Purification of Nuclear Proteins That Bind to Cisplatin-damaged DNA
IDENTITY WITH HIGH MOBILITY GROUP PROTEINS 1 AND 2*

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The biochemical processes responsible for the recognition and repair of cisplatin-damaged DNA in human cells are not well understood. We have developed a damaged DNA affinity precipitation technique that allows the direct visualization and characterization of cellular proteins that bind to cisplatin-damaged DNA. The method separates damaged DNA-binding proteins from complex radiolabeled cell mixtures and further resolves them into individual polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This technique is complementary to gel retardation and Southwestern blotting analyses that have been previously used to identify cellular components that specifically bind to cisplatin-damaged DNA. Using this technique, we have characterized a set of HeLaS3 nuclear proteins of 26.5, 28, 90, and 97 kDa that specifically bind to cisplatin-DNA adducts. Competition studies with soluble cisplatin-damaged DNA confirmed these findings. The major cisplatin-damaged DNA-binding proteins of 26.5 and 28 kDa recognized adducts of DNA modified with cisplatin but not with its trans isomer or with UV radiation. These proteins were purified 450-fold to near homogeneity by ion-exchange and cisplatin-damaged DNA affinity chromatography. Amino-terminal sequence analysis showed that the 26.5- and 28-kDa proteins were identical to high mobility group (HMG) proteins HMG-2 and HMG-1, respectively.

Cis-Diaminedichloroplatinum (II) or cisplatin has emerged as one of the most widely prescribed chemotherapeutic agents used in combination with other antineoplastic drugs and with ionizing radiation to treat patients with locoregionally advanced malignancies (1–4). Cellular DNA is generally thought to be the critical biologic target for cisplatin-mediated cell killing (5). A number of different intrastrand and interstrand DNA adducts are formed following cisplatin exposure of mammalian cells (6–10). Although correlations between cell survival and DNA adduct removal have been reported (11–15), the biochemical mechanisms responsible for recognition and processing of these lesions have not been well-characterized.

An important step in elucidating these processes has been the recent discovery of intracellular factors that bind to damaged DNA restriction fragments. By use of gel retardation analysis, Chu and Chang (16) identified an XPE-binding factor as a nuclear protein that recognizes UV-irradiated DNA, undamaged ss'DNA, cisplatin-damaged DNA, and undamaged dsDNA with decreasing affinities. XPE-binding factor expression is increased in cisplatin-resistant HeLa and HT1080 cells. Chu and Chang (17) also described a cisplatin-cross-linked (CCD) binding factor present in the cytoplasm and nucleus of HeLa and HT1080 cells. In contrast to XPE-binding factor, CCD binding expression is not increased in cisplatin-resistant cells. Toney et al. (18) used Southwestern blotting to identify HeLa cell cytosolic factors of 28 and 100 kDa that selectively bind to DNA damaged by chemotherapeutically active platinum (II) compounds, but not to undamaged DNA or DNA modified with clinically ineffective platinum compounds. The 100-kDa protein apparently binds to 1,2-intrastrand d(GpG) and d(ApG) cross-links formed by cisplatin (19). By use of the same techniques, Andrews and Jones (20) identified nuclear proteins of 26, 28, and 97 kDa that bind to cisplatin-damaged and 1,2-diaminocyclobexane platinum (II)-damaged DNA; the expression of these proteins was similar in sensitive and resistant human ovarian carcinoma cells. The correspondence between these proteins identified by Southwestern and gel retardation analysis is unknown.

In this report we describe an assay that allows the direct visualization and characterization of cellular proteins that specifically bind to damaged DNA and/or interact with a protein-nucleic acid complex. This damaged DNA affinity precipitation assay is analogous to immunoprecipitation/gel electrophoretic techniques and is complementary to current molecular biologic techniques that identify sequence-specific transcription factors and damaged DNA-binding factors (21, 22). By use of this assay, we have characterized a set of HeLaS3 nuclear proteins that specifically bind to cisplatin-damaged DNA but not to undamaged DNA. The major cisplatin-damaged DNA-binding proteins of 26.5 and 28 kDa were preparatively purified to near homogeneity by ion-exchange and cisplatin-damaged DNA affinity chromatography. Sequence analysis of 21 NH2-terminal amino acids showed that the 26.5-kDa protein is identical to high mobility group (HMG) protein -2 while the 28-kDa protein is identical to HMG-1. These results suggest that HMG-1 and -2 proteins preferentially recognize CDDP-damaged DNA. Hence, these proteins may participate in the repair of CDDP-damaged DNA, in addition to their many postulated functions which include general chromatin assembly proteins and specific transcriptional activators (23–27).

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Cis-Diaminedichloroplatinum (II); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CCD, cisplatin cross-linked; HMG, high mobility group.

1The abbreviations used are: ss, single-stranded; ds, double-stranded; CDDP, cis-diaminedichloroplatinum (II); TDPP, trans-diaminedichloroplatinum (II); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CCD, cisplatin cross-linked; HMG, high mobility group.
Experimental Procedures

Chemicals—EN'HANCE and L-[35S]methionine (>1000 Ci/mmol) were purchased from American Type Culture Collection (Rockville, MD). Calf thymus ds- and ssDNA cellulose were obtained from Sigma. Prestained protein standards were purchased from Sigma and Bio-Rad. Q Sepharose was obtained from Pharmacia LKB Biotechnology Inc. P-11 phosphocellulose was provided by Du Pont-New England Nuclear. Calf thymus ds- or ssDNA cellulose were obtained from Aldrich Chemical Co. Fetal bovine serum came from Hazelton Biologies (Lenexa, KS).

Cell Culture Techniques—The HeLaS3 line was obtained from the American Type Culture Collection (Rockville, MD). Monolayer cell cultures were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in a 5% CO2 humidified atmosphere. Suspension cultures were grown in Joklik's minimal essential medium lacking methionine but supplemented with 10% fetal bovine serum. The HeLaS3 cell line was routinely found free of Mycoplasma contamination (28).

Cell Labeling and Preparation of Extracts—Subconfluent monolayers of cells were washed three times each with 25 ml of warm Hank's balanced salt solution and incubated for 2 h at 37 °C with 4 ml of Eagle's minimal essential medium lacking methionine but containing 20 mM HEPES, pH 7.4, and 125 µCi/ml [35S]methionine (C1000Ci/mmol, Du Pont-New England Nuclear). Subsequently, the cells were washed twice in cold Hank's balanced salt solution. Nuclear and cytoplasmic extracts were prepared by the method of Dignam et al. (29) except that buffer A contained 0.5% Nonidet P-40 and 1 mM phenylmethylsulfonyl fluoride. Instead of the Dounce homogenization step, cells were digested by vortex for 10 s. Protein was determined by the bicinchoninic acid method (30). Nuclear and cytoplasmic extracts contained approximately 0.3 and 1.2 mg/ml protein, respectively, with a specific activity of 1×10⁶ cpm/mg protein.

Whole cell extracts were prepared by incubating cells in 20 mM HEPES, pH 7.9, 0.42 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 25% glycerol, and 0.5% Nonidet P-40 for 30 min on ice. Extracts were clarified by centrifugation at 13,000×g for 30 min at 4 °C.

Preparation of Cisplatin-damaged DNA Cellulose—Aliquots of calf thymus ds- or ssDNA cellulose were suspended in 1 mM phosphate, pH 7.4, 3 mM NaCl, and different concentrations of cisplatin. The DNA concentration of the cellulose suspension was 1.05×10⁻³ M nucleotide-phosphate as measured by the method of Alberts and Henner (31). The initial cisplatin concentration ranged from 1×10⁻⁶ M to 1×10⁻⁴ M. Following incubation with gentle rocking at 37 °C for 24 h in the dark, the DNA cellulose suspensions were washed five times in 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 500 mM NaCl, and 0.5 mM dithiothreitol. The DNA cellulose suspensions were washed once in 20 mM Tris-HCl, pH 8.0, and 50 mM NaCl, and stored at 4 °C. Experiments with trans-diaminodichloroplatinum were performed under identical conditions.

The molar ratio of free cisplatin to nucleotide-phosphate at the onset of incubation, R₀, determines the molar ratio of bound platinum to nucleotide-phosphate (32). The concentrations of cisplatin and nucleotide-phosphate for typical experiments yielded an R₀ value of 0.05, unless otherwise indicated.

Cisplatin modification of calf thymus ds- or ssDNA was performed as described (18). The DNA preparations were sheared prior to use in competition assays by the method of Chu and Berg (33).

Cisplatin-damaged DNA Affinity Precipitation Assay—Extracts (3.0×10⁶ cpm or 300 µg of cell protein) from cells biosynthetically labeled with [35S]methionine were preabsorbed (0 °C, 60 min) with 600 µg of undamaged dsDNA cellulose. The suspension was clarified by centrifugation at 13,000×g (4 °C, 5 min). The supernatant (3×10⁶ cpm) was mixed (0 °C, 60 min) with a 30-µl pellet of DNA cellulose containing 10.5 mg of damaged DNA, whose nonspecific protein-binding sites had been blocked by incubation in 20 mM Tris-HCl, pH 8.0, 1% bovine serum albumin, and 5% nonfat dry milk (25 °C, 50 min). The supernatant buffer was 20 mM HEPES, pH 7.9, 0.2 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 25% glycerol. The suspension was washed five times in 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 500 mM NaCl, 0.5 mM dithiothreitol, and 10% glycerol. The suspension was washed once in 20 mM Tris-HCl, pH 8.0, and 50 mM NaCl, and stored at 4 °C. Experiments with cisplatin-damaged DNA cellulose were performed under identical conditions.

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (34) was carried out with 10% gels (0.75×160×180-mm gels, Figs. 1–3, and 5), with 12.5% gels (Figs. 6–8), or with 12.5% minigels (0.75×75×100 mm, Fig. 4). Fluorography was performed with EN'HANCE according to the manufacturer's instructions. The preassembled protein standards, β-galactosidase (Mr = 116,000), phosphorylase b (Mr = 16,000), fructose-6-phosphate kinase (Mr = 84,000), bovine serum albumin (Mr = 68,000), ovalbumin (Mr = 49,500), furamase (Mr = 48,500), carbonic anhydrase (Mr = 32,500), soybean trypsin inhibitor (Mr = 24,000), and lysozyme (Mr = 18,500) were used to calculate the apparent molecular weights of the DNA-binding proteins. The numbers on the left indicate the apparent molecular weights (Mr) of prestained protein standards.

FIG. 1. Analysis of HeLaS3 nuclear proteins precipitated by different cellulose matrices. Aliquots of nuclear extracts (3.5×10⁶ cpm) were added to the indicated cellulose matrices. [35S] Methionine-labeled proteins precipitated by each preparation were analyzed on 10% polyacrylamide gels as described under "Experimental Procedures." Lane 1, 1.3×10⁶ cpm of crude nuclear extract; lane 2, proteins precipitated with undamaged dsDNA cellulose; lane 3, proteins precipitated with dsDNA cellulose damaged by incubation with CDDP; lane 4, proteins precipitated with dsDNA cellulose damaged by incubation with cisplatin; lane 5, proteins precipitated with dsDNA cellulose damaged by incubation with trans-diaminodichloroplatinum; lane 6, proteins precipitated with dsDNA cellulose damaged by incubation with cisplatin and CDDP; lane 7, proteins precipitated with dsDNA cellulose damaged by incubation with cisplatin and trans-diaminodichloroplatinum; lane 8, proteins precipitated with dsDNA cellulose damaged by incubation with cisplatin, CDDP, and trans-diaminodichloroplatinum.
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Fig. 2. Correlation between extent of protein binding and level of cisplatin-DNA adduct formation. dsDNA cellulose was incubated with different concentrations of cisplatin at 37 °C for 24 h in the dark. The [35S]methionine-labeled HeLaS3 nuclear proteins precipitated by the damaged DNA affinity assay were visualized as described in Fig. 1. Lane 1, 2 × 10⁸ cpm of crude nuclear extract; lanes 2–8, inclusive show the proteins precipitated by dsDNA cellulose damaged by increasing concentrations of cisplatin; lane 2, 0.001 mM; lane 3, 0.003 mM; lane 4, 0.01 mM; lane 5, 0.03 mM; lane 6, 0.1 mM; lane 7, proteins precipitated by undamaged ds DNA cellulose; lane 8, proteins precipitated by cellulose alone incubated with 1.0 mM cisplatin. The arrows on the right indicate the positions of the 26.5-, 28-, 90-, and 97-kDa DNA-binding proteins.

Fig. 3. Specificity of the major cisplatin-damaged DNA-binding proteins of 26.5 and 28 kDa. [35S]Methionine-labeled HeLaS3 nuclear extracts (3 × 10⁶ cpm) were added to each of the following DNA cellulose matrices: 1) dsDNA cellulose damaged by incubation with 0.03 mM CDDP; 2) ssDNA cellulose damaged with 0.05 mM CDDP; 3) undamaged dsDNA cellulose; 4) undamaged ssDNA cellulose; 5) dsDNA cellulose damaged by incubation with 0.3 mM trans-diamininedichloroplatinum (TDDP); 6) dsDNA cellulose damaged with 0.03 mM TDDP; 7) dsDNA cellulose damaged by UV radiation of 9000 J/m² with a General Electric 15-W germicidal lamp (maximum output at 254 nm) calibrated with a YSI-Kettering radiometer at a flux of 5 J/(m²s). The [35S]methionine-labeled proteins precipitated by each damaged DNA cellulose preparation were visualized as described in Fig. 1. The arrows indicate the positions of the 28- and 26.5-kDa proteins in this section of the autoradiogram.

Fig. 4. Competitive binding with soluble cisplatin-damaged or undamaged DNA. DNA competition assays were performed by adding different amounts of soluble competitor DNA to the binding reactions of the standard cisplatin-damaged DNA affinity precipitation assay before the addition of [35S]methionine-labeled HeLaS3 nuclear extracts (3 × 10⁶ cpm). Soluble competitor DNA and DNA cellulose were modified to B values of 0.03. Proteins were analyzed with 12.5% minigels. The amounts of cisplatin-damaged DNA (Pt-DNA) or undamaged DNA added as competitor, are indicated. The arrows indicate the positions of the 26.5- and 28-kDa proteins. A and B, samples mixed with cisplatin-damaged dsDNA cellulose containing dsDNA as competitor (A) or ssDNA as competitor (B); C and D, samples mixed with cisplatin-damaged ssDNA cellulose with dsDNA as competitor (C) or ssDNA as competitor (D). Quantitative tracings of individual lanes of the same fluorogram were performed with an LKB Ultrascan XL densitometer.

RESULTS

Identification of Human Cell Proteins That Bind to Cisplatin-damaged DNA—The interaction of HeLaS3 cellular proteins with genomic DNA damaged by cisplatin was studied by use of the damaged DNA affinity precipitation technique. Fig. 1 shows the pattern of HeLaS3 nuclear proteins that bound to different cellulose matrices containing native and damaged DNA. Comparison of these complex protein patterns defined five classes of binding proteins: 1) major proteins of 26.5 and 28 kDa substantially enriched by binding to cisplatin-damaged DNA; 2) minor proteins of 90 and 97 kDa that bound to cisplatin-damaged DNA, as well as a group of lower molecular weight proteins that migrated near the dye front; 3) “nonspecific” major proteins of 58 and 105 kDa, and minor proteins of 43, 50, and 66 kDa that were precipitated by each of the different matrices; 4) major proteins of 20, 25, and 31 kDa enriched by binding to cellulose alone treated with 30-fold excess cisplatin; 5) a minor protein of 70 kDa precipitated by...
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**Fig. 5.** Cellular localization of the cisplatin-damaged DNA-binding proteins. Aliquots (3 \times 10^6 cpm) of nuclear extracts (lanes 2–4) and cytoplasmic extracts (lanes 6–8) from [\textsuperscript{35}S]methionine-labeled HeLa-S3 cells were incubated with different cellulose matrices. The precipitated proteins were visualized as described in Fig. 1. Lane 1, 1.5 \times 10^6 cpm crude nuclear extract; lane 5, 1.5 \times 10^6 cpm crude cytoplasmic extract; lanes 2 and 6, proteins precipitated by dsDNA cellulose damaged with 0.03 mM cisplatin; lanes 3 and 7, proteins precipitated by cellulose alone damaged with 0.03 mM cisplatin; lanes 4 and 8, proteins precipitated by undamaged dsDNA cellulose.

**Fig. 6.** Panel A, chromatography of HeLaS3 nuclear proteins on phosphocellulose. Approximately 120 ml of a 420 mM NaCl extract of HeLaS3 nuclei was applied to a P-11 phosphocellulose column. The column was washed and eluted as described under "Experimental Procedures". Fractions of 9 ml each were collected (fractions 1–30), followed by fractions of 3 ml. Panel B, electrophoretic analysis. Fractions from phosphocellulose chromatography were analyzed by electrophoresis in a 12.5% gel with 0.1% sodium dodecyl sulfate under reducing conditions (34). The gel was stained with Coomassie Brilliant Blue R250. The samples were from the corresponding column fractions.

Cisplatin-damaged DNA-binding Proteins—A series of competitive binding experiments was performed to study further the specificity of binding of the 26.5-, 28-, 90-, and 97-kDa proteins. For these experiments, limiting amounts of [\textsuperscript{35}S]methionine-labeled HeLaS3 nuclear extract were incubated with a fixed amount of cisplatin-damaged DNA cellulose in the presence of increasing amounts of soluble competitor DNA. Specific protein binding to cisplatin-damaged dsDNA cellulose was effectively competed by increasing quantities of soluble ds- or ssDNA modified by cisplatin to the same extent (Fig. 4, A and B). The 90- and 97-kDa proteins were poorly resolved in the minigel system. To obtain a quantitative estimate of the amount of protein bound, the autoradiograms were scanned with a densitometer. Binding of the 26.5- and 28-kDa proteins was inhibited by 50% at a greater than 5-fold molar excess of competitor dsDNA modified by cisplatin, while complete inhibition of 26.5- and 28-kDa protein binding was observed at a 100-fold excess of competitor cisplatin-damaged DNA (Fig. 4, A and B). Likewise, 26.5- and 28-kDa protein binding to cisplatin-damaged ssDNA cellulose was specifically inhibited by increasing quantities of soluble ds- or ssDNA modified by...
precipitation of HeLaS3 nuclear proteins with the cisplatin-protein had the same binding specificity as the 28-kDa nuclear protein. It remains to be established whether the proteins were localized in the nucleus and absent in the cytoplasm (Fig. 5). This distribution was independent of whether the cisplatin-damaged DNA affinity precipitation assay was performed. The fractions of interest were pooled, concentrated by negative pressure dialysis, and analyzed with the protein competition assay, as described under "Experimental Procedures." The fractions that eluted with 450 and 465 mM NaCl washed were combined and subjected to ion-exchange chromatography on Q-Sepharose. This step resulted in further purification as well as separation of the 26.5- and 28-kDa proteins in their native state was achieved by binding to cisplatin-damaged DNA cellulose followed by elution with KI. Gel electrophoretic analysis of the proteins eluting with KI demonstrated major polypeptides of 26,500 and 28,000 daltons (Fig. 8). There was minor cross-contamination of the 28-kDa fraction with the 26.5-kDa protein. A minor proteolytic degradation fragment of 25 kDa was occasionally found in the 26.5-kDa protein fraction. We estimated that the overall recovery of the 26.5- and 28-kDa proteins was approximately 65%; thus, each cisplatin-damaged DNA-binding protein has been purified about 450-fold from the nuclear extract (Table I).

**Cellular Localization of the Cisplatin-damaged DNA-binding Proteins**—In HeLaS3 cells, the 26.5-, 90-, and 97-kDa proteins were localized in the nucleus and absent in the cytoplasm (Fig. 5). This distribution was independent of whether the cisplatin-damaged DNA affinity precipitation assay was normalized for protein content or [35S]methionine incorporation. The cytosolic 28-kDa cisplatin-damaged DNA-binding protein had the same binding specificity as the 28-kDa nuclear protein. It remains to be established whether the proteins found in the nuclear and cytosolic extracts are identical.

**Purification of the 26.5- and 28-kDa Proteins**—Preparative precipitation of HeLaS3 nuclear proteins with the cisplatin-damaged DNA affinity technique revealed that the 26.5- and 28-kDa proteins were the major polypeptide components (data not shown). Chromatography with phosphocellulose was a critical step in which the 26.5- and 28-kDa proteins were enriched 130-fold from the crude HeLaS3 nuclear extract. The cisplatin-damaged DNA-binding proteins of 26.5 and 28 kDa were eluted with 450, 465, and 550 mM NaCl (Fig. 6). The individual column fractions were analyzed by gel electrophoresis. Fractions of interest were pooled, concentrated by negative pressure dialysis, and analyzed with the protein competition assay, as described under "Experimental Procedures." The fractions that eluted with 450 and 465 mM NaCl washes were combined and subjected to ion-exchange chromatography on Q-Sepharose. The samples were from the corresponding column fractions.

cisplatin (Fig. 4, C and D). In contrast, undamaged competitor DNA had much less effect on protein binding. The specific binding of the 26.5- and 28-kDa proteins was only partially inhibited at a 100-fold excess of undamaged competitor DNA (Fig. 4, A–D).

**Amino Acid Sequence Analysis**—To establish the identity of the 26.5- and 28-kDa cisplatin-damaged DNA-binding proteins (purified by ion-exchange chromatography followed by binding to cisplatin-damaged DNA cellulose, see above) NH2-terminal amino acid sequence analysis of the purified proteins was performed. The sequence of these proteins is shown in Fig. 9. A search of GenBank revealed that the 26.5-kDa protein is identical to HMG protein 2 and the 28-kDa protein to HMG-1. The primary structures of HMG-1 and -2 have been determined previously (36, 37). There was a significant decrease in yield in sequencing cycles 13 and 14, suggesting
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**TABLE 1**

Purification of the 26.5- and 28-kDa cisplatin-damaged DNA-binding proteins

| Purified fraction | Volume | Total protein | Specific activity | Cisplatin-damaged DNA binding activity |
|-------------------|--------|---------------|-------------------|----------------------------------------|
| Crude nuclear extract | 120 ml | 259 mg | unit/mg x 10^-4 | 1.5 x 10^4 |
| Phosphocellulose | 77 ml | 1.62 mg | 1.9 x 10^4 | 3.85 x 10^4 |
| Q-Sepharose 26.5 kDa | 3.1 mg | 0.25 mg | 5.6 x 10^4 | 1.4 x 10^4 |
| 28 kDa Cisplatin-DNA cellulose | 5.0 mg | 0.27 mg | 5.2 x 10^4 | 1.4 x 10^4 |
| 26.5 kDa | 0.6 mg | 0.18 mg | 6.7 x 10^4 | 1.25 x 10^4 |
| 28 kDa | 0.6 mg | 0.19 mg | 6.7 x 10^4 | 1.27 x 10^4 |

**Fig.** NH2-terminal amino acid sequences of the purified cisplatin-damaged DNA-binding proteins. The partially purified 26.5- and 28-kDa proteins from Q-Sepharose chromatography were separated by polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane and sequenced with a model 475B gas-phase sequencer connected to a model 20A PTH analyzer (Applied Biosystems, Inc. Foster City, CA). The NH2-terminal sequences of HMG-1 and -2 are shown for comparison. Within the initial 21 amino acids, HMG-1 and -2 differ only at position 6.

that an unusual amino acid feature (e.g. phosphorylation) was present at these serine residues.

**DISCUSSION**

A set of HeLaS3 nuclear proteins that bind to cisplatin-damaged DNA has been characterized by use of a damaged DNA affinity precipitation technique. Major proteins of 26.5 and 28 kDa and minor proteins of 90 and 97 kDa were found to bind specifically to adducts of cisplatin-damaged DNA. Specificity for the 26.5- and 28-kDa protein binding to cisplatin-damaged DNA was established by several criteria: 1) competition for binding was observed with cisplatin-damaged DNA, but not undamaged DNA; 2) a correlation between the extent of protein binding with level of DNA adduct formation; and 3) these proteins did not recognize DNA adducts formed by treatment with trans-diaminodichloroplatinum (II) or UV irradiation. The lack of binding to DNA modified by the trans-isomer is particularly important since trans-diaminodichloroplatinum (II) cannot form intrastrand cross-links between adjacent nucleotides and is chemotherapeutically ineffective (35). Similar levels of 26.5- and 28-kDa protein binding to ds- and ssDNA cellulose damaged by cisplatin was observed, suggesting that these proteins specifically recognize intrastrand adducts. Amino acid sequence analysis of the purified polypeptides revealed that the 26.5- and 28-kDa cisplatin-damaged DNA-binding proteins are identical to HMG-2 and -1, respectively. Interestingly, cisplatin has been shown to selectively cross-link HMG-1 and -2 to DNA in micrococcal nuclease accessible regions of chromatin in isolated nuclei (38).

By use of molecular biologic techniques, several investigators have identified human cellular factors that recognize cisplatin-damaged DNA. Chu and colleagues (16, 39) identified XPE-binding factor as a nuclear protein of unspecified size that is absent in some, but not all, xeroderma pigmentosum group E cells. Biochemical and genetic studies showed that XPE-binding factor is homologous to yeast photolyase (40). Hirschfeld et al. (41) found a protein with similar properties that is synthesized at higher levels in UV-irradiated primate cells. Although expression of XPE-binding factor is increased in the cisplatin-resistant HeLa-R1 and HeLa-R3 cells, it preferentially recognizes UV-damaged DNA (17). These findings were confirmed by Chao et al. (42). The binding specificity of XPE factor distinguishes it from the major 26.5-kDa (HMG-2) and 28-kDa (HMG-1) binding proteins found in the HeLaS3 nuclear compartment. In addition, preliminary studies have demonstrated similar expression of the 26.5- and 28-kDa proteins in xeroderma pigmentosum groups A, B, C, D, E, and H lymphoblastoid cells. Chu and colleagues (17) and Fujiwara et al. (43) also identified cisplatin-cross-linked DNA (CCD) binding factor by gel mobility shift assays with a cisplatin-damaged DNA probe. The major 28-kDa cisplatin-damaged DNA-binding protein (HMG-1) had a number of features in common with CCD-binding factor, including equivalent distribution in nuclear and cytoplasmic subcellular fractions, preferential binding to DNA damaged with cisplatin but not UV radiation, and similar expression in cisplatin-resistant HeLa-R1 and HeLa-R3 cells.

The 26.5-, 28-, 90-, and 97-kDa proteins are likely the same as those proteins in the 28-100 kDa range which have been identified with Southwestern blotting in HeLa cells (18) and the 26-, 28-, and 97-kDa proteins in human ovarian carcinoma cells (20). Whether the 90-kDa protein represents a proteolytic degradation product of the 97-kDa protein or a distinct gene product remains to be determined. In contrast to these studies, the 26.5- and 28-kDa proteins were the major species identified by the damaged DNA affinity precipitation technique. The recovery of bound proteins was complete, as treatment of the protein-DNA complexes with DNase I prior to sodium dodecyl sulfate-polyacrylamide gel electrophoreses resulted in identical findings (data not shown). The different results obtained between the Southwestern blotting method and the cisplatin-damaged DNA affinity precipitation technique may be attributed to several factors including: 1) the number of methionine residues in the individual proteins; 2)
altered rates of specific protein turnover; 3) preferential binding of native versus denatured/renatured protein to cisplatin-damaged DNA; 4) differential electrotor flow of the 90- and 97-kDa proteins onto nitrocellulose membranes; 5) use of genomic versus restriction fragment length DNA; 6) cooperative binding as a result of native protein-protein or protein-DNA interactions; and 7) the ability of the 26.5- and 28-kDa protein binding to withstand elution with buffers of ionic strength equivalent to 500 mM NaCl.

The characteristics of the 26.5-kDa (HMG-2) and 28-kDa (HMG-1) cisplatin-damaged DNA-binding proteins also distinguish them from a number of other base damage recognition proteins, including factors that bind to DNA modified by depurination (44-46), N-acetoxyacetylaminofluorene, methylnitrosourea, and methylnitrosourea (47), UV radiation, x radiation, nitrous acid, and sodium bisulphite (48), UV radiation and N-acetoxyacetylaminofluorene (49), and x radiation (50) treatment.

The structure, function, and intracellular localization of high mobility group proteins have been extensively studied (25). HMG-1 and -2 have been shown to be associated with nucleosomes of active genes (24), to participate in nucleosome assembly (25), and to stimulate transcription of class II and III genes (25, 27). These properties are consistent with their capacity to bind more strongly to single-stranded than double-stranded DNA (51), to destabilize or unwind double-stranded DNA (52), and to induce negative supercoiling in relaxed plasmids (53). The ability of HMG-1 and -2 to preferentially recognize cisplatin-modified native DNA suggests a role for such proteins in excision repair. For example, the binding of HMG-1 and -2 to cisplatin-damaged DNA may result in local melting with displacement of histones allowing increased accessibility of DNA repair enzymes. By analogy, HMG-1 and -2 have been shown to stimulate transcription in a nonspecific manner, perhaps by alteration of the DNA template in order to permit increased accessibility by RNA polymerases II and III as well as other transcription factors (26). The ability of HMG-1 and -2 to preferentially bind to single-stranded DNA may be important in this regard (51). Another single-stranded DNA-binding protein, SSB (also called RP-A and RF-A), has been shown to be required for human DNA excision repair in a cell-free system (54). A role for DNA-binding proteins in the damage recognition step that precedes incision has been suggested by Hélène and co-workers (55) on the basis of the relative affinities of T4 gene 32 protein for unmodified native DNA and DNA modified with chemical adducts, including cisplatin. The T4 gene 32 protein is also involved in DNA replication, recombination, and the repair of UV damage (56). Alternatively, the clinical efficacy of cisplatin may be associated with the sequestration of HMG-1 and -2 by the preferential binding of these proteins to cisplatin-damaged DNA, thereby restricting their participation in replication and/or transcription.

In summary, the development of a damaged DNA affinity precipitation assay has led to the identification and characterization of a set of human intracellular proteins that specifically bind to cisplatin-damaged DNA. The major proteins recognizing such damage were identified as HMG-1 and -2 by amino-terminal sequence analysis of the purified proteins, suggesting a possible role for these abundant proteins in DNA repair. Based upon our protein purification results (Table 1), we estimated that there were $6 \times 10^5$ HMG-1 molecules/cell capable of binding to cisplatin-damaged DNA. A similar number of HMG-2 molecules/cell was also calculated, and the findings are in keeping with previously reported determinations of HMG-1 and -2 amounts of $10^5$-10$^6$/cell (23). General application of the method provides a new and powerful tool for studies of protein-damaged DNA interactions. First, considerable biochemical and cell biological information about individual proteins in their native state can be obtained without resorting to antibody production or gene cloning. Second, damaged DNA affinity chromatography can be used for the preparative isolation of binding factors for further biochemical, immunologic, and genetic studies.

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