Role of GRP58 in Mitomycin C-induced DNA Cross-Linking

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ABSTRACT

Mitomycin C (MMC) is an anticancer drug that requires reductive activation to exert its toxicity. MMC is known to cross-link DNA that contributes significantly to the cytotoxicity and consequent cell death. Cytosolic NADPH:quinone oxidoreductase 1 (NQO1) and microsomal enzymes have been shown to mediate MMC-induced DNA cross-linking. However, NQO1 plays only a minor role, indicating presence of other cytosolic enzymes/proteins that contribute to this process. In this study, we have characterized a unique cytosolic activity in NQO1-null mice that catalyzed MMC-induced DNA cross-linking. This activity was cofactor independent and dicoumarol insensitive. The unique cytosolic activity was purified to homogeneity. The peptide sequencing of the purified protein identified the unique cytosolic activity as GRP58 (Mₙ, 58,000 glucose-regulatory protein), also known as GRp57/ER60/ERp61/HIP-70/Q2 and CPT. Immunodepletion of NQO1-null mice liver cytosol and partially purified fractions with anti-GRP58 antibody led to a complete loss of GRP58 protein and consequent significant reduction of MMC-induced DNA cross-linking. Mouse cDNA encoding GRP58 was isolated and sequenced. Chinese hamster ovary cells permanently overexpressing GRP58 showed increased MMC-induced DNA cross-linking and increased cytotoxicity on exposure to MMC. Bacterially expressed and purified GRP58 increased the MMC-induced DNA cross-linking when added to mouse cytosolic samples. A tissue array analysis indicated that GRP58 is ubiquitously expressed among mouse tissues, although at different levels. Expression analysis using matched human tumor/normal array revealed an up-regulation of GRP58 in breast, uterus, lung, and stomach tumors compared with normal tissues of similar origin.

INTRODUCTION

Bioreductive chemotherapy is the most successful treatment for certain types of cancer. It is based on two major factors (1): the first is to develop drugs that are activated by specific proteins, and the second is to identify tumor types that are rich in those proteins (1). In addition, bioreductive drug development is based on differences in oxygen content and cellular pH between normal and tumor tissues (1–7). Several natural and synthetic quinones, including MMC,3 indoloquinone E09, diaziqumone, methyl-diaziqumone, and S-(azirin-1-yl)-2,4-dinitrobenzamide (CB1954), have found an application in bioreductive chemotherapy because of their potential to undergo reduction by different cellular enzymes in aerobic and hypoxic conditions (1).

MMC is a prototypical bioreductive alkylating antitumor agent that is effective against several tumor tissues including colon, breast, lung, head, and neck (8). MMC is activated metabolically to cause DNA alkylation, monofunctional adducts formation, and DNA cross-linking that leads to cytotoxicity and cell death (9, 10). Several enzymes, including cytosolic (NQO1 and other unknown cytosolic proteins) and microsomal (P-450 reductase, cytchrome b₅ reductase, xanthine oxidoreductase, and dehydrogenase), have been shown to catalyze MMC activation, leading to DNA cross-linking and cytotoxicity (11–17). MMC activation by cytosolic proteins far exceeds the activation by microsomal proteins (16). NQO1 has been studied the most and has been shown to play only a minor role in cytosolic activation of MMC (16, 17). This suggested the presence of additional enzymes/proteins that contribute to the cytosolic activation of MMC, leading to DNA cross-linking.

GRP58, also known as ERp57, is a Mr, 58,000 glucose-regulatory protein with significant homology to protein disulfide isomerase (18, 19). GRP58 has two thioredoxin-like domains that are responsible for its thiol-dependent oxidoreductase activity (18–20). In the endoplastic reticulum, GRP58 specifically interacts with glycoproteins such as calnexin and calreticulin, playing an important role as a molecular chaperone of glycoprotein biosynthesis (21). Recently, GRP58 has been shown to be involved in the assembly of newly synthesized MHC class I molecules, a process that also involves the chaperones calnexin and calreticulin (21). The presence of GRP58 has also been shown in cytosolic and nuclear fractions (22–24). In the nucleus, GRP58 has been indicated as a component of the subset of nuclear matrix proteins that are responsible for DNA attachment to the nuclear matrix and for the formation of DNA loops caused by GRP58 binding capacity to DNA (22, 23). In the cytosol, GRP58 has been identified as a chaperone for the signal transducer and activator of transcription signaling in which STAT3 protein function in the cytoplasm as complexes with novel accessory scaffolding protein (24). The biological role of GRP58, except its chaperone function, remains unknown.

In the present study, we have further characterized and identified the unique cytosolic activity that catalyzes MMC-induced DNA cross-linking. This activity was found cofactor independent, dicoumarol insensitive, and optimum at physiological pH. The unique cytosolic activity that catalyzes MMC-induced DNA cross-linking was purified to homogeneity from NQO1-null mice liver and identified as GRP58. GRP58 is expressed ubiquitously but at varying levels among the various mouse tissues. Human tumor/normal expression array analysis demonstrated higher expression of GRP58 in human breast, lung, uterus, and stomach tumors compared with normal tissues of similar origin.

MATERIALS AND METHODS

Materials

The Chinese hamster ovary (CHO) cells were obtained from American Type Culture Collection (ATCC CRL9096; Manassas, VA). Cell culture reagents were obtained from Life Technologies, Inc. (Gaithersburg, MD). MMC was a gift from Bristol-Myers Squibb (Princeton, NJ) and was also purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Polyclonal antibodies against purified full-length GRP58 protein (SPA-580) were purchased from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada). All other reagents used in the experiments were of the highest purity available commercially.

NQO1-Null and NQO2-Null Mice

We used a gene-targeting method in the past to disrupt the NQO1 gene and generated NQO1−/− mice that do not express NQO1 RNA, protein, and activity (25). A similar strategy was also used to generate NQO2−/− mice that...
were deficient in NQO2 RNA, protein, and activity (26). Analysis of NQO1-null and NQO2-null mice also indicated that levels of other drug-metabolizing enzymes, including glutathione S-transferase, xanthine oxidoreductase, cytochrome P-450 reductase, and cytochrome-b5 reductase, remained unchanged because of the loss of expression of the NQO1 gene. The wild-type, NQO1-null, and NQO2-null mice were bred separately in the animal colony at Baylor College of Medicine to generate sufficient mice for all experiments listed below.

**MMC-induced DNA Cross-linking Assay**

 MMC-induced DNA cross-linking assays were performed by procedure, as described (11, 16, 17). Briefly, the two strands of the 23 bp of oligonucleotides containing the MMC-binding site were selected from plasmid pBR322, synthesized, and used for the cross-linking experiments. The nucleotide sequence of the 23-bp oligonucleotide is: 5’-CTACATCGTGTACGACAGGAT-3’. The complementary strands were mixed in equal amounts and annealed by heating to 70°C for 15 min and slowly cooling to room temperature. The 3’-end of the top strand was selectively labeled with DNA polymerase I large (Klenow) fragment (Promega, Madison, WI) and [α-32P]dCTP (New England Nuclear, Boston, MA). The labeled oligonucleotides were purified on a 15% nondenaturing polyacrylamide gel, gel eluted, and concentrated by ethanol precipitation. 32P-labeled oligonucleotides were used to detect MMC-induced DNA cross-linking by incubation with 0.5 mg of cytosolic proteins, 100 mM phosphoprotein buffer (pH 5.8), 1 mM NADPH, 150 µM MMC, 6 µM FAD, 0.01% Tween 20, and 0.18 mg of BSA, unless specified differently. Reactions were incubated for 1 h at 37°C and terminated by the addition of ethanol, 10 mM MgCl2, and 1.5 mM ammonium acetate. The samples were frozen at −70°C, and DNA was pelleted by centrifugation at 13,700 rpm for 30 min at 4°C. The supernatant was removed, and the precipitated oligonucleotides were centrifuged in Speed-Vac until dry. The precipitated oligonucleotides were resuspended in DNA sequencing dye-containing formamide and denatured by heating at 95°C for 15 min, and then rapidly cooled on ice. The samples were analyzed on a 15% denaturing polyacrylamide gel containing 8 M urea, and cross-linked and unmodified (free) oligonucleotides were detected by autoradiography performed on Kodak Bio-max MS films using intensifying screens at ~70°C for 18 h.

**Characterization of Unique Cytosolic Activity and Comparison of MMC-induced DNA Cross-linking in the Various Tissues from NQO1-null and Wild-Type Mice**

Wild-type and NQO1-null mice were starved overnight, anesthetized, and sacrificed by cervical dislocation. The liver, colon, lung, kidney, spleen, and testis were removed by surgery and homogenized in buffer containing 0.25 M sucrose, 0.05 M Tris (pH 7.4), 1 mM EDTA, 2 µg/ml antipain, and 2 µg/ml leupeptin to yield 20% (w/v) homogenates. The various tissue homogenates were centrifuged at 12,000 × g at 4°C for 30 min. The supernatants were collected and centrifuged at 105,000 × g at 4°C for 1.2 h. The cytosolic fractions were collected and used in MMC-induced DNA cross-linking assays to further characterize the unique cytosolic activity. The liver and colon cytosolic fractions were used to determine optimum pH, dicumarol inhibition, and requirement for cofactors for unique cytosolic activity that catalyzes MMC-induced DNA cross-linking in NQO1-null mice. The pH was varied by the addition of phosphate or Tris-HCl buffers, followed by MMC-induced DNA cross-linking. The cytosolic fractions were incubated with a range of dicumarol concentrations for 30 min at room temperature to determine dicumarol inhibition of unique activity. Dicumarol was dissolved in a 2% NaOH (v/v) solution (pH 12). Reaction mixture including 100 mM phosphate buffer was added, and the DNA cross-linking assay was performed. The cytosolic extracts were incubated with the reaction mixture in the presence or absence of cofactors, such as NADPH, NRH, FAD, BSA, and Tween 20, to determine the cofactor requirement for MMC-induced DNA cross-linking. To eliminate the possibility that endogenous NADPH and FAD were present in the cytosolic extracts, samples were dialyzed overnight in homogenate buffer at 4°C or washed 40 times and concentrated in Y-10 Centricon columns to remove molecules smaller than Mw 10,000. Dialyzed fractions were analyzed for the DNA cross-linking assay, as described above.

The cytosolic fractions from the various tissues from wild-type (NQO1+/+) and NQO1-null mice were absolutely free of microsomal contamination, because no activity of microsomal specific enzyme NADPH:cytochrome P450 reductase could be detected (data not shown). The cytosolic fractions from wild-type mice containing 1 unit of NQO1 activity (equivalent to 1 μmol of 2,6-dichlorophenolindophenol reduced in 1 min) and similar amounts of cytosolic proteins from NQO1-null mice were incubated with the reaction mixture for the DNA cross-linking assay, as described above.

**Purification of Unique Cytosolic Activity That Catalyzes MMC-induced DNA Cross-linking**

The unique cytosolic activity was purified from NQO1-null mouse livers. The purification was monitored by SDS-PAGE/silver-staining analysis and MMC-induced DNA cross-linking assay. The mice livers cytosol was prepared by procedures described previously.

**Saltling-Out Fractionation.** Mouse liver cytosolic fraction was fractionated with ammonium sulfate to (a) 40% salt saturation and (b) the supernatant containing 40% saturation to 60% salt saturation. The addition of salt to the liver cytosolic extract was performed very slowly at 4°C under agitation. The sample was incubated for an additional 10–15 min under agitation for complete salt homogeneity. Samples were centrifuged at 12,000 × g at 4°C for 1 h to separate insoluble from soluble proteins. The 40% and 60% pellets were homogenized separately in a small volume of homogenization buffer. All of the fractions were analyzed for proteins by 10% SDS PAGE/silver-staining and MMC-induced DNA cross-linking. The 60% salt supernatant containing 3-fold enrichment of GRP58 was saved for further purification.

**Phenyl-Sepharose Column Chromatography.** The supernatant collected from 60% salt precipitation was purified by hydrophobic interaction on a phenyl-Sepharose column HI-prep 16/10 using the AKTA-fast protein liquid chromatography system (Amersham Pharmacia Biotech). The column was equilibrated with 2.5 mM (NH4)2SO4, 0.05 mM phosphate buffer (pH 6.5) and washed by gradient to 0.05 mM phosphate buffer (pH 6.8). A second gradient was applied using 0.25 mM sucrose, 1 mM EDTA, 10% glycerol, and 0.01 M phosphate buffer (pH 6.8) as buffer A and buffer B plus 10% ethylene glycol as buffer B. Fractions eluted using second gradient were dialyzed against homogenate buffer and analyzed for proteins by 10% SDS-PAGE/silver-staining and MMC-induced DNA cross-linking. Active fractions were pooled and used for additional purification.

**Affinity Chromatography by Heparin-Sepharose Column.** Active fractions from the phenyl-Sepharose column were applied on a high-trap heparin-Sepharose column associated with the AKTA-fast protein liquid chromatography system (Amersham Pharmacia Biotech) and eluted with a salt gradient using homogenate buffer containing 2 mM NaCl. Active fractions were eluted between 0.6 M and 1.2 M NaCl and dialyzed against 0.05 mM NaCl/0.05 M 4-morpholinopropanesulfonic acid (pH 6.0) and analyzed for proteins by 10% SDS-PAGE/silver-staining and MMC-induced DNA cross-linking. Active fractions were pooled and used for additional purification.

**Affinity Chromatography by MMC-Agarose Column.** The MMC-agarose column was prepared by coupling MMC (Sigma-Aldrich Chemical Company) with NHS-activated Sepharose 4 Fast Flow (Amersham Pharmacia Biotech). Briefly, 10 mg of MMC were dissolved in 5 ml 0.05 M phosphate buffer (pH 7.0) and mixed with 1 mM HCI prewashed NHS-Sepharose at the ratio 1:0.5. The mixture was protected from light and incubated overnight, shaking at 4°C. Nonreacted groups were blocked with the addition of 3 ml of 1 M Tris-HCl (pH 6.8) and incubated, shaking at 4°C for 2 h. The column was packed and washed six times with alternating buffers, A [0.1 M Tris (pH 8.5)] and B [0.1 M acetate buffer (pH 4.0)]. Approximately 30 μmol of MMC were bound to the NHS-agarose. The column was equilibrated with 0.05 mM NaCl/0.05 mM MES (pH 6.0). Active fractions from the previous step were applied to the MMC-agarose affinity column and eluted with 0.1 M Tris (pH 8.5). Eluted fractions were concentrated on Centricon Y-10 by centrifugation at 3500 rpm at 4°C. Concentrated fractions were saved for additional protein analysis by 10% SDS-PAGE/silver-staining and MMC-induced DNA cross-linking.

**Identification of Unique Cytosolic Activity as GRP58**

**Peptide Sequencing and Alignments.** Fractions obtained from the protein purification steps were analyzed by SDS-PAGE. Gel was stained with 0.05% Coomassie Blue for 30 min, followed by destaining in 5% acetic acid and 10% methanol. Proteins to be identified were isolated from the gel and digested with
lysC. Digested peptides were purified and sequenced by mass spectrophotometry at Baylor College of Medicine Protein Chemistry Core Facility.

**Immunoprecipitation Assay.** To obtain GRP58-immunodepleted samples, mouse liver cytosolic fractions and other partially purified fractions (60% ammonium sulfate supernatant and phenyl-Sepharose and heparin-Sepharose fractions) were incubated with rabbit polyclonal anti-GRP58 antibodies (StressGen Biotechnologies Corp.) overnight at 4°C, under shaking conditions. The samples incubated with rabbit preimmune serum were used as negative control. Protein AG-PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was washed twice with homogenate buffer (0.25 M sucrose, 0.05 M Tris (pH 7.4), and 0.001 M EDTA) and spun at 2000 g for 10 min to remove supernatant with impurities. Approximately 120 ml of the Protein AG-PLUS-Agarose suspension was added to each preincubated sample/purified fraction and incubated overnight at 4°C, under shaking conditions. The samples were centrifuged at 2000 g at 4°C, and the immunodepleted supernatant was carefully removed for analysis. The precipitate was washed twice with homogenate buffer, as described above. Bound proteins were eluted with a one-step wash by incubating the precipitate with 4M NaCl in 0.05M Tris (pH 7.4) solution for 30 min, shaking at 4°C. The incubation mixture was centrifuged at 2000 g at 4°C for 15 min. The supernatant containing eluted proteins was removed and dialyzed against 4 liters of homogenate buffer overnight at 4°C and kept for additional analysis. The precipitate was boiled in 20 ml of SDS-loading buffer for Western blot analysis. The various fractions were analyzed for immunodepletion of GRP58 by Western blot analysis and MMC-induced DNA cross-linking.

**Cloning of Mouse GRP58 cDNA**

The full-length mouse GRP58 cDNA was amplified by RT-PCR using synthesized forward (5′-ACCATGGCCCTTCAGTGCCCTTGC-3′) and reverse (5′-TTTGGGCTCTTGGTGAAGGCTTCTTGA-3′) primers and mouse liver Marathon cDNA library (Invitrogen, Carlsbad, CA). The PCR product was cloned into the pcDNA3.1/V5/HisTOPO vector to generate mouse GRP58-pcDNA plasmid. This clone was sequenced. The sequences matched 100% to mouse GRP58 (accession no. BC003285).

**CHO/DHFR™ Cells Stably Expressing cDNA-derived GRP58 and Cytotoxicity Assays**

The CHO/DHFR™ cells were grown in monolayer culture in Iscove’s modified Dulbecco’s medium with 4 ml 1-glutamine adjusted to contain 1.5 g/liter sodium bicarbonate and supplemented with 0.1 mM hypoxanthine/0.016 mM thymidine and 10% fetal bovine serum. The cells were grown in 5% CO2 medium containing different MMC concentrations. After 24 h, the medium was changed to medium containing different MMC concentrations. The cells were trypsinized, and plated in medium containing 400 mg/ml ampicillin. The resulting ampicillin-resistant clones were picked, verified by digestion, and sequenced. An overnight culture of DH5α E. coli (pProEx-HTA-GRP58) was diluted 1:50 (with 100 μg/ml ampicillin) in LB media, grown at 37°C with vigorous shaking until OD₆₀₀ of 0.6 was reached, and induced for another 4–5 h by the addition of isopropyl β-D-thiogalactoside to 1 mM. Cells were harvested by centrifugation at 4,000 × g for 20 min at 4°C and frozen. Bacteria were lysed by freeze/thaw in lysis buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole (pH 8.0), followed by sonication. The lysate was cleared by centrifugation at 10,000 × g for 30 min at 4°C. The supernatant was filtered through 1.2-μm Acrodiscs (Gelman Sciences) and applied to a column of Ni-NTA-agarose to purify 6xHis-tagged-GRP58. The column was washed with 50 mM NaH₂PO₄, 300 mM NaCl, and 50 mM imidazole (pH 8.0), and then eluted with 50 mM NaH₂PO₄, 300 mM NaCl, and 250 mM imidazole (pH 8.0). Fractions including 6xHis-tagged-GRP58 were dialyzed against 0.25 mM sucrose, 0.001 mM EDTA, and 0.05 mM Tris (pH 7.4). To remove the 6xHis affinity tag, the dialyzed fractions were incubated overnight at 4°C with recombinant Tobacco Etch Virus (rTEV) protease (Life Technologies, Inc.), 1 mM DTT, 0.5 mM EDTA, and 50 mM Tris-HCl (pH 8.0). The reaction mixture was dialyzed against 0.25 mM sucrose, 0.001 mM EDTA, and 0.05 mM Tris (pH 7.4), concentrated in Centricon Y-30, and the bacterially expressed mouse GRP58 was maintained at 4°C for additional analysis.

Bacterially expressed and purified GRP58 thiol-reductase activity was evaluated by insulin reduction assay, as described previously (18). Briefly, 50 μl of insulin [1 mg/ml 100 mM potassium acetate (pH 7.5) and 2 mM EDTA] plus bacterially expressed mouse GRP58 and water to a final volume of 60 μl were mixed in a cuvette. The reaction was started by adding 2 μl of DTT (10 mM). The nonenzymatic insulin reduction by DTT was registered simultaneously as a control. Thiorodoxin (Sigma-Aldrich Chemical Company) was used as positive control. Reactions were followed in the spectrophotometer (600 nm) for 60 min.

Different concentrations of bacterially expressed and purified GRP58 protein were used in MMC-induced DNA cross-linking assay to study the role of GRP58 in MMC activation and DNA cross-linking. In a related experiment, 0.5 mg of cytosolic proteins from NQO1-null liver cytosol or similar amounts of 60% ammonium sulfate fractionation were mixed with 10 μg of albumin or similar amount of bacterially expressed GRP58 and analyzed for MMC-induced DNA cross-linking. The samples were analyzed on a 15% denaturing polyacrylamide gel, and cross-linked and unmodified (free) oligonucleotides were detected by autoradiography.

**Tissue-specific Expression of Mouse GRP58**

RNA dot-blot (catalogue no. 7771-1), containing normalized load DNA from different 22 mouse tissues and mouse embryos at different stages of development, was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). A specific mouse GRP58-pcDNA 3′-P-labeled-fragment (–1.5 kb) was hybridized overnight with the RNA blot by procedures described in the manufacturer’s protocol. The blot was washed and exposed for 24 h to Biomax MS X-ray film with an intensifying screen (Eastman-Kodak Co., Rochester, NY) and autoradiographed. The blot was stripped of bound radioactivity and hybridized with 32P-labeled glyceraldehyde-3-phosphate dehydrogenase cDNA, a housekeeping gene, according to the manufacturer’s protocol.

**Expression Analysis Using Tumor/Normal Human Tissue Array**

A matched tumor/normal human expression array (Clontech Laboratories, Inc.), containing 68 cDNAs prepared from untreated human tumorigenic and
corresponding normal tissue, was used. Each pair was independently normalized based on the expression of three housekeeping genes and immobilized in separate dots. A human cDNA clone encoding GRP58 (IMAGE clone no. 4692794, ResGen; Invitrogen Corp., Huntsville, AL) was grown and characterized by sequencing. The clone containing full-length cDNA encoding human GRP58 was digested with SfiI to generate an approximate 1.5-kb fragment. The DNA fragment, isolated from a preparative agarose gel using a gel extraction kit, was radiolabeled using Ready-to-go-DNA labeling beads (Amersham Pharmacia Biotech). The expression array was hybridized with 32P-labeled human GRP58 cDNA, washed, and autoradiographed by procedures described on the Clontech protocol. The array was stripped of bound radioactivity and hybridized with 32P-labeled ubiquitin cDNA according to the manufacturer’s protocol.

RESULTS

Characterization of Unique Cytosolic Activity That Catalyzes MMC-induced DNA Cross-linking. The unique cytosolic activity showed a pH optimum at 5.8 (Fig. 1A). A significant reduction in activity was observed at pH 4.0 and pH 8.0 (Fig. 1A). The dicoumarol failed to inhibit unique cytosolic activity that catalyzed MMC-induced DNA cross-linking in NQO1-null mice (Fig. 1B). The inhibition observed with 100 μM dicoumarol concentration was caused by alkaline pH required to dissolve dicoumarol (Fig. 1B). The addition of exogenous cofactors NADH, NADPH, and/or FAD had no effect on MMC-induced DNA cross-linking (Fig. 1C, left). Depletion of endogenous NADH and FAD from NQO1-null mouse liver, colon, and lung cytosol by dialysis and filtration also had no effect on MMC-induced DNA cross-linking (Fig. 1C, right). Similar results were observed with NQO2-null mice. NQO2-null mice showed presence of MMC-induced cross-linking activity that was unaffected by the addition of cofactors such as NRH or NADH (Fig. 1D). Altogether, these results suggested that the unique cytosolic activity that catalyzed MMC-induced DNA cross-linking is cofactor independent. A comparison of the cytosolic fractions from the various mouse tissues (liver, colon, lung, kidney, spleen, and testis) revealed a significant increase in MMC-induced DNA cross-linking in NQO1-null mice, as compared with wild-type mice (Fig. 2).

Purification and Identification of Unique Cytosolic Activity That Catalyzes MMC-induced DNA Cross-linking. NQO1-null mouse liver cytosol was the selected source for the purification of the unique cytosolic activity that catalyzed MMC-induced DNA cross-linking. After screening a number of separation methods, the following were adopted to purify the unique cytosolic activity. The NQO1-null mouse liver cytosol was fractionated with ammonium sulfate to (a) 40% salt saturation and (b) the supernatant of 40% saturation to

Fig. 1. Characteristics of the unique cytosolic activity that stimulates MMC-induced DNA cross-linking. Samples containing 0.5 mg of cytosolic proteins from NQO1-null or NQO2-null mice liver and colon were incubated with 32P-labeled, 23 bp of oligonucleotide containing the MMC binding site and the cofactors, and the DNA cross-linking assay was performed by procedures described in “Materials and Methods.” The samples were analyzed on a 15% denaturing polyacrylamide gel, and cross-linked and unmodified (free) oligomers were detected by autoradiography. A, effect of pH. NQO1-null mice cytosolic fractions were incubated at different pH (4.0, 5.0, 5.8, 7.0, 8.0, and 9.0). B, effect of dicoumarol. NQO1-null mice cytosolic fractions from liver (Lanes 1–4) and colon (Lanes 5–8) were preincubated for 30 min at room temperature with different dicoumarol concentrations (0, 20, 50, and 100 μM) or a 2% NaOH solution (v/v). The amount of NaOH used in Lane 7 was the same as in Lane 5 to obtain a 100-μM concentration of dicoumarol (left to right). After dicoumarol treatment, samples were directly used for MMC-induced DNA cross-linking assay. C, cofactor requirement. Left, NQO1-null mouse liver and colon cytosolic proteins were incubated with different combinations of cofactors. The cofactors indicated on each lane correspond only to those physically added to the reaction mixture for the DNA cross-linking assay. Right, NQO1-null mouse liver and colon cytosolic proteins (0.35 mg) were not treated (original), dialyzed, or concentrated on Y-10 Centricron columns as described in “Materials and Methods.” D, cofactor requirement. NQO1-null mice liver cytosolic fractions were evaluated in the presence of different cofactors.

Fig. 2. Comparison of NQO1-null and wild-type mice tissues for cofactor-independent MMC-induced DNA cross-linking. Cytosolic proteins (0.5 mg) from the various tissues of NQO1-null and wild-type mice were incubated with 32P-labeled oligonucleotide and MMC, and the DNA cross-linking experiment was performed by procedure as described in “Materials and Methods.” The samples were analyzed on a 15% denaturing polyacrylamide gel, and cross-linked and unmodified (free) oligomers were detected by autoradiography.
Fig. 3. Purification of unique cytosolic activity that catalyzes MMC-induced DNA cross-linking. The MMC-induced $^{32}$P-DNA cross-linking assay and SDS-PAGE were used to monitor the purification of unique cytosolic activity from NQO1-null mice liver cytosol. BSA was used in place of cytosolic fractions for negative control. A, the NQO1—that mice liver cytosol was fractionated with ammonium sulfate in two steps to obtain 40% and 60% salt-saturated fractions (supernatant and pellet fractions). Each sample (0.5 mg) was applied for activity assay. B, the 60% salt-saturated fractions containing the majority of unique cytosolic activity were pooled and applied on a phenyl-Sepharose column. Each sample (0.2 mg) was applied for activity assay. C, fractions 21–31 eluted from the phenyl-Sepharose column were pooled and affinity purified by the heparin-Sepharose column. The unique cytosolic activity was detected in fractions 31–41, eluted between 0.6 and 0.8 M NaCl. Each sample (0.2 mg) was applied for activity assay. D, fractions 33–41 from the heparin-Sepharose column were pooled and applied on a MMC-agarose affinity column. The unique cytosolic activity was highly enriched in fractions 38–42, eluted with 0.1 M Tris (pH 8.5). Ten micrograms of each sample were applied for activity assay. E, SDS-PAGE and silver-staining analysis of the eluted fraction from the MMC-agarose column showed MMC binding of $M_r$ 58,000 (p58) and $M_r$ 60,000 (p60) proteins. p58 and p60 were cut out from the gel and digested with lysC. The digested peptides were purified by mass spectrophotometry and sequenced.

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<th>Steps of purification</th>
<th>Total activity (cpm)</th>
<th>Total protein (mg)</th>
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<th>Fold of purification</th>
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<th>Table 1 Purification of the unique cytosolic activity that catalyzes MMC-induced DNA cross-linking</th>
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<td>Liver cytosol from NQO1-null mice was used to purify the unique cytosolic activity. The MMC-induced $^{32}$P-DNA cross-linking assay was used to monitor the purification. Cross-linked DNA was cut off from the gel, and cpm were detected in a scintillation counter. Specific activity was calculated as cpm/mg of protein for quantification purposes.</td>
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60% salt saturation (Fig. 3A). The precipitation steps led to a significant increase in specific enzyme activity (2.5-fold) but a recovery of only 69.1% of the total unique cytosolic activity (Table 1). It is unclear whether the loss of activity results from the inhibition of the activity by residual salt or is caused by a loss of enzymatic protein. The 60% salt-saturated fraction was applied directly to a phenyl-Sepharose column. This step desalted the proteins and resulted in a 3.1-fold increase in specific unique enzymatic cytosolic activity (Fig. 3B, Table 1). Fractions 27 and 28 concentrated 38% of the unique cytosolic activity (Fig. 3B) and an enhanced amount of a cytosolic activity (Fig. 3B, Table 1). Fractions 27 and 28 concentrated 38% of the unique cytosolic activity in two steps to obtain 40% and 60% salt-saturated fractions (supernatant and pellet fractions). Each sample (0.5 mg) was applied for activity assay. B, the 60% salt-saturated fractions containing the majority of unique cytosolic activity were pooled and applied on a phenyl-Sepharose column. Each sample (0.2 mg) was applied for activity assay. C, fractions 21–31 eluted from the phenyl-Sepharose column were pooled and affinity purified by the heparin-Sepharose column. The unique cytosolic activity was detected in fractions 31–41, eluted between 0.6 and 0.8 M NaCl. Each sample (0.2 mg) was applied for activity assay. D, fractions 33–41 from the heparin-Sepharose column were pooled and applied on a MMC-agarose affinity column. The unique cytosolic activity was highly enriched in fractions 38–42, eluted with 0.1 M Tris (pH 8.5). Ten micrograms of each sample were applied for activity assay. E, SDS-PAGE and silver-staining analysis of the eluted fraction from the MMC-agarose column showed MMC binding of $M_r$ 58,000 (p58) and $M_r$ 60,000 (p60) proteins. p58 and p60 were cut out from the gel and digested with lysC. The digested peptides were purified by mass spectrophotometry and sequenced.

Liver cytosol from NQO1-null mouse was used to purify the unique cytosolic activity. The MMC-induced $^{32}$P-DNA cross-linking assay was used to monitor the purification. Cross-linked DNA was cut off from the gel, and cpm were detected in a scintillation counter. Specific activity was calculated as cpm/mg of protein for quantification purposes.
(p58) and M, 60,000 (p60), to the MMC affinity column (Fig. 3E). HMG1, as expected from previous studies, did not bind to the MMC-agarose column. Both p58 and p60 were identified by mass spectrophotometry. The sequenced peptides of p60 matched to antithrombin III (data not shown). Antithrombin III, available from commercial sources (Sigma-Aldrich Chemical Company), failed to catalyze MMC-induced DNA cross-linking, suggesting to have no further relevance for these studies. In contrast, three (peptides A, C, and D) of the four sequenced peptides of p58 matched 100% to mouse glucose-regulatory protein GRP58 (Fig. 4). The fourth peptide (peptide B) also matched to GRP58, with the exception of one amino acid that was not clear from sequencing results (Fig. 4). GRP58 is also known as GRP57, ERP60, ERP61, Q2, HIP-70, and CPT (20, 31). GRP58 is activated in glucose deprivation and has been shown to be active as thiol-dependent oxidoreductase (18). However, a clear role of this protein remains unknown and is still a subject of investigation. The amino acid alignment sequence of mouse, rat, bovine, and human GRP58 revealed a greater than 92% homology across the species (Table 2). This indicated high conservation of GRP58.

**GRP58 Mediated MMC-induced DNA Cross-Linking.** Immuno-depletion of GRP58 from mouse liver cytosol, 60% ammonium sulfate supernatant, and phenyl-Sepharose and heparin-Sepharose fractions resulted in a complete loss of the GRP58 protein and a significant reduction of MMC-induced DNA cross-linking (Fig. 5). In the same experiment, the preimmune serum failed to remove GRP58 protein from the various samples and did not alter the MMC-induced DNA cross-linking activity (Fig. 5). GRP58 protein plus anti-GRP58 antibodies were recovered by salt elution from the agarose beads, leading to partial recovery of MMC-induced DNA cross-linking activity (Fig. 5). These results corroborate that GRP58 plays a major role in the MMC-induced DNA cross-linking.

We generated CHO cell clones permanently overexpressing higher levels of GRP58 to test the hypothesis that the higher expression of GRP58 results in increased MMC-induced DNA cross-linking that leads to cell death. Western analysis revealed that several CHO cell clones expressed 3- to 4-fold higher levels of GRP58 than wild-type CHO cells (Fig. 6A). The CHO cells overexpressing cDNA-derived GRP58 demonstrated increased MMC-induced DNA cross-linking (Fig. 6B). In addition, the cytotoxicity assays revealed that CHO cells overexpressing cDNA-derived GRP58 were more sensitive to MMC toxicity compared with CHO cells expressing endogenous levels of GRP58 (Fig. 6C). The IC_{50} dose for CHO-15 cells expressing overexpressed GRP58 protein was active (Fig. 7A). However, bacterially expressed GRP58 protein was not sufficient to be quantified by protein assay (<1 pg).

Additional evidence that GRP58 activates MMC-induced DNA cross-linking was observed from studies using bacterially expressed and purified mouse GRP58. GRP58 has thiol-dependent reductase activity that catalyzes the reduction of insulin disulfites by DTT. The two conserved Trp-Cys-Gly-His-Cys-Lys motifs of GRP58 are indicated to be the active sites for this catalytic activity because of its similarity to the single motif of all thioredoxins of prokaryotes and eukaryotes. Insulin reduction produces free disulfite bonds and consequent formation of a white insoluble precipitate (18). Bacterially expressed and purified mouse GRP58 cross-reacted with antibodies against GRP58 (Fig. 5). The bacterial GRP58 also efficiently catalyzed reduction of insulin, indicating that bacterially expressed GRP58 protein was active (Fig. 7A).

**Table 2**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>KTEKFPTEK</td>
</tr>
<tr>
<td>B</td>
<td>HIGGIGS</td>
</tr>
<tr>
<td>C</td>
<td>KTEKFPTEK</td>
</tr>
<tr>
<td>D</td>
<td>HIGGIGS</td>
</tr>
</tbody>
</table>

**Fig. 4.** Alignment of p58 peptides to the GRP58 amino acid sequence. Four peptides (A–D) were aligned to GRP58. All of the peptides matched 100% with the GRP58 sequence, with the exception of peptide B. Peptide B had one of nine amino acids that was not clearly identified by mass spectroscopy and did not match to GRP58.
Table 2  Percentage of nucleotide and amino acid homology sequences of GRP58 from mouse, rat, bovine, and human

<table>
<thead>
<tr>
<th></th>
<th>GRP58 cDNA homology (%)</th>
<th>GRP58 protein homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Rat</td>
<td>93.5</td>
<td>92.5</td>
</tr>
<tr>
<td>Bovine</td>
<td>90.1</td>
<td>91.4</td>
</tr>
<tr>
<td>Human</td>
<td>92.9</td>
<td>92.9</td>
</tr>
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</table>

Expression of GRP58 in Normal and Tumor Tissues. The hybridization of mouse tissue RNA array with GRP58 cDNA revealed that GRP58 is expressed at different levels (higher, moderate, and lower) among the various tissues (Fig. 8, A–C). Mouse epididymus, submaxillary gland, thyroid, ovary, and uterus demonstrated higher GRP58 expression than other tissues studied. Liver, kidney, spleen, pancreas, testis, prostate, thymus, and smooth muscle showed moderate GRP58 expression. GRP58 expression was more or less not detected in brain, eye, lung, heart, and skeletal muscle. Interestingly, GRP58 was expressed at much higher levels in 7-day-old embryos than in 11-, 15-, and 17-day-old embryos. These results corroborate the similar findings on human tissue distribution of GRP58 (31). The hybridization of a human tumor/normal tissue array revealed a significant up-regulation of GRP58 in human breast (seven of nine samples), uterus (five of seven), lung (two of three), and stomach (six of eight) tumors compared with normal tissues of similar origin (Fig. 8, D–F). However, GRP58 was suppressed in 9 of 11 tumors derived from colon.

DISCUSSION

MMC and other bioreductive drugs are chemotherapeutic agents that require biological activation to exert toxicity (1–8). The complex structure of MMC allows this molecule to form multiple metabolites. Alkylation and/or redox-cycling of these metabolites depend on the environment surrounding it at the time of reduction and the availability of the enzyme/protein involved in the activation. The cytosolic NQO1 and several microsomal enzymes are known to activate MMC to metabolites that bind to DNA, leading to DNA-adduct formation, cross-linking of DNA, cytotoxicity, and consequent cell death (9–17). NQO1 catalyzes the reduction of MMC to 2,7-diaminomitosene, a metabolite that binds to the DNA, leading to its cross-linking (15). Recent studies have concluded that NQO1 is a minor player in cytosolic activation of MMC, leading to DNA cross-linking (16, 17). The same studies also suggested the presence of a unique cytosolic activity in NQO1-null mouse liver (or other tissues) that catalyzed MMC-induced DNA cross-linking (17). In this study, we have further characterized and identified the unique cytosolic activity. This activity was dicoumarol insensitive and cofactor independent. The absence of cofactors (NADH, NADPH, NRH, and FAD) in the assay mixture, as well as removal of endogenous cofactors from mouse liver homogenate, had no effect on MMC-induced DNA cross-linking activity. This activity was optimum at pH 5.8 and was inhibited by acidic and alkaline pH. The NQO1-null mice tissues showed higher amounts of MMC-induced DNA cross-linking, compared with wild-type mice. The reasons for this increase in the absence of NQO1 are not known. However, NQO1 is known to reduce cellular oxidative and electrophilic stress (25). Therefore, it is possible that increased stress in NQO1-null mice up-regulated the unique cytosolic activity leading to increased MMC-induced DNA cross-linking.

The unique cytosolic activity was purified from the NQO1-null mice liver cytosol to homogeneity. Four peptides from the purified
protein folding and posttranscriptional modifications are extremely active site is located in a hydrophobic surface of GRP58 implies that MMC close to DNA for binding and cross-linking. The fact that the important role in this process, by reducing MMC and/or bringing (one each on N terminus and COOH terminus) may possibly play an linking remains uncertain (18–21, 32). The redox-active disulfide precision biological role in MMC activation leading to DNA cross-linking. It is noteworthy that the magnitude of MMC-induced DNA cross-linking and cytotoxicity in CHO cells overexpressing GRP58 was also lower than expected. It is possible that GRP58 requires the thioredoxin/thioredoxin reductase system for keeping its thioredoxin moieties in the reduced form that may be required for MMC reductive activation and consequent DNA cross-linking. In other words, the endogenous thioredoxin/thioredoxin system may not be sufficient to meet with demands of CHO cells overexpressing cDNA-derived GRP58, thus leading to a lower magnitude of MMC activation and DNA cross-linking. An additional and important mechanism associated with GRP58 may act exclusively to the transport of MMC to the nucleus, acting more like a catalyst or chaperone for the DNA cross-linking. GRP58 is localized in the cytosol and nucleus (22–24). We have shown here that GRP58 strongly binds to MMC, and previous studies reported GRP58 interaction with DNA (23, 33). Thus, GRP58 may be the MMC carrier from the cytosol to the nucleus in which it brings MMC close to the DNA, inducing DNA covalent cross-linking. Additional investigations are required for a more precise mechanism.

GRP58 is expressed at higher levels in ovary, prostate, epididymis, uterus, submaxillary gland, and thyroid and at moderate levels in liver.

Fig. 6. Development of CHO cells permanently expressing cDNA-derived GRP58 and the role of GRP58 in MMC-induced DNA cross-linking and cytotoxicity. A. Western analysis. The cDNA encoding mouse GRP58 was subcloned in mammalian expression vector pcDNA to generate pcDNA-GRP58. CHO cells were transfected with pcDNA-GRP58 and selected in the presence of G418. CHO clones expressing endogenous level of GRP58 (CHO-WT) and overexpressing cDNA-derived mouse GRP58 (CHO-9 through CHO-16) were grown in monolayer culture, scraped, and homogenized in appropriate buffer, and cytosis was prepared by standard procedures. Seventy-five micrograms of the cytosolic proteins were separated on 10% SDS-PAGE, Western blotted, and probed with GRP58 antiserum. B. DNA cross-linking. Cytosolic protein (0.5 mg) from CHO-WT, CHO-10, and CHO-15 were used to analyze MMC-induced DNA cross-linking. Cross-linked and free oligonucleotides were detected by autoradiography. C. MMC cytotoxicity. CHO-WT expressing endogenous GRP58 (●) and CHO-15 overexpressing GRP58 (□) cells were exposed for 24 h to different concentrations of MMC. The percentage of cell survival was detected by colony formation assay.

and enriched cytosolic activity fraction matched 100% to mouse GRP58. The following results supported the conclusion that GRP58 catalyzes MMC, leading to DNA cross-linking. The unique cytosolic activity that catalyzed MMC-induced DNA cross-linking was independent of external cofactors, and so is GRP58. GRP58 does not require cofactors for its activity (21, 22). GRP58 and antithrombin III were the only two proteins that bind with MMC in a MMC affinity column. Additional analysis failed to associate MMC-induced DNA cross-linking with antithrombin III, thus leaving only GRP58 a potential candidate as a catalyst for MMC-induced DNA cross-linking. The immunodepletion of NQO1-null mice fractions led to the loss of GRP58 protein and a significant reduction of MMC-induced DNA cross-linking activity. The CHO cells overexpressing mouse GRP58 showed increased MMC-induced DNA cross-linking and cytotoxicity. The addition of bacterially expressed and purified mouse GRP58 to mouse cytosolic samples showed a proportional increase of the MMC-induced DNA cross-linking.

The identification of GRP58 as unique cytosolic activity raised interesting questions regarding the mechanism by which GRP58 catalyzes MMC-induced DNA cross-linking. GRP58 is a highly conserved protein that belongs to the superfamily of Cysteine Glycine Histidine Cysteine (CGHC)-containing proteins. Although the presence of two thioredoxin-like (Try-Cys-Gly-His-Cys-Lys) active sites leaves little doubt that GRP58 functions as an oxidoreductase, the precise biological role in MMC activation leading to DNA cross-linking remains uncertain (18–21, 32). The redox-active disulfide (one each on N terminus and COOH terminus) may possibly play an important role in this process, by reducing MMC and/or bringing MMC close to DNA for binding and cross-linking. The fact that the active site is located in a hydrophobic surface of GRP58 implies that protein folding and posttranscriptional modifications are extremely important for the efficient enzymatic activity. Therefore, it may be possible that GRP58 requires modifications to stay in the cytosol and/or to catalyze MMC activation. Although it has been shown that the GRP58 reduct state is important for DNA binding (22–24), the nature of protein modifications remains unknown. These properties of GRP58 may rationalize the reason(s) for the failure of bacterially expressed GRP58, or for the in vitro-translated GRP58, in inducing significant MMC activation leading to DNA cross-linking. It is noteworthy that the magnitude of MMC-induced DNA cross-linking and cytotoxicity in CHO cells overexpressing GRP58 was also lower than expected. It is possible that GRP58 requires the thioredoxin/thioredoxin reductase system for keeping its thioredoxin moieties in the reduced form that may be required for MMC reductive activation and consequent DNA cross-linking. In other words, the endogenous thioredoxin/thioredoxin system may not be sufficient to meet with demands of CHO cells overexpressing cDNA-derived GRP58, thus leading to a lower magnitude of MMC activation and DNA cross-linking. Additional investigations are required for a more precise mechanism.

GRP58 is expressed at higher levels in ovary, prostate, epididymis, uterus, submaxillary gland, and thyroid and at moderate levels in liver.

Fig. 7. Bacterial expression, purification, and role of GRP58 in insulin reduction and MMC-induced DNA cross-linking. The mouse GRP58 cDNA was subcloned in bacterial expression vector to produce histidine-tagged GRP58 that was purified on a nickel column. A, GRP58 catalyzed reduction of insulin. Two micrograms (●) and 4 μg (■) of bacterially expressed and purified GRP58 were used to determine time-dependent insulin reduction. The cytosolic proteins (100 μg) from CHO-10 clone (●) and CHO-15 (□) overexpressing GRP58 and 4 μg of purified thioredoxin (○) were included as positive controls. B, cytosolic proteins (0.5 mg) from NQO1-null liver cytosol (Lanes 1 and 2) or similar amounts of 60% ammonium sulfate fractionation (Lanes 3 and 4) were mixed with 10 μg of albumin (Lanes 1 and 3) or similar amount of bacterially expressed GRP58 (Lanes 2 and 4) and analyzed for MMC-induced DNA cross-linking. The samples were analyzed on a 15% denaturing polyacrylamide gel, and cross-linked and unmodified (free) oligomers were detected by autoradiography.
kidney, smooth muscle, pancreas, thymus, spleen, and testis. The GRP58 expression was very low in brain, eye, lung, heart, and skeletal muscle. GRP58 expression was higher in 7-day-old mouse embryos compared with 11-, 15-, and 17-day-old embryos for unknown reasons. A higher expression of GRP58 in human breast, lung, uterus, and stomach tumors may, in part, explain the beneficial use of MMC for treatment of these tumors. GRP58 is significantly activated in cells deprived of glucose (18). It is also known that a core of tumor tissues are not only hypoxic but also deprived of glucose (34). This may lead to selective activation of GRP58 and killing of cells that generally escape radiation treatment and are points of recurrence of tumor growth. Enhanced expression of GRP58 in association with oncogenic transformation (by ras or src) in mammalian cells (18) or by mitogens and phorbol esters in Jurkat cells (35) has been described.

In conclusion, we have characterized and identified a unique cytosolic activity that activated MMC, leading to DNA cross-linking. The unique cytosolic activity is cofactor independent and dicoumarol insensitive. The unique cytosolic activity that catalyzes MMC-induced DNA cross-linking was purified to homogeneity and identified as GRP58. GRP58 was found expressed at different levels in many tissues. A higher expression of GRP58 in human breast, lung, uterus, and stomach tumors was observed.

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GRP58 IN MMC-INDUCED DNA CROSS-LINKING


Role of GRP58 in Mitomycin C-induced DNA Cross-Linking

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