Distinct Nucleotide Binding/Hydrolysis Properties and Molar Ratio of MutSα and MutSβ Determine Their Differential Mismatch Binding Activities*

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MutSα (MSH2/MSH6) and MutSβ (MSH2/MSH3) are eukaryotic mismatch recognition proteins that preferentially process base-base and small insertion/deletion (ID) mispairs, respectively, despite the fact that cells contain a MutSα:MutSβ ratio of 10:1. To explore the mechanism underlying the differential mismatch recognition by these two proteins, purified human MutSα and MutSβ were analyzed individually and competitively for their abilities to interact with a T-G and an ID substrate. We show that MutSα has $K_D$ values of 26.5 and 38.2 nm for the G-T and ID substrates, respectively, and that MutSβ has $K_D$ values of 76.5 and 23.5 nm for G-T and ID, respectively. Consistent with these results, competitive binding assays revealed the following relative binding affinities: MutSβ-ID > MutSα-T-G > MutSα-ID $\gg$ MutSβ-T-G. Interestingly, binding of MutSβ to ID heteroduplexes is greatly stimulated when the MutSα:MutSβ ratio is $\approx$10. Distinct ATP/ADP binding and ATPase activities of MutSα and MutSβ were also observed. In the absence of DNA, ADP binding and ATPase activities of MutSβ are significantly higher than those of MutSα. However, interaction with DNA significantly stimulates the MutSα ATPase activity and reduces the MutSβ ATPase activity, the consequence being that both proteins exhibit the same level of hydrolytic activity. We conclude that the preferential processing of base-base and ID heteroduplexes by MutSα and MutSβ is determined by their significant differences in ATPase activity, ADP binding activity, and high cellular MutSα:MutSβ ratio.

DNA mismatch repair (MMR)* plays an important role in maintaining genome stability by primarily correcting both base-base mismatches and insertion/deletion (ID) mispairs generated during DNA replication (1). The MMR system is highly conserved as both prokaryotes and eukaryotes use a similar group of protein factors and a similar repair mechanism for correcting mispairs. MMR reactions in *Escherichia coli* and human cells have been reconstituted with purified proteins (2–4), which essentially involve mismatch recognition by MutS family proteins, removal of mispaired base by nucleases in a manner dependent on MutL family proteins and several other protein factors, and DNA repair synthesis by replicative DNA polymerases in concert with factors involved in DNA replication (1).

Mismatch recognition is a critical step of MMR. 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 heterodimeric complex. Both genetic and biochemical studies suggest that MutSα and MutSβ have 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 (5–8). A recent genetic study in yeast suggests that MutSβ may also play some role in the repair of base-base mismatches (9). Interestingly, cells make $\sim$10-fold more MutSα than MutSβ, and overexpression of MSH3 results in a strong mutator phenotype (10, 11), 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.

In addition to the mismatch binding activity, all MutS proteins, from *E. coli* to humans, contain an ATPase activity and ATP and ADP binding activities (12–14). Both the nucleotide binding and ATPase activities of the MutS family proteins are essential for MMR (15–19), but how these activities work in MMR is not fully understood. MutS family proteins can simultaneously bind ATP and ADP (20, 21) and under ADP exchange (8, 22–25) to induce MutS conformational changes, signaling downstream repair events. Mazur et al. (26) have recently demonstrated that two subunits of yeast MutSα exhibit differential nucleotide binding ability: 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 (27) or to verify MutS mismatch binding and authorize the eventual repair reaction (18). 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

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2 The abbreviations used are: MMR, mismatch repair; ID, insertion/deletion mismatch; nt, nucleotide.
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 in base-base and ID mismatch recognition, 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 involvement of these novel properties and activities in differential mismatch recognition by MutSα and MutSβ is discussed.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant MMR Proteins**—Human MutSα and MutSβ were expressed in insect cells using the baculovirus system. Baculovirus stocks for the human MSH2, MSH6, and MSH3 genes were generous gifts of Josef Jiricny (University of Zurich). MutSα and MutSβ were overexpressed and purified essentially as described previously (4, 17). The recombinant proteins were purified to near homogeneity. Protein concentrations were determined by the Bio-Rad protein assay kit.

**Gel Shift 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 dithiothreitol, [32P]-labeled oligonucleotide heteroduplexes, and MutSα and/or MutSβ in the presence of a 10-fold excess amount of unlabeled oligonucleotide homoduplex. The reactions were incubated on ice for 20 min, followed by the addition of 5 μl of 50% (w/v) sucrose. Samples were loaded on and separated by electrophoresis through a 6% nondenaturing polyacrylamide gel in buffer containing 50 mM Tris borate (pH 7.5) and 1 mM EDTA. The buffer was recirculated during electrophoresis (28). The gel was dried and analyzed by a Storm PhosphorImager (GE Healthcare).

**Nucleotide UV Cross-linking and ATPase Analyses**—The nucleotide cross-linking assays were performed essentially as described (26). Reactions were assembled and incubated on ice in nucleotide binding buffer containing 50 mM Tris-HCl (pH 8.0), 110 mM NaCl, 2 mM dithiothreitol, 100 mg/ml bovine serum albumin, 0.5 mM EDTA, and 5% glycerol in the presence or absence of 5 mM MgCl₂. Where specified, DNA heteroduplex or homoduplex was added 10 min prior to addition of nucleotide. MutSα or MutSβ was mixed with [α-32P]ATP, [α-32P]ADP, or [γ-32P]ATP and incubated for 10 min. Samples were then subjected to 10 min of UV cross-linking (Stratalinker), followed immediately by fractionation by 8% SDSPAGE. Radiolabeled bands were quantified using a PhosphorImager. [α-32P]ADP was generated by incubating [α-32P]ATP with hexokinase and purified as described (26). 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-32P]ATP, and the indicated amount of proteins and DNA substrates. After incubation at 37 °C for 10 min, the reactions were terminated and fractionated through a 20% denaturating polyacrylamide gel. 32P-containing species were detected and quantified by PhosphorImager.

**RESULTS**

**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 (Fig. 1A). 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 or MutSβ. 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.

**FIGURE 1. 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.
was co-incubated with the G-T and ID DNA substrates in competition with each other by gel shift analysis (Fig. 2). 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 Fig. 2A, although the increase in the ID/G-T ratio is associated with the increased 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 (Fig. 2A, 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 (Fig. 2B), the ID DNA substrate is bound preferentially in the examined conditions. When the two DNA substrates are equal in concentration (Fig. 2B, 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 (Fig. 2B, lanes 9–11). Comparing the corresponding reactions in Fig. 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-nM ID substrate, and the reaction products were analyzed by gel shift analysis. Protein-DNA complexes with MutSα and MutSβ were distinguished by their ability 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 (Fig. 3 and data not shown). Surprisingly, increasing amounts of MutSα stimulate binding of MutSβ to the ID DNA substrate. When a reaction contained a MutSα:MutSβ ratio ≥10 (Fig. 3A, 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 lane 6). No supershifted products were detected in the same reactions without MutSβ (Fig. 3B), 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 (Fig. 3C). 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 (18) or signaling protein translocation along the DNA molecule to initiate mismatch excision (23, 27). ATP hydrolysis by the MutS family ATPase requires two important cofactors: DNA and Mg2+ (21, 24, 26, 29). To explore whether MutSα and MutSβ possess differential ATP/ADP binding and hydrolysis activities, which may contribute to their distinct mis-
match recognitions, purified MutSα and MutSβ were incubated in the presence of [γ-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 (Fig. 4A, lanes 1–4), consistent with previous observations for yeast MutSα (26). In contrast, both the MSH2 and MSH3 subunits of MutSβ are cross-linked to ATP with similar efficiency (Fig. 4B, 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 (Fig. 4A, lanes 1–4), they significantly reduce the MutSβ-ATP interaction (compare lane 1 with lanes 2–4 in Fig. 4B). When the reactions were performed in the presence of Mg2+, which supports ATP hydrolysis, little 32P-labeled MutSα was detected in reactions containing [γ-32P]ATP (Fig. 4A, lanes 5–8), consistent with the fact that the 32P-labeled phosphate (at the γ-position) is hydrolyzed by MutSα ATPase activity (26). However, under the same conditions, enhanced cross-links were observed in the MSH2 subunit of MutSβ in the presence of DNA (Fig. 4B, lanes 6–8). This result suggests that MutSβ, 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 (Fig. 4B, lanes 6–8).

Similar cross-linking experiments were performed by substituting [γ-32P]ATP with [α-32P]ATP (Fig. 4, C and D). As expected, in the absence of Mg2+ (i.e. no ATP hydrolysis), the amount of ATP cross-links to individual subunits of MutSα or MutSβ is essentially the same as observed in reactions with [γ-32P]ATP (Fig. 4, compare lanes 1–4 in A and C for MutSα and in B and D for MutSβ). Under conditions that support ATP hydrolysis (i.e. in the presence of Mg2+), the MSH2 subunit but not the MSH6 subunit of MutSα was preferentially labeled (Fig. 4C, lanes 5–8), consistent with the observation with yeast MutSα (26). In the case of MutSβ, both subunits were well labeled, with a better cross-link for MSH2 (Fig. 4D, lanes 5–8). Apparently, DNA plays an inhibitory role in MutSβ cross-linking with [α-32P]ATP, as judged by the fact that much intense labeling was detected for both MSH2 and MSH3 in the absence of DNA substrates (Fig. 4D, lane 5). Because Mg2+ stimulates ATP hydrolysis and because DNA substrates selectively block Mg2+-provoked ATP hydrolysis by MutSβ (Fig. 4B, lanes 6–8), the 32P-labeled proteins in Fig. 4D could result from cross-linking to [α-32P]ATP (without hydrolysis), [α-32P]ADP (with hydrolysis), or both.

To distinguish these possibilities, cross-linking experiments were conducted in the presence of [α-32P]ADP. As shown in Fig. 4E, only the MSH2 subunit of MutSα interacts with ADP. Interestingly, this interaction is greatly enhanced in the presence of Mg2+ (compare lanes 5–8 with lanes 1–4, respectively, in Fig. 4E), and the enhancement is more pronounced in reactions containing heteroduplexes (lanes 7 and 8). The cross-linking experiments performed with MutSβ reveal that in the absence of Mg2+, the protein behaves similarly to MutSα, i.e. only the MSH2 subunit cross-links to ADP (Fig. 4F, lanes 1–4); however, addition of Mg2+ 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 Mg2+ and the absence of DNA (Fig. 4F, lane 5); DNA greatly reduces the affinity of MSH3 but not that of MSH2 for ADP (lanes 6–8). Similar to the interaction between MutSα and ADP, there appeared to be a little more ADP binding to the MSH2 subunit of MutSβ in the reaction containing the ID substrate (Fig. 4F, lane 8). These results may explain why Mg2+ is required for mismatch binding by MutSβ (29) and why MutS proteins in their ADP-bound form possess a higher affinity for heteroduplexes (18, 30). Comparing data in Fig. 4 (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 (17, 19). It has also been shown that the ATPase activity of MutSβ could be stimulated by homo- or heteroduplex DNA although to different extents (20, 31, 32). 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 [γ-32P]ATP in the presence or absence of DNA substrates. The 32P-containing species, i.e. the unreacted [γ-32P]ATP and the hydrolyzed [γ-32P]phosphate, were detected after gel electrophoresis (Fig. 5, 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 (Fig. 5, C and D). However, DNA substrates, regardless of a homoduplex and a heteroduplex, significantly stimulated the ATPase activity of MutSα (Fig. 5D; also compare lane 7 with lanes 8–10 in Fig. 5A), consistent with previous observations. Surprisingly, DNA substrates were found to inhibit MutSβ ATPase activity.
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by ~ 20% (Fig. 5D; also compare lane 7 with lanes 8–10 in Fig. 5B). These results differ somewhat from those of Fishel and co-workers (8) 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 Fig. 5D), suggesting that the DNA-associated ATPase activity of MutS proteins is not related to mismatch binding specificity but to the downstream signaling of MMR.

DISCUSSION

This study investigates molecular mechanisms by which MutSα and MutSβ preferentially process base-base mismatches and ID mispairs using purified human MMR proteins. Some interesting observations have been made in this study, including enhancement of the MutSβ-ID interaction by excess amount of 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 of the puzzling phenomena in MMR is that MutSβ dominantly binds and directs repair of small ID mispairs, despite the fact that the amount of MutSβ in human cells is only one-tenth the amount of MutSα (10) and that MutSα also recognizes ID heteroduplexes (reviewed in Ref. 1). Our work presented here provides some significant insights into this question. 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, 26.5, 38.2, 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 for ID heteroduplexes (Fig. 3). This finding explains why cells have to 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 against each other for MSH2 in vivo. Previous studies show that overexpression of MSH3 greatly reduces the MutSα:MutSβ ratio, leading to a mutator phenotype (10, 11). This is apparently because base-base mismatches, which are poor substrates of MutSβ (Fig. 1) (4), are left 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 stimulating MutSβ activity.

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 (4), 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 (4). 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 Mg2+ and 110 mM NaCl), MutSβ pos-
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 environment in living cells (which includes many other components and conditions that are different from those in reconstituted assays with purified components) may modulate MMR in a more 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.

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