Identification of a Novel p53 Functional Domain That Is Necessary for Mediating Apoptosis*

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The ability of p53 to induce apoptosis requires its sequence-specific DNA binding activity; however, the transactivation-deficient p53(Gln1–42-Ser54) can still induce apoptosis. Previously, we have shown that the region between residues 23 and 97 in p53 is necessary for such activity. In an effort to more precisely map a domain necessary for apoptosis within the N terminus, we found that deletion of the N-terminal 23 amino acids compromises, but does not abolish, p53 induction of apoptosis. Surprisingly, p53(Δ1–42), which lacks the N-terminal 42 amino acids and the previously defined activation domain, retains the ability to induce apoptosis to an even higher level than wild-type p53. A more extensive deletion, which eliminates the N-terminal 63 amino acids, renders p53 completely inert in mediating apoptosis. In addition, we found that both p53(Δ1–42) and p53(Gln1–22-Ser23) can activate a subset of cellular p53 targets. Furthermore, we showed that residues 53 and 54 are critical for the apoptotic and transcriptional activities of both p53(Δ1–42) and p53(Gln22–Ser23). Taken together, these data suggest that within residues 43–63 lie an apoptotic domain as well as another transcriptional activation domain. We therefore postulate that the apoptotic activity in p53(Gln22–Ser23) and p53(Δ1–42) is still transcription-dependent.

The p53 tumor suppressor protein serves as a checkpoint in maintaining genome stability (1–3). Several different biological responses that could play a role in maintaining genome stability have been strongly correlated with wild-type p53 function (1, 3). Following stress conditions such as in the presence of damaged DNA or insufficient growth and survival factors, the cellular levels of p53 increase. This leads to one of at least three well understood cellular responses as follows: cell cycle arrest, differentiation, or apoptosis. Several factors have been shown to determine how a cell responds to the accumulation of p53, e.g. cell type and the presence of several cellular and viral proteins (4–8). In addition, the levels of p53 in a given cell can dictate the response of the cell such that lower levels of p53 result in cell cycle arrest (9) or differentiation (10), whereas higher levels result in apoptosis (9, 10).

The functional domains of p53 have been subjected to extensive analysis (1, 3, 4). A transcriptional activation domain has been shown to lie within N-terminal residues 1–42 (11, 12). Within this region there are a number of acidic and hydrophobic residues, characteristics of the acidic activator family of transcriptional factors (13). Indeed, a double point mutation of the two hydrophobic amino acids at residues 22 and 23 renders p53 transcriptionally inactive (14). These two residues presumably are required for the interaction of the activation domain with the TATA box binding protein and/or TATA box binding protein-associated factors (15–18).

It is well established that as a transcriptional activator, p53 up-regulates p21, a cyclin-dependent kinase inhibitor (19–21), which leads to p53-dependent G1 arrest. However, it is not certain what function(s) of p53 is required for apoptosis. The transactivation function of p53 was shown to be required in some experimental protocols (22–24). There are several candidate genes that play roles in apoptosis that can be activated in response to p53 induction, such as BAX (25), IGFBP3 (26), PAG608 (27), KILLER/DR5 (28), and several redox-related PIGs genes (29). Several other studies, including our own observations, have provided evidence that p53 might have a transcription-independent function in apoptosis (9, 30–32). Recently, the proline-rich region between residues 60 and 90, which comprises five “PXXP” motifs (where P represents proline and X any amino acid), was found to be necessary for efficient growth suppression (33) and apoptosis (34) and to serve as a docking site for transactivation-independent growth arrest induced by GAS1 (35).

Previously, we showed that the region between residues 23 and 97 is necessary for apoptosis (9). To more precisely map such a domain in the N terminus necessary for apoptosis, we have made several new mutants. Analyses of these mutants lead to identification of a novel domain between residues 43 and 63 that can mediate apoptosis and activate cellular p53 targets. We also found that a double point mutation at residues 53 and 54 completely abolishes both the transcriptional and apoptotic activities mediated by this novel domain. Thus, we hypothesize that a transcriptional activity located in this novel domain regulates a subset of cellular p53 targets that are responsible for apoptosis.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—Mutant p53 cDNAs were generated by polymerase chain reaction using the full-length wild-type p53 cDNA as a template. To generate p53(Δ1–23), the pair of primers used were as follows: forward primer N24, GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TAA ACT ACT TCT TCG TGA A; and reverse primer C393, GAT CGA ATT CTC ATG CTT AGT AGT CAG GCC CTT. To generate p53(Δ1–42), the pair of primers used were as follows: forward primer N24, GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TAA ACT ACT TCT TCG TGA A; and reverse primer C393, GAT CGA ATT CTC ATG CTT AGT AGT CAG GCC CTT. To generate p53(Δ1–54), the pair of primers used were: forward primer N64, GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TTT GAT GCT GTC CCC G; and reverse primer C393. To generate p53(Δ1–63), the pair of primers used were: forward primer N64, GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TCT CAG CAG AAT GCC AGA GCC T; and reverse primer C393. To generate p53(Gln1–22-Ser23/Gln22–Ser23), cDNA fragments encoding amino acids 1–59 and 60–393 were amplified in-

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p53-dependent Apoptosis

J. Zhu, W. Zhou, J. Jiang, and X. Chen, manuscript in preparation.

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2. The abbreviation used is: FACS, fluorescence-activated cell sorter.
In addition, both trypan blue exclusion assay and FACS analysis showed that approximately 50–68% of cells underwent apoptosis (Fig. 2C; Table I). Similar results were obtained from several other cell lines. These results suggest that the entire previously defined activation domain within the N-terminal 42 amino acids is dispensable for apoptosis. In fact, deletion of this region enhanced the ability of p53 to induce apoptosis (Table I).

To delineate further the domain in the N terminus required for apoptosis, we generated seven inducible cell lines expressing p53(D1–63) which lacks the N-terminal 63 amino acids but contains an intact proline-rich region. Three representative cell lines, p53(D1–63)-14, -22, and -27, were shown in Fig. 3A, and the activity of p53(D1–63) was analyzed as above. The results showed that p53(D1–63) was unable to activate p21 expression (Fig. 3A), and p53(D1–63)-14 cells, a high p53 producer, continued to multiply when p53(D1–63) was induced (Fig. 3B). Furthermore, both FACS analysis and trypan blue exclusion assay showed that neither apoptosis nor cell cycle arrest was observed in cells expressing p53(D1–63) (Fig. 3C and Table I).

Within Residues 43–63 Lies Another Activation Domain That Overlaps with the Domain Necessary for Mediating Apoptosis—The ability of transactivation-deficient p53(Gln22-Ser23) to induce apoptosis leads to the hypothesis that p53 has transcription-independent apoptotic activity (9, 31, 33). Since p53(D1–42) lacks the previously defined activation domain and only

### Table I

| Characteristics of various mutant p53 proteins |
|-----------------------------------------------|
| Wild-type p53                                      |
| p53(Gln22-Ser23)                                  |
| p53(Gln22-Ser23)/Gln53-Ser54)                    |
| p53(D1–23)                                       |
| p53(D1–42)                                       |
| p53(D1–42/Gln53-Ser54)                           |
| p53(D1–63)                                       |
| Arrest*                                        |
| Apoptosis*                                      |
| + + + +                                           |
| 45                                              |
| 30                                              |
| + + + +                                          |
| >60                                             |
| + + + +                                          |
| 15–18                                           |
| +/-                                             |
| 50–68                                           |
| -                                               |

* Arrest was assayed by the relative growth rate of cells and the number of cells in S phase.

* Apoptosis was assayed at day 3 by the percentage of cells staining with trypan blue and by determination of the sub-G1 phase cells using the ModFit program.
minimally activates p21 as determined by Western blot analysis (Fig. 2A), it appears that it can induce apoptosis in a transcription-independent manner. To ascertain whether p53(D1–42) contains a transcriptional activity, the expression patterns of four well defined cellular p53 targets, p21, MDM2, GADD45, and BAX, were analyzed in cells expressing p53(D1–42) by Northern blot analysis (Fig. 4A). The expression levels of these genes in cells with or without p53 were quantitated by PhosphorImage scanner, and the fold increase of their relative mRNAs was calculated after normalization to glyceraldehyde-3-phosphate dehydrogenase mRNA levels (Table II). The results showed clearly that p53(D1–42) significantly activated MDM2 (8-fold), GADD45 (7.03-fold), and BAX (3.9-fold) but only minimally activated p21 (1.83-fold). As expected, wild-type p53 but not mutant p53(Gln 22–Ser23) activated these cellular p53 targets (Fig. 4A; Table II). As a control, p53(D64–91), which lacks all of the five PXXP motifs, was examined. The proline-rich domain in p53 is dispensable for transactivation (33, 34). As expected, p53(D64–91) activated these p53 targets (Fig. 4A and Table II). Since p53(D1–63) failed to activate any of these p53-regulated genes (data not shown), the results suggest that another activation domain lies within residues 43–63. For clarity, we designate the originally defined activation domain located within residues 1–42 as activation domain I and this novel domain as activation domain II.

The above observations raise the following question: why does p53(Gln22–Ser23) fail to activate these well-defined p53 transcriptional targets (Fig. 4A; Table II) despite the fact that it still contains an intact activation domain II? One of the
A Northern blot was prepared using 10 μg of total RNA isolated from uninduced cells (−) or cells induced to express (+) wild-type p53, p53(Δ1–42), p53(Δ62–91), or p53(Gln22-Ser23). The blots were probed with p21, MDM2, GADD45, BAX, and GAPDH cDNAs, respectively. B, a Northern blot was prepared using 10 μg of total RNA isolated from uninduced cells (−) or cells induced to express (+) wild-type p53, p53(Δ1–42), p53(Δ62–91), p53(Δ364–393), or p53(Gln22-Ser23). The blot was probed with MCG14 cDNA. C, a Northern blot was prepared using 10 μg of total RNA isolated from uninduced cells (−) or cells induced to express (+) wild-type p53, p53(Gln22-Ser23)/Gln53-Ser54, or p53(Δ1–42)/Gln53-Ser54. The blot was probed with MCG14 cDNA.

FIG. 4. Within residues 43–63 lies another activation domain. A, Northern blots were prepared using 10 μg of total RNA isolated from uninduced cells (−) or cells induced to express (+) wild-type p53, p53(Δ1–42), p53(Δ62–91), or p53(Gln22-Ser23). The blots were probed with p21, MDM2, GADD45, BAX, and GAPDH cDNAs, respectively. B, a Northern blot was prepared using 10 μg of total RNA isolated from uninduced cells (−) or cells induced to express (+) wild-type p53, p53(Δ1–42), p53(Δ62–91), p53(Δ364–393), or p53(Gln22-Ser23). The blot was probed with MCG14 cDNA. C, a Northern blot was prepared using 10 μg of total RNA isolated from uninduced cells (−) or cells induced to express (+) wild-type p53, p53(Gln22-Ser23)/Gln53-Ser54, or p53(Δ1–42)/Gln53-Ser54. The blot was probed with MCG14 cDNA.

Table II: Transcriptional activities of various mutant p53 proteins

|          | p21 | mdm2 | gadd45 | bax |
|----------|-----|------|--------|-----|
| Wild-type p53 | 6.79 | 40.6 | 9.45   | 4.2 |
| p53(Δ1–42)  | 1.83 | 8.8  | 7.03   | 3.9 |
| p53(Δ62–91) | 3.65 | 6    | 7      | 2.7 |
| p53(Gln22-Ser23) | 1.43 | 1.2  | 1.44   | 1.3 |

*Fold increase in relative mRNA*
intact activation domain II, we hypothesized that it might still contain transcriptional activity. Indeed, we found that p53(Gln22-Ser23) can activate one of the putative p53 targets, MCG14. Furthermore, a double point mutation at residues 53 and 54 completely abolishes the ability of both p53(Gln22-Ser23) and p53(D1–42/Gln53-Ser54) to activate MCG14 and induce apoptosis. Consistent with our results, Candau et al. (46) recently showed that within residues 40–83 lies a sub-activation domain, which can activate a reporter gene under control of a promoter with a p53-responsive element. Since both p53(1–42/Gln53-Ser54) to activate MCG14 and a double point mutation at residues 53 and 54 abolished the transcriptional activity of the sub-activation domains. These results suggest that p53 has two independent activation domains. A second activation domain within a transcription factor is not without precedent. Herpes simplex virus protein VP16 also contains two independent activation domains (47). Thus, it appears that in response to various stress conditions and their subsequent modifications, the two independent activation domains might serve as an intrinsic factor of p53 that determines whether a given p53 target is activated. Although BAX, MDM2, and GADD45 are the activation domain II-regulated gene products, these cellular p53 targets might not mediate the p53-dependent apoptosis on the basis of two observations: (i) these genes were not activated by p53(Gln22-Ser23) which is competent in inducing apoptosis (Fig. 4A; Table II); (ii) these genes were activated by p53(D1–42) which is defective in inducing apoptosis (Fig. 4A; Table II). Since cell type has been shown to influence the cellular response (cell cycle arrest or apoptosis) to p53 (1, 4, 8), cellular genetic background might then determine the modification of the two activation domains. Therefore, the results obtained in H1299 cells need to be confirmed in other cell types.

It is intriguing that although p53(Gln22-Ser23) contains an intact activation domain II, it fails to activate BAX, GADD45, and MDM2 (Fig. 4A). Since both p53(D1–23) and p53(D1–42) can activate these p53 targets, it suggests that the presence of the first 23 amino acids may mask the ability of the activation domain II in p53(Gln22-Ser23) to activate these cellular p53 targets. Alternatively, it is also possible that when the activation domain I is inactivated by a double point mutation at residues 22 and 23, the N-terminal 42 residues might then inhibit or block interaction of a co-activator (or an adaptor) with the activation domain II that is required for activation of some p53 targets, such as MDM2, p21, BAX, and GADD45, but not for activation of other p53 targets, such as MCG14. It is important to note that although the activation domain I is primarily responsible for activation of p21, the level of p21 in cells expressing either p53(D1–23) and p53(D1–42) was slightly increased upon p53 induction (Figs. 1A and 2A), suggesting that the activation domain II can weakly activate p21. Furthermore, our preliminary studies showed that activation of p21 was compromised by a double point mutation at residues 53 and 54 when p53(Gln22-Ser23) was expressed at a low to intermediate level, consistent with the idea that the activation domain II contributes to the activation of p21. Since several clones that express various expression levels of the target genes are required for determining the function of the targets (48), these results remain to be confirmed.

Previously, it was shown that overexpression of p21 can protect human colorectal carcinoma RKO cells from prosta-glandin A2-mediated apoptosis (49). Lack of p21 expression due to homologous deletion of the p21 gene also renders HCT116 colorectal cancer cells susceptible to apoptosis following treatment with either γ-radiation or chemotherapeutic agents (50). In addition, a significant fraction of tumors in mice deriving from p21−/− HCT116 cancer cells were completely cured, and all tumors deriving from p21−/− cancer cells underwent re-growth after treatment with γ-radiation (50). It is interesting to note that p53(Gln22-Ser23) and p53(D1–42), both of which lack a functional activation domain I, cannot significantly activate p21 (Fig. 2 and 4; Table II) but can induce apoptosis (Table I). The strong apoptotic activity conferred by p53(D1–42) might be due to its failure of activating p21. Thus, we have generated a mutant, p53(D1–42), that might be better than wild-type p53 in the elimination of cancer cells and therefore a potential candidate for gene therapy.

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