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A homogeneous resonance energy transfer-based assay to monitor MutS/DNA interactions

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ABSTRACT

Probing the interactions of the DNA mismatch repair protein MutS with altered and damaged DNA is of great interest both for the understanding of the mismatch repair system function and for the development of tools to detect mutations. Here we describe a homogeneous time-resolved fluorescence (HTRF) assay to study the interactions of *Escherichia coli* MutS protein with various DNA substrates. First, we designed an indirect HTRF assay on a microtiter plate format and demonstrated its general applicability through the analysis of the interactions between MutS and mismatched DNA or DNA containing the most common lesion of the anticancer drug cisplatin. Then we directly labeled MutS with the long-lived fluorescent donor molecule europium tris–bipyridine cryptate ([TBP(Eu^{3+})]) and demonstrated by electrophoretic mobility shift assay that this chemically labeled protein retained DNA mismatch binding property. Consequently, we used [TBP(Eu^{3+})]-MutS to develop a faster and simpler semidirect HTRF assay.

The *Escherichia coli* MutS protein plays a central role in the activity of the DNA mismatch repair (MMR)[1] system that is implicated both in the repair of mismatches formed during DNA replication and/or recombination and in the signaling of DNA damage. In particular, this protein recognizes mispaired and unpaired DNA bases from single mismatches up to five nucleotide insertion/deletion loops [1]. In addition, MutS exhibits binding activity to DNA that has been damaged by anticancer drugs, such as cis-diamminedichloridoplatinum(II) (cisplatin), which is widely used in human chemotherapy for the treatment of many solid tumors (reviewed in Ref. [2]). The N-terminal part of MutS is implicated in DNA mispaired base recognition, whereas the C-terminal part is responsible for oligomerization and contains two ATPase domains [3,4].

Several technical approaches have been proposed to study specific MutS/DNA interactions and to develop MutS-based tools to detect mutations [5–16]. Among them, heterogeneous format assays, which make use of electrophoretic mobility shift assay (EMSA) and surface plasmon resonance (SPR), have been largely reported, although such techniques are time-consuming and, in the case of EMSA, are also characterized by poor robustness [5–9]. Other approaches are based on a homogeneous format, for example, the MutS exonuclease protection assay (reviewed in Ref. [11]). This test is simple to perform but is prone to unavoidable fluorescence interference due to the fluorescence background caused by the contaminating DNA present in the exonuclease preparations [12].

Besides DNA labeling or staining techniques to reveal MutS/DNA complex formation, MutS labeling has also been reported. Indeed, MutS detection has been possible by using fluorescent cyanine-labeled MutS [13] or by fusing MutS to a reporter domain such as a biotinylated tag [14] or a green fluorescent protein (GFP) [15]. Although fusion of MutS to a reporter protein should not impair MutS/DNA binding properties, we have noticed that the brightest GFP/MutS chimera exhibited relatively lower affinity towards mismatched DNA than did wild-type MutS; in addition, the fluorescence properties are influenced by the type of MutS/GFP construct [15], and biotinylated MutS has not received other use to our knowledge. Thus, it appears that the design of a fully homogeneous format assay would be of great interest for the study of MutS/DNA interactions. For that purpose, a tool able to discriminate between the molar fraction of specifically bound molecules and the population of excess unbound molecules is required. There

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[1] *Abbreviations used:* MMR, mismatch repair; EMSA, electrophoretic mobility shift assay; SPR, surface plasmon resonance; GFP, green fluorescent protein; ODN, oligodeoxyribonucleotide; RET, resonance energy transfer; ANS, anilino naphthalensulfonic acid; HTRF, homogeneous time-resolved fluorescence; [TBP(Eu^{3+})], europium tris–bipyridine cryptate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; SA, streptavidin; KF, potassium fluoride; DMSO, dimethyl sulfoxide; NHS, *N*-hydroxysuccinimide; CV, coefficient of variation.

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are two main types of homogeneous format assays. The first one relies on fluorescence anisotropy measurements that allow the discrimination between DNA bound to a protein (low anisotropy, large molecule) and free DNA (high anisotropy, small molecule). However, results are strongly related to the differences in fluorescence anisotropy, which depend mostly on the relative size of each interacting partner, and labeling of short DNA fragments, such as oligodeoxyribonucleotides (ODNs), is generally preferred to labeling of MutS [9]. The second homogeneous format relies on proximity assays, such as resonance energy transfer (RET), to probe MutS binding to mismatched DNA. Interestingly, fluorescence quenching of anilino naphthalen-sulfonic acid (ANS) arising from a conformational switch of ANS-labeled MutS dimer upon DNA binding was recently reported [16]. In this assay, 30% fluorescence quenching was observed in the presence of ANS-labeled MutS and unlabeled DNA substrates with a C/T mismatch, whereas single-stranded DNA yielded a nonspecific signal of 50% quenching. In conclusion, it can be deduced that labeling only one interacting species presents some limitations; therefore, an assay designed to have a double selectivity by labeling both MutS and DNA should give a higher signal-to-noise ratio. The use of a time-resolved method, such as the robust homogeneous time-resolved fluorescence (HTRF) [17], should enable the probing of MutS/DNA interactions. To use such a technique, one needs to label the biomolecules with a luminescent tag such as a lanthanide complex. Europium cryptates are particularly attractive due to their unique long-lived fluorescence and complete stability even in the presence of potentially competing divalent cations, which are often necessary for protein activity. Indeed, europium tris–bipyridine cryptate ([TBP(Eu^{3+})]) [18] has been used as the long-lived fluorescent donor in combination with a modified allophycocyanin (named XL665) as the acceptor for the design of the HTRF method [19]. Such assay relies on the dual wavelength detection of the cryptate emission at 620 nm and of the acceptor emission at 665 nm. The long-lived 665-nm fluorescent signal, which is specific to the interacting molecular species, is then readily distinguished from the short-lived fluorescence background through both spectral and temporal selectivity. The HTRF technique has a long established history [17,19], commercially available HTRF immunoassays are well known, and many of them are routinely used in clinical laboratories. In the nucleic acid field, HTRF was recently used for the detection of mutations using either a ligation-based discrimination technique [20] or minisequencing [21]. Here we report the development of an HTRF-based assay to detect MutS/DNA interactions that displays the high sensitivity, robustness, and throughput that are characteristic of HTRF.

Materials and methods

Single- and double-stranded DNA

Two sets of single-stranded ODNs purchased from Eurogentec (France) were used. Biotinylated ODNs were labeled with a 1-*O*-(6-biotinamido-hexyl)-2-deoxyribose unit (dR–biotin) and were HPLC purified. The first set of ODNs contained the codon 248 (CGG, Arg) of the *p53* tumor suppressor gene (Table 1). Also, 21- or 51-mer ODNs with or without biotin moiety at their 5 end were used for duplex formation (Table 1). Homoduplexes and heteroduplexes were obtained by annealing the corresponding complementary strands in phosphate-buffered saline (PBS) buffer at 37 °C for 30 min. The second set was made up of 24-mer ODNs with or without a 3′ biotin label that were first purified on denaturing polyacrylamide gels. The preparation and purification of 24-mer ODNs containing a single 1,2-d(GpG) cisplatin intrastrand cross-link (G*G*) has been described previously [6].

Indirect DNA/MutS HTRF assays

Typically, 0.3 to 1.5 pmol of a biotinylated duplex in 10 µl of binding buffer (100 mM phosphate [pH 8.0], 0.1% bovine serum albumin [BSA], 0.5 M NaCl, and 5% glycerol) was mixed with 1.5 pmol [22] of hexameric histidine (His6)-tagged MutS protein (see Supplementary material) in 3 µl of binding buffer at 20 °C for 15 min. Following the recognition step, either the XL_{665}-labeled anti-His6-tag monoclonal antibody (1.5 pmol) in combination with streptavidin–[TBP(Eu^{3+})] (SA–[TBP(Eu^{3+})]) (0.3 pmol) (Fig. 1A) or the [TBP(Eu^{3+})]-labeled anti-His6-tag monoclonal antibody (0.3 pmol) in combination with SA–XL_{665} (0.3 pmol) (Fig. 1B) was added to a final volume of 200 µl of measuring buffer (50 mM Hepes [pH 7.2], 0.1% BSA, and 0.4 M potassium fluoride [KF]). All conjugates were purchased from CIS Bio International (France) and diluted in measuring buffer. Measurements were performed after 0, 0.5, 1, and 2 h incubation at 20 °C. The assays were carried out in duplicate in 96-well black microtiter plates and measured on a Rubystar reader (BMG Labtechnique, Germany, http://www.bmglabtech.com) using the recommended settings (delay time t_d = 50 µs, gating time t_g = 400 µs). For each series of experiments, blank, positive, and negative samples were analyzed in parallel. Blanks contained all of the reagents, but the ODNs and negative controls (nonspecific signal) contained homoduplexes and positive samples (specific signal) heteroduplexes. For each point, the long-lived 665-nm fluorescence signal from the acceptor was standardized using the 620-nm signal from the donor, and the R ratio = (665-nm signal / 620-nm signal) × 10^4 was computed. This value was independent from the measuring conditions. The relative corrected (ΔF) values were then calculated, ΔF (%) = [(R assay–R blank)/R blank] × 10^2, to allow the comparison between independent experiments. ΔF values were calculated for both positive and negative assays. Assays with DNA duplexes containing cisplatin compound lesions were carried out in the same way.

[TBP(Eu^{3+})] labeling of MutS

MutS protein (2.3 µM in 50 mM Hepes [pH 8.0], 350 mM NaCl, and 10% glycerol) was labeled at 4 °C using [TBP(Eu^{3+})] (1 mM in 5 µl of dimethyl sulfoxide [DMSO]) activated as an *N*-hydroxysuccinimide (NHS) ester ([TBP(Eu^{3+})] labeling kit (CIS Bio Interna-

Table 1
Sequences of oligodeoxynucleotides used in this study for formation of various DNA duplexes

Name	Sequence (5′ to 3′)
CGG-21	Bio-GGCATGAA<u>CCG</u>GAGGCCCATC[a]
CCG-21	GATGGGCCT<u>CCG</u>GTTCATGCC
TGG-21	Bio-GGCATGAAC**T**<u>GG</u>AGGCCCATC[b]
CGG-51	Bio-CAGTTCCTGCATGGGCGGCATGAAC<u>CGG</u>AGGCCCATCCT-CACCATCAC
CCG-51	GTGATGGTGAGGATGGGCCT<u>CCG</u>GTTCATGCCGCCCATGCAG-GAACTG
TGG-51	Bio-CAGTTCCTGCATGGGCGGCATGAAC<u>TGG</u>AGGCCCATCCT-CACCATCAC
GG-24	CTTACTCTCTC<u>GG</u>TCTCTACTCCT[c]
G*G*-24	CTTACTCTCTC<u>G*G*</u>TCTCTACTCCT[d]
CC-24	AGGAGTAGAGA<u>CC</u>GAGAGAGTAAG-Bio
TC-24	AGGAGTAGAGA<u>T**C**</u>GAGAGATAAG-Bio

[a] The 21- and 51-mer ODNs containing the codon 248 of *p53* are named according to the central underlined sequence. For example, the biotinylated duplex formed by hybridization of the CGG-21 ODN with its complementary strand CCG-21 is referred to as 21-CGG/GCC in the text.

[b] The thymine nucleotides shown in bold indicate the location of a G/T mismatch after hybridization to the corresponding complementary strand.

[c] The 24-mer ODN abbreviated GG-24 is a pyrimidine-rich strand that contains a central GG site (underlined) for modification by cisplatin. For instance, hybridization to the complementary biotinylated purine-rich strand CC-24 yields a biotinylated homoduplex that is named GG/CC in the text.

[d] G*G* denotes the presence of a 1,2-d(GpG) cisplatin intrastrand cross-link.