BIOCHEMICAL CHARACTERIZATION OF HUMAN MISMATCH RECOGNITION PROTEINS MUTSα AND MUTSβ

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ABSTRACT OF DISSERTATION

Lei Tian

The Graduate School
University of Kentucky
2010
BIOCHEMICAL CHARACTERIZATION OF HUMAN MISMATCH RECOGNITION PROTEINS MUTα AND MUTβ

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

Lei Tian
Lexington, Kentucky

Director: Dr. Guo-min Li, Professor of Toxicology
Lexington, Kentucky
2010

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ABSTRACT OF DISSERTATION

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The integrity of an organism's genome depends on the fidelity of DNA replication and the efficiency of DNA repair. The DNA mismatch repair (MMR) system, which is highly conserved from prokaryotes to eukaryotes, plays an important role in maintaining genome stability by correcting base-base mismatches and insertion/deletion (ID) mispairs generated during DNA replication and other DNA transactions. Mismatch recognition is a critical step in MMR. Two mismatch recognition proteins, MutSα (MSH2-MSH6 heterodimer) and MutSβ (MSH2-MSH3 heterodimer), have been identified in eukaryotic cells. MutSα and MutSβ have partially overlapping functions, with MutSα recognizing primarily base-base mismatches and 1-2 nt ID mispairs and MutSβ recognizing 2-16 nt ID heteroduplexes. The goal of this dissertation research was to understand the mechanism underlying differential mismatch recognition by human MutSα and MutSβ and to characterize the unique functions of human MutSα and MutSβ in MMR.

In this study, recombinant human MutSα and MutSβ were purified. Binding of the proteins to a T-G mispair and a 2-nt ID mispair was analyzed by gel-mobility assay; ATP/ADP binding was characterized using a UV cross-linking assay; ATPase activity was measured using an ATPase assay; MutSα and MutSβ’s mismatch repair activity was evaluated using a reconstituted in vitro MMR assay.

Our studies revealed that the preferential processing of base-base and ID heteroduplexes by MutSα and MutSβ, respectively, is determined by the significant differences in the ATPase and ADP binding activities of MutSα and MutSβ, and the high ratio of MutSα:MutSβ in human cells. Our studies also demonstrated that MutSβ interacts similarly with a (CAG)_n hairpin and a mismatch, and that excess MutSβ does not inhibit (CAG)_n hairpin repair in vitro.

These studies provide insight into the determinants of the differential DNA repair specificity of MutSα and MutSβ, the mechanism of mismatch repair initiation, and the mechanism of (CAG)_n hairpin processing and repair, which plays a role in the etiology and progression of several human neurological diseases.
Key Words: Genome instability, mismatch repair, MutSα, MutSβ, Trinucleotide repeat,
BIOCHEMICAL CHARACTERIZATION OF HUMAN MISMATCH RECOGNITION PROTEINS MUTSα AND MUTSβ

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DISSEYATION

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By

Lei Tian

Lexington, Kentucky

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2010

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This dissertation is dedicated to my parents, Changqi Tian & Jinying Ma
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CHAPTER ONE

INTRODUCTION

Genome instability and mismatch repair

The integrity of an organism’s genome depends on the fidelity of DNA replication and the efficiency of DNA repair. Usually mutations are rare events, occurring spontaneously at a frequency of 1 per $10^9$-$10^{10}$ base pairs per cell division (Drake JW. 1991; Drake JW. 1999). However, there are at least three ways in which unpaired and mispaired bases arise in DNA: (1) misincorporation of nucleotides and strand slippage during DNA replication, which generate mismatched base pairs and insertion/deletion (ID) of nucleotides, respectively (Modrich, P 1989; Modrich, P 1997); (2) physical or chemical damage to DNA and its precursors, such as deamination of 5-methyl-cytosine resulting in thymine (Fishel, R. 1998); and (3) genetic recombination during which two different parental DNA sequences form heteroduplexes (Holliday, R.A. 1964). There are two types of mispairs: base-base mismatches and insertion-deletions. If unrepaired, these mismatches have the potential to generate mutations in the genome of somatic or germline cells, which can alter cellular phenotype and ultimately lead to dysfunction and disease.

Among DNA repair systems, mismatch repair (MMR) is a conserved DNA repair pathway that corrects base-base and ID mismatches. MMR enhances the fidelity of DNA replication 100–1000 fold (Kolodner R 1996; Schofield MJ. et al., 2003; and Ravi R. et al., 2006). Defects in the MMR greatly increase the spontaneous mutation rate and result in a strong mutator phenotype.

A hallmark of MMR-deficient cells is to display microsatellite instability (MSI). Microsatellites are simple 1-6 bp DNA sequences found throughout the genome in tandem arrays of up to 100 repeats. They are inherently hypermutable because of their propensity for strand slippage during DNA replication, which gives rise to small ID mispairs (or loops) that are normally repaired by MMR. In cells with mutations in DNA repair genes such as MMR genes, some of these sequences accumulate errors and become longer or shorter, which are referred to as MSI. MSI has been widely used as a diagnostic marker for loss of MMR function in certain cancers, including colorectal cancers such as Hereditary nonpolyposis colon cancer (HNPCC) (or Lynch syndrome) (Lynch & de la Chapelle, 1999; Umar et al., 2004).
HNPCC is a cancer predisposition syndrome that accounts for about 5% of all colorectal cancers. HNPCC is characterized by early development and increased risk of cancer of the colon, endometrium, stomach, small intestine, and ovary. HNPCC patients usually carry one defective allele and one wild-type allele of a MMR gene (i.e., MSH2, MLH1, MSH6, or PMS2) (Lynch & de la Chapelle, 1999; Peltomaki et al., 1997). Subsequent mutation of the functional allele in some cells inactivates MMR, which strongly increases the risk of secondary mutations leading to cellular transformation and tumor development. Besides HNPCC, approximately 15% of sporadic colorectal cancers also exhibit high-level MSI (Bellizzi AM et al., 2009). The phenotypes of these patients have considerable overlap with HNPCC. In these cases MLH1 genes are usually silenced at transcription level because of promoter hypermethylation (Kane MF et al., 1997; Herman JG et al., 1998).

Addition to repair of mismatched DNA, MMR also plays an important role in DNA damage-induced cell cycle arrest and apoptosis (Li, GM 2008, Stojic L. 2004). MMR defects render mammalian cells resistant to the cytotoxic effects of several DNA damaging agents, including methylating agent N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). MMR proteins also participate in a number of other cellular processes, including homologous recombination and somatic hypermutation in immunoglobulin genes (Hsieh P and Yamane K. 2008).

In summary, the MMR system involves a wide variety of DNA transactions, and it is very important for maintaining genome stability.

Mechanisms of mismatch repair

MMR proteins and mechanism are highly conserved in prokaryotes and eukaryotes (Table1.1). MMR reactions in Escherichia coli (E. coli) and human cells have been reconstituted with purified proteins (Lahue, 1989; Zhang, 2005; Constantin, 2005).

Mismatch repair in E. coli

The E. coli MMR has been reconstituted in vitro using MutH, MutL, MutS, UvrD (helicase II), DNA polymerase III holoenzyme, single-stranded DNA binding protein (SSB), one of the single-stranded DNA exonucleases (Exo I, Exo VII, ExoX or RecJ) and DNA ligase (Lu et al., 1983; Su et al., 1986; Welsh et al., 1987; Grilley et al., 1989; Lahue et al., 1989; Cooper et al., 1993). The E. coli MMR targets seven
Table 1.1 Proteins involved in MMR pathway

<table>
<thead>
<tr>
<th>E. coli</th>
<th>Human</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>(MutS)2</td>
<td>hMutSα (MSH2-MSH6)</td>
<td>DNA mismatch/damage recognition</td>
</tr>
<tr>
<td></td>
<td>hMutSβ (MSH2-MSH3)</td>
<td></td>
</tr>
<tr>
<td>(MutL)2</td>
<td>hMutLα (MLH1-PMS2)</td>
<td>Molecular matchmaker; endonuclease; termination of mismatch-provoked excision</td>
</tr>
<tr>
<td></td>
<td>hMutLβ(MLH1-PMS1)</td>
<td>Function unknown</td>
</tr>
<tr>
<td></td>
<td>hMutLγ(MLH1-MLH3)</td>
<td>Suppresses ID mutations; participates in meiosis</td>
</tr>
<tr>
<td>MutH</td>
<td>?</td>
<td>Nicks nascent unmethylated strand at hemimethylated GATC sites</td>
</tr>
<tr>
<td>UvrD</td>
<td>?</td>
<td>DNA helicase</td>
</tr>
<tr>
<td>Exol,ExoVII,ExoX,RecJ</td>
<td>Exol</td>
<td>DNA excision; mismatch excision</td>
</tr>
<tr>
<td>Pol III holoenzyme</td>
<td>Polδ, PCNA</td>
<td>DNA re-synthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Initiation of MMR; DNA re-synthesis</td>
</tr>
<tr>
<td>SSB</td>
<td>RPA</td>
<td>ssDNA binding/protection; stimulating mismatch excision; termination of DNA excision</td>
</tr>
<tr>
<td></td>
<td>HMGB1</td>
<td>Mismatch-provoked excision</td>
</tr>
<tr>
<td></td>
<td>RFC</td>
<td>PCNA loading; 3’nick-directed repair; activation of MutLα endonuclease</td>
</tr>
<tr>
<td>DNA ligase</td>
<td>DNA ligase I</td>
<td>Nick ligation</td>
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of eight possible base-base mismatches except C:C mispairs, and IDs of up to four bases. MMR is initiated by MutS recognizing and binding mispaired nucleotides (Su and Modrich 1986). It has been suggested that MutS is recruited to mismatches by interaction with β-clamp (López de Saro F J. and O'Donnell, M. 2001). The β-clamp is a specific DNA clamp and a subunit of the DNA polymerase III holoenzyme. It has two beta subunits which are assembled around the DNA, and serves as a processivity-promoting factor in DNA replication. The clamp protein binds DNA polymerase and prevents the polymerase from dissociating from the template DNA strand. After MutS binding to a mismatch site, MutL interacts with MutS at the mismatch (Grilley et al., 1989), stimulating an ATP-dependent translocation of the MutS–MutL complex (Allen et al., 1997) towards a hemi-methylated dGATC site bound by MutH (Welsh et al., 1987). In E. coli, DNA is fully methylated at the N⁶ position of adenine in dGATC sequences. During replication, the daughter strand is transiently unmethylated. MutH is a member of the type II family of restriction endonucleases (Ban C et al., 1998). Upon its recruitment and activation by MutS and MutL in the presence of ATP, MutH specifically incises the unmethylated daughter strand at hemimethylated dGATC sites located within about 1 kb of the mismatch. This methyl-directed nicking by MutH directs the MMR in E. coli to the newly synthesized DNA strand that contains the error. And this strand-specific nick, which can be either 3’ or 5’ to the mismatch, works as the entry point for DNA helicase II, single-strand DNA binding protein as well as one of four single-stranded exonucleases (Rec J, Exo I, Exo VII, ExoX), which degrade the newly replicated strand and generate a single-stranded DNA (ssDNA) gap. This excision removes the error and allows highly accurate DNA polymerase III to correctly resynthesize the strand. DNA ligase seals the nick to complete MMR.

Three key features of this important pathway are: 1) the repair is dependent on MutS, MutL, and MutH; 2) the repair is always targeted to the newly synthesized DNA strand (strand specificity); 3) the repair is bi-directional, i.e., the mismatch can be removed from 5’→3’ or 3’→5’.

**Mismatch repair in human cells**

The mechanism of MMR is highly conserved. Both prokaryotic and eukaryotic MMR are targeted to the newly synthesized strand and dependent on a nick which can
be either 5’ or 3’ to the mismatch site. However, there are notable differences between *E. coli* and human MMR pathways.

**Proteins involved in human MMR**

Eukaryotic MutS and MutL homologs are heterodimers composed of two related, but distinct protein subunits. In human cells, five MutS homologs (MSH2, MSH3, MSH6, MSH4, MSH5) (Bocker T. et al., 1999; Drummond, J. T., et al., 1995; Genschel, J. et al., 1998; Kneitz, B., et al., 2000) and four MutL Homologs (MLH1, MLH3, PMS2, PMS1) (Li, G.M. et al., 1995; Porter, G., et al., 1996; Wang, T.F. et al., 2002) have been identified. MSH2 interacts with MSH6 and MSH3 to form MutSα and MutSβ, respectively; MSH4 forms a heterodimer with MSH5; and MLH1 forms heterodimers with PMS2 (MutLα), PMS1 (MutLβ), or MLH3 (MutLγ). Previous studies have shown that only MutSα, MutSβ and MutLα are involved in strand-specific MMR. MSH4-MSH5 may play a role in meiotic DNA recombination (de Vries SS et al., 1999; Kneitz, B et al., 2000; Snowden, T et al., 2004); MutLγ plays a role in meiosis, and no specific biological role has been identified for MutLβ.

Eukaryotic homolog of *E. coli* MutH has not yet been identified. Thus, although human MMR is thought to be nick-directed, a MutH-like protein may not be required to generate a strand-specific nick during MMR. This is consistent with the fact that GATC sites are not hemi-methylated in eukaryotic DNA. It has been proposed that lagging strand nicks or discontinuities in between Okazaki fragments act as strand discrimination signals in eukaryotic cells (Lopez, de Saro FJ et al., 2001).

In eukaryotic MMR, excision is performed by ExoI. Several other proteins such as replication proteins A (RPA), proliferating cell nuclear antigen (PCNA), replication factor C (RFC), and high mobility group box 1 protein (HMGB1) are also required for MMR. DNA resynthesis is catalyzed by an aphidicolin-sensitive polymerase, DNA polymerase δ (Table 1.1). PCNA is a multifunctional protein that plays a role in several DNA repair processes. In MMR, PCNA is thought to participate in both the initiation and resynthesis steps. PCNA interacts with MutSα/MutSβ and MutLα, and may help recruit MMR proteins to mismatches. PCNA may also stimulate the mismatch binding specificity of MSH2-MSH6, and participate in excision and DNA repair synthesis by virtue of its association with DNA polymerase δ. HMGB1 is a non-histone chromatin protein that facilitates protein-protein interactions and bends
DNA molecules (Bustin M., 1999). Studies from Li’s lab showed that human HMGB1 is involved in the initial damage recognition steps of heteroduplex repair. It is able to interact with the MMR proteins MSH2 and MLH1. Recombinant human HMGB1 also works together with RPA to mediate EXO1-catalyzed DNA excision, and that HMGB1 can replace RPA’s activity in a reconstituted human MMR system (Yuan F, et al., 2004; Zhang Y, et al., 2005).

The initiation of mismatch repair in human cells

Mismatch recognition and initiation in human cells are conducted by MutS and MutL homologs. Unlike *E. coli* MutS, human MutSα preferentially recognizes all eight base-base mismatches and ID mispairs of 1 or 2 nucleotides, while MutSβ preferentially recognizes larger ID mispairs up to 16 nucleotides. Our understanding of mismatch recognition by human MutS is incomplete. Crystal structures of human MutSα binding to a variety of mismatched DNAs suggest a universal recognition mechanism that is conserved in MutS homologs (Figure 1.1 B). Mismatch recognition by MutSα maps to the MSH6 subunit. The N-terminal domain of MSH6 includes a conserved Phe-X-Glu motif. The carboxyl moiety of Glu434 forms a hydrogen bond with the mismatched base, which is sandwiched between Phe432 and Met459. These interactions, along with nonspecific contacts between the protein and sugar-phosphate backbone of flanking DNA regions, widen the DNA minor groove in the vicinity of the mispair and kink the DNA towards a narrowed major groove at the mismatch (Warren, JJ et al., 2007; Obmolova G et al., 2000). This Phe-X-Glu binding model is conserved between *E. coli* MutS and human MutSα. However, MSH3 and MSH6 share little sequence homology in their DNA binding domains. MutSβ seems to have no Phe-X-Glu motif, which suggests that MutSα and MutSβ may recognize mispairs in different ways.

Besides DNA binding domain, MutS homologs contain ATPase domains at their C-terminals. Both MutSα and MutSβ belong to the ATP-binding cassette (ABC) transporters superfamily. ABC transporters superfamily is one of the largest and most ancient families. It contains transmembrane proteins that utilize the energy of ATP hydrolysis to carry out translocation of various substrates, including metabolic products, lipids and sterols, and drugs, across extra- and intracellular membranes. This family also includes proteins involved in non-transport-related processes such as
Figure 1.1 Structure of human MutSa (From Warren JJ et al., 2007). A. Ribbon diagram of the structure of a MutSa/ADP/G•T mispair complex. Blue, MSH6; red, MSH2; green ribbon, DNA; yellow, ADP; and green spheres, Mg$^{2+}$ ions. Positions of the ABC ATPase domains and the two channels in MutSa are indicated. B. Interactions between a G-T mispair and an adjacent base pair with MSH6 domain 1.
translation of RNA and DNA repair. Proteins are classified as ABC transporters based on the sequence and organization of their ATP-binding cassette (ABC) domain(s). The typical ABC domain consists of two domains, the catalytic core domain and the helical domain. The catalytic core domain consists of the catalytic Walker A motif (GXXGXGKS/T where X is any amino acid) or P-loop and Walker B motif (ΦΦΦΦD, of which Φ is a hydrophobic residue). The helical domain consists of three or four helices and the ABC signature motif. ATP usually induces the dimer formation of the two ABC domains. In MutSα, two molecules of ATP are positioned at the interface of the dimer, sandwiched between the Walker A motif of one subunit and the ABC signature motif of the other (Figure 1.1A). The roles of ATPases of MutSα/MutSβ in mismatch repair are not fully understood. It has been shown that MutS family proteins can simultaneously bind ATP and ADP (Bjornson, K. P et al. 2000; Blackwell, L. J et al. 1998) and undergo ADP → ATP exchange (Wilson, T. et al. 1999; Acharya, S et al. 2003; Gradia, S et al. 1997; Gradia, S. et al. 1999; Mendillo, M. L. et al. 2005) to induce MutS conformational changes, signaling downstream repair events. Increasing evidence also showed that ATP binding modulates DNA substrate binding. Challenging DNA bounded MutSα with ATP leads to dissociation of MutSα from the mispair and movement along the helix contour (Drummond JT, et al., 1995; Alani E, et al., 1997; Gradia S et al., 1999). These studies suggested that mismatched DNA recognition and adenosine nucleotide binding/hydrolysis are closely linked in MutS homologs.

In the mismatch recognition step, MutLα can associate with either MutSα or MutSβ at the mismatch, and participates in repair of base-base or ID mispairs. However, MutLα is a weak ATPase and binds nonspecifically to DNA. Although many studies have been performed to explore the structure and function of the MutS-MutL-DNA ternary complex (Kunkel TA et al., 2005), the MutS-MutL interaction is not yet understood and remains an important area of study. In a reconstituted human MMR system, MutLα regulates termination of mismatch-provoked excision (Zhang, Y et al., 2005). Other studies show that MutLα possesses a PCNA/replication factor C (RFC)-dependent endonuclease activity that plays a critical role in 3’ nick-directed MMR involving EXO1 (Kadyrov, FA, et al., 2006).

The mismatch-provoked excision step in human MMR initiates at a preexisting nick or gap, proceeds along the shortest path to the mismatch, and terminates
approximately 150 nucleotides beyond the mismatch. A long-term mystery in the field is the mechanism by which MutSα/MutSβ triggers EXO1-catalyzed excision at a site distant from the mismatch. Based on the observations about MutS protein’s mismatch recognition and ATPase activity, several alternative models have been proposed for mismatch repair initiation. The “trans” or “stationary” model proposes that interactions among MMR proteins induce DNA bending or looping that brings the two distant sites together, while MutS (or MSH heterodimers) remains bound at the mismatch (Guarne A.et al. 2004; Junop, M. S et al. 2001). In this model, the ATPase activity of MutS (or MSH heterodimers) is required for mismatch recognition and MutS (or MSH heterodimers) interaction with proteins that operate downstream like MutL and MutH (or MutLα) (Wang and Hays, 2003, 2004). Another type of model called “cis” or “moving” models suggest that MutS-MutL (or MutSα/β-MutLα) complexes load at a mismatch site and then travel along the DNA helix until they encounter a signal, i.e. a strand break possibly in the context of other auxiliary proteins such as PCNA and/or RFC in eukaryotic cells. There are two moving models. In one so called “translocation” model (Allen D.J. et al. 1997), ATP reduces the mismatch-binding affinity of MutS or the MSH heterodimers, and the energy of ATP hydrolysis is used to fuel a unidirectional translocation of MutS away from the mismatch site. DNA is threaded through the protein complex until the latter reaches a strand discrimination signal in either orientation, a process that forms a DNA loop (Allen D.J. et al. 1997). In an alternate model which called “switch model”, an ADP-bound MutS (or the MSH heterodimer) binds to mismatched DNA and triggers a conformational change that allows an ADP to ATP exchange, resulting in the transformation of MutS from a mismatch-binding protein to a sliding clamp that diffuses away from the mismatch in search of other repair protein. In this model, it is the binding of ATP, not ATP hydrolysis that signals downstream events including formation of ternary complex with MutL (or MLH heterodimers) and sliding of the ternary complex from the mismatch to the strand break (Fishel. R.1998; Gradia S, et al. 1997; Jiang J. et al. 2005; Mendillo ML et al. 2005). To date, additional studies are still needed to address the molecular mechanism of MMR initiation.

Mismatch recognition initiated excision in human cells

In 5′-directed MMR (reconstituted in vitro), MutSα/MutSβ activates 5′ → 3′ excision by EXO1, a 5′→3′ exonuclease that interacts with MSH2. EXO1-catalyzed
excision is terminated by concerted interactions among EXO1, MutLα and RPA upon mismatch removal (Zhang, Y et al., 2005).

Since no human MutH homolog has been identified and nascent DNA is not transiently hemi-methylated in human cells, the nature of the strand discrimination signal in human cells has been a matter of investigation and debate. Furthermore, in vitro reconstituted 3′-nick-directed MMR requires EXO1, which is a 5′→3′ exonuclease instead of a 3′→5′ exonuclease, adding to questions about the mechanism of human MMR. Recently, an endonuclease activity in the eukaryotic PMS2 subunit of MutLα has been identified (Kadyrov, FA et al., 2006, Kadyrov, FA et al., 2007). Since MLH1 can interact with PCNA, which loads onto the 3′-end of Okazaki fragments or the 3′-end of the leading strand with the help of RFC, the following model for EXO1-catalyzed 3′-nick-directed repair has been proposed (Hsieh, P et al., 2008): (1) mismatch activated MutLα endonuclease makes an incision 5′ to the mismatch in a strand-specific manner (directed to the daughter strand by the presence of PCNA and RFC); (2) EXO1 performs 5′→3′ excision from the MutLα-incision site through and beyond the site of the mismatch. This model proposes that the interaction between MutLα and PCNA as well as lagging DNA strand associated nicks act as the strand discrimination signal(s) for human MMR.

**Research Objectives**

MMR proteins such as MutSα and MutSβ have been suggested to involve in cancer development and cancer therapy. Defect in MSH2, MSH6 or MSH3 genes would render cells’ defective MMR and reduced replication fidelity, which greatly enhance the spontaneous mutation rates and carcinogenesis. MutSα or MutLα might also help to signal cell damage under the treatment of certain chemotherapeutic drugs such as temozolomide, procarbazine, or cisplatin (Li, GM, 2008). That MMR-deficient cells are resistant to these drugs has significant impacts on cancer treatments. It is therefore very important to explore the characterization of MMR proteins and the mechanism of MMR machinery.

In this study, the biochemical characterization of MutSα and MutSβ were evaluated. Previous structural models of bacterial and human MutS proteins have revealed that MutS homologs have conserved DNA binding and ATPase domains that are structurally coupled. It is unknown how this conformation coupling contributes to
mismatch recognition and initiation. This study explored the underlying mechanism by which MutSα and MutSβ perform the specific mismatch recognition. Especially, the DNA binding and ATPase activities of purified MutSα and MutSβ were evaluated. Additionally, the potential role of MutSβ in trinucleotide repeat repair was tested, which contributed to the efforts for revealing the mechanism of trinucleotide repeat instability that related to Huntington disease as well as other neurodegenerative and neuromuscular diseases.
CHAPTER TWO

MATERIALS AND METHODS

Chemicals and regents
Amersham: ECL Detection Reagent, Sephacry S-300
Perkin Elmer: \( [\gamma^{\text{32}}P]\)-ATP, \( [\alpha^{\text{32}}P]\)-ATP, \( [\text{35}S]\)-ATP-\(\gamma\)-S
FisherBiotech: 1- Butanol, Iso-propanol, KH\(_2\)PO\(_4\) (Potassium Phosphate Monobasic), K\(_2\)HPO\(_4\) (Potassium Phosphate Dibasic), NaH\(_2\)PO\(_4\) (Sodium Phosphate Monobasic), Na\(_2\)HPO\(_4\) (Sodium Phosphate Dibasic), SDS (Sodium Dodecyl Dulfate), Potassium Acetate, Tween-20, P.E.G-8000, NaOH (Sodium Hydroxide), KOH (Potassium Hydroxide)
New England Biolab: restriction enzymes
Santa Cruze: MSH2, MSH3 polyclonal antibodies
Bethyl: MSH6 polyclonal antibody
Roche: ATP (Adenosine Triphosphate), dNTP (deoxy Nucleotides Triphosphate), DTT (Dithiothreitol), Nonidet P-40
Sigma: Acrylamide, Aphidicolin, Boric Acid, MgCl\(_2\) (Magnesium Chloride), NaCl (Sodium Chloride), KCl (Potassium Chloride), N,N’-Methhylene-bis-Acrylamide, USB: Ammonium Sulfate, Ammonium Persulfate, Agarose, CsCl (Cesium Chloride), ExoV, EDTA, Ethidium Bromide, Glycine, HEPES, Urea, T4-PNK, Tris, Phenol, etc.

Agarose gel electrophoresis
Agarose gel electrophoresis was run in TAE (Tris/Acetate/EDTA) buffer containing 40 mM Tris-Acetate and 2 mM EDTA. DNA samples were prepared for analysis by the addition of 10x agarose gel loading buffer containing 40% (w/v) sucrose, 0.5% (v/v) xylene cyanol, 0.05% (v/v) bromophenol blue, 20 mM EDTA and 0.2% sodium dodecyl sulfate (SDS). After running, gels were stained for 20 mins using ethidium bromide (EtBr) solution (0.5 μg/ml) and then rinsed for 20 mins in ddH\(_2\)O. Gels were visualized on an ultraviolet transilluminator. The images were captured using the Kodak Image Station 2000R system.

SDS-PAGE gel electrophoresis
Slab gels containing an 8% acrylamide separating gel (0.375 M Tris-HCl pH 8.8, 0.1% SDS) and 4% acrylamide stacking gel (0.125 M Tris-HCl pH 6.8, 0.1% SDS)
were prepared from a 30% acrylamide stock solution (acrylamide: bis-acrylamide = 37.5:1). The samples were prepared by adding 6x loading buffer (125 mM Tris-HCl pH 6.8, 2% SDS, 20% glycerol, 0.2% bromophenol blue) and heated at 95°C for 3 mins. After loading, the gel was run at 150 volts in gel running buffer which contained 2.5 mM Tris, 0.2 M Glycine and 0.1% SDS.

**Preparation of radioactive DNA substrates**

DNA oligonucleotides are synthesized by Integrated DNA Technologies (IDT). 31 mer heteroduplex containing a mismatch was generated by annealing of two complementary 31-mer synthetic oligonucleotides

(A: 5'-GCTAGCAAGCTTTCGATTCTAGAAATTGC-3';
B: 5'-GCGAATTTCCTAGAATCTGAAAGCTTGCTAGC-3'), which generates a G·T mismatch at position 14. 50 mer heteroduplex containing GT insertion was generated by annealing of two complementary 50-mer synthetic oligonucleotides (C: 5’-GCAGATCTGGCCTGGTACTCCTCCTGGGCGGCGGTAAACAGTACGTAGTC-3’;
D: 5’-GACTACGTACTGTTAACCGCCGAGAGGTACCAGGCGGAGCAGATCTG-3’).

One strand of each heteroduplex was radiolabeled with $[\gamma-^{32}P]$-ATP (3000 Ci/mmol, PerkinElmer) by T4 PNK (USB). After purification through Sephadex G25 column (Roche), radiolabeled oligonucleotides were annealed to complementary strands by putting the radiolabeled strand and the complement strand (1:1 ratio) in the buffer containing TE and 100 mM NaCl. The samples were heated to 75°C and cooled down gradually to room temperature.

**Gel-shift/EMSA Analysis**

Gel-Shift assays were performed in 20-µl reactions containing 10 mM HEPES-KOH (pH 7.5), 110 mM KCl, 1 mM EDTA, 1 mM DTT. DNA substrate oligonucleotide and MutS protein were added at the indicated concentration, and non-specific unlabeled oligonucleotide DNA was included at a concentration of 10–30 pmol. The reactions were incubated on ice for 20 min, specific antibody was added for another 20 min (if needed for supershift), followed by the addition of 5 µl 50% (w/v) of sucrose. Samples were loaded on and separated by electrophoresis through a 6% non-denaturing polyacrylamide gel in buffer containing 6.7 mM Tris–acetate (pH 7.5) and 1 mM EDTA. The buffer was recirculated during electrophoresis. The gel was dried and analyzed by autoradiography and/or phosphorimager. The affinity of
protein-DNA binding (Kd) was calculated by Hill plot analysis as described previously (Yong, Y et al., 1995).

**Purification of MutSα/MutSβ proteins from overexpressed High Five cells**

Baculovirus stocks for the human MSH2, MSH6, and MSH3 genes were generous gifts of Josef Jiricny (University of Zurich). MSH2NA, MSH2ND and MSH2GA baculovirus were made from pFastbac1-MSH2N653A, pFastbac1-MSH2N653D and pFastbac1-MSH2G674A respectively following the manual of Invitrogen (Bac-to-Bac Expression System).

Insect cells (High Five) were purchased from Invitrogen. The cells were cultured in TNM-FH medium (US biological) containing 10% FBS (Hyclone).

Insect cells were inoculated (at least seven 175 flasks of mid-log cells) with MSH2- and MSH6- viruses and cultured for about 48-72 hrs. Cells were collected by centrifugation at 1,000 rpm for 5 min, and washed once by PBS. After that the cells were resuspended with minimum volume of Buffer A (25 mM HEPES, pH 8.0, 1 mM EDTA, 2 mM DTT and 1x proteinase inhibitor including 100 mM PMSF, 191.5 mM Benzamidine, 0.05 g/l Pepstatin A, 0.05 g/l Leupeptin, 0.05 g/l Antipain, and 200 μM Bestatin), broken by glass homogenizer, and followed by sonication for 15-20 seconds. 1/10 volume of glycerol was added then and mixed well followed by adding 1/10 volume of saturated (NH₄)SO₄ solution. The mixture was stand on ice for 30 min. Then the cells were cleared by centrifugation at 20,000 rpm in 4°C for 60-90 min. The protein supernatant was added with 1/10 volume of 5 M NaCl and loaded to a Q sepharose column (GE healthcare) that was equilibrated with Buffer B (25 mM HEPES, pH 8.0, 1 mM EDTA, 2 mM dithiothreitol (DTT), 1 M NaCl and 1x proteinase inhibitor). The flowthrough was collected and dialyzed in Buffer A to conductance equivalent to 250 mM KCl. After that, the protein sample was load to a 5 ml Heparin column (GE healthcare) which was equilibrated with 200 mM NaCl in Buffer A. The protein was eluted with 200 mM-750 mM NaCl gradient and the protein fractions were verified by SDS-PAGE protein gel. The fractions that contain MutSα were combined and diluted with Buffer A to reach conductance equivalent to 150 mM KCl. After that the protein pool was loaded onto a 1ml Mono Q column (GE healthcare) which is balanced with 15% Buffer B (150 mM NaCl in Buffer A). The protein was eluted with 15%-50% Buffer B in 30 ml at 1 ml/min for collection. The
target protein would be eluted at around 30% Buffer B followed by being concentrated to 300 μl using centrifugal filter units (at 6,500 rpm). The sample was then loaded to a Superdex™ 200 column (GE healthcare) which is equilibrated by 15% Buffer B. The protein was eluted with 25 ml of 15% of Buffer B, and collected according to SDS-PAGE verification. After measuring protein concentration by the Bradford assay, MutSα was stored in the presence of 1 mg/ml BSA and 10% sucrose. The aliquots of the protein were flash frozen and stored at -80°C.

MutSβ purification is similar to MutSα except that insect cells were inoculated with MSH2 and MSH3 baculovirus; and using Mono S column (GE healthcare) instead of Mono Q. The salt gradient used for Mono S column is as same as Mono Q column.

UV crosslinking experiments

The UV crosslinking experiments were performed essentially as described by Mazur et al., (Mazur DJ, et al., 2006), with some minor modifications. 10 Ci/mmol [γ-32P]-ATP, 800 Ci/mmol [α-32P]-ATP and 65 Ci/mmol [35S]-ATP-γ-S were purchased from PerkinElmer. Briefly, reactions were performed in binding buffer containing 50 mM Tris buffer (pH 8.0), 110 mM NaCl, 2 mM DTT, 100 mg/ml BSA, 0.5 mM EDTA, 5% glycerol and with or without 5 mM MgCl2. Where specified, 1 μM mispaired or paired DNA were added 10 min prior to addition of nucleotide. Proteins were added at the indicated concentration, mixed with [γ or α-32P]-ATP or [35S]-ATP-γ-S, and incubated on ice for 10 min. Samples were then subjected to 5 min of crosslinking (UVP Crosslinkers) followed immediately by fractionation by 8% SDS-PAGE gel. Gels were dried and radiolabeled bands were detected by PhosphorImager (Molecular Dynamics) and quantified by Kodak Image Station 2000. All experiments were performed at least 3 times.

ATPase analyses

ATPase activity of MutSα and MutSβ was assayed in 20-μl reactions containing 50 mM Tris-HCl (pH 8.0), 110 mM NaCl, 0.5 mM EDTA, 5 mM MgCl2, [γ-32 P]-ATP, and the indicated amount of proteins and DNA substrates. After incubation at 37 °C for 10 min, the reactions were terminated by adding 6x loading dye and fractionated through a 20% denaturing polyacrylamide gel. 32P-containing species were detected by PhosphorImager and quantified by Kodak Image Station 2000.
Nuclear extract preparation

Hela-S3 cells were cultured in RPMI-1640 medium containing 10% Fetal Bovine Serum (FBS). N6 cells were maintained in DMEM medium containing 10% FBS.

Extracts of nuclear protein from mammalian cells were prepared as previously described (Holmes et al., 1990). All steps were performed on ice or at 4°C using ice-cold solutions and pre-cooled bottles and centrifuge rotors. All solutions contained 0.1% (v/v) PMSF, 1 µg/ml leupeptin and 1 µg/ml pepstatin A. For cells grown in suspension, cultures of 5-6L were grown to a density of 1.0 x 10^6 cells/ml and then harvested. The cell suspension was chilled to ≤ 10°C with ice-water slurry, and then collected by centrifugation for 10 min at 3000 g. The supernatant was discarded and the cell pellet was suspended in buffer A (20 ml per L of culture) that contained 20 mM HEPES•KOH (pH 7.5), 5 mM KCl, 0.5 mM MgCl₂, 0.5 mM DTT, and 0.2 M sucrose. Cells were grown as a monolayer in roller bottles (10-40 bottles/extract). 20 ml buffer A was added per bottle and the cells were dislodged using a cell scraper (Bellco Glass, Vineland). The slurry of cells was transferred to a centrifuge bottle and the roller bottle rinsed with an additional 15 ml buffer A. Whether from suspension or monolayer cultures, steps after the cells were suspended in buffer A were the same.

Cells were then harvested by centrifugation for 5 min at 3300 g. The cell pellet was suspended in buffer B (2.78 ml per gram of cell pellet) that contained 20 mM HEPES•KOH (pH 7.5), 5 mM KCl, 0.5 mM MgCl₂, and 0.5 mM DTT and then incubated on ice for 10 min. Cells were lysed by Dounce homogenization using a type B pestle (Bellco Glass, Vineland). (Ausubel et al., 1987) Progress of cell lysis was monitored with a light microscope observing the decrease in intact cells and increase in free nuclei. The number of strokes required to lyse approximately 80% of cells (with ≤ 10% of nuclei lysed) was 3-15 and varied depending on cell line. Nuclei were recovered by centrifugation of cell lysates for 5 min at 2000 g. The pellet was suspended in buffer C (1.39 ml per gram of starting cells) that contained 50 mM HEPES•KOH (pH 7.5), 10% (w/v) sucrose, and 0.5 mM DTT. The volume of the nuclei solution was measured and 0.031 vol of 5 M NaCl was added. This solution was then rotated on a LabQuake shaker (Barnsted-Thermolyne, Dubuque) for 60 min and centrifuged for 20 min at 14,500 g. The pellet was discarded and the volume of the supernatant was measured. Ammonium sulfate (0.42 g/ml) was slowly added
(spanning ~ 20 min) while the solution was stirred on ice. The solution was stirred for an additional 20 min and then centrifuged for 20 min at 15,800 g.

The supernatant was decanted and the pellet was slowly suspended in a small volume (~20-30 µl per gram of starting cells) of buffer D containing 25 mM HEPES•KOH (pH 7.6), 50 mM KCl, 0.1 mM EDTA and 2 mM DTT. The protein slurry was dialyzed in buffer D until the conductivity of the protein solution was measured using a 1:400 dilution of extract in ddH$_2$O until a value of ~50 µS/cm was obtained. The dialyzed extract was cleared of precipitated protein by centrifugation for 10 min at 19,600 g at 4°C. Aliquots (30-40 µl) of the supernatant were frozen in liquid nitrogen and stored at -80°C. Protein concentrations were measured by the Bradford method (Ausubel et al., 1987) using duplicated reactions from two different dilutions.

**Preparation of DNA substrate**

**Large scale isolation of phage double-stranded DNA (dsDNA)**

Unless otherwise indicated all enzymes were purchased from New England Biolabs (Rockville). The bacteriophages used were derivatives of the MR series described previously (Su et al., 1988). flMR1, flMR3, flMR23 and flMR24 were kind gifts of Dr. Paul Modrich, Duke University.

3 L of 2X-YT medium was pre-warmed to 37°C and inoculated with 50 ml O/N cultured XL-Blue cells which were selected by tetracycline (2µg/ml). The culture was shaken at 37°C at 220 rpm until the OD595 reached 0.3. Total cells at this OD were approximately 1.5x10$^{12}$. A 10 fold excess of phage virions (1.5x10$^{13}$) was added and the culture was shaken for additional 7 hours at 37°C at 220 rpm. To harvest the DNA, the cultures were chilled on ice for 20 min followed by centrifugation for 30 min at 4500 g. The supernatant was kept for later use. The cell pellet was suspended in 60 ml of ice-cold Buffer A containing 25 mM Tris- HCl (pH 8.0), 10 mM EDTA, 0.9% glucose (w/v) and 5 mg/ml lysozyme. The cell suspension was incubated at RT for 10 min and then on ice for 10 min. 120 ml of freshly prepared Buffer B that contained 0.2 N NaOH and 1% SDS was added while stirring gently in a single direction with a 10 ml plastic pipette. The solution was then incubated on ice for 10 min. Buffer C was prepared freshly by mixing together 60 ml of 5 M potassium acetate, 11.5 ml glacial (17.4 M) acetic acid and 28.5 ml ddH$_2$O. 90 ml of Buffer C was added to the lysed
cell mixture while stirring as described in the previous step. After 10 min incubation on ice, the solution was centrifuged for 30 min at 13,000 g at 4°C.

The supernatant was filtered through 4 layers of cheesecloth. The volume was measured and 0.6 vol of Iso-propanol was then added to precipitate DNA at room temperature (RT) for 30 min. DNA was precipitated by centrifugation for 30 min at 16000 g at 4°C. The pellet was washed with 70% ice-cold ethanol, and dried in air for 15-30 min. The DNA was suspended in 20 ml TE solution (10 mM Tris-HCl pH 8.0, 1 mM EDTA), and the solution was weighed. CsCl (1.05 g/g solution) and 10 mg/ml EtBr (50 µl/g solution) was added and supercoiled DNA was isolated by CsCl/EtBr equilibrium centrifugation at 45,000 rpm at 25°C for 18 hr using Beckman NVT65 rotor (Ausubel et al., 1987). The band of supercoiled dsDNA was removed from the centrifuge tube using a syringe with an 18 gauge needle. EtBr was removed from DNA by 4-6 times of water-saturated n-Butanol extraction. The aqueous solution containing the supercoiled DNA was then dialyzed by TE buffer (pH 8.0). The concentration and purity of DNA was determined by measuring absorption at 260 and 280 nm.

Large scale isolation of phage single-stranded DNA (ssDNA)

Phage particles were precipitated from the culture supernatant by adding NaCl (36 g/l) and PEG-8000 (50 g/l) into the culture supernatant. The solution was stirred at RT for 45 min, and centrifuged for 30 min at 4,500 rpm at 4°C. The pellet was suspended in 23 ml 10 mM Tris-HCl (pH 8.0), incubated for 2 hours at 37°C at 220 rpm, and centrifuged for 10 min at 14,500 g at 4°C. The pellet was discarded and the weight of the supernatant was measured. Phage particles were concentrated by CsCl (0.4342 g/g) equilibrium centrifugation as described above. The band of phage particles was removed from the centrifuge tube using a syringe with an 18 gauge needle, and dialyzed using TE buffer (pH 8.0). ssDNA was extracted from phage particles using TE (pH 7.6)-balanced phenol (55°C, 4 min), and ethyl-ether (2 times). Centrifugation of the extraction was performed at 25°C for 5 min at 12,000 g. After the ethyl-ether phase was evaporated, the solution was dialyzed using TE buffer (pH 8.0). The concentration and purity of DNA were measured by ultraviolet absorption at 260 and 280 nm.
Preparation of circular mismatch substrate

The substrate was prepared by annealing linear dsDNA with circular single-stranded DNA containing a one or two base(s) difference to yield a circular dsDNA heteroduplex. The product contained a mismatch at 5’ to the mismatch. The 5’-nicked base-base (G-T) mismatch substrate was constructed by using the dsDNA and ssDNA purified from flMR1 and flMR3. The 5’-nicked two nucleotides insertion/deletion (AC insertion/deletion) mismatch substrate was constructed by using the dsDNA and ssDNA purified from flMR23 and flMR24 DNA and an appropriate restriction enzyme to linearize circular dsDNA (Figure 2.1 A) are listed in Table 2.1.

The molar ratio of linear dsDNA and ssDNA is 1:5 during annealing (Figure 2.1 B). dsDNA was denatured in a 30 ml solution containing 50 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA, and 0.3 N NaOH (at room temperature for 5 min). The mixture was neutralized by adding 3.0 ml of 2.9 N acetic acid, and adjusted with 1.35 ml of 3.0 M KCl and 3.7 ml 1.0 M Na-Pi (pH 7.4 at 100 mM). The C strand of linear dsDNA was annealed to circular ssDNA (V strand) by incubating the mixture at 65°C for 30 min, and gradually cooling to 37°C in a large volume (1-2L) water bath (around 5 hrs). After being incubated at 37°C for 30 min, the solution was stored on ice. The solution at this point contained nicked circular heteroduplex DNA (the desired species), excess circular ssDNA, re-annealed linear homoduplex, and linear ssDNA of the displaced V strand (Figure 2.1 C). The efficiency of annealing was determined by analysis of samples using 0.8% agarose gel electrophoresis. Linear dsDNA and ssDNA migrate at distinct positions in the gel, and both type of DNA migrate faster than nicked circular dsDNA. Therefore, the appearance of a third, slower migration band in the “post-annealing” sample indicated that heteroduplex substrates were produced.

Nicked circular dsDNA was separated from ssDNA and linear dsDNA through multiple steps of chromatography. First (Figure 2.1 D), Hydroxyapatite resin (Biorad, 1-1.2 g/mg of total DNA) was equilibrated in 30 mM Na-Pi (pH 6.9) by gentle swirling in 20-30 ml of buffer and then incubated for 10-15 min at RT. The supernatant was decanted along with the “fine” particles that did not settle. This process was repeated 3-4 times. A column with a diameter of 2.5 cm was poured and washed with 2 volumes of 30 mM Na-Pi (pH 6.9) at a flow rate of 1-1.3 volume/hour. The annealed substrate was loaded onto the column slowly. The resin bed was then washed with 6 volumes of 30 mM Na-Pi (pH 6.9), 160 mM Na-Pi (pH 6.9), and 420
Table 2.1 DNA used in mismatch substrate preparation

<table>
<thead>
<tr>
<th>Substrate</th>
<th>ssDNA</th>
<th>dsDNA</th>
<th>Enzyme cutting dsDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’G/T nick</td>
<td>flMR1</td>
<td>flMR3</td>
<td>Sau96I</td>
</tr>
<tr>
<td>5’ ID nick</td>
<td>flMR23</td>
<td>flMR24</td>
<td>Sau96I</td>
</tr>
</tbody>
</table>
Figure 2.1 Flow diagram of substrate construction and purification  

A. Circular dsDNA (black) is linearized with Sau96I.  
B. Linearized dsDNA is then denatured and reannealed in the presence of excess circular ssDNA (green) from derivative phage.  
C. After annealing, 4 types of product are present: nicked circular heteroduplex, circular ssDNA, linear homoduplex and linear ssDNA.  
D. Hydroxyapatite is used to separate the majority ssDNA from dsDNA.  
E. *E.coli* ExoV digests linear DNA to short (1-5nt) fragments which are separated from heteroduplex DNA by Sepharose S300 size exclusion chromatography.
mM Na-Pi (pH 6.9) respectively, and 1.0 ml fractions were collected with a fraction collector (Pharmacia). ssDNA was eluted from the column by 160 mM Na-Pi and dsDNA was eluted by 420 mM Na-Pi. 3 μl of each fraction was mixed with 7 μl of EtBr (1 μg/ml) on plastic wrap and viewed on a UV transilluminator to visualize the peak of DNA (EtBr spot test). Fractions from the 420 mM Na-Pi (pH 6.9) elution that contained the highest concentrations of dsDNA were pooled and concentrated 3-4 fold by n-butanol and dialyzed in TE (pH 7.6) at 4°C. The concentration of dsDNA was measured by UV absorbance at 260 nm (pre-ExoV).

Linear homoduplex dsDNA was removed from the substrate preparation using *E.coli* ExonucleaseV (ExoV) (Figure 2.1 E). Digestion was performed in 66.7 mM Glycine, 5 mM MgCl₂, 8.3 mM β-ME, and 0.5 mM ATP. ExoV was added to 0.2 U/μg total DNA. The solution was incubated at 37°C for 60 min, and was then placed on ice (post-ExoV). The aliquots of pre-ExoV and post-ExoV samples were run on a 1% agarose gel to evaluate the efficiency of the digestion. A disappearing, fast migrating linear band indicated the removal of linear dsDNA from the preparation. The reaction solution was then extracted once with phenol and concentrated to 0.5 ml by n-butanol extraction.

Free nucleotides from the ExoV digestion were separated from the circular DNA substrates (6.4 kb) by Sephacryl S300 (Pharmacia) column chromatography. The beads were equilibrated in TES buffer containing 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 0.3 mM NaCl and poured into a glass column (45 cm × 1.2 cm). The resin bed was washed using 3 volumes of TES. The concentrated ExoV digested DNA (0.5 ml) was loaded onto the column. After the entire DNA entered the resin bed, TES buffer was added to the top of resin bed. The flow rate through the S300 column was 12 ml/hr. Once DNA was loaded onto the column, 1.0 ml fractions were collected with a fraction collector (Pharmacia). Groups of 10 fractions were tested as they became available by the EtBr spot test described above. The substrate would typically be present around the 12th fraction. The purity of the substrate in the fractions was checked by using 1% agarose gel electrophoresis. If only a single band was evident in the gel (which corresponded to nicked circular dsDNA), the fractions were pooled and concentrated 8 fold by n-butanol extraction. After being dialyzed in TE (pH 7.6), the concentration of the substrate was measured by UV absorbance at 260nm. The S300
column purification provided a 5’ or 3’ nicked substrate that was free of both ssDNA (either circular or linear) and linear homoduplex dsDNA.

**DNA MMR assay with circular substrate**

The MMR assay was performed in a 20 μl reaction mixture containing 30 fmol of heteroduplex DNA (base-base or insertion/deletion mismatch substrate), 70 μg of nuclear extract proteins (Hela-S3 cell nuclear extract or NALM-6 cell nuclear extract plus extra MutS protein), 10 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 100 mM KCl, 1.5 mM ATP, 1 mM Glutathione, 50 μg/ml BSA and 0.1 mM dNTPs. After incubation at 37°C for 15 min, 30 μl proteinase K solution (2.5 mM Tris-HCl pH 7.5, 5 mM CaCl₂, 12.5% glycerol, 5 mg/ml proteinase K, 6.7% SDS, 0.25 M EDTA) was added to the reaction and incubate at 37°C for another 20 min. Then the DNA samples were recovered by two times phenol extraction and followed by ethanol precipitation. The DNA was dried by speed vacuum and dissolved in ddH₂O. After that, the restriction enzyme digestion was performed (see Table 2.2 and Figure 2.2). The products were analyzed by 1% agarose gel. The two faster migrating bands (at 3.1 kb and 3.3 kb) are corresponded to the repair products.
Table 2.2 Restriction Enzymes for DNA mismatch repair assay

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' G/T nick</td>
<td>HindIII + ClaI</td>
<td>3.1kb, 3.3 kb</td>
</tr>
<tr>
<td>5' ID nick</td>
<td>XcmI + ClaI</td>
<td>3.1kb, 3.3 kb</td>
</tr>
</tbody>
</table>
Figure 2.2 Principle of in vitro mismatch repair assay. Circular double strand DNA substrates were constructed to contain a G-T mismatch (upper panel) or AC insertion/deletion (bottom panel) and a strand break at 5’ of the mismatch site. The mismatch is located within the overlapping recognition sites of two restriction enzymes so that repair of the mismatch can be scored with these enzymes. Incubate this DNA with Hela-S3 cell nuclear extract which contains complete mismatch repair machinery will trigger the repair process on this circular DNA substrate in vitro and result in a closed circular homoduplex DNA. Since human MMR is nick directed, the repair product has recovered HindIII or XcmI site. To score the mismatch repair, HindIII / XcmI and ClaI double digestion would be performed. The repaired DNA would be cut to two smaller fragments.
CHAPTER THREE*

DISTINCT NUCLEOTIDE BINDING/HYDROLYSIS PROPERTIES AND
MOLAR RATIO OF MUTSα AND MUTSβ DETERMINE THEIR
DIFFERENTIAL MISMATCH BINDING ACTIVITIES

*This research was originally published in J Biol Chem. Tian L, Gu L, Li GM. Distinct nucleotide binding/hydrolysis properties and molar ratio of MutSalpha and MutSbeta determine their differential mismatch binding activities. J Biol Chem. (2009) 284(17): 11557-62. © the American Society for Biochemistry and Molecular Biology

Introduction

Mismatch recognition is a critical step in the MMR pathway. In E. coli, recognition of both base-base and ID mismatches is conducted by the MutS protein. However, at least two mismatch recognition proteins, MutSα (the MSH2-MSH6 heterodimer) and MutSβ (the MSH2-MSH3 heterodimer), have been identified in eukaryotic cells and each of them is a heterodimer. Both genetic and biochemical studies suggest that MutSα and MutSβ have different lesion specificities but also partially overlapping functions, with MutSα targeting base-base mismatches and 1-2-nucleotide (nt) ID mispairs and MutSβ targeting $\geq 2$-nt but $\leq 16$-nt ID heteroduplexes (Genschel, J. et al., 1998; McCulloch, SD et al., 2003; Palombo, F et al., 1996; Wilson, T et al., 1999). A recent genetic study in yeast suggests that MutSβ may also play some role in the repair of base-base mismatches (Harrington, J.M. et al., 2007). Interestingly, cells make 10-fold more MutSα than MutSβ, and overexpression of MSH3 results in a strong mutator phenotype (Drummond, J. T et al., 1997; Marra, G. et al., 1998), presumably because the excess MSH3 saturates the pool of MSH2, essentially depleting MutSα in cells. It is unclear how MutSα and MutSβ, at a 10:1 ratio, partition in cells to specifically process their favored substrates.

A series of structural and functional studies have been done on prokaryotic MutS homodimer and eukaryotic MutSα heterodimer to explore the basis for mismatch recognition. It has been shown that DNA binding is asymmetric for both the MutS and the MutSα proteins, with one subunit in direct contact with a mispaired base and
the other making nonspecific contacts with the phosphodiester backbone (Lamers, M.H. et al., 2000; Obmolova G et al., 2000). Both proteins contain a conserved Phe-X-Glu motif in which the phenylalanine residue stacks onto the mispaired DNA base (Lamers, M.H. et al., 2000; Obmolova G et al., 2000; Alani, E. et al., 2003; Warren JJ et al., 2007) and is essential for efficient repair (Iyer, R.R et al., 2006; Kunkel, T.A. et al., 2005; Schofield, M.J.et al., 2003; Yamamoto, A et al., 2000). For MutSβ (MSH2-MSH3), although it shares a common MSH2 subunit with MutSα, MSH3 and MSH6 have little sequence homology in their DNA binding domains. At the analogous position of Phe-X-Glu in MSH6, human MSH3 has a lysine instead of phenylalanine, and lysine or arginine instead of glutamate (Iyer, R.R et al., 2006; Kunkel, T.A. et al., 2005; Schofield, M.J.et al., 2003; Lamers, M.H. et al., 2000; Obmolova G. et al., 2000; Alani, E. et al., 2003; Warren JJ et al., 2007). Thus, repair by MSH2-MSH3 and MSH2-MSH6 is unlikely to occur by the same mechanism even for the same lesion (Palombo F. et al., 1996). A study of yeast Msh2–Msh6 shows that replacement of the DNA binding domain of Msh6 with the equivalent domain of Msh3 results in a chimera that is functional for mismatch repair and possesses the DNA binding specificity. The effect is not reversible: a chimeric MSH2–MSH3 protein harboring the DNA binding domain of yeast Msh6 does not complement a yeast Msh6-deletion mutant Msh3 (Shell, S.S et al., 2007). These data suggest that the Msh3 and Msh6 subunits are structurally and functionally different. And until now the underlying basis for lesion specificity between the two MMR complexes is still poorly understood.

In addition to the mismatch binding activity, all MutS proteins, from E. coli to humans, belong to ABC transporter ATPase superfamily which contains an ATPase activity (Galio, L. et al., 1999; Gradia, S. et al., 1999; Gradia, S. et al., 2000). They have two conserved Walker-type nucleotide binding domains, both of which form as a composite site comprising the C terminus of each subunit. Both the nucleotide binding and ATP hydrolysis of the MutS family proteins are essential for MMR (Alani, E. et al., 1997; Dufner, P. et al., 2000; Iaccarino, I. et al., 1998; Junop, M. S. et al., 2001; Studamire, B. et al., 1998), but how these activities work in MMR is not fully understood. MutS family proteins can simultaneously bind ATP and ADP (Bjornson, K. P et al., 2000; Blackwell, L. J et al., 1998) and undergo ADP → ATP exchange (Wilson, T.et al., 1999; Acharya, S et al., 2003; Gradia, S et al., 1997;
Gradia, S. et al., 1999; Mendillo, M. L. et al., 2005) to induce MutS conformational changes, signaling downstream repair events. Mazur et al., (Mazur, D. J. et al., 2006) have recently demonstrated that two subunits of yeast MutSα exhibit differential nucleotide binding abilities: the MSH6 subunit has a higher affinity for ATP binding than the MSH2 subunit, but the MSH2 subunit exhibits a higher affinity for ADP binding than the MSH6 subunit. ATP hydrolysis by MutS proteins is thought to promote translocation of these proteins along DNA helixes (Allen, D. J. et al., 1997) or to verify MutS mismatch binding and authorize the eventual repair reaction (Junop, M. S. et al., 2001). Although much of the work concerning ATP/ADP binding and hydrolysis is conducted with bacterial MutS and eukaryotic MutSα, it is not known whether or not these activities in MutSβ are different from those of MutSα and whether or not they contribute to specific recognition of ID heteroduplexes by MutSβ.

To address these issues, purified human MutSα and MutSβ were analyzed individually and competitively for their ability to recognize base-base and ID mismatches, ATP/ADP binding, and ATP hydrolysis. We identified some hitherto unknown properties of these two mismatch recognition proteins and their striking differences in nucleotide binding and ATPase activities. The possible importance of these novel properties and activities in differential mismatch recognition by MutSα and MutSβ is discussed.

**Results**

**Purification and functional tests of MutSα and MutSβ**

Recombinant human MutSα and MutSβ were overexpressed in insect cells and purified to homogeneity (Figure 3.1 A). In order to test the activities of these proteins, in vitro mismatch repair assays were performed. MutSα or MutSβ was incubated with NALM-6 cell nuclear extract and a DNA substrate that contains G-T mismatch or AC insertion/deletion (ID) (as described in Chapter II). The cell line NALM-6 was derived from a 12-year-old patient with acute lymphoblastic leukemia (ALL). NALM-6 cells are pre-B cells that carry a homozygous mutation for MSH2 mismatch repair protein that renders the absence of both MutSα and MutSβ in these cells (Matheson, E. C. and Hall, A. G. 2003). The nuclear extract of NALM-6 cells is not able to repair the mismatched DNA (Figure 3.1 B and C, lane 2). When the NALM-6 nuclear extract was complemented with wild type MutSα, the efficiency of repair of
Figure 3.1 Purification and functional tests of recombinant human MutSα and MutSβ. A, recombinant human MutSα and MutSβ after purification (see Chapter II). Both proteins showed ~ 98% purity. B and C, *in vitro* mismatch repair assays were performed to detect the activities of MutSα and MutSβ. The 20 μl reactions contain 70 μg Hela or NALM-6 cell nuclear extract, 200ng MutSα or MutSβ as indicated and 30 fmol of heteroduplex DNA substrates (B, nicked 5’-GT substrate. C, nicked 5’-ID substrate). NE-nuclear extract; HL-Hela cell nuclear extract; N6- NALM-6 cell nuclear extract.
the GT mismatch substrate can be recovered to around 70% (Figure 3.1 B lane 3), which is close to the repair efficiency of Hela-S3 nuclear extract (around 60% repair, Figure 3.1 B, lane 1). The repair efficiency of NALM-6 nuclear extract to insertion/deletion substrate was only <5% (Figure 3.1 C, lane 2). MutSβ could increase the repair efficiency to 40% which is similar to the repair by Hela-S3 nuclear extract (Figure 3.1 C, lane 3 and lane 1). The increased mismatch repair of DNA substrates by adding MutSα or MutSβ into NALM-6 nuclear extract suggested that the purified protein has the repair activities in vitro.

Binding of a G-T and a 2-nt ID DNA Substrate by MutSα and MutSβ

To examine the molecular basis by which MutSα and MutSβ play differential roles in the repair of base-base mismatches and ID mispairs, purified recombinant human MutSα and MutSβ were examined for their ability to interact with a 31-mer oligonucleotide duplex containing a G-T mismatch and a 50-mer duplex containing a 2-nt ID mispair, which are referred to as G-T and ID, respectively (Figure 3.2 A). In initial studies, the binding constants were determined for each protein/DNA substrate pair. Binding reactions were carried out in reactions with a constant DNA substrate concentration (5 nM) and a variable concentration of MutSα (7.5-75 nM) or MutSβ (16-48 nM). Reaction products were visualized by the gel shift method and quantified using a PhosphorImager. Representative gel shift assays are shown in Figure 3.2 (B and D). The fraction of bound and unbound DNA substrate was determined, and the values were plotted (Figure 3.2, C, E, F and G).

Steady-state binding analysis showed that the Kd values of MutSα for the G-T and ID substrates were 26.5 nM and 38.2 nM, respectively. For MutSβ, the Kd values for G-T and ID DNA substrates were 76.5 nM and 23.5 nM, respectively. Thus, MutSα has a 1.5-fold higher affinity for the base-base mismatched substrate than for the ID substrate, whereas MutSβ has a 3-fold higher affinity for the ID substrate than for the base-base mismatched substrate. To confirm this result, each protein was co-incubated with the G-T and ID DNA substrates in competition with each other by gel shift analysis (Figure 3.3). Binding reactions were carried out in reactions with 6 nM MutSα and different ratios of the two DNA substrates (i.e. 5 nM G-T and 0-40 nM ID substrate or 5 nM ID substrate and 0-40 nM G-T DNA substrate). As shown in Figure 3.3 A, although the increase in the ID: G-T ratio is associated with the increased
A

G-C

5' GCTAGCAAGCTCTGCATTCTAGAAATTCCGC
3' CGATCGTTCCGAGCTAAGATCTTTAAAGCG

G-T

5' GCTAGCAAGCTTTGCATTCTAGAAATTCCGC
3' CGATCGTTCCGAGCTAAGATCTTTAAAGCG

ID

5' GCAGATCTGGCTGTTACTCCCTCTCTCTC-GGGCGCGGGTTAACAGTAGAGTC
3' CGTCTAGACCGCGGACCATGAGAGGAGA CCCGCGCCAATGTCTATGAGCAG

B

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<td>0.8</td>
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<tr>
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<td>0.25</td>
<td>0.8</td>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
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C

DNA bound vs MutSα (nM) graph.

D

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<th>MutSβ (nM)</th>
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E

DNA bound vs MutSβ (nM) graph.
Figure 3.2 Interactions of MutSα and MutSβ with G-T and ID heteroduplexes. 

A, oligonucleotide duplexes used in this study. B and C, interactions of MutSα with G-T and ID substrates. D and E, interactions of MutSβ with G-T and ID substrates. Protein-DNA interactions were performed in 20-μl reactions containing the indicated concentration of MutS proteins and 5 nM oligonucleotide duplex. The reactions were incubated on ice for 20 min, and the products were analyzed by gel shift assays. Representative gel shift analyses for MutSα and MutSβ are shown in B and D, respectively, and the relative binding activities of individual reactions determined using the average binding value of two independent experiments are plotted in C and E. F and G are Hill plot of C and E, respectively.
Figure 3.3 Competitive binding of MutSα or MutSβ to G-T and ID heteroduplexes. Gel shift analyses were performed as described under Chapter II using the indicated proteins and heteroduplexes. A, competitive binding of G-T and ID to MutSα; B, competitive binding of G-T and ID to MutSβ.
amount of free G-T substrate (lanes 5-8), only <50% of the free G-T probe is seen in
the presence of an 8-fold excess amount of the ID substrate (compare the amount of
free G-T substrate between lanes 1 and 8), indicating that at least 50% of the G-T
substrate remains bound under this condition. When the substrate ratio was reversed
(Figure 3.3 A, lanes 9-11), unbound ID substrate was almost kept at the same level as
the input (compare the amount of free ID substrate in lanes 9-11 with that in lane 2).
These results are consistent with the Kd values of MutSα for these two substrates
described above and support the notion that MutSα preferentially binds to base-base
mismatches.

In competitive binding reactions with MutSβ (Figure 3.3 B), the ID DNA
substrate is bound preferentially in the examined conditions. When the two DNA
substrates are equal in concentration (Figure 3.3 B, lane 5), the amount of unbound ID
substrate is about the same (if not less) as that in the reaction containing only the ID
substrate (lane 4); almost all G-T substrates exist in unbound form when excess
amounts of ID are present (lanes 6-8). In contrast, a molar excess of the G-T substrate
only slightly reduces the fraction of the ID DNA substrate bound by MutSβ (Figure
3.3 B, lanes 9-11). Comparing the corresponding reactions in Figure 3.2 (A and B)
also draws a clear conclusion that the preferred substrate for MutSα or MutSβ is the
G-T or ID heteroduplex, respectively. These results are consistent with the Kd values
for the enzyme/DNA substrate pairs noted above.

**High MutSα: MutSβ Ratios Stimulate MutSβ Binding to ID Substrates**

Cells express both MutSα and MutSβ, and the two proteins may compete for
binding to the same DNA heteroduplexes, especially those that are well recognized by
both proteins. To simulate the situation in vitro, MutSα and MutSβ were co-incubated
with a 2-nt ID substrate, and the reaction products were analyzed by gel shift analysis.
Protein-DNA complexes with MutSα and MutSβ were distinguished by their abilities
to be “supershifted” by an anti-MSH3 antibody, which specifically supershifts the
MutSβ-DNA complex but not the DNA substrate and/or the MutSα-DNA complex
(Figure 3.4). Surprisingly, increasing amounts of MutSα stimulate binding of MutSβ
to the ID DNA substrate. When a reaction contained a MutSα:MutSβ ratio ≥ 10
(Figure 3.4 A, lanes 9 and 10), >3-fold MutSβ-DNA complex (see arrow)
supershifted by the anti-MSH3 antibody was observed (compare lanes 9 and 10 with
Figure 3.4 High MutSα:MutSβ ratios stimulate MutSβ-ID interaction. Unless otherwise specified, gel shift assays were performed (see Chapter II) using 20 nM MutSα and 16 nM MutSβ. An antibody (Ab; 400 ng) against the MSH3 subunit of MutSβ was used, as indicated, to supershift the MutSβ-ID complex. A, stimulation of the MutSβ-ID interaction (arrow) by high concentrations of MutSα. The MutSα concentrations used were 40, 80, 160, and 240 nM in lanes 7-10, respectively. B, the MSH3 antibody does not supershift the MutSα-ID complex. The MutSα concentrations used were 20, 40, 60, 80, and 100 nM in lanes 3-7, respectively. C, bovine serum albumin (BSA) at high concentrations does not stimulate the MutSβ-ID interaction. The bovine serum albumin concentrations used were 80, 160, and 320 nM in lanes 5-7, respectively.
lane 6). No supershifted products were detected in the same reactions without MutSβ (Figure 3.4 B), consistent with the fact that the antibody is highly specific to MutSβ. The enhanced interaction between MutSβ and the ID heteroduplex appears to be specifically mediated by MutSα because the addition of bovine serum albumin, regardless of the amount of protein used, did not promote binding of MutSβ to the ID substrate (Figure 3.4 C). Therefore, these observations suggest that a MutSα:MutSβ ratio ≥10 is necessary to stimulate MutSβ affinity for its preferred DNA substrates.

**MutSα and MutSβ Possess Distinct Nucleotide Binding Activities**

MutS protein family members share a conserved ATP/ADP-binding site and ATPase activity. Previous studies have shown that binding to ATP/ADP and hydrolysis of ATP by MutS or MutSα play a crucial role in MMR, including verifying mismatch recognition and authorizing the repair (Junop, M. S. et al., 2001) or signaling protein translocation along the DNA molecule to initiate mismatch excision (Gradia, S. et al., 1997; Allen, D. J. et al., 1997). ATP hydrolysis by the MutS family ATPase requires two important cofactors: DNA and Mg$^{2+}$ (Blackwell, L. J. et al., 1998; Gradia, S. et al 1999; Mazur, D. J. et al., 2006; Biswas, I. et al., 1996). To explore whether MutSα and MutSβ possess differential ATP/ADP binding and hydrolysis activities, which may contribute to their distinct mismatch recognitions, purified MutSα and MutSβ were incubated in the presence of [$\gamma$-32P]-ATP with or without DNA substrates. Bound ATP was immobilized by UV cross-linking, and reaction products were resolved by SDS-PAGE and visualized by a PhosphorImager. Under these conditions, the MSH6 subunit of MutSα is cross-linked much more efficiently to ATP than the MSH2 subunit of the protein (Figure 3.5 A, lanes 1-4), consistent with previous observations for yeast MutSα (Mazur, D. J. et al., 2006). In contrast, both the MSH2 and MSH3 subunits of MutSβ are cross-linked to ATP with similar efficiency (Figure 3.5 B, lanes 1-4). Interestingly, whereas DNA duplexes, regardless of homoduplex (G-C) or heteroduplex (G-T or ID), have little effect on ATP binding to MutSα (Figure 3.5 A, lanes 1-4), they significantly reduce the MutSβ-ATP interaction (compare lane 1 with lanes 2-4 in Figure 3.5 B). When the reactions were performed in the presence of Mg$^{2+}$, which supports ATP hydrolysis, little 32P-labeled MutSα was detected in reactions containing [$\gamma$-32P]-ATP (Figure 3.5
Figure 3.5 Binding of MutSα and MutSβ to ATP, ADP or ATP-γ-S. MutSα (A, C, E and G) or MutSβ (B, D, F and H) was incubated with [γ-32P]-ATP, [α-32P]-ATP, [α-32P]-ADP or [35S]-ATP-γ-S, as indicated, in the presence or absence of the indicated DNA duplexes and 5 mM MgCl₂, followed by UV cross-linking and SDS-PAGE as described under Chapter II. The 32P-cross-linked subunits were detected by a Storm PhosphorImager.
A, lanes 5-8), consistent with the fact that the $^{32}$P-labeled phosphate (at the $\gamma$-position) is hydrolyzed by MutS$\alpha$ ATPase activity (Mazur, D. J. et al., 2006). However, under the same conditions, enhanced cross-links were observed in the MSH2 subunit of MutS$\beta$ in the presence of DNA (Figure 3.5 B, lanes 6-8). This result suggests that MutS$\beta$, when interacting with DNA duplexes, has adapted a conformation in favor of ATP binding but not hydrolysis, and this seems to apply only to MSH2 but not MSH3 (Figure 3.5 B, lanes 6-8).

Similar cross-linking experiments were performed by substituting $[\gamma^{32}$P$]$-ATP with $[\alpha^{32}$P$]$-ATP (Figure 3.5 C and D). As expected, in the absence of Mg$^{2+}$ (i.e. no ATP hydrolysis), the amount of ATP cross-links to individual subunits of MutS$\alpha$ or MutS$\beta$ is essentially the same as observed in reactions with $[\gamma^{32}$P$]$-ATP (Figure 3.5, compare lanes 1-4 in A and C for MutS$\alpha$ and in B and D for MutS$\beta$). Under conditions that support ATP hydrolysis (i.e. in the presence of Mg$^{2+}$), the MSH2 subunit but not the MSH6 subunit of MutS$\alpha$ was preferentially labeled (Figure 3.5 C, lanes 5-8), consistent with the observation with yeast MutS$\alpha$ (Mazur, D. J. et al., 2006). In the case of MutS$\beta$, both subunits were well labeled, with a better cross-link for MSH2 (Figure 3.5 D, lanes 5-8). Apparently, DNA plays an inhibitory role in MutS$\beta$ cross-linking with $[\alpha^{32}$P$]$-ATP, as judged by the fact that much intense labeling was detected for both MSH2 and MSH3 in the absence of DNA substrates (Figure 3.5 D, lane 5). Because Mg$^{2+}$ stimulates ATP hydrolysis and because DNA substrates selectively block Mg$^{2+}$-provoked ATP hydrolysis by MutS$\beta$ (Figure 3.5 B, lanes 6-8), the $^{32}$P-labeled proteins in Figure 3.5 D could result from cross-linking to $[\alpha^{32}$P$]$-ATP (without hydrolysis), $[\alpha^{32}$P$]$-ADP (with hydrolysis), or both.

To distinguish these possibilities, cross-linking experiments were conducted in the presence of $[\alpha^{32}$P$]$-ADP and non-hydrolysable ATP analog $[^{35}$S$]$-ATP- $\gamma$-S. As shown in Figure 3.4 E, only the MSH2 subunit of MutS$\alpha$ interacts with ADP. Interestingly, this interaction is greatly enhanced in the presence of Mg$^{2+}$ (compare lanes 5-8 with lanes 1-4, respectively, in Figure 3.5 E), and the enhancement is more pronounced in reactions containing heteroduplexes (lanes 7 and 8). The cross-linking experiments performed with MutS$\beta$ reveal that in the absence of Mg$^{2+}$, the protein behaves similarly to MutS$\alpha$, i.e. only the MSH2 subunit cross-links to ADP (Figure 3.5 F, lanes 1-4); however, addition of Mg$^{2+}$ to the reaction not only stimulates the
MSH2-ADP interaction but also promotes the MSH3 subunit to interact with the nucleotide (lanes 5-8). ADP appears to bind equally well to MSH2 and MSH3 in the presence of Mg\(^{2+}\) and the absence of DNA (Figure 3.5 F, lane 5); DNA greatly reduces the affinity of MSH3 but not that of MSH2 for ADP (lanes 6-8). Similar to the interaction between MutS\(\alpha\) and ADP, there appeared to be a little more ADP binding to the MSH2 subunit of MutS\(\beta\) in the reaction containing the ID substrate (Figure 3.5 F, lane 8). For cross-linking experiments performed using non-hydrolysable ATP analog \([^{35}\text{S}]\text{-ATP-}\gamma\text{-S}\), in the absence of Mg\(^{2+}\), MSH6 subunit of MutS\(\alpha\) is cross-linked much more efficiently to \(\text{ATP-}\gamma\text{-S}\) than the MSH2 subunit (Figure 3.5 G, lane 1-4), which is as the same as observed in reactions with \([\gamma-^{32}\text{P}]\text{ATP}\) or \([\alpha-^{32}\text{P}]\text{-ATP}\). In the presence of Mg\(^{2+}\), ATP-\(\gamma\text{-S}\) is cross-linked more to MSH2 than MSH6, which indicates that under hydrolysis condition more ATP would be bound and hydrolyzed in MSH2 nucleotide binding pocket (Figure 3.5 G, lane 5). These ATP may come from free nucleotides pool surround or be transferred from MSH6 as suggested by a yeast study (Mazur, D. J. et al., 2006). DNA substrates slightly enhanced ATP-\(\gamma\text{-S}\) binding under hydrolysis condition (Figure 3.5 G, lane 5-8). For MutS\(\beta\), in the absence of Mg\(^{2+}\), ATP-\(\gamma\text{-S}\) cross-linked to both MSH3 and MSH2 similar to but weaker than \([\gamma-^{32}\text{P}]\text{ATP}\) or \([\alpha-^{32}\text{P}]\text{-ATP}\) (Figure 3.5 H, lane 1). DNA substrates can significantly inhibit both subunits’ cross-linking efficiency (Figure 3.5 H, lane 1-4). Interestingly, under hydrolysis condition (in the presence of Mg\(^{2+}\)), both MSH3 and MSH2 have greatly enhanced cross-linking to \(\text{ATP-}\gamma\text{-S}\) (Figure 3.5 H, lane 5) and all DNA substrates except ideal ID substrate only have marginal inhibition of the cross-linking. The ID substrate can inhibit more cross-linking amount (Figure 3.5 H, lanes 5-8) to MSH3. These results suggest that under hydrolysis condition both subunits of MutS\(\beta\) have higher affinity to ATP binding than non-hydrolysis condition. DNA binding does not inhibit ATP binding of MSH2 (Figure 3.5 H, lanes 5-8) but inhibits the MSH2’s ATP hydrolysis (Figure 3.5 B, lanes 5-8). And DNA binding inhibit MSH3’ ATP binding (Figure 3.5 H, lanes 5-8) as well as its hydrolysis ability (Figure 3.5 B, lanes 5-8).

These results may explain why Mg\(^{2+}\) is required for mismatch binding by MutS (Biswas, I. et al., 1996) and why MutS proteins in their ADP-bound form possess a higher affinity for heteroduplexes (Junop, M. S et al., 2001; Fishel, R. 1999). Comparing data in Figure 3.5 (D and F), it appears that the cross-links in D (lanes 5-8)
contain components of both ADP and ATP. These observations suggest that DNA stimulates the ATPase activity of MutSα, but it slightly inhibits the ATPase activity of MutSβ.

**MutSα and MutSβ Possess Distinct ATPase Activities**

The ATPase activity of MutS proteins is essential for their functions in MMR (Iaccarino, I et al., 1998; Studamire, B. et al., 1998). It has also been shown that the ATPase activity of MutSα could be stimulated by homo- or heteroduplex DNA although to different extents (Bjornson, K. P. et al., 2000; Bowers, J. et al., 1999; Gradia, S. et al., 2000). To determine whether there is any difference in ATPase activity between MutSα and MutSβ, which may contribute to their preferential mismatch recognition, the purified human MutS heterodimers were assayed for their ability to hydrolyze $[^{32}P]\text{-ATP}$ in the presence or absence of DNA substrates. The $^{32}$P-containing species, i.e. the unreacted $[^{32}P]\text{-ATP}$ and the hydrolyzed $[^{32}P]$-phosphate, were detected after gel electrophoresis (Figure 3.6, A and B). The results indicate that in the absence of DNA substrates, MutSβ exhibited a much more active ATPase activity than MutSα at all concentrations and time points tested (Figure 3.6, C and D). However, DNA substrates, regardless of a homoduplex and a heteroduplex, significantly stimulated the ATPase activity of MutSα (Figure 3.6 D; also compare lane 7 with lanes 8-10 in Figure 3.6 A), consistent with previous observations. Surprisingly, DNA substrates were found to inhibit MutSβ ATPase activity by $\sim 20\%$ (Figure 3.5 D; also compare lane 7 with lanes 8-10 in Figure 3.6 B). These results differ somewhat from those of Fishel and co-workers (Wilson, T. et al., 1999) who reported stimulation of MutSβ ATPase activity by ID substrates. Although the exact reason for this discrepancy is unknown, we did notice that a His-tagged MutSβ and a nontagged MutSβ were used in the previous study and this study, respectively, which may have an impact on MutSβ ATPase activity. Interestingly, despite the stimulation of MutSα activity and the reduction of MutSβ activity by DNA substrates, both proteins exhibited the same level of ATPase activity upon their interactions with DNA (see Figure 3.6 D), suggesting that the DNA-associated ATPase activity of MutS proteins is not related to mismatch binding specificity but to the downstream signaling of MMR.
**Figure 3.6 ATPase analysis of MutSα and MutSβ.** Unless otherwise specified, ATPase activity of MutSα or MutSβ was assayed in reactions containing 50 nM proteins, [γ-32P]-ATP, and 5 mM MgCl2 in the presence or absence of the indicated DNA substrates. The reactions were incubated at 37°C for the indicated times, followed by electrophoresis as described under Chapter II. 32P-Labeled species were detected and quantified by a PhosphorImager. A and B, representative ATPase assays for MutSα and MutSβ, respectively; C, titration of ATPase activity of MutSα and MutSβ; D, ATPase activity in a time course. Pi, [32P] phosphate.
Discussion

This study investigates molecular mechanisms by which recombinant purified MutSα and MutSβ preferentially process base-base mismatches and ID mispairs. Interesting observations made during this study include enhanced binding of MutSβ to ID mispairs in the presence of excess MutSα and significant differences between MutSα and MutSβ in DNA substrate recognition, ATP/ADP binding, and ATP hydrolysis. These differences may influence the functional roles of these two proteins in MMR in vivo.

One puzzling phenomenon in human MMR is that MutSβ binds and directs repair of small ID mispairs in vivo, even though its concentration in human cells is only one-tenth the concentration of MutSα (Drummond, J. T. et al., 1997) (Li, G-M. 2008). Our work presented here provides an explanation for this phenomenon. First, our steady-state in vitro DNA binding studies reveal that MutSα and MutSβ display distinct specificities for base-base and ID heteroduplex binding and have the following hierarchy of binding affinities: MutSβ-ID > MutSα-G-T > MutSα-ID ≫ MutSβ-G-T (Kd values were 23.5 nM, 26.5 nM, 38.2 nM, and 76.5 nM, respectively). Second, we surprisingly find that MutSα at a high concentration does not inhibit but stimulates the binding activity of MutSβ to ID heteroduplexes (Figure 3.4). This finding explains why cells maintain a 10:1 MutSα:MutSβ ratio and why MutSβ at a low concentration is capable of efficiently processing ID heteroduplexes. Because MSH2 is shared between MutSα (MSH2/MSH6) and MutSβ (MSH2/MSH3), the distinct MSH6 and MSH3 subunits compete with each other for MSH2 in vivo. Previous studies show that overexpression of MSH3 greatly reduces the MutSα:MutSβ ratio, leading to a mutator phenotype (Drummond, J. T. et al., 1997; Marra, G. et al., 1998). This is apparently because base-base mismatches, which are poor substrates for MutSβ (Figure 3.2) (Zhang, Y. et al., 2005), go unrepaired under conditions of insufficient MutSα. Thus, the high ratio of MutSα to MutSβ appears to be a mechanism ensuring efficient repair of both base-base and ID heteroduplexes, i.e. a high level of MutSα not only guarantees the efficient processing of base-base mismatches but also promotes the efficient repair of ID mispairs by MutSβ. However, the molecular basis as to how MutSα stimulates the MutSβ affinity for ID
heteroduplexes is unclear. Because multiple molecules of MutS proteins are required for processing a single mismatch (Zhang, Y. et al., 2005), one possibility is that binding of MutSα to homoduplex DNA regions (i.e. unlabeled noncompetitive DNA in the case of the gel shift reactions) allows MutSβ to focus on ID binding, resulting in a dramatic increase in the local concentration of MutSβ for an efficient repair. It is also possible that MutSα and MutSβ may physically interact with each other, and abundant MutSα proteins can facilitate the MutSβ-ID heteroduplex interaction by initially localizing the ID mispairs and passing them to MutSβ for a specific and efficient repair of the ID heteroduplexes (Zhang, Y. et al., 2005). Further studies are required to define the molecular mechanism by which a high MutSα concentration enhances the MutSβ-ID interaction.

Another important observation of this study is that the ATP/ADP binding and ATP hydrolysis characteristics of MutSα and MutSβ are significantly different. Under the experimental conditions (pH 7.5; 5 mM Mg<sup>2+</sup> and 110 mM NaCl), MutSβ possesses a higher ADP binding activity than MutSα in the presence or absence of DNA, reflecting the properties of the MSH3 subunit (see Figure 3.5, lanes 5-8, compare C with D and E with F). Interestingly, the ADP binding activity of MutSα and MutSβ appears to be correlated with their ATPase activity, as MutSβ has a more active ATPase activity than MutSα in the absence of DNA (Figure 3.6 C). This correlation suggests that prior to interacting with DNA, MutSβ has a high tendency to bind ADP, which favors heteroduplex binding (Fishel, R. 1999). This explains why the MutSβ binds ID mispairs with higher affinity than MutSα. The differential biochemical activities of MutSα and MutSβ may be determined by their ternary structures. The MutSα-DNA co-crystal structures revealed that the MSH2 nucleotide-binding site is surrounded by two well organized Walker P-loops, but the corresponding P-loops in MSH6 are partially disorganized (Warren, J. J. et al., 2007), which may explain why MSH2 has a higher ADP binding activity than MSH6. Although the crystal structure of MutSβ is not available at this time, we predict that the P-loops are better organized in MSH3 than in MSH6. We also find that binding to DNA reduces MutSβ ATPase activity but stimulates MutSα ATPase activity; as a result, both proteins exhibit the same level of ATPase activity (Figure 3.6 D). These results suggest that whereas the ATPase of MutS proteins enhances their heteroduplex
affinity by converting the ATP-bound proteins to their ADP-bound form before interacting with DNA, the hydrolytic activity, upon binding of MutS proteins to a heteroduplex, acts to signal downstream repair events, including recruiting other MMR proteins and authorizing the repair reaction (Li, GM. 2008, Junop, M. S et al., 2001; Fishel, R. 1999).

In summary, this study demonstrates significant differences in the in vitro DNA binding, ATP/ADP binding, and ATP hydrolysis characteristics of human MutSα and MutSβ. These differences, together with a high MutSα:MutSβ ratio, are likely responsible for the preferential recognition and repair of base-base and ID mispairs by MutSα and MutSβ, respectively. Although the micro-environment in living cells (which differs from reconstituted in vitro assays with purified recombinant proteins and synthetic DNA substrates) may modulate MMR in a complex manner, the biochemical characteristics of MutSα and MutSβ, as well as their relative concentrations in cells, appear to play an important role in determining their functions in vivo.
CHAPTER FOUR*

MISMATCH RECOGNITION PROTEIN MUTSβ DOES NOT HIJACK
(CAG)n HAIRPIN REPAIR IN VITRO


Introduction

CAG/CTG trinucleotide repeats instability is an important and unique form of mutation that is linked to more than 17 neurodegenerative and neuromuscular diseases, such as Huntington disease and myotonic dystrophy, whose clinical symptoms are directly linked to expansion of CAG and CTG repeats, respectively (Lahue R. S. and Slater DL. 2003; Mirkin S. M 2007; Pearson C. E. et al., 2005). Each of these diseases is clinically distinct and involves expansion of a trinucleotides repeat (TNR) at a unique site either in the coding or non-coding region in the human genome. Normal individuals typically harbor <30 repeats, whereas unstable disease-causing alleles can have 35–6,550 repeats. Large tracts of trinucleotides can cause disease in several ways: by affecting gene expression; by producing toxic RNA species; or by altering the function of the resultant protein (Dion V and Wilson JH. 2009; Orr HT and Zoghbi HY 2007). Longer tracts are more likely to undergo an expansion mutation than shorter tracts. Thus the severity and onset of disease also depend on the number of CAG/CTG repeats. The precise mechanisms by which TNR expansion occurs and the factors that promote it are not fully understood, but most models explaining TNR expansions involve slipped-DNAs. It has been proposed that CAG and CTG repeats form thermostable hairpins that include A-A and T-T mispairs in the hairpin stem (Gacy A. M. et al., 1995; Pearson C. E. et al., 2002). In vivo analysis has revealed that the secondary structure formation in TNR increases the frequency of expansion in yeast 5–1,000-fold (Miret, JJ et al., 1998; Miret, J.J. et al., 1997; Moore, H et al., 1999). While equivalent TNR lacking the capacity to form secondary structure do not expand in vivo and the mutation frequency cannot be distinguished from background.
Therefore, it is possible that cellular mechanisms that process DNA hairpin/loop structures and/or A-A or T-T mispairs may influence TNR stability.

Recent studies have identified and characterized a DNA hairpin repair (HPR) system in human cells that promotes CAG/CTG repeat stability (Hou C. et al., 2009; Panigrahi G. B. et al., 2009). The mechanism of human HPR involves incision and removal of CAG/CTG repeat hairpins in a nick-directed and proliferating cell nuclear antigen-dependent manner, followed by DNA resynthesis using the continuous strand as a template (Hou C. et al., 2009). In addition to human HPR, the human mismatch repair (MMR) system is well known for its role in stabilizing simple repetitive sequences called microsatellites, which are prone to forming small loops or insertion/deletion (ID) mispairs. In human cells, MutSα (MSH2–MSH6) and MutSβ (MSH2–MSH3) both bind to 1–2-nt ID mispairs, but MutSβ has higher affinity for these small loops (see Chapter III). Defects in MMR genes cause microsatellite instability and predisposition to cancer (Li, GM 2008), demonstrating that MMR is essential for genetic stability in human cells. Surprisingly, genetic studies in mice suggest that MutSβ promotes (CAG)_n expansion and TNR instability. These studies show that expansion of a heterologous (CAG)_n tract occurs in wild type and MSH6^{−/−} mice but that expansion of the (CAG)_n tract is suppressed in MSH2^{−/−} and MSH3^{−/−} mice (Manley K. et al., 1999; Owen B. A. et al., 2005). Recently, Owens et al., (Owen B. A. et al., 2005) reported that binding to a (CAG)_n hairpin influences the protein conformation, nucleotide binding, and hydrolysis activities of MutSβ so that they are different from what has been reported for MutSα during mismatch recognition. It is therefore hypothesized that (CAG)_n hairpins, through their ability to alter the biochemical properties of MutSβ, hijack the MMR process, leading to CAG repeat expansion instead of CAG hairpin removal (Owen B. A. et al., 2005). However, it is not clear why MMR, a major genome maintenance system, would promote TNR instability instead of TNR stability. We, therefore, have developed a novel functional assay and examined the validity of this hypothesis. Our results reveal that MutSβ displays normal biochemical activities when binding to CAG hairpins and does not inhibit (CAG)_n hairpin repair. The observations presented here provide novel thoughts on whether or not or how MutSβ is involved in CAG repeat instability in human cells.
Results

MutSβ Does Not Inhibit CAG/CTG Hairpin Repair

Repair of DNA hairpins formed within CAG and CTG TNRs has recently been characterized in human cells (Hou C.et al., 2009, Panigrahi G. B. et al., 2009). The HPR system removes (CAG)_n or (CTG)_n hairpins by incisions in a nick-directed, proliferating cell nuclear antigen-dependent, and error-free manner (Hou C.et al., 2009). To determine whether MutSβ hijacks (CAG)_n HPR, by collaboration with Dr. Caixia Hou in the lab, a functional in vitro assay was performed to examine the catalytic competence of MutSβ in repair of a (CAG)_{25} hairpin and a (CTG)_{25} hairpin by Hela-S3 nuclear extracts (Tian L, et al., 2009). In this assay, the DNA substrate is incubated with Hela-S3 nuclear extracts in the presence or absence of excess exogenous human MutSβ. The results showed that, as expected, both substrates were efficiently repaired by Hela-S3 nuclear extracts. Surprisingly, when excess exogenous human MutSβ, which is very active in repair of insertion/deletion mispairs in a defined MMR system (Zhang, Y et al., 2005), was preincubated with the DNA substrate prior to assembling the complete reaction, there was no reduction or inhibition of either (CAG)_{25} or (CTG)_{25} HPR. Instead, the repair was 1.1–1.7-fold higher in the presence of MutSβ. This result suggests that MutSβ facilitates (CAG)_n and (CTG)_n HPR, likely through interactions with these hairpins. In addition, the extent of repair did not decrease when the DNA substrate was incubated with MutSβ and Hela-S3 nuclear extract at the same time. Similar results were also obtained with MutSα. These observations show that neither MutSα nor MutSβ inhibits (CAG)_n or (CTG)_n HPR in the in vitro assay.

MutSβ Binds CAG/CTG Hairpins and ID Mispairs in Similar Manners

To determine whether MutSβ interacts with (CAG)_n and (CTG)_n hairpins, electrophoretic mobility shift analysis was performed using purified MutSβ and a (CAG)_{13} hairpin (Owen B. A. et al., 2005) and a (CTG)_{13} hairpin substrates. As shown in Figure 4.1, MutSβ binds both (CAG)_{13} and (CTG)_{13} hairpins with a Kd of 26 nM and 22 nM, respectively, which is similar to the Kd (23 nM) for an ID substrate. It is known that mismatch binding by MutSα leads to an ATP-provoked conformational change that allows the protein to be released from the DNA (Mendillo,
Figure 4.1 MutSβ binds to CAG and CTG hairpins as it does to an ID mispair. A Gel-shift analysis was performed as described in Chapter II using 1 pmol of MutSβ, 1 pmol of a (CAG)$_{13}$ hairpin substrate, a (CTG)$_{13}$ hairpin (CTG), or a GT-dinucleotide ID mispair (ID). B. The Kd of MutSβ binding to different substrates were determined as described in Chapter II.
M. L et al., 2005; Gradia, S et al., 1999; Drummond, J. T. et al., 1995). However, little is known about the MutSβ activities during its mismatch recognition. To determine whether hairpin binding alters MutSβ biophysical properties as proposed (Owen B. A. et al., 2005), gel-shift analysis were performed in the presence of ATP. The results showed that ATP inhibits both the MutSβ-ID and the MutSβ-(CAG)_{13} hairpin interactions suggesting that MutSβ undergoes an ATP-induced conformational change whether it is bound to a (CAG)_{13} hairpin or to an ID mispair.

**MutSβ Exhibits Identical Nucleotide Binding and ATPase Activities When Interacting with Hairpin and ID Heteroduplexes**

All MutS proteins possess a weak ATPase activity and a nucleotide (ATP and ADP) binding activity (Li, GM 2008). Previous studies have shown that binding of MutSα to a mismatch enhances its ATPase and ATP binding activities (Mendillo, M. L et al., 2005; Gradia, S et al., 1999). Interestingly, the MutSβ-ID interaction reduces MutSβ ATPase activity (Chapter III). We therefore examined the effects of ID and hairpin heteroduplexes on MutSβ ATPase. As shown in Figure 4.2 A, MutSβ displays an identical reduction in ATPase activity (from 100% in the absence of DNA to 66% in the presence of heteroduplexes) regardless of its interaction with a (CAG)_{13} hairpin, a (CTG)_{13} hairpin, or an ID heteroduplex (compare lane 2 with lanes 4–6). Kinetic studies revealed that although the k_{cat} value (13.9 min^{-1}) for the ID substrate is higher than that (11.3 min^{-1}) for the CAG or CTG hairpin substrate (Figure 4.2 A), which appears to be in agreement with the data reported previously (Owen B. A. et al., 2005), the catalytic efficiencies, k_{cat}/k_{m}, for the individual DNA substrates used are almost the same (3.32 for CAG hairpin, 3.39 for CTG hairpin, and 3.5 for ID heteroduplex) (Figure 4.2 A), suggesting that the reduction in MutSβ ATPase activity induced by DNA is not specific or unique to the (CAG)_{13} or (CTG)_{13} hairpin structure.

MutSβ nucleotide binding affinity was determined by performing UV cross-linking experiments (Mazur DJ et al., 2006 and Chapter III). The results show that all DNA heteroduplexes, including a CAG hairpin and an ID mispair, inhibit binding of MutSβ to ATP by 60% in the absence of Mg^{2+} (Figure 4.2 B, upper panel, also see quantitative data in Figure 4.2 D). In the presence of Mg^{2+}, DNA substrates no longer inhibit MutSβ-ATP interactions (Figure 4.2 B, lower panel), leading to an enhanced (2–3-fold) ATP binding (see Figure 4.2 D). This is consistent with the fact that DNA
A

DNA   K cat   km    Kcat/km  
    [m⁻¹]  [M×10⁵]  [10⁵ m⁻¹M⁻¹]  
None     5.1±0.6  1.25±0.29  4.08  
Homo    10.0±0.7  2.99±0.37  3.34  
ID      13.9±2.4  3.97±0.27  3.5  
CAG    11.3±2.2  3.40±0.12  3.32  
CTG    11.3±3.9  3.33±0.33  3.39  

B

C

D

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<th>Nucleotide</th>
<th>Subunit</th>
<th>- Mg²⁺</th>
<th>- DNA</th>
<th>Homo</th>
<th>ID</th>
<th>CAG</th>
<th>CTG</th>
<th>+ Mg²⁺</th>
<th>- DNA</th>
<th>Homo</th>
<th>ID</th>
<th>CAG</th>
<th>CTG</th>
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<td>N.D.</td>
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<td>40</td>
<td>39</td>
<td></td>
<td></td>
<td>100</td>
<td>N.D.</td>
<td>230</td>
<td>216</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>MSH2</td>
<td>100</td>
<td>N.D.</td>
<td>42</td>
<td>42</td>
<td>43</td>
<td></td>
<td></td>
<td>100</td>
<td>N.D.</td>
<td>340</td>
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<td>MSH3</td>
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Figure 4.2. Analysis of MutSβ nucleotide binding and hydrolysis activities. A), ATPase activity. [γ-32P]-ATP was incubated with MutSβ (0.2 μM) and 5 mM MgCl₂ for 10 min in the presence or absence of the indicated DNA substrates, and samples were electrophoresed in an 20% SDS-PAGE gel as described (Chapter II). Relative ATPase activity (R.A.) was determined by dividing the amount of 32P-phosphate (Pi) with the amount of 32P-phosphate in the reaction without DNA and multiplying by 100. ATPase assays were also performed by incubating MutSβ with 4.0 pmol of individual DNA substrates and varying concentrations of ATP. The resulting data were fit to the Michaelis-Menten equation. k_{cat} and k_M values and standard deviations were calculated from three independent experiments. Homo, a perfect matched oligonucleotide duplex DNA; CAG, a (CAG)₁₃ hairpin; CTG, a (CTG)₁₃ hairpin; ID, a GT-dinucleotide insertion/deletion mispair. m⁻¹ and M⁻¹ stand for min⁻¹ and molarity⁻¹, respectively. B) and C), nucleotide binding activity. MutSβ (0.2 μM) was incubated with either [γ-32P]-ATP (B) or [α-32P]-ADP (C) in the presence or absence of DNA duplexes and 5 mM MgCl₂, as indicated, followed by UV cross-linking and SDS-PAGE (Chapter II). D) Quantification of data derived from gels shown in B) (ATP binding) and C) (ADP binding). Relative binding affinity was calculated by dividing the 32P intensity of individual MSH2 or MSH3 subunits with that of the same subunit in non-DNA-containing reactions and multiplying by 100. N.D., not determined; UD, undetectable.
substrates inhibit MutSβ ATPase activity (Figure 4.2 A) (Chapter III). Figure 4.2 C shows similar analysis for ADP. Again, the type of DNA substrates has no effects on ADP binding, but Mg$$^{2+}$$ stimulates binding of MutSβ to ADP, particularly the MSH3 subunit (Figure 4.2 C, compare lanes 6–10 with lanes 1–5, also see Figure 4.2 D), which differs from MutSα and its MSH6 subunit (Mazur DJ et al., 2006 and Chapter III). These data strongly suggest that binding to a (CAG)$_n$ or a (CTG)$_n$ hairpin does not alter the nucleotide binding and ATPase activities of MutSβ, which are associated with its function in MMR.

**Discussion**

A previous study (Owen B. A. et al., 2005) reported that “CAG-hairpin binding inhibits the ATPase activity of Msh2–Msh3 and alters both nucleotide (ADP and ATP) affinity and binding interfaces between protein and DNA.” These alterations are considered “critical functional defects in the Msh2–Msh3-CAG hairpin complex that could misdirect the DNA repair process,” i.e. “the aberrant enzymatic and/or structural properties of the Msh2–Msh3-hairpin DNA complex may divert the repair process to other non-MMR pathway, leading to expansion instead of repair” (Owen B. A. et al., 2005). However, the results presented here demonstrate that MutSβ exhibits identical biochemical and biophysical activities, including nucleotide binding and hydrolysis (Figure 4.2), and ATP-induced conformational change and protein translocation/sliding when MutSβ interacts with a favored ID mispair or a CAG/CTG hairpin. More convincingly, functional *in vitro* HPR assays reveal that excess MutSβ does not inhibit CAG/CTG hairpin removal (Tian L et al., 2009). Therefore, binding to CAG hairpins does not alter MutSβ MMR activities and does not inhibit CAG HPR.

Although the discrepancy between these studies requires further investigation, we did identify the following differences: (i) the previous study was performed with a recombinant His-tagged MutSβ, whereas the present study was performed with a preparation of MutSβ that lacks an epitope tag and (ii) the MutSβ protein used in the present study is active in a functional MMR assay (Zhang Y. et al., 2005), but the MutSβ protein used in the previous study was not tested for its MMR function. These factors may have contributed to the difference in these studies. We also found that data were analyzed differently in these two studies. For example, $k_{cat}$ and $k_{cat}/k_{m}$ were
used to evaluate MutSβ ATPase activity in the previous and current studies, respectively. Despite the fact that both studies show different $k_{cat}$ values for MutSβ ATPase activity when incubated with different DNA substrates, a much smaller difference was measured when $k_{cat}/k_m$ values were used. A good example is that although Owens et al., (Owen B. A. et al., 2005) observed a $k_{cat}$ value of $6.3 \pm 0.2$ and $5.0 \pm 0.2$ min$^{-1}$ for a homoduplex and a CAG hairpin, respectively, the $k_{cat}/k_m$ values for both substrates are essentially the same in our hands ($1.9 \times 10^5$ min$^{-1} \text{m}^{-1}$), indicating that there is little difference in MutSβ ATPase activity when the protein interacts with these DNA substrates. It is worth mentioning that although $k_{cat}$ is frequently used to express enzyme activity, the term $k_{cat}/k_m$, referred to as the catalytic efficiency, is more often employed as a specificity constant to compare the relative rates of the same enzyme reacting with different substrates (Eisenthal R. et al., 2007; Johnson K. A. 1992; Radzicka A. et al., 1995; Takamatsu S. et al., 1996). We found that $k_{cat}/k_m$ values accurately reflect the observed rate of ATP hydrolysis when MutSβ is incubated with different DNA substrates (Figure 4.2 A).

We also realize that different interpretations of the existing data contribute to the distinct conclusions in these two studies. Both studies show that specific DNA substrates can inhibit MutSβ ATPase, which completely differs from MutSα or Escherichia coli MutS, whose ATPase activity is stimulated by similar DNA substrates (Bjornson, K. P et al., 2000; Bowers, J. et al., 1999; Gradia, S et al., 2000). We report here significant differences in the biochemical functions of MutSα and MutSβ during recognition and interaction with base-base and ID mismatches. For example, MutSβ binds ADP with higher affinity than MutSα, and DNA substrates partially inhibit MutSβ ATPase activity but stimulate MutSα ATPase activity (Chapter III). A recent study by Owens et al. (Owen B. A et al., 2009) also revealed similar differences in the properties of MutSα and MutSβ. Taken together, we believe that the distinct properties of MutSβ and MutSα explain the selective specificity of MutSβ for ID heteroduplexes (Chapter III). And we do not support the hypothesis that MutSβ interacts in a unique manner with CAG hairpins, as proposed in the hijacking model (Owen B. A. et al., 2005, McMurray C. T. 2008).

In summary, our previous and current studies dispute the idea that binding of (CAG)$_n$ hairpin to MutSβ inhibits (CAG)$_n$ HPR in vitro or in vivo and questions the validity of the proposed hijacking model (Owen B. A. et al., 2005, McMurray C. T.
2008; Manley K. et al., 1999; Owen B. A. et al., 2005). Further, our results raise the following question: Do transgenic mouse models for Huntington's disease reproduce the phenomenon of CAG repeat expansion in human cells; Does human MutSβ promote CAG repeat expansion in human cells? If it does, why do human MSH2 defects result in microsatellite instability (i.e., MSI)? A recent study by Lin et al., (Lin Y. et al., 2006) suggests that MutSβ may influence CAG repeat instability via transcription; however, the mechanism is unclear. Therefore, thorough investigations are required to elucidate the mechanism of TNR expansion in specific human diseases, as well as the potential in vivo role of MutSβ or other DNA repair proteins in this process.
CHAPTER FIVE

SUMMARY AND DISCUSSION

Summary of findings

The research described here characterizes several important biochemical properties of human MutSα and MutSβ. First, the mechanistic basis of preferential processing of base-base and ID heteroduplexes by MutSα and MutSβ was elucidated and shown to involve significant differences in ATPase activity, ADP binding activity, and depend on the MutSα:MutSβ ratio. Second, the role of MutSβ in (CAG)_n hairpin repair was investigated, revealing that MutSβ displays identical biochemical and biophysical activities when interacting with a (CAG)_n hairpin and a mismatch, suggesting that MutSβ does not hijack (CAG)_n hairpin repair \textit{in vitro or in vivo}.

Mismatch recognition

Our work indicates that MutSα and MutSβ display distinct specificities in binding to base-base and ID heteroduplexes and have the following hierarchy of binding affinities: MutSβ-ID > MutSα-G-T > MutSα-ID ≫ MutSβ-G-T (Kd values were 23.5 nM, 26.5 nM, 38.2 nM, and 76.5 nM, respectively). It is interesting that human cells express MutSα at a 10-fold higher concentration than MutSβ. Our results suggest that MutSα at such a high concentration does not inhibit, but stimulates binding of MutSβ to ID heteroduplexes. Because MutSα (MSH2/MSH6) and MutSβ (MSH2/MSH3) both include MSH2, MSH6 and MSH3 compete for binding to MSH2 \textit{in vivo}; thus, the relative concentrations of all three subunits are critical for balancing the concentration and functions of MutSα and MutSβ in the cell. Consistent with this, overexpression of MSH3 significantly lowers the concentration of MutSα, leading to a mutator phenotype, likely because base-base mismatches, which are poor substrates of MutSβ, are repaired inefficiently under these conditions. Thus, these data explain how the 10:1 MutSα:MutSβ ratio in human cells ensures efficient repair of both base-base and ID heteroduplexes.
ATPase activity of MutSα and MutSβ

MutSα and MutSβ are both ABC transporter family proteins, and have two composite nucleotide binding pockets, but their ATP/ADP binding and ATP hydrolysis activities display significant differences. Under conditions that do not support ATP hydrolysis, the MSH6 nucleotide binding pocket in MutSα binds ATP, while the MSH2 nucleotide binding pocket binds ADP. Under conditions that support ATP hydrolysis, MSH2 binds ATP, but still with lower affinity than ADP. In contrast, under conditions that do not support ATP hydrolysis, MSH3 and MSH2 in MutSβ bind ATP, although MSH3 has a higher affinity for ATP than MSH2; and under conditions that support ATP hydrolysis, ATP occupancy increases in both subunits, especially in MSH2. However, MutSβ has higher affinity for ADP than MutSα. Interestingly, the ADP binding activities of MutSα and MutSβ appear to be correlated with their ATPase activities, as MutSβ ATPase is more active than MutSα ATPase in the absence of DNA (Figure 3.5 C). This correlation suggests that prior to interacting with DNA, MutSβ exists predominantly in an ADP-bound state, which favors heteroduplex binding. This explains why MutSβ binds ID mispairs with higher affinity than MutSα.

DNA binding modulates the nucleotide binding and ATP hydrolysis activities of MutSα and MutSβ. DNA binding stimulates ATP hydrolysis and ADP binding by MSH2 but not by MSH6 in MutSα, while it inhibits ATP hydrolysis and ATP/ADP binding by MSH3 in MutSβ. However, the ATPase activities of MutSα and MutSβ are comparable in the presence of DNA (see Figure 3.6 D), suggesting that the DNA-dependent ATPase activity of MutSα or MutSβ does not depend on mismatch binding, and is likely to play a role in downstream signaling by MMR.

The role of MutSβ in (CAG)n repair

CAG and CTG repeats form thermostable hairpins that include A-A and T-T mispairs in the hairpin stem and a small loop at the end of the hairpin. Genetic studies in mice suggest that MutSβ promotes (CAG)n expansion and TNR instability. To explain this phenomenon, a recent in vitro study proposed that (CAG)n hairpins, through their ability to alter the biochemical properties of MutSβ, hijack the MMR process, leading to CAG repeat expansion instead of CAG hairpin removal (Owen B.
A. et al., 2005). However, our studies show that when MutSβ interacts with an ID mispair or a CAG/CTG hairpin, MutSβ exhibits identical biochemical and biophysical activities, including nucleotide binding and hydrolysis (Figure 4.2), ATP-induced conformational change and protein translocation/sliding. More convincingly, in collaboration with Dr. Caixia Hou in the lab, we showed that excess MutSβ does not inhibit CAG/CTG hairpin removal (Tian L et al., 2009). In summary, these studies indicate that binding of (CAG)$_n$ hairpins by MutSβ does not interfere with (CAG)$_n$ hairpin repair in vitro and is unlikely to do so in vivo. Thus, another explanation for (CAG)$_n$ expansion in the context of mouse models for triplet repeat diseases is needed. Further investigations are also needed to determine whether MMR proteins play a role in TNR expansion in human diseases such as Huntington's disease.

**Future directions**

The research described here explores the biochemical characteristics of human MutSα and MutSβ and provides better understanding of the specificity of MMR. It is important to continue to dissect the roles of nucleotide binding, ATP hydrolysis, and DNA binding by both subunits of MutS heterodimers in modulating the various steps in MMR, especially the steps needed to identify and use the strand discrimination signals. We will construct MutSα or MutSβ mutants that contain mutations in ATPase domains. The resulting mutant protein will be tested for mismatch recognition and ATP/ADP binding activities. In vitro MMR assays will also be performed. And single molecule fluorescence resonance energy transfer (smFRET) experiments will be used to analyze protein-protein and protein-DNA interactions during MMR initiation. In this analysis, MutSα or MutSβ and a nicked DNA substrate with a centrally-located G-T or ID heteroduplex will be labeled with different fluorophores, and migration of MutSα/MutSβ along the DNA substrate will be monitored using Single-Molecule Total Internal Reflection Fluorescence Microscopy (TIRFM). Experiments will be conducted in the presence or absence of MutLα, PCNA, RPA, and RFC. These experiments should reveal the roles of the MutS ATPase activity and MutS sliding clamp in MMR initiation.

MMR is an important cellular mechanism that maintains genome stability. Defects in this pathway cause predisposition to cancer. In addition, MMR is also implicated in
cancer chemotherapy, because tumor cells defective in MMR are highly resistant to many chemotherapeutic drugs including procarbazine, temozolomide, or cisplatin. Therefore, understanding the molecular mechanisms of MMR will provide opportunities for developing new therapeutic approaches for cancer treatment and prevention.

It will also be of great interest to understanding how MutSβ promote (CAG)$_n$ expansion in vivo, if it does not inhibit (CAG)$_n$ hairpin repair in vitro. To explore the role of MutSβ in (CAG)$_n$ expansion/stability, in vitro hairpin repair will be performed in the presence of MutSβ, with or without other MMR and chromatin-binding proteins that might influence repair efficiency. These studies will lead to better understanding of the role of MMR proteins in maintaining the stability of TNR sequences associated with neurodegenerative and neuromuscular diseases such as Huntington disease.
ABBREVIATION

EtBr, ethidium bromide
ECL, enhanced chemiluminescent
EMSA, electrophoretic mobility shift assay
HNPCC, hereditary nonpolyposis colon cancer
HPR, hairpin repair
ID, insertion/deletion
LS, Lynch syndrome
MMR, mismatch repair
MSI, microsatellite instability
SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
smFRET, single molecule fluorescence resonance energy transfer
TNR, trinucleotide repeats
REFERENCES


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