Germ-Line-derived Hinge Domain p53 Mutants Have Lost Apoptotic but not Cell Cycle Arrest Functions

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ABSTRACT

The protein p53 is a critical tumor suppressor, as demonstrated by its frequent mutation in human cancers. Overexpression of the wild-type form of the p53 tumor suppressor gene in human cancer cell lines has been shown to lead to either cell cycle arrest or apoptosis. A study of two Li-Fraumeni syndrome-derived p53 hinge domain mutants shows that both mutants retain the ability to arrest cell growth but are significantly impaired for the induction of apoptosis in human p53-null cell lines. This indicates that the hinge domain may be important in the regulation of p53-dependent apoptosis.

INTRODUCTION

The p53 tumor suppressor gene is a critical regulator of tumorigenesis, as demonstrated by the loss of functional p53 in greater than half of human cancers (1). In mice, homozygous deletion of the p53 gene results in tumor formation within 3–6 months after birth (2). On the molecular level, p53 is important in the cellular response to DNA damage events and has been shown to carry this out in a cell type-specific manner by one of two mechanisms: (a) cell cycle arrest; or (b) apoptosis (3–5). These two responses have been shown to be, in part, a result of the transcriptional regulation of the effector genes of these pathways, because p53 has been shown to act as a transcriptional activator of genes with promoters containing p53-binding sites (6).

However, there is recent evidence that the p53-dependent cell cycle arrest and apoptotic pathways may be regulated by p53 through mechanisms other than transcriptional activation (7–10). In these studies, p53 mutants have retained the ability to induce apoptosis but not cell cycle arrest (10) or vice versa (7–9). Each of the mutants used in these studies was a missense substitution localized to the sequence-specific DNA-binding domain of the protein (as are a vast majority of tumorigenic mutations of p53; Ref. 1). This report focuses on two mutations derived from two respective patients with germ-line mutations to p53 that lie outside of the DNA-binding domain and within the hinge domain that joins the DNA-binding domain to the tetramerization domain. We have found that they both retained the ability to induce cell cycle arrest but are significantly impaired in their ability to induce apoptosis. This is the first characterization of germ-line-derived p53 mutations behaving in such a manner and underscores the need for a functional p53-dependent apoptotic pathway for controlling tumorigenic growth. This study also suggests that the p53-dependent apoptotic pathway and the cell cycle arrest pathway may require differential protein-DNA and/or protein-protein interactions for their regulation.

MATERIALS AND METHODS

Cell Lines and Conditions. Non-small cell lung carcinoma Calu 6 cells were obtained from Dr. C. C. Harris (NCI-NIH, Bethesda, MD) and are null for endogenous p53. EB cells are colon cancer cell lines obtained from the lab of Dr. P. Shaw (Institute of Pathology, Lausanne, Switzerland) and are null for p53. The overexpression of transfected wt p53 in these cells results in apoptosis (4). The human osteosarcoma cell line Saos-2 is also null for p53. All cell lines were grown in DMEM with 10% FBS.

Transcriptional Regulation Assays and Western Blot Analysis. The WAF1 promoter (WWP-luc) and Bax promoter (Bax-luc) constructs were gifts from Drs. B. Vogelstein (The Johns Hopkins University, Baltimore, MD) and J. C. Reed (Ludwig Institute for Cancer Research, La Jolla, CA), respectively. Calu 6 cells (6 x 10^5) were plated into each well of a 6-well plate. The next day, 3 μg of either WWP-luc or Bax-luc were cotransfected with 1 μg of a p53 expression plasmid using the Lipofectin procedure (Life Technologies, Inc.). The p53 expression plasmid consists of wt or mutant p53 cDNA cloned into the BamHI site of pRE4 (Invitrogen). Also cotransfected with the p53 expression plasmid and the firefly luciferase reporter (e.g., WWP-luc or Bax-luc) is an internal control plasmid, pCMV-RL, expressing renilla luciferase (Promega). Each transfection of the different p53 expression plasmids was done in duplicate for each experiment, and the experiment was repeated at least four times with the indicated mutants and controls. The ratio of firefly:renilla luciferase was calculated for each transfection, and the results were expressed as the -fold transactivation relative to wt p53 averaged over the set of experiments.

Western blots were performed by collecting a set of cell pellets from one of the transient transfections used for studying transactivation of the WWP-luc construct. Cells were lysed in a buffer containing 150 mM NaCl (Fisher), 1% NP40 (Sigma), 0.5% deoxycholic acid (Sigma), 0.1% SDS (Fisher), 50 mM Tris (pH 8.0), Sigma), and 1 mM phenylmethylsulfonyl fluoride (Sigma). A 10% SDS-polyacrylamide gel was loaded with 100 μg of the indicated cell lysate. The protein was then immunoblotted onto Immobilon-P nylon membrane (Millipore), incubated with DO-1 anti-p53 antibody (Santa Cruz; 1:500 dilution) followed by peroxidase-conjugated antigoat IgG (Santa Cruz), and developed with the Amersham enhanced chemiluminescence detection system according to the manufacturer’s protocol.

Colony Formation Assays. Cells (2 x 10^5) were plated in 6-well tissue culture plates and allowed to adhere overnight. The next day, the cells were transfected with 3 μg of a pRE4-derived expression vector (encoding a hygromycin resistance gene) using the Lipofectin procedure and allowed to incubate for 16 h. Selection for hygromycin-resistant colonies was started 48 h posttransfection. After 12–14 days of selection, hygromycin-resistant colonies were fixed and stained with crystal violet and 50% methanol. Colonies of approximately 10 or more cells were counted.

Transfection of Saos-2 and EB Cells for FACS Analysis. The day before transfection, 1 x 10^5 Saos-2 cells or 3 x 10^6 EB cells were seeded into 60-mm tissue culture flasks. The next day, the cells were transfected with various p53 expression vectors identified above via the Lipofectin procedure. Serum-containing medium was added to the cells 4 h posttransfection. Floating and adherent cells were then harvested at the indicated times and fixed in 100% methanol for at least 2 h at 20°C. The cells were then rehydrated in PBS and incubated with 150 μg/ml RNase A for 30 min at room temperature, pelleted, gently resuspended in a 1:100 dilution of mouse anti-p53 DO-1 primary antibody (Santa Cruz), diluted in PBS and 2.5% BSA, and incubated at room temperature for 30 min. After two PBS washes, the cells were resuspended in a 1:60 dilution of goat antimouse FITC-conjugated secondary antibody (DAKO) in PBS and...
trum). Samples were analyzed on a FACScan cell scanner (Becton Dickinson), expressed p53 protein.

transiently transfected CaLu 6 cells used in a shows that there are detectable levels of plasmid containing the indicated p53 cDNA into p53-null CaLu 6 cells. The p53 mutants promoter, a. the reporter plasmid pWWP-luc, which contains the /2/WAP"l/CIIM promoter and the ApoTAG Direct kit (Oncor) and used according to the manufacturer's solution.

immunocytochemistry Analysis. Saos-2 cells were plated onto sterile acid-washed glass coverslips. The next day, cells were transfected with 2 /µg/ml propidium iodide. The samples were then filtered through Spectra Mesh filters (Spectrum). Samples were analyzed on a FACSscan cell scanner (Becton Dickinson), and the data were analyzed by ModFIT/LT software (Verity Software). Cells were gated to remove doublets and very small debris and gated for high FITC fluorescence.

Immunocytochemistry Analysis. Saos-2 cells were plated onto sterile acid-washed glass coverslips. The next day, cells were transfected with 2 /µg/ml propidium iodide. The samples were then filtered through Spectra Mesh filters (Spectrum). Samples were analyzed on a FACSscan cell scanner (Becton Dickinson), and the data were analyzed by ModFIT/LT software (Verity Software). Cells were gated to remove doublets and very small debris and gated for high FITC fluorescence.

RESULTS

Two LFS-derived p53 mutants, one at codon 305 (Lys to Met substitution) and one at codon 325 (Gly to Val substitution), were initially analyzed for their phenotypes in functional assays with the non-small cell lung adenocarcinoma cell line Calu 6 that is null for p53 (11). The germ-line mutation at codon 305 was derived from a proband that had anaplastic astrocytoma at age 35 years (12). The proband with the mutation at codon 325 had non-Hodgkin’s lymphoma at age 17 years and colon carcinoma at age 26 years (13). Both of the mutations lie within an amino acid linker, hereafter referred to as the hinge domain, that links the sequence-specific DNA-binding domain (spanning residues 102–292) to the tetramerization domain that encompasses residues 326–354 (14, 15). The cancerous phenotypes associated with these two germ-line mutations prompted the characterization of the functional impairment of each mutation.

Initially, we analyzed the ability of the p53 mutants to elicit cell cycle regulation responses. Overexpression of p53 is associated with growth arrest at the G1-S- and G2-M-phase checkpoints of the cell cycle (16, 17). wt p53 is a transcription-regulatory protein containing a potent transactivation domain in its amino terminus. Genes regulated by p53 serve as the effectors of phenotypes associated with p53 overexpression. One gene activated by wt p53 is p21/WAF1/CIP1, which itself binds to and inactivates cyclin-cyclin-dependent kinase complexes (18, 19). The p21/WAF1/CIP1 protein is thus a critical effector of cell cycle control initiated by p53 (20). We analyzed the ability of the p53 mutants to transactivate a reporter construct consisting of the p21/WAF1/CIP1 promoter cloned upstream of a firefly luciferase reporter gene (Fig. 1a) in p53-null Calu 6 cells. The two LFS mutants retained the ability to transactivate the promoter to levels comparable to that of wt p53: mutant Met205 displayed an average transactivation of 77 ± 3.0% of wt p53; whereas mutant Val225 showed an average transactivation of 106 ± 15.8% of wt p53. By contrast, transfection of the missense mutant at codon 143 (Val to Ala substitution), a sporadic tumor-derived mutation, lacked this transactivating property, in agreement with earlier reports (21). The relative activities of the hinge domain mutants and the Ala143 mutant relative to wt p53 did not change over a range of plasmid concentrations (50 ng–1 µg; data not shown).

To investigate whether the hinge domain mutants that retained the ability to transactivate a gene important in the inhibition of cell cycle progression also exhibited an overall arrest of cell growth, we performed colony inhibition assays in Calu 6 cells (Table 1). Expression vectors for the various p53 cDNAs bearing a hygromycin resistance gene were transfected into the cells. After about 2 weeks of selection, the vector control transfection yielded an average of about 100 colonies of >10 cells, wt p53 yielded only an average of 4 colonies/ transfection. However, transfection of the sporadic cancer-derived mutant Ala143 resulted in an average of 70% of the colonies of the vector control. The hinge domain LFS mutants retained all of the growth-inhibiting activity of wt p53. The LFS mutants have therefore displayed two major characteristics associated with the ability of p53 to regulate the cell cycle: (a) p21/WAF1/CIP1 transactivation; and (b) the ability to inhibit cell growth.

We then investigated the capability of the hinge domain mutants to elicit an apoptotic response. wt p53 is activated in response to DNA damage events and is required for G1 arrest after γ-irradiation (16, 22). For some cell types, the activation of p53 results in apoptosis (4, 2191

Table 1  p53-dependent inhibition of colony formation

<table>
<thead>
<tr>
<th>Expression vector</th>
<th>Average no. of colonies</th>
<th>SD</th>
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<tbody>
<tr>
<td>wt p53</td>
<td>4</td>
<td>±1</td>
</tr>
<tr>
<td>Vector control</td>
<td>101</td>
<td>±19</td>
</tr>
<tr>
<td>Ala143</td>
<td>71</td>
<td>±16</td>
</tr>
<tr>
<td>Val225</td>
<td>2</td>
<td>±1</td>
</tr>
<tr>
<td>Met205</td>
<td>9</td>
<td>±4</td>
</tr>
</tbody>
</table>
p53 HINGE DOMAIN IN APOPTOTIC RESPONSE

23). An important effector of the p53-mediated apoptotic pathway is the bax gene, which is itself transactivated by wt p53 (24, 25). In a study of tissue-specific tumor growth induction of choroid plexus epithelial cells in transgenic mice, it was shown that bax-null mice developed tumors at a rate nearly five times that of bax+/+ counterparts, and that bax induction in response to tumor growth was wt p53 dependent (26), pointing out not only the need for Bax protein-mediated apoptosis in these cells, but also the need for p53-mediated apoptosis in curtailing tumor growth in vivo.

We tested the ability of the hinge domain mutants to transactivate a luciferase reporter construct containing the bax promoter cotransfected into CaLu 6 cells (Fig. 2). Transfection of the sequence-specific DNA-binding domain mutant Ala326 completely lost the ability to transactivate the bax promoter, consistent with a previous study performed in H1299 cells (8). The two hinge domain mutants showed a noticeable but not total loss of transactivation of the bax promoter relative to wt p53, showing a 50% decrease in transactivation. There is evidence that Bax is needed for the apoptosis of only certain cell types. For example, mouse thymocytes null for bax are still able to undergo p53-dependent apoptosis (27), yet choroid plexus epithelial cells show a dependence on p53 and bax gene products (26). Additionally, in the transgenic mouse study involving Bax and p53-dependent apoptosis in choroid plexus epithelial cells, bax-null but p53+/+ animals showed less apoptosis (and faster tumor growth) than bax+/+ but p53-null animals, indicating that there are multiple p53-responsive genes involved in the p53-dependent apoptotic pathway (26). Although bax is a major gene through which wt p53 induces an apoptotic pathway, it is most likely not the only p53-responsive gene involved in the pathway.

Because of this, it was important to test the ability of the hinge domain p53 mutants to induce apoptosis in the p53-null cell lines Saos-2 and EB, osteosarcoma and colon carcinoma cell lines, respectively (Fig. 3), that have been well-characterized as undergoing apoptosis in response to wt p53 overexpression (4, 9). Expression vectors bearing either of the two hinge domain mutants or controls were transfected into either Saos-2 or EB cells; samples were collected at 24, 48, and 72 h posttransfection; and the cells were subjected to FACS analysis. As seen in Fig. 3a, there is a consistently higher degree of apoptotic cells (cells containing a sub-G, DNA content) in the population of Saos-2 cells expressing exogenous wt p53 when compared with that in a vector control transfection. There is no appreciable increase in the number of apoptotic cells over that seen in the vector control in transfections with either mutant Ala143 or the two hinge domain mutants Met305 and Val325 over the indicated time course. Fig. 3b shows a representative panel of DNA profiles of transiently transfected Saos-2 cells at 72 h posttransfection and a summary of the percentage of cells at each stage of the cell cycle. Those cells transfected with wt p53 or the hinge mutants have a significantly higher percentage of cells in G1 compared to the mutant Ala143 p53. This is consistent with a G1 arrest. It should be noted that there are cells in S phase in all cases. This is due to the fact that the cells are transiently transfected, and that the G1 arrest in transfected cells is not complete and irreversible. As seen in Table 2, overexpression of wt p53, on average, caused more than twice the amount of apoptosis at the 72-h time point in Saos-2 cells than the two hinge domain mutants (19.83% apoptotic cells with wt transfection versus 8.95 and 8.52% apoptosis with Met305 and Val325 transfections). Also noteworthy is the observation that the two hinge domain mutants retained the G1 arrest characteristics of wt p53 when nocodazole-pretreated Saos-2 cells were electroporated with expression vectors and cell cycle characteristics were observed over a 12-h period (data not shown), giving more evidence that the hinge domain mutants are impaired for apoptosis but not for cell cycle arrest.

The differences in the ability of the hinge domain mutants to initiate apoptosis compared to that of wt p53 are also illustrated in Fig. 4. Here we see that wt p53-transfected Saos-2 cells show both a signal for p53 expression (Fig. 4A) and for DNA fragmentation, indicative of apoptosis, through use of the TdT assay (Fig. 4B). The DAPI staining of this cell (Fig. 4C) demonstrates the extent of DNA fragmentation compared to that of the surrounding untransfected cells. Cells expressing the hinge domain mutants, however, do not show positive staining for the TdT assay over background levels (Fig. 4, E and H). The ratio of apoptotic Saos-2 cells that are p53 positive:total number of p53-positive cells was quantitatively assessed through confocal microscopy, and these results are summarized in Table 3. wt p53 overexpression resulted in a ñ3-fold increase in this ratio when compared to that of the hinge domain mutants.

DISCUSSION

This study of two germ-line p53 mutants at codons 305 and 325 indicates a possible new regulatory domain for p53-mediated apoptosis located in the hinge domain that connects the DNA-binding domain to the tetramerization domain. Aberrant localization of the two mutant proteins does not occur, based on the findings presented here (measurable transactivation in Figs. 1b and 2; nuclear staining in Fig. 4) and on an earlier study on one of the p53 nuclear localization signals that does lie within the hinge domain (residues 313–322; Ref. 28) that showed that only these residues are necessary for nuclear localization. From the studies of both the core sequence-specific DNA-binding domain and the tetramerization domain (14, 15), it is evident that the hinge domain must remain flexible for there to be proper alignment of the respective tetramerization motifs (residues 326–354; Ref. 15) of individual p53 polypeptides. Furthermore, mutations that affect the flexibility of the hinge domain may have adverse effects on the conformational changes that the p53 tetramer is hypothesized to undergo on DNA binding and thus may hinder binding to some sites such as the bax promoter, as seen in Fig. 2. One must remember, however, that Bax may not be needed for p53-dependent...
Fig. 3. Hinge domain mutants are impaired in their ability to induce apoptosis in Saos-2 cells. a, the p53-null cell lines Saos-2 and EB were transfected with an expression vector containing the indicated p53 cDNAs. Samples were taken at the indicated time points and immunostained for p53 expression and DNA content as described in “Materials and Methods.” The samples were then sorted via FACS analysis. The cells were gated on the p53-positive (FITC-positive) population, and the percentage of apoptotic cells (with a sub-G1 level of DNA content) was determined from this gated population. These experiments were done at least three times with similar results. The averaged quantitative results of the Saos-2 experiments are shown in Table 2. b. DNA profiles of transfected Saos-2 samples taken from the 72-h time point are shown. The sub-G1 DNA population of cells of each profile is highlighted. The sub-G1 profile in the wt transfection is more pronounced than that in any of the mutant transfections. The cell cycle characteristics of the cells at this time point are shown below the profiles.
Table 2  FACS analysis of sub-G1 DNA content of transiently transfected Saos-2 cells

<table>
<thead>
<tr>
<th>Time Posttransfection (h)</th>
<th>wt p53</th>
<th>Vector</th>
<th>Met&lt;sup&gt;305&lt;/sup&gt;</th>
<th>Val&lt;sup&gt;125&lt;/sup&gt;</th>
<th>Ala&lt;sup&gt;143&lt;/sup&gt;</th>
</tr>
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<tr>
<td>24</td>
<td>3.28 ± 1.61</td>
<td>1.96 ± 0.2</td>
<td>2.32 ± 0.85</td>
<td>2.31 ± 0.65</td>
<td>2.01 ± 0.81</td>
</tr>
<tr>
<td>48</td>
<td>13.10 ± 4.47</td>
<td>4.19 ± 2.18</td>
<td>3.79 ± 3.51</td>
<td>4.51 ± 0.12</td>
<td>1.58 ± 0.01</td>
</tr>
<tr>
<td>72</td>
<td>19.83 ± 6.34</td>
<td>5.19 ± 2.8</td>
<td>8.95 ± 1.68</td>
<td>8.52 ± 2.61</td>
<td>4.78 ± 0.83</td>
</tr>
</tbody>
</table>

Table 3  Quantitation of apoptotic Saos-2 cells

<table>
<thead>
<tr>
<th>Expression vector</th>
<th>Ratio of apoptotic: total p53-expressing cells</th>
<th>% of apoptotic cells</th>
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<tbody>
<tr>
<td>wt p53</td>
<td>121:328</td>
<td>36.9</td>
</tr>
<tr>
<td>Met&lt;sup&gt;305&lt;/sup&gt;</td>
<td>41:356</td>
<td>11.5</td>
</tr>
<tr>
<td>Val&lt;sup&gt;125&lt;/sup&gt;</td>
<td>38:329</td>
<td>13.1</td>
</tr>
<tr>
<td>Ala&lt;sup&gt;143&lt;/sup&gt;</td>
<td>33:405</td>
<td>8.1</td>
</tr>
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This study shows that the two germ-line-derived missense mutations in the hinge domain can significantly impair the apoptotic response in cells without the loss of phenotypes associated with the arrest of the cell cycle. This inability to elicit an efficient apoptotic response presumably contributes to the predisposition of affected
members of the LFS families to cancer. The interactions of the respective mutant p53 proteins with apoptotic factors and how they impair the p53-dependent apoptotic response as a whole remain to be elucidated.

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