A Movable and Regulable Inactivation Function within the Central Region of a Temperature-sensitive p53 Mutant*

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The tumor suppressor p53 protein is an important negative regulator of cell proliferation (1). Reintroduction of the wild type p53 gene into transformed cells blocks cell proliferation (2) and causes these cells to accumulate in the late G2 phase of the cell cycle (3). Loss of p53 function results in genome instability (4, 5) and eliminates growth arrest in response to inadequate or detrimental growth conditions at the G1 phase (6). p53 functions as a typical eukaryotic transcription factor; p53 binds to specific DNA sequences termed p53-responsive elements and stimulates transcription of the target genes (7). Paradoxically, p53 also represses the transcription of many viral and cellular genes that apparently do not have p53-responsive elements (8).

In structure, p53 is organized into three functional domains: an N-terminal region involved in transcriptional activation, a central region mediating specific DNA binding, and a C-terminal region responsible for digimerization, transcriptional repression, and non-specific DNA binding (7, 9). The natural forms of p53 are predominantly of missense type, resulting in loss of the protein’s specific DNA binding activity (7).

Mostly they are single missense point mutations located within the conserved regions 2–5 of p53 protein, resulting in loss of the protein’s specific DNA binding activity (7).

Conditional mutations such as chimeras between the ligand-binding domain (LBD) of steroid receptors and non-receptor proteins (for a review, see Ref. 10) are frequently used to manipulate the activity of proteins and thereby identify their role in various cellular processes. Nonetheless, use of LBD chimeras has at least three disadvantages. First, steroid hormones have receptor-independent activity (11, 12). Second, the hormone-binding domain possesses transactivation and trans-repression activities (13, 14). Third, a receptor-free assay system may not always be available. Therefore, a new means of creating conditional mutations should be desirable. Here we demonstrate that the central region (CR) containing residues 101–318 of p53N247I, a temperature-sensitive mutant of the tumor suppressor p53 (15), confers thermal regulability on chimeras between that region and a variety of proteins. Moreover, the CR exhibits no other activity, and its function is independent of endogenous p53 status. This result strongly suggests that the CR can be used as a movable regulatory cassette, a powerful tool for thermal regulation of chimeric proteins.

**MATERIALS AND METHODS**

Plasmid Constructions—Plasmids pgE1bCAT, pSGVP, pSG424, pL6EC, and pLex-VP16 have been previously described (16, 17). pGAL4-p53(1–318) and pGAL4-p53N247I(1–318) were constructed by inserting wild-type and mutant p53 DNA fragments between the EcorI and BamHI sites of pG424, respectively. Likewise, pGAL4VP16-p53(101–318) and pGAL4VP16-CR were constructed by inserting wild-type and mutant p53 DNA fragments between the EcorI and BamHI sites of pG424. pe4CAT was constructed by replacing the HindIII/BamHI fragment of pe1bCAT (17) with a DNA fragment containing the adenovirus E4 sequences -330 to -20 from the transcription initiation site. pe1aCD67 was constructed by replacing the HindIII/EcorI fragment of pSG424 with a DNA fragment encoding amino acids 1–222 of the adenovirus E3S E1a protein (17). pe1aCD67-CR was constructed by inserting the EcorI/BamHI fragment of pGAL4VP16-CR between the EcorI and BamHI sites of pe1aCD67. pL6EC-C was constructed as described below first. The SalI/BamHI fragment of pe1bCAT was replaced with the NdeI/BamHI fragment of pL6EC to make pL6EC-BSK. Then, three copies of a p53-binding site oligo, 5′-AGCTAGGTGCAT-3′ (18), were inserted into the 5′ end of pL6EC-BSK to complete the construction of pL6EC-C. pp53(51–363) was constructed by replacing the HindIII/BamHI fragment of pSG424 with a DNA segment encoding residues 51–363 of p53 protein.

Transient Transfection and CAT Assay—Saos-2, HeLa, H1299, and HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. CHO cells were maintained in McCoy’s 5A medium with 10% fetal bovine serum. Unless specified, calcium phosphate-mediated and DEAE-mediated DNA transfections were performed as described (17, 19). The relative CAT activity was generally below 23% for this work. For temperature-sensitive assays, the incubation temperature was switched to 30 °C after a 12-h transfection at 37 °C.

The abbreviations used are: LBD, ligand-binding domain; CR, central region; CHO, Chinese hamster ovary; CAT, chloramphenicol acetyltransferase; PBS, p53 binding site.

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Western Blotting Analysis—The anti-GAL4 antibody was purchased from UBI, New York. GAL4-p53 chimeras were detected by immunoblotting as described (21, 22).

RESULTS AND DISCUSSION

To test whether the naturally occurring p53 mutations affect the protein’s transactivation function, we measured the transcriptional activity of chimeras between p53 and GAL4 DNA-binding domain. To eliminate complication by p53 C-terminal oligomerization (7) and repression (9, 22) from our study, only residues 1-318 of p53 were fused to the GAL4 DNA-binding domain. Chimera GAL4-p53(1–318) stimulated transcription in transfected Saos-2 cells quite well at 37°C; in contrast, chimera GAL4-p53N247I(1–318) did not (Fig. 1A, compare lane 7 to lane 8). Thus, the mutation not only caused p53 to lose its DNA binding activity (7, 15) but repressed, at least in the background of a GAL4 chimera, the p53 N-terminal activation domain. In support, most analogous chimeras between GAL4 and the “hot spot” p53 mutants (7), including mutations at codons 143, 175, 248, 249, and 273, failed to activate transcription.

Since p53N247I is temperature-sensitive as regards DNA binding (7, 15), we also examined the repression activity at different temperatures. Indeed, when the temperature was shifted from nonpermissive (37°C) to permissive (30°C), the repression activity vanished (Fig. 1A, compare lane 4 to lane 8), indicating that this activity was also temperature-sensitive. Control experiments demonstrated that the two GAL4 chimeras were expressed at similar levels (Fig. 1B) and were localized in the nucleus2 regardless of the assay temperature.

Next, we defined the region mediating the p53N247I repression activity. As shown in Fig. 2, the region containing residues 101–318 of p53N247I, but not of wild-type p53, conferred thermal regulability on a heterologous activator, GAL4VP16 (23) (panel 1, compare lanes 7 and 8 to lanes 3 and 4). Notably, however, the two chimeric proteins were expressed approximately equally at either assay temperature (data not shown), which, in conjunction with data in Fig. 1B, suggested that fusion of the mutant p53 fragment to a peptide seemed not to affect expression of the resultant chimera. Furthermore, the temperature-sensitive repression activity of the p53 fragment was not restricted to Saos-2 cells. The same phenomenon was observed in CHO (see below) and in HeLa, H1299, and HepG2 cells (Fig. 2, panels II, III, and IV). Thus, we reached three conclusions. First, the thermally regulable inactivation function of p53N247I was confined to a region encompassing residues 101–318 (called the central region or CR). Second, this function of the CR was independent of protein context (Figs. 1A and 2; also see below). Third, the endogenous p53 status had little influence on the CR’s function. For instance, Saos-2 and H1299 cells expressed no p53 protein, whereas HeLa and HepG2 cells, although containing the wild-type p53 gene, differed dramatically in p53 protein level (5, 24). However, the CR’s function was similar in all four cell lines (Fig. 2).

All studies described above used GAL4 derivatives. To test the generality of the CR’s inactivation function we fused it to a totally unrelated protein, the adenovirus 13S E1a protein, which exerts complex regulatory effects on various viral and cellular promoters, such as the adenovirus E4 promoter (25). As shown in Fig. 3, E1aCD67 (26), an E1a derivative, stimulated E4 transcription at either temperature (compare lanes 1 and 2 to lanes 4 and 5) in CHO cells. Remarkably, the chimeric protein, E1aCD67-CR, stimulated transcription in a completely temperature-dependent manner (compare lanes 1 and 3 to lanes 4 and 6). Thus, these results parallel those obtained with GAL4VP16-CR and demonstrate that the transactivation activity of E1aCD67 can be thermally repressed by fusing E1aCD67 to the CR.

Results obtained with GAL4VP16-CR and E1aCD67-CR are consistent with our hypothesis that the CR temperature sensitivity represses other activities present on the same polypeptide chain without strict regard to the configuration of that polypeptide. Nonetheless, ideally to provide a movable and regulable inactivation function, the CR should have as few other activities as possible. Therefore, the binding of a wild-type p53 fragment containing residues 102–292 to DNA in vitro
(27) seems to pose a concern about the CR as an optimal approach to creating conditional mutants. Accordingly, we determined whether the CR within a chimera possesses DNA binding activity. In this assay, p53 derivatives binding sites (or PBS hereafter) were placed immediately downstream of the reporter's TATA box. Thus, binding of p53 derivatives would block assembly of the transcription initiation complex on the promoter and therefore reduce CAT activity. p53N247I, a derivative providing only p53 DNA binding activity (7, 9) but not GAL4VP16-CR, reduced transcription of the reporter containing PBS at 30°C (Fig. 4, compare lane 3 to lane 4). The reduction was specific because transcription of a corresponding reporter lacking PBS was not affected (Fig. 4, compare lanes 2 and 3 to lanes 7 and 8). Thus, in the context of a chimera the CR showed no detectable affinity to PBS. Besides, the inability to bind PBS was not a peculiarity of GAL4VP16-CR, since another chimera, E1aCD67-CR, was also unable to bind PBS (Fig. 4, compare lane 3 to lane 5).

We have demonstrated that the CR possesses a movable and thermally regulable inactivation function toward several distinct proteins. Interestingly, among human temperature-sensitive p53 mutants (15), p53N247I is unique in having a CR endowed with such a function. How does the CR regulate other activities present on the same polypeptide chain? We speculate that the conformational state of the CR may play a role, since the codon 247 mutation is located within the p53 conformation-sensitive region (7). It is possible that within a chimera the change of the CR's conformation could propagate to the neighboring region. This could account for loss of function of the chimera. Alternatively, the change of the CR's conformation facilitates the protein's interaction with some other protein which, as illustrated by the interaction between LBD and heat shock protein 90 (10), inactivates the chimera by steric hindrance. Further studies are required to distinguish these hypotheses.

It was puzzling to observe that the CR failed to bind DNA (Fig. 4) at the permissive temperature where the CR is supposed to assume the DNA binding conformation of p53. Perhaps this discrepancy reflects two aspects. First, p53N247I's Central Region Has Autonomous Regulatory Function 23901
from which the CR is derived, retains a small percentage of p53's transactivation activity at 30 °C, apparently due to its low DNA binding affinity (15). Second, the protein concentrations (p53 versus CR) required for DNA binding may differ. If, as seems likely, the oligomerization domain within the context of p53 helps the CR to bind DNA, then the CR alone would need to be expressed to a higher concentration than that achievable in vivo to drive the protein-DNA interaction.

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