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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 first year of funding we have began to address Tasks 1 and 2. We have established that the interaction of p53 with 14-3-3 is critical for the ability of p53 to induce cell cycle arrest (Task 2), but appears to not affect the DNA binding activity of p53 (Task 1). Overall, the results support the initial hypothesis that the interaction of p53 with 14-3-3 activates the tumor suppressor function of p53, but also indicate that the mechanism might not be through activation of the sequence-specific DNA binding activity of p53, as originally thought.

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Thanos Hilarianetis 5-11-00

PI - Signature Date

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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 have initiated work to address Tasks 1 and 2. As described below, using DNA binding assays of the type described in the proposal, we have not seen an effect of association of p53 with 14–3–3 proteins on the sequence–specific DNA binding activity of p53 (Task 1). Because these DNA binding assays measure the DNA binding activity of p53 after it is isolated from cells, the possibility exists that these assays do not accurately reflect the DNA binding activity of p53 in the cell. We are therefore developing new methods to analyze the DNA binding activity of p53 in the cell (Chromatin–Immunoprecipitation Assays) with the intention to reanalyze the effect of the association of p53 with 14–3–3 proteins on the DNA binding activity of p53. Our efforts to address Task 2 have led to definitive answers. Specifically, we have demonstrated that the cell cycle arrest function of p53 is dependent on its interaction with 14–3–3 proteins, thereby supporting our hypothesis that this interaction underlies the increase in p53 functional activity after DNA damage. To complete the experiments described in Task 2, we need to study the apoptotic activity of p53, which will be done during the second year of USARMRC research funding. A more detailed description of the research accomplished during the first year of funding is described below.

Task 1: Determine whether the interaction of p53 with 14–3–3 affects the sequence–specific DNA binding activity of p53.

As a first step to evaluate the physiological significance of the interaction of p53 with 14–3–3, we generated p53 mutants that cannot interact with 14–3–3. The region of p53 that is recognized by 14–3–3 proteins includes Ser376, Thr377 and Ser378. Using in

vitro assays to monitor the interaction of p53 with 14–3–3 proteins, we had shown that serines 376 and 378 are important for the interaction of p53 with 14–3–3 proteins, whereas Thr377 is not important. Accordingly, we generated the following p53 mutants: Ser376 to Ala (A376), Thr377 to Ala (A377), Ser378 to Ala (A378) and a mutant that has all these three amino acid substitutions (A376–8).

Because of the need to study these mutants in cancer cell lines that have endogenous p53 protein, all the amino acid substitutions described above were generated in the context of p53IND, a p53 protein that has a modified tetramerization domain. p53IND forms homotetramers and functions indistinguishably from wild–type p53, but it does not hetero–oligomerize with endogenous p53 proteins (Stavridi et al., 1999), and thus can be used in cell lines that express endogenous p53. In addition, p53IND was engineered with an N–terminal hemaglutinin (HA) tag, so that it could be distinguished from endogenous p53 using monoclonal antibodies that recognize the HA tag.

The p53IND proteins with wild–type (wt) or mutant 14–3–3 binding sites were expressed in a variety of cancer cell lines of breast and non–breast origin. The levels of expression of p53INDwt were not affected by exposure of cells to DNA damage (5 Gray (Gy) of ionizing radiation (IR)) (Fig. 1A) and all p53IND mutants were expressed at levels equivalent to those of p53INDwt (Fig. 1C). As we had previously demonstrated for endogenous wild–type p53 of breast cancer cells (Waterman et al., 1998), p53INDwt interacted with endogenous 14–3–3 proteins only in irradiated cells (Fig. 1B). The p53IND proteins with mutant 14–3–3 binding sites interacted with endogenous 14–3–3 to a variable degree: p53INDA377 interacted as well as p53INDwt; p53INDA376 and p53INDA376–8 showed no interaction; and p53INDA378 interacted weakly with endogenous 14–3–3 (Fig. 1D).

To study the functional significance of the interaction of p53 with 14–3–3, the above described p53IND mutants were examined for their ability to bind sequence–specific DNA. Cells expressing p53IND proteins with wt or mutant 14–3–3 binding sites were either not irradiated or exposed to IR; cell extracts were prepared 1 hr later and the p53IND proteins in these extracts were examined for their ability to bind to beads coated with oligonucleotides containing the specific p53 DNA binding site or a non–specific DNA site. p53INDwt bound to the beads coated with the specific oligonucleotide, but not to beads coated with the non–specific oligonucleotide, establishing that the assay measures the sequence–specific DNA binding activity of p53 (Fig. 2A). Exposure of cells to IR did not affect the sequence–specific DNA binding of p53INDwt (Fig. 2A). The p53IND proteins with mutant 14–3–3 binding sites bound the specific DNA as efficiently p53INDwt (Fig. 2B). Thus, the association of p53 with 14–3–3 proteins does not affect the sequence–specific DNA binding activity of p53 in this assay.

Because the DNA binding assay was performed using cell extracts, there is a concern that it may not accurately reflect binding of p53 to the promoters of its target genes in the cell. A recently–described assay, the chromatin immunoprecipitation (CHIP) assay, allows us to study binding of p53 to its target genes in cells. We are therefore currently developing the CHIP assay to study binding of the various p53IND proteins to the promoter of the p53–target gene p21/cip1/waf1. The CHIP assay is a recently–described assay and was, therefore, not included in our original proposal. Nevertheless, we feel that it is important to use this new assay, since it allows us to study p53 DNA binding in cells, whereas the previously available assays could only study p53 DNA binding in the context of cell extracts.

Task 2: Determine whether the interaction of p53 with 14–3–3 modulates the cell cycle arrest and/or apoptotic functions of p53.

To study whether association of p53 with 14–3–3 affects the ability of p53 to induce cell cycle arrest in G1 in response to irradiation we used the same experimental system, as described above for sequence–specific DNA binding. First, we monitored the response of non–transfected cells to DNA damage (5 Gy IR). The DNA profile of non–irradiated cells shows many cells in G1, S and G2/M, consistent with cycling cells. 12 hrs after exposure to IR, most cells were arrested in G2 and a few cells were arrested in G1. Practically no cells were in S phase (Fig. 3A). Cells expressing a tumor–derived p53 mutant (p53W248) behaved essentially like the non–transfected cells (Fig. 3B). Cells expressing p53INDwt showed a peak of cells arrested in G1 after exposure to IR, indicating that p53INDwt, like wild–type p53, induces cell cycle arrest. The p53IND mutants that do not interact with 14–3–3 proteins (based on our analysis shown in Fig. 1D) were unable to induce G1 arrest (Fig. 3B); whereas p53INDA377, which binds to 14–3–3 proteins as efficiently as p53INDwt (Fig. 1D), induced G1 arrest in cells exposed to IR (Fig. 3B). These results support our hypothesis that the association of p53 with 14–3–3 proteins is important for p53 function.

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 and their ability to induce cell cycle arrest in response to DNA damage.

REPORTABLE OUTCOMES

We are currently preparing a manuscript describing the cell cycle defect of the p53 mutant proteins that fail to interact with 14–3–3 proteins. While the experiments that will be described in this manuscript have been completed, we are still in the process of writing this manuscript, and therefore cannot yet provide a copy.

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.

The second conclusion supports 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.

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Figure 1
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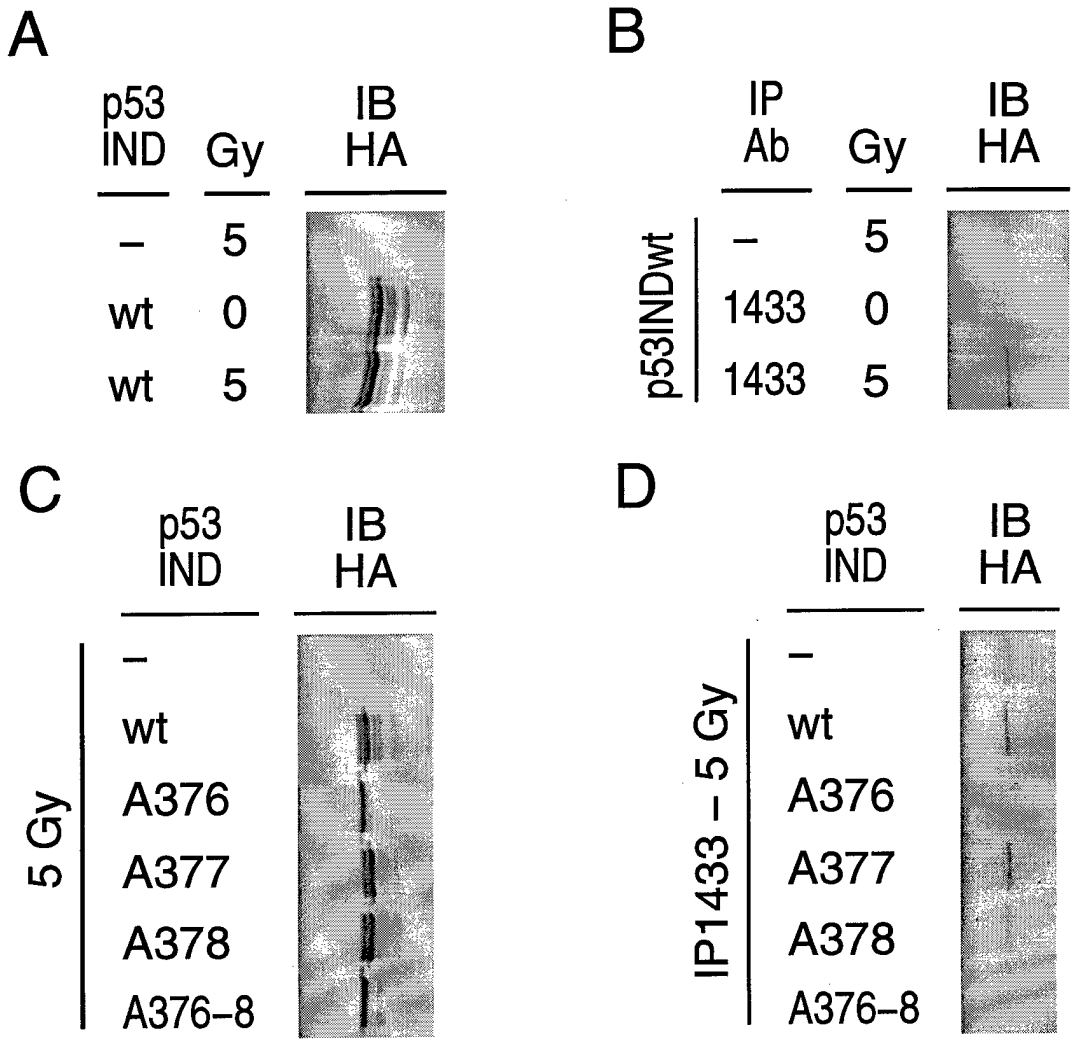

Characterization of the expression and interaction with 14–3–3 proteins of p53 proteins with amino acid substitutions targeting the 14–3–3 binding site of p53.
A. Protein levels of p53IND with a wild–type (wt) 14–3–3 binding site in non–irradiated (0 Gy) and irradiated (5 Gy) cells. Protein levels were determined by immunoblotting (IB) with an antibody that recognizes the HA tag of p53IND.
B. Interaction of 14–3–3 with p53IND with a wild–type (wt) 14–3–3 binding site in non–irradiated (0 Gy) and irradiated (5 Gy) cells. The interaction was determined by immunoprecipitation (IP) of 14–3–3 proteins with an antibody (Ab) that recognizes 14–3–3 (1433), followed by IB with an antibody that recognizes the HA tag of p53IND.
C. Protein levels of p53IND proteins with wt or mutant 14–3–3 binding sites.
D. Interaction of 14–3–3 with p53IND with wt or mutant 14–3–3 binding sites.

Figure 2
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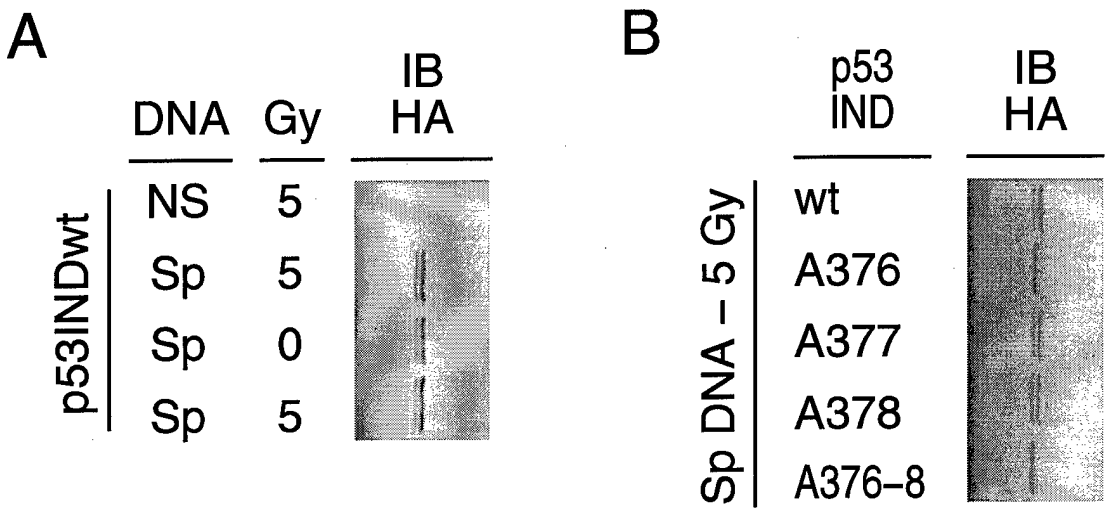

DNA binding activity of p53 proteins with amino acid substitutions targeting the 14–3–3 binding site of p53.
A. Non–specific (NS) and sequence–specific (Sp) DNA binding activity of p53IND with a wild–type (wt) 14–3–3 binding site extracted from non–irradiated (0 Gy) or irradiated (5 Gy) cells. p53IND bound to beads coated with NS or Sp DNA was detected by immunoblotting (IB) with an antibody that recognizes the HA tag of p53IND.
B. Sequence–specific (Sp) DNA binding activity of p53IND with wt or mutant 14–3–3 binding sites extracted from irradiated (5 Gy) cells. p53IND bound to beads coated with Sp DNA was detected by immunoblotting (IB) with an antibody that recognizes the HA tag of p53IND.

Figure 3
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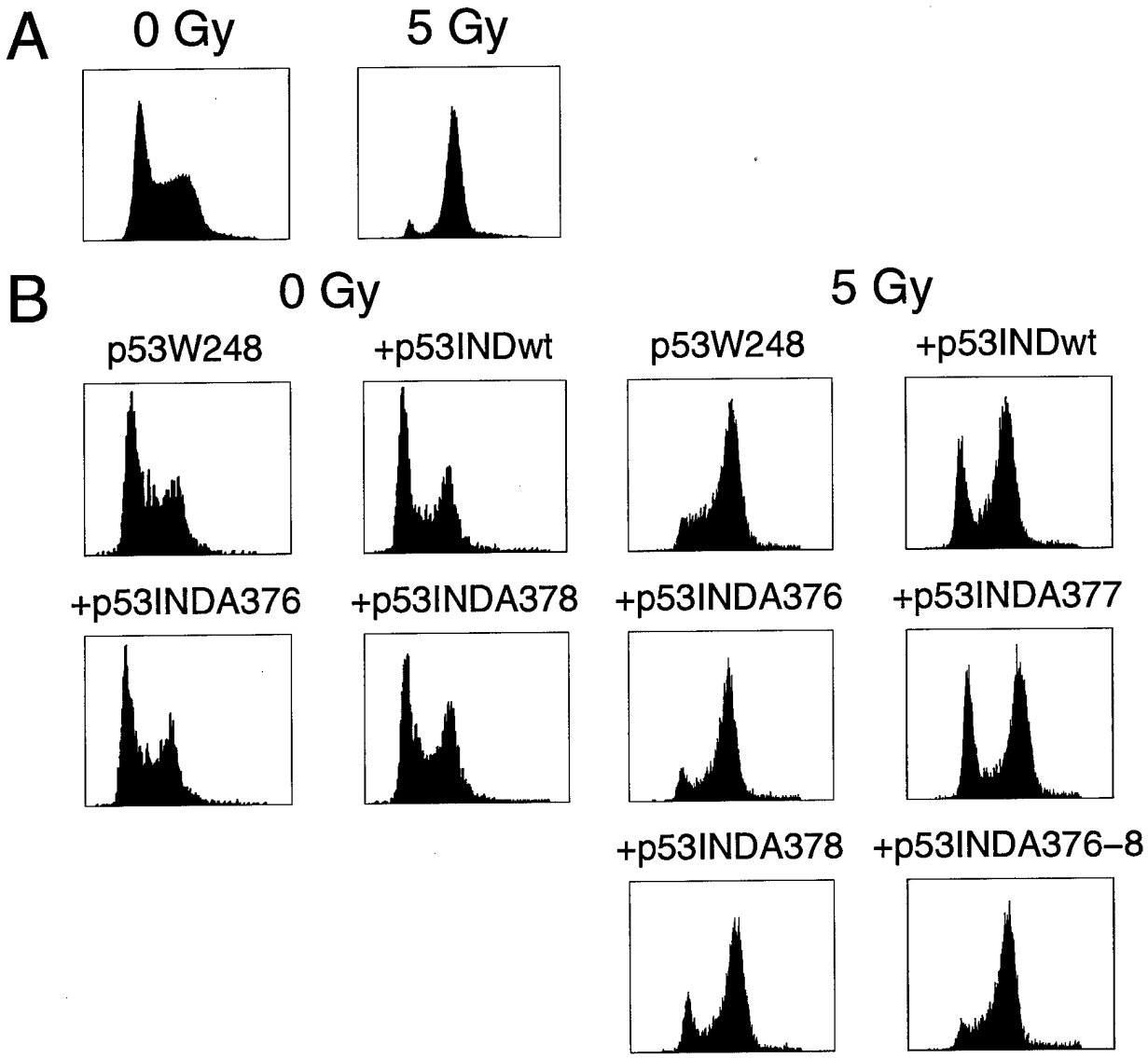

Cell cycle arrest activity of p53 proteins with amino acid substitutions targeting the 14–3–3 binding site of p53.
A. Cell cycle profile of non–irradiated (0 Gy) and irradiated (5 Gy) non–transfected cells.
B. Cell cycle profile of non–irradiated (0 Gy) and irradiated (5 Gy) cells transfected with plasmids expressing a tumor–derived p53 mutant (p53W248) or p53IND proteins with wild–type (wt) or mutant 14–3–3 binding sites.