DDB accumulates at DNA damage sites immediately after UV irradiation and directly stimulates nucleotide excision repair

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Abstract

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•HR23B, TFIIH, XPF•ERCC1 and XPG, up to 17-fold for CPDs and approximately 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 combined technique of recently developed micropore 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.
Introduction

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 (XPA through XPG 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 to have mutations in p48 gene (7, 12, 16, 17), although Ddb + 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 non-transcribed genomic DNA and the non-transcribed strand of expressed genes. Chu and colleagues have demonstrated that Chinese hamster cells lack DDB activity (13) and that the cells have a defect in the GGR of cyclobutane pyrimidine dimers (CPDs), but not (6-4)
photoproducts (6-4PPs) (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 \textit{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 \textit{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 applied to a heparin-sepharose (Pharmacia) column. After extensive washing, proteins were eluted by a linear gradient from 0.1 M to 1 M KCl in buffer A. DDB was then purified by anti-FLAG affinity gels (Sigma) as described previously (20). The other six repair factors, XPA, RPA, XPC•HR23B, TFIIH, XPF•ERCC1 and XPG which are necessary for the reconstitution experiment were prepared basically as described (21); all the factors except TFIIH were recombinant proteins.

In Vitro Reconstituted Excision Repair Assay. The assay measures release of 24-32 nt-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) photoproduct in the center and ³²P-label at 4th 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% denaturing polyacrylamide gels, and either visualized by autoradiography or quantified by exposing the gels to Bas2000 imaging screens and measuring the intensities with a Fuji Bas2000 Bioimaging Analyzer.

**Generation of human cell lines stably expressing FLAG-tagged p48.** The SV40-transformed normal human cell line, WI38VA13, and various XP cell lines listed below were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS): XP2OSSV (XP group A), XP4PASV (XP group C), and XP2YOSV (XP group F). p48 cDNA with FLAG epitope sequences was excised from pFASTBac1-Fp48 used for baculovirus/insect cell system (20) and subcloned into a mammalian expression vector, pcDNA3.1 (Invitrogen). Human cells were transfected with the pcDNA-Fp48 plasmid using the Effectene transfection reagent (Qiagen) according to the manufacture’s instructions and selected in DMEM supplemented with 10% FBS containing 400 - 600 µg/ml of geneticin (Sigma).

**In Vivo Repair Assay.** Human cells were irradiated with 10 or 40 J/m² of UV light (254 nm) from a germicidal lamp (Toshiba, GL-10) and incubated for various periods. Genomic DNAs were purified with the DNeasy kit (Qiagen) and the amounts of CPDs and 6-4PPs were determined by an enzyme-linked immunosorbent assay (ELISA) using specific monoclonal antibodies, TDM-2 or 64M-2, respectively, as described
previously (24).

**Micropore UV irradiation and Immunostaining.** Micropore UV irradiation was carried out essentially as described (25). Cells expressing FLAG-tagged p48 were inoculated at 2 - 8 x 10^5 cells in 35-mm glass-bottom dishes (MatTek Corp.). After 1- or 2-day incubation, cells were covered with an isopore polycarbonate membrane filter (Milipore Corp., pore size 8 µm in diameter) and exposed to 100 J/m² of UV light. The cells were fixed with 4% formaldehyde (Wako) at 4°C for 15 min, permeabilized with ice-cold detergent solution (0.5% Triton X-100, 1% BSA in PBS) on ice for 5 min, and treated with 20% FBS in PBS at 37°C for 30 min for masking non-specific binding sites of antibodies. After incubation with anti-FLAG M2 antibody (Sigma) and sequentially Alexa Fluor™ 488 goat anti-mouse IgG (H+L) conjugate (Molecular Probes), fluorescence images were obtained with a Leica DMIRBE microscope equipped with a cooled CCD camera (CoolSNAP HQ, Photometrics).
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 AA8 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 failed to stimulate the excision of 6-4PPs and rather inhibited the reaction by ~2-fold (data not shown, also see Fig. 1B, Lane 4). However, the excision of 6-4PPs could be stimulated ~2-fold when 0.1-1 ng of DDB was used (Fig. 1B). We also observed an inhibitory effect or no effect of DDB on 6-4PP excision in a concentration range of 4.4-280 ng per 25 µl reaction using the crude system (20). Optimum DDB concentrations for stimulation of 6-4PP repair appear to be much lower than for that of CPD repair, probably due to the much stronger affinity of DDB for 6-4PPs...
vs cys-syn CPDs (10, 11, 20). We speculate that non-physiological excess amounts of DDB to 6-4PPs may produce abnormal complexes that are non-productive for excision.

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 hr, the repair of 6-4PPs is more significantly enhanced, while at 4 - 24 hr, 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 gene also exhibited partial deficiency in (6-4) 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 hr or later following the UV irradiation (data not shown). We considered that the loss of the localized staining might reflect the rapid turnover of DDB in the NER reaction. We generated the XP2YOSV cell line, which belongs to XP group F (XP-F) and therefore carries a defect in the 5' endonuclease, and also expresses FLAG-tagged p48. When this repair-deficient cell line was subjected to the micropore UV irradiation, the localized staining of p48 could be observed even at 24 hr after UV (Fig. 4). These results suggest that the association of DDB at the damaged sites appears to be productive for
the subsequent NER process.

To address the question of 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 hr 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 found that CPDs are hardly recognized by XPC•HR23B, which binds to 6-4PPs with high affinity (30). We previously showed that DDB enhances the binding of XPA to CPD-containing DNA and that it forms a complex with XPA or XPA and replication protein A (RPA) on damaged DNA (20). Hence XPA or XPA•RPA seems to be the repair factor that is attracted to the DNA damage sites by DDB, but the precise mechanism remains to be elucidated.

Another role suggested for DDB is to mediate chromatin modification and/or remodeling at the damaged sites (13, 31, 32). 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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References


Footnotes

¹The 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)photoproduct; CFE, cell-free extract; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay.
Figure Legends

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 of time 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. DNAs were 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-hr incubation. Bars indicate the standard errors.  
B, Three fmol of internally-labeled 136-bp substrates containing a 6-4PP were incubated for 2 hr 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 standard errors.

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-mer
$^{32}$P-labeled DNA probes containing a 6-4PP and analyzed by autoradiography after
electrophoresis on a 5% non-denaturing 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/m$^2$ of UV 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 standard deviations.

**Fig. 3. DDB accumulation at the damaged sites after UV irradiation.** A, WI38VA13/pcDNA-Fp48 (C-5) cells or WI38VA13/pcDNA3.1 (V-3) cells were fixed with
4% formaldehyde, permeabilized and stained with anti-FLAG M2 antibody and Alexa
Fluor™ 488 goat anti-mouse IgG conjugate. B and C, WI38VA13/pcDNA-Fp48 (C-5)
cells were irradiated with 100 J/m$^2$ of UV light through an isopore membrane and
immediately fixed with 4% folmaldehyde. After permeabilization, the cells were
stained with TDM-2 antibody specific for CPDs (B) or anti-FLAG M2 antibody (C), and
with Alexa Fluor™ 488 goat anti-mouse IgG conjugate (green). DAPI, 4',6'-diamidino-
2-phenylindole, was also used for nuclear counter staining (blue). D, WI38VA13/pcDNA-Fp48 (C-5) cells were locally irradiated with UV and fixed without
incubation. For double immunostaining, anti-FLAG polyclonal antibody and Alexa
Fluor™ 488 goat anti-rabbit IgG conjugate (green) were used for p48 staining and CPDs
were visualized by TDM-2 antibody and Alexa Fluor™ 594 goat anti-mouse IgG
conjugate (red) after denaturation of the DNAs as described (25).
Fig. 4. Effect of *XPA*, *XPC* or *XPF* mutations upon accumulation of DDB.

XP2OSSV/pcDNA-Fp48 (XP-A), XP4PASV/pcDNA-Fp48 (XP-C) or XP2YOSV/pcDNA-Fp48 (XP-F) cells were irradiated with 100 J/m² of UV through an isopore membrane filter, and incubated for 0.5 or 24 hr in the culture medium. Cells were fixed, permeabilized and stained with anti-FLAG M2 antibody and Alexa Fluor™ 488 goat anti-mouse IgG conjugate.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
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