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13. ABSTRACT *(Maximum 200 Words)*

The p53 tumor suppressor protein is a sequence-specific DNA binding transcription factor that induces cell cycle arrest or apoptosis in response to DNA damage. We had previously demonstrated that ionizing radiation (IR) leads to association of p53 with 14-3-3 in breast cancer cells and hypothesized that this association activates the tumor suppressor function of p53. To test this hypothesis, we had proposed to determine whether the interaction of p53 with 14-3-3 affects:

1. the sequence-specific DNA binding activity of p53 (months 1-12);
2. the cell cycle arrest and/or apoptotic functions of p53 (months 13-24); and
3. p53 intracellular localization and half-life (months 25-36).

During the three years of funding we showed that the interaction of p53 with 14-3-3 proteins does not affect p53 sequence-specific DNA binding activity in vivo (Task 1); that the interaction of p53 with 14-3-3 is critical for the ability of p53 to induce cell cycle arrest and that 14-3-3 proteins affect p53 function by regulating the transcriptional activity of p53 (Task 2); and that the interaction of p53 with 14-3-3 proteins does not affect p53 intracellular localization or half-life (Task 3).

Overall, the results support the initial hypothesis that the interaction of p53 with 14-3-3 activates the tumor suppressor function of p53, although the mechanism turns out to be different than what we had expected. The 14-3-3 proteins affect the transcriptional activity of p53, rather than its DNA binding activity.

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INTRODUCTION

The p53 tumor suppressor protein, a transcription factor for genes that induce cell cycle arrest or apoptosis, is a critical factor for the response of mammalian cells to DNA damage (Maltzman and Czyzyk, 1984; Kastan et al., 1991; Kuerbitz et al., 1992). DNA damage leads to increased p53 protein levels and increased p53 functional activity (Maltzman and Czyzyk, 1984; Kastan et al., 1991; Haapajarvi et al., 1997). The mechanism by which DNA damage increases p53 protein levels involves dissociation of p53 from Mdm2 (Shieh et al., 1997; Unger et al., 1999; Chehab et al., 1999), a protein that targets p53 for degradation (Haupt et al., 1997; Kubbutat et al., 1997; Midgley and Lane, 1997); whereas the mechanism by which DNA damage increases the functional activity of p53 has not been established. DNA damage leads to dephosphorylation of Ser376 of p53, which in turn leads to binding of p53 to 14–3–3 proteins (Waterman et al., 1998). The subject of the USARMRC–funded research is to establish whether the binding of p53 to 14–3–3 proteins underlies the increase in p53 functional activity after DNA damage. Because DNA damaging agents are used to treat breast cancer and because their efficacy depends on their ability to activate p53 (Fisher, 1994), understanding the molecular mechanism by which p53 function is upregulated after DNA damage will help the development of novel therapies to treat this disease.

BODY

The Tasks outlined in the approved Satement of Work for the entire research funding period are:

1. Determine whether the interaction of p53 with 14–3–3 affects the sequence–specific DNA binding activity of p53 (months 1–12);
2. Determine whether the interaction of p53 with 14–3–3 modulates the cell cycle arrest and/or apoptotic functions of p53 (months 13–24); and
3. Determine whether the interaction of p53 with 14–3–3 affects p53 intracellular localization and half–life (months 25–36).

During the first year of funding we addressed Task 1. We showed that the association of p53 with 14–3–3 proteins does not affect the sequence–specific DNA binding activity of p53 isolated from cells. This result was unexpected, because 14–3–3 enhances the DNA binding activity of recombinant p53 expressed in bacteria. Nevertheless, we are now confident that 14–3–3 proteins do not enhance the DNA binding activity of p53 in vivo, because in another study we did that looks at the effects of acetylation on p53 DNA binding we obtained similar results, i.e. that acetylation of Lys382 of p53 enhances the DNA binding activity of p53 expressed in bacteria, but did not affect DNA binding of p53 in vivo.

During the second year of funding we addressed Task 2. We showed that the interaction of p53 with 14–3–3 proteins is required for p53 to induce cell cycle arrest. These experiments are described in detail in the accompanying manuscript (Stavridi et al., 2001), which has been submitted for publication to the journal "Cancer Research". The results pertaining to the cell cycle arrest properties of wild–type p53 and the p53 mutants that fail to interact with 14–3–3 proteins are shown in Figure 5 of the attached publication (Stavridi et al., 2001).

During the third year of funding we addressed Task 3. We generated fusion proteins of the p53 mutants described above with green fluorescent protein. Plasmids expressing these proteins were stably transfected into U2OS cells. These cells were either not irradiated or were exposed to ionizing radiation (typically 5 Gray). At 1, 4 and 12 hours after irradiation the cells were fixed with paraformaldehyde, stained with DAPI and

visualized using a fluorescence microscope. As Figure 1 indicates, all the p53 proteins localized exclusively in the nucleus. Significantly, the protein with alanines at positions 376–378, which does not bind 14–3–3 at all and is defective in its ability to induce cell cycle arrest and activate transcription, still localized in the nucleus. Thus, the impaired ability to activate transcription is not due to exclusion of the protein from the nucleus.

We also monitored the half–life of these proteins. Briefly, U2OS cells expressing the various p53 mutants were non–irradiated or exposed to 5 Gray ionizing radiation, cycloheximide was added immediately after irradiation to inhibit protein synthesis and p53 levels were monitored over time by western blotting. The wild–type and mutant p53 proteins exhibited similarly prolonged half–lifes after irradiation. This result is consistent with our previous observation that p53 mutants that fail to interact with 14–3–3 proteins are stabilized after irradiation, as efficiently as wild–type p53 (Chehab et al., 1999).

Our initial hypothesis was that the association of p53 with 14–3–3 proteins is important for p53 function. This hypothesis is confirmed by our findings showing that p53 mutants that do not interact with 14–3–3 proteins are defective in cell cycle arrest. However, we anticipated that the mechanism by which 14–3–3 proteins enhance p53 function would involve enhancement of the sequence–specific DNA binding activity of p53. As described above (Task 1), this is not so. Instead, we found that 14–3–3 proteins enhance the transcriptional activity of p53 (see Task 2, above and our appended publication – Stavridi et al., 2001). We also found that 14–3–3 proteins do not regulate the intracellular localization and half–life of p53 (Task 3)

KEY RESEARCH ACCOMPLISHMENTS

We have constructed a panel of p53 mutants which are defective in their ability to interact with 14–3–3 proteins.

We have expressed these p53 mutants in a variety of cancer cells lines of breast and non–breast origin and studied their DNA binding properties, their ability to activate transcription and induce cell cycle arrest in response to DNA damage, their intracellulat localization and half–life.

REPORTABLE OUTCOMES

We published a manuscript describing our findings in the journal "Cancer Research". A copy of the publication, which lists the support provided by the DOD, is appended.

We have developed plasmids directing the expression of mutant p53 proteins that do not associate with 14–3–3 proteins in breast cancer cell lines.

CONCLUSIONS

Our key conclusions can be summarized as follows:

1. The interaction of p53 with 14–3–3 proteins does not affect the sequence–specific DNA binding activity of p53 when assayed using cell extracts.
2. The interaction of p53 with 14–3–3 proteins is required for the ability of p53 to induce cell cycle arrest in G1 in response to DNA damage.
3. The interaction of p53 with 14–3–3 proteins is required for the ability of

p53 to activate transcription of its target genes.

4. The interaction of p53 with 14–3–3 proteins does not affect the intracellular localization of p53 or its half–life.

The second and third conclusions support our hypothesis that the interaction of p53 with 14–3–3 proteins is important for the functional activity of p53. These findings are therefore important, because they suggest that modulating the interaction of p53 with 14–3–3 proteins is a viable mechanism to regulate p53 activity in patients with breast cancer. Augmenting p53 function in patients with breast cancer could have a therapeutic effect equivalent to the one currently obtained using DNA damaging agents, but without the toxicity of the latter.

In conclusion we successfully completed the Tasks described in our original application. We believe we have a better understanding of the mechanisms leading to regulation of p53 in breast cancer cells and are grateful to the DOD for their support of these important studies.

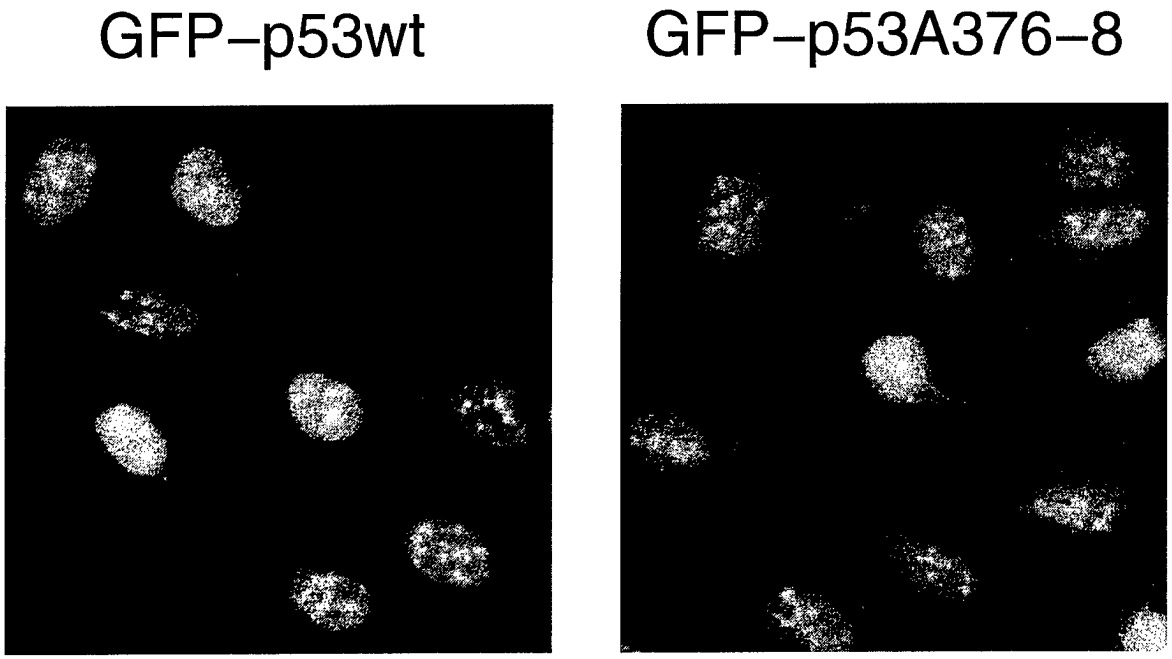

Figure 1. Binding of p53 to 14–3–3 does not affect the subcellular localization of p53 after DNA damage. Wild–type (wt) p53 and p53 containing substitutions of residues 376–378 with alanines (A376–8) were fused to green fluorescent protein (GFP) and their intracellular localization was studied in response to irradiation. Under all conditions tested, both p53 proteins localized exclusively in the nucleus. The figure shows cells exposed to 5 Gy ionizing radiation and processed 1 hour after irradiation.

REFERENCES

Chehab,N.H., Malikzay,A., Stavridi,E.S. and Halazonetis,T.D. (1999) Phosphorylation of Ser20 mediates stabilization of human p53 in response to DNA damage. Proc. Natl. Acad. Sci. USA, 96, 13777–13782.

Fisher, D.E. (1994) Apoptosis in cancer therapy: crossing the threshold. Cell, 78, 539–542.

Haapajarvi,T., Pitkanen,K., Tsubari,M. and Laiho,M. (1997) p53 transactivation and protein accumulation are independently regulated by UV light in different phases of the cell cycle. Mol. Cell. Biol., 17, 3074–3080.

Haupt,Y., Maya,R., Kazaz,A. and Oren,M. (1997) Mdm2 promotes the rapid degradation of p53. Nature, 387, 296–299.

Kastan,M.B., Onyekwere,O., Sidransky,D., Vogelstein,B. and Craig,R.W. (1991) Participation of p53 protein in the cellular response to DNA damage. Cancer Res., 51, 6304–6311.

Kubbutat,M.H., Jones,S.N. and Vousden,K.H. (1997) Regulation of p53 stability by Mdm2. Nature, 387, 299–303.

Kuerbitz,S.J., Plunkett,B.S., Walsh,W.V. and Kastan,M.B. (1992) Wild–type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl Acad. Sci. USA, 89, 7491–7495.

Maltzman,W. and Czyzyk,L. (1984) UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. Mol. Cell. Biol., 4, 1689–1694.

Midgley,C.A. and Lane,D.P. (1997) p53 protein stability in tumour cells is not determined by mutation but is dependent on Mdm2 binding. Oncogene, 15, 1179–1189.

Shieh,S.Y., Ikeda,M., Taya,Y. and Prives,C. (1997) DNA damage–induced phosphorylation of p53 alleviates inhibition by MDM2. Cell, 91, 325–334.

Stavridi,E.S., Chehab,N.H., Malikzay,A. and Halazonetis,T.D. (2001) Substitutions that compromise the ionizing radiation–induced association of p53 with 14–3–3 proteins also compromise the ability of p53 to induce cell cycle arrest. Cancer Research, 61, 7030–7033.

Unger,T., Juven–Gershon,T., Moallem,E., Berger,M., Sionov,R.V., Lozano,G., Oren,M. and Haupt,Y. (1999) Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2. EMBO J., 18, 1805–1814.

Waterman,M.J., Stavridi,E.S., Waterman,J.L. and Halazonetis,T.D. (1998) ATM–dependent activation of p53 involves dephosphorylation and association with 14–3–3 proteins. Nature Genet., 19, 175–178.

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Advances in Brief

Substitutions That Compromise the Ionizing Radiation-induced Association of p53 with 14-3-3 Proteins Also Compromise the Ability of p53 to Induce Cell Cycle Arrest[1]

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Abstract

Ionizing radiation (IR) induces an increase in the levels and activity of the p53 tumor suppressor protein. The increased activity is attributed to IR-induced posttranslational modifications, some of which regulate the interaction of p53 with other proteins. One of these modifications is dephosphorylation of Ser^{376}, which leads to association of p53 with 14-3-3 proteins. To establish the significance of this interaction, we examined the function of mutant p53 proteins that do not interact with 14-3-3 proteins *in vivo*. These p53 mutants retained sequence-specific DNA binding activity. However, their ability to activate transcription of the endogenous *p21/waf1/cip1* gene and to induce G_1 arrest was compromised, suggesting that the dephosphorylation of Ser^{376} and the association of p53 with 14-3-3 proteins contribute to the activation of p53 in response to IR.

Introduction

The p53 tumor suppressor protein is a critical component of the DNA damage checkpoint machinery. p53 transactivates genes that induce cell cycle arrest and apoptosis and genes involved in DNA repair (1, 2). The p53-regulated genes that induce cell cycle arrest include *p21/cip1/waf1* and *14-3-3* σ (3, 4). *p21/cip1/waf1* encodes an inhibitor of cyclin-dependent kinases. *14-3-3* σ is one of the 14-3-3 protein isoforms; it induces cell cycle arrest in G_2 by sequestering in the cytoplasm proteins required for entry into mitosis (5, 6).

The mechanisms by which DNA damage activates p53 have been the subject of intense study. It appears that most DNA-damaging agents, including IR, lead to posttranslational modifications of p53 that regulate the interaction of p53 with other proteins or otherwise regulate p53 function. One of these modifications is phosphorylation of p53 on Ser^{20} (7–9). This modification leads to increased p53 protein levels by inducing dissociation of p53 from Mdm2 (7, 10, 11), a protein that targets p53 for degradation through the ubiquitin pathway (12–14). Other modifications induced in response to DNA damage include phosphorylation of Ser^6, Ser^9, Ser^{15}, Ser^{33}, Ser^{37}, Ser^{46}, Ser^{392}, dephosphorylation of Ser^{376}, and acetylation of Lys^{320}, Lys^{373}, and Lys^{382} (1, 15).

One of the modifications, the functional significance of which is unclear, is dephosphorylation of Ser^{376} of p53. This modification creates a binding site for 14-3-3 proteins and leads to an association of p53 with 14-3-3 (16). *In vitro*, 14-3-3 proteins enhance the sequence-specific DNA binding activity of p53, but *in vivo* their effect on p53 function is not known. We also do not know which 14-3-3 isoforms bind to p53 *in vivo*. If 14-3-3 σ binds to p53 and enhances its activity, then there is potential for a positive-feedback loop driving p53 activation, because p53 transactivates the gene encoding 14-3-3 σ (4). Here, we address the functional significance of the interaction of p53 with 14-3-3 proteins and explore which of the 14-3-3 isoforms interact with p53 in irradiated cells.

Materials and Methods

Interaction of p53 with 14-3-3 *in Vitro*. GST[4]/14-3-3 fusion proteins expressed in *Escherichia coli* were incubated with glutathione Sepharose 4B beads (Pharmacia, Piscataway, NJ) in 1× IP buffer [25 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 100 mM EDTA, 200 ng/ml BSA, and 0.1% Tween 20). The beads were then incubated with ^{35}S-labeled *in vitro* translated p53 (17) for 1 h at 4°C. p53 that remained bound to the beads after washing was visualized by autoradiography.

Interaction of p53 with 14-3-3 *in Vivo*. U2OS osteosarcoma cells were either mock irradiated or exposed to 9 Gy of IR or 50 J/m^2 UV light. Whole cell extracts were prepared 2 h after exposure to IR or 16 h after exposure to UV light by lysis in 1× extraction buffer [50 mM Tris (pH 8), 120 mM NaCl, 0.5% NP40, 1 mM DTT, 0.4 μg/ml Pefabloc SC, 2 μg/ml pepstatin, 0.2 μM wortmannin, 0.1 μM staurosporine, 15 mM NaF, and 1 mM sodium vanadate]. 14-3-3 was precipitated using isoform-specific antibodies or antibody K19, which recognizes all 14-3-3 isoforms (Santa Cruz Biotechnology, Santa Cruz, CA). Coprecipitated p53 was detected by immunoblotting with antibody DO7 (Calbiochem, San Diego, CA). The interaction of HA-tagged p53IND proteins with endogenous 14-3-3 was performed using U2OS cells transiently transfected with 2.5 μg of plasmids encoding p53 and 27.5 μg of pBC12/PLseap carrier plasmid (7). Antibody Y11 (Santa Cruz Biotechnology) was used to recognizes HA-tagged p53IND that coprecipitated with 14-3-3.

DNA Binding Assay. U2OS cells were transfected with 2.5 μg of plasmid encoding p53 and 27.5 μg of pBC12/PLseap carrier plasmid (7). Twenty-four h later, the cells were exposed to 9 Gy of IR or were mock irradiated, and 1 h later, the cells were lysed using 1× extraction buffer. Oligonucleotides BCV4A and TT3 (18) with biotin tags at their 5-prime ends were coupled to streptavidin-agarose beads and incubated with the cell lysates for 1 h at 4°C in 1× extraction buffer containing a single-stranded oligonucleotide as nonspecific competitor DNA (18). HA-tagged p53 bound to the beads was detected by immunoblotting with antibody Y11.

Transcription Activation Assays. Saos2 cells were transfected by calcium phosphate precipitation with 0.1 μg of plasmid expressing p53 and 29 μg of pBC12/PLseap carrier plasmid (7). Twenty-four h later, the cells were exposed to 9 Gy of IR, and 24 h later, the cells were lysed by scraping in 0.5 ml of 2× RIPA buffer [40 mM Tris (pH 7.4), 2 mM EDTA, 300 mM NaCl, 20 mM KCl, 2% NP40, 0.2% Triton-X, and 0.2% SDS]. p21/cip1/waf1 protein levels were monitored by immunoblotting using a specific monoclonal antibody (Calbiochem, San Diego, CA). Alternatively, cells were transfected with 1 μg of plasmid expressing p53 and 29 μg of the p53-specific reporter plasmid pEp21-TK-SEAP. Alkaline phosphatase activity was determined 48 h later (19).

Cell Cycle Arrest. U2OS osteosarcoma cells were transfected by calcium phosphate precipitation with 2.5 μg of a plasmid expressing p53IND, 5 μg of

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[4] The abbreviations used are: GST, glutathione *S*-transferase; IR, ionizing radiation; HA, hemagglutinin.

a plasmid expressing a dominant-negative p53 mutant (p53Trp248), 1 μg of a plasmid expressing green fluorescent protein (as a marker), and 24 μg of pBC12/PLseap carrier plasmid (7, 10). Twenty-four h later, the cells were exposed to 5 Gy of IR or were mock irradiated. The cells were harvested 12 h later, resuspended in 200 μl of 0.4% paraformaldehyde in PBS, and incubated for 12 min at 37°C and subsequently for 10 min on ice. The fixed cells were overlaid with 1800 μl of cold (−20°C) methanol with gentle vortexing. After a 10-min incubation on ice, the cells were washed in 1× PBS-TF (PBS with 0.1% Tween 20 and 2% fetal bovine serum) and incubated in 1 ml of PBS-TF containing 20 μl of RNase (Life Technologies, Inc., Grand Island, NY) and 10 μl of propidium iodide (Boehringer Mannheim, Indianapolis, IN) for 1 h at 37°C. Flow cytometry analysis was performed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Results

14-3-3 Isoform Specificity. The question of whether all or specific 14-3-3 isoforms bind to p53 was addressed both *in vitro* and *in vivo*. The *in vitro* studies examined the seven known human genes encoding 14-3-3 isoforms. We generated plasmid vectors that allowed each *14-3-3* gene product to be expressed as a GST fusion protein; the GST/14-3-3 fusion proteins were immobilized on glutathione beads and examined for their ability to capture full-length wild-type p53. Because the interaction between p53 and 14-3-3 proteins requires Ser^{378} of p53 to be phosphorylated, we used a p53 protein bearing three amino acid substitutions, including substitution of Ser^{378} with Ala as a negative control. ^{35}S-labeled wild-type p53 translated *in vitro* was captured specifically by the GST/14-3-3 γ isoform, whereas the mutant p53 protein was not captured by any of the GST/14-3-3 fusion proteins (Fig. 1*A*).

In vivo, the isoform specificity of the interaction between p53 and 14-3-3 was examined by coimmunoprecipitation analysis of the endogenous wild-type p53 and 14-3-3 proteins using 14-3-3 isoform-specific antibodies and lysates prepared from U2OS osteosarcoma cells that were either not irradiated or exposed to IR or UV light. p53 and 14-3-3 interact in cells exposed to IR but not in nonirradiated cells or in cells exposed to UV light (10). Thus, for each 14-3-3 isoform we had a negative control. The γ, ε, and τ isoforms exhibited an IR-specific interaction with wild-type p53, whereas no interaction could be detected with the σ and ζ isoforms (Fig. 1*B*). The K19 antibody, which recognizes all 14-3-3 isoforms, served as a positive control (Fig. 1*B*), and the isoform-specificity of the antibodies, which were obtained commercially, was verified by immunoblotting using recombinant GST/14-3-3 fusion proteins (Fig. 1*C*).

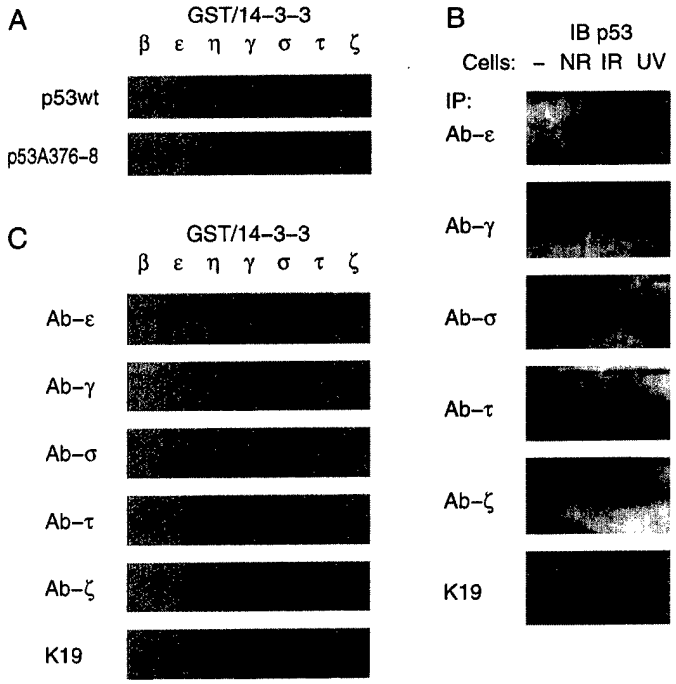

Fig. 1. Interaction of p53 with specific 14-3-3 isoforms. *A*, interaction of GST/14-3-3 isoforms with ^{35}S-labeled *in vitro* translated wild-type p53 or p53 with alanines at positions 376–8. The latter protein serves as a negative control, because it does not bind to 14-3-3. *B*, coimmunoprecipitation of p53 with specific 14-3-3 isoforms. Lysates prepared from nonirradiated cells (*NR*) or cells exposed to IR or UV light were immunoprecipitated (*IP*) with 14-3-3 isoform-specific antibodies or antibody K19 and coprecipitated p53 was detected by immunoblotting (*IB*). As a control, buffer only (*Cells* −) was used instead of the cell lysates. *C*, specificity of 14-3-3 isoform-specific antibodies. GST/14-3-3 isoforms were immunoblotted with the isoform-specific antibodies or with antibody K19, which recognizes all 14-3-3 isoforms.

Functional Significance of the p53/14-3-3 Interaction. To examine the functional significance of the p53/14-3-3 interaction, we assembled a panel of COOH-terminal p53 mutants that interacted with 14-3-3 proteins to varying degrees and examined their function in tissue culture cells. The panel consisted of p53 mutants with single substitutions of Ser^{376} to Ala (A376), Thr^{377} to Ala (A377) or Ser^{378} to Ala (A378) and a p53 mutant with all of these three amino acid substitutions (A376–8). These mutants were selected because the interaction between p53 and 14-3-3 *in vitro* and *in vivo* is regulated by the phosphorylation states of Ser^{376} and Ser^{378} (16).

The functional properties of the COOH-terminal p53 mutants were first examined in U2OS osteosarcoma cells, which have been used extensively to study p53 activation in response to DNA damage (7, 10, 16). Because U2OS cells express wild-type p53, the COOH-terminal p53 mutants were modified in two ways (Fig. 2*A*): (*a*) an NH_2-terminal HA tag was inserted to distinguish them from endogenous p53; and (*b*) seven amino acid substitutions were introduced in the tetramerization domain. The modified domain, hereafter referred to as IND (independent), allows the COOH-terminal p53 mutants to form tetramers but prevents hetero-oligomerization with endogenous p53 (20). Transient transfection of U2OS cells with 0.1 μg of plasmid DNA encoding HA-tagged p53IND with a wild-type (p53INDwt) or mutant (p53INDA376, A377, A378, and A376–8) COOH terminus led to low levels of p53 protein expression, which increased significantly in response to IR or UV light (data not shown; Ref. 7). The DNA damage-induced p53 stabilization was a handicap for this study, which focuses on regulation of p53 functional activity. However, transfecting the cells with 2.5 μg of plasmid DNA led to higher levels of p53 protein, which did not increase further in response to DNA damage (Fig. 2*B*), allowing us to study the effects of DNA damage on p53 activity independently of its effects on p53 protein levels.

The ectopically expressed p53INDwt protein behaved similarly to endogenous wild-type p53 in that it interacted with 14-3-3 proteins only in cells exposed to IR (Fig. 2*C*). The COOH-terminal substitutions had no effect on p53 protein expression (Fig. 2*D*), but as expected, some of the substitutions interfered with the ability of p53 to interact with 14-3-3 proteins (Fig. 2*E*). Specifically, the interaction of p53 with 14-3-3 was abrogated by substitution of Ser^{376}, unaffected by substitution of Thr^{377} and weakened by substitution of Ser^{378}. Thus, these mutants, which differ in their ability to associate with 14-3-3 proteins *in vivo*, were used to study the functional significance of the p53/14-3-3 interaction.

Sequence-specific DNA binding was examined by transfecting the panel of p53 COOH-terminal mutants in U2OS cells, preparing cell lysates, and analyzing the DNA binding activities of the ectopically expressed p53 proteins in these lysates. The lysates were prepared 1 h after exposure of the cells to IR or from mock-irradiated cells. The HA-tagged p53IND proteins were examined for their ability to bind to beads coated with oligonucleotides containing the specific p53 DNA binding site or a nonspecific DNA site. p53INDwt bound to beads coated with the specific oligonucleotide but not to beads coated with

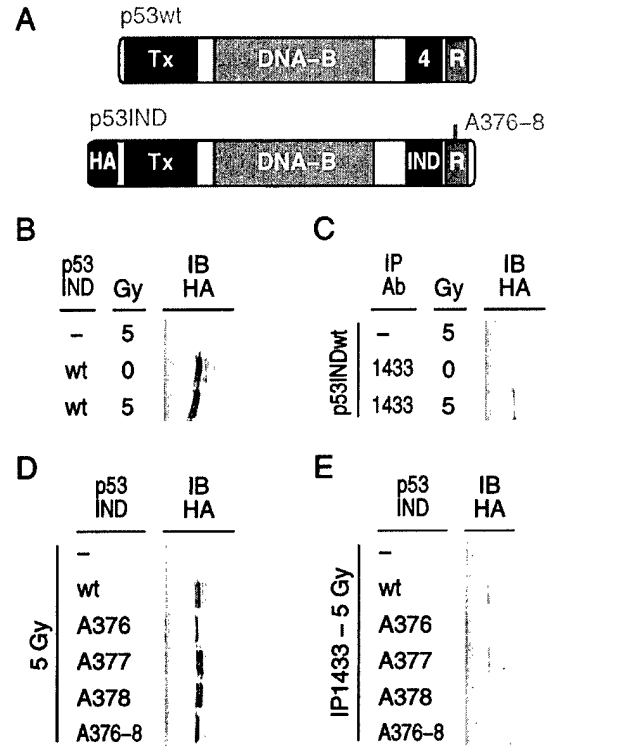

Fig. 2. Interaction of p53 COOH-terminal mutants with 14-3-3 proteins in U2OS cells. *A*, diagrammatic representations of wild-type p53 (*p53wt*) and p53IND. *HA*, hemagglutinin tag; *Tx*, transactivation domain; *DNA-B*, sequence-specific DNA binding domain; *4*, native tetramerization domain; *IND*, modified independent tetramerization domain; *R*, COOH-terminal regulatory region, where the alanine substitutions targeting residues 376–378 map. *B*, protein levels of p53INDwt in mock-irradiated (0 Gy) and irradiated (5 Gy) U2OS cells. *IB*, immunoblot. *C*, interaction between p53INDwt and endogenous 14-3-3 in mock-irradiated and irradiated U2OS cells. *IP Ab*, immunoprecipitation antibody; *IB*, immunoblot. *D*, protein levels of p53INDwt and COOH-terminal p53IND mutants in irradiated (5 Gy) U2OS cells. Note that the COOH-terminal mutants with Ala at position 376, unlike p53INDwt and the rest of the mutants, do not migrate as doublets. The basis for this difference in electrophoretic migration is not understood. *IB*, immunoblot. *E*, interaction between p53INDwt and COOH-terminal p53IND mutants with endogenous 14-3-3 in irradiated U2OS cells. *IB*, immunoblot.

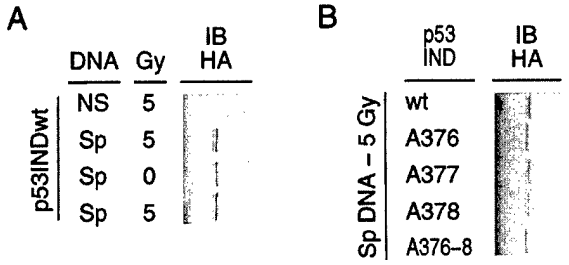

Fig. 3. Sequence-specific DNA binding of wild-type and mutant p53 proteins expressed in U2OS cells by transient transfection. *A*, cell lysates containing HA-tagged p53INDwt prepared from nonirradiated (0 Gy) or irradiated (5 Gy) cells were incubated with beads coated with oligonucleotides containing the specific (*Sp*) p53 binding site or a nonspecific (*NS*) site. Bound p53 was detected by immunoblotting. *B*, cell lysates containing HA-tagged p53IND proteins with wild-type or mutant COOH termini were incubated with beads coated with oligonucleotides containing the specific (*Sp*) p53 binding site. Bound p53 was detected by immunoblotting.

the nonspecific oligonucleotide, establishing the sequence specificity of the assay (Fig. 3*A*). Exposure of the cells to IR did not affect the sequence-specific DNA binding activity. Furthermore, the p53IND proteins with mutant 14-3-3 binding sites bound the specific DNA as efficiently as p53INDwt (Fig. 3*B*). Thus, the association of p53 with 14-3-3 proteins did not affect the sequence-specific DNA binding activity of p53 in this assay.

We subsequently examined the ability of the p53 COOH-terminal mutants to activate expression of the endogenous *p21/cip1/waf1* gene. For these studies, expression of the endogenous wild-type p53 protein in U2OS cells complicated the analysis, because only a subset of the transfected cells actually expressed the p53 COOH-terminal mutants, and both the endogenous and ectopically expressed p53 could contribute to *p21/cip1/waf1* expression. We therefore decided to pursue analysis of the transcriptional activity of the p53 COOH-terminal mutants in Saos2 osteosarcoma cells, which do not express endogenous p53. The mutants interacted with endogenous 14-3-3 in irradiated Saos2 cells as described above for U2OS cells (Fig. 4*A*), and their ability to activate transcription of the endogenous *p21/cip1/waf1* gene correlated with their ability to interact with 14-3-3 (Fig. 4*B*). In contrast, all p53 COOH-terminal mutants activated transcription from a reporter plasmid (Fig. 4*C*), suggesting that the effect of 14-3-3 on p53 transcriptional activity is not evident in transient reporter assays.

The ability of the p53 COOH-terminal mutants to induce cell cycle arrest was studied in U2OS osteosarcoma cells. The research design involved coexpressing three proteins in transiently transfected cells: green fluorescent protein to mark the efficiently transfected cells; a dominant-negative, tumor-derived p53 mutant to inactivate endogenous wild-type p53; and one of the p53IND proteins described above. Parental (nontransfected) U2OS cells arrested predominantly in G_2 after exposure to IR, whereas cells expressing p53INDwt showed a significant fraction of cells arresting in G_1 (Fig. 5). The COOH-terminal mutants that did not interact with 14-3-3 proteins were unable to induce G_1 arrest; p53INDA378, which interacted weakly with 14-3-3 proteins, induced partial G_1 arrest; and p53INDA377, which interacted strongly with 14-3-3 proteins, induced G_1 arrest as efficiently as p53INDwt (Fig. 5).

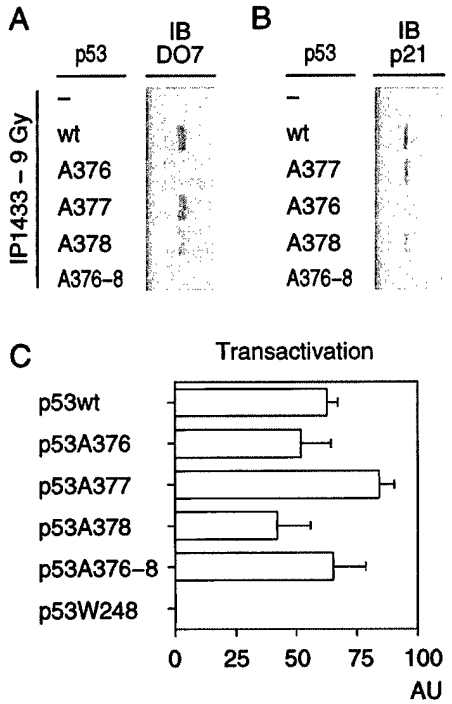

Fig. 4. Transcriptional activities of wild-type p53 and p53 COOH-terminal mutants in Saos2 cells. *A*, interaction of p53 COOH-terminal mutants with endogenous 14-3-3 proteins in irradiated Saos2 cells. *IB*, immunoblot. *B*, transactivation of the endogenous *p21/cip1/waf1* gene, as indicated by determining the levels of p21/waf1/cip1 protein in cells transiently transfected with the indicated p53 proteins. *IB*, immunoblot. *C*, transcriptional activities of the same p53 mutants using a reporter plasmid that contains the p53 binding site present in the *p21/waf1/cip1* gene. The tumor-derived p53 mutant p53W248 serves as a negative control.

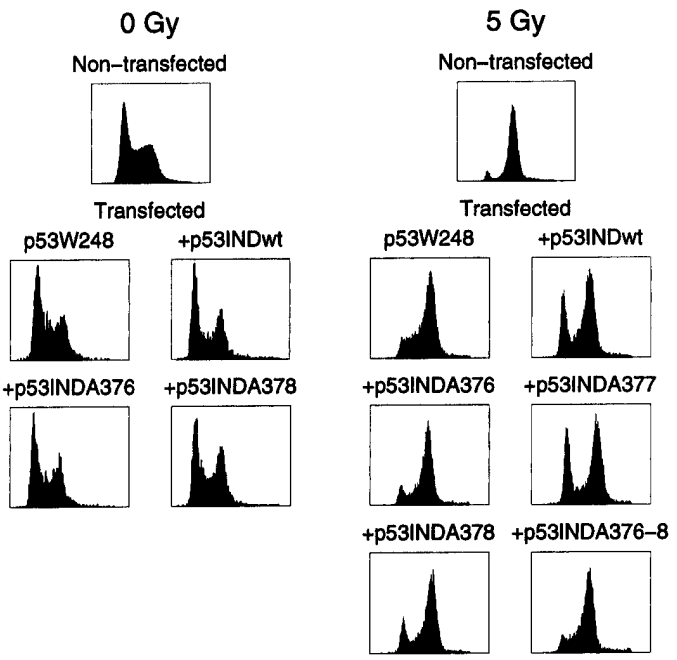

Fig. 5. Cell cycle arrest activities of wild-type p53 and p53 COOH-terminal mutants in U2OS cells. The cells were either not transfected or transfected with plasmids encoding the indicated proteins. All transfected cells express a tumor-derived p53 mutant (*p53W248*), and some transfected cells additionally express a p53IND protein, as indicated. The cells were either mock-irradiated (*0 Gy*) or exposed to 5 Gy IR.

Discussion

The first question that we addressed in this study is that of isoform specificity in the interaction between p53 and 14-3-3. There are seven distinct genes encoding 14-3-3 isoforms in mammals, and these are highly related to each other at the amino acid sequence level. Yet, despite the high similarity, the isoforms differ significantly in their affinity for specific ligands (21). Indeed, we observed that *in vitro* the γ isoform bound p53 with significantly higher affinity than the other isoforms. *In vivo*, we observed an interaction of p53 with 14-3-3 γ but also with isoforms ϵ and τ. This could reflect different posttranslational modifications of p53 or 14-3-3 *in vitro* and *in vivo*, differences in the relative abundance of the 14-3-3 isoforms *in vivo*, and/or heterodimerization of different 14-3-3 isoforms *in vivo*. Interestingly, we did not observe a significant interaction between p53 and 14-3-3 σ. If these two proteins interacted, then we would have the potential for a positive feedback loop leading to p53 activation, because p53 induces expression of the 14-3-3 σ gene (4) and binding of 14-3-3 to p53 enhances its functional activity.

The second question that we wanted to address is whether the interaction between p53 and 14-3-3 proteins is functionally significant. Analysis of the function of p53 mutants that are defective in their ability to interact with 14-3-3 suggests that 14-3-3 enhances p53 function. However, we cannot exclude the possibility that the substitution we introduced affected not only the interaction of p53 with 14-3-3 but also some other p53-protein interaction or some p53 posttranslational modification. Thus, it is formally possible that the functional defects were not attributable to the disruption of the p53/14-3-3 interaction. Nevertheless, we think that the p53/14-3-3 interaction is functionally important, because p53/14-3-3 binding and p53 activity correlated well in our panel of p53 mutants. The mechanism by which 14-3-3 proteins could enhance p53 function remains elusive. We used an *ex vivo* DNA binding assay, and it appears that 14-3-3 proteins do not affect the sequence-specific DNA binding activity of p53. However, this needs to be examined more carefully using chromosome-immunoprecipitation assays. 14-3-3 proteins also did not appear to affect the intracellular localization of p53 (data not shown). Instead, the results raise the possibility that 14-3-3 enhances the transcriptional activity of p53. Interestingly, a similar role for 14-3-3 proteins has been proposed in plant cells, where 14-3-3 proteins have been shown to facilitate interaction of sequence-specific DNA binding transcription factors with the basal transcription machinery (22). Further analysis of the p53 mutants described in this study may help elucidate the mechanisms by which p53 exerts its tumor suppressor effect.

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References

1. Vogelstein, B., Lane, D., and Levine, A. J. Surfing the p53 network. Nature (Lond.), *408:* 307–310, 2000.
2. Zhou, B. B., and Elledge, S. J. The DNA damage response: putting checkpoints in perspective. Nature (Lond.), *408:* 433–439, 2000.
3. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. Cell, *75:* 817–825, 1993.
4. Hermeking, H., Lengauer, C., Polyak, K., He, T. C., Zhang, L., Thiagalingam, S., Kinzler, K. W., and Vogelstein, B. 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. Mol. Cell, *1:* 3–11, 1997.
5. Chan, T. A., Hermeking, H., Lengauer, C., Kinzler, K. W., and Vogelstein, B. 14-3-3 sigma is required to prevent mitotic catastrophe after DNA damage. Nature (Lond.), *401:* 616–620, 1999.
6. Chan, T. A., Hwang, P. M., Hermeking, H., Kinzler, K. W., and Vogelstein, B. Cooperative effects of genes controlling the G(2)/M checkpoint. Genes Dev., *14:* 1584–1588, 2000.
7. Chehab, N. H., Malikzay, A., Stavridi, E. S., and Halazonetis, T. D. Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. Proc. Natl. Acad. Sci. USA, *96:* 13777–13782, 1999.
8. Unger, T., Juven-Gershon, T., Moallem, E., Berger, M., Sionov, R. V., Lozano, G., Oren, M., and Haupt, Y. Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2. EMBO J., *18:* 1805–1814, 1999.
9. Shieh, S. Y., Taya, Y., and Prives, C. DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, Ser20, requires tetramerization. EMBO J., *18:* 1815–1823, 1999.
10. Chehab, N. H., Malikzay, A., Appel, M., and Halazonetis, T. D. Chk2/hCds1 functions as a DNA damage checkpoint in G1 by stabilizing p53. Genes Dev., *14:* 278–288, 2000.
11. Hirao, A., Kong, Y. Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S. J., and Mak, T. W. DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science (Wash. DC), *287:* 1824–1827, 2000.
12. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. Mdm2 promotes the rapid degradation of p53. Nature (Lond.), *387:* 296–299, 1997.
13. Kubbutat, M. H., Jones, S. N., and Vousden, K. H. Regulation of p53 stability by Mdm2. Nature (Lond.), *387:* 299–303, 1997.
14. Midgley, C. A., and Lane, D. P. p53 protein stability in tumour cells is not determined by mutation but is dependent on Mdm2 binding. Oncogene, *15:* 1179–1189, 1997.
15. Giaccia, A. J., and Kastan, M. B. The complexity of p53 modulation: emerging patterns from divergent signals. Genes Dev., *12:* 2973–2983, 1998.
16. Waterman, M. J., Stavridi, E. S., Waterman, J. L., and Halazonetis, T. D. ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins. Nat. Genet., *19:* 175–178, 1998.
17. Waterman, M. J., Waterman, J. L., and Halazonetis, T. D. An engineered four-stranded coiled coil substitutes for the tetramerization domain of wild-type p53 and alleviates transdominant inhibition by tumor-derived p53 mutants. Cancer Res., *56:* 158–163, 1996.
18. Waterman, J. L., Shenk, J. L., and Halazonetis, T. D. The dihedral symmetry of the p53 tetramerization domain mandates a conformational switch upon DNA binding. EMBO J., *14:* 512–519, 1995.
19. Liu, L., Scolnick, D. M., Trievel, R. C., Zhang, H. B., Marmorstein, R., Halazonetis, T. D., and Berger, S. L. p53 sites acetylated *in vitro* by PCAF and p300 are acetylated *in vivo* in response to DNA damage. Mol. Cell. Biol., *19:* 1202–1209, 1999.
20. Stavridi, E. S., Chehab, N. H., Caruso, L. C., and Halazonetis, T. D. Change in oligomerization specificity of the p53 tetramerization domain by hydrophobic amino acid substitutions. Protein Sci., *8:* 1773–1779, 1999.
21. Rosenquist, M., Sehnke, P., Ferl, R. J., Sommarin, M., and Larsson, C. Evolution of the 14-3-3 protein family: does the large number of isoforms in multicellular organisms reflect functional specificity? J. Mol. Evol., *51:* 446–458, 2000.
22. Pan, S., Sehnke, P. C., Ferl, R. J., and Gurley, W. B. Specific interactions with TBP and TFIIB *in vitro* suggest that 14-3-3 proteins may participate in the regulation of transcription when part of a DNA binding complex. Plant Cell, *11:* 1591–1602, 1999.