GPX2, a Direct Target of p63, Inhibits Oxidative Stress-induced Apoptosis in a p53-dependent Manner*

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The p53 family consists of p53, p63, and p73, each of which has multiple isoforms due to transcription at two separate promoters and alternative splicing. Although p53 is a bona fide tumor suppressor, p63 appears to have a Janus-faced function as a tumor suppressor and an oncogene. To address the two opposing functions of p63, we analyzed its target genes. Here, we found that GPX2, which encodes a glutathione peroxidase, is up-regulated by p63 but not p53. Accordingly, a unique responsive element was found in the promoter of the GPX2 gene that can be activated and bound by p63 but not p53. We also found that upon overexpression, GPX2 alleviates the apoptotic response of MCF7 cells to oxidative stresses. Interestingly, the protective function of GPX2 is p53 dependent. Likewise, we showed that a deficiency in GPX2 renders MCF7 cells susceptible to oxidative stress-induced apoptosis. Given that some isoforms of p63 serve as a pro-survival factor by up-regulating GPX2 to reduce the p53-dependent oxidative stress-induced apoptotic response.

GPX2, a member of the glutathione peroxidase (GPX) family, is a selenoprotein and a glutathione peroxidase (1). GPX2 is a tetrameric enzyme localized in cytosol and capable of reducing hydrogen peroxide and alkyl hydroperoxides. GPX2 appears to be expressed in selected tissues, including mammary tissue and the gastrointestinal tract, and thus was originally designated GPX-GI (2). Because of its tissue-specific expression, it is hypothesized that GPX2 in the gastrointestinal tract plays a critical role in protecting mammals from the toxicity, including cancer formation, by ingested lipid hydroperoxides or reactive oxygen species derived from inflammation (1). Indeed, a deficiency in Gpx2 together with Gpx1 predisposes mice susceptible to bacteria-associated inflammation and cancer (3), suggesting that peroxidative stress plays a role in ileal and colonic pathology and inflammation.

As a selenoprotein, the expression of GPX2 can be regulated post transcriptionally by the availability of selenium (1, 2). The level of GPX2 is found to oscillate rapidly in response to selenium deprivation and supplementation, suggesting that GPX2 is a major selenoprotein for cell survival (4). In addition, GPX2 is found to be transcriptionally regulated by oxidants and antioxidants (1, 5). For example, GPX2 is regulated by antioxidants via Nrf2 transcription factor (6) and retinoic acids via three putative retinoic acid-responsive elements in the GPX2 promoter (7). Furthermore, increased expression of GPX2 in the small intestine coincides with rapid growth of the intestine, consistent with its potential regulation by β-catenin/T cell factor-4 in colonic epithelium (1).

p63, a member of the p53 family, is expressed as two major groups, the TA and ΔN variants (8–10). The TA variant, which is expressed from the upstream promoter, contains an activation domain similar to the first activation domain in p53 and thus has a strong transcriptional activity (10). Like p53, TA p63 is capable of inducing cell cycle arrest and apoptosis when overexpressed (8–10). However, the ΔN variant, which is expressed from the cryptic promoter in intron 3, lacks an activation domain conserved in p53 but obtains 14 unique residues at the N terminus (10). These 14 amino acids together with the adjacent residues constitute an activation domain for the ΔN variant (11). Thus, the ΔN variant also possesses a transcriptional activity and is capable of inducing cell cycle arrest and apoptosis, albeit much weaker than the TA variant (11). Interestingly, the ΔN, but not the TA, variant is highly expressed in the basal epithelial cells and often amplified in tumor cells (12, 13). In addition, the ΔN variant has a dominant negative activity toward p53, although wild-type p63 and p53 are incapable of forming a heterotetramer (14). Although ΔNp63 may compete with p53 to bind to the p53-responsive element in, and thus attenuate the induction of, some p53 target genes, only a small subset of the promoters of the p53 target genes are recognized by ΔNp63 (15). Thus, the mechanism by which ΔNp63 inhibits p53 merits further study.

In this study, we found that p63, especially ΔNp63, up-regulate GPX2, which inhibits the activation of p53 by reducing the extent of oxidative stresses and oxidative stress-induced apoptosis in a p53-dependent manner. Thus, we have provided a novel mechanism by which ΔNp63 regulates its own unique target genes, which serve as a mediator of ΔNp63 to antagonize the activity of p53.

EXPERIMENTAL PROCEDURES

Plasmids—For inducible expression of GPX2, a cDNA fragment that contains a hemagglutinin (HA) epitope sequence at the 5′-end, GPX2 open reading frame, and 304-bp 3′-untranslated region, was amplified from an Expressed Sequence Tag clone (GenBank accession code BG468501) with the forward primer (5′-AAG CTT ACC ATG GAG TAC CCA TAC GAC GTA CCA GAT TAC GCT GCC TCC TTC CAC GAG TCT CTA CTA CCA GCA GAG TCG GTC CAC GAG CAG GC-3′) and the reverse primer (5′-CCT GAG TGA TGA CTC CAG GAC TCT TGT TGA TGA GAG CAG GTC GAG GTC CAG GCT-3′), confirmed by DNA sequencing, and then cloned into pcDNA4 tetracycline-inducible expression vector (Invitrogen).

To generate constructs that express p53 small interfering RNA (siRNA), a DNA oligonucleotide as reported previously (16) was synthesized and cloned into pBabe-U6 at BamHI and XhoI sites. The PolIII promoter-driven plasmid pBabe-U6 was described previously (17). To generate a construct that express GPX2 siRNA under the control of tetracycline, one pair of oligos was cloned into pBabe-H1 at HindIII and BglII sites, and the resulting construct was designated pBabe-GPX2. pBabe-H1 is a PolIII promoter-driven plasmid with a tetracycline operator sequence inserted before the transcriptional starting site (18). The
siRNA oligos cloned in pBabe-GPX2 are sense, 5'-GAT CCC CGG TAG ATT TCA ATA CGT TCT TCA AGA GAG AAC GTA TTG AAA TCT ACC TTT TTG GAA A-3', and antisense, 5'-AGC TTT TCC AAA AAG GTA GAT TTC AAT ACG TTC TCT CTT GAA GAA CGT ATT GAA ATC TAC C GGG-3', with the siRNA targeting region shown in bold.

The luciferase reporter under the control of the p21 promoter was as previously described (19). A 162-bp fragment from the GPX2 promoter (from nt −140 to +22) was amplified from the genomic DNA purified from MCF7 cells with a forward primer, 5’-CTC GAG CTT ACA GGT GGG GAC CTG TTT TTG-3, and a reverse primer, 5’-AAG CTT TGA AGC GCA GAG TGA GCC CCG-3', and confirmed by sequencing. The fragment was cloned into a promoter-less luciferase reporter vector pGL2 (Promega, Madison, WI), and the resulting plasmid is designated pGL2-GPX2 (−140/+22). pGL2-GPX2 (−140/+22) M was generated with the same GPX2 promoter fragment that contains two point mutations in the potential p53 response element (from nt −129 to −101); that is, the Cat nt −126 and the Cat nt −107 were replaced with A and T, respectively.

Cell Culture—MCF7 cell lines, which inducibly express various isoforms of the p53 family proteins, were cultured as described previously (11, 20–22). MCF7-GPX2 cell lines, which inducibly express GPX2 under the control of the tetracycline-regulated promoter, were generated as described (23). To generate inducible GPX2 knockdown cell lines, pBabe-H1-GPX2 siRNA was transfected into MCF7 cells in which a tetracycline repressor is expressed by pcDNA6. The resulting GPX2 knockdown cell lines were selected with puromycin and confirmed by Northern blot analysis.

Affymetrix GeneChip Assay and Northern Blot Analysis—Total RNA was isolated from cells using TRIzol reagent (Invitrogen). U133 plus GeneChip, which contains oligos representing 37,000 unique human transcripts, was purchased from Affymetrix (Santa Clara, CA). GeneChip analysis was performed according to the manufacturer’s instruction. Northern blot analysis and preparation of p21 and glyceraldehyde-3-phosphate dehydrogenase probes were as described previously (24). The GPX2 probe was prepared from an Expressed Sequence Tag clone (GenBank™ accession code BG468501).

Luciferase Assay—A dual luciferase assay was performed in triplicate according to the manufacturer’s instructions (Promega). Briefly, 0.25 μg of a luciferase reporter, 0.25 μg of pcDNA3 or pcDNA3 that expresses a p53 family protein divided by that induced by an empty pcDNA3 (Promega) were cotransfected into H1299 cells. The fold increase in relative luciferase activity is a product of the luciferase activity induced by a p53 family protein divided by that induced by an empty pcDNA3 vector. The pGL2-p21A luciferase reporter under the control of the two p53-responsive elements in the p21 promoter was used as a positive control (19).

Chromatin Immunoprecipitation (ChIP) Assay—ChIP was performed as previously described (17, 25). MCF7 cells, which were uninduced (−) or induced (+) to express p63y or ΔNp63y for 24 h, were cross-linked by 1% formaldehyde for 10 min at room temperature. Nuclear extracts were prepared, and chromatin was sonicated to generate 200- to 1000-bp DNA fragments. Protein-DNA complexes were immunoprecipitated with various antibodies. The DNA-protein cross-links were reversed by heating at 65 °C for 4 h. After phenol and chloroform extraction, DNA was purified by ethanol precipitation. To amplify the region from nt −340 to −120 in the GPX2 promoter, PCR was performed with the forward primer 5’-GTA GGA GTC AAT CCT GCA TCC-3’ and the reverse primer 5’-AAC AGG TCC CCA CCT GTA AG-3’. To amplify the region from nt −2312 to −2131 in the p21 promoter, PCR was performed with the forward primer 5’-CAG GCT GTG CTC ATT GG-3’ and the reverse primer 5’-TTC AGA GTA ACA GGC TAA GG-3’.

Cell Survival Assay—To determine the cytotoxicity induced by H2O2, cells seeded at a density of 5 × 104 cells/well in a 6-well plate, were induced with or without tetracycline (1.0 μg/ml) for 72 h, treated with various concentrations of H2O2 for 2.5 h, and then rinsed with and cultured in fresh medium. At the times indicated, cells were rinsed with phosphate-buffered saline twice to remove dead cells and debris. Live cells on the plate were trypsinized and collected separately. Cells from each plate were counted at least three times using the Coulter cell counter (Beckman Coulter, Fullerton, CA). The average number of cells from three wells was used for survival ratio determination. The percentage of live cells on day 0 was set as 100%. The percentage of live cells after various days of treatment was the product of total live cells divided by total number of cells on day 0.

Western Blot Analysis—Mouse anti-Mdm2 monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against p53, p21, HA, and Myc epitopes and actin were described previously (26). Anti-DR5 was kindly provided by Dr. T. Zhou (The University of Alabama at Birmingham).

DNA Histogram Analysis—Cells were seeded at 4 × 105/10-cm plate, induced with or without tetracycline for 72 h, treated with various concentrations of H2O2 for 2.5 h, and rinsed with and cultured in fresh medium for 24 h. Both floating dead cells in the medium and live cells on the plate were collected and fixed with 10 ml of 70% ethanol for 24 h. The fixed cells were centrifuged and resuspended 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, G0/G1, S, and G2–M phases were determined using the CELLQuest program (BD Biosciences).

RESULTS

Identification of GPX2 as a Unique Target Gene of p63—To identify novel target genes regulated by the p53 family, Affymetrix GeneChip assay was performed. Total RNA was isolated from MCF7 cells that were uninduced and induced to express p63y by withdrawal of tetracycline from the culture medium. cRNA was synthesized and used to hybridize the Affymetrix U133 plus GeneChip. We found that GPX2, a potential novel target gene regulated by p53 family members, was highly induced in cells expressing p63y. To confirm the microarray study, we performed Northern blot analysis. The level of p21 was examined as a positive control and found to be induced by p53 and p63y (Fig. 1), consistent with our previous report (11, 20–22). The level of glyceraldehyde-3-phosphate dehydrogenase was determined as a loading control (Fig. 1). Here, we found that GPX2 was highly induced by p63y and ΔNp63y, weakly by p63a and ΔNp63a, but not by p53 and p53(R249S). We also found that GPX2 is not induced in cells upon DNA damage (data not shown). This indicates that GPX2 is a unique target of p63, but not p53.

As a member of the p53 family, p63 is capable of transactivating a promoter that contains a p53-responsive element (RRRC(A/T)(A/T)GYYY; R represents purine, whereas Y represents pyrimidine) (27), although not all of the p53 target genes can be regulated by p63 (15). A recent study showed that in addition to recognizing the p53-responsive element, p63 selectively transactivates a promoter that contains a unique sequence (RRRC(TG)GGYY) (28). However, no consensus p53- or p63-responsive element was found in the genomic locus encoding the GPX2 gene. Nevertheless, we cloned the promoter of the GPX2 gene in...
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FIGURE 1. Up-regulation of GPX2 by p63. Northern blots were prepared using total RNAs isolated from MCF7 cells that were uninduced (−) and induced (+) to express various p53 family proteins for 24 h. The blots were probed with cDNAs derived from the GPX2, p21, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes, respectively.

front of a luciferase reporter for luciferase assay (Fig. 2A). Interestingly, we found that the GPX2 promoter from nt −140 to +22 was activated highly by p63y and ΔNp63y, weakly by p63α and ΔNp63α, but not by mutant ΔNp63y(R149W), wild-type p53, and mutant p53(R249S) (Fig. 2B). Arg-149 in p63 corresponds to Arg-175 in p53, and mutation of this residue alters the overall structure of p53 and renders p53 incapable of binding to DNA (29). R149W mutation in ΔNp63 was found in ectodactyly, ectodermal dysplasia, cleft lip and palate (EEC) syndrome (30). Thus, the ability of p63y and ΔNp63y to transactivate GPX2 is consistent with their ability to regulate the endogenous GPX2 gene. As a positive control, we showed that the luciferase reporter under the control of the p21 promoter was activated highly by p53 and p63y, weakly by p63α and ΔNp63y, but not by p53(R249S), ΔNp63α, and ΔNp63y(R149W) (Fig. 2, C and D).

Up further searching for a potential responsive element within the promoter from nt −140 to +22, we found a sequence (−129 GGACCTGT/TgtcggatCATCCTGGG −101) that shares some common nucleotides (in bold) with the canonical p53-responsive element and recently reported unique p63-responsive element. To determine whether this element is required, we mutated two nucleotides predicted to be critical for p53- and p63-responsive elements, that is, the first C in both half site was replaced with A and T, respectively. The resulting reporter was designated pGL2-GPX2(−140/+22)M. We found that the luciferase activity for pGL2-GPX2(−140/+22)M was not significantly increased by p63y and ΔNp63y (Fig. 2B). This result suggests that RRRRCCCTGGY is a novel responsive element for p63y and ΔNp63y.

Next, we performed ChIP assay to determine whether p63y and ΔNp63y directly bind to the novel responsive element in the GPX2 gene in vivo. The p63-DNA complexes were immunoprecipitated with anti-Myc antibody. We found that the captured fragment with the novel responsive element in the GPX2 gene was highly enriched upon inducible expression of p63y or ΔNp63y (Fig. 2F). No DNA fragment was enriched by the control antibody anti-FLAG (Fig. 2F). As a positive control, the fragment with the upstream p53-responsive element in the p21 gene was enriched upon inducible expression of p63y or ΔNp63y (Fig. 2G).

Overexpression of GPX2 Decreases the Sensitivity of MCF7 Cells to Oxidative Stress-induced Cell Death—To determine whether GPX2 has an effect on cell proliferation, we generated MCF7 cell lines that express GPX2 tagged with HA at the N terminus under the control of a tetracycline-regulated promoter. Four representative clones are shown in Fig. 3A. Next, we performed growth rate analysis with the MCF7-GPX2–31 cell line and found that GPX2 had no effect on the growth rate of MCF7 cells over a 5-day period (Fig. 3B). Thus, expression of GPX2 alone has little, if any, effect on cell proliferation.

Based on the expression pattern of GPX2 in the gastrointestinal tract, it is hypothesized that GPX2 may play a role in protecting cells from apoptosis induced by food-borne alkyl hydroperoxide (5). Thus, we examined whether overexpression of GPX2 inhibits oxidative stress-induced apoptosis in MCF7 cells. Upon addition of tetracycline to induce GPX2, MCF7 cells were treated with various concentrations of H2O2 for 2.5 h. 24 h following the treatment, surviving cells were counted. We found that the number of surviving cells was significantly increased by induction of GPX2 (Fig. 3C). As a control, the parental MCF7 cells in the presence or absence of tetracycline were similarly treated with H2O2 for 2.5 h. We found that tetracycline itself had no effect on cell survival (Fig. 3D). To further determine the effect of GPX2 on the survival rate of cells exposed to oxidative stresses, we examined the number of surviving cells at various times following treatment with 75 μM H2O2 for 2.5 h. We found that upon induction of GPX2, MCF7 cells began to proliferate on day 2 after H2O2 treatment. In contrast, in the absence of GPX2, MCF7 cells failed to proliferate over the 4-day testing period (Fig. 3E).

To determine the underlying mechanism by which GPX2 is able to alleviate the toxicity of H2O2 to MCF7 cells, apoptosis was examined by DNA histogram analysis. In control cells without treatment with H2O2, GPX2 has no effect on apoptosis as less than 0.3% of cells underwent apoptosis (Fig. 3F), suggesting that endogenous GPX2 is sufficient to eliminate the toxic effect of endogenous oxidative stresses. However, upon treatment with H2O2, exogenous GPX2 reduced the level of apoptosis from 7.82 to 1.30% (Fig. 3F). These data are consistent with that obtained from the cell survival assay.

GPX2-mediated Protection of Oxidative Stress-induced Apoptosis Is p53 Dependent—The importance of p53 in mediating hydrogen peroxide-induced apoptosis has been well established, as elimination of p53 function enhances the survival rate of H2O2-treated cells (31, 32). In addition, p21, a target of p53, is shown to play a role in H2O2-induced growth suppression and replicative senescence (33). To determine the role of p53 in the GPX2-mediated protection of cells from H2O2-induced apoptosis, the stability and transcriptional activity of p53 were examined in MCF7 cells, which were untreated or treated with 75 μM H2O2 for various times as indicated in the presence or absence of GPX2 (Fig. 4A). We found that GPX2 mitigated the effect of H2O2 to stabilize p53 and subsequently the ability of p53 to induce p21 (Fig. 4A).

To determine whether p53 is required for GPX2 to mitigate the effect of H2O2 on apoptosis, we generated MCF7 cell lines in which p53 is stably knocked down by siRNA and GPX2 is inducibly expressed by the tetracycline-inducible expression system. One representative cell line, M7-p53KD-GPX2–2, was chosen for further studies (Fig. 4B). In M7-GPX2–31, which carries endogenous wild-type p53 and inducibly expresses GPX2, p53 was stabilized upon treatment with H2O2, which then led to induction of p53 target genes such as p21, DR5, and MDM2 (Fig. 4B). In addition, GPX2 mitigated the effect of H2O2 on p53 stability and induction of p21, DR5, and Mdm2 (Fig. 4B). In contrast, in M7-p53KD-GPX2–2, p53 and its target genes p21 and MDM2 were undetectable upon treatment with H2O2 regardless of the expression of GPX2, suggesting that endogenous p53 was nearly completely knocked down (Fig. 4B). Interestingly, DR5 was slightly induced by H2O2, in the absence of detectable p53, but its expression was attenuated by GPX2 (Fig. 4B). Next, we examined the survival rate of both the control or p53 knockdown MCF7 cells in the presence or absence of exogenous GPX2 upon treatment with H2O2. The control MCF7 cells were sensitive to H2O2-induced cell death, which was substantially inhibited by GPX2
In contrast, p53 knockdown MCF7 cells were resistant to H2O2-induced cell death (Fig. 4C). In addition, the ability of GPX2 to mitigate H2O2-induced cell death in p53 knockdown MCF7 cells was markedly decreased (Fig. 4C).

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To determine whether GPX2 plays an obligatory role in protecting cells from H2O2-induced apoptosis, we generated multiple MCF7 cell lines in which GPX2 is inducibly knocked down by siRNA under the control of the tetracycline-regulated promoter. One representative cell line, MCF7-GPX2-KD-7, was chosen for further studies. First, to demonstrate that GPX2 is efficiently knocked down, the level of GPX2 induced by H2O2, retinoid acid, or both was examined in the absence or presence of siRNA against GPX2. We found that GPX2 induced by H2O2 or retinoid acid was efficiently suppressed by GPX2 siRNA (Fig. 5A, compare lanes 3 and 5 with lanes 4 and 6, respectively). Interestingly, GPX2 was highly induced by a combined treatment of H2O2 and retinoid acid, which was still suppressed by GPX2 siRNA (Fig. 5A, compare lane 7 with lane 8). To examine whether p63

**FIGURE 2. Identification of a p63-responsive element in the GPX2 promoter.** A, schematic presentation of the GPX2 locus and luciferase reporter constructs. A potential p63-responsive element is located at nt −129 to −101 in the promoter of the GPX2 gene. The reporter construct, pGL2-GPX2 (−140/+22M), carries two nucleotide substitutions in which the first C in both half sites was substituted with A and T, respectively. B, the potential p63 binding site in the GPX2 promoter is responsive to p63γ and ΔNp63γ, weakly to p63α and ΔNp63α, but not to p53, mutant p53(R249S), and mutant ΔNp63γ(R149W). C, schematic presentation of the p21 luciferase reporter construct. D, the p21 promoter is responsive to p53 and p63γ, weakly to p63α and ΔNp63γ, but not to mutant p53(R249S), ΔNp63α, and mutant ΔNp63γ. E, schematic presentation of the GPX2 and p21 promoter and the location of the potential p53 family-responsive element(s) and PCR primers used for ChIP assay. F, p63γ directly binds to the GPX2 and p21 promoter in vivo. p63γ-DNA complexes were captured with anti-Myc. Anti-FLAG antibody was used as a control. G, ΔNp63γ directly binds to the GPX2 and p21 promoter in vivo. The experiments were performed as described in panel F.
FIGURE 3. Overexpression of GPX2 decreases the sensitivity of cells to H$_2$O$_2$-induced cell death. A, the level of HA-tagged GPX2 in four representative MCF7 cell lines was quantified by Western blot analysis with anti-HA antibody. Extracts were collected from cells uninduced (−) or induced (+) to express GPX2 for 24 h. B, expression of GPX2 alone has little effect on MCF7 cell proliferation. Growth rate of MCF7 cells uninduced or induced to express GPX2 over a 5-day period. C, GPX2 protects MCF7 cells from H$_2$O$_2$-induced cell death. MCF7 cells were uninduced (control) or induced (GPX2) to express GPX2 for 3 days and then treated with 0–250 μM H$_2$O$_2$ for 2.5 h. 24 h following the treatment, surviving cells were counted. D, tetracycline has no effect on H$_2$O$_2$-induced cell death. The experiment was performed as in panel C. E, GPX2 protects MCF7 cells from H$_2$O$_2$-induced cell death. MCF7 cells were uninduced (control) or induced (GPX2) to express GPX2 for 3 days and then treated with 75 μM H$_2$O$_2$ for 2.5 h. The surviving cells were determined over a 4-day period. F, GPX2 protects MCF7 cells from H$_2$O$_2$-induced apoptosis. MCF7 cells were uninduced (control) or induced (GPX2) to express GPX2 for 3 days and then treated with 0 or 75 μM H$_2$O$_2$ for 2.5 h. 24 h following 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.

FIGURE 4. GPX2-mediated protection of oxidative stress-induced apoptosis is p53 dependent. A, the level of GPX2, p53, p21, and actin in MCF7 cells uninduced (−) and induced (+) to express GPX2 for 3 days followed by treatment with 75 μM H$_2$O$_2$ for 0–10 h. B, the level of p53, p21, DR5, MDM2, GPX2, and actin in the control and p53 knockdown MCF7 cells uninduced (−) and induced (+) to express GPX2 for 3 days followed by treatment with 0 or 75 μM H$_2$O$_2$ for 24 h. C, the rate of cell survival was determined for the control and p53 knockdown MCF7 cells uninduced (−) and induced (+) to express GPX2 for 3 days followed by treatment with 75 μM H$_2$O$_2$ for 2.5 h. 24 h following the treatment, surviving cells were counted.
plays a role in the induction of GPX2 in MCF7 cells upon treatment with H2O2, we analyzed the expression level of both the TA and ΔN isoforms of p63. We found that H2O2 did not directly up-regulate p63 to induce GPX2 in MCF7 cells (Fig. 5B, lanes 1–4).

Next, we examined the effect of GPX2 on the activity of p53 induced by H2O2. We found that GPX2 knockdown alone led to weak accumulation of p53, but the level of p53 was still not sufficient to induce Mdm2 (Fig. 5C, compare lanes 1 and 2). Upon treatment with H2O2, p53 was stabilized and activated to induce Mdm2 (Fig. 5C, compare lanes 1–3). However, GPX2 knockdown enhanced the effect of H2O2 on p53 stabilization, which led to a stronger induction of Mdm2 (Fig. 5C, compare lanes 3 and 4). Finally, we examined the effect of GPX2 knockdown on H2O2-induced cell death by measuring the extent of cell survival and apoptosis. We found that the rate of cell survival was inhibited by GPX2 knockdown (Fig. 5D). Likewise, we found that the extent of H2O2-induced apoptosis was increased from 3.67% to 8.55% by GPX2 knockdown (Fig. 5E).

**DISCUSSION**

GPX2 glutathione peroxidase is a potent detoxifier of reactive oxidants (1). Because of its tissue-specific expression in the gastrointestinal tract, GPX2 is a major enzyme to remove ingested lipid hydroperoxides and reactive oxygen species derived from both pathogenic and non-pathogenic bacteria in the gut (1, 2). In addition, lack of Gpx2 along with Gpx1 results in frequent bacteria-associated inflammations and increased cancer risks in mice (3). Thus, these characteristics would classify GPX2 as a potent anti-carcinogenic agent. However, these same properties would also classify GPX2 as a pro-carcinogenic agent because removal of hydroperoxides helps cells, especially damaged cells that have progressed into a precancerous status, to evade apoptosis (1). Consistent with this idea, GPX2 has been found to be overexpressed in human colorectal adenoma and carcinoma, Barrett’s esophageal mucosa, and during neoplastic transformation of squamous epithelial cells (34–38). However, the mechanism by which GPX2 is overexpressed in tumor cells is not clear. In this study, we found that GPX2 is regulated by p63, especially ΔNp63γ. We also found that upon overexpression, GPX2 alleviates the apoptotic response of MCF7 cells to oxidative stresses. Interestingly, the protective function of GPX2 is p53 dependent. Likewise, we showed that a deficiency in GPX2 renders MCF7 cells susceptible to oxidative stress-induced apoptosis. Given that the ΔN isoform of p63 is frequently overexpressed in tumor cells and dominant negative over p53 (12, 13), the observations here provide an insight into the mechanism by which some isoforms of p63 serve as a pro-survival factor by up-regulating GPX2 to inhibit the p53-dependent oxidative stress-induced apoptotic response.

In addition to its unique tissue-specific expression in the gastrointestinal tract, the expression gradient of GPX2 was found to be increased from the apical part of the crypt to the crypt ground in the colon (5). Furthermore, a deficiency of Gpx2 in the knock-out mice results in a phenotype characterized by shortened villi in the small intestine and altered levels of undifferentiated cells throughout the gut. These data suggest that GPX2 may play a role in maintenance of the undifferentiated mucosal epithelium (1). Interestingly, the expression of ΔNp63, which is preferentially expressed in undifferentiated basal epithelium cells, is suppressed in differentiated cells (39). Thus, it is hypothesized that ΔNp63 is associated with, and serves as a marker for, undifferenti-
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...in its promoter. Through mutational analysis, we found one nucleotide

GPX2 is a unique p63 target gene that contains a novel p63 binding site

that this novel p63 binding site is responsive to p63, not to p53, p63

strong activation domain. This suggests that the extreme C terminus

CCTG sequence of

promoter of the

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REFERENCES

1. Chu, F. F., Esworth, R. S., and Doroshow, J. H. (2004) Free Radic. Biol. Med. 36, 1481–1495
2. Chu, F. F., Doroshow, J. H., and Esworth, R. S. (1993) J. Biol. Chem. 268, 2571–2576
3. Chu, F. F., Esworth, R. S., Chu, P. G., Longmate, J. A., Huey, M. M., Wilczynski, S., and Doroshow, J. H. (2004) Cancer Res. 64, 962–968
4. Wingler, K., Bocher, M., Flohe, L., Kollmus, H., and Brigelius-Flohe, R. (1999) Eur. J. Biochem. 259, 149–157
5. Brigelius-Flohe, R., Muller, C., Menard, J., Florian, S., Schmehl, K., and Wingler, K. (2001) Biofactors 14, 101–106
6. Banning, A., Deubel, S., Kluth, D., Zhou, Z., and Brigelius-Flohe, R. (2005) Mol. Cell. Biol. 25, 4914–4923
7. Chu, F. F., Esworth, R. S., Lee, L., and Wilczynski, S. (1999) J. Nutr. 129, 1846–1854
8. Osada, M., Ohba, M., Kawahara, C., Ishioka, C., Kanamaru, R., Katoh, I., Ikawa, Y., Nimura, Y., Nakagawara, A., Obinata, M., and Ikawa, S. (1998) Nat. Med. 4, 839–843
9. Trink, B., Okami, K., Wu, L., Sriranganp, V., Jen, J., and Sidransky, D. (1998) Nat. Med. 4, 747–748
10. Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dotsch, V., Andrews, N. C., Caput, D., and McKeon, F. (1998) Mol. Cell. 2, 305–316
11. Dohn, M., Zhang, S., and Chen, X. (2001) Oncogene 20, 3193–3205
12. DiComo, C. J., Urist, M. J., Babayan, I., Drobnjak, M., Hedvat, C. V., Teruya-Feldstein, J., Pohar, K., Hoos, A., and Cordon-Cardo, C. (2002) Clin. Cancer Res. 8, 494–501
13. Hibi, K., Trink, B., Patturajan, M., Westra, W. H., Caballero, O. L., Hill, D. E., Ratafiaszki, L. A., Jen, J., and Sidransky, D. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 5462–5467
14. Davison, T. S., Vagner, C., Kaghad, M., Ayed, A., Caput, D., and Arrasmith, C. H. (1999) J. Biol. Chem. 274, 18709–18714
15. Harms, K., Nozell, S., and Chen, X. (2004) Cell. Mol. Life Sci. 61, 822–842
16. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) Science 296, 550–553
17. Liu, G., Xia, T., and Chen, X. (2003) J. Biol. Chem. 278, 17557–17565
18. el-Deiry, W. S., Kern, S. E., Pieters, J. A., Kinzler, K. W., and Vogelstein, B. (1992) Nat. Genet. 1, 45–49
19. Nozell, S., and Chen, X. (2002) Oncogene 21, 1285–1294
20. Liu, G., Nozell, S., Xiao, H., and Chen, X. (2004) Mol. Cell. Biol. 24, 487–501
21. Nozell, S., Wu, Y., McNaughton, K., Liu, G., Paik, J., and Chen, X. (2003) Oncogene 22, 4333–4347
22. Zhu, J., Zhang, S., Jiang, J., and Chen, X. (2000) J. Biol. Chem. 275, 39972–39974
23. Dohn, M., Nozell, S., Willis, A., and Chen, X. (2003) Methods Mol. Biol. 223, 221–235
24. Chen, X., Bargonetti, J., and Prives, C. (1995) Cancer Res. 55, 4257–4263
25. Harms, K. L., and Chen, X. (2005) Mol. Cell. Biol. 25, 2014–2030
26. Zhu, J., Zhou, W., Jiang, J., and Chen, X. (1998) J. Biol. Chem. 273, 13030–13036
27. el-Deiry, W. S., Kern, S. E., Pieters, J. A., Kinzler, K. W., and Vogelstein, B. (1992) Nat. Genet. 1, 45–49
28. Osada, M., Park, H. L., Nagakawa, Y., Yamashita, K., Fomenkov, A., Kim, M. S., Wu, G., Nomoto, S., Trink, B., and Sidransky, D. (2005) Mol. Cell. Biol. 25, 6067–6089
29. Willis, A., Jung, E. I., Wakefield, T., and Chen, X. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 2330–2338
30. Brunner, H. G., Hamel, B. C., and Van Bokhoven, H. (2002) J. Med. Genet. 39, 377–381
31. Kitamura, Y., Ota, T., Tsubo, K., Yamaoka, I., Kimura, H., Kohzuma, S., Noma, T., Gibrilie-Flohe, R., and Shiraki, H. (1999) Biochem. J. 347, 543–551
32. Florian, S., Wingler, K., Schmehl, K., Jacobba, G., Kreuzer, O. J., Meyerhoff, W., and Gibriloe-Flohe, R. (2001) Free Radic. Res. 35, 655–663
33. Lin, Y. M., Furukawa, Y., Tsunoda, T., Yone, C. T., Yang, A., and Nakamura, Y. (2002) Oncogene 21, 4120–4128
34. Mork, H., al-Taie, O. H., Bahr, K., Zierer, A., Beck, C., Scheurlen, M., Jakob, F., and Kohrle, J. (2000) Int. J. Cancer 86, 154–164
35. Yim, Y., Terunouchi, Y., Solomon, G. G., Aizawa, S., Banaragajan, P. N., Yazaki, Y., Kadomatsu, T., and Barret, J. C. (1998) Oncogene 17, 707–710
36. Chen, Q. M., Liu, J., and Merrett, J. R. (2000) Biofactors 22, 293–302
37. Serevko, M. M., Popa, C., Duhler, A. L., Smith, L., Stratton, G. M., Coman, W., Dicker, A. J., and Saunders, N. A. (2002) Cancer Res. 62, 3759–3765
38. Yang, A., Schweitzer, B., Sun, D., Kaghad, M., Walker, N., Bronson, R. T., Tabin, C., Sharpe, A., Caput, D., Crum, C., and McKeon, F. (1999) Nature 398, 714–718
39. Yang, A., Kaghad, M., Caput, D., and McKeon, F. (2002) Trends Genet. 18, 90–95
40. Chiavone, P., Bolognesi, F., Dufif, P. H., Van Bokhoven, H., Mantovani, R., and Guerini, L. (2002) Mol. Cell. Biol. 22, 8659–8668
41. Serber, Z., Lai, H. C., Yang, A., Ou, H. D., Sigal, M. S., Kelly, A. E., Dartimont, B. D., Dufif, P. H., Van Bokhoven, H., McKeon, F., and Dotsch, V. (2002) Mol. Cell. Biol. 22, 8601–8611

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