Mutational Analysis of the p53 Core Domain L1 Loop* S

Andrew Zupnick and Carol Prives

From the Department of Biological Sciences, Columbia University, New York, New York 10027

The p53 tumor suppressor gene acquires missense mutations in over 50% of human cancers, and most of these mutations occur within the central core DNA binding domain. One structurally defined region of the core, the L1 loop (residues 112–124), is a mutational “cold spot” in which relatively few tumor-derived mutations have been identified. To further understand the L1 loop, we subjected this region to both alanine- and arginine-scanning mutagenesis and tested mutants for DNA binding in vitro. Select mutants were then analyzed for transactivation and cell cycle analysis in either transiently transfected cells or cells stably expressing wild-type and mutant proteins at regulatable physiological levels. We focused most extensively on two p53 L1 loop mutants, T123A and K120A. The T123A mutant p53 displayed significantly better DNA binding in vitro as well as stronger transactivation and apoptotic activity in vivo than wild-type p53, particularly toward its pro-apoptotic target AIP1. By contrast, K120A mutant p53, although capable of strong binding in vitro and wild-type levels of transactivation and apoptosis when transfected into cells, showed impaired activity when expressed at normal cellular levels. Our experiments indicate a weaker affinity for DNA in vivo by K120A p53 as the main reason for its defects in transactivation and apoptosis. Overall, our findings demonstrate an important, yet highly modular role for the L1 loop in the recognition of specific DNA sequences, target transactivation, and apoptotic signaling by p53.

The tumor suppressor p53 is central to a complex cellular network that mediates signals from various forms of stress and numerous cellular outcomes (1). p53 is a nuclear phosphoprotein that undergoes extensive modification following stress (2,3) and has been implicated to have a role in cell cycle arrest, apoptosis, genomic stability, DNA repair, and angiogenesis (reviewed in Refs. 4–7). Underscoring its importance as a tumor suppressor, p53 is found mutated in over 50% of all human cancers (8,9), and mice deficient for p53 expression are highly tumor-prone (10,11). In fact, p53 was the first commercial gene therapy treatment to be approved (12).

TP53 possesses a number of functional domains as follows: an acidic N-terminal region with a bipartite transactivation domain (residues 20–40 and 40–60), a proline-rich region with a proposed role in apoptosis (residues 60–90), a core sequence-specific DNA-binding domain (residues 100–300), a tetramerization domain (residues 325–355), and a basic C-terminal region that possesses sequence nonspecific nucleic acid binding properties and facilitates linear diffusion along DNA (reviewed in Ref. 57). Highlighting the major function of p53 as a transcription factor is the observation that the great majority of tumor-derived mutations reside within the core domain of p53, with six of them clustering into “hot spot” missense point mutations (Fig. 1A). Core domain mutations have effects that include global unfolding, local perturbations, or loss of an essential DNA contact residue, as evidenced by biochemical and structural data (13–15). Frequently, the end result is a decrease in affinity of p53 for many if not virtually all versions of its consensus sequence, which consists of two copies of the 10-bp motif 5′-RRRC(A/T)(T/A)GYYY-3′ separated by 0–13 bp (16–18). As a result, many tumor-related mutant forms of p53 are impaired or completely prevented from transactivating most p53 downstream target genes. p53 also has been shown to bind to and repress a range of promoters, yet the exact mechanism underlying this process is still poorly understood (19–22).

The L1 loop (residues 112–124) is part of the loop-sheet-helix motif of the core that contains the only three residues that contact DNA bases directly (see Fig. 1, A and B). Surprisingly, even though it contains Lys-120 that makes both direct base and backbone contact with DNA, this region is not mutated in a significant number of cancers. Furthermore, individual L1 loop residues have arisen in a number of yeast screens that sought to isolate mutations with enhanced transcriptional activation, a toxic phenotype, or second-site suppressor characteristics (23–27). With the exception of T123P, which behaves like wild-type p53, and S121F, which was reported to exhibit altered sequence specificity and an increased ability to induce apoptosis (28,29), yeast screen-derived L1 loop mutants have not been extensively characterized in mammalian systems.

Recently, molecular dynamics simulations identified a super-stable mutation within the L1 loop and challenged the classical interpretation of the crystal structure of p53 bound to DNA, due mostly in part to the predicted stability of the loop when in a dimerized state (6,30,31). Additionally, crystal structures of mouse p53, Caenorhabditis elegans p53, a quadruple mutant of human p53, and NMR solution structures have revealed a significant structural difference in this region when comparing DNA-free and DNA-bound states, indicating a potential conformational shift upon DNA recognition and binding (32–36).

Based on these findings we have performed a systematic mutational analysis of the L1 loop by alanine and arginine scanning followed by characterization of select mutants in vitro and
in vivo to reveal more information about this interesting region of p53. Our data indicate a role for this small segment of the core domain in the recognition of specific DNA sequences by p53 and its apoptosis-inducing function.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Site-specific Mutagenesis, Protein Expression, and Purification**—Alanine and arginine point mutations were introduced into pRSETb-p53 (37) using the QuikChange II site-directed mutagenesis kit (Stratagene). Mutations were confirmed by sequencing from the T7 promoter (GeneWiz). Mammalian expression vectors for transfection were similarly created using pCDNA3-p53. Vectors for the inducible cell line were made by subcloning the NheI-EcoRV fragment from pRSETb-p53 into pTRE2-hyg (Invitrogen), which removed the His tag but maintained an Omni epitope. His-tagged p53 proteins were induced with 84 μM isopropyl 1-thio-β-d-galactopyranoside in a BL21(DE3)/pLysS strain of Escherichia coli at 22 °C for 4 h and then harvested. Solubilized protein was isolated by stepwise lysis with hypotonic (50 mM Tris-HCl, 25% sucrose) and hypertonic buffers (1 M KCl, 0.2% Triton X-100, 50 mM Tris-HCl, 20 mM imidazole, 20 mM β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitors (1 μM benzamidine, 3 μg/ml leupeptin, 0.1 μg/ml bacitracin, 1 μg/ml macroglobulin)) followed by sonication and centrifugation. The proteins were then bound to nick-el-nitrilotriacetic acid-agarose beads (Qiagen), packed into a column, and washed with 40 column volumes of medium salt buffer (MSB) (600 mM KCl, 50 mM Tris-HCl, 20% glycerol, 20 mM imidazole, 0.1% Triton X-100, 10 mM β-mercaptoethanol) followed by 20 column volumes of MSB with 40 mM imidazole. His-tagged proteins were eluted with 250 mM imidazole in MSB, and the fractions most concentrated and active for DNA binding as judged by Bradford protein analysis (Bio-Rad) and EMSA were pooled and dialyzed overnight in BC100 (20 mM Tris-HCl, 100 mM KCl, 0.1 mM EDTA, 20% glycerol, 1 mM dithiothreitol). Proteins translated in vitro were expressed by adding 1 μg of the same pRsetb-p53 plasmid to the TNT® T7 quick-coupled reticulocyte lysate system (Promega).

**Electromobility Shift Assay**—EMSA reaction mixtures (20 μl) contained 20 μl HEPES (pH 7.9), 25 mM KCl, 0.1 mM EDTA (pH 8.0), 2 mM MgCl2, 10% glycerol, 0.025% Nonidet P-40, 4 mM spermidine, 2 mM dithiothreitol, 0.2 μg of bovine serum albumin, 50X mutant GADD45 oligonucleotide (5’-AGCTG-ATATCGAATTCTCGAGCAGAAAATTTCTAAGAATTC-3’), and 4 ng of [32P]-labeled oligonucleotide. After incubation at room temperature for 20 min, the mixtures were subjected to electrophoresis on a 4% polyacrylamide gel (29:1 acrylamide/bisacrylamide) containing 0.5% Triton borate/EDTA buffer at 165 V for 1.25 h at room temperature. The gel was dried and exposed to Kodak BioMax MR film. DNA-protein complexes were quantified by phosphorimaging with ImageQuant software. The 44-bp blunt oligonucleotide probes (Invitrogen) used had the following sequences (deviations from the p53 consensus sequence (18) are indicated in lowercase): p21, 5’-AGCTAGTAGACGGAA- CATGTCCcAACATGTGTGCGTGCACG-3’; Killer/-DR5, 5’-AGCTAGTAGACGGGCCATGCCGCGGAAGC- gGCGTGTCGACG-3’; AIP1, 5’-AGCTAGTAGAAGCtctCT- TGCCCGGGCTTGTGCGTGTCGACG-3’; and PUMA, 5’-AGCTAGTAGAAGCGCAGAAGACTTGTGCAGCG- TGCTGCACG-3’. Experiments with bacterially expressed p53 used 80 and 160 ng of protein, and in vitro translation experiments contained 4 and 8 μl of the 50-μl reaction mixture. Supershifts were achieved by addition of 1 μl of affinity-purified monoclonal mouse pAb 421 antibody.

**Transfection and Western Blot Analysis**—Transfections were performed in 6-well plates using a 1:2 ratio of plasmid to Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cell lysis and immunoblotting were performed as described previously (38). p53 protein was visualized by separating 4 μg of whole-cell lysate (40 μg for inducible cells) or an indicated amount of reticulocyte lysate on a 10% polyacrylamide gel, transferring to nitrocellulose, followed by immunoblotting with a mixture of anti-p53 antibodies (1801 and DO-1) and 1:4500 for 45 min at room temperature. Other antibodies used were anti-p21 (40 μg of whole-cell lysate loaded) at a 1:500 dilution (C19, Oncogene Research Products) and anti-actin at a 1:3000 dilution (Sigma). For in vitro translation, 0.5, 1, and 2 μl of the reaction mixtures were run on a 10% SDS gel prior to transfer and immunoblotting as above.

**RT-PCR**—RNA was extracted from cell pellets using RNeasy mini kit (Qiagen) and quantitated by ultraviolet spectrophotometry. cDNA was created using oligo(DT)-primed reverse transcriptase using 4 μg of total RNA and the Superscript III first strand synthesis system for RT-PCR (Invitrogen). PCR was then performed using a dNTP mixture (Roche Applied Science) and Taq 2000 DNA polymerase (Stratagene). Conditions for linear amplification were established through template and cycle curves. The cycling conditions were as follows: a denaturation step at 95 °C for 5 min followed by 20 cycles (for p21, glyceraldehyde-3-phosphate dehydrogenase, and PIG3) or 31 cycles (for AIP1) at 95 °C for 30 s, 56 °C for 30 s (58 °C for AIP1), and 70 °C for 30 s, with a final extension of 72 °C for 7 min. PCR products (40% of reaction mixture) were then separated on 2.5% agarose gels, and bands were visualized with ethidium bromide and imaged with a Gel Logix 100 imaging system (Eastman Kodak Co). Sequences for the primers used are listed as follows: p21 forward, 5’-GTTCCTTGTGGGAGCCGGAGC-3’; reverse, 5’-GTTACAGACAGTGACAGGTC-3’; pIG3 forward, 5’-GGCCCTAACAACAAATG-GCCAGTTCACGACG-3’; reverse, 5’-GCCTATGGTTCAGGGTGC-3’; PIG3 forward, 5’-GCCTATGGTTCAGGGTGC-3’; reverse, 5’-GCCTATGGTTCAGGGTGC-3’; and AIP1 forward, 5’-GGCCCCTAACAACAAATG-AGG-3’; and reverse, 5’-ACCAAGATCCAGCGAGA-3’.

**Tet-off Inducible Cell Lines**—H1299-derived inducible cell lines were created using a two-step tetracycline-regulated system and clonally selected with 400 μg/ml hygromycin B (Invitrogen), as described previously (39). The cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supple-

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2 The abbreviations used are: EMSA, electrophoretic mobility shift assay; FACs, fluorescence-activated cell sorter; pAb, polyclonal antibody; RT, reverse transcription; DRS, death receptor 5; AIP1, apoptosis-inducing protein 1; PUMA, p53-up-regulated mediator of apoptosis.
mented with 10% fetal bovine serum, 5 μg/ml tetracycline, 100 μg/ml hygromycin B, and 200 μg/ml G418.

**FACS Analysis**—For cell cycle analysis, H1299 cells were plated to a density of 90% after 16 h in a 6-well plate, and following transfection fresh medium was added after 5 h. For inducible lines, 2 × 10^5 cells were seeded per 60-mm plate without tetracycline. 22 h after plating, the cells were washed, and tetracycline was added as needed to equilibrate protein levels. 5-Fluorouracil (0.33 mM) was added 24 h after induction as indicated, and 48 h later, cells were harvested by trypsinization and fixed overnight with −20 °C methanol as described previously (40). Fixed cells and fragmented DNA were spun down for 5 min at 2400 rpm (1660 × g), resuspended with 1 ml of cold phosphate-buffered saline, and rehydrated for 30 min on ice (41). After another spin, cells were resuspended in 0.9 ml of phosphate-buffered saline solution containing RNase (50

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**FIGURE 1.** DNA binding properties of p53 L1 loop alanine substitution mutants. **A**, shown are domains of p53 indicating the location and frequency of tumor-derived mutations as well as the primary sequence of the L1 loop region. **B**, crystal structure of the core domain of p53 (14) is zoomed in around the residues of the L1 loop, shown as ball-and-stick model. The residue that contacts the DNA is Lys-120. Image was produced using Protein Explorer. **C**, silver-stained gel of two side-by-side bacterially purified protein preparations showing purity. **D**, p53-DNA complexes visualize by EMSA. Reaction mixtures with 32P-labeled linear 44-bp oligonucleotides containing p53-binding sites from p21, Killer/DR5, AIP1, or PUMA promoters as indicated were incubated with wild-type (WT) or mutant p53 protein (80 or 160 ng). The p53-DNA complexes are indicated by the black arrow and the super-shifted p53-DNA-chromatin immunoprecipitation 421 complexes (80 ng of p53 protein) are indicated by the red arrow. **E**, summary chart showing the fold binding by four mutants relative to wild-type p53 as quantified by phosphorimaging. Note that the values for wild-type p53 are set as one and indicated by the black bar. Error bars show range of values obtained from three independent experiments.
Alanine scan Arginine scan

| L1 loop mutant | Relative to wild-type p53 DNA binding | Sequence specificity | L1 loop mutant | Relative to wild-type p53 DNA binding | Sequence specificity |
|----------------|---------------------------------------|---------------------|----------------|---------------------------------------|---------------------|
| G112A          | ↓                                     | –                   | G112R          | ↓                                     | –                   |
| F113A          | ↓                                     | –                   | F113R          | ↓                                     | –                   |
| L114A          | ↓                                     | –                   | L114R          | ↓                                     | –                   |
| H115A          | ↓                                     | –                   | H115R          | ↓                                     | –                   |
| S116A          | ↓                                     | –                   | S116R          | ↓                                     | –                   |
| G117A          | ↓                                     | –                   | G117R          | ↓                                     | –                   |
| T118A          | ↓                                     | –                   | T118R          | ↓                                     | –                   |
| A119           | NA                                    | NA                  | A119R          | ↔                                     | +                   |
| K120A          | ↑                                     | +                   | K120R          | ↔                                     | +                   |
| S121A          | ↑                                     | +                   | S121R          | ↔                                     | +                   |
| V122A          | ↑                                     | +                   | V122R          | ↔                                     | +                   |
| T123A          | ↑                                     | +                   | T123R          | ↔                                     | +                   |
| C124A          | ↓                                     | –                   | C124R          | ↓                                     | –                   |
| T125A          | ↓                                     | –                   | T125R          | ↓                                     | –                   |

Because p53 core domain mutants vary in their affinities for different versions of the p53 consensus sequence (Ref. 27 and references therein), we tested oligonucleotide probes with binding sites derived from p53 downstream targets involved in apoptosis. Three mutants (L114A, K120A, and V122A) possessed lesser affinity for DR5, AIP1, and PUMA site oligonucleotides than to the p21 5’ site, with K120A and V122A binding significantly worse than wild-type p53 to DR5 and AIP1 (Fig. 1D). By contrast, T123A showed markedly enhanced binding to DR5 and AIP1 and, in contrast to the other versions of p53, also bound these sites to levels even greater than those of p21. Averages and standard deviations of three independent EMSA experiments for fold binding over wild-type of all four mutants on the four different targets are shown in Fig. 1E.

It was reported that substitution to positively charged residues within the p53 core domain could both create novel DNA contacts and rescue binding of hotspot mutants (44). We therefore mutated each L1 loop amino acid to arginine and similarly tested DNA binding of purified bacterially expressed p53 proteins. Unexpectedly, this set of mutants yielded only one variant (K120R) with slightly enhanced DNA affinity when compared with wild-type p53 (data not shown and summarized in Table 1). Although their overall binding was weak, as was seen with the alanine mutants, a number of L1 loop arginine mutations exhibited altered DNA affinities when tested against apoptotic probes. In particular A119R, K120R, and V122R displayed nearly negligible binding to DR5 and AIP1, although T123R had properties similar to T123A in that it bound to DR5 and AIP1 better than p21 (data not shown).

To further characterize the DNA-binding properties of the K120A, V122A, and T123A mutants, we examined them as proteins translated in vitro in rabbit reticulocyte lysates. After checking to make sure equal protein amounts were loaded (Fig. 1A), we performed as described previously (42). Input chromatin samples were diluted 1:10 to remain in the linear range for PCR. Samples were resolved on 2.5% agarose gels and visualized as described above. Primer sequences are listed as follows along with their respective annealing temperatures and cycles in parentheses: p21-5’ (56°C, 29 cycles) forward, 5’-CTGGAACGGCCACTCCTGTCGCT-3’, and reverse, 5’-CTCCATCATCCGCTTCTCTCACT-3’, and p21-neq (56°C, 29 cycles) forward, 5’-GGGCACTCTTGTC-3’.

**RESULTS**

Alanine and Arginine Scanning of the p53 L1 Loop Reveals Mutations with Enhanced DNA Binding and Altered Sequence Specificity—We first examined the role of the L1 loop in the DNA binding activity of p53 by individually mutating each amino acid (residues 112–124; Fig. 1C) to alanine. In each set of experiments two mutant proteins expressed in and purified from bacteria were tested alongside wild-type p53 to internally control protein activity. Protein concentrations were equilibrated (Fig. 1C), and the DNA-binding properties of p53 variants were tested using the EMSA in which DNA binding was assayed against a 44-bp oligonucleotide probe containing the 5’ p53 response element from the p21 promoter. The results for all mutants are summarized in Table 1. Based on the relatively rare occurrence of tumor-derived L1 loop mutants, it was somewhat unexpected that mutation to alanine of the majority of L1 loop residues (G112A, F113A, H115A, S116A, G117A, T118A, C124A, and T125A) either partially or completely ablated the affinity of the protein for DNA. Nevertheless, four L1 loop p53 mutants (L114A, K120A, V122A, and T123A) demonstrated unique DNA-binding properties in that they each possessed a greater affinity (2–3-fold) for the p21 probe than wild-type p53. Results from a typical purification and EMSA are shown in Fig. 1D, with assays always being performed using an excess of labeled probe (data not shown). These findings were recapitulated when p53 was “supershifted” with the C terminus-specific monoclonal antibody pAb 421 demonstrating that the increased binding was a function of the core domain, as opposed to a nonspecific or structure-specific effect of the C terminus (43).
K120A, V122A, and T123A p53 proteins failed to possess an affinity for the p21 site oligonucleotide that was more than 2-fold greater than that of wild-type p53. However, as seen with bacterially expressed proteins, K120A and V122A p53 variants each displayed much weaker binding to AIP1 than did wild-type p53. Although V122A approximated wild-type p53 in binding to DR5, K120A was essentially inert in interacting with either DR5 or AIP1, suggesting a possible function of this lysine in recognizing weaker promoters. Binding to PUMA by all three mutants as in vitro translated proteins was more robust than to p21 with V122A possessing 5-fold greater binding than wild-type p53.

Once again, T123A possessed DNA-binding properties unique from the other mutants, binding p21 to a similar extent as wild-type but having a significantly greater affinity for DR5 and PUMA, averaging 5-fold higher. Most striking was binding of T123A to AIP1. Although this mutant had demonstrated a 4-fold enhancement when expressed bacterially, here it bound upwards of 25 times better than wild-type p53. In fact, although AIP1 has traditionally been a very weak target of p53 (45), binding of T123A to AIP1 was even greater than the recognition of a p21 probe by wild type. Thus L1 mutants K120A, V122A, and T123A display unique sequence-specific variations in their interactions with DNA when compared with each other and to wild-type p53. Additionally, the source of p53 protein (bacterial or reticulocyte lysate) has a significant impact on their relative sequence selectivity.

Another goal of generating the L1 loop mutants in reticulocyte lysates was to determine whether or not the enhanced DNA-binding properties of T123A could be used to rescue binding by two p53 hot spot tumor-derived mutants (R175H and R273H) by co-translationally expressing the two different proteins (differentially tagged) to create a mixed tetramer. Unfortunately, even though hetero-oligomers were efficiently formed between either wild-type or T123A p53 and either R175H or R273H, in no cases were the resulting p53 complexes capable of binding better than either of the hot spot mutants alone to p21 or AIP1 probes (data not shown). Thus, as shown previously (46), the dominant-negative properties of these tumor-derived hot spot mutants are too great to allow for their rescue even by a super-binder such as T123A.

Transcriptional Activities of Transfected L1 Loop p53 Proteins—We then turned to examine the behavior of the three alanine substitution mutants (K120A, V122A, and T123A) in transiently transfected human cell lines. Constructs were generated in which these mutants were expressed under control of a cytomegalovirus promoter. In H1299 cells, although the p53 proteins were efficiently expressed at equal concentrations (Fig. 3A, top panel), they conferred different effects on endogenous targets. Under these experimental conditions, K120A behaved almost exactly like wild-type p53 by induc-
Mutational Analysis of the p53 Core Domain L1 Loop

AIP1-binding site. Indeed, whereas T123A induced wild-type levels of p21 protein (Fig. 3A) and p21 and PIG3 mRNA (Fig. 3B), it reproducibly induced 2–3-fold greater endogenous AIP1 mRNA than did wild-type p53 (Fig. 3B). This property was most striking in RKO cells (Fig. 3D) and was also recapitulated in transfected Saos-2 cells (Fig. 3F). The ability of T123A to preferentially activate the AIP1 promoter is consistent with its unique DNA binding properties.

Exogenous Expression of T123A Induces Increased Apoptosis—During the previous experiments, it was noticed that cultures transfected with T123A contained many more floating cells than those transfected with wild-type p53, a property indicative of apoptosis. To test whether L1 loop mutants imparted a physiological effect divergent from the apoptotic functions of the wild-type p53, H1299 cells were transfected and analyzed by flow cytometry (Fig. 4). All versions of p53 were expressed at approximately equivalent levels (Fig. 4A). Transfectants were specifically selected by gating for green fluorescent protein-positive cells, and cellular DNA content was measured by propidium iodide fluorescence. As a potential positive control, we created a construct expressing S121F p53, as this mutation had been shown previously to cause enhanced apoptosis and relatively reduced expression of p21 (29). In line with these previously published results, S121F p53 was deficient at inducing p21 protein (Fig. 4A, bottom panel).

To assess apoptosis we examined the sub-G₁ DNA content of the cells as described previously (47) (Fig. 4B). Transfection of vector alone was able to induce apoptosis, although expression of wild-type p53 significantly increased the extent of sub-G₁ DNA in the transfected cell population. Consistent with results examining expression of endogenous p53 targets in transiently transfected cells, ectopically expressed K120A produced a similar extent of cell death as seen with wild-type p53. Also consistent with the DNA binding and transactivation data, the T123A mutant p53 was superior to wild-type p53 at killing cells. Over a number of experiments, T123A induced upwards of twice as much death as wild-type p53 (Fig. 4C).

Unexpectedly the previously characterized L1 loop mutant S121F was actually somewhat defective in causing cell death, when compared with wild-type p53 after normalizing for vector-induced toxicity (Fig. 4C). We do not know whether differences in the delivery system or cell type are an explanation for the discrepancy between the described super-apoptotic properties of this mutant and our result. In the previous study, ectopic S121F p53 was delivered into Saos-2 cells via adenovirus (29).

As transfection of vector alone can have effects on cell cycle distribution, we therefore decided to examine protein functions in a more physiological setting. To do so, we attempted to generate H1299 cell lines expressing either wild-type p53, K120A, or T123A p53 protein under a tetracycline-regulated promoter. It was extremely difficult to isolate any cell lines expressing T123A (4% positive) even after three independent attempts screening a total of 52 clones, whereas clones expressing inducible wild-type p53 were much more readily obtainable (35% positive). Although expression is supposed to be silenced in the presence of tetracycline (48, 49), it is likely that some leakiness occurs. This suggests that even a relatively small amount of
Mutational Analysis of the p53 Core Domain L1 Loop

T123A p53 might be toxic enough to prevent the isolation of cells expressing this mutant. Of the two clones isolated that inducibly expressed T123A, one expressed p53 protein at extremely low levels, and the other produced protein at levels near that of wild-type p53 (data not shown). These low expressing clones was markedly pro-apoptotic in response to DNA damage, whereas the other clone induced apoptosis only mildly better than wild-type (data not shown). Unfortunately, because only two clones of T123A could be isolated and they did not have consistent apoptotic activities, little more could be concluded about T123A in vivo other than its ability to resist selection using the Tet-Off system. Additionally, these findings highlight the importance of testing multiple clones and the potential negative impacts of clonal variation.

K120A Expressed at Physiological Levels Is Impaired in Inducing Target Gene Expression and Apoptosis—In stark contrast to results seen with T123A and even wild-type p53, K120A-expressing inducible “Tet-Off” clones were very easy to isolate (67% positive). Furthermore, when expressed at fully induced levels (i.e. by complete withdrawal of tetracycline from the culture media), all clones tested displayed at least twice as much K120A protein (Fig. 5A) as wild-type p53 expressing clones (one being our previously characterized untagged line (39)). By regulating tetracycline levels, we equalized expression of wild-type and K120A protein and then examined the response to these two versions of p53. It should be noted that tetracycline-regulated wild-type p53 when fully induced in stable clones is well within the levels of p53 detected after inducible transfection of wild-type p53 (28, 39, 50). Remarkably, in contrast to results seen with p21, PIG3, and PUMA mRNA (data not shown).

Consistent with its reduced target transactivation ability, K120A was unable to elicit a phenotypic response. Upon equivalent protein induction, in response to damage by 5-fluorouracil, two clones expressing wild-type p53 underwent significant apoptosis in a p53-dependent manner (Fig. 5C). Two clones of K120A, on the other hand, failed to trigger any cell death. 5-Fluorouracil was chosen as it was shown to potentiate its effects in a p53-dependent fashion in vivo (40). Thus, when expressed at physiological levels, K120A p53 is considerably deficient at activating transactivation and the apoptotic regimen of p53.

Decreased Binding to Target Genes in Vivo by K120A Mutant p53—The DNA-binding property of p53 expressed in the same set of inducible clones was assessed in vivo by using the chromatin immunoprecipitation assay. Following protein expression, formaldehyde cross-linking, and DNA shearing by sonication, lysates were immunoprecipitated with an anti-p53 antibody, and cross-links were reversed, and PCR was performed to show the relative amounts of p53 bound to the p21, AIP1, or PIG3 promoter (Fig. 6 and supplemental Fig. 2). p53 protein expression levels were regulated by tetracycline so that their concentrations were roughly equivalent (Fig. 6, input lanes) and immunodepletion was nearly 100% (supplemental Fig. 2A, compare 10% IP lanes to Post-IP lanes), allowing us to compare relative DNA binding across samples. Additionally, immunoprecipitation and DNA shearing were efficient, as evidenced by negligible binding by p53 to a sequence ~2 kb upstream of the p21 5’-response element (supplemental Fig. 2B, p21-neg panels). The results showed that on a per mol basis,
significantly less K120A than wild-type p53 protein was bound to the p21 5’ p53-binding site (supplemental Fig. 2B, p21–5’ panels). When normalized to the input DNA and the amount of protein we estimate that the relative binding by K120A was ~30% that of wild-type p53. Our data indicated that a defect in DNA binding explains the impairment of K120A in inducing p21 expression in H1299 cells.

To support this assumption, we compared the DNA binding and transactivation activities of varying levels of wild-type and K120A mutant p53 protein. This was accomplished by regulating the amount of tetracycline in the cell cultures (Fig. 6A). Under these conditions wild-type p53 was again superior to K120A p53 in binding to and transactivation of different p53 target promoters over the range of p53 concentrations tested. At equivalent levels of p53 protein, binding of K120A was again about 30% of wild-type p53 (Fig. 6, A and B, compare lanes 4 and 7). Accordingly, wild-type p53 elicited significant activation of the p21 promoter, whereas K120A was virtually inert for p21 mRNA induction (Fig. 6C, lanes 4 and 7). Of note is the observation that the fold difference in binding to DNA, transactivation, and p21 protein induction between wild-type and K120A p53 at equivalent protein levels is roughly the same, indicating a direct relationship between DNA binding and protein production. Importantly, however, under conditions of equivalent binding by either form of p53 (Fig. 6B, lanes 3 and 8), K120A was able to induce p21 mRNA to the same fold extent as wild-type p53 (Fig. 6C, lanes 3 and 8). Furthermore, quantitation of the p21 Western blot (Fig. 6A, lanes 3 and 8) shows roughly similar protein levels under these conditions (201 arbitrary units versus 172 arbitrary units by densitometry). This result indicates that the defect in K120A p53 is largely if not exclusively in target promoter binding.

Consistent with previous findings, we also found that binding by either wild-type or K120A to its site within the p21 promoter is much better (~6-fold) than to p53-binding sites within either the PIG3 or AIP1 promoters (28, 51, 52). Taken together, our results demonstrate an essential role of Lys-120 in p53 for a complete apoptotic response via its DNA binding and transactivation properties.
Mutational Analysis of the p53 Core Domain L1 Loop

Differences between Transfection and Inducible Expression; a Cautionary Note—In an attempt to understand the discrepancy between transfection and inducible cell line data for K120A, we introduced p53 expression plasmids into the inducible cells. As shown in Fig. 6D, inducible expression of K120A was again shown to be deficient for transactivation of p21 (lane 8 versus lane 2). Additionally, transfection of vector alone did not significantly interfere with the induction or activity of the inducible proteins (Fig. 6D, lanes 4 and 10). Upon transfection of untagged wild-type p53 plasmid with inducible p53 expression silenced (Fig. 6D, lane 5), p21 protein was present at the same level as with inducible p53 alone (compare with lane 4), even though p53 levels were massively higher. Upon removal of tetracycline, the activity of inducible plus transfected p53 was roughly additive, as the level of p21 protein rose even further (Fig. 6D, lane 6). Exogenous K120A, however, did not display this ability to cooperate with induced p53 because, as seen before, transfected K120A was as active as wild-type p53 (Fig. 6D, compare lane 11 to lane 5), and the combination of the two failed to further up-regulate p21 expression (lane 12). These results suggest that the process of transfection, although stressful to the cell, does not itself alter the activity of p53. Massively overexpressing p53 as occurs upon transient transfection can overcome the deficiencies of some mutated versions of p53.

DISCUSSION

A systematic mutational analysis of the L1 loop yielded some unexpected observations. First, mutation of several L1 loop residues abrogates DNA binding completely, which makes it somewhat surprising that they are not more frequently mutated in cancers. Second, consistent with data from yeast screens, some L1 loop mutants when mutated to alanine but not arginine display greater than wild-type binding to at least one p53-binding site but lesser binding to others. Third, dramatic differences are obtained between assays measuring DNA binding, transfection, and expression at more normal levels of p53.

Our results indicate a role for the L1 loop in p53 target DNA sequence recognition, transactivation, and downstream function. It has been reported that the L1 loop might be part of either a dimerization interface (30, 32, 35, 36) or involved in intermolecular contacts with other binding partners such as Rad51 and 53BP2 (53). In addition, 4 of the 13 residues of the L1 loop are either serine or threonine, and the DNA contact residue 120 is a lysine, raising the possibility for a number of post-translational modifications among this region. Thus, it is appealing to hypothesize that inter/intra-molecular binding, phosphorylation, or acetylation of the L1 loop could alter the ability of p53 to recognize various DNA sequences and could be a signal by which p53 selectively triggers apoptosis, similar to the case with phosphorylation at serine 46 (45, 54) or binding by apoptosis-stimulating protein of p53 (ASPP) (55). It was at first surprising that alanine mutations in this region produced increased activity and that arginine mutations that add a positive charge presumably useful for DNA contact almost always impaired function (Table 1). However, this finding is in line with the mutational cold spot property of the L1 loop, in that perturbations of this region rarely confer a loss of p53 function and thus are not found to be associated with tumorigenesis. In our study, we focused on results with mutation of Lys-120 and Thr-123 that are discussed individually below.

Lys-120—Although the Lys-120 contact is not necessary for overall tumor suppressor function of p53, because it is rarely found mutated in tumors, it might play a role in recognizing specific, weaker binding sites usually associated with those targets involved in apoptosis (51, 56). In the crystal structures of the unbound C. elegans, mouse, and human core domain of p53, Lys-120 is not in a conformation capable of fitting into the major groove of DNA as shown with the human DNA-bound form (14, 32, 33). It has been proposed that a small structural change in the L1 loop is necessary to shift from the DNA-free state of p53 to the DNA-bound form (34). Perhaps mutation of the residues within the L1 loop can either facilitate or block the conformational change needed, leading to the various DNA affinities we observed.

Further characterization of the in vivo properties of K120A supported the significance of DNA binding by p53. Our experiments also highlight both the importance of examining p53 at physiological levels and the danger of interpreting results from in vitro binding assays without confirmation in cell-based systems. On one hand, K120A behaved identically to wild-type p53 when overexpressed by transient transfection (Fig. 3), yet when expressed at much lower concentrations via an inducible cell line, its overall function was severely impaired (Figs. 5 and 6 and supplemental Fig. 2). This obvious contradiction is quite intriguing and indicates that a plateau of DNA binding can be reached by much lower amounts of wild-type p53. Thus, the effects of the K120A mutation are masked in the overexpression system. Furthermore, the results for inducible K120A also did not exactly reciprocate those seen with in vitro binding data; it would have matched well if K120A was found at the p21 promoter but not the AIP1 promoter. One speculation is that p53 protein may be modified and folded in mammalian cells in such a way that the differences seen with bacterially expressed protein or translated in vitro would be less evident. It is particularly noteworthy that although K120A p53 bound significantly worse than wild-type p53 on a per mol basis, under conditions of equivalent binding by both proteins, similar levels of transactivation were achieved. This suggests that a threshold amount of overall DNA binding by p53 is required to elicit downstream transactivation, a supposition that bears further investigation.

Thr-123—In vitro bacterial and reticulocyte DNA binding assays on the T123A p53 mutant clearly showed a strikingly different and stronger affinity for p53-binding sites, including the AIP1 response element, which was reflected by greater endogenous AIP1 mRNA induction in a number of cell lines. This was consistent with the observation that T123A causes significantly more apoptosis than wild-type p53 in transfected cells. It was unfortunate that we were unable to determine the effects of T123A in cells stably expressing this protein at physiological levels although the extreme difficulty of isolating inducible clones of T123A also implicates an enhanced apoptotic phenotype. We hope to eventually elucidate the mechanism by which T123A confers increased apoptosis. Our results implicate a potential role of AIP1, yet it is unlikely that a single downstream target can be solely attributed to the apoptotic...
effects of p53. If inducible cells could be isolated, possibly by using a more tightly repressible promoter so as to block any leaky protein expression, it would be worthwhile to perform a microarray analysis to obtain a more global view of how T123A imparts its pro-apoptotic function. Furthermore, this approach could potentially isolate some novel genes involved in p53-mediated apoptosis, as well as highlight the targets that can be specifically modulated by the L1 loop. Biophysical and structural studies with these mutants would likely provide further insight, and a crystal structure of wild-type p53 versus T123A bound to an AIP1-derived sequence would undoubtedly shed light on the mystery of how p53 binds apoptotic sequences and whether or not the conformation of and contacts conferred by the L1 loop are utilized as a determining factor. Thus, more research would be beneficial to fully understanding the important mechanistic role of the L1 loop in the DNA binding, transactivation, and apoptotic functions of p53.

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