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# Small Peptides Activate the Latent Sequence-Specific DNA Binding Function of p53

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## Summary

Normal cells contain p53 protein in a latent state that can be activated for sequence-specific transcription by low levels of UV radiation without an increase in protein levels. Microinjection of cells with an antibody specific to the C-terminal negative regulatory domain can activate the function of p53 as a specific transcription factor in the absence of irradiation damage, suggesting that posttranslational modification of a negative regulatory domain in vivo is a rate-limiting step for p53 activation. Small peptides derived from the negative regulatory domain of p53 have been used as biochemical tools to distinguish between allosteric and steric mechanisms of negative regulation of p53 tetramer activity. Presented is the development of a highly specific peptide activation system that is consistent with an allosteric mechanism of negative regulation and that forms a precedent for the synthesis of novel low molecular mass modifiers of the p53 response.

## Introduction

p53 appears to play a central role in the cellular response to irradiation damage by activating an apoptotic or growth arrest pathway in proliferating cells (Maltzman and Czyzyk, 1984; Kastan et al., 1992; Kuerbitz et al., 1992; Hall et al., 1993; Lu and Lane, 1993; Zhan et al., 1993; Clarke et al., 1993; Lowe et al., 1993; Merritt et al., 1994; Yonish et al., 1991). The precise mechanism by which p53 is activated by cellular stress is of intense interest and may involve both increases in p53 protein level and in the specific activity of p53 by covalent modification. Although direct activation of the latent sequence-specific DNA binding activity of human p53 can occur through ultraviolet (UV) light and serum-responsive signaling pathways in insect cell systems (Hupp and Lane, 1995), upstream intracellular signaling pathways involved in the direct activation of the biochemical function of p53 in mammalian cells following irradiation have not been clearly delineated, nor have the actual enzymes activating p53 function in vivo been identified. Biochemical analysis of p53 has shown that it interacts with many proteins implicated in regulation

of p53 protein function, including protein kinases and phosphatases, heat shock proteins, and DNA binding proteins. The biochemical activity of p53 may also be regulated by interaction of the C-terminus with single stranded RNA or DNA (Oberosler et al., 1993; Bakalkin et al., 1994; Jayaraman and Prives, 1995). The activity of p53 most tightly linked to its tumor suppressor activity is the ability of the protein to bind to DNA sequence-specifically (Kern et al., 1992; El-Deiry et al., 1992). Inactivating point mutations usually map within the active site for sequence-specific DNA binding or within the central core DNA binding domain (Cho et al., 1994; Halazonetis and Kandil, 1993; Bargonetti et al., 1993). Thus, sequence-specific DNA binding is a biologically relevant function of p53, and understanding its regulation may reveal mechanisms whereby the cell regulates a key damage-responsive pathway.

Biochemical analysis of the regulation of wild-type p53 sequence-specific DNA binding has shown that the unphosphorylated tetramer has a cryptic sequence-specific DNA binding activity (Hupp et al., 1992). This cryptic or latent state of p53 depends upon a C-terminal negative regulatory domain, which locks the unphosphorylated tetramer in an inactive state. Phosphorylation of the C-terminal negative regulatory domain of latent p53 by either protein kinase C or casein kinase II (Hupp and Lane, 1994b; Delphin and Baudier, 1994; Takenaka et al., 1995), or deletion of this regulatory domain (Hupp et al., 1992), activates sequence-specific DNA binding. In addition, a monoclonal antibody or bacterial heat shock protein 70 (Hsp70), whose binding sites reside in the C-terminal negative regulatory domain, mimic the affects of protein kinases and activate latent p53 through a concerted transition of subunits in the tetramer (Hupp and Lane, 1994a). Thus, neutralization of this negative regulatory domain by covalent or noncovalent modification is an important stage in the activation of p53.

To demonstrate directly that this activation pathway operates in mammalian cells, we show that the activation of p53 as a sequence-specific transcription factor following UV irradiation does not require increases in protein level and can be mimicked in vivo by the intranuclear microinjection of antibody directed to the C-terminal negative regulatory domain of p53. In light of our results, we propose a model in which each C-terminal negative regulatory domain interacts with a motif in the core of the tetramer and must be displaced to permit the specific DNA binding activity of the protein. A prediction of this model, that small peptides derived from the C-terminal negative regulatory domain might interfere with this intra or intermolecular interaction and activate the DNA binding function of latent p53, is confirmed. Definition of the molecular details of this control pathway that regulates the cellular response to irradiation damage suggests the possibility of rational design of low molecular mass modifiers of the p53 response.

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## Results

### UV-Induced Activation of the Transcriptional Function of p53 Does Not Require an Increase in p53 Protein Levels: Support for a Model of p53 Activation by Posttranslational Modification

The levels of p53 protein rise dramatically in some cells and tissues exposed to DNA-damaging radiation (Maltzman and Czyzyk, 1984; Kastan et al., 1991; Hall et al., 1993; Midgley et al., 1995). This rise in p53 protein following irradiation is associated with enhanced transcription of p53-responsive genes (Lu and Lane, 1993; Zhan et al., 1993). Although the mechanism by which p53 levels increase in the environment of the irradiated cell is not known, the increase in p53 protein concentration provides a simple explanation for the increase in transcription from p53-responsive genes. However, an alternate mechanism for the regulation of p53-dependent transcription has been suggested by a series of biochemical studies on the regulation of the sequence-specific DNA binding function of p53. These studies show that p53 can be produced in a biochemically latent state that can be activated for specific DNA binding after the modification of a C-terminal negative regulatory domain by distinct enzymes in vitro (Hupp and Lane, 1994b). Furthermore, signaling pathways that respond to UV radiation or growth factors in serum can directly activate sequence-specific DNA binding function of p53 in cells (Hupp and Lane, 1995). According to this model, then, covalent modification of p53 without increase in protein level may be sufficient to activate the p53 response following DNA damage in normal cells.

To test these two models, we used a wild-type p53 containing murine fibroblast cell line with a stably integrated p53-responsive β-galactosidase reporter gene. When these cells are exposed to intermediate doses of UV light (20

and 40 J/m²) two events are observed: p53 levels rise, as judged by a sensitive enzyme-linked immunosorbent assay (ELISA) (Figure 1A), and expression of the β-galactosidase reporter gene is activated as determined by cell staining (Figure 1B). Notably, the dose–response curve shows that at lower doses of UV irradiation (10 J/m²), p53-dependent transcription is activated without any detectable increase in p53 protein level (Figures 1A and 1B). These results imply that the specific activity of p53 as a transcription factor can be altered by irradiation without increasing p53 protein concentrations. In addition, these results suggest that unirradiated fibroblasts contain a pool of latent p53 that can be activated by a factor in response to DNA damage. To establish the existence of this pool of latent p53 in unirradiated cells, we sought to activate it independent of DNA damage. The antibody PAb421 is a potent activator of the DNA binding function of latent p53 in vitro, so we tested the capacity of the PAb421 antibody to activate p53-dependent transcription in vivo by microinjection.

### Activation of the Sequence-Specific Transcriptional Response of p53 In Vivo by Microinjection of Antibody PAb421 Establishes the Existence of a Pool of Latent p53 in Mammalian Cells

Strikingly, microinjection of the antibody PAb421 activates the p53 transcriptional response, as shown by the production of β-galactosidase enzyme in the injected reporter cells (Figure 2ii). Buffer alone (Figure 2i), or an inhibitory antibody, PAb246 (Halazonetis and Kandil, 1993), does not give rise to a transcriptional response (Figure 2iii). A quantitative summary of two independent experiments is shown in Table 1. These results reflect an important concept, that a latent pool of p53 can be activated in vivo in the absence of irradiation damage by a posttranslational
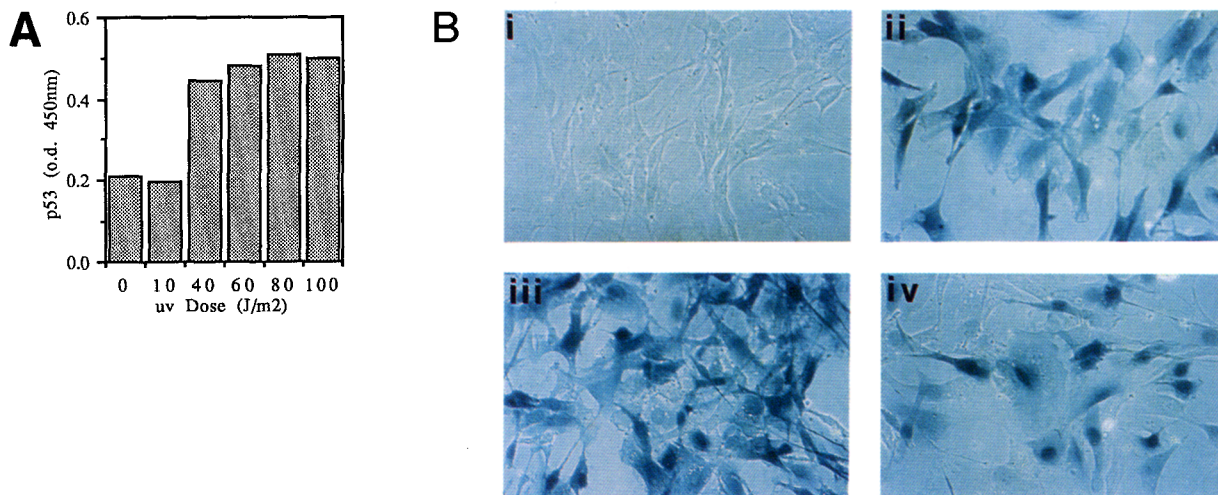


Figure 1. UV-Induced Activation of the Transcriptional Function of p53 Does Not Require an Increase in p53 Protein Levels

(A) The level of p53 protein in extracts of the irradiated and unirradiated mouse fibroblast reporter cells was measured 16 hr after irradiation at the doses indicated by using a two-site ELISA in which p53 is captured with solid phase monoclonal antibody PAb248 and detected with rabbit anti-p53 antibody CM1 and an enzyme-labeled second antibody. The ELISA was standardized by titration of pure murine p53 protein.
(B) The level of β-galactosidase produced by p53 transcriptional activity was assessed at the single cell level by using an immunohistochemical method and the X-Gal substrate: (Bi), 0 J/m²; (Bii), 10 J/m²; (Biii), 20 J/m²; (Biv), 40 J/m².
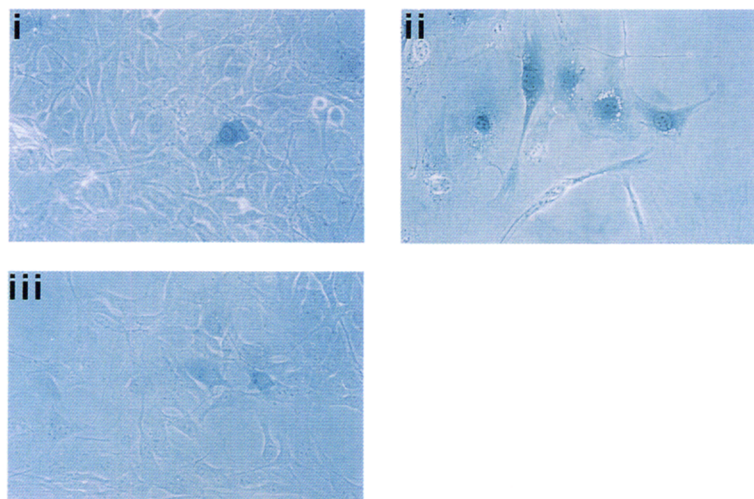
Figure 2. Activation of the Sequence-Specific Transcriptional Response of p53 In Vivo by Microinjection of PAb421

Cells were microinjected with control buffer (2i), the activating antibody PAb421 (2ii), or the inhibiting antibody PAb246 (2iii), incubated for 16 hr, and then stained for β-galactosidase production as in Figure 1. The complete results of two similar experiments are detailed in Table 1.

event catalyzed by a specific agent targeting the C-terminal negative regulatory domain. Furthermore, they provide an explanation of an earlier paradox in the field. While the viability of p53-null mice establishes unequivocally that p53 function is not required for the cell cycle, microinjection of anti-p53 antibodies into the nuclei of quiescent cells has been shown to block entry of these cells into S phase following serum stimulation (Mercer et al., 1982, 1984; Deppert et al., 1990). These results had been interpreted previously to show that p53 was required for entry into S, as it was reasonably assumed at the time that the antibodies acted as antagonists blocking an essential p53 function required for G1 exit. Our current results, based on our in vitro and in vivo studies, clearly demonstrate that these antibodies can instead act as agonists, activating the transcriptional activity of latent p53 in vivo, and would therefore be expected to induce a p53-dependent G1 block in the same way as ionizing irradiation. Given the relevance of this activation reaction in vivo, we have focused on determining the mechanism of p53 activation with a view toward identifying small molecules that can be used to regulate the p53 pathway.

## A Synthetic Peptide Derived from the C-Terminal Regulatory Domain Activates Latent p53

Our current model of how the C-terminal negative regulatory domain of p53 acts to control the DNA binding function of p53 is illustrated in Figure 8 (q.v.). Negative regulation of p53 may occur through amino acid side-chain interactions between the regulatory site and another domain within the tetramer. According to this allosteric model, competitive disruption of the regulatory site amino acid contacts by synthetic peptides derived from the C-terminus would lead to the conformational changes that activate p53. In contrast, according to a steric model of negative regulation, small peptide mimetics of the C-terminus would actually bind to the active site and inhibit sequence-specific DNA binding of p53. To discriminate between these two possibilities, we sought to determine whether p53 function would be inhibited or activated by small peptides derived from its negative regulatory domain.

A panel of low molecular mass, overlapping polypeptides derived from the C-terminal regulatory domain was used for these studies (Figure 3A). Peptide $C_1$ (C369–383), which harbors both the protein kinase C site and the

Table 1. Quantitative Summary of Two Independent Experiments

| Injected Material | β-Galactosidase-Positive Cells | Number of Cells Injected | Percent β-Galactosidase-Positive Cells |
| --- | --- | --- | --- |
| Experiment 1 | | | |
| Buffer | 15 | 105 | 14 |
| Buffer | 3 | 117 | 3 |
| PAb 421 | 54 | 119 | 45 |
| PAb 421 | 75 | 181 | 41 |
| PAb 246 | 12 | 215 | 6 |
| PAb 246 | 6 | 70 | 9 |
| Experiment 2 | | | |
| Buffer | 17 | 216 | 8 |
| Buffer | 14 | 184 | 7 |
| PAb 421 | 90 | 223 | 40 |
| PAb 421 | 47 | 166 | 28 |
| PAb 246 | 0 | 140 | 0 |
| PAb 246 | 3 | 204 | 2 |
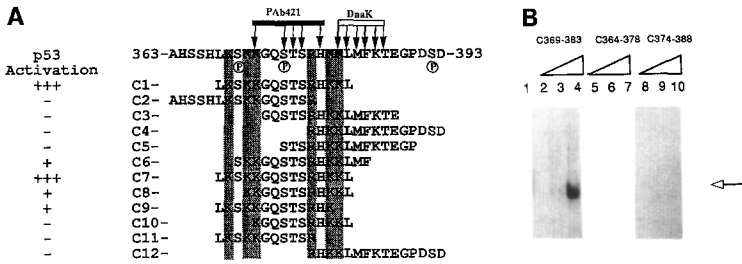
**A**



| p53 Activation | | 363-AHSSHL...SKKGQSTSRHKKLMFKTEGPDSD-393 |
|---|---|---|
| +++ | C1- | LKSKKGQSTSRHKKL |
| - | C2- | AHSSHLKSKKGQSTSR |
| - | C3- | GQSTSRHKKLMFKTE |
| - | C4- | RHKKLMFKTEGPDSD |
| - | C5- | STSRHKKLMFKTEGP |
| + | C6- | SKKGQSTSRHKKLMF |
| +++ | C7- | LKSKKGQSTSRHKKL |
| + | C8- | KKGQSTSRHKKL |
| + | C9- | LKSKKGQSTSRHK |
| - | C10- | KKGQSTSRHKKL |
| - | C11- | LKSKKGQSTSR |
| - | C12- | RHKKLMFKTEGPDSD |

**B**

(A) Binding sites of activating proteins within the C-terminal regulatory domain of p53. The high affinity amino acid contacts required for PAb421 (Stephen et al., 1995) and DnaK binding (data not shown) and the sites of phosphorylation of protein kinase C and casein kinase II are as indicated. The overlap and sequence of synthetic peptides $C_1$–$C_{12}$, relative to the C-terminus of p53, are also as indicated. Peptides $C_1$–$C_4$ are not biotinylated, while peptides $C_5$–$C_{12}$ contain an N-terminal biotin group linked to the amino acid sequence SGSG. The shaded amino acids depict high affinity amino acid contacts required for potent p53 activation, which is summarized on the left, from data in Figure 7.

(B) Incubation of latent p53 tetramers with synthetic peptides derived from the C-terminal regulatory domain. p53 protein (50 nM) was assembled in activation reactions with a panel of synthetic peptides as follows. Lane 1, no peptide; lanes 2–4, 1, 10, and 100 μM peptide $C_1$; lanes 5–7, 1, 10, and 100 μM peptide $C_2$; lanes 8–10, 1, 10, and 100 μM peptide $C_3$. After a 30 min incubation at 30°C, sequence-specific DNA binding was assayed at 0°C as indicated in the Experimental Procedures.

PAb421 epitope, activates p53 at peptide concentrations of 100 μM (Figure 3B, lanes 2–4 versus lane 1). Synthetic peptides $C_2$ (C364–378, lanes 5–7), $C_3$ (C374–388, lanes 8–10) or $C_4$ (data not shown) at concentrations up to 100 μM do not activate p53 function, indicating that activation by peptide $C_1$ is highly specific. These results are consistent with the model whereby specific amino acid contacts between the regulatory site and a surface subdomain can be disrupted to give rise to the allosteric activation of p53.

### Synergistic Activation of Latent p53 by Use of Casein Kinase II and a Synthetic Peptide Derived from the Negative Regulatory Domain

The activation of the latent sequence-specific DNA binding function of p53 seen with peptide $C_1$ is modest. We sought to find conditions where the effect was enhanced so that we could examine the specificity of the activating peptides in more detail. Specifically, we have taken advantage of the fact that, under specific conditions, stoichiometric

phosphorylation of wild-type p53 can be uncoupled from its activation (see below, Figure 8).

The rate of p53 activation by casein kinase II was analyzed by using catalytic levels of recombinant casein kinase II (10-fold lower molar equivalents); under these reaction conditions, p53 becomes progressively more active from 2–10 min at 30°C (Figure 4A, lanes 1–5), despite the fact that stoichiometric phosphorylation has occurred within 30 s (Figure 4B). These results indicate that phosphorylation by recombinant casein kinase II is a very rapid step and that subsequent conformational changes leading to p53 activation are rate limiting.

Under these conditions, in which there is a lower level of p53 activation, but stoichiometric phosphorylation of the protein, the subsequent addition of peptide $C_1$ at 0°C dramatically stimulated p53 sequence-specific DNA binding (Figure 4A, lanes 7–11) to achieve a specific activity similar to PAb421-activated p53 (data not shown). The N-terminal antibody DO-1 shifts the migration of this pro-
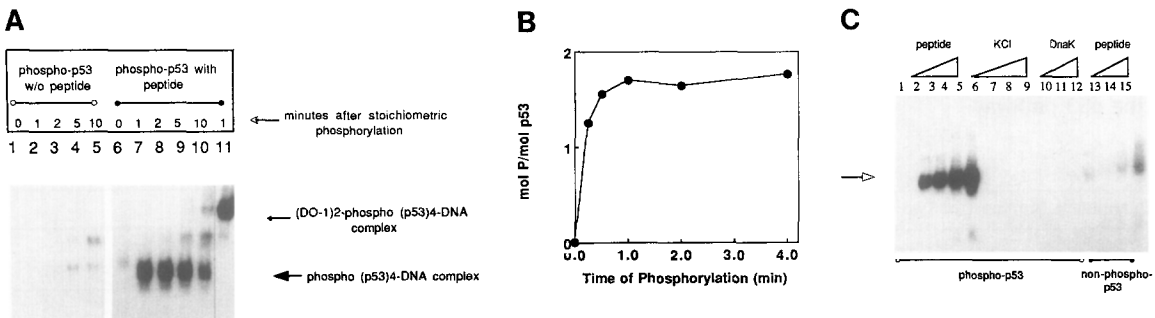
**A**



**B**



**C**



Figure 4. Synergistic Activation of p53 by Phosphorylation and C-Terminal Peptide Addition

(A) Synergistic activation of phospho-p53 with a synthetic peptide derived from the C-terminal regulatory domain. p53 (50 nM) was left unphosphorylated (lanes 1 and 6) or phosphorylated at 30°C with recombinant human casein kinase II (Hupp et al., 1993), 2 ng for 1 min. After phosphorylation, the reactions were incubated further for 1, 2, 5, and 10 min at 30°C. Subsequently, peptide $C_1$ (20 μM) was added (lanes 6–11) during DNA binding at 0°C and products were analyzed as indicated in the Experimental Procedures. The N-terminal antibody DO-1 was added to a parallel reaction (lane 11 versus lane 7) to demonstrate p53 specificity and tetrameric nature when bound to DNA.

(B) Rate of p53 phosphorylation by casein kinase II. p53 (50 nM) was incubated with recombinant human casein kinase II for the indicated time in reactions containing [γ-$^{32}$P]ATP, and phosphate incorporated into p53 was determined as indicated previously (Hupp et al., 1992).

(C) Peptide titration in activation of phospho-p53 tetramers. p53 (50 nM) was phosphorylated with casein kinase II (2 ng) for 1 min at 30°C (lanes 1–12) or was left unphosphorylated (lanes 13–15). Subsequently at 0°C, either peptide $C_1$ (lanes 2–5; 5, 10, 20, or 40 μM), potassium chloride (lanes 6–9; 50, 100, 150, and 200 mM), DnaK (lanes 10–12; 1.4, 2.8, or 5.6 μM protein) or peptide $C_1$ (lanes 13–15; 10, 20, or 40 μM) were added, and DNA binding was assayed as indicated in Experimental Procedures.

tein–DNA complex (lane 11 versus lane 7), demonstrating the p53 specificity of the reaction. In addition, the tetrameric nature of this peptide-activated form of p53 (lane 11) is clear from the expression of four DO-1 epitopes bound by two DO-1 monoclonal antibodies during native gel electrophoresis, as described previously (Hupp and Lane, 1994a).

Alterations in ionic strength (Figure 4C, lanes 6–9 versus lane 1) or addition of bacterial Hsp70 (lanes 10–12 versus lane 1) could not lead to a stimulation of sequence-specific DNA binding of latent, phospho-p53 intermediate complexes. Under these same conditions, a prior, rapid, and stoichiometric phosphorylation of p53 was essential to unmask the highest level of stimulation of sequence-specific DNA binding by the synthetic peptide (lanes 2–5 versus lanes 13–15).

### Synergistic Activation of Latent p53 by Use of DnaK and a Synthetic Peptide Derived from the Negative Regulatory Domain

To gain further insight into the activation of p53 by synthetic peptides, we investigated whether a prior modification of the C-terminal regulatory site of p53 by a nonkinase activator could also give rise to synergistic activation by peptides. This second assay takes advantage of the facts first that the Escherichia coli Hsp70 protein (DnaK) can also interact with latent p53 tetramers to unmask the cryptic sequence-specific DNA-binding activity efficiently in vitro (Hupp et al., 1992, 1993); second, that DnaK targets the C-terminal negative regulatory domain at a site within peptide $C_3$ (data not shown); and third, that activation of p53 by DnaK can now be divided into two stages; a latent DnaK–p53 intermediate complex can be stably isolated at 0°C (see below).

Activation of latent p53 by DnaK requires an incubation at 30°C, as activation of p53 does not occur when reactions are incubated at 0°C (Figure 5, lane 6). As DnaK does not activate p53 at 0°C, the ability of peptide $C_1$ to synergize in concert with DnaK under these conditions was examined. Importantly, and in contrast with peptide $C_3$, the activating peptide $C_1$ does not bind to DnaK (data
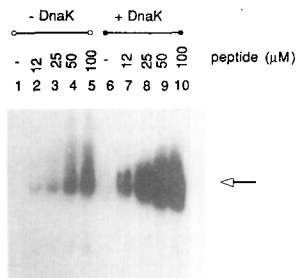


Figure 5. Activation of Latent p53 with Peptide $C_1$ and DnaK

p53 protein (50 nM) was assembled in activation buffer with increasing amounts of peptide $C_1$ alone (lanes 1–5; 0 μM, 12 μM, 25 μM, 50 μM, and 100 μM, respectively) or with DnaK (2.8 μM) and increasing amounts of peptide $C_1$ (lanes 6–10; 0 μM, 12 μM, 25 μM, 50 μM, and 100 μM, respectively) at 0°C for 30 min. After the addition of DNA binding buffer and radiolabeled DNA, products were analyzed as indicated in Experimental Procedures.

not shown). As seen previously, a titration of peptide $C_1$, in the absence of DnaK at 0°C, gives rise to a modest activation of latent p53 (Figure 5, lanes 1–5). In contrast, a titration of peptide $C_1$, in the presence of DnaK at 0°C (Figure 5, lanes 7–10 versus lane 6), gives rise to a dramatic activation of latent p53 with an apparent $K_m$ of peptide activation being 32 μM. The specific activity of DnaK–peptide-activated p53 at 0°C was identical to that of DnaK-activated p53 at 30°C (data not shown). Alterations in the pH, ionic strength, or time of incubation does not lead to a DnaK-dependent activation of p53 at 0°C in the absence of peptide (data not shown).

### Alanine Scan of the Activating Peptide Defines Specific Amino Acids Required to Activate Latent Forms of p53

Truncation of the activating peptide defines endpoints required for activation of p53. A panel of biotinylated peptides that contain N-terminal or C-terminal deletions of the activating peptide $C_7$ (a biotinylated version of the activating peptide $C_1$; see Figure 3) was used to define the end limits required for peptide activation with DnaK at 0°C. A titration of peptide $C_7$ activates p53 by using DnaK with an apparent $K_m$ of activation being 9 μM (Figure 6), which is approximately 4-fold lower than that obtained with the nonbiotinylated peptide $C_1$ in the presence of DnaK. Truncated derivatives of the full-length peptide, peptides $C_8$ and $C_9$, could also coactivate with DnaK, but the apparent $K_m$ of activation was slightly higher at 30 μM. However, further truncation from the N-terminus (peptide $C_{10}$ or $C_{12}$) or from the C-terminus (peptide $C_{11}$) prevented peptide activation, thus defining N- and C-terminal endpoints required for high affinity activation of p53.

A series of peptides with single alanine substitutions at every position from amino acids 369–383 was used to define critical amino acids within the full-length peptide that function in the activation of p53 (Figure 7). These results define a consensus polypeptide sequence that plays an essential role in the rate-limiting activation of p53. Specifically, substitution of any of the positively charged amino acids (K370, K372, K373, R379, K381, or K382) with alanine dramatically reduces the ability of the 19 amino acid synthetic peptide to activate p53 potently. Sub-
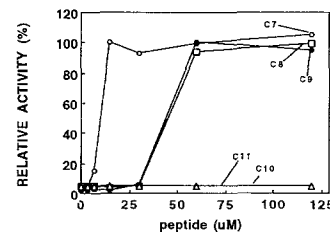


Figure 6. Truncation of Activated Peptide Defines Endpoints Required for High Affinity Activation

Activation reactions were assembled containing p53 (50 μM), DnaK (2.8 μM), and the indicated synthetic peptides containing truncations (as indicated in Figure 3) at 0°C for 30 min. After the addition of DNA binding buffer and radiolabeled DNA, products were analyzed as indicated in Experimental Procedures.

**A**



```
  1  2  3  4  5  6   7  8  9 10  11 12 13 14 15  16  17
```

◀——(DO-1)2-(p53)4-DNA
◁——(p53)4-DNA

**B**

```
ALANINE SUBSTITUTED PEPTIDES
 1-AKSKKGQSTSRHKKL
 2-LASKKGQSTSRHKKL
 3-LKAKKGQSTSRHKKL
 4-LKSAKGQSTSRHKKL
 5-LKSKAGQSTSRHKKL
 6-LKSKKAQSTSRHKKL
 7-LKSKKGASTSRHKKL
 8-LKSKKGQATSRHKKL
 9-LKSKKGQSASRHKKL
10-LKSKKGQSTARHKKL
11-LKSKKGQSTSAHKKL
12-LKSKKGQSTSRAKKL
13-LKSKKGQSTSRHAKL
14-LKSKKGQSTSRHKAL
15-LKSKKGQSTSRHKKA
16-LKSKKGQSTSRHKKL
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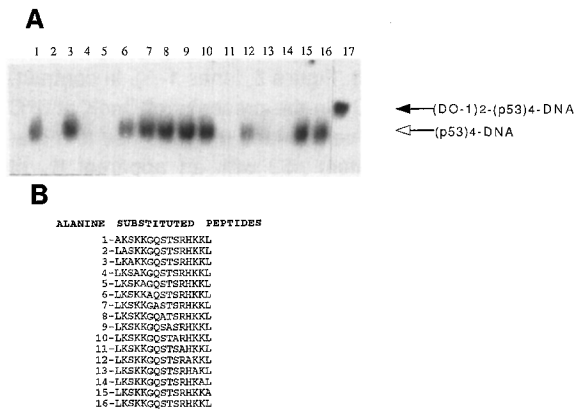
Figure 7. Alanine Substitution of the Activating Peptide Defines Essential Amino Acid Contacts

Activation reactions were assembled containing p53 (50 nM), DnaK (2.8 μM), and synthetic peptides (30 μM) containing alanine substitutions at the indicated positions of 1 through 16). Lane 17 represents peptide-16/DnaK-activated p53–DNA complexes shifted by DO-1, demonstrating the tetrameric nature of p53.

stitutions of the remaining nine amino acids with alanine are tolerated and do not dramatically reduce the apparent $K_m$ of activation. These results begin to define the exact molecular requirements and assay conditions to develop low molecular mass regulators of the p53 allosteric activation pathway.

## Discussion

### A Rate-Limiting Step in the Activation of p53 Sequence-Specific Transcriptional Function In Vivo

Prior to these studies, key chemical steps involved in directly activating p53 protein following DNA damage in mammalian cells have been undefined. However, the ability to activate the sequence-specific transcriptional function of wild-type p53 in vivo, by using a monoclonal antibody (PAb421) known to activate the specific DNA binding function of p53 in vitro, underscores the rate-limiting role of this allosteric activation reaction in modulating the p53 pathway. These data establish the presence of a pool of latent p53 that can be activated posttranslationally in normal cells and demonstrate that the activation of p53-responsive reporter genes can occur in the absence of other signals that might be mediated by DNA damage within the cells, i.e., direct effects on the template DNA. Indeed, a p53-dependent superinduction of p21[CIP1] mRNA was observed upon serum stimulation of quiescent wild-type fibroblasts in the presence of cycloheximide (Macleod et al., 1995), suggesting that activation of p53 transcriptional function can occur in the absence of protein synthesis. In addition, the fact that modification of p53 in the C-terminal negative regulatory domain resulting in loss of the antibody PAb421 epitope (Milner, 1984; Ullrich et al., 1992; Hupp and Lane, 1995) or alternative splicing of mouse p53 mRNA resulting in removal of the negative regulatory domain occurs in tissue culture systems (Arai

et al., 1986; Kulesz-Martin et al., 1994) indicates that modification of the negative regulatory domain can occur in vivo in the absence of DNA damage.

That activation of the transcriptional activity of p53 can occur without an increase in p53 protein levels is consistent with the existence of distinct cellular signaling pathways capable of modulating the conversion between latent and activated forms of p53 (Hupp and Lane, 1995). The results also explain how microinjection of antibodies to the C-terminus of p53 can block cell cycle progression (Mercer et al., 1982, 1984; Deppert et al., 1990), by virtue of activating the biochemical function of endogenous p53 protein. As such, determining the mechanism of p53 activation may have future therapeutic relevance. Selective activation of wild-type p53 by agents that do not induce DNA damage may differentially alter the response of normal tissue as compared with that of p53-negative tumor cells to therapeutic agents toxic only to rapidly cycling tumor cells. On the other hand, one class of mutation in p53 results in proteins that cannot be activated after phosphorylation, but can be activated in vitro by binding of the activating monoclonal antibody PAb421 (Hupp et al., 1993; Niewolik et al., 1995). The latter study is notable, as almost one third of the mutant forms of p53 synthesized in human tumor cell lines can be activated in vitro by the binding of PAb421, suggesting that a considerable proportion of human tumors might respond to allosteric activators by rescuing their p53 response.

### An Allosteric Model for the Activation of p53

The mechanism of p53 latency and the mechanism whereby the protein is activated after covalent modification are not known. In addition, neither the crystal structure of the full-length unphosphorylated p53 tetramer nor that of the phosphorylated tetramer are known, thus precluding predictions on the mechanisms of latency and activation. However, using synthetic peptides derived from the C-terminal negative regulatory domain of p53, we have defined a small molecule that can activate latent p53 function, and this information has led to a specific biochemical model reflecting a possible mechanism of p53 latency and a mechanism whereby phosphorylation alters polypeptide structure. The key elements of this model are that the C-terminal tails of p53 interact with the core of the molecule (Figure 8). This interaction locks the core into a conformation that is inactive for DNA binding. When this tail–core interaction is disrupted by covalent modification, noncovalent modification, or deletion, the core is then able to adopt the active form. Complete activation of p53 is not accomplished effectively by synthetic polypeptides alone, suggesting that negative regulatory domain interactions are not easily disrupted. The stability of the negative regulatory domain interactions are also manifested in the ability to purify to homogeneity latent p53 tetramers by using three sequential chromatographic matrices (Hupp and Lane, 1994a); i.e., a spontaneous shift in equilibrium from the latent to activated state during purification would have reduced latent tetramer yield if p53 negative regulatory domain–tetramer interactions were of low affinity and subject to destabilization.
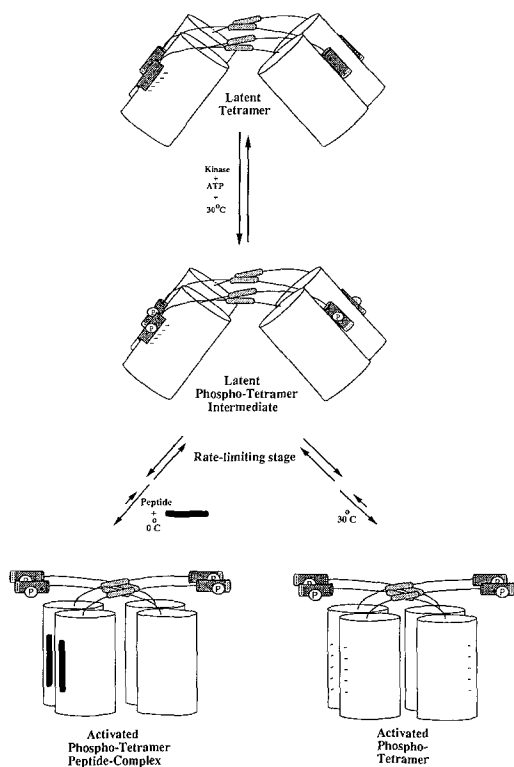
Figure 8. Model for Peptide Activation of Latent p53 Tetramers

p53 exists stably in the latent state owing to interactions between the basic negative regulatory domain amino acid side chains (shaded cylinders) and a peptide-binding pocket within the tetramer. Deletion of the regulatory domain or its phosphorylation and subsequent incubation at high temperature (30°C) disrupts regulatory domain interactions and converts the latent tetramer to an activated tetramer through a concerted transition of subunits. Activation by posttranslational modification at the regulatory site can now be separated into two stages. The first step is a rapid event in which the regulatory domain is modified (i.e., by covalent modification), but the energy barrier required to disrupt the regulatory domain–tetramer interaction is not overcome. As a result, a stable and latent phospho-intermediate can be isolated. However, in a rate-limiting stage, the activating peptide (darkened cylinder) can effectively compete with the phosphorylated regulatory domain binding site at 0°C, promoting the dissociation of the phosphorylated C-terminus from its binding site and thus inducing the conformational change required to activate the tetramer for sequence-specific DNA binding.

Although the activating peptide was not able to activate latent p53 potently, a striking decrease in the apparent $K_m$ for peptide activation and an increase in the specific activity of p53 was accomplished by a prior modification of the C-terminus by casein kinase II or interactions with the heat shock protein DnaK under conditions in which activation is rate limiting. This modification presumably primes p53 protein and decreases the energy barrier to peptide activation by shifting the equilibrium to a state more favorable to stabilize synthetic peptide–tetramer interactions (Figure 8). This model is also supported by the existence of mutant forms of p53 that can be activated from their latent state by the PAb421 monoclonal antibody, but cannot be activated after phosphorylation of its negative regulatory domain (Hupp et al., 1993). Thus, a single point mutation in the core domain of p53 can stabilize

C-terminal negative regulatory domain interactions and hinder activation by covalent modification.

Peptide synergism with activating factors also questions the reliability of using immunoaffinity-purified p53 to interpret biochemical studies on its posttranslational regulation. Immunoaffinity purification of p53 using a PAb421 antibody–linked matrix utilizes a peptide similar to the activating peptide $C_1$ (Figure 3) to elute p53 from the antibody column (Bargonetti et al., 1993). That specific peptides can synergize with p53-activating factors (Figures 4–6) indicates that conclusions based on studies of regulation of p53 by enzymes may be misleading, owing to contamination of protein preparations with the peptides.

The allosteric model of negative regulation can also be used to predict that reduction in net basic charge in the vicinity of the C-terminal regulatory site would be destabilizing and could displace the negative regulatory domain from its binding site. Apart from phosphate modification, three other independent experiments support this prediction. First, site-directed mutagenesis producing the substitution of four basic amino acid residues with hydrophobic amino acids from the C-terminal negative regulatory domain has already been shown to increase strikingly the specific activity of p53 as a sequence-specific transcriptional activator in vivo (Tarunina and Jenkins, 1993), presumably by allowing spontaneous activation of p53 during tetramer assembly in cells. These latter results are striking, especially as this quadruple regulatory site mutant form of p53 actually has a higher specific activity than p53 activated by deletion of its negative regulatory domain. Second, a monoclonal antibody, which binds to the acidic domain in the extreme C-terminus, cannot activate p53 and appears actually to stabilize negative regulatory domain interactions (Hupp and Lane, 1994a). Third, by an elegant approach, random mutagenesis of the C-terminus of human p53 and subsequent screening for mutants that have lost sequence-specific transcriptional activity in yeast model systems resulted in acquisition of two independent point mutations in the casein kinase II site at amino acid 393 that increased net basic charge (Ishioka et al., 1995), an expected result if maintenance of net basic charge is an important element in contributing to p53 latency.

In conclusion, peptide-dependent activation provides the opportunity to formulate biochemical models of p53 latency that can be tested experimentally and thus shed new light on the role phosphorylation plays in allosterically altering polypeptide conformation. These studies also hold promise for the design of low molecular mass agents that can also activate mutant forms of p53 defective in allosteric activation by protein kinases, as activation of wild-type p53 can now be shown in vivo by use of a monoclonal antibody.

## Experimental Procedures

### Reagents, Enzymes, and Proteins

specific DNA-binding reactions, ELISA, DnaK and p53 peptide-binding reactions, and phosphorylations were performed as indicated in the figure legends. Recombinant human casein kinase II was obtained from Boehringer Mannheim. Synthetic peptides were obtained from Pfizer and Chiron Mimitopes.

### Activation of p53

p53 (indicated amounts) was added to 10 μl of activation buffer (10% glycerol, 1.0 mg/ml BSA, 0.05 M KCl, 0.1 mM EDTA, 5 mM DTT, 0.05% Triton X-100, 10 mM $MgCl_2$, 0.5 mM ATP [or 50 μM ATP when activated with protein kinases] and 25 mM HEPES [pH 7.6]), followed by incubation at 30°C for 30 min with indicated activating factor or peptide. Reactions were placed at 0°C with 10 μl of a DNA binding buffer (20% glycerol, 1.0 mg/ml BSA, 0.05 M KCl, 0.1 mM EDTA, 5 mM DTT, 0.05% Triton X-100, 10 mM $MgCl_2$, 0.5 mM ATP, 5 ng of radiolabeled consensus site oligonucleotide [Hupp et al., 1992]) and 100 ng of supercoiled pBluescript competitor DNA. Reaction products were processed by using native gel electrophoresis as indicated previously (Hupp et al., 1992).

### DNA Transfections and UV Treatment

RGCΔFos LacZ plasmid DNA (10 μg) and DOR neo2 plasmid DNA (1 μg) were cotransfected into $5 \times 10^5$ cells (β-galactosidase cells) by calcium phosphate precipitation followed by a 15% glycerol shock. The transfected cells were cultured in selection medium supplemented with 1 μg/ml G418 (β-galactosidase cells) to select cells containing neo plasmid. Fourteen resistant cells were cloned by cloning ring and expanded. The β-galactosidase activity was measured 12 hr after the cells were exposed to 10 $J/m^2$ of UV to induce the endogenous wild-type p53, and the most responsive clone was used to carry out further study. When cells had grown to 80% confluence, in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1 μg/ml G418, culture medium was removed, and the cells were exposed to UV in an XL-1500UV cross-linker (Spectronics Corporation). The energy of UV light delivered was precisely controlled by the cross-linker. After exposure, the cells were grown in fresh culture medium for 16 hr before LacZ expression was determined. Cells were then washed with phosphate-buffered saline (PBS) and fixed with 2% formaldehyde, 0.2% glutaraldehyde in PBS for 5 min on ice. Cells were washed with PBS and overlaid with 0.25 mg/ml X-Gal in reaction mix (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride in PBS). Cells were incubated at 37°C for 16 hr and β-galactosidase-positive cell numbers determined.

### Murine p53 ELISA

Levels of murine p53 were determined in a two-site immunoassay as follows. Falcon microtiter dish wells were coated with 50 μl of purified mouse monoclonal antibody PAb248 at 30 μg/ml in carbonate coating buffer for 8 hr at 4°C. The capture antibody was then removed and the plates blocked with 200 μl of 3% BSA in PBS overnight at 4°C. The plates were washed (1 × PBS, 2 × 0.1% NP-40 in PBS, 1 × PBS) and 50 μl of serially 2-fold-diluted cell extract added for 3 hr at 4°C. Cell extracts were prepared by lysis in ice-cold NET buffer (50 mM Tris–HCl [pH 8.0], 150 mM NaCl, 5 mM EDTA, 1% NP-40) containing 2 mM phenylmethylsulfonyl fluoride, for 30 min at 4°C. Debris was removed from the lysate by centrifugation at 14,000 rpm in a refrigerated Eppendorf centrifuge. Cell lysates were removed, avoiding cross-contamination, and the plates washed as before. Captured murine p53 was detected by using 50 μl of the rabbit anti-p53 serum CM1 diluted 1:1000 in 1% BSA in PBS for 2 hr at 4°C. The plates were washed, and 50 μl of horseradish peroxidase–conjugated swine anti-rabbit IgG diluted 1:1000 in 1% BSA in PBS was added for 2 hr at 4°C. Following washing, bound p53 was visualized with 50 μl of TMB substrate per well. The color reaction was stopped by the addition of 50 μl of 1 M $H_2SO_4$ per well and the optical density at 450 nm measured. The murine p53 ELISA was standardized by including known quantities of recombinant murine p53 expressed in E. coli and resolubilized from inclusion bodies.

### Microinjection

Prior to microinjection (24 hr beforehand), the cells were trypsinized, and approximately 200 cells were spotted as 5 μl drops onto tissue culture dishes. Cells were allowed to adhere for 1 hr before culture medium was added. Microinjection was performed with an Eppendorf microinjection system (Microinjector 5242, Micromanipulator 5170) mounted to an Axiovert 35 M with heated stage. Injections were intranuclear. Purified mouse monoclonal antibodies PAb421 and PAb246 were injected in microinjection buffer (100 mM glutamic acid, 140 mM KOH, 1 mM $MgSO_4$ [pH 7.2]) at a concentration of 2 mg/ml. Following microinjection, fresh medium was added to the cell cultures, and they were incubated 24 hr before being fixed and assayed for LacZ expression with X-Gal.

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