Purification of a HeLa cell nuclear protein that binds selectively to DNA irradiated with ultra-violet light

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ABSTRACT

Ultraviolet (UV) light induces a variety of lesions in DNA of which the pyrimidine dimer represents the major species. Pyrimidine dimers exist as both a cyclobutane type and a 6-4' (pyrimidine-2'-one) photoproduct. We have purified a protein of M\(_r\) ~ 125,000 from HeLa cell nuclei which binds efficiently to double-stranded DNA irradiated with UV light but not to undamaged DNA. This protein was designated UVBP1 (UV damage binding protein 1). UVBP1 did not recognise DNA damaged by cisplatin. Using oligonucleotides with a single dipyrimidine site for induction of UV photoproducts, binding of UVBP1 to a TC-containing substrate was shown to be more efficient than to substrates containing a TT, a CT or a CC pair. This binding specificity implies selective recognition of the 6-4' photoproduct. Further evidence for this was provided by the finding that hot alkali treatment of the substrate (which selectively hydrolysates 6-4' photoproducts) abrogated binding of UVBP1, whereas incubation with DNA photolyase to remove cyclobutane dimers did not. No detectable DNA helicase, ATPase or exonuclease activity was associated with the purified protein. We suggest that UVBP1 may be involved in the lesion recognition step of DNA excision repair and could contribute to the preferential repair of 6-4' photoproducts from the DNA of UV-irradiated mammalian cells.

INTRODUCTION

Ultraviolet (UV) light generates a variety of lesions in cellular DNA which can be both cytotoxic and mutagenic. The major species, the pyrimidine dimer, comprises both the cyclobutane type and the 6-4' (pyrimidine-2'-one) photoproduct. Several minor species are also generated, including pyrimidine hydrates and DNA:protein cross-links (1, 2).

Pyrimidine dimers are removed from DNA by the nucleotide excision repair (NER) pathway (for reviews, see Refs. 3 – 6). Detailed biochemical knowledge of NER is only available in E.coli where the participation of at least six distinct proteins is required for efficient repair (4, 5). Pyrimidine dimer recognition is carried out by a complex consisting of 2 UvrA and 1 UvrB subunits. Following binding of 1 subunit of UvrC, the DNA is cleaved at the 8th phosphodiester bond 5' and the 4/5th bond 3' to the dimer. The lesion-containing oligonucleotide is then removed, the gap refilled and the nick sealed by the combined actions of DNA helicase II (UvrD), DNA polymerase I and DNA ligase. Mutants defective in any one of the Uvr proteins are highly sensitive to killing by UV light (3).

An alternative route for dimer repair is catalysed by DNA photolyase. This enzyme directly reverses cyclobutane pyrimidine dimers, without cleavage of the DNA phosphodiester backbone, using the energy of visible light (7). This process is termed photoreactivation. DNA photolyase can also enhance the efficiency of the UvrABC-mediated NER pathway (8).

Far less is known about the details of NER in human cells, although recent work indicates that the process operates via the excision of an oligonucleotide containing the UV adduct in an analogous way to NER in E.coli (9). Studies on the autosomal recessive condition xeroderma pigmentosum (XP), of which there are at least 7 different genetic complementation groups, suggest that a multi-enzyme complex is required for efficient NER in human cells. XP is characterised by extreme sunlight sensitivity and predisposition to UV-induced skin cancers (reviewed in Ref. 10). Cells from XP patients display differing degrees of NER deficiency ranging from near total, such as in XP complementation group A, to very mild (50% reduced), as in XP-E.

Several proteins from mammalian cells have been identified which bind selectively to DNA damaged either by radiation or by cytotoxic drugs. Primate cells have been shown to contain nuclear proteins with strong binding specificity for UV-damaged DNA (11 – 16). However, the gene(s) encoding these proteins have yet to be identified. It has been suggested that a nuclear protein which binds UV-damaged DNA is absent from protein extracts of XP group E cells (14, 17, 18). However, this does not seem to be a feature of all XP-E cell lines (19, 20).

Here, we describe the purification to apparent homogeneity of a nuclear protein from the human cervical carcinoma cell line, HeLa, which binds to UV-damaged DNA. The 6-4' photoproduct appears to be the lesion recognised preferentially.

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MATERIALS AND METHODS

Nuclear extract preparation
A 0.3M NaCl nuclear extract was prepared from ~ 3 x 10^10 HeLa S3 cells. All steps were performed at 4°C. The following protease inhibitors were added to all buffers: 0.5mM PMSE, 1μg/ml leupeptin, 0.5mM benzamidine, 1μg/ml aprotinin and 1μg/ml pepstatin. Cells were harvested, washed sequentially with PBS and buffer A (10mM Tris—HCl, pH 7.5, 5mM MgCl₂, 0.5 M sucrose), and the pellet resuspended in 100μl buffer A containing 0.25% Triton X-100. The nuclei were harvested, washed three times with buffer A and resuspended in 100μl buffer A containing 0.3M NaCl. After 10 mins, the extract was centrifuged for 15 mins at 27,000g and the supernatant diluted to 500μl with buffer B (10μM Hepes, pH 7.9, 5mM MgCl₂, 0.1mM EDTA, 1mM DTT) containing 0.03M KPO₄ (pH 8.0).

Protein purification
The nuclear extract was loaded onto a 300ml phosphocellulose P11 column, pre-equilibrated with buffer B containing 0.1M KPO₄, pH 8.0. The column was washed with 600ml of equilibration buffer and a linear gradient applied of 0.1M to 0.8M KPO₄ in buffer B. Fractions were analysed using a gel retardation assay. Active fractions, eluting between 0.15M and 0.2M KPO₄, were made 0.1M with NaCl and then loaded onto a 70ml heparin sepharose column. The column was washed with 140 ml buffer containing 0.1M NaCl, and a linear gradient applied of 0.1M to 0.75M NaCl. Active fractions, eluting between 0.4M and 0.5M NaCl, were diluted with buffer B to a final NaCl concentration of 0.1M and then loaded onto a 1ml FPLC MonoQ column. After washing with buffer B containing 0.1M KPO₄, pH 8.0, a 0.1 to 0.5M KPO₄ gradient was applied. Active fractions, eluting between 0.22M and 0.25M KPO₄, were pooled, diluted with buffer B to a final KPO₄ concentration of 0.1M, and loaded onto a 1ml FPLC MonoQ column. This column was processed as for the MonoS column. Active fractions eluted from MonoQ between 0.3M and 0.35M KPO₄. These fractions were pooled, glycerol was added to a final concentration of 20%, and the protein stored at -80°C in aliquots.

Protein gel electrophoresis
Proteins were resolved by SDS-PAGE using the discontinuous buffer system of Laemmli (21). Proteins were prepared in SDS lysis buffer (0.125M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue, 10% 2-mercaptoethanol).

Gel retardation assay
100ng double-stranded oligonucleotide was 32P end-labeled using polynucleotide kinase and [γ-32P]-dATP. Radiolabeled oligonucleotide (1 ng) was incubated for 20 mins at 20°C with 1μg poly dl:dC, 1-4μl column fraction or purified protein in a 20μl assay containing 25mM Tris—HCl, pH 7.5, 1μM EDTA, 5mM MgCl₂ and a final concentration of 5% glycerol. The mixture was then loaded directly (without boiling) onto a 5% polyacrylamide gel and electrophoresis performed in 0.5xTBE buffer. Electrophoresis was monitored by running a sample of gel tracking dye in an adjacent lane.

South Western blotting
Proteins were separated on a 12% polyacrylamide gel containing SDS and transferred to nitrocellulose filters (Hybond-C super, Amersham) as described (22). Electrophoretic blotting was carried out in transfer buffer (25mM Tris, 200mM glycine, 20% v/v methanol), for 16 hours at 4°C. Prehybridization, hybridization and washing of filters was as described by Borelli et al. (23). The probe used was UV-irradiated oligoR (see below), which had been 5'-end labelled using polynucleotide kinase and [γ-32P]-dATP. Prehybridisation and hybridisation solutions (10ml) contained 10 μg unlabelled, undamaged oligoR (see below) and 100 μg poly dl:dC.

Oligonucleotides
The following oligonucleotides (all written 5'-3'), together with their complementary strands, were used in this study:

<table>
<thead>
<tr>
<th>OligoR</th>
<th>OligoC</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAT CAT TGG</td>
<td>ACG CAT GCA TGC CCG</td>
</tr>
<tr>
<td>ATT CCC TCT</td>
<td>CCG GTC GTA TA</td>
</tr>
<tr>
<td>ACT TCT GCA</td>
<td>ACG CAT GCA TGC GCG</td>
</tr>
<tr>
<td>GCC TAT A</td>
<td>TCG CCG GTA TA</td>
</tr>
<tr>
<td>AGT TTC ACT</td>
<td>ACG CAT GCA TGC CCG</td>
</tr>
<tr>
<td>GCC TAT A</td>
<td>CCG GTC GTA TA</td>
</tr>
<tr>
<td>CCG GTC GTA</td>
<td>ACG CAT GCA TGC CCG</td>
</tr>
<tr>
<td>GCC TAT A</td>
<td>ACG CAT GCA TGC CCG</td>
</tr>
</tbody>
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Dipyrimidine sequences present in the strand shown are underlined.

Treatment of oligonucleotides
Double stranded oligonucleotides were UV-irradiated on ice using a Stratalinker TM 1800 (Stratagene) with a dose of 36 kJ/m² unless stated otherwise.

Hot alkali treatment of both undamaged and UV-irradiated double-stranded oligoR was carried out in 0.1M NaOH at 90°C for 30 mins, before cooling on ice for 5 mins. The samples were then neutralized with HCl.

DNA photolyase from A. nidulans (kindly provided by Dr R. Wood) was incubated on ice with undamaged or UV-irradiated oligoR in 50 mM Tris—HCl, pH 7.6, 10 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol in a total reaction volume of 100 μl for 30 mins in the dark. Photoreactivation was performed for 1 hour at room temperature using a fluorescent lamp as described (24).

For cisplatin treatment, 4μg double-stranded oligonucleotide was incubated overnight in the dark at 37°C with 2μg cisplatin in a 20μl reaction volume. The reaction was stopped by addition of 2μl 5M NaCl and 2μl 3M NaOH. After phenol/chloroform extraction and ethanol precipitation, the pellet was dissolved in TE buffer, pH 8.0, at a final concentration of 100μg/ml. Nitrogen mustard treatment of oligonucleotides was as described by Robins et al. (25). Baseless (AP) sites in DNA were induced by acid/heat treatment as described by Robson et al. (26).

Identification of the mode of DNA binding
Following binding of UVRB1 to radiolabelled oligonucleotide and electrophoresis on a 5% polyacrylamide gel, the retarded band was excised from the gel and the DNA:protein complex eluted either with TNE buffer (10mM Tris—HCl, pH 8.0, 0.1M NaCl, 1mM EDTA) alone, with TNE containing 0.1% SDS, or TNE with both 0.1% SDS and proteinase K (100 μg/ml). The labelled oligonucleotide was then re-run on a 20% gel (with and without heat-denaturation) and its mobility compared to that of native or single stranded oligonucleotide.

Helicase assay
The helicase substrate, consisting of M13 viral form DNA to which an oligonucleotide was annealed and extended in the
presence of [α-32P]-dATP, was prepared as described by Matson and George (27) and Sambrook et al. (28). Reaction mixtures in a total volume of 20μl contained 20ng of 32P-labelled substrate in 40mM Tris–HCl, pH 7.5, 10mM MgCl2, 1mM DTT, 2mM ATP, 50ng BSA and 1–5μl purified protein (or UvrD protein as a control). The reaction was incubated for 10 mins at 37°C and the mixture separated on a 5% polyacrylamide gel. Labelled DNA was visualised by autoradiography.

Exonuclease assay

Reaction mixtures (20μl total volume) contained 66mM Tris–HCl, pH 7.5, 10mM MgCl2 (unless omitted), 10mM ATP (unless omitted) and 1mM 2-mercaptoethanol, together with 1–5μl purified protein (or up to 10 units E.coli exonuclease III protein) and 32P end-labelled, double-stranded oligonucleotide. The mixture was incubated for 30 mins at 37°C prior to electrophoresis on a 20% polyacrylamide gel.

ATPase assay

ATPase activity was assayed as described previously by Hickson et al. (29). Several DNA cofactors were used, including native and denatured salmon sperm DNA, and native, UV-treated (36kJ/m²) salmon sperm DNA.

RESULTS

Purification of a protein that binds to UV-damaged DNA

Using a gel retardation assay, a protein was identified in HeLa cell nuclear extracts that bound to UV-irradiated oligonucleotide of essentially random sequence (designated oligoR, see Methods). This oligonucleotide contains multiple potential sites for pyrimidine dimer formation. The protein was purified using conventional chromatography and FPLC. Active fractions from the MonoQ FPLC column contained a predominant protein of Mₚ ~ 125,000 as determined by SDS-PAGE (Figure 1a, left panel). The low yield of the purified protein precluded loading of more protein to the SDS gel and thus it could not be excluded that the active species was a minor contaminant not visible in Figure 1. However, two lines of evidence indicated that the 125 kDa protein was the active UV damage-specific binding protein. Firstly, South Western blotting of partially purified (from the heparin sepharose column) nuclear extracts using a UV-damaged oligonucleotide probe revealed a single band of Mₚ ~ 125,000 (Figure 1a, right panel). Secondly, the level of DNA binding activity in different fractions off the final column reflected the amount of the 125 kDa protein observed on an SDS-polyacrylamide gel (Figure 1b). This protein was designated UVBP1 (UV-damage binding protein 1).

The overall yield could not be determined accurately both because the gel retardation assay as used was only semi-quantitative, and because the detection of the UV damage-specific binding activity in the crude cell extract was masked by numerous other proteins with DNA binding activities. Overall, approximately 20μg of protein were purified from 3×10¹⁰ HeLa cells. In all subsequent assays, the highly purified UVBP1 was used.

Dose dependence of binding

Using oligonucleotides irradiated with increasing fluences of UV light, it was shown that the binding of UVBP1 was dependent upon UV dose (Figure 2), with maximal binding at 18 kJ/m² or above.

Specificity of binding

In a gel retardation assay using oligoR as substrate, UVBP1 showed specificity for UV-irradiated double-stranded DNA and did not recognise efficiently either UV-irradiated single-stranded DNA or undamaged single- and double-stranded DNA (Figure 3). In the presence of lower levels of poly dI:dC
Analysis of the UV photoproduct recognised by UVBPl
To identify whether UVBPl preferentially recognised specific UV photoproducts, oligonucleotides containing only a single potential site for the formation of a pyrimidine dimer were synthesised. Five oligonucleotides were used containing either no adjacent pyrimidines or a single TT, TC, CT or CC pyrimidine pair (see Materials and Methods). The efficiency of binding was then determined using the gel retardation assay. The TC-containing oligonucleotide was recognised with high efficiency, while those containing a TT, a CT or a CC pair produced a lower level of binding (Figure 4). Binding to the substrate lacking adjacent pyrimidines was not observed (Figure 4). Consistent with this apparent binding specificity, the TC-containing oligonucleotide competed more efficiently than did the TT, CT or CC-containing oligonucleotides for binding of UVBPl to oligoR, which contains multiple sites for induction of UV dimers (data not shown). Based on these competition assays, it was concluded that UVBPl recognised the TC-containing oligonucleotide approximately 5-fold more efficiently than the CT, CC or TT-containing substrates.

The 6-4' photoproduct is induced preferentially at TC dipyrimidine sites. To show further that UVBPl recognised 6-4' photoproducts preferentially over cyclobutane dimers, oligoR was UV-irradiated and then treated either with hot alkali (which selectively hydrolyzes 6-4' photoproducts; references 30, 31) or with DNA photolyase (to monomerize cyclobutane dimers) and a gel retardation assay performed. Figure 5 shows that treatment of the substrate with hot alkali completely abrogated UVBPl binding while photolyase treatment did not significantly reduce the extent of UVBPl binding. The photolyase preparation was shown both to be active in control experiments and to bind to the hot alkali-treated substrate (data not shown).
Binding specificity of this protein indicate that it is probably the monkey cell homologue of UVBP1.

associated with a protein of 126 kDa. The size and suggested et al. (13) and shown to be recently purified by Abramic (16) reported the identification a DNA binding Hirschfeld et al. in placenta which recognises UV damaged DNA. Subsequently, consistent with the proposal that UVBP1 preferentially (or possibly exclusively) recognises 6-4' photoproducts. The 6-4' photoproduct is formed preferentially at this site, whereas the cyclobutane-type dimer predominately arises at TT sites. Consistent with this apparent binding specificity, we have shown that removal of cyclobutane dimers has little or no effect on the binding of UVBP1 to its substrate, whereas elimination of 6-4' photoproducts greatly reduced binding. These data are consistent with the suggestion that UVBP1 selectively to UV-damaged double-stranded DNA. This activity is associated with a protein of subunit Mr 125,000. The Mr of the major UV-specific DNA binding protein in HaLa cells was also confirmed as being 125,000 using South Western blotting.

We have shown that UVBP1 binds with high affinity to an oligonucleotide containing a TC dinucleotide site. The 6-4' photoproduct is formed preferentially at this site, whereas the cyclobutane-type dimer predominately arises at TT sites. Consistent with this apparent binding specificity, we have shown that removal of cyclobutane dimers has little or no effect on the binding of UVBP1 to its substrate, whereas elimination of 6-4' photoproducts greatly reduced binding. These data are consistent with the proposal that UVBP1 preferentially (or possibly exclusively) recognises 6-4' photoproducts. The 6-4' photoproduct is induced only 2-fold less efficiently at CC sites than at TT sites (35) and thus it may have been expected that the CC-containing oligonucleotide would have been recognised almost as efficiently as the TC substrate. The reason for this slight discrepancy is not clear, although it is possible that UVBP1 also recognises weakly a lesion other than the 6-4' photoproduct.

Several groups have identified proteins which bind specifically to DNA damaged by UV light or cisplatin. Feldberg and co-workers (11,12) identified a 120 kDa protein from human placenta which recognises UV damaged DNA. Subsequently, Hirschfeld et al. (16) reported the identification a DNA binding protein from monkey kidney CV-1 cells that recognises UV-damaged DNA and concluded that the most probable target site was the 6-4' pyrimidine photoproduct. This binding activity was recently purified by Abramic et al. (13) and shown to be associated with a protein of 126 kDa. The size and suggested binding specificity of this protein indicate that it is probably the monkey cell homologue of UVBP1.

Two proteins reported to recognise cisplatin-damaged DNA are both apparently distinct from UVBP1 both in size (~90 kDa and ~28 kDa versus 125 kDa for UVBP1) and in binding specificity (15, 33, 34).

It has been reported that primary skin fibroblasts (GM02415) and lymphoblastoid (GM02450D) cells representing XP complementation group E are deficient in a binding protein with specificity for UV- or cisplatin-damaged DNA (14,18). This binding protein apparently shares some features with DNA photolyase from yeast cells (14). However, Kataoka and Fujiwara (19) showed that XP-E fibroblasts from four Japanese patients were not deficient in this nuclear UV binding protein, even though these XP-E cells were phenotypically very similar to those lacking the nuclear factor. Keeney et al. (20) confirmed the lack of association between the presence or absence of the UV-binding protein and the clinical characteristics of individual XP-E patients.

At present, it is unclear whether the observed deficiency in this DNA damage recognition protein is related to the phenotype of XP-E cells. If DNA damage recognition were implicated in the DNA repair deficiency of XP-E, it would be interesting to see if UVBP1 could correct this deficiency.

Recently, Treiber et al. (32) reported that a HeLa cell protein (UV-DRP), apparently identical to the activity deficient in some XP-E cell lines (14,18), bound primarily to 6-4' photoproducts. It would be interesting to determine whether UV-DRP is identical to UVBP1 described here.

The level of a UV-damaged DNA binding activity is enhanced in UV-irradiated monkey cells (16) and thus this activity may constitute part of an inducible response to UV stress. It is not known at this stage whether the increased DNA binding activity following UV irradiation results from transcriptional activation or from changes in post-translational modification of the protein.

Recent evidence indicates that it is the 6-4' photoproduct which is selectively repaired in mammalian cell DNA immediately after UV irradiation, while repair of the cyclobutane pyrimidine dimer is delayed (36,37). Presumably, at least one DNA repair enzyme is responsible for the enhanced recognition and/or excision of the 6-4' photoproduct. The apparent specificity of UVBP1 for 6-4' photoproducts suggests a possible role for this protein in the lesion-recognition step of NER at early times after UV-irradiation. However, no direct evidence of a role for UVBP1 in DNA excision repair has been provided by the experiments described here.

In sharp contrast to the apparently selective recognition by UVBP1 of 6-4' photoproducts, the UvrABC excision repair pathway in E.coli shows a broad substrate specificity (for review, see Ref. 3). In addition to both classes of UV dimer photoproduct, the UvrABC pathway deals with bulky adducts generated by cisplatin, N-acetoxy-2-acetylaminofluorine and many other chemical carcinogens. Indeed, this pathway can repair, albeit with low efficiency, small lesions such as baseless sites and modified DNA bases (38, 39).

Several E.coli and yeast DNA repair enzymes (reviewed in refs. 3, 40) have been shown to exhibit ATPase activity (seen with E.coli UvrA and UvrD proteins), DNA helicase activity (seen with UvrD and yeast Rad3 proteins) or nuclease activity. Based upon sequence comparisons, it has also been suggested that the human NER proteins ERCC2 and ERCC3 are also DNA helicases (41, 42). With the exception of UV photoproduct recognition, we have been unable to detect any of the activities commonly associated with DNA repair enzymes.
Our data indicate that binding of UVBP1 to DNA is non-covalent and does not result in nicking of the phosphodiester backbone of the oligonucleotide substrate. We cannot exclude the possibility that the bond between the dimerized pyrimidines is cleaved or that our substrate is not sufficiently long to be incised properly. However, by comparison with the NER pathway in E.coli, it seems likely that a multienzyme complex will be required for the incision step to take place. We have purified the oligonucleotide from the retarded DNA–protein complex and shown that UVBP1 will still bind to it specifically, indicating that the interaction of UVBP1 with a UV photoproducct does not result in elimination of the lesion from the DNA (unpublished data), as is seen with DNA photolyase.

In summary, we have purified a protein of Mₘ ~ 125,000 from HeLa cell nuclei which binds selectively to UV-irradiated DNA. The 6-4' UV photoproducct appears to be the strongly preferred substrate. UVBP1 may play a key role in cellular protection against the lethal and mutagenic effects of UV light. Work is in progress to isolate cDNA's encoding this protein.

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