The Interaction of DNA Mismatch Repair Proteins

with Human Exonuclease I

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ABSTRACT

Exonucleolytic degradation of DNA is an essential part of many DNA metabolic processes including DNA mismatch repair (MMR)\(^1\) and recombination. Human exonuclease I (hExoI) is a member of a family of conserved 5’→3’ exonucleases which are implicated in these processes by genetic studies. Here, we demonstrate that hExoI binds strongly to hMLH1, and we describe interaction regions between hExoI and the MMR proteins hMSH2, hMSH3, and hMLH1. In addition, hExoI forms an immunoprecipitable complex with hMLH1/hPMS2 \textit{in vivo}. The study of interaction regions suggests a biochemical mechanism of the involvement of hExoI as a downstream effector in MMR and/or DNA recombination.

INTRODUCTION

DNA is susceptible to exogenous and endogenous damaging agents as well as spontaneous hydrolysis resulting in nucleotide damage, abasic (AP) sites, and DNA strand breaks (1). In addition, DNA sequence alterations may be caused by nucleotide misincorporation errors during DNA replication (2) and recombination between divergent DNA parents (3). Highly conserved DNA repair mechanisms have been identified, and inactivation of these pathways has been linked to a variety of diseases including cancer (4). DNA mismatch repair (MMR) is one of the best studied repair pathways (5-7), and inactivating mutations in the human MMR genes are causally involved in the development of sporadic and hereditary cancers such as the common cancer susceptibility syndrome Hereditary Non-Polyposis Colon Cancer (HNPCC) (8).

\(^1\) Abbreviations: hExoI, human exonuclease I; MMR, DNA mismatch repair; HNPCC, hereditary nonpolyposis colorectal cancer; PMSF, phenylmethylsulfonylfluoride; GST, glutathione S-transferase; IVTT, \textit{in vitro} transcription translation; DTT, dithiothreitol; PBS, phosphate buffered saline.
The biochemistry of MMR has been most extensively studied in *E. coli*. Eight protein fractions (MutS, MutL, MutH, DNA helicase II (UvrD), single-strand DNA binding protein, exonuclease I, DNA ligase and the DNA polymerase III holoenzyme complex), ATP and the four deoxynucleotide triphosphates are sufficient to carry out the complete MMR process *in vitro* (9). Several human MutS homologs (MSH) have been identified including the nuclear MMR proteins hMSH2, hMSH3, hMSH6 as well as the meiosis specific proteins hMSH4 and hMSH5. In eukaryotes, the MutS homologs function as heterodimers, which recognize single base mismatches, insertion/deletion loop-type (IDL) mismatches, and some types of nucleotide damage (5,10). The mechanism for the initial step(s) of MMR continues to be controversial (6,11). However, there has been significant progress in deciphering the molecular basis for mispair recognition (12,13) and the role of ATP binding and hydrolysis by the MutS homologs (14-18). In contrast, the steps following the recognition of a mismatch or nucleotide lesion remain unclear. MutL homologs appear to be involved in signal transfer to downstream effectors (19-23). In *E. coli*, MutL stimulates the endonucleolytic activity of MutH at GATC sites (24,25). The resulting strand break functions as a signal for UvrD and exonucleases to remove the nascent strand starting at the GATC site and ending several nucleotides past the mismatch (26,27). Four single-strand DNA exonucleases appear to maintain overlapping and redundant requirements for MMR: ExoI, RecJ, ExoVII and ExoX. The 5'→3' exonuclease activity of either RecJ or ExoVII is sufficient to excise a mismatch in a circular plasmid when a nick is present 5' to the lesion (28). Bacterial ExoI (and presumably ExoX) can remove the mismatch through its 3'→5' exonuclease activity in cases where the nick is 3' of the mismatch (27-31).

While eukaryotic members of the MutS and MutL protein family appear to function similarly compared to their *E. coli* orthologs (5), experimental results suggest a slightly different pathway for down-stream events in eukaryotes. First, a homolog for MutH or a DNA helicase activity linked to MMR has not been identified. Second, a class of proteins with double-strand 5'→3' exonuclease and flap-endonuclease activities has been described which include *S. cerevisiae*
EXO1 (32), S.pombe EXO1 (33), the D.melanogaster TOSCA protein (34), human and yeast FEN1 (35-37), and the human hExoI (32,38,39). S.pombe EXO1 degrades DNA starting at both double-strand ends and DNA nicks, and an exo1- strain exhibited a 4- to 13-fold increased mutation rate in a G→T transversion assay (33). While the mutation rate of S.cerevisiae msh2 mutants was several-fold higher than exol mutants, this rate was not significantly elevated in exol msh2 double mutants (40,41). These results suggested that msh2 and exol were likely to be epistatic (although additivity was not entirely ruled out). Yeast strains harboring a replicative DNA polymerase δ defective in its 3’→5’ proofreading exonuclease (pol3-01) in combination with a deletion of exol or msh2 display similarly elevated mutation rates (41), and haploid yeast strains with a DNA polymerase δ proofreading defect combined with a deletion of exol or msh2 are lethal (41). Taken as a whole, these results suggest yeast EXO1 plays a role in mutation avoidance and supports a model in which the 5’→3’ activity of EXO1 and the 3’→5’ exonuclease activity of Pol δ participate in a bidirectional MMR (41). Several lines of evidence also indicate that EXO1 functions in additional pathways. EXO1 expression is UV inducible, and EXO1-mutant yeast strains display a mild UV sensitivity (42). Biochemical and genetic evidence in yeast (43) and the observation of high hExoI/HEX1 expression in testis (44) suggests the involvement of these proteins in meiotic and mitotic recombination. hExoI also shows RNAse H activity, and it may function as backup for FEN1 in RNA primer removal during lagging strand DNA synthesis (45).

We have recently shown that hExoI interacts directly with hMSH2 in vitro (38). hExoI has been shown to functionally complement a yeast exol mutant strain, and the truncated version of hExoI, termed HEX1 (or hExoIa), has both single-stranded and double-stranded 5’→3’ exonuclease activity in vitro (45). In this study, we examined the interactions of hExoI with other human MutS and MutL homologs. Characterization of the interaction regions suggests a mechanism for recognition, signaling and excision of the damage. Our results support a role for
hExoI in down-stream events associated with MMR and/or other metabolic processes involving MMR enzymes.

MATERIALS AND METHODS

Protein-Protein Interaction - The GST/IVTT protein-protein interaction assay was performed essentially as described (38). Briefly, hExoI, hMSH2, hMSH3, hMSH6, hMLH1, hPMS2 and relevant fragments of these constructs were subcloned into pGEX-4T-2 (Pharmacia Biotech), which allows high expression of a glutathione S-transferase (GST) fusion protein, and were transformed into *E. coli* XL1-blue. A 10ml overnight culture was diluted with LB medium to 800ml and grown at 37°C until the OD$_{600}$ of the culture was 0.5. Adding IPTG to a final concentration of 1 mM and incubation for 2 hr at 30°C induced protein expression. The culture was harvested by centrifugation at 2000 xg for 15 min at 4°C. The bacterial pellet was resuspended in PBS containing 1mM phenylmethylsulfonylfluoride (PMSF), 2µg/ml leupeptin, and 2µg/ml pepstatin. The cells were lysed by 2 freeze-thaw cycles in the presence of 100µg/ml lysozyme and Triton X-100 (0.2%) and 5 mM dithiothreitol (DTT). DNAse I was then added to a final concentration of 20µg/ml. Lysates were incubated on ice for 30 min and cellular debris removed by centrifugation. The cleared supernatant was incubated with glutathione agarose beads (SIGMA) for 1 h at 4°C under gentle continuous agitation. Under these conditions, approximately 20-50ng of protein was bound to 25µl beads. Samples were pelleted for 1 min at 300 xg and each pellet was washed 3 times with 500µl of binding buffer (20mM Tris pH7.5, 10% glycerol, 150mM NaCl, 5mM EDTA, 1mM DTT, 0.1% Tween 10, 0.75mg/ml BSA, 0.5mM PMSF, 1µg/ml leupeptin, and 1µg/ml pepstatin). Lysates containing the unmodified pGEX vector were treated similarly and used as a negative control. GST and GST-fusion protein binding to the beads was verified by denaturing gel electrophoresis (SDS-PAGE) and quantitated using BSA as a standard as previously described (46). To produce $^{35}$S-labeled proteins, hExoI, hMSH2,
hMSH3, hMSH6, hMLH1, hPMS2 and fragments thereof where subcloned into pET24d (Novagen), and 1µg of these constructs was used in *in vitro* transcription/translation (IVTT) (TNT Coupled Reticulocyte Lysate System; Promega). The labeled proteins were quantitated as described (46). Equivalent amounts of protein from each IVTT mix was added to the suspension of protein-bound glutathione beads in 500µl of binding buffer. The mixture was incubated at 4°C for 1 h under gentle agitation. Subsequently, the beads were washed 3 times with binding buffer and then resuspended in SDS-PAGE loading buffer. Proteins were resolved on a 10% SDS-PAGE gel, visualized and quantified using a Molecular Dynamics PhosphorImager System.

**Quantitation of Protein Interactions** - The GST-IVTT interaction assay system is not absolutely quantitative and is likely to depend on the relative association constant (k_assoc) of the individual interacting proteins. Thus, subtle changes in the relative concentration of interacting peptides may influence the ultimate measure of interaction. In order to provide modest control between experiments for such concentration-dependent processes, we determined the approximate molar concentrations of the GST-fusion protein and the IVTT protein as previously described (46). Interaction experiments were designed to contain an equivalent ratio of the test peptides. Relative interaction (Int_rel) was determined as the fraction of the peptide interaction-ratio (IR_p) divided by the wild type interaction-ratio (IR_wt). IR_p was determined by quantitating the amount of interacting peptide [Molecular Dynamics PhosphorImager with ImageQuant software (Sunnyvale, CA)] and dividing this number by the quantitated amount of IVTT protein. These quantifications takes the number of ^35^S-Met in the peptides into consideration. The wild type interaction-ratio (IR_wt) was calculated similarly by quantifying the amount of wild type IVTT protein precipitated in an interaction experiment and dividing it by the IVTT expression control. Thus, each interaction precipitation was normalized to the amount of protein expressed by IVTT and introduced into the interaction experiment. It is important to note that the quatitation of peptide interaction is always relative to the full-length wild type protein. In several cases, the quantitation of truncated peptides revealed relative interaction greater than the wild type protein. In these cases,
the interaction is set at 100% with an asterisk above the bar graph to indicate that it was more efficient than the full-length peptide.

**Immunoprecipitation** – An hExoI construct containing a deletion of the N-terminal catalytic domain (amino acids 1-128) was overproduced in *E.coli* and used to generate a rabbit polyclonal antibody (Strategic Biosolutions). This antibody recognizes recombinant hExoI protein. Immunoprecipitations were performed using HeLa total cell extract. Protein A beads (SIGMA) were exposed over night at 4°C to antibodies specific for hExoI, hMLH1, or hPMS2, respectively, in a solution of 25mM HEPES pH 7.5, 150 mM NaCl, 5mM EDTA, 20mM DTT, and protease inhibitors. The beads were then washed and incubated with HeLa cell extract for 2 h at 4°C under gentle agitation. Subsequently, the beads were washed 3 times, and the proteins were separated by PAGE and detected by Western blotting.

**RESULTS**

We have previously demonstrated that hExoI interacts with hMSH2 (38), suggesting a role in human MMR and/or recombination repair processes that involve the MMR lesion-recognition processes. To further explore the role of hExoI, we examined its interaction with several other human MMR proteins using the glutathione-S-transferase-fusion (GST)/in vitro transcription translation (IVTT) bait-prey interaction system as previously described (38,46). A ^35^S-Met-labeled IVTT reaction containing the interacting partner (or specified truncation mutations) was prepared, quantitated as a function of ^35^S-Met-specific activity, and equivalent molar quantities introduced into each interaction mix. Specific interaction(s) were distinguished from nonspecific binding by quantitating the relative interaction (Int$_{rel}$) of the ^35^S-labeled IVTT mix (prey) between the GST-fusion protein (bait) compared to the GST moiety (control) alone (38,46). As an additional control, we have observed that none of the interaction deficiencies appeared to be the result of protein degradation in the interaction mix, since no altered peptide sizes or fragments were detected.
following incubation and direct examination (data not shown). Any background of smaller-than-predicted peptide fragments present in the IVTT reaction and/or interaction experiments were found to represent internal translation start sites contained in the IVTT subclone which are generated in the IVTT reaction (data not shown). In addition, treatment of the GST/IVTT extracts with DNase did not alter the results suggesting that contaminating DNA does not mediate any of the observed interactions (data not shown). While it is possible that our peptide-deletion constructs do not adopt an entirely wild type conformation, it is important to note that the interaction regions identified for hMSH2, hMSH3 and hMSH6 by this methodology (46) have been largely confirmed by comparison to the crystal structure of bacterial MutS (12,13).

Using the GST/IVTT methodology, we observed strong interactions relative to GST-hExoI with IVTT-hMSH2 (Int\textsubscript{rel-hMSH2}) between GST-hExoI with IVTT-hMLH1 and IVTT-hMSH3, while only weak interactions were detected between GST-hExoI with IVTT-hMSH6, IVTT-hPMS1, or IVTT-hPMS2, respectively (Figure 1). An identical pattern was found by performing the reciprocal interaction(s) where interaction between GST-MMR proteins and IVTT-hExoI was examined (data not shown). The robust interaction(s) between hExoI with hMSH2, hMSH3, and hMLH1 allowed the identification of detailed interaction regions.

The interaction regions between hExoI and hMSH2

We determined the interaction region between GST-hMSH2 with IVTT-hExoI (Figure 2A). The truncations of amino acids 1-128 [hExoI(del 1)] and amino acids 1-261 [hExoI(del 2)] as well as a central deletion of amino acids 267-490 [hExoI(del 3)] did not significantly diminish the hExoI-peptide IVTT interaction with full length GST-hMSH2, relative to the full-length hExoI (Int\textsubscript{rel-hExoI}). However, the Int\textsubscript{rel-hExoI} of the hExoI-peptide IVTT interaction was substantially reduced when amino acids 388-680 [hExoI(del 4)] or the C-terminal amino acids 640-846 [hExoI(del 5)] were removed and completely abolished when amino acids 604-846 [hExoI(del 7)] were deleted. These results suggest that the carboxy-terminal amino acids 604-846 of hExoI were likely involved in the specific interaction with hMSH2 (see shaded area of Figure 2A). This
conclusion was confirmed with the observation of a specific association between GST-hMSH2 and IVTT amino acids 600-846 of hExoI [hExoI(del 9)]. However, there may be several sub-interaction regions within amino acids 603-846 of hExoI, since the Intrel-hExoI of the IVTT-peptide containing an internal deletion of amino acids 603-744 [hExoI(del 8)] and amino acids 603-787 [hExoI(del 10)] continue to display binding to GST-hMSH2.

We similarly determined the reciprocal interaction region of the IVTT-hMSH2 protein with GST-hExoI (Figure 2B). The hMSH2 protein was initially divided in 3 overlapping IVTT peptide fragments. The IVTT amino-terminal [hMSH2(del 1)] and carboxy-terminal [hMSH2(del 3)] regions of hMSH2 displayed extremely weak interaction with GST-hExoI relative to full-length hMSH2 (Intrel-hMSH2). However, a strong Intrel-hMSH2 was obtained with IVTT amino acids 251-750 [hMSH2(del 2)]. The hMSH2 protein was further divided into 4 non-overlapping IVTT peptide fragments [hMSH2(del 4-del 7)], none of which displayed significant interaction: although amino acids 251-498 [hMSH2(del 5)] and amino acids 499-750 [hMSH2(del 6)] displayed a weakly detectable association. The interaction with GST-hExoI appeared to be completely restored when IVTT-hMSH2 amino acids 162-771 [hMSH2(del 8)] and amino acids 370-760 [hMSH2(del 9)] was included. These results suggested that a strong hExoI interaction region was likely to exist between amino acids 370-760 of hMSH2 [hMSH2-(aa 370-760)]. Interestingly, subdivision of hMSH2-(aa 370-760) into IVTT-peptides containing amino acids 460-760 [hMSH2(del 10)] or amino acids 460-707 [hMSH2(del 11)] resulted in a severe reduction in Intrel-hMSH2 binding to GST-hExoI, while IVTT-peptides containing amino acids 370-669 [hMSH2(del 12)], 306-669 [hMSH2(del 13)], 370-707 [hMSH2(del 14)], 261-669 [hMSH2(del 15)] did not significantly reduce the interaction with GST-hExoI. These results imply that amino acids 261-669 of hMSH2 are essential for its interaction with hExoI. Interestingly, deletion of 71 amino acids (aa 601-671) from a nearly full-length IVTT-hMSH2 (aa134-934) nearly abolished the interaction with GST-hExoI [hMSH2(del 16)]. We conclude that amino acids 601-671 of hMSH2 are necessary for interaction with hExoI, and that hMSH2-(aa 261-600) may further stabilize this interaction and/or allow enhanced peptide-folding of the interaction region (see shaded area of Figure 2B).
The interaction regions between hExoI and hMSH3

The interaction region between IVTT-hExoI and GST-hMSH3 was determined (Figure 3A). An N-terminal truncation of 128 amino acids [hExoI (del1)], internal deletion of amino acids 388-680 [hExoI (del 4)] or a C-terminal truncation of amino acids 604-846 [hExoI (del 7)] within hExoI did not diminish interaction with GST-hMSH3 relative to the full-length hExoI (Intrel-hExoI). A deletion of the N-terminal 261 amino acids [hExoI (del 2)], or the internal deletion of amino acids 267-490 [(hExoI(del 3)], amino acids 603-744 [hExoI(del 8)], or amino acids 603-787 [hExoI(del 10)] moderately reduced the Intrel-hExoI between the IVTT hExoI-peptide and GST-hMSH3. However, a peptide containing amino acids 600-846 [hExoI(del 9)] displayed no interaction with GST-hMSH3. Taken together, these results suggest that the C-terminus of hExoI is not required for interaction with hMSH3 and that the minimal N-terminal interaction region encompasses amino acids 129-387 of hExoI (see shaded areas of Figure 3A).

The interaction region between IVTT-hMSH3 and GST-hExoI was determined to be also N-terminal (Figure 3B). Of the subdivided hMSH3 overlapping peptides, only the peptide containing amino acids 1-617 [hMSH3(del 1)] was found to significantly interact with GST-hExoI (note: the interaction was actually greater than the full-length protein). Subdivision of amino acids 1-297 of hMSH3 [hMSH3(del 4-6)] resulted in further definition of the interaction region with hExoI. Since all three peptides displayed a significant Intrel-hMSH3, we conclude that the minimal hExoI interaction region of hMSH3 spans amino acids 75-297 (see shaded area of Figure 3B). The lack of an additional interaction region outside hMSH3-(aa 75-297) was confirmed with the hMSH3(del 7) IVTT-peptide. It appears that del7 has a slightly increased mobility (compare del4, 297 amino acids to del 7, 334 amino acids). This may be due to region of high polarity in this protein fragment or due to a slightly longer electrophoresis of the gel containing del6 and del7.
The interaction regions between hExoI and hMLH1

Our initial studies suggest that hExoI displayed a more robust interaction with hMLH1 compared to hMSH2 (Figure 1). We further defined the region(s) of IVTT-hExoI interaction with GST-hMLH1 (Figure 4A). Deletion of amino acids 1-128 [hExoI(del 1)] and amino acids 1-261 [hExoI(del 2)] of hExoI did not significantly affect its interaction with GST-hMLH1. Moreover, determination of the Intrel-hExoI suggested that the interaction of hExoI(del 1) and hExoI(del 2) was significantly greater than the full-length hMLH1 protein. These results suggest that the extreme amino terminus of hExoI may modulate its interaction with hMLH1. Furthermore, deletion of the C-terminal amino acids 640-846 [hExoI(del 5)] or amino acids 604-846 [hExoI(del 7)] as well as the N-terminal amino acids 1-599 [hExoI(del 9)] significantly reduced its interaction with GST-hMLH1. In addition, the internal deletion of amino acids 388-680 [hExoI(del 4)] or amino acids 267-490 [hExoI(del 3)] as well as a peptide containing only amino acids 129-390 [hExoI(del 6)] of hExoI was also deficient for interaction with hMLH1. In contrast, the internal deletion of amino acids 603-744 [hExoI(del 8)] or amino acids 603-787 [hExoI(del 10)] of the hExoI-(aa 129-846) interaction peptide [hExoI(del 1)] display a strong association with hMLH1. These results imply that there are two regions of hExoI that interact with hMLH1. These two regions encompass amino acids 388-490 and amino acids 787-846 of hExoI (see shaded areas of Figure 4A).

The reciprocal interaction of IVTT-hMLH1 with GST-hExoI was examined (Figure 4B). We found that deletion of amino acids 501-756 [hMLH1(del 1)] eliminated the interaction of hMLH1 with hExoI, while deletion of amino acids 1-252 [hMLH1(del 2)] did not affect this interaction. Dividing hMLH1 into three non-overlapping peptides (aa 1-250; aa 253-500; aa 506-756, [hMLH1(del 3-5)]) also significantly reduced or eliminated its interaction with hExoI. However, deletion of 25 amino acids from hMLH1 completely eliminated this residual interaction [hMLH1(del 6)]. A significant interaction with GST-hExoI was observed with the hMLH1 IVTT-peptide containing amino acids 410-700 [hMLH1(del 7)]. Moreover, deletion of 25 [hMLH1(del 8)] or 50 amino [hMLH1(del 9)] acids from the hMLH1-(410-700) peptide [hMLH1(del 7)] did not affect its interaction with GST-hExoI. These results suggest that the interaction region of
hMLH1 with hExoI is located between amino acids 410-650 of hMLH1 (see shaded areas of Figure 4B).

**Interaction between hExoI and hMLH1 is affected by mutations found in HNPCC**

MLH1 is essential for MMR in eukaryotic cells (47,48). Mutational inactivation or abolished expression by promoter hypermethylation in mammalian cells leads to a mutator phenotype (49-52), and up to 50% of all HNPCC cases show mutations in hMLH1 (8). We have previously demonstrated that missense mutations of hMLH1 found in well-defined HNPCC kindreds affect the interaction of hMLH1 with both hPMS1 and hPMS2 suggesting a pathogenic consequence of these mutations ((53); unpublished results). We tested the effect of several of these missense mutations of hMLH1 on its interaction with hExoI (Figure 4C). We found that four of these missense mutations (hMLH1-L574P; hMLH1-K616Δ; hMLH1-R659P; hMLH1-A681T) reduced the interaction with hExoI >80%. These missense mutations also affected the interaction of hMLH1 with hPMS1 and hPMS2. However, the interaction between hMLH1 and hPMS1 or hPMS2 appeared to be more reduced by these missense mutations than the interaction between hMLH1 and hExoI [compare ref. (53) and Figure 4C]. A missense mutation (hMLH1-L582V) that did not affect the interaction of hMLH1 with either hPMS1 or hPMS2, also did not affect the interaction between hMLH1 and hExoI. These results suggest that hMLH1 mutations found in HNPCC kindreds, which affect its interaction with its heterodimeric partners hPMS1 or hPMS2, may also affect its interaction with hExoI. These results have significant implications when considering the functional effects of HNPCC mutations.

**Interaction between hExoI and hMLH1 does not compete with binding to hPMS2**

A summary of the interaction regions between the human MMR proteins and hExoI are shown in Figure 5. Because the interaction between hExoI and hMLH1 overlaps the C-terminal interaction region for hPMS2 (53), it was formally possible that hExoI might interfere with the MutL homolog heterodimer interactions. To test this possibility and to determine the physiological
relevance of the hExoI and hMLH1 interaction we performed co-immunoprecipitation studies using HeLa total cell extracts (Figure 6). Antibodies for hExoI, hMLH1, and hPMS2, each reciprocally co-precipitated all three proteins (Figure 6, Lanes 1-3). In contrast, these proteins were not precipitated when no antibody or pre-immune serum was present in the incubation mixture (Figure 6, Lane 4; data not shown). The hExoI antibody was found to recognize the hExoI protein but not hMLH1 or hPMS2 (Lane 5). Similar specific recognition was demonstrated with the hMLH1 and hPMS2 antibodies (data not shown). While we cannot exclude that hExoI binds individually to hMLH1 and hPMS2 in the cell, the weak interaction between hExoI and hPMS2 in vitro supports the concept that some fraction of the hExoI, hMLH1 and hPMS2 proteins exist as an immunoprecipitable complex in the cell. In addition, our results support the conclusion that hExoI can interact with the hMLH1-hPMS2 heterodimer. We were unable to reciprocally co-immunoprecipitate hMSH2 or hMSH6 with the hExoI antibody under the same conditions (data not shown). Because hMSH2 may exist in at least three different conformational states that depend on its binding to adenosine nucleotides (16,54), it is possible that the cellular interaction of hMSH2 with hExoI requires a specific form of hMSH2 and/or its heterodimeric partners, or that other factors alter this interaction.

DISCUSSION

The excision of mismatched or damaged nucleotides during MMR as well as other DNA metabolic processes such as DNA recombination requires an exonucleolytic enzyme activity. Genetic and biochemical evidence suggests that the 5'→3' double-strand DNA exonuclease, hExoI, is involved in these processes in eukaryotic cells (45). hExoI interacts strongly with the MMR protein hMSH2 in the GST/IVTT assay (38), and the S.cerevisiae orthologs have also been shown to interact by yeast two-hybrid analysis (40). Here we demonstrate that hExoI interacts with the MMR protein hMLH1 in vitro, and that hExoI may exist in a complex with hMLH1-hPMS2 in vivo. It is important to reiterate that the interaction regions described here could be
substantially influenced by inappropriate or altered peptide folding associated with the deletion constructs. However, our studies clearly provide a framework for further analysis.

The global interaction of hExoI with MMR proteins suggests both a specific and mechanistic association. We have previously proposed a model for MutS homolog function in which mismatched nucleotides, lesions, or DNA structures provoke ADP→ATP exchange and the formation of a hydrolysis-independent sliding clamp capable of diffusion along the DNA backbone (16). The sliding clamp model proposes that a C-terminal “hinge” region maintains a stable contact between hMSH2 and hMSH3 while an N-terminal “clasp” domain changes conformation in response to mismatch provoked ADP→ATP exchange and clamp formation (16,46,55). The crystal structure of the *E.coli* MutS and *Taq* MutS homodimers bound to a G/T mismatch has recently been solved (12,13). Comparison of these structures with corresponding regions in the human homologs have largely confirmed the stable contact between the C-terminus “hinge domain” of hMSH2 with hMSH3 or hMSH6. The overall structure resembles a “pair of praying hands” (13) or a clamp (16) surrounding the mismatched DNA. While the “clasp” region identified for hMSH2 fits well with the structural data, the similar interaction regions identified on hMSH3 and hMSH6 appear to encompass other functional domains (46,55). It is possible that the structural transitions associated with ATP-binding by the MutS homologs will identify additional interaction regions (46).

We have observed that the interaction regions for hExoI with hMSH2 and hMSH3 appear to cover, but are not identical to, the N-terminal “clasp” regions identified between hMSH2 with hMSH3 (Figure 5). These interactions may reflect the limitations of the GST/IVTT interaction system, as well as the two-hybrid interaction system, where partial peptides may fold and/or interact inappropriately. With this substantial caveat in mind, the core domain for interaction with hExoI that we have identified here (hMSH2 aa 600-672) covers the lower portion of the Walker A/B nucleotide-binding domain. The “stabilizing region” that we have identified runs along the outer surface to the “clasp” domain identified in the MutS crystal structures. These regions are
anchored to the Walker A/B domain, which is likely to trigger large conformational transitions that depend on binding to adenosine nucleotide. It is tempting to speculate that such conformational transitions may drive the interaction with hExoI.

The interaction region(s) of hExoI with hMSH3 and the N-terminal interaction regions of hMSH3 with hMSH2 (see Figure 5) are difficult to reconcile with the present crystal structures. For MutS to form a sliding clamp, the mismatched DNA must move upwards towards the internal C-terminal “hinge” region while the upper DNA binding domains must move out of the core region. It is important to note that the reported MutS structures have been solved for the nucleotide free and ADP-bound forms. Substantial conformational transitions of hMSH2-hMSH3 and hMSH2-hMSH6 appear to be associated with ATP-binding (16,54). Moreover, infusion of ATP or ATPγS disrupted the MutS crystals (12,13). It is possible that the conformational transitions associated with ATP-binding may result in an adjustment of N-terminal interaction regions and a re-positioning of the DNA which may also bring the catalytic N-terminal hExoI exonuclease domain into register with the mismatched DNA. The lack of an identifiable co-immunoprecipitate between hExoI and hMSH2 could then be attributed to its specific interaction with only the ATP-bound form.

Another attractive possibility would suggest that the hExoI 5’→3’ exonuclease activity is regulated by hMLH1-hPMS2. The structure of the N-terminal portion of bacterial MutL has been solved (25), however, this structure does not include the projected interaction domain between the MutL homologs nor the interaction region with hExoI. There is growing evidence that the human and yeast MLH1-PMS2 proteins only interact with the ATP-bound form of MSH2-MSH6 ((21,56-59); S.A. and R.F. unpublished). Based on the crystal structure of bacterial MutL, it is likely that MutL homologs undergo conformational transitions associated with binding to ATP similar to the MutS homologs (25,60,61). Perhaps the interaction of hExoI with the human MutL homologs will influence the extent and/or nature of conformational transitions associated with these proteins. We have previously demonstrated equivalent C-terminal interaction regions between hMLH1 and
hPMS2 that are affected by missense mutations found in HNPCC (53). Interestingly, HNPCC mutations, which affect the interaction between hMLH1 and hPMS2, also affect the interaction with hExoI (Figure 4C).

There are a number of caveats associated with peptide interactions. However, the identification of HNPCC mutations, which affect the interaction between full-length hMLH1 and hExoI in addition to the demonstration of immunoprecipitable complexes of these proteins from human cells, appears to provide a biological significance to these studies. A comprehensive biochemical study of the functions associated with the purified MMR proteins and hExoI is in progress.

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FIGURE LEGENDS

**Fig.1:** Interactions of hExoI with MMR proteins. Fusion products of hExoI with glutathione-S transferase (GST-hExoI) were bound to glutathione beads and incubated with 35S-labeled MMR proteins generated by *in vitro*-transcription-translation (IVTT) (see Materials and Methods). As a control for nonspecific binding to the beads, the IVTT proteins were also incubated with GST alone. After washing, the proteins were eluted from the beads, separated by PAGE and visualized by autoradiography. Quantitative analysis of the interaction relative to hMSH2 (Intrel-hMSH2) is shown below.

**Fig.2:** Interaction domain of hExoI with hMSH2. Panel A) 35S-labeled hExoI full length and deletion peptides were made by IVTT and precipitated with GST-hMSH2 bound to
glutathione beads (see Materials and Methods). As a control for nonspecific binding to the beads, the IVTT proteins were also incubated with GST alone. After washing, the proteins were eluted from the beads, separated by SDS-PAGE and visualized by autoradiography. Quantitation was performed as previously described (46). Double lines in the grid above the gels indicate separate gels. Lower panels illustrate the constructs and summarize the interaction results ($\text{Int}_{\text{rel}}$). The shaded area marks the interaction region of hExoI with hMSH2. Panel B) $^{35}$S-labeled hMSH2 full length and deletion peptides were precipitated with GST-hExoI, and the interaction was analyzed as in Panel A. The dark shaded area marks the region essential for the interaction of hMSH2 with hExoI while the lighter shaded area illustrates the region stabilizing the interaction.

**Fig. 3:** Interaction domain of hExoI with hMSH3. Panel A) $^{35}$S-labeled hExoI full length and deletion peptides were precipitated with GST-hMSH3 (see legend to Figure 2 and Materials and Methods). As a control for nonspecific binding to the GST or glutathione beads, the IVTT proteins were also incubated with GST+glutathione beads alone. Double lines in the grid above the gels indicate separate gels. Lower panels illustrate the constructs used, and the shaded area marks the consensus interaction region of hExoI with hMSH3. Interaction between truncated peptides that displayed greater that 100% $\text{Int}_{\text{rel-hExoI}}$ (relative to the wild type hExo1 protein) are marked with a star (*). Panel B) $^{35}$S-labeled hMSH3 full length and deletion peptides were similarly precipitated with GST-hExoI, and the interaction was analyzed as above. The shaded areas mark the consensus interaction regions of hMSH3 with hExoI. Interaction between truncated peptides that displayed greater that 100% $\text{Int}_{\text{rel-hMSH3}}$ (relative to the wild type hMSH3 protein) are marked with a star (*).

**Fig. 4:** Interaction domains of hExoI with hMLH1. Panel A) $^{35}$S-labeled hExoI full length and deletion peptides were precipitated with GST-hMLH1 (see legend to Figure 2 and Materials and Methods). As a control for nonspecific binding to the GST or glutathione beads, the IVTT proteins were also incubated with GST+glutathione beads alone. Double lines in the grid
above the gels indicate separate gels. Lower panels illustrate the constructs used, and the shaded area marks the consensus interaction region of hExoI with hMLH1. Interaction between truncated peptides that displayed greater than 100% $\text{Int}_{\text{rel-hExoI}}$ (relative to the wild type hExoI protein) are marked with a star (*). Panel B) $^{35}$S-labeled hMLH1 full length and deletion peptides were precipitated with GST-hExoI (see legend to Figure 2 and Materials and Methods). The shaded areas mark the consensus interaction regions of hExoI with hMLH1. Panel C) hMLH1 point mutants were generated as described (53), precipitated with full length GST-hExoI, and interaction analyzed as above. The lower panel illustrates the quantification of interaction relative to wild type hMLH1 ($\text{Int}_{\text{rel-hMLH1}}$). Interaction between truncated peptides that displayed greater than 100% $\text{Int}_{\text{rel-hMLH1}}$ (relative to the wild type hMLH1 protein) are marked with a star (*).

**Fig. 5:** Summary of interaction Regions between MMR proteins and hExoI.

**Fig. 6:** Immunoprecipitation of hExoI, hMLH1, and hPMS2. Specific antibodies to hExoI, hMLH1, and hPMS2 were used to immunoprecipitate MMR proteins from HeLa cell extract (Lanes 1-3). The immunoprecipitated proteins were separated by SDS-PAGE and identified by Western analysis using antibodies to the respective proteins as indicated on the left. Antibodies for hExoI, hMLH1, and hPMS2 were able to co-precipitate all three proteins suggesting that they exist as a cellular complex. As a control, no signal was generated in the absence of a precipitating antibody or with pre-immune serum (Lane 4; data not shown). The specificity of the hExoI, hMLH1, or hPMS2 antibodies was tested by Western analysis using purified recombinant hExoI (Lane 5).
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Fig. 1

| IVTT | hMSH2 | hMSH3 | hMSH6 | hMLH1 | hPMS1 | hPMS2 |
|------|-------|-------|-------|-------|-------|-------|
| GST-hExoI | + | + | + | + | + | + |
| GST | + | + | + | + | + | + |

![Image of gel electrophoresis and bar chart showing protein interactions]
Fig. 2A

| IVTT hExoI | full | del1 | del2 | del3 | del4 | del5 | del6 | del7 | del8 | del9 | del10 |
|------------|------|------|------|------|------|------|------|------|------|------|-------|
| GST-hMSH2  | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +     |
| GST        | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +     |

| hExo1   | Amino Acids          |
|---------|----------------------|
| full:   | 1-846                |
| del1:   | 129-846              |
| del2:   | 262-846              |
| del3:   | Δ267-490             |
| del4:   | Δ388-680             |
| del5:   | 1-639                |
| del6:   | 129-390              |
| del7:   | 1-603                |
| del8:   | 129-846Δ603-744      |
| del9:   | 600-846              |
| del10:  | 129-846Δ603-787      |

Int\text{rel-hExo1}
Fig. 2B

| IVTT hMSH2 | full | del1 | del2 | del3 | del4 | del5 | del6 | del7 | del8 | del9 | del10 | del11 | del12 | del13 | del14 | del15 | del16 |
|------------|------|------|------|------|------|------|------|------|------|------|-------|-------|-------|-------|-------|-------|-------|
| GST-hExoI  | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +     | +     | +     | +     | +     | +     | +     |
| GST        | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +     | +     | +     | +     | +     | +     | +     |

hMSH2 | Amino Acids
---|---
full: | 1-934
del1: | 1-498
del2: | 251-750
del3: | 499-934
del4: | 751-934
del5: | 162-771
del6: | 261-669
del7: | 306-669
del8: | 370-707
del9: | 370-669
del10: | 460-707
del11: | 460-760
del12: | 460-760
del13: | 261-934
del14: | 134-600/672-934

Int_hExo-hMSH2

0 50 100
Fig. 3A

| IVTT hExoI | full | del1 | del2 | del3 | del4 | del9 | del7 | del8 | del10 |
|-------------|------|------|------|------|------|------|------|------|-------|
| GST-hMSH3   | +    | +    | +    | +    | +    | +    | +    | +    | +     |
| GST         | +    | +    | +    | +    | +    | +    | +    | +    | +     |

hExoI Amino Acids

| hExoI | Amino Acids         |
|-------|---------------------|
| full: | 1-846               |
| del1: | 129-846             |
| del2: | 262-846             |
| del4: | Δ388-680            |
| del3: | Δ267-490            |
| del9: | 600-846             |
| del7: | 1-603               |
| del8: | 129-846Δ603-744     |
| del10:| 129-846Δ603-787     |

$\text{Int}_{\text{rel-HexoI}}$
### Fig. 3B

| IVTT hMSH3 | full | del1 | del2 | del3 | del4 | del5 | del6 | del7 |
|------------|------|------|------|------|------|------|------|------|
| GST-hExoI  | +    | +    | +    | +    | +    | +    | +    | +    |
| GST        | +    | +    | +    | +    | +    | +    | +    | +    |

**hMSH3 Amino Acids**

- **full**: 1-1128
- **del1**: 1-617
- **del2**: 283-912
- **del3**: 603-1128
- **del4**: 1-297
- **del5**: 1-250
- **del6**: 75-297
- **del7**: 284-617

**Int_{rel-hMSH3}**

![Graph showing Int_{rel-hMSH3} values for different hMSH3 lengths](image)
| IVTT hExoI  | full | del1 | del2 | del5 | del4 | del3 | del9 | del6 | del7 | del8 | del10 |
|-------------|------|------|------|------|------|------|------|------|------|------|-------|
| GST-hMLH1   | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +     |
| GST         | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +     |

**hExo1 Amino Acids**

- **full**: 1-846
- **del1**: 129-846
- **del2**: 262-846
- **del5**: 1-639
- **del4**: Δ388-680
- **del3**: Δ267-490
- **del9**: 600-846
- **del6**: 129-390
- **del7**: 1-603
- **del8**: 129-846Δ603-744
- **del10**: 129-846Δ603-787

**Intel-hExo**

- 0
- 50
- 100

*Note: Asterisks indicate specific modifications or conditions.*
**Fig. 4B**

| IVTT hMLH1 | full | del1 | del2 | del3 | del4 | del5 | del6 | del7 | del8 | del9 |
|-------------|------|------|------|------|------|------|------|------|------|------|
| GST-ExoI    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| GST         | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |

**hMLH1 Amino Acids**

- full: 1-756
- del1: 1-500
- del2: 253-756
- del3: 1-250
- del4: 253-500
- del5: 506-756
- del6: 531-756
- del7: 410-700
- del8: 410-675
- del9: 410-650

**Int**

- Int<sub>rel-hMLH1</sub> (0-100)
|          | IVTT hMLH1 | full | L574P | L582V | L616Δ | R659P | A681T |
|----------|------------|------|-------|-------|-------|-------|-------|
| GST-hExoI| +          | +    | +     | +     | +     | +     | +     |
| GST      | +          | +    | +     | +     | +     | +     | +     |

**Fig. 4C**

![Image of gel electrophoresis and bar graph showing relative intensities of different variants of hMLH1 proteins](http://www.jbc.org/Downloadedfrom)
| Western antibody | hExo1 | hMLH1 | hPMS2 | hExo1 (recombinant) |
|------------------|-------|-------|-------|--------------------|
| hExo1            | ![Western Blot Band](#) | ![Western Blot Band](#) | ![Western Blot Band](#) | ![Western Blot Band](#) |
| hMLH1            | ![Western Blot Band](#) | ![Western Blot Band](#) | ![Western Blot Band](#) | ![Western Blot Band](#) |
| hPMS2            | ![Western Blot Band](#) | ![Western Blot Band](#) | ![Western Blot Band](#) | ![Western Blot Band](#) |

Fig.6
The interaction of DNA mismatch repair proteins with human exonuclease I
Christoph Schmutte, Margaret M. Sadoff, Kang-Sup Shim, Samir Acharya and Richard Fishel

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