotide excision repair, characterized by a high incidence of skin cancer upon exposure to ultraviolet (UV) light and by defective nucleotide excision repair, characterized by a high incidence of skin cancer upon exposure to ultraviolet (UV) light and by defective nucleotide excision repair, characterized by a high incidence of skin cancer upon exposure to ultraviolet (UV) light and by defective nucleotide excision repair, characterized by a high incidence of skin cancer upon exposure to ultraviolet (UV) light and by defective nucleotide excision repair, characterized by a high incidence of skin cancer upon exposure to ultraviolet (UV) light and by defective nucleotide excision repair.

Evidence that DDB is involved in DNA repair is generally indirect (9–12). However, microinjection of purified DDB protein into XP-E cells restores repair DNA synthesis to normal levels in XP-E Ddb− strains, while not affecting that of XP-E Ddb+ strains or of cells from other XP groups (13). A feasible explanation for these observations would be that while a mutation(s) in DDB is responsible for defective nucleotide excision repair in XP-E, Ddb− and Ddb+ strains have mutations in different subunits or different domains of a given subunit of DDB. p127 was the most likely candidate for the location of the mutations, since the large subunit alone apparently is capable of DNA binding (7, 12, 14–16). In the present study, we sequenced cDNAs of both subunits of DDB, but observed mutations to be only in the p48 subunit of Ddb− XP-E strains. Individual and co-expression of the DDB subunits in insect cells demonstrated that p48 is required to obtain DNA binding activity.

EXPERIMENTAL PROCEDURES

Cell Strains and Culture—S9 insect cells (CRL 1711) and IMR-90 normal human fibroblasts (CRL 1268) were purchased from the American Type Culture Collection, Rockville, MD. The European XP-E fibroblast strain GM2415 (XP2RO) (2–4, 13) was obtained from the Human Genetic Mutant Repository, Camden, NJ. XP3RO (3, 4), an XP-E fibroblast strain from the second cousin of XP2RO in a family with possible earlier intermarriages (17), was a gift from Drs. D. Bootsma and J. Hoeijmakers, Erasmus University, The Netherlands. The XP-E fibroblast strains XP82TO, XP89TO, XP93TO, and XP95TO (3, 4, 11, 18) were from unrelated Japanese patients. The normal and XP-E fibroblast strains were cultured as described previously (4). S9 cells were cultured in EX-CELL 400 (JRH) with 2% fetal bovine serum and 1% Fungizone (Life Technologies, Inc.) at 27°C.

RT-PCR and Sequence Analysis of DDB1 and DDB2—RNA isolated from nontransformed human fibroblasts strains IMR-90, XP2RO, XP82TO, XP89TO, XP93TO, and XP95TO by a LiCl-ethanol precipitation method (19) was reverse-transcribed (RT), and the cDNA products were amplified by PCR as described by Kawasaki and Wang (20). The coding sequences, 37 bp of 5′-UTR and 158 bp of 3′-UTR of DDB1 (p127) cDNA were amplified as four overlapping regions of approximately 1 kb each. The coding sequences, 132 bp of 5′-UTR and 268 bp of 3′-UTR of DDB2 (p48) cDNA were amplified as two overlapping regions of approximately 1 kb each. The RT-PCR products were gel-purified (Qiaex II, Qiagen), and both strands were thermal cycle sequenced (dsDNA Cycle Sequencing System, Life Technologies, Inc.) and analyzed on an 8% polyacrylamide gel. To exclude artifacts, the original cDNA preparations from all cell strains were independently reamplified by PCR. In addition, RT-PCR was repeated with the original RNA preparations. All PCR products were analyzed by thermal cycle sequencing.

Analysis of Genomic DNA to Confirm Mutations—Genomic DNA was isolated from the diploid human fibroblasts by the salting out method (21). The fragment of DDB2 that contained the coding sequence corresponding to cDNA nucleotides 645–899 was amplified by PCR and 1 µg of the ~660-bp PCR product was gel-purified, digested with 10 units of HhaI at 37°C for 1 h, and analyzed on a 1% agarose gel.

Southern Blot Analysis—Genomic DNA was isolated (22) from the diploid human fibroblasts, and 20 µg from each strain was digested in triplicate with 20 units of BglII at 37°C for 3 h and then electrophoresed on an 0.8% agarose gel. The DNA was denatured, transferred to a nitrocellulose filter (22), and hybridization was carried out in 50% formamide, 6 × SSC, 150 µg/ml salmon sperm DNA, 0.5% SDS at 42°C for 20 h (1 × SSC contains 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The hybridization probes, used simultaneously, were the complete human ribosomal protein S5 (rps5) cDNA (GenBank™ U14992) and DDB2 cDNA nucleotides 702–754 labeled with [α-32P]dCTP by run-off primer extension (22). After hybridization, the filters were washed with 2 × SSC, 0.5% SDS at room temperature for 5 min, 2 × SSC, 0.1% SDS at room temperature for 15 min, 0.1 × SSC, 0.5% SDS at 37°C for 1 h, and 0.1 × SSC, 0.5% SDS at 64°C for 15 min, and then scanned and...
analyzed with the ImageQuant and MultiQuant programs of a Molecular Dynamics PhosphorImager. The ratios of the \( \text{rpS3} \) to the \( \text{DDB2} \) signals were determined in order to normalize the \( \text{DDB2} \) signal to the amount of DNA in each lane.

**RESULTS AND DISCUSSION**

We have determined the sequences of the \( \text{DDB1} \) and \( \text{DDB2} \) cDNAs in five nontransformed diploid XP-E fibroblast strains which are either \( \text{Ddb}^+ \) (XP2RO and XP82TO) or \( \text{Ddb}^- \) (XP89TO, XP93TO, and XP95TO). RNA was isolated from these cells and from a normal fibroblast strain, IMR-90, and reverse-transcribed, and then the resulting cDNAs were amplified by the PCR. Thermal cycle sequencing of both strands of the PCR products detected unique changes in the cDNA of the \( \text{p48} \) subunit of each of the two \( \text{Ddb}^- \) strains (Fig. 1). In XP2RO, a G \( \rightarrow \) A transition at nucleotide 818 produces an R273H substitution, whereas in XP82TO an A \( \rightarrow \) G transition at nucleotide 730 produces a K244E substitution. Except for these changes, the \( \text{DDB1} \) and \( \text{DDB2} \) cDNA sequences of all six cell strains were in complete agreement with the previously re-
portions of activity, a 4-fold enhancement was obtained when p127 was coexpressed with p48, p127 and p48, p127.

**Table I**

**Overexpression of p127 and p48 in insect cells**

| Extract added  | Femtomoles bound to UV-DNA | Femtomoles bound to non-UV-DNA | Units per μg | Protein band visible |
|----------------|---------------------------|-------------------------------|--------------|---------------------|
| **Experiment I** |                           |                               |              |                     |
| 0.72 μg HeLa DDB | 11.56                     | 0                             | 16.3         |                     |
| 1.4 μg p127-1   | 0.73                      | 0.24                          | 0.4          | None                |
| 1.4 μg p127-8   | 0.92                      | 0.12                          | 0.6          | p127                |
| 1.5 μg p127-12  | 1.36                      | 0.07                          | 0.9          | p127                |
| 0.83 μg p48-1   | 0.77                      | 0.43                          | 0.4          | None                |
| 0.44 μg p48-2   | 0.94                      | 0.78                          | 0.3          | None                |
| 0.76 μg p48-4   | 0.92                      | 0.03                          | 1.2          | None                |
| 0.22 μg p48-5   | 1.48                      | 0.54                          | 4.3          | p48                 |
| 0.43 μg p48-9   | 2.89                      | 0.95                          | 4.5          | p48                 |
| 0.89 μg p48-11  | 5.78                      | 6.5                           | p48          |                     |
| 0.89 μg p48-11 + 1.4 μg p127-1 | 6.78 | 0.75 | p48 only |
| 0.89 μg p48-11 + 1.4 μg p127-8 | 4.91 | 0.73 | p48, p127 |
| 0.89 μg p48-11 + 1.5 μg p127-12 | 5.93 | 0.24 | p48, p127 |
| **Experiment II** |                           |                               |              |                     |
| 0.72 μg HeLa DDB | 10.23                     | 0                             | 14.2         |                     |
| 0.47 μg p48-9   | 1.70                      | 0.27                          | 3.0          | p48                 |
| 0.75 μg p48-11  | 2.80                      | 0.50                          | 3.1          | p48                 |
| 2.28 μg p127-8  | 1.60                      | 0.62                          | 0.43         | p127                |
| 1.74 μg p127-12 | 1.41                      | 1.20                          | 0.12         | p127                |
| 1.39 μg p48-9 with p127-12 | 17.24 | 0.50 | 12.0 | p48, p127 |
| 0.76 μg p48-11 with p127-8 | 12.96 | 0.55 | 16.4 | p48, p127 |

Discussion is unlikely. When BgII digests of genomic DNA from the Ddb− XP-E strains were quantitatively probed by Southern analysis in the DDB2 region and compared to those from normal and Ddb− XP-E fibroblasts, there was neither a change in the band migrations nor in the relative intensity of the signals. Since the cells used in this study are nontransformed, diploid strains, apparent homozygosity would not have arisen by gene duplications, segregations, or deletions. Hence, the three Ddb− individuals would appear to have been homozygous for the changes observed.

Both the p127 and the p48 peptides were overexpressed in a baculovirus-insect cell system (Fig. 3). Previous studies had suggested that p127 was the DNA binding subunit of DDB (7, 12, 14–16). However, cells containing overexpressed p127 had no increased DDB activity (Table I), although the overexpressed p127 was soluble and migrated with the expected Mₐ of 127,000 during SDS-polyacrylamide gel electrophoresis (Fig. 3). On the other hand, overexpressed p48 migrated with a Mₐ of 41,000, as previously noted for p48 purified from HeLa cells, and, unexpectedly, extracts from cells containing the overexpressed p48 had increased DNA damage binding activity (Table I). Moreover, while the combination of extracts containing the individually overexpressed subunits showed no enhancement of activity, a 4-fold enhancement was obtained when p127 was co-expressed with p48 (Table I). The above results could indicate either that p48 is necessary to activate nascent p127 (and/or perhaps an insect cell peptide) or that p48 itself binds to damaged DNA but is stimulated by nascent p127 or an insect peptide.

Several lines of evidence suggest that there is a direct relationship between the amount of p48 and the level of DDB activity. Quantitative Northern blot analysis by Takao et al. (14) demonstrated no differences in the levels of p127 mRNA in crude extracts from XP2RO (Ddb− XP-E), XP95TO (Ddb− XP-E), and wild type strains. Kazantsev and Sancar2 probed Western blots with p127 antibodies and found equivalent expression of p127 protein in Ddb− XP-E, Ddb− XP-E, and wild

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2 A. Kazantsev and A. Sancar, personal communication.
Mutations Specific to XP-E Ddb

type strains. Therefore, there does not appear to be a p127 defect in XP-E Ddb− or Ddb+ at the level of mRNA or protein expression, in addition to sequence. Finally, Vaisman et al. (23) observed that p48 mRNA levels were induced up to 4.5-fold following cisplatin treatment of human cells, while no alterations in p127 mRNA levels were detected. A quantitatively similar increase in DDB activity had also been noted following cisplatin treatment of human cells (23) and UV irradiation of monkey cells (14).

Since no mutations were found in the Ddb+ XP-E strains, what is the relation of DDB to XP-E? DDB does not restore excision repair activity in vitro to either Ddb− or Ddb+ XP-E cell-free extracts (24). While it is feasible that the absence of experiments or that Ddb− genetic loci which fail to complement each other in cell-fusion experiments or that different XP-E patients may have mutations at distinct genetic loci which fail to complement each other in cell-fusion experiments or that Ddb+ XP-E strains have mutations in a third locus which codes for a peptide which functionally interacts with p48 (and p127). Since the microinjection experiments utilized the p127/p48 heterodimer, the effect of p48 alone is not known.

In conclusion, we have identified mutations in the gene of the p48 subunit of DDB in cell strains of xeroderma pigmentosum complementation group E which lack specific DNA damage binding activity. These findings potentially challenge previous reports that suggested that the p127 subunit was responsible for the damage-specific binding. The fact that no mutations were found in XP-E strains that have DDB activity implies that XP-E might contain two subclasses of mutations. Although the role of DDB is unknown, it is possible that the heterodimer might act in a novel manner such as inhibiting repair (as it does in vitro) (24) so as to modulate the rate of excision or allowing damage bypass by DNA polymerase (23, 25). One of the subunits individually might then be more directly involved in DNA repair. We plan to use overexpressed p48 and p127 purified from insect cells to study these and other possibilities.

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