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Phosphorylated hMSH6: DNA mismatch versus DNA damage recognition

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Abstract

DNA mismatch repair (MMR) maintains genomic integrity by correction of mispaired bases and insertion-deletion loops. The MMR pathway can also trigger a DNA damage response upon binding of MutSα to specific DNA lesions such as O⁶methylguanine (O⁶meG). Limited information is available regarding cellular regulation of these two different pathways. Within this report, we demonstrate that phosphorylated hMSH6 increases in concentration in the presence of a G:T mismatch, as compared to an O⁶meG:T lesion. TPA, a kinase activator, enhances the phosphorylation of hMSH6 and binding of hMutSα to a G:T mismatch, though not to O⁶meG:T. UCN-01, a kinase inhibitor, decreases both phosphorylation of hMSH6 and binding of hMutSα to G:T and O⁶meG:T. HeLa MR cells, pretreated with UCN-01 and exposed to MNNG, undergo activation of Cdk1 and mitosis despite phosphorylation of Chk1 and inactivating phosphorylation of Cdc25c. These results indicate that UCN-01 may inhibit an alternative cell cycle arrest pathway associated with the MMR pathway that does not involve Cdc25c. In addition, recombinant hMutSα containing hMSH6 mutated at an N-terminal cluster of 4 phosphoserines exhibits decreased phosphorylation and decreased binding of hMutSα to G:T and O⁶meG:T. Taken together, these results suggest a model in which the amount of phosphorylated hMSH6 bound to DNA is dependent on the presence of either a DNA mismatch or DNA alkylation damage. We hypothesize that both phosphorylation of hMSH6 and total concentration of bound hMutSα are involved in cellular signaling of either DNA mismatch repair or MMR-dependent damage recognition activities.

Keywords

DNA mismatch repair; hMutSα; hMSH6; MMR-dependent damage-signaling; alkylation damage

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1.0 INTRODUCTION

DNA mismatch repair (MMR) plays a crucial role in maintaining genomic integrity in all living cells. The MMR pathway corrects mispairs and insertion-deletion loops (IDLs), resulting from errors during replication, recombination and other DNA metabolic processes. Loss of MMR activity is associated with hereditary non-polyposis colorectal cancer and several sporadic cancers [1–5].

Repair of a DNA mismatch within human cells is initiated when hMutS α (hMSH2-hMSH6) binds to the mismatch [6–9]. hMutL α (hMLH1-hPMS2) then acts as a “matchmaker” between the hMutS α -DNA complex and downstream enzymatic components responsible for excision and resynthesis of the DNA strand containing the incorrect base [3,10,11]. The regulation of overall MMR activity within the cell is not as well understood as binding interactions of purified hMutS α with various DNA constructs. Highest MMR activity occurs during S phase of the cell cycle [12]. The localization of hMutS α within DNA replication factories during DNA synthesis likely contributes to the high fidelity of MMR during S phase [12,13]. Upon exposure of the cell to low, chemotherapeutic levels of alkylating agents however, there is a rapid and prolonged relocation of hMutS α from active replication factories to other areas of the chromosome [14,15]. Thus, MMR activity is regulated differently during chromosomal replication as opposed to a response to DNA damage. Monofunctional alkylating agents such *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) produce several alkylated DNA adducts, the majority of which are repaired efficiently by the base excision repair pathway (BER) [16–18]. The O⁶meG modification is directly repaired by the covalent transfer of O⁶methyl to Methylguanine Methyltransferase (MGMT) [19]. If MGMT is not expressed or DNA replication occurs before repair of O⁶meG, thymidine is frequently misinserted opposite the damaged guanine [20,21]. The hMutS α complex binds to O⁶meG:T, initiating a DNA damage response, rather than effective repair [4,22–25]. This binding is required for recruitment and activation of ATR-ATRIP, phosphorylation of Chk1 and subsequent activation of the DNA damage-signaling pathway [24]. Alkylation-induced DNA damage checkpoint arrest in G₂ and apoptosis is decreased in MMR-deficient cells [4,26–29]. In addition, murine Msh2 and Msh6 have been mutated in their ATPase domains to demonstrate genetic separation-of-function. Mouse cells harboring these altered proteins have deficient MMR but retain an intact DNA damage-signaling response to alkylating agents [22,23].

Twenty-two distinct phosphorylation sites have been identified in hMSH6 by phosphoproteome mass spectrometry. The majority of these sites are located in six clustered regions within the N-terminus of this protein [30–37]. The functional significance of these clustered phosphorylation sites is unknown, although both increased MMR activity and hMSH6 phosphorylation have been demonstrated after kinase activation or alkylation treatment [38–41]. In this study, we demonstrate that MSH6, but not MSH2, is phosphorylated in nuclear extracts and in intact cells. The phorbol ester, TPA, a classical kinase activator, increases hMSH6 phosphorylation and hMutS α binding to a G:T mismatch, although not to an O⁶meG:T lesion. The kinase inhibitor, UCN-01, decreases hMSH6 phosphorylation and hMutS α activity for both G:T and O⁶meG:T. Further, less hMutS α is bound to G:T than is bound to O⁶meG:T. Therefore phosphorylation of hMSH6 and the quantity of hMutS α bound to DNA may be different cellular mechanisms that regulate activity between mismatch repair and DNA damage-signaling activity.

2.0 MATERIALS AND METHODS

2.1 Materials

Monoclonal antibodies against hMSH6 (BD Biosciences), hMSH2 (Calbiochem), phosphoserine (Calbiochem), Chk1 (Santa Cruz Biotechnology), phospho-Chk1 Ser³⁴⁵ and Ser³¹⁷ (Cell Signaling), phospho-Cdc25c Ser²¹⁶ (Santa Cruz Biotechnology), Cdc2 (Cdk1) (Cell Signaling), and phospho-Cdc2 (pCdk1) Tyr¹⁵ (Cell Signaling) were purchased from the indicated suppliers. Protease inhibitor cocktails were purchased from Calbiochem. E 64 protease inhibitor, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), 7-hydroxystaurosporine (UCN-01), and 12-O-tetradecanoylphorbol 13-acetate (TPA) were from Sigma. [³²P]-orthophosphoric acid ([³²P]-H₃PO₄) was purchased from MP Biomedicals and lambda protein phosphatase (λPPase; serine-threonine phosphatase) from New England Biolabs. [α-³²P]-dATP and [γ-³²P]-ATP were purchased from Amersham. Unlabeled adenosine 5'-triphosphate (ATP) was from Calbiochem. Protein A/G beads and dialysis cassette were obtained from Pierce Biotechnology and PVDF membrane from Millipore. All other reagents were purchased from Sigma unless otherwise noted. Synthetic oligomers of 69 bases containing the coding strand sequence 5'-AATTCACGGAATATAAGCTGGTGGTGGTGGGCGCCG**G**CGGTTGGGCAAGAGTGGCGTGACCATCCAGG-3', and complementary noncoding oligomers were obtained from Operon. The above 69 base-pair oligomer is a portion of the coding sequence of human *H-ras* DNA [42]. The bolded **G** represents *H-ras* codon 12 middle G position. Complementary strands containing either **C** or **T** opposite codon 12 middle base **G**, were annealed in equimolar ratio at a final concentration of 0.2 μg per μL 1 mM Tris-HCL and 1 mM MgCl₂, pH 7.5. For O⁶meG oligomer, bold **G** in the coding strand was replaced with **O⁶-meG**. *hMSH6* cDNA in Bluescript KS plasmid and *hMSH2* cDNA in pCITE3b plasmid were kind gifts from Dr. Josef Jiricny. The cDNAs were excised and cloned into a pFastBacDual vector (Invitrogen) for stable co-expression of the proteins as hMutSα. The KpnI and XhoI restriction enzyme sites flanking *hMSH2* cDNA and the BamHI and NotI restriction enzyme sites flanking *hMSH6* cDNA were used for cloning into pFast-BacDual vector. Recombinant baculoviruses with *hMSH2* and *hMSH6* cDNAs were generated following the manufacturer's protocol (Invitrogen). The presence and orientation of each gene was confirmed by polymerase chain reaction and DNA sequencing. Quick-change II XL (Stratagene) was used to make phosphorylation site-specific mutants following the manufacturer's protocol. Baculoviruses with empty pFastBacDual plasmids (control) also were generated.

2.2 Purification of hMSH2/hMSH6

Sf9 insect cells were infected with either control baculovirus or MSH2/MSH6 recombinant baculovirus (5:1 multiplicity of infection). hMutSα was purified as follows. On day 1 after infection, E 64 protease inhibitor was added to the culture (1 μL per ml medium). On day 3, the cells were harvested, and whole cell extracts prepared as described below. Cell extract was dialyzed overnight in Buffer A (25 mM HEPES, 1 mM DTT, 0.1 mM EDTA, 200 mM NaCl and 20% glycerol). The dialyzed extract was centrifuged at 10,000 rpm for 10 min. The resulting supernatant was diluted to 150 mM NaCl in Buffer A and passed through a pre-equilibrated phosphocellulose column (Whatman). Proteins were eluted with buffer A in a linear salt gradient ranging from 0.2 M to 1 M NaCl. The peak phosphocellulose elution fractions were pooled, diluted to 250 mM NaCl, and passed through a pre-equilibrated heparin-sepharose column (GE healthcare). Proteins were eluted with Buffer A using a linear salt gradient ranging from 0.3 M to 1 M NaCl. The peak fractions from the heparin column were diluted to 250 mM NaCl and passed through a PBE 94 column (Pharmacia). Proteins were eluted with buffer A with a linear salt gradient ranging from 0.25 M to 1 M NaCl. Peak protein fractions were pooled, diluted to 100 mM NaCl, and passed through a

pre-equilibrated S-column (Biorad). Proteins were eluted with 10 ml of a linear salt gradient of 0.15 M – 0.5 M NaCl in Buffer A. The fractions containing the pure proteins (as determined by SDS-PAGE with Commassie staining) were pooled, dialyzed extensively against Buffer A, and stored in aliquots at -80°C . All buffers for protein preparation contained protease inhibitors; Pepstatin (1mg/ml), Leupeptin (1mg/ml) and 0.1M phenylmethanesulfonyl fluoride. Protein concentrations in eluted fractions were determined by Bradford protein analysis. Supplement Figure 3 is a demonstration of the purity and activity of recombinant hMutS α used for the experiments described below.

2.3 Cell culture

NIH 3T3 cells (ATCC) were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). HeLa S3 (ATCC) and Hec59 cells (hMutS $\alpha^{-/-}$; ATCC) were grown in Iscove's Dulbecco's Medium with 10% FBS. HeLa MR cells (kind gift from Dr. Sankar Mitra) were grown in DMEM/Ham's F12 50/50 mix with 10% FBS. HeLa MR cells do not express MGMT [14]. All cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere.

Nuclear extracts, whole cell lysates and cross-linked chromatin were prepared as previously described [14,15,43]. Protein concentrations were determined by Bradford protein analysis. DNA concentrations were determined by the ratio of absorbance at 260/280 nm. One hundred μL aliquots were snap-frozen in pre-chilled 1.5 ml tubes on dry ice and stored at -80°C .

2.4 Immunoblot analysis

Immunoblots of proteins separated by SDS or nondenaturing polyacrylamide gel electrophoresis (PAGE) were performed as described [14,15]. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) reagent (0.1M Tris HCl, p-Coumaric acid, 3-Aminophthalhydrazide, 30% H_2O_2) and exposure to X-ray film. Protein bands were quantified from ECL activity by Alpha Innotech FluorChem HD2. Each immunoblot experiment was repeated at least three times.

2.5 hMSH6 phosphorylation in HeLa and NIH 3T3 cells

During early proliferation (50–60% confluent), the medium was replaced with phosphate- and serum-free DMEM for 5 h to deplete endogenous phosphate. The medium was then replaced with fresh phosphate-free DMEM supplemented with 1% dialyzed FBS and [^{32}P]- H_3PO_4 (specific activity: 285 Ci/mmol) at a final concentration of 0.5 mCi/ml. After 15 h, cells were harvested in 400 mM NaCl, 1mM EDTA, 0.1% Triton X 100 and 10 mM Tris, pH 8.0 with phosphatase and protease inhibitors. The extracts were centrifuged briefly, hMSH6 was immunoprecipitated and subjected to SDS-PAGE [14]. The dried gel was exposed to X-ray film, and phosphorylation of immunoprecipitated hMSH6 was quantified with a phosphorimager. Each cellular radioactive labeling experiment was performed in triplicate.

2.6 hMSH6 phosphorylation within nuclear extracts

Recombinant hMutS α was added to MMR-proficient nuclear extracts or MMR-deficient cell lysates and incubated with 50 μCi of [γ - ^{32}P]-ATP and 250 μM unlabeled ATP at 30°C for 30 min. Equimolar concentrations of unlabeled G:C, G:T or O⁶meG:T 69mers were added to reactions as noted. For dephosphorylation measurements, phosphorylated solutions were incubated with λ PPase for 20 min at 30°C . All reactions were carried out in 40 mM Tris pH 7.6, 10 mM Mg Cl_2 , 0.2 M KCl, 100 $\mu\text{g/ml}$ bovine serum albumin, phosphatase and protease inhibitors. After incubation, hMSH6 was immunoprecipitated and subjected to

SDS-PAGE [14]. The dried gel was exposed to X-ray film and phosphorylation of immunoprecipitated hMSH6 was quantified with a phosphorimager. Nuclear extract radioactive labeling experiments were repeated a minimum of three times.

2.7 DNA binding activity and electrophoretic mobility-shift assay (EMSA)

Protein-DNA binding assays were performed using $0.8 - 1.0 \times 10^5$ cpm of 69mer and 2–5 μ g nuclear extract in an equal volume of 2 X electrophoretic mobility shift reaction buffer (12% glycerol, 2 mM $MgCl_2$, 10 μ M EDTA, 10 μ M DTT, 0.1 M NaCl, 20 mM Tris-HCL pH 7.5, 0.25 mg/ml PolydI-dC, 1 mg/ml BSA). Nuclear extracts were pre-incubated with 100 X molar excess non-radioactive homoduplex (G:C) 69mer in the same buffer for 20 min on ice. Electrophoretic mobility shifts were performed using nuclear extracts or recombinant hMutS α . Annealed oligomers were radioactively labeled by a fill-in reaction, using [α - ^{32}P]-dATP and Klenow polymerase, per manufacturer's protocol (Invitrogen). ^{32}P -labeled 69mer duplexes containing either a G:C, G:T, or O⁶meG:T were added to the reactions, and incubation continued for 15 min at room temperature. Each experiment also included reactions without oligomers, with 100X unlabeled G:T oligomer, or with antibody to hMSH6. Final reactions contained 1 mM $MgCl_2$, 5 μ M EDTA, 5 μ M DTT, 50 mM NaCl, 10 mM Tris-HCL, pH 7.5, 0.125 mg/ml poly[dI:dC], 0.5 mg/ml bovine serum albumin and 6% glycerol in a volume of 20 μ l. The reactions were subjected to electrophoresis in a 6% non-denaturing polyacrylamide gel using 0.5 X TBE buffer at 30 mA for 2–3 h at 4°C. Radioactivity was measured using a phosphorimager, and gel-shifted bands were visualized by exposure to X-ray film. Each experiment using radioactively labeled oligomers was performed a minimum of three times. Protein-DNA binding reactions for electrophoretic mobility shift-immunoblots (EMSA-immunoblot) analyses were performed as described above but with nonradioactive oligomers. After nondenaturing PAGE, proteins were transferred to a PVDF membrane and subjected to immunoblot analysis of hMSH6. Protein bands were quantified from ECL activity by Alpha Innotech FluorChem HD2. Each protein immunoblot experiment using unlabeled oligomers was performed a minimum of three times at different protein concentrations.

To determine if either kinase activator or inhibitor alters the DNA binding activity of endogenous hMutS α , proliferating HeLa MR cells were pre-incubated for 12 h with either 200 nM TPA or 200 nM UCN-01. Nuclear extracts were then incubated with radioactively labeled G:T oligomers and subjected to EMSA using nondenaturing polyacrylamide gels. Each experiment involving TPA or UCN-01 pre-incubation was performed at least three times.

2.8 Cell cycle synchronization and flow cytometry

Cells were synchronized at late G₁/early S by double thymidine block (DTB) [14]. When present, 2 μ M MNNG was added to the medium immediately after release from DTB. Distribution of cells in different phases of the cycle was monitored by staining nuclei with propidium iodide followed by analysis of DNA concentration in a Beckman/Coulter EPICS Elite flow cytometer [13]. The data were analyzed by Multicycle software (Phoenix Flow Systems) and reported as the percentage of cells in G₁, S, or G₂ phase. Cell cycle synchronization experiments by DTB have been performed numerous times in our laboratory and are highly reproducible[14,15].

2.9 Chromatin cross-linking and immunoprecipitation analyses

Chromatin was cross-linked by fixing cells within the culture plates, as described previously [14]. Purification of chromatin and immunoprecipitation with anti-hMSH2 antibody, SDS-PAGE and immunoblot analysis with anti-hMSH6 was also described previously [14].

3.0 RESULTS

Phosphorylation of hMSH6 occurs in whole cells incubated with [^{32}P]- H_3PO_4 and nuclear extracts incubated with [γ - ^{32}P]-ATP. Phosphorylation of hMSH2 was undetectable (Supplement Figure 1). Twenty-two distinct phosphorylated sites have been identified within hMSH6 by mass spectrometry studies of HeLa nuclear extracts [30–37]. Twenty of these sites are clustered into six groups of 2–5 amino acids within the first 348 residues of the unstructured N-terminal region of hMSH6 (Supplement Figure 2). We therefore asked if the cellular environment could affect hMSH6 phosphorylation and, in turn, if alterations in hMSH6 phosphorylation could affect hMutS α mismatch repair or activities that respond to alkylation-induced DNA damage. We hypothesized that mismatched (G:T), alkylated ($\text{O}^6\text{meG:T}$) oligomers, kinase activator (TPA), and kinase inhibitor (UCN-01) would affect hMSH6 phosphorylation and activities of hMutS α in different manners.

Using recombinant hMutS α (Supplement Figure 3), we found that a G:T mismatch stimulates recombinant hMSH6 phosphorylation in the nuclear environment. Specifically, in Hec59 (hMutS $\alpha^{-/-}$) cell lysates the amount [^{32}P]-labeled recombinant hMSH6 immunoprecipitated in the presence of the G:T mismatch was three times the levels of radioactively-labeled hMSH6 immunoprecipitated in the absence of oligomer or in the presence of the G:C oligomer (Supplement Figure 3D).

In HeLa MR cells TPA causes a significant increase in binding of hMutS α to G:T relative to that in untreated cells whereas UCN-01 decreases hMutS α G:T binding activity significantly below that of untreated cells (Figure 1A). Amount of hMSH6 and hMSH2 protein was not significantly different in nuclear extracts from control or treated HeLa cell populations indicating that neither TPA-provoked increase nor UCN-01-provoked decrease in mismatch binding activity was due to altered concentrations of hMutS α (Figure 1B). When average hMutS α binding activity was normalized to hMSH6 protein concentration, TPA increased hMutS α mismatch binding activity (ratio of 1.21) and UCN-01 decreased binding by over one-half (ratio of 0.48) (Figure 1C).

Is phosphorylation of endogenous hMSH6 altered by the presence of a mismatch or correlated to changes in kinase activity? Incubation of HeLa nuclear extracts with a G:T oligomer increased phosphorylation of endogenous hMSH6 significantly (Figure 2A). TPA further increased phosphorylation of hMSH6 significantly in the presence of both the G:C and G:T oligomer (Figure 2A). Treatment of HeLa cells with UCN-01 significantly decreased phosphorylation of endogenous hMSH6 by an average of 55% (Figure 2B), consistent with its effect on mismatch binding activity (Figure 1A). Addition of G:C or G:T oligomers did not have an effect on this result (results not shown).

The hMutS α heterodimer binds to mismatches and triggers activation of the MMR pathway. This heterodimer also binds to $\text{O}^6\text{meG:T}$ and triggers the DNA damage-signaling pathway [4]. We hypothesized that phosphorylation of hMSH6 is affected by the type of DNA lesion, and is involved in signaling either activation of MMR or the DNA damage-signaling pathway. To determine if endogenous kinase activity affects the amount of $\text{O}^6\text{meG:T}$ oligomer bound by endogenous hMutS α , we determined DNA binding activity using HeLa MR nuclear extracts purified from cells incubated with either TPA or UCN-01. The amount of $\text{O}^6\text{meG:T}$ shifted by nuclear extract is not significantly different in HeLa MR cells incubated with or without TPA (Figure 3A). However, treatment of cells with UCN-01 significantly decreased the amount of $\text{O}^6\text{meG:T}$ shifted (Figure 3A). TPA had no significant effect on the increased phosphorylation of hMSH6 in the presence of $\text{O}^6\text{meG:T}$ (Figure 3B). In contrast TPA increased phosphorylation of hMSH6 with G:C or G:T oligomer present (Figures 2A and 3B). These results indicate that TPA does not increase phosphorylation of

endogenous hMSH6 in the presence of O⁶meG:T nor does hMutS α have increased binding to alkylated lesions in the presence of TPA (Figure 3A). However, UCN-01 significantly decreased binding of hMutS α to this alkylated lesion (Figure 3A), consistent with the decreased electrophoretic mobility shift of the G:T oligomer (Figure 1A) and decreased phosphorylation of hMSH6 (Figure 2B) caused by UCN-01.

EMSA demonstrates that more G:T than O⁶meG:T radioactive oligomer is bound (shifted) in the presence of equal amounts of hMutS α (Figure 4A). These results are in agreement with others [44,45]. An EMSA-immunoblot measures the amount of hMSH6 protein bound to unlabeled oligomer (rather than the amount of shifted radioactive oligomer). In contrast to the results with labeled oligomers, we consistently observe significantly more hMSH6 bound to the shifted O⁶meG:T oligomer as compared to the shifted G:T (Figure 4B). Therefore, in the presence of equimolar concentrations of G:T and O⁶meG:T, our results agree with others in that hMutS α binds less O⁶meG:T oligomer than G:T oligomer (Figure 4A). In contrast, the amount of hMSH6 bound to O⁶meG:T from equal amounts of total nuclear protein is statistically higher ($P=0.04$) than to G:T (Figure 4B).

To determine if decreased phosphorylation of endogenous hMSH6 alters the amount of hMutS α bound to either the G:T or O⁶meG:T oligomer, we used HeLa MR nuclear extracts from the cells incubated with UCN-01 to perform additional EMSA-immunoblots. UCN-01 decreased the amount of HeLa hMSH6 protein bound to both types of oligomers, in the presence of equal hMSH6 protein concentrations in treated and untreated nuclear extracts (Figure 5A). Therefore, UCN-01 decreases the amount of hMutS α bound to both types of oligomers, in agreement with less DNA shifted (Figure 3A), and less phosphorylation of hMSH6 after UCN-01 treatment of cells (Figure 2B). To determine if UCN-01 inhibits binding of hMSH6 to O⁶meG within chromosomes, synchronized HeLa MR cells were pre-incubated with UCN-01 and exposed to MNNG 2 h later. Protein-bound chromosomal DNA was purified 5 h later (late S, early G₂ phase [14]). Similar to inhibition of hMSH6 binding to oligomers containing G:T or O⁶meG:T, UCN-01 decreases binding of hMSH6 to alkylation-treated chromosomes, as well as decreasing constitutive binding to untreated chromosomes (Figure 5B).

The UCN-01-induced decrease in DNA binding activity for both G:T and O⁶meG:T appeared similar to the effects of ATP in DNA binding activity experiments. DNA-bound hMutS α releases the mismatched oligomer in an “ATP-dependent” manner (Supplementary Figure 4A and references [46,47]). However UCN-01 does not cause the same decrease in hMSH6 as that caused by ATP. ATP causes release of hMSH6 and hMSH2, which migrate as intact hMutS α below the gel-shifted oligomer in the nondenaturing polyacrylamide gel (Supplementary Figure 4B). In UCN-01-treated HeLa MR cells, a much smaller amount of hMSH6 is initially bound to either G:T or O⁶meG:T, and unbound hMSH6 migrating below the DNA-bound protein is undetectable (Figure 5C). Therefore, a different mechanism, in addition to dephosphorylation of hMSH6, appears to be inhibiting the binding of hMutS α to both oligomers, rather than ATP-dependent release.

UCN-01 directly inhibits activation of Chk1 kinase that, in the presence of sufficient DNA damage, normally triggers a DNA damage response and cell cycle arrest [15,48,49]. We therefore assessed the effect of UCN-01 on activation of Chk1 kinase in the MNNG-induced DNA damage response pathway. After synchronization of HeLa MR cells by double thymidine block (DTB), we exposed the cells to 2 μ M MNNG and 12 h later either added UCN-01 or left the cells untreated. This concentration of MNNG results in phosphorylated Chk1 at 24 – 32 h after alkylation damage (Figure 6A and reference [29]). At 12 h – 24 h after additional UCN-01 treatment (24 h – 32 h after MNNG), the phosphorylation of chromatin-crosslinked Chk1 was not inhibited, in agreement with the literature that UCN-01

does not inhibit ATR activity [49]. Also, UCN-01 does not appear to inhibit MNNG-induced Chk1 kinase activity, as evidenced by persistence of phosphorylated Cdc25c in cytoplasmic extract. This is not consistent with *in vitro* experiments reported by others [50]. Several other kinases are known to phosphorylate Cdc25c at S216 as well, therefore inhibition of Chk1 cannot be ruled out by these results [51]. However UCN-01 does inhibit phosphorylation of Cdk1 at tyrosine 15 within cells exposed to MNNG, therefore allowing the cell to escape the G₂ arrest occurring after MNNG treatment alone (Figure 6A & 6B and reference [48]). The combined MNNG and UCN-01 treatment subsequently resulted in 100% cell death (Figure 6B at 48 h, and 0% colony survival, results not shown). Flow cytometric analysis of the cell cycle demonstrated that UCN-01 treatment alone did not alter cell cycle kinetics (Figure 6B) and colony survival was 100% (results not shown). Taken together, these results suggest that activation of Chk1 may not play a primary role in the inhibition of hMSH6 phosphorylation and decrease in activity caused by UCN-01. Further experiments will be required to fully elucidate kinase pathway activation/inhibition by UCN-01 after DNA alkylation damage. It is possible that inhibition of hMSH6 phosphorylation by UCN-01 may inhibit the activation of a different DNA damage-signaling pathway. This notion agrees with the fact that only one of the 22 identified phosphorylation sites on hMSH6 is an ATM/ATR recognition motif (Supplement Figure 2).

Several of the phosphorylation sites located in the N-terminus of hMSH6 contain CK2 recognition motifs. We therefore generated recombinant hMutS α with a mutant hMSH6 containing a cluster of four site-specific mutations located at S252A, S254A, S256A, and S261A, all of which have recently been identified by phospho-proteome studies as CK2 phosphorylation motifs (Supplementary Figure 2 and references [30–37]). The mutated heterodimer binds to both [³²P]-G:T and [³²P]-O⁶meG:T oligomers more poorly than the wildtype heterodimer (Figure 7A). Mutation of the four serines inhibits binding activity to [³²P]-O⁶meG:T less than to [³²P]-G:T. Similar to endogenous hMSH6 (Figure 4), recombinant wildtype hMutS α shifts approximately 2/3rd of the amount of [³²P]-O⁶meG:T compared to its shift of [³²P]-G:T, even at twice the recombinant protein concentration (20 nM for O⁶meG:T vs 10 nM for G:T). In addition, there is a decreased amount of phosphoserine within the recombinant mutant hMSH6 protein, as compared to an equal concentration of the recombinant WT hMSH6 (Figure 7B).

4.0 DISCUSSION

There remains much to learn about regulation of the activity of hMutS α in the cell. Technical difficulties have inhibited efforts to express exogenous hMSH6 in mammalian cells, therefore there are few reports describing phosphorylation of hMutS α and associated cellular activities. Increased phosphorylation, nuclear translocation, and mismatch and chromosomal binding activity is stimulated after cells are exposed to MNNG [13,14,39]. Increased G:T binding activity is associated with increased phosphorylation of hMutS α within nuclear extracts and, additionally, phosphorylation of recombinant hMutS α can be stimulated by PKC and CK2 *in vitro* [39]. MMR protein expression, repair activity and sensitivity to 6-thioguanine are increased by TPA [41]. Further, hMutS α phosphorylation by the PKC ζ pathway protects against ubiquitin-dependent proteasomal degradation within human cell lines [40]. As well, endogenous hMSH6 undergoes decreased phosphorylation, and MMR proteins are decreased in expression after cellular exposure to different kinase inhibitors having a wide range of activity [39,41].

We show here, by several different approaches, that hMSH6, but not hMSH2, phosphorylation occurs within both intact cells and nuclear extracts. Our results agree with data from several large studies that have collectively identified 22 distinct phosphorylated sites within the N-terminus of hMSH6 by phospho-proteome mass spectrometry. Eighteen

of these phosphorylation sites are clustered into six distinct clusters of amino acids within the first 348 residues of the unstructured N-terminal region of hMSH6 (Supplementary Figure 1 and references [30–37]. The majority of phosphorylation sites contain CDK, MAPK, PKC or CK2 recognition motifs [52]. Serine 348 is the only residue that has been reported as differentially phosphorylated (after gamma irradiation), and is also the only phosphorylation site that is an ATM/ATR recognition motif [37]. CK2 has the highest number of potential recognition motifs within the N-terminus of hMSH6 (8 sites; Supplement Figure 2). CK2 is constitutively active in human cells and has been called the master regulator of cellular function, with over 300 substrates identified to date [53,54]. We have created a recombinant mutant hMSH6 containing four S→A alterations in the fifth cluster of phosphorylation sites (S252–S261) within the N-terminus. This cluster contains only CK2 recognition motifs. This alteration caused decreased binding of both G:T and O⁶meG:T-containing DNA suggesting a role for CK2-catalyzed phosphorylation of these sites in post-translational control of hMutSα activity. Much work remains to be accomplished with eighteen phosphorylation sites yet to be investigated for their roles in differential signaling of hMutSα. All of the above kinases are potential effectors of hMutSα activity, and they could operate in a number of different kinase signaling pathways. Taken together, these observations suggest a complicated set of cellular signaling pathways that control both quantitative and qualitative hMSH6 phosphorylation patterns, in turn affecting hMutSα activity.

The N-terminal region is a proteolytically-sensitive unstructured domain that tethers hMSH6 to PCNA [55,56]. Upon deletion of the PCNA recognition motif (PIP box), deletion-mutant hMutSα still exhibit *in vitro* MMR activity, indicating that PCNA may be used to tether hMutSα to the DNA during replication [56]. The N-terminal portion of the yeast Msh6 confers sensitivity to MNNG; however this purified N-terminal region does not specifically recognize O⁶meG [57]. Human MSH6 that lacks the N-terminal 340 amino acids has been co-crystallized with hMSH2 as the hMutSα heterodimer. This truncated heterodimer was also crystallized bound to several heteroduplex DNA structures (G:T, O⁶meG:T, G:U) without notable structural differences [58]. Each of these three DNA structures elicits activation of different cellular pathways (MMR, DNA damage-signaling, and somatic immunoglobulin hypermutation, respectively). These diverse functions activated by DNA-bound hMutSα require different signaling pathways. Phosphorylation/dephosphorylation of binding proteins is a well known mechanism of cellular signaling. Differential phosphorylation at multiple sites within a protein is now recognized as an elegant and essential mechanism for modulation of activity, structure, and interaction of cellular substrates within a large variety of metabolic pathways [59–63].

In this report, we have investigated how phosphorylation of hMSH6 is regulated by binding of hMutSα to G:T and O⁶meG:T lesions that activate MMR or DNA damage-signaling, respectively. The degree of phosphorylation of hMSH6 and DNA binding activity of hMutSα is different in the presence of G:T than in the presence of O⁶meG:T. A G:T mismatch elicits increased phosphorylation of hMSH6, whereas O⁶meG:T does not. More G:T than O⁶meG:T radioactive oligomer is shifted after incubation with equal amounts of nuclear extract, in agreement with other reports [44,45]. However, comparison of hMSH6 protein bound to each shifted oligomer indicates more protein bound to O⁶meG:T than to the G:T oligomer. These oligomers are identical, except for either G or O⁶meG opposite T, and relatively short at 69 bp, therefore the observed effects may be minimized in our system. These results may indicate that both the degree of hMSH6 phosphorylation and differential binding activity of hMutSα may be required to trigger specific cellular pathway responses.

We also probed the relationship between changes in phosphorylation of hMSH6 and changes in the binding of hMutSα to G:T and O⁶meG:T. TPA is a phorbol ester that

activates all PKC isoenzymes. The majority of PKC isoenzymes are located at the plasma membrane or within the cytosol [64]. PKC phosphorylates hMutS α and stimulates MMR functions [39]. The atypical PKC ζ isoenzyme phosphorylates hMutS α and inhibits degradation of this heterodimeric protein [40]. Incubation of HeLa MR cells with TPA increases hMSH6 phosphorylation in the presence of an oligomer containing a G:T mismatch, concomitant with a small increase in binding of hMutS α to G:T. However, TPA does not stimulate binding activity to O⁶meG:T, nor is hMSH6 phosphorylated to an increased extent by TPA in the presence of O⁶meG:T. Based on these results, one or more of the PKC isoenzymes may operate within a cellular signaling network that increases phosphorylation of hMSH6 and activates hMutS α in the presence of a DNA mismatch but not in the presence of DNA alkylation damage. These results point toward differential kinase regulation of the mismatch repair system that is dependent on the type of lesion bound by hMutS α .

Conversely, the staurosporine analogue UCN-01 inhibits hMSH6 phosphorylation and hMutS α binding to both G:T and O⁶meG:T. This effect is not due to decreased amounts of hMSH6 in nuclear extracts of these cells. UCN-01 is a broad range kinase inhibitor that has been reported, by *in vitro* methods, to be a potent inhibitor of a number of different cell cycle-dependent kinases, including Chk1, at the concentration used in this study [49,65]. Treatment with 200 nM UCN-01 alone is nontoxic to HeLa MR cells. However when UCN-01 is used in combination with exposure to a low level of MNNG, the MNNG-induced second cell-cycle arrest does not occur and the cell population dies after undergoing mitosis. Phosphorylation of Chk1 is not inhibited by UCN-01 after MNNG exposure, demonstrating as previously reported that UCN-01 does not inhibit upstream ATR activity [50,66]. Phosphorylation of Cdk1 at tyrosine 15 is inhibited by UCN-01 after MNNG treatment, therefore allowing the cell to escape G₂ arrest after alkylation damage, despite that Cdc25c is still phosphorylated at S216, rendering this cell cycle kinase inactive [51]. Kinases other than activated Chk1 phosphorylate Cdc25c at S216, such as Chk2, C-TAK1, and Plk3, therefore inhibition of Chk1 activity cannot be ruled out in these studies [49]. Chk2 and C-TAK1 have been reported to be sensitive to this level of UCN-01 within cells, it is not known if Plk3 activity is affected by UCN-01 within the cell [48,49]. Overall, this suggests that another DNA damage-signaling pathway, other than ATR-Chk1, may be induced within the cell by activation of hMutS α in the presence of alkylation damage. Further investigations within this area are needed. Several clinical trials with UCN-01 in combination with chemotherapeutic DNA damaging agents are currently ongoing. These trials are based on the assumption that UCN-01 inhibits ATR-Chk1 check point signaling pathway, therefore driving the damaged tumor cell to continue cycling and subsequent cell death.

5.0 CONCLUSION

Based on our current results and all other evidence to date, we propose a mechanistic model for differential activation of hMutS α to trigger either the DNA mismatch repair pathway or the DNA damage-signaling pathway (Figure 8). According to this model, hMutS α recognizes both undamaged mismatched bases (G:T) and specifically damaged mismatches (O⁶meG:T). If a mismatch occurs during replication, hMutS α binds and triggers the mismatch repair pathway and the mismatch is rapidly and correctly repaired. This pathway requires less bound hMutS α containing more highly phosphorylated hMSH6. Cells exposed to monofunctional alkylators contain O⁶methylG that is frequently replicated incorrectly with insertion of thymidine opposite the damaged guanine, thereby creating O⁶meG:T. This mismatched lesion is bound by more hMutS α molecules, and does not trigger hMSH6 phosphorylation above constitutive levels. These two effects initiate the MMR-induced DNA damage-signaling pathway. Differentially phosphorylated hMSH6 within the hMutS α population may bind to different lesions or differential phosphorylation of hMSH6 may take

place after binding to specific lesions. We hypothesize that the cell controls signaling for MMR versus DNA damage response by hMSH6 phosphorylation (higher for G:T *versus* O⁶meG:T) and binding stoichiometry of hMutSα to different DNA constructs (lower for G:T *versus* O⁶meG:T), providing an elegant mechanism for the cell to differentiate between mismatch repair and the DNA damage-signaling pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

List of Abbreviations used

MMR	DNA mismatch repair
MNNG	<i>N</i> -methyl- <i>N'</i> -nitro- <i>N</i> -nitrosoguanidine
UCN-01	7-hydroxystaurosporine
TPA	12-O-tetradecanoylphorbol 13-acetate
MGMT	Methylguanine Methyltransferase
FBS	fetal bovine serum
DTB	double thymidine block

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A. hMutS α EMSA (G:T) w/wo TPA, UCN01

B. hMutS α nuclear protein concentration w/wo TPA, UCN01

C. Ratio of average hMutS α activity versus hMutS α concentration w/wo TPA, UCN01

Untreated	TPA	UCN01
1.0	1.21	0.48

Figure 1. Pretreatment with TPA increases hMutS α binding to G:T, pretreatment with UCN-01 decreases hMutS α binding to G:T

A. HeLa MR cells were incubated with 200 nM TPA or 200 nM UCN-01 for 12 h, and nuclear extracts were prepared. Electrophoretic mobility shifts (EMSA) were performed after incubation of 4 μ g of each of the HeLa MR nuclear extracts with 32 P-G:T oligomer and either 100X unlabeled G:C or 100X G:T oligomer. Non-denaturing polyacrylamide gels were dried and subjected to autoradiography, with a typical autoradiograph shown. The bar graph depicts relative ratios of each condition from three experiments subjected to phosphorimager quantification of hMutS α -shifted radioactive DNA bands. Untreated control (–) values were all normalized to “1”. Asterick (*) indicate statistically significant difference from untreated control (Student t-test, $P < 0.05$). **B.** 30 μ g of each nuclear extract (HeLa MR NE) was subjected to SDS-PAGE, transferred to PVDF and immunoblotted

using antibodies against hMSH6 and hMSH2. Chemiluminescence activity of the hMSH6 band in each lane was captured using Alpha Innotech Fluorochem HD2 and is represented as a bar graph below. Numbers at the bottom of each bar are normalized ratios to the untreated control (set to equal 1). There were no significant differences between control or treated samples. **C.** Ratios of normalized electrophoretic mobility shift radioactive phosphorimager activity [A] to total hMSH6 protein as measured by chemiluminescence activity [B]. Control (untreated) ratio is equal to 1.

Figure 2. Pretreatment with TPA and/or G:T mismatch increases hMSH6 phosphorylation, pretreatment with UCN-01 decreases hMSH6 phosphorylation

A. HeLa MR cells were untreated or treated with 200 nM TPA for 12 h, and nuclear extracts were incubated with or without 200 ng nonradioactive G:C or G:T oligomer and 50 μ Ci of [γ - 32 P]-ATP for 30 min. hMSH6 was immunoprecipitated, subjected to SDS PAGE, dried and subjected to autoradiography, with a typical autoradiograph shown. The MSH6 immunoblot indicates comparable amounts of hMSH6 protein in each preparation. The bar graph depicts relative ratios of each condition (untreated controls normalized to a value of 1) from three experiments subjected to phosphorimager quantification of hMSH6 radioactive bands. Asterick (*) indicate statistically significant difference from untreated control (Student t-test, $P < 0.05$). **B.** HeLa MR cells were incubated with or without 200 nM UCN-01 for 12 h and nuclear extracts were subjected to *in vitro* phosphorylation as above. hMSH6 was immunoprecipitated, subjected to SDS-PAGE and subsequent autoradiography of the dried gel, with a typical autoradiograph shown. The MSH6 immunoblot indicates comparable amounts of hMSH6 protein in each preparation. The bar graph depicts relative ratios of each condition from three experiments subjected to phosphorimager quantification of hMSH6 radioactive bands. Untreated control (-) values were all normalized to "1". Asterick (*) indicate statistically significant difference from untreated control (Student t-test, $P < 0.05$).

Figure 3. Pretreatment with TPA does not alter hMutS α binding to O⁶meG:T, and does not increase hMSH6 phosphorylation in the presence of O⁶meG:T. Pretreatment with UCN-01 decreases hMutS α binding to O⁶meG:T

A. HeLa MR cells were incubated with 200 nM TPA or 200 nM UCN-01 for 12 h and nuclear extracts were prepared. Electrophoretic mobility shifts (EMSA) were performed after incubation of 4 μ g of nuclear extract with [³²P]-O⁶meG:T oligomers (plus 100X cold G:C oligomer). The non-denaturing polyacrylamide gels were dried and subjected to autoradiography, with a typical autoradiograph shown. The bar graph depicts relative ratios of each condition from three experiments subjected to phosphorimager quantification of hMutS α -shifted radioactive bands. Untreated control (–) values were all normalized to “1”. Asterick (*) indicate statistically significant difference from untreated control (Student t-test, P<0.05). **B.** HeLa MR nuclear extracts (200 μ g) were incubated with 200 nM TPA, 200 ng nonradioactive G:C or O⁶meG:T oligomer and [γ ³²P]-ATP for 30 min. hMSH6 was

immunoprecipitated, subjected to SDS-PAGE, dried and subjected to autoradiography. Arrows indicate differences in density of radioactive bands after incubation of nuclear extracts and TPA with G:C *versus* O⁶meG:T oligomers in a typical autoradiograph. The MSH6 immunoblot below the radioactive bands indicate comparable amounts of hMSH6 protein in each preparation. The bar graph depicts relative ratios of each condition from three experiments subjected to phosphorimager quantification of radioactively-labeled hMSH6. Untreated control (–) values were all normalized to “1”. Asterick (*) indicate statistically significant difference from untreated control (Student t-test, P<0.05).

A. hMutS α EMSA of [32 P]-G:T and [32 P]-O 6 meG:T

B. EMSA-immunoblot of hMSH6 bound to G:T and O 6 meG:T

Figure 4. More G:T than O 6 meG:T oligomers are shifted by equimolar hMutS α . More hMSH6 is bound to equimolar O 6 meG:T than to G:T

A. Electrophoretic mobility shifts (EMSA) were performed after incubations of equal concentrations (2 ng) and cpm of [32 P]-labeled G:T or O 6 meG:T oligomers (plus 100X cold G:C oligomer) with 3 μ g of HeLa MR nuclear extract. The non-denaturing polyacrylamide gel was dried and subjected to autoradiography. The bar graph represents phosphorimager quantification of each protein-shifted radioactive DNA band. **B.** EMSAs were performed using 8, 12, or 16 μ g of HeLa MR nuclear extract (HeLa NE), and 5 ng of unlabeled G:T or O 6 meG:T oligomer. Proteins from nondenaturing polyacrylamide gels were transferred to PVDF and blotted with antibody to hMSH6 and chemiluminescence activity was captured

using Alpha Innotech Fluorochem HD2. The immunoblot on the right is a typical immunoblot from these experiments. The bar graph on the left depicts relative ratios of G:T and O6meG:T bound hMSH6 from three experiments subjected to chemiluminescence quantification. G:T-bound hMSH6 values were all normalized to “1”. Asterick (*) indicate statistically significant difference (Student t-test, $P < 0.05$).

A. EMSA-immunoblot of hMSH6 bound to G:T and O⁶meG:T w/wo UCN01

B. HeLa MR ChIP w/wo MNNG or UCN01

C. EMSA-immunoblot of hMSH6 bound to G:T and O⁶meG:T w/wo UCN01, ATP

Figure 5. Pretreatment with UCN-01 decreases binding of hMSH6 to both G:T and O⁶meG:T oligomers, and to alkylation damaged chromosomal DNA. This effect is not the same as the ATP-dependent release of hMutSa from DNA

A. HeLa cells were incubated with or without 200 nM UCN-01 for 12 h and nuclear extracts prepared. Electrophoretic mobility shifts (EMSA) were performed after incubating 10 µg of HeLa MR nuclear extract with 5 ng of unlabeled G:T or O⁶meG:T oligomer. Proteins from the nondenaturing polyacrylamide gels were transferred to PVDF and blotted with antibody to hMSH6. Equal portions of each electrophoretic mobility shift reaction were also subjected to SDS-PAGE and immunoblotted for hMSH6 and hMSH2 (lower immunoblot). **B.** HeLa MR cells were synchronized by DTB, released and exposed immediately to 200 nM UCN-01, and 2 µM MNNG at 2 h. Cells were harvested for cross-linked chromatin at 7 h. Equal amounts of cross-linked chromatin were subjected to SDS-PAGE, transferred to PVDF membrane and immunoblotted with antibody to hMSH6. **C.** HeLa MR cells were incubated with or without 200 nM UCN-01 for 12 h and nuclear extracts prepared. EMSAs were performed after incubating 12 µg HeLa MR nuclear extract with 5 ng of unlabeled G:T or O⁶meG:T oligomer, with or without 2.5 mM ATP. Proteins from nondenaturing polyacrylamide gels were transferred to PVDF and immunoblotted for hMSH6.

A. Chk1, Cdc25c and Cdk1 phosphorylation w/wo UCN01, MNNG

B. UCN01 & MNNG effects on cell cycle

Figure 6. After exposure to MNNG, UCN-01 increases activation of Cdk1 (dephosphorylation) without decreasing pChk1 or pCdc25c levels. This effect of UCN-01 releases cells from MNNG-induced G₂ arrest into cell death

HeLa MR cells were synchronized by DTB and released into media with or without 2 μ M MNNG. At 12 h, cells were further incubated with or without 200 nM UCN-01. Cells were harvested at indicated times for cross-linked chromatin, cytoplasmic extract, or flow cytometry. **A.** Equal amounts of cross-linked chromatin were subjected to SDS-PAGE, transferred to PVDF membrane and immunoblotted with antibody to phosphorylated Chk1 (S345 and S317). Equal amounts of HeLa MR cytoplasmic extract were subjected to SDS-PAGE, transferred to PVDF membrane and immunoblotted with antibodies to phosphorylated Cdc25c (S216) and GAPDH (loading control), or phosphorylated Cdk1 (T15) and total Cdk1. **B.** HeLa MR cells were harvested at indicated time points to monitor cell cycle progression by flow cytometric analysis of DNA content.

Figure 7. Recombinant hMutS α containing 4 mutated phosphoserine sites within hMSH6 (4M) have decreased binding to both G:T and O⁶meG:T, and decreased phosphoserine content
A. Electrophoretic mobility shifts (EMSA) were performed after incubating equal cpm of 32 P-G:T or 32 P-O⁶meG:T oligomer (plus 50X unlabeled G:C oligomer) with equal concentration of wild type (WT) and 4M (hMSH6) recombinant hMutS α (10 nM each for G:T and 20 nM each for O⁶meG:T). The non-denaturing polyacrylamide gels were dried and subjected to autoradiography. The bar graph below represents phosphorimager quantification of each protein-shifted radioactive DNA band. Note that both recombinant hMutS α proteins were doubled in concentration for the O⁶meG:T electrophoretic mobility shift experiments. **B.** Equal quantities of recombinant WT hMutS α and mutant 4M hMutS α were subjected to SDS-PAGE, transferred to PVDF membrane and immunoblotted with antibody against phosphoserine. Chemiluminescent activity of each band was quantitated using Alpha Innotech Fluorochem HD2 and presented in the bar graph below the phosphoserine immunoblot.

Figure 8. Model of pathway differentiation between MMR and DNA damage-signaling
 This model depicts that the mismatch repair pathway is differentiated from the DNA damage-signaling pathway by both the amount of hMutS α bound to the mismatched or damaged DNA and the degree of phosphorylation of hMSH6 within the DNA-bound hMutS α . A simple mismatch, such as G:T, requires less mismatch-bound hMutS α to signal the MMR pathway, but hMSH6 contains more phosphorylated N-terminal amino acids than hMutS α bound to a complicated lesion, such as O⁶meG:T, that signals the DNA damage pathway.