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ATP binding and hydrolysis by *Saccharomyces cerevisiae* Msh2–Msh3 are differentially modulated by mismatch and double-strand break repair DNA substrates

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ABSTRACT

In *Saccharomyces cerevisiae*, Msh2–Msh3-mediated mismatch repair (MMR) recognizes and targets insertion/deletion loops for repair. Msh2–Msh3 is also required for 3′ non-homologous tail removal (3′NHTR) in double-strand break repair. In both pathways, Msh2–Msh3 binds double-strand/single-strand junctions and initiates repair in an ATP-dependent manner. However, we recently demonstrated that the two pathways have distinct requirements with respect to Msh2–Msh3 activities. We identified a set of aromatic residues in the nucleotide binding pocket (FLY motif) of Msh3 that, when mutated, disrupted MMR, but left 3′NHTR largely intact. One of these mutations, *msh3Y942A*, was predicted to disrupt the nucleotide sandwich and allow altered positioning of ATP within the pocket. To develop a mechanistic understanding of the differential requirements for ATP binding and/or hydrolysis in the two pathways, we characterized Msh2–Msh3 and Msh2–msh3Y942A ATP binding and hydrolysis activities in the presence of MMR and 3′NHTR DNA substrates. We observed distinct, substrate-dependent ATP hydrolysis and nucleotide turnover by Msh2–Msh3, indicating that the MMR and 3′NHTR DNA substrates differentially modify the ATP binding/hydrolysis activities of Msh2–Msh3. Msh2–msh3Y942A retained the ability to bind DNA and ATP but exhibited altered ATP hydrolysis and nucleotide turnover. We propose that both ATP and structure-specific repair substrates cooperate to direct Msh2–Msh3-mediated repair and suggest an explanation for the *msh3Y942A* separation-of-function phenotype.

1. Introduction

DNA mismatch repair (MMR) is a highly conserved DNA repair pathway that is critical for maintaining genome stability [1–3]. MMR is best known for recognizing and directing repair of nucleotide misincorporation or DNA slippage events that occur at the replication fork. MMR is initiated when replication errors are recognized and bound by MutS homologs, or Msh proteins. Prokaryotes encode a single MutS protein whereas most eukaryotes, including *Saccharomyces cerevisiae*, contain two distinct Msh complexes, Msh2–Msh3 and Msh2–Msh6, with separate but overlapping specificities. Msh2–Msh3 primarily binds and directs repair of both small (1 nucleotide) and larger (up to 17 nucleotide) insertion/deletion loops (IDLs) [4,5]. Msh2–Msh6 primarily directs repair of misincorporation events and small (1–2 nucleotide) IDLs [4,6]. Msh2–Msh3 also recognizes and binds some mispairs, particularly C–C mispairs [7]. Once bound to a mismatch (mispair or IDL), the Msh complex recruits the downstream MutL homolog (Mlh) complex, primarily Mlh1-Pms1 in yeast. The ternary complex formation is dependent on ATP-binding by the Msh complex and triggers subsequent steps in MMR, including helicase and exonuclease enzymes to remove the mismatch. Repair is completed by DNA resynthesis of the nascent strand and ligation of the DNA [1,2,6].

In addition to MMR of IDLs, *S. cerevisiae* Msh2–Msh3 is also required during genetic recombination [8–10]. It is required for the prevention of homeologous recombination, *i.e.* recombination between divergent sequences in which loop structures are formed [8,9,11]. Repair of large unpaired loops that can occur during meiotic recombination also requires Msh2–Msh3 as well as the structure-specific endonuclease Rad1–Rad10, which is part of the nucleotide excision repair (NER) pathway [12,13]. Rad1–Rad10

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cleaves at double-strand (ds)/single-strand (ss) DNA junctions with 3′ ssDNA tails [14–16]. Msh2–Msh3 and Rad1–Rad10 are also both required in a specialized pathway of double-strand DNA break repair (DSBR) that involves recombination intermediates with 3′ non-homologous tails (3′ NHTs), such as single strand annealing (SSA) and some gene conversion events [10,17,18]. DNA synthesis is required to complete repair, but DNA polymerases cannot prime from unannealed 3′ hydroxyl group. Therefore the 3′ NHTs must be removed to allow synthesis and subsequent ligation. Rad1–Rad10 is responsible for cleaving the tails, but requires partner proteins Msh2–Msh3 and Saw1 to be recruited to the 3′ NHTs [17,19,20]. Msh2–Msh3 has been proposed to stabilize the recombination intermediate to promote cleavage by Rad1–Rad10 [17,20].

In MMR, DNA-binding and ATP-binding activities of bacterial MutS and yeast and human Msh2–Msh6 complexes have been well-studied and demonstrated to be coordinated; DNA-binding leads to conformational changes in MutS and Msh2–Msh6 that are transmitted to the ATP-binding domain *via* the transmitter region [21–28]. Analogous conformational changes likely occur in Msh2–Msh3; mutations in the putative transmitter region of Msh3 lead to defects in both MMR and 3′NHTR *in vivo* [29]. Furthermore, it has been shown that the DNA-binding domains of MutS and Msh2–Msh6 modulate activity and conformational changes within the ATPase domain and *vice versa* [21,30–37]. The presence of DNA stimulates steady-state ATP hydrolysis, an effect that is abrogated when there are no free DNA ends [30,38,39]. Therefore dissociation from DNA is thought to provoke hydrolysis. In turn, ATP-binding reduces MutS and Msh2–Msh6 complex binding to specific DNA substrates and is predicted to promote the formation of a sliding clamp conformation that allows the complex to move away from the mismatch.

Fewer studies have examined the relationship between Msh2–Msh3 DNA-binding and ATPase activities[40–43]. Nonetheless, the coordinated regulation of DNA-binding and ATP-binding and hydrolysis by Msh2–Msh3 is thought to be critical for proper MMR function [40,42]. However, while there are similarities, the ATP binding and hydrolysis activities of human Msh2–Msh6 and human Msh2–Msh3 are distinct [41,42]. Furthermore, the requirements for ATP binding and/or hydrolysis in the Msh3 subunit are distinct for MMR and 3′NHTR [29]; mutations within the conserved FLY motif of Msh3 [44] predicted to alter the nucleotide binding pocket exhibited a strong defect in MMR but had much milder effects on 3′NHTR *in vivo* [29]. These observations led us to hypothesize that the type of DNA substrate (MMR *versus* 3′NHTR) might further regulate ATP-binding and/or hydrolysis by Msh2–Msh3.

To develop a mechanistic understanding of the differential requirements for ATP binding/hydrolysis *in vivo* in MMR and 3′NHTR, we performed an *in vitro* analysis of Msh2–Msh3 ATP-binding and ATP hydrolysis activities in the presence of distinct DNA substrates and then compared them to the activities of Msh2–msh3Y942A under the same conditions. This mutation changes the Y (tyrosine) of the FLY motif, which is predicted to form half of a nucleotide (ATP/ADP) sandwich in the Msh3 nucleotide binding pocket, stacking with the adenine [44]. Replacing the Tyr with Ala is predicted to widen the adenine-binding portion of the pocket and lead to fewer constraints on the positioning of ATP or ADP in the pocket. We used (a) a homoduplex DNA substrate to mimic non-specific DNA-binding, such as Msh2–Msh3 would encounter during a target search, (b) an MMR-specific substrate (a +8 loop; $(GT)_4$), (c) a splayed or (d) a 3′ flap substrate to mimic 3′NHTR intermediates (Fig. 1a). We found that the kinetics of ATP hydrolysis by Msh2–Msh3 were substrate-dependent, supporting a model in which distinct DNA substrates promote signature Msh2–Msh3 ATPase activity. This regulation was disrupted in Msh2–msh3Y942A and therefore required an intact Msh3 nucleotide binding pocket. Based on these data, we suggest a possible mechanistic explanation for the *msh3Y942A* separation-of-function phenotype *in vivo*.

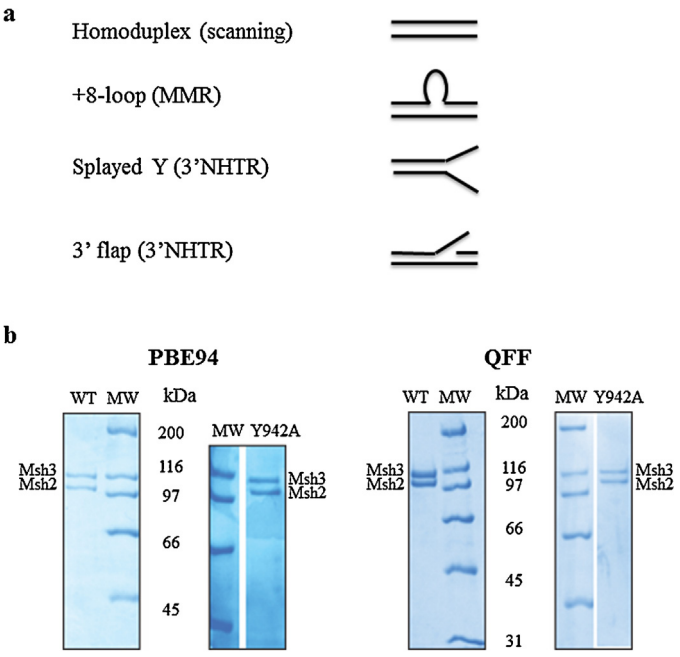

Fig. 1. DNA substrates and purified Msh2–Msh3 and Msh2–msh3Y942A. (a) The four different synthetic DNA substrates used in this study were homoduplex (non-specific), +8-loop (MMR), splayed Y and 3′ flap (3′NHTR) substrates. (b) Purified Msh2–Msh3 and Msh2–msh3Y942A (1.5 μg complex each) using the PBE94 purification (left) or the Q-Sepharose Fast Flow purification protocol (right). The protein complexes were analyzed by SDS-PAGE (8%) and stained with Coomassie Blue. Msh2 and Msh3 are indicated. The sizes of molecular weight markers (MW; Bio-Rad, broad range) are indicated alongside the gels in kDa.

2. Materials and methods

2.1. Reagents

Phosphoenol pyruvate (PEP), FTE nicotinamide adenine dinucleotide (NADH), pyruvate kinase, lactate dehydrogenase were obtained from Sigma. A 200 mM stock solution of PEP was made in 0.5 M Tris–acetate (pH 7.5). NADH was dissolved in 10 mM Tris–acetate (pH 7.5) and the concentration was determined spectrometrically, using an extinction coefficient of 6250 M^{-1} cm^{-1}. ATP was obtained from Amersham Pharmacia Biotech and was dissolved in 0.5 M Tris–HCl (pH 7.5), with the concentration determined spectrophotometrically using an extinction coefficient of 1.54×10^5 M^{-1} cm^{-1}. Oligonucleotides used to construct homoduplex, +8 loop, 3′ flap and splayed substrates were purchased from Integrated DNA Technologies, Coralville, IA and have been described previously [43].

Msh2–Msh3 was initially purified as described previously [45]. Over the course of this study, it became necessary to modify our purification protocol due to the fact that PBE94 is no longer available. In place of PBE94, we used Q-Sepharose Fast Flow (GE) as the first chromatography step. Induced cells were resuspended and frozen in 1 × MSH buffer (25 mM Tris–HCl pH 7.5, 1 mM EDTA) with 200 mM NaCl. The Q-Sepharose column was loaded at 200 mM NaCl and eluted with a linear gradient to 1 M NaCl. Msh2–Msh3 eluted at approximately 250 mM NaCl. The final PBE94 column was similarly replaced with Q-Sepharose, loaded at 200 mM NaCl and eluted at 500 mM NaCl. Msh2–msh3Y942A purified exactly as the wild-type Msh2–Msh3 complex (Fig. 1b). The *in vitro* activities of both Msh2–Msh3 and Msh2–msh3Y942A were indistinguishable in side-by-side comparisons of the two purification protocols (data