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Ser 524 is a phosphorylation site in MUTYH and Ser 524 mutations alter 8-oxoguanine (OG):A mismatch recognition

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Abstract

MUTYH-associated polyposis (MAP) is a colorectal cancer predisposition syndrome that is caused by inherited biallelic mutations in the base excision repair (BER) gene, *MUTYH*. MUTYH is a DNA glycosylase that removes adenine (A) misinserted opposite 8-oxo-7,8-dihydro-2'-deoxyguanosine (OG). In this work, wild type (WT) MUTYH overexpressed using a baculovirus-driven insect cell expression system (BEVS) provided significantly higher levels of enzyme compared to bacterial overexpression. The isolated MUTYH enzyme was analyzed for potential post-translational modifications using mass spectrometry. An *in vivo* phosphorylation site was validated at Serine 524, which is located in the C-terminal OG recognition domain within the proliferating cell nuclear antigen (PCNA) binding region. Characterization of the phosphomimetic (S524D) and phosphoablating (S524A) mutants together with the observation that Ser 524 can be phosphorylated suggest that this residue may play an important regulatory role *in vivo* by altering stability and OG:A mismatch affinity.

Keywords

MUTYH; MAP; 8-oxoguanine; adenine glycosylase; phosphorylation; posttranslational modification

1. Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) are two well-studied inherited forms of colorectal cancer (CRC) caused by mutations in the mismatch repair (MMR) and adenomatous polyposis coli (*APC*) genes, respectively [1,2]. The latest entrant in the field of inherited CRC syndromes is MUTYH associated polyposis (MAP), an autosomal recessive condition that develops due to biallelic inherited mutations in *MUTYH* [3,4]. Bacterial MutY and its human homologue MUTYH are adenine glycosylases of the base excision repair (BER) pathway that play important roles in the prevention of mutations associated with the oxidative product of guanine, 8-oxo-7,8-dihydro-2'-deoxyguanosine (OG) [3,5]. The mutagenic potential of OG arises from its frequent mispairing with A during DNA replication. Failure to intercept OG:A mismatches prior to further replication events results in G:C→T:A transversion mutations [6]. MUTYH

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plays a unique role in finding OG:A mismatches and removing the misinserted adenine, thereby providing another opportunity for proper removal of OG from an OG:C bp by the human OG glycosylase hOGG1.

Since the first discovery of the connection between MUTYH and CRC in 2002 [7], many mutations have been discovered in *MUTYH* that correlate with a polyposis phenotype leading to a designation of MAP [4]. The two most common variants of *MUTYH* found in approximately 70–80% of Caucasian MAP patients are Y165C and G382D *MUTYH* [8]. Functional assays carried out by our laboratory on the corresponding variants in *E. coli* MutY (Y82C and G253D MutY) demonstrated that the variants were catalytically compromised [7,9] providing support for the hypothesis of the disease mechanism of MAP: colonic cells harboring *MUTYH* variants are deficient in OG:A mismatch repair and accumulate mutations in the “gatekeeper” *APC* gene leading to the inactivation of the APC protein. Enzymatic analyses of the *MUTYH* enzyme have been limited due to low levels of overexpression and related toxicity in bacterial cells. To date functional information is available on only 10 of more than 70 different missense variants of *MUTYH* identified in MAP patients [7,9–14]. Much of the information obtained from studies with partially-purified human enzyme or the corresponding *E. coli* (Ec) or mouse variant proteins has been conflicting. This may be due in part to the low levels of active enzyme produced in bacterial expression systems that can vary considerably among different preparations, even of the same enzyme form. We have recently reported that by correcting for active enzyme fraction of the expressed protein when analyzing the adenine glycosylase activity of WT *MUTYH* and MAP variants, fluctuations associated with different enzyme preparations can be removed [14], thus more fully revealing consequences in adenine excision catalysis due to an amino acid alterations.

Different conclusions have also been drawn based on studies of MAP variants obtained from bacterial overexpression systems relative to those in eukaryotic expression systems [15,16] or on the basis of experiments performed in eukaryotic cell lines [10,17–19]. The discrepancies observed between bacterial and eukaryotic overexpression systems may be due to superior folding and presence of post-translational modifications (PTMs) in the enzyme when overexpressed from the latter. Several reports suggest that *MUTYH* is phosphorylated *in vivo* [15,20]. Based on differential mobility on SDS-PAGE of *MUTYH* isolated from HeLa cells compared to that isolated from bacteria and the fact that the differential migration was removed upon treatment of the former with alkaline phosphatase, Gu and Lu suggested that the native *MUTYH* was phosphorylated *in vivo* [15]. In another study, Parker *et al.* linked faulty phosphorylation of *MUTYH* to decreased OG:A repair activity in CRC cell lines [20]. In a series of CRC cell lines that exhibited low levels of *MUTYH* protein and adenine glycosylase activity, the adenine excision activity was increased by treatment of the cell extracts with several kinases suggesting an increase in glycosylase activity of *MUTYH* upon phosphorylation. Though both of these previous reports point to a potential role for *MUTYH* phosphorylation, no specific phosphorylation sites in *MUTYH* have been reported.

The studies to date prompted us to overexpress recombinant *MUTYH* as an N-terminal maltose-binding protein (MBP) fusion using a baculovirus expression vector in Sf9 cells; this recombinant enzyme form is designated henceforth as *MUTYH* (I) to distinguish it from the bacterially-expressed form, *MUTYH* (E). The active enzyme concentration of *MUTYH* (I) was considerably higher than that typically obtained with bacterially-overexpressed *MUTYH* (E). Phosphorylated peptides derived from WT *MUTYH* (I) were detected by mass spectrometry and a phosphorylated serine at position 524 in the PCNA-binding motif of *MUTYH* (I) was confirmed. The phospho-mimetic and phospho-inhibiting mutants (S524D and S524A, respectively) were generated and overexpressed in a manner similar to WT

MUTYH (I). *In vitro* glycosylase assays with an OG:A-containing duplex and the two phosphomutants showed that the intrinsic rate of adenine removal was not affected by changing the serine residue to either aspartic acid or alanine. However, dissociation constants (K_d) measured via electrophoretic mobility shift assays (EMSA) with an OG:FA (where FA = 2'-fluoroadenosine)-containing DNA duplex demonstrated that the binding affinity of both phosphomutants was approximately 10-fold lower than WT MUTYH (I). Interestingly, Ser 524 lies in the PCNA binding motif of MUTYH. Taken together with the functional data, this suggests that phosphorylation at Ser 524 may be an important mechanism for regulating MUTYH-mediated OG:A repair activity in cells.

Materials and Methods

2.1. Chemicals and reagents

The analogue 9-(2'-deoxy-2'-fluoro- β -D-arabinofuranosyl) adenine (FA β) and (3R,4R)-4-(hydroxymethyl)pyrrolidin-3-ol (1-aza-dR or 1N) phosphoramidite monomers were synthesized using literature procedures [21,22]. Oligonucleotides were synthesized at the University of Utah Core Facility (University of Utah Medical School) with standard 2'-deoxynucleotide- β -cyanoethyl (CE) phosphoramidites and the 8-oxo-dG-CE phosphoramidite from Glen Research. Oligonucleotides used for PCR were purified using oligonucleotide purification cartridges (OPC) from Invitrogen. All other oligonucleotides were purified via HPLC on a Beckman Gold Nouveau system with a C₁₈ RCM column from Waters. PCR was performed on a GeneAmp PCR system 2400 from Perkin Elmer. Radiolabeling was done using [γ -³²P]ATP purchased from ICN with T4 polynucleotide kinase from New England BioLabs. Labeled oligonucleotides were purified using ProbeQuant G-50 spin columns from Amersham Pharmacia according to the manufacturer's protocol. Storage phosphor autoradiography was performed on a Typhoon 9400 phosphorimager system, with data analysis using ImageQuaNT 5.2 and GraFit 5 software. All plasmid DNA was purified using the QIAprep Spin Miniprep kit from Qiagen according to the manufacturer's protocol. Bacmid DNA for transfection was isolated using the QIAGEN Mini Kit following the manufacturer's protocol. Gel purification of DNA from agarose gels was performed using a Qiagen Gel Extraction kit using the protocol provided by the manufacturer for use with a centrifuge. All restriction endonucleases and the Quick Ligase used in this study were purchased from New England BioLabs (NEB). All other chemicals, buffers, and reagents used for these experiments were purchased from VWR, Fisher, or Sigma.

2.2. Cloning and vector construction of WT, S524A and S524D MUTYH

The Bac-to-Bac Baculovirus Expression System (Invitrogen) was used to express the 535 amino acid form of MUTYH with a N-terminal maltose-binding protein (MBP) tag. The *MUTYH* gene [14] and *malE* were sequentially cloned into the MCS II of the *pFastBacDual* vector (Invitrogen). Specifically, the *MUTYH* gene was subcloned into the EcoRI and HindIII sites of the *pFastBacDual* vector to generate the pFBDMUTYH construct. The *malE* gene encoding for the Maltose Binding Protein was PCR-amplified using the *pMAL-c2x* vector (NEB) as the template and oligonucleotides which amplified the region between the start codon of the MBP and the EcoRI site. The amplified *malE* gene included the factor Xa protease cleavage site and the entire linker region. The amplified product was digested using RsrII and EcoRI, purified and cloned into the RsrII and EcoRI sites of the pFBDMUTYH construct. The primers used for the PCR reaction of the *malE* gene are as follows:

Forward primer 5'- GCG CGC CGG ACC GCA TAT GAA AAT CG- 3'

Reverse primer 5'- GCG CGC GAA TTC TGA AAT CCT TCC CCT C- 3'

All ligation reactions were carried out at a vector to insert ratio of 1:3. The full construct when expressed gives an ORF of 927 amino acids with the fusion protein expressed with an N-terminal MBP tag (*pFBDmaleMUTYH*). The ligation mixtures were transformed into DH5 α cells and plated onto LB agar plates containing 100 μ g/ml ampicillin. Colonies were initially screened using restriction mapping and then further confirmed by sequencing to identify the recombinant *pFBDmaleMUTYH* plasmid. The recombinant bacmid was generated by transformation into chemically competent DH10Bac *E. coli* cells. Colonies were screened for the recombinant bacmid using a blue-white screening assay followed by a PCR assay according to manufacturer's protocol. This bacmid was developed into a baculovirus (BvMBP-MUTYH) using the Bac-to-Bac kit protocols. Supernatants of transfections or amplifications of the baculovirus were assayed by dot blot using an anti-gp64 monoclonal antibody (eBioscience) to detect the presence of baculovirus. Baculovirus titer was estimated by comparison of cleared supernatants with baculovirus of known titer. Sf9 cells were infected at a multiplicity of infection (MOI) of three for protein expression.

Mutations in *MUTYH* coding for S524A and S524D *MUTYH* were made by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene) and the *pFBDmaleMUTYH* plasmid as the template. The following primers were used to generate the required changes:

S524A-1: 5'- GGATAATTTCTTTTCGGTCTCACATCGCAACTGATGCAC-3'

S524A-2: 5'- GTGCATCAGTTGCGATGTGAGACCGAAAGAAATTATCC-3'

S524D-1: 5'- CGGTCTCACATCGACACTGATGCACAC-3'

S524D-2: 5'- GTGTGCATCAGTGTGCGATGTGAGACCG-3'

The constructs were fully sequenced to confirm the mutation of interest and integrity of the gene. These constructs were then similarly developed into bacmids and baculovirus as described for WT *MUTYH*.

2.3. Transfection and dot blot assays

Transfection experiments were carried out following the Bac-to-Bac kit protocol. Briefly, 6-well tissue culture plates were set up for transfection of the different constructs. One $\times 10^6$ Sf9 cells were added to each well and allowed to attach for one hour at room temperature. Two different transfection reactions were set up in the culture plates with 2 and 6 μ L DNA aliquots respectively. DNA was added to 100 μ L of Hyclone SfX media which had already been supplemented with Gentamycin (final concentration 25 μ g/ml) and then incubated for 30 min with 6 μ L of Cellfectin reagent mixed in 100 μ L media. Mock transfection mixtures were set up by adding no DNA. Media was removed from the attached cells and the transfection mixture was added along with 800 μ L of additional media. The cells were incubated at 27°C for 5 h, followed by aspiration of the transfection mixture, addition of fresh media and incubation for 72 h in a humidified incubator at 27°C to generate the first generation of virus particles. The cells were observed under a microscope to verify that the transfection procedure was successful. The supernatants were then collected and centrifuged at 500 g for 5 min to remove any cells or debris. The clear supernatants were then transferred to fresh 15 ml conical tubes and stored at 4°C. Two μ L of this supernatant was used to perform the dot blot assays.

A baculovirus of known high titer was used as a standard to determine the multiplicity of infection (MOI). Several serial dilutions of this standard were made in PBS. Similar serial dilutions were made for the supernatants obtained from the transfection reactions. For the dot blot assay, 2 μ L from each serial dilution of the standard, a mock transfection supernatant (negative control) and the transfection/viral amplification supernatants were dotted on the nitrocellulose membrane. The membrane was allowed to sit for a few minutes

to make sure all the proteins adhered. The membrane was then blocked by incubating in blocking solution (5% milk in PBST) for 15 min at room temperature with shaking. The blot was then incubated with mouse anti-gp64 antibody (eBiosciences, CA, USA), diluted 1:1,000 in blocking solution for 1 h, then followed by normal western blotting procedures for detection using the GE Healthcare western blotting kit with ECF substrate.

2.4. Protein analysis

SDS-PAGE was performed according to standard protocols [23]. Briefly, samples were fractionated and run on 12% SDS- polyacrylamide gel for 45 min at 200 V. Gels were stained with Sypro Orange. Electrophoretic transfer was performed onto a PVDF membrane at 100 V for 1h. Immunodetection was carried out using the ECF western blotting reagent pack from GE Healthcare Life Sciences and following the manufacturer's protocol. The anti-MBP antibody from NEB was used as the primary antibody at a 1:10,000 dilution.

2.5. Protein purification

Eight hundred ml exponentially growing Sf9 cells at more than 95% viability were seeded at a concentration of 1×10^6 cells/ml in a suspension culture and then infected with an empirically determined amount of the baculovirus of interest (MOI ~3). Media used was supplemented with gentamycin at a final concentration of 25 µg/ml. The cells were harvested 65 h post infection and spun down at 600 g for 10 min, washed and resuspended in 10 ml wash buffer [20 mM TRIS-HCl, pH 7.6, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1× phosphatase and protease inhibitors each (Roche)]. The cells were lysed using a freeze-thaw method (3× at 37°C alternated with 3× at −80°C.) All of the following steps were then carried out at 4°C unless otherwise noted. The cell lysate was subjected to sonication (2×, 10 s each with a 10 s interval) using a Branson sonifier250 at 70% power. After sonication, an 18% PEG solution containing 1M NaCl and 10% glycerol was added dropwise to the cell lysate with continuous stirring for 30 min to precipitate out genomic DNA. The cell lysate was then centrifuged at 15,000 g using a SS-34 rotor in a Sorvall centrifuge to pellet the cellular debris and genomic DNA for 30 min. The supernatant was removed and amylose resin (1 mL) was added and incubated together for 2 h with end-over-end rotation to bind the MBP-protein to the resin. The mix was then loaded onto a column and washed with 10 column volumes of chilled wash buffer. The first two washes were carried out in wash buffer containing 200 mM NaCl. The next four washes were carried out using buffer containing 1000 mM NaCl. The last four washes were carried out using wash buffer containing 200 mM NaCl. All the flow through and wash fractions were collected and analyzed to check for loss of protein in these steps. Five ml of elution buffer (wash buffer + 10 mM Maltose) was added to the column and incubated for 30 min. The eluate was concentrated approximately 5-fold using an Amicon concentrator (YM 10 membrane), aliquoted into single-use fractions and stored at −80°C.

2.6. DNA Substrates

The following oligonucleotide duplex was used for the adenine glycosylase assays and electrophoretic mobility shifts assays:

- d(5'-CGATCATGGAGCCAC(**Y**)AGCTCCCGTTACAG-3'
- d(3'-GCTAGTACCTCGGTG (**X**)TCGAGGGCAATGTC-5')

where **Y**= **G** or **OG** (8-oxo-7,8-dihydro-2'-deoxyguanosine) and **X**=A, FA, 1N or THF

The X-containing strand (2.5 pmol) was radiolabeled on the 5' end with [³²P]-ATP using T4-polynucleotide kinase (PNK). For glycosylase assays under multiple-turnover conditions additional unlabeled A-containing oligonucleotide strand was added such that the final

concentration would contain approximately 5% [^{32}P]-phosphate labeled DNA. The complementary OG or G-containing strand was then added in 10% excess to the A-containing strand in annealing buffer (20 mM Tris-HCl pH7.6, 10 mM EDTA, 150 mM NaCl). The DNA duplex was then annealed by heating to 90 °C for 5 min followed by slow cooling to room temperature overnight. For glycosylase assays under single-turnover conditions and binding experiments, 10% excess of the OG or G-containing strand was added to 100% labeled A-containing DNA in the annealing buffer. For EMSA experiments using MUTYH enzymes, a 20% excess of the complement OG strand was added to 100 % radiolabeled FA/ 1N/ THF (X)-containing DNA and the duplex was annealed as mentioned previously.

2.7. Glycosylase assays: active site titrations, stability assays and determination of rate constants

Adenine glycosylase activity assays of WT MUTYH and the phosphomutants S524A and S524D MUTYH were performed similarly to previous reports for MUTYH by our laboratory [14]. In general, the enzyme activity is monitored by assessing the amount of strand scission at the abasic site produced by MUTYH at the A opposite OG or G in the 30 bp duplex. Reactions were performed in two buffer systems with either 60 or 150 mM NaCl: our standard MutY assay buffer (40 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.1 mg/mL BSA) or an optimized MUTYH buffer (40 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.1 mg/mL BSA, 5 μM ZnCl_2 and 5 mM MgCl_2). Enzyme and DNA substrate were incubated at 37 °C and aliquots were removed at various times (20 s to 60 min). These aliquots were quenched with 0.2 M NaOH and heated to 90 °C for 5 min. An equal volume of formamide loading dye (0.025% bromophenol blue, 0.025% xylene cyanol, and 80% formamide in 1 $\times$ TBE) was added and the samples were heated at 90 °C for an additional 5 min. The samples were resolved on a 15% (19:1) denaturing polyacrylamide gel in 1 $\times$ TBE at 1500 V for 1.5 h and visualized by storage phosphor autoradiography.

For active site titration (AST) and multiple-turnover experiments, the removal of adenine from 5 to 10 nM OG:A-containing DNA substrate was monitored with the amount of MUTYH adjusted to give a burst amplitude in a detectable range, typically 20% of the DNA concentration. Active site concentrations used were determined from AST experiments performed with standard MutY assay buffer containing 60 mM NaCl. For the single-turnover (STO) experiments, the DNA substrate concentration was 1-5 nM and the enzyme concentration (based on AST) was at least 10 nM. Several enzyme (10–20 nM) and DNA (1–5 nM) concentrations were tested to insure the maximum k_{obs} and allow for the $k_2 = k_{\text{obs}}$ approximation. The STO experiments were performed using both standard MutY assay buffer containing 150 mM NaCl and optimized MUTYH buffer at both 60 and 150 mM NaCl. The analysis of the data and determination of the rate constants is analogous to that previously reported for MUTYH(E) and *E. coli* MutY [14,24].

2.8. Electrophoretic Mobility Shift Assay

Binding studies of WT MUTYH and variants were performed as described previously by our laboratory for the murine Mutyh form [25]. Briefly, serial dilutions of WT, S524A and S524D MUTYH (I) were made in dilution buffer (20 mM Tris-HCl pH 7.5, 10 mM EDTA, and 20 % glycerol) to a concentration range of between 200 nM and 0.05 nM at 4 °C. The appropriate DNA duplex (10 nM) was incubated with increasing concentrations of enzyme for 30 min at 25 °C. The samples were run on a 6 % non-denaturing polyacrylamide gel (29:1 acrylamide:bis), at 4 °C in 0.5X TBE buffer, at 120V, for 2 h. Gels were dried and exposed to a storage phosphor screen overnight. Analysis of the gel was performed as described previously by our laboratory using ImageQuant 5.2 and GraFit 5.0 software, in

which the binding curve generated (plotted as percent bound substrate versus log[Enz]) was fit to a one-site ligand binding equation.

2.9. Mass spectrometry of MUTYH peptides

The MUTYH enzyme was precipitated by the Wessel precipitation method [26], resuspended in 1X SDS loading buffer and applied to a 12% pre-made BIORAD SDS gel. While preparing the sample it was absolutely necessary to make sure that all apparatus used were rinsed with 70 % ethanol, nitrile gloves were used and fresh buffers made to avoid keratin contamination. The gel was stained with the mass spectrometry compatible total protein stain G250 Coomassie BioSafe stain (Bio-rad).

MUTYH peptides were prepared for MS analysis using standard reduction, alkylation, and tryptic digest procedures [27]. Peptides were dried down in a vacuum concentrator after digestion, then resolubilized in 2% acetonitrile/ 0.1 % trifluoroacetic acid for LC-MS/MS analysis. Digested peptides were separated by reversed-phase chromatography using a Waters nanoACQUITY UPLC system (Milford, MA), with a Waters BEH C18 1.7 μ m, 100 μ m $\times$ 10 cm column. A gradient was utilized of 1% to 10% acetonitrile in 5 min, 10% to 35% in 65 min, 35% to 70% in 5.2 minutes, hold 0.8 min, 70% to 1% in 0.2 min, hold 13.8 min, with 0.1 % formic acid constant throughout LC runs. Separated peptides were analyzed in a ThermoElectron LTQ-FT mass spectrometer (San Jose, CA) with a Michrom Advance Plug and Play nano-electrospray ionization source at a flow rate of 1 μ L/min. MS and MS/MS spectra were acquired using a top 4 method, where the four most abundant ions in the MS scan were selected for automated low energy CID, with a 30 s exclusion time and repeat count of two. The FTMS scan was obtained for the m/z range 300–1400 Da. An isolation width of 2.5 was used for ITMS, and normalized collision energy of 35% was used for the fragmentation.

Tandem mass spectra were extracted with Xcalibur version 2.0.7. All MS/MS samples were analyzed using X! Tandem (version TORNADO (2008.02.01.2)). X! Tandem was set up to search the human_e.fasta.pro database (version 3.52, 47648 entries) assuming the digestion enzyme trypsin. X! Tandem was searched with a fragment ion mass tolerance of 1 Da and a parent ion tolerance of 1.5 Da. Iodoacetamide derivative of cysteine was specified in X! Tandem as a fixed modification. Oxidation of methionine and acetylation of the N-terminus were specified in X! Tandem as variable modifications. Scaffold (version Scaffold_2_01_01, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability as specified by the Peptide Prophet algorithm [28]. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [29]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

All MS/MS samples were analyzed for phosphorylation using SEQUEST (Bioworks, version 3.3; ThermoFinnigan, San Jose, CA). SEQUEST was set up to search a FASTA file of MUTYH and an assorted database of common protein contaminants, assuming the digestion enzyme trypsin. SEQUEST was searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 1.5 Da. Phosphorylation of serine, threonine and tyrosine was specified in SEQUEST as variable modifications. DTASelect software was used to filter out low score matching. The filtering criteria consisted of: Cross-correlation (xcorr) values larger than 1.5 for singly charged ions, 2.0 for doubly charged ions, and 2.5 for triply charged ions, for both half or fully tryptic peptides. Manual validation was carried out for all peptides with possible posttranslational modifications (PTMs). Criteria for

manual validation consisted of a high quality MS/MS spectrum with fragment ions above base-line noise, a readily identifiable neutral-loss peak, minimum of four continuous y or b series ions observable, and intense y and/or b ions on the N-terminal cleavage of Pro, but weak on the C-terminal cleavage, except where Pro is the second residue from the peptide N terminus. All the major intense ions also must fit the criteria of being interpretable with ProteinProspector (prospector.ucsf.edu). Scaffold was used to further validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 80.0% probability, assuming they had already passed the SEQUEST filters above. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides.

Results

3.1. A recombinant baculovirus driven Sf9 expression system for MUTYH

The 535 amino acid form of the human adenine glycosylase MUTYH with both an N-terminal hexa-His or maltose binding protein (MBP) tag was expressed in Sf9 insect cells using a baculovirus expression vector system (BEVS). Similar to that reported by Shinmura *et al.* [16] in Sf21 insect cells, we observed low expression levels of the full-length His₆-tagged MUTYH in Sf9 cells. However, previously we observed that expression of an N-terminal MBP fusion construct of *E. coli* MutY[30] and MUTYH[14] significantly increased expression levels in bacteria compared to His₆-tagged forms. Use of MBP-fusion constructs have been shown to improve expression in insect cells [31]. Thus, the *malE* and a *MUTYH* gene construct that contained seven altered arginine codons to improve expression[14] were sequentially cloned into the pFastBacDual vector to yield a construct with an ORF (*pFBDmalEMUTYH*). The recombinant bacmid was generated by transformation into chemically competent DH10Bac cells. This bacmid was developed into a baculovirus, *bvWT-MBPMUTYH*. Denaturing SDS-polyacrylamide gel electrophoresis (PAGE) of whole cell lysates from *bvWT-MBPMUTYH*-infected Sf9 cells showed the presence of a new protein band at the correct molecular weight when compared to whole cell lysates prepared from uninfected cells (Figure 1). Large-scale overexpression of MUTYH was achieved by infecting suspension cultures of Sf9 cells with the recombinant virus at an MOI of 3 for 65 hours. The enzyme was purified using amylose affinity chromatography.

Western blot analysis revealed that the full length MBP-MUTYH (I) protein was expressed successfully at the expected molecular weight. Additionally, SDS-PAGE analysis of MUTYH (I) enzyme shows that translation termination after the MBP coding region is minimal in the BEVS (Figure 1: right panel, lane 2 versus lanes 5 and 6). The potential that the protein was phosphorylated was examined by analyzing an SDS-PAGE gel with Pro-Q diamond stain, a stain specific for phospho-proteins (Supplementary Figure 1). This indicated that the isolated MUTYH (I) was phosphorylated and prompted the mass spectrometry analysis to detect sites of phosphorylation.

3.2. MUTYH expressed in Sf9 cells is phosphorylated at Ser 524

Peptides produced from trypsin in-gel digestion of MUTYH (I) were run on a calibrated LTQ-FT to give accurate masses for post translation modification (PTM) analysis. Analysis of the LC-MS/MS data using Sequest and X!Tandem software identified peptides derived from trypsin digestion of MUTYH as the dominant protein in the samples. In one sample, appropriate analysis with X!Tandem Scaffold X indicated trypsin fragments representing approximately 73 % sequence coverage of the whole protein using a database containing the MBP-MUTYH protein sequence (Figure 2).

Initial analysis of the LC-MS/MS data using Sequest software with a file that contained the MUTYH sequence and approximately 50 of the known contaminants in protein samples yielded a group of possible peptides containing different potential phosphorylation sites (Figure 3A). In this initial analysis, the peptide, SHIpSTDAHSLNSAAQ (**1**), had the highest Xcorr value. Subsequent analysis of this data with the Modiro programs indicated two phosphopeptides, SHIpSTDAHSLNSAAQ (**1**) and pSHISTDAHSLNSAAQ (**2**). However, using the same data file and constraints, X!Tandem showed the presence of only phosphopeptide **1**. After confirming this phosphopeptide with three separate search engines, we next used Protein Prospector and manual validation to confirm that the correct sequence for phosphopeptide **1** is SHIpSTDAHSLNSAAQ (Figure 3). This validation entailed confirming the presence in the raw data file that the dominant peak from the spectrum was the phosphate neutral loss peak for the molecular ion (761.10126, $[M + 2H]^{+2} - P$). We also found correct masses corresponding to the majority of B and Y ions expected for phosphopeptide **1**, using the theoretical fragmentation algorithm in Protein Prospector, and another strong neutral loss peak for B14 (688.02045, $[B14 + 2H]^{+2} - P$) (Figure 3B). The phosphorylated serine in the phosphopeptide **1** corresponds to Serine 524 in MUTYH (Figure 2).

3.3. Active site titration and adenine glycosylase activity of WT MUTYH (I)

We have previously shown that MBP-MUTYH expressed in *E. coli* displays “burst” kinetics with OG:A-containing substrates under conditions of multiple-turnover ($[DNA] > [Enzyme]$), similar to the behavior observed with other MutY enzymes [24,25,32]. WT MUTYH (I) exhibits the same type of biphasic kinetic profile displayed by MUTYH (E) (Figure 4A) and therefore we utilized the same kinetic scheme (Figure 4C) to analyze the kinetic parameters associated with WT MUTYH (I). In initial kinetic experiments, buffer conditions were used similar to those used with MUTYH (E) containing either lower (60 mM) or higher (150 mM) NaCl. Active site titration (AST) experiments were performed that utilize the burst amplitude to determine active enzyme fraction in a given enzyme preparation. In AST analysis of several different WT MUTYH (I) preparations, the active protein yields were found to range from 10–28 ug per liter culture (Table 1) indicating a significantly increased amount of active enzyme than obtained from bacterial overexpression of the analogous MBP-MUTYH construct. Stability assays based on the change in the burst amplitude with time indicate loss of activity in the glycosylase buffer similar to that observed with murine Mutyh [25]. The loss of enzyme activity is less pronounced if non-specific DNA is present which may mimic the conditions used to measure rate constants more closely. However, due to the loss of activity with time for MUTYH (I), the observed rate determined from the steady-state phase of the kinetic plot under conditions of multiple-turnover is reported as k_{to} (Table 1) and not equated with the microscopic rate constant k_3 . Fitting of the linear phase of the plots generated from adenine glycosylase assays performed under conditions of multiple-turnover yields a rate of turnover (k_{to}) of $0.02 \pm 0.01 \text{ min}^{-1}$. The value of k_{to} for WT MUTYH (I) is similar to the rate constant k_3 obtained for *E. coli* MutY at 150 mM NaCl concentrations [33]. Similarly, the value of k_{to} at 150 mM NaCl concentrations with MUTYH (I) is approximately 100-fold faster than that determined using a lower salt concentration (60 mM) in the assay buffer ($k_{to} = 0.0002 \pm 0.0001$).

The rate constants for adenine excision (k_2) from an OG:A containing duplex were determined from glycosylase assays performed under single-turnover (STO) conditions (Figure 4B), where $[Enzyme] > [DNA]$. These conditions allow for isolation of the intrinsic rate constant for step(s) associated with the base excision without influence from product release. If the enzyme concentration is above K_d , then it is valid to equate the observed rate constant (k_{obs}) to the true rate constant (k_2) for the base removal steps (Figure 4C)[24]. The

rate constant k_2 for WT MUTYH (I) was determined to be $0.5 \pm 0.1 \text{ min}^{-1}$ using our standard MutY assay buffer containing 150 mM NaCl (Table 1). Surprisingly, under similar conditions, we observed minimal reaction of WT MUTYH (I) with a G:A containing duplex. Due to the potential that the buffer conditions may be more adversely affecting the reaction with the G:A substrate as observed with the bacterial MutY enzyme [13], we examined both substrates using an optimized buffer for MUTYH reported previously [34] that contains both MgCl_2 and ZnCl_2 and lower NaCl concentration (60 mM). Using this buffer system, a measurable rate of reaction with the G:A substrate of $0.2 \pm 0.1 \text{ min}^{-1}$ was observed (Table 1). Interestingly, a two-fold increase in k_2 with the OG:A substrate was observed in this buffer system; we attribute this enhancement to addition of MgCl_2 and ZnCl_2 , since similar reaction rates are observed in this buffer containing 150 mM and 60 mM NaCl concentrations.

3.4. Adenine glycosylase activity of phospho-mimetic and phospho-ablated forms of MUTYH (I)

MUTYH (I) as expressed likely contains only a small fraction of protein that is phosphorylated at Ser 524 [35,36]. This is also consistent with the observation of both phosphorylated and unphosphorylated versions on the peptides in LC-MS/MS. A useful approximation of the phosphoenzyme is the mutated form containing an Asp residue at the location of the Ser residue in order to mimic the negative charge of the phosphorylated serine. The serine at position 524 in MUTYH (I) was also replaced with alanine to prevent phosphorylation at this site. Isolation of bacmid DNA, generation of the baculovirus, and protein expression and purification of these two mutated enzymes were carried out in a manner as described for the WT enzyme. Total protein expression as determined from Western analysis with antibodies to the MBP-tag (data not shown) was similar for S524A, S524D and WT MUTYH (I). Both S524A and S524D retain the biphasic kinetic profile similar to WT MUTYH (I), prompting the use of the same kinetic approach and determination of active fraction via AST experiments. The active yield of S524A and S524D MUTYH (I) are within error of that of WT MUTYH (I) (Table 1).

Stability assays were carried out on the phosphomutants and compared to WT MUTYH (I) (Figure 5A). MUTYH (I) was incubated in the glycosylase assay buffer (150 mM NaCl) at 37°C. Aliquots were removed at various times and added to the OG:A-substrate duplex, and the amplitude to the burst from multiple turnover experiments was determined. Based on the decrease in the burst amplitude, MUTYH (I) and the two phosphomutants show a decreased active fraction with time under these conditions. S524D MUTYH (I) follows the WT enzyme in its stability assay profile; both retain approximately 40% of the initial activity at the end of a five-minute incubation period. Additionally, at the end of 10 minutes, both enzymes are approximately 20 % active. S524A MUTYH (I), on the other hand, rapidly loses its active fraction and is only 20% active at the end of five minutes. The enzyme is completely inactive at the end of the 10 minute incubation period, illustrating that the phosphoinhibiting S524A MUTYH (I) is more unstable than the phosphomimetic form, S524D MUTYH (I) or WT MUTYH (I).

The rate of adenine excision from the OG:A containing substrate was examined for S524A and S524D MUTYH (I) using the adenine glycosylase assay under conditions of single-turnover (Figure 5B, Table 1) in both the standard MutY buffer and the optimized MUTYH buffer. The k_2 values determined using the standard MutY buffer for S524A and S524D MUTYH (I) were 0.4 ± 0.1 and $0.5 \pm 0.1 \text{ min}^{-1}$ respectively. Both these numbers are similar to the k_2 value determined for WT MUTYH (I) ($0.5 \pm 0.1 \text{ min}^{-1}$). As we have seen previously with WT MUTYH (I), the determination of the rate constant associated with product release (k_3) may be partially obscured by the loss of activity of the enzyme. The phospho-mutants also undergo a loss of active fraction during the course of the reaction;

hence, the rate of turnover (k_{to}) was determined under conditions of multiple-turnover (Table 1). The values of k_{to} with the OG:A substrate determined for S524A MUTYH (I) ($0.03 \pm 0.01 \text{ min}^{-1}$) and S524D MUTYH (I) ($0.02 \pm 0.01 \text{ min}^{-1}$) were similar to that of WT MUTYH (I). Similarly, both S524A and S524D MUTYH exhibited similar k_2 rate constants as WT MUTYH with the OG:A and G:A substrate in the optimized MUTYH buffer (Table 1).

3.5. Mutations at Ser 524 reduce the affinity of MUTYH for substrate analogue-containing duplexes

In order to measure and compare the relative dissociation constants (K_d) of MUTYH to substrate, transition state, and product analogues, electrophoretic mobility shift assays (EMSA) were performed. The noncleavable substrate analogue 9-(2-deoxy-2-fluoro- β -D-arabinofuranosyl) adenine (FA^β) (Figure 6A) was used instead of A to determine the affinity of MUTYH for a substrate-like base pair that would be resistant to enzymatic processing. We also evaluated the affinity with duplexes containing the transition state analogue, 1-aza-deoxyribose (1N), and product analogue, tetrahydrofuran (THF), nucleotides paired opposite to OG in the 30-bp duplex sequence context used for kinetic studies (Figure 6A). Previous work with Ec MutY has established high affinity binding to these substrate, transition state and product mimics [37]. The dissociation constants determined by EMSA at 25°C (Figure 6B) are compared in Table 1. The presence of a specific DNA-MUTYH complex was observed in the EMSA using all three synthetic analogues. WT MUTYH (I) binds to the OG:FA-containing duplex with high affinity as evidenced by K_d value of approximately 150 pM (Figure 6C). WT MUTYH (I) also exhibits extremely high affinity to both the product analogue THF and the transition state analogue-containing duplex (Table 1). In both cases the DNA was completely bound at all enzyme concentrations and therefore an upper-limit for the K_d value ($< 50 \text{ pM}$) was estimated based on the lowest enzyme concentration used. The K_d values determined for WT MUTYH (I) are comparable to those measured for Ec MutY with these synthetic analogues [37,38], consistent with the high similarity between the bacterial and human forms of the enzyme.

In order to measure and compare the relative binding affinities (K_d) of the phosphomutants, similar EMSA experiments were performed with the 30-bp duplex containing OG base paired to the noncleavable substrate analogue, FA^β , at 25°C. A representative graphical plot of the percent protein bound to DNA versus the enzyme concentration from the EMSA experiments is shown in Figure 6D. Surprisingly, both S524A and S524D MUTYH (I) lose the tight binding affinity displayed by WT MUTYH to the OG:FA-containing substrate. Both phosphomutants show an approximately 10-fold decrease in the K_d value (Table 1). This suggests that the presence of a hydroxyl group at this position is important for high affinity binding of MUTYH with its DNA substrate such that its substitution with the nonpolar Ala residue or the charged Asp, reduces DNA affinity.

Discussion

Recently, several research laboratories have used baculovirus infected Sf9 insect cells to overexpress and purify recombinant forms of DNA repair enzymes that have inherently low expression levels otherwise [39,40]. One of the advantages of insect cell expression is that high levels of the foreign protein of interest can be produced while retaining the native fold of the protein and associated post-translational modifications. Many examples have illustrated that proteins expressed in insect and human cells have equivalent phosphorylation sites, indicating that the native and the recombinant proteins undergo similar processing [41–45]. The BEVS developed herein for production of MBP-MUTYH provides superior amounts of high quality enzyme compared to the bacterial overexpression system [14]. Notably, the MUTYH (I) isolated from this eukaryotic expression system is a

phosphoprotein. Using mass spectrometry techniques, we identified for the first time a site of *in vivo* phosphorylation in MUTYH, specifically Ser 524. In order to assess the functional significance of phosphorylation at this site, S524A and S524D MUTYH (I) variants were analyzed in terms of the kinetics of adenine removal, binding affinity and stability compared to WT MUTYH (I). These studies indicate a potential role for the C-terminal end of MUTYH in modulating the affinity for the damaged substrate via phosphorylation.

Our laboratory has previously exploited the use of non-cleavable analogues to reveal fundamental features concerning recognition of bacterial and mammalian MutY enzymes [9,18,25,37,38,46]. In previous studies, the FA^α nucleotide, where α designates the ribo sugar conformation, was used in place of A while in the present study, the FA^β analogue, where β designates the arabino sugar conformation, was used. The FA^β analogue may more closely mimic the sugar conformation of DNA and was recently used in structural studies with *Bacillus stearothermophilus* (Bs) MutY [47]. EMSA indicates a slightly higher affinity of Ec MutY for duplexes containing OG paired with FA^β over FA^α analogue [48]. Importantly, like Ec MutY and murine Mutyh, WT MUTYH (I) binds with picomolar affinity to the 30 bp duplex containing FA^β opposite OG. MUTYH (I) also exhibits extremely high affinity for the transition state mimic 1N and the product analogue THF similar to that observed with the bacterial enzyme [38,49]. Taken together, the EMSA results suggest that the human protein recognizes the OG:A mispair, reaction coordinate intermediates and the product in a manner similar to that of the bacterial enzyme. The rate constant k_2 for adenine excision by MUTYH (I) is also similar to values obtained for murine Mutyh [25] and MUTYH (E)[14]. However, in all cases the mammalian MutY enzymes have a reduced rate constant k_2 (approximately 10-fold) for adenine removal *in vitro* compared to Ec MutY [9,50]. It is interesting that despite the similarities in OG:A mismatch affinity, the rate of adenine excision k_2 is reduced for the mammalian enzymes. This may be a reflection of the increased complexity of the human BER pathway and the potential modulation of MUTYH activity by other proteins.

Several different post-translational modifications (PTMs), such as acetylation, phosphorylation, sumoylation, and ubiquitination have been detected in BER proteins including BER glycosylases [51]. PTMs have been shown to alter subcellular localization [52,53], turnover rates [52,54–57], binding affinity towards substrates [56,58], and protein-protein interactions [59,60], thereby affecting the overall efficiency of BER proteins. Though previous work hinted that MUTYH may be phosphorylated, [15] there were no reports of specific phosphorylation sites of MUTYH. Using mass spectrometry, a phosphorylation site at Ser 524 has now been identified. As a first step towards understanding the effects of phosphorylation at Ser 524, we replaced this serine residue in MUTYH (I) with alanine or aspartic acid. The aspartic acid residue mimics the negative charge that is created upon phosphorylation while the alanine substitution prevents phosphorylation. Both phosphomutants had levels of expression of total protein comparable to WT MUTYH (I). Stability assays revealed a higher stability of S524D and WT MUTYH over S524A MUTYH (I). This suggests that phospho-mimetic modifications of Ser 524 are tolerated while removal of the polar hydroxyl group with alanine is not well-tolerated. This suggests that the S524A mutation may not be an innocent mutation that simply prevents phosphorylation but may also be altering the conformation of the C-terminal end of MUTYH that may be important for stability.

The adenine glycosylase rate constant k_2 determined under conditions of single-turnover with both OG:A and G:A substrate are similar for the two phosphomutants and WT MUTYH (I). This suggests that under these conditions, the modifications do not affect the intrinsic adenine glycosylase activity of MUTYH. This result initially may seem in contrast to previous results reported by Gu and Lu [15], where superior adenine glycosylase activity

of phosphorylated MUTYH isolated from HeLa cells was observed [15]. In the previous report, it was also observed that the activity on G:A substrates was more adversely affected by dephosphorylation of native MUTYH. However, the sites of phosphorylation of MUTYH in HeLa cells were not identified and therefore a direct comparison between the previous and present studies is not possible. Moreover, it is possible that MUTYH isolated from HeLa cells may harbor a variety of phosphorylation sites that are phosphorylated at sub-stoichiometric levels. Indeed, this underscores the usefulness of our approach in identifying specific phosphorylation sites and using phosphomimetic mutations to gauge the affects of site-specific phosphorylation of MUTYH. Indeed, there may potentially be many different phosphorylated forms of MUTYH that are present under different cellular conditions. Another complicating factor in comparing this work with previous studies is related to the difference in methods used for analyzing the glycosylase activity of MutY enzymes. In the present study of MUTYH, the kinetic analysis has been performed to determine the relevant rate constants with use of the active site concentration to insure the appropriate conditions for the analysis. If the native phosphorylated protein is present at a higher active concentration than the unphosphorylated recombinant enzyme, using similar amounts of total protein and analysis at a single time point (as was performed in the previous study) may be the reason that a higher amount of product is formed rather than an increase in the intrinsic catalytic activity of the enzyme. Thus the approach outlined herein allows for distinguishing these potential differential effects due to phosphorylation or modification of specific amino acid residues in MUTYH.

Remarkably, both S524A and S524D MUTYH (I) exhibited a 10-fold lower affinity for the OG:FA^β duplex compared to WT MUTYH (I). Though affinity is reduced for the substrate analogue mismatch, an altered k_2 in the glycosylase assays under single-turnover conditions ($[MUTYH(I)] > K_d$) is not observed consistent with the expectation that the microscopic rate constant k_2 for adenine removal should not be altered by changes in k_1 or k_{-1} (K_d for substrate) (Figure 4). Since binding affinity is related to the efficiency of finding the mismatch, binding defects will reduce the extent of OG:A repair in cells. Indeed, we recently showed using unnatural substrates with Ec MutY that defects in binding affinity are magnified in a cellular setting in terms of the overall levels of repair of the mismatch, while defects in catalysis result are less deleterious to repair [33]. We reasoned that this is due to competition between repair and replication such that binding defects translate into missed opportunities to repair the OG:A mismatch prior to its conversion to a normal T:A bp. In contrast, slow base excision may be tolerated as long as the OG:A mismatch is captured and sequestered from the replication machinery. Further investigation into the biological activity of the Ser 524 MUTYH phosphomutants within a cellular environment will be necessary to fully understand the role of phosphorylation with respect to BER *in vivo*.

Ser 524 is located in the C-terminal domain of MUTYH that is unique to MutY enzymes and not found in other members of the BER superfamily of DNA glycosylases [3,61,62]. Structural studies [47,62] and functional studies of MutY enzymes have established the important role of the C-terminal domain in OG recognition while the N-terminal domain harbors catalytic residues needed for catalysis [38,63]. Since the human enzyme is much larger than *Bs* MutY and the C-terminal tail end containing Ser 524 is not present in the *Bs* MutY structures, structure-based predictions on the effects of phosphorylation at Ser 524 are not possible. The location of Ser 524 at the very end of the C-terminal domain, rather than the catalytic N-terminal domain may explain, in part, the lack of a difference in the intrinsic base excision reaction between the two phosphomutants and WT MUTYH (I). However, our observed reduced affinity of S524D and S524A MUTYH (I) with the substrate analogue-containing duplex further implicates the dominant role of the C-terminal domain in damage recognition, and suggests that the extended tail region of the C-terminal domain in the human enzyme indirectly or directly controls DNA affinity. The region containing Ser 524,

or the Ser side chain itself, may contact the DNA duplex such that Ala or Asp substitution reduces affinity. Phosphorylation events also are known to induce conformational changes in proteins [64–66]. In this case, a change in conformation of the C-terminal region due to substitution of Ser 524 or phosphorylation may alter the ability of the C-terminal domain to fully engage upon the OG of the OG:A mismatch, thus resulting in reduced affinity for the OG:A mismatch.

Interestingly, though the region harboring Ser 524 is not present in the bacterial enzyme, this region is highly conserved among mammalian MutY enzymes (Figure 7). Notably, in the few mammals (mouse, rat) that do not contain Ser or Thr at position 524, there are also many other amino acid changes in this region. MUTYH is known to interact with many other replication and repair proteins such as RPA, PCNA, MSH6 and the 9-1-1 complex [67–69]. The sequence of MUTYH harboring Ser 524 is within the identified proliferating cell nuclear antigen (PCNA)-binding region (505–527) of MUTYH [70]. PCNA participates in long patch base excision repair as a processivity factor to mediate protein and DNA interactions [71]. Previous work has established that disruption of the PCNA binding motif in MUTYH abrogates repair *in vivo*, indicating a critical role for replication in MUTYH-mediated repair [72]. However, based on the observation that this region of MUTYH physically interacts with PCNA [70], it may be speculated that the C-terminal end is not tucked within the protein and is accessible for interactions with other proteins and/or DNA. Phosphorylation at the C-terminal tail of MUTYH may play dual roles in modulating the interaction with PCNA during replication-coupled repair and coordinating the recognition of the lesion-containing strand.

Parker *et al.* correlated defective OG:A repair in CRC cell lines to defective phosphorylation of MUTYH by establishing increased levels of repair by treatment with protein kinase A (PKA), protein kinase C (PKC) and casein kinase II (CKII) [20]. In this study, they also demonstrated that MUTYH could be phosphorylated by PKC *in vitro* which resulted in improved OG:A repair activity [20]. These results are intriguing in light of the role of inherited defects in MUTYH in MAP, suggesting another mechanism leading to CRC may precipitate from defective phosphorylation of MUTYH. The newly identified phosphorylation site Ser 524 is a predicted phosphorylation site using prediction software (NetPhos [73], KinasePhos [74] and GPS 2.0[75]); however, based on the sequence alone a clear top candidate kinase does not emerge, though many give reasonable scores in these algorithms (Chk1, PKC, ATM). Thus, further work will be needed to identify kinases capable of mediating phosphorylation of MUTYH.

In this work, we identified a phosphorylation site present under normal conditions of expression in insect cells without performing procedures to enrich phospho-peptides. Other sites of phosphorylation may be identified by taking advantage of enrichment methods, as well as by isolating enzyme under stress conditions that may induce phosphorylation. The observation of MUTYH phosphorylation suggests that MUTYH activity may be modulated *in vivo* by post-translational modifications and is consistent with the notion that MUTYH plays important roles in the complex response of cells to oxidative damage. These complex roles for MUTYH also suggest that there are multiple pathways by which MUTYH variants may participate in the etiology of MAP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. SDS-PAGE (left panel) and Western blot (right panel) analysis of WT MUTYH (I)
 Lane 1: Control *E. coli* MBP MutY, Lane 2: MUTYH (E), Lane 3: Marker, Lane 4: Uninfected control Sf9 cells, Lane 5: Pellet fraction from whole cell lysates infected with MUTYH baculovirus, Lane 6: Supernatant from whole cell lysate infected with WT bvMBP MUTYH baculovirus. Left Panel: Samples were run on a 12 % SDS-PAGE gel and stained with Sypro orange. The arrow in lane 4 marks the missing band in uninfected cells, which appears in the infected cells, lanes 5 and 6. The band for MUTYH (I) is present between the 97–116 kDa markers thus falling in the expected region (~102 kDa). Right panel: A second SDS-PAGE gel was run with identical samples and then western blotted with an anti-MBP antibody.

TPLVSRLSRLWAIMRKPRAAVGS GHRKQAASQEG
RQKHAKNNSQAKPSACDGLARQPEEVVLQASVSS
YHLFRDVAEVTAFRGSLLSWYDQEKRDLPWRRRA
EDEMDDLDRRAYAVWVSEVMLQQQTQVATVINYYTG
WMQKWPTLQDLASASLEEVNQLWAGLGYYSRGR
RLQEGARKVVEELGGHMPRTAETLQQLLPGVGRY
TAGAIASIAFGQATGVVDGNVARVLCRVRAIGADP
SSTLVSQQLWGLAQQLVDPARPGDFNQAAAMELGA
TVCTPQRPLCSQCPVESLCRARQRVEQEQLLASGS
LSGSPDVEECAPNTGQCHLCCLPPSEPWDQTLGVV
NFPRKASRKPPREESSATCVLEQPGALGAQILLVQ
RPNSGLLAGLWEFPSVTWEPSEQLORKALLQELQR
WAGPLPATHLRHLGEVVHTFSHIKLTYQVYGLALE
GQTPVTTVPPGARWLTQEEFHTAAVSTAMKKVFR
VYQGQOPGTCMGSKRSQVSSPCSRKKPRMGQQVL
DNFFRSHISTDAHSLNSAAQ

Figure 2. Sequence coverage of MUTYH obtained by LC-MS/MS analysis

Sequence underlined is the peptide coverage obtained. Peptide identified that upon validation identified Ser 524 as phosphorylated site is shown boxed.

Figure 3. Mass Spectrometry Analysis leading to validation of a phosphorylation of Ser 524 of MUTYH

(A) List of potential phosphorylated peptides based on masses observed in LC-MS/MS analysis. Phosphopeptide 1 shown in bold was validated as the correct site of phosphorylation. The lower case p indicates the following serine is phosphorylated. (B) Product ion spectra of the $[M + 2H]^{2+}$ ion from SHIpSTDAHSLNSAAQ acquired by ESI-FTICR. (C) Fragmentation table for SHIpSTDAHSLNSAAQ. Orange and blue highlighted m/z values indicate the presence of standard b- and y-type fragment ions, respectively, whereas green-highlighted product ions are indicative of special losses from the b and y ions.

Figure 4. Adenine Glycosylase activity of WT MUTYH (I)

(A) A representative plot of product formation as a function of time under multiple-turnover conditions with WT MUTYH (I) [1.25 nM] on a OG:A-containing DNA duplex [20 nM] at 37 °C. The graph illustrates a biphasic behavior for MUTYH (I) with an initial burst of product formation followed by a slower steady-state phase. (B) A representative plot of product formation as a function of time under single-turnover conditions with WT MUTYH (I) [11 nM] on a OG:A-containing DNA duplex [1 nM] at 37 °C. (C) Minimal kinetic scheme for MUTYH (I). All enzyme concentrations listed are active enzyme concentrations based on active site titration experiments.

A.

B.

Figure 5. Adenine Glycosylase Activity of S524D and S524A MUTYH

(A) Bar graph comparing the stability of the phosphomutants with WT MUTYH (I); WT (black), S524D (gray), S524A (vertical stripes). Activity was measured by incubation of WT MUTYH (I) and phosphomutants in assay buffer. Aliquots were removed at intervals of 0, 5 and 10 min for adenine glycosylase assays under multiple-turnover conditions. The activity of MUTYH (I) without prior incubation was normalized to 1. The bars at 5 and 10 min represent the average % loss of activity on incubation with respect to the normalized data. The error is one standard deviation from the average. (B) Representative plots of product formation as a function of time under single-turnover conditions with phosphomutants S524A (closed squares) and S524D (open circles) MUTYH on a OG:A-containing DNA duplex [5 nM] at 37 °C. Rate constants determined from appropriate fitting of the data and averaged from several experiments are listed in Table 1.

Figure 6. DNA Mismatch Affinity of WT, S524D and S524A MUTYH

Representative storage phosphor autoradiogram and graphical fitting of EMSA data for WT MUTYH bound to a 30-mer DNA duplex containing FA, 1N and THF. (A) Synthetic analogues used for EMSA studies. (B) Gel image of EMSA data for WT MUTYH bound to a 30-mer duplex with a central OG:A mispair. (C) Plot of percent WT MUTYH bound to duplex containing a central OG:FA (open circles), OG:1N (closed circles) or OG:THF (open squares) mispair. The % bound has been normalized to maximum amount bound with a given duplex. [DNA] = 0.01 nM, [MUTYH] = 0.05 to 21 nM. (D) Representative graphical plot of percent WT (open circles), S524A (closed circles) and S524D (open squares) MUTYH (I), bound to a DNA duplex containing a central OG: FA β mispair. The % bound has been normalized to maximum amount bound with a given enzyme. The K_d values from at least 4 separate experiments are listed in Table 1

Figure 7. Sequence alignment of different mammalian MutY homologues in the region corresponding to 511 to 525 in MUTYH
The alignment shows the high level of conservation of Ser 524 (indicated in bold) identified as an *in vivo* phosphorylation site.

Table 1
Active yield, rate constants and dissociation constants for S524A, S524D and WT MUTYH (I)^a

Enzyme form	Active Yield (µg/L culture)	Kd (mM) ^{a,c}	OG:FA	k _{to} (min ⁻¹) ^{b,c}	OG:A ^b	k ₂ (min ⁻¹) ^c	
						OG:A ^d +MgCl ₂ & ZnCl ₂	G:A ^d +MgCl ₂ & ZnCl ₂
WT							
MUTYH (I)	10,28,28,18	0.2 ± 0.1		0.02 ± 0.01	0.5 ± 0.1	1.0 ± 0.1	0.2 ± 0.1
S524A							
MUTYH (I)	8,19,16	1.5 ± 0.1		0.03 ± 0.01	0.4 ± 0.1	0.9 ± 0.1	0.2 ± 0.1
S524D							
MUTYH (I)	22, 26,55	1.8 ± 0.1		0.02 ± 0.01	0.5 ± 0.1	0.9 ± 0.1	0.2 ± 0.1
MUTYH (E) ^e	0.15, 0.20, 0.85	Not measured		0.011 ± 0.005	1.6 ± 0.2	Not measured	Not measured

^a Dissociation constants were determined with an OG:FAβ-containing 30-mer DNA duplex at 25 °C and 100 mM NaCl-containing assay buffer.

^b Rate constants were determined with an OG:A containing 30-mer DNA duplex at 37 °C and 150 mM NaCl-containing assay buffer. Rate constants *k_{to}* were determined under conditions of multiple-turnover ([DNA] > [Enzyme]) while rate constants *k₂* were determined under single-turnover conditions ([DNA] < [Enzyme]).

^c Rate constants and dissociation constants reported have been averaged over at least three separate experiments and the error reported is one standard deviation from the average.

^d These experiments were performed with the OG:A or G:A substrate in an optimized MUTYH assay buffer (see methods). In the case of the G:A substrate, the NaCl concentration was lowered to 60 mM NaCl in order to observe glycosylase activity.

^e These values were previously reported in Ref. 14.