p53 Pre- and Post-Binding Event Theories Revisited: Stresses Reveal Specific and Dynamic p53-Binding Patterns on the p21 Gene Promoter

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Abstract

p53 is a master transcription factor that prevents neoplasia and genomic instability. It is an important target for anticancer drug design. Understanding the molecular mechanisms behind its transcriptional activities in normal cells is a prerequisite to further understand the deregulation effected by mutant p53 in cancerous cells. Currently, how p53 coordinates transcription programs in response to stress remains unclear. One theory proposes that stresses induce pre-binding events that directly bind to specific response elements, whereas a second posits that, in response to stress, p53 binds most response elements and post-binding events then regulate transcription initiation. It is critical to establish the relevance of both theories and investigate whether stresses induce specific p53-binding patterns correlated with effector gene induction. Using unique in cellulo genomic footprinting experiments, we studied p53 binding to the five response elements of p21 in response to stresses and monitored p21 mRNA variant transcription. We show clear footprints of p53 bound to response elements in living cells and reveal that the binding of p53 to response elements is transient, subject to dynamic changes during stress responses, and influenced by response element pentamer orientations. We show further that stresses lead to specific p53-binding patterns correlated with particular p21 mRNA variant transcription profiles and that p53 binding is necessary but not sufficient to induce p21 transcription. Our results indicate that pre- and post-binding events act together to regulate adapted stress responses; this paves the way to the unification of pre- and post-binding event theories. [Cancer Res 2009;69(21):8463–71]

Introduction

The modulation of tumor suppressor activity remains one promising approach for cancer treatment. The p53 gene is mutated in 50% of human cancers and has often been targeted for cancer treatment (1, 2). Understanding the mechanism of gene expression regulation by wild-type and mutated p53 is thus a prerequisite to designing rational strategies for new therapeutic approaches.

p53 is a master transcriptional regulator that maintains genome integrity and cellular homeostasis by adapting programs of gene expression in response to stresses. p53 transcriptional activity is mediated by the process of binding to response elements. p53 response elements contain two inverted pentameric sequences generally found in tandem (→ ← ←). The decamer consensus sequence (→ ←) follows the pattern 5′-RRRC(A/T)(A/T)GYYY-3′ (R = purine and Y = pyrimidine; ref. 3). p53 binding to response elements regulates the transcription of genes involved in many cellular pathways and leads to either cell cycle arrest, apoptosis, senescence, or differentiation (4–6). p53 transcriptional activity is an important cellular mechanism that is commonly deregulated in human tumors mainly by mutations located in the DNA-binding domain (1). The gene deregulation effected by p53 mutations has severe consequences and is implicated in carcinogenesis and resistance to cancer treatments (7, 8).

Studies have clearly shown that p53 triggers stress-adapted gene regulation (9, 10). However, the mechanism behind this phenomenon is not fully understood and two theories have been proposed. The pre-binding event theory suggests that stress induces p53 binding only to genes that need to be regulated. This is thought to occur through pre-binding events (post-translational modifications, targeting proteins, and p53 concentration) that direct p53 to bind to specific response elements (11–16). However, this model must be accompanied by some caveats. First, no studies have shown stress-specific p53-binding patterns because most of them have focused on only one stress or one response element. Second, the role of post-translational modifications is not clear because Nutlin-3 induces gene regulation even if p53 is not phosphorylated on six residues (17, 18). Additionally, how can targeting proteins direct p53 to bind to DNA when they themselves interact through the DNA-binding domain of p53 (19)? Finally, a concentration-based explanation of the regulation of p53 binding does not explain how different gene expression programs are triggered. Ultimately, the Achilles’ heel of the pre-binding event theory is that, following 5-fluorouracil (5-FU) treatment, only a subset of effector genes bound by p53 are regulated (20). Moreover, HCT116 and U2OS presented identical p53-binding patterns when exposed to different stresses (21). This suggests that p53 binding is not necessarily correlated with transcriptional activities and that stress-adapted responses might not be triggered by specific p53-binding patterns. Based on these observations, Espinos proposed the post-binding event theory: following stress, p53 binds most of its response elements; then, post-binding events operate as filters to allow transcription of specific genes (22).

Although there is clear evidence of the existence of post-binding events involved in the regulation of p53 effector genes (23, 24), it remains to be determined whether stresses induce specific p53-binding patterns and what their effect is on gene transcription.
To investigate p53-binding activities, we performed in cellulo DNase I genomic footprinting experiments coupled to ligation-mediated PCR. This technique was chosen over chromatin immunoprecipitation because it maps protein-DNA interactions at single nucleotide resolution with high sensitivity and allows for the measurement of binding to response elements that are close together. p53-binding patterns were investigated on the p21 gene, which features five p53 response elements located at +657, −1,354, −2,241, −3,969, and −11,708 bp from the customary transcription start site (TSS; Fig. 1; refs. 20, 25–27). The p21 gene was also selected because it is a major regulator of the cell cycle and its regulation is mainly p53 dependent (28). In addition to multiple p53 response elements, p21 also contains three TSS yielding eight p21 transcript variants: p21V1, p21V2, p21Vn, p21Vc, p21alt-a, p21alt-a, p21alt-b, and p21alt-c (Fig. 1; refs. 27, 29). Questions arise about the role of multiple p53 response elements in p21 regulation. Are they differentially bound by p53 following stress? Is the extent of p53 interaction with response elements correlated with the p21 mRNA induction level? Is the regulation of p21 mRNA variants affected by p53 binding in a stress-dependent manner?

In this article, we show that, in a normal human cellular context, stresses trigger specific and dynamic p53-binding patterns correlated with p21 mRNA transcription profiles. We also show that p53 binding is necessary but not sufficient to induce effector gene transcription. These results pave the way to the unification of the pre- and post-binding event theories.

Materials and Methods

Cells and cell culture. Human normal primary skin fibroblasts and human Li-Fraumeni skin fibroblasts (LF041 strain, a gift of M. Tainsky, The University of Texas M. D. Anderson Cancer Center) were grown in DMEM containing 10% fetal bovine serum, 0.2 units/mL penicillin G, and 100 μg/mL streptomycin (all from Wisent Bioproducts). LF041 fibroblasts have lost one p53 allele and carry a frameshift mutation at codon 184 in the remaining copy.

Cell treatments. The 500 J/m² UVB irradiation was done with two FS20T12/UVB/BP fluorescent tubes (Philips); wavelengths below 290 nm were filtered by a Kodacel TA-407 clear 0.015 inch (Eastman Kodak). The 500 J/m² γ-irradiation was carried out using a cobalt-60 γ-cell. For nucleotide imbalances, stressed control slides. Exonuclease I was then inactivated at 80°C for 20 min. DNA fragments were labeled by a five-cycle extension step using a third primer (DNA) and Alexa Fluor 488 (p53) intensities were quantified and corrected using automatic photomultiplier tube and background correction functions. Cell cycle phase identification was based on G1 and G2 peak positions on "4′,6-diamidino-2-phenylindole max pixel" scattergrams as suggested by the company. Threshold levels for p53-positive cells were set to include 10% positive cells in non-stressed control slides.

Ligation-mediated PCR. DNase I footprinting reaction was carried out as published previously (31). The ligation-mediated PCR extension, ligation, and amplification steps were done on a TGradient Thermocycler (Roche Applied Science), and Taq DNA polymerase (Roche Applied Science), and T4 DNA ligase (Roche Applied Science), respectively, as described previously (31). Following amplification, samples were treated for 30 min at 37°C with 1 unit exonuclease I (New England BioLabs). Exonuclease I was then inactivated at 80°C for 20 min. DNA fragments were labeled by a five-cycle extension step using a third primer with automatic photomultiplier tube and background correction functions. Cell cycle phase identification was based on G1 and G2 peak positions on "4′,6-diamidino-2-phenylindole max pixel" scattergrams as suggested by the company. Threshold levels for p53-positive cells were set to include 10% positive cells in non-stressed control slides.

Footprint analysis. We obtained gel band intensity profiles using sequencing TIF files and ImageQuant 5.0 (Molecular Dynamics). Band intensity profile ratios and 50-pixel interval mobile averages were calculated using Excel. Negative footprint intensity averages were calculated by averaging the mobile average values encompassed in the regions of negative footprints. Negative footprint sequence alignments were done using Genious 4.0.4 (Biomatters).

Results and Discussion

To study p53 transcriptional binding activities in a normal cellular context, experiments were conducted on normal human primary fibroblasts. Fibroblasts were exposed to 20 Gy γ-irradiation, 375 μmol/L 5-FU, 500 J/m² UVB, or 5 μmol/L Nutlin-3; then, cells were harvested over a 24-h time course. Nutlin-3 was used as a
Figure 2. Assessment of p53, p21 mRNA, and cell cycle arrest induction following stress treatments; determination of time conditions for measuring p53 transcriptional binding. A, induction kinetics of nuclear p53, p21 mRNA, and percentage of S-phase cells in wild-type fibroblasts exposed to 500 J/m² UVB, 20 Gy γ-irradiation, 375 μmol/L 5-FU, and 5 μmol/L Nutlin-3. p53 nuclear induction was measured by indirect immunofluorescence and quantified by laser scanning cytometry. p21 mRNA induction was quantified by quantitative reverse transcription-PCR and percentage of cells in S phase was determined by laser scanning cytometry using 4′,6-diamidino-2-phenylindole staining of nuclear DNA. Experiments were done in triplicate. Mean ± SD. B, induction kinetics of p53-positive cells in wild-type fibroblasts exposed to 500 J/m² UVB, 20 Gy γ-irradiation, 375 μmol/L 5-FU, and 5 μmol/L Nutlin-3. p53 nuclear induction was measured by indirect immunofluorescence and quantified by laser scanning cytometry. Threshold levels for p53-positive cells were set to include 10% positive cells in nonstressed control conditions. Experiments were done in triplicate and data are presented as mean. To facilitate reading, SD are not presented, but their average is 10%. C, measurement of p21 mRNA induction kinetics by quantitative reverse transcription-PCR in wild-type (full lines) and LF041 (dotted lines) fibroblasts exposed to 500 J/m² UVB, 20 Gy γ-irradiation, 375 μmol/L 5-FU, and 5 μmol/L Nutlin-3. Primer set amplified all variants except p21-alt 1 and 2. Arrows, induction times retained to measure p53 transcriptional binding activities. Experiments were done in triplicate. Mean ± SD.
positive control to induce p53 in the absence of stress (17). All the treatments induced similar levels of p53 nuclear accumulation and p53-positive cells (Fig. 2A and B). Induction of p21 transcription was observed following each treatment and correlated with G1-S cell cycle arrest, except for UVB treatment (Fig. 2A). Interestingly, although the p53 nuclear amounts induced by stresses were quite similar, we observed different induction levels of p21 mRNA. This indicates that there is no direct correlation between p53 nuclear amounts and transcription levels of p21.

Subsequently, to determine the time conditions needed to study p53 transcriptional binding activities, we compared the induction profiles of p21 mRNA in LFO41 (p53-deficient) with those obtained with wild-type fibroblasts (Fig. 2C). p21 mRNA induction was p53 dependent, except in the case of UVB treatment. This accords with the results obtained by Loignon and colleagues (28). However, in the case of γ-irradiation, p21 mRNA induction was p53 dependent only until 6 h post-treatment. Thus, we decided to measure p53 binding at early and late stages of p21 mRNA induction: 2 and 4 h for γ-irradiation, 2 and 6 h for Nutlin-3, 4 and 8 h for 5-FU, and 8 and 12 h for UVB.

We then assessed the in cellulo binding of p53 to the five response elements of p21 at selected times. Wild-type and LFO41 fibroblasts were exposed to stresses or not and then treated with DNase I. The DNA fragments generated were subsequently amplified by ligase-mediated PCR (Fig. 3; Supplementary Figs. S1-S3). Interestingly, the band patterns of nontreated wild-type and LFO41 fibroblasts were identical, indicating that p53 did not bind to response elements in nontreated cells. For each condition, band intensity profiles were quantified and expressed as the ratios of treated to nontreated (Fig. 3B). p53 binding was typically characterized by a negative footprint in the vicinity of the response element surrounded by positive footprints. We did not observe any footprint in LFO41 (Fig. 3B; Supplementary Figs. S1-S3).

To obtain a qualitative perspective on p53-binding patterns, we calculated the average intensity of each negative footprint (Fig. 3C). We observed that stresses induce specific p53-binding patterns on p21, which supports the pre-binding event theory. The 5-FU treatment induced p53 binding to the −1,354, −2,241, and −11,708 bp response elements as observed by Wei and colleagues (20).

Figure 3. p53 binding to its response elements located on the p21 gene. A, in cellulo DNA ligase-mediated PCR footprinting of the p21 promoter region containing the −2,241 bp response element obtained in LFO41 and wild-type fibroblasts (F wt) at 8 and 12 h following exposure to 500 J/m² UVB or nontreated (NT). The first four lanes show standard Maxam-Gilbert sequencing reactions. The sequence of the response element is presented with pentamer orientations; negative footprints are indicated by a bar and positive footprints by an asterisk. B, gel band intensity profile ratios of treated/nontreated, obtained with wild-type and LFO41 fibroblasts at 8 and 12 h, following 500 J/m² UVB for the −2,241 bp response element. Black line, 50-pixel interval mobile averages of ratios. When there is no footprint, the gel band intensities of the nontreated sample are equal to those of the treated sample, resulting in a ratio of 1, as observed with LFO41. If the nontreated sample presents band intensities superior to those of the treated sample, the ratio is below 1 and reflects a negative footprint as observed with wild-type fibroblasts. Finally, if the nontreated sample presents band intensities inferior to those of the treated sample, a ratio superior to 1 is obtained, reflecting a positive footprint, as observed with wild-type fibroblasts. C, p53-negative footprint intensities obtained for the −1,354, −2,241, and −11,708 bp response elements on the p21 gene in wild-type fibroblasts following exposure to 500 J/m² UVB, 20 Gy γ-irradiation, 375 μmol/L 5-FU, and 5 μmol/L Nutlin-3. The footprint intensities were calculated by averaging the mobile average values encompassed in the region of negative footprints. The SD corresponds to the average SE of three independent DNase I ligation-mediated PCR footprints used as reference and is equal to 3.6%.
Nutlin-3 treatment led to p53 binding to the -1,354 and -2,241 bp response elements, whereas γ-irradiation induced p53 binding to the -2,241 bp response element only. We did not observe p53 binding to the -3,969 bp response element, a binding reported to occur in MCF-7 cells treated with 300 μM/L 5-FU for 6 h, which is close to our conditions (26). This suggests that p53 binding may be specific to this cell line or linked to cell transformation. Also, we did not measure p53 binding to the +657 bp response element, although Nozell and Chen showed in vitro that p53 can bind this response element (27). Because we did not measure any footprints in LF041, we concluded that the binding patterns were p53-specific (Fig. 3B; Supplementary Figs. S1-S3). They were also not linked to the p53 concentration, because each treatment induced similar p53 nuclear accumulation (Fig. 2A).

These p53-binding patterns give rise to interesting observations: Nutlin-3 induces p53 accumulation in the absence of stress and leads to p53 binding to the -1,354 and -2,241 bp response element. We propose that, following γ-irradiation, there exists a stress-dependent mechanism that inhibits p53 binding to the -1,354 bp response element. On the other hand, following UVB and 5-FU treatments, stress-dependent mechanisms that favor p53 binding to the -11,708 bp response element should operate as well. Moreover, we never observed binding to a response element at only one time. This indicates that the inhibition of p53 binding to a specific response element was constitutive during our experimental time conditions.

Our data support the notion that p53 binds response elements in a stress-dependent manner (11–13, 32). Recently, Shaked and colleagues made similar observations in peripheral blood mononuclear cells exposed to γ-rays using ChIP-on-chip (21). However, they obtained the opposite result in HCT116, FF-T, and U2OS. In these cell lines, p53 was bound to response elements similarly before and after stresses, and no binding dynamic was observed when tested in HCT116. It is astonishing that cell lines from different origins (HCT116, FF-T, and U2OS derived from colon carcinoma, foreskin, and osteosarcoma, respectively) present similar binding patterns, whereas only primary cells differ in terms of p53-binding activities. These observations strongly suggest that the absence of binding patterns might be related to cell transformation. Cell lines generally present a high basal level of p53 and could have p53 constitutively activated by comparison with normal cells (21). This may explain why p53 is bound to most response elements constitutively in cell lines. Moreover, because the majority of studies were done with cell lines, this could explain why stress-specific p53-binding patterns were not observed until now. Altogether, this underlines the fact that, although cell lines are a good tool for studying p53 activity in a cancer cell context, they have limitations when it comes to attaining conditions as close as possible to physiologic.

To determine the p53-binding position on response elements, we performed alignments of DNA sequences comprised within negative footprints observed at the early stage of p21 mRNA induction (Fig. 4). p53-negative footprints covered 26 to 29 nucleotides. Interestingly, the -2,241 and -11,708 bp response elements with canonical pentamer orientation (→→→→) were bound by p53 in a centered manner. In contrast, the atypically oriented pentamers of the -1,354 bp response element (→→→→→) were bound asymmetrically on only three pentamers (→→→→→), suggesting that the first two pentamers are not involved in the interaction. This corroborates the theory proposed by Ma and Levine, that is, that p53 tetramers can use only two monomers to bind two response element quarter sites (→→→→→), ref. 33. We also consider that the real recognized sequence may contain an additional pentamer AGATT on the 3′ end of the response element (→→→→→), making it a canonical response element.

Interestingly, the strongest p53 binding was observed at the early stage of p21 mRNA induction, when the nuclear levels of p53 were not at their maximum and when only ~50% of cells were p53 positive (Figs. 2A and B and 3C). Less intense p53 binding was measured at a late p21 mRNA induction stage, when p53 levels were the highest and when ~80% of cells were p53 positive (Figs. 2A and B and 3C). This indicates that the p53 interactions with response elements were transient. In the case of a persisting interaction, we would have predicted an increase in footprint

**Figure 4.** p53 footprint consensus sequences. p53 footprint consensus sequences (large letters) were obtained by aligning the DNA sequences encompassed in negative footprint regions of the -1,354, -2,241, and -11,708 bp response elements at an early transcription-induction stage following exposure to 500 J/m² UVB, 20 Gy γ-irradiation, 375 μmol/L 5-FU, and 5 μmol/L Nutlin-3. Arrows, response element pentamer orientations; dotted arrow, a possible sixth pentamer.

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intensities as more cells become p53 positive and the amount of p53 increases, but this was not observed. Moreover, a transient interaction also explains why we only observed negative footprints with intensities below 50% (Fig. 3C), because the p53 time response to stress of each cell in the cell population is not synchronized (34). The transient nature of p53 interactions with DNA suggests that p53 is essential mainly during the initiation stage of gene transcription (e.g., for recruiting cofactors). Moreover, the observation that p53 is free from interactions at certain times during the stress response indicates that p53 must remain available for alternative pathways.

We also noted that p53-binding intensities were not necessarily correlated with effector gene induction. γ-Irradiation, 5-FU, and Nutlin-3 treatments induced weak response element occupancy (Fig. 3C) but led to higher p21 mRNA induction (Fig. 2C). In contrast, UVB treatment led to the most intense footprints (Fig. 3C), but the p21 expression was almost the same between wild-type and LF041 fibroblasts (Fig. 2C). Thus, the induction of p21 transcription by UVB was mainly p53 independent despite the presence of p53 on the promoter. We propose that BRCA1 might be responsible of transcription induction, because this protein is both activated following DNA damage and able to regulate p21 (35, 36). Moreover, our data confirm that p53 binding to response elements is necessary but not sufficient to trigger effector gene transcription and that post-binding events are also necessary to induce p21 transcription. In harmony with our results, Donner and colleagues have shown that the CDK8 cofactor is recruited to the p21 promoter to activate transcription elongation following 5-FU, γ-irradiation, and Nutlin-3 treatments but not following UV exposure (24). Taken together, these data support the view expressed by Espinosa that post-binding events constitute a critical mechanism in the modulation of p53 transcriptional activities (22).

Finally, we observed that p53 binding to specific response elements was dynamically modulated during the stress response. Following 5-FU treatment, p53 binding to the −2,241 bp response element decreased by ~50% between 4 and 8 h, whereas it only slightly decreased at the −11,708 bp response element (Fig. 3C). In the case of UVB treatment, between 8 and 12 h, p53 binding decreased by only ~30% at the −2,241 bp response element and by ~50% at the −11,708 bp response element (Fig. 3C). We think that the binding dynamic reflects the ability of the cell to constantly reevaluate the stress level and modulate p53 binding during the stress response. As suggested by others, this may be achieved by the pulsating nature of the p53 response (34). By degrading and renewing the p53 protein pool, the cell can induce a new set of post-translational modifications and adapt the p53 response (37). To determine the possible function of p53-binding patterns and the p53-binding dynamics, we measured p21 mRNA variant expressions following stress treatments. Wild-type and LF041 fibroblasts were exposed to treatment and harvested over a 24-h time course. We monitored one p21 mRNA variant per p21 TSS: p21alt-a, p21V1, and p21VC (Figs. 1 and 5). In wild-type fibroblasts, we observed that the γ-irradiation and Nutlin-3 treatments strongly induced p21alt-a, p21V1, and p21VC in a p53-dependent manner. Following 5-FU treatment, the induction of p21alt-a and p21V1 was also p53-dependent but less pronounced than
Figure 6. Unification of pre- and post-binding event theories. In the absence of stress, response element occupancy is very low due to weak and rapidly reversible interactions of p53 (A). In stress contexts, pre-binding events trigger strong p53 binding on certain response elements, leading to stress-specific binding patterns (B-D). The binding of p53 to its response elements is necessary but not sufficient to induce p21 mRNA variants (B). Stress-specific post-binding events (post-translational modifications, presence of RNA polymerase, recruitment of coactivators such as CDK8, etc.) are also required to switch on transcriptional activities (C and D). Thus, pre- and post-binding events are not mutually exclusive; rather, they are complementary.
after γ-irradiation or Nutlin-3 treatment, and there was no p53-dependent induction of p21<sup>VC</sup>. Finally, on UVB irradiation, p21 mRNA variant induction levels were not p53 dependent, because induction profiles obtained in LF041 were superior or equal to those observed in wild-type fibroblasts. These data clearly indicate that p21 mRNA variant transcription is stress and p53 dependent.

We propose that stress-specific p53-binding patterns and the dynamics of response element occupancy are involved in the different p21 mRNA variant induction profiles observed. Indeed, 5-FU and UVB treatments resulted in the strongest footprints and were the only treatments that induced binding by p53 to the −11,708 bp response element (Fig. 3C). Paradoxically, they presented the lowest p21 mRNA variant induction levels, particularly for p21<sup>VC</sup> (Fig. 5).

Moreover, on UVB irradiation, p21 transcription increased during the interval between 8 and 12 h, surpassing the level observed in LF041 (Fig. 2C). During the same interval, p53 binding to the −11,708 bp response element decreased by −50%. The binding by p53 to the −11,708 bp response element is thus associated with a repressive effect that moderates p21 mRNA variant transcription, indicating that post-binding events play a role. Taken together, these data reveal that the p53 response element transcription profiles obtained in LF041 were superior or equal to those observed in wild-type fibroblasts.

## Discussion of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## References


Correction: p53 Pre- and Postbinding Event Theories Revisited: Stresses Reveal Specific and Dynamic p53-Binding Patterns on the p21 Gene Promoter

In this article (Cancer Res 2009;69:8463–71), which was published in the November 1, 2009, issue of Cancer Research (1), one of the p53 response elements (RE) and its related transcription site (TSS) were named incorrectly. "+657 bp RE" should be labeled as "+3253 bp RE" and "+774 TSS" should be labeled as "+3370 TSS". The authors regret this error.

Reference
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