Glyoxalase II, a Detoxifying Enzyme of Glycolysis Byproduct Methylglyoxal and a Target of p63 and p73, Is a Pro-survival Factor of the p53 Family*

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The p53 family proteins are transcription factors and have both common and distinct functions. p53 is a classic tumor suppressor, whereas p63 and p73 have fundamental functions in development. To gain an insight into the functional diversities among the p53 family, target genes specifically regulated by p63 and p73 were examined. Here, we found that the GLX2 gene, which encodes glyoxalase II enzyme, is up-regulated by p63 and p73. Accordingly, a specific responsive element was found in intron 1 of the GLX2 gene, which can be activated and bound by p63 and p73. We also found that, upon overexpression, the cytosolic, but not the mitochondrial, GLX2 inhibits the apoptotic response of a cell to methylglyoxal, a by-product of glycolysis. Likewise, we showed that cells deficient in GLX2 are hypersensitive to methylglyoxal-induced apoptosis. Interestingly, a deficiency in GLX2 also enhances the susceptibility of a cell to DNA damage-induced apoptosis in a p53-dependent manner. These observations reveal a novel link between the p53 family and the glyoxalase system. Given that methylglyoxal is frequently generated under both physiological and pathological conditions, we postulate that GLX2 serves as a pro-survival factor of the p53 family and plays a critical role in the normal development and in the pathogenesis of various human diseases, including cancer, diabetes, and neurodegenerative diseases.

p53, a sequence-specific transcription factor, transactivates an array of target genes that mediate p53 functions, such as cell cycle arrest, apoptosis, and DNA repair (1). The other two p53 family members, p63 and p73, share considerable structural homology with p53 and can also bind to p53-responsive elements (p53-REs)² to transactivate some p53 target genes, such as p21, MDM2, FDxr, and POLH (2–5). Thus, p63 and p73 appear to have overlapping functions with p53 (1). However, unlike p53 as a classic tumor suppressor, p63 and p73 are rarely mutated in cancer and their roles in tumor suppression remain controversial (6). Furthermore, mice deficient in either p63 or p73 exhibit profound developmental defects without increased susceptibility to spontaneous tumor formation (7, 8). The diverse physiological functions of p63 and p73 suggest that a distinct set of transcriptional targets are regulated by p63 and p73.

Glyoxalase II (GLX2), also known as hydroxyacylglutathione hydrolase, together with glyoxalase I (GLX1), constitutes the glyoxalase system (9). The major function of the system is to detoxify α-ketoaldehydes, especially the potent and cytotoxic methylglyoxal (MG) (10). MG, a by-product of glycolysis, is produced through nonenzymatic phosphate elimination from the glycolytic intermediates, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. MG is converted to S-β-lactoylglutathione (SLG) by GLX1 with reduced glutathione as a cofactor, and SLG in turn is hydrolyzed to β-lactate along with regeneration of reduced glutathione by GLX2. GLX1, which uses MG as the preferred substrate, is found selectively overexpressed in leukemia cells, and GLX1 inhibitor is shown to be a potential anti-cancer agent (11). Overexpression of GLX1 is capable of inhibiting the formation of hyperglycemia-induced advanced glycation end products (AGEs) in bovine endothelial cells, indicating that GLX1 has a protective role in diabetic microangiopathy (12). Recent studies revealed that GLX1 has additional roles in the pathogenesis of Alzheimer disease and anxiety (13, 14). All these studies indicate that the glyoxalase system plays an important role in the pathogenesis of various human diseases. However, very little is known about GLX2, including its role in the glyoxalase system and its physiological significance in the stress response.

In this study, we showed that GLX2 is a novel target of p63 and p73. We also showed that the cytosolic, but not the mitochondrial, GLX2 protects cells from MG-induced cell death, whereas a deficiency in GLX2 promotes MG-induced cell death. Furthermore, we showed that GLX2 knockdown sensitizes cells to DNA damage-induced apoptosis in a p53-dependent manner, implying that GLX2 has a novel function in the DNA damage response. Taken together, our data reveal a novel link between the p53 family and the glyoxalase system, which plays an important role in the normal development and in the pathogenesis of various human diseases, such as cancer, diabetes, and Alzheimer disease.

EXPERIMENTAL PROCEDURES

Plasmids—The cDNAs encoding both the cytosolic and mitochondrial GLX2 were generated by reverse transcription-PCR

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² The abbreviations used are: RE, responsive element; GLX1, -2, glyoxalases I and II; MG, methylglyoxal; SLG, S-β-lactoylglutathione; AGE, advanced glycation end product; HA, hemagglutinin; siRNA, small interference RNA; nt, nucleotide(s); ChIP, chromatin immunoprecipitation; PARP, poly(ADP-ribose) polymerase.

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**Note:** The text above is the natural representation of the provided document.
using total RNAs purified from MCF7 cells. The primers used to generate the cytosolic GLX2 were forward primer cGLX2-F (5'-AAGCTTGCACCATGAAAGTGAAGTGCTGCTGCTGCCTG-3') and reverse primer GLX2-R (5'-CTCAGATGAAGGTTGGGCGGCGAGCTGCTGCTGCCAGGCG-3') and reverse primer GLX2-R. The cDNA encoding the C-terminally HA-tagged cytosolic GLX2 was amplified with forward primer cGLX2-F and reverse primer GLX2–3HA-R. The cDNA encoding the N-terminally HA-tagged cytosolic GLX2 was amplified with forward primer GLX2–5HA-F (5'-AAGCCTTGCCACCATGTAACCATGCAAGCG-3') and reverse primer GLX2-R. The cDNA encoding the N-terminally HA-tagged GLX2 was amplified with forward primer GLX2–5HA-F (5'-AAGCCTTGCCACCATGTAACCATGCAAGCG-3') and reverse primer GLX2-R. The cDNA encoding the N-terminally HA-tagged GLX2 was amplified with forward primer GLX2–5HA-F (5'-AAGCCTTGCCACCATGTAACCATGCAAGCG-3') and reverse primer GLX2-R. The cDNA encoding the N-terminally HA-tagged GLX2 was amplified with forward primer GLX2–5HA-F (5'-AAGCCTTGCCACCATGTAACCATGCAAGCG-3') and reverse primer GLX2-R. The cDNA encoding the N-terminally HA-tagged GLX2 was amplified with forward primer GLX2–5HA-F (5'-AAGCCTTGCCACCATGTAACCATGCAAGCG-3') and reverse primer GLX2-R. The cDNA encoding the N-terminally HA-tagged GLX2 was amplified with forward primer GLX2–5HA-F (5'-AAGCCTTGCCACCATGTAACCATGCAAGCG-3') and reverse primer GLX2-R. The cDNA encoding the N-terminally HA-tagged GLX2 was amplified with forward primer GLX2–5HA-F (5'-AAGCCTTGCCACCATGTAACCATGCAAGCG-3') and reverse primer GLX2-R. The cDNA encoding the N-terminally HA-tagged GLX2 was amplified with forward primer GLX2–5HA-F (5'-AAGCCTTGCCACCATGTAACCATGCAAGCG-3') and reverse primer GLX2-R. The cDNA encoding the N-terminally HA-tagged GLX2 was amplified with forward primer GLX2–5HA-F (5'-AAGCCTTGCCACCATGTAACCATGCAAGCG-3') and reverse primer GLX2-R. The cDNA encoding the N-terminally HA-tagged GLX2 was amplified with forward primer GLX2–5HA-F (5'-AAGCCTTGCCACCATGTAACCATGCAAGCG-3') and reverse primer GLX2-R. The cDNA encoding the N-terminally HA-tagged GLX2 was amplified with forward primer GLX2–5HA-F
Glyoxalase II, a Pro-survival Factor of the p53 Family

were purified by using a Qiagen column. PCR was performed to visualize the enriched DNA fragments. Primers designed to amplify the region from nt +3779 to +3969 in the GLX2 intron 1 were forward primer (5'-CTGAGACAGGAGACGGCT-GAACC-3') and reverse primer (5'-CTGAGTAGGAGGACT-GCTTGAGG-3'). Primers designed to amplify the region from nt -2312 to -2131 in the p21 promoter were forward primer (5'-CAGGCTGTGGCCTGATTGG-3') and reverse primer (5'-TTCCAGATACGGCTAAGG-3').

Colony Formation Assay—RKO cells seeded at 300 per well and MCF7 cells seeded at 500 per well in a 6-well plate were incubated in the absence or presence of tetracycline (1.0 μg/ml) for 72 h, then treated with various concentrations of MG for 1 h followed by repetitive wash with phosphate-buffered saline to remove MG. The cells were maintained in fresh medium for next 12–14 days and then stained with crystal violet.

Western Blot Analysis—Chicken anti-GLX2 polyclonal IgY antibody was purchased from GenWay Biotech (San Diego, CA). Mouse anti-PARP monoclonal antibody was purchased from BD Pharmingen. Antibodies against p53, p21, HA, and Myc epitopes and actin were described previously (23). Whole cell extracts were prepared by lysing cells with 2× SDS sample buffer. Proteins were separated on 7–10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the indicated antibodies followed by ECL detection.

DNA Histogram Analysis—Cells were seeded at 2 × 10^5 per 6-cm plate with or without tetracycline for 72 h, and then treated with MG or a DNA damage agent for various times. Both floating dead cells in the medium and live cells on the plate were collected and fixed with 1 ml of 100% ethanol for at least 30 min. The fixed cells were centrifuged and re-suspended in 0.5 ml of phosphate-buffered saline solution containing 50 μg/ml each of RNase A and propidium iodide (Sigma). The stained cells were analyzed in a fluorescence-activated cell sorter within 4 h. The percentages of cells in the sub-G1, G1, S, and G2-M phases were determined using the CellQuest program (BD Biosciences).

Immunofluorescence Microscopy—Cells grown on slides were fixed with 3% paraformaldehyde in phosphate-buffered saline for 45 min at room temperature and treated with 0.5% Triton X-100 for 5 min, blocked with 1% bovine serum albumin for 1 h, and stained with anti-HA to detect HA-tagged GLX2 or with chicken anti-GLX2 to detect endogenous GLX2 at room temperature for 1 h. Slides were washed and then incubated with fluorescein isothiocyanate-conjugated secondary antibodies against mouse or chicken immunoglobulin G (Jackson ImmunoResearch and Molecular Probes), followed by treatment with 4',6-diamidino-2-phenylindole (Sigma) to stain nuclei. For MitoTracker (Molecular Probes) staining, cells were incubated in 50 nM MitoTracker for 30 min and then washed with prewarmed growth medium followed by paraformaldehyde fixation.

RESULTS

Identification of GLX2 as a Target Gene of p63 and p73—To identify novel target genes regulated by the p53 family, an Affymetrix GeneChip assay was performed. Total RNAs were isolated from MCF7 cells that were uninduced and induced to express p63α, p63γ, p73α, p73β, Np63α, Np63γ, and Np73β (Fig. 1A). The level of p21 was examined as a positive control and found to be induced by p63γ and p73β. The level of glyceraldehyde-3-phosphate dehydrogenase was determined as a loading control. We also found that

![FIGURE 1. Up-regulation of GLX2 by p63 and p73. A, Northern blots were prepared using total RNAs isolated from MCF7 cells that were uninduced (−) or induced (+) to express various p63 and p73 proteins for 24 h. The blots were probed with 32P-labeled cDNAs derived from the GLX2, p21, and glyceraldehyde-3-phosphate dehydrogenase genes, respectively. B, the experiments were performed similarly as in A except that RNA samples were purified from MCF7 cells that were uninduced (−) or induced (+) to express p53 for 24 h, or from MCF7, RKO, and HCT116 cells that were mock-treated (−) or treated (+) with 250 nm camptothecin for 24 h. C, Western blots were prepared using extracts from MCF7 cells that were uninduced (−) or induced (+) to express various p63 and p73 proteins for 24 h. The blots were analyzed by anti-GLX2, anti-Myc to detect p63, anti-HA to detect p73, anti-p21, and anti-actin, respectively. D, the experiments were performed similarly as in C except that extracts were purified form MCF7 cells that were uninduced (−) or induced (+) to express wild-type or mutant p53 for 24 h, or from MCF7, RKO, and HCT116 cells that were mock-treated (−) or treated (+) with 250 nm camptothecin for 24 h.

express p63γ by withdrawal of tetracycline from the culture medium and used to prepare probes for the Affymetrix U133 Plus GeneChip. We found that GLX2 was highly induced in cells expressing p63γ. To confirm and extend the microarray result, we performed Northern blot analysis and found that GLX2 was highly induced by p63γ and p73β and little if any by p63α, ΔNp63α, ΔNp63γ, and ΔNp73β (Fig. 1A). The level of p21 was examined as a positive control and found to be induced by p63γ and p73β. The level of glyceraldehyde-3-phosphate dehydrogenase was determined as a loading control. We also found that
GLX2 was only slightly induced by p53 but not significantly by DNA damage in cells that carry endogenous wild-type p53 (Fig. 1B). Furthermore, consistent with the increase in the mRNA level, Western blot analysis showed that GLX2 protein was significantly induced by p63\& and p73\& but little if any by p63\&a, \&Np63\&a, \&Np63\&y, \&Np73\&B, wild-type p53, and mutant p53 in MCF7 cells (Fig. 1, C and D). These results suggest that both GLX2 mRNA and protein are preferentially up-regulated by p63\&y and p73\& but little if any by p53.

As members of the p53 family, p63 and p73 are capable of transactivating a promoter that contains a p53-responsive element (p53-RE), \&R\&RC(A/T)(A/T)GY\&YY, in which R represents purine, whereas Y represents pyrimidine (24). Thus, we searched for and found a potential p53-RE in the first intron (from nt +3901 to +3934) of the GLX2 gene (Fig. 2A). The localization of p53-RE in the intron of a target gene is not unusual, because several well established p53-REs are found in the introns of p53 targets, such as PML and PUMA (25, 26). To determine whether this potential p53-RE is functional, we cloned the intron fragment as well as several mutant fragments in front of a luciferase reporter (Fig. 2B). We showed that the fragment from nt +3419 to +4127 was highly responsive to p63\&y and p73\& (Fig. 2, B and C). Similarly, the fragment from nt +3850 to +4127 was also responsive (Fig. 2, B and C). However, the fragment from nt +3954 to +4127 and the internal deletion mutant, \&A\&+3879/+3935, were only weakly responsive (Fig. 2, B and C), suggesting that the potential p53-RE from nt +3901 to +3934 is necessary. Next, point mutations at the sites predicted to be critical for p53 binding were made in the potential p53-RE. Two nucleotide substitutions, C3904T and C3928A, were made for GLX2-(+3910/+4127)M2, whereas four nucleotide substitutions, C3904T, G3907T, C3928A, and G3931A were made for GLX2-(+3910/+4127)M4 (Fig. 2A). We found that, like the deletion of the potential p53-RE, mutations also made the p53-RE less responsive to p63 and p73 (Fig. 2, D and E). These data imply that the p53-RE from nt +3901 to +3934 is responsible for p63\&y and p73\& induction of the GLX2 gene.

To determine whether p63\&y and p73\& directly bind to the p53-RE in the GLX2 gene in vivo, we performed ChIP assays. The Myc-tagged p63\&y-DNA complexes were immunoprecipitated with anti-Myc antibody, whereas anti-HA was used as a control. The HA-tagged p73\&-DNA complexes were immunoprecipitated with anti-HA antibody, whereas anti-Myc was used as a control. To visualize the enriched DNA fragments, PCR was performed to amplify the region spanning the p53-RE in the GLX2 gene along with the p53-RE1 within the p21 promoter as a positive control with primers shown in Fig. 2F. We found that the captured fragment containing the p53-RE in the GLX2 gene was highly enriched upon inducible expression of p63\&y or p73\& (Fig. 2, G and H, GLX2 panels). No DNA fragment was enriched by the control antibody (Fig. 2, G and H). As a positive control, the fragment containing the p53-RE1 in the p21 gene was also enriched upon inducible expression of p63\&y or p73\& (Fig. 2, G and H, p21 panels). Taken together, these data indicate that GLX2 is a direct transcriptional target of p63 and p73.

Subcellular Localization of GLX2 Protein—In yeast and higher plants, the cytosolic and mitochondrial isoforms of GLX2 are encoded by two separate genes, whereas in human both isoforms are encoded by the single GLX2 gene (27). As shown in Fig. 3A, the human GLX2 gene encodes two isoforms of the protein through alternate translational start sites: the mitochondrial GLX2 translated from the first ATG codon located in exon 1 and the cytosolic GLX2 from the second ATG codon, an internal ribosome entry site in exon 2. To characterize the function of both isoforms, we generated multiple RKO cell lines that inducibly express GLX2 under the control of the tetracycline-inducible promoter. The cytosolic GLX2 was tagged with an HA epitope at either the N or C termini. The mitochondrial GLX2 was tagged with HA only at the C terminus, because an N-terminal HA might interfere with the mitochondrial targeting signal located at the N terminus. One representative clone from each cell line, that is, RKO-GLX2–5HA-3, which inducibly expresses the N-terminally HA-tagged cytosolic GLX2, RKO-GLX2–3HA-16, which inducibly expresses the C-terminally HA-tagged cytosolic GLX2, and RKO-mGLX2–3HA-4, which inducibly expresses the C-terminally HA-tagged mitochondrial GLX2, are shown in Fig. 3B. Next, the intracellular localizations of these GLX2 isoforms were determined by immunofluorescence microscopy. We found that endogenous GLX2 detected by anti-GLX2 was localized primarily in cytosol with some mitochondrial distribution (Fig. 3C, top panel). The HA-tagged cytosolic GLX2 was localized primarily in cytosol (Fig. 3C, second and third panels). However, the C-terminally HA-tagged mitochondrial GLX2 was found to be localized exclusively in mitochondria as it colocalized well with the Mitotracker Red (Fig. 3C, fourth panel). Interestingly, the N-terminally HA-tagged GLX2 was localized in cytosol (Fig. 3C, bottom panel), suggesting that the N-terminal HA tag masked the mitochondrial localization signal.

The Cytosolic GLX2 Protects Cells from MG-induced Cell Death—MG is the preferred substrate of GLX1, and the product of GLX1 enzymatic reaction, SLG, is the substrate for GLX2. Currently, it is not clear whether GLX2 may impact on the detoxification process of MG, and even less is known about which isoform is active in the MG detoxification pathway. Thus, we performed a colony formation assay to determine the effect of GLX2 on MG-induced growth inhibition (Fig. 4A). For cells without MG treatment, GLX2 overexpression had little effect on cell proliferation (Fig. 4A, control panel). Following MG treatment, most cells died as only a few colonies formed (Fig. 4A, bottom panel, no GLX2 expression). Upon induction of GLX2, the number of colonies was substantially higher than the group without GLX2 expression, regardless of GLX2 that was tagged with HA at its N or C termini (Fig. 4A, bottom panel and first and second columns). In contrast, there was no difference in colony formation between control cells and cells induced to express the mitochondrial GLX2 (Fig. 4A, bottom panel and third column). These data indicate that the cytosolic but not mitochondrial GLX2 confers cell resistance to MG-induced growth inhibition.

Because MG is capable of inducing apoptosis (28–30), we performed DNA histogram analysis to determine whether the protection against MG-induced growth inhibition by the cytosolic GLX2 is due to a decrease in apoptosis. We found that overexpression of either the cytosolic or the mitochon-
Glyoxalase II, a Pro-survival Factor of the p53 Family

A

+1 +450 +3419 +4127
exon1
+3419 +4127
+3850 +4127
+3954 +4127
Δ+3879 +3935
+3419 +3901
+3901 +4127
+3901 +4127 M2
+3901 +4127 M4
consensus p53-RE

B

Relative fold increase

+3419 +4127
+3850 +4127
+3954 +4127
Δ+3879 +3935
peDNA3 p63γ

C

Relative fold increase

+3419 +4127
+3850 +4127
+3954 +4127
Δ+3879 +3935
peDNA3 p73β

D

Relative fold increase

+3901 +4127
+3901 +4127 M2
+3901 +4127 M4
peDNA3 p63γ

E

Relative fold increase

+3901 +4127
+3901 +4127 M2
+3901 +4127 M4
peDNA3 p73β

F

GLX2 intron1

+3779 p53-RE +3969 +4127
p53-REF 191-bp p53-RER

p21 promoter

-2312 p53-RE1 182-bp p53-RE1R
p53-RE1F

G

MCF7-p63γ-19

input α-HA α-myc induction

- + - + GLX2 (191-bp)
- + - - p21 (182-bp)

H

MCF7-p73β-33

input α-myc α-HA induction

- + - + GLX2 (191-bp)
- + - - p21 (182-bp)
drial GLX2 alone had no effect on the cell cycle, because the cell cycle profile was nearly identical to that for control groups (Fig. 4, B, D, and F, compare control panels with GLX2 panels). Upon treatment with MG, a substantial number of cells, ranging from 25 to 54%, underwent apoptosis (Fig. 4, B, D, and F, MG panels). Interestingly, the cytosolic GLX2 decreased the number of apoptotic cells from 25 to 6.9% in RKO-GLX2–5HA-3 cells (Fig. 4B, compare MG

FIGURE 2. GLX2 is a direct target of p63 and p73. A, schematic presentation of the GLX2 genomic locus containing intron 1 and the pGL2 luciferase reporter constructs. A potential p53-responsive element is located between nt + 3901 and + 3934 in intron 1 of the GLX2 gene. The reporter constructs, + 3901/+ 4127 M2 and + 3901/+ 4127 M4, carry two and four nucleotide substitutions at the critical nucleotides (in bold) in the potential p53-responsive element, respectively. B and C, the potential p53-responsive element in intron 1 is responsive to p63 and p73. The luciferase assay was carried out as described under “Experimental Procedures.” D and E, mutations at the critical nucleotides abrogate the potential p53-responsive element in intron 1. F, schematic presentation of the GLX2 intron 1 and p21 promoter with the location of the p53-responsive element and PCR primers used for ChIP assay. G and H, p63 and p73 bind directly to the p53-responsive element in the GLX2 intron 1 and p21 promoter in vivo.

FIGURE 3. Subcellular localization of GLX2. A, schematic presentation of the human GLX2 genomic locus with exons and two translational start sites. The mitochondrial GLX2 is translated from the first ATG codon in exon 1. The cytosolic GLX2 is translated from the second ATG codon, an internal ribosomal entry site in exon 2. B, generation of RKO cell lines that inducibly express GLX2. Western blots were prepared using extracts from RKO cells that were uninduced (−) or induced (+) to express the N-terminally HA-tagged cytosolic GLX2 (GLX2–5HA), the C-terminally HA-tagged cytosolic GLX2 (GLX2–3HA), or the C-terminally HA-tagged mitochondrial GLX2 (mGLX2–3HA) for 24 h. The blots were analyzed with anti-GLX2 and anti-actin antibodies, respectively. C, subcellular localization of endogenous GLX2 (first panel), the N-terminally HA-tagged cytosolic GLX2 (second panel), the C-terminally HA-tagged cytosolic GLX2 (third panel), the C-terminally HA-tagged mitochondrial GLX2 (fourth panel), and the N-terminally HA-tagged mitochondrial GLX2 (bottom panel) in RKO cells. Endogenous GLX2 was detected by anti-GLX2 antibody (GenWay Biotech, San Diego, CA), and HA-tagged GLX2 was detected by anti-HA antibody. Immunofluorescence microscopy was performed as described under “Experimental Procedures.” GLX2 is stained as green, nuclei as blue (4′,6-diamidino-2-phenylindole), and mitochondria as red (MitoTracker).
Glyoxalase II, a Pro-survival Factor of the p53 Family

FIGURE 4. Overexpression of the cytosolic GLX2 inhibits MG-induced cell death. A, colony formation assay was performed using RKO cells uninduced (−) or induced (+) to express the N-terminally HA-tagged cytosolic GLX2 (first column), the C-terminally HA-tagged cytosolic GLX2 (second column), or the C-terminally HA-tagged mitochondrial GLX2 (third column) for 72 h, which were then treated with 2 mM of MG for 1 h, and washed with and cultured in fresh medium for 12 days. B, D, and F, the cytosolic, but not the mitochondrial, GLX2 protects RKO cells from MG-induced apoptosis. RKO cells were uninduced (−) or induced (+) to express GLX2 for 72 h and then treated with 1 mM of MG for 8 h. Following the treatment, both floating dead cells in the medium and live cells on the plate were collected and stained with propidium iodide for DNA histogram analysis. C and E, MG-induced PARP cleavage was inhibited by the cytosolic GLX2. Western blots were prepared using extracts from RKO cells uninduced (−) or induced (+) to express the C-terminally HA-tagged GLX2 (C) or the N-terminally HA-tagged GLX2 (E) for 24 h, which were then untreated (lanes 1 and 2) or treated with 2 mM MG for 4, 8, and 12 h, respectively. The blots were analyzed with antibodies against PARP, GLX2, and actin, respectively. G, MG-induced PARP cleavage was not inhibited by the mitochondrial GLX2. The experiment was performed similarly as in C and E except MG treatment for 8 h.

panel with MG+ GLX2 panel) and from 54.1 to 10% in RKO-GLX2–3HA-16 cells (Fig. 4D, compare MG panel with MG+ GLX2 panel). In contrast, the mitochondrial GLX2 was inactive in inhibiting MG-induced apoptosis, because the number of apoptotic cells was not substantially decreased (Fig. 4F, compare MG panel with MG+ GLX2 panel).
Poly(ADP-ribose) polymerase (PARP) is cleaved during apoptosis and used as an apoptotic marker. Thus, PARP cleavage was measured in RKO cells following treatment with MG for various times in the absence or presence of GLX2 expression. We showed that PARP was cleaved upon treatment with MG (Fig. 4, C and E, lanes 5 and 7; Fig. 4G, lanes 3 and 7). However, PARP cleavage was substantially inhibited when the cytosolic GLX2 was induced (Figs. 4, C and E, compare lanes 5 and 7 with lanes 6 and 8, respectively; Fig. 4G, compare lanes 3 and 4). In contrast, PARP cleavage was not inhibited by the mitochondrial GLX2 (Fig. 4G, compare lane 7 with lane 8). Taken together, these data suggest that the cytosolic, but not the mitochondrial, GLX2 is able to protect cells from MG-induced apoptosis.

**Knockdown of GLX2 Sensitizes Cells to MG-induced Cell Death**—To further address the physiological significance of GLX2 in the MG detoxification pathway, we generated multiple RKO and MCF7 cell lines in which endogenous GLX2 was inducibly knocked down by siRNA under the control of the tetracycline-inducible H1 promoter. Four representative cell lines, RKO-GLX2-KD-19, RKO-GLX2-KD-27, MCF7-GLX2-KD-1, and MCF7-GLX2-KD-6, along with the control RKO and MCF7 cell lines, are shown in Fig. 5A. We showed that, upon induction of siRNA against GLX2, the level of GLX2 protein was significantly decreased (Fig. 5A). Next, we examined whether GLX2 knockdown had an effect on the sensitivity of cells to MG-induced growth inhibition by colony formation assay. We found that, in the absence of MG treatment, GLX2 knockdown had little effect on the colony-forming capability in MCF7 and RKO cells (Fig. 5, B and C, top panels). However, upon treatment with MG, the ability of RKO and MCF7 cells to form colonies was inhibited, which was further reduced by GLX2 knockdown as fewer colonies were formed in cells treated with MG in the presence of GLX2 knockdown than in the absence of GLX2 knockdown (Fig. 5, B and C). The tetracycline had no effect as the sensitivity of the control RKO and MCF7 cells to MG remained the same (Fig. 5, B and C, left panel). Similarly, DNA histogram analysis showed that the number of MG-induced apoptotic cells was increased by GLX2 knockdown in RKO cells from 8.6 to 29.8% and in MCF7 cells from 1.1 to 10.5% (Fig. 5, D and F, compare MG panels with MG + GLX2-KD panels). We also measured the extent of PARP cleavage by Western blot analysis and found that, upon treatment with MG, PARP cleavage was increased by GLX2 knockdown in both RKO and MCF7 cells (Fig. 5E, compare lanes 5 and 6; Fig. 5G, compare lanes 3 and 5 with lanes 4 and 6, respectively). Taken together, these data indicate that GLX2 knockdown sensitizes both MCF7 and RKO cells to MG-induced apoptosis and that the protective function of GLX2 is not cell type-specific.

**GLX2 Knockdown Sensitizes Cells to DNA Damage-induced Cell Death**—p73 is known to play a role in the DNA damage response and thus GLX2 may serve as a mediator of p73 in such a response. To test this, we performed DNA histogram analysis to examine DNA damage-induced apoptosis in two RKO cell lines in which GLX2 is inducibly knocked down. We showed that, upon treatment with doxorubicin, the extent of apoptotic response was substantially enhanced by GLX2 knockdown as the number of apoptotic cells was increased from 25.9 to 38.9% in RKO-GLX2-KD-19 cells (Fig. 6A, compare Dox panel with Dox + GLX2-KD panel) and from 21.3 to 41.4% in RKO-GLX2-KD-27 cells (Fig. 6B). Similarly, the extent of PARP cleavage was also enhanced by GLX2 knockdown, whereas the level of p53 was not changed (Fig. 6C, compare lanes 3 and 7 with lanes 4 and 8, respectively). We also tested the sensitivity of RKO cells to cisplatin-induced apoptosis. We found that the extent of apoptotic response was substantially enhanced by GLX2 knockdown as the number of apoptotic cells was increased from 16.4 to 25.0% in RKO-GLX2-KD-19 cells (Fig. 6D, compare DDP panel with DDP + GLX2-KD panel). In addition, the extent of PARP cleavage was also enhanced by GLX2 knockdown, whereas the level of p53 was not changed (Fig. 6E, compare lanes 3 and 4 with lane 8). These results suggest that GLX2 has a novel protective role in the DNA damage response.

Because p53 is a critical determinant in the DNA damage response, we wanted to determine whether the protection of DNA damage-induced apoptosis by GLX2 is p53-dependent. To test this, we generated RKO cell lines in which p53 is stably knocked down and GLX2 is inducibly knocked down by the tetracycline-inducible siRNA expression system. One representative cell line, RKO-p53-KD-GLX2-KD-4, was chosen for further studies. As shown in Fig. 6F, p53 was undetectable upon treatment with doxorubicin, suggesting that p53 was efficiently knocked down (p53 panel, compare lanes 1 and 2 with lanes 3 and 4). Upon induction of GLX2 siRNA, the level of GLX2 was markedly decreased (Fig. 6F, GLX2 panel, compare lanes 1 and 3 with lanes 2 and 4, respectively). Next, we performed DNA histogram analysis and found that, upon treatment with doxorubicin, the number of apoptotic cells was slightly increased (from 17.4 to 22.9%) when GLX2 was induced to knocked down in stable p53 knockdown RKO cells (Fig. 6G, compare Dox panel with Dox + GLX2-KD panel). Similarly, PARP cleavage was also slightly increased upon GLX2 knockdown (Fig. 6F, PARP panel, compare lanes 3 and 4). These results suggest that GLX2-mediated protection against DNA damage-induced apoptosis is primarily p53-dependent, but p53-independent DNA damage-induced apoptosis is also enhanced by GLX2 knockdown. We note that in the absence of p53, DNA damage-induced G1 arrest was nearly abolished (Fig. 6G), which is consistent with an early report (31).

Finally, we examined whether overexpression of GLX2 is able to inhibit DNA damage-induced apoptosis in two separate cell lines and showed that doxorubicin-induced apoptosis was not inhibited by overexpressed exogenous GLX2 (Fig. 7, A and B, compare Dox panels with Dox + GLX2 panels). Similarly, the extent of PARP cleavage was not reduced by overexpressed exogenous GLX2 (Fig. 7C, PARP panel, compare lanes 3 and 7 with lanes 4 and 8, respectively).

**DISCUSSION**

As a highly reactive by-product of glycolysis, MG, which is continuously formed in cells, can modify arginine and lysine residues of cellular proteins to AGEs (Fig. 8). MG can also attack guanine residues of DNA, leading to DNA glycation (Fig. 8). Thus, excessive MG is toxic and interferes with normal cell functions (10). At normal physiological conditions, MG is maintained at low levels mainly by the glyoxalase sys-
tem (Fig. 8). Although GLX1 catalyzes the initial conversion of MG to less toxic SLG, GLX2 is also required for MG detoxification. For example, as the enzymatic reaction undergoes a process of equilibrium, the accelerated conversion of SLG to D-lactate by GLX2 decreases the level of SLG, which would then accelerate MG into GLX1-catalyzed reaction (Fig. 8). In fact, enzyme kinetic analysis showed that GLX2 is the rate-limiting enzyme of the glyoxalase system (32). In addition, regeneration of GSH from oxidized glutathione produced by GLX1-catalyzed reaction, which is necessary for subsequent conversion of MG to SLG and for the maintenance of an appropriate redox state within the cell,
requires GLX2. However, the overall effect of GLX2 on MG detoxification is not known. Here we showed that GLX2 is regulated by p63 and p73. Overexpression of the cytosolic GLX2 inhibits MG-induced cell death, whereas knockdown of GLX2 enhances it. Therefore, we uncovered a novel link between p63/p73 and the glyoxalase system (Fig. 8).
Glycolysis is a conserved metabolic pathway throughout the living organisms (33). Due to limited supply of oxygen at some stages during the progression of tumor formation, cancer cells adapt to the local hypoxic environment by increasing the level of aerobic glycolysis, which produces sufficient energy required for rapid growth (34) as well as accumulation of high levels of MG in cancer cells. Thus, it is not surprising that both the level and the activity of GLX1 and GLX2 are found to be increased in breast and bladder cancers (35, 36). Because p63 and p73 are also found to be overexpressed in some types of cancers (37), we hypothesize that cancer cells may evolve to counteract the MG toxicity through accumulation of p63 and p73 that up-regulate the level of GLX2. High glycolytic activity and high levels of MG are also found in rapidly proliferating cells during development (38). Therefore, up-regulation of GLX2 by p63 and p73 may contribute to their essential roles in normal development.

In addition to cancer, MG-induced AGEs are closely related to the pathogenesis of other human diseases, including diabetes and Alzheimer’s disease (Fig. 8). The characteristics of diabetes are chronic hyperglycemia and persistent diabetic microangiopathy in the retina, kidney, and peripheral nerve (39). As a result, the level of MG is elevated by 3- to 5-fold in the blood and tissues of diabetic patients (40). In fact, MG is considered as a marker for glycemic fluctuation (41), and increased formation of MG-induced AGEs is one of the major mechanisms underlining the microvascular complications of diabetes (39). Alzheimer disease is characterized by formation of neurofibrillary tangles assembled from microtubule-associated protein Tau, aggregation of which is facilitated by AGEs modification (13). Therefore, based on the essential role of GLX2 in MG detoxification, modulation of GLX2 through p63 and p73 to inhibit AGEs formation deserves further exploration, which could lead to novel therapeutic strategies for managing diabetes and Alzheimer disease. Although it is not clear whether GLX2 can be induced by metabolic stresses, a recent study showed that MG can impair glucose metabolism, leading to intracellular ATP depletion and mitochondrial dysfunction (42). Thus, GLX2 may play a pro-survival role in the metabolic stress response through detoxifying MG to maintain the energy balance.

In addition to its role in MG detoxification, we found that GLX2 has a protective role in the DNA damage response as GLX2 knockdown sensitizes RKO cells to DNA damage-induced apoptosis in a p53-dependent manner. One possibility is that GLX2 knockdown leads to increased accumulation of endogenous MG, which is shown to act synergistically with cisplatin to induce apoptosis (43). However, unlike the early study that overexpression of GLX1 renders human leukemia cells resistant to chemotherapeutic agents (11), overexpression of GLX2 alone has little if any effect on DNA damage-induced apoptosis.
Unlike GLX1, GLX2 activity was detected in both cytosol and mitochondria. The cytosolic GLX2 was estimated to be 85–90%, with the remainder being for the mitochondrial GLX2 (27). In yeast, the mitochondrial GLX2 complements the cytosolic form in MG detoxification (44). However, the precise role of human mitochondrial GLX2 in the glyoxalase system is not clear, because the mitochondrial GLX2 does not function as a major MG-detoxifying enzyme. First, the mitochondrial GLX2 is unable to inhibit MG-induced cell death when overexpressed. Second, MG is mainly produced in the cytosol where the glycolysis takes place. Third, GLX1 appears to be absent in the mitochondria, and thus SLG is not synthesized in the mitochondria (27). Because the mitochondria are the major source for reactive oxygen species production, it is possible that the mitochondrial GLX2 is involved in the regulation of the redox state, which deserves further investigation.

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