Damaged DNA-binding Protein DDB Stimulates the Excision of Cyclobutane Pyrimidine Dimers in Vitro in Concert with XPA and Replication Protein A*

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Human cells contain a protein that binds to UV-irradiated DNA with high affinity. This protein, damaged DNA-binding protein (DDB), is a heterodimer of two polypeptides, p127 and p48. Recent in vitro studies suggested that DDB is involved in global genome repair of cyclobutane pyrimidine dimers (CPDs), but the mechanism remains unclear. Here, we show that in vitro DDB directly stimulates the excision of CPDs but not (6–4)photoproducts. The excision activity of cell-free extracts from Chinese hamster AA8 cell line that lacks DDB activity was increased 3–4-fold by recombinant DDB heterodimer but not p127 subunit alone. Moreover, the addition of XPA or XPA + replication protein A (RPA), which themselves enhanced excision, also enhanced the excision in the presence of DDB. DDB was found to elevate the binding of XPA to damaged DNA and to make a complex with damaged DNA and XPA or XPA + RPA as judged by both electrophoretic mobility shift assays and DNase I protection assays. These results suggest that DDB assists in the recognition of CPDs by core NER factors, possibly through the efficient recruitment of XPA or XPARPA, and thus stimulates the excision reaction of CPDs.

Nucleotide excision repair (NER)1 is the major mechanism for removing bulky DNA lesions including cyclobutane pyrimidine dimers (CPDs) and (6–4)photoproducts induced by sun light in humans (1–4). The basic reaction mechanism of NER is highly conserved from yeast to human. The core reaction from DNA damage recognition to excision of the damage is accomplished by six repair factors (XPA, RPA, XPC-HR23B, TFIIH) of the six core repair factors (17–19). DDB activity has not been clearly demonstrated.

NER process, presumably in a damage recognition step. However, DDB is clearly not essential for NER in several in vitro reconstituted systems, and its precise role in the damage recognition process has not been demonstrated so far (24–27). However, recent in vitro studies clearly showed that XP-E cells and rodent cells lacking DDB activity are selectively defective in global genome repair (GGR) of CPDs, and transfection of the p48 gene into the rodent cells complements the deficiencies (28, 29), suggesting that DDB may be involved in GGR especially for CPDs.

For this study, we have overproduced DDB in a baculovirus/insect cell system, purified it to near homogeneity, and tested its effect upon in vitro excision reactions with DNA substrates containing either a single CPD or a single (6–4)photoproduct. Indeed the recombinant DDB stimulated the excision rate of CPDs by cell-free extracts (CFEs) prepared from AA8 Chinese hamster ovary cell lines that lack DDB activity, and the stimulatory effect was enhanced in the presence of XPA or XPA + RPA. We also observed that DDB forms a complex with XPA and RPA at the damage sites based on electrophoretic mobility shift assays and DNase I protection assays. These results provide direct biochemical evidence that DDB can be involved in the recognition process of CPDs in NER.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—To overproduce FLAG-tagged DDB subunits in insect cells, 5′-terminal portions of cDNAs with FLAG epitope se-
**RESULTS**

**Overproduction and Purification of DDB Heterodimer and p127 Subunit—**To investigate the effect of DDB on *in vitro* excision repair reaction, we have overproduced DDB heterodimer (p127 and p48) or p127 subunit alone in baculovirus/insect cell system. An 8-amino acid FLAG epitope fused at the N terminus of both polypeptides (p127 and p48) facilitated the purification of the recombinant proteins. After a two-step separation with SP-Sepharose and an anti-FLAG M2 affinity gel, the near homogeneous proteins (Fig. 1A) were tested for DDB activity by electrophoretic mobility shift assay (EMSA) (Fig. 1B). The heterodimeric DDB specifically bound to a 56-bp duplex containing a (6–4)photoproduct (lanes 7 and 8), and the mobility of the complex was identical to that with the native DDB (lanes 3 and 4) from HeLa cell-free extracts. However, the recombinant p127 protein alone did not bind to DNA either.
with or without a (6–4)photoproduct (lanes 9 and 10), consistent with previous reports (36, 37). We also compared the binding ability of the DDB to a 56-mer duplex DNA containing a single CPD or a (6–4)photoproduct (Fig. 1C). As expected, DDB showed a clear preference for the DNA duplex with a (6–4)photoproduct (lanes 10–12) over the undamaged DNA probe (lanes 2–4), and it also showed a moderate preference for the DNA probe containing the CPD (lanes 6–8).

Effects of DDB on in Vitro Nucleotide Excision Repair Reaction with CHO AA8 Cell-free Extracts—To test the effect of the recombinant DDB on an in vitro excision reaction with a CPD-containing DNA substrate, we prepared CFEs from CHO AA8 cells, which have been shown to lack p48 expression and, consequently, DDB activity (29, 36). Fig. 1B (lanes 5 and 6) confirmed that there is no binding activity in AA8 CFEs. The 136-bp substrate with a single CPD was preincubated with DDB heterodimer or p127 alone (as a control) and subsequently incubated with AA8 CFE (Fig. 2A). DDB (lane 2), but not p127 alone (lane 3), stimulated the excision reaction by AA8 CFEs 3-fold, in agreement with in vivo data that when CHO V79 cells were transfected with the p48 gene DDB activity reappeared along with GGR of CPDs (29). This stimulation was also dependent on the concentration of DDB (Fig. 2B). On the other hand, when the DNA substrate containing a (6–4)photoproduct was used, the same amount of DDB (280 ng) greatly inhibited the excision reaction (Fig. 2A, lanes 4 and 5). Since DDB binds to a (6–4)photoproduct more efficiently than a CPD, as shown in Fig. 1C, a similar experiment with less DDB was carried out (Fig. 2C). Under this condition, DDB up to 13.2 ng did not affect the excision level of (6–4)photoproducts by AA8 CFEs. It should be noted that the excision amount of over, the addition of specific antibodies to XPA (lane 4, to −7-fold) or XPA + RPA (lane 5, to −10-fold) but not with RPA (lane 3) or XPC-HR23B (lane 6). The individual repair factors show some stimulatory effect in the absence of DDB (lanes 7–10), and the effects of DDB and XPA or XPA + RPA appear to be additive. Interestingly, when the DNA substrates with a (6–4)photoproduct were used (Fig. 3B), the inhibitory effect of DDB on the excision reaction was apparently reversed by XPA (lane 4) or XPA + RPA (lane 5) but not by RPA (lane 3) or XPC-HR23B (lane 6). These results suggest that XPA and RPA might be directly involved in the DDB-stimulated excision reaction of CPDs.

Complex Formation of DDB with XPA and RPA on a Damaged Site—To determine whether DDB forms a complex with XPA and/or RPA on a damaged DNA, we first employed EMSA with various pairwise combinations of DDB, XPA, and RPA (Fig. 4). Since we had a special interest in the possible ability of DDB to recruit XPA or RPA onto a damaged DNA, we chose conditions under which XPA or RPA alone barely bound to the DNA probes (Fig. 4, A and B, lane 2). A combination of DDB and XPA (Fig. 4A) produced a shifted band (lane 4) that migrated more slowly than the DDB-DNA complex (lane 3). Moreover, the addition of specific antibodies to XPA (α-FLAG, lane 5) or DDB (α-FLAG, lane 6) supershifted all of this band. These data indicate that DDB enhances the binding of XPA to CPD-
containing DNA and makes a ternary complex of DDB-XPA-DNA. On the other hand, another combination, DDB and RPA (Fig. 4B), gave a more intense band compared with RPA alone (lane 2 versus lane 4). This band appears to contain RPA-DNA complex and DDB-RPA-DNA complex since the band was partially supershifted by α-FLAG antibody (lane 6). These data indicate the potential complex formation of DDB-RPA-DNA, which shows nearly the same mobility as the RPA-DNA complex, consistent with a previous report (38).

Finally, we mixed all three factors with the DNA probe to test whether a higher order complex forms. As shown in Fig. 4C, only one retarded band was observed (lane 5), which was supershifted by all of the specific antibodies to these factors (lanes 6–8), indicating that this retarded band contains DDB-XPA-RPA-DNA. We believe that the band in lane 5 is different from the band in lane 4 for the following reason. When we added α-His antibody to the reaction containing RPA and DDB, as in lane 4, we could not observe any supershifted band.

**Fig. 3.** Cooperative stimulation of excision reaction of CPDs by DDB, XPA, and RPA. Six or 3 fmol of internally labeled 136-bp substrates containing a CPD (A) or a (6–4) photoproduct (B) was preincubated at 30 °C for 10 min with DDB (210 or 70 ng) in the presence of XPA (360 ng), RPA (300 ng), XPA (360 ng) + RPA (300 ng), or XPC-HR23B (20 ng). Fifty μg of AA8 CFE was added to the reaction mixture, and incubation was continued for 45 min. The DNA was then extracted, separated on 8% sequencing gels, and detected by autoradiography. The relative excision levels were determined by an image analyzer and are shown in the bottom panels. Bars indicate S.D. from three independent experiments.
However, α-His antibody supershifted more than 90% of the complex as shown in lane 6.

To determine whether the three factors make a complex at a damaged site or bind independently, we conducted a DNase I protection assay (39) using DNA probes containing a (6–4) photoproduct and internal $^{32}$P label at the 4th phosphate bond 5' to the lesion. This fragment was chosen for these studies due to the inability of CPD-containing substrates to show clear signals in the DNase I protection assay (data not shown). After incubation with DDB and/or XPA + RPA, the probes were extensively digested with DNase I to determine the size of the DNA region that was protected from DNase I digestion (Fig. 5). DDB alone conferred a specific and distinctive band of 9 nucleotides (lane 4) in addition to nonspecific bands of 4–6 nucleotides, indicating the presence of a DDB-DNA complex around a damaged site. On the other hand,
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In Vitro Effect of DDB on the Excision of a (6–4)Photoprod-
uct—Could DDB not be involved in the repair of (6–4)-
photoproduts? In vivo repair studies (28, 47, 48) do not support
this idea. The repair of (6–4)photoproduts in XP-E cells lack-
ing DDB activity is significantly slower compared with normal
cells, indicating the partial involvement of DDB in the repair of
(6–4)photoproduts in vivo. In intact cells, DDB may be
required for the recognition of (6–4)photoproduts at some parts
of the genome. Alternatively, a possible role of DDB in the
repair of DNA in chromatin may be implicated, as suggested by
others (26). Why DDB inhibits the excision of (6–4)phot-
oproduts at higher concentrations (Fig. 2) is not known at
present. A similar inhibitory effect of DDB also has been re-
ported in a reconstituted system under XPA- or RPA-limiting
conditions (25). DDB has an ~10-fold higher affinity for (6–
4)photoproduts compared with CPDs (18), and at high concen-
trations, the second band, with slower migration, increases
relative to the first band in EMSA (Fig. 1C). This form of the
complex might inhibit the binding of core NER factors and the
complex formation of human excision nuclease.

Functional Interaction between DDB, XPA, and RPA—An
other noteworthy finding in this study is that the stimulatory
effect of DDB on the excision of CPDs was further enhanced in
the presence of XPA or XPA + RPA (Fig. 3A). Interestingly, the
inhibitory effect of DDB on the excision of (6–4)photoproduts
was also suppressed by the presence of these factors (Fig. 3B).
These observations have a good correlation with the complex
formation detected by EMSA (Fig. 4). XPA, which readily made
a complex with DDB on a damaged DNA, could enhance the
stimulatory effect of DDB, whereas RPA, which partially made
a complex with DDB, could not. Analogously, the combination
of XPA and RPA made a complex with DDB as well as en-
hanced the stimulation of DDB. Furthermore, the binding of
XPA to damaged DNA was profoundly enhanced in the pres-
ence of DDB (Fig. 4A). These results strongly suggest a func-
tional link between DDB and XPA in the efficient repair of
CPDs in vitro. Taken together, we propose the following model.
DDB binds to a CPD in DNA, providing the kink or bending of
DNA, as shown by others (18, 19), and facilitates the recruit-
ment of XPA or XPA together with RPA to the CPD site possi-
ibly through protein-protein interaction with XPA. The complex
formation at a damaged site by these three factors may lead to
the subsequent excision reaction.

In this study, we failed to obtain an indication of a functional
interaction between DDB and XPC-HR23B based on the in
vitro excision assays (Fig. 3) and EMSA (data not shown). This
observation is consistent with the recent report (49) that DDB
and XPC-HR23B bind to UV-damaged DNA independently as
judged by EMSA. These results were rather unexpected be-
cause XPC-HR23B has been already known to be involved in
GGR of CPDs (50, 51) and was also proposed to be a primary
damage recognition factor (45). Other unidentified factors sug-
gested by others (28) or XPA-RPA might be needed to link DDB
and XPC-HR23B. The further analysis is needed to verify this
model and clarify the higher order complex formation.

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