The mismatch-repair protein hMSH2 binds selectively to DNA adducts of the anticancer drug cisplatin

Jill A Mello[1], Samir Acharya[2], Richard Fishel[2*] and John M Essigmann[1*]

Background: The antitumor drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP or cisplatin) exerts its cytotoxic effects through the formation of covalent DNA adducts. A family of proteins possessing a common HMG box motif that binds specifically to cisplatin DNA adducts has been previously suggested to be important in the clinical efficacy of the drug.

Results: We have shown that the human mismatch-repair protein, hMSH2, also binds specifically to DNA containing cisplatin adducts and displays selectivity for the DNA adducts of therapeutically active platinum complexes. Moreover, hMSH2 is overexpressed in testicular and ovarian tissue; tumors in these tissues are most effectively treated by cisplatin.

Conclusions: Our results suggest a role for hMSH2 in mediating cisplatin toxicity. Supporting this view, previous studies in *Escherichia coli dam*$^-$ strains demonstrate that mutations in mismatch-repair proteins confer resistance to cisplatin toxicity. Mismatch-repair deficiency is also correlated with tolerance to O^6-methylguanine, a cytotoxic DNA lesion formed by methylating agents. A current model ascribes O^6-methylguanine toxicity to unsuccessful attempts at repair of this lesion by mismatch-repair proteins, resulting in a futile cycle of incision and synthesis, leading ultimately to lethal DNA-strand breaks. We propose that mismatch repair may contribute to cisplatin toxicity by a similar mechanism. Alternatively, hMSH2 may shield cisplatin adducts from repair, allowing adducts to persist, thus enhancing lethality.

Addresses: [1]Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, USA and [2]Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107, USA.

Correspondence: J Essigmann or R Fishel
e-mail: jessig@mit.edu
rfishel@hendrix.jci.tju.edu

Key words: cisplatin, cisplatin-binding proteins, hMSH2, mismatch repair

Received: **May 17 1996**
Revisions requested: **May 29 1996**
Revisions received: **Jul 8 1996**
Accepted: **Jul 8 1996**

Chemistry & Biology July 1996, **3**:579–589

© Current Biology Ltd ISSN 1074-5521

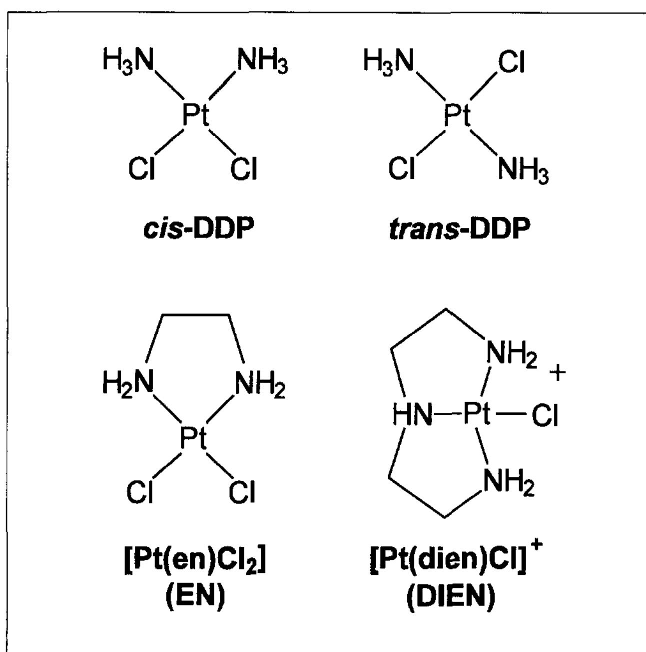
Introduction

The spectacular success of cisplatin (*cis*-diamminedichloroplatinum(II) or *cis*-DDP; Fig. 1) in the treatment of testicular cancer and the impressive activity of the drug in delaying the development of ovarian and other cancers [1] has stimulated interest in its biochemical mechanism(s) of action. It is well established that cisplatin kills cells through processes triggered by its reaction with DNA [2]. Cisplatin–DNA lesions formed by such reactions include intrastrand 1,2-d(GpG), 1,2-d(ApG), 1,3-d(GpNpG) (where N is any nucleotide), and interstrand crosslinks [3,4]. These DNA lesions are proposed to mediate cell death by inhibiting DNA and RNA synthesis [2] and ultimately inducing apoptosis [5]. To date, however, the precise biochemical mechanism underlying the therapeutic activity and organotropism of cisplatin remains elusive and is likely to be more complex than the simple inhibition of polymerases in rapidly proliferating tumor cells.

Of possible importance to the cytotoxic mechanism of cisplatin is the observation that various cellular proteins bind to cisplatin–DNA adducts [6]. Recently, a family of cisplatin-adduct binding proteins possessing a common high mobility group (HMG) box motif has been identified [7–9]. Significantly, these HMG box proteins display a selective affinity for the DNA adducts of therapeutically active platinum compounds [8,10], suggesting a role for such proteins in potentiating cisplatin cytotoxicity. The HMG box domain is known to recognize bent DNA structures, such as four-way junctions and cruciform DNA [11–13]. It is thus believed that duplex DNA bending and unwinding induced by cisplatin adducts [14,15] provide a structural signal for HMG box protein recognition [7,16].

Several models to explain the possible involvement of HMG box proteins in the mechanism of action of cisplatin have been proposed. One model suggests that binding of these proteins to platinum adducts precludes access to the lesions by DNA repair enzymes. Indeed, evidence has been presented both *in vitro* and *in vivo* in support of the view that HMG box proteins sensitize cells to cisplatin by shielding cisplatin adducts from repair [9,17,18]. A second model stems from the identification of human upstream binding factor (hUBF) as one of the HMG box proteins attracted to therapeutic platinum–DNA adducts. hUBF is an important regulator of ribosomal RNA synthesis, which is essential for proliferating cells. hUBF binds to a cisplatin adduct (apparent dissociation constant, $K_{d(app)}$, 60 pM) and to its cognate rRNA promoter sequence ($K_{d(app)}$ = 18 pM) with comparable affinities, leading to

Figure 1



The structures of cisplatin and cisplatin analogs. Cisplatin (*cis*-DDP) and $[Pt(en)Cl_2]$ (en, ethylenediamine), both therapeutically active platinum complexes, have chloride ligands in the *cis* geometry. The *trans* isomer of cisplatin, *trans*-DDP, and [Pt(dien)Cl]Cl (dien, diethylenetriamine) are clinically ineffective cisplatin analogs.

the proposal that cisplatin adducts may act as molecular decoys for the transcription factor *in vivo*. Thus, by a 'transcription factor hijacking' mechanism, cisplatin may deplete cells of a necessary resource for growth [19].

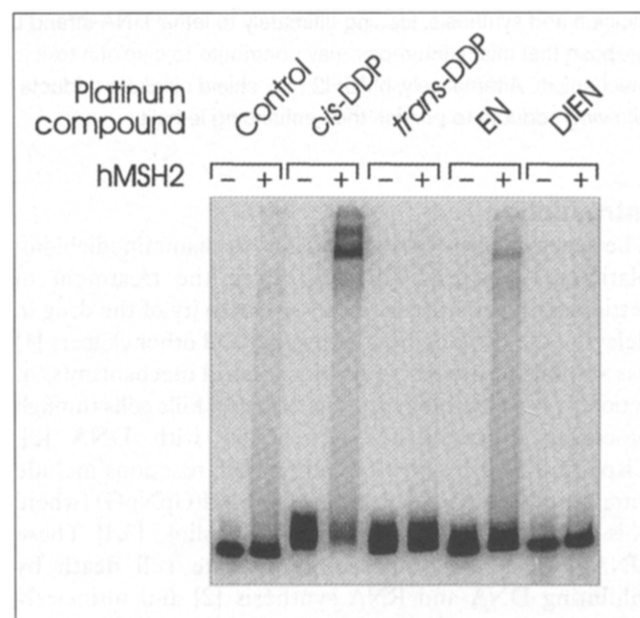
To date the models advanced to explain the cytotoxicity of cisplatin have failed to explain an important feature of its pharmacological effects, namely its organotropic specificity for testicular and, to a lesser extent, ovarian tumors. We have recently reported that testicular tissue has elevated expression levels of the mRNA for the human mismatch repair protein, hMSH2 [20]. Mutations in the hMSH2 gene are a predisposing factor in the development of hereditary non-polyposis colorectal carcinomas [21,22]. A role for the *Escherichia coli* mismatch-repair proteins in sensitivity to the cytotoxic effects of cisplatin has been demonstrated by Marinus and coworkers [23]. Moreover, the observation that purified hMSH2 recognizes insertion/deletion mispairs as well as single-base mismatches [24,25] suggests that hMSH2 may recognize structural distortions in DNA. These observations, taken together, suggested to us that hMSH2 may interact with DNA that is damaged by cisplatin. Here, we demonstrate that purified hMSH2 recognizes and binds specifically to DNA containing cisplatin adducts *in vitro* and, moreover, displays selective affinity for the DNA adducts of therapeutically active platinum compounds. These results suggest that mismatch repair may be involved in the sensitization of human cells to an important anticancer drug.

Results

hMSH2 binds to platinum-crosslinked DNA

To test the hypothesis that hMSH2 might recognize cisplatin-modified DNA, purified hMSH2 was used in an electrophoretic mobility shift assay with DNA globally modified by cisplatin. Binding of hMSH2 to a radiolabeled 162-base pair (bp) DNA probe containing cisplatin–DNA adducts was readily observed by the retarded migration of the radiolabeled probe through the gel (Fig. 2, lane 4). Unlabeled nonspecific competitor DNA (chicken erythrocyte DNA of homogeneous length) was required in the binding reactions to observe discrete bands. Under identical conditions the protein did not cause a shift of unmodified 162-bp probe (Fig. 2, lane 2). A hallmark of the interaction of HMG box proteins with platinum-damaged DNA is the selectivity for adducts of therapeutically active platinum compounds [8,10]. To determine whether hMSH2 displays similar selectivity,

Figure 2



Selectivity of hMSH2 for DNA modified with therapeutically active platinum compounds. A radiolabeled 162-bp DNA probe was modified with cisplatin (lanes 3 and 4), *trans*-DDP (lanes 5 and 6), $[Pt(en)Cl_2]$ (EN, lanes 7 and 8), or [Pt(dien)Cl]Cl (DIEN, lanes 9 and 10) at drug-to-nucleotide ratios (r_b) of 0.018, 0.036, 0.012, and 0.020, respectively. Unmodified 162-bp probe was used as a control (lanes 1 and 2). DNA probes were incubated in the absence (–) or presence (+) of hMSH2 (100 nM). Cisplatin-modified probes migrate slower than unmodified DNA, as described previously [16]. Two discrete, shifted bands were observed only when hMSH2 was incubated with DNA containing adducts of the therapeutically active complexes cisplatin or $[Pt(en)Cl_2]$ (lanes 4 and 8, respectively).

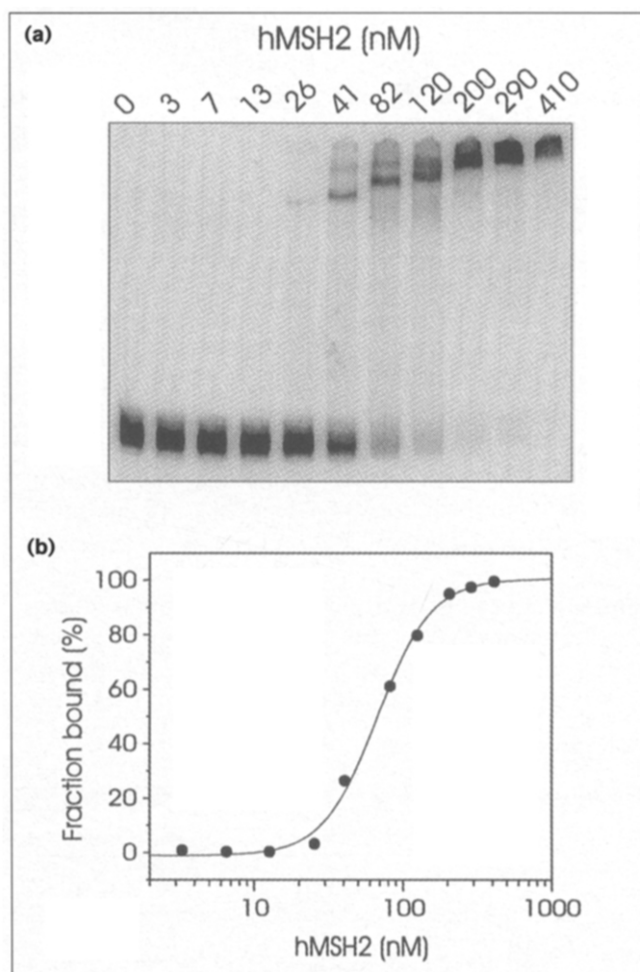
we examined the interaction of hMSH2 with DNA modified by various cisplatin analogs (Fig. 1). hMSH2 bound to DNA modified by the therapeutically active platinum analog $[Pt(en)Cl_2]$ (Fig. 2, lane 8), although to a lesser extent than was observed for cisplatin-modified DNA. By contrast, hMSH2 had no affinity for DNA that contained adducts of the inactive platinum complexes *trans*-diamminedichloroplatinum(II) (*trans*-DDP) and $[Pt(dien)Cl]^+$ (Fig. 2, lanes 6 and 10, respectively).

Cisplatin and $[Pt(en)Cl_2]$ differ from the two clinically inactive compounds in that the chloride ligands have a *cis* geometry, enabling the formation of 1,2-intrastrand crosslinks at adjacent nucleotides. These crosslinks, formed at GG and AG sites, comprise greater than 90 % of all adducts formed by cisplatin [3,4]. Our binding results suggest that hMSH2 may recognize either or both of the 1,2-intrastrand platinum adducts. Less binding was observed to DNA modified with $[Pt(en)Cl_2]$ compared to that with cisplatin. These two platinum complexes are believed to form the same spectrum of adducts [3]. This differential binding may be due in part to a slight difference in modification level of the two DNA probes (r_b = 0.018 vs 0.012). These results may also indicate that substitution of the two amine groups of cisplatin with a bidentate ligand (ethylenediamine) negatively affects hMSH2–cisplatin adduct interactions.

Specificity of hMSH2 binding to cisplatin DNA adducts

To characterize the nature of the interaction between hMSH2 and cisplatin-modified DNA further, hMSH2 protein was titrated into binding reactions containing a constant concentration of cisplatin-modified DNA probe. At hMSH2 concentrations approximating half-maximal binding, two distinct bands were consistently observed (Fig. 3a). The addition of increasing amounts of protein caused the complex to be proportionately retarded through the gel, presumably because multiple proteins bound to the multi-platinated probe. The binding isotherm (Fig. 3b) reveals that the fraction of bound probe increases to saturation over a narrow range of hMSH2 concentrations, consistent with positive cooperative hMSH2 binding (Hill constant, n_H = 2.4). The 162-bp probe used in this experiment contained an average of six platinum adducts per duplex molecule (one platinum adduct per 27 bp). The apparently cooperative binding behavior may thus be a consequence of multiple platinum adducts situated in relatively close proximity in the duplex DNA; the binding of one hMSH2 molecule to a platinum adduct may render the subsequent binding of a second hMSH2 molecule to a nearby platinum adduct more favorable. Alternatively, hMSH2 may be binding as a dimer or some higher-ordered complex, as has been previously proposed [25]. Generation of the binding isotherm yielded a $K_{d(app)}$ = 67 nM. Neither the active fraction of our hMSH2 preparation nor the aggregation

Figure 3



Binding isotherm describing the interaction between hMSH2 and cisplatin-modified DNA. **(a)** hMSH2 protein was titrated into binding reactions containing 124 pM of a radiolabeled 162-bp probe that contained an average of six cisplatin DNA adducts (r_b = 0.018). Cisplatin-modified probe in the absence of hMSH2 is shown in lane 1. **(b)** The fraction of bound probe in each lane was quantitated by PhosphorImager analysis and is presented as a function of the concentration of hMSH2 present in the binding reactions. The binding curve was generated by fitting these binding data to the Hill equation.

state of the protein have been established, and thus our estimation of $K_{d(app)}$ assumes that hMSH2 binds as a monomer and that 100 % of the protein is active in cisplatin-adduct binding. These considerations, taken together with the observed complex nature of hMSH2 binding to the multi-platinated probes, dictate that the $K_{d(app)}$ be considered only an approximation of the affinity of hMSH2 for a platinum adduct.

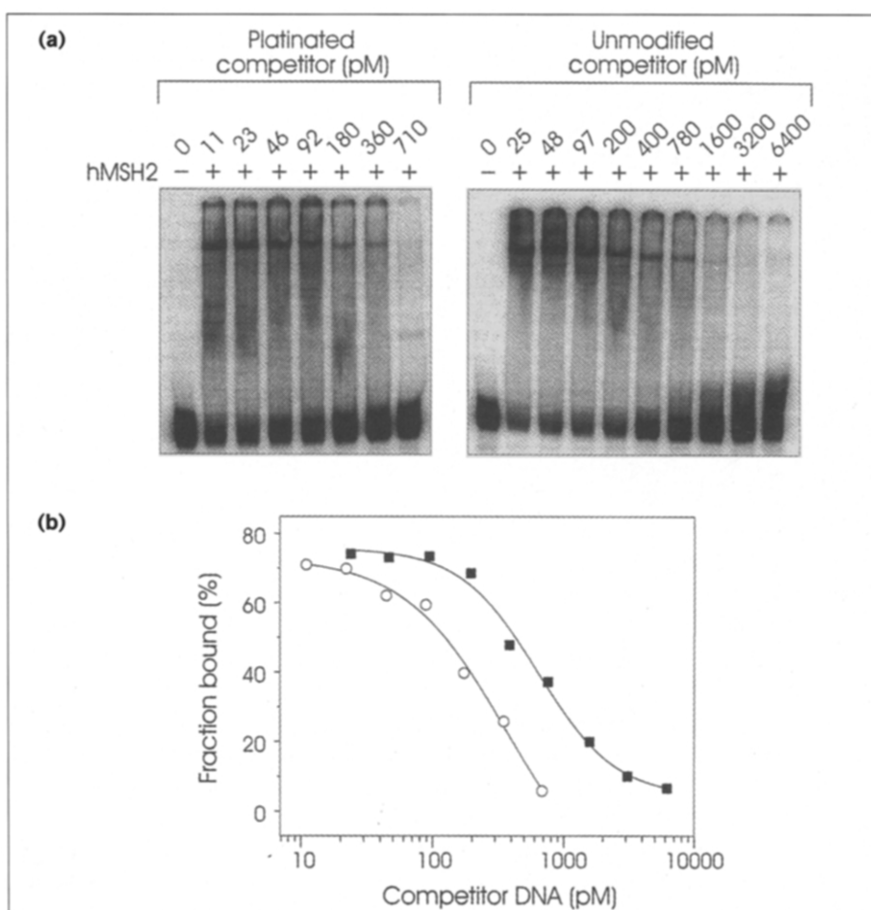
The specificity of the interaction between hMSH2 and cisplatin-modified DNA was examined by an analysis of the competition between [^{32}P]-platinated DNA and non-radioactive platinated or unmodified supercoiled plasmid

DNA for hMSH2 binding. Increasing concentrations of unlabeled competitor DNA, either modified with cisplatin (r_b = 0.043) or unmodified control, were titrated into binding reactions containing hMSH2 and radiolabeled, platinated, 162-bp probe (r_b = 0.018). A representative experiment is shown in Figure 4. A 400-fold molar excess of competitor platinum adducts was required to achieve complete competition of hMSH2 binding. An approximately five-fold greater concentration of unmodified competitor DNA compared with cisplatin-modified DNA was required to reduce binding by 50 % (Fig. 4b). This differential affinity is comparable to that observed when examining the binding of the *Saccharomyces cerevisiae* MSH2 protein to a G–T mismatch, four-base loop, or 14-nucleotide palindromic insertion loop relative to homoduplex DNA [26]. Previous studies of the interaction of hMSH2 with DNA probes containing one-base mismatches or insertion/deletion loops also found similar selectivity over homoduplex DNA [24,25].

hMSH2 binds to DNA with low levels of cisplatin adducts

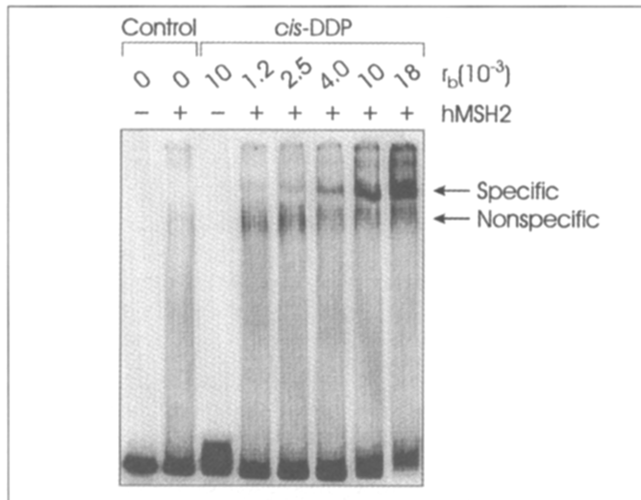
The degree of DNA modification by cisplatin used in the above experiments, approximating one DNA adduct per 27 bp, is significantly higher than would be encountered *in vivo*. To examine whether hMSH2 binds to DNA containing levels of cisplatin modification more closely approximating those found in cellular DNA, binding assays were carried out with hMSH2 and 162-bp probes modified to a range of r_b levels. Discrete shifted bands that represented specific binding to a cisplatin modified DNA probe of r_b = 0.018 (Figs 2,3,4 and Fig. 5, lane 8) were also observed when hMSH2 was included in binding reactions with DNA probes of r_b = 0.010 (Fig. 5, lane 7), 0.0040 (lane 6), 0.0025 (lane 5) and 0.0012 (lane 4). The fraction of probe bound diminished proportionately as the cisplatin modification level decreased, reinforcing the specific nature of this shifted band. The appearance of an increased fraction of unshifted material was probably a reflection of an increasing population of unmodified DNA. The lowest modification level, r_b = 0.0012, corresponded to an average of 0.4 cisplatin adducts per probe molecule. The Poisson distribution predicts that 27 % of this DNA population would contain a single adduct, 6 % would have two adducts, and that the remainder of the population would be unmodified. These data thus suggest that hMSH2 can bind to DNA containing a single cisplatin adduct.

Figure 4



Specificity of hMSH2 binding to cisplatin-modified DNA. Unlabeled duplex DNA, either cisplatin-modified or unmodified control, was used to compete with a radiolabeled 162-bp cisplatin-modified DNA probe for association with hMSH2. **(a)** ^{32}P-labeled 162-bp probe modified with cisplatin (r_b = 0.018, 150 pM) was incubated in the presence of hMSH2 (100 nM) and 0–710 pM unlabeled cisplatin-modified DNA (r_b = 0.043) (left panel), or 0–6400 pM unlabeled unmodified DNA (right panel). Cisplatin-modified probe in the absence of hMSH2 is shown in lane 1 for either competitor. **(b)** The fraction of bound probe in each lane was quantitated by PhosphorImager analysis and is presented as a function of the concentration of competitor DNA present in the binding reactions. ○, platinated DNA; ■, unmodified DNA.

Figure 5



Binding of hMSH2 to cisplatin-modified DNA at different bound ratios of platinum to nucleotide. Radiolabeled 162-bp probe modified with cisplatin at drug-to-nucleotide ratios of 0.0012 (lane 4), 0.0025 (lane 5), 0.0040 (lane 6), 0.010 (lane 3 and 7) and 0.018 (lane 8), corresponding to an average of 0.4, 0.8, 1.3, 3.4 and 6 cisplatin adducts per probe molecule, respectively, were incubated in the absence (–) or presence (+) of hMSH2 (130 nM). Unmodified probe was used as a control (lanes 1 and 2). Retarded bands representing specific binding of hMSH2 to the DNA probe of $r_b = 0.018$ (Figs 2, 3 and 4 and Fig. 5, lane 8) were also present at the lower levels of platinum modification. Specific binding diminished as the degree of cisplatin modification decreased. The broad, faster-migrating band present in all lanes containing protein represents nonspecific binding to the 162-bp probe that was incompletely competed out by nonspecific competitor DNA present in the binding reactions.

hMSH2 binds to the major cisplatin adduct

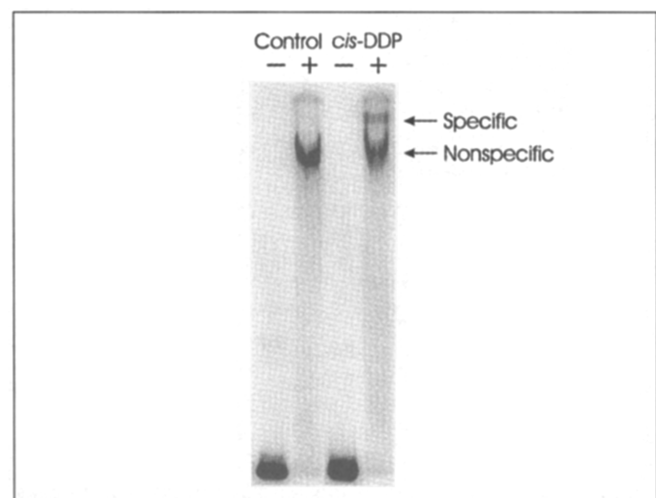
The 1,2-intrastrand d(GpG) cisplatin crosslink represents 65% of all adducts formed by this compound [2]. The observation that hMSH2 binds to globally modified DNA in which a maximum of one to two cisplatin adducts were present suggested that hMSH2 may recognize this 1,2-d(GpG) crosslink. The specificity of hMSH2 for platinum compounds that uniquely form 1,2-intrastrand crosslinks also supported this view. We therefore examined binding by hMSH2 to a 100-bp probe containing a single d(GpG) adduct. Binding assays revealed a discrete band that was specific to the platinated probe (Fig. 6), demonstrating that hMSH2 recognizes the major cisplatin crosslink. Nonspecific binding to the 100-bp probe was high in these gel-shift assays, precluding our ability to perform thermodynamic analysis of the interaction with confidence. The 1,2-d(GpG) adduct present in a shorter DNA probe and in a different sequence context may be useful in such studies.

hMSH2 is overexpressed in human testicular and ovarian tissues

The expression of hMSH2 was examined by Western analysis of protein extracts prepared from five different human tissues using an hMSH2-specific monoclonal antibody. The same amount of total protein was analyzed for each tissue examined. A protein band of relative molecular mass (M_r) 105 000 was observed in all five human tissues, corresponding to full length hMSH2 (Fig. 7a). In addition, a lower band of ~ M_r 75 000 that was of approximately equal intensity to the full length hMSH2 band was observed in all tissues except the ovary, where the lower band was present but faint. This lower band was seen both when using a monoclonal antibody (Fig. 7) and when using a polyclonal antibody to hMSH2 (data not shown) and was not a nonspecific signal due to secondary antibody. This protein band is believed to be a specific degradation product of hMSH2 present in these tissue extracts. As a control, these same five human tissue extracts were analyzed by Western analysis for α-tubulin expression (Fig. 7b). A sharp band of M_r 50 000 corresponding to α-tubulin was observed. Essentially similar levels of α-tubulin were found in all five protein extracts, demonstrating similar total protein content for each tissue extract.

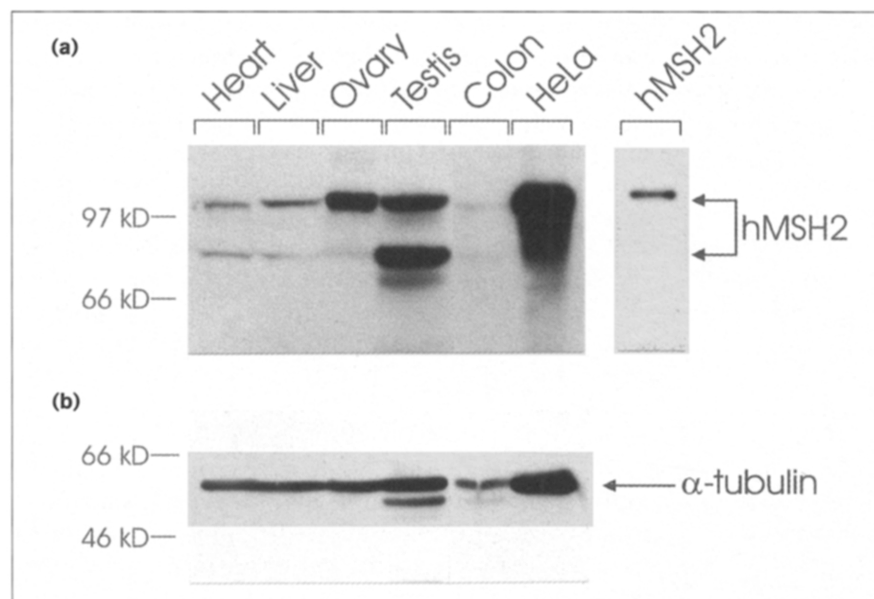
High levels of hMSH2 protein were found in both testicular and ovarian tissue, whereas lower levels were observed in tissue of the liver, heart, and colon. Quantitative analysis of hMSH2-specific signals and normalization to that of α-tubulin for each tissue examined indicated that this enrichment of hMSH2 in testicular and ovarian tissue is approximately five-fold. The observed overexpression of hMSH2 protein in the

Figure 6



Binding of hMSH2 to a 100-bp probe containing a single, site-specific 1,2-d(GpG) cisplatin intrastrand crosslink. hMSH2 (160 nM) was incubated with a 100-bp probe that contained the major cisplatin adduct or with its unplatinated control (100 pM). A discrete, shifted band was visible, which was specific for the platinated probe, while a faster migrating band, representing nonspecific binding, was observed with both the platinated and unplatinated probes.

Figure 7



Western blot analysis of hMSH2 expression in human tissue. **(a)** Protein extracts derived from human tissues were separated by electrophoresis on SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with monoclonal antibody to hMSH2. Nuclear extract of HeLa cells and purified hMSH2 were included as positive controls. Lane 1, heart; lane 2, liver; lane 3, ovary; lane 4, testis; lane 5, colon; lane 6, HeLa nuclear extract; lane 7, purified hMSH2 from a separate loading. The upper band corresponds to full length hMSH2 protein, M_r 105 000. The lower band is believed to be a specific degradation product of hMSH2 present in these tissue extracts. **(b)** Protein extracts from human tissues were blotted and probed with monoclonal antibody to α-tubulin, demonstrating similar total protein content for each tissue sample.

testis is consistent with previous results showing that hMSH2 is overexpressed in human testicular tissue at the RNA level [20]. However, elevated levels of hMSH2 RNA were not previously found in ovarian tissues [20], suggesting that posttranscriptional regulatory mechanisms may be responsible for the elevated levels found in these tissues. Tumors that arise in tissues of the testis and ovary are those best treated by cisplatin [1]. The correlation of the enrichment of hMSH2 in these tissues with tumor response to cisplatin treatment suggests that the protein could be involved in sensitizing these tissues to cisplatin toxicity.

Discussion

We have shown that the human mismatch repair protein hMSH2 recognizes and binds specifically to duplex DNA containing cisplatin DNA adducts. hMSH2 also binds to DNA containing adducts of the clinically effective cisplatin analog [Pt(en)Cl_2], but not to DNA modified with the therapeutically inactive platinum complexes *trans*-DDP or [Pt(dien)Cl]$^+$. Binding specificity for DNA adducts of therapeutically active platinum complexes has previously been observed for a family of HMG box proteins. To date, however, no correlation between specific HMG proteins and the sensitivity of tissues to cisplatin has been found. Western analysis of hMSH2 protein in the human tissues tested corroborates previous results showing that this protein is overexpressed in tissue of the testis and indicates for the first time that hMSH2 is also present at elevated levels in ovarian tissue. These observations are significant in that cancers occurring in testicular and ovarian tissues are those most successfully treated by cisplatin. These results suggest that mismatch-repair proteins could be involved in the antitumor activity and organotropism of cisplatin.

The selective affinity of hMSH2 for DNA modified with cisplatin or [Pt(en)Cl_2], but not for DNA modified with other platinum complexes, suggested that this protein may recognize the 1,2-intrastrand crosslinks formed uniquely by these two compounds. Binding of hMSH2 to globally modified probes that contained a maximum of one to two cisplatin adducts further suggested that at least one of the 1,2-intrastrand crosslinks, which comprise ~90 % of the total adduct spectrum of the drug, were recognized by hMSH2. Setting precedent for such specificity is HMG1, which binds selectively to cisplatin intrastrand 1,2-d(GpG) and 1,2-d(ApG) crosslinks, but lacks specificity for 1,3-d(GpNpG) crosslinks [8]. Although it has been shown that HMG1 can bind to the interstrand crosslink of cisplatin, but not to that formed by *trans*-DDP [27], the infrequency with which the cisplatin interstrand adduct is formed (2 %) [2] suggested that recognition of this adduct was less likely. Binding assays using a 100-bp probe containing a single, site-specific 1,2-d(GpG) crosslink (Fig. 6) demonstrated that this major adduct (65 %) is indeed recognized by hMSH2. This result does not exclude the possibility that the less abundant 1,2-d(ApG) crosslink may also be recognized.

It is not yet clear how the structures of mispairs in the DNA helix affect recognition by hMSH2. It is possible, however, that cisplatin adducts distort DNA in a manner that mimics the presence of either a single-base mismatch or insertion/deletion mispair. Both *S. cerevisiae* and human MSH2 display greater selectivity for palindromic and loop

insertion mispairs compared to single-base mismatches *in vitro* [25,26]. HMG box proteins are known to bind DNA containing sharp angles, such as cruciforms and four-way junctions [11–13]. Of note is the resemblance between the cruciform DNA structures bound by HMG proteins and the palindromic insertion structures for which MSH2 proteins display greatest affinity [25,26]. Further studies probing the nature of the interaction of hMSH2 with cisplatin DNA adducts are clearly warranted.

Although binding by hMSH2 to a single, site-specific 1,2-d(GpG) cisplatin adduct was observed, high non-specific binding precluded thermodynamic analysis of this interaction. The apparent dissociation constant for the binding of hMSH2 to a DNA fragment containing multiple cisplatin adducts, however, was estimated to be 67 nM. It must be emphasized that due to the complex nature of binding to the multi-platinated probe in our experiments (see above), this $K_{d(app)}$ is only an approximation of the affinity of hMSH2 for a platinum adduct. To date, affinity constants have not been reported for mispair binding with either *S. cerevisiae* or human MSH2.

Competition studies demonstrated a five-fold greater affinity for DNA containing cisplatin adducts as compared to unmodified DNA. Previous mispair-binding studies with both the *S. cerevisiae* and human MSH2 proteins revealed a similar level of specificity for DNA containing one-base mismatches or insertion/deletion loops over homoduplex DNA [24–26]. This relatively modest selectivity of MSH2 proteins for DNA mispairs *in vitro* seems at variance with their efficient repair *in vivo*. The recent discovery that hMSH2 can be purified as a complex with another human MutS homolog, GTBP/p160 [28,29], may provide a partial explanation for binding selectivity. The published evidence suggests that hMSH2 and GTBP/p160 are required for the recognition of single- and one-base mispairs, but that hMSH2, either alone or in complex with an as yet unidentified protein, is capable of recognizing larger loops [29–31]. In *S. cerevisiae*, Marsischky *et al.* [31] have recently shown that MSH2 forms a heterodimer not only with MSH6, the homolog of GTBP/p160, but also with yet another MutS homolog, MSH3. Their results indicate that the MSH2–MSH3 complex has a greater affinity for insertion/deletion loops than for single-base mispairs. Similar results have been found with the human homologs (S.A. *et al.*, unpublished data). Given that hMSH2 alone is capable of binding mispairs, it is possible that GTBP/p160 and the human homolog hMSH3 (DUG) serves to modulate binding specificity. Future studies will address the question of how these partner proteins affect the binding of hMSH2 to cisplatin adducts.

The present results are the first demonstration that purified hMSH2 can bind to DNA lesions other than mispaired bases or loops. This binding of hMSH2 to cisplatin adducts could reflect the involvement of mismatch-repair proteins in the removal of cisplatin adducts from DNA. This possibility is underscored by the discovery of an association of the genes involved in mismatch correction with transcription-coupled nucleotide excision repair in both *E. coli* [32] and eukaryotes [33]. We note, however, that the selective affinity of hMSH2 for DNA adducts of clinically effective platinum complexes suggests an opposing role for hMSH2, that of mediating cisplatin toxicity. Interestingly, a connection between mismatch repair and cisplatin lethality has existed in the literature for some time. Early work by Marinus and coworkers [23,34] shows that *dam*⁻ strains of *E. coli*, which have decreased adenine methylase activity and thus are unable to distinguish between parental and newly replicated DNA strands, are hypersensitive both to the methylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and to cisplatin. Significantly, the introduction of a mutation in either *mutS* or *mutL*, which inactivates mismatch-repair activity in these cells, abrogates hypersensitivity to both agents [23,34,35]. *E. coli dam*⁻ and wild type strains are not differentially sensitive to the therapeutically inactive platinum compound *trans*-DDP.

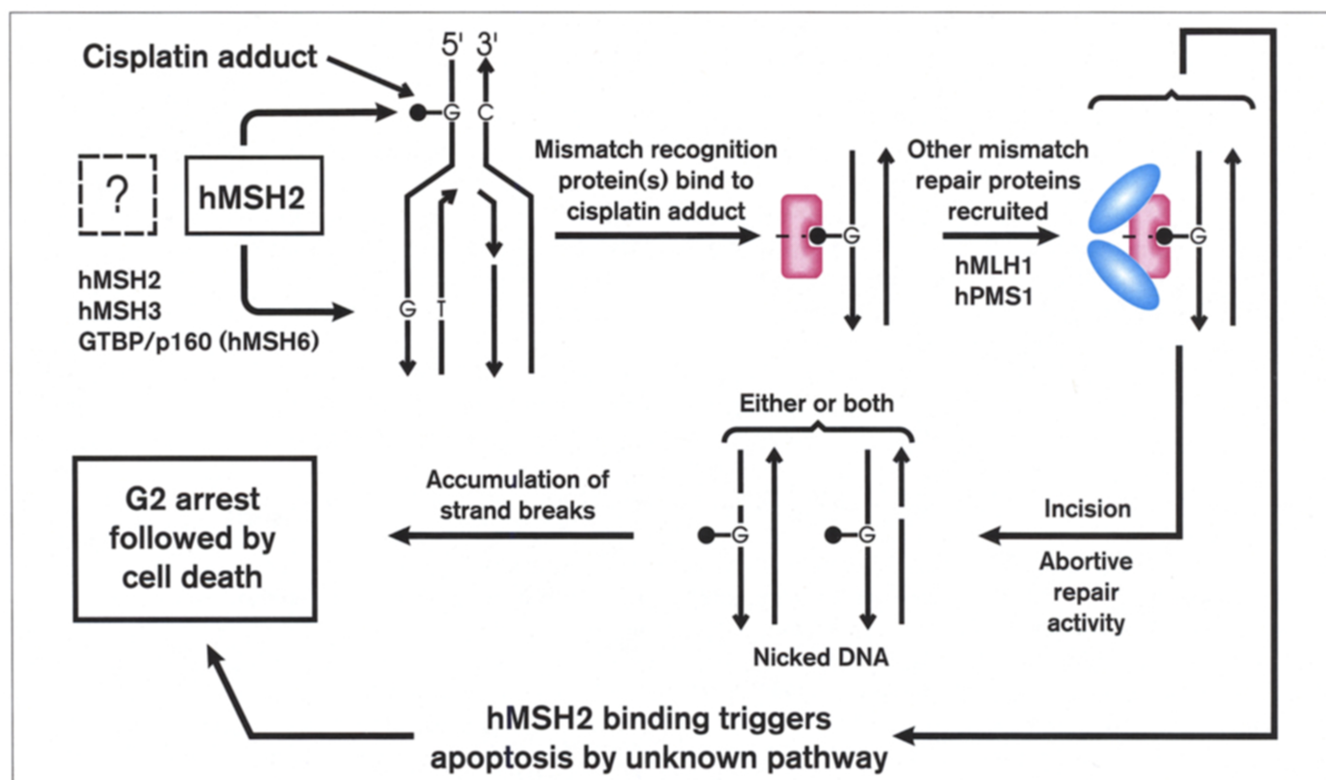
Connections between resistance to MNNG and defective mismatch repair have also been observed in higher eukaryotes. Goldmacher *et al.* [36] isolated an MNNG-resistant derivative of the human lymphoblastoid cell line TK6. This variant cell line exhibits a mutator phenotype, and extracts from this variant line have defective mismatch-repair activity *in vitro* [37]. The observed MNNG-resistant phenotype is not due to increased repair of O^6-methylguanine (O^6-MeGua), but is instead an acquired ability to tolerate the presence of the methylated base in DNA [38]. Independent examples of methylation-tolerant cell lines that are defective in mismatch repair subsequently have been reported [39–41]. A model to explain this apparently paradoxical correlation of a defect in repair with the tolerance of methylation damage proposes a futile cycle of excision repair [34,36]. In this model, O^6-MeGua lesions formed in DNA are presumed to be recognized by the mismatch-repair system due to the absence of a good complementary match for the methylated base. Repair attempts at O^6-MeGua-containing base pairs would be directed at the newly synthesized strand after DNA replication; failure to find a correct base pair for O^6-MeGua would invoke a futile cycle of incision and resynthesis in the opposing strand, resulting in the accumulation of lethal DNA strand breaks. Tolerance results when a defect in mismatch repair precludes initiation of this abortive repair. Supporting this hypothesis is the demonstration that a G–T mismatch-binding activity in cell extracts recognizes and binds to O^6-MeGua-T base pairs *in vitro* [42]. Moreover, a study examining the processing of O^6-MeGua in plasmid DNA by human cell extracts demonstrates that

O[6]-MeGua can be processed by a pathway involving excision and resynthesis of DNA that is not associated with the *O*[6]-MeGua-DNA methyltransferase enzyme [43].

Our present findings, viewed together with the original observations of Marinus, suggest that a mechanism analogous to that proposed for *O*[6]-MeGua could be operative in cellular processing of cisplatin adducts. By this model (summarized in Fig. 8) the mismatch-repair system, at least one component of which (hMSH2) is overly expressed in testis and ovary, recognizes cisplatin-modified bases in DNA. Binding by hMSH2 would trigger the recruitment of other mismatch-repair proteins and the consequent initiation of misdirected repair attempts at sites of cisplatin damage; abortive repair activity could provide, perhaps through the accumulation of DNA strand breaks, a signal for apoptosis. DNA strand breaks have been observed in cells after treatment with cisplatin, although it is unclear whether they originated from repair activity or as a consequence of apoptosis [44]. Based on current evidence, it is possible that recognition of cisplatin adducts by hMSH2 in complexed form with partner proteins would be required for this model to be operative. Future studies examining whether these proteins, GTBP/p160 and hMSH3, modulate the affinity of hMSH2 for cisplatin adducts will be useful in assessing the feasibility of this model.

A second and equally plausible mechanism by which hMSH2 could contribute to the therapeutic activity of cisplatin again derives from the abundance of this protein in ovarian and testicular tissue. Evidence exists supporting the view that HMG box proteins sensitize cells to cisplatin by shielding cisplatin adducts from repair [9,17,18]. To date, however, no correlation between the levels of HMG proteins and the sensitivity of target tissues to cisplatin has been found. The discovery that hMSH2, which lacks an HMG box, is able to bind cisplatin adducts and is also overexpressed in testicular and ovarian tissue suggests the possibility that it may form a complex with cisplatin adducts that is refractory to nucleotide excision repair. Slow repair would result in selective retention of the signal

Figure 8



The abortive repair model for the role of hMSH2 in potentiating cisplatin cytotoxicity. In this model, hMSH2, either alone or in complex with other mismatch-recognition proteins, recognizes structural distortions induced in DNA by cisplatin DNA adducts. This initial binding provides a signal for the recruitment of other mismatch-repair proteins, including hMLH1 and hPMS1. Misdirected repair attempts at or near sites of cisplatin damage could lead to G2 arrest and the induction of apoptosis, perhaps through the accumulation of DNA strand breaks. The initial recognition of cisplatin adducts by mismatch-repair proteins could also provide a signal for apoptotic cell death by some other direct or indirect pathway. Another non-mutually exclusive model, repair shielding, proposes that hMSH2 and possibly its partner proteins shield the cisplatin adduct from DNA-repair enzymes (see text).

that triggers cell death. In principle, the involvement of other mismatch-repair partner proteins would not be required for this mechanism to be operative. Future experiments will be directed at determining whether hMSH2, either alone or in combination with partner proteins, shields cisplatin adducts from repair.

The two mechanisms outlined above could both be active in the organ-specific cytotoxicity of cisplatin. One complication with both models, however, is the possibility that HMG box proteins, which are abundant in the cell, may interfere with the ability of hMSH2 to gain access to the cisplatin adducts. The most likely HMG protein to interfere with hMSH2 binding is hUBF, which binds to cisplatin adducts three to four orders of magnitude more tightly than other known HMG proteins [19]. We note, however, that hUBF is localized in the nucleolus [45], whereas hMSH2 is likely to be more generally distributed in the nucleus [46]. It is thus not necessarily the case that hUBF would compete with hMSH2 for cisplatin adducts in nuclear DNA.

Finally, there is a growing body of evidence implicating the mismatch-repair machinery as a modulator of the cytotoxic response of cells to cisplatin. Evidence to date comes from the analysis of cisplatin-resistant human cell lines. Some tumor cell lines selected for resistance to cisplatin following exposure to the drug have been found to contain defects in mismatch-repair genes [47]. This phenotype appears to be common in other cisplatin-resistant lines, since it has also been found that such selection can result in cells that are deficient in G–T mismatch binding activity [48]. Also of possible relevance is the recent observation that a mismatch repair deficient cell line lacks the ability to arrest at the G2 checkpoint upon exposure to the base analog 6-thioguanine. This observation led to the proposal that mismatch repair may be involved, either directly or indirectly, in triggering G2 arrest [49]. Interestingly, cisplatin-induced G2 arrest prior to apoptosis has been observed in numerous cell lines [5,50–53]. Mismatch repair could thus contribute to cisplatin cytotoxicity through the provision of either a direct or indirect signal for G2 arrest. All of the results detailed above predict that the inactivation of the mismatch-repair pathway would lead to tumors that are relatively refractory to treatment by cisplatin. Such observations may have profound clinical importance in determining optimum treatment regimens for cancer patients.

Significance

Chemotherapy for human cancer is clinically a far from ideal option because few cytotoxic drugs show an adequate selectivity for tumor cells, while sparing normal tissues. One notable exception is the treatment of testicular cancer which, according to recent estimates, is 93 % curable by a combination of surgery followed by chemotherapy with a regimen involving cisplatin as the principal cytotoxic agent [54]. Several models have been advanced to explain the therapeutic efficacy of cisplatin, including the possibility that HMG box proteins, which bind to cisplatin adducts, might shield DNA lesions from repair. In the present work we have identified hMSH2 as a novel platinum-adduct-binding protein, and find that it binds selectively to the adducts of platinum-based drugs that show clinical efficacy or potential.

The significance of the work is threefold. First, hMSH2 is highly expressed in testis and ovary, the two tissues in which tumors are best treated by cisplatin. These observations suggest that hMSH2 may contribute to the organotropism of the drug. Second, our demonstration of an interaction between hMSH2 and cisplatin DNA adducts *in vitro* complements a growing body of literature correlating mismatch-repair activity with both cisplatin and methylating-agent cytotoxicity. Together, these observations are consistent with a model whereby mismatch repair actively enhances the toxicity of DNA lesions. Moreover, because mismatch-repair deficiency correlates with resistance to alkylating agents, our results may provide insight into a heretofore undiscovered mechanism by which tumor cells may acquire resistance to cisplatin. Finally, the demonstration that hMSH2 binds to DNA lesions other than single-base mismatches and insertion/deletion mispairs may indicate that the range of substrates for the mismatch-repair system is broader than previously imagined, and that mismatch recognition proteins may have multiple roles.

Materials and methods

Overexpression and purification of hMSH2

Overexpression and purification of hMSH2 was by a modification of the procedures described by Fishel *et al.* [24]. A significant increase in the overexpression of hMSH2 was achieved by insertion of the gene into a baculovirus vector followed by infection of Sf9 insect cells. The protein coding sequence was modified with six histidine residues on the amino terminus. In a typical preparation, 250 ml of Sf9 insect cells were infected at a multiplicity of infection of ~10 with baculovirus containing the hMSH2 gene under the control of the polyhedron promoter. All manipulations of cells were performed at 4 °C. After 72 h, the cells were harvested and washed once with phosphate-buffered saline and resuspended in lysis buffer containing 50 mM potassium phosphate (pH 7.5), 300 mM NaCl, 10 % glycerol (buffer A) plus 1 mM EDTA, 1 mM PMSF and 0.8 $\mu g\ ml^{-1}$ each of the protease inhibitors pepstatin A and leupeptin. The cells were lysed by a single freeze/thaw followed by Dounce homogenization. The lysate was then cleared by centrifugation at 100 000 x g and the supernate poured into a well rinsed beaker. Ammonium sulfate (45 % v/v) was added while stirring at 4 °C over a 30 min period and the mixture allowed to stir for an additional 30 min. The precipitate was harvested by centrifugation and resuspended in 4 ml buffer A plus 1 mM PMSF and 0.8 $\mu g\ ml^{-1}$ each of pepstatin A and leupeptin. The protein was diluted to a conductivity equivalent to 0.5 M NaCl, applied to a 1 ml Ni-Chelex column at 0.05 ml min^{-1} and washed with 10 column volumes of buffer A plus protease inhibitors. The bound proteins were eluted with 10 column volumes of buffer A plus 25 mM imidazole followed by a linear gradient between 25 mM and 250 mM imidazole. hMSH2 eluted between 80 mM and 100 mM imidazole and was collected into tubes containing

100 μg ml^{-1} BSA. Peak fractions were dialyzed into 50 mM potassium phosphate (pH 7.5), 50 mM NaCl, 20 % glycerol, 0.1 mM EDTA, 1.0 mM dithiothreitol, aliquotted, quick frozen in dry ice/ethanol and stored at −80 °C. hMSH2-dependent mispair binding activity was stable for at least three months. The protein was generally found to be greater than 99 % homogeneous as judged by SDS-PAGE following silver staining for protein. However, occasionally the protein was less homogeneous and a second Heparin-Sepharose chromatography step was performed similar to that described by Fishel *et al.* [24].

Preparation of platinum-modified DNA probes

cis-DDP, *trans*-DDP, $[Pt(en)Cl_2]$ and [Pt(dien)Cl]Cl were prepared as described [55–57]. Restriction enzyme digestion of pSTR3 with *Cla*I and *Eco*RV yielded 162-bp and 4205-bp restriction fragments. Platination reactions of the restriction fragments were carried out in 3 mM NaCl, 1 mM Na_2HPO_4 (pH 7.4) with 100 μg ml^{-1} DNA and appropriate platinum compound:DNA molar ratios by incubating at 37 °C for 16 h. Unreacted platinum compound was removed by dialysis (24 h) against 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (TE). Levels of platinum modification were determined by flameless atomic absorption spectroscopy on a Varian AA1475 instrument equipped with a GTA95 graphite furnace. The r_b value determined for DNA modified with [Pt(dien)Cl]Cl was approximate. DNA probes of 162-bp were radiolabeled with [α-^{32}P]dCTP (6000 Ci $mmol^{-1}$, New England Nuclear), purified from the 4205-bp restriction fragment on native 5 % polyacrylamide gels, and resuspended in TE to 5000–10 000 counts per minute (cpm) per μl.

A 100-bp DNA probe containing a single, centrally located *cis*-$[Pt(NH_3)_2\{d(GpG)\text{-}N7(1),N7(2)\}]$ intrastrand crosslink (referred to above as 1,2-d(GpG)) and the analogous unmodified control were constructed as previously described [8], with minor modifications. All six deoxyoligonucleotides used in the construction were purified by polyacrylamide gel electrophoresis. The central 20-base oligonucleotide containing the *cis*-$[Pt(NH_3)_2\{d(GpG)\text{-}N7(1),N7(2)\}]$ crosslink was resolved from unreacted DNA on a 7 M urea, 20 % polyacrylamide gel. Following 5'-phosphorylation with ATP of the appropriate oligonucleotides and subsequent annealing of complementary strands, ligations were conducted for 12 h at 16 °C. Full length probe was purified by electrophoresis from unligated material on a 7 M urea, 8 % polyacrylamide gel. Following reannealing the 100-bp duplex DNA was purified from single-stranded DNA by electrophoresis on a native 8 % polyacrylamide gel. Duplex DNA was eluted in 1x TE, ethanol precipitated, air dried and resuspended in 1x TE. Probes were radiolabeled with [(α-^{32}P]ATP (6000 Ci $mmol^{-1}$, New England Nuclear), passed over a Sephadex G-50 Quick Spin column, and stored at −20 °C.

Binding assays

Binding assays typically contained radiolabeled 162-bp DNA probes (present at 100–200 pM, 5000–10 000 cpm) either unmodified or modified with platinum compounds, and purified hMSH2 present at 0–500 nM. Binding reactions were carried out in 15 μl reactions containing 25 mM potassium phosphate (pH 7.5), 25 mM NaCl, 0.5 mM dithiothreitol, 0.05 mM EDTA, 50 μg ml^{-1} BSA, 5 % glycerol, and 50 ng of nonspecific chicken erythrocyte competitor DNA. Binding was performed at 35 °C for 10 min. Samples were then loaded onto 4 % (29:1; acrylamide:bis) native polyacrylamide gels containing 1x TBE (90 mM Tris (pH 8.0), 2.0 mM EDTA, 90 mM boric acid) and 5 % glycerol, and separated by electrophoresis at 4 °C in 1x TBE at 25 mA for 2 h. Binding assays with 100-bp probes (100 pM, 8000 cpm) were similarly conducted except that 10 ng of nonspecific chicken erythrocyte competitor DNA was present in the reactions. Amounts of bound and unbound radiolabeled probe were determined by quantitative analysis of dried gels using a Molecular Dynamics PhosphorImager. The $K_{d(app)}$ was determined by a nonlinear least squares fitting of the binding data to the standard Hill equation [58].

Competition assays

Competition assays were carried out by titrating increasing amounts of unlabeled competitor DNA into binding reactions that contained constant amounts of both hMSH2 (present at 130 nM) and ^{32}P-labeled, 162-bp, cisplatin-modified (r_b = 0.018) DNA probe (present at 150 pM). Unlabeled competitor was pBR322 that was either unmodified or modified with cisplatin (r_b = 0.043).

Western blot analysis

Nuclear extracts from HeLa cells were prepared as described [59]. Pre-prepared protein extracts from adult human tissues were obtained from Clontech. For Western blot analysis of hMSH2, 400 μg of protein from human tissues, 75μg of protein from HeLa nuclear extract, and 10 ng of purified hMSH2 were resolved on an 8 % SDS-polyacrylamide gel and electroblotted to nitrocellulose. For Western blot analysis of α-tubulin, 100 μg of protein from human tissues and 75 μg of nuclear HeLa extract were used. The blots were incubated with a primary mouse monoclonal antibody either to hMSH2 (Oncogene Sciences) or to α-tubulin (Oncogene Sciences) followed by incubation with a horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology, Inc.). Antigen-specific signals were visualized using enhanced chemiluminescence (Amersham) according to the manufacturer's recommendations. Autoradiograms of Western blots were scanned using a Hewlett–Packard Scanjet IIp and scanned images were analyzed using the NIH Image 1.6 program. Relative intensities of hMSH2 signal were determined after normalization to that for α-tubulin.

Acknowledgements

We thank S. Lippard for the gift of platinum complexes, E. Trimmer and X.Q. Zhai for platinum-modified DNAs, M. Kartalou for technical assistance, P. Dedon for analysis of scanned images and D. Harrington for help with preparation of Figures. We thank E. Trimmer, D. Wang, M. Kartalou and T. Williams for valuable discussions and reading of the manuscript. This investigation was supported by the National Institutes of Health (Grants CA52127 to J.M.E. and CA67007 to R.F.).

References

1. Loehrer, P.J. & Einhorn, L.H. (1984). Cisplatin. *Ann. Intern. Med.* **100**, 704–713.
2. Bruhn, S.L., Toney, J.H. & Lippard, S.J. (1990). Biological processing of DNA modified by platinum compounds. *Prog. Inorg. Chem.* **38**, 477–516.
3. Eastman, A. (1983). Characterization of the adducts produced in DNA by *cis*-diamminedichloroplatinum(II) and *cis*-dichloro(ethylenediamine)-platinum(II). *Biochemistry* **22**, 3927–3933.
4. Fichtinger-Schepman, A.M.J., van der Veer, J.L., den Hartog, J.H., Lohman, P.H. & Reedijk, J. (1985). Adducts of the antitumor drug *cis*-diamminedichloroplatinum(II) with DNA: formation, identification, and quantitation. *Biochemistry* **24**, 707–713.
5. Sorenson, C.M., Barry, M.A. & Eastman, A. (1990). Analysis of events associated with cell cycle arrest at G_2 phase and cell death induced by cisplatin. *J. Natl Cancer Inst.* **82**, 749–755.
6. Chu, G. (1994). Cellular responses to cisplatin. *J. Biol. Chem.* **269**, 787–790.
7. Bruhn, S.L., Pil, P.M., Essigmann, J.M., Housman, D.E. & Lippard, S.J. (1992). Isolation and characterization of human cDNA clones encoding a high mobility group box protein that recognizes structural distortions to DNA caused by binding of the anticancer agent cisplatin. *Proc. Natl. Acad. Sci. USA* **89**, 2307–2311.
8. Pil, P.M. & Lippard, S.J. (1992). Specific binding of chromosomal protein HMG1 to DNA damaged by the anticancer drug cisplatin. *Science* **256**, 234–237.
9. Brown, S.J., Kellett, P.J. & Lippard, S.J. (1993). Ixr1, a yeast protein that binds to platinated DNA and confers sensitivity to cisplatin. *Science* **261**, 603–605.
10. Toney, J.H., Donahue, B.A., Kellett, P.J., Bruhn, S.L., Essigmann, J.M. & Lippard, S.J. (1989). Isolation of cDNAs encoding a human protein that binds selectively to DNA modified by the anticancer drug *cis*-diamminedichloroplatinum(II). *Proc. Natl. Acad. Sci. USA* **86**, 8328–8332.
11. Bianchi, M.E., Falciola, L., Ferrari, S. & Lilley, D.M.J. (1992). The DNA binding site of HMG1 protein is composed of two similar segments (HMG boxes), both of which have counterparts in other eukaryotic

regulatory proteins. *EMBO J.* **11**, 1055–1063.
12. Ferrari, S., Harley, V.R., Pontiggia, A., Goodfellow, P.N., Lovell-Badge, R. & Bianchi, M.E. (1992). SRY, like HMG1, recognizes sharp angles in DNA. *EMBO J.* **11**, 4497–4506.
13. Lilley, D.M.J. (1992). HMG has DNA wrapped up. *Nature* **357**, 282–283.
14. Bellon, S.F. & Lippard, S.J. (1990). Bending studies of DNA site-specifically modified by cisplatin, *trans*-diamminedichloroplatinum(II) and *cis*-[Pt(NH_3)$_2$(N3-cytosine)Cl]$^+$. *Biophys. Chem.* **35**, 179–188.
15. Bellon, S.F., Coleman, J.H. & Lippard, S.J. (1991). DNA unwinding produced by site-specific intrastrand cross-links of the antitumor drug *cis*-diamminedichloroplatinum(II). *Biochemistry* **30**, 8026–8035.
16. Donahue, B.A., *et al.*, & Essigmann, J.M. (1990). Characterization of a DNA damage-recognition protein from mammalian cells that binds specifically to intrastrand d(GpG) and d(ApG) DNA adducts of the anticancer drug cisplatin. *Biochemistry* **29**, 5872–5880.
17. Huang, J.-C., Zamble, D.B., Reardon, J.T., Lippard, S.J. & Sancar, A. (1994). HMG-domain proteins specifically inhibit the repair of the major DNA adduct of the anticancer drug cisplatin by human excision nuclease. *Proc. Natl. Acad. Sci. USA* **91**, 10394–10398.
18. McA'Nulty, M.M. & Lippard, S.J. (1996). The HMG-domain protein Ixr1 blocks excision repair of cisplatin–DNA adducts in yeast. *Mutat. Res.* **362**, 75–86.
19. Treiber, D.K., Zhai, X., Jantzen, H.-M. & Essigmann, J.M. (1994). Cisplatin–DNA adducts are molecular decoys for the ribosomal RNA transcription factor hUBF (human upstream binding factor). *Proc. Natl. Acad. Sci. USA* **91**, 5672–5676.
20. Wilson, T.M., *et al.*, & Kelley, M.R. (1995). Differential cellular expression of the human MSH2 repair enzyme in small and large intestine. *Cancer Res.* **55**, 5146–5150.
21. Fishel, R., *et al.*, & Kolodner, R. (1993). The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell* **75**, 1027–1038.
22. Leach, F.S., *et al.*, & Vogelstein, B. (1993). Mutations of a *mutS* homolog in hereditary nonpolyposis colorectal cancer. *Cell* **75**, 1215–1225.
23. Fram, R.J., Cusick, P.S., Wilson, J.M. & Marinus, M.G. (1985). Mismatch repair of *cis*-diamminedichloroplatinum(II)-induced DNA damage. *Mol. Pharmacol.* **28**, 51–55.
24. Fishel, R., Ewel, A. & Lescoe, M.K. (1994). Purified human MSH2 protein binds to DNA containing mismatched nucleotides. *Cancer Res.* **54**, 5539–5542.
25. Fishel, R., Ewel, A., Lee, S., Lescoe, M.K. & Griffith, J. (1994). Binding of mismatched microsatellite DNA sequences by the human MSH2 protein. *Science* **266**, 1403–1405.
26. Alani, E., Chi, N.-W. & Kolodner, R. (1995). The *Saccharomyces cerevisiae* Msh2 protein specifically binds to duplex oligonucleotides containing mismatched DNA base pairs and insertions. *Genes Dev.* **9**, 234–247.
27. Kašparková, J. & Brabec, V. (1995). Recognition of DNA interstrand cross-links of *cis*-diamminedichloroplatinum(II) and its *trans* isomer by DNA-binding proteins. *Biochemistry* **34**, 12379–12387.
28. Palombo, F., *et al.*, & Jiricny, J. (1995). GTBP, a 160-kilodalton protein essential for mismatch-binding activity in human cells. *Science* **268**, 1912–1914.
29. Drummond, J.T., Li, G.-M., Longley, M.J. & Modrich, P. (1995). Isolation of an hMSH2–p160 heterodimer that restores DNA mismatch repair to tumor cells. *Science* **268**, 1909–1912.
30. Papadopoulos, N., *et al.*, & Vogelstein, B. (1995). Mutations of GTBP in genetically unstable cells. *Science* **268**, 1915–1917.
31. Marsischky, G.T., Filosi, N., Kane, M.F. & Kolodner, R. (1996). Redundancy of *Saccharomyces cerevisiae* MSH3 and MSH6 in MSH2-dependent mismatch repair. *Genes Dev.* **10**, 407–420.
32. Mellon, I. & Champe, G.N. (1996). Products of DNA mismatch repair genes *mutS* and *mutL* are required for transcription-coupled nucleotide-excision repair of the lactose operon in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **93**, 1292–1297.
33. Mellon, I., Rajpal, D.K., Koi, M., Boland, C.R. & Champe, G.N. (1996). Transcription-coupled repair deficiency and mutations in human mismatch repair genes. *Science* **272**, 557–560.
34. Karran, P. & Marinus, M.G. (1982). Mismatch correction at O^6-methylguanine residues in *E. coli* DNA. *Nature* **296**, 868–869.
35. Jones, M. & Wagner, R. (1981). *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine sensitivity of *E. coli* mutants deficient in DNA methylation and mismatch repair. *Mol. Gen. Genet.* **184**, 562–563.
36. Goldmacher, V.S., Cuzick Jr, R.A. & Thilly, W.G. (1986). Isolation and partial characterization of human cell mutants differing in sensitivity to killing and mutation by methylnitrosourea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *J. Biol. Chem.* **261**, 12462–12471.
37. Kat, A., Thilly, W.G., Fang, W.-H., Longley, M.J., Li, G.-M. & Modrich, P. (1993). An alykylation-tolerant, mutator human cell line is deficient in strand-specific mismatch repair. *Proc. Natl. Acad. Sci. USA* **90**, 6424–6428.
38. Karran, P. & Bignami, M. (1992). Self-destruction and tolerance in resistance of mammalian cells to alkylation damage. *Nucleic Acids Res.* **20**, 2933–2940.
39. Branch, P., Aquilina, G., Bignami, M. & Karran, P. (1993). Defective mismatch binding and a mutator phenotype in cells tolerant to DNA damage. *Nature* **362**, 652–654.
40. Branch, P., Hampson, R. & Karran, P. (1995). DNA mismatch binding defects, DNA damage tolerance, and mutator phenotypes in human colorectal carcinoma cell lines. *Cancer Res.* **55**, 2304–2309.
41. Aquilina, G., Hess, P., Fiumicino, S., Ceccotti, S. & Bignami, M. (1995). A mutator phenotype characterizes one of two complementation groups in human cells tolerant to methylation damage. *Cancer Res.* **55**, 2569–2575.
42. Griffin, S., Branch, P., Xu, Y.-Z. & Karran, P. (1994). DNA mismatch binding and incision at modified guanine bases by extracts of mammalian cells: implications for tolerance to DNA methylation damage. *Biochemistry* **33**, 4787–4793.
43. Karran, P., Macpherson, P., Ceccotti, S., Dogliotti, E., Griffin, S. & Bignami, M. (1993). O^6-Methylguanine residues elicit DNA repair synthesis by human cell extracts. *J. Biol. Chem.* **268**, 15878–15886.
44. Sorenson, C.M. & Eastman, A. (1988). Mechanism of *cis*-diammine-dichloroplatinum(II)-induced cytotoxicity: role of G_2 arrest and DNA double-strand breaks. *Cancer Res.* **48**, 4484–4488.
45. Roussel, P., André, C., Masson, C., Géraud, G. & Hernandez-Verdun, D. (1993). Localization of the RNA polymerase I transcription factor hUBF during the cell cycle. *J. Cell Sci.* **104**, 327–337.
46. Leach, F.S., *et al.*, & Vogelstein, B. (1996). Expression of the human mismatch repair gene hMSH2 in normal and neoplastic tissues. *Cancer Res.* **56**, 235–240.
47. Aebi, S., *et al.*, & Howell, S.B. (1996). Loss of DNA mismatch repair in acquired resistance to cisplatin. *Cancer Res.*, in press.
48. Anthoney, D.A., McIlwrath, A.J., Gallagher, W.M., Edlin, A.R.M. & Brown, R. (1996). Microsatellite instability, apoptosis, and loss of p53 function in drug-resistant tumor cells. *Cancer Res.* **56**, 1374–1381.
49. Hawn, M.T., *et al.*, & Koi, M. (1995). Evidence for a connection between the mismatch repair system and the G_2 cell cycle checkpoint. *Cancer Res.* **55**, 3721–3725.
50. Sorenson, C.M. & Eastman, A. (1988). Influence of *cis*-diammine-dichloroplatinum(II) on DNA synthesis and cell cycle progression in excision repair proficient and deficient Chinese hamster ovary cells. *Cancer Res.* **48**, 6703–6707.
51. Piacentini, M., Fesus, L. & Melino, G. (1993). Multiple cell cycle access to the apoptotic death programme in human neuroblastoma cells. *FEBS Lett.* **320**, 150–154.
52. Evans, D.L. & Dive, C. (1993). Effects of cisplatin on the induction of apoptosis in proliferating hepatoma cells and nonproliferating immature thymocytes. *Cancer Res.* **53**, 2133–2139.
53. Shinomiya, N., Shinomiya, M., Wakiyama, H., Katsura, Y. & Rokutanda, M. (1994). Enhancement of CDDP cytotoxicity by caffeine is characterized by apoptotic cell death. *Exp. Cell Res.* **210**, 236–242.
54. Feuer, E.J., Brown, L.M. and Kaplan, R.S. (1993) in SEER Cancer Statistics Review: 1973–1990 (Miller, B.A., *et al.*, & Edwards, B.K., eds) pp. XXIV.1–XXIV.13, NIH Publication No. 93-2789: National Cancer Institute, Bethesda, MD.
55. Watt, G.W. & Cude, W.A. (1968). Diethylenetriamine complexes of platinum(II) halides. *Inorg. Chem.* **7**, 335–338.
56. Dhara, S.C. (1970). A rapid method for the synthesis of *cis*-[Pt(NH_3)$_2$$Cl_2$]. *Indian J. Chem.* **8**, 193–194.
57. Lippard, S.J., Ushay, H.M., Merkel, C.M. & Poirier, M.C. (1983). Use of antibodies to probe the stereochemistry of antitumor platinum drug binding to deoxyribonucleic acid. *Biochemistry* **22**, 5165–5168.
58. Creighton, T.E. (1993). Interactions with other molecules. In *Proteins: Structures and Molecular Properties.* (2nd edn), pp. 367–382, Freeman and Company, New York.
59. Dignam, J.D., Lebovitz, R.M. & Roeder, R.G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**, 1475–1489.