

Phosphorylation of methylated-DNA–protein-cysteine S-methyltransferase at serine-204 significantly increases its resistance to proteolytic digestion

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In a previous paper [Lim, Park, Jee, Lee and Paik (1999) *J. Cancer Res. Clin. Oncol.* **125**, 493–499], we showed two major forms of active DNA-6-*O*-methylguanine:protein-L-cysteine S-methyltransferase (MGMT; EC 2.1.1.63) in the liver with *N*-nitrosodiethylamine (DEN)-induced carcinogenesis: these were 26 and 24 kDa species. Here we show that a 2 kDa C-terminal fragment was cleaved from the 26 kDa species *in vitro* by thrombin or microsomal fractions isolated from DEN-treated rat livers. When Ser²⁰⁴ of the 26 kDa protein was replaced with Ala by site-directed mutagenesis, phosphorylation of the protein

was completely abolished, indicating Ser²⁰⁴ to be the site of phosphorylation. We also show that the phosphorylation was performed by Ca²⁺-independent protein kinase isoenzymes, and that the phosphorylated rat MGMT protein was resistant to digestion by protease(s) whose activity was increased during DEN-induced hepatocarcinogenesis and also by digestion with endopeptidase Glu-C (V8 protease).

Key words: endopeptidase Glu-C, DNA repair, hepatocarcinogenesis, rat.

INTRODUCTION

Methylated-DNA–protein-cysteine S-methyltransferase (MGMT; DNA-6-*O*-methylguanine:protein-L-cysteine S-methyltransferase, EC 2.1.1.63) is a ubiquitous protein that repairs *O*⁶-alkylguanine via a unique mechanism in that it accepts the alkyl group on a Cys residue in a single-step stoichiometric reaction, thereby itself being inactivated [1,2]. However, it has been reported that the *O*⁶-methylguanine repair capacity of H4 rat hepatoma cells can be increased after a single treatment with alkylating agents by a process different from the adaptive response found in *Escherichia coli* [3]. Indeed, cells capable of repairing *O*⁶-alkylguanine are much less susceptible to the cytotoxic effects of *N*-nitrosourea or *N*-methyl-*N'*-nitrosoguanidine than MGMT-deficient cells [4,5]. Whereas MGMT is expressed in all normal human cell types and tissues, approx. 20–30% of human tumours are completely deficient in MGMT expression [6]. The protective effect of MGMT towards carcinogen-induced hepatocarcinogenesis was clearly demonstrated in transgenic mice containing *E. coli ada* attached to the metallothionein I gene promoter [7] or in tumorigenesis induced by methyl nitrosourea in MGMT knock-out mice [5].

MGMT cDNA species were cloned from mouse [8,9], human [10–12] and rat [13,14] with only a single kind of cDNA; however, the apparent molecular masses of the products were 24, 22 and 26 kDa respectively. When the amino acid sequences of the bacterial Ada, human and rat MGMT proteins are compared, the C-terminal tail of approx. 2 kDa is absent from Ada (Ada C) (Figure 1), and human and rat MGMT proteins do not share any significant sequence similarities at the C-terminal end. In addition, only rat MGMT has a potential Ca²⁺-dependent protein kinase (PKC) phosphorylation site of -Ser/Thr-Xaa-Arg/Lys- [15,16] at the C-terminal region. These findings strongly suggest a possibility of post-translational processing of the protein such as side-chain modification or proteolytic conversion of the rat

MGMT proteins.

During studies on the role of MGMT in *N*-nitrosodiethylamine (DEN)-induced hepatocarcinogenesis in rat, we observed two major forms of active MGMT proteins, demonstrated by Western-blot analysis and gel slice assay: these had molecular masses of 26 and 24 kDa [17]. To investigate the possible modification of MGMT proteins during hepatocarcinogenesis, the modification and proteolytic conversion of MGMT were examined *in vivo* and *in vitro*, with rat recombinant MGMT protein (rMGMT) and its polyclonal and C-terminal peptide antibodies. Here we report for the first time that rMGMT was cleaved off by proteases *in vitro*, generating a 24 kDa species from the 26 kDa protein, and that the Ser²⁰⁴ residue in the 2 kDa fragment located in the C-terminal region was phosphorylated. Phosphorylation of Ser²⁰⁴ was mediated by PKC isoenzymes. Phosphorylated MGMT protein (MGMT-P) was more resistant to liver microsomal proteases and endopeptidase Glu-C than the unmodified MGMT, whereas its sensitivity to trypsin or chymotrypsin was unaffected. These findings indicate strongly that conformational changes might be induced not only by DNA binding or the alkylation of MGMT protein but also by the phosphorylation of Ser²⁰⁴. However, in contrast with the rapid digestion of MGMT protein by DNA binding or alkylation changes, the phosphorylation of MGMT endows the protein with resistance to proteases such as endopeptidase Glu-C (V8 protease) induced in liver during DEN-induced carcinogenesis.

EXPERIMENTAL

Materials

N-[methyl-³H]-*N*-Nitrosourea (20 Ci/mmol), [γ -³²P]ATP (3000 Ci/mmol) and enhanced chemiluminescence (ECL[®]) reagent were obtained from Amersham Life Science Products (Little Chalfont, Bucks., U.K.). *N*-Diethylnitrosamine (catalogue no. N-0756), dithiothreitol, thrombin [bovine plasma (T-7513)], phos-

Abbreviations used: DEN, *N*-nitrosodiethylamine; ECL, enhanced chemiluminescence; LV-TLE, low-voltage thin-layer electrophoresis; MGMT, DNA-6-*O*-methylguanine:protein-L-cysteine S-methyltransferase (EC 2.1.1.63); PKC, protein kinase C; rMGMT, rat recombinant MGMT protein; rMGMT-P, phosphorylated rMGMT.

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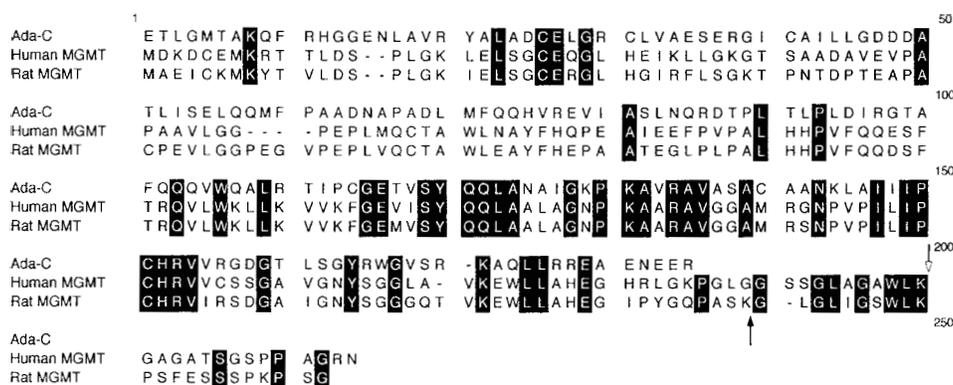


Figure 1 Comparison of amino acid sequences between the bacterial Ada-C, human and rat MGMT proteins

Similar amino acids are shown on a black background. The C-terminal tail is absent from Ada-C but present in the human and rat proteins. The C-terminal regions are less similar between the human and rat proteins. The Cys residue in the highly similar IPCHRV sequence is the alkyl accepting site, K¹⁸⁷G¹⁸⁸ in rat MGMT is the possible thrombin digestion site (solid arrow) and the SS²⁰⁴PK sequence in rat MGMT is the potential PKC phosphorylation site. The open arrow indicates the reported trypsin digestion site in human MGMT protein.

phoamino acids [phosphoserine (P-0753), phosphothreonine (P-1003) and phosphotyrosine (P-5024)], tosylphenylalanyl-chloromethane ('TPCK')-treated trypsin (T-8642), tosyl-lsylchloromethane ('TLCK')-treated chymotrypsin (C-3142) and endopeptidase Glu-C (P-6181) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glutathione Sepharose[®] 4B was purchased from Pharmacia Biotech (Uppsala, Sweden). Indolocarbazole G66976 (no. 365250), bisindolymaleimide G66850 and PKC-M (no. 539513) were purchased from Calbiochem-Novabiochem Co. (San Diego, CA, U.S.A.) and protein assay kit from Bio-Rad (Hercules, CA, U.S.A.). Histone H1 was from Boehringer Mannheim (Mannheim, Germany). Sprague-Dawley rats were obtained from the Korea Research Institute of Bioscience and Biotechnology (DaeJeon, Chung Nam, Korea). Other chemicals were of the highest purity available.

Maintenance of rats and preparation of microsomal and cytosolic fractions

Male Sprague-Dawley rats were maintained in a specific pathogen-free condition with an irradiated and microbe-controlled diet (PicoLab Rodent Diet 20, 5053; PMI Feeds, Richmond, IN, U.S.A.) and autoclaved water *ad libitum*. The environmental condition of the animal housing was controlled at a constant temperature (22 ± 1 °C) and relative humidity (55 ± 5 %). The room was ventilated 17 times per hour and illuminated for 12 h per day.

Seven-week-old male Sprague-Dawley rats, weighing approx. 200 g, were injected intraperitoneally with DEN (200 mg/kg body weight) and killed 3 days later. The sources of proteases were prepared by the published method [18] from DEN-treated rat livers; in brief, 10 % (w/v) homogenates in 50 mM Hepes buffer, pH 7.8, containing 0.25 M sucrose were centrifuged at 12000 *g* for 20 min; the supernatant was further centrifuged at 105000 *g* for 1 h. The final precipitate was resuspended in 50 mM Tris/HCl, pH 7.8, and used as microsomal proteases in the following experiments.

Various organs from age-matched control rats were excised and the cytosolic fractions were prepared as the source of a non-receptor-type protein kinase in accordance with the published method [19–22]. Thus each organ was homogenized at 10 % (w/v) in a solution containing 25 mM Hepes buffer, pH 7.4, 0.25 M sucrose and PMSF as protease inhibitor. The homogenate

was centrifuged at 12000 *g* for 20 min and the supernatant obtained was further centrifuged at 105000 *g* for 1 h to obtain the cytosolic fraction, which was used as the source of protein kinase.

Preparation of anti-(C-terminal peptide) antibodies

Recombinant MGMT protein and the polyclonal antibodies against rMGMT were prepared with pGSTRMGMT plasmid by the method described previously [17]. Anti-(C-terminal peptide) antibody (corresponding to residues 194–209) was prepared by the addition of keyhole-limpet haemocyanin to the N-terminus of the SWLKPSFESSSPKPSG (single-letter amino acid codes) peptide synthesized by Research Genetics (Huntsville, AL, U.S.A.). The peptide linked to keyhole-limpet haemocyanin by glutaraldehyde and carbodi-imide was injected into New Zealand White rabbits. After two boosters, the whole serum containing the highest antibody titre against the C-terminal peptide was obtained in 10 weeks, and Protein A-Sepharose[®] (CL-4B)-bound IgG was purified as the antibody against MGMT C-terminal peptide for the following experiment.

Phosphorylation reaction of MGMT proteins

Reaction mixtures for phosphorylation contained rMGMT (3 µg), cytosolic fraction (5 µg), [γ -³²P]ATP (9 µCi) (diluted with 0.1 mM unlabelled ATP), 1 mM CoCl₂ and 50 mM Hepes buffer, pH 7.4, in a total volume of 60 µl; the mixtures were incubated at 22 °C for 30 min. The reaction was terminated by the addition of 20 mM EDTA. Autoradiography was performed after resolving the total incubation mixture by SDS/PAGE and by exposing the gel to X-ray film for 4 h (or 10–12 h when liver kinase was used) at –70 °C with intensifying screen.

Determination of phospho amino acids

To identify phospho amino acid(s) after the phosphorylation reaction of MGMT, the protein was incubated with [γ -³²P]ATP by the procedure described above and the reaction mixture was separated by SDS/PAGE. To elute the phosphorylated rMGMT (rMGMT-P), γ -³²P-labelled protein band on SDS/PAGE was cut out and the gels were incubated in 1 ml of 50 mM NaHCO₃/0.5 % SDS/5 mg/ml dithiothreitol for 16 h at 20 °C. Eluted

protein was hydrolysed in 6 M HCl at 110 °C for 90 min after the removal of salt by dialysis against distilled water; the hydrolysate was dried with a Speedvac. The hydrolysate was resuspended in 10 μ l of water, and was resolved together with standard phosphoserine, phosphothreonine and phosphotyrosine by one-dimensional low-voltage thin-layer electrophoresis (LV-TLE; 17 V/cm) [23] on silica-gel plates in a solvent system containing pyrimidine/acetic acid/water (1:10:189, by vol.) at pH 3.5. The phosphorylated amino acids on the plate were identified by ninhydrin spray and autoradiography.

Site-directed mutagenesis with mega-primer

To prepare a mutated rMGMTS204A plasmid, mega-primer (132 bp) was first produced by PCR amplification with *Pfu* DNA polymerase (Stratagene, La Jolla, CA, U.S.A.) with S204A primer (5'-GAGTCTTCGCCCAAAG-3'), pGEX 3'-primer (5'-CCGGGAGCTGCATGTGTCAGAGG-3'; Pharmacia Biotech) and wild-type pGSTRMGMT plasmid as template prepared in our laboratory [17]. Another round of PCR amplification was performed with the amplified double-strand mega-primer MGMT upstream primer (5'-GGGGAATTCATGGCTGAGATCTGCAAA-3') and the wild-type pGSTRMGMT plasmid as template; the full-length mutated MGMTS204A DNA strands were then inserted into the *EcoRI* site in the pGEX-4T-1 expression vector. MGMTS194A, MGMTS199A, MGMTS194/199A and MGMTS202/203A were also prepared by the same procedure as above, with the use of the primers 5'-TCTGAT-TGGGGCCTGGCTCAAG-3' for S194A, 5'-CTCAAGCCAGCCTTCGAGTC-3' for S199A and 5'-TTCGAGGCTGCCAGCCCA-3' for the S202/203A double mutant. The MGMTS194/199A double mutant was prepared with the S199A primer and the MGMTS194A mutant plasmid as template DNA. Mutations of the prepared plasmids were confirmed by automatic DNA sequence analysis (ABI377; Perkin-Elmer, Foster, CA, U.S.A.).

Effect of PKC inhibitors on MGMT phosphorylation

To identify which PKC isoenzymes were involved in MGMT phosphorylation, a specific PKC inhibitor indolocarbazole Gö6976 was employed for inhibiting Ca²⁺-dependent PKC isoenzymes and bisindolymaleimide Gö6850 was employed for inhibiting total PKC isoenzymes, including PKC- α , PKC- β 1, PKC- γ , PKC- ϵ , PKC- δ and PKC- ζ [24–26]. Each PKC inhibitor was added, at a final concentration of 20 μ M, to the phosphorylation reaction mixture containing [γ -³²P]ATP and spleen cytosolic fraction. The reaction was terminated by the addition of 20 mM EDTA, rMGMT was separated by SDS/PAGE [12% (w/v) gel] and the gel was exposed to X-ray film (4 h with the cytosolic fraction from spleen, but 12 h with the liver fraction) for autoradiography with intensifying screen. As a positive control for PKC inhibitors, up to 2 μ M of each PKC inhibitor was added to the incubation mixture containing 5 μ g of histone H1, 1 m-unit of PKC-M (1 unit being defined as the amount of enzyme transferring 1.0 nmol of phosphate to histone H1 per min at 30 °C), 10 μ M ATP plus [γ -³²P]ATP and 50 mM Hepes buffer, pH 7.4, and the mixture was incubated at 30 °C for 10 min. Histone H1 was separated by SDS/PAGE and the gel was exposed to X-ray film for 4 h with an intensifying screen at -70 °C for autoradiography.

Proteolysis of MGMT protein by thrombin or liver microsomal protease

To investigate the degradation of MGMT proteins, 100 ng of rMGMT was incubated with 5 units of thrombin in PBS, pH 7.4,

in a total volume of 120 μ l at 25 °C for 12 h. During incubation, aliquots (20 μ l) were taken at the indicated times and were separated by SDS/PAGE [12% (w/v) gel]. To confirm whether the C-terminal fragment of rMGMT was cleaved off during carcinogenesis, 200 ng of rMGMT was incubated with 10 μ g of the microsomal protease and 10 mM CaCl₂ in 50 mM Tris/HCl, pH 7.4, in a total volume of 200 μ l, at 37 °C. At 0, 1, 2 and 6 h, a 40 μ l aliquot was taken for Western-blot analysis and autoradiography. For evaluating the effect of proteolysis on the phosphorylation, the above aliquots were further incubated with rat spleen cytosolic fraction by the method described above in the section on the phosphorylation reaction of MGMT proteins.

Effect of phosphorylation of MGMT on the protease resistance

To test the effect of phosphorylation on protease digestion, the phosphorylated and unphosphorylated rMGMTs (100 ng of each) were incubated with trypsin (enzyme-to-substrate ratio 1:100), chymotrypsin (enzyme-to-substrate ratio 1:100) or Glu-C (enzyme-to-substrate ratios 1:50 and 1:10) in sodium bicarbonate buffer, pH 7.8, at 30 °C for 1 h. Aliquots of the reaction mixture were taken 0, 10, 20 and 60 min later. Degradation of the C-terminal region by proteases was determined by Western-blot analysis with the anti-(C-terminal peptide) antibody and the ECL kit.

Western-blot analysis and autoradiography

MGMT proteins were diluted in 2 \times SDS sample buffer [62.5 mM Tris/HCl (pH 6.8)/2% (w/v) SDS/5% (v/v) 2-mercaptoethanol/10% (v/v) glycerol/0.002% Bromophenol Blue], boiled for 5 min and resolved by SDS/PAGE [12% (w/v) gel]. The protein bands were transferred to nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) and the blot was hybridized with anti-rMGMT IgG antibody or anti-(C-terminal peptide) antibodies prepared as described above. ECL reagent was applied to reveal the protein bands. [γ -³²P]ATP-labelled MGMT proteins were measured by autoradiography after separation by SDS/PAGE [12% (w/v) gel] and exposure to X-ray film at -70 °C.

RESULTS

Conversion of 26 kDa MGMT protein into 24 kDa species *in vitro* and *in vivo*

In confirmation of our earlier results [17], MGMT protein in rat liver was significantly induced during the DEN-treated hepatocarcinogenesis; the presence of two MGMT proteins as 26 and 24 kDa species was confirmed by Western-blot analysis with anti-MGMT polyclonal antibody (Figure 2A). Because cancer development is frequently accompanied by increased intracellular protease activity [18], we considered a possible involvement of proteases in the appearance of the protein with two molecular sizes and in the conversion of the 26 kDa species into the 24 kDa species. Therefore, as a first step, rMGMT was incubated with thrombin *in vitro* and the conversion was followed by Western-blot analysis with an anti-MGMT polyclonal antibody. Indeed, as shown in Figure 2(B), the 26 kDa MGMT protein began to decrease after 1 h of incubation and the decrease continued until 12 h, with a concomitant increase in the 24 kDa species. When rMGMT was pretreated with thrombin for 12 h, during which time almost half of the 26 kDa species was converted into the 24 kDa species (Figure 2B), and the sample was assayed for MGMT activity by reaction with [*methyl*-³H]DNA for 1 h at 37 °C to assess remaining activity by the established method [27],

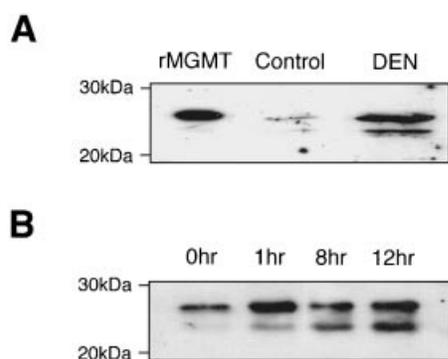


Figure 2 Western-blot analyses showing the conversion of 26 kDa rMGMT into 24 kDa

(A) Induction and proteolytic modification of MGMT protein during DEN-induced carcinogenesis, demonstrated by anti-MGMT polyclonal antibodies. rMGMT in the first lane indicates the recombinant rat MGMT protein (10 ng), control indicates the MGMT level in normal liver, and DEN indicates MGMT proteins found in the DEN-treated rat livers; 250 μ g of protein was loaded in each lane. Note, in the DEN lane, the induced level of the protein and the partial conversion of the MGMT protein from 26 into 24 kDa during the carcinogenesis. (B) Thrombin-induced conversion of 26 kDa rMGMT. rMGMT (100 ng) was incubated with 5 units of thrombin in a total volume of 120 μ l at 37 °C; aliquots (20 μ l each) were taken at the indicated times. The samples were resolved by SDS/PAGE [12% (w/v) gel], then hybridized with anti-rMGMT polyclonal antibody. Note the increasing ratio of 24 kDa to 26 kDa rMGMT as the incubation continued.

the amount of *methyl*- 3 H transferred to rMGMT was the same as that in the control, which had not been treated with thrombin (results not shown). This indicated that conversion of the 26 kDa species into the 24 kDa species (Figure 2B) did not diminish the *methyl*- 3 H acceptability of the protein; that is, both the 26 and 24 kDa species of MGMT had an equal capacity for accepting a methyl group from [*methyl*- 3 H]DNA.

Phosphorylation of serine residue of 26 kDa rMGMT

Because rMGMT protein contains a potential PKC phosphorylation site in the C-terminal region as mentioned above, post-translational phosphorylation of rMGMT protein was evaluated. First, to obtain active protein kinases, cytosolic fractions of several normal rat organs were prepared and were subjected to kinase assay with [γ - 32 P]ATP and rMGMT. As shown in Figure 3(A), spleen, brain and testes revealed high activity; low activity was found in liver. Next, to answer the question of whether or not both the 26 and 24 kDa species of MGMT protein could be phosphorylated, rMGMT was first incubated with [γ - 32 P]ATP with the use of the rat spleen cytosolic fraction; the radiolabelled rMGMT was resolved by SDS/PAGE followed by autoradiography. As shown in Figure 3(B), when pretreated with thrombin, subsequent phosphorylation occurred only in the 26 kDa rMGMT species, not in the smaller one (lane 1 shows staining with Coomassie Blue and lane 2 shows autoradiography). To identify the amino acid residue phosphorylated in the 26 kDa rMGMT, 32 P-labelled rMGMT was hydrolysed with 6 M HCl and the hydrolysate was resolved by LV-TLE as described in the Experimental section. As shown in Figure 3(C), Ser was the only amino acid found to be phosphorylated.

Ser²⁰⁴ phosphorylated by Ca²⁺-independent PKC isoenzymes

Next, we undertook the definition of the site of phosphorylated serine in the rMGMT-P. To identify the general location of the

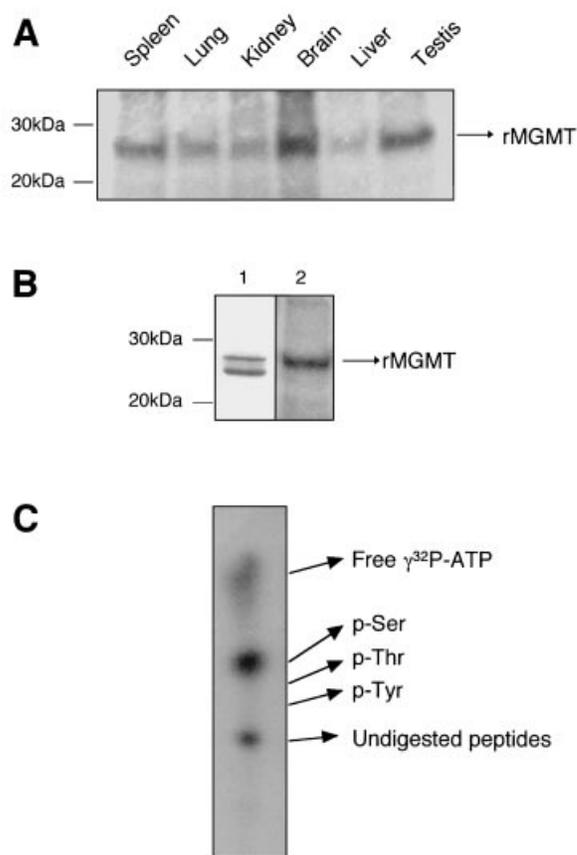


Figure 3 Phosphorylation of the serine residue of the 26 kDa rMGMT protein

(A) Autoradiography of rMGMTs incubated with [γ - 32 P]ATP and the cytosolic fractions of various rat organs. Note high kinase activity in brain, spleen and testes, intermediate activity in lung and kidney, and the lowest in liver. (B) Coomassie Blue staining and autoradiogram of [γ - 32 P]ATP-labelled rMGMTs. Phosphorylation was performed by incubating 3 μ g of rMGMT with [γ - 32 P]ATP and spleen cytosolic fraction as described in the Experimental section. The reaction mixture was resolved by SDS/PAGE [12% (w/v) gel], stained with Coomassie Blue (lane 1) and then autoradiographed (lane 2). Note the phosphorylation signal in the 26 kDa rMGMT only. (C) Analysis of phospho amino acid residue by LV-TLE. To identify phosphorylated amino acid residues, 32 P-labelled rMGMT was hydrolysed in 6 M HCl, the hydrolysate was resolved by LV-TLE procedure and autoradiography was performed. The autoradiogram was aligned with the migration of unlabelled phosphoserine (p-Ser), phosphothreonine (p-Thr) and phosphotyrosine (p-Tyr) as standards.

phosphoserine, 32 P-labelled 26 kDa protein was subjected to treatment with CNBr in formic acid. The digest was resolved and autoradiographed at -70 °C for 5 h. Of the five polypeptides produced, arising from the four methionine residues in rMGMT (Figure 1), only the 8 kDa fragment corresponding to residues 139–209 contained 32 P label (results not shown). This fragment, which corresponded to the C-terminal region of rMGMT, contained the active site of MGMT protein (PCHRV; single-letter amino acid codes) as well as ten Ser residues, two Thr residues and one Tyr residue (Figure 1). Therefore, by means of site-directed mutagenesis, the Ser residues present in the C-terminal region of rMGMT were replaced with Ala to yield the mutants S194A, S199A, S194/199A, S202/203A and S204A. When these MGMT proteins were isolated and reacted for phosphorylation, only the S204A mutant was absolutely inert towards phosphorylation (Figure 4A), thus confirming Ser²⁰⁴ to be the phosphorylation site in the rat MGMT protein. At this

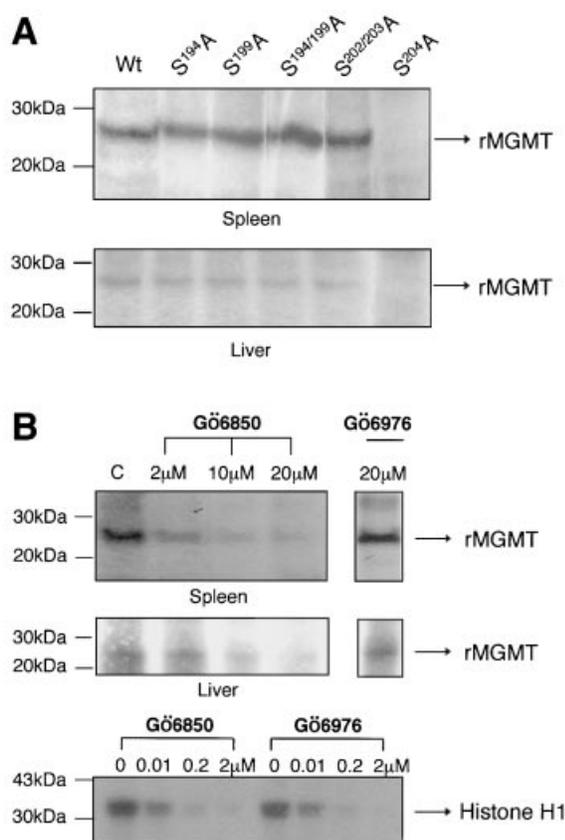


Figure 4 Phosphorylation of Ser²⁰⁴ by novel PKC (Ca²⁺-independent) isoenzymes

(A) Autoradiogram confirming the phosphorylation of Ser²⁰⁴ in rMGMT by site-directed mutagenesis. Six Ser residues in the C-terminus of the 26 kDa rMGMT were replaced with Ala by site-directed mutagenesis. The wild-type and mutant rMGMTs were overexpressed in BL21(DE3) cells and subjected to phosphorylation reaction with [γ -³²P]ATP. Only the Ser²⁰⁴ mutant revealed a loss of phosphorylation signal, proving Ser²⁰⁴ to be the only site in rMGMT that is phosphorylated by an MGMT kinase in rat spleen (upper panel) and in liver (lower panel). (B) Autoradiogram demonstrating the involvement of Ca²⁺-independent PKC isoenzymes of spleen (top panel) and liver tissue (middle panel) in the phosphorylation of MGMT. Gö6850 and Gö6976 are inhibitors of total PKC isoenzymes and Ca²⁺-dependent PKC isoenzymes respectively. Bottom panel: phosphorylation of histone H1 protein by PKC-M, which is the catalytic subunit of purified PKC, and the inhibition of its phosphorylation by Gö6850 and Gö6976, as a positive control.

point it was concluded that the C-terminal 2 kDa fragment of rMGMT was cleaved off by thrombin and that Ser²⁰⁴ was the phosphorylation site.

To delineate the protein kinase isoenzymes that were possibly involved in phosphorylating rMGMT, specific PKC inhibitors were employed. As shown in Figure 4(B), phosphorylation of the rMGMT by spleen or liver cytosolic fractions was inhibited by Gö6850 at 2 μ M, whereas it was not inhibited by Gö6976 even at 20 μ M. The result clearly indicated that phosphorylation of the rMGMT was performed by Ca²⁺-independent PKC isoenzymes present in the cytosolic fraction of rat spleen and liver tissues [24].

Degradation of 26 kDa rMGMT by liver microsomal preparation during DEN-induced carcinogenesis

To confirm that the MGMT protein could be degraded by proteases induced during hepatocarcinogenesis and that the

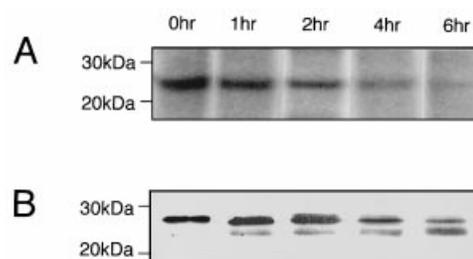


Figure 5 Degradation of the 26-kDa rMGMT by a liver microsomal preparation during DEN-induced carcinogenesis

(A) Autoradiogram of rMGMT-P proteins showing the effect of prior proteolytic processing on the subsequent phosphorylation of rMGMT. The reaction mixture for proteolysis contained 1 μ g of rMGMT and 5 μ g of microsomal proteins isolated from the liver of DEN-treated rat; the mixtures were incubated at 37 °C for the indicated durations. Subsequently, a phosphorylation reaction was performed by the addition of 5 μ g of rat spleen cytosolic fraction, 1 mM CoCl₂ and 9 μ Ci of [γ -³²P]ATP at 22 °C for 30 min. The control (0 h) contained all the reaction mixtures but had no prior treatment with microsomal fraction, to indicate the initial total phosphorylation level of the rMGMT. (B) Western-blot analysis with anti-rMGMT polyclonal antibodies. The same blot as that used in (A) was hybridized with anti-rMGMT polyclonal antibodies to reveal the proteolytic processing of the 26 kDa MGMT protein by microsomal proteins. Because the C-terminus contains the phosphorylation site, the densities of the autoradiogram (A) and the 26 kDa MGMT band start to decrease and a new 24 kDa band appears within 1 h of the start of treatment (B).

resulting products could also be phosphorylated *ex vivo*, rMGMT was first incubated with microsomal proteins prepared from DEN-treated rat livers and phosphorylation was subsequently performed with spleen cytosolic fraction in the presence of [γ -³²P]ATP. As shown in Figure 5(A), the phosphorylation of rMGMT was markedly diminished after 4 h of treatment with microsomal fraction (proteolysis). When the samples were hybridized with polyclonal anti-MGMT antibody, a decrease in the 26 kDa species was clearly visible, with a concomitant increase in the 24 kDa MGMT band (Figure 5B). This indicated strongly that the protease(s) that increased during the DEN-induced carcinogenesis cleaved off a peptide fragment that contained a putative phosphorylation site (Ser²⁰⁴) from rMGMT.

Phosphorylated MGMT protein is resistant to digestion with endopeptidase Glu-C and DEN-induced liver proteases

To investigate the biological significance of MGMT phosphorylation, rMGMT was first phosphorylated by spleen cytosolic fraction with or without unlabelled ATP at 22 °C for 30 min. Subsequently, the rMGMT-P and unphosphorylated proteins were incubated with microsomal proteins isolated from DEN-treated rat livers at 30 °C and analysed by Western-blot hybridization with anti-(C-terminal peptide) antibody. This antibody was expected to quantify MGMT protein with the C-terminal region intact. As shown in Figure 6(A), rMGMT-P revealed more resistance to C-terminal cleavage than the unphosphorylated one. To elucidate the kind of proteases involved in the rMGMT degradation during DEN-induced carcinogenesis in rat, digestions with trypsin, chymotrypsin or Glu-C were performed at 30 °C for 60 min. As shown in Figure 6(B), the rMGMTs were relatively resistant to digestions with trypsin and chymotrypsin for 1 h. Moreover, the degree of sensitivity of the MGMT proteins to trypsin and chymotrypsin was independent of phosphorylation. In contrast, as determined with two different

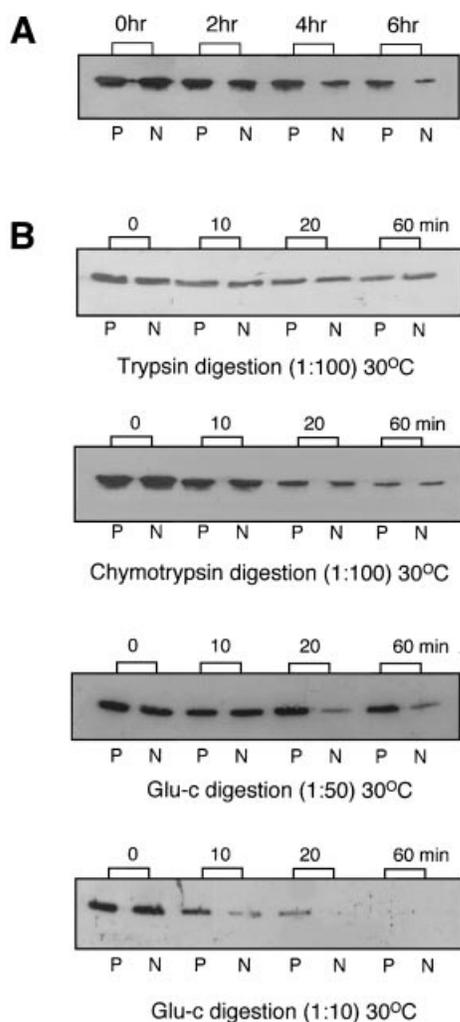


Figure 6 Western-blot analyses showing the effect of phosphorylation of rMGMT on its sensitivity to various proteolytic enzymes

rMGMT-P (P) and unphosphorylated rMGMT (N) were prepared as described in the Experimental section and in the legend to Figure 5. Subsequently, the above phosphorylation reaction mixtures at 22 °C were subjected to the actions of the liver microsomal fraction of DEN-treated rat liver at 37 °C (A), or trypsin, chymotrypsin or Glu-C (V8 protease) at 30 °C (B). The final reaction mixtures were resolved by SDS/PAGE [12% (w/v) gel] and transferred to nitrocellulose membrane, then hybridized with anti-(C-terminal peptide) antibody. The enzyme-to-substrate ratios are indicated in parentheses. Note the significant protection of the rMGMT-Ps against digestion by microsomal proteases and by Glu-C. rMGMT (150 ng) was used for the chymotrypsin assay; 100 ng was used for the others.

concentrations of Glu-C, rMGMT-P was much more resistant to digestion with Glu-C than rMGMT (Figure 6B).

DISCUSSION

The biological significance of MGMT is to remove the *O*⁶-alkylguanine adduct in DNA formed either by exogenous environmentally important genotoxic alkylating agents or by endogenous *S*-adenosyl-*L*-methionine [2,28–30]. If not repaired, the persistent presence of *O*⁶-methylguanine in DNA could ultimately lead to mutagenesis and/or carcinogenesis by either interfering with the G–C hydrogen-bonding in DNA [31–34] or by mismatching owing to its preference for pairing with thymine during DNA replication [35–37].

Ada-C underwent conformational changes in its structure on binding to DNA, thereby exposing the active site to the alkyl group on the DNA substrate; the conformational changes consistently activated the protein [38]. Native human MGMT protein was found to have very limited accessibility to proteases in its native state; however, the sensitivity to digestion by trypsin or Glu-C (V8 protease) was significantly increased in response to DNA binding or alkylation [39], indicating that the protein undergoes conformational changes under these conditions. Hubbard et al. [40] suggested that the proteases generally cleave peptide sequences only in the regions that can adopt rather extended conformations. It should be noted that mammalian MGMT protein has also been known to bind to both double-stranded DNA and single-stranded DNA [41–43], and more tightly to the *O*⁶-methylguanine DNA adduct [44].

In the present study we demonstrated two post-translational modifications of rMGMT during DEN-induced carcinogenesis: the cleavage of a C-terminal 2 kDa fragment containing a potential phosphorylation site at Ser²⁰⁴, and the phosphorylation of Ser²⁰⁴, which made rMGMT more resistant to cleavage by proteases induced during DEN-induced carcinogenesis *in vivo* or by Glu-C. Because amino acid sequence analysis revealed that only rMGMT contained the C-terminal region with a potential PKC phosphorylation site, we explored the best source of PKC activity in normal rat organs and found that brain, spleen and testes had the highest activity, followed by lung and kidney; liver had the lowest (Figure 3A). This pattern of phosphorylating activity in the various rat organs was well correlated with a report by Wetsel et al. [45] on the distribution and concentration of PKC isoenzymes determined by Western-blot analyses. In the present study we therefore chose spleen cytosolic fraction as a source of protein kinase. Because phosphorylation was strongly inhibited by 2 μM Gö6850, which inhibited the PKC-α, PKC-βI, PKC-δ, PKC-ε and PKC-ζ isoenzymes, but not by 20 μM Gö6976, which inhibited the Ca²⁺-dependent PKC-α, PKC-βI and PKC-μ isoenzymes (Figure 4B), it was highly likely that the phosphorylation was performed primarily by Ca²⁺-independent PKC isoenzymes such as PKC-δ, PKC-ε, PKC-ζ and possibly another uncharacterized kinase in the liver cytosolic fraction [24–26].

A site-directed mutagenesis study (Figure 4A) revealed that the phosphorylation site was absolutely specific; when the six serine residues present in the C-terminal region of rMGMT were mutated to alanine one at a time (S194A, S199A, S204A) or in pairs (S194/199A, S202/203A), only the S204A mutant showed a complete loss of phosphorylation potential. This Ser²⁰⁴-Pro-Lys sequence corresponded exactly to PKC consensus sequences reported previously [15,16]; this phosphorylation sequence was present only in rat MGMT protein, not in the human, mouse or Ada-C proteins (Figure 1).

Phosphorylation of the C-terminal fragment that can be cleaved off was confirmed by autoradiography and Western-blot analyses with anti-MGMT polyclonal antibodies (Figure 5). Microsomal proteins isolated from the DEN-treated rat livers significantly degraded 26 kDa rMGMT (Figure 5A) with a concomitant increase in the 24 kDa species during a 6 h incubation (Figure 5B). In addition, the overall level of phosphorylation was significantly diminished when rMGMT was pretreated with the liver microsomal fraction; a 2 kDa peptide fragment was cleaved off from the 26 kDa protein during the pretreatment. This indicated that the C-terminal region of rMGMT was phosphorylated by PKC isoenzymes and that this region could be cleaved off by the microsomal protease that increased in the DEN-treated rat liver. The notion that proteolysis was induced in DEN-treated rat liver was supported by the following findings.

The administration of liver tumour promoters to male rats for 1 week induced Ca²⁺-dependent serine protease activity 3–5-fold and the enzyme was found particularly in the smooth endoplasmic reticulum [18].

Before we investigated the site of hydrolysis of rMGMT by thrombin (Figure 2B), ³²P-labelled 26 kDa rMGMT was treated with CNBr in formic acid to define its general location; the digest was resolved by SDS/PAGE followed by autoradiography. Only an 8 kDa fragment corresponding to residues 139–209 was ³²P-labelled (results not shown). Thrombin is one of the highly specific proteolytic enzymes to hydrolyse the peptide bond between Lys and Gly present up-stream of the hydrophobic amino acids [46]. On the basis of our results it was therefore highly possible that thrombin cleaved rMGMT at Lys¹⁸⁷-Gly¹⁸⁸, producing a 22-residue peptide. It is of interest that the most likely potential trypsin cleavage site has also been suggested to be Lys¹⁹³-Gly¹⁹⁴ in human MGMT [39]. Our finding that thrombin-digested rMGMT retained all the methyltransferase activity is in good agreement with the result of Elder et al. [47] that the rates of methyltransferase activity of full-length and C-terminal-truncated human MGMT proteins were virtually identical. Furthermore, full activity was retained in human MGMT mutants with 8 and 31 residues deleted from the N-terminus and the C-terminus respectively [44]. It should again be noted that the cleaved fragment corresponded to the extended C-terminal region of rMGMT, which was absent from yeast, rabbit and bacterial MGMTs [38,48,49]. In contrast, Morgan et al. [49] reported that although both the full-length MGMT and the protein with 28 residues deleted were active at 37 °C, only the former was active at 4 °C. They also observed different substrate specificities between them, even though the DNA repair activities were the same.

It has been shown in the present study that the phosphorylation of the C-terminal Ser²⁰⁴ of rMGMT has a significant effect on its resistance to proteases *in vivo* as well as *in vitro* (Figure 6). rMGMT-P is clearly more resistant to digestion than the unphosphorylated protein. Therefore, although Glu-C is naturally found in bacteria, this endowment of protease resistance on rMGMT by phosphorylation might have some significance during carcinogenesis in liver by alkylating agents. These results indicate that conformational changes are induced not only by the binding of DNA and the alkylation of MGMT but also by the phosphorylation of rMGMT.

In conclusion, we suggest that the treatment of a rat with alkylating agents might induce Glu-C-like proteolytic enzymes in liver during the carcinogenic process and that the phosphorylation of MGMT can induce a conformational change in the protein, thereby protecting it from digestion by induced proteases. Because alkylated MGMT protein can be ubiquitinated [50] and digested by uncharacterized proteases *in vivo* for eventual disposal, further studies on this metabolic fate of rMGMT-P *in vivo* are needed.

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