Functional four-base A/T gap core sequence CATTAG of P53 response elements specifically bound tetrameric P53 differently than two-base A/T gap core sequence CATG bound both dimeric and tetrameric P53

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ABSTRACT
The consensus sequence of p53 is repeated half sites of PuPuPuC(A/T)(A/T)GPyPyPy. GtAGCAttAG CCCAGACATGTCC is a 14-3-3p promoter p53 regulator site; the first core sequence is CAttAG, and the second is CATG. Both mutants GtAGgAttAGCCCAGACATGTCC and GtAGCAttAGCCCAGACATcTCC can be activated by p53 as a 1.5-fold half site. The original p53 regulated site on the 14-3-3p promoter is a whole site, and CATTAG is a functional core sequence. The p53-binding affinity and the activity of CATTAG were lower than for the mutant CATATG core sequence. Wild-type p53 acts as a tetramer to bind to the whole site; however, it also can bind to a half site by one of its dimers. Wild-type p53 can only bind to a half site with core sequence CATG but not to CATATG. The 1.5-fold half site or whole site with core sequence CATATG can be bound by wild-type p53. A p53 mutant, A344, forms dimeric p53; it can only bind to CATG, and not to CATATG. Therefore, tetrameric and dimeric p53 can bind to a two-base A/T gap core sequence, but only tetrameric p53 can bind to a four-base A/T gap core sequence.

INTRODUCTION
p53 is a key regulator of the cell cycle. After DNA damage, p53 can be activated to block cell-cycle progression and mediate multiple check points. The p53 target genes, p21 and 14-3-3σ, can be induced by activated p53 to arrest the G1-to-S phase and G2-to-M phase of the cell cycle, respectively (1,2). The p53 consensus sequence is 10-bp repeat of PuPuPuC(A/T)(A/T)GPyPyPy, separated by a spacer with up to 13 bases (3). C(A/T)(A/T)G is the core sequence, and the purine (pu) and pyrimidine (py) bases comprise the flanking sequence (Figure 1). The whole p53-binding sequence is the 10-base direct repeated sequence. Only 10 and 5 bases comprise the half site and quarter site of the p53-binding site, respectively. The p53-binding sequence with a half site plus a quarter site is equal to 1.5-fold of the p53 half site (4). A half site of the p53-binding site possesses p53-binding affinity (4); however, it can only be activated by p53 in a sequence-specific manner or with other required cofactors (5–7). About 1.5-fold of the p53 half site can be activated by p53, but its activity is much lower than for the whole p53-binding site (5,7). Both p21 and 14-3-3σ have two p53 consensus sequences on their promoter (8). The p21 promoter at 5’ site one (GAACATGTCCcAACATGTTg) and 3’ site two (GAagAAGaCTGGGCATGTCT) can be activated by p53. The 14-3-3σ promoter 3’ site two (GtAGCAttAGCCCAGACATGTCC) can be activated by p53 but not that at 5’ site one (AGGCATGTgCcAcC ATGCCC) (9). However, the p21 and 14-3-3σ promoter p53-binding sites presented some mismatched bases (the lowercase letters note the mismatch bases). There are three mismatch bases in the flanking sequence of the p53-binding site in 14-3-3σ site one, but only one mismatch base in 14-3-3σ site two. There are two bases of the A/T gap in the core sequence of the p53-binding site in p21 site one and 14-3-3σ site one, but there are four bases in 14-3-3σ site two. Although the entire p53 consensus sequence comprises the whole p53-binding site, a 1.5-fold half site of the p53-binding site could be activated by p53, similar to p21 site two (7). In order to identify

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whether the p53-regulated site on the 14-3-3-3σ promoter is a whole site or a 1.5-fold half site, different mutant core sequences were cloned and tested for their functional activities. Interestingly, the p53-binding site on the 14-3-3σ promoter contained four bases of the A/T gap CATTAG core sequence. It differed from the p53 consensus by two bases of A/T gap CATG core sequence. To clarify how many bases of the A/T gap in the p53 core sequence are tolerant for p53-activating genes, basic promoter vectors with different A/T gap p53 response elements were constructed. The promoter activities and binding affinities were measured in our experiments.

p53 binding to the whole p53 response element is tetrameric and it can bind to the p53 half site by one of its dimers. A dimeric form of p53 mutant p53A344 also can bind to the p53 half site (4). To understand the four-base A/T gap CATTAG p53 core sequence binding character to p53, a dimeric form of p53 mutant p53A344 and a tetrameric p53 were used in a DNA affinity purification assay (DAPA).

MATERIALS AND METHODS

Construction of reporter vectors with p53 response element sequence

Oligonucleotides (20–34 bp) with p53 response element sequences were synthesized and annealed to pGL3 promoter vector (Promega). The vector and insert sequences were cloned and tested for their functional activities. Interestingly, the p53-binding site on the 14-3-3σ promoter contained four bases of the A/T gap CATTAG core sequence. It differed from the p53 consensus by two bases of A/T gap CATG core sequence. To clarify how many bases of the A/T gap in the p53 core sequence are tolerant for p53-activating genes, basic promoter vectors with different A/T gap p53 response elements were constructed. The promoter activities and binding affinities were measured in our experiments.

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RESULTS

The original p53-regulated site on the 14-3-3σ promoter is a p53 whole site response element, and CATTAG is a functional core sequence

Designed p53 response element sequences were constructed in the pGL3 promoter digested by SmaI (Figure 2A). These designed p53 response element sequences with core response mutants are listed in Figure 2B. The whole p53 site is as a perfect match sequence for the p53 consensus sequence (p53 2-x), and the fourth quarter with a TcTCT mutation is a 1.5-fold half site of the p53-binding site (p53 1.5-x). Deletion of the first and second quarter produces a half site of the p53-binding site (p53 1-x). After cotransfection with the p53 expression vector, the reporter activity of the whole site (p53 2-x) is higher than that of the 1.5-fold half site (p53 1.5-x), but there is no activity in a half site (p53 1-x) construct compared to that for the pGL3 promoter vector only (pr) (Figure 2C). The p53 response element on the 14-3-3σ promoter, its first half site with a four-base A/T gap (2,7), may have created using the following primers: CAAGGCTTCT CGCTTCTCGGAACATC and GAACTCAAGGAT GCCAGCGCTTG. All constructs were checked by DNA sequencing.

DNA affinity purification assay

Nuclear extract (100 μg) was prepared from 5 x 10^6 H1299 cells transfected with 16 μg of p53Δ364–393, p53A344Δ364–393 or p53A344 plasmid. The nuclear extracts were precleared by incubation with 40 μl streptavidin–agarose beads (4%) with a 50% slurry at 4°C for 1 h with rotation. They were then centrifuged at 4000 x g for 1 min at 4°C. The supernatant was collected as the pre-cleared nuclear extract. The binding reaction was performed by mixing the precleared nuclear extract proteins with the annealing probe (0.3 nmol), 5 x binding buffer [100 mM HEPES, pH 7.6, 5 mM EDTA, 50 mM (NH4)2SO4, 5 mM DTT, 1% Tween 20 (w/v), 150 mM KC1], 10 μl sonicated salmon sperm DNA (1 μg/μl) and the 40 μl streptavidin–agarose beads (4%) with a 50% slurry in 1 ml. This was incubated at room temperature for 1 h with rotation. The beads were washed three times with 1 x PBS. The binding proteins were eluted with 20 μl of 5 x loading dye, then separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS–PAGE) followed by western blot analysis, and were probed with p53 antibody (p53 Ab-2, Lab Vision). Nuclear extract (10 μg) was mixed with 20 μl 5 x loading dye as the 10% ‘input’ sample. To differentiate binding affinities of various p53 proteins to the p53 response element, either the low or high dosage of nuclear extract was used in the DAPA assay. The 30 or 300 μg of nuclear extract was used in each binding reaction, with 10 μg of nuclear extract used as an ‘input’ loading control, which was 33% or 3.3% of the sample, respectively.

LUCIFERASE ACTIVITY ASSAY

H1299 cells were grown in 12-well tissue culture dishes to 60% confluence and then cotransfected with 0.5 μg of pcDNA 3.0 (Invitrogen), pcDNA 3.0 p53, pcDNA 3.0 p53F46, or pcDNA 3.0 p53A344Δ364–393 in the presence of 0.5 μg of pGL3 promoter firefly luciferase (Promega). The vector and insert sequences were ligated using a 1:4500 ratio at 22°C for 15 min then transformed at 37°C overnight. The positive clones were checked by DNA sequencing.

Luciferase activity assay

H1299 cells were grown in 12-well tissue culture dishes to 60% confluence and then cotransfected with 0.5 μg of pcDNA 3.0 (Invitrogen), pcDNA 3.0 p53, pcDNA 3.0 p53F46, or pcDNA 3.0 p53A344Δ364–393 in the presence of 0.5 μg of pGL3 promoter firefly luciferase (Promega) or pGL3 promoter with a p53 response element sequence and 10 ng of pCMV renilla luciferase plasmids (Promega). Twenty-four hours post-transfection, cells were harvested in 0.25 ml reporter lysis buffer and were subjected to a dual luciferase assay according to the manufacturer’s protocol (Dual-Luciferase Reporter Assay System, Promega). Firefly luciferase activity was normalized to renilla luciferase activity and data are presented as the mean ± SD of three independent experiments, each performed in triplicate.
no function. We supposed that the full 14-3-3\(\mu\) p53 response element sequence is a 1.5-fold half site similar to that of p53 1.5\(\times\). Therefore, the first-quarter mutation (14-3-3 \(\mu\) m1) activities were the same as for the full 14-3-3\(\mu\) sequence, and the fourth-quarter mutation (14-3-3 \(\mu\) m4) had no activity. The promoter activity of the full 14-3-3\(\mu\) p53 response element sequence is higher than 14-3-3 \(\mu\) m1. The 14-3-3 \(\mu\) m1 is also higher than 14-3-3 \(\mu\) m4 activity. The first- and second-quarter double-deleted mutants 14-3-3 \(\mu\) d12 is no promoter activity at all (Figure 2C). Therefore, the original p53-regulated site on the 14-3-3\(\mu\) promoter acts as a whole site, with CATTAG as a functional core sequence.

Four-base A/T gap acts as p53 core sequence response element

To evaluate if the A/T base gap in the p53 core sequence can be a functional core sequence of the p53 response element, one to five A/T base gaps were synthesized and cloned into 1.5-fold half sites (Figure 3A). The promoter activities were examined by luciferase activity assay. Only two- and four-base gaps can be activated by p53 (Figure 2B). When comparing two-base A/T gap core sequences with four-base A/T gaps, the activities were higher in two-base A/T gaps than in four-base A/T gaps. However, the A/T base gap CATATG activity was higher than CATTAG (Figure 3B).

The whole p53 response element sequence activation by p53 was higher than for the 1.5-fold half site (5). Is the four-base A/T gap core sequence as functional in the whole site as in the 1.5-fold half site as previously demonstrated? The four-base A/T gap mutants in the whole p53 response element sequence were cloned, and the promoter activity was examined by a luciferase activity assay (Figure 4A). The results indicated that the core CATATG activity activated by p53 is higher than for CATTAG in both the whole site and the 1.5-fold half site (Figure 4B). Two super p53 mutants, p53F46 and p53A364–393 (10,11), also can activate the p53 whole site with the CATATG core sequence, even more so than wild-type p53 (Figure 5).

Tetrameric and dimeric p53 can bind to the two-base A/T gap core sequence, but only tetrameric p53 can bind to the four-base A/T gap core sequence

By using DAPA to investigate the p53-binding character to a two-base A/T core sequence and to a four-base
A/T core sequence (Figure 6B), three p53 mutants, p53/C1364–393, p53A344/C1364–393 and p53A344, were constructed and expressed in H1299 cells (Figure 6A). The p53/C1364–393 mutant, consisting of the last 30 amino acids of a C-terminal deletion p53 mutant, avoided non-specific binding and sliding-off effects (5,11). First, 50-labeled biotin probes with 2–4 gaps of A/T bases in a p53 half-site sequence were used to examine the binding character of p53/C1364–393. The results indicated that p53/C1364–393 can only bind to two-base-gap CATG core sequences but not to three-base-gap CATAG, four-base-gap CATATG, or CATTAG core sequences (Figure 6C). Surprisingly, with probes with two to four gaps of A/T bases in a 1.5-fold p53 half-site sequence, p53/C1364–393 not only can bind to two-base-gap CATG, but also can bind to four-base-gap CATATG or CATTAG core sequences (Figure 6D). However, p53/C1364–393 cannot bind to a three-base-gap CATAG core sequence.

The binding affinity patterns in DAPA correlated to the function assay of the sequence activated by p53 (Figures 3B and 6D). The p53-binding affinity and activity of two four-base-gap CATATG and CATTAG core sequences were different; CATATG-binding affinity and activity was higher than for CATTAG (Figures 3B and 6D). p53A364–393 can bind two-base-gap CATG in both p53 half sites and 1.5-fold half sites as probes, but it can only bind to four-base-gap CATATG or CATTAG core sequences in 1.5-fold p53 half sites, not to p53 half sites (Figure 6C and D). p53A364–393 can also bind to a p53 whole site with four-base A/T gaps in each core sequence (Figure 6E). To evaluate the binding-pattern discrepancy between two-base-gap CATG to four-base-gap CATATG, perhaps due to dimeric or tetrameric-binding character differences, dimeric p53A344/C1364–393 and p53A344 were used (4,12). The DAPA results show that both dimeric p53 mutants, p53A344 and p53A344/C1364–393, can only bind to two-base-gap CATG core sequences in a p53 half site, but cannot bind to four-base-gap CATATG core sequences in a 1.5-fold or 2-fold half site (Figure 6F and G). Because of weak binding of dimeric p53A344/C1364–393 to the two-base-gap CATG...
core sequences in a p53 half site (Figure 6F), different dosages of nuclear extract were used in the DAPA reaction to compare the affinity of the dimeric and the tetrameric p53 binding with two- or four- base-gap CATG core sequences. In the low-dosage DAPA assay, dimeric p53A344A364–393 was not detected in two-base-gap CATG core sequences as a p53 half site, but tetrameric p53A364–393 bound both to two-base-gap CATG core sequences in a p53 half site and four-base-gap CATATG core sequences in a p53 whole site (Figure 6H). In the high-dosage DAPA assay, dimeric p53A344A364–393 bound to two-base-gap CATG core sequences in a p53 half site but could not bind to four-base-gap CATATG core sequences in 2-fold half site (Figure 6I). According to the results, we believed that the four-base A/T gap can be bound only by tetrameric p53, but not by dimeric p53.

**DISCUSSION**

Based on the functional assay and DAPA results, except for the two-base A/T gap core sequence of the p53 response element, the four-base A/T gap core sequence also acts as a p53 response element. The p53 target gene, the 14-3-3ε core sequence of p53 response element GtAGCATTTGCCCAGACATGTGC, is thought to be a whole p53 response element. We tested two four-base A/T gap variant core sequences of p53 response elements, CATATG and CATTAG. Both p53-binding affinity and activity of CATATG were higher than for CATTAG (Figures 3B, 4B and 6D). It has been demonstrated that two-base A/T gap core sequences in different A/T arrays had different responses for p53 activation (13). The p53-induced core sequence activity with AT or TA was higher than for AA or TT. It may explain how the four-base A/T gap CATATG activity was higher than for CAT TAG in our experiments. However, A/T arrays in the core sequence with four-base gaps may have 16 variants; different A/T arrays in the core sequence with four-base gaps may have different p53 responses. It needs further investigation.

Sauer et al. (11) reported p53 binding to p53 response elements with the electrophoretic mobility shift assay (EMSA). An oligonucleotide probe less than 66 bp may cause non-specific binding and sliding-off to reduce specific binding. Once the C-terminal 30 amino acids of p53 were deleted, the specific binding affinity was recovered (11). For DAPA, from our designed probes of 20–34 bp
p53 half sites can bind dimeric p53 mutants (Figure 6F and G). Therefore, dimeric p53 can only bind to two-base but not to four-base A/T gap p53 response elements.

p53 is composed of an N-terminal transactivation domain, a DNA-binding core domain and a C-terminal oligomerization domain (Figure 6A) (14). The oligomerization domain is composed of a turn followed by a β-sheet, a turn and an α-helix (15,16). The β-sheet has a monomer dimerization motif, and the α-helix has a dimer–dimer interface motif. The oligomerization domain of p53 is also involved in DNA binding to the supercoil DNA and enhances core domain DNA-binding affinity (17,18). In our experiments, the dimeric p53A344Δ364–393 mutant bound to two-base gap core sequences of the p53 response element half site, but the affinity was much lower than for tetrameric p53A364–393 (Figure 6I; 1 × 2 g AT compares to input). It is indicated that the α-helix dimer–dimer interface of the p53 oligomerization domain enhanced core domain DNA binding. With an extended core sequence from two-base A/T gaps to four-base A/T gaps, not dimeric p53, but only tetrameric p53 can bind to both 1.5-fold p53 half sites and p53 whole sites (Figure 6E, F and G). Therefore, the α-helix dimer–dimer interface of the p53 oligomerization domain seems necessary for binding to four-base A/T core sequences of p53 response elements in both 1.5-fold p53 half sites and p53 whole sites (Figure 7).

According to McLure and Lee (4), the dissociation constant ($K_d$) of tetrameric or dimeric p53 binding to the p53 whole site or half site varied. The binding $K_d$ pattern was: tetrameric to whole site < dimeric to whole site < tetrameric to half site < dimeric to half site (4). Therefore, the highest p53-binding affinity was tetrameric binding to the whole site, followed by dimeric binding to the whole site, tetrameric binding to the half site, and the lowest p53-binding affinity is dimeric binding to the half site. There were similar results in our DAPA assay; the binding affinity of tetrameric p53A364–393 was higher than dimeric p53A344Δ364–393 bound to two-base A/T gap core sequence of the p53 half site (Figure 6I). Once the four-base A/T gap core sequence was substituted for the two-base A/T gap core sequence in the whole site, it could be bound only by tetrameric p53A364–393, but not by dimeric p53A344Δ364–393 (Figure 6I). Therefore, the integrity of the tetrameric state of p53 is necessary for p53 binding to the four-base A/T gap core sequence. It has been indicated that several other factors in cells can associate with the p53 oligomerization domain to affect the tetramerization state of p53. S100 family proteins and apoptosis repressors with caspase recruitment domains (ARC) can associate with the p53 oligomerization domain to inhibit p53 tetramerization and to repress the trans-activation function of p53 (19–23). The 14-3-3 family proteins can also associate with the p53 oligomerization domain to enhance p53 tetramerization (24). Therefore, the 14-3-3 family, S100 family and ARC may be important regulators for influencing the affinity and activity of p53 through the core sequence with a four-base A/T gap.

We identified a non-canonical p53 response element and found that a four-base A/T gap core sequence can be bound only by tetrameric p53.
be activated by p53. Also, the different A/T arrays in a four-base A/T gap core sequence may affect the p53-binding affinity and activation function. We also clarified that the p53-binding character for four-base A/T gap core sequence is different from the canonical p53 response element.

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