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Discrimination of Lesion Removal of N-Methylpurine-DNA Glycosylase Revealed by a Potent Neutralizing Monoclonal Antibody

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Abstract

N-methylpurine-DNA glycosylase (MPG), a ubiquitous DNA repair enzyme, initiates excision repair of several *N*-alkylpurine adducts, induced by alkylating chemotherapeutics, and deaminated and lipid peroxidation-induced purine adducts. We have generated monoclonal antibodies (moAbs) against human MPG. Twelve independent hybridoma clones were characterized, which, except 520-16A, are identical based on epitope exclusion assay. Four moAbs, including 520-2A, 520-3A, 520-16A, and 520-26A, have high affinity ($K_D \sim 0.3$ –1.6 nM), and their subtypes were IgG_{2a}, IgG₁, IgG_{2a}, and IgG_{2b}, respectively. moAb 520-3A recognizes the sequence ⁵²AQAPCPRERCLGPP⁶⁶T, an epitope exclusively present in the N-terminal extension of human MPG. We found that moAb 520-3A significantly inhibited MPG's enzymatic activity towards different substrates, such as hypoxanthine, 1,N⁶ethenoadenine and methylated bases, which represent different classes of DNA damage, however, with different efficiencies. Real-time binding experiments using surface plasmon resonance (SPR) spectroscopy showed that the pronounced inhibition of activity was not in the substrate binding step. Single turnover kinetics (STO) revealed that the inhibition was at the catalytic step. Since we found that this antibody has an epitope in the N-terminal tail, the latter appears to have an important role in substrate discrimination, however, with a differential effect on different substrates.

1. Introduction

Cellular DNA is continuously exposed to endogenous or exogenous chemical or physical agents that induce DNA lesions. DNA base damage threatens genomic stability and cellular viability. Multiple DNA repair pathways exist in all organisms—from bacteria to humans—to preserve the integrity of the genome [1]. Damaged bases, if not repaired, can be mutagenic [2] and/or trigger cell death [3].

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In all organisms, repair of DNA-containing small adducts, as well as altered and abnormal bases, occurs primarily via the base excision repair (BER) pathway, beginning with cleavage of the base by a DNA glycosylase [1,2]. Mechanistically, DNA glycosylases are classified as: mono- or bifunctional DNA glycosylases. Monofunctional DNA glycosylases, such as MPG and Uracil DNA-glycosylase, use an activated water molecule as a nucleophile to generate an apurinic or apyrimidinic (AP) site in DNA. Bifunctional DNA glycosylases/AP lyases, such as NTH1 and OGG1, use an activated amino group (Lys) or imino group (Pro) as the nucleophile to create a Schiff-base intermediate that coordinates base removal and subsequent strand incision (AP lyase) 3' to the AP site [4,5]. Mammalian MPG is known to excise at least 17 structurally diverse modified bases from DNA, which are induced by alkylating chemotherapeutics, deamination and lipid peroxidation [6]. The lesion substrates primarily include the purine derivatives, such as 3-alkylpurines, 7-alkylguanine, 1,*N*⁶-ethenoadenine (ϵ A), *N*²,3-ethenoguanine, and hypoxanthine (Hx) [7-12]. Moreover, the base alterations are located in both the major and minor grooves of duplex DNA. Mammalian MPG orthologs in *Escherichia coli* (AlkA) and yeast (MAG) have overlapping, but not identical, substrate ranges. Nonetheless, in spite of this functional similarity, mammalian MPG and *E. coli* (AlkA) do not share significant sequence similarity or structural homology [13,14], despite 3-methyladenine being a preferred substrate for both. MPG excises ϵ A and Hx more efficiently than AlkA and MAG [11], but unlike AlkA, it cannot excise *O*²-alkylpyrimidines [15,16] and oxidized bases, such as 5-formyluracil and 5-hydroxymethyluracil [17] from DNA. MAG does not excise *O*²-methylthymine either [6,18]. Although MPG can excise different modified bases, the specificity of MPG towards all substrates is not the same. In our previous study, we showed that MPG is organized into three distinct domains with a protease hypersensitive region at the amino terminus [19]. The non-conserved, N-terminal extension plays a role in excision of some alkylation damage and 1,*N*²-ethenoguanine (1,*N*²- ϵ G), although 1,*N*²- ϵ G is yet to be detected in genomic DNA [9,20]. Studies with hybrid recombinant proteins containing N- and C-terminal halves of human and mouse glycosylases showed that the N-terminal extension of MPG could be critical for its recognition of 3-methylguanine and 7-methylguanine adducts in DNA [9]. Hx and ϵ A are two other very important substrates of MPG. Hx was shown to be significantly mutagenic [21,22]. Besides all of this, how distinct those substrates are in respect to MPG is not yet known. We attempted to generate some highly specific and characterized antibodies against MPG to be used as probes for exploring substrate specificity, catalytic mechanisms and structure-function relationships.

Here we report that we raised several anti-MPG monoclonal antibodies and characterized them for binding specificity towards MPG. These antibodies significantly inhibited MPG's enzymatic activity. Surface plasmon resonance studies and single turnover studies showed that inhibition is not in the binding step but in the chemistry step. However, using three different DNA substrates which represent reaction products of three important different endogenous and environmental DNA damaging agents, we found that the degree of inhibition varied for different substrates. Furthermore, the antigenic epitope for the antibodies resides at the N-terminal extension of MPG and thus raises the possibility for the N-terminal playing a critical role in MPG's substrate specificity.

2. Materials and methods

2.1 Construction of expression plasmids for human MPG lacking variable first exon

MPG has two splice variants: Exon I is variable with 7 (*I_a*) or 13 (*I_b*) amino acid residues, while the rest of the exons are identical. We intended to express and purify Exon I less hMPG in order to use it for raising antibody that may recognize hMPG in human cells independent of splice variance. The plasmid pRR15, harboring full-length cDNA human MPG, was used as a template for PCR to generate a 184 bp human MPG cDNA fragment with the deletion of the

first 7 amino acid residues constituting Exon I_a at the N-terminus and inclusion of *Bam*HI and *Nde*I sites at the 5' end. The 3' of the PCR product ended with MPG's internal *Eco*47III site and had no change in sequences. The PCR product was digested with *Bam*HI and *Eco*47III and ligated in the plasmid, pPG23 [23], linearized with *Bam*HI and *Eco*47III. The resulting construct was digested again with *Nde*I and *Eco*RI, and the MPG cDNA fragment, encoding all the exons except I, was religated into pRSETB linearized with *Nde*I and *Eco*RI to generate the final expression construct, pRR17, whose identity was confirmed by sequencing.

2.2 Purification of human MPG lacking the variable first exon

E. coli BL21(DE3) carrying pRR17 was grown in magnificent broth (MacConnell Research, CA) at 37 °C until the absorbance at 600 nm reached 0.6. The culture was cooled to 25 °C and, after the addition of IPTG to 0.5 mM, was grown at 25 °C for 16 h, prior to chilling to 0 °C. All subsequent procedures were carried out at 4 °C. After the bacteria were harvested by centrifugation, they were resuspended in buffer A (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 0.1% Triton X-100, 5% glycerol) and then sonicated (10 × 45 s) on ice at full power using a Braun-Sonic U. After centrifugation of the cell lysate (2×30 min at 15,000 ×g), the supernatant was applied to ion-exchange columns, Q Sepharose (5 ml) and SP Sepharose (1 ml) (Amersham Pharmacia Biotech, Piscataway, NJ) connected in tandem. The columns were pre-equilibrated with buffer A. After washing with buffer A, the Q Sepharose column was removed, and the protein bound with SP Sepharose was eluted with a linear gradient of NaCl (50 to 450 mM) in buffer A. The electrophoretically pure peak fractions were pooled and kept at -80 °C in aliquots. On average, 8-10 mg of human MPG was recovered from 1 L of *E. coli* cells.

2.3 Monoclonal antibody production and subtyping

The purified hMPG lacking variable first exon was used for immunization. The protein was dialyzed against phosphate buffered saline and emulsified in either complete Freund's (first immunization) or incomplete Freund's (subsequent immunizations) adjuvant before subcutaneous injection of Balb/c mice. A mouse with high titer was selected for monoclonal antibody production as described previously [24,25]. Cultures were screened using an ELISA assay with the immunizing protein as the target [26]. Positive cultures were recloned by limit dilution, and the clones were tested for stable production of antibody. Antibodies were purified from ascites fluid by ammonium sulfate precipitation followed with ion exchange chromatography on DEAE cellulose (DE52) [26]. Antibodies were analyzed for IgG subclasses using a commercial capture-detection ELISA kit.

2.4 Epitope competition assay and binding affinity (K_D) analysis

Purified monoclonal antibodies were radioiodinated and tested for direct binding to the immunizing protein attached to 96 well format Immulon 4 snap apart wells. Experiments were performed with 1 µg target protein per well, and multiple concentrations of radioiodinated antibody, diluted in PBS containing 5 mg/mL BSA, were tested for binding (1 hr at 25 °C in duplicate). Wells were washed and counted in a gamma scintillation counter. The K_D values were calculated from double reciprocal plots [27]. Competition binding experiments to assess epitope competition were performed as described previously [28] using the same binding format. Briefly, 50 ng of radioiodinated monoclonal antibody was mixed with different unlabelled antibodies in 50-fold molar excess. Binding data were collected, and the amount of competition for binding determined.

2.5 SDS-PAGE and Western Blot Analysis

Purified MPG (50 ng) or nuclear extracts from human or mouse cells (50 µg soluble protein) were separated by SDS-PAGE (15% polyacrylamide) and stained with Coomassie brilliant blue. For Western Blot analysis, proteins were transferred to a nitrocellulose membrane, and

the MPG bands were visualized with the anti-human MPG monoclonal antibodies, 1:500-1000 dilution for cell extracts or 500 ng for purified protein, using enhanced chemi-luminescence protocol (Amersham Life Sciences, Piscataway, NJ).

2.6 Identification of Epitope Sequence Recognized by 520-3A moAb

To determine the hMPG epitope recognized by the monoclonal antibody 520-3A, we generated a library of bacterial clones expressing short peptides derived from hMPG. The library was constructed using DNase I in the presence of Mn^{2+} to cause double-strand breaks in the hMPG cDNA generating fragments, averaging 50 to 100 bp according to a published method [29]. Following standard colony hybridization techniques [30], 34 positive colonies were identified using 520-3A monoclonal antibody (1:1000 dilution) and enhanced chemi-luminescence protocol. Six out of 34 colonies yielded DNA inserts, and the cell-free extracts from all 6 colonies were tested by Western analysis using 520-3A antibody. Four of them showed expression of MPG peptides. All 4 of those DNA inserts were then sequenced for epitope sequence determination.

2.7 Competition of the hMPG Antibody with a Synthetic Epitope Peptide Corresponding to Residues 52-82 of hMPG

Individual membrane strips containing 50ng of purified hMPG protein were processed for Western Blot analysis by incubating with 500ng of 520-3A moAb, which was preincubated with varying amounts (0-9 μ g) of peptide corresponding to residues 52-82 or 9 μ g of control peptide containing unrelated sequence at 25°C for 3 hrs.

2.8 Preparation of Substrates

Hx or ϵ A containing 50-mer oligonucleotides with the sequence 5'-TCGAGGATCCTGAGCTCGAGTCGACG~~X~~TCGCGAATTCTGCGGATCCAAGC-3' (where X represents Hx, or ϵ A) were purchased from Operon Technologies (Alameda, CA) and Gene Link (Hawthorne, NY). The complementary oligonucleotide containing T opposite Hx was synthesized by the Recombinant DNA Laboratory Core Facility at the University of Texas Medical Branch (Galveston, TX). The oligonucleotides were purified on a polyacrylamide sequencing gel. The Hx oligonucleotide was labeled at the 5' end using T4 polynucleotide kinase and γ - 32 P-ATP and annealed to complementary oligonucleotide to prepare 32 P-end-labeled duplex oligonucleotide as described previously [31]. [3 H]-labeled methylated calf thymus DNA substrate (~370 c.p.m./ μ g, 109 fmol 7-methylguanine and 20 fmol 3-methyladenine in a total of 960 pmol adenine and 640 pmol guanine per μ g DNA) was prepared as described previously [9].

2.9 MPG-Mediated Excision Activity Assay

The hMPG protein (5-10 nM) was incubated in the presence of MPG (0-29 nM) and APE (control, 10 nM) antibodies. The standard reaction mixtures contained 25 mM HEPES-KOH, pH 7.9, 0.5 mM DTT, 150 mM NaCl, 10 μ g/ml nuclease-free BSA, and 10% glycerol in a total volume of (20-200 μ l). Incubation was at 37°C for 10 min, if not otherwise stated. For methylated substrates, the reaction was terminated by the addition of 200 μ g of carrier calf thymus DNA and the large DNA fragments' precipitation with cold ethanol in the presence of 0.3M sodium acetate. The radio-labeled base adducts in the ethanol-soluble fraction were quantified directly by liquid scintillation counting [9]. For Hx and ϵ A, the reaction was stopped by inactivating the enzyme at 75°C for 5, min and the products were analyzed as described previously [32].

2.10 Binding Studies Using Surface Plasmon Resonance

We examined the modulation of MPG binding to Hx or ϵ A by hMPG moAb using a Biacore-1000 (Biacore, Uppsala, Sweden). A 50-mer duplex oligonucleotide, containing an Hx, ϵ A or adenine (control) at the 26th position from the 5' end of one strand, was used for measuring enzyme-substrate DNA interactions. Oligonucleotides were biotinylated and immobilized on streptavidin-coated Biacore chips. Previously, we showed Mg^{2+} can inhibit the binding of Hx towards MPG by surface plasmon resonance [32]. Under similar conditions of MPG-Hx interactions, we tested the effect of 520-3A antibody on MPG's (40-125 nM) binding towards Hx, ϵ A or unmodified oligonucleotide in the presence of various concentrations of antibody (0-1000 nM).

2.11 Single Turnover (STO) Kinetic Study

The hMPG (40-62.5 nM) was incubated with various concentrations (0-60 nM) of 520-3A antibody and a fixed concentration (2 nM) of 5' ³²P-labeled Hx or ϵ A-containing duplex oligonucleotide substrates at 37°C in an assay buffer containing 25 mM HEPES-KOH, pH 7.9, 10 μ g/ml nuclease-free BSA, 0.5 mM DTT, 150 mM NaCl and 10% glycerol in a total volume of 100 μ l. Aliquots of 5 μ l were removed at different times (0-20 min) and heat inactivated at 80°C in a preheated microcentrifuge tube. The products containing the AP-sites were quantitatively cleaved into smaller fragments, followed by resolving on denaturing gels, and radioactivity in the incised oligonucleotide was also quantified as described previously [32].

3. Results

3.1 Purification of human MPG lacking variable first exon

The Δ Exon I hMPG protein was purified to homogeneity as shown by SDS-PAGE (Fig. 1). The human enzyme showed cleavage of Hx with specific activity similar to that of mouse MPG as described earlier [9]. The human protein was dialyzed in phosphate buffered saline and used for immunization and antibody production.

3.2 Production and characterization of anti-MPG monoclonal antibodies

Twelve independent hybridoma clones were isolated and extensively characterized. Among them, 4 moAbs (520-2A, 520-3A, 520-16A, and 520-26A) were of high affinity ($K_D \sim 0.3$ -1.6 nM; Table 1), and the rest were of lower affinity. The high affinity moAbs were subtyped. The 520-2A, 520-3A, 520-16A, and 520-26A were IgG_{2a}, IgG₁, IgG_{2a}, and IgG_{2b} respectively (Table 1). Epitope competition assay showed that, unlike 520-16A, the other 3 moAbs competed for binding with each other (Table 1), and homologous competition always resulted in > 80% reduction in binding. Then we selected 520-3A, a high affinity antibody, for epitope identification. Upon sequencing, all 4 of those DNA inserts which showed expression of MPG peptides were found to be identical and matching the nucleotide sequences 156 to 246 from human MPG. moAb 520-3A recognizes the sequence ⁵²QAPCPRERCLGPPT⁶⁷TPGYPYSIYFSSPKGH⁸², which corresponds to the residues 156 to 246 in the hMPG coding sequence. In Western Blot analysis, the antibody (520-3A) cross-reacted with a polypeptide of 34 kDa in the cell-free extract of a variety of human cell lines (Fig. 2), and the protein sizes were similar to those reported by others using polyclonal antibodies [10]. However, this moAb does not cross-react with MPG in cell extracts from mouse cells (Fig. 2). Therefore, the segment ⁶⁷T through ⁸²H may be excluded from the above epitope sequence, and the epitope could be further narrowed down to ⁵²AQAPCPRERCLGPP⁶⁶T, which is a unique human sequence. Thus, it appears that the moAb 520-3A and possibly others require at least 15 residues to bind to MPG in human cells. However, an epitope peptide containing residues 52-82 successfully inhibited ~70% of the antibody's MPG binding (Fig. 3), whereas the shorter peptide (52-66) failed to inhibit activity

(data not shown). Probably the extra 16 residues at the carboxy terminus are required to retain the minimum conformation for the antibody to recognize the epitope.

3.3 Inhibition of MPG activity by the antibody

The activity of the purified hMPG was measured in the presence of 0-29 nM antibody using different MPG substrates. The IC_{50} is strikingly different for ϵ A compared to Hx or methylated substrates (Fig. 4 A-D). An unrelated control mouse moAb against human APE did not have any effect on MPG's activity (Fig. 4C). However, the antibody inhibits MPG's activity with ~ 5 fold more efficiency towards Hx and methylated substrates than ϵ A, despite the fact that Hx is neutral, whereas methylated bases are positively charged substrates. Moreover, unlike Hx or methylated adducts inhibition of ϵ A removal follows a bi-phasic curve. Therefore, this antibody is particularly useful in understanding the discriminating properties of MPG's substrate specificity.

3.4 Mechanism of antibody inhibition of MPG reaction

To elucidate the mechanism of inhibition, we tested various reaction steps of MPG. Unlike alkylated calf thymus DNA, Hx and ϵ A are more defined substrates, and, therefore, we used this oligo substrate to analyze the detailed mechanisms of antibody-mediated inhibition of MPG activity using pre-steady-state kinetics. We have discussed previously [32] how pre-steady-state kinetic analysis provides the opportunity to identify the intermediate step(s) that might be affected by the antibody. Briefly, the effect of moAb was assessed on substrate binding [measured by surface plasmon resonance (SPR)] and chemistry [catalysis; measured by single turn over (STO) kinetics] steps.

3.5 Surface plasmon resonance (SPR) to analyze substrate binding

The 50-mer oligonucleotide containing Hx or ϵ A was biotinylated and immobilized on streptavidin-coated chips. An unmodified oligonucleotide with a similar sequence was used as a control. Using SPR, one can follow real-time DNA-protein interactions compared with other commonly used methods, such as gel shift assay. We established the binding conditions for oligonucleotides as before [32]. Then, we tested the effect of antibody on MPG's binding to the Hx- or ϵ A- containing oligonucleotide and found an increase in MPG's affinity for Hx at lower concentrations of antibody and a small decrease at higher moAb concentrations. For ϵ A we found a steady increase at lower concentrations, and it reached near saturation at higher concentrations (Fig. 5B and C). The antibody, at a 1:2 molar ratio with MPG, enhanced binding of the enzyme onto Hx and ϵ A, while inhibiting its activity significantly. Notably, the antibody also increased the nonspecific binding of MPG towards the control oligo (Fig 5A). These results suggest that in order to affect MPG's activity the antibody does not abrogate MPG's substrate binding step.

3.6 STO Kinetics

Prompted by the observation that antibody can inhibit product formation, we tested whether antibody affected its catalytic reaction other than the binding step. We conducted STO kinetics with MPG proteins to measure the k_{chem} . The reaction was performed at substrate and enzyme concentrations of 2 and 40-62.5 nM, respectively, with or without antibody (0-60 nM). Data were analyzed using the first-order rate equation:

$$[P_t] = A_0 \{ 1 - \exp(-k_{obs}t) \} \quad (1)$$

where A_0 represents the amplitude of the exponential phase, and k_{obs} is the observed rate constant associated with the reaction process. Under the STO conditions ($[E] \gg [S]$), all the

substrate molecules should remain bound by enzymes. The binding step should not affect the rate of product formation; hence, under these conditions k_{obs} can be considered as k_{chem} . In fact, at [S] : [E] of 1:20 or 1:30, the k_{chem} did not alter, ensuring the enzyme reactions are following the STO conditions properly (Fig 6 A and B). Under similar conditions, incubation of antibody with hMPG showed pronounced inhibition (Fig. 6 C, D and E), indicating that the antibody was inhibiting the catalytic reaction at the chemistry step of MPG reaction. The k_{chem} for MPG-mediated Hx or ϵ A catalysis decreased with increasing antibody concentration, albeit, Hx removal was more affected. Thus, the new MPG moAb, which is extensively characterized for its immunologic properties, neutralizes MPG activity by impeding its chemistry (catalysis) step without abrogating the binding step.

4. Discussion

We have identified a specific antibody inhibitor of MPG, a DNA glycosylase, with recognition and cleavage specificity for a wide variety of structurally diverse alkylated, deaminated, and etheno DNA adducts. Despite the importance of DNA repair glycosylases in repairing mutagenic and toxic DNA base adducts caused by endogenous and exogenous sources, such as oxidative and nitrosative stress, replication errors, and cigarette smoke, there are not many well-characterized inhibitors for this important class of BER enzymes. UGI, a viral protein, has been shown to inhibit UDG, which excises uracils in DNA generated from replication misincorporations [33]. Speina et al. [34] screened a battery of compounds, including various base analogs and tryptophan pyrrolisate (Trp-P-1), a mutagenic DNA intercalator heterocyclic amine found in cooked food, targeting a number of *E. coli* and human DNA glycosylases. The 2-thioxanthine was effective for *E. coli* Fpg protein and was shown to inhibit its excision activity, whereas Trp-P-1 inhibited multiple DNA glycosylases, such as AlkA, TagA, MPG, NTH, and Fpg by altering the DNA secondary structures.

In a previous paper, we showed that Mg^{2+} could inhibit MPG's activity by abrogating its substrate binding and decreasing the active enzyme concentration [32]. However, Mg^{2+} -mediated inhibition might be better considered as a regulator for balanced BER than an inhibitor as all the downstream BER enzymes, other than MPG, require Mg^{2+} for their optimum repair activity.

We have shown before, by systematic deletion analysis of MPG from N- and C-termini, that a minimally sized polypeptide (N Δ 100C Δ 18) lacking 100 and 18 amino acid residues from the amino and carboxyl termini, respectively, and wild-type enzyme had similar kinetic and binding properties for ϵ A [7]. Since then, there were several reports on the crystallographic structures of the similarly truncated protein in complex with ϵ A or control DNA [13,14,35]. Using recombinant chimeric proteins, containing N- and C-terminal halves of human and mouse MPG, we found that the N-terminal half is critical for the recognition of 3-methylguanine and 7-methylguanine [9]. Dr. Jacques Laval's group also showed that N-terminal tail is important for the catalysis of 1, N^2 - ϵ G-DNA (20). From this study, however, it is apparent that MPG's modes of action towards different substrates are strikingly different. As ϵ A appears less inhibited at lower concentrations of antibody compared to higher concentrations, it clearly shows a bi-phasic reaction, which indicates the possibility of two different mechanisms for the inhibitions. We also do not rule out the possibility of inhibition of catalysis by unspecific binding of the antibody at higher concentrations. Nonetheless, the structural information of ϵ A bound to truncated MPG available from literature [13,14,35] does not adequately reveal the full scenario for other DNA adducts, including Hx and methylated bases.

MPG, being a broad substrate enzyme, must be flexible for DNA binding in order to recognize DNA lesions of varied structures. In fact, for similar reasons T. Ellenberger and his colleagues

proposed that a “nonspecific catalytic mechanism” must be met for an enzyme to succeed as a generalist as one of the major criteria, which comes “at the expense of catalytic power” [23]. For this study, we have compared three different substrates which represent the products of three major classes of DNA damaging agents of both endogenous and environmental origins: alkylating chemotherapeutics (methylated substrates), deamination (Hx) and lipid peroxidation (ϵ A) -induced purine adducts. So, from the major differences in the IC_{50} s for inhibition of at least three different substrates by an antibody with an epitope in the N-terminal tail it is clearly indicated that the mechanism of reaction varies for structurally different substrates. This inhibition can be attributed to two different scenarios: (1) the N-terminal tail is important and involved in MPG reaction, but its importance may depend on the substrate type, as the excision of methylated substrates and Hx are 5 times more inhibited than ϵ A; (2) The antibody when binding to the N-terminal tail may affect the structural integrity and perhaps the catalytic core of MPG by posing spatial steric hindrance with different degrees for different substrate types. Thus, this affects their excision accordingly and generates substrate specificity. In fact, the first 70 amino acid residues comprising the N-terminal extension of hMPG are indispensable for 1, N^2 - ϵ G excision reaction [20]. The differences in IC_{50} s for N-terminal-specific antibody-mediated inhibition of three different substrates for human MPG again suggest that the N-terminal tail can be a critical regulatory domain for the activity of human MPG towards different substrates which remains to be tested. Since the N-terminal extension is present in all the MPG's from higher eukaryotes, it suggests an evolutionarily conserved regulatory function of the N-terminal tail for MPG's activity.

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Abbreviations

BER	base excision repair
AP	Apurinic / apyrimidinic
MPG	N-Methylpurine DNA-glycosylase
APE	Apurinic / apyrimidinic endonuclease
Hx	Hypoxanthine
εA	1,N ⁶ ethenoadenine
moAb	monoclonal antibody
m³A	3-methyl adenine
m⁷G	7-methyl guanine

Figure 1. Purification of Δ Exon I hMPG

The Δ Exon I hMPG protein was purified to near homogeneity. The details of the purification are described in “Materials and Methods.”

Figure 2. Western Blot analysis of MPG by monoclonal antibody in different human and mouse cell lines

(A) Cell-free extracts from human or mouse cells (50 µg of soluble protein) were subjected to Western Blot analysis. The details of the reaction conditions are described in “Materials and Methods.” The band intensities for MPG and β-actin were quantified by densitometric image analysis, and the data obtained for MPG were normalized with those of β-actin.

hMPG (ng)	50	50	50	50	50
hMPG Ab (ng)	500	500	500	500	500
Epitope peptide (μ g)	0	1	3	9	-
Control peptide (μ g)	-	-	-	-	9

Figure 3. Competition of the hMPG moAb, 520-3A with a synthetic epitope peptide corresponding to residues 52-82 of hMPG

50 ng of purified protein was loaded onto the SDS-PAGE and the synthetic peptide inhibits ~70% of the binding. The details of the experiments are described in "Materials and Methods."

Figure 4. Modulation of MPG activity by 520-3A moAb

The activity of the purified hMPG (5 nM) was measured in the presence of 0-29 nM antibody concentration for Hx (A) and εA (B). A comparison of Hx and εA with methylated substrate measured by using [³H]-labeled methylated calf thymus DNA is shown in (C). Data taken from panel C are used for IC₅₀ determination and presented in (D). IC₅₀ denotes 50% of the remaining MPG activity. The details of the reaction conditions are described in "Materials and Methods." Data represent mean values with standard error derived from at least three independent experiments.

Figure 5. Effect of 520-3A moAb on MPG binding to control (A), Hx (B) and εA (C) -containing oligonucleotides

The DNA–protein interactions were carried out using (40-125) nM MPG in the presence of different moAb concentrations (0-1000 nM). The details of the experiments are described in “Materials and Methods.”

Figure 6. Effect of 520-3A moAb on MPG reaction with Hx (A) and εA (B) under single turnover conditions

The reaction was performed using substrate and enzyme concentrations of 2 and 40 - 62.5 nM, respectively, as described in "Materials and Methods." Effect of moAb on pre-steady-state kinetic parameters under single turnover conditions for Hx (C) or εA (D). (E) Data derived from Panel (C) or (D) were analyzed using the first-order rate equation: $[P]_t = A_0 \{ 1 - \exp(-k_{\text{obs}} t) \}$ as described in "Results." k_{chem} , catalytic constant at the chemistry step.

Table 1

Characterization of anti-human MPG monoclonal antibodies

Hybridoma clones	Antigen Binding affinity (K_D , nM)	Antibody subclass	Epitope recognition	Epitope sequence in hMPG (see text for details)
520-2A	1.0	IgG _{2a}	Same	
520-3A	0.32	IgG ₁	Same	⁵² AQAPCPRERCLGPP ⁶⁶ T
520-16A	0.76	IgG _{2a}	Different	
520-26A	1.6	IgG _{2b}	Same	