DDB Accumulates at DNA Damage Sites Immediately after UV Irradiation and Directly Stimulates Nucleotide Excision Repair*

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Mitsuo Wakasugi‡, Aki Kawashima‡, Hiroshi Moroi‡, Stuart Linn**, Aziz Sancar**, Toshio Morit‡, Osamu Nikaido‡, and Tsukasa Matsunaga‡‡‡

From the ‡Facility of Pharmaceutical Sciences, Kanazawa University, Takara-machi, Kanazawa 920-0934, Japan, the ‡Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo 060-0812, Japan, the ∆Division of Biochemistry and Molecular Biology, University of California, Berkeley, California 94720-3202, the ∆Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599-7260, and the §Radioisotope Research Center, Nara Medical University, Kashihara, Nara 634-8521, Japan

Damaged DNA-binding protein, DDB, is a heterodimer of p127 and p48 with a high specificity for binding to several types of DNA damage. Mutations in the p48 gene that cause the loss of DDB activity were found in a subset of xeroderma pigmentosum complementation group E (XP-E) patients and have linked to the deficiency in global genomic repair of cyclobutane pyrimidine dimers (CPDs) in these cells. Here we show that with a highly defined system of purified repair factors, DDB can greatly stimulate the excision reaction reconstituted with XPA, RPA, XPC, ERCC1, TFIH, XPF, and TFIIH and up to 17-fold for CPDs and ~2-fold for (6-4) photoproducts (6-4PPs), indicating that no additional factor is required for the stimulation by DDB. Transfection of the p48 cDNA into an SV40-transformed human cell line, WI38VA13, was found to enhance DDB activity and the in vivo removal of CPDs and 6-4PPs. Furthermore, the technique of recently developed microarray of UV irradiation and immunostaining revealed that p48 (probably in the form of DDB heterodimer) accumulates at locally damaged DNA sites immediately after UV irradiation, and this accumulation is also observed in XP-A and XP-C cells expressing exogenous p48. These results suggest that DDB can rapidly translocate to the damaged DNA sites independent of functional XPA and XPC proteins and directly enhance the excision reaction by core repair factors.

Xeroderma pigmentosum (XP) is a rare autosomal recessive disease characterized by sun sensitivity, pigmentation abnormalities, and high incidence of skin cancer (1, 2). XP is genetically heterogeneous and mutations in eight different genes (XP-A through XP-G and XPV) are known to cause this disease. All XP gene products, except XPV, are involved in nucleotide excision repair (NER), which removes a wide variety of DNA damages by dual incisions on both sides of the lesion (3–5). However, the function of damaged DNA-binding protein (DDB), which is linked to XP group E, is poorly understood.

DDB was originally identified as a nuclear factor that binds to UV-damaged DNA and has been shown to recognize a wide spectrum of DNA lesions (6–11). It is a heterodimer of p127 and p48, and both subunits are required for the activity (9, 12, 13). It has been reported that the mRNA levels of p48, but not of p127, strongly depend on the tumor suppressor p53 and increase further after DNA damage in a p53-dependent manner (14). Correspondingly, the protein levels and the activity of DDB also increase after UV irradiation (8, 15). A subset of cell strains from XP group E patients were found to be deficient in the DDB activity (Ddb−/−XP-E) and have mutations in p48 gene (7, 12, 16, 17), although Ddb1 strains may have been misclassified as XP-E (18). The recent in vivo studies showed that XP-E cells are selectively defective in global genomic repair (GGR) (14), which repairs lesions from both nontranscribed genomic DNA and the nontranscribed strand of expressed genes. Chu and colleagues have demonstrated that Chinese hamster cells lack DDB activity and that the cells have a defect in the GGR of cyclobutane pyrimidine dimers (CPDs), but not (6-4) photoproducts (6-4PPs) (13, 19). Furthermore, transfection of the p48 gene into Chinese hamster cells conferred the DDB activity as well as GGR activity for CPDs (19). Recently we found that DDB stimulates the excision of CPDs, but not of 6-4PPs, with cell-free extracts (CFEs) from Chinese hamster cells (20). All of these data suggest that DDB can be involved in the recognition process of CPDs in GGR. In this study we have employed a defined system to test the direct effect of DDB on the in vitro excision reaction using purified repair proteins. Furthermore, we have established the SV40-transformed human cell lines expressing FLAG-tagged p48 and investigated a role of DDB in NER in vivo.

EXPERIMENTAL PROCEDURES

Preparation of DDB and Other Repair Factors—DDB heterodimer was overexpressed in a baculovirus/insect cell system and extensively purified by a modification of the procedure described previously (20). Briefly, after chromatographic separation on a SP-Sepharose column, the fractions containing DDB heterodimer were combined and dialyzed against buffer A (25 mM Hepes-KOH (pH 7.9), 12 mM MgCl2, 1 mM EDTA, 2 mM dithiothreitol, 10% glycerol) containing 0.1 M KCl and 1 The abbreviations used are: XP, xeroderma pigmentosum; NER, nucleotide excision repair; DDB, damaged DNA-binding protein; Ddb−, absence of DDB activity; Ddb+, presence of DDB activity; GGR, global genomic repair; CPD, cyclobutane pyrimidine dimer; 6-4PP, (6-4) photoprod...
applied to a heparin-Sepharose (Amersham Biosciences Inc.) column. After extensive washing, proteins were eluted by a linear gradient from 0.1 to 1 M KCl in buffer A. DDB was then purified by anti-FLAG affinity chromatography from a heparin-Sepharose column. After extensive washing, proteins were eluted by a linear gradient from 0.1 to 1 M KCl in buffer A. DDB was then purified by anti-FLAG affinity chromatography from a heparin-Sepharose column.

In Vitro Reconstituted Excision Repair Assay—The assay measures release of 24–32-nucleotide-long oligomer by dual incision of damaged DNA (22). The substrates for the excision repair assay were a 136-bp duplex-DNA containing a cis-syn-cyclobutane thymine-thymine dimer or a thymine-thymine (6-4) photoprotein in the center and 32P-label at fourth phosphodiester bond 5’ to the lesion (23). The substrate (3 or 6 fmol) was incubated at 30 °C for the indicated periods with six repair factors (60 ng of XPA, 300 ng of RPA, 20 ng of XPC-HR23B, 75 or 150 ng of TFIIH, 20 ng of XPF-ERCC1, and 10 ng of XPG) in 25 μl of reaction buffer (32 mM Hepes-KOH (pH 7.9), 64 mM KCl, 6.44 mM MgCl2, 0.16 mM dithiothreitol, 0.16 mM EDTA, 2 mM ATP, and 4% glycerol). To investigate the effect of DDB on the reconstituted system, various amounts of DDB were added to the reaction. After incubation, DNA was extracted with phenol/chloroform, separated on 8% sequencing gel, and detected by autoradiography. The quantitative analysis from two separate experiments is shown in the right panel and represents the relative values to the percent excision in the absence of DDB. Bars indicate the S.E.

FIG. 1. Stimulation of the excision of CPDs and 6-4PPs by DDB in the reconstituted system. A, six fmol of internally labeled 136-bp substrate containing a CPD were incubated for the indicated periods with purified repair factors (60 ng of XPA, 300 ng of RPA, 20 ng of XPC-HR23B, 150 ng of TFIIH, 20 ng of XPF-ERCC1, and 10 ng of XPG) in the absence (lanes 1–3) or presence (lanes 4–6) of 100 ng of DDB. DNA was extracted, separated on an 8% sequencing gel, and detected by autoradiography. The right panel shows quantitative analysis by an image analyzer from two separate experiments and represents the relative values to the percent excision after 1-h incubation. Bars indicate the S.E. B, three fmol of internally labeled 136-bp substrates containing a 6-4PP were incubated for 2 h with the indicated amounts of DDB and the same amounts of repair factors as A (except that 75 ng of TFIIH was used), and excision products were analyzed as described above. The quantitative analysis from two separate experiments is shown in the right panel and represents the relative values to the percent excision in the absence of DDB. Bars indicate the S.E.

Effect of DDB on the reconstituted system, various amounts of DDB were added to the reaction. After incubation, DNA was extracted with phenol/chloroform, separated on 8% sequencing gel, and detected by autoradiography. The quantitative analysis from two separate experiments is shown in the right panel and represents the relative values to the percent excision in the absence of DDB. Bars indicate the S.E.

RESULTS AND DISCUSSION

DDB Directly Stimulates the Excision of CPDs and 6-4PPs in a Defined System—We previously reported that DDB stimulates the excision of CPDs by CFEs prepared from AAS Chinese hamster ovary cells that lack DDB (20). However, we did not exclude the possibility that DDB titrated out unknown inhibitory factors in CFEs or that other factors in CFEs mediated the stimulation. To clarify these points, we have now utilized a defined system with purified repair proteins. DNA substrates containing a CPD were incubated with purified DDB (100 ng) and appropriate amounts of six core NER factors, XPA, RPA, XPC-HR23B, TFIIH, XPF-ERCC1, and XPG (Fig. 1A). To our surprise, DDB could greatly enhance the excision of CPDs in the defined system, and the stimulatory effect (5–17-fold) was much higher than that observed in the crude system (3–4-fold) (20), clearly demonstrating that no additional factor is required for the stimulation of CPD repair by DDB in vitro.

Similar experiments were conducted to determine the effect of DDB on the excision of 6-4PPs in the defined system. In contrast to the results with CPD substrates, 100 ng of DDB
WI38VA13/pcDNA3.1 clone 3 (V-3) cells were irradiated with 10 or 40 J/m² UV light and incubated for the indicated periods. Genomic DNAs were
autoradiography after electrophoresis on a 5% nondenaturing polyacrylamide gel.

C

Excision in a concentration range of 4.4×10⁻¹⁴ M, we observed an inhibitory effect or no effect of DDB on 6-4PP repair. We previously
examined the repair of 6-4PPs in individual drug-resistant clones and tested for the expression of FLAG-p48 by Western blotting with anti-FLAG M2 antibody (A) and DDB activity as measured by EMSA (B). For the EMSA, nuclear extracts were incubated with 56-bp ³²P-labeled DNA probes containing a 6-4PP and analyzed by autoradiography after electrophoresis on a 5% nondenaturing polyacrylamide gel. C, WI38VA13/pcDNA-Fp48 clones 5 (C-5) and 18 (C-18) or WI38VA13/pcDNA3.1 clone 3 (V-3) cells were irradiated with 10 or 40 J/m² UV light and incubated for the indicated periods. Genomic DNAs were purified and used for the determination of CPD and 6-4PP content by an ELISA. Each point represents the mean of three independent experiments, and bars show the S.D. values.

Enhancement of the DDB Activity and the Removal of CPDs and 6-4PPs after Transfection of the p48 Gene into SV40-transformed Human Cells—The in vitro stimulation prompted us to test whether DDB also acts as a stimulatory factor for NER in vivo. We transfected the p48 gene with FLAG epitope sequences (pcDNA-Fp48) into the SV40-transformed human cell line, WI38VA13, which exhibits relatively low DDB activity in vitro and repair activity in vivo (data not shown), probably due to the inactivation of p53 function by the SV40 large T antigen. The protein levels of FLAG-p48 in each stable clone correlate well with their DDB activity (Fig. 2A and B), indicating that the exogenous FLAG-tagged p48 forms a heterodimeric complex with endogenous p127, since both subunits are required for DDB activity (12, 13). Two clones (C-5 and C-18) showing high FLAG-p48 expression and DDB activity exhibited enhanced repair capacity for both types of photolesions (Fig. 2C). It should be noted that, at 2 h, the repair of 6-4PPs is more significantly enhanced, while at 4–24 h, CPDs are repaired faster than the WI38VA13/pcDNA3.1 (V-3) control cells, suggesting that 6-4PPs are the early target, and CPDs are a later target for DDB. Since transfection of p48 into the rodent cells lacking DDB showed no effect on the 6-4PP repair (19), human cells may require DDB for the efficient repair of 6-4PPs more than do rodent cells, which is supported by the fact that XP-E cells carrying the p48 mutations also exhibited partial deficiency in 6-4PP repair (14, 26).

Rapid Accumulation of DDB at Damaged Sites Independent of XPA and XPC after UV Irradiation—To determine whether DDB actually translocates to the damaged sites in vivo, we have employed the micropore UV irradiation technique that enables us to induce photoproducts in a localized area of the cell nucleus (25, 27) (also see Fig. 3B). Without UV irradiation, homogeneous fluorescence staining was observed in the nucleus of WI38VA13/pcDNA-Fp48 (C-5) cells, but not of WI38VA13/pcDNA3.1 (V-3) control cells (Fig. 3A), consistent with the previous reports that p48 subunit alone or DDB heterodimer localizes within the nucleus (7–9, 28, 29). The micropore UV irradiation dramatically changed the localization of p48 from homogeneous staining to localized foci within the nucleus, which can be observed even in the cells fixed without incubation after UV (Fig. 3C). Furthermore, the localized staining pattern of p48 was completely merged with CPD localization (Fig. 3D). These results indicate that p48 (probably in the form of DDB heterodimer) translocates to the damaged DNA sites very rapidly, in agreement with the notion that DDB acts at a very early step of NER. However, we failed to observe the localized staining of p48 after 2 h or later following the UV irradiation (data not shown). We considered that the loss of the localized staining might reflect the rapid turnover of p48 from homogeneous staining to localized foci within the nucleus.

To determine whether the rapid intranuclear translocation of DDB requires other NER factors, we conducted similar experiments with the XP2OSSV (XP-A) and XP4PASV (XP-C) cell lines expressing FLAG-p48 (Fig. 4). Accumulation of DDB could be observed in both cell lines at 30 min as well as 24 h after the micropore UV irradiation, indicating that XPA and XPC proteins are not required for the translocation of DDB to the damaged sites. These results together with the in vitro data and the literature lead us to propose that most of CPDs and some of 6-4PPs are recognized by DDB, which can recruit other repair factors including XPA and XPC. Consistently, it has been reported that CPDs are hardly recognized by

Fig. 2. Increased DDB activity and repair ability of CPDs and 6-4PPs in WI38VA13 cells highly expressing p48. WI38VA13 cells were transfected with the pcDNA-Fp48 or the pcDNA3.1 vector and selected in medium containing Geneticin. Nuclear extracts were prepared from individual drug-resistant clones and tested for the expression of FLAG-p48 by Western blotting with anti-FLAG M2 antibody (A) and DDB activity

by an EMSA (B). For the EMSA, nuclear extracts were incubated with 56-bp ³²P-labeled DNA probes containing a 6-4PP and analyzed by autoradiography after electrophoresis on a 5% nondenaturing polyacrylamide gel. C, WI38VA13/pcDNA-Fp48 clones 5 (C-5) and 18 (C-18) or WI38VA13/pcDNA3.1 clone 3 (V-3) cells were irradiated with 10 or 40 J/m² UV light and incubated for the indicated periods. Genomic DNAs were purified and used for the determination of CPD and 6-4PP content by an ELISA. Each point represents the mean of three independent experiments, and bars show the S.D. values.
were irradiated with 100 J/m² UV light through an isopore membrane.

PCDNA3.1 (V-3) cells were fixed with 4% formaldehyde, permeabilized, and irradiation. For p48 staining and CPDs were visualized by TDM-2 antibody and anti-FLAG M2 antibody (C) or r and immediately fixed with 4% formaldehyde. After permeabilization, and Alexa Fluor® 488 goat anti-rabbit IgG conjugate (6, H11032). 4

RPA CPD-containing DNA and that it forms a complex with XPA or preX2OSSV/pcDNA-Fp48 (XP-A), XP4PASV/pcDNA-Fp48 (XP-F) cells were irradiated with XPA, WI38VA13/pcDNA-Fp48 (C-5) cells or WI38VA13/ XPC, or XPF, WI38VA13/pcDNA-Fp48 (C-5) cells and stained with anti-FLAG M2 antibody and Alexa Fluor® 488 goat anti-mouse IgG conjugate.

Another role suggested for DDB is to mediate chromatin modification and/or remodeling at the damaged sites. It is, however, unlikely that the stimulation of in vitro excision reaction by DDB in this study is related to the chromatin modification, since we used the pure system with naked DNA and purified repair proteins. DDB might play multiple roles in the NER reaction in vivo, damage recognition, and the recruitment of other repair factors as well as of chromatin modification factors to the damaged DNA sites.

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