Transcriptional Activation of the Human Glutathione Peroxidase Promoter by p53*

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Glutathione peroxidase (GPX) is a primary antioxidant enzyme that scavenges hydrogen peroxide or organic hydroperoxides. We have recently found that GPX is induced by etoposide, a topoisomerase II inhibitor and a p53 activator. In a search for a cis-element that confers potential p53 regulation of GPX, we identified a p53 binding site in the promoter of the GPX gene. This site bound to purified p53 as well as p53 in nuclear extract activated by etoposide. A luciferase reporter driven by a 262-base pair GPX promoter fragment was transcriptionally activated by wild type p53 in a p53 binding site-dependent manner. The same reporter was also activated in a p53 binding site-independent manner by several p53 mutants. The p53 binding and transactivation of the GPX promoter were enhanced by etoposide in p53-positive U2-OS cells. Etoposide-induced transactivation was blocked by a dominant negative p53 mutant, indicating that endogenous wild type p53, upon activation by etoposide, transactivated the GPX promoter. Furthermore, expression of endogenous GPX was induced significantly at both mRNA and enzyme activity levels by etoposide in U2-OS cells but not in p53-negative Saos-2 cells. This is the first report demonstrating that GPX is a novel p53 target gene. The finding links the p53 tumor suppressor to an antioxidant enzyme and will facilitate study of the p53 signaling pathway and antioxidant enzyme regulation.

Human p53 is a 393-amino acid nuclear phosphoprotein and transcription factor. Structurally, the p53 molecule consists of three major domains: an oligomerization domain in its carboxyl terminus which mediates homotetramer formation; a DNA binding domain in the central portion of the molecule which specifically binds to its consensus binding site; and a transactivation domain in the amino terminus which mediates transactivation of many downstream target genes (1–3). As a typical tumor suppressor, p53 has been shown to inhibit tumor cell growth and suppress transformation through induction of G1 arrest or apoptosis (4–6). As a "genome guard," p53 prevents growth and suppress transformation through induction of G1 arrest or apoptosis (4–6). As a "genome guard," p53 prevents growth and suppress transformation through induction of G1 arrest or apoptosis (4–6).

Most of the functions of p53 involve its activity as a transcription factor. p53 binds to its consensus binding sequence (two copies of the 10-bp motif 5′-PuPuPuC(A/T)(T/A)Py-3′, separated by 0–13 bp) (13) and transactivates expression of target genes. Several biologically significant genes were found to contain this consensus sequence and to be subjected to p53 regulation. Among those commonly studied are Waf-1/p21 (14), Mdm2 (15), Gadd45 (16), Bax (17), and the genes encoding proliferating cell nuclear antigen (18, 19), cyclin G (20), epidermal growth factor receptor (21), thrombospordin (22), and matrix metalloproteinases-2 (23), among others. The p53 mutations found in many human cancers were clustered in the specific DNA binding domain of the p53 molecule (24). This leads to an inactivation of p53 function through abolishing p53-specific DNA binding and transactivation.

Cellular glutathione peroxidase, GPX (EC 1.11.1.9), is one of the primary antioxidant enzymes that scavenges hydrogen peroxide and organic hydroperoxides with glutathione as the hydrogen donor (25). The enzyme was first described in 1957 and is found mainly in cytoplasm (26). GPX is a selenium-dependent enzyme that exists as a homotetramer with each 22-kDa subunit containing a selenium atom incorporated within a catalytically active selenocysteine residue (27, 28). There are three other members of the selenium-dependent GPX family, although cytosolic GPX (GPX1) is the predominant form (29). The gene encoding GPX was mapped on chromosome 3q11–13 (30). Because GPX decomposes hydrogen peroxide and organic hydroperoxides produced during normal metabolism and after oxidative insults, GPX prevents peroxide-induced DNA damage, lipid peroxidation, and protein degradation (31).

Using DNA chip technology, we have found recently that GPX is inducible by etoposide, a topoisomerase II inhibitor, an apoptosis inducer, and a p53 activator (32). We report here the characterization of GPX as a novel p53 target gene, determined by assays for DNA binding, transcriptional activation, and endogenous gene induction. This finding links p53 to an antioxidant enzyme for the first time and may shed light on a better understanding of p53 signaling pathways and redox regulation.

MATERIALS AND METHODS

Cell Culture and Drug Treatment

Two human osteogenic sarcoma cell lines, U2-OS and Saos-2, were grown in 10% McCoy or 10% Dulbecco’s modified Eagle’s medium, respectively. U2-OS cells harbor a wild type p53 (23), whereas Saos-2 cells have the p53 gene deletion (33). The doubling time is about 24 h for U2-OS and 40 h for Saos-2 under these culture conditions. For drug treatment, U2-OS and Saos-2 cells were exposed to etoposide (25 μM, Sigma) for various periods of time up to 48 h.

Gel Shift Assay

The assay was performed as described previously (34, 35). Briefly, a 20-bp synthetic oligonucleotide, GPX 1 (5′-GGGCGAGGACGACAT-oxidase; GPX W/p53BS, GPX promoter containing p53 binding site; GPX W/O p53BS, GPX promoter without p53 binding site; pAb, polyclonal antibody; PCR, polymerase chain reaction; ROS, reactive oxygen species.

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‡ The abbreviations used are: bp, base pair(s); GPX, glutathione peroxide; GPX W/p53BS, GPX promoter containing p53 binding site; GPX W/O p53BS, GPX promoter without p53 binding site; pAb, polyclonal antibody; PCR, polymerase chain reaction; ROS, reactive oxygen species.
The results are presented as the fold activation of the empty excess of unlabeled specific or nonspecific oligonucleotide were also amounts of proteins (60 μg aprotinin. The lysate was centrifuged at 14,000 rpm for 30 min. Equal detected by horseradish peroxidase-conjugated secondary antibody coupled as SDS-polyacrylamide gel and run at 100 V for 2 h. The proteins were made as follows.

**Luciferase Reporter Constructions**

The luciferase reporter constructs driven by the GPX promoter were made as follows.

**GPX W/p53BS—A 262-bp DNA fragment of the GPX promoter containing a p53 binding site was generated by PCR amplification of human placenta DNA (Oncor) with primers GPX01 and GPX04 (**GGGGCGAATTCTTGGCGAAGC-3′ and 5′-GCTGCTCCTTCCGGCTTAGG-3′**) and GPX04. The PCR fragments were gel purified, subcloned into a TA cloning vector (Invitrogen), and seven independent clones were sequenced by an automatic DNA sequenator to verify the orientation and freedom of mutations. The recombinant clones were digested with HindIII and XhoI and ligated into a predigested pGL-Basic luciferase reporter-3 (Promega).

**DNA Transfection and Luciferase Assay**

Dispersed cells were seeded into 24-well plates at a cell concentration of 10^5/well (for Saos-2) or 2 × 10^5/well (for U2-OS) 16–24 h before transfection. The calcium phosphate method was used for transient transfection of Saos-2 cells as described previously (35), whereas the LipofectAMINE method (Life Technologies, Inc.) was used for U2-OS transfection according to the manufacturer’s instructions. The luciferase reporters described above, along with the control plasmid, were cotransfected with a β-galactosidase construct in the presence or absence of constructs expressing wild type or mutant p53 proteins. The ratio of the DNA amounts for p53-expressing vector versus luciferase reporter was 1.1 or 1.2. 38 h post-transfection, cells were lysed and assayed for luciferase/β-galactosidase activities as detailed previously (35). The results are presented as the fold activation of the empty reporter after normalization with β-galactosidase activity.

**Northern Analysis**

U2-OS and Saos-2 cells were treated with 25 μM etoposide, a known 25 μM etoposide for 6 or 24 h. As shown in Fig. 1, a 20-bp oligonucleotide, consisting of the p53 binding site found in the GC-rich region in the published data. Computer analysis was used as loading controls for densitometric quantitation.

**Western Blot Analysis**

Subconfluent human Saos-2 cells were cotransfected with GPs Wip53BS construct and various constructs expressing wild type or mutant p53 proteins along with the vector control by the calcium phosphate method as described (35). Cells were harvested 38 h post-transfection and lysed on ice for 30 min in a lysis buffer (phosphate-buffered saline containing 2% Nonidet P-40, 0.5% SDS, 1 mM sodium orthovanadate, 5 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 0.2 unit/ml aprotinin. The lysate was centrifuged at 14,000 rpm for 30 min. Equal amounts of proteins (60 μg) in supernatant were loaded onto a 12% SDS-polyacrylamide gel and run at 100 V for 2 h. The proteins were transferred onto a nitrocellular membrane and probed with p53 antibodies pAb421 and pAb240 (1:500 dilution). The p53 proteins were detected by horseradish peroxidase-conjugated secondary antibody coupled with Enhanced Chemiluminescence (ECL) Western blotting detection reagents (Amersham Pharmacia Biotech).
p53 Regulation of GPX

among others (data not shown).

p53-dependent Transactivation of the GPX Promoter—To examine potential p53-dependent transactivation of the GPX promoter, we transfected luciferase reporters driven by the GPX promoter sequence with (GPX W/p53BS) or without (GPX W/O p53BS) p53 binding site into p53-negative Saos-2 cells along with p53 expression plasmid or an empty vector as the control. As shown in Fig. 3, the absence of p53 (vector control) the PCR-cloned promoter fragment contains a strong promoter activity. An 80-fold higher luciferase activity was observed compared with the empty vector, pGL-Basic-3. The p53/Ab-supershifted bands are indicated by the arrows. The band shown on the bottom of the gel is free-labeled probe. Also, in nuclear extracts, several nonspecific bands were detected which do not respond to etoposide treatment.

Fig. 1. p53 binds to a putative p53 binding site in the promoter of the GPX gene. The synthetic oligonucleotides of the p53 binding consensus sequence (GPX01: 5'-GGCCAGACAGACATGCTT-3') and its complementary strand were annealed and labeled with 32P using T4 polynucleotide kinase and [γ-32P]ATP. The gel shift assay was performed as detailed under “Materials and Methods.” Lanes 1–4, partially purified p53 (3 μg) with or without pAb421 antibody; lanes 5–12, nuclear extracts (8.5 μg) prepared from cells treated with etoposide (25 μM) for 0 h (lanes 5 and 6), 6 h (lanes 7 and 8), and 24 h (lanes 9–12). The nonspecific oligonucleotide is mT3SF (5'-GGGGTTGGCTTGAA-GAGCCGTC-3') (74). The p53/Ab-supershifted bands are indicated by the arrows. The band shown on the bottom of the gel is free-labeled probe.

Fig. 2. Comparison of the PCR-cloned GPX promoter with a published sequence. The GPX promoter was cloned by PCR amplification of human placenta DNA using GPX01 and GPX04, as detailed under “Materials and Methods.” The cloned GPX promoter was sequenced and compared with a published sequence (Accession No. M83094) using a GCG program.

Fig. 3. p53-dependent activation of the GPX promoter. Two luciferase reporter constructs driven by the GPX promoter with (GPX W/p53BS) or without (GPX W/O p53BS) p53 binding site were transiently cotransfected, respectively, with a p53-expressing plasmid or an empty vector as the control. The recipient cells are p53-negative human Saos-2 cells. The luciferase activity was measured. The results were expressed as fold activation ± S.E. derived from three independent transfections and assays, each run in duplicate, after normalization with β-galactosidase activity for transfection efficiency. The vector controls in the absence of p53 were set arbitrarily as 1 to calculate the fold activation.

Constructs

p53 Binding Site-independent Transactivation of the GPX Promoter by p53 Mutants—We next examined whether the GPX promoter was also regulated by p53 mutants. Plasmid DNAs encoding p53 mutant proteins were individually cotransfected with luciferase reporters into Saos-2 cells. They are the p53-143A, p53-175H, p53-248W, p53-273H, p53-281G, five of the most commonly found p53 mutants in human cancers (23, 24), and p53-280T, a dominant negative p53 mutant found in nasopharyngeal carcinomas (42–44). As shown in Fig. 4, top panel, compared with the vector control, some p53 mutants, such as 248W, 273H, and 281G induced a 6-9-fold activation of the GPX promoter. Mutant 143A conferred a 3-fold activation, comparable to wild type p53. Other mutants (175H and 280T) had little effect. Moreover, activation by these p53 mutants was p53 binding site-independent because it can be detected at a comparable level in both p53 site-containing or -deleted luciferase constructs. It is noteworthy that there is no significant difference in β-galactosidase activity when cotransfected with either wild type p53 or various p53 mutants, indicating a similar transfection efficiency (not shown). The results demonstrated that some p53 mutants can also transactivate the GPX promoter and that transactivation was mediated by a downstream sequence from the p53 binding site.
Materials and Methods. Equal amounts of cellular protein (60 μg) were loaded. The location of the p53 protein is indicated by an arrow.

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**p53-dependent Transactivation of the GPX Promoter Was Enhanced by Etoposide, a p53 Activator**—Etoposide has been shown previously to activate p53 in U2-OS cells (23). We therefore examined whether etoposide would induce p53-dependent transactivation of the GPX promoter. As shown in Fig. 5, transactivation of the luciferase reporter driven by the p53 site-containing promoter (GPX/W/p53BS) was induced by etoposide, and a 9-fold induction was achieved at 24 or 48 h. Slight induction (3-fold) of luciferase activity was also seen in the control, the empty vector was used. As shown in Fig. 5, etoposide started to increase 6 h after etoposide treatment, gradually being induced thereafter, and reached a peak at 12–24 h (first five lanes). This is consistent with the results shown in Fig. 5 and clearly demonstrates that expression of the endogenous GPX is regulated by p53.

**Induction of GPX mRNA Expression by Etoposide in p53-positive, but Not p53-negative Cells**—To examine whether endogenous GPX is subjected to p53 regulation, we treated p53-positive U2-OS cells and p53-negative Saos-2 cells with etoposide. As shown in Fig. 6, both cells express a detectable basal level of GPX mRNA with a higher level in U2-OS cells (first and seventh lanes from left). In U2-OS cells, expression of GPX started to increase 6 h after etoposide treatment, gradually being induced thereafter, and reached a peak at 12–24 h (first five lanes). A bigger induction (2.7-fold, sixth lane) was seen at 48 h which, however, might be error-prone because of the decline in mRNA synthesis resulting from cell death. No significant induction of GPX expression was observed in Saos-2 cells up to 24 h (seventh through ninth lanes). This is consistent with the results shown in Fig. 5.

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**Induction of GPX Enzymatic Activity by Etoposide in p53-positive, but Not p53-negative Cells**—Lastly, we examined GPX enzymatic activity after etoposide treatment in U2-OS and Saos-2 cells. As shown in Table I, etoposide caused a 78% increase in GPX activity in U2-OS cells at 24 h after etoposide removal. The groups were statistically different at the p < 0.05 level. In contrast, no increase was measured in the Saos-2 cells.
induces expression of a protective antioxidant enzyme, GPX, which has been shown to protect cells from oxidative damage and apoptosis (49–52). The fact that induction of GPX immediately followed p53 activation (Figs. 1, 5, and 6) suggests that p53 may induce GPX expression at an early stage of apoptosis. It is known that p53-induced ROS generation is a rather later event (48). Thus, p53 may regulate cellular redox status in a time-dependent manner: it increases antioxidant synthesis at an early stage followed by an increase in ROS generation.

The regulation of GPX expression is rather complex and is controlled at several levels. First, GPX is expressed differentially in a wide range of tissues (53). Second, GPX is subjected to developmental and hormonal regulation (54–56). Third, GPX activity can be induced by oxygen tension, diet, and some xenobiotics (25, 54, 57) but is inactivated by superoxide (58) and nitric oxide (59). A 3.0-kilobase promoter fragment of the GPX gene has been cloned and characterized. Three possible cis-acting regulatory regions as well as oxygen-responsive elements were defined (40, 60). We showed here that a 262-bp fragment located immediately upstream from the translational initiation site of GPX has strong promoter activity and that p53 positively regulates this promoter fragment. Our findings, along with a recent identification of GPX as a p53-responsive gene in a DNA microarray screening experiment by others (61), indicated that in addition to those listed above which regulated GPX, p53 also positively regulates GPX. Transactivation of GPX by p53 links the p53 signaling pathway to the antioxidant pathway. Because p53 is activated by DNA-damaging agents, this finding would implicate a role of antioxidant enzymes in the cellular response not only to oxidative stress, but also to DNA damage.

p53 mutations have been found to be the most common genetic alteration in human cancers, and the majority of p53 mutations were clustered in the DNA binding domain (24). p53 mutants either gain oncogenic function, lose tumor suppressor function, or function dominant negatively to suppress wild type function (43). Most p53 mutants lose activity to bind to a p53 consensus binding site as well as lose transcriptional activity (62, 63). Intriguingly, we showed here that several p53 mutants commonly found in human cancers also transactivate the GPX promoter, and transactivation is, however, independent of the p53 binding site. Some p53 mutants confer a 9-fold activation of the GPX promoter in a p53 binding site-dependent manner. Third, p53 binding and transactivation of the GPX promoter were enhanced by etoposide, a p53 activator. Fourth, etoposide/p53-induced transactivation of the GPX promoter can be blocked by a dominant negative p53 mutant. Fifth, expression of endogenous GPX was induced significantly by etoposide only in p53-positive U2-OS cells but not in p53-negative Saos-2 cells. In addition, some of the p53 mutants commonly found in human cancers also transactivate the GPX promoter, but in a p53 binding site-independent manner.

p53, after being activated by DNA-damaging reagents, has been shown either to induce G1 growth arrest or apoptosis (5, 6). The p53 target genes that mediate or associate with p53-induced apoptosis include Bax (45), Fas/APO1 (46), KILLER/DR5 (47) as well as those involving generation of ROS (48). We have previously shown that etoposide could activate p53 and subsequently induce apoptosis in U2-OS cells (23, 32). Now we have identified and characterized that GPX, an antioxidant enzyme, is also induced by p53. It appears paradoxical that p53, on one hand, induces genes responsible for ROS generation, which mediates apoptosis (48), and on the other hand, induces expression of a protective antioxidant enzyme, GPX, which has been shown to protect cells from oxidative damage and apoptosis (49–52). The fact that induction of GPX immediately followed p53 activation (Figs. 1, 5, and 6) suggests that p53 may induce GPX expression at an early stage of apoptosis. It is known that p53-induced ROS generation is a rather later event (48). Thus, p53 may regulate cellular redox status in a time-dependent manner: it increases antioxidant synthesis at an early stage followed by an increase in ROS generation.

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The biological importance of GPX regulation by p53 mutants is not clear at the present time. However, it has been observed that elevated GSH or GPX is associated with acquired drug resistance (67–70), and some human cancers with an increased GPX expression are more resistant to chemotherapy (71, 72). Because cancer cells with p53 mutations are often more resistant to chemotherapeutic drugs (73), GPX could mediate drug resistance conferred by some p53 mutants. Moreover, the activity of GPX is altered in many human cancers, being either elevated or decreased (31). It will be of interest to correlate GPX activity with p53 mutations in these cancers. Upon establishment of such a correlation, the p53 mutation as well as GPX level may serve as an index for potential drug resistance.
