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Mutational Analysis of the Nucleotide Binding Domain of the Mismatch Repair Enzyme hMSH-2

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The genes involved in postreplicative DNA mismatch repair are a highly conserved family of proteins. In humans, germline mutations in these genes (hMSH-2, hMLH-1, hPMS-1, and hPMS-2) have been implicated in hereditary nonpolyposis colorectal cancer (HNPCC). We have previously shown that a region of high homology between the members of this class of proteins in different species contains a type A nucleotide binding site consensus sequence which has ATPase activity and is sufficient to bind DNA containing specific mismatched residues (1). To identify residues which are necessary for this activity, we have created a range of mutants containing amino acid substitutions within the nucleotide binding domain of hMSH-2. These mutants have been expressed and assessed for ATPase activity and their ability to identify mismatch-containing DNA. Here we demonstrate that a variant protein which has the conserved residue Lys 675 within the nucleotide binding consensus sequence altered to an alanine has severely impaired ATPase activity and is unable to bind DNA containing specific mismatched residues.

Colorectal cancer (CRC) is one of the most common forms of human malignacy especially in industrialised countries and its incidence is increasing (2). There are two major inherited forms of CRC predisposition. The first, familial adenomatous polyposis (FAP) accounts for approximately 1% of CRC cases in the western world (3). The second, more common form is hereditary non-polyposis colorectal cancer (HNPCC) which accounts for 4-13% of all CRC in developed nations (3). HNPCC kindreds are characterised by a high incidence of instability in microsatellite sequences (4). Cell lines which have been derived from HNPCC tumours have been shown to be highly unstable genetically (5). Germline mutations in HNPCC families have been found in the human genes homologous to those bacterial proteins involved in mismatch repair.

Four genes have been implicated in HNPCC, hMSH-2 which is a homologue of the *Escherichia coli* (*E. coli*) Mut S and hMLH1, hPMS1 and hPMS2, which are *E. coli* Mut L homologues (6-11). In *E. coli*, Mut S and Mut L or their homologues in yeast MSH1, PMS1 and PMS2, are involved in the initial stages of mismatch repair (reviewed in 12). Mut S specifically binds to base mismatches and loops of up to four unpaired nucleotides, in DNA. The Mut L gene product then interacts with Mut S to increase the stability of the DNA-protein complex. Mut H, an endonuclease, then initiates excision of the newly formed unmethylated strand at a hemi-methylated GATC site. Resynthesis of the excised DNA then occurs followed by religation to repair the mismatch.

In humans, mismatch binding is mediated by hMutSα, a heterodimer of hMSH-2 and G/T binding protein (GTBP) (13-14). hMSH-2 alone has also been shown to bind DNA containing

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mismatches *in vitro* (15-16). hMSH-2 is of particular importance as mutations in hMSH-2 are thought to underlie approximately 40% of HNPCC cases (10,21). Tumour-derived cell lines defective in these genes accumulate spontaneous mutations resulting in the 'mutator phenotype' (17-19). Recent data has also shown that hMSH-2 dimerises with a third protein, hMSH3 to form the complex hMutSβ, which binds loops of up to 4 base pairs (20). However, to date only mutations in hMSH-2 have been shown to segregate with HNPCC, suggesting GTBP and hMSH3 are non-essential in loop repair (20-21).

We have previously shown that the C-terminal region of hMSH-2 and other MutS family members contains a region of high homology, incorporating a nucleotide binding domain. This C-terminal hMSH-2 domain has been shown to display ATPase activity and is sufficient to bind specific mismatched oligonucleotides (1). In this paper, we have created a range of mutant proteins containing amino acid substitutions within the nucleotide binding domain of hMSH-2. These mutants have been expressed and assessed for ATPase activity and their ability to identify DNA containing mismatched residues.

METHODS

Construction of mutant hMSH-2 nucleotide binding domain expression vectors. DNA fragments expressing the C-terminal DNA binding domain sufficient to bind specific mismatched oligonucleotides and mutants 1-5 shown in figure 1 were generated by polymerase chain reaction (PCR) using 10ng of plasmid pBShMSH-2 DNA, and 250ng of the following forward primers dCCG GGA TCC TTC CAC ATC ATT ACT GGC CCC AAT ATG GGA GGT AAA TCA; dCCG GGA TCC TTC CAC ATC **GGT** ACT GGC CCC AAT ATG GGA GGT AAA TCA; dCCG GGA TCCTTC CAC ATC ATT ACT **GCC** CCC AAT ATG GGA GGT AAA TCA; dCCG GGA TCC TTC CAC ATC ATT ACT GGC CCC AAT ATG GGA **GCT** AAA TCA; dCCG GGA TCC TTC CAC ATC ATT ACT GGC CCC AAT ATG GGA GGT **GCA** TCA; dCCG GGA TCC TTC CAC ATC ATT ACT GGC CCC AAT ATG GGA GGT AAA **GCA** and the reverse primer dGCG GGA TCC TCT TTC CAG ATA GCA CTT CTT TGC TGC (changes shown in bold type). These oligonucleotides incorporated *Bam*HI restriction sites for convenient cloning of the PCR products. The reaction was performed with 4 units of *Pfu* DNA Polymerase (Stratagene) in the buffer recommended by the supplier. After 30 cycles (1 min, 92 °C, 1 min 60°C, 1 min 72°C), the DNA produced was phenol/chloroform extracted, ethanol precipitated, digested with *Bam*HI and cloned into the corresponding site of pET21a (Novagen) to derive pEThMSH-2 and pETΔ1-5 respectively. The integrity of each insert was confirmed by DNA sequencing (data not shown).

Production of hMSH-2 nucleotide binding domain mutants as bacterial fusion proteins. The wild type and mutant proteins encoding the amino acid 663-877 hMSH-2 domain in the pET bacterial expression vector, were used to transform *E. coli* strain BL21(DE3). A fresh overnight culture of transformed *E. coli* was diluted 1 in 20 with LB medium containing ampicillin (100 μg/ml). After growth at 37°C for 2 hours, the culture was induced with IPTG (1 mM) and grown at 37°C for a further 5 hours. The cells were harvested by centrifugation at 3200g for 10 minutes and resuspended in 0.1 volume lysis buffer (100mM Tris-HCl, pH 8.0, 1mM EDTA) and incubated on ice with 3 mg/ml of lysozyme for 30 minutes. The cells were then sonicated and lysed by the addition of Tween 20 lysis buffer (100 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.3 mg/ml phenylmethylsulphonyl fluoride, 0.8 μg/ml pepstatin, 1 mM DTT, 1% Tween 20). Cellular debris was pelleted by centrifugation at 4,000g.

Detection of fusion protein by SDS-PAGE. Protein extracts were mixed with 2$\times$ reducing sample buffer (50mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulphate (SDS), 5 mM EDTA, 10% β-mercapthoethanol, 1 mM DTT and 0.01% bromophenol blue). After boiling for 3 minutes, samples were fractionated on a 12% SDS polyacrylamide gel. Following electrophoresis the gel was stained with Coomassie blue solution (25% v/v isopropyl alcohol, 10% v/v acetic acid and 0.25% w/v Coomassie blue).

ATPase assay. The assay was performed at 37°C in 20 mM Tris-HCl, pH 7.6, 0.5 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM DTT, 100 μg/ml BSA, 0.1 mM EDTA with 150 ng of wild type or mutant hMSH-2 domains. Assays were performed using 2, 2.5, 3.3, 5 and 10 μM ATP. Hydrolysis of [α-^{32}P]ATP by the wild type and each mutant carboxy terminal domain was assayed by thin layer chromatography. The radioactive counts for ATP and its hydrolysis products were quantified using a scintillation counter (Packard).

Functional binding assay. Mismatch recognition was detected by a nitrocellulose binding assay of labelled oligonucleotides followed by autoradiography as described previously (1). Briefly, oligonucleotides (dCGG ATC CGG A**X**G TCA TGG AAT TCC and dGGA ATT CCA T**X**A CAT CCG GAT CCG) were annealed to produce either a perfect matched double-stranded molecule or a single mismatch (position shown in bold type). Oligonucleotides were mixed to a final concentration of 100 pmole/μl each in 100 μl STM (100 mM NaCl, 10 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 5 mM DTT) heated to 95°C and cooled to 25°C over 2 hours. End-labelling of double-stranded DNA (100 pmole) in STM buffer was performed with polynucleotide kinase. After incubation at 20°C for 10 minutes the

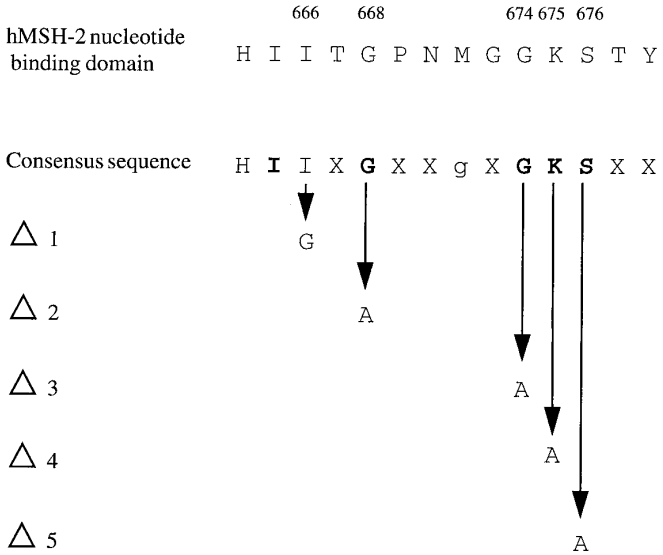

FIG. 1. Homology between hMSH-2 DNA binding domain and the ''Type A'' consensus sequence. Bold type indicates conserved residues between both proteins. The mutants produced are indicated showing the alteration of each conserved residue.

unincorporated label was removed using a Sephadex NAP 5 column. The labelled DNA was diluted to 0.2 pmole/μl. The binding assay used 1 pmole of DNA with 150 ng of wild type or each mutant hMSH-2 domain in a total volume of 10 μl. After 1 hour on ice the mixture was slowly filtered over pure prewetted nitrocellulose (Millipore, 0.45μm) and washed in STM buffer. The filter was then allowed to air dry and bound DNA was detected by autoradiography.

RESULTS

PCR amplification and cloning. A fragment of the hMSH-2 cDNA sequence which encodes amino acid residues 637 to 877 has been shown to bind oligonucleotides containing mismatches (1). In order to determine which specific residues are important in this domain, mutant proteins have been produced which alter specific residues within the putative nucleotide binding region (Fig.1). DNA fragments which encode the nucleotide binding domain of hMSH-2 were amplified using PCR, incorporating *Bam*HI restriction sites for convenient cloning. Each product was ligated to the expression vector pET21a, in phase with respect to the ATG translational start codon immediately upstream of the multiple cloning site (MCS) and also in frame with the C-terminal coding sequence immediately downstream of the MCS to ensure proper fusion to the C-terminal HisTag.

Expression of the hMSH-2 nucleotide binding domain mutants. To confirm their integrity, each mutant hMSH-2 nucleotide binding domain was cloned into the bacterial expression vector pET21a. Expression of the mutant hMSH-2 fusion proteins resulted in 30 kDa species detected using SDS-PAGE comprising the hMSH-2 domain (containing a type A nucleotide binding site consensus sequence) coupled to the HisTag peptide at its carboxy terminus. (Fig. 2). We designate these mutant fusion proteins Δ1 to Δ5. All mutant proteins were expressed at comparable levels to the wild type fusion protein.

ATPase analysis of mutant fusion proteins. It has been shown that the carboxy terminal domain of hMSH-2 contains ATPase activity (1). In order to determine whether these mutants hydrolyse ATP to ADP and Pi, [α-^{32}P]ATP was incubated with each mutant fusion protein and separated using TLC. To determine K_m and k_{cat} values for the mutants, ATPase activity was measured in the presence of various concentrations of ATP (Fig. 3). The results show

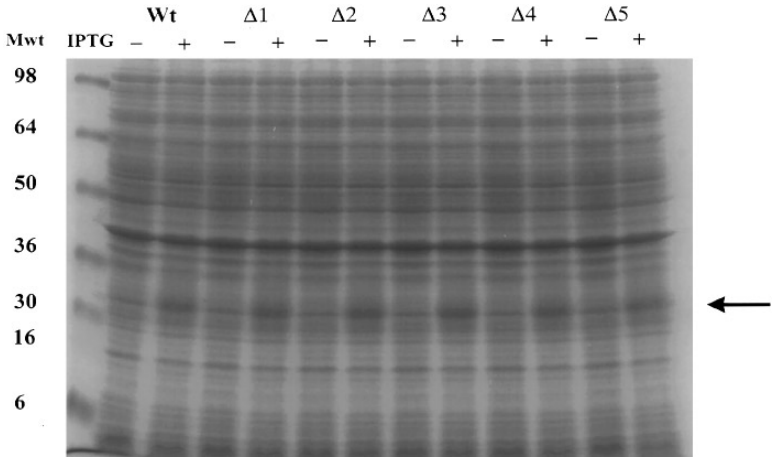

FIG. 2. Analysis of expression of mutant fusion proteins. Coomassie blue-stained SDS-PAGE gel showing protein extracts analysed 4 hours postinduction with 1 mM IPTG. Lanes 1-2, hMSH-2 domain uninduced and induced; Lanes 3-4, Δ1 uninduced and induced; Lanes 5-6, Δ2 uninduced and induced; Lanes 7-8, Δ3 uninduced and induced; Lanes 9-10, Δ4 uninduced and induced; Lanes 11-12, Δ5 uninduced and induced, respectively.

that Δ1, Δ2, Δ3 have limited effects on the ATPase activity of the domain. Wild type K_m and k_{cat} values were 8.33 μM and 0.55 s^{-1}, respectively, compared to Δ2 values of 5.88 μM and 0.633 s^{-1}, respectively. However, Δ5 had a reduced activity with K_m and k_{cat} values of 3.6 μM and 0.65 s^{-1} and Δ4 which alters the codon 675 from a lysine to an alanine has a marked effect upon ATP hydrolysis, effectively reducing it to zero. In a control experiment, nonenzymatic hydrolysis of ATP in the absence of the wild type expressed domain was less than 5%.

Functional analysis of the mutant fusion proteins. A mismatch binding assay was developed

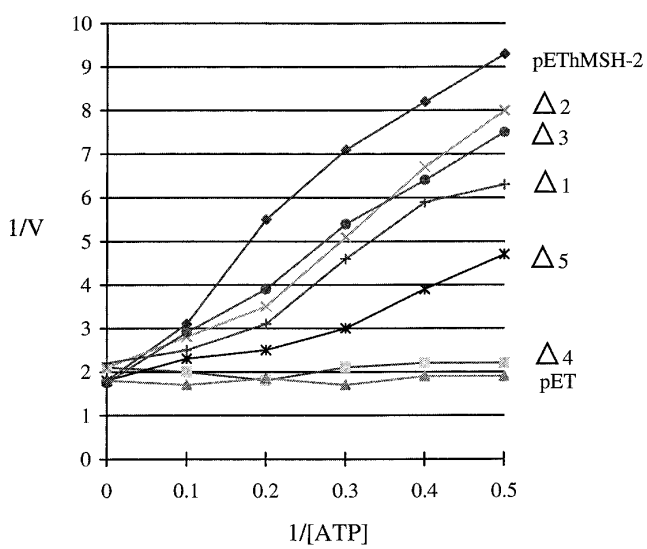

FIG. 3. ATPase analysis of the mutant bacterial fusion proteins. Hydrolysis of various substrate concentrations of [α-^{32}P]ATP by the carboxy terminal domain of hMSH-2, pET, and Δ1-5 were assayed by thin layer chromatography and quantified using a scintillation counter.

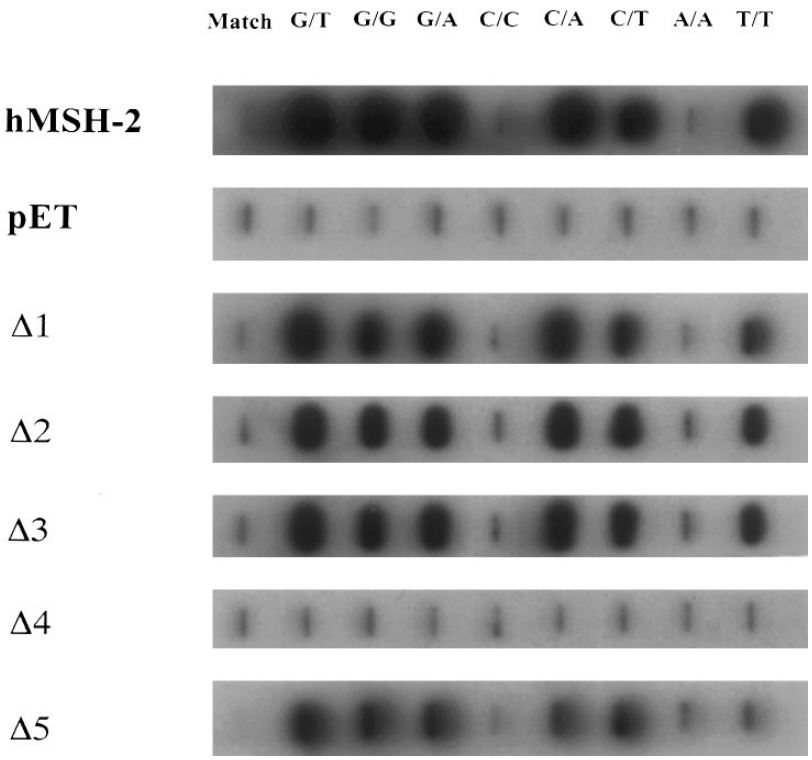

FIG. 4. Functional analysis of the mutant bacterial fusion proteins. Oligonucleotides containing either a perfect match or a range of single mismatches were radiolabelled using polynucleotide kinase. One picomole of labelled DNA was incubated for 1 hour with protein extracts of hMSH-2, pET, and Δ1-5. After the incubation period the mixtures were slowly filtered over prewet nitrocellulose. Washed and bound DNA was detected using autoradiography.

to measure the hMSH-2 C-terminal domains activity (1). Mismatch recognition was detected by nitrocellulose binding of labelled oligonucleotides containing a mismatch at position 11 within the context of a double-stranded 24-mer oligonucleotide pair. We found that the wild type C-terminal domain of hMSH-2 selectively bound all specifc mismatches apart from A/A and C/C, in agreement with results described previously (1). The pET control did not bind to any labelled oligonucleotide pair. Applying this assay to the mutant proteins, we found that Δ1, Δ2 and Δ3 bound the same specific mismatches as the wild type domain albeit to a somewhat lesser extent. This may be due to the amino acid substitutions reducing recognition of the mismatches or a reduced affinity of these proteins once bound to a mismatch resulting in separation from the mismatch in the washing procedures of the assay. Δ5 which alters Ser 676 to an Ala has further reduced affinity for these mismatches and Δ4 was found to have no selective binding to any of the specific mismatches (Fig. 4).

DISCUSSION

We have reported previously that the C-terminal domain of hMSH-2 containing a type A nucleotide binding motif, exhibits ATPase activity and is sufficient to bind mismatched oligonucleotides (1). Herein, we have produced and studied a range of mutations within the nucleotide binding motif and have shown that the alteration of an invariant lysine, in the nucleotide binding domain consensus sequence to an alanine produced a protein without the ability to bind mismatched oligonucleotides and with no ATPase activity.

As reported earlier, the domain produced only remains soluble in the presence of greater than 1 mM DTT, and as yet this protein has proved refractory to complete purification (1). Solubilisation and purification studies on the mutant fusion proteins produced herein were again unsuccessful. However, binding of mismatched oligonucleotides and ATPase activites observed from crude extracts from induced cultures demonstrates the specificity of the activity as no activity was detected in uninduced cells, suggesting these activities are generated from the construct alone.

The inability of $\Delta 4$, which alters the codon 675 from a lysine to an alanine, to identify DNA containing mismatches suggests that this Lys 675 residue is important for the binding function. It is unlikely that the mutation alters the structure of this domain significantly so as to reduce stability in *E. coli* as the expression level of this mutant is comparable to that of wild type hMSH-2 nucleotide binding domain. Thus the deficiency is not due to a gross structural instability. At present the role of Lys 675 within the nucleotide binding site in hMSH-2 is not known. However, similiar motifs in other proteins have been analysed. Structural studies have shown that a 'type A' sequence is a flexible loop bounded by a β sheet with an α helix on either side (22-26). This flexible loop allows the protein to undergo conformational change, thus controlling the accessibility of substrate binding or binding site affinities (27). Further studies have shown that an analogous lysine plays an important role in ATP-dependent function of these proteins (27-28).

The key role of Lys 675 is also emphasised by the fact that mutations at residues 666, 668 and indeed 674 (the residue next to the critical lys residue) have minimal effects on mismatch recognition and ATPase activity. Furthermore, mutation of the conserved Ser 676, the residue immediately C-terminal of Lys 675 still provides a protein which retains 40% of it's normal activity. These observations suggest that structural factors alone may not fully explain the importance of Lys 675 and perhaps this basic cationic residue is involved more directly, for example in recognising the phosphate backbone at the mismatch point in mispaired DNA. Introduction of an Arg 675 residue or if a Lys residue was placed at positions 674 or 676 may be of help in answering these questions. Such experiments could be particularly worthwhile if they produce variant hMSH-2 domains which prove to recognise C/C or A/A mismatches, a feature which is lacking in hMSH-2 itself.

To date, mutations within HNPCC kindreds have been detected in hMSH-2 and three Mut L homologs. Mutations within hMSH-2 account for approximately 30-40% of HNPCC mutations (21,29). Analysis of these mutations predicts substantial protein changes occurring from large deletions, truncations or missense mutations. The mutations identified to date show no significant mutation 'hot spot' within the coding region of hMSH-2. However, mutations have been described within exon 12 and 13 of hMSH-2 which both overlap the nucleotide binding domain (29). These comprise large frameshift deletions of codons 638-669 and 668-736, respectively, confirming that this region is essential for the functioning of the protein.

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