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Role of MutSα in the Recognition of Iododeoxyuridine in DNA[1]

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ABSTRACT

We have previously demonstrated that both the MLH1 and MSH2 status impact the DNA levels of the halogenated thymidine (dThd) analogues iododeoxyuridine (IdUrd) and bromodeoxyuridine (BrdUrd), and thereby radiosensitization induced by these analogues, indirectly implicating both mismatch repair (MMR) proteins in the removal of these bases from DNA. More recent data from our group demonstrate that base excision repair (BER) also impacts IdUrd-DNA levels, supporting a role for the BER pathway in IdUrd removal as well. In this study, we have examined more direct interactions between the MSH2 protein and the processing of IdUrd incorporated in DNA. Our data demonstrate that the MutSα (MSH2/MSH6) complex binds specifically to DNA containing an IdUrd-G mismatch, using both purified human MutSα as well as nuclear extracts from *Msh2*-proficient and-deficient mouse cell lines. MutSα binding to a IdUrd-G is better recognized than a G-T mismatch in the same sequence context. In addition, MSH2 protein can be found colocalized with IdUrd-DNA using confocal microscopy in G_1 synchronized cells after treatment with IdUrd. Consistent with our recent publication, coadministration of IdUrd and a chemical inhibitor of BER, methoxyamine (MX), also increases the extent of MSH2 nuclear colocalization with IdUrd. Furthermore, we show that the extent of MSH2 colocalization with IdUrd in G_1-synchronized human tumor cells varies with MLH1 status, suggesting a role for the MLH1 protein in stabilizing the interaction between the MSH2 protein and DNA containing IdUrd. These data, both *in vitro* and *in vivo*, suggest direct involvement of MSH2 in processing IdUrd in DNA.

INTRODUCTION

The initial step in postreplicational DNA MMR[3] involves recognition of mismatches and IDLs, resulting from replication errors, by the MutS homologues. In eukaryotes, there are multiple homologues of the MutS protein that exist as heterodimers in the cell and that have different recognition specificities for mismatched substrates in DNA. The MSH2 protein, which is, by far, the most commonly mutated of the MutS homologues in families with HNPCC (1), dimerizes with one of two additional MutS homologues, stabilizing the dimeric protein partner. MSH2 forms the MutSβ complex with MSH3, which preferentially recognizes large IDLs in DNA (2). The MSH2 protein can also dimerize with MSH6, forming the MutSα complex, to bind smaller IDLs, single base-pair mismatches (1, 3), cisplatin adducts (4, 5), O^6-methylguanine and O^4-methylthymine-containing base pairs (4), aminofluorene adducts (6), and can also recognize some larger IDLs as well, albeit with much lower efficiency (7).

The repair specificities of MutSα and MutSβ correspond to their binding affinities (2, 7, 8). The repair efficiency of MutSα for single base-pair mismatches is much greater than that of MutSβ (9), whereas purified MutSβ has greater specificity for correcting IDLs ranging from 3 to 10 extra bases (7). In addition, the binding affinity and repair efficiency of MutSα or MutS directly correlate among various single base-pair mismatch substrates. The MSH2/MSH6 complex has the highest affinity to bind a G-T mismatch, and also repairs this mismatch most efficiently (7–9), with decreasing binding affinity and repair efficiency for six different single base-pair mismatches (9).

Localization of the MMR proteins within the cell also corresponds to *in vitro* binding data and repair assays. *In vivo* studies have recently shown that both the MSH3 and MSH6 dimeric partners of MSH2 can be localized to DNA replication foci (10), consistent with a role for both MutSα and MutSβ in the repair of replicational errors, and suggesting that it is possible to visualize MMR proteins that localize to areas requiring MMR activity. Additionally, the components of the MutSα complex were found to relocate in the cell after treatment with MNNG (11), which is known to be selectively cytotoxic to MMR-proficient cells and results in O^6-methylguanine-T mispairs in DNA that are recognized by MutSα in EMSA studies (4).

Previously, we have shown indirect evidence implicating both the MLH1 and MSH2 proteins in processing the halogenated dThd analogues, IdUrd, and BrdUrd in DNA, using multiple genetically matched human and murine cell lines that differ in MLH1 or MSH2 status (12, 13). Our initial studies demonstrated lower levels of IdUrd and BrdUrd in the DNA of MMR-proficient cells, consistent with removal of the drug from DNA by MMR, similar to repair of single base-pair mismatches. However, no significant cytotoxicity nor cell cycle effects were detected after IdUrd treatment, unlike the response to other nucleoside analogues such as 6-thioguanine (12, 13). We now show that human and murine MutSα directly bind DNA containing an IdUrd-G mispair with high affinity. We also show nuclear colocalization of MSH2 and IdUrd-DNA in G_1-synchronized cells after prior treatment with IdUrd. These data suggest that the nuclear colocalization of MSH2 with IdUrd represents a specific interaction rather than the coincidental presence of the MMR proteins in replication foci with IdUrd. Because recent data from our group also indicate that BER impacts IdUrd-DNA incorporation (14), we also studied the effects of a BER inhibitor, MX, on the colocalization of MSH2 with IdUrd. The addition of MX increases the extent of MSH2 colocalization with IdUrd in both MLH1-proficient and MLH1-deficient cell lines. Finally, we also demonstrate that the degree of MSH2 colocalization with IdUrd in G_1-synchronized cells correlates with MLH1 status, implicating the MLH1 protein in the stabilization of MSH2 on DNA.

MATERIALS AND METHODS

Cell Lines. HCT116 human colon cancer cells are deficient in MMR because of mutations in both alleles of the *Mlh1* gene. HCT116/3-6 human colon cancer cells are proficient in MMR because of chromosome transfer of a human chromosome 3 (15), containing a wild-type copy of the *Mlh1* gene, to the parental HCT116 cell line. Both HCT116 and HCT116/3-6 cells have been previously characterized in our laboratory (12, 16). The $Msh2^{+/+}$ and $Msh2^{-/-}$ murine embryonic stem cells from *Msh2* wild-type and knockout mice were transformed with E1A and have been described previously by our laboratory (13). Cells were grown in DMEM (Mediatech, Inc., Herndon, VA) complemented with 10% defined FBS (Hyclone, Logan, UT), 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Inc., Rockville, MD), 0.1 mM nonessential amino acids (Life Technologies, Inc.), and grown in 90% air-10% CO_2 at 37°C. HCT116/3-6 cells were maintained in 400 μg/ml G418 (Life Technologies, Inc., NY) to select for cells continuing to carry a copy of

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[3] The abbreviations used are: MMR, mismatch repair; dThd, thymidine; IdUrd, iododeoxyuridine; BrdUrd, bromodeoxyuridine; BER, base excision repair; IDL, insertion-deletion loop; MNNG, *N*-methyl-*N′*-nitro-*N*–nitrosoguanidine; EMSA, electrophoretic mobility shift assay; MX, methoxyamine; HPLC, high-performance liquid chromatography; dsDNA, double-stranded DNA.

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Table 1 *Oligonucleotides used in EMSA analysis*

Set A		
Top strands		
a	5′-GCTAGCAAGC **C** ATCGATTCTAGAAATTCGGC-3′	Complementary
b	5′-GCTAGCAAGC **T** ATCGATTCTAGAAATTCGGC-3′	G-T mismatch
c	5′-GCTAGCAAGC **I** ATCGATTCTAGAAATTCGGC-3′	IdUrd-G mismatch
d	5′-GCTAGCAAGC (**CA**)$_5$ ATCGATTCTAGAAATTCGGC-3′	(CA)$_5$ IDL
Bottom strand		
e	3′-CGATCGTTCG **G** TAGCTAAGATCTTTAAGCCG-5′	
Set B		
Top strand		
c	5′-GCTAGCAAGC **I** ATCGATTCTAGAAATTCGGC-3′	
Bottom strands		
f	3′-CGATCGTTCG **A** TAGCTAAGATCTTTAAGCCG-5′	IdUrd-A complementary
g	3′-CGATCGTTCG **C** TAGCTAAGATCTTTAAGCCG-5′	IdUrd-C mismatch
h	3′-CGATCGTTCG **T** TAGCTAAGATCTTTAAGCCG-5′	IdUrd-T mismatch

chromosome 3, but experiments were carried out in the absence of the selection agent.

Oligonucleotides. Thirty-one-bp complementary oligonucleotides were obtained from Oligos Etc. Inc. (Wilsonville, OR). They contained a sequence interrupted by either a G-T mismatch or an IdUrd residue paired with various natural bases or a sequence with a G-C complementary base pair, as indicated in Table 1.

EMSA. Purified human MutSα and MutSβ were generously provided by Dr. Paul Modrich (Duke University Medical Center, Durham, NC). MutSα was diluted to a concentration of 50 ng/μl in a buffer containing 25 mM HEPES-KOH (pH 7.5), 330 mM KCl, 1 mM EDTA, 1 mg/ml BSA, and 1 mM DTT. MutSβ was diluted to 50 ng/μl in a buffer containing 25 mM HEPES-KOH (pH 7.5), 100 mM KCl, 0.1 mM EDTA, and 2 mM DTT. Nuclear extracts from murine E1A-transformed ES cells from *Msh2*$^{-/-}$ knockout mice or their *Msh2*$^{+/+}$ wild-type siblings were prepared as described previously (13). Reactions containing either purified MutSα, purified MutSβ, or nuclear extract from *Msh2*$^{+/+}$ and *Msh2*$^{-/-}$ cells were preincubated with 1 μg of poly(deoxy-iodine-deoxycytidine) on ice for 5 min in a reaction buffer containing 10 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 50 mg/ml BSA, 1 mM EDTA, and 0.4% glycerol. For cold competition experiments with purified MutSα, 10–300 ng of unlabeled dsDNA were added during the 5-min preincubation period, and for cold competition with cell extracts, 150 ng of unlabeled dsDNA of the same sequence were added for 5 min at room temperature before the addition of labeled DNA. For supershift of the band visible specifically after incubation with *Msh2*$^{+/+}$ nuclear extract, 1 ng of MSH6 (GTBP) monoclonal antibody (Transduction Laboratories, Lexington, KY) was preincubated with nuclear extract from *Msh2*$^{+/+}$ cells for 5 min at 37°C, followed by the addition of the labeled probe. Approximately 20 ng of ^{32}P-substrate was then added to the reaction, for a total volume of 20 μl. Samples were then incubated on ice for 10 min (MutSα and cell extracts) or 30 min (MutSβ), followed by the addition of a loading buffer containing 0.25% bromphenol blue, 0.25% xylene cyanole FF, and 30% glycerol to stop the reaction, and were subsequently loaded onto a 6% nondenaturing polyacrylamide gel at 4°C. Samples were then subjected to electrophoresis at 10 V/cm at 4°C, and dried on a gel dryer at 80° for 2 h. Dried polyacrylamide gels were then exposed to a Molecular Dynamics PhosphorImager Cassette (Molecular Dynamics, Inc., Sunnyvale, CA) at room temperature overnight. Bands were quantitated using Image Quant software (Molecular Dynamics, Inc.).

Nuclear and Cytoplasmic Extract Preparations for Msh2$^{+/+}$ and Msh2$^{-/-}$ Cells. Approximately 4 × 10^6 exponentially growing cells were harvested and centrifuged, and subsequently washed in 1 ml of PBS, then were centrifuged again. The pellet was then washed in a buffer containing 10 mM HEPES (pH 7.4), 10 mM KCl, and 0.5 mM DTT, followed by centrifugation. Cells were then lysed at 4°C in 20 μl of a buffer containing 10 mM HEPES (pH 7.4), 10 mM KCl, 0.5 mM DTT, and 1.0% NP40 for 10 min on ice, with rocking. The nuclei were then collected by centrifugation at 2,000 × *g* for 5 min, and the supernatant was considered the cytoplasmic extract. The nuclei were then lysed at 4°C for 15 min in a high-salt buffer containing: 20 mM HEPES (pH 7.9), 500 mM NaCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 1.5 mM $MgCl_2$, and 20% glycerol, with the use of a Dounce homogenizer as needed, checking for lysis with trypan blue staining under a light microscope. The lysate was then spun down at 12,000 × *g* for 10 min at 4°C, then was aliquoted and was stored at −80°C.

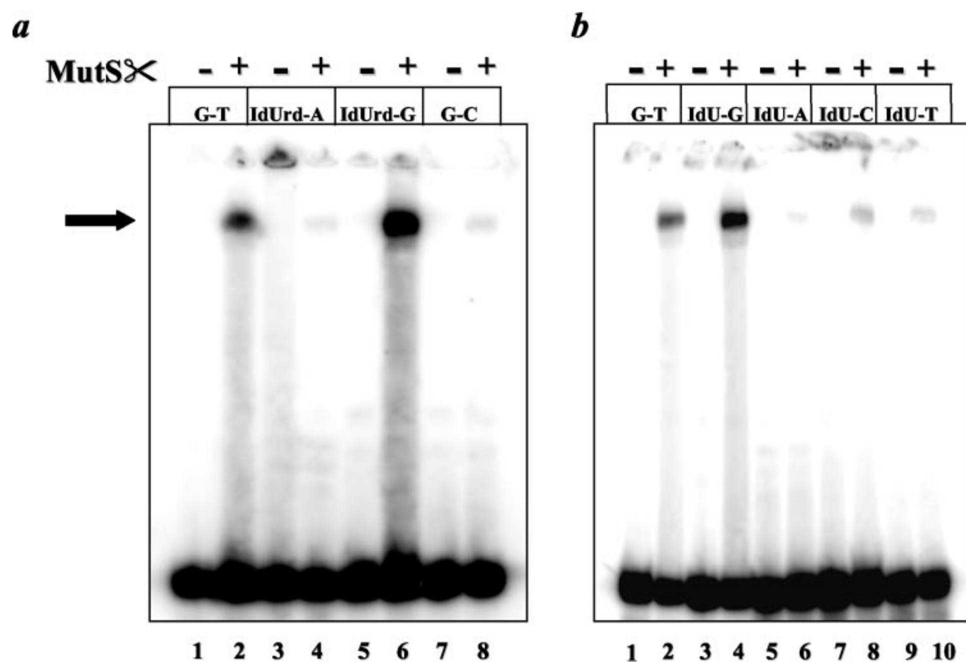

Fig. 1. MMR dimer recognizes the halogenated dThd analogue IdUrd in DNA. *a*, purified MutSα was incubated with 31-bp dsDNA oligonucleotides containing a G-T mismatch (*Lanes 1* and *2*), as a positive control for binding; a G-C complementary base pair, in the same sequence, as a negative control (*Lanes 7* and *8*); or IdUrd paired to an adenine (*Lanes 3* and *4*) or mispaired to a guanine (*Lanes 5* and *6*), in the same sequence context. *b*, MutSα has low affinity for IdUrd paired to a cytosine (*Lanes 7* and *8*) or to a thymine (*Lanes 9* and *10*), which are not known to occur after incorporation of IdUrd into DNA.

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Fluorescent Staining for IdUrd and MMR Proteins in Synchronized Cells. Cells were grown to confluence in the presence of 10% FBS or dialyzed FBS (−dThd) with either 10 μM IdUrd alone or 10 μM IdUrd plus 6 mM MX. Cell synchronization in G_1 phase was accomplished by incubation with DMEM, supplemented with either 0.1% FBS or 0.1% dialyzed FBS, with treatment as above for 48 h. Cells were released from synchronization by plating onto coverslips in a 6-well dish with DMEM supplemented with 10% FBS. After 5 h, the coverslips were washed with PBS, fixed in 4% formalin solution (Sigma, St. Louis, MO) for 10 min at room temperature, washed three times with PBS, permeabilized by 0.25% Triton X (in PBS) for 5 min at room temperature, washed once with PBS, and then stored at 4°C in PBS before staining. Incubations for blocking and treating with antibodies were done at room temperature for 1 h in PBS/0.3% Tween 20. Coverslips containing fixed cells were blocked in 1% BSA, incubated with a 1:150 dilution of IdUrd monoclonal antibody (CALTAG Laboratories, Burlingame, CA), incubated in antimouse secondary antibody Alexa 568 (Molecular Probes, Eugene, OR) at a dilution of 1:250, incubated with a 1:50 dilution of MSH2 monoclonal antibody (Ab-2; Oncogene Research, Cambridge, MA), and incubated in antimouse secondary antibody Alexa 488 (Molecular Probes) at a dilution of 1:250; wells were washed in PBS/0.3% Tween 20 between each incubation. The coverslips were then mounted on slides with gel-mounting medium containing a nucleus-specific stain (Vector Laboratories, Burlingame, CA) and were placed at −20°C until viewing. The confocal images were obtained at ×67 with a Zeiss LSM510 inverted confocal microscope system (Zeiss, Oberkochen, Germany) equipped with a tunable T-Sapphire laser (Mira-F-V5-XW-220) with a diode pump laser (Verdi 5 W) to obtain the images with the different dyes at the Confocal Core Microscopy Facility at Case Western Reserve University. Image analysis was carried out with Adobe Photoshop software (Adobe Systems, San Jose, CA).

RESULTS

MutSα Recognizes the dThd Analogue IdUrd in DNA. Although *Thermus aquaticus* (*Taq*) MutS has previously been shown not to recognize homoduplex DNA containing IdUrd base-paired to adenine (17), it is not known whether bacterial MutS or MutS eukaryotic homologues recognize IdUrd-A or whether IdUrd is recognized by MMR proteins when paired to other Watson-Crick bases. We, therefore, tested the ability of the human MutSα to bind to dsDNA containing the physiologically relevant base pair IdUrd-A and IdUrd-G (18, 19), as well as IdUrd-C and IdUrd-T (Fig. 1). A 31-bp dsDNA probe containing a G-T mismatch in the middle, shown previously to be bound with high affinity by MutSα (20), was used as a positive control for binding. The same sequence was also used to compare MutSα binding to a G-C complementary base-pair or a base pair containing IdUrd, at the same position as the G-T mismatch (Table 1). In Fig. 1*a*, *Lane 2*, incubation of MutSα with heteroduplex DNA results in an intense band, representing the specific shift of the ^{32}P-probe, compared with the very low level of background binding to a complementary base pair within the same sequence (Fig. 1*a*, *Lane 8*). Similar to results with *Taq* MutS (17), human MutSα does not bind dsDNA containing IdUrd paired with adenine with greater affinity than to homoduplex DNA in the same sequence context (Fig. 1*a*, *Lane 4*). However, when IdUrd is mispaired with a G, the MutSα dimer shows a very strong shift (Fig. 1*a*, *Lane 6*), indicating high-affinity binding. A single-stranded oligonucleotide containing IdUrd (oligonucleotide *c*, Table 1) was also incubated with purified MutSα to determine whether IdUrd alone was recognized by the MMR complex, but MutSα did not show any binding to this substrate (data not shown). Binding of MutSα was also tested for a 31-mer containing IdUrd-C and IdUrd-T base pair (Table 1), which are thought to occur infrequently when IdUrd is incorporated into DNA (19, 21). MutSα recognizes both of these substrates to a degree comparable with homoduplex DNA in the same sequence context (Fig. 1*b*), suggesting that the substrate that the MutSα complex recognizes after incorporation of IdUrd into DNA is an IdUrd-G mismatch. This correlates with the high affinity of this complex both for naturally arising G-T mismatches (3) and for other chemically modified bases and drug-induced DNA adducts, including O^6-methylguanine-T pairs in DNA (4), S^6-methylthioguanine-T pairs in DNA (22), cisplatin adducts (4, 5), and aminofluorene adducts (6).

Although MutSβ has not previously been shown to recognize any drug-induced DNA adducts that are specifically cytotoxic to MMR-proficient cells, there is a degree of overlap in the activity of MutSα and MutSβ, as mutation spectrums indicate (23), and repair of some single-base mismatches by MutSβ can occur in *in vitro* repair assays (9). As a result, we also used EMSA analysis with IdUrd in the same sequence context used for the MutSα binding, using dsDNA containing a $(CA)_5$ IDL (Table 1) as a positive control for binding and found that MutSβ does not recognize IdUrd-G pairs in DNA (data not shown).

An IdUrd-G mismatch is also selectively recognized by proteins in

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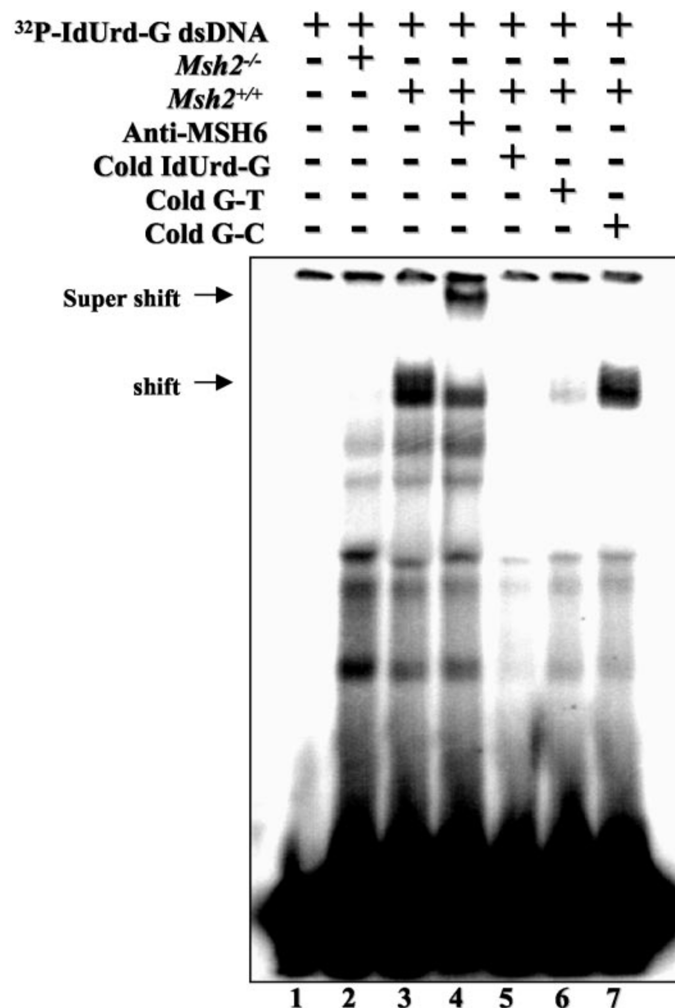

Fig. 2. An IdUrd-G substrate is selectively recognized by proteins from MSH2-proficient nuclear extracts. There are two shifted bands after incubation of *Msh2*$^{+/+}$ nuclear extract with a dsDNA probe containing an IdUrd-G mispair in the center (*Lane 3*), which are not present after incubation of this probe with *Msh2*$^{-/-}$ nuclear extract (*Lane 2*). One of these bands migrates much slower after the addition of a monoclonal antibody to the MSH6 protein (*Lane 4*), one component of the MutSα dimer. In addition, preincubation of the *Msh2*$^{+/+}$ nuclear extract with "cold," unlabeled DNA containing either an IdUrd-G mismatch, or a G-T mismatch in the same sequence context as the labeled probe, and the gel retardation assays with purified MutSα, abolishes both retarded bands specific to the *Msh2*$^{+/+}$ nuclear extract (*Lanes 5* and *6*). However, preincubation with unlabeled DNA containing a G-C complementary base pair in the same sequence context abolishes shifted bands common to both *Msh2*$^{-/-}$ and *Msh2*$^{+/+}$ nuclear extracts, but not the two bands specific to the *Msh2*$^{+/+}$ nuclear extracts (*Lane 7*).

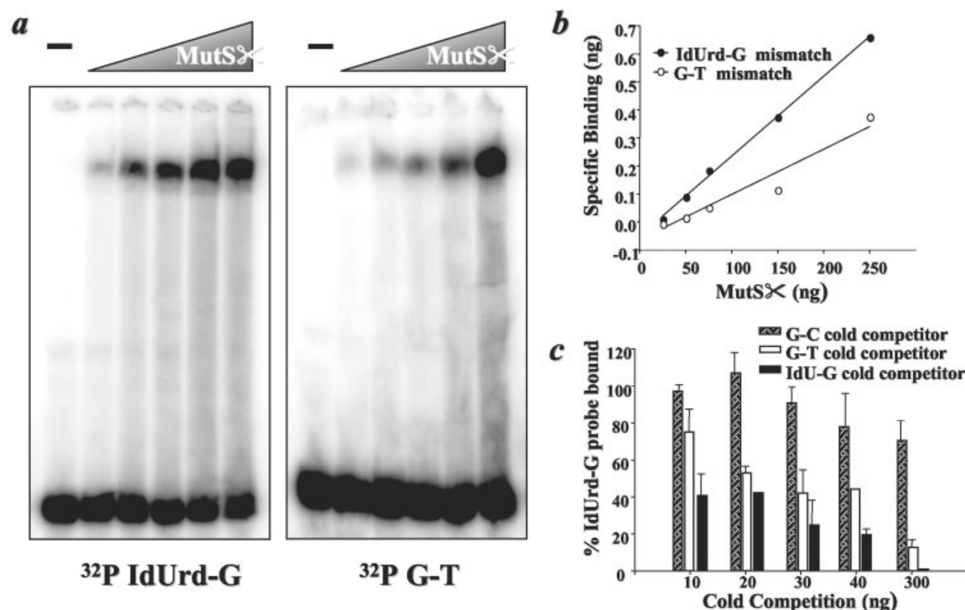

Fig. 3. Specific binding of MutSα for a G-T mismatch and an IdUrd-G mismatch. *a,* purified MutSα, in various amounts, was incubated with a 31-bp dsDNA probe containing either a G-T or an IdUrd-G mismatch in the same sequence context. *b,* bands were quantitated using Image Quant software and the points on the graph represent the average of three experiments. *c,* effect of preincubation with unlabeled cold DNA on the binding of MutSα to a ^{32}P-labeled dsDNA probe containing an IdUrd-G mismatch, representing the average of three separate experiments.

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nuclear extracts of murine MMR-proficient cells (Fig. 2). We found that there are two retarded bands after incubation of a double-stranded fragment of DNA containing an IdUrd-G mismatch with *Msh2*$^{+/+}$ nuclear extract that are not present after incubation with *Msh2*$^{-/-}$ nuclear extract (Fig. 2, *Lanes 2* and *3*). In addition, one of these bands is clearly immunoreactive with the antibody to the MSH6 protein (*Lane 4*), suggesting that MSH6 is present in a complex with dsDNA containing an IdUrd-G pair, causing the ^{32}P-probe to migrate more slowly. Cold competition with excess unlabeled dsDNA of the same sequence context, containing an IdUrd-G base pair, or a G-T mismatch (Table 1), also abolishes the retarded bands specific to the *Msh2*$^{+/+}$ nuclear extract (*Lanes 5* and *6*), with cold IdUrd-G more effectively abolishing the two bands than cold G-T. This also corresponds to Fig. 3, which demonstrates that MutSα has a higher affinity for an IdUrd-G pair in this sequence context than for a G-T pair in this sequence. Excess unlabeled dsDNA of the same sequence, but containing a G-C base pair rather than an IdUrd-G pair, does not eliminate the two retarded bands specific to the *Msh2*$^{+/+}$ nuclear extract (Fig. 2, *Lane 7*), further supporting the possibility that the two retarded bands specific to the *Msh2*$^{+/+}$ nuclear extract are the result of MMR proteins binding to the dsDNA containing an IdUrd-G base pair. Also, the addition of all three unlabeled dsDNA fragments results in the abolishment of bands common to both *Msh2*$^{-/-}$ and *Msh2*$^{+/+}$ extracts, demonstrating that these retarded bands are not the result of specific recognition by the MMR proteins (*Lanes 5–7*).

Binding Affinity of the MutSα Complex for a G-T Mismatch and an IdUrd-G Mismatch in DNA. To directly compare the affinity of purified MutSα for an IdUrd-G mispair with that of the recognition complex for a G-T mismatch, we used various amounts of purified MutSα with equivalent amounts of each 31-mer. In three separate reactions, the MutSα complex consistently demonstrated higher specific binding activity for an oligonucleotide containing an IdUrd-G mismatch than for a G-T mismatch (Fig. 3, *a* and *b*). This can also be demonstrated using unlabeled oligonucleotides containing a complementary G-C base pair, a G-T mismatch, or an IdUrd-G mismatch to compete for binding by MutSα to a ^{32}P-labeled 31-mer containing an IdUrd-G pair. Although preincubation with unlabeled G-C does not affect MutSα binding to an IdUrd-G mismatch (Fig. 3*c*), preincubation with both unlabeled and heteroduplex oligonucleotides quickly eliminates the binding to IdUrd-G. However, cold IdUrd-G competes for binding at lower concentrations than does cold G-T; and at the highest concentrations, preincubation with cold IdUrd-G prevents all binding to the labeled IdUrd-G oligonucleotide, whereas preincubation with the same concentration of cold G-T does not. It is likely that the alteration in DNA, and possibly the charge distribution created by an IdUrd-G mispair, is slightly different from that caused by the presence of a G-T mismatch, affecting the binding specificity of MutSα for these different substrates.

Nuclear Colocalization of MSH2 with IdUrd. The endogenous MSH2 protein is distributed throughout the nucleus and cytoplasm of intact HCT116/3-6 and HCT116 human colon cancer cells in the absence of treatment (Fig. 4, *b* and *k,* respectively). These data are in contrast with previous publications in which these proteins were found to be exclusively nuclear in intact cells among multiple tissue types (24, 25) but are consistent with *in vitro* MMR assays using cytoplasmic extract (10), as well as with a more recent report that demonstrates the presence of MSH2 and MSH6 in the cytoplasm of HeLa cells using both Western blot analysis and immunocytochemical staining of intact cells (11). Because a short pulse (15 min) with the halogenated dThd analogue BrdUrd has been used to colocalize MSH3 and MSH6 to replication foci, we have looked for MSH2 colocalization with IdUrd in a synchronized population of cells in G_1. We found that MSH2 remains colocalized with IdUrd during G_1 (Fig. 4), in which cell cycle status was confirmed with flow cytometry (data not shown), indicating that the colocalization cannot be attributed exclusively to the presence of MSH2 in replication foci. We also found that MSH2 colocalized with IdUrd in both MLH1-deficient and MLH1-proficient cells, although the extent of colocalization is greater in MLH1-proficient HCT116/3-6 cells (compare Fig. 4*o* with Fig. 4*f*). These data demonstrate that MLH1 is not necessary for the nuclear colocalization of MSH2 and IdUrd but suggest that MLH1 may be necessary for a prolonged interaction with, or for a stable MMR complex assembly on, DNA that contains IdUrd.

Finally, our group has recently demonstrated that the BER status of cells also impacts IdUrd-DNA levels and subsequently IdUrd-induced radiosensitization (14). Therefore, we have also examined the impact of a general BER inhibitor, MX, on MSH2 colocalization with IdUrd. The addition of MX during treatment with IdUrd results in an increase in the extent of MSH2 colocalization with IdUrd (Fig. 4), suggesting that there are greater numbers of IdUrd residues available for binding

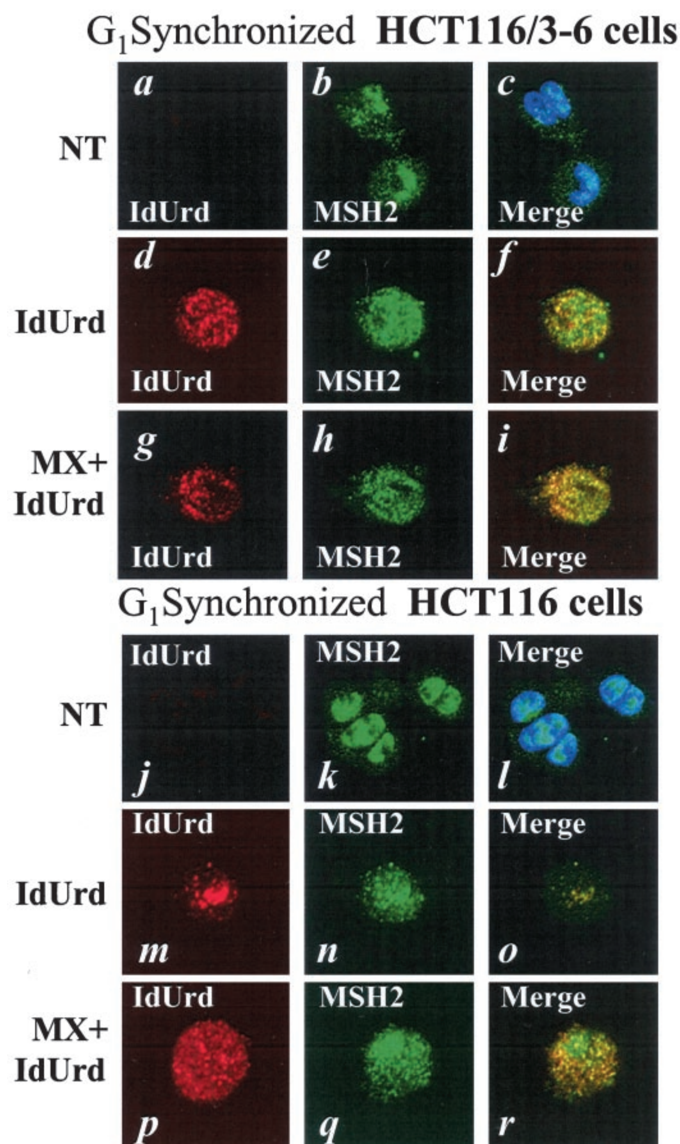

Fig. 4. MSH2 colocalization with IdUrd during G_1. HCT116/3-6 and HCT116 cells were exposed to drug-free medium (*a–c, j–l*) or to 10 μM IdUrd, in the absence or presence of 6-mM BER inhibitor MX,(*d–i, m–r*) for 3 days. The cells were synchronized in G_0-G_1 by incubation in medium supplemented with 0.1% FBS and drug treatment as mentioned in the previous sentence for another 48 h. Cells were released from synchronization and samples were fixed 5 h after release. All of the samples were incubated with antibody to IdUrd and MSH2. The MSH2 protein can be seen colocalized with IdUrd in G_1-Phase cells (*f, i, o,* and *r*). Also, colocalization is clear in both MLH1-proficient and -deficient cells during G_1, although there appears to be more colocalization of MSH2 and IdUrd in MLH1-proficient cells (compare *f* and *o*). Additionally, colocalization occurs to a greater extent in the presence of MX in both MLH1-proficient and -deficient cells (*i* and *r*).

by MSH2 in the presence of MX, which would otherwise be bound by BER proteins. These data on combined IdUrd + MX treatment are consistent with our recently published study showing higher levels of IdUrd-DNA incorporated (as measured by HPLC) compared with IdUrd alone (14).

DISCUSSION

Our data demonstrate the recognition of IdUrd in dsDNA by MutSα and nuclear colocalization of the MSH2 protein with IdUrd, supporting a direct role for the MSH2 protein in the recognition of IdUrd in DNA. In agreement with previous studies of the *Taq* MutS protein (17), we find that human MutSα does not recognize DNA that contains IdUrd-A pairs but does recognize IdUrd-G mispairs in the same sequence context with high affinity, indicating that this is the primary substrate for the MMR-associated differences in IdUrd levels in DNA (12, 13). Previous studies using chemically modified bases and single base-pair mismatches in the same location, within the same sequence context that we have used in our EMSA analysis, have demonstrated that the human MutSα complex recognizes a G-T mismatch with greater specificity than do aminofluorene and 2-acetylaminofluorene adducts (6), cisplatin adducts (5), and O^6-methylguanine- or O^4-methylthymine-containing pairs (4) at the same position. Therefore, an IdUrd-G mismatch appears to be unique in that it is recognized by the human MutSα complex in this same sequence context more efficiently than a G-T mismatch. Of the possible base pairings between Watson-Crick bases and IdUrd, an IdUrd-G pair would bear the greatest similarity to a G-T mismatch in DNA. However, the van-der-Waals radius of iodine is just slightly larger than that of the methyl group at the same position of dThd (26), which may cause a greater distortion of the DNA helix containing IdUrd-G than would a G-T mismatch and could explain the higher affinity of MutSα for DNA that contains IdUrd-G than for DNA that contains G-T in the same sequence context. Previous studies with the structurally similar dThd analogue, BrdUrd, suggest that base pairing of this analogue with guanine does occur *in vivo* in λ phage and *Escherichia coli*, and plays a role in BrdUrd-induced mutagenesis (19, 21).

We have also shown that MutSα has very little affinity for IdUrd paired with adenine, cytosine, or thymine in the same sequence context, suggesting that MMR is not involved in processing these pairs in DNA. This would also correlate with our previous data, in which we have shown that, although the levels of IdUrd in DNA decrease over time in MMR-proficient cells, approximately 5.0% of the dThd remains substituted with IdUrd in most cell lines that we have studied (12, 13). It is likely that IdUrd pairs with other bases in addition to guanine, part of the time, and we speculate that these pairs probably account for the analogue that remains in the DNA of MMR-proficient cells.

Our binding data indicate that MMR proteins can recognize DNA that contains IdUrd-G pairs with high affinity and specificity, and this is supported by protein colocalization of MSH2 and IdUrd in nuclear foci of synchronized cells in G_1. Our data from the synchronous G_1 cells indicates that the nuclear localization of MSH2 is not the result of the incidental presence of the protein in replication foci. This is interesting also because these G_1 foci may be indicative of active MMR of DNA that contains IdUrd, because it has been demonstrated that strand-specific DNA MMR can be carried out in cells at various stages of the cell cycle, such as in G_2-synchronized cells (27), in G_1 synchronized cells (28), and in extracts of senescent, mitotic, and postmitotic tissues from *Drosophila* (29).

It is also important to consider the potential role of BER in processing IdUrd in DNA, based on our recent publication (14). In the present study, we demonstrate that treatment with the BER inhibitor MX increases the extent of MSH2 colocalization with IdUrd (Fig. 4). Although MX is known to inhibit single-nucleotide BER by blocking repair of the AP site after glycosylase-mediated removal of a base (30), this MX/AP stable intermediate is known to be a substrate for long-patch BER (31). Our results using confocal microscopy in this study and using HPLC analysis in the prior study (14) show that there are a greater number of IdUrd residues in DNA after cotreatment with MX, which suggests that, although IdUrd does not appear to be a substrate for removal by single-nucleotide BER, it is likely a substrate for long-patch BER. Consistent with this, addition of MX during treatment with IdUrd results in an increase in the extent of MSH2 colocalization with IdUrd. This would indicate that long-patch BER

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also plays a role in binding IdUrd in DNA and thereby prevents MSH2 recognition of some IdUrd residues.

In conclusion, although BER also is likely to play a role in the recognition and processing of IdUrd in DNA, these data demonstrate that MMR proteins directly interact with DNA that contains IdUrd, and that IdUrd-G mispairs are likely to be the primary substrate for recognition by MutSα. Our findings also demonstrate that the interaction of the MMR proteins with IdUrd is prolonged and can occur during G_1, and that MLH1 is not necessary for the specific interaction of MSH2 and IdUrd but may stabilize it. These data provide more direct evidence for the interaction of MMR proteins with IdUrd and shed light on one mechanism for the cellular processing of IdUrd after incorporation into DNA.

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