The mutagen and carcinogen cadmium is a high-affinity inhibitor of the zinc-dependent MutLα endonuclease

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MutLα (MLH1-PMS2 heterodimer), which acts as a strand-directed endonuclease during the initiation of eukaryotic mismatch repair, has been postulated to function as a zinc-dependent enzyme [Kosinski J, Plotz G, Guarné A, Bujnicki JM, Friedhoff P (2008) *J Mol Biol* 382:610–627]. We show that human MutLα copurifies with two bound zinc ions, at least one of which resides within the endonuclease active site, and that bound zinc is required for endonuclease function. Mutagenic action of the carcinogen cadmium, a known inhibitor of zinc-dependent enzymes, is largely due to selective inhibition of mismatch repair [Jin YH, et al. (2003) *Nat Genet* 34:326–329]. We show that cadmium is a potent inhibitor (apparent $K_i \sim 200$ nM) of MutLα endonuclease and that cadmium inhibition is reversed by zinc. We also show that inhibition of mismatch repair in cadmium-treated nuclear extract is significantly reversed by exogenous MutLα but not by MutSα (MSH2-MSH6 heterodimer) and that MutLα reversal depends on integrity of the endonuclease active site. Exogenous MutLα also partially rescues the mismatch repair defect in nuclear extract prepared from cells exposed to cadmium. These findings indicate that targeted inhibition of MutLα endonuclease contributes to cadmium inhibition of mismatch repair. This effect may play a role in the mechanism of cadmium carcinogenesis.

mismatch repair | MutLalpha | cadmium | carcinogen | zinc metalloenzyme

Inactivation of human MutLα (MLH1-PMS2 heterodimer, MLH1-PMS1 in yeast) results in a large increase in mutation production and strong cancer predisposition in humans (1, 2). Biochemical experiments have shown that MutLα plays an essential role in the initiation of eukaryotic mismatch repair (MMR). In physiological salt–Mg^{2+} buffer, MutLα functions as a strand-directed endonuclease that depends on a mismatch, MutSα (MSH2-MSH6 heterodimer) or MutSβ (MSH2-MSH3 heterodimer), and DNA-loaded proliferating cell nuclear antigen (PCNA) for activation, although endonuclease function is demonstrable in the absence of other proteins provided that the ionic strength is low and Mn^{2+} substituted for Mg^{2+} (3–7). The endonuclease active site resides within the MutLα C-terminal dimerization domain (CTD) and depends on integrity of conserved **DQHA**$(X)_2$**E**$(X)_4$**E**, **ACR**, and **CPHGRP** motifs within the PMS2 subunit (PMS1 in yeast) (3, 4, 8, 9). These motifs were postulated to comprise a binding site for a Zn^{2+} ion (8), and structural study of the yeast MutLα CTD revealed presence of two bound zinc ions (10). One zinc is stabilized by His703 and Glu707 of the PMS1 ^{701}**DQHA**SDEKYNF**E** sequence element and by interaction with Cys817 of the ^{816}**ACR** motif and the C-terminal Cys769 of MLH1. PMS1 Glu707 and MLH1 Cys769 also interact with the second zinc, which is further stabilized by interaction with Cys848 and His850 of the ^{848}**CPHGRP** motif (10).

Cadmium, which can replace zinc in a number of metalloenzymes (11–13), has been classified as a human carcinogen by the International Agency for Research on Cancer due to its link to lung cancer and possible involvement in cancers of the kidney and prostate (14, 15). The metal is an industrial pollutant but is also concentrated from the soil by certain plants, including tobacco, resulting in elevated blood Cd^{2+} levels in smokers (16).

Gordenin and coworkers (17) provided seminal insight into the mode of cadmium action with the demonstration that exposure of *Saccharomycies cerevisiae* to low-micromolar concentrations results in extreme hypermutability, an effect largely due to selective inhibition of MMR. Cadmium is also a mutagen in mammalian cells with about one-half the potency of activated benzo[α]pyrene (18), and as in the case of yeast, interference with MMR may contribute to this effect. Thus, cadmium disrupts MMR-dependent checkpoint activation in cultured human cells after treatment with an S_N1 DNA methylator (19), and injection of mice with $CdCl_2$ (1 mg/kg) results in microsatellite instability (20), which is diagnostic for MMR deficiency (2). Such findings prompted several reports attributing these effects to Cd^{2+} inhibition of the ATPase and mismatch recognition functions of MutSα (21–23). However, the Cd^{2+} concentrations required for MutSα inhibition are quite high, with apparent K_i values in the 10- to 200-μM range. This issue was clarified by Wieland et al. (23), who showed that Cd^{2+} inhibition of MutSα is nonspecific in nature and involves binding of about 100 Cd^{2+} ions to the MSH2-MSH6 heterodimer.

Given the structural evidence that MutLα endonuclease function may be Zn^{2+} dependent (10) and the fact that Cd^{2+} is a known inhibitor of a number of Zn^{2+} metalloenzymes (11, 12), we have addressed the involvement of zinc in human MutLα function and tested the possibility that Cd^{2+} inhibition of MMR may reflect targeted inhibition of MutLα. We show here that

Significance

MutLα (MLH1-PMS2 heterodimer) is an endonuclease that acts during an early step of eukaryotic mismatch repair. We show that human MutLα endonuclease copurifies with two equivalents of bound zinc, at least one of which resides within the endonuclease active site. We also show that cadmium, a known inhibitor of zinc-dependent enzymes and a potent mutagen and carcinogen, is a high-affinity inhibitor of MutLα endonuclease and that exogenous MutLα significantly reverses the mismatch repair defect in cadmium-treated human cell nuclear extract or nuclear extract prepared from cadmium-treated cells. Because the mutagenic action of cadmium is largely due to the selective inhibition of mismatch repair, these findings suggest that MutLα is a primary cadmium target for mutagenesis and presumably, carcinogenesis as well.

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MutLα copurifies with two Zn^{2+} ions (at least one of which resides in the endonuclease active site), that bound Zn^{2+} is involved in endonuclease function, that Cd^{2+} is a high-affinity inhibitor of endonuclease action, and that this effect at least partially accounts for Cd^{2+} inhibition of MMR.

Results

Human MutLα Is a Zinc Metalloenzyme. Structural analysis of the yeast MutLα CTD has shown presence of two bound Zn^{2+} ions (10). To evaluate zinc association with human MutLα, the protein was isolated using trace metal-grade reagents in the absence of chelator and DTT, and metal content was determined by inductively coupled plasma MS (ICP-MS) (24). Zn coelutes with MutLα during the final purification step (*SI Appendix*, Fig. S1*A*), and analysis of multiple samples of the wild-type protein showed the presence of 2.4 ± 0.3 zinc equivalents per MLH1-PMS2 heterodimer (Table 1).

Amino acid substitutions D699N or E705K within the conserved PMS2 $^{699}DQHA(X)_2E(X)_4E$ active site of human MutLα abolish endonuclease activity and MutLα function in MMR (3, 4, 8). The corresponding D701 residue within yeast PMS1 is not involved in zinc coordination, but E707 interacts with both active site Zn^{2+} ions in the yeast MutLα CTD structure (*SI Appendix*, Fig. S1*B*) (10). ICP-MS analysis showed that human D699N MutLα retains both zinc ions, but approximately one Zn equivalent is lost in the E705K variant (Table 1), indicating that at least one of two bound metal ions is located within the endonuclease active site.

Metal Dependence of the Basal MutLα Endonuclease Activity. Involvement of bound Zn^{2+} in MutLα endonuclease function was evaluated by treatment of the protein with the Zn^{2+}-selective chelator *N*,*N*,*N*′,*N*′-tetrakis(2-pyridlmethyl) ethylenediamine (TPEN). After removal of the chelator by gel filtration (*SI Appendix*, Fig. S1*C*), TPEN-treated and untreated control samples were tested at high concentration (0.5 μM) for endonuclease activity on supercoiled DNA in the absence of exogenous divalent metal (*Materials and Methods*). As shown in Fig. 1 (red squares) untreated native MutLα displays low but detectable endonuclease activity under these conditions. We attribute this activity to MutLα endonuclease function, because it is reduced by 80% with the endonuclease-defective D699N protein (Fig. 1, red diamonds). Prior TPEN treatment dramatically reduces the endonuclease activity of wild-type MutLα, but activity is restored to about 25% of control levels by low-micromolar concentrations of $ZnSO_4$ (Fig. 1, black circles), which have little if any effect on the activity of the untreated native protein. Because $ZnCl_2$ activates TPEN-treated MutLα to a similar degree (*SI Appendix*, Fig. S2*A*), this is a Zn^{2+} effect. $MgCl_2$ has no demonstrable effect on the TPEN-treated protein under these conditions, although $MnCl_2$ modestly activates at micromolar concentrations. As discussed above, 1 mM Mn^{2+} has been shown to activate the MutLα nuclease under low-salt conditions in the absence of other proteins (3).

Table 1. Zinc content of MutLα

MutLα	Zn equivalents per mole (determinations)
Wild type	2.4 ± 0.3 (30)
D699N endo dead	2.5 ± 1.2 (7)
E705K endo dead	0.8 ± 0.2 (6)

Samples of isolated MutLα were digested with analytical nitric acid and zinc content determined by ICP-MS (*Materials and Methods*). Three different ICP-MS instruments were used for the metal determinations summarized here. A breakdown of these results by instrument is shown in *SI Appendix*, Table S1. Coelution of Zn and MutLα from a MonoS column is shown in *SI Appendix*, Fig. S1. Errors are ±1 SD.

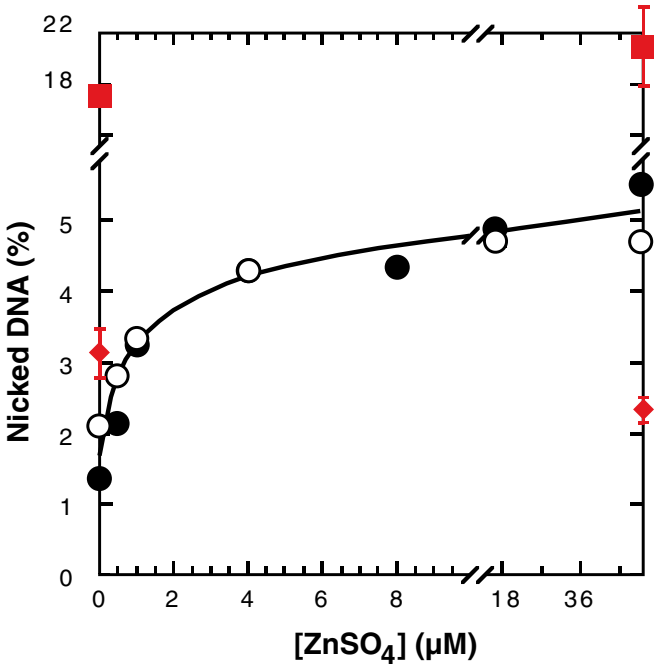

Fig. 1. TPEN-treated MutLα endonuclease is activated by Zn^{2+}. Incision of supercoiled DNA by 0.5 μM TPEN-treated MutLα (black circles) was determined in 60 mM KCl as a function of $ZnSO_4$ concentration (trace metal grade) in the absence of ATP, BSA, and other exogenous metals as described in *Materials and Methods*. Open and closed symbols correspond to two independent experiments. Controls are 0.5 μM wild-type (red squares) or endonuclease-deficient D699N MutLα (red diamonds), which were not treated with TPEN and are the average of three independent experiments (±1 SD). Note the breaks in abscissa and ordinate scales.

In view of these relatively selective metal effects on the TPEN-treated protein, we also tested the response of native MutLα to the three metals at 0.5 μM protein concentration, where the basal nuclease is readily evident in the absence of other factors (Fig. 1). As shown in *SI Appendix*, Fig. S2*B* (red circles) Mg^{2+} has no effect on basal endonuclease activity over a wide range of concentration from submicromolar to millimolar; Mn^{2+} (*SI Appendix*, Fig. S2*B*, blue circles) significantly activates at ∼100 μM to 1 mM, whereas Zn^{2+} (*SI Appendix*, Fig. S2*B*, gray circles) dramatically activates over the latter concentration range. Under these conditions, no significant endonuclease activity was observed with endo-dead D699N MutLα (*SI Appendix*, Fig. S2*B*, open circles). Because the endonuclease active site is fully occupied by Zn^{2+} at low-micromolar metal concentrations (Fig. 1 and *SI Appendix*, Fig. S1*A*), this nuclease activation at elevated Zn^{2+} concentrations is presumably a consequence of metal interaction with secondary sites on the protein, DNA, or both.

During the course of these experiments, we discovered an unexpected mixed metal effect that is evident at micromolar Zn^{2+} concentrations when Mn^{2+} is also present. The Zn^{2+} dependence of basal MutLα endonuclease activity scored in the presence of 1 mM Mg^{2+} is essentially identical to that in the absence of Mg^{2+} (*SI Appendix*, Fig. S2*C*, light blue circles; compare with *SI Appendix*, Fig. S2*B*, gray circles). However, basal MutLα endonuclease is activated by 1 mM Mn^{2+}, and the Mn^{2+}-activated nuclease responds differently to low Zn^{2+} concentrations (*SI Appendix*, Fig. S2*C*, gray circles). Although unaffected by 0–1 μM Zn^{2+}, endonuclease activity in the presence of 1 mM Mn^{2+} is inhibited by Zn^{2+} in the 1- to 10-μM range, and this inhibitory phase was followed by an activation phase at higher concentrations, similar to that observed in the presence of Zn^{2+} alone (compare gray circles in *SI Appendix*, Fig. S2 *B* and *C*). Similar

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patterns of Zn^{2+} inhibition and activation are observed in the presence ATP, which modestly enhances DNA incision at low zinc concentrations (*SI Appendix*, Fig. S2*C*, black circles). Because constitutive activation of basal MutLα endonuclease has been observed only in the presence of Mn^{2+} and because inhibition of the endonuclease by 1–10 μM Zn^{2+} does not occur in the presence of Mg^{2+} (*SI Appendix*, Fig. S2*C*) or the absence of a secondary metal (*SI Appendix*, Fig. S2*B*), these findings suggest that Zn^{2+} and Mn^{2+} are competing for a common site. We suggest that Mn^{2+} activation of basal MutLα endonuclease is a consequence of Mn^{2+} substitution for one or both active site zinc ions and that inhibition of the activated nuclease by low concentrations of exogenous Zn^{2+} is due to reversal of this effect.

Cd^{2+} Is a High-Affinity Inhibitor of Human MutLα Endonuclease. The mutagenic action of Cd^{2+} is largely due to selective inhibition of MMR (17). Because Cd^{2+} is a known inhibitor of Zn^{2+}-dependent enzymes (11, 12), we tested Cd^{2+} effects on MutLα endonuclease and ATPase in low-salt–Mn^{2+} buffer where both activities can be scored in the absence of other proteins (3, 7). As shown in Fig. 2*A*, the MutLα endonuclease function is exquisitely sensitive to Cd^{2+} inhibition, with an apparent K_i of 0.20 ± 0.05 μM. This value is only threefold higher than the 80 nM MutLα concentration used in the assays, implying that endonuclease inhibition is near stoichiometric. By contrast, the apparent K_i for MutLα ATPase inhibition is 40-fold higher (8.2 ± 2.7 μM), a value comparable with that observed for Cd^{2+} inhibition of MutSα ATPase at a similar protein concentration (23). Because Cd^{2+} inhibition of MutSα ATPase is nonspecific in nature, this may be the case for MutLα ATPase as well.

The curves shown in Fig. 2*A* are best fits to a Hill equation. Hill coefficients for both endonuclease and ATPase inhibition are less than 1 (0.43 and 0.33, respectively), indicating apparent anticooperativity. Cd^{2+} is expected to interact with other reaction components, including DNA, ATP, and secondary protein sites (23, 25, 26). Furthermore, because glutathione is present at millimolar concentrations in mammalian cells (27), all assays described here were done in the presence of the antioxidant, which is known to bind both cadmium and zinc (28). It, therefore, seems likely that the apparent anticooperative effects are the consequence of Cd^{2+} sequestration by secondary ligands.

The selective inhibition of MutLα endonuclease suggested that Cd^{2+} may target this zinc-dependent active site. We have been unable to directly show cadmium association with MutLα by ICP-MS after treatment of the protein with Cd^{2+} (17 μM MutLα, 50 μM $CdCl_2$) followed by gel filtration, indicating that MutLα affinity for Cd^{2+} is substantially less than that for zinc. As an alternate approach, we asked whether exogenous Zn^{2+} would compete with the inhibitory effect of 2 μM Cd^{2+} on endonuclease function. In fact, cadmium inhibition is reversed by low Zn^{2+} concentrations, with the effect peaking at a Zn^{2+} concentration comparable with that of the 80 nM MutLα concentration used in the assays (Fig. 2*B*). However and in contrast to the Zn^{2+} activation profile observed with TPEN-treated MutLα in the absence of other metals (Fig. 1), higher Zn^{2+} concentrations are inhibitory (Fig. 2*B*). The experiments shown in Fig. 2 were done in the presence of 23 mM KCl, 0.38 mM ATP, and 0.5 mg/mL BSA, but similar results were obtained in 60 mM KCl in the absence of ATP and BSA (*SI Appendix*, Fig. S3). We think that the inhibitory effects of higher Zn^{2+} concentrations in these experiments are the likely consequence of a mixed metal effect similar to that shown in *SI Appendix*, Fig. S2*C* and discussed above.

Exogenous MutLα, but Not MutSα, Significantly Reverses MMR Inhibition in Cd^{2+}-Treated Nuclear Extract and in Extract Prepared from Cd^{2+}-Treated Cells. Cadmium has been shown to inhibit MMR in extracts of human cells, with repair reduced 80% when extract is pretreated with 50 μM Cd^{2+} (17). We have confirmed

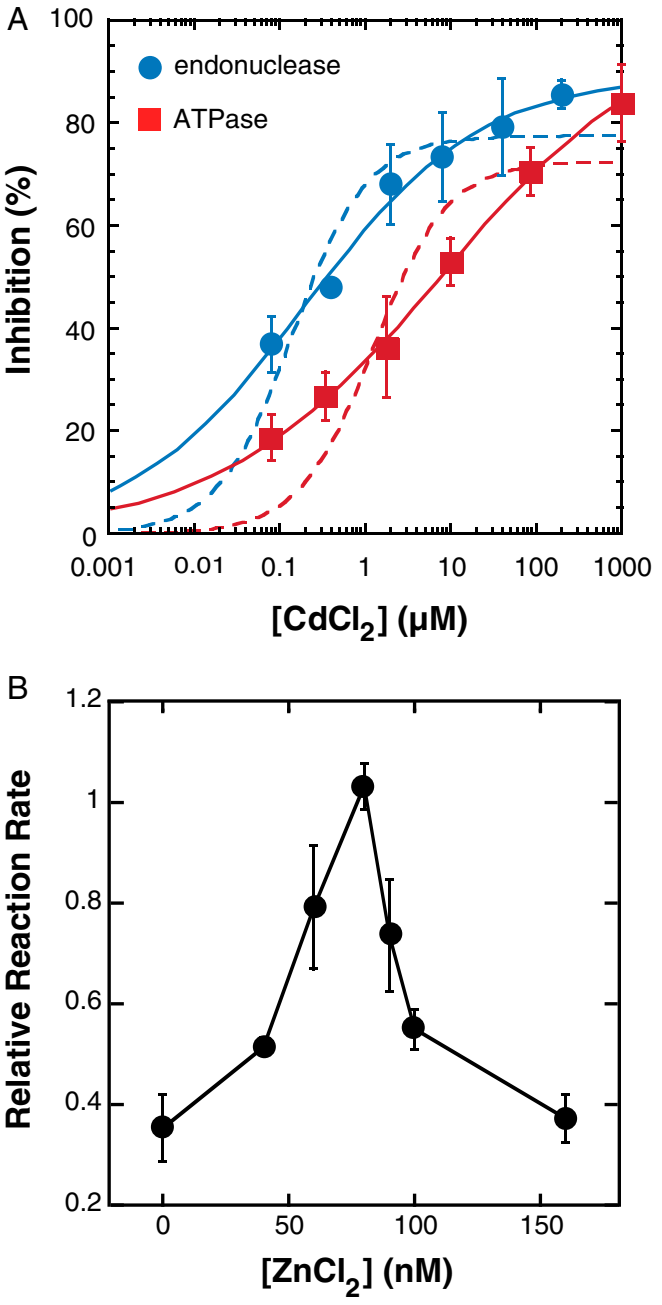

Fig. 2. Cd^{2+} preferentially inhibits MutLα endonuclease, and inhibition is reversed by Zn^{2+}. (*A*) $CdCl_2$ inhibition of incision of supercoiled DNA (blue circles) by 80 nM MutLα was determined in 23 mM KCl and 1 mM Mn^{2+} buffer containing 0.38 mM ATP and 0.5 mg/mL BSA. $CdCl_2$ inhibition of ATP hydrolysis (red squares) was determined under similar conditions, except that ATP concentration was 0.5 mM. $CdCl_2$ was premixed with other reaction components, and hydrolysis was initiated by MutLα addition (*Materials and Methods*). Values shown are the average of three independent experiments (±1 SD). Curves shown are nonlinear best fits to a Hill equation. Apparent K_i values for Cd^{2+} inhibition are 0.20 ± 0.05 μM with a Hill coefficient of 0.43 ± 0.07 for endonuclease and 8.2 ± 2.7 μM with a Hill coefficient of 0.33 ± 0.02 for ATPase. For comparison, data were also fit to a Hill equation with $n = 1$ (dashed lines). (*B*) Endonuclease reactions in the presence of ATP were as in *A*, except that $CdCl_2$ was present at 2 μM and $ZnCl_2$ as indicated. Initial reaction rates are normalized relative to that observed for MutLα incision in the absence of exogenous $CdCl_2$ and $ZnCl_2$ (initial rate 0.018 ± 0.0036 nM/min). Values are the average of three independent experiments (±1 SD). Similar results were obtained in the absence of ATP and BSA (*SI Appendix*, Fig. S3).

this finding and have asked whether inhibition can be rescued by exogenous MutLα or MutSα. As shown in Fig. 3*A*, supplementation of untreated nuclear extract from 293T Lα cells (29) with 25 nM MutLα or MutSα has only a small effect on MMR activity. However, supplementation of Cd^{2+}-treated extract with MutLα results in significant restoration of repair, but addition of MutSα does not, and extract activity when supplemented with both proteins is indistinguishable from that observed with MutLα alone. Furthermore, rescue of the MMR defect in Cd^{2+}-treated extract requires MutLα endonuclease function, as endonuclease-defective E705K MutLα does not suffice in this regard (Fig. 3).

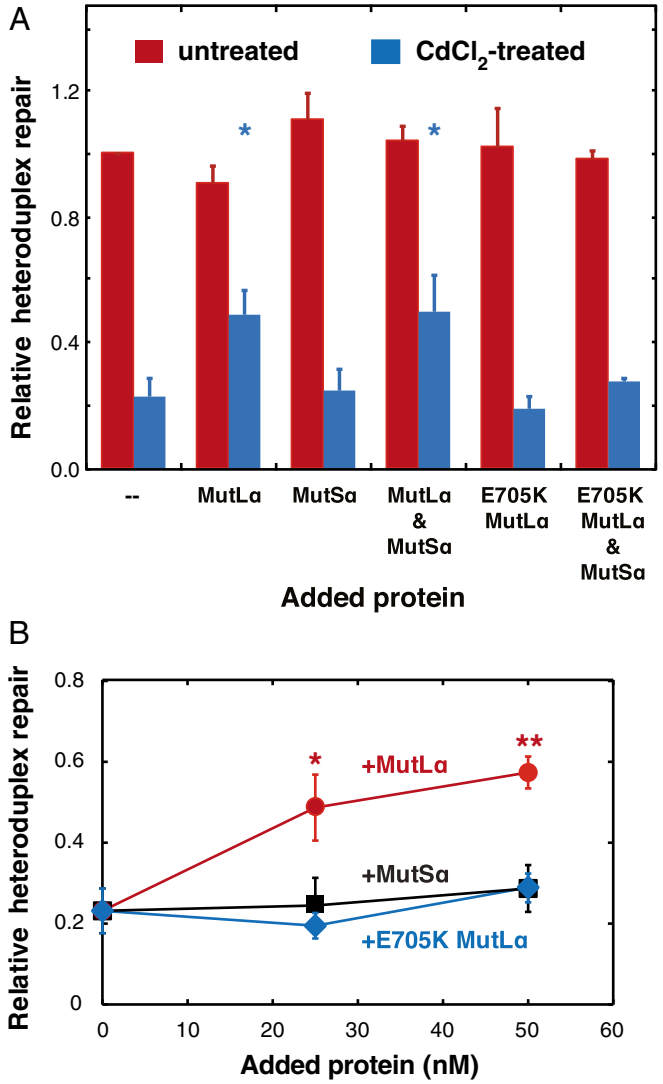

Fig. 3. Exogenous MutLα significantly reverses MMR inhibition in Cd^{2+}-treated nuclear extract. (*A*) Nuclear extract from 293T Lα cells (50 μg) was untreated (red bars) or pretreated with 50 μM $CdCl_2$ (blue bars) before supplementation as indicated with 25 nM MutSα, 25 nM MutLα, 25 nM endonuclease-defective E705K MutLα, 25 nM MutSα + 25 nM MutLα, or 25 nM MutSα + 25 nM E705K MutLα (*Materials and Methods*). Values for 3′ G-T heteroduplex repair activity are the mean of three determinations (±1 SD) and are expressed relative to that observed with untreated, unsupplemented extract. Significant reversal of Cd^{2+} inhibition (asterisks) was only observed up supplementation with MutLα ($P = 0.013$) or MutLα + MutSα ($P = 0.021$). (*B*) As in *A* except that extract supplementation was with indicated concentrations of MutSα (black squares), MutLα (red circles), or E705K MutLα (blue diamonds). Rescue was observed with 25 nM ($P = 0.013$) and 50 nM ($P = 2 \times 10^{-4}$) MutLα.

Biological studies have shown that Cd^{2+} treatment inhibits mammalian MMR in vivo (19, 20), and we have found that MMR in extracts prepared from 293T Lα cells treated with 5 μM Cd^{2+} for 4 h is reduced about 80% relative to that of extracts prepared from untreated control cells (Fig. 4). As observed with Cd^{2+}-treated extracts, the repair defect in extracts prepared from Cd^{2+}-treated cells is significantly rescued by exogenous MutLα but not by MutSα. Western blot analysis indicates that the MSH6 subunit of MutSα and the PMS2 subunit of MutLα are both present in extracts prepared from cadmium-treated cells, although PMS2 levels may be modestly reduced after Cd^{2+} exposure (*SI Appendix*, Fig. S4).

Discussion

$DQHA(X)_2E(X)_4E$, ACR, and CPHGRP motifs of PMS2 (PMS1 in yeast) together with the C-terminal Cys of MLH1 comprise a Zn^{2+} binding site and define the endonuclease center of the MutLα CTD (3, 6, 8, 10). Although initial estimates suggested presence of a single Zn^{2+} ion (8), crystallographic analysis of the yeast MutLα CTD revealed presence of two bound Zn ions (10), and we have confirmed presence of two Zn equivalents in native human MutLα. Because E705K substitution within the $DQHA(X)_2E(X)_4E$ motif, which inactivates endonuclease function, results in loss of approximately one Zn equivalent (Table 1), one of the two bound metals presumably resides within the CTD endonuclease active site. Additional evidence for Zn^{2+} involvement in MutLα function is provided by the fact that TPEN chelation of the metal reduces intrinsic endonuclease activity by 90%, which can be partially reversed by exogenous Zn^{2+} (Fig. 1).

MutLα endonuclease is subject to constitutive activation in the absence of a mismatch, and other repair proteins provided that Mn^{2+} is substituted for Mg^{2+} (3, 4), but the basis of this effect has been unclear. We show here that Mn^{2+}-dependent activation can be suppressed by low-micromolar Zn^{2+} concentrations (*SI Appendix*, Fig. S2*C*). This suggests that activation by millimolar Mn^{2+} is the result of substitution for one or both endogenous zinc ions and that this effect can be reversed by low concentrations of exogenous Zn^{2+}. The endonuclease motifs described above are found in many but not all bacterial MutL proteins (3, 30), where they are also believed to comprise Zn^{2+} binding endonuclease active sites that are subject to Mn^{2+} activation (31). NMR analysis of the *Aquifex aeolicus* MutL CTD has shown that Mn^{2+} binds in the proximity of the $DQHA(X)_2E(X)_4E$ and CPHGRP zinc coordination motifs (32), which is consistent with our findings based on functional assays.

Cadmium mutagenesis in yeast is largely a consequence of the selective inhibition of MMR (17), and the mutagenic action of Cd^{2+} in this organism is efficiently suppressed by Zn^{2+} (22). Although cadmium responses in higher organisms are likely to be more involved due, for example, to tissue differences and sequestration of the metal by inducible metallothionein (33), similar genotoxic cadmium effects have been documented in mammalian cells. Cd^{2+} treatment of cultured human cells abolishes the MMR-dependent checkpoint response to DNA methylator damage, an effect that is also reversed by Zn (19), and injection of mice with $CdCl_2$ results in testicular microsatellite instability (20). Such genotoxic effects presumably contribute to cadmium's action as a carcinogen (13, 15). Although Cd^{2+} inhibits mismatch recognition and ATP hydrolysis by MutSα (21, 22), these effects seem to be nonspecific in nature, involving binding of about 100 Cd equivalents per MSH2-MSH6 heterodimer (23).

The results described here show that Cd^{2+} selectively targets the endonuclease function of MutLα and suggest that this effect contributes significantly to selective inhibition of MMR by the metal. We have found that Cd^{2+} inhibits MutLα endonuclease with a submicromolar K_i and that inhibition is reversed by Zn^{2+} (Fig. 2), suggesting that the two metals compete for the same site(s). This idea is consistent with structural study of the *A. aeolicus* MutL

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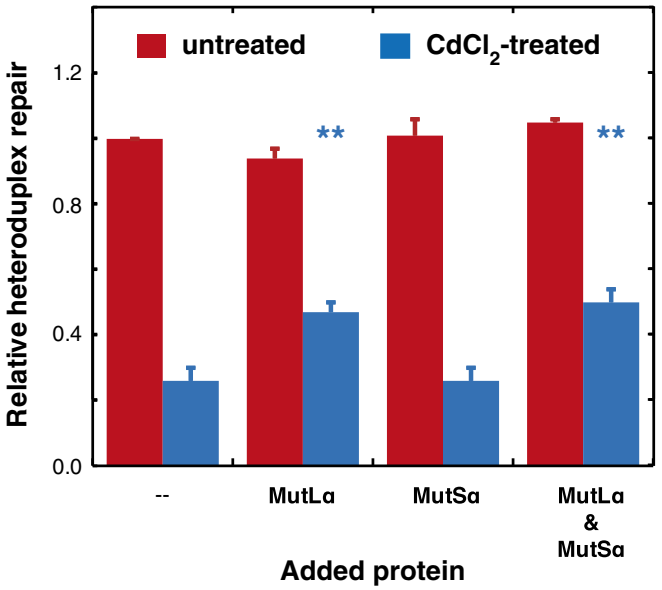

Fig. 4. MutLα partially rescues the MMR defect in extracts prepared from Cd^{2+}-treated cells. Nuclear extracts were prepared from 293T Lα cells, which were untreated (red bars) or treated for 4 h with 5 μM $CdCl_2$ (blue bars) before harvest (*Materials and Methods*). Extracts (50 μg) were supplemented as indicated with 25 nM MutSα and/or 25 nM MutLα; 3′ G-T heteroduplex repair is normalized relative to that observed with unsupplemented extract from untreated cells. Values shown are the mean of three determinations (±1 SD). Significant rescue of the repair defect (asterisks) was only observed on supplementation with MutLα ($P = 6.5 \times 10^{-9}$) or MutLα + MutSα ($P = 6.6 \times 10^{-5}$).

CTD crystallized in the presence of Cd^{2+} (30). Of the three Cd^{2+} ions found in the structure, two are coordinated by $DQHA(X)_2E(X)_4E$, ACR, and CPHGRP motifs in a manner identical to the two Zn atoms in the yeast MutLα CTD (10).

Perhaps the most compelling argument that selective MutLα inhibition contributes to Cd^{2+} mutagenesis is the finding that exogenous MutLα significantly reverses MMR inhibition in Cd^{2+}-treated nuclear extract and partially rescues the MMR defect in extracts prepared from Cd^{2+}-treated cells (Figs. 3 and 4). MutSα is without significant effect when added to such extracts, and MMR rescue by exogenous MutLα depends on integrity of the endonuclease active site. However, MMR rescue by exogenous MutLα in these extract experiments is incomplete. This may indicate that cadmium inhibition of other MMR activities contributes to pathway disruption, although MutSα seems an unlikely target, because MutSα and MutLα together are no more effective with respect to extract rescue than MutLα alone (Fig. 3*A*). However, although it seems likely that the bulk of the cadmium in treated extracts is bound to cellular components, the availability of free or exchangeable Cd^{2+} may be sufficient to inhibit the added MutLα to an extent that precludes complete reversal of the MMR defect in treated extracts as judged by biochemical assay.

Materials and Methods

DNA Substrates, Proteins, and Nuclear Extracts. Phagemid pGEM-3Zf(-) (Promega) was modified by site-directed mutagenesis at positions 3072 and 3073 to introduce a unique BbvCI site at position 3070 and designed as pGB31. This DNA was further modified by standard methods to yield 3.2-kb pGB31-MR1, which contains a 33-residue insert (5′-AGCTGCTAGCAAGCTTTCGAGTCTAGAAATTCG) in the top strand between positions 56 and 57 of pGB31. Replicative form pGB31-MR1 DNA was isolated as previously described (34); 6.4-kb G-T f1 heteroduplex DNAs for MMR assays were prepared using phages f1MR59 and f1MR60 (strand break 141 bp 3′ to the mismatch) (35).

Recombinant human MutSα and MutLα and MutLα variants with a D699N or E705K substitution in the PMS2 endonuclease active site (3) were isolated from baculovirus-infected SF9 cells as described (36), except that EDTA was omitted and 1 mM glutathione was substituted for DTT in all buffers. In some cases, buffers used for the last Mono S column were prepared using ultrapure water and trace metal-grade components, but this did not alter Zn content of final MutLα preparations. Concentrations of purified proteins were determined as previously described (37).

MutLα for preparation of the Zn-free protein was isolated in a similar manner, except that the order the last two columns (Mono Q and Mono S) was reversed and that the elution buffer for the final Mono Q column was prepared using trace metal-grade components [H_2O (omniTrace Ultra; EMD Millipore), Hepes (BioUltra; Sigma-Aldrich), KCl (TraceSelect; Sigma-Aldrich), glycerol (Ultrapure; Affymetrix), HCl (OPTIMA; Fisher), and KOH (TraceSelect; Sigma-Aldrich)]. Fractions were collected into metal-free 0.5-mL microfuge tubes that had been sequentially prerinsed with 10 mM EDTA, 10% nitric acid (OmniTrace; EMD Millipore), and ultrapure water (38). To prepare the Zn-free protein, MutLα (480 μg) was incubated on ice for 5 min in 150 μL of trace metal-grade 20 mM Hepes-KOH, pH 7.5, 0.2 M KCl, 1 mM glutathione, and 10% glycerol containing the Zn-selective chelator TPEN (10 mM; Sigma-Aldrich). Zn-free MutLα was resolved from TPEN by gel filtration on a 3-mL Sephacryl S-100 column equilibrated with the TPEN-free buffer (*SI Appendix*, Fig. S1*C*).

The 293T Lα cells, with a stably integrated h*MLH1* minigene under Tet-Off control (29), were cultured in roller bottles in DMEM containing 10% Tet-screened FBS (HyClone), 300 μg/mL Hygromycin B (Invitrogen), and 100 μg/mL Zeocin (Invitrogen). For Cd^{2+} treatment, 293T Lα cells were subcultured for 67–75 h before supplementation with 5 μM $CdCl_2$ (99.999% trace metals basis, 439800; Sigma-Aldrich), and treatment continued up to 8 h. Nuclear extracts were prepared as described (39), except that 1 mM glutathione replaced DTT in all buffers.

Extract protein concentrations were determined by Bradford assay (Bio-Rad). Western blot analysis (37) utilized antibodies against PMS2 (c-20; Santa Cruz), MSH6 [rabbit polyclonal (40)], and Lamin B1 (loading control, H-90; Santa Cruz). Immune complexes were visualized with IRDye-conjugated secondary antibodies (Li-Cor) and visualized with an Odyssey CLx Imager (Li-Cor).

ICP-MS Analysis. Metal content of MutLα samples was determined by ICP-MS (38). Samples were digested overnight at room temperature with HNO_3 (final concentration: 35% vol/vol; OmniTrace; EMD Millipore) in metal-free centrifuge tubes, heated at 85 °C for 30 min, and submitted for analysis on a ThermoFisher VG PlasmaQuad-3 (Duke University), Perkin-Elmer Elan DRCII (North Carolina State University), or Perkin-Elmer Nexion 300D (University of North Carolina, Chapel Hill). Elution buffer for the final column was used as background control in each case. Quantitation of metal content was based on use of certified standards (Fluka TraceSELECT) of known concentration in 2% HNO_3.

MMR Reactions. MMR in 293T Lα nuclear extract was determined in 20-μL reactions containing 20 mM Hepes-KOH, pH 7.5, 125 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 2 mM glutathione, 0.2 mM each dNTP, 5% (vol/vol) glycerol, 50 ng (0.6 nM) 3′ f1 G-T heteroduplex DNA, and 50 μg nuclear extract protein. After incubation at 37 °C for 30 min, reactions were terminated, and products were scored as described previously (3). For Cd^{2+} inhibition experiments, all reaction components except extract were premixed with $CdCl_2$ (mock treatment for controls) followed by extract addition and preincubation on ice for 5 min. MutLα or MutSα was then added, and reactions were immediately transferred to 37 °C. The ability of MutLα or MutSα to rescue the MMR defect in extracts prepared from $CdCl_2$-treated cells was evaluated by a slightly different procedure; in this case, 50 μg of extract on ice was supplemented with MutLα or MutSα immediately before mixing with other reaction components and transfer to 37 °C.

MutLα Endonuclease and ATPase Assays. For determination of Cd^{2+} effects on Mn^{2+}-dependent MutLα endonuclease (3), 38.8 μL of 20 mM Hepes-KOH, pH 7.6, 23 mM KCl, 0.38 mM ATP, 0.5 mg/mL BSA, 2% (vol/vol) glycerol, 1 mM glutathione, 100 ng (1.2 nM) pGB31-MR1 supercoiled DNA, 1 mM $MnSO_4$, and $CdCl_2$ as indicated were premixed on ice. Reactions were initiated by addition of 1.2 μL MutLα to yield a final concentration of 80 nM. Incubation was at 37 °C, the reaction was sampled as a function of time for rate determination, hydrolysis was quenched, and products were scored as previously described (3). $CdCl_2$ inhibition results were fit to a Hill equation by nonlinear regression using KaleidaGraph (Synergy Software):

$$[\text{Inhibition } \%] = \frac{\text{Maximum inhibition} \times [CdCl_2]^n}{K_i^n + [CdCl_2]^n}.$$

The ability of Zn^{2+} to reverse Cd^{2+} inhibition was tested by a similar procedure, except that reaction mixtures were supplemented with 2 μM $CdCl_2$ and $ZnCl_2$ as indicated before initiation by addition of MutLα.

Metal activation of untreated or TPEN-treated MutLα was determined in a similar manner except that $MnSO_4$ and BSA were omitted, and 10-μL reactions contained KCl and ATP as indicated, 50 ng (2.4 nM) pGB31-MR1 supercoiled DNA, and 0.5 μM MutLα. Trace metal-grade $ZnSO_4$, $ZnCl_2$, $MnCl_2$, or $MgCl_2$ was present as specified, metal-free microfuge tubes were used, and incubation was at 37 °C for 110 min.

For ATPase determination, 7 μL of 20 mM Hepes-KOH, pH 7.5, 46 mM KCl, 2 mM glutathione, 35 ng pGB31-MR1 (1.2 nM final) supercoiled DNA, 2 mM trace metal-grade $MnCl_2$, 1 mg/mL BSA, 5% (vol/vol) glycerol, 160 nM MutLα, and 0–2 mM $CdCl_2$ were prewarmed to 37 °C for 2 min. Hydrolysis was initiated by addition of 7 μL 1 mM [γ-^{32}P]ATP (3.5 Ci/mmol) in 20 mM Hepes-KOH, pH 7.5, and 5% (vol/vol) glycerol; 2-μL samples were removed as a function of time, reactions were quenched, and hydrolysis was determined as described (41).

Statistical Methods. Errors shown are ±1 SD. *P* values were calculated by two-tailed Student *t* test using Mathematica software (Wolfram).

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