A biotinylated MutS fusion protein and its use in a rapid mutation screening technique

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Abstract

The Escherichia coli DNA mismatch repair protein, MutS, binds single base pair mismatches and short deletions in vivo and in vitro. To adapt this protein for mutation detection, a fusion protein of E. coli MutS with a biotinylated peptide domain has been constructed (MutSb). The biotinylation tag facilitates MutS detection and binding by avidin without significantly altering the DNA mismatch binding properties of MutS in vitro. We describe a novel and rapid mutation detection method with MutSb using streptavidin-coated magnetic beads and demonstrate that MutSb can also be used to remove mismatch containing DNA fragments from a mixture of DNA fragments in solution.

Keywords: Heteroduplex DNA; Mismatch-binding; Mutation detection; MutS

1. Introduction

Screening for unknown single base pair mutations is one of the more time consuming and expensive elements of identifying disease causing genes in humans and other organisms. Several useful screening methods exist, but those in widespread use such as heteroduplex analysis and single strand conformation polymorphism analysis (SSCP) have a disadvantage in that they depend on changes in gel mobility caused by mutations and hence require gel electrophoresis. No single condition is more than 80% sensitive and a combination of methods is often necessary [1,2]. Thus, new rapid mutation detection techniques, especially those that are potentially automatable, would be useful tools.

The mismatch repair proteins of Escherichia coli offer potentially powerful reagents for mutation detection in eukaryotes and prokaryotes [3,4]. In vitro, MutS binds preferentially to single base pair (bp) mismatched duplex DNA and can recognize deletions or mismatches of up to 4 bp [4–6], although certain mismatches such as C:C are poorly recognized [7,8]. DNA mobility shift experiments show that although MutS binds to mismatch-free DNA, it exhibits 5-fold to 17-fold greater binding to mismatch containing double stranded oligonucleotides [5]. The potential to use MutS as a mutation detection reagent has also been demonstrated by several investigators using different methods, including gel retardation, nuclease protection, a filter binding assay, and most recently in combination with MutH and MutL [8–11]. Relying on this property of prefential mismatch binding, MutS has been used together with MutH and MutL, as the cornerstone of a novel genetic linkage technique called genomic mismatch scanning, that enriches for identical by descent, genomic regions shared by individuals with a disease or trait in common [12].
We have constructed a MutS fusion protein containing a peptide domain that is biotinylated in *E. coli*. The addition of this 13 kD polypeptide to the N-terminus of MutS does not disrupt mismatch binding. It is a useful tool for protein purification, immobilization and detection and can be used to enrich mismatch containing DNA from a mixture of DNAs. Using streptavidin-coated magnetic beads, MutSb is used to extract mismatch containing heteroduplex DNA from solution in the presence of other non-mismatch containing DNA fragments. MutSb is also employed to screen for mutations in a 420 bp polymerase chain reaction (PCR) product, in a non-gel based assay.

2. Methods

2.1. Construction of MutSb overexpression plasmid

pMutSb was constructed from three plasmids pMS312 [4], pT7.7 [13], and PinPoint Xa-1 (Promega). pMS312 was digested with ApoI and BglII, and the 3126 bp fragment containing muts was inserted into the PinPoint XaI polylinker between the NruI site and the BglII site using a NruI/ApoI synthetic linker oligonucleotide (5'CATTGATGCAATAAGAA3'/5'AATTTTCTA- TTGCACACTG3'). This created the fusion gene, mutsb, downstream from a tac promoter. Base pairs 1–378 of mutsb encode the biotinylation sequence (biotinylated lysine codon is 262–264 bp), 379–390 encode a factor Xa cleavage site, and 391–2952 encode the 853 amino acids of *E. coli* muts. This construct which used the tac promoter did not allow sufficient overexpression in JM101 cells. Thus, the entire muts fusion gene was excited on a PstI–HindIII 3069 bp fragment which retained the ribosome binding site but excluded the tac promoter of PinPoint XaI. This fragment was inserted between the PstI and HindIII sites of the polylinker of pT7.7 immediately downstream from the T7 promoter to create the 5533 bp plasmid pMutSb. The plasmid contains a ColE1 origin and beta lactamase gene.

2.2. MutSb overproduction and purification

pMutSb was transfected into BL21(DE3) cells and MutSb protein was overproduced by induction with IPTG according to standard methods [14]. From a 1 l culture, 5 g of cell paste was suspended in 20 ml of 50 mM Hepes pH 7.8, 50 mM KCl, 1 mM DTT, and 0.1 mM EDTA (MutSb buffer) and lysosome (100 µg/µl), on ice. All further processing was carried out at 4°C. Cells were lysed by sonication and insoluble material was pelleted by centrifugation for 20 min at 12 000 × g. Ammonium sulfate was slowly added to the soluble fraction until it was 31% saturated, and the solution was stirred for 45 min. The pellet containing the majority of the MutS (AS) was obtained by centrifugation at 15 000 × g for 30 min and was resuspended in 2 ml of MutSb buffer. A one step column purification was accomplished using monomeric avidin coupled to agarose beads (Soft-link and Ultra-Link; Pierce) according to the directions of the manufacturer, with a few modifications. Binding was performed in batch mode, while elution was performed in column mode. The AS fraction was diluted to a final volume of 10 ml and incubated with 5 ml of beads. After washing, approximately 60–75% of the MutSb remained bound to the beads. Elution buffer contained 0.2 M NaCl in addition to 20 mM biotin. Active protein was eluted at a concentration of 50–200 ng/µl. Recentrifugation and dialysis into MutSb buffer was accomplished using the Micro Pro Dicon Vacuum Dialyzer with a 25 000 MW cut off (Spectrum). Protein concentration was determined using the Bradford protein assay (Biorad). To check for nuclease activity, 1 µl of MutSb (35 pmol) was incubated with 1 µg of HindIII digested linearized pBluescript (SK—) at 4°C and 37°C for 1 h. Neither a change in original band intensity nor digestion products indicative of degradation by nucleases was observed.

2.3. Oligonucleotide preparation

Oligonucleotides of the following sizes and sequences, as well as their complementary strands were synthesized. The complementary strand is shown as an example for the 20 mer:

(1) 20 mer: 3'-TAAAAAGATTACGTGAGTAC-5'; 5'-AATTTTCTAXTGCACTCATG-3' (X = G, A).

(2) 64 mer: 5'-GACTGTCAAGTTCACGATG-GATCCACATGTGCACCGXGCTCAG-GTCTCTGGAATACTAACG-Y (X=G or A).

(3) 75 mer: 5'-TAGGTTGAGCTATAAGATACCGG- GAAATTCCXGAATGCGTAATCATGGT- CATAGCTGTTTCTGTTGAAATTTG3'- (X = C or T). Complementary strand contains G at the X position.

Oligonucleotides were end-labeled with 32P, using T4 polynucleotide kinase unincorporated nucleotide was removed, and oligonucleotides were annealed with the appropriate unlabeled complementary strand in a ratio of 4:5 [5]. Oligonucleotides greater than 20 bp, as well as the 420 bp PCR product described below were denatured at 95°C for 3 min and allowed to reanneal by slowly coming to room temperature over 2 h and subsequently stored at −20°C. No single stranded products were seen on non-denaturing gels, indicating complete renaturation of the labeled product.
2.4. Analysis of KCNA1 mutations

A 420 base pair region of KCNA1 (Genbank accession L02750; [15,16]) that included a polymorphism was amplified by PCR using primers: 5'-AGGAATGTAGCCACACATTG-3' (forward) and 5'-GAGGCATGGGA-GAAGTTCC-3' (reverse). The PCR mixture contained 40 ng of human genomic DNA, 5 units AmpliTaq DNA polymerase (Perkin-Elmer/Cetus), 1 \( \mu \)M of each primer, 200 \( \mu \)M dNTPs, 10 mM Tris (pH 9.0), 50 mM KCl, 1.5 mM MgCl\(_2\), 0.1% Triton X-100 and 0.01% gelatin in a final volume of 50 \( \mu \)l. PCR was performed in a Perkin Elmer 480 thermocycler using an initial 5 min denaturation at 95°C, 30 cycles of a 30 s denaturation step at 94°C, annealing for 40 s at a temperature of 58°C and extension at 72°C for 1 min. This was followed by a final 5 min extension cycle at 72°C. Unincorporated primers and nucleotides were removed using the Qiaquick spin column (Qiagen). Primers 5'-GTGATGTCGCGAGGAGCTG-3' (forward) and 5'-TTAACACCCTGTCAGATGCTG-3' (reverse) were used to PCR amplify the 1482 bp KCNA1 gene from 40 ng of human genomic DNA of heterozygotes and homozygotes using touchdown PCR according to Brown et al. [15]. In both the 420 and 1482 bp products from heterozygotes, the polymorphism results in a C to T transition at position 684, resulting in half of the reannealed heterozygote DNA containing a GT or CA mismatch.

2.5. Band shift assays

One microliter of the AS fraction (35 pmol), purified MutSb (6.0 pmol) or purified MutS (17 pmol), purchased from USB, was added to a mixture of 32P end-labeled mismatch-containing (GT) or mismatch-free (AT) oligonucleotides (0.2 pmol), 1 \( \mu \)l of non-labeled AT (40-200 pmol) oligonucleotide, in buffer containing 20 mM Tris HCl (pH 7.8), 5 mM MgCl\(_2\), and 1 mM DTT in a total volume of 5 \( \mu \)l and incubated on ice for 30 min after thorough mixing. 1.7 \( \mu \)l of 50% sucrose was added, and 3 \( \mu \)l of the mixture was run on a 6% non-denaturing polyacrylamide gel [5].

2.6. Magnetic bead mismatch separation and detection

Magnetic beads were used to bind mismatches in solution in two ways. In the first, 1 \( \mu \)l of the AS fraction was mixed with 0.01–0.4 pmol of radiolabeled double-stranded oligonucleotide and an excess of unlabeled competitor in a total volume of 5 \( \mu \)l, as previously outlined, for 10–15 min. Two to five microliters of streptavidin coupled magnetic beads, consisting of 50–250 pmol of streptavidin (MPG Streptavidin, CPG) equilibrated in MutSb buffer were added and the mixture was incubated on ice for an additional 10 min.

In the second procedure, magnetic beads were pre-bound with MutSb in batches by incubating 100 \( \mu \)l of beads with 40 \( \mu \)l of AS in a total volume of 200 \( \mu \)l for 15 min at 4°C, followed by 3 rinses in MutSb buffer and resuspension in 100 \( \mu \)l of MutSb buffer. At this stage, the beads may be frozen at \(-70°C\) with retention of mismatch-binding activity for at least 1 thaw cycle. For mismatch detection, 5 \( \mu \)l of MutSb magnetic beads were incubated with radiolabeled oligonucleotide or PCR product and unlabeled AT oligonucleotide in a 7 \( \mu \)l reaction for 10–15 min on ice. In both cases, after DNA binding the beads were washed three times by resuspension in MutSb buffer and resuspended in a final volume of 200 \( \mu \)l. The beads were captured by holding the microfuge tube to a magnet to allow aspiration of the wash solution. The final wash contained MutSb buffer with 200 mM KCl. Bound, labeled DNA was quantitated in a scintillation counter. Counts were normalized for the counts present in 2 \( \mu \)l of the starting pre-bound oligonucleotide solution. Mismatch-specific DNA binding is expressed as the proportion of the starting counts remaining bound to the beads in the heteroduplex DNA (GT) divided by the proportion of the starting counts bound to the beads in the homoduplex DNA (AT or GC) run concurrently: heteroduplex/homoduplex ratio = \([\text{GT}_{\text{bound}}/\text{GT}_{\text{starting}}]/[\text{AT}_{\text{bound}}/\text{AT}_{\text{starting}}]\).

3. Results

3.1. Purification of MutSb

MutSb was overexpressed and partially purified by sequential streptomycin sulfate and ammonium sulfate precipitation (see experimental protocol). MutSb comprised 8–10% of the protein in the initial cell lysate, but was the dominant protein (35%–40% by laser densitometry; \( n = 3 \)) in the 31% saturated ammonium sulfate pellet fraction (AS). The cell lysate and the pellet from a 31% saturated ammonium sulfate precipitation are shown in Fig. 1. Protein blotting with streptavidin coupled alkaline phosphatase (Promega) demonstrated that the MutSb fusion protein is the major biotin containing product in the AS fraction (data not shown). MutSb was further purified by binding to a column of avidin-coated agarose beads, and comprised more than 90% of protein present after elution (Fig. 1). Although 60–75% of the MutSb present in the AS fraction bound to the beads, only about 10% was eluted off in an active form. The yield from the monomeric avidin column, starting with 2–5 mg of MutSb in the AS fraction, was 100–400 \( \mu \)g. Since purification to homogeneity was not necessary for either of the mismatch binding and detec-
tion methods described below, the AS fraction was used unless otherwise noted.

3.2. MutSb retains its specific mismatch binding activity

A gel mobility shift assay demonstrates that the AS fraction is capable of complete band-shifting of the mismatch containing double-stranded 20 mer oligonucleotide (GT; Fig. 2(a)). This binding is largely blocked by the addition of a 200-fold excess of unlabeled GT, but not by a 200-fold excess of the unlabeled non-mismatched oligonucleotide (AT). In addition, the AS fraction results in complete gel retardation of radiolabeled GT, and minimal retardation of labeled AT (lanes 7 and 8), similar to what has been previously demonstrated with native MutS[5]. Unlabeled AT was used to block non-specific binding at different concentrations up to 40 μM. The highest signal to noise ratio was detected using between 200–500 fold excess cold competitor (8–20 μM). The same specificity of binding was seen for incubations of 10–30 min. Fig. 2(b) demonstrates that about 6 pmol of column-purified MutSb results in band shifting of 0.2 pmol of GT and no shifting of AT, even with two-fold the MutSb concentra-
tion (lane 5). The specificity of the MutSb for heteroduplex oligonucleotide is greater than 10-fold that for the homoduplex oligonucleotide, similar to what has been reported previously for native MutS in solution (5–17 fold; Refs. [6,8]) and to native MutS purchased from USB run concurrently (lane 6). Specific band-shifting occurred over a wide range of GT concentrations ranging from 120 nM to 120 pM, indicating a wide range for mismatch detection (not shown). Thus, despite the addition of a 13 kD biotinylated peptide domain to the N-terminus of MutS, mismatch specific DNA-bind-
ing activity is preserved.

3.3. Mutation detection with MutSb

MutSb coupled to streptavidin coated magnetic beads was used to detect single base pair mismatches in heteroduplex DNA in solution. Specific binding was assessed by the ratio of binding of radiolabeled GT to radiolabeled AT duplex DNA in the presence of unlabeled 20 mer AT. The optimal ratio of heteroduplex to homoduplex binding was observed in the presence of 200-fold or greater excess unlabeled AT. Binding to radiolabeled GT was blocked in the presence of 8 μM unlabeled 20 mer GT competitor (about 200-fold excess). An experiment comparing the specificity of binding of MutSb for GT versus the AT oligonucleotide done in duplicate is presented in Table 1. Over a series of such experiments, the MutSb bound to GT with an average of 10-fold greater specificity than AT (n = 10; range 5–18). The lower ratios were seen in the initial experiments, with a trend towards increased specificity of binding with beads that were pre-bound with MutSb, rather than added to the MutSb after DNA binding, as was done in the first experiments. These improved results with batch pre-binding of MutSb to the beads are probably due to more uniform MutSb concentra-
tions, since the same batch was used for both AT and GT comparisons. Therefore, in most of the experiments involving DNA targets greater than 20 mers, the MutSb AS fraction was bound to the streptavidin-coated beads prior to incubation with the DNA. After rinsing, the major protein bound to the beads was MutSb (Fig. 1). More consistent heteroduplex binding was also seen when 5 μl (250 pmol) rather than 2 μl (50–100 pmol) of beads were used, but there was no additional advantage to using more than 5 μl of beads. The addition of ATP did not alter specific binding (data not shown).

To determine whether the MutSb fusion protein binds specifically to larger mismatch containing molecules, a 64 bp oligonucleotide was used in this simple binding assay, an example of which is depicted in Table 1. The average specificity of mismatch binding (GT:AT) using this longer oligonucleotide was 5.6-fold (range 3.8–8.8), still easily distinguishable from non-mismatch binding (AT:AT ratio = 1.1).
As a demonstration of the utility of MutSbs for a potentially rapid mutation detection system, known human homozygote and heterozygotes were screened for a single base pair polymorphism in KCNA1 with MutSb [15,16]. The radiolabeled 420 bp PCR products amplified from genomic DNA were incubated individually with MutSb bound to streptavidin-coated magnetic beads, and the rinsed beads were resuspended in buffer and counted in a scintillation counter. One such experiment is illustrated in Table 1. The heterozygotes were bound an average of 3-fold over the homozygotes (n = 7; range 1.6–4.1). The PCR product concentration ranged between an estimated 2–40 nM without significantly affecting the results. Any ratio of binding of a mismatch containing double-stranded DNA product that is greater than 2 should be readily distinguishable from homozygotes, since comparison of non-mismatched products run concurrently resulted in a binding ratio close to 1 (1.1 ± 0.2 (s.d.); n = 9; also see Table 1). The entire mutation detection scheme beginning with the incubation of the PCR product with the MutSb and beads in solution, to scintillation counting, takes about 1 h.

3.4. MutSb can extract heterozygotes/mutant duplex DNA from solution

Many of the unique applications of this technology involve MutS extraction of heteroduplex DNA from a mixture of DNA in solution. To assess the potential for MutSb to enrich for a mismatch containing molecule out of a mixture of DNA, double-stranded 20, 64 and 75 mer oligonucleotides were mixed in equimolar amounts in solution. In each mixture, only one of the oligonucleotides contained a GT mismatch. In this way, the length of the mismatch containing oligonucleotide was varied to insure that the selection was not based on size alone. The starting mixture was bound to MutSb streptavidin coated magnetic beads as described in the experimental protocol. Fig. 3 demonstrates that MutSb is capable of significantly enriching each of the different sized mismatch-containing heteroduplexes from this mixture. The range of enrichment for the mismatch containing 75 mer was 18–36 fold, the 64 mer was 8–19 fold, and the 20 mer was 2.6–3.2 fold.

4. Discussion

The results show that the biotinylated MutS fusion protein, MutSb, retains the mismatch binding specificity of the native MutS protein as demonstrated by gel-retardation in vitro. We have adapted this reagent for use in a rapid mutation detection scheme that requires no gel electrophoresis. This mutation detection scheme employing magnetic beads coupled via streptavidin to MutSb is rapid when compared with methods that require nuclease digestion or electrophoresis.
Table 1
Mismatch specific extraction in a mixture of different sized DNA by MutSb

<table>
<thead>
<tr>
<th>DNA product (bp)</th>
<th>Heteroduplex bound (cpm)</th>
<th>Homoduplex bound (cpm)</th>
<th>Ratio (heteroduplex/homoduplex)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>28413</td>
<td>2494</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>27416</td>
<td>2124</td>
<td>12.9</td>
</tr>
<tr>
<td>64</td>
<td>3057</td>
<td>752</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>2422</td>
<td>638</td>
<td>3.8</td>
</tr>
<tr>
<td>420</td>
<td>7700</td>
<td>1979</td>
<td>3.9</td>
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<td></td>
<td>6929</td>
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<td>1.3</td>
</tr>
<tr>
<td></td>
<td>548</td>
<td>458</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Data from a set of magnetic bead experiments done in duplicate for each sized DNA are shown. The heteroduplex/homoduplex binding ratios range between 3.8 and 12.9 for products up to 420 bp. There is a trend towards decreased mismatch specific binding with larger products. Starting (and bound) counts vary between experiments with different sized DNA fragments due to the differential efficiency of end-labeling and the amount and specific activity of radionucleotide used in labeling each oligonucleotide or PCR product. Within each experiment, counts are normalized to counts present in 2 µl of the starting pre-bound oligonucleotide.

such as SSCP, chemical cleavage or heteroduplex analysis. A recently described method using native MutS to detect heteroduplex DNA by filter binding is also rapid relative to gel-based methods [8]. Two other recently described methods employing native MutS in mutation detection schemes share the disadvantage of requiring electrophoresis, and it appears that each may need to be optimized carefully for different sized DNAs [9,10].

This MutSb magnetic bead assay has the additional benefit that the same binding conditions may be used for a wide range of target DNA sizes (20–420 bp) and concentrations (120 nM to 120 pM). Preliminary results indicate that other intermediate sized targets (e.g. a 200-mer PCR product derived from plasmid DNA) and deletions up to four bases may be specifically detected under the same conditions. Additionally, avidin coupled to agarose beads rather than magnetic beads has been used in some experiments with similar results (data not shown).

The upper size limit of the screening capabilities of the MutSb is unknown, but previous results suggest that native MutS is not reliable for mutation detection in vitro on fragments larger than 450 base pairs [8,9]. This may be due to non-mismatch-specific DNA interactions that increase with the size of the DNA target. Our results indicate that this is true for MutSb as well. As Table 1 indicates, MutSb screening of the entire intact 1.5 Kb KCNA1 gene has not been successful.

We also demonstrate that MutSb can also be used to extract mutation containing DNA from a simple mixture in solution, which is then analyzed using electrophoresis. Although MutSb was able to distinguish the mismatch containing from the non-mismatch containing molecules in each case, there is a trend towards greater enrichment when the mismatch containing fragment is larger than the other fragments in solution. This implies that this technique will work best when the mismatch containing fragments are not much smaller than the other fragments in solution. Preliminary experiments suggest that DNA fragments that differ more than 5-fold in size can be distinguished from their respective homoduplexes and each other using this technique. This ability to pull mismatch containing DNA fragments out of a mixture gives this method the potential to scan large regions of a restriction-digested gene for a number of sequence differences at once.

Fig. 3. MutSb can extract mismatch containing DNA from a mixture. Three separate mixtures, each containing 3 double-stranded oligonucleotides 75, 64 and 20 bp (0.1 pmol each), were incubated with MutSb coated magnetic beads, separated from the beads by denaturation (Sequenase Stop solution, USB) and resolved on a denaturing 12% polyacrylamide minigel (Hoeffer) containing 7M Urea in 1 x TBE. In each mixture only one of the oligonucleotides contains a mismatch (labeled 75, 64 and 20 M at the top of the gel lanes). Each experiment was performed in triplicate. The initial unbound starting material containing the three oligonucleotides used for each set of experiments is displayed next to each set (S). Band intensity was quantified by Phosphor Imager (Molecular Dynamics). Enrichment of the heteroduplex was calculated by dividing the ratio of its intensity to other DNAs bound to the magnetic beads, to its relative intensity in the starting mixture. For example, in the first case (75 M), enrichment was calculated as: [75 mer/(64 mer + 20 mer)]bound/[75 mer/(64 mer + 20 mer)]starting.
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