Escherichia coli Fpg Protein and UvrABC Endonuclease Repair DNA Damage Induced by Methylene Blue Plus Visible Light In Vivo and In Vitro

HANNA CZECZOT; BARBARA TUDEK, BERNARD LAMBERT, JACQUES LAVAL, AND SERGE BOITEUX*

Groupe "Réparation des lésions radio-chimioinduites," UA158 CNRS, U140 INSERM, Institut Gustave-Roussy, 94800 Villejuif, France

Received 10 December 1990/Accepted 15 March 1991

The Fpg protein of Escherichia coli was initially identified as a DNA glycosylase which excised the imidazole ring-opened form of N7-methylguanine (2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine or Fapy) residues in DNA (2, 9, 13). This enzyme was shown also to liberate the imidazole ring-opened form of adenine residues from DNA treated with ionizing radiation (10) and of guanine residues modified at the N-7 position with bulky alkyating agents (12). The molecular cloning of the fpg* gene of E. coli (8), the purification of the Fpg protein (9), and the isolation of a bacterial mutant defective in Fpg protein (4) were critical steps for studies of the biological significance of this protein. The Fpg protein exhibits a large substrate specificity, including imidazole ring-opened purines modified at the C-8 position by N-hydroxy-2-aminofluorene (3). The Fpg protein is a metalloprotein containing one zinc atom per monomer (9) and is endowed with a nicking activity which incises DNA at apurinic-apyrimidinic (AP) sites (38). The biological importance of this enzyme is suggested by the fact that Fapy-DNA glycosylase activities have been conserved in prokaryotes (7) and eukaryotes (29, 33) and that the imidazole ring-opened form of N7-methylguanine residues in DNA is a potentially lethal lesion (6, 37). However, the lack of sensitivity of the E. coli mutant defective in Fpg protein to methylating agents suggested that imidazole ring-opened N7-methylguanine is not a biological substrate and/or that these residues are excised from cellular DNA by an alternative repair pathway (4). Such an alternative repair pathway may implicate nucleotide excision repair mediated by the UvrABC complex, which has been shown to repair a variety of damage induced by ionizing radiation and oxidizing agents such as AP sites and thymine glycols (24, 31, 46) as well as bulky adducts such as pyrimidine dimers (41, 42), platinum adducts (22), or psoralen cross-links (23, 47).

Recently, we have shown that the photosensitizer methylene blue plus visible light (MB-light) induces DNA base damage which is recognized and incised by the Fpg protein of E. coli (36). Under cell-free conditions, MB-light generates DNA damage which is attributed to singlet oxygen as the ultimately reactive species (18, 19). This damage is specific for guanine residues (39, 44, 45). The damage induced by singlet oxygen in bacterial, plasmid, and bacte riophage DNAs is lethal and/or mutagenic (16, 17, 21, 34). Although the only damaged base that has been identified in MB-light-treated DNA is 8-hydroxyguanine (43), the formation of other lesions cannot be excluded (11).

In this study, pBR322 DNA was modified with MB-light to generate DNA base damage and used for transformation experiments into E. coli hosts defective in excision repair activities. The results showed that both the base and nucleotide excision repair pathways mediated by Fpg protein and UvrABC endonuclease contribute to the repair of lethal base damage induced by MB-light in pBR322 DNA in vivo and in vitro.

MATERIALS AND METHODS

Bacterial strains and plasmids. All the strains used derived from E. coli K-12: AB1157, thr-1 leu-6 proA2 his-4 argE3 thi-1 lacY1 galK2 ara-14 xyl-5 mit-1 tsx-33 rpsL31 supE44; BH20, as AB1157 but fpg-1::Kn* (4); BH200, as AB1157 but uvrA::Tn10 (P1 SR1601; uvrA::Tn10 [27] × AB1157); BH190, as AB1157 but fpg-1::Kn* uvrA::Tn10 (this work);

* Corresponding author.
† Present address: Department of Biochemistry, Warsaw Medical Academy, Banacha 1, 02-097 Warsaw, Poland.
GC4803, as AB1157 but tagA alkA (5). AB1157/pBR322 (Amp' Tet') was from our laboratory stock.

**Media.** Rich medium was LB broth (35), supplemented when required with 50 μg of ampicillin per ml, 40 μg of kanamycin per ml, or 12.5 μg of tetracycline per ml. Solid media contained, in addition to LB broth, 1.5% agar (Difco), supplemented when required with 100 μg of ampicillin per ml.

**Chemicals.** Methyl methanesulfonate (MMS) was obtained from Aldrich Chemical Co. MB was obtained from Sigma Chemical Co. Antibiotics were purchased from Boehringer Mannheim.

**Preparation of pBR322 DNA.** pBR322 DNA was prepared from AB1157/pBR322 bacteria by a standard sodium dodecyl sulfate lysis method (32), purified by a CsCl density gradient, and stored at -20°C in TE buffer (10 mM Tris-Cl [pH 8], 1 mM Na2 EDTA). Purification of DNA repair proteins. Fpg protein and Nfo protein were purified to apparent homogeneity from overproducing strains harboring plasmid pFPG230 (9) or pBW21 (15) as previously described (30). UvrA, UvrB, and UvrC proteins purified to more than 95% purity as described previously (49) were a kind gift from A. T. Yeung.

**Treatment with MB-light.** pBR322 DNA was diluted to 0.1 mg/ml in TE buffer or 10 mM phosphate buffer (pH 7.4) (100-μl final volume) in a 96-well microtiter plate and illuminated with 2 × 100-W lamps (Phillips). The microtiter plate was placed on ice at a distance of 20 cm from the bulb. The MB was diluted in distilled water and stored in the dark at 4°C. The MB-light-treated DNA was ethanol precipitated and resuspended in 50 μl of TE buffer.

**Treatment with MMS.** pBR322 DNA was diluted to 0.1 mg/ml in 100 mM Tris-HCl buffer (pH 7.4) (100-μl final volume) and incubated for 60 min in the presence of MMS. The pBR322 DNA was precipitated and resuspended in TE buffer as described above.

**Treatment with radiation.** pBR322 DNA was diluted to 0.1 mg/ml in TE buffer (100-μl final volume) and irradiated with a Sylvania germicidal lamp with a maximum output at 254 nm. Alternatively, pBR322 DNA (100 μl in 10 mM phosphate buffer [pH 7.4]) was irradiated with a 125Cs source (Commissariat Energie Atomique) at a dose rate of 300 rads/min.

**Preparation of competent cells and transformation.** The bacteria were made competent by the CaCl2 method (28, 32). Competent cells (50 μl) were transformed with 50 ng of pBR322 DNA and incubated for 20 min at 0°C. The suspension was heat shocked for 4 min at 42°C, supplemented with 750 μl of LB broth medium, and incubated for 30 min at 37°C. Transformants were selected on LB plates containing 100 μg of ampicillin per ml.

**Incision by Fpg protein and Nfo protein.** The standard reaction mixture (25-μl total volume) contained 70 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-KOH [pH 7.6], 100 mM KCl, 2 mM Na2 EDTA, 0.2 μg of pBR322 DNA, and 10 ng of homogeneous Fpg protein or Nfo protein. The reactions were performed at 37°C for 10 min. The DNA's were either analyzed by 0.8% agarose gel electrophoresis or transformed into competent E. coli cells.

**Incision by UvrABC endonuclease.** The standard reaction mixture (25 μl) contained 40 mM potassium morpholinosopropane sulfonate (MOPS) [pH 7.6], 85 mM KC1, 1 mM thiotreitol, 15 mM MgSO4, 0.2 μg of modified pBR322 DNA, 2 mM ATP, and 500 fmol each of UvrA, UvrB, and UvrC proteins (26). The reaction was performed at 37°C for 25 min and stopped by the addition of 50 mM Na2 EDTA.

**RESULTS**

Survival of pBR322 DNA treated with MB-light. The treatment of phage PM2 DNA with MB-light generates DNA base modifications which are incised in vitro by the Fpg protein but not the Nth protein (endonuclease III) or the Nfo protein (endonuclease IV) of E. coli (36). This observation prompted us to study the survival of plasmid pBR322 DNA modified with MB-light when transformed into bacterial mutants defective in excision repair pathways. Figure 1 shows that the survival of MB-light-treated pBR322 was nearly the same in either the uvrA or fpg-1 single mutant as in the wild type. In contrast, the survival of pBR322 DNA was greatly reduced in the uvrA fpg-1 double mutant compared with that in the wild-type strain (Fig. 1). This reduction in the survival of pBR322 in the double mutant carrying uvrA fpg-1 was proportional to the concentration of MB and to the illumination time (Fig. 1). This synergistic effect showed that the
base excision repair and the nucleotide excision repair pathways mediated by Fpg protein and UvrABC endonuclease, respectively, are both involved in the elimination of genotoxic lesions formed in pBR322 DNA after treatment with MB-light.

Control experiments showed that the transformation efficiency is identical for the four isogenic strains used. Inactivation of the pBR322 DNA required both MB and light, since neither illumination with visible light for up to 20 min nor incubation of the DNA with MB in the dark resulted in the formation of detectable amounts of lethal lesions in transformation assays (data not shown).

The inactivation of pBR322 DNA transforming isogenic strains harboring uvrA and/or fpg-1 mutations was analyzed after treatment with UV light at 254 nm (Fig. 2A), ionizing radiation (Fig. 2B), MMS (Fig. 2C), and acid depurination (data not shown). Two of these treatments (ionizing radiation and MMS) are supposed to generate imidazole ring-opened purine residues in DNA (1, 2, 10). In contrast to MB-light-damaged DNA (Fig. 1), none of these treatments significantly decreased the survival of pBR322 DNA when it was transformed into the pBR322 DNA treated after preincubation at 37°C. These results show that neither ionizing radiation nor methylating agents introduced detectable amounts of imidazole ring-opened purines in pBR322 DNA treated under our conditions.

pBR322 DNA treated with MB-light is a substrate for Fpg protein. Figure 3 shows that Fpg protein efficiently incises MB-light-treated pBR322. The number of incisions made by Fpg protein or Nfo protein increased linearly with the illumination time (Fig. 3). The rate of formation of Fpg-sensitive sites, however, was 10 times higher than that of AP sites (defined as Nfo protein-sensitive sites) (Fig. 3). Since Fpg protein is endowed with an AP-nicking activity, these results imply that most of the sites induced by MB-light in pBR322 are damaged bases and not AP sites. The most probable mechanism for DNA strand cleavage is the excision of a modified base by the DNA glycosylase activity of the Fpg protein, which yields an AP site which is subsequently incised by the associated β-lyase activity of the Fpg protein. Therefore, we cannot exclude a direct incision of the DNA without initial cleavage of the glycosyl bond. The number of Fpg protein- and Nfo protein-sensitive sites in pBR322 DNA treated with UV light at 254 nm, ionizing radiation, or MMS is nearly identical (data not shown). This in turn is in agreement with the fact that the fpg-1 mutation has no effect on the survival of pBR322 treated by these DNA-damaging agents (Fig. 2).

In vitro repair of MB-light-damaged pBR322 DNA by Fpg protein results in increased transformation efficiency in the fpg-1 uvrA mutant. The results reported above show that Fpg protein is involved in repairing MB-light-induced DNA damage in vivo and incised MB-light-treated DNA in vitro. Therefore, incubation of MB-light-damaged pBR322 DNA with homogeneous Fpg protein in vitro should remove damage from the DNA and consequently increase the surviving fraction of transformants in the fpg-1 uvrA mutant. Figure 4 shows that the preincubation of MB-light-treated pBR322 DNA with Fpg protein increased the survival of plasmid molecules transformed into the pBR322 DNA treated under our conditions.

FIG. 2. Survival of UV-irradiated, γ-irradiated, and MMS-damaged pBR322 DNA when transformed into E. coli wild type and excision repair mutants. pBR322 DNA was treated with UV light at 254 nm (A), ionizing radiation (B), or MMS (C) and used for transformation into competent E. coli cells as described in Materials and Methods. The symbols are the same as in Fig. 1.

FIG. 3. Incision of MB-light-treated pBR322 DNA by Fpg protein and Nfo protein. pBR322 DNA in TE buffer was supplemented with 0.25 μg of MB per ml and illuminated as a function of time. The modified DNA was incubated for 10 min at 37°C either in the absence of enzyme (●) or with Fpg protein or Nfo protein. The average number of nicks per molecule was determined after electrophoresis as described in Materials and Methods. Each datum point represents the mean of three to five independent experiments.
FIG. 4. Survival of MB-light-treated pBR322 DNA incised in vitro by Fpg protein when transformed into the wild type and an fpg-J uvrA mutant. pBR322 DNA was illuminated for 10 min in the presence of increasing concentrations of MB (MB-light) and incubated or not with Fpg protein. These modified DNAs were used for transformation into AB1157 (wild type) or BH190 (fpg-J uvrA). Preincubation of undamaged pBR322 DNA with Fpg protein had no effect on the transformation efficiency. pBR322 treated with MB-light was used to transform AB1157 (○) or BH190 (△), or pBR322 treated with MB-light and incubated with Fpg protein was used to transform AB1157 (★) or BH190 (△).

Incised with either Fpg protein or UvrABC endonuclease and then transforming the uvrA fpg-J mutant was less than the survival of the same plasmids transforming the wild-type strain (Fig. 4; data not shown). This observation suggests that repair was incomplete or that residual lesions not processed by Fpg protein or UvrABC endonuclease were present. Furthermore, preincubation with Fpg protein (Fig. 4) or UvrABC endonuclease (data not shown) reduced the surviving fraction of pBR322 when it was transformed into the wild-type strain. A possible explanation is that the nicked or gapped DNAs were more susceptible to nuclease attack than were superhelical molecules. These results showed that the recognition of DNA base modifications by Fpg protein and/or UvrABC endonuclease is the initial event in the repair of MB-light-induced damage in vivo (Fig. 1).

pBR322 DNA treated with MB-light is a substrate for UvrABC endonuclease. Although transformation data presented above show that the uvrA mutation greatly reduced the survival of pBR322 DNA modified with MB-light in an fpg-J background, the recognition and incision of the damaged DNA by UvrABC endonuclease were not demonstrated. To clarify this point, we used pBR322 DNA treated with MB-light as a substrate for the UvrABC purified complex. Figure 5 shows that the number of sites incised by the UvrABC complex is identical to the number of Fpg-sensitive sites (Fig. 5). Neither UvrABC endonuclease in the absence of ATP nor UvrAB complex incised pBR322 DNA damaged by MB-light (Fig. 5). The observation that the two enzymes make the same number of incisions suggests that they recognize the same lesions. This hypothesis was confirmed, since the number of incisions generated in MB-light-treated pBR322 by the successive action of Fpg protein and UvrABC endonuclease was not significantly different from the number of incisions resulting from Fpg protein alone (data not shown). These in vitro results support the hypothesis that nucleotide excision repair contributes in vivo to the repair of DNA damage induced by MB-light.

### DISCUSSION

In this report, we showed that extracellular modification of pBR322 DNA with MB-light results in the formation of DNA base damage which is lethal if not repaired. These lethal lesions were repaired by both the nucleotide and base excision repair pathways in E. coli. The synergistic effects of uvrA and fpg-J mutations on the survival of MB-light-treated pBR322 DNA suggest that the two repair systems are involved in the elimination of the same lesions. This conclusion is further supported by the fact that MB-light-treated pBR322 DNA was incised efficiently by Fpg protein and UvrABC endonuclease in vitro. If we assume that base modifications recognized by Fpg protein are responsible for the lethal effect, we can conclude that these lesions are moderately toxic compared to those generated by other DNA-damaging agents. In similar studies, three cis-platinum (22) or five N-AAA (20) adducts were required to produce a lethal hit in a uvrA mutant, whereas seven Fpg-sensitive sites are required to produce a lethal hit in the uvrA fpg-J mutant. The number of bases damaged per lethal hit in the wild-type strain confirms that MB-light-induced DNA damage is less toxic than pyrimidine dimers or cis-platinum...
adducts. Alternatively, the toxicity induced by MB-light treatment might be due to various types of DNA damage recognized by Fpg protein and UvrABC endonuclease, some of them being lethal and others promutagenic.

To date, the glycosylase function of the Fpg protein has been reported to excise only imidazole ring-opened purine residues from DNA (3, 10). The formation of formamidopyrimidine residues in DNA by photosensitizers plus light via the generation of active oxygen species such as hydroxyl radicals cannot be excluded (1). However, the only modified base identified in MB-light-treated DNA is 8-hydroxyguanine (43). This base is moderately toxic, since a single 8-hydroxyguanine residue per single-strand viral genome reduces survival by 10 to 50% in E. coli (48) and allows some translesion synthesis in vitro (25). Furthermore, the ratio between strand breaks induced by MB-light and Fpg-sensitive sites observed in this study is similar to the ratio between single-strand breaks and 8-hydroxyguanine residues observed by Schneider et al. (43). Finally, Fpg protein excised 8-hydroxyguanine residues from MB-light-treated calf thymus DNA (1a). Therefore, Fpg protein and the 8-hydroxyguanine endonuclease recently described by Chung et al. (14) might be identical. Although 8-hydroxyguanine is formed in MB-light-treated DNA and excised by Fpg protein, the contribution of other lesions to the toxic effects of photosensitizers plus visible light cannot be excluded.

In this study, we report the first direct biological evidence for a role of Fpg protein in cellular DNA repair mechanisms. Since the ultimately reacting species generated by MB-light is most likely singlet oxygen, Fpg protein should be considered as a protein protecting DNA from the deleterious effects of reactive oxygen species as well as those of alkylating agents (2, 3, 10, 12).

ACKNOWLEDGMENTS

H.C. and B.T. were supported by INSERM fellowships. This work was supported by CNRS, INSERM, and Association pour la Recherche sur le Cancer.

We are very grateful to J. Felzenswalb (this laboratory) for the kind gift of Nfo protein and to A. T. Yeung (Fox Chase Research Center, Philadelphia, Pa.) for the kind gift of UvrABC proteins. We thank T. R. O’Connor for helpful discussion and his work on the manuscript. We thank P. Auffret Van der Kemp for her excellent technical assistance.

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