A Yeast Two-Hybrid Assay Provides a Simple Way to Evaluate the Vast Majority of hMLH1 Germ-Line Mutations

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ABSTRACT

Germ-line mutations in the hMLH1 gene are the most frequent cause of hereditary nonpolyposis colorectal cancer and are characterized by missense mutations at high frequency. We found a yeast two-hybrid assay to be an extremely useful and simple tool for evaluating the biological significance of such hMLH1 germ-line missense mutations; 78% (18 of 23) of the missense ones can be recognized as causative for nonpolyposis colorectal cancer. In addition, two of five variants not recognized as causative were thought to be rare polymorphisms. However, we could not detect any differences between wild-type hMLH1 and any of the nine already known polymorphisms causing amino acid alterations. Additional analysis demonstrated that the two-hybrid assay not only detected the dysfunctions at the COOH terminus of the hMLH1 protein necessary for the interaction with associated proteins but also detected a conformational change at the NH2 terminus carrying ATPase activity. Thus, this method provides a simple and reliable system for accurate diagnosis of hMLH1 alterations.

INTRODUCTION

HNPCC1 is the most common form of familial colorectal cancer and is associated with germ-line mutations in one of six DNA MMR genes, hMLH1, hMLH3, hMSH2, hMSH6, hPMS1, and hPMS2. Mutations in these genes have been reported in ~70% of kindreds with HNPCC, the tumor DNAs of which show microsatellite instability, a phenotypic manifestation of MMR deficiency (1). The germ-line mutations of more than 650 HNPCC kindreds had been registered in the International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer database (2) by February 2003; 55.6% of these germ-line mutations occurred in hMLH1, 36.2% in hMSH2, 5.6% in hMSH6, and 1.7% in hMLH3. Only six kindreds have been reported to carry mutations in the other two genes; five in hPMS2 and one in hPMS1.

The prominent feature of hMLH1 germ-line mutations is the relatively high frequency of missense mutations; about one-third of all of the mutations are of this type. It is important to clarify whether these missense mutations are in fact pathogenic. In general, pathogenic mutations can be distinguished from polymorphisms by segregation studies. However, such studies are not always available because of ethical issues, limitations of family size, and unavailability of specimens. Therefore, effective evaluation methods are indispensable. Two novel methods using Saccharomyces cerevisiae (3, 4) have been developed on the basis of the assumption that the MMR system is evolutionarily conserved between humans and yeast. An essay examining the MMR ability of hMLH1 variant proteins in a human expression system has also been developed recently (5, 6). However, it is somewhat laborious to use these methods. A simpler system is necessary for accurate assessment of the biological significance of missense mutations; such a system can be highly useful for daily clinical diagnosis.

In this study, we performed the yeast two-hybrid assay with hPMS2 or hEXO1 and hMLH1 variants; in addition to 23 missense variants, we examined 13 truncating variants caused by six deletions, four insertions, and three nonsense mutations, one in-frame deletion caused by a 3-bp deletion, one extension type of COOH terminus caused by a 4-bp insertion at the stop codon, and nine polymorphisms. These variant proteins were predicted from the literature (2). Although the wild-type hMLH1 and all nine polymorphisms did not show any significant differences in their β-gal activities, the specimens corresponding to the vast majority of hMLH1 germ-line mutations showed significantly reduced β-gal activities in this simple assay. This phenomenon was attributable to either or both of two major structural defects in the hMLH1 protein at the NH2 and the COOH termini.

MATERIALS AND METHODS

Strains and Plasmid Constructions. Escherichia coli strain DH5α2 was used to propagate all plasmids. Isolation of hMLH1 and hPMS2 cDNA clones was described previously (7). The hEXO1 cDNA was PCR amplified by using the pooled DNA of the human colon cDNA library (Stratagene, La Jolla, CA) as the template, and the primer sequences were designed on the basis of the sequence database (GenBank accession no. NM_130398). The yeast two-hybrid vectors, pBTM116 and pVP16, and the reporter strain of S. cerevisiae, L40, were kindly provided by Dr. Stanley M. Hollenberg (Oregon Health Sciences University, Portland, OR). The other yeast strain used in the repression assay, EGY48, was purchased from Invitrogen (Carlsbad, CA). The GST fusion protein of hPMS2 was made using a pGEX-2TK vector (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Site-directed Mutagenesis. Site-directed mutagenesis was performed using the overlapping PCR method described previously (8) with some modifications. For the first PCR, two fragments were amplified with mutagenic oligonucleotides and an upstream or a downstream oligonucleotide by using 10 ng of the PBS-hMLH1 plasmid as the template (7), 1 μM primer pair, 1 mM MgSO4, 0.2 mM deoxynucleotide triphosphates, and 0.5 units of KOD DNA polymerase (Toyobo, Osaka, Japan). Reactions were carried out in a Perkin-Elmer 9700 Thermocycler for 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. For the second PCR, the two overlapping PCR-amplified fragments were used as the templates, and reactions were carried out by the same cycling parameters used in the first PCR, except that the internal upstream and downstream oligonucleotides were used in place of the first PCR primers. Products of the second PCR were cut with appropriate restriction enzymes and used to replace the wide-type fragment in pBTM1 and pVPd-hMLH1. The sequences of all of the constructs of variant hMLH1 cDNAs were confirmed by BigDye Terminator FS Ready Reaction kit and the ABI Prism 310 DNA Sequencer (PE Applied Biosystems, Foster City, CA). Nucleotide sequences of the PCR primers used are available upon request from the authors.

Yeast Two-Hybrid Assay. Yeast transformation was performed by the polyethylene glycol-lithium acetate method (9). For the β-gal assay using the nylon filter, freshly transformed colonies were streaked onto filters layered over agar plates containing the selection medium. The filters were frozen and thawed using liquid nitrogen, placed on Whatman 3MM papers presoaked in Z buffer [60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4 (pH

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3 The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal cancer; MMR, mismatch repair; h-, human; MLH, MutL homologue; MSH, MutS homologue; PMS, postmitotic segregation; β-gal, β-galactosidase; GST, glutathione S-transferase; IVTT, in vitro transcription and translation; EMSA, electrophoretic mobility shift assay; BD, binding domain; AD, activation domain.

4 Internet address: http://www.nfdht.nl/.

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7.0)5'-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (0.02%) solution, and incubated at 30°C. For the β-gal assay in liquid culture using α-nitrophenyl-1-thio-β-D-galactopyranoside as the substrate, three independent colonies were cultured overnight and refreshed in YPD medium at 30°C until the A600 was reached at 1.0–1.5. Then an aliquot of 1.5 ml of each culture was centrifuged at 12,000 × g for 30 s, washed once with Z buffer, and resuspended in 0.3 ml of Z buffer. To measure the β-gal activity, the cells derived from 0.1 ml of the suspension were disrupted with 0.1 ml of glass beads using a vortex mixer, added to 0.7 ml of Z buffer containing 1.9 μl of 2-mercaptoethanol, combined with 160 μl of 4 mg/ml α-nitrophenyl-1-thio-β-D-galactopyranoside in Z buffer, and incubated at 30°C. Reactions were then stopped by the addition of 400 μl of 1 m Na2CO3. The reaction tubes were centrifuged at 12,000 × g for 5 min, and the supernatants were collected to measure the A420. The β-gal activity was calculated by the following formula: β-gal units = 1000 × A420 nm(λ) × V × A600 nm(λ), where t = time of reaction (min), and V = volume of culture used in the assay (ml).

The protein concentration was measured by the DC protein assay reagent (Bio-Rad, Hercules, CA) with BSA as the standard.

**GST-IVTT Assay.** GST-IVTT assays with hMLH1 cDNAs containing wild-type or 1 of 40 variants cloned into pcDNA3.1/V5-His vector (Invitrogen) were performed as described previously (10). GST-fused hPMS2 protein-associated glutathione beads were prepared as described previously (11). Briefly, IVTT reactions with 35S-methionine (Promega, Madison, WI) were performed with wild-type or 1 of 40 variant hMLH1 cDNAs. Each IVTT-hMLH1 protein was added to a tube that contained the GST-fused hPMS2 protein-associated glutathione beads. They were then incubated for 1 h at 4°C on a rocker. The beads were washed three times with the binding buffer (20 mM Tris-HCl (pH 7.5), 10% glycerol, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.1% Tween 20, 0.75 mg/ml BSA, 0.5 mM phenylmethylsulfonyl fluoride, 0.8 μg/ml leupeptin, and 0.8 μg/ml pepstatin) and resuspended in 50 μl of SDS loading buffer (62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.005% bromophenol blue). Samples were resolved in a 6% SDS-PAGE, imaged by a BAS-1500 (FujiFilm, Tokyo, Japan), and analyzed by a LAS-1500 Plus (FujiFilm). The relative interaction (IRv) of each hMLH1 variant with GST-hPMS2 was determined as the fraction of the variant interaction ratio (IRv) divided by the wild-type interaction ratio (IRv), as described previously (10). The IRv was determined by quantifying the amount of IVTT-hMLH1 variant that interacted with GST-hPMS2 and dividing this number by the original amount of IVTT-hMLH1 variant added to the reaction. This quantification was determined for each experiment that contained an hMLH1 variant protein as well as the wild-type protein control on a single SDS-PAGE. The IRv was calculated similarly by quantifying the amount of wild-type IVTT protein precipitated in an interaction experiment and dividing it by the IVTT control. Results are presented as the mean and SD of three separate experiments.

**Immunoblotting.** Yeast cell lysates were prepared as described previously (3). A total of 100 μg of the total protein was subjected to 5% SDS-PAGE and electroblotted onto a Clear blot membrane-P (Atto, Tokyo, Japan). The LexA-hMLH1 variant proteins were detected using a rabbit anti-LexA, DNA binding site polyclonal antibody (Upstate, Lake Placid, NY) and visualized using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

**Yeast Repression Assay.** The yeast repression assay was performed using three independent transformants containing one of the pBTMD-hMLH1 constructs and the pJK101 plasmid described previously (12). The pBTMD-hMLH1 constructs contain various LexA-fused hMLH1 variant cDNAs, whereas pJK101 contains two LexA operators-LacZ genes under the control of GAL1 upstream-activating sequences as shown in Fig. 4A. The pBSTM-DLlexA plasmid is a vector that does not encode any LexA protein; it was used as a negative control. The β-gal assay was performed using galactose as the carbohydrate source.

**EMSA.** Four LexA-hMLH1 fusion genes, LexA-WT, LexA-68N, LexA-I107R, and LexA-ES578G, used as the bait constructs were cloned into HindIII and XhoI sites of pcDNA3.1/V5-His vector for the IVTT reaction. EMSA using the IVTT mixture was performed as described previously (13). Briefly, the lop site, a double-stranded, blunt-ended oligonucleotide in which the plus strand sequence is 5′-CCCTATA[CGT]TATATATACGATACACATCCC-3′, was the wild-type LexA operator for binding in the assay (14); in this sequence, the LexA-binding site is in brackets. A mutant sequence, 5′-CCCTATACGGTATATATACGATACACATCCC-3′ (15), and +1 operator sequence, 5′-CCCTATGTTATATTGACGTAATCC-3′ (14), were used as negative controls. Single strand DNA was synthesized, annealed, and end-labeled with 32P using T4 polynucleotide kinase. An aliquot of 5 μl of IVTT mixture was incubated with 2 μg of poly(dCopolymerase-deoxyoxydicylic acid) and 30,000 cpm of 32P-end-labeled oligonucleotides in 20 μl of DNA binding buffer [0.1% Triton X-100, 4% glycerol, 1 mM EDTA, 5 mM DTT, 20 mM Tris-HCl (pH 7.2), and 80 mM NaCl] at 4°C for 20 min. Protein/DNA complexes were run on 6% nondenaturating polyacrylamide gels in 0.5x Tris-borate EDTA buffer. The gel was dried, and labeled binding complexes were visualized by autoradiography.

**RESULTS**

Our previous analysis had indicated that the minimal region conserving the strong interacting activity of hMLH1 with hPMS2 corresponded to a region between residues 492 and 756 (10). On the basis of this result, we excluded the analysis of truncating mutants in which the breakpoints resided between residue 492 and the NH2 terminus because such mutants lack the domain of hMLH1 interacting with hPMS2. Hence, we constructed 47 hMLH1 variants as shown in Table 1 [11 frameshifts and 1 in-frame 3-bp deletion, 3 nonsense mutations, and 23 missense mutations as described previously (2)]. Nine polymorphisms, I32V, V213M, R217C, I219V, I219L, R265H, S406N, H718Y, and L729V, which are all known hMLH1 polymorphisms causing amino acid alterations, were also included as the experimental controls. As shown in Table 1 and Fig. 1A, the two-hybrid assay between DNA BD-fused hMLH1 constructs containing frameshift or nonsense mutations and a transcriptional AD-fused hPMS2 construct showed extremely low levels of β-gal activities in comparison with those that used a wild-type hMLH1, regardless of the position of the mutation. Among the missense mutations, on the other hand, 14 (60.9%) of 23 mutations displayed almost no β-gal activity (Table 1).

The amino acid alterations showing low levels of β-gal activity were clustered in the NH2-terminal region; P28L, M35R, S44F, G67R, I68N, C77Y, I107R, T117M, T117R, A128P, V185G, and G244D are all NH2-terminal mutations from residue 244. Although alterations such as L574P and R659P that are in the domain of hMLH1 (between residues 492 and 756) interacting with hPMS2 caused suppression of the β-gal activity, other alterations such as A492T, V506A, Q542L, E578G, L582V, K618T, K618A, and A681T did not show any significant reduction in the β-gal activity. To confirm these results, we used reversely cloned plasmids: missense constructs of hMLH1 cDNA in the AD-plasmid and hPMS2 cDNA in the BD-plasmid. The results were consistent; low levels of β-gal activities were observed (Table 1).

We additionally used hEXO1 as the hMLH1 partner; this protein also interacts with hMLH1 in the COOH-terminal region to play a role in the human MMR system (16). When hEXO1 was used as the bait, 4 missense mutations (V506A, K618T, K618A, and A681T) were also detected in addition to the 14 previously detected mutants; all of them were in the COOH-terminal region (Table 1 and Fig. 1B). In contrast to these hMLH1 germ-line mutations, all nine polymorphisms carrying amino acid alterations of hMLH1 protein showed levels of β-gal activities quite similar to that observed in the wild-type hMLH1 (Table 1 and Fig. 1C). With the two-hybrid assay using hPMS2 and hEXO1, 33 (86.8%) of 38 germ-line mutations (18 of 23 missense, 11 of 11 frameshifting, 3 of 3 nonsense, and 1 of 1 in-frame deletion mutations) were selected as candidate pathogenic mutations.

To clarify whether the low levels of β-gal activities in the two-hybrid assay were caused by defects in the physical interaction between hMLH1 variants and a wild-type hPMS2, we next performed in vitro GST-IVTT assays using 40 hMLH1 variant proteins (38 germ-line mutants and two polymorphic variants, R217C and R265H). These results are shown in Fig. 2. All of the hMLH1 variant proteins
that showed no reduction in the β-gal activity interacted strongly with GST-hPMS2. However, all of the hMLH1 variant proteins produced by frameshift and nonsense mutations indicated that there were defects in the physical interaction with hPMS2. In addition, the COOH-terminal hMLH1 missense variants tended to show defects in their physical interactions with hPMS2. The 12 NH2-terminal missense variants with the low levels of β-gal activity did not exhibit any change in their physical interaction with hPMS2.

Analysis of the conserved NH2-terminal region in bacterial MutL, LN40, based upon the sequence homology between E. coli and humans suggested that this region has four ATP-binding motifs and that the majority of the NH2-terminal missense mutations in the hMLH1 gene are located in and around the ATP-binding pocket (17). In addition, the crystal structures of an apoprotein and a nonhydrolyzable ATP analogue, the ADPnP-bound form of LN40, are extremely different, and this structural transformation could be inhibited by the NH2-terminal missense mutations (18). Therefore, we sought the cause(s) of the low β-gal activities in the NH2-terminal missense mutations of hMLH1 seen in the two-hybrid assay by examining three different aspects: protein expression; cellular localization; and LexA-binding ability.
To determine the expression level of hMLH1 variant proteins, we used immunoblotting and found no significant differences between the expression levels of any of the hMLH1 missense variants and that of wild-type (Fig. 3). We also examined the cellular localization of 40 hMLH1 variant proteins immunocytochemically (data not shown). Again, the hMLH1 variant proteins and the wild-type hMLH1 were all localized at the nucleus. Therefore, we considered other possibilities for explaining the low levels of β-gal activity in the NH₃-terminal missense variant proteins; perhaps these proteins could neither bind to the LexA-binding sites (in the case of BD constructs) nor activate transcription from the promoter region of the LacZ gene (in the case of AD constructs) in the two-hybrid assay.

We tested the binding ability to the LexA operator sequences of the hMLH1 variants inserted into the BD plasmid by a yeast repression assay. If the LexA⁵'-fused hMLH1 variant protein could bind to the LexA operators in the promoter region of the LacZ gene in the reporter plasmid pJK101, expression of LacZ would be repressed (Fig. 4A, b). In fact, a LexA⁵'-fused wild-type hMLH1 protein was able to repress the β-gal activity up to 40% in comparison with the plasmid construct lacking LexA. As shown in Fig. 4B, all of the 12 NH₃-terminal missense variants that exhibited suppression in the two-hybrid assay showed <40% repression of the β-gal activity compared with the wild-type hMLH1; these variant proteins had very likely lost their binding affinity to the LexA operator. This notion was confirmed by EMSA (Fig. 4C). A LexA⁵'-fused wild-type hMLH1 protein could bind to a LexA operator sequence and shifted its mobility on the gel. In contrast, two LexA⁵'-fused hMLH1 variant proteins, I68N and I107R, lost their binding abilities to the LexA operator sequence and therefore could not shift their mobilities on the gel. Similarly to the LexA⁵'-fused wild-type hMLH1 protein, a LexA⁵'-fused hMLH1 variant protein, E578G, which showed a strong repression of the β-gal activity, could bind to the LexA operator sequence efficiently. These results are consistent with those in the repression assay. Surprisingly, except for two insertion mutations, 497 ins 1 bp and 519 ins 1 bp, the vast majority of hMLH1 germ-line mutations with suppression of β-gal activity in the two-hybrid assay using hPMS2 as the partner protein, even including those that cause alterations in the COOH-terminal region, nicely correlated with the inhibition of the LexA-binding activities.

**DISCUSSION**

Recent studies have identified and characterized two critical domains in the hMLH1 protein, one at the NH₂ terminus and the other at the COOH terminus. hMLH1 forms a stable heterodimer with hPMS2 (hMutLα) in the cell; hPMS2 has been demonstrated to be one of the essential players in the MMR pathway by a complementation study using hMLH1-deficient cell extracts (19). Studies using the in vitro GST-IVTT assay (11) and the yeast two-hybrid assay (10) showed that hPMS2 interacts with the COOH-terminal region of hMLH1. In addition, a double-stranded DNA-specific 5'-3' exonuclease, hEXO1, originally identified as a molecule that interacts with hMSH2, also interacts with the COOH-terminal domain of hMLH1 (16). In S. cerevisiae, exo1 mutants show a moderate mutator phenotype, and the mutations are mainly single base deletions and substitutions (20). On the other hand, the NH₂-terminal region of MutL in E. coli contains four putative ATP-binding motifs that are found in the type II topoisomerases, the HSP90 heat shock proteins, and histidine kinases known as the GHKL (gyrase, Hsp90, histidine kinase, MutL) class of ATPases (17, 18). This ATPase domain in MutL is also conserved in hMLH1 and hPMS2, and, in fact, hMutLα has been recently demonstrated to have ATPase activity (21, 22). hMutLα variants containing mutations in either the ATP-binding or the hydrolysis domain showed defective mismatch repair activities in vitro, suggesting that this ATPase activity is essential for the MMR system. In support of this notion, although hMLH1 germ-line mutations are quite evenly distributed throughout the coding region of the gene, >50% of the missense mutations are located within the coding region for the conserved ATP-binding pocket.

In this study, we found that the yeast two-hybrid assay can evaluate hMLH1 germ-line mutations very efficiently. This method is the simplest developed to date. We analyzed 14 truncating mutations (11 frameshift and 3 nonsense mutations) as well as 1 in-frame 3-bp deletion that harbor some or most of the COOH-terminal residues.

Fig. 1. Yeast two-hybrid assay of a wild-type hMLH1 and 47 variants. The filter β-gal assays are shown. The filters on 3MM papers containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were incubated at 30°C. A, filter β-gal assay of each yeast cell carrying a LexA DNA BD fused hMLH1 variant containing a frameshift or nonsense mutation and a VP16 transcriptional AD-fused hPMS2. None of the mutations exhibits visible β-gal activity. B, filter β-gal assay of each yeast cell carrying a BD-fused hEXO1 and an AD-fused hMLH1 variant containing 1 of 23 missense mutations or an in-frame deletion (K616del) mutation. Only five mutations, V326A, A492T, Q542L, E578G, and L582V, showed detectable β-gal activities. C, filter β-gal assay of each yeast cell carrying a BD-fused hEXO1 and an AD-fused hMLH1 variant containing one of nine polymorphisms, I32V, V213M, R217C, I219V, I219L, R265H, S406N, H718Y, or L729V. All of these polymorphisms showed levels of β-gal activities quite similar to the one using wild-type hMLH1 (WT).
from the 492nd amino acid and found that all of them showed <30% of the wild type ß-gal activity (Table 1) in the quantitative assay using liquid cultures. It is notable that 1 insertion mutation, a 4-bp insertion at the stop codon (756 ins 4 bp) causing additional 34 amino acid residues, showed almost no ß-gal activity. Eighteen (78.3%) of 23 missense mutations also showed decreased ß-gal activity. Altogether, 33 (86.8%) of 38 hMLH1 germ-line mutations showed significant decreased ß-gal activities (Table 1). It should also be noted that the use of the filter ß-gal assay makes judgment much easier (Fig. 1).

Interestingly, the two-hybrid assay can assess hMLH1 germ-line mutations not only by detecting the defects in the interaction with hMLH1-associated proteins (hPMS2 or hEXO1 in this study) at the COOH-terminal region, which is normally expected as the result of this method, but also by detecting conformational change(s) at the NH₂-terminal region. Both the NH₂ and the COOH terminus are important regions for hMLH1 function, and the two-hybrid assay can detect abnormalities in both regions.

To distinguish between pathogenic mutations and polymorphisms in hMLH1 variants, four assays have been developed thus far. Among these, two assays use the yeast MMR system. The first assay is based on interference of the human MLH1 protein with the yeast MMR system (3). In this assay, the wild-type hMLH1, as well as variants carrying nonpathogenic alterations, show a dominant mutator effect when overexpressed, whereas pathogenic mutations have no effect.

**Fig. 2.** Influence of the hMLH1 germ-line mutations on the physical interaction with hPMS2. A, each 35S-labeled IVTT product of hMLH1 variant protein was resolved on 6% SDS-PAGE and imaged with a BAS-1500. B, 35S-labeled hMLH1 proteins shown in A were pretreated with either GST alone or GST-hPMS2. Samples were resolved on 6% SDS-PAGE and examined by BAS-1500. Results of the GST fusion protein assay are presented as the mean and SD of three independent experiments in parentheses. The COOH-terminal mutations of hMLH1 protein tended to reduce the interacting activity with hPMS2.

**Fig. 3.** Detection of LexA-hMLH1 variant proteins expressed in the yeast L40 strain. Both LexA-wild-type hMLH1 and representative LexA-hMLH1 variants were expressed from the pBTM116 vector and analyzed by immunoblotting with an anti-LexA DNA-binding region antibody. Note that the expression level of these variant proteins was equivalent to that observed for wild-type hMLH1 (WT).
Because this dominant mutator effect is produced by complex interactions between hMLH1 variants and the yeast MMR system, it might be difficult to judge all of the hMLH1 mutations properly. The second yeast assay examines the phenotype of mlh1 strains that express yeast mlh1 genes carrying mutations corresponding to hMLH1 amino acid residues altered in HNPCC kindreds (4, 23). Human and yeast MLH1 proteins share a significant homology in both their NH2- and the COOH-terminal regions. However, the amino acid sequence of the central region in the MLH1 protein is less conserved; therefore, it is difficult to evaluate mutations in this region. The third assay uses an in vitro GST fusion protein interaction system in which 35 S-labeled hMLH1 variants are tested for their ability to interact with hPMS2 (11, 24). However, as shown in Fig. 2, mutations that do not affect the heterodimer formation would have no particular phenotype. The fourth assay described recently uses a homologous human MMR system and is probably the most sophisticated (5, 6). The merit of this assay is that it eliminates any problems caused by the use of a heterologous yeast system. However, because each hMLH1 variant protein must be purified by the use of baculovirus-infected insect cells and the assay system itself has to be adjusted properly, a great deal of effort is needed to analyze many types of variants. In addition, the overexpression of hMLH1 variants by transient transfection experiments might eliminate their subtle effects on the MMR system. In contrast, the two-hybrid assay described here can provide a simple and effective way to detect the basic conformational changes caused by hMLH1 germ-line mutations.

Among the five missense mutations not detected by the two-hybrid assay, variant hMLH1 proteins carrying V326A had the ability to complement MMR-deficient 293T cell extracts (6). In addition, the V326A and Q542L mutations did not abolish the dominant mutator effects in yeast (3). These results suggest that these two mutations, V326A and Q542L, represent rare polymorphisms. However, we still cannot exclude the possibility that these variants may have some effects on MMR or that they might affect some other unknown
function(s) of hMLH1. Additional analyses have to be done to clarify this point. The other three missense mutations not detected by the two-hybrid assay (A492T, E578G, and L582V) could abolish the dominant mutant effect (3). Therefore, they are likely to be pathogenic. To evaluate such missense mutations, other downstream factors of MMR associated with hMLH1 have to be used as the bait construct. One possible candidate, methyl CpG-binding domain protein 4, which is known to interact with the COOH-terminal region of hMLH1 and is suggested to be involved in MMR, was used as the bait in the two-hybrid assay, but no significant reduction of the β-gal activity was observed (data not shown).

Our present study first discovered the conformational defect(s) at the NH2-terminus of most hMLH1 variant proteins. This is one of the most striking features caused by mutations of hMLH1. On the basis of the repression assay, 28 (73.7%) of 38 hMLH1 germ-line mutations could not repress the β-gal activity, suggesting possible structural defects at the NH2-terminus. All of the members of the MutL family share the conserved NH2-terminal region of ~300 amino acid residues corresponding to four putative ATP-binding sites. This region has a low ATPase activity and is essential for MMR function in E. coli MutL. Ban et al. (18) suggested that the flexible, yet conserved, loops surrounding NH2-terminal ATP-binding sites undergo conformational changes upon ATP hydrolysis, thereby modulating interactions between MutL and other components of the repair machinery. Two recent articles also reported that hMutLa variants carrying defects in either ATP-binding or the hydrolysis domain in one subunit showed defective mismatch repair activities in vitro (21, 22). In addition, they suggested that the ATPase activity of hMutLa is required downstream from mismatch recognition because the ATP binding in hMutLa was not required for the formation of ternary complexes with hMutSα bound to a mispair (22). In combining these previous data and our present study, we propose the notion that most hMLH1 germ-line mutations cause conformational change(s) at the NH2-terminal domain of hMLH1 protein and may disrupt the structural transformation by ATP binding and hydrolysis activities, leading to the inhibition of interaction with downstream factors essential for MMR activity. Additional analysis is needed to clarify the relationship between the structural abnormalities at the NH2-terminus of hMLH1 caused by germ-line mutations and their roles in the MMR defects.

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