High mobility group proteins 1 and 2 recognize chromium-damaged DNA

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Cr(III) covalently binds to DNA, producing DNA strand breaks and 8-hydroxyguanosine (3). Free radical production, resulting from Cr(VI) reduction, also results in NF-κB activation in intact cells (9). Cr(III) covalently binds to DNA, producing DNA and DNA → protein cross links (2,3,6). Further, Cr bound to DNA increases non-specific DNA and RNA polymerase activity and results in premature DNA polymerase arrest, which is thought to result from the production of ‘Cr-mediated’ interstrand crosslinks (10). Hence, the genotoxic activity of Cr likely results from: (i) the production of reactive oxygen species accompanying the reduction of Cr(VI) to Cr(V), Cr(IV) and Cr(III) species; (ii) the direct binding of Cr(III) and possibly Cr(V) to DNA.

The high mobility group (HMG) proteins are a family of low molecular weight chromatin proteins which are widespread and highly conserved in eukaryotic organisms (11). While the functions of these proteins have not been fully elucidated, they are believed to play an important role in chromatin structure/function and affect the binding of H1 histone to DNA (11). HMG1 and HMG2 are structurally similar and members of the HMG box family of proteins. HMG boxes are DNA binding domains containing ~80 amino acids which have been identified in a large number of proteins involved in the control of gene expression (reviewed in 12). A characteristic feature of all HMG box proteins is their ability to recognize bent DNA structures (12). Previous work has demonstrated that HMG1, HMG2 and other HMG box-containing proteins recognize DNA modified by the cancer chemotherapy drug cisplatin (13,14). However, the ability of these proteins to recognize DNA damaged by other agents has not been extensively examined. In the current study, we have investigated the interaction of HMG proteins with chromium-damaged DNA (Cr-DNA). Our results demonstrate that these proteins bind Cr-DNA, suggesting that they are involved in the recognition of Cr-DNA in intact cells.

**Materials and methods**

**Chemicals**

DNA → cellulose, CrCl3 and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma (St Louis, MO).

**Preparation of Cr-DNA**

CrCl3 was dissolved in water at a concentration of 0.1 mM and the solution allowed to stand overnight at 20°C. Aliquots of 175 mg DNA → cellulose (4 mg DNA/g cellulose) were incubated with 0–100 μM CrCl3 (pH 6.8) in a total volume of 10 ml overnight. The DNA-cellulose was washed twice in distilled water, twice in 20 mM Tris, pH 6.8, 500 mM NaCl, 10% glycerol, 0.1% NP-40, 0.5 mM dithiothreitol (DTT) and finally in 10 mM Tris, pH 7.5, and stored at 4°C. The Cr-DNA preparation is stable for at least 1 week.

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fetal bovine serum and subsequently treated with 0, 10, or 100 μM potassium chromate for 3 h. After treatment, the cells were pelleted by centrifugation, resuspended in 5 ml 25 mM Tris, pH 7.5, 25 mM EDTA, 100 mM NaCl. The cells were lysed by addition of SDS (final concentration 0.5%) and incubated with proteinase K (10 μg/ml) for 2 h on a rocker platform. Samples were extracted with phenol/chloroform and the DNA was precipitated by the addition of 2 vol ethanol.

### Preparation of labeled DNA

A 100 bp fragment containing the multiple cloning region and 50 bp of 5’ flanking region of pUC18 was amplified by PCR and cloned into a TA cloning vector. For preparation of probe, plasmid DNA was digested with EcoRI, end-filled with [α-32P]dATP at the 5’ overhang using Klenow fragment, run on a 1% agarose gel and subsequently purified from the gel using a GenElute agarose spin column (Supelco, Bellefonte, PA). Five nanograms of the labeled fragment were incubated with CrCl3 and subsequently spun through a Sephadex G50 spin column to remove unbound Cr. The DNA samples were run on 5% polyacrylamide gels, using a high ionic strength buffer system (50 mM Tris–HCl, pH 8.5, 0.38 M glycine and 1 mM EDTA), the gels dried and autoradiographed (16).

### HMG protein purification and preparation of antiserum

Calf thymus tissue was obtained from a local slaughter house and immediately frozen in liquid nitrogen. HMG proteins were purified from calf thymus by perchloric acid extraction, acetone precipitation and ion exchange chromatography as described (17). Polyclonal antiserum to HMG1 was prepared in chickens using purified protein as antigen and purified by affinity chromatography (18,19).

### Preparation of tissue extracts

Calf thymus tissue extracts were prepared by homogenization in 20 mM HEPES, pH 7.9, 0.2 mM EDTA, 450 mM NaCl, 0.5 mM DTT, 1.5 mM MgCl2, 25% glycerol and 0.5 mM PMSF and clarified by centrifugation (12,000 g for 45 min at 4°C). The supernatant was dialyzed against 20 mM Tris, pH 7.5, 50 mM NaCl and 0.1 mM DTT and centrifuged (10,000 g for 30 min at 4°C). Samples were stored at −80°C until use.

### Damaged DNA binding assay

Protein samples (purified protein and tissue extracts) were mixed with undamaged DNA− and Cr-DNA−cellulose and incubated (17 h at 4°C) on a rotating platform, positioned 60° from the horizontal axis, and subsequently centrifuged (3000 g for 2 min). The pellets were washed three times with 3 ml wash buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 0.5 mM DTT, 10% glycerol, 0.1% NP-40) containing 0.15–0.5 M NaCl, followed by one wash with 10 mM Tris, pH 7.5 (13,17). Protein bound was extracted from the DNA−cellulose pellets by incubation with SDS gel sample loading buffer [125 mM Tris, pH 6.8, 10% 2-mercaptoethanol, 4% SDS and 20% glycerol (100°C for 3 min)] and analyzed on 12.5% SDS−polyacrylamide gels (20). Dissociation constants were determined by Scatchard analysis (17,21).

### Protein binding was also assessed using a solid phase binding protocol. DNA was isolated from cells which were untreated or treated with K2CrO4 as described above. The DNA was sheared by repeated passage through a 21 gauge needle and applied to a polysine-coated 96-well microtiter plate (10 μg/well; 22). The plates were incubated overnight at 4°C and washed with phosphate-buffer(10 mM Tris−HCl, pH 8.5, 0.38 M glycine and 1 mM EDTA) buffer containing 1% bovine serum albumin (BSA) and subsequently centrifuged at 3000 g for 2 min. The pellets were washed three times with PBS containing 0.1% Tween 20 and blocked with Tris-buffered saline (TBS) containing 5% bovine serum albumin (BSA) and incubated with anti-HMG antibody (diluted 1:1000 in TBS, 1% BSA) for 2 h. The plates were washed and incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-chicken IgG. Bound antibody was detected using o-phenylenediamine dihydrochloride (OPD) as substrate. Stock solutions of OPD (0.5 mg/ml) were prepared in 50 mM citrate−phosphate buffer, pH 6.0, 0.15% H2O2 being added prior to use. The plates were read in a microplate reader at 405 nm.

### Western immunoblots

Protein samples were run on 12.5% SDS−polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked in TBS (blocking solution: TBS containing 5% powdered milk and 1% BSA) for 30 min and then incubated with anti-HMG antibody (diluted 1:1000 in TBS, 1% BSA) at 4°C overnight (19). The membrane was washed and incubated with HRP-conjugated rabbit anti-chicken IgG (diluted 1:1000 in TBS containing 20 μl/ml normal rabbit serum) for 30 min at room temperature. Bound antibody was detected using 3,3′-diaminobenzidine tetrahydrochloride (DAB) as substrate. Stock solutions of DAB were prepared at a concentration of 0.5 mg/ml in TBS, 0.02% H2O2 being added prior to use.

### Results

The first series of experiments were designed to assess Cr binding to DNA. Sheared DNA was incubated with increasing concentrations of CrCl3, after which the samples were extensively dialyzed to remove unbound Cr and subsequently analyzed by inductively coupled plasma/mass spectrometry. We observed dose-dependent binding of Cr to DNA (Figure 1). To examine further the binding of Cr to DNA, a 32P-labeled 100 bp DNA fragment was incubated with increasing concentrations of CrCl3 overnight and then analyzed on a 5% polyacrylamide gel. DNA treated with higher Cr concentrations had decreased mobility in the gel (Figure 2). The Cr-induced changes in electrophoretic mobility of DNA suggest a change in DNA structure, which may result from the production of Cr-induced DNA crosslinks.

To determine whether HMG proteins recognize Cr-DNA, purified protein was incubated with unmodified or Cr-modified DNA−cellulose. After incubation, the protein−DNA−cellulose complexes were washed and bound protein was eluted and analyzed on SDS−polyacrylamide gels. Under these conditions, HMG2 binds to Cr-DNA−cellulose, but does not bind cellulose, Cr−cellulose or DNA−cellulose (Figure 3). Under these conditions, protein binding was Cr lesion density−dependent and maximal binding of both proteins was observed when DNA was modified with 100 μM CrCl3 (Figure 4). Under the conditions of the assay, HMG proteins bound Cr-DNA with a minimum lesion density of ~10 Cr adducts/103 nucleotides as determined by inductively coupled plasma/mass spectrometry analysis.

The goal of the next experiments was to assess protein affinity for Cr-DNA. Increasing concentrations of HMG2 were incubated with a fixed amount of Cr-DNA, the samples were washed and bound proteins were eluted and run on SDS−polyacrylamide gels. The amount of protein bound was...
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Fig. 4. HMG binding as a function of Cr lesion density. Purified HMG1 (upper) or HMG2 (lower) (12.5 µg) was incubated with untreated or Cr-treated DNA for 18 h. Bound protein was extracted and analyzed on SDS–polyacrylamide gels. Lane 1, input protein (0.25 µg); lanes 2–7, protein incubated with DNA treated with 0–100 µM CrCl$_3$ respectively. Numbers on left, $M_r$ in kDa. Maximal protein binding was to DNA treated with 100 µM CrCl$_3$ (lane 7).

Fig. 2. A $^{32}$P-labeled 100 bp DNA fragment was incubated with 0–100 µM CrCl$_3$ (lanes 1–8) for 4 h and the samples were analyzed on a 5% polyacrylamide gel. The gel was dried and autoradiographed. Note decreased mobility of the DNA fragment as a function of input CrCl$_3$ concentration.

Fig. 3. Binding of HMG2 to Cr-DNA. Purified HMG2 (15 µg) was incubated with untreated DNA (lane 2), DNA treated with 100 µM CrCl$_3$ (Cr-DNA; lane 3), untreated cellulose (lane 5) or cellulose treated with 100 µM CrCl$_3$ (lane 6) for 18 h. Bound protein was extracted and analyzed on SDS–polyacrylamide gels. Lanes 1 and 4, input HMG2 (0.25 µg). Numbers on left, $M_r$ in kDa.

Fig. 5. Scratched analysis of HMG2 binding to Cr-DNA. Increasing amounts of purified HMG2 were mixed with Cr-DNA–cellulose in 10 mM Tris, pH 7.5, 50 mM NaCl and incubated for 15 h at 4°C. The samples were pelleted by centrifugation and washed as described in Materials and methods. Bound proteins were extracted and analyzed on SDS–polyacrylamide gels. The amount of protein present in each lane was quantitated with a densitometer. Standard curves were generated by scanning lanes of gels containing known concentrations of HMG2. The calculated $K_d$ for HMG2 was $10 \pm 5 \times 10^{-9}$ M. Results presented are from a representative experiment.

assessed by scanning the gels in a densitometer. Analysis of the binding data on Scatchard plots (21) indicated that HMG2 binds Cr-DNA with a $K_d$ of $\sim 10^{-9}$ M (Figure 5).

The previous studies revealed that purified HMG1 and HMG2 will selectively bind Cr-DNA. We also determined whether these proteins are functionally active Cr-DNA binding proteins in tissues. For these studies, extracts from calf thymus tissue were prepared and incubated with Cr-DNA. After incubation, the protein–DNA–cellulose complexes were washed and bound proteins were eluted and analyzed on SDS–polyacrylamide gels. HMG protein binding was deter-
mined on Western blots. Under these conditions, we observed that HMG proteins bind to Cr-DNA (Figure 6). As was the case with the purified proteins (see Figure 4), HMG binding was Cr lesion dependent.

We next determined whether HMG proteins will recognize Cr-DNA adducts formed in cells exposed to Cr. For these experiments, HL60 cells were incubated in the absence or presence of K₂CrO₄ (mammalian cells are unable to take up CrCl₃; 7 and 8). After incubation, the cells were washed and DNA was isolated and used as substrate in a solid phase DNA binding assay. We observed increased HMG protein binding to DNA obtained from cells treated with chromate (Figure 7). These results provide direct evidence that HMG proteins are capable of recognizing Cr-DNA adducts arising in chromate-treated cells. In addition, we find that HMG proteins obtained from chromate-treated cells are functionally active as Cr-DNA binding proteins (data not shown).

**Discussion**

Cr(III) is a well-established DNA damaging agent. Incubation of DNA with CrCl₃ resulted in dose-dependent binding of Cr. In addition, at concentrations >20 µM CrCl₃, altered mobility of a 100 bp DNA fragment was observed in a gel mobility shift assay. In the course of this work, we also found that HMG1 and HMG2 bind to Cr-DNA. Protein binding was both Cr lesion and time dependent. HMG–Cr-DNA complexes are stable in >0.5 M NaCl and HMG2 binds Cr-DNA with a K_d of ~10⁻⁹ M.

HMG1, HMG2 and other HMG box proteins specifically recognize bent DNA structures (12), which is probably a reflection of their normal in vivo function. For example, they bind cruciform and four-way junction DNA and have also been shown to bind cisplatin-modified DNA (12,13). Cisplatin is known to form well-characterized intrastrand crosslinks between adjacent purines in genomic DNA. The resulting adducts induce a bend of 32–34° towards the major groove (23–25). HMG proteins dock in the widened minor groove of cisplatin-modified DNA and upon binding, induce additional bending.

Our results suggest that Cr(III) binding to DNA produces distortions in DNA structure which are recognized by HMG1 and HMG2. Cr alters DNA structure, which may result from the production of Cr-induced interstrand crosslinks (which can be visualized in Figure 1). Relevant to this point are findings that Cr bound to DNA alters non-specific DNA and RNA polymerase activity and results in premature DNA polymerase arrest (10). These effects are likely the result of Cr adduct formation and/or resulting changes in DNA structure. The ability of HMG proteins to bind Cr-DNA suggests that Cr binding produces structural alterations in DNA which are similar to those obtained with cisplatin. Although HMG proteins bind tightly to Cr-DNA (HMG–Cr-DNA complexes are stable in 0.5 M NaCl) the affinity of HMG2 for Cr-DNA (K_d ~10⁻⁹ M) is ~10-fold less than for cisplatin-modified DNA (K_d ~10⁻¹⁰ M; 17).

Our studies, using whole tissue extracts as input protein for binding assays, indicate that the ability of HMG proteins to selectively recognize Cr-DNA is not just an in vitro finding. Further, these problems also recognize Cr-DNA lesions formed under normal physiological conditions, as they bound DNA isolated from chromate-treated cells. Taken together, these findings suggest that HMG proteins are also functionally active damage recognition proteins in vivo. In addition to recognition, HMG1 and HMG2 may also be involved in guiding the cellular response following the formation of Cr adducts in DNA. For example, these proteins could be involved in the repair of
these lesions or directing cells down alternative pathways, one of which may result in apoptosis (26).

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References


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