行政院國家科學委員會補助專題研究計畫成果報告

計畫名稱: 小球單胞藻 DNA 修補蛋白之親和性純化與功能鑑定
計畫類別: 個別型計畫
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一、 中文摘要

本研究先以化學藥物 cisplatin 處理過之質體 DNA 為修補基質，建立在小球單胞藻蛋白抽取液中檢測核酸切割修補作用之體外 DNA 修補試驗。由於先前已發現小球藻抽取液中有一含 72，80 及 90-kDa 蛋白之 UV 辨識活性，我們將 UV 照射過之 DNA 固定在小顆粒上，再以親和性吸附法吸取小球藻抽取液中之 DNA 修補蛋白; 抽取液在吸附後幾乎無法修補受 cisplatin 處理之質體，而未吸附之抽取液與經正常 DNA 吸附後之抽取液都有極明顯之修補作用，強度約為吸附後抽取液之 40 倍。以蛋白質電泳分析附著在正常與 UV 照射過 DNA 上之蛋白，發現有一 72 kDa 對受傷 DNA 有特別強之親和力，因為此蛋白與修補活性下降之關聯，此一 72 kDa 之蛋白應參與小球藻之核酸切割修補作用。

二、Abstract

A repair synthesis assay monitoring nucleotide excision repair (NER) was established in cell-free extracts of unicellular alga *Chlorella pyrenoidosa*. Based on the technique of gel retardation assay, a UV-damaged-DNA binding activity composed of a 72-kDa, a 80-kDa and a 90-kDa polypeptide had been detected in *C. pyrenoidosa* extracts. Therefore, a duplex UV-irradiated or unirradiated DNA probe was immobilized on a solid matrix and affinity adsorption of NER proteins from *C. pyrenoidosa* extracts was performed to identify NER proteins. The extracts post incubation with unirradiated DNA and the control extracts showed a repair capacity about 40-fold of those post incubation with UV-damaged DNA. A polypeptide estimated to be 72 kDa in molecular mass was found to bind much more strongly to the damaged DNA than to the unirradiated DNA after analyzing the proteins bound to the solid matrix by SDS-PAGE, and this polypeptide is believed to to play a role in NER in *C. pyrenoidosa*.

二、 計畫緣由與目的

核酸切割修補作用(NER) 所參與之蛋白及其互相間之作用在動物細胞中已

相當清楚 [1,2]，但植物中 NER 作用方式仍有待了解。本實驗室先前在小球單胞藻蛋白抽取液中已測得NER 切割活性 [3]，更由抽取液中純化出一可辨識UV傷害 DNA 之結合活性，其中包括三個分子量各為 72，80 及 90 kDa 之蛋白，推測可能是 NER 修補蛋白 [4]。本計畫利用親和性吸附法吸取小球藻抽取液中可辨識UV 傷害DNA之蛋白，再用體外DNA 修補試驗檢測吸附後之抽取液NER活性是否明顯下降，對照抽取液中對受傷 DNA，有較強親力之蛋白，便可提供更有力之證據說明上述三個辨識蛋白有無參與小球藻中NER 作用。

四・結果與討論

Cisplatin 所產生之 DNA 傷害目前已知僅能藉 NER 進行切除修補，而體外 DNA 修補試驗顯示小球藻抽取液中含有NER 活性，受 16.8 pmole cisplatin 作用之質體所獲之放射性 dATP 強度為未受處理質體之 9.9 倍。小球藻抽取液經固定化之正常DNA 進行親和性吸附仍有一般抽取液83 %之修補活性，而以 UV (27 KJ/m^2) 照射後之 DNA 進行吸附，所得之抽取液其修補活性僅達一般抽取液 2.2 %，故以正常及受傷DNA 吸附後之修補強度約為40:1，也明白顯示親和性吸附幾可移除小球藻中之全部 NER 蛋白。SDS-PAGE 分析比較吸附在正常及受傷 DNA 上之蛋白，發現有一 70 至 72 kDa 者對受傷 DNA 有特別強之親和力，對照此一蛋白之分子量應為前所純化之一 UV 辨識活性中之 72 kDa 蛋白 [4]，因為吸附後之抽取液 NER 能力劇降，更支持此一蛋白參與小球藻 NER 之可能性。

五・計畫成果自評

本計畫成果達到預期目標，成果已發表在國際期刊 Plant Science 156:95-102 於 2000 年 7 月份出刊[5]。

六・參考文獻

[1] R. D. Wood, DNA repair in eukaryotes, Annu. Rev. Biochem. 65 (1996) 135-167.

[2] A. S. Balajee, A. May, V. A. Bohr, Fine structural analysis of DNA repair in mammalian cells, Mutat. Res. 404 (1993) 3-11.

[3] C.-W. Chang, J.-C. Ho, T. Hsu, Thymine-dimer dependent incision on ultraviolet light damaged-DNA in cell-free extracts of *Chlorella pyrenoidosa*, Biosci. Biotech. Biochem. 60 (1996) 490-492.

[4] T. Hsu, J.-C. Ho, C.-C. Chao, Purification of a UV-damaged-DNA binding activity from cell-free extracts of unicellular alga *Chlorella pyrenoidosa*, Plant

Sci. 138 (1998) 137-147.

[5] T. Hsu, R.-C. Sheu, Y.-S. Lai, Possible involement of a 72-kDa polypeptide in nucleotide excision repair of *Chlorella pyrenoidosa* identified by affinity adsorption and repair synthesis assay, Plant Sci. 156 (2000) 95-102.

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Possible involvement of a 72-kDa polypeptide in nucleotide excision repair of *Chlorella pyrenoidosa* identified by affinity adsorption and repair synthesis assay

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Abstract

A DNA repair synthesis assay monitoring nucleotide excision repair (NER) was established in cell-free extracts of unicellular alga *Chlorella pyrenoidosa* using cisplatin- or mitomycin C-damaged plasmid DNA as the repair substrate. The algal extracts promoted a damage-dependent increase in ^{32}P-dATP incorporation after normalization against an internal control. To identify the proteins responsible for NER, a biotin-labeled duplex 27 mer (2 μg) irradiated with or without UV (27 kJ m^{-2}) was immobilized on streptavidin-conjugated agarose beads and incubated with *C. pyrenoidosa* extracts (50 μg) to pull down repair proteins. The extracts post incubation with beads carrying unirradiated 27 mer promoted an eightfold increase in repair synthesis in plasmid DNA (1 μg) damaged by 16.8 pmol of cisplatin. The extracts obtained after affinity adsorption with UV-damaged DNA ligand, however, failed to repair plasmid DNA treated with cisplatin, reflecting that some proteins crucial to NER had been sequestered by the damaged 27 mer. A polypeptide ~70–72 kDa in molecular mass was found to bind much more strongly to the damaged DNA than to the control DNA after analyzing the proteins bound to the beads by SDS-PAGE, and this polypeptide is believed to play a role in NER in *C. pyrenoidosa*.

Keywords: Affinity; Algae; Cisplatin; *Chlorella pyrenoidosa*; Nucleotide excision repair; Ultraviolet light

1. Introduction

Nucleotide excision repair (NER) is an ATP-dependent and a multistep DNA repair pathway that plays an important role in removing DNA damage produced by UV irradiation, alkylating agents like cisplatin or reactive oxygen species [1,2]. In NER, DNA lesions are first recognized by damage-recognition proteins, and an endonucleolytic incision is introduced at both sides of the damaged region. The incised DNA fragment is then excised and the gap generated after incision and excision is filled by synthesis of new DNA that is finally joined to preexisting DNA [1,3,4]. The proteins responsible for NER in *Escherichia coli*, yeast and human cells have been well characterized [1,3].

Based on the excision of UV-induced pyrimidine dimers from plant DNA, NER has been found to operate in *Arabidopsis thaliana* [5], unicellular green alga *Chlamydomonas reinhardtii* [6], carrot protoplasts [7] and some water plants [8]. UV-specific endonucleases acting on pyrimidine dimer-containing DNA have been partially purified from spinach and suspension cultures of the carrot *Daucus carota* [9,10]. Recently, a cDNA homologous to the human Xeroderma pigmentosum group B (XPB) gene was cloned from *A. thaliana* [11]. XPB gene encodes a DNA-dependent ATPase whose DNA helicase motifs are known to participate in

Abbreviations: 6-4PPs, (6-4)photoproducts; CPDs, cyclobutane pyrimidine dimers; ds DNA, double-stranded DNA; DTT, dithiothreitol; MMC, mitomycin C; NER, nucleotide excision repair; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; UV, ultraviolet light.

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unwinding the damaged DNA region before the dual incision step. Moreover, two isoforms of yeast rad23 gene were identified in a carrot cDNA library when this library was used to complement a UV-sensitive yeast mutant that is deficient in a single-stranded DNA binding protein associated with DNA unwinding [12]. Although some information regarding NER proteins in higher plants has been obtained, little is known about the nature of NER proteins in lower plants and the way they interact with each other.

In vitro assays detecting damage-specific incision of DNA (the DNA incision assay) and damage-dependent DNA repair synthesis (the repair synthesis assay) developed in cell-free extracts have been recognized as valuable tools for studying the biochemical mechanisms, including the cutting site introduced by the 5′ or 3′ incision endonuclease, the size of the excised fragment, the cofactors required for repair and the substrate specificity, of NER in *E. coli* and human cells [13–17]. Unicellular algae are ideal model systems for exploring the biochemical mechanisms of NER in lower plants, because they can be cultured and handled easily in the laboratory. We have developed an in vitro DNA incision assay in cell-free extracts of unicelluar alga *C. pyrenoidosa*, which clearly revealed the cutting position introduced at the 3′ side of a pyrimidine dimer by the extracts [18]. Following the development of incision assay, a UV-damaged-DNA binding activity composed of three polypeptides, p72, p80 and p90, was purified from *C. pyrenoidosa* extracts, and these three polypeptides were found to bind directly to UV-damaged DNA as they could be extracted from gel shift bands produced by the crude extracts or more purified protein fractions [19]. This binding activity may participate in the damage-recognition step of NER, since it recognized both UV and cisplatin-damaged DNA in the absence of ATP. The goal of this research was to identify the proteins involved in NER in *C. pyrenoidosa* by functional analysis. An in vitro DNA repair synthesis assay monitoring NER was established and an affinity adsorption of binding proteins from the algal extracts was performed with the same UV-damaged DNA probe [19] immobilized on a solid phase. The effects of affinity adsorption on the capacity of NER were determined by the repair synthesis assay and a polypeptide ~72 kDa in molecular mass that preferentially binds to damaged DNA is believed to play a crucial role in NER.

2. Materials and methods

2.1. Plant materials and cell-free extracts

C. pyrenoidosa kindly provided by Dr Jiunn-Tzong Wu (Institute of Botany, Academia Sinica, Taipei, Taiwan, ROC) was grown in a synthetic salt medium [20] at 25°C under illumination of a fluorescent light and gentle shaking. Algal growth was monitored by absorbance at 685 nm. Algal cells (1000 ml) at mid-log growth ($5.5–7.0 \times 10^7$ cells/ml) were collected by centrifugation at $5000 \times g$ and the pellet was washed twice with ice-cold distilled water and once with extraction buffer (20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol). Algal cells were then suspended in 4 ml hypotonic buffer (40 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM DTT) containing protease inhibitors (2 mM PMSF, 4 μg/ml leupeptin, 1 μg/ml pepstatin), and the swollen cells were broken on ice by pulsed sonication. After centrifugation at $14\,000 \times g$ for 10 min at 4°C, the supernatant was transferred to a beaker on ice and nucleic acids were removed by stirring in the presence of 1.5% (w/v) streptomycin sulfate for 15 min. After centrifugation at $16\,000 \times g$ for 90 min, the supernatant was withdrawn and ammonium sulfate was added to 55% saturation to fractionate repair proteins. The protein precipitate was dissolved in 1 ml dialysis buffer (20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 2 mM DTT, 0.1 M KCl, 12 mM $MgCl_2$, 17% (v/v) glycerol), and dialyzed against the same buffer for 12 h at 4°C. The dialyzed protein solution was concentrated with Centricon-10 (Amicon, USA) and used as the cell-free extract for the repair synthesis assay. The protein concentration in the extract was measured by a protein assay kit (Bio-Rad, USA) based on the method developed by Bradford [21].

2.2. Preparation of immobilized DNA ligands for affinity adsorption

A 27-mer oligonucleotide, 5′-GAC CGA GCT GGG TTA CGA CGC GAC GCC-3′ and its complementary strand were synthesized and la-

beled with biotin at the 5′ end by a commercial source. Both strands at equal concentrations (1 μg/μl) were mixed and the mixture was placed in a PCR thermocycler (Perkin Elmer, Norwalk, CT, USA). The temperature was raised to 95°C and decreased to 25°C at a rate of 1°C/min. After filtering the mixture on a microcon-10 concentrator (Amicon, Beverly, MA, USA), the annealed 27 mer retained on the membrane was collected for preparing UV-damaged DNA. The duplex 27 mer was pipetted onto a piece of parafilm, and UV (254 nm) irradiation was performed in an XL-1500 UV crosslinker (Spectronics, Westbury, NY, USA). UV irradiation was expected to induce the formation of CPDs and 6-4PPs between adjacent TT or CT on the 27 mer [22]. UV-irradiated or unirradiated 27 mer (2 μg) was then incubated with a suspension (20 μl) of streptavidin-conjugated agarose beads (1–3 mg streptavidin/ml, Merck, Germany), and the oligonucleotide was immobilized on the beads through the strong affinity between biotin and streptavidin.

2.3. Affinity adsorption of repair proteins

To pull down repair proteins from cell-free extracts of *C. pyrenoidosa*, the algal extract containing 50 μg proteins in 20 μl dialysis buffer was incubated at 30°C for 20 min with 20 μl agarose beads carrying 2 μg UV (27 kJ m^{-2})-irradiated 27 mer. The suspension was centrifuged at 5000 × *g* at 4°C, and a small fraction of the extract supernatant was taken for the determination of protein concentration. An aliquot of the extract containing 30-μg proteins was used for the repair synthesis assay. The repair capacity in the extract incubated with beads carrying unirradiated 27 mer or no 27 mer was also tested as a comparison.

2.4. DNA substrates for the repair synthesis assay

Plasmid DNA damaged by the alkylating agent cisplatin or MMC was used as the substrate for the repair synthesis assay. pBR 322 (4.3 kb) and pGEM IV (2.8 kb) plasmid DNA were isolated from *E. coli* JM 109 by a plasmid DNA preparation kit (Qiagen, Valencia, CA, USA), and the closed circular form DNA was purified by CsCl gradient centrifugation. The concentration of DNA in TE buffer was determined by the absorbance at 260 nm. Alkylating agent-damaged DNA was prepared by incubating pGEM IV plasmid (0.1 μg/μl in TE buffer) with the same volume of aqueous solution containing different amount of cisplatin or mitomycin C at 37°C overnight in the dark. The damaged DNA was precipitated with ethanol and solubilized in distilled water. Untreated pBR 322 was used as an internal control in each repair reaction mixture.

2.5. In vitro DNA repair synthesis assay

The standard repair reaction mixture (50 μl) contained 45 mM Tris–HCl (pH 7.8), 4 mM EDTA, 70 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 40 mM creatine phosphate-Tris (pH 7.7), 2.5 μg phosphocreatine kinase, 2 mM ATP, 18 μg bovine serum albumin, 50 μM each of dGTP, dCTP, dTTP, dATP, 2 μCi [α-^{32}P]dATP (3000 Ci/mmol), 1 μg pBR 322 plasmid DNA, 1 μg control or damaged pGEM IV plasmid DNA, and 30 μg algal extract proteins. After incubating the reaction mixture at 30°C for 2 h, the repair reaction was terminated by the addition of EDTA to a final concentration of 25 mM. The extract proteins were removed by proteinase K (200 μg/ml) digestion in the presence of 0.5% SDS. The mixture was then extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and DNA products were precipitated with ethanol. Purified DNA products were linearized with Pst I and electrophoresed on a 1.2% agarose gel. After ethidium bromide staining, the gel was vacuum dried and exposed to a Kodak XAR-5 X-ray film with an intensifying screen. Band intensities on the autoradiograph were quantitated using a Amersham/Pharmacia Imagemaster documentation system. Damage-stimulated repair synthesis was expressed as repair factor determined by dividing the band intensity of the damaged DNA by that of the internal control.

2.6. SDS-PAGE analysis of proteins captured by affinity adsorption

To analyze the proteins that were captured on the beads, agarose beads were washed thoroughly with 20 mM KH_2PO_4 (pH 7.5) containing 0.15 M NaCl to remove loosely bound proteins. The beads were then boiled for 10 min in 2 × gel loading buffer to release the tightly bound proteins. The proteins present in the boiled mixtures were analyzed by 12.5% SDS-PAGE and

silver staining. Non-specific binding proteins washed from the beads were also analyzed on the same gel for comparison.

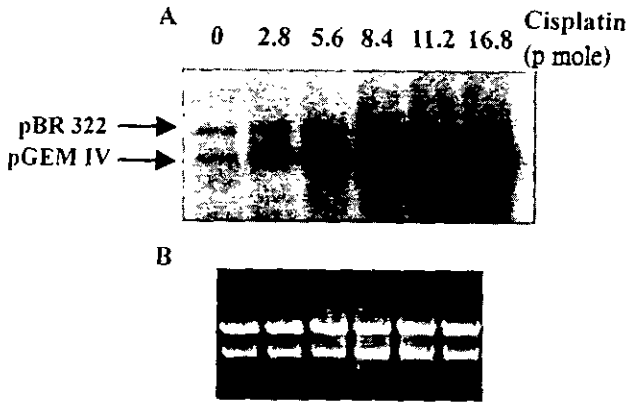

Fig. 1. Cisplatin dose-dependent DNA repair synthesis in cell-free extracts of *C. pyrenoidosa*. Cisplatin-damaged or non-damaged pGEM IV plasmid DNA (1 μg) was incubated at 30°C for 2 h with algal extract proteins (30 μg) in a 50-μl reaction mixture containing dNTPs, [α-^{32}P]dATP, ATP, an ATP-regenerating system and all other required cofactors. pBR 322 DNA (1 μg) was included in each assay as an internal control. The repair reaction was terminated by the addition of EDTA and proteinase K. The DNA products were purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The purified DNA products were linearized with Pst I, and electrophoresed on a 1.2% agarose gel. (A) Autoradiogram of the dried gel and (B) ethidium bromide staining of the gel are shown.

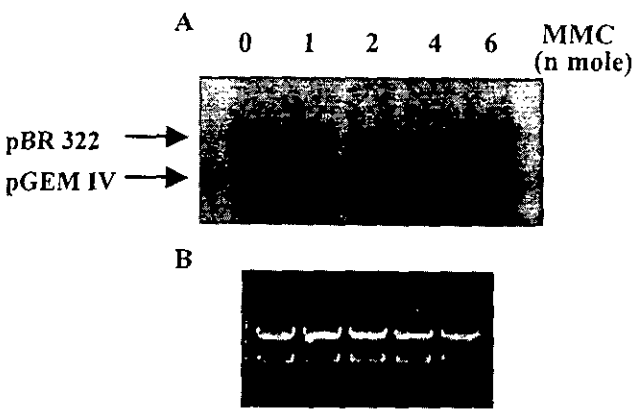

Fig. 2. MMC dose-dependent DNA repair synthesis in cell-free extracts of *C. pyrenoidosa*. The excision repair of MMC-damaged pGEM IV DNA was monitored under the repair condition as described in Fig. 1. (A) Autoradiogram of the dried gel and (B) ethidium bromide staining of the gel are shown.

2.7. Chemicals

PMSF, leupeptin, pepstatin, streptomycin sulfate, cisplatin and mitomycin C were purchased from Sigma (St Louis, MO, USA).Other reagent grade chemicals were obtained from J.T. Baker (Phillipsburg, NJ, USA). DTT and streptavidin-conjugated agarose beads were obtained from Boehringer Mannheim (Mannheim, Germany). [α-^{32}P]dATP (3000 Ci mmol^{-1}) was a product of Amersham (Little Chalfont, Amersham, Buckinghamshire, UK). Biotin-labeled oligonucleotides were synthesized by Perkin Elmer (Norwalk, CT, USA).

3. Results

3.1. Damage-stimulated DNA repair synthesis in cell-free extracts of C. pyrenoidosa

When cisplatin-damaged DNA was incubated with algal protein extracts, a clear dose-dependent increase in dAMP incorporation into damaged DNA was detected after normalization against the internal control, indicating the presence of a major body of NER proteins in the extracts (Fig. 1). Quantitative analysis of DNA band intensities showed that the repair factors for plasmid DNA damaged by cisplatin at 0, 2.8, 5.6, 8.4, 11.2 and 16.8 pmol were 1.0, 2.1, 4.4, 6.2, 6.8 and 7.1, respectively. A linear increase in repair factor was found for pGEM IV DNA treated with 0–8.4 pmol cisplatin and NER capacity in the extracts seemed to be saturated by DNA lesions induced by higher concentrations of cisplatin. The excision repair of cisplatin-damaged DNA was absolutely protein-dependent as no repair synthesis could be detected in the absence of extract proteins. Damage-specific dAMP incorporation was increased with increasing amount of extract proteins and all our repair assays were performed in the presence of 30 μg extract proteins because proteins at this level already gave a significant degree of damage-dependent repair synthesis. The optimal level of repair synthesis was found in the reaction mixture containing 7–10 mM Mg^{2+} (data not shown). An MMC dose-dependent increase in repair synthesis was also promoted by *C. pyrenoidosa* protein extracts (Fig. 2). The ratios of damage-stimulated to control dAMP incorporation were 1.1, 3.4, 7.3, 7.7

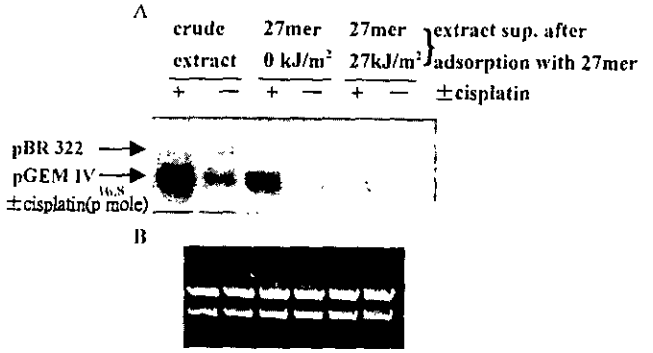

Fig. 3. In vitro excision repair of cisplatin (16.8 pmol)-damaged DNA in the algal extracts collected after affinity adsorption. The extract (50 μg) was incubated at 30°C for 20 min with UV (27 kJ m^{-2})-irradiated or unirradiated 27 mer dsDNA immobilized on the beads, and the beads were spun down at 5000 × *g* at 4°C for 10 min. An aliquot of the supernatant containing 30 μg proteins was tested for its ability to repair cisplatin-treated DNA. The extract (30 μg) incubated with only streptavidin-conjugated beads was also subjected to the repair synthesis assay as a comparison. (A) Autoradiogram of the dried gel and (B) ethidium bromide staining of the gel are shown.

and 8.3 for plasmid DNA damaged by MMC at 0, 1, 2, 4 and 6 nmol, respectively. MMC has been shown to react almost exclusively with the N-2 of guanines to form monoadducts and crosslinks. Unlike the DNA crosslinks formed by cisplatin, the crosslinks induced by MMC do not significantly disturb the DNA helical structure [23]. Therefore, plasmid DNA had to be treated with MMC in the nanomole range in order to generate a detectable repair signal for in vitro DNA repair synthesis assay.

3.2. Affinity adsorption of repair proteins

To test if the repair proteins in cell-free extracts of *C. pyrenoidosa* could be pulled down by affinity adsorption, excision repair of cisplatin-damaged DNA was monitored in the extracts post incubation at 30°C for 20 min with UV (27 kJ m^{-2})-irradiated or unirradiated 27 mer ds DNA insolubilized on the beads. This incubation condition had been proved to be appropriate for specific recognition proteins in *C. pyrenoidosa* extracts to interact with UV-damaged DNA [19]. Extracts incubated with only streptavidin-conjugated agarose beads and extracts collected after incubation with beads carrying unirradiated 27 mer ds-DNA displayed a significant level of damage-dependent repair synthesis. Both extracts showed about an eight to ninefold increase in repair factor after incubation with DNA damaged by cisplatin at 16.8 pmol. In contrast, cisplatin-stimulated repair was almost undetectable in the extracts post incubation with beads carrying UV-damaged 27 mer ds DNA as the repair factor was only 1.2, which signified that very little or no damage-stimulated incorporation of radioactive nucleotides could be detected (Fig. 3, Table 1). When untreated pGEM IV plasmid DNA was incubated with the extracts post incubation with beads carrying UV-irradiated DNA, unirradiated DNA or no ligand as the control for cisplatin-independent semiconservative DNA synthesis, the level of radioactive dAMP incorporated into this plasmid was very close to that incorporated into pBR322 DNA (Fig. 3) with ratios ranging from 0.9 to 1.2 in several experiments. We occasionally found that the extracts preincubated with beads carrying UV-damaged 27 mer gave a compara-

Table 1
Quantitative analysis of cisplatin-induced repair synthesis in *C. pyrenoidosa* protein extracts after affinity adsorption[a]

DNA	DNA band intensity determined by image analysis[a]		
	Crude extracts[b]	Extracts incubated with control 27 mer	Extracts incubated with UV-damaged 27 mer
pBR 322 internal control	1.0	0.82	0.48
Cisplatin (16.8 pmol) -damaged pGEM IV	9.9	6.7	0.58
DNA repair factor (pGEM IV/pBR 322)	9.9 (100%)	8.4 (83%)	1.2 (2.2%)

[a] Each data point represents the average of three separate determinations.
[b] Crude extracts represent the algal extracts that incubated with only streptavidin-conjugated agarose beads.

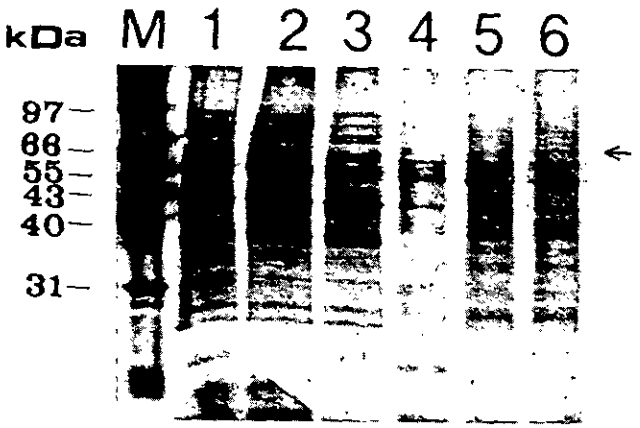

Fig. 4. SDS-PAGE analysis of the proteins captured by affinity adsorption. Biotin-labeled 27 mer irradiated with 0 or 27 kJ m^{-2} of UV were linked to streptavidin-conjugated agarose beads, and the beads were incubated at 30°C for 20 min with algal extracts containing 50 μg protein. After centrifugation at 5000 × *g* at 4°C for 10 min, the supernatant was taken for the repair synthesis assay. The beads were first washed with 20 mM KH_2PO_4 (pH 7.5) containing 0.15 M NaCl at room temperature, and then boiled in 2 × SDS gel loading buffer for 10 min. The proteins in the boiled mixtures were electrophoresed on a 12.5% SDS-polyacrylamide gel and the gel was silver stained. The proteins washed from ligand-free beads and from beads carrying unirradiated or UV-irradiated 27 mer dsDNA are shown in lanes 1, 2 and 3, respectively. M indicates a protein marker composed of proteins with known molecular weights. The proteins bound to ligand-free beads and to beads carrying control or damaged DNA ligand are shown in lanes 4, 5 and 6, respectively.

tively lower level of background incorporation into pBR 322 DNA (Fig. 3), implying that a certain fraction of DNA polymerases might have been adsorbed onto the damaged DNA.

3.3. SDS-PAGE analysis of proteins captured by affinity adsorption

Before analyzing the extract proteins that actually bound UV-irradiated or unirradiated 27 mer dsDNA immobilized on the beads, the beads were washed thoroughly to remove non-specific binding proteins. Subsequently, the beads were boiled in SDS loading buffer to release specific binding proteins. SDS-PAGE analysis and silver staining showed that the polypeptide patterns for non-specific binding proteins washed from the ligand-free beads and from the beads carrying different ligands were very similar (Fig. 4, lanes 1–3). Among the proteins captured by affinity adsorption, a polypeptide with a molecular mass of ~70–72 kDa was found to bind preferentially to UV-damaged DNA (Fig. 4, lane 6, indicated by an arrow) as this polypeptide p72 was captured only on the beads carrying UV-damaged DNA (Fig. 4, lanes 4–6). Since the extracts collected after affinity adsorption with immobilized damaged DNA showed a dramatic decrease in NER capacity, the polypeptide p72 was highly speculated to play a role in NER. Although a polypeptide of ~65 kDa was also found to bind preferentially to the damaged DNA ligand after affinity adsorption, this preferential binding was not stably detected.

4. Discussion

NER is a wide-spectrum DNA repair pathway found in almost all living organisms. Studies of the mechanisms of NER have focused largely on bacteria, yeast and humans, with very minor work on plants. Efficient DNA repair systems, however, must have evolved in plants due to the inability of most plants to minimize environmental exposure to noxious agents like UV [24]. Thus, we are interested in revealing the mechanism of NER in plant cells using unicellular alga *C. pyrenoidosa* as the model system. Since NER requires a coordinated action of several proteins, the repair process would be interfered with if any component of the repair system were lacking. In this study UV-damaged duplex DNA immobilized on agarose beads was incubated with cell-free extracts of *C. pyrenoidosa* to pull down damage-dependent binding proteins and the importance of these proteins in NER was determined by monitoring the difference in NER capacity in the extracts post incubation with insolubilized control and damaged DNA.

Proteins other than those involved in NER, like photolyases responsible for the photoreactivation repair and high mobility group (HMG) proteins, have been shown to bind preferentially to UV-irradiated DNA containing CPDs and 6-4PPs [25,26]. Photoreactivation repair functions in many prokaryotic and eukaryotic cell types, specifically eliminating CPDs or 6-4PPs present in UV-damaged DNA [26]. In this repair pathway, photolyases bind the dipyrimidine photoproducts in the dark, and the covalent bonds connecting two adjacent pyrimidines are opened via the action of photolyases activated by light stimulation. HMG proteins are abundant eukaryotic chromosomal

proteins, which bind more tightly to cisplatin-damaged DNA than to unmodified DNA [27] except the preferential binding of some HMG proteins to UV-damaged DNA [25]. Insolubilized UV-irradiated DNA was selected as the ligand to pull down NER proteins from the algal extracts because cisplatin-treated DNA was not anticipated to give a better specificity in capturing NER proteins. Cisplatin-damaged DNA, however, was considered as a more specific substrate than UV-damaged DNA for monitoring NER by the repair synthesis assay, since radiolabel incorporation detected in UV-damaged DNA may result from repair synthesis linked to UV excision repair and base excision repair. UV excision repair (UVER) is a DNA repair system recently discovered in fungi and bacteria that cleaves only UV-induced CPDs and 6-4PPs [28]. Like NER, the gap produced after incision and excision in UVER is filled by DNA polymerization. DNA polymerization also functions in base excision repair to fill the abasic sites generated after excising minor DNA lesions such as hydrated and ring-fragmented pyrimidines from UV-irradiated DNA by DNA glycosylases [29,30]. In contrast, cisplatin-induced DNA damage was found to be removed primarily by NER in *E. coli* and human cell extracts [31,32], and no repair enzymes specifically cutting cisplatin-damaged DNA have been reported at present. Cisplatin-damaged plasmid DNA was therefore employed as the major repair substrate in this study to avoid detecting repair activity other than NER.

The algal extracts post incubation with beads carrying UV-irradiated ds DNA showed no repair capacity for cisplatin-damaged plasmid DNA, indicating that some NER factors had been depleted by UV-damaged DNA fixed on the beads. Therefore, affinity adsorption with insolubilized UV-damaged DNA should be a simple and an efficient way to capture NER proteins from a cell-free system. When the proteins bound to the insolubilized DNA were analyzed by SDS-PAGE, a polypeptide ~ 72 kDa in molecular mass displayed an apparently higher affinity for damaged DNA than for non-damaged DNA. We previously purified a UV-damaged-DNA binding activity composed of three polypeptides, p72, p80 and p90 from cell-free extracts of *C. pyrenoidosa* [19]. Because the irradiated DNA used for affinity adsorption was the same one used as the probe for mobility shift assay detecting the binding activity except the biotin labeled at the 5′ end, the polypeptide p72 captured by the immobilized DNA should be no different from the p72 contained in the UV-damaged-DNA binding activity. The extracts post incubation with UV-damaged DNA tended to promote a lower absolute radiolabel incorporation into the control pBR 322 DNA than those incubated with unirradiated DNA, suggesting that a fraction of DNA polymerases were pulled down along with the polypeptide p72.

Previous studies revealed that NER in an eukaryotic system like human cells is initiated by the binding of damage-recognition proteins XPA and RPA to the damaged region without the presence of ATP, and the localization of these proteins recruits the binding of a DNA helicase that utilizes the energy released from ATP to unwind the damaged region and the binding of incision proteins to introduce a dual incision at both sides of a lesion [1,3]. The exact function of the polypeptide p72 in NER remains to be determined, yet the inverse correlation between the increase in the amount of p72 captured by the damaged ligand and the drastic reduction in cell-free NER capacity after affinity adsorption strongly suggests its participation in NER. The preferential binding of this polypeptide to the damaged DNA within a short incubation period in the absence of ATP implies that it may play a role in the stage of damage recognition. The UV-damaged DNA ligand used for affinity adsorption has also been shown to be a suitable substrate for *C. pyrenoidosa* extract proteins to introduce a NER-like nucleotide incision in the presence of ATP. The combination of affinity adsorption with in vitro assays detecting DNA incision should be very helpful in identifying the nucleotide incision complex composed of damage-recognition proteins and incision proteins in *C. pyrenoidosa* extracts.

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References

[1] R.D. Wood, DNA repair in eukaryotes, Annu. Rev. Biochem. 65 (1996) 135–167.

[2] P. Møller, H. Wallin, Adduct formation, mutagenesis and nucleotide excision repair of DNA damage produced by reactive oxygen species and lipid peroxidation product, Mutat. Res. 410 (1998) 271–290.

[3] A. Sancar, Excision repair in mammalian cells, J. Biol. Chem. 270 (1995) 15915–15918.

[4] A.S. Balajee, A. May, V.A. Bohr, Fine structural analysis of DNA repair in mammalian cells, Mutat. Res. 404 (1998) 3–11.

[5] A.B. Britt, J.-J. Chen, D. Wykoff, D. Mitchell, A UV-sensitive mutant of *Arabidopsis* defective in the repair of pyrimidine-pyrimidinone (6-4) dimers, Science 261 (1993) 1571–1574.

[6] G.D. Small, Repair systems for nuclear and chloroplast DNA *Chlamydomonas reinhardtii*, Mutat. Res. 181 (1987) 31–35.

[7] G.P. Howland, Dark-repair of ultraviolet-induced pyrimidine dimers in the DNA of wild carrot protoplasts, Nature 254 (1975) 160–161.

[8] N. Degani, E. Ben-Hur, E. Riklis, DNA damage and repair: induction and removal of thymine dimers in ultraviolet light irradiated intact water plants, Photochem. Photobiol. 31 (1980) 31–36.

[9] A.G. McLennan, A.C. Eastwood, An endonuclease activity from suspension cultures of *Daucus carota* which acts upon pyrimidine dimers, Plant Sci. 46 (1986) 151–157.

[10] P.W. Doetsch, W.H. McCray Jr, M.R. Valenzuela, Partial purification and characterization of an endonuclease from spinach that cleaves ultraviolet light-damaged duplex DNA, Biochim. Biophys. Acta 1007 (1989) 309–317.

[11] D.T. Ribeiro, C.R. Machado, R.M.A. Costa, U.M. Praekelt, M.A. Van Sluys, C.F.M. Menck, Cloning of a cDNA from *Arabidopsis thaliana* homologous to the human XPB gene, Gene 208 (1998) 207–213.

[12] A. Strum, S. Lienhard, Two isoforms of plant RAD23 complement a UV-sensitive rad23 mutant in yeast, Plant J. 13 (1998) 815–821.

[13] K. Sugasawa, C. Masutani, F. Hanaoka, Cell-free repair of UV-damaged simian virus 40 chromosomes in human cell extracts I. Development of a cell-free system detecting excision repair of UV-irradiated SV 40 chromosomes, J. Biol. Chem. 268 (1993) 9098–9104.

[14] S. Tateishi, N. Hori, E. Ohtsuka, M. Yamaizumi, Human nucleotide excision nuclease incises synthetic double-stranded DNA containing a pyrimidine dimer at the phosphodiester linkage 3′ to the pyrimidine dimer, Biochemistry 32 (1993) 1541–1547.

[15] E. Braithwaite, X. Wu, Z. Wang, Repair of DNA lesions induced by polycyclic aromatic hydrocarbons in human cell-free extracts: involvement of two excision repair mechanisms in vitro, Carcinogenesis 19 (1998) 1239–1246.

[16] J.-C. Huang, D.L. Svoboda, J.T. Reardon, A. Sancar, Human nucleotide excision nuclease removes thymine dimers from DNA by incising the 22nd phosphodiester bond 5′ and the 6th phosphodiester bond 3′ to the photodimer, Proc. Natl. Acad. Sci. USA 89 (1992) 3664–3668.

[17] E. Seeberg, J. Nissen-Meyer, P. Strike, Incision of ultraviolet-irradiated DNA by extracts of *E. coli* requires three different gene products, Nature 263 (1976) 524–526.

[18] C.-W. Chang, J.-C. Ho, T. Hsu, Thymine-dimer dependent incision on ultraviolet light damaged-DNA in cell-free extracts of *Chlorella pyrenoidosa*, Biosci. Biotech. Biochem. 60 (1996) 490–492.

[19] T. Hsu, J.-C. Ho, C.-C. Chao, Purification of a UV-damaged-DNA binding activity from cell-free extracts of unicellular alga *Chlorella pyrenoidosa*, Plant Sci. 138 (1998) 137–147.

[20] J.D. Weinstein, R.W. Howell, R.D. Leverette, S.Y. Grooms, P.S. Brignola, S.M. Mayer, S.I. Beale, Heme inhibition of δ-aminolevulinic acid synthesis is enhanced by glutathione in cell-free extracts of *Chlorella*, Plant Physiol. 101 (1993) 657–665.

[21] M.M. Bradford, A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[22] D.E. Brash, UV mutagenic photoproducts in *Escherichia coli* and human cells: a molecular genetics perspective on human skin cancer, Photochem. Photobiol. 48 (1988) 59–66.

[23] A.J. Warren, M.A. Ihnat, S.E. Ogdon, E.E. Rowell, J.W. Hamilton, Binding of nuclear proteins associated with mammalian DNA repair to the mitomycin C-DNA interstrand crosslink, Environ. Mol. Mutagen. 31 (1998) 70–81.

[24] E.J. Vonrax, H.L. Mitchell, R. Karthikeyan, I. Chatterjee, B.A. Kunz, DNA repair in higher plants, Mutat. Res. 400 (1998) 187–200.

[25] G.B. Sancar, DNA photolyases: physical properties, action mechanism and roles in dark repair, Mutat. Res. 236 (1990) 147–160.

[26] E.A. Pasheva, I.G. Pashev, A. Favre, Preferential binding of high mobility group 1 protein to UV-damaged DNA, J. Biol. Chem. 273 (1998) 24730–24736.

[27] P.M. Pil, S.J. Lippard, Specific binding of chromosomal protein HMG 1 to DNA damaged by the anticancer drug cisplatin, Science 256 (1992) 234–237.

[28] A. Yasui, S.J. McCready, Alternative repair pathways for UV-induced DNA damage, Bioessays 20 (1998) 291–297.

[29] R.P. Cunningham, DNA glycosylases, Mutat. Res. 383 (1997) 189–196.

[30] R. Stephen Lloyd, Base excision repair of cyclobutane pyrimidine dimers, Mutat. Res. 408 (1998) 159–170.

[31] I. Husain, S.G. Chaney, A. Sancar, Repair of cis-platinum-DNA adducts by ABC excinuclease in vivo and in vitro, J. Bacteriol. 163 (1985) 817–823.

[32] S. Ullah, I. Husain, W. Carlton, A. Sancar, Human nucleotide excision repair in vitro: repair of pyrimidine dimers, psoralen and cisplatin adducts by HeLa cell-free extract, Nucleic Acids Res. 17 (1989) 4471–4484.