Identification of the protein components of mismatch binding complexes in human cells using a gel-shift assay

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Abstract In eukaryotes, mismatch recognition is thought to be mediated by two heterodimers, hMutSα (hMSH2+hMSH6), which preferentially binds to base-base mismatches and hMutSβ (hMSH2+hMSH3), which binds to insertion/deletion loops. We studied these mismatch binding activities in several human cell lines with a gel-shift assay using various mismatch oligonucleotides as substrates. Both hMutSα and hMutSβ activities could be detected in various human cell lines. In cells with amplified copies of the *hMSH3* gene, a large increase in hMutSβ and a reduction in hMutSα were observed. To identify the composition of each mismatch binding complex, the protein-DNA complexes were transferred from gel-shift polyacrylamide gel to a polyvinylidene difluoride membrane and were subjected to immunoblot analysis with an enhanced chemiluminescence protein detection system. The results clearly demonstrated that hMutSα detected by the gel-shift assay was composed of hMSH2 and hMSH6, while hMutSβ was composed of hMSH2 and hMSH3. Our data, therefore, support a model whereby formation of hMutSα and hMutSβ is mutually regulated. Combination of a gel-shift assay with immunoblotting (shift-Western assay) proved to be a highly sensitive technique and should be useful for studying the interactions between DNA and binding proteins, including DNA mismatch recognition.

Key words: DNA repair; Gel-shift; hMSH3; Protein-DNA complex; Western blotting; Human HL60 cell

1. Introduction

Mismatch repair (MMR) is important for maintaining the genome stability in many species [1,2]. Its mechanism has been extensively studied in *Escherichia coli*, where it was observed that MutS has a mismatch binding activity and is an essential component of mismatch recognition, the first step in the MMR pathway. In human, a number of MutS homologues (hMSH) have been identified [3–8]. Using a baculovirus expression system, we previously showed that hMutSα, a heterodimer composed of hMSH2 and hMSH6, preferentially binds to DNA heteroduplexes with base-base mismatches and one nucleotide insertion/deletion loops (IDLs), whereas hMutSβ, composed of hMSH2 and hMSH3, binds to DNA heteroduplexes with 2–4 nucleotide IDLs [9]. These heterodimers were recently purified from HeLa cells [10] and hMutSα was shown to restore base-base mismatch and one nucleotide IDL repair activity to extracts derived from hMSH2-deficient LoVo cells, while hMutSβ restored only the latter [10,11]. A similar restoration of MMR activity was observed when recombinant hMutSα or hMutSβ was added to extracts from MMR-deficient HEC59 cells [12]. Thus, hMutSα and hMutSβ appear to be essential for both mismatch recognition and repair in human cells.

The first human gene encoding a MutS homologue, *hMSH3*, was isolated in our laboratory [5]. It is located immediately upstream of the gene encoding dihydrofolate reductase (DHFR) and is bidirectionally transcribed from the opposite strand of the promoter [13]. Because of their close proximity, the *DHFR* and *hMSH3* genes are often co-amplified when cells are treated with increasing concentrations of methotrexate (MTX), a specific inhibitor for DHFR. Recent studies have shown that overexpression of hMSH3 sequesters hMSH2 into hMutSβ and inhibits the formation of hMutSα, resulting in a defective base-base mismatch repair [10,12] and suggesting that the formation of hMutSα and hMutSβ are mutually regulated in human cells.

In order to study MMR in various cancer cells, we wanted to develop a rapid system for detecting mismatch binding complexes using a gel-shift assay without having to purify the complexes. While the existence of mismatch binding activity has been previously demonstrated in cell extracts using the gel-shift assay [12], the identity of each component was not completely characterized. In the present study, we establish the shift-Western assay which could be used to detect individual components of mismatch binding complexes. This method should enable study of the interaction between DNA and binding proteins in crude cell lysates.

2. Materials and methods

2.1. Cell lines and nuclear extracts

MTX-resistant cell lines (HL60R, HeLaR and K562R) were maintained as described previously [5]. Each cell line contains more than 100 copies of the *DHFR* gene per cell. Nuclear extracts were prepared from 1 liter cultures of cells according to the method of Dignam et al. [14], dialyzed against buffer D containing 20 mM HEPES-OH (pH 7.8), 100 mM KCl, 0.2 mM EDTA, 1.0 mM dithiothreitol (DTT), 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 0.05% NP-40 and 20% glycerol and were stored in small aliquots at −80 °C. Protein concentrations were measured with Protein Assay Reagent (Bio-Rad) using bovine serum albumin as a standard.

2.2. Expression and purification of recombinant hMutSβ

Baculovirus vectors carrying cDNA inserts encoding hMSH2 or hMSH3 were used to infect cultures of Sf9 cells. Recombinant hMutSβ was then purified as described previously [9].

2.3. Antibodies (Abs)

Anti-hMSH2 Ab (Santa Cruz) was raised in rabbits against a peptide corresponding to amino acids 2–21. Anti-hMSH6 Ab, a gift from Dr Josef Jiricny, was raised in rabbit against an overexpressed fragment of hMSH6 corresponding to amino acids 750–928 [15]. Ab against the full-length hMSH3 was prepared as follows: insoluble

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full-length hMSH3 expressed in a baculovirus system was solubilized in 4 M guanidine HCl and dialyzed against buffer containing 0.5 M NaCl, 0.05 M Tris-Cl (pH 7.5), 1 mM DTT, 1 mM EDTA, 1 mM PMSF and sequentially reduced concentrations of urea (4–0 M). The dialyzed protein was then used to raise Abs in rabbits. The IgG fraction was purified from antiserum using E-Z-SEP Ab purification kits (Amersham Pharmacia Biotech). Non-specific binding in the anti-hMSH3 IgG fraction was removed using a Hi-Trap NHS-activated column (Amersham Pharmacia Biotech) on which *E. coli*-expressed hMSH3 (Novagen, pET-15b) was immobilized.

2.4. Western analysis

Protein samples (50 μg) were subjected to electrophoresis in 10% SDS-polyacrylamide gels and then transferred to activated polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore) for 15 h at 4 °C and 30 V. The transfer buffer contained 25 mM Tris, 192 mM glycine and 20% methanol. Membranes were then placed in buffer containing 10 mM Tris, 150 mM NaCl, 0.05% Tween 20 (TBS-T) and 5% skim-milk and labelled with anti-hMSH2, anti-hMSH3 or anti-hMSH6 Abs. The resultant bands were stained with goat anti-rabbit IgG(H+L) conjugated with horseradish peroxidase (Promega), detected using the ECL Plus Western blot detection system-II (Amersham Pharmacia Biotech) and exposed to X-ray film (Fuji) for less than 2 min.

2.5. Oligonucleotides and labelling

The following oligonucleotides were used. A synthetic 34-mer oligonucleotide, 5′-AATTCCCGGGGATCCGTCGACCTGCAGCCA-AGCT-3′, was labelled with [γ-^{33}P]ATP at the 5′ end using Megalabel (Takara) and was annealed to its complementary oligonucleotide, 5′-AGCTTGGCTGCAGGTYGACGGATCCCCGGGAATT-3′, where Y represents pyrimidine (C, T: [16]). The labelled oligonucleotide was also annealed with modified complementary oligonucleotides, where Y = C and either A, AC or ACGT was inserted between T-15 and C-16, respectively, creating +1, +2 and +4 IDLs. Non-labelled heteroduplexes were prepared in the same way and used for the shift-Western assay described below.

2.6. Gel-shift assay

The binding reaction was carried out in 20 μl of buffer containing 25 mM HEPES-OH (pH 8.0), 0.5 mM EDTA, 10% glycerol, 0.1 mM $ZnCl_2$, 0.5 mM DTT and 2 μg calf thymus DNA (Gibco BRL). Nuclear extracts (10 μg) or recombinant hMutSβ protein (1 μg) were added to the buffer and incubated for 10 min at room temperature, after which 400 fmol of radiolabelled heteroduplexes was added and further incubated for 30 min at room temperature. Then, 2 μl of 20% (w/w) ficoll was added to the reaction mixtures and half of each sample was loaded onto a 5% polyacrylamide gel (160×160×2 mm) that was pre-electrophoresed at 110 V for 90 min. Electrophoresis was carried out in 0.25×TBE buffer (110 V, 4 °C) until the bromophenol blue dye in a side lane migrated 9 cm (4.0–4.5 h). The gels were subsequently dried and exposed to X-ray film (Fuji) for 2 days. To examine the effect of the Abs on the binding reaction, an IgG fraction or antiserum was added to the reaction mixture and incubated for 40 min at 4°C before the addition of radiolabelled substrates.

2.7. Shift-Western assay

Samples were prepared with unlabelled heteroduplexes and fractionated by polyacrylamide gel electrophoresis as described in the Section 2.6. After the electrophoresis, the gels were blotted on PVDF membranes at room temperature for 3 h at 150 mA in a buffer containing 25 mM Tris, 192 mM glycine, 5% methanol using a semidry blotting apparatus (Amersham Pharmacia Biotech). For immunoreactive detection, membranes were blocked overnight at 4°C in TBS-T containing 5% skim milk. Bands were visualized using enhanced chemiluminescence (ECL) as described for the Western analysis. Ab stripping was carried out according to the manufacturer's instructions with the ECL Plus system.

3. Results

3.1. Mismatch binding activities in human cells

The nuclear extracts from HL60, HeLa and K562 (data not shown) cells were incubated with radiolabelled heteroduplexes and analyzed using the gel-shift assay (Fig. 1B, odd number lanes). At least five shifted bands were detected. Band 3 was seen with a control homoduplex (Fig. 1B, lane 1), but the other four bands were mismatch-specific and were completely blocked in the presence of a 25-fold excess of non-labelled substrate (data not show). The faint band 1 was seen with heteroduplexes containing 1–4 nucleotide IDLs in HL60 and HeLa cells (Fig. 1B, lanes 5, 7, 9 and 13), while band 2 was clearly detected in all three cell types with heteroduplexes containing G/T mismatches and one nucleotide IDLs (Fig. 1B, lanes 3, 5 and 11). This binding pattern suggests that bands 1 and 2 correspond to binding complexes containing hMutSβ and hMutSα, respectively.

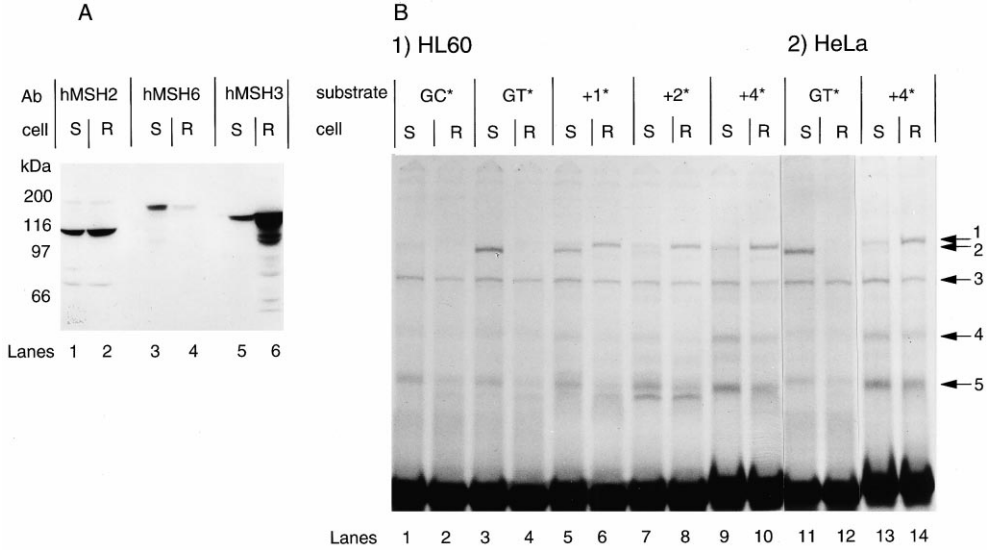

Fig. 1. Mismatch binding activity in human MTX-sensitive (S) and -resistant (R) cells. (A) Western blot analysis of three MutS homologues. Nuclear extracts (50 μg) from HL60S and HL60R cells were analyzed. Molecular weight markers (kDa) are drawn on the left. (B) Gel-shift assay. Nuclear extracts (10 μg) from HL60S/R (lanes 1–10) and HeLaS/R (lanes 11–14) cells were incubated with various radiolabelled substrates and analyzed as described in Section 2.

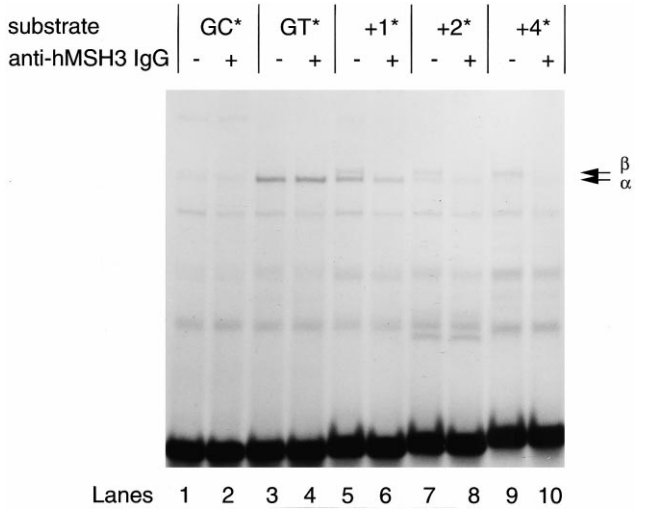

Fig. 2. The effect of anti-hMSH3 IgG on hMutSβ formation in a gel-shift assay. Ab was added to the reaction mixtures in the even-numbered lanes. The arrows on the right indicate the mismatch binding complexes, hMutSα (α) and hMutSβ (β), respectively.

3.2. Mismatch binding activities in MTX-resistant cells

We next studied mismatch binding activities in MTX-resistant cells, where the *hMSH3* and *DHFR* genes were co-amplified about 100-fold [5]. Overexpression of hMSH3 in MTX-resistant cells was confirmed by Western analysis. As compared to MTX-sensitive HL60 cells (HL60S), a large increase in hMSH3 and a concomitant reduction in hMSH6 were detected in MTX-resistant HL60 cells (HL60R). There was no substantial difference in hMSH2 levels between the two cell lines (Fig. 1A). When the nuclear extracts from MTX-resistant cells were incubated with heteroduplexes, there was a remarkable increase in the density of band 1 (Fig. 1B, lanes 6, 8, 10 and 14). Band 2, by contrast, was barely detectable after incubation with heteroduplexes containing G/T mismatches and one nucleotide IDLs (Fig. 1B, lanes 4, 6 and 12).

3.3. Components of mismatch binding complexes

The components of mismatch binding complexes were studied by exposing the complexes to the respective Ab against each MutS homologue. Addition of anti-hMSH3 IgG diminished band 1 (Fig. 2, lanes 6, 8 and 10), whereas band shift patterns were partially affected by anti-hMSH2 IgG or anti-hMSH6 Ab (data not shown).

To identify the protein components of the complexes directly, we used the shift-Western assay in which shifted bands were transferred to membranes and stained with the Abs. Nuclear extracts were incubated with radiolabelled or unlabelled substrates and applied side by side to the same gel. The recombinant hMutSβ protein expressed in the baculovirus system was then added to the right hand side of each gel half as a control (Fig. 3). After electrophoresis, the gel was transferred to a membrane which was cut in two and the membrane half containing radiolabelled substrates was exposed to a X-ray film. The recombinant hMutSβ showed a mismatch-specific band at a position identical to band 1 (Fig. 3A). The gel half containing unlabelled substrates was sequentially incubated with anti-hMSH2 Ab (Fig. 3B-1), anti-hMSH3 IgG (Fig. 3B-2) and anti-hMSH6 Ab (Fig. 3B-3), after stripping the previously applied Ab. Proteins were detected by staining with secondary Ab using the enhanced chemiluminescence system. Incubation with Ab against hMSH2 yielded two bands at positions identical to bands 1 and 2 in Fig. 3B-1, whereas incubation with Ab against hMSH3 or hMSH6 each yielded a single band at a position identical to band 1 or band 2, respectively (Fig. 3B-2 and -3). The recombinant hMutSβ was stained only with Ab against hMSH2 or hMSH3 (Fig. 3B-1 and -2). The two other mis-

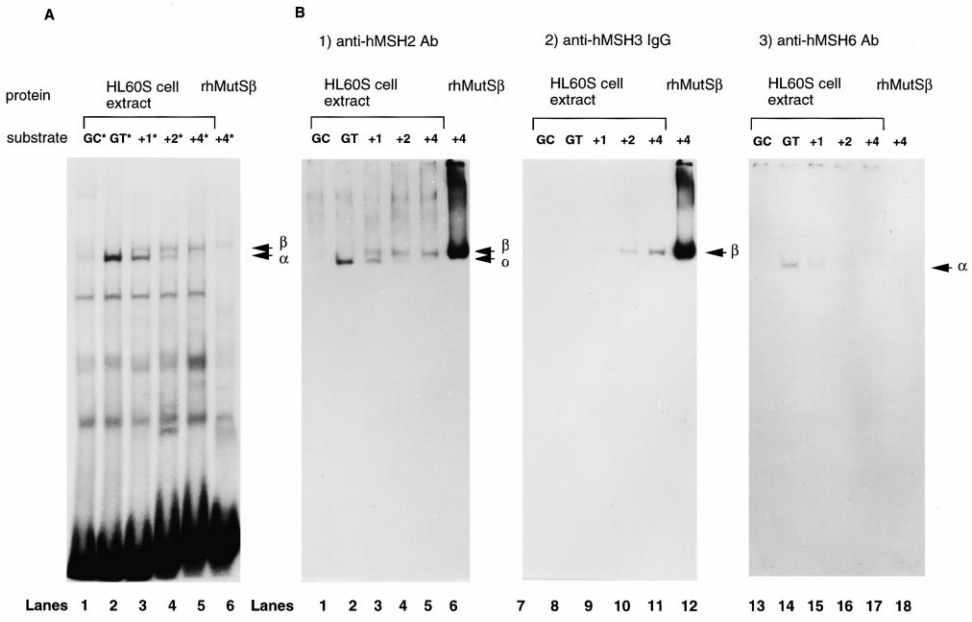

Fig. 3. Identification of hMutSα and hMutSβ binding activities using the shift-Western assay. (A) Gel-shift assay. Nuclear extracts from HL60S cells or recombinant hMutSβ (rhMutSβ: lane 6) were incubated with various radiolabelled substrates. (B) Shift-Western assay. A similar mixture of non-labelled substrates was simultaneously incubated and loaded onto the remaining half of the same gel. Protein-DNA complexes were transferred to a PVDF membrane and the membrane was sequentially treated with anti-hMSH2 (B-1), anti-hMSH3 (B-2) and anti-hMSH6 Abs (B-3).

match-dependent bands (bands 4 and 5 in Fig. 1) were unlabelled, indicating that those complexes did not contain hMSH2, hMSH3 or hMSH6 and may not be involved in MMR [17,18].

The above results directly demonstrate that band 1 corresponds to hMutSβ (hMSH2+hMSH3), while band 2 corresponds to hMutSα (hMSH2+hMSH6). They also reveal the mismatch binding-specificity of the two MutS heterodimers in the cell: hMutSα (band 2) binds to G/T mismatches and one nucleotide IDLs, while hMutSβ (band 1) binds to heteroduplexes with 1–4 nucleotide IDLs. Redundant binding by hMutSα and hMutSβ was also observed with 1 and 2 nucleotide IDL substrates (Fig. 3A, lanes 3 and 4), as has been seen with recombinant complexes [9].

4. Discussion

In the present study, we established the shift-Western assay and analyzed mismatch binding complexes. Our results clearly demonstrate that the G/T mismatch and one nucleotide IDL binding complex is composed of hMSH2 and hMSH6 in human cells, while the 1–4 nucleotide IDL mismatch binding complex is composed of hMSH2 and hMSH3. This method enabled us to quickly distinguish multiple mismatch binding complexes even when their molecular masses are very similar (e.g. hMutSα is 265 kDa and hMutSβ is 232 kDa). Thus, the shift-Western assay should be useful for studying the MMR activity in a variety of cell types.

Gel-shift assays are widely used to study protein-DNA interactions. This method is simple to perform and suitable for quantitative analysis of large numbers of samples. However, its drawbacks are that there may be numerous, non-specific band shifts and the electrophoretic patterns are easily affected by the experimental conditions. Consequently, identification of the protein components in the shifted bands is essential for interpretation of the results and Abs against putative protein components are included in the reaction mixtures. Nonetheless, the results are frequently inconsistent. Ab-protein binding may result in either inhibition of protein-DNA binding or supershift of the complexes, depending upon the location of the epitope on the protein and nature of the Ab. Moreover, the affinity of some Abs appears to be too low for them to be useful. In our experiments, complete depletion of the hMutSβ-IDL complex was observed when anti-MSH3 IgG was included. However, only partial reduction of the intensities of hMutSβ-IDL and hMutSα-G/T and one nucleotide IDL were seen when anti-MSH2 and anti-MSH6 Abs were used.

The shift-Western assay was designed to detect the presence and the relative abundance of proteins within specific protein-DNA complexes [19]. Combination with an ECL detection system significantly increased the sensitivity of the assay and enabled the identification of proteins even when the binding affinity of the Ab was low. The anti-hMSH2 and anti-hMSH6 Abs used in our study were unable to supershift protein-DNA complexes, but were sufficient for detection of protein components in a shift-Western assay. Thus, this technique should be useful for studying protein-DNA complexes containing multiple protein components or multiple binding sites on DNA fragments.

The data strongly support a model recently proposed by Drummond et al. [10], which postulates that formation of hMutSα and hMutSβ are mutually regulated and that hMutSα and hMutSβ heterodimers compete for a limited quantity of hMSH2. In the presence of excess hMSH3, available hMSH2 would be used up through generation of hMutSβ and would, therefore, be unavailable for generation of hMutSα. Lack of hMutSα activity was previously found to result in defective repair of base-base mismatches in HL60R cells [10,12]. Our experiments show that this phenomenon also occurs in other MTX-resistant HeLaR and K562R cells.

The first *mutS* homologue discovered in humans was *hMSH3* [5], but the function of its putative product has remained uncertain until recently. Now, there are several findings suggesting that hMSH3 may be involved in carcinogenesis. First, decreased expression of *hMSH3* was found in bone marrow cells of some patients with hematological malignancies [20]. Second, one base deletion and insertion at the $(A)_8$ track in exon 7 of the *hMSH3* gene were found in various human tumors and in cell lines with microsatellite instability [21–23]. Thus, it is possible that this mutation causes the genomic instability at the 1–4 nucleotide IDLs seen in at least some cancer cells [22]. Mutation of the *hMSH3* gene may also be the result of an earlier MMR error. Once the mutation is introduced, the defective hMSH3 activity may enhance genomic instability and accelerate tumor progression. Finally, our study suggests a third possibility. MTX is a widely used anti-cancer agent, but its efficacy is often diminished by the development of cell-resistance due to amplification of the *DHFR* gene [13,24]. It may, therefore, be that secondary tumors developing after treatment of the primary cancer derive from MTX-resistant cells in which overexpression of hMutSβ has resulted in a defective hMutSα activity.

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