Ca\textsuperscript{2+}-mediated inhibition of a nuclear protein that recognizes UV-damaged DNA and is constitutively overexpressed in resistant human cells: DNA-binding assay

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ABSTRACT

A nuclear protein that recognizes UV-damaged DNA was detected from HeLa cells using DNA-binding assay. Treatment of cells with Ca\textsuperscript{2+} ionophore (A23187) caused a dramatic inhibition of the damage-recognition activity. In contrast, in vitro treatment of nuclear extracts with agents that affect protein conformation (such as urea, NP40 and Ca\textsuperscript{2+}) did not significantly affect on the damage-recognition activity. The Ca\textsuperscript{2+}-mediated inhibition of UV damage recognition was reconstituted by the addition of the cytosolic extracts, suggesting that the Ca\textsuperscript{2+} effect does not directly act on the UV damage-recognition protein. The expression of the detected nuclear protein was increased in UV-resistant HeLa cells. In contrast, the level of this protein was dramatically reduced in UV-sensitive xeroderma pigmentosum group A cells. In addition, UV damage-recognition protein is resistant to RNase, and is independent of the previously identified proteins that bind cisplatin-DNA adduct. These findings implied that the recognition of UV-DNA adduct is modulated by the intracellular level of Ca\textsuperscript{2+}.

INTRODUCTION

Ultraviolet (UV) light causes a cyclobutane-type pyrimidine dimer as well as other DNA base adducts on cellular DNA, which are normally repaired in human cells through excision repair (1). It has also been demonstrated that the removal of the major cisplatin adducts involves nucleotide excision repair in bacterial (2–4) and in mammalian cells (5–6). These results argued that the repair process of cisplatin-adduct is probably the same, at least in part, as that of ‘UV-type’ excision repair. This is supported by the observation of cross-resistance and enhanced plasmid reactivation of cisplatin-resistant HeLa cells to UV damage (7–8). In addition, it has been shown that nuclear extracts isolated from mammalian and yeast mutants in DNA repair fail to interact with damaged DNA (9–10). Since UV-type excision repair involves multiple steps which require different enzymes and accessory proteins that presumably must have access to the damaged DNA for effective repair to occur (1, 11), it is reasonable to think that one or more of these factors can be identified through DNA-binding activity. To date, little is known about the damage recognition and its regulation in cells.

We have previously shown that a nuclear protein which binds to cisplatin adduct is overexpressed in a HeLa cell line resistant to cisplatin (12). The same resistant cell line also showed cross resistance to UV (7). In this study, we have identified a nuclear protein from HeLa cells using DNA-binding assay. This protein recognizes UV-modified DNA and its activity is modulated by Ca\textsuperscript{2+} ionophore A23187. The expression of this damage-recognition protein (DRP) is increased in cisplatin-resistant HeLa cells, and reduced in xeroderma pigmentosum cells. These data have important implication in that the modulation of DNA damage recognition in cells by Ca\textsuperscript{2+} may play a role in DNA repair and subsequently affect cellular sensitivity or resistance to DNA-damaging agents.

MATERIALS AND METHODS

Cell lines

HeLa and VA13 (a WI-13 subline) were maintained according to the supplier’s specifications (American Type Culture Collection). XP fibroblasts (XP12RO, SV40 transformed xeroderma pigmentosum complementation group A from Dr. P. Hanawalt, Stanford University) and a cisplatin-resistant HeLa derivative cell line were maintained as previously described (13). In brief, cells were cultured in Dulbecco’s modified Eagle’s medium (GIBCO, Gaithersburg, MD) containing 10% (v/v) fetal bovine serum, 100 µg/ml streptomycin and 100 units/ml penicillin, and incubated at 37°C in a humidified atmosphere of 5% (v/v) CO\textsubscript{2} in air. Inhibition of cell growth was assayed by the colorimetric MTT method as previously described (13). For some cases, cells were treated with 7 µM of Ca\textsuperscript{2+} ionophore A23187 (Sigma Chemical Co., St. Louis, MO) for specified time periods.
UV-irradiation and platination of DNA
The f130 DNA (see below) at a concentration of 100 \( \mu \text{g/ml} \) was irradiated with UV germicidal lamps as previously described (14). DNA was irradiated with a fluence rate 25 \( \text{J/m}^2/\text{s} \) from a VL-100C UV irradiation unit (Vilbert Lourmat, France). The fluence rate was measured by a VLX-254 radiometer (Vilbert Lourmat, France). The f103 DNA (see below) at a concentration of 100 \( \mu \text{g/ml} \) (3 \( \times \) 10\(^{-4} \) M-nucleotide phosphate) was treated with cisplatin in 3 mM NaCl, and 1 mM sodium phosphate (pH 7.4) at 37°C for 18–24 h as previously described (12). According to Ushay et al. (15) this treatment generated an estimated molar ratio of free cisplatin to nucleotide phosphate of \( r_f = 0.08 \). Following this treatment, \( r_p \), the molar ratio of bound cisplatin to nucleotide phosphate, is \( \sim 0.8 \times r_f \).

DNA probes
The DNA fragment f130 was originally isolated from plasmid pSVT (16) by restriction with Sph I-Bgl I. This 130 bp fragment was ligated to Sph I and Sma I opened vector pBS (+) (Stratagene) at Sph I site, the 5' recessed Bgl I site of f130 was then filled with Klenow DNA polymerase (New England Biolabs, Inc.), and ligated to the Sma I site of the vector. The Hind III-Eco RI f130 fragment containing 17 bp da/dT rich region is a potential target for UV modification. The DNA fragment f103 with protruding ends was the 103 bp Stu I-Ava II fragment from pCD-\( \alpha \)-globin (17), filled with Klenow DNA polymerase, and attached to Eco RI and Xba I linkers, respectively, and cloned into pBS(+). The f103 fragment containing the 14 bp dG.dC rich region is a potential target for cisplatin modification. Hind III and Eco RI generated f103 and f130 fragments were \(^{32}\text{P}[\text{dCTP}]-\)labeled (3 \( \times \) 10\(^4 \) cpm/ng DNA) using Klenow DNA polymerase and purified in spin columns by standard methods (18).

Cell extracts and gel mobility shift assay
Nuclear and cytosolic extracts were prepared according to Dignam et al. (19). The protein concentration was measured by the method of Bradford (20) and visualized by SDS-polyacrylamide gel electrophoresis (21). Gel mobility shift assay was performed in buffer containing 15% glycerol-12 mM Hepes (pH 7.9)-100 mM KCl-5 mM Tris.HCl-1 mM EDTA-1 mM dithiothreitol-300 \( \mu \text{g/ml} \) BSA as previously described (22). Briefly, the DNA probe (0.3 ng) was incubated with cell extracts in 15 \( \mu \text{l} \) at 30°C for 30 min. For some cases, cell extracts were treated with proteinase K or RNase before the binding reaction. The reaction mixtures were then subjected to a 4% polyacrylamide gel electrophoresis under low ionic strength (6.7 mM Tris.HCl, pH 7.9-3.3 mM sodium acetate-1 mM EDTA) at 25°C and 15 mA constant current. The resolved gels were dried and exposed to Kodak XAR-5 X-ray film at -70°C with an intensifying screen. The intensity of the shifted DNA bands was determined by scanning the X-ray film through a scanning densitometer (Hoefer GS 300).

RESULTS
Detection of a UV DRP in HeLa nuclear extracts
Under the standard DNA-binding conditions (including 2 \( \mu \text{g of poly[dI-dC]} \) with the indicated amounts of HeLa nuclear extracts, a UV DRP was detected. As shown in Fig. 1A, the labeled probe (f130-UV) was bound by a nuclear factor or factors that increases with the level of extracts (lanes 2–4, indicated with 'b'). In contrast, only the free probe was detected in the extract-free reaction (lane 1, indicated with 'f'). This DRP is not the same as that which recognizes cisplatin-damaged DNA (12) as judged by the gel mobility of the bound DNA. When cisplatin-damaged DNA (i.e., f103-pt) was used as a probe, a binding activity indicated with 'B' was identified. At high concentration of nuclear extract, another bound form which migrates slower than 'B' and with about the same rate as the UV DRP was detected (lane 6–8). f103-pt alone did not show any retarded band (lane 5). The slowly migrating protein complex is probably the dimer or oligomer of 'B', since the cisplatin DRP (i.e., 'B') of 10 \( \mu \text{g extract} \) appeared to be the same as that detected from the 2 \( \mu \)g extract (compare lanes 7 and 8). The data suggest that the level of UV DRP was not saturated by the level of damage induced by 1000 \( \text{J/m}^2 \) of UV. The data suggest that the detected UV DRP is different from cisplatin DRP. This was supported by competition assays, and the data are shown in Fig. 1B. Increasing the concentration of the undamaged f103 (lanes 4–6) or cisplatin-damaged f103 (i.e., f103-pt, lanes 10–12) up to \( \sim 100\)-fold molarity (i.e., 30 ng)

Figure 1. Identification of a nuclear factor which interacts with UV-damaged DNA. (A) Nuclear extracts (N.E.) with varying amounts indicated on top were incubated with 1000 \( \text{J/m}^2 \) UV-irradiated probe f130-UV (lanes 1–4) or \( r_f \) 0.08 cisplatin-treated probe f103-Pt (lanes 5–8). (B) Competition of UV DRP binding activity. Two \( \mu \text{g of nuclear extracts} \) were incubated with f130-UV in the presence of different amounts of competitor indicated on top: unirradiated f130 (lanes 4–6), f130-UV (lanes 7–9) or f103-Pt (lanes 10–12). Control lanes are: probe without N.E. (lane 1), unirradiated f130 with N.E. (lane 2), without competitor (lane 3). Comp., competitor; b, bound f130-UV probe; B, bound f103-Pt probe; f, free probe.
did not significantly compete for the UV DRP (indicated with an arrowhead) compared with the one without competitor (lane 3). In contrast, increasing the concentration of UV-damaged DNA (i.e., f130-UV) as a competitor effectively competed for UV DRP (lanes 7–9). Most of the UV DRP was competed by 30 ng of specific competitor (compare lanes 3 and 9). The control lanes are probe alone (lane 1) and unirradiated f130 with nuclear extract (lane 2). It should be noted that the detected UV DRP did not bind single-stranded DNA, neither its binding activity was competed by single-stranded DNA (data not shown), suggesting that the binding is specific for duplex DNA.

To localize the DRP in cells, partially purified nuclear (N) or cytosolic (C) extracts were used for the DNA-binding assay. As shown in Fig. 2, most of the cisplatin DRP appeared in the nuclear fraction (lane 2, indicated with ‘B’). However, cytosolic extract also had a lower amount of cisplatin DRP (lane 3) probably because of cross contamination of the extract. Similarly, most of the UV DRP was localized in the nuclear fraction (lane 6, indicated with ‘b’). The cytosolic fraction also showed slight binding (lane 7). This UV DRP is extremely sensitive to protease K (lane 8), but is resistant to RNase (lane 9), suggesting that the UV DRP is a protein or protein complex. For control, undamaged f130 incubated with nuclear or cytosolic extract is shown in lane 4 and lane 5, respectively.

Modulation of UV DRP by agents that modify protein conformation

The UV DRP was assayed for its DNA binding activity in vitro under the conditions containing agents that modify protein conformation. Urea, Nonidet-P-40 (NP-40), and Ca\(^{2+}\) were used because their chemical nature has been demonstrated to modify the conformation of hsp70, and subsequently modulate its binding activity to the consensus heat shock element (23). In our hands, these agents were also able to affect the binding activity of a human nuclear factor to the promoter region of a glyceraldehyde-3-phosphate dehydrogenase gene (Chao, C.C.-K., unpublished data). The results of UV DRP assays are shown in Fig. 3A. Two µg of nuclear extract was used in the binding reaction containing the indicated concentrations of urea, NP-40, or Ca\(^{2+}\). The quantitated densitometric data are shown in Table 1. The results indicate that urea (lanes 4–6) and NP-40 (lanes 7–9) in the tested concentrations did not significantly affect UV DRP activity as compared to the modifier-free reaction (lane 3). Similarly, Ca\(^{2+}\) concentration up to 10 mM did not affect UV DRP (lanes 10–12). In contrast, 100 mM of Ca\(^{2+}\) lowered the binding activity of UV DRP by 82% (compare lanes 13 and 3). Control lanes are UV-damaged probe alone (lane 1) and undamaged DNA with nuclear extract (lane 2). Since 100 mM of Ca\(^{2+}\) is at least 100-fold higher than physiologic concentration, the observed inhibition is probably not due specifically to Ca\(^{2+}\), but rather to the ionic effect in general. However, this speculation was not supported by in vivo study (see below). The nuclear extracts from HeLa cells which have been treated with Ca\(^{2+}\) ionophore (A23187) were assayed for UV DRP, and the results are shown in Fig. 3B. The quantitated data are shown in Table 2. Treatment of cells with A23187 (7 µM) for 5 h inhibited the DRP activity by 52% compared to the

![Figure 2](image-url)  
**Figure 2.** UV-damage recognition of nuclear (N) and cytosolic (C) extracts, and its sensitivity to proteinase and RNase. Two µg of cell extracts were untreated or treated with proteinase K (P+, lane 8) or RNase (R+, lane 9) before the binding reaction with the probe that was unirradiated (lanes 1–5) or irradiated with 1000 J/m\(^2\) UV (lanes 6–9). Control lanes are: f103-Pt (τ\(_{ft}\) = 0.08) without cell extracts (lane 1) or with cell extracts (lanes 2–3). Symbols are as in Fig. 1.

![Figure 3](image-url)  
**Figure 3.** Modulation of UV DRP binding activity by urea, NP-40 or Ca\(^{2+}\). (A) Two µg of nuclear extracts were mixed with the indicated concentration of urea (lanes 4–6), NP-40 (lanes 7–9) or CaCl\(_2\) (lanes 10–13), and incubated for the binding reaction. Control lanes are: unirradiated f130 with N.E. (lane 1), probe f130-UV without N.E. (lane 2), without the addition of modulating agents (lane 3). (B) Nuclear extracts were isolated from cells pretreated with 7 µM of A23187 for 0 h (lane 2), 5 h (lane 3) or 15 h (lane 4). Control lane is: probe f130-UV without N.E. (lane 1). Symbols are as in Fig. 1.
Table 1. In vitro inhibition of UV DRP binding activity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>b/b + f*</th>
<th>% binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.22 ± 0.008</td>
<td>100</td>
</tr>
<tr>
<td>Urea, M</td>
<td>0.26 ± 0.004</td>
<td>118</td>
</tr>
<tr>
<td>0.5</td>
<td>0.25 ± 0.009</td>
<td>114</td>
</tr>
<tr>
<td>2</td>
<td>0.29 ± 0.01</td>
<td>132</td>
</tr>
<tr>
<td>NP-40, %</td>
<td>0.3 ± 0.001</td>
<td>136</td>
</tr>
<tr>
<td>0.02</td>
<td>0.22 ± 0.005</td>
<td>100</td>
</tr>
<tr>
<td>0.2</td>
<td>0.29 ± 0.003</td>
<td>132</td>
</tr>
<tr>
<td>CaCl₂, mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0.26 ± 0.001</td>
<td>118</td>
</tr>
<tr>
<td>1</td>
<td>0.24 ± 0.001</td>
<td>109</td>
</tr>
<tr>
<td>10</td>
<td>0.26 ± 0.008</td>
<td>118</td>
</tr>
<tr>
<td>100</td>
<td>0.04 ± 0.001</td>
<td>18</td>
</tr>
</tbody>
</table>

*The relative levels of binding activity (mean ± SD) were determined by scanning densitometry of the X-ray films of three experiments.

Table 2. Inhibition of UV DRP binding activity in HeLa cells by Ca²⁺ perturbation with 7 μM of A23187.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>b/b + f*</th>
<th>% binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>0.167 ± 0.019</td>
<td>100</td>
</tr>
<tr>
<td>5 h</td>
<td>0.08 ± 0.037</td>
<td>48</td>
</tr>
<tr>
<td>15 h</td>
<td>0.058 ± 0.005</td>
<td>35</td>
</tr>
</tbody>
</table>

*The relative levels of binding activity (mean ± SD) were determined by scanning densitometry of the X-ray films of three experiments.

The relative levels of binding activity (mean ± SD) were determined by scanning densitometry of the X-ray films of three experiments.

untreated cells (compare lanes 2 and 3). Prolonging the treatment (15 h) caused further inhibition by 65% (lane 4). These results raise a possibility that the in vitro study of Ca²⁺ effect on UV DRP probably needs additional factors that are not available in the nuclear extracts. One way to test this idea is by adding the cytosolic extracts to the reaction to reconstitute the Ca²⁺ effect. The results are shown in Fig. 4. DNA-binding reactions were set up under a series of Ca²⁺ concentration (0.1–100 mM) without (Fig. 4, lanes 3–6) or with (lanes 7–10) cytosolic extract, or with cytosolic extract but without nuclear extract (lanes 11–14). It appeared that the addition of cytosolic extract totally blocked the UV DRP activity. There was no UV DRP activity detected in the cytosolic fraction. The control lanes are probe alone (lane 1), and the standard binding reaction without adding Ca²⁺ (lane 2). The results suggest that inhibition of UV DRP activity is mediated by the Ca²⁺ concentration in the cells, and this negative effector is present in the cytosol.

Overexpression of UV DRP in resistant cells

The sensitivity of resistant and parental HeLa cells to UV was analyzed using colorimetric MTT assays (see Experimental for details). Known repair-competent VA13 cells (VA) and repair-deficient XP complementation group A cells (XP) (13) were included as controls. A typical dose-response curve for cellular sensitivity to UV is shown in Fig. 5. The D50, UV fluence causing 50% inhibition of cell growth, for each cell line is indicated. Resistant HeLa cells has a 2.3-fold resistance to UV. XP cells are 8- and 5-fold more sensitive to UV than VA and HeLa cells, respectively. Nuclear extracts from these cells were prepared for DNA-binding assays. Two μg of nuclear extracts from these cells were used to detect the UV DRP using DNA probe irradiated with 0, 1000 or 3000 J/m². As shown in Fig. 6, the resistant cell nuclear extracts (panel R, lanes 5–7, indicated with 'b') showed a 3–4 fold increase of the UV DRP as contrasted to parental extracts (panel P, lines 2–4). Probe alone was included as a control (lane 1). These data also indicate a correlation of UV damage to the DRP. A significant UV DRP was also observed in VA cells (panel VA, lanes 8–10). In contrast, XP cells showed a reduced level of UV DRP (panel XP, lines 11–13). The amount of protein in the extracts used was the same based on SDS-PAGE analysis as well as the colorimetric (Bradford) assay. It is interesting to note that a second retarded band, although lesser in amount, was observed when the UV damage is increased (see lanes 7 and 10). However, we have no idea whether this complex
involves different protein or a dimer of the same protein. The association of UV DRP and the cellular sensitivity to UV suggests that the identified UV DRP may play a functional role in the sensitivity or resistance of cells to UV.

Similarity of UV DRP between sensitive and resistant cells
To further verify whether the identified UV DRP in the sensitive and resistant cell lines is the same, a competition experiment was carried out. As shown in Fig. 7A, with increasing amounts of competitors the competition became apparent. Very little competition effect, if any, was detected if the amount of competitor is less than 10-fold (i.e., 3 ng) for both sensitive parental (panel P, compare lanes 2-4) and resistant HeLa cells (panel R, compare lanes 7-9). For both cell lines (lanes 5 and 10) significant competition was seen when competitors were increased by 100-fold (i.e., 30 ng). All of the UV DRP from sensitive or resistant cells was competed off by a 1000-fold competitors (lanes 6 and 11). Binding reaction without nuclear extract is shown in lane 1 as a control. Therefore, the overexpression of the identified DRP in resistant cells is specific for UV damage. In our hands, there is a ~15% deviation in routinely performed DNA-binding assay. Allogather the three independent experiments, the quantitated data of the X-ray films by densitometry are shown in Fig. 7B. The slopes of the two response patterns are nearly the same, suggesting that the detected UV DRP from the two cell lines is the same in terms of damage recognition. It should be noted that a 100-fold molarity of the competitor inhibited about 50% of the binding activity, implying that the chemical nature of DNA binding is different from the promoter-binding proteins such as those of human glucose-regulated protein gene promoter to which a near 100% inhibition is observed with the same molarity of sequence-specific competitor (Chao, C.C.-K. et al., unpublished data).

DISCUSSION
In this study, we have identified a cellular protein that recognizes UV-damaged DNA. The majority of this DRP is localized in the nucleus, and is probably conserved in human cells. The resistant HeLa cells constitutively expressed a greater level of UV DRP than the sensitive parental cells. In contrast, XP cells showed significantly less UV DRP than resistant cells. It is known that XP cells are extremely sensitive to UV and chemical damages (1). In addition, the resistant HeLa cells were originally selected for cisplatin resistance and revealed cross-resistance to some alkylating agents (13). These findings may have an implication in that the identified protein is potentially important for the sensitivity or resistance of cells to DNA-damaging agents. We have previously shown that the resistant HeLa cells acquired 2-fold enhanced plasmid reactivation, accompanying a 2-fold increase in UV DRP (7). These data may imply a correlation between UV DRP and DNA excision repair. Our preliminary data suggest that the repair synthesis rate and incision of damage in our resistant cells is the same as the sensitive parental cells (Chao, C.C.-K. et al., unpublished data), suggesting that damage
constitutive DRPs is important but it may not necessarily correlate of monkey cells with UV fluence (25). Altogether, the level of cytosolic extract with proteinase K dramatically reduced the effect on UV DRP is probably mediated by the latter the Ca\(^{2+}\) increases in the cell is at least 100-fold lower than the in vitro function of UV DRP with the addition of 10 mM of Ca\(^{2+}\). In addition, the physiologic level of Ca\(^{2+}\) in the cell is at least 100-fold lower than the in vitro conditions, it is suggested that the inhibition of UV damage recognition is not due to the Ca\(^{2+}\) concentration alone. This inhibition phenomenon could result directly from the Ca\(^{2+}\)-dependent inactivation of UV DRP, or it could be mediated by Ca\(^{2+}\)-associated cytosolic proteins. Since the in vitro data did not support the former hypothesis, the Ca\(^{2+}\) effect on UV DRP is probably mediated by the latter pathway. In fact, we have recently found that pretreatment of cytosolic extract with proteinase K dramatically reduced the inhibition of the UV DRP activity (Chao, C.C.-K. et al., unpublished data). These findings suggest a multifactorial modulation of the function of UV DRP in cells including a Ca\(^{2+}\)-mediated pathway. It has also recently been reported by others that a constitutive UV DRP can be induced by pretreatment of monkey cells with UV fluence (25). Altogether, the level of constitutive DRPs is important but it may not necessarily correlate with the sensitivity or resistance of cells to DNA damaging agents. One should also consider the inducible level of DRPs in pondering the potential importance of cellular proteins to cytotoxicity. Therefore, the observed Ca\(^{2+}\)-mediated modulation of UV DRP may be potentially relevant in cellular response to damaging agents.

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