**DUSP1** Is Controlled by p53 during the Cellular Response to Oxidative Stress

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Abstract

p53 controls the cellular response to genotoxic stress through multiple mechanisms. We report here that p53 regulates **DUSP1**, a dual-specific threonine and tyrosine phosphatase with stringent substrate specificity for mitogen-activated protein kinase (MAPK). **DUSP1** is a potent inhibitor of MAPK activity through dephosphorylation of MAPK. In a colon cancer cell line containing inducible ectopic p53, **DUSP1** protein level is significantly increased upon activation of p53, leading to cell death in response to nutritional stress. In mouse embryonic fibroblast cells, **DUSP1** protein abundance is greatly increased after oxidative stress in a p53-dependent manner and also when apoptosis is triggered. We show that p53 induces the activity of a human **DUSP1** regulatory region. Furthermore, p53 can physically interact with the **DUSP1** regulatory region in vivo, and p53 binds to a 10-bp perfect palindromic site in this **DUSP1** regulatory region. We show that overexpression of **DUSP1** or inhibition of MAPK activity significantly increases cellular susceptibility to oxidative damage. These findings indicate that p53 is a transcriptional regulator of **DUSP1** in stress responses. Our results reveal a mechanism whereby p53 selectively regulates target genes and suggest a way in which subgroups of those target genes might be controlled independently. (Mol Cancer Res 2008;6(4):624–33)

Introduction

p53 is a tumor suppressor that regulates cell cycle progression and the programmed cell death response to DNA damage (1, 2). The protein functions primarily as a transcription factor, targeting and activating multiple downstream genes that ultimately regulate these pathways. Genes controlled by p53 contain DNA consensus recognition sites primarily in their promoters. These sites are bound with specificity by the p53 protein, leading to the transcriptional control of these target genes (3). For example, p21, a gene critical for progression of cells from the G1-S phase of the cell cycle, was identified early on as a target for p53 (4), and the precise promoter sequence bound by p53 was also defined (4). Subsequently, many other genes have been identified within the p53 control network (1, 2), and a p21-like consensus sequence has usually been identified within their promoter regions or introns (5-8). However, at least one target gene, *PAC1*, contains an unorthodox yet effective promoter consensus sequence bound and used by p53 (9). In addition, **DUSP1** has a p53 binding site within an intron and an exon that can regulate transcription of the gene (10, 11).

The mitogen-activated protein kinase (MAPK) cascade is a predominant pathway for cell growth and proliferation. MAPKs can be grouped into three families: extracellular signal-regulated kinase (ERK), c-Jun-NH2 kinase, and p38 (12). The activity of MAPKs is regulated by dual phosphorylation on their tyrosine and threonine residues. MAPKs are activated via phosphorylation on threonine and tyrosine and are inactivated by a unique family of dual specificity phosphatases, MAPK phosphatases, which are encoded by immediate early genes and induced in response to environmental stressors and growth factor stimulation. **DUSP1** is a phosphatase, which has also been called CL100, MKP1, 3CH134, or Erp. **DUSP1** was originally cloned as CL100 by subtration hybridization using cells treated with hydrogen peroxide (13, 14). **DUSP1** encodes a dual threonine/tyrosine phosphatase that specifically dephosphorylates and inactivates MAPKs (15). Upon activation, MAPKs, including ERK1 and ERK2, translocate into the nucleus where they phosphorylate transduction targets, including transcription factors (16). MAPKs are activated upon dual phosphorylation by **MAPK kinase**, which in turn is activated by the Ras/Raf pathway (17). In contrast, MAPKs are inactivated upon dephosphorylation of tyrosine and threonine residues by MAPK phosphatases, including **DUSP1**, **MKP2**, and **PAC1** (18, 19).

There is evidence that **DUSP1** is controlled at the transcriptional level by p53 during cell cycle progression (10). However, conditions relevant for the induction of one **DUSP1**-mediated function in vivo versus another are not yet well defined. In this report, we show that **DUSP1** is a transcriptional target of p53 in signaling apoptosis after...
oxidative stress. Furthermore, we identify a palindromic sequence in the promoter region of DUSP1 that binds p53 and can mediate DUSP1 expression. The effect of this finding in the context of the cellular response to stress is presented.

Results
Selective Regulation of DUSP1 by p53 under Stress Conditions

A wide variety of mutagenic agents are toxic to cells, but the mechanisms of toxicity can vary. Some agents irreversibly induce growth arrest, whereas others induce cell death by apoptosis or necrosis. For example, γ radiation causes cell cycle arrest in mouse embryo fibroblasts (MEF) in a p53-dependent manner (20), whereas oxidative stress induces cell death by apoptosis in the same type of mouse cells and that also depends on p53 (21). Similarly, we have observed that normal human fibroblasts undergo G1 arrest in response to radiation-induced DNA damage, but cell death after oxidative stress was induced by H2O2 (22). The molecular basis for differential regulation of cell cycle arrest versus cell death by p53 is not known. One possibility is that p53 mediates distinct stress responses by differentially regulating its target genes. To address this, our first approach was to identify p53 target genes involved in the p53-mediated apoptotic process.

Using gene expression microarray screening, we previously identified >60 p53-regulated genes, including DUSP1 (23). DUSP1 functions to inactivate ERK1 and ERK2 kinase activity through dephosphorylation of ERK1/2 (18). We used the EB-1 system to confirm the data from the microarray. The EB cell line was derived from a human colon cancer with mutant p53. EB-1 is a stable clone containing a wild-type (wt) p53 transgene under control of the metallothionein promoter and expressing wt p53 upon administration of zinc chloride (ZnCl2; ref. 24). These cells undergo apoptosis after serum deprivation in the presence of p53 (24). p21 is a universal inhibitor of cyclins/cyclin-dependent kinases and a direct transcriptional target of p53 (4,25). As expected, p21 is absent in EB-1 without wt p53 (Fig. 1A, middle, lane 1) but increased after wt p53 expression induced by ZnCl2 (middle, lanes 2 and 3). As shown in Fig. 1A, the DUSP1 transcript is undetectable in EB-1 without p53 (top, lane 1). Interestingly, DUSP1 is not increased in EB-1 cells with ZnCl2 (lane 1 versus lane 2), which induces p21 (middle, lane 2) and causes cell cycle arrest (9). However, DUSP1 expression is greatly increased in the EB-1 cells treated with ZnCl2 and starved for serum (Fig. 1A, top, lane 3), which activates p53 and induces apoptosis (9, 24). As expected, DUSP1 is not induced by serum starvation in the absence of p53 (lane 4). Also, in the presence of p53, γ irradiation does not induce DUSP1 (lane 5), whereas H2O2 increases DUSP1 transcript levels (lane 6) and also causes apoptosis (9). Correspondingly, the abundance of DUSP1 protein is increased by p53 upon serum starvation (Fig. 1B, lane 3) or under...
oxidative stress (lane 6). These results suggest that DUSP1 transcription can be regulated by p53, and induction of DUSP1 is dependent on p53 status and influenced by stress conditions.

To determine whether DUSP1 expression is responsive to stress conditions in a different cell system, normal and p53 null MEFs (p53+/+ and p53−/− MEFs) were treated with γ-rays, which causes cell cycle arrest at G1, (26), or H2O2, which induces cell death by apoptosis (22). As shown in Fig. 1C, DUSP1 is low in p53−/− MEFs and is not induced by γ irradiation (top, lanes 1 and 2). However, DUSP1 is markedly increased after oxidative damage by H2O2 (Fig. 1C, top, lane 1 versus lane 3). Furthermore, the transcriptional response of DUSP1 to oxidative damage is completely eliminated in p53−/− MEFs (lane 3 versus lane 8). As a comparison, p21 is significantly induced by p53 after γ irradiation, serum starvation, UVC exposure or oxidative stress (Fig. 1C, middle, lanes 2-5). This is supported by the lack of DUSP1 induction in p53−/− MEFs, especially after γ irradiation and H2O2 treatment (Fig. 1C, middle, lanes 6-10). Interestingly, DUSP1 is not

FIGURE 2. Activation of the DUSP1 promoter by p53. A. Sequence of the human DUSP1 promoter. Nucleotide sequence upstream of the translation start site is annotated from a PCR-amplified and cloned regulatory region of human DUSP1. The TATA box is boxed, and the approximate transcription start site is shown as an arrow. A 10-bp palindromic site is bolded and underlined. Numbering is with respect to the translation initiation site. B. Transactivation of the DUSP1 promoter by p53. Luciferase assays were carried out for induction of promoter activities of DUSP1 and p21 by wt p53. H1299 cells were transiently transfected with respective plasmids described below using LipofectAMINE in OPTI-MEM (Invitrogen). Cell extracts were assayed for luciferase activity on a Berthold Autolumat LB953 Rack Luminometer. The luciferase activity readout is expressed as means ± SD of triplicate cultures and transfections. The transfection groups are as follows (from the left to right in the figure): (a) empty pCMV vector plus pGL3-Basic; (b) pCMV/wtp53 plasmid plus pGL3-Basic; (c) pCMV vector plus DUSP1-luc; (d) pCMV/wtp53 plus DUSP1-luc; (e) mutant p53 vector (pCS3-248) plus DUSP1-luc; (f) pCMV/wtp53 plus p21-luc; (g) pCMV/wtp53 plus a mutated DUSP1 reporter DUSP1-mt-luc.
induced by either serum starvation or a moderate dose of UVC exposure (Fig. 1C, lanes 4 and 5), which causes cell cycle arrest (27). Western blot analysis indicates that DUSP1 protein abundance coincides with RNA levels detected (compare Fig. 1C and D). These observations suggest that DUSP1 is inducible by oxidative stress in a p53-dependent manner, when apoptosis is triggered, and that p53 can selectively regulate DUSP1 in response to oxidative damage. Because serum starvation causes induction of DUSP1 expression in EB-1 cells, which undergo apoptosis, but not in p53+/−/− MEFs, which are under cell cycle arrest, we propose that cell type also influences fate of stressed cells. The different patterns of DUSP1 induction indicate that p53 may regulate DUSP1 through different mechanisms.

Transactivation of the Regulatory Region of the Human DUSP1 Gene by p53

To understand the molecular basis for regulation of DUSP1 by p53, we cloned the human DUSP1 gene regulatory region upstream of the translation start site. We used a 340-bp fragment flanking the 5′ untranslated region and partial coding sequence of DUSP1 as a probe to screen a human genomic library in the pWE15 cosmid (Stratagene) for the promoter of the DUSP1 gene. Screening was done according to the manufacturer’s protocol. The resulting positive clones were subjected to secondary screening, and a clone containing the 2.4-kb region upstream of the start of DUSP1 translation was isolated. However, sequence analysis of this region reveals no known established p53 consensus binding site [i.e., 5′-PuPuPuPuC(A/T)(T/A)GPyPyPy-3′; ref. 3]. Interestingly, there is a 10-bp perfect palindromic sequence from −369 to −360 (5′-GTGACGTCAC-3′) in this putative DUSP1 regulatory region (Fig. 2A).

To determine whether the DUSP1 fragment is regulated by p53, part of that region of the gene (−826 to −15), which contains the palindromic sequence (Fig. 2A), was amplified. This 812-bp sequence was ligated into vector pGL3-basic (Promega), adjacent to a luciferase reporter gene, resulting in plasmid DUSP1-luc. To examine luciferase activity, the DUSP1 luciferase reporter and other reporters were transfected into p21 promoter reporter is induced by wt p53, but not by the plasmid pC53-248 (mtp53; column 4 versus column 7). Induction of the DUSP1 promoter by wt p53 was significantly reduced (Fig. 2B, column 7). These results provide strong evidence that the palindromic site is critical for transactivation of the regulatory region of DUSP1 by p53.

Physical Interaction between p53 and the DUSP1 Promoter In vivo

Because p53 can transactivate the regulatory region of DUSP1, it is possible that p53 can interact with this region in vivo. To test this possibility, we did a chromatin immunoprecipitation (ChIP) assay using an anti-p53 antibody to detect the physical interaction between p53 and the DUSP1 regulatory region. We first chose the p53 inducible system, EB-1 cells, which express wt p53 on June 17, 2017. © 2008 American Association for Cancer Research. mcr.aacrjournals.org Downloaded from

FIGURE 3. ChIP analysis of physical association of p53 with chromatin containing the regulatory regions of DUSP1. A. Physical association of p53 with a human DUSP1 regulatory fragment under different stresses. EB-1 cells were treated as Fig. 1A for 2 h and processed for ChIP using an anti-p53 antibody (PAb1801) and a ChIP assay kit (UBI) according to the manufacturer’s protocol. The DUSP1 promoter fragment from p53 immunoprecipitates was amplified by PCR using the corresponding primers. The regulatory fragment was directly amplified by PCR of genomic DNA from each group as input. B. Stress-induced interaction between endogenous p53 and the regulatory fragment of mouse DUSP1. Exponentially growing MEF cells were exposed to γ irradiation (6 Gy), H2O2 (100 μM), serum starvation (0.1% FBS), or UV irradiation (10 J/m2), respectively, as indicated for 2 h. Cells were processed for ChIP with an anti-p53 antibody (Systems Biomed) plus an anti-phosphorylated p53 (Ser15). The p53 bound mouse DUSP1 promoter fragment was amplified by PCR using the corresponding primers. The same regulatory fragment directly amplified from genomic DNA was used as input.
EB-1, for induction of p53. As shown in Fig. 3A, the DUSP1 regulatory region was PCR amplified from the ChIP products pulled out by the p53 antibody in group 3 where EB-1 cells were treated by 100 μmol/L ZnCl2 plus serum deprivation (lane 3) or in group 6 where EB-1 was treated with hydrogen peroxide (H2O2) in the presence of 100 μmol/L ZnCl2 (lane 6). However, there is no evidence for p53 binding to the same region when cells were γ irradiated (lane 5). Next, we examined the possible association of endogenous p53 with the regulatory fragment of mouse DUSP1. As shown in Fig. 3B, p53 is associated with the mouse DUSP1 regulatory fragment when MEFs were under oxidative stress but not after incurring damage or exposed to stress by treatment with irradiation, serum starvation, or UV irradiation. These results indicate that oxidative signals may result in p53 modification that influences p53 binding to chromatin containing the regulatory region of DUSP1.

To determine whether p53 physically binds to the palindromic site in this region, we synthesized a 30-bp oligonucleotide containing the palindromic sequence of interest. This DUSP1 oligo is called 30W and is presented in Fig. 4 (bottom). This oligo and others were labeled and used as probes for electrophoretic mobility shift assay experiments. As shown in Fig. 4, two prominent shifted bands were detected when this 30-bp radiolabeled DUSP1 oligo (30W) was incubated with recombinant human wt p53 (lane 1 versus lane 2). Formation of the p53-protein-30W oligo complex was completely inhibited by addition of an excess amount of the same but unlabeled DUSP1 30W oligonucleotide as a competitor (lane 3), whereas a nonspecific 30-bp oligo (NS30) had no effect on complex formation (lane 4). To confirm p53 binding specificity, PAb421, an anti-p53 monoclonal antibody, was included in the reaction and that resulted in formation of a supershifted band (lane 5), which was not detected when cold DUSP1 30W was added as a competitor (lane 6). Because the higher of the two p53-DNA complex bands supershifted, the results suggest that the lower band is either an artifact, nonspecific, or cannot bind antibody, perhaps because of special conformational constraints. There was a greatly reduced shift band formed when p53 was incubated with 30M, a mutated form of the 30W oligo containing a T-G conversion in the palindromic site (lane 7), and no band at all was detected with the unrelated oligo NS30W (lane 8). These data indicate that the palindromic sequence is critical for p53 binding. As a positive control, we included p21-30W, a 30-bp synthetic oligo containing a consensus p53 binding motif from the p21 promoter in the electrophoretic mobility shift assay reactions. As expected, p53 bound this oligo (lane 9), which was supershifted in the presence of the p53 antibody PAB421 (lane 10). The size of the p21-30W shifted band (lane 9) is similar to that of the top DUSP1 30W shifted band (lane 2), providing further evidence that the top band is a true p53-DUSP1 DNA complex. Our results show that p53 specifically binds to the oligo containing the palindromic site, which is present in the DUSP1 promoter.

The Role of DUSP1 in Signaling Apoptosis after Oxidative Stress

We showed previously that p53 is required for the cellular apoptotic response to oxidative stress (21). Our preliminary results indicate that DUSP1 is up-regulated by p53 in response to serum deprivation and oxidative stress, suggesting that DUSP1 may be involved in signaling the cellular response to environmental stresses, in particular when apoptosis is induced. To determine more directly the role of DUSP1 in the apoptotic process, we constructed a human DUSP1 expression vector by cloning a 1.4-kb human DUSP1 cDNA containing the full coding sequence in frame with the human cytomegalovirus promoter in pcDNA3/hygro (Invitrogen). The resulting DUSP1 expression vector, pcDNA3/DUSP1, or an empty vector, pcDNA3, was transfected into DLD-1, a colon cancer cell line containing mutant p53 and a low level of DUSP1, which were then challenged with hygromycin B for isolating stable clones of DLD-1/DUSP1. We chose DLD-1/pcDNA3 cells and DLD-1/DUSP1 cells containing a high level of ectopic DUSP1 expression (Fig. 5A) for functional studies. The phosphorylation status of myelin basic protein, a MAPK substrate, was measured as an indication of the activity of MAPK ERK1/2. As shown in Fig. 5B, myelin basic protein is highly phosphorylated by ERKs in parental DLD-1 cells with the empty vector (lane 1). However, the phosphorylation of myelin basic protein
is detectably inhibited in the DLD-1 cells containing a high level of DUSP1 (Fig. 5B, lane 2), indicating that MAPK activity is effectively inhibited by DUSP1 in these DLD-1/DUSP1 cells. To determine whether DUSP1 can mediate cell death, we examined the efficiency of cell killing in both DLD-1/pcDNA3 cells and the DLD-1/DUSP1 cells under oxidative stress. Indeed, whereas DLD-1/pcDNA3 cells are resistant to oxidative stress, the DLD-1/DUSP1 population is highly

FIGURE 5. DUSP1 inhibits MAPK activity and mediates apoptosis. A. Ectopic expression of DUSP1 in DLD-1 cells. DLD-1 cells were transfected with either pcDNA3 or pcDNA3/DUSP1 and selected for stable clones. Indicated clones as DLD1/pcDNA3 and DLD1/MKP1 were cultured under normal growth conditions. Equal amounts of total RNA were resolved on 1.2% formaldehyde gels and transferred for Northern blotting using the corresponding [α-32P]dCTP-labeled cDNA probes. B. Inhibition of MAPK activity by DUSP1 in DLD-1 cells. Indicated cell lysates from A, as above, were immunoprecipitated by anti-ERK1/2 antibodies and the immunoprecipitated complex was incubated with myelin basic protein in the presence of [γ-32P]ATP as described elsewhere (40). Phosphorylated myelin basic protein was resolved by 15% SDS-PAGE and visualized by autoradiography. Actin was used as a loading control. C. TUNEL analysis of DUSP1-mediated apoptosis. The indicated cells (1 x 10⁶) were treated with H₂O₂ (200 μmol/L) for 12 h. The cells were harvested together with supernatant for TUNEL staining using an *in situ* apoptosis detection kit (Intergen). The cells were visualized under fluorescent microscope. Double-colored cells were counted as apoptotic cells. D. Viability of DLD-1 cells with or without ectopic DUSP1 expression under oxidative stress. Exponentially growing DLD1/pcDNA3 and DLD1/DUSP1 cells (10⁶ cells per group) as indicated were treated with H₂O₂ (200 μmol/L) for 24 h. The populations were scored for numbers of viable and nonviable cells by trypan blue exclusion. The data are presented as means ± SD of three independent experiments of duplicate cultures. E. Induction of cell death by combination of MAPK inhibition and oxidative damage. DLD1/pcDNA3 cells were treated by MAPK inhibitor Apigenin (25 μmol/L), H₂O₂ (200 μmol/L), or both for 48 h. Viability was evaluated as D. F. Survival of colon cancer cells under oxidative stress. Exponentially growing cells indicated were plated at the density of 6 x 10⁵ per well and treated with H₂O₂ (200 μmol/L) for 24 h. Colonies with a diameter of >1.0 mm were counted after 2 wk of culture. The values are the means ± SD of three independent experiments. G. Mediation of cell killing by DUSP1 after indomethacin treatment. DLD1/pcDNA3 and DLD1/DUSP1 cells were treated with indomethacin (200 μmol/L) for various times as indicated. The rate of cell death was determined by TUNEL. The values are the means ± SD of three independent experiments.
susceptible to cell killing by \( \text{H}_2\text{O}_2 \) treatment, which is reflected either as increased frequency of apoptotic cells (Fig. 5C) or decreased cell viability (Fig. 5D). To determine whether inhibition of MAPK activity causes the same biological effects as increasing DUSP1 expression, we used a MAPK inhibitor, Apigenin, to determine whether it can cause the same biological effects as DUSP1 does. As shown in Fig. 5E, Apigenin alone fails to induce cell death in the absence of oxidative damage. However, Apigenin plus \( \text{H}_2\text{O}_2 \) causes significant cell killing. These results thus show that activation of DUSP1 or inhibition of MAPK is necessary but insufficient to induce cell death. Damage signals are needed to initiate cell death. These results indicate that DUSP1 functions as a cell death mediator in the cellular response to oxidative stress. Consistently, the frequency of colony formation is much lower for DLD-1/DUSP1 cells than for DLD-1/pcDNA3 cells after treatment with \( \text{H}_2\text{O}_2 \) (Fig. 5F).

Because DUSP1 is involved in the cellular response to oxidative stress and it mediates cell death after oxidative damage, it is possible that DUSP1 mediates cell killing by chemotherapeutic treatment through oxidative signaling. We thus treated cells with indomethacin and tested susceptibility to drug treatment in the absence or presence of DUSP1. Indomethacin is a nonsteroidal antiinflammatory drug that has been shown to cause cancer cell death by apoptosis (30). It has been reported that reactive oxygen species levels are increased in some cancer cells treated with indomethacin (31). Therefore, we determined whether the cells with ectopically expressing DUSP1 are more sensitive to treatment with indomethacin. As shown in Fig. 5G, the DLD-1/DUSP1 cell population has a higher percentage demonstrating apoptosis when compared with the cells not expressing DUSP1 (DLD-1/pcDNA3) and similarly treated with indomethacin. These results suggest that DUSP1 mediates cell killing of DLD-1 cells by indomethacin.

**Discussion**

DUSP1 is a dual threonine/tyrosine phosphatase that inactivates MAPKs (15) by dephosphorylation. This reaction is important for the cellular response to environmental stress as part of the MAPK signaling network. DUSP1 (CL100) was cloned as an early response gene after oxidative stress. DUSP1 is critical for the inactivation of p38 MAPK and c-Jun-NH\(_2\) kinase after stimulation with serum, anisomycin, and osmotic stress (32). However, the mechanism of DUSP1 regulation is largely unknown. It was reported previously that p53 regulates transcription of DUSP1 and that reaction is important for progression of cells through the G\(_1\) phase of the cell cycle after serum-starved cells are stimulated by growth factors (10, 11). We have reported previously that p53 is essential for the cellular response to oxidative stress. In this paper, we show that p53 is the primary regulator of DUSP1 in signaling the cellular response to oxidative stress. In particular, we show that p53 can physically interact with the regulatory region of the DUSP1 gene in vivo not under normal growth conditions but under stresses. Consistently, DUSP1 is highly induced in response to these stresses. DUSP1 is also highly expressed in MEFs treated with hydrogen peroxide to induce oxidative stress. This regulation of DUSP1 expression was p53 dependent. Therefore, stress conditions that induce apoptosis preferentially cause p53-mediated transcriptional activation of DUSP1.

We observed that DUSP1 can mediate the cellular response to oxidative stress or chemotherapeutic treatment leading to apoptosis. It is well known that p53 is essential for the cellular apoptotic response to oxidative damage (21). We found that oxidative damage induces MAPK phosphatases and mainly inactivates ERK1/2 in a p53-dependent manner (ref. 9; data not shown). In this paper, we show that DUSP1 directly inactivates ERK1/2 activity and mediates apoptosis in response to oxidative stress. Thus, DUSP1 may act as an inhibitor of cell death as a downstream target of p53 and suppresses a predominant cell survival pathway through inactivation of ERK1/2 in mediating apoptosis. To evaluate the role of phosphatase activity of DUSP1 in signaling cell death, we used a pharmacologic agent, Apigenin, which is an inhibitor of MAPK activity, to determine whether it can cause the same biological effects as DUSP1 does. As shown in Fig. 5E, Apigenin cannot induce cell death in the absence of oxidative damage. However, Apigenin induces cell killing when cells are treated together with \( \text{H}_2\text{O}_2 \). These results indicate that activation of DUSP1 or inhibition of MAPK alone is necessary but insufficient to induce cell death. Damage signals are needed to initiate the cell death process. There are other possible mechanisms for the involvement of DUSP1 in cell death. Because ERK1/2 is a cell survival factor, it is possible that ERK1/2 controls protective oxidative stress response signaling. Thus, when ERK1/2 is inhibited by DUSP1, the protective response induced by exposure to oxidative stress is blunted, thereby sensitizing the cells to oxidative stress. Alternatively, overexpression of p53 up-regulates proline oxidase PIG3, which may generate metabolic reactive oxygen species (33-35). It is likely that increased DUSP1 level prevents an oxidative stress response by suppressing MAPK signaling, which leads to apoptosis.

There is no conventional p53 consensus binding sequence in the promoter of DUSP1, although this promoter can be transactivated by p53, indicating that p53 may use other mechanisms to activate the promoter. We show that p53 can bind a palindromic 10-bp sequence in the regulatory region of DUSP1 and that region is important for p53-mediated induction of DUSP1 transcription. This sequence is upstream of the start of DUSP1 transcription and close to a TATA box, suggesting that it is influential in terms of regulating DUSP1 transcription in vivo. Indeed, we show p53 physically interacts with the DUSP1 promoter in vivo and that p53 transactivates the promoter through binding to this palindrome. Furthermore, a computer search of the human genome indicates that ~100 other genes contain this palindromic sequence and might thus also be regulated by p53. For example, there is a 10-bp palindromic sequence in the promoter of cytochrome \( c \), which is identical to the palindromic sequence in the DUSP1 promoter. Cytochrome \( c \) is located in the mitochondrial membrane, and it plays an essential role in the activation of caspase-related protease cascade during apoptosis (36). We found that p53 physically binds to this palindromic site and
transactivates the cytochrome c promoter.\textsuperscript{4} We found that overexpression of DUSP1 in a colon cancer cell line, DLD-1, induced apoptosis at a high frequency and significantly reduced cellular viability. Therefore, these findings provide evidence that the transcriptional activation of DUSP1 by p53 is a mechanism for triggering programmed cell death in response to certain types of stress.

It is well established that p53 regulates the apoptotic and cell cycle delay response to stress by activating transcription of downstream target genes. We identified a palindrome that can be bound by p53 and used for transcriptional activation of DUSP1. In addition, we show the selective p53-dependent transcription of DUSP1 under conditions that cause apoptosis. A fundamental question in the field of p53 investigation is how p53 distinguishes different signals and directs cells to either cell cycle arrest or cell death. We sought to identify a novel mechanism whereby p53 only induces certain targets under certain stress conditions. We reasoned that p53 does so through regulating subsets of its targets and the mechanism could be that p53 has options to choose different binding sites in response to different types of damage. We have identified several palindrome motifs as novel p53 binding sites (9, 37, 38). The great difference between this palindrome-related mechanism and the conventional mechanism is that binding of this site is stress dependent. For example, the promoters containing a canonical p53 binding site seem to be ready for transactivation, and the involved genes are often induced as long as p53 is present. For example, whenever p53 is expressed, whether it is ectopically expressed or induced by DNA damage, p21 is always induced, no matter what kind of damage is incurred (Fig. 1; ref. 9). Instead, the genes containing palindrome sites are inducible by p53 only in the presence of stresses, in our case, oxidative stress. Therefore, we believe that there must be different mechanisms involved. We hypothesized that some p53 target genes are only stress inducible because p53 gets access to this different kind of regulatory site only when cells are under stress that likely results in p53 modification. We have observed that p53 becomes phosphorylated at serine 36 and serine 46 during serum starvation that induces DUSP1 and apoptosis in the EB-1 system (data not shown). We also found that p53 is phosphorylated at serine 37 when MEFs are under oxidative stress and at serine 18 when MEFs are subjected to γ irradiation.\textsuperscript{5} Interestingly, p53 can bind to the regulatory region of DUSP1 only under conditions that induce apoptosis (Fig. 3). These observations lead to our hypothesis that p53 has to be modified to get access to chromatin where the palindrome binding sites are located and to transactivate the genes containing these palindromic sites. Therefore, p53 may selectively regulate its target genes that contain either the canonical p53 binding sites or palindrome motifs based on the nature of damage or environmental stresses that cause p53 protein modifications. Indeed, our results suggest that p53 can modulate individual cellular responses to specific types of stress by selectively activating transcription of a subset of downstream target genes. Therefore, the identification of all genes regulated by p53, as well as the conditions in vivo that induce their transcriptional activation, should provide important insight into the molecular pathways used by cells to respond to environmental stress.

Materials and Methods

Cell Culture and Plasmid Transfection

EB and EB-1 cells have been described previously (23). MEFs were described elsewhere (21). Cancer cell lines were purchased from American Type Culture Collection. These cell lines were maintained in Earle’s MEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO\textsubscript{2}. Transfection was done using LipofectAMINE reagent (Life Technologies) according to the manufacturer’s instruction. For selection of stable clones, transfected cells were treated with hygromycin B until the clones were formed and grown for confirmation of ectopic DUSP1 expression.

Plasmid Construction

The promoter region of DUSP1 was amplified by PCR using the following primers: 5'−CAAGTCTTCCGGGCCCAACAGCTAGGAA−3'; 5'−TCCGACACAGCCCAAATGTCCCTTCGCAG−3'. The PCR product was ligated into the pGL3-basic reporter (Promega), resulting in pGL3/DUSP1-luc. pGL3/DUSP1mt-luc was generated by changing the palindromic sequence from 5'-GGTGACGTAC-3' to 5'-GGTGACGTTC-ACG-3' using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. To construct the human DUSP1 expression vector, the cDNA sequence, including the coding region of human DUSP1, was ligated into pcDNA3.1 (Invitrogen), resulting in pcDNA3/DUSP1.

ChIP

Growing cells were treated as indicated and processed for ChIP using a ChIP assay kit (UBI) according to the manufacturer’s protocol. Briefly, cells were cross-linked by 1% formaldehyde and cell lysates were incubated with the p53 antibodies. Immune complexes were precipitated and the promoter regions were amplified by PCR using the primers spanning the potential p53 binding site.

Cellular Viability and TUNEL Assay

Cultured cells were seeded at an equal density (5 × 10\textsuperscript{5} /100-mm dish), and the exponentially growing cells were treated by indicated conditions and scored for numbers of viable and nonviable cells by trypan blue dye exclusion. For the TUNEL assay, cells were cultured exponentially on chamber slides. The slides were fixed with 4% formaldehyde incubated with biotinylated dUTP and terminal transferase (Boehringer Mannheim Biochemica) in TDT buffer [30 mmol/L trizma base (pH 7.2), 140 mmol/L Na cacodylate, and 1 mmol/L cobalt chloride] for 1 h. The slides were processed using fluorescein in situ apoptosis detection kit (Intergen).

Luciferase Assay

Cells were transiently transfected with plasmids as described above. A pCMV-β-gal reporter plasmid was cotransfected to...
normalize transfection efficiency. Cell extracts were processed using the dual-light kit (Tropix) according to the manufacturer’s instructions. Luciferase activity was measured with a Berthold Autolumat LB953 Rack Lumimeter. Luciferase values were normalized against β-galactosidase activity. Luciferase readout was always obtained from triplicate transfections and averaged.

**RNA Isolation, Northern Blot, and Western Blot**

Total RNA was isolated from growing cells using TRizol Reagent (Life Technologies) according to the instructions of the manufacturer. For Northern analysis, 20 µg RNA was run on a 1.2% formaldehyde gel and transferred to a Nybond-N autoradiography. Anti-p53 antibodies (Systems Biomed). The reaction mixtures deoxycytidylic acid). A super shift was detected by adding 20 mmol/L Tris-HCl (pH 7.5), 4% Ficoll-400, 2 mmol/L (2/C2 a baculovirus vector expressing human wt p53 and partially binant p53 protein was produced in insect cells infected with kinase and [α-32P]dCTP (Amersham) using the Prime-It RmT Random Primer Labeling kit (Stratagene). The membrane was hybridized with labeled DNA probes in the NB Hyb (Systems Biomed) at 65 °C for 2 h and developed for autoradiography. For Western blotting, growing cells at 60% to 70% confluence were lysed in cold Nonidet P-40 buffer with protease/phosphatase inhibitors. The samples were resolved by 7.5% SDS-polyacrylamide gels and then transferred onto a nitrocellulose filter after protein separation. Immunoblots were incubated with primary antibodies and then incubated with peroxidase-conjugated rabbit anti-mouse IgG as secondary antibody. The signals were detected with enhanced chemiluminescence (Systems Biomed).

**Electrophoretic Mobility Shift Assay**

Oligonucleotides in pairs of sense and antisense were annealed and labeled with [32P] by using T4 polynucleotide kinase and annealed and labeled with 32P by using T4 polynucleotide kinase. The reaction mixtures containing 20% C for 2 hand developed for autoradiography. For Western blotting, growing cells at 60% to 70% confluence were lysed in cold Nonidet P-40 buffer with protease/phosphatase inhibitors. The samples were resolved by 7.5% SDS-polyacrylamide gels and then transferred onto a nitrocellulose filter after protein separation. Immunoblots were incubated with primary antibodies and then incubated with peroxidase-conjugated rabbit anti-mouse IgG as secondary antibody. The signals were detected with enhanced chemiluminescence (Systems Biomed).

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**References**


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