p53 Suppresses the Nrf2-dependent Transcription of Antioxidant Response Genes*

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Cells respond to the shift of intracellular environment toward pro-oxidant conditions by activating the transcription of numerous "antioxidant" genes. This response is based on the activation of the Nrf2 transcription factor, which transactivates the genes containing in their promoters the antioxidant response cis-elements (AREs). If the oxidative stress provokes DNA damage, a second response of the cell takes place, based on the activation of p53, which induces cell cycle arrest and/or apoptosis. Here we have explored the cross-talk between these two regulatory mechanisms. The results show that p53 counteracts the Nrf2-induced transcription of three ARE-containing promoters of the x-CT, NQO1, and GST-α1 genes. Endogenous transcripts of these antioxidant genes accumulate as a consequence of Nrf2 overexpression or exposure to electrophile diethylmaleate, but these effects are again blocked by p53 overexpression or endogenous p53 activation. Chromatin immunoprecipitation experiments support the hypothesis that this p53-dependent trans-repression is due to the direct interaction of p53 with the ARE-containing promoters. Considering that p53-induced apoptosis requires an accumulation of reactive oxygen species, this negative control on the Nrf2 transactivation appears to be aimed to prevent the generation of a strong antioxidant intracellular environment that could hinder the induction of apoptosis.

The shift of the intracellular environment toward pro-oxidant conditions, due to the accumulation of reactive oxygen species (ROS) or to other electrophilic insults, induces a prompt response of the cells. One of the aims of this response is of course that of preventing the possible harmful effects of the oxidative stress, through the scavenging of the ROS, before they reach concentrations sufficient to induce oxidative damages to cellular molecules, mainly to DNA. To do this, cells activate the transcription of more than 200 genes encoding "antioxidant proteins" (for a review, see Ref. 1). These proteins include, for example, those involved in the generation and metabolism of GSH, a very effective scavenger of ROS and electrophiles, such as heavy and light chains of γ-glutamylcysteine synthetase (2), the x-CT (subunit of the cystine/glutamate transporter) component of the cystine/glutamate exchange transport system (3), the glutathione S-transferases (GSTs), and the glutathione peroxidase (4, 5).

The expression of most of these antioxidant proteins is regulated through the interaction of the NF-E2-related factor (Nrf2) transcription factor with a cis-element present, often in multiple copies, in their cognate gene promoters, named ARE (antioxidant response element). Nrf2 is a potent transactivator, which is regulated through different mechanisms. The most studied mechanism concerns the regulation of the nuclear availability of Nrf2 by Keap1. Early results supported the hypothesis that Keap1 functions as an extranuclear anchor site for Nrf2, and more recently, experimental evidence suggested that Keap1 promotes Nrf2 degradation. In fact, Keap1 directly binds Nrf2 and the actin cytoskeleton, thus sequestering Nrf2 in the cytoplasm (6) and, on the other hand, it controls Nrf2 ubiquitination, which marks this protein for degradation by the 26 S proteasome (7, 8). Upon the addition of electrophiles, the Nrf2-Keap1 complex dissociates, the Keap1-dependent ubiquitination of Nrf2 is blocked (9), and Nrf2 accumulates into the nucleus, where it forms transcriptionally active complexes on the AREs (10).

In parallel with the described "antioxidant approach," cells counteract the consequences of an oxidative stress by attempting to repair the ROS- and/or electrophile-induced damages. A key role in the cellular response is played by p53. This is a transcription factor activated by the DNA damage, which regulates the expression of many target genes, leading to cell cycle arrest aimed to allow time for the repair of DNA damage (for a review, see Ref. 11). On the other hand, p53 also has a fundamental role in the induction of apoptosis of cells where DNA damage remains un repaired.

The apoptosis induced by p53 appears to be at least in part dependent on the accumulation of ROS (12). In fact, a subset of the genes regulated by p53, named PIGs (p53-induced genes),

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‡ The abbreviations used are: ROS, reactive oxygen species; ARE, antioxidant response element; GST, glutathione S-transferase; DEM, diethylmaleate; PM5F, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; WT, wild type; FACS, fluorescence-activated cell sorter.
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Encodes proteins that could collectively induce an increase in ROS concentration (12). The crucial role of the p53-dependent ROS accumulation is supported by several observations; for example, antioxidants, such as N-acetylcysteine, diphenyleneiodonium chloride, and pyrrolidine dithiocarbamate, counteract ROS accumulation caused by p53 and, at the same time, prevent p53-induced apoptosis (12, 13).

The possible coexistence of the Nrf2-mediated antioxidant response with the regulation of apoptosis by p53 raises an apparent paradox. At least in principle, the induction of a very efficient ROS scavenging machinery by the Nrf2-dependent response could hamper, or at least interfere with, the p53-induced apoptosis that on the contrary requires the accumulation of ROS. Therefore, it is expected that a cross-talk between the Nrf2- and p53-induced responses should exist.

Here we demonstrate that p53 suppresses the Nrf2-dependent transcription of ARE-containing promoters. This result is achieved by a direct inhibitory effect of p53 on these promoters.

**EXPERIMENTAL PROCEDURES**

**Cells and Culture Conditions**—The mouse hepatocarcinoma cell line Hepa1–6 was purchased from the American Type Culture Collection (ATCC, CRL-830, Manassas, VA) and was grown in Dulbecco’s modified essential medium (Invitrogen) containing 10% fetal bovine serum (Cambrex, Bio Science Vierviers, Belgium). Human lung carcinoma Calu-6 cell line (ATCC, HTB-56; ICLC, HTL97003) was cultured in Dulbecco’s modified essential medium containing 10% fetal bovine serum with 2 mM l-glutamine and 0.1 mM non-essential amino acids (Invitrogen). Human osteosarcoma cell line Saos-2 (ATCC, HTB-85) and HEK293 cells were grown in Dulbecco’s modified essential medium containing 10% fetal bovine serum. HCT116 cells (p53WT and p53−/−) were kindly provided by G. Blandino and were grown in Dulbecco’s modified essential medium supplemented with 10% fetal bovine serum. All the media were supplemented with penicillin 10 units/ml and streptomycin 10 mg/ml at 37 °C under 5% CO2 atmosphere. Before each experiment, cells were subcultured at a density of 3 × 10⁶/100-mm diameter dish in complete medium and incubated overnight at 37 °C. Diethylmaleate (DEM), etoposide, and cisplatin (Sigma) were used at the indicated concentrations.

**Plasmids and Transfections**—The expression vector for FLAG-Nrf2 was constructed by inserting into the EcoRV/BamHI-digested p3XFLAG-CMV 7-1 plasmid (Sigma) the cDNA encoding human Nrf2 (I.M.A.G.E.: 4548874). The insert was subcloned in-frame with FLAG epitope at the N terminus. The expression vector for p53 (pCMV-p53) contains the human wild-type full-length cDNAs inserted in pCMV-neo and was described previously (14).

The promoter region of mouse x-CT gene from −235 to +15 and of the mouse glutathione S-transferase-α1 (GST-α1) from −940 to +41 were isolated by PCR from genomic DNA from NIH3T3 cells (15). These fragments were cloned into PGL3 Basic vector (Promega, Madison, WI). All the deletion constructs were made by PCR using x-CT (−235 to +15) promoter as template and cloned in PGL3 Basic vector. For the constructs mutΔ-48-31, mutΔ-33-19, and mutΔ-48-19, the AREs-containing upstream region was amplified by PCR and ligated to double-stranded oligonucleotides designed on the basis of the promoter sequence from −31 to +15 or from −19 to +15. The fragments were cloned in PGL3 Basic vector. The promoter region of human NAD(P)H quinone oxidoreductase (NQO1) gene (from −923 to +111) (16) was amplified by PCR from genomic DNA prepared from HEK293 using the following primers (forward, 5′-TCCGGGTCAAGCGATTCTCTCTGGCCTCACGTAATTTTGT-3′, and reverse, 5′-GGCTCTGGTGCAGTCCGGGGCTCAGTGGGACTTTGAC-3′) and then cloned in PGL3 Basic vector. All plasmid constructs were sequenced.

For transient transfections of luciferase constructs, cells were seeded in 6-well dishes 24 h before experiments, and each transfection was performed in triplicate. Cells were co-transfected by using the calcium phosphate method with reporter plasmids (2 μg) and/or p53 and/or Nrf2 expression vectors (0.15 μg of each) in the presence of 0.2 μg of pRLSV40 encoding Renilla luciferase (Promega). DNA concentration was kept constant with empty vector. After overnight incubation with the precipitation reaction mixture, the medium was changed and, where indicated, the cells were treated with etoposide, cisplatin, or DEM. 36 h after the transfection, cells were harvested and analyzed for luciferase activity. The luciferase assay was performed with a Dual-Luciferase reporter assay system (Promega) according to the manufacturer’s instructions. The firefly luciferase activity was normalized to Renilla internal control luminescence. The Lipofectamine Plus transfection reagent (Invitrogen) was used to perform the transfections in human HEK293 and Calu-6 following the suggestions of manufacturer’s procedures.

**RNA Preparation and Quantitative Real-time Reverse Transcription-PCR**—Total RNA was isolated by using the Qiagen total RNA isolation kit (Qiagen, Valencia, CA). For quantitative real-time PCR, cDNAs were synthesized in a Gene AMP PCR system 9700 from 1 μg of total RNA in a 20-μl reaction containing 1× reverse transcriptase buffer with 5 mM MgCl₂, 10 mM 1.4-dithiothreitol, 5 mM random exonmers, 1 mM dNTP, 1 unit/ml RNase inhibitor, and 10 units/ml reverse transcriptase (Moloney murine leukemia virus reverse transcriptase, Invitrogen). The reaction was incubated at 70 °C for 10 min and then at 25 °C for 10 min followed by 42 °C for 45 min and 99 °C for 3 min. Aliquots of cDNA (10 μl of reverse transcription reactions) were used in real-time PCRs. SYBR Green-based real-time PCR was used to determine cDNA levels. Aliquots of cDNA were amplified in an iCycler iQ real-time PCR detection system (Bio-Rad) using IQTM SYBR Green Supermix in triplicate in 25-μl reaction volumes. The sequences of the primer pairs used with mouse samples were: c-Abi (oncogene homologue of abelson murine leukemia virus, used as the internal control), 5′-GGTATGAAAGGAGGTGTGACA-3′ and 5′-GGTGAATACACTGACGAGTGTGTA-3′; Gadd45, 5′-AGACCCCGGACCTGCAC-3′ and 5′-CCGGCAAACAAATTAAGTTGACT-3′; GST-α1, 5′-CAGGGTGCTCTAGGTCGA-3′ and 5′-GGTCTGCGCCAGCTTCA-3′; NQO1, 5′-CCCTCA-ACATCTTGAGCCCAT-3′ and 5′-GGCTAGTGTGAATGTTGCTCTTCTGTA-3′. The sequences of the primer pairs used with human samples were: c-Abi, 5′-TGGGATATACACTCTGAAGCTATACTAAAGGT-3′ and 5′-GATGATGTCTTGGGACC-
CA-3'; NQO1, 5'-GATATTCGGAGTCCCTATGTTG3' and 5'-AAAGCCTGCTTTGTCATGGGG3'; Gadd45, 5'-AGACCCGACCTGCTTCGAC-3' and 5'-CCGGCAAACAAGATGTGAC-3'.  PCR cycling conditions were: 95 °C for 30 s and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.  Expression levels were calculated relative to c-Abl mRNA levels as endogenous control.  Relative expression was calculated as 2^ΔΔCt (17).

Nuclear Protein Preparation and Western blot Analysis—For nuclear protein preparation, cells were treated with the indicated amounts of DEM or with Me2SO (at the same concentration as the DEM solution) for 1 h, washed twice with cold PBS, solubilized at 4 °C with 300 μl of lysis buffer (10 mm Hepes, 60 mm KCl, 1 mm EDTA, 1 mm 1.4-dithiothreitol, 1 mm phenylmethylsulfonyl fluoride (PMSF), 1 mm Na3VO4, 0.2% Nonidet P-40, and 10 μg/ml aprotinin, leupeptin and pepstatin), and centrifuged at 2500 rpm for 5 min at 4 °C.  The nuclear pellet was washed with lysis buffer without Nonidet P-40 and resuspended in 300 μl of the same buffer.  This volume was added to 300 μl of lysis buffer with 30% saccharose and without Nonidet P-40 and centrifuged at 6000 rpm for 15 min at 4 °C.  Nuclear pellet was resuspended in 50 μl of a buffer containing 250 mm Tris-HCl, pH 8.0, 60 mm NaCl, 1 mm EDTA, 1 mm 1.4-dithiothreitol, 1 mm Na3VO4, 1 mm PMSF, and 10 mg/ml aprotinin, leupeptin and pepstatin.  Nuclei were lysed with three cycles freezing (−80 °C)/defrosting (37 °C).  Finally, nuclear extracts were obtained by centrifugation at 9500 rpm for 10 min at 4 °C.  15 μl of supernatant from each sample were heated at 95 °C for 5 min, separated by electrophoresis on 10% SDS-polyacrylamide, and transferred to Immobilon-P transfer membranes (Millipore). Membranes were washed and blocked with 5% nonfat dry milk in PBS containing 0.1% Tween 20 at room temperature for 1 h.  Nrf1 and Nrf2 levels were analyzed by Western blotting using 1 μg/ml anti-Nrf1 and anti-Nrf2 rabbit polyclonal antibodies (H-285 and H-300, respectively; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at room temperature for 2 h.  After incubation with rabbit horseradish peroxidase-conjugated secondary antibody at room temperature for 30 min, the blots were developed using enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden).

For Western blot on total extracts, the cells were washed with cold PBS and solubilized at 4 °C for 30 min in lysis buffer containing phosphate buffer, 0.1 mm EDTA, 1 mm Na3VO4, 50 mm NaF, 0.5% Nonidet P-40, 1 mm PMSF, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin.  The cell lysates were cleared by centrifugation at 4000 rpm for 10 min at 4 °C.  Western blots were performed as described above with antibodies against FLAG (FLAG M2 from Sigma), p53 (rabbit polyclonal FL-393), and mouse monoclonal DO-1 (from Santa Cruz Biotechnology), pATM (from Cell Signaling Technology, Inc, Beverly, MA), and tubulin (from Santa Cruz Biotechnology), and the signals were detected by using the ECL kit (Amersham Biosciences).

Chromatin Immunoprecipitation Assay—Hepa1–6 and Calu-6 cells were grown in 100-mm dishes; Calu-6 cells were transfected with FLAG-Nrf2 or pCMV p53 and/or with the deletion mutants of the x-CT gene promoter by using the Lipofectamine transfection reagent.  24 h after transfection, the cells were fixed using 1% formaldehyde in medium for 10 min at room temperature.  The cells were treated with 0.125 m glycine for 5 min to stop fixation and washed twice with ice-cold PBS.  The cells were scraped in PBS, collected by centrifugation, and lysed in lysis buffer (5 mm Pipes, pH 8.0, 85 mm KCl, 0.5% Nonidet P-40, 1 mm PMSF supplemented with protease inhibitors).  After centrifugation, nuclear pellet was resuspended in nuclear lysis buffer (50 mm Tris-HCl, pH 8.0, 10 mm EDTA, 0.8% SDS, 1 mm PMSF supplemented with protease inhibitors) and sonicated for four cycles (30-s pulse and 30-s rest on ice).  The sonication conditions were optimized to determine generation of DNA fragments between 500 and 1000 base pairs in length.  Sheared chromatin was diluted to 8 ml, and aliquots of 300 μl were incu
RESULTS

p53 Overexpression Interferes with the Nrf2-mediated Induction of ARE-containing Gene Promoters—The x-CT gene transcription is activated by the electrophilic agent DEM, through a mechanism based on the recruitment of Nrf2 transcription factor on the four AREs present in the 235 bp upstream of the transcription start site (3) (Fig. 1A). As expected, transient transfection of Nrf2-encoding vector in Hepa cells induced the activation of the x-CT gene promoter. A similar effect was observed in cells exposed to DEM, an electrophile that also provokes an accumulation of ROS by inducing GSH depletion. However, the exposure to DEM of cells previously transfected with FLAG-Nrf2 led to a significant decreased induction of the promoter (Fig. 1A). This phenomenon was observed in various cell lines but not in Calu and Saos cells, in which the exposure to DEM did not modify the response of the promoter to the transfection of Nrf2 (Fig. 1A). The most striking difference between Calu and Saos and the other cell lines tested is that both Calu and Saos are p53-null (19, 20). Therefore, we explored whether the overexpression of p53 does affect the Nrf2-dependent activation of the x-CT gene promoter. As shown in Fig. 1B, the co-transfection of Nrf2 with increasing amounts of a p53-encoding vector led us observe a dose-dependent inhibition of the Nrf2-dependent activation. This phenomenon was dramatic in p53-deficient cells Calu and Saos, in which the transfection of a very low amount of the p53 vector completely abolished the transcription from the x-CT gene promoter (Fig. 1B). To exclude that genetic differences, other than the absence of active p53, among Hepa, Calu, and Saos cells were responsible for their different behaviors, we performed the same experiment reported in Fig. 1A in HCT116 cells in which WT p53 is expressed and in isogenic HCT116 cells in which p53 gene is inactivated by gene targeting (21). As shown in Fig. 1C, only in the WT HCT cells and not in the p53−/− counterpart, DEM treatment significantly decreases the Nrf2-dependent induction of the x-CT gene promoter. We then explored whether p53 overexpression also suppresses the Nrf2-mediated activation of other ARE-containing promoters, namely those of GST-a1 and NQO1 genes (22, 23) (Fig. 2, A and B).

To evaluate the effects of Nrf2 and p53 on the transcription of endogenous x-CT and NQO1 genes, HEK293 cells were transfected with Nrf2 alone or with Nrf2 and p53, and the mRNA levels of these genes were measured by real-time PCR. As shown in Fig. 3, Nrf2 overexpression increased the x-CT and NQO1 mRNA levels about 3-fold, and the co-expression of p53 almost completely abolished the effects of Nrf2. Gadd45 mRNA was measured as a control and, as expected, its levels were not affected by Nrf2. On the contrary, p53 induced the accumulation of Gadd45 mRNA, and this induction was not affected by Nrf2.

DNA Damage Counteracts the Nrf2-mediated Antioxidant Response through a p53-dependent Mechanism—The results reported in Fig. 1 can be explained by hypothesizing that ROS accumulation, induced by DEM treatment, provokes the DNA damage-dependent activation of p53. Thus, we asked whether

50 mM KCl, 10 mM MgCl₂, 18 mM CaCl₂, and 10 µl of propidium iodide. FACS analysis was performed with standard protocols.
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![Graphs and images showing luciferase activity and Western blots for GST-α1-Luc and NQO1-Luc constructs in Hepa and Calu cells with and without p53 and DEM treatments.](image)

**FIGURE 2.** p53 suppresses the Nrf2-dependent activation of the GST-α1 and NQO1 gene promoters. A, Hepa and Calu cells were transfected with GST-α1-Luc construct in which the luciferase gene is under the control of 940 bp upstream of the transcription start site of the mouse GST-α1 gene. They were also transfected with Nrf2 expression vector and/or with 0.125 μg of p53 vector or with an equivalent amount of empty vector. 30 h after the transfection, the cells were treated for 3 h with 200 μM DEM in MeSO or with MeSO as indicated. Luciferase activity was measured as described under “Experimental Procedures.” B, Hepa and Calu cells were transfected and treated, as described in panel A, with NQO1-Luc construct in which the luciferase gene is under the control of 923 bp upstream of the transcription start site of the human NQO1 gene. All the values are reported as the mean relative luciferase activities of triplicate experiments, setting as 1 the values obtained in control experiments. S.D. is reported. The Western blots report the amounts of FLAG-Nrf2 and of p53 in a typical experiment. α-Tubulin Western blots were made as a control of loading.

![Graph showing relative mRNA levels for x-CT, NQO1, and GADD45 in Hepa and Calu cells with and without p53 and DEM treatments.](image)

**FIGURE 3.** p53 suppresses the Nrf2-dependent accumulation of the x-CT and NQO1 mRNAs. HEK293 cells were transfected with FLAG-Nrf2 expression vector and/or with 1 μg of p53 vector or with an equivalent amount of empty vector. Total RNA was prepared 36 h after transfection and used for real-time PCR measurement of the indicated mRNAs as described under “Experimental Procedures.” All the values are reported as the mean relative mRNA abundance in triplicate experiments, setting as 1 the values obtained in control experiments. The S.D. is reported. The Western blots of FLAG-Nrf2 and of p53 are reported in the inset.

Taken together, these results suggest an articulate response of the cells to ROS; the activation of p53 counteracts the Nrf2-mediated activation of antioxidant response genes. To get results not biased by the overexpression of Nrf2, we treated Hepa cells with etoposide to induce the activation of p53, and then the same cells were exposed for 1 h to DEM to induce the Nrf2-dependent antioxidant response. In these conditions, p53 was activated by etoposide, as demonstrated by the accumulation of the Gadd45 mRNA (Fig. 5A) and of p53 itself (Fig. 5C), and DEM induces a significant accumulation of Nrf2 in the nucleus (Fig. 5D). In these cells, we first explored the transcription from the x-CT gene promoter. The results showed that whereas DEM activated the transcription of the reporter gene, etoposide treatment had no effect. On the contrary, the activation of the transcription induced by DEM was significantly decreased in cells previously exposed to etoposide (Fig. 5A). The response of the endogenous genes was explored in the same experimental conditions. Both x-CT and NQO1 mRNAs were induced by DEM treatment, but the pretreatment with etoposide significantly suppressed the response to DEM. On the contrary, etoposide had no effects in Calu cells both on the transcription of the reporter construct and on the endogenous levels of the x-CT mRNA (Fig. 5B), thus confirming the role of p53 in the observed phenomena.

We then explored the behavior of isogenic HCT(p53WT) and HCT(p53−/−) cells exposed to cisplatin. As observed in Hepa cells treated with etoposide, cisplatin induced the activation of p53 in HCT(p53WT) (Fig. 5G), and DEM treatment resulted in the nuclear translocation of Nrf2 in both HCT(p53WT) and HCT(p53−/−) cells (Fig. 5H). In these con-
ditions, the transcription of the x-CT-Luc reporter and the accumulation of endogenous x-CT mRNA, induced by DEM, were inhibited only in HCT(p53WT) and not in HCT(p53−/−) cells (Fig. 5, E and F).

p53 Suppresses the Nrf2-dependent Transactivation by Interacting with and Inhibiting the x-CT Gene Promoter—The mechanism underlying the observations reported above could be based on the direct interaction of p53 with the promoters we examined. To address this possibility, we explored the possible association of this protein with the x-CT gene. Fig. 6A shows that, in Calu cells transfected with FLAG-Nrf2, the x-CT promoter chromatin is immunoprecipitated with anti-FLAG antibodies. The same chromatin region is also immunoprecipitated with anti-p53 antibody in cells transfected with p53, thus indicating that this region interacts with the transcription factor. When the cells are co-transfected with both Nrf2 and p53, the chromatin associated with p53, whereas only a barely detectable signal was observed with the anti-FLAG antibody.

To identify the region(s) of the x-CT gene promoter involved in the interaction with p53, we generated several deletion constructs of the promoter and analyzed their interaction with transfected p53 in Calu cells. Fig. 6B shows that the constructs
lacking the AREs and the AP1 sites present in the promoter are still able to interact with p53, whereas the constructs lacking the region upstream of the transcription start site (−33−19), which also contains the TATA box, did not. No evident p53 cis-elements are present in this region.

To study the association of endogenous Nrf2 and p53 to the x-CT gene promoter, we performed chromatin immunoprecipitation experiments in Hepa cells, exposed or not to DEM. This experiment allowed us to observe that also, in basal conditions, p53 is present in chromatinized complexes associated with the x-CT gene. In cells treated with DEM, the amount of chromatin associated with endogenous Nrf2 clearly increased, whereas that immunoprecipitated with anti-p53 antibody slightly decreased (Fig. 6C).

p53 Deficiency Reduces the Sensitivity of the Cells to DEM-induced Apoptosis—The observation that the antioxidant response of the cells mediated by Nrf2 is down-modulated by p53 suggests that in the absence of this p53-dependent regulation, the sensitivity of the cells to ROS-induced apoptosis should be decreased. To examine this possibility, we measured the apoptosis induced by increasing concentrations of DEM in HCT(p53WT) and HCT(p53−/−) cells. As shown in Fig. 7, the absence of p53 strongly decreased the sensitivity of the HCT cells to the oxidative stress.

DISCUSSION

The cells respond to the accumulation of ROS in two ways. The first one is aimed at strengthening the antioxidant scavenging systems to dispose of the excess of ROS and is based on the activation of ARE-containing “antioxidant genes” by the transcription factor Nrf2. With the second one, the cells respond to the ROS-induced DNA damages by blocking cell cycle progression and, in the presence of unreparable damages, by inducing cell apoptosis. p53 is the main transcriptional mediator of this pathway. In this study, we addressed whether these two responses to the oxidative stress are integrated. We found that the Nrf2-dependent activation of antioxidant genes, such as x-CT, GST-α1, and NQO1, is down-modulated by p53. These
results demonstrate the existence of a cross-talk between the two mechanisms.

The involvement of p53 in the regulation of the redox homeostasis has been suggested by numerous results. It, in fact, activates the transcription of several genes whose products have either pro-oxidant or anti-oxidant functions (12, 24–27). Furthermore, low levels of p53 appear to be associated with the activation of several antioxidant genes and with the consequent decrease of intracellular ROS (28). On the contrary, DNA damage-dependent activation of p53 induces a significant accumulation of ROS that is crucial for the p53-dependent apoptosis (12, 13). Our findings indicate the existence of a new mechanism through which p53 affects intracellular redox conditions.

p53 could regulate the ARE-containing promoters by modifying the expression of key proteins that control these genes. Nrf2 and Keap1 expression is not affected by p53 overexpression or by the activation of endogenous p53 induced by DNA damage (data not shown). On the contrary, our results suggest that p53 could regulate these genes by functioning as a transcription repressor. There are numerous mechanisms that have been proposed for the p53-dependent trans-repression (for a review, see Ref. 29), but this point remains to be definitively addressed. Among the various genes whose expression is suppressed by p53, one class of genes encodes proteins regulated in the G1/M phase of the cell cycle (30–33). p53 is stably associated with these promoters in the absence of recognizable p53 cis-elements by using the CCAAT box-binding protein nuclear factor Y as an anchor site. Upon DNA damage, p53 is acetylated, and this modification probably unmasks its ability to repress the transcription of the associated genes (34). Therefore, in these cases, p53 targets the promoters containing the CCAAT box. The three promoters we examined do not contain any recognizable CCAAT boxes; thus, it is unlikely that p53 affects their activity through the nuclear factor Y-dependent mechanism. Similarly, these promoters do not contain any obvious p53 cis-elements. However, in the case of the x-CT gene promoter, our results show that p53 is stably associated in chromatinized complexes also in the absence of DNA damage, as already demonstrated for other gene promoters (35). The dissection of the x-CT gene promoter allowed us to demonstrate that the region involved in the interaction of p53 is located just upstream of the transcription start site, where no obvious p53 cis-elements are present (Fig. 6B). This means that p53 could directly interact with atypical DNA cis-elements or, indirectly, through still unidentified DNA-binding proteins.

In cells not exposed to DNA-damaging agents, p53 bound to these promoters does not seem to affect the basal levels of transcription. In fact, when compared with the efficiency of constitutively active promoters, such as that of cytomegalovirus, the basal transcription efficiency of the three promoters we studied is very similar in all the cell lines used, regardless the p53 genotype. This suggests that, as in the case of the CAA1T-containing promoters mentioned above, p53 represses these promoters only upon regulatory events, e.g. phosphorylation and/or acetylation, induced by the DNA damage.

The results reported in Fig. 6A apparently suggest that p53 represses the transcription by displacing Nrf2 bound to the x-CT gene promoter. However, the chromatin immunoprecipitation experiments exploring the endogenous proteins (Fig. 6C) do not support this hypothesis. In fact, endogenous p53 was found to be associated with the promoter in basal conditions. Furthermore, the interaction of Nrf2 with the promoter, induced by the oxidative stress, appears to be compatible with the contemporary occupancy of the promoter by p53. These results support the speculation that the p53-dependent trans-repression is due to the activation of p53, perhaps as a consequence of its phosphorylation and/or acetylation and not to the recruitment of the protein on the promoter. The results that emerged from the dissection of the x-CT gene promoter, indicating that the region involved in the interaction with p53 also contains the TATA box (Fig. 6B), support the possibility that activated p53 could act by interfering with the assembly of basal transcription machinery. However, other possibilities cannot be excluded, such as the recruitment of histone deacetylase or of other chromatin remodeling factors by activated p53.

The findings we report in this study gave further support to the hypothesis that the Nrf2-Keap1-based antioxidant machinery could have a relevant role in cancer. It was recently found that genetic variants of the Keap1 gene are associated with lung cancer (18). The amino acids affected by these mutations lie in the region involved in the binding of Keap1 to Nrf2; thus, in the examined cancer cells, Keap1 function is defective, and this results in a constitutive activation of Nrf2. How this phenomenon contributes to the development of the neoplastic phenotype is still not clear. However, the observation that p53, among its various functions, is also responsible for the suppression of the Nrf2 activity further suggests that the antioxidant response of the cell is taken under a strict control because of its possible harmful consequences.

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