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In addition to binding DNA in a sequence-specific manner, p53 can interact with nucleic acids in a sequence-independent manner. p53 can bind short single-stranded DNA and double-stranded DNA containing nucleotide loops; these diverse associations may be critical for p53 signal transduction in breast cells. In this study, we are analyzing p53 binding to DNA fragments containing insertion/deletion mismatches (IDLs). p53 required an intact central domain and dimerization domain for high affinity complex formation with IDLs. From saturation binding studies we determined that the K_D of p53 binding to IDLs was 2.1 nM as compared to a K_D of 0.3 nM for p53 binding to DNA fragments containing a consensus binding site. Consistent with these dissociation constants, p53-IDL complexes were dissociated with relatively low concentrations of competitor consensus site-containing DNA. The relative number and availability of each form of DNA in a cell immediately after DNA damage may promote p53 interaction with DNA lesions. Understanding how the sequence-specific and non-specific DNA binding activities of p53 are integrated will contribute to our knowledge of how signalling cascades are initiated after DNA damage in breast cells.

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INTRODUCTION

As stated in the original grant proposal, the global focus of my project is to reach a better understanding of the cellular response to DNA damaging agents. Specifically, I proposed to determine the effects of genotoxic agents on the cell cycle kinetics and G2/M cell cycle signalling pathways in both normal and tumor-derived breast epithelial cells. **I hypothesized that a difference between the cell cycle signalling in normal and tumorigenic cells could be manipulated to favor apoptosis in tumorigenic breast cells, thus potentially providing rational targets for therapeutic intervention.**

I initially proposed to use mammary glands isolated from mice as a source of normal breast cells. However, our collaborator for that procedure left Vanderbilt University. Thus, I investigated other sources of normal mammary cells. I chose the MCF-10A cell line which was established from mammary tissue and has a normal karyotype and intact mammary biology (1). For this and other cell lines to be used in the study, I optimized protocols for synchronizing cell cycle (mimosine treatment for tumorigenic cell lines, EGF and insulin withdrawal for MCF-10A; See Appendix 1); by collecting a population of cells representing a specific phase of the cell cycle, the biochemistry of that particular phase could be accurately studied. Furthermore, I optimized a cytoplasmic/nuclear fractionating protocol to study localization-specific biochemistry of specific protein complexes which regulate the cell cycle. However, disappointment was on the horizon.

After DNA damage, the MCF-10A cells exhibited a G2/M cell cycle arrest, the same phenotype observed in established tumorigenic breast cell lines post-DNA damage. Thus, the premise of our study was thwarted, and further studies were deemed to be ultimately unproductive. In fact, another study carried out in the laboratory also showed that normal primary human keratinocytes and fibroblasts exhibit the same phenotypes with respect to cell cycle kinetics and biochemistry as tumorigenic cell lines of similar origin (2). After serious consideration, my Ph.D. committee advised me to pursue a related avenue of research which I have since been following

full-time.

Like my grant proposal, my other avenue of work involves studying the cellular response to DNA damaging agents. The mechanism(s) that elicits the response includes the activation of the p53 tumor suppressor protein. **Importantly, p53 mutations occur in approximately 40% of sporadic breast tumors** (3). My study of p53 involves an extensive characterization and comparison of the interaction of p53 with its consensus DNA binding site and DNA lesions. The vast majority of all tumor-derived mutant forms of the protein have point mutations in the central DNA binding domain, abrogating the ability of p53 to bind DNA(4). Most studies have focused on the sequence-specific interaction of p53 with DNA; however, recent findings extend the types of DNA fragments with which p53 can interact (5,6). The regulation of this binding by various mechanisms, including p53 binding to other proteins and post-translational modifications of p53, are being investigated.

The results of these studies will enable us to determine if p53 binding to DNA lesions is physiologically relevant, and if this phenomenon can be fit into a biochemical pathway of a breast cell in response to stress. Indeed, in the myriad of functions attributed to p53, the direct interaction of p53 with DNA or its binding to DNA-associated proteins is a common theme. These interactions may constitute the upstream events that trigger the activation and stabilization of the p53 protein. A more thorough understanding of how these properties of p53 are integrated will contribute to our understanding of how checkpoints and signalling cascades are initiated upon the onset of DNA damage in breast cells, both normal and tumor-derived.

BODY

Revised Background and Hypothesis/Purpose

Exposure of normal breast cells to agents that damage DNA initiates a p53 signal transduction cascade, resulting in either cell cycle arrest or apoptosis (7). The interaction of p53 with DNA is thought to be critical for its signalling since the majority of tumor-derived forms of p53 have

mutations in the central DNA binding domain (8), abrogating the ability of p53 to bind its consensus DNA sites (9). To date, many studies have focused on the sequence-specific interaction of p53 with DNA, including those that have identified downstream transcriptional targets and studies that describe post-translational modifications that activate p53 consensus site binding.

The ability of p53 to function as a transcriptional activator is believed to be integral for its growth suppressive properties (10,11). Sequence-specific transactivation is one of the most well-understood biochemical activities of p53. After cellular stress such as DNA damage (12), hypoxia (13), viral infection (14), or activation of oncogenes such as *ras* (15) and *myc* (16), p53 becomes transcriptionally active. Once active, p53 induces, among many genes, p21 (17), an inhibitor of cyclin dependent kinases (cdk) thought to be necessary for the p53-dependent G1/S cell cycle arrest (18-21). The increase in p53-mediated transcriptional activity may be due to elevated levels of p53 in the cell (12,22) or increased sequence-specific binding ability. Post-translational modifications of p53, including phosphorylation by S and G2/M phase cdk/cyclin complexes (23,24), DNA-dependent protein kinase (25), protein kinase C (26), and casein kinase II (27), as well as C-terminal acetylation (28), have been found to enhance sequence-specific DNA binding *in vitro*.

In addition to binding DNA containing consensus sites, p53 can interact with nucleic acids in a sequence-independent manner. p53 can bind RNA (29), short single-stranded DNA (ssDNA)[1] (30-32), and double-stranded DNA containing nucleotide loops (5); these diverse associations may be critical to p53 signal transduction. The ability of p53 to bind ssDNA is of interest since this form of DNA is an intermediate of both DNA damage and repair. Studies have correlated p53 signalling activation with both the timing and amount of DNA strand breaks. Nelson *et al.* have shown that p53 levels increased after electroporation of enzymatically active restriction endonucleases into cells (33). Microinjection of single-stranded circular phagemid or

circular DNA with a large gap into nuclei of normal human fibroblasts induced a p53-dependent G1 arrest (34). Jayaraman *et al.* reported stimulation of p53 consensus site binding *in vitro* in the presence of short ssDNA fragments (35). Further proof that p53 may directly interact with damaged DNA was provided in a study reporting p53 binding to DNA fragments containing insertion/deletion lesions (5). Also, confocal microscopy studies have shown co-localization of p53 protein with sites of damaged DNA in histological sections of human skin exposed to UV light (36). Collectively, these reports suggest that p53 may be directly or indirectly regulated by DNA damage intermediates. A direct interaction of p53 with either DNA lesions or with proteins that bind damage intermediates may be a relevant upstream event in the biochemical engagement of the protein. **We hypothesize that after DNA damage, p53 protein half-life and/or biochemical activity are increased due to p53 interaction with DNA damage intermediates.**

Revised Technical Objectives

Specific Aim 1. To characterize p53 binding to both sequence-specific and non-sequence-specific DNA fragments.

COMPLETED. See manuscript in Appendix. Submitted to "Journal of Biological Chemistry". p53 binding to DNA fragments was characterized and the following were determined:

a) The types of DNA lesions to which p53 can bind.

b) The domain of p53 responsible for its binding to DNA lesions.

c) The affinity of p53 for a triple cytosine insertion/deletion mismatches (IDL) and its consensus site.

d) The effect of the presence of one DNA fragment on the kinetics of p53 binding to another DNA site.

Specific Aim 2. To determine if other proteins modulate the p53/DNA interaction.

Background and Rationale

The nanomolar dissociation constant that I observed for p53 binding to IDLs provides evidence for potential *in vivo* relevance. A working hypothesis is that p53 interaction with IDLs may be important in repair processes or p53 may be activated, through post-translational modification, at sites of DNA damage. Several reports suggest that p53 is an important determinant in nucleotide excision repair (NER) (37,38). Using cells derived from patients with Li-Fraumeni syndrome, Ford and Hanawalt showed that the efficiency of global NER was dependent on p53 status (37). Compared to cells heterozygous for p53, homozygous mutant p53 cells exhibited global NER deficiency; however, transcription-coupled repair was unaffected by the p53 status. The ability of p53 to bind replication protein A (RPA) and subunits of TFIIH, both of which are essential components of NER, suggests that p53 may play a direct role in NER. These protein associations are among those that define similarities between p53 and XPA, the damage recognition and binding component of NER. XPA can bind to various damage lesions, and we have observed p53 binding to DNA fragments containing a cholesterol adduct, an artificial DNA lesion used in *in vitro* DNA repair assays (data not shown). XPA binds DNA with RPA cooperatively and once complexed with DNA recruits TFIIH to the site of damage.

Experimental Design

For these protein-DNA binding studies, I will be using Surface Plasmon Resonance offered by the Pharmacia BIACore instrument in the Molecular Recognition Core Lab at Vanderbilt University. The advantages of this system over the McKay assay are the sensitivity, ease, precision, and rapidity with which data can be obtained once the conditions are worked out. The binding studies to be performed with the BIACore include:

a) Comparison of XPA versus p53 binding to C_3.

b) Effect of RPA on p53 binding to C_3.

I will test if p53 binding to C_3 is affected by RPA or XPA. I have obtained purified RPA protein

from John Turchi (Wright State University) and a bacterial clone that expresses XPA from Richard Wood (Imperial Cancer Research Fund). I am currently optimizing XPA expression in BL21 cells.

Specific Aim 3. <u>To determine the effect of post-translational modification on p53 DNA binding affinity.</u>

Background and Rationale

p53 has many serines and threonines which can be phosphorylated *in vitro*. Casein kinase II and protein kinase C can phosphorylate p53 carboxy terminal residues Serine 392 and Serine 370/Threonine 372, respectively, increasing its DNA binding activity in *in vitro* assays(39). Furthermore, the Prives laboratory observed that phosphorylation of p53 by specific cyclin/cdk complexes enhanced p53 binding to the p21 and GADD45 promoter sites(23). p53 has DNA-activated protein kinase (DNA-PK) sites on its amino terminus at Serines 15 and 37, which are probably phosphorylated only when DNA-PK is adjacently bound on DNA; interestingly, Ku and RPA are also phosphorylated by DNA-PK(40). The amino terminus of p53 is also phosphorylated following UV irradiation by the c-Jun kinase (JNK1) at Serine 34(41). Whether these phosphorylation events represent a level of regulation or are basal post-translational modifications of the protein remains unclear.

In addition to phosphorylation, a recent report from Roeder's group demonstrates that p53 can be acetylated by p300 at its carboxyl terminus(28). This acetylation event appears to increase p53 sequence-specific DNA binding activities, suggesting that acetylation may be an upstream event of p53 activation(28).

The human papilloma virus type 16 (HPV-16) partially exerts its tumorigenic action through its E6 protein which binds and inactivates the p53 tumor suppressor protein(42). While studying this interaction, Howley's group noticed that when co-expressed in rabbit reticulocyte lysates, the p53 protein was degrading over time(43). From this observation, they determined that

upon forming a complex with the E6 oncoprotein and the E6-associated protein (E6-AP), p53 is targeted for degradation by ubiquitin(43,44). The rapid turnover of p53 *in vivo* may be due to degradation by the ubiquitin proteolytic system, and the stability of the protein observed post-DNA damage could indicate an over-ride of this modification. This may occur by formation of a p53/DNA complex; Molinari and Milner have reported that p53 is resistant to ubiquitin-dependent proteolysis when bound to its DNA consensus site(45).

Experimental Design

a) The effect of ubiquitination. The p53 half-life in a DNA (consensus site and C_3) bound vs. unbound state can be determined using an *in vitro* degradation assay. The HPV-16 E6 protein and p53 can be expressed from the T7 promoter using Promega's *in vitro* transcription and translation reagents. The assay will be set up such that p53 is allowed to bind DNA before the addition of E6. If no protection is afforded when p53 is pre-bound to C_3 DNA, this result would suggest that p53 likely adopts a different conformation depending on the DNA substrate to which it's bound.

b) The effect of acetylation. I received the HAT domain of p300 from Robert Roeder at Rockefeller University. I purified active acetyltransferase activity from BL21 cells and human p53 from baculovirus-infected Sf9 cells. Once the optimal assay condition is determined, the effect of acetylation on p53 binding to C_3 can be investigated.

CONCLUSIONS

Currently, there is limited information available regarding the biochemical signals that activate p53 after DNA damage and whether or not these signal pathways involve a direct interaction between p53 and DNA damage intermediates. In this study, we analyzed p53 binding to DNA fragments containing IDLs. We found that full-length p53 requires an intact central domain and dimerization capability in order to bind IDLs. Tumor-derived mutant forms of p53 lost IDL binding and C-terminal truncation mutants still bound IDLs if the protein maintained

dimerization capability. The nanomolar dissociation constant that we observed for p53 binding to IDLs provides evidence for potential *in vivo* relevance.

Previously, the C-terminus of p53 (amino acids 311-393) was shown to bind IDLs (36). Reed *et al.* also found that the C-terminus of p53 bound non-specifically to dsDNA (6). These data contribute to a model suggesting that the p53 C-terminus negatively regulates the sequence-specific binding activity of the protein. When the p53 carboxyl-terminal end is deleted, phosphorylated, or bound to antibody, peptide, or single-stranded DNA, p53 binding to its consensus site is stimulated (35,46). Although we demonstrated that the C-terminus of murine p53 could bind IDLs, an equimolar amount of murine p53 lacking the C-terminus had greater affinity for IDLs than the C-terminal fragment alone.

Our finding that the C-terminus of p53 is not required for IDL binding is in agreement with the study of Parks *et al.* demonstrating that p53 binding to IDLs was unaffected by PAb421, a monoclonal antibody that binds the C-terminal amino acids 371-380 (47). Also, Bakalkin *et al.* suggested that the domain of p53 responsible for non-specific DNA binding depends on the DNA substrate; the C-terminus of p53 binds single-stranded ends of DNA, whereas the central DNA binding domain of p53 binds internal ssDNA segments (30). The identity of the p53 binding domain(s) for various forms of DNA damage may suggest distinct roles for p53 in DNA damage-signalling pathways or DNA repair.

Several reports suggest that p53 is an important determinant in nucleotide excision repair (NER). Using cells derived from patients with Li-Fraumeni syndrome, Ford and Hanawalt showed that the efficiency of global NER was dependent on p53 status (37). Compared to cells heterozygous for p53, homozygous mutant p53 cells exhibited global NER deficiency; however, transcription-coupled repair was unaffected by the p53 status. The ability of p53 to bind RPA (48) and subunits of TFIIH (49), both of which are essential components of NER, suggests that p53 may play a direct role in NER. These protein associations are among those that define similarities

between p53 and XPA, the damage recognition and binding component of NER (50). XPA binds DNA cooperatively with RPA (51), and once complexed with DNA recruits TFIIH to the site of damage (52). p53 binding to IDLs may also be stimulated by RPA. XPA can bind to various damage lesions (50), and we have observed p53 binding to DNA fragments containing a cholesterol adduct (data not shown), an artificial DNA lesion used in *in vitro* DNA repair assays (53).

Although we show that p53 has a 7-fold higher affinity for its consensus site as compared to a DNA lesion, the temporal availability of p53 consensus binding sites must be considered. Wu *et al.* have reported that the p21 gene is the first measurable target of p53 transactivation after high-dose UV-irradiation (54). However, p53-mediated induction of p21 gene expression does not occur until 2 h to 5 h post-irradiation. In agreement with this result, using *in vivo* footprinting, Chin *et al.* did not observe significant DNaseI cleavage protection of the p21 promoter until 2 h after exposure of cells to 20 Gy of ionizing radiation (55). After exposure of cells to one Gy of ionizing radiation, 2 to 8 double-strand breaks per genome have been shown to occur (56). In separate studies, Ji *et al.* reported that treatment of cells with malondialdehyde (an endogenous product of lipid peroxidation) resulted in the formation of three-hundred M_1G-DNA adducts per genome and subsequent elevation of p53 activity several hours later (57). Thus, immediately after exposure of cells to genotoxic agents, the number of DNA lesions in a cell would likely exceed accessible p53 consensus sites and p53 binding to DNA lesions may occur. The nanomolar dissociation constant that we report for p53 binding to IDLs is consistent with the physiological concentrations of both p53 and DNA lesions in the cell after DNA damage. However, once DNA repair is initiated, the availability of consensus sites would likely increase and the higher affinity of p53 for these sites would shift the binding equilibrium.

Our understanding of the biochemical activities that are required for p53 tumor suppressive

activities has increased enormously in recent years. However, the role of DNA damage intermediates and the network of signalling pathways by which cells activate p53 in the overall response to DNA damage is not well defined. Determining if p53 is directly activated by DNA damage intermediates and understanding how the sequence-specific and sequence-nonspecific DNA binding activities of p53 are integrated will contribute to our knowledge of how human cells respond to a wide range of DNA lesions.

STATEMENT OF WORK
("Month 1" refers to July 1998.)

Specific Aim 1. To characterize p53 binding to both sequence-specific and non-sequence-specific DNA fragments.
COMPLETE

Specific Aim 2. To determine if other proteins modulate the p53/DNA interaction.
Months 1-2: Optimization of p53 running conditions for Surface Plasmon Resonance on the Pharmacia BIACore instrument in the Molecular Recognition Core Lab at Vanderbilt University.
Months 1-3: Optimization of XPA growth and purification conditions.
Months 4-6: Optimization of XPA conditions on the BIACore instrument.
Month 7: Comparison of XPA and p53 binding to C_3 DNA fragment.
Months 8-10: DNA binding experiments on the BIACore using RPA.

Specific Aim 3. To determine the effect of post-translational modification on p53 DNA binding affinity.
Months 1-6: Assay p53 half-life in a DNA (consensus site and C_3) bound vs. unbound state using an *in vitro* degradation assay.
Months 7-9: Optimize assay conditions for acetylating p53 *in vitro* with the HAT domain of p300.
Months 10-12: DNA binding experiments using acetylated p53.

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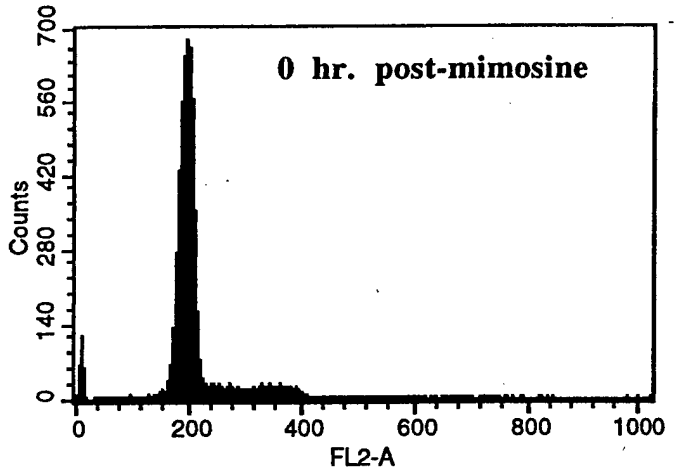
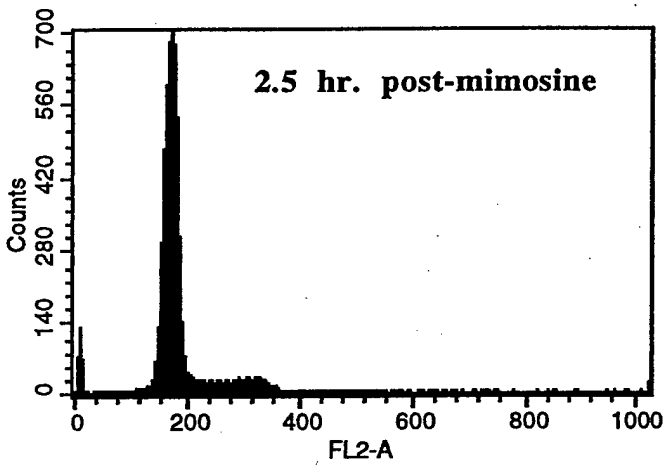
APPENDIX 1

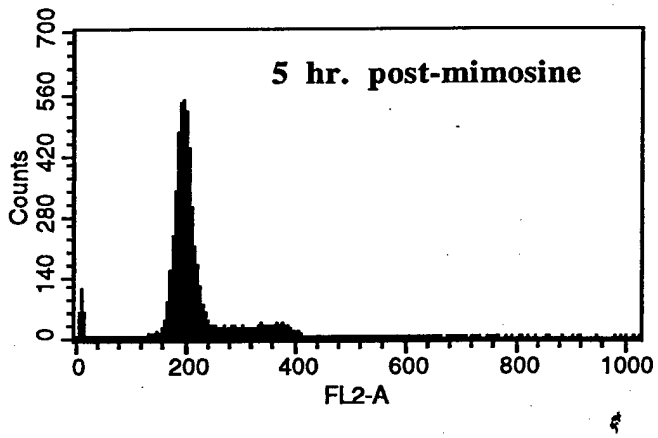
This appendix includes figures showing the cell cycle synchronization of two representative breast cell lines; BT474 is a tumor-derived breast cell line, and MCF10A is a breast cell line derived from normal breast tissue.

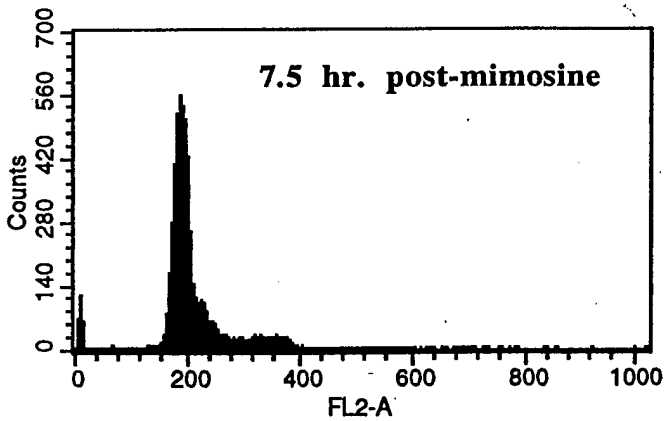
The third figure shows the DNA damage response of the MCF10A cells.

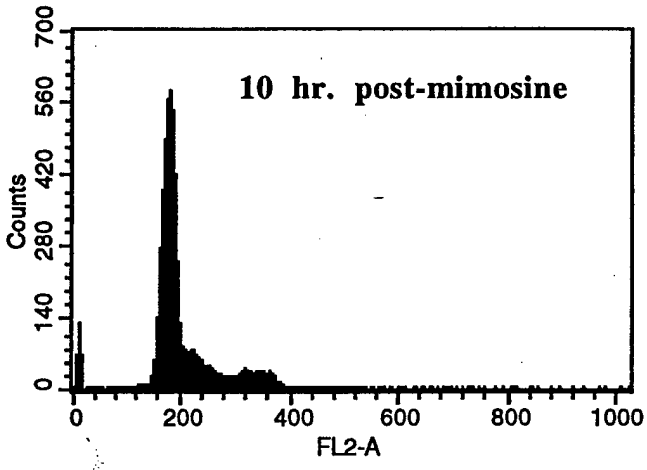
BT 474 cells

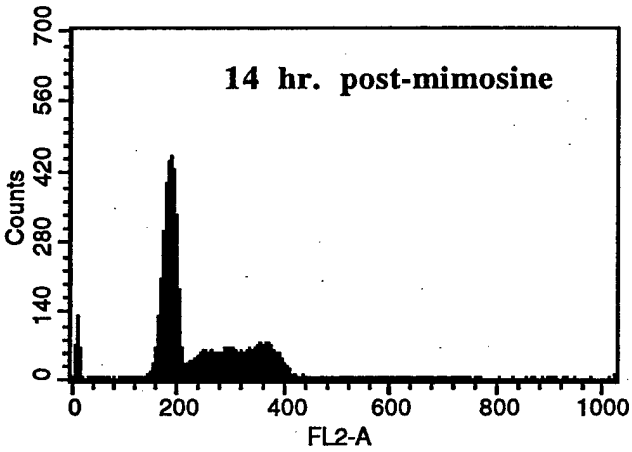












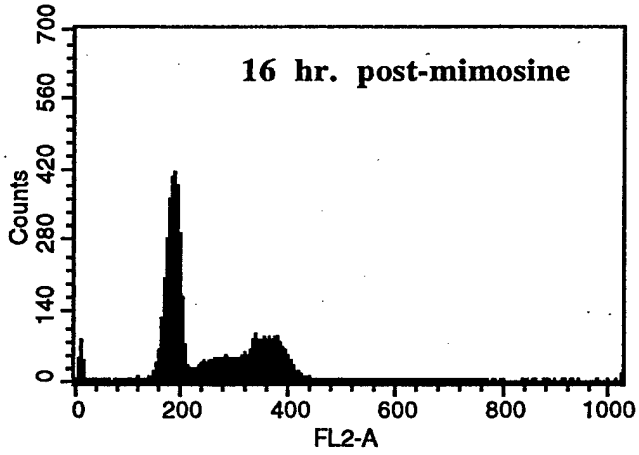
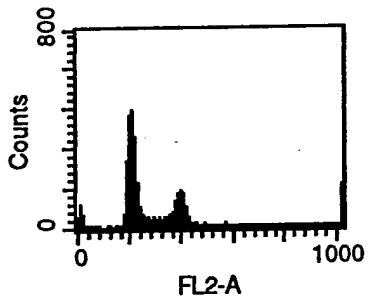
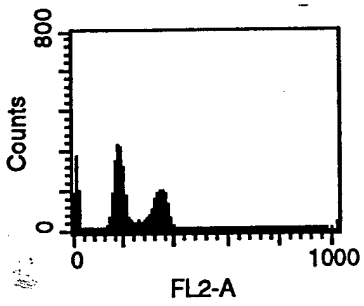


Figure A1: Mimosine Synchronization of BT474 Breast Cells
BT474 cells were treated with mimosine for 48 hours to synchronize cells in the G1 phase of the cell cycle. After mimosine removal, cells began cycling synchronously. Flow cytometry analysis shows the cell cycle position post mimosine withdrawal.

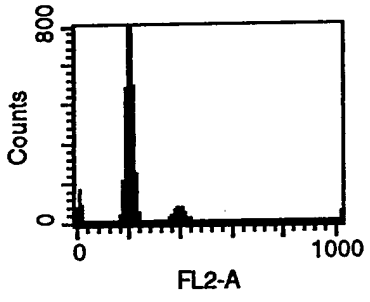
MCF-10A Cells:



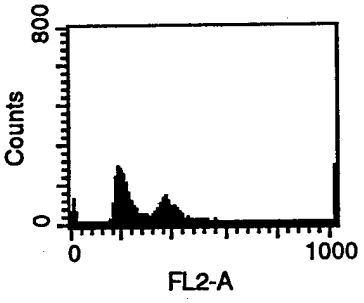
Control



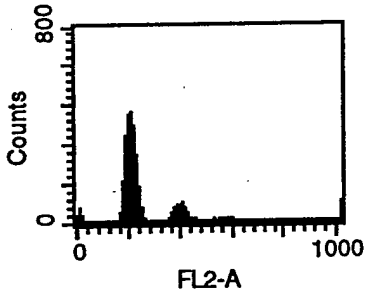
32 hr



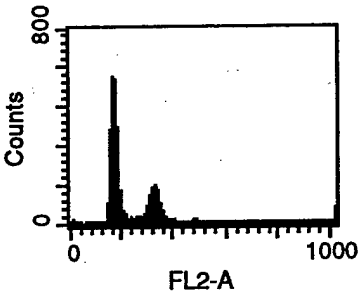
Starved



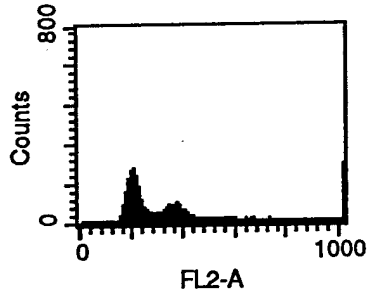
50 hr



8 hr post restimulate



76 hr



24 hr post restimulate

Figure A2: Starvation Synchronization of MCF10A Breast Cells
Both EGF and Insulin were removed from the MCF10A cells' medium; the effect is that the cells arrest at G1. After 36 hours, fully supplemented medium is added to the cells, allowing them to cycle again. Times show the cell cycle position after restimulation of the cells post starvation. Note the synchronous movement of the cells through the cell cycle.

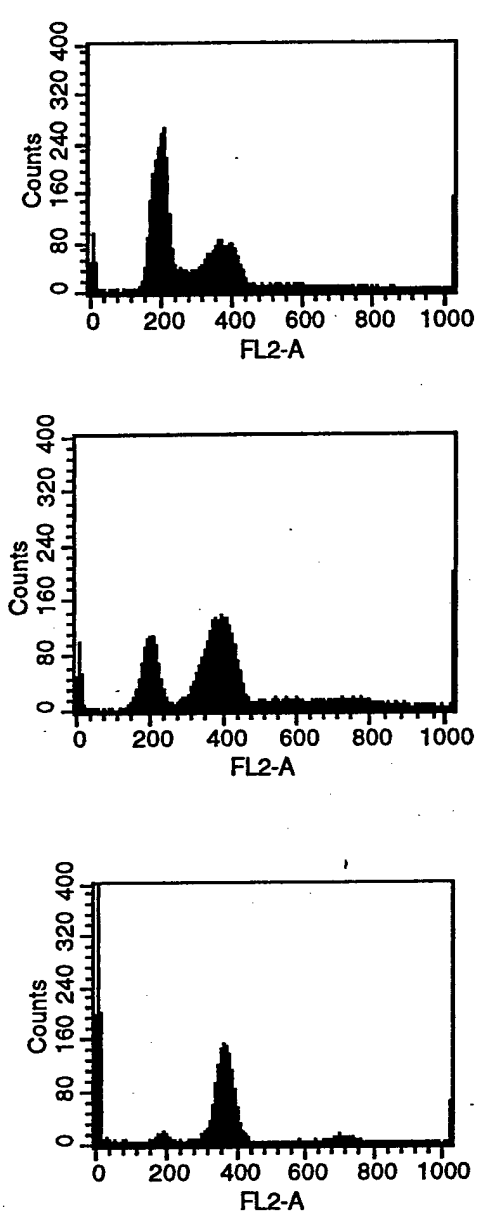


Figure A3: DNA Damage-Induced G2/M Cell Cycle Arrest
MCF10A cells were exposed to 8 Gray of ionizing radiation, and the cell cycle position determined at the times post damage. Note the accumulation of cells at the G2/M phase of the cell cycle.

APPENDIX 2

This appendix includes the manuscript which represents the completion of Specific Aim 1. It has been sent to the "Journal of Biological Chemistry" for review.

High Affinity Insertion/Deletion Lesion Binding by p53: Evidence for a Role of the p53 Central Domain*

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Running Title: High Affinity IDL Binding by p53

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[1]The abbreviations used are: wt, wild-type; IDLs, DNA fragments containing insertion/deletion lesions; ss, single-stranded; ds, double-stranded.

In addition to binding DNA in a sequence-specific manner, p53 can interact with nucleic acids in a sequence-independent manner. p53 can bind short single-stranded DNA and double-stranded DNA containing nucleotide loops; these diverse associations may be critical for p53 signal transduction. In this study, we analyzed p53 binding to DNA fragments containing insertion/deletion mismatches (IDLs). p53 required an intact central domain and dimerization domain for high affinity complex formation with IDLs. In fact, the C-terminus of p53 (amino acids 293-393) was functionally replaceable with a foreign dimerization domain in IDL binding assays. From saturation binding studies we determined that the K_D of p53 binding to IDLs was 2.1 nM as compared to a K_D of 0.3 nM for p53 binding to DNA fragments containing a consensus binding site. Consistent with these dissociation constants, p53-IDL complexes were dissociated with relatively low concentrations of competitor consensus site-containing DNA. Although p53 has a 7-fold higher affinity for DNA with a consensus site as compared to IDLs, the relative number and availability of each form of DNA in a cell immediately after DNA damage may promote p53 interaction with DNA lesions. Understanding how the sequence-specific and non-specific DNA binding activities of p53 are integrated will contribute to our knowledge of how signalling cascades are initiated after DNA damage.

Exposure of normal cells to agents that damage DNA initiates a p53 signal transduction cascade, resulting in either cell cycle arrest or apoptosis (7). The interaction of p53 with DNA is thought to be critical for its signalling since the majority of tumor-derived forms of p53 have mutations in the central DNA binding domain (8), abrogating the ability of p53 to bind its consensus DNA sites (9). To date, many studies have focused on the sequence-specific interaction of p53 with DNA, including those that have identified downstream transcriptional targets and studies that describe post-translational modifications that activate p53 consensus site binding.

The ability of p53 to function as a transcriptional activator is believed to be integral for its growth suppressive properties (10,11). Sequence-specific transactivation is one of the most well-understood biochemical activities of p53. After cellular stress such as DNA damage (12), hypoxia (13), viral infection (14), or activation of oncogenes such as *ras* (15) and *myc* (16), p53 becomes transcriptionally active. Once active, p53 induces, among many genes, p21 (17), an inhibitor of cyclin dependent kinases (cdk) thought to be necessary for the p53-dependent G1/S cell cycle arrest (18-21). The increase in p53-mediated transcriptional activity may be due to elevated levels of p53 in the cell (12,22) or increased sequence-specific binding ability. Post-translational modifications of p53, including phosphorylation by S and G2/M phase cdk/cyclin complexes (23,24), DNA-dependent protein kinase (25), protein kinase C (26), and casein kinase II (27), as well as C-terminal acetylation (28), have been found to enhance sequence-specific DNA binding *in vitro*.

In addition to binding DNA containing consensus sites, p53 can interact with nucleic acids in a sequence-independent manner. p53 can bind RNA (29), short single-stranded DNA (ssDNA)[1] (30-32), and double-stranded DNA containing nucleotide loops (5); these diverse associations may be critical to p53 signal transduction. The ability of p53 to bind ssDNA is of

interest since this form of DNA is an intermediate of both DNA damage and repair. Studies have correlated p53 signalling activation with both the timing and amount of DNA strand breaks. Nelson *et al.* have shown that p53 levels increased after electroporation of enzymatically active restriction endonucleases into cells (33). Microinjection of single-stranded circular phagemid or circular DNA with a large gap into nuclei of normal human fibroblasts induced a p53-dependent G1 arrest (34). Jayaraman *et al.* reported stimulation of p53 consensus site binding *in vitro* in the presence of short ssDNA fragments (35). Further proof that p53 may directly interact with damaged DNA was provided in a study reporting p53 binding to DNA fragments containing insertion/deletion lesions (5). Also, confocal microscopy studies have shown co-localization of p53 protein with sites of damaged DNA in histological sections of human skin exposed to UV light (36). Collectively, these reports suggest that p53 may be directly or indirectly regulated by DNA damage intermediates. A direct interaction of p53 with either DNA lesions or with proteins that bind damage intermediates may be a relevant upstream event in the biochemical engagement of the protein.

In this study, we analyzed the interaction of p53 with DNA fragments containing insertion/ deletion lesions. In contrast to many studies that have shown p53 C-terminus binding to ssDNA or DNA lesions, we demonstrate that an intact central domain and dimerization capability are required for wild-type (wt) human p53 binding to IDLs. The results of our binding analyses demonstrate that the affinity of p53 for DNA fragments containing either an IDL or a consensus site is in the low nanomolar range. Competition binding assays revealed that p53-IDL complexes were dissociated with relatively low concentrations of consensus site-containing DNA. However, the number and availability of each DNA site immediately after DNA damage may promote p53 binding to DNA lesions in lieu of sequence-specific DNA binding.

EXPERIMENTAL PROCEDURES

Expression of p53 Proteins—Sf9 cells were infected with either wt p53 or mutant $p53^{273}$-expressing recombinant baculovirus (kindly provided by C. Prives, Columbia University). Protein extracts of infected cells were harvested in lysis buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol (DTT), 0.5 mM PMSF (Sigma), 1 μM E-64, antipain (10 μg/ml), leupeptin (10 μg/ml), pepstatin A (10 μg/ml), chymostatin (10 μg/ml) (Sigma), and 4-(2-aminoethyl)-benzenesulfonylfluoride (200 μg/ml) (Calbiochem-Novabiochem Corp.)], sonicated, and extracts incubated on ice for 30 min. The protein lysates were centrifuged at 12,000 x g for 20 min at 4°C. Supernatant was collected and stored at -80°C.

Extracts from Sf9 cells infected with baculoviruses expressing various forms of histidine-tagged murine p53 (generously provided by P. Tegtmeyer, SUNY) were prepared as outlined above except the lysis buffer used was DNA Binding Buffer (DBB: 20 mM Tris-HCl pH 7.2, 100 mM NaCl, 10% glycerol, 1% NP-40, 1 mM DTT).

p53 expression vectors (10) were transformed into *Saccharomyces cerevisiae* strain YPH681. Expression was induced by substituting galactose for dextrose in liquid cultures. Protein extracts were obtained by adding DBB and glass beads to the yeast pellets. Yeast were lysed using a bead beater and extracts clarified by centrifugation at 12,000 x g for 10 min at 4°C.

DNA Fragments—The following oligonucleotides were used, underlined sequences represent the triplet cytosines. $3C_3$: 5'-CGAACCCGTTCTCGGAGCACCCCTGCCCCAG CCCAACCGCTTTGGCCGCCGCCCAGCC-3'; C_3: 5'-CGAACCCGTTCTCGGAGCACTG CCCCAGAACCGCTTTGGCCGCCGCCCAGCC-3'; Consensus Binding Site [nucleotides 2293-2332 of the p21 promoter (17), consensus site is underlined]: 5'-TGGCCATCAG GAACATGTCCCAACATGTTGAGCTCTGGCA-3'. Oligonucleotides were purified on 10%

PAGE/1X Tris Borate-EDTA, 7M urea gels, and end-labelled with [$\gamma -^{32}$P]-ATP using T4 Polynucleotide Kinase (New England BioLabs). Complimentary DNA strands were then annealed and duplexes purified using 10% PAGE with 1X Tris Acetate/Borate-EDTA (TAEB).

DNA Binding Assay—To study the p53-IDL interaction, we used an *in vitro* protein-DNA binding assay developed by McKay (58) that allows for quantitative analysis. A monoclonal antibody that recognizes the N-terminus of p53, PAb1801, was chemically cross-linked to Protein A Sepharose (PAS) with 52 mM dimethyl pimelimidate (Pierce). For assays using murine p53 (amino terminally tagged with six histidine residues), a monoclonal Penta-His antibody (Qiagen) was cross-linked to Protein G Sepharose (PGS). The antibody-PAS/PGS complex was added to yeast or baculoviral protein extracts and mixed end-over-end for 1.5 h at 4°C in 250 µl of DBB. Immunoprecipitated p53 was washed once with DBB, followed by a 5 min end-over-end wash with 0.5 M NaCl in Buffer B (5X Buffer B contains: 100 mM Tris-HCl pH 8.0, 5 mM EDTA, 50% glycerol), and finally by a 5 min end-over-end wash in DBB. Subsequent analysis of these samples by SDS-PAGE and silver staining showed immunopurification of p53 to ~95-98% homogeneity. The immunopurified protein was then mixed end-over-end for one hour with 3.5 fmoles of [^{32}P]-end-labelled DNA fragments in 250 µl of DBB for 1 h at room temperature. After three washes with DBB, protein components of the complex were digested with SDS/Proteinase K (VWR Scientific) in TE8 (20 mM Tris pH 8.0, 10 mM EDTA). DNA was phenol-chloroform extracted, ethanol precipitated, and electrophoresed in 1X TAEB on a 10% PAGE at 100 V. Gels were fixed in 5% methanol/5% acetic acid before drying and exposure to film. DNA was quantified using a 445-SI PhosphorImager (Molecular Dynamics) and an Instant Imager (Packard Instruments). Alternative processing of the protein-DNA complexes for protein detection involved adding Laemmli sample buffer to the final complex and subjecting samples to 10% SDS-PAGE electrophoresis. Gels were stained with GelCode Blue Stain Reagent (Pierce).

Saturation Binding Assays—DNA binding assays were performed as outlined above, using the indicated amounts of wt p53 protein immunopurified from Sf9 cells and 3.5 fmoles of either the consensus or C_3 DNA fragment. After final washes, the tubes were counted in a scintillation counter.

Competition Binding Assays—DNA binding assays were performed as outlined above. Equilibrium DNA binding conditions were used (0.3 nM p53 for consensus site DNA; 2.1 nM for C_3 DNA). After counting the initial bound CPM, radiolabelled DNA bound to p53 was competed by the addition of the indicated amounts of unlabelled DNA in 250 µl of DBB. After 1 h incubation at room temperature, complexes were washed three times with DBB, and the tubes were counted in a scintillation counter.

Prism software (GraphPad) was used for analyses of binding data. The percent incorporation of each radiolabelled DNA fragment, the background CPM, and radioactive decay rate of the DNA fragments were corrected for in the analyses.

RESULTS

To study p53-IDL interactions, we used an *in vitro* protein-DNA binding assay developed by McKay (58) that allows for quantitative analysis. The DNA lesions in this study were the same that Lee *et al.* used to demonstrate p53 binding to IDLs through gel shift and electron microscope analyses (5). Random-sequence, double-stranded DNA fragments with either one (C_3) or three ($3C_3$) sets of triplet cytosines in one of the strands were used; the extra bases caused a nucleotide loop in the DNA duplex.

Full-Length p53 Binds to DNA Fragments Containing IDLs—p53 protein produced in either baculoviral or yeast overexpression systems bound to DNA fragments containing IDLs at a level proportional to the number of triple cytosine loops in the DNA (compare middle and right

panel of Fig. 1). Comparable p53 binding activity was also seen using DNA fragments of different sequence context that contained a triple cytosine loop (data not shown). p53 did not bind to a control DNA duplex without triplet cytosines (see first panel of Fig. 1). There was minimal DNA binding activity detected with control immunoprecipitates from yeast and Sf9 extracts lacking p53 protein (Fig. 1, see "Protein Con" lanes). The $p53^{273}$ tumor-derived mutant form of p53 was also tested in the assay and did not exhibit significant binding to DNA fragments containing IDLs.

Tumor-Derived Mutant Forms of p53 Lack C_3 Binding Activity—The lack of IDL binding by the $p53^{273}$ protein prompted us to screen other p53 proteins with point mutations in the central domain. Four mutant p53 proteins representative of tumor-derived forms were produced in yeast, immunopurified, and equivalent amounts of protein analyzed in the binding assay (Fig. 2B). We found that all four mutant p53 proteins had <5% of the wt p53 binding activity to the C_3 IDL (Fig. 2A). These data suggest that full-length p53 requires an intact central domain to bind DNA fragments containing IDLs. However, these results do not rule out the possibility that the p53 C-terminus, as a separate entity, can also bind IDLs as previously reported by Lee *et al.* (5).

Deletion of the C-Terminal 40 Amino Acids of p53 Abrogates C_3 Binding—To study the role of the p53 C-terminus in IDL binding, engineered proteins with successive deletions of the C-terminus were overexpressed in yeast and equivalent amounts of proteins tested for C_3 DNA fragment binding (Fig. 3B). Up to 40 amino acids could be deleted from the C-terminus of p53 without loss of protein binding to IDLs (Fig. 3A; see 1-353). In fact, deletion of 20 amino acids from the C-terminus increased binding activity by more than 2-fold over wt p53 (Fig. 3A; see 1-373). However, deletions of 50 or 60 amino acids resulted in 90% loss of IDL binding (Fig. 3A; see 1-343 and 1-333).

The loss of IDL binding observed with deletions of 50 or more amino acids from the C-

terminus suggested that either the intrinsic C-terminal sequence was required for IDL binding or the oligomerization domain, which is encompassed within residues 312-365 of human p53 protein (59), must be intact for this p53 activity. In order to determine which of these two properties was necessary for p53-IDL binding, we used chimeric proteins containing various C-terminally truncated p53 fused to the coil-coil (CC) dimerization domain of the yeast transcription factor GCN4 (residues 249-281) (60) for further analyses (Fig. 4A). p53-CC fusion proteins with deletions up to 100 amino acids from the p53 C-terminus were able to bind IDLs (Fig. 4B). In fact, the p53-CC fusion proteins 293CC, 323CC, 333CC, 343CC displayed binding activities 2- to ~4-fold greater than wt protein. However, despite the enhanced binding seen with these fusion proteins, a single point mutation in the central domain of the p53 portion of the chimeric protein resulted in 90% loss of IDL binding (Fig. 4A, compare 343CC to $347CC^{175}$). Fusion proteins with p53 C-terminal deletions greater than 100 amino acids (273CC and 283CC) had less than 10% of wt binding activity; these p53 deletions disrupted the central DNA binding domain which is encompassed by residues 100-300 (61). Thus, the data suggest that full-length p53 binding to IDLs requires an intact central domain and that the entire C-terminus of p53 is functionally replaceable with a foreign dimerization domain in these assays

C_3 Binding Activity of Full-Length p53 Versus the C-Terminal Fragment—Previous studies have reported that the C-terminus alone can bind IDLs (5). In order to determine the relative binding activities of full-length p53 and the C-terminal fragment, we assayed the C_3 binding properties of wt human p53 and three forms of murine p53 produced in Sf9 cells: full-length p53, amino acids 1-360, and amino acids 315-390 (62). McKay assays were performed with equimolar amounts of p53 protein incubated with the C_3 DNA fragment (Fig. 5). Murine and human wt p53 had equal C_3 binding activity. Similar to our results with human p53 C-terminal

truncation mutants (Fig. 3A), partial deletion of the C-terminus of murine p53 significantly lowered C_3 binding ability. Consistent with the results of Lee *et al.* (5), we found that the C-terminal fragment of p53 could bind C_3, albeit with less than 10% of full-length p53 IDL-binding activity.

Saturation Binding of p53 to DNA Fragments Containing Consensus Sites or IDLs—The significance of p53 binding to its consensus DNA site is supported by many biological studies (63) and the report of nanomolar affinities for p53 binding to derivatives of its consensus site (64). The lack of significant C_3 binding by tumor-derived mutant forms of p53 suggests this activity, like the p53 consensus binding, may be biologically relevant. To determine if IDL binding occurs in a physiologic range, we performed saturation binding assays to compare the affinity of full-length, wt human p53 for its consensus site with that for C_3. The p53 binding site in the p21 promoter (nucleotides 2293-2332) was used for consensus binding analyses. With 3.5 fmol of DNA fragment, we determined that the K_D for p53 binding to its consensus site was 0.3 nM (Fig. 6A), and the K_D for p53 binding to the C_3 DNA was 2.1 nM (Fig. 6B). Although a 7-fold difference in the equilibrium dissociation constants was identified, the maximal binding of each DNA fragment by p53 was relatively equivalent (~2 fmoles).

Competition of p53 DNA Binding—To confirm the relative affinities determined using saturation binding curves and to study the DNA binding property of p53 when both a consensus DNA site and IDL DNA site were available, competition binding assays were performed. Under equilibrium conditions, p53 was first bound to radiolabelled DNA fragments containing either a consensus site or C_3. Increasing concentrations of either unlabelled consensus site or C_3 DNA were added to assess competition of the pre-bound radiolabelled DNA. When p53 was initially bound to a consensus DNA site, the EC_{50} for competition using consensus site DNA was 0.7 nM

(Fig. 7A). In contrast, a 31-fold higher amount of C_3 was required for competition of p53 off its consensus site; the EC_{50} for this reaction was 22.1 nM. Similarly, if p53 was pre-bound to the C_3 DNA fragment, it was efficiently competed off in the presence of consensus site DNA with an EC_{50} of 0.3 nM. In these assays, ssDNA was not an effective competitor as compared to C_3 (data not shown). The EC_{50} for the C_3 DNA fragment competition of pre-bound C_3 DNA was 1.3 nM. These data demonstrate that p53 has a higher affinity for its consensus site as compared to an IDL site and will likely bind to the former if both DNA sites are present, with all other variables remaining constant.

DISCUSSION

Currently, there is limited information available regarding the biochemical signals that activate p53 after DNA damage and whether or not these signal pathways involve a direct interaction between p53 and DNA damage intermediates. In this study, we analyzed p53 binding to DNA fragments containing IDLs. We found that full-length p53 requires an intact central domain and dimerization capability in order to bind IDLs. Tumor-derived mutant forms of p53 lost IDL binding and C-terminal truncation mutants still bound IDLs if the protein maintained dimerization capability. The nanomolar dissociation constant that we observed for p53 binding to IDLs provides evidence for potential *in vivo* relevance.

Previously, the C-terminus of p53 (amino acids 311-393) was shown to bind IDLs (36). Reed *et al.* also found that the C-terminus of p53 bound non-specifically to dsDNA (6). These data contribute to a model suggesting that the p53 C-terminus negatively regulates the sequence-specific binding activity of the protein. When the p53 carboxyl-terminal end is deleted, phosphorylated, or bound to antibody, peptide, or single-stranded DNA, p53 binding to its

consensus site is stimulated (35,46). Although we demonstrated that the C-terminus of murine p53 could bind IDLs, an equimolar amount of murine p53 lacking the C-terminus had greater affinity for IDLs than the C-terminal fragment alone.

Our finding that the C-terminus of p53 is not required for IDL binding is in agreement with the study of Parks *et al.* demonstrating that p53 binding to IDLs was unaffected by PAb421, a monoclonal antibody that binds the C-terminal amino acids 371-380 (47). Also, Bakalkin *et al.* suggested that the domain of p53 responsible for non-specific DNA binding depends on the DNA substrate; the C-terminus of p53 binds single-stranded ends of DNA, whereas the central DNA binding domain of p53 binds internal ssDNA segments (30). The identity of the p53 binding domain(s) for various forms of DNA damage may suggest distinct roles for p53 in DNA damage-signalling pathways or DNA repair.

Several reports suggest that p53 is an important determinant in nucleotide excision repair (NER). Using cells derived from patients with Li-Fraumeni syndrome, Ford and Hanawalt showed that the efficiency of global NER was dependent on p53 status (37). Compared to cells heterozygous for p53, homozygous mutant p53 cells exhibited global NER deficiency; however, transcription-coupled repair was unaffected by the p53 status. The ability of p53 to bind RPA (48) and subunits of TFIIH (49), both of which are essential components of NER, suggests that p53 may play a direct role in NER. These protein associations are among those that define similarities between p53 and XPA, the damage recognition and binding component of NER (50). XPA binds DNA cooperatively with RPA (51), and once complexed with DNA recruits TFIIH to the site of damage (52). p53 binding to IDLs may also be stimulated by RPA. XPA can bind to various damage lesions (50), and we have observed p53 binding to DNA fragments containing a cholesterol adduct (data not shown), an artificial DNA lesion used in *in vitro* DNA repair assays (53).

Although we show that p53 has a 7-fold higher affinity for its consensus site as compared

to a DNA lesion, the temporal availability of p53 consensus binding sites must be considered. Wu *et al.* have reported that the p21 gene is the first measurable target of p53 transactivation after high-dose UV-irradiation (54). However, p53-mediated induction of p21 gene expression does not occur until 2 h to 5 h post-irradiation. In agreement with this result, using *in vivo* footprinting, Chin *et al.* did not observe significant DNaseI cleavage protection of the p21 promoter until 2 h after exposure of cells to 20 Gy of ionizing radiation (55). After exposure of cells to one Gy of ionizing radiation, 2 to 8 double-strand breaks per genome have been shown to occur (56). In separate studies, Ji *et al.* reported that treatment of cells with malondialdehyde (an endogenous product of lipid peroxidation) resulted in the formation of three-hundred M_1G-DNA adducts per genome and subsequent elevation of p53 activity several hours later (57). Thus, immediately after exposure of cells to genotoxic agents, the number of DNA lesions in a cell would likely exceed accessible p53 consensus sites and p53 binding to DNA lesions may occur. The nanomolar dissociation constant that we report for p53 binding to IDLs is consistent with the physiological concentrations of both p53 and DNA lesions in the cell after DNA damage. However, once DNA repair is initiated, the availability of consensus sites would likely increase and the higher affinity of p53 for these sites would shift the binding equilibrium.

Our understanding of the biochemical activities that are required for p53 tumor suppressive activities has increased enormously in recent years. However, the role of DNA damage intermediates and the network of signalling pathways by which cells activate p53 in the overall response to DNA damage is not well defined. Determining if p53 is directly activated by DNA damage intermediates and understanding how the sequence-specific and sequence-nonspecific DNA binding activities of p53 are integrated will contribute to our knowledge of how human cells respond to a wide range of DNA lesions.

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FIG. 1. **p53 binds DNA containing insertion/deletion mismatches.** Binding assays were performed with the DNA fragments shown and p53 protein preparations from Sf9 or yeast cells. "Control DNA" represents a random-sequence, double-stranded DNA fragment. The DNA fragments containing IDLs were formed by inserting either three extra cytosines ("C_3") or three sets of three extra cytosines ("$3C_3$") into one of the DNA strands. "Protein Control" represents an immunoprecipitate of a crude protein lysate from Sf9 or yeast cells not engineered to express p53 protein. $p53^{273}$ represents the arg-->his^{273} mutant. An equivalent amount of p53 protein was used in each assay and the results are representative of five independent experiments.

FIG. 2. **Tumor-derived mutant p53 proteins lack C_3 binding activity.** *A*, the results of binding assays performed with the C_3 DNA fragment and protein immunopurified from yeast cells engineered to express the following p53 point mutants: val^{143}-->ala, arg^{175}-->his, arg^{248}-->trp, and arg^{273}-->his. Quantification of the bound radiolabelled DNA is presented in the histogram. *B*, level and purity of the proteins used in the assay as visualized on a Coomassie blue-stained SDS polyacrylamide gel. Results are representative of five independent experiments.

FIG. 3. **Deletion of the C-terminal 40 amino acids of p53 abrogates C_3 binding.** *A,* the results of binding assays performed with the C_3 DNA fragment and C-terminally truncated p53 proteins immunopurified from yeast cells. Quantification of the bound radiolabelled DNA is presented in the histogram. *B,* level and purity of the proteins used in the assay as visualized on a Coomassie blue-stained SDS polyacrylamide gel. Results are representative of five independent experiments.

FIG. 4. **The dimerization domain of p53 can be replaced by the coiled-coil domain of the yeast transcription factor GCN4.** *A,* schematic representing the construction of C-terminal deletions of p53 fused to the coiled-coil (CC) dimerization domain of the yeast transcription factor GCN4. I-V represent evolutionarily conserved regions in p53. *B,* the results of binding assays performed with the C_3 DNA fragment and protein immunopurified from yeast cells engineered to express the indicated proteins. Quantification of the bound radiolabelled DNA is presented in the histogram. *C,* level and purity of the proteins used in the assay as visualized on a Coomassie blue-stained SDS polyacrylamide gel. Results are representative of five independent experiments.

FIG. 5. **C_3 binding activity of full-length p53 and the C-terminal fragment.** The results of binding assays performed with the C_3 DNA fragment and protein immunopurified from a crude extract of Sf9 cells engineered to express the indicated proteins. Quantification of the bound radiolabelled DNA is presented in the histogram. Results are representative of three independent experiments.

FIG. 6. **p53 affinity for DNA fragments containing IDLs.** Binding assays were performed by incubating increasing amounts of p53 with 3.5 fmoles of either *A*, p53 consensus site representing nucleotides 2293-2332 of the p21 promoter, or *B*, C_3 DNA fragment. K_D represents the concentration of p53 required to reach half-maximal binding of the DNA. B_{max} is the maximal binding. The equation used to plot the data represents a rectangular hyperbolic function, indicative of binding which follows the mass action law: $Y=B_{max}*X/(K_D+X)$. Results are representative of four independent experiments.

FIG. 7. **Dissociation of p53-IDL complexes by competitor DNA.** Competition assays were performed at equilibrium binding conditions for p53 binding to *A*, consensus site, and *B*, C_3. After binding the indicated [^{32}P]-end-labelled DNA fragment, increasing concentrations of either unlabelled consensus site or C_3 were incubated with the complex. EC_{50} represents the concentration of competitor that reduced p53-DNA binding by 50%. Competition assays were analyzed using GraphPad Prism software. The equation used to plot the data is indicative of competition of binding to a single site: Y=Bottom + (Top-Bottom)/(1+10^(X-LogEC$_{50}$)).

Figure 1:

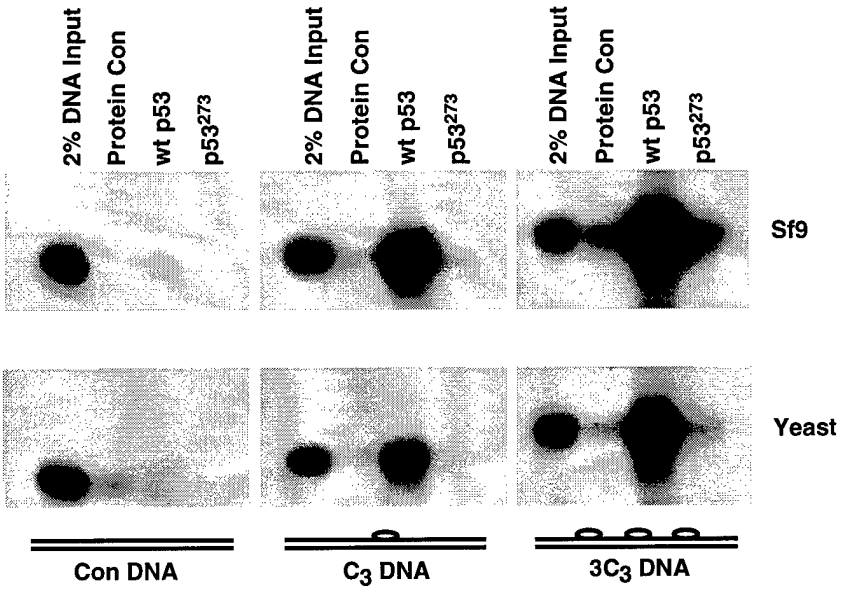

2% DNA Input
Protein Con
wt p53
p53273
2% DNA Input
Protein Con
wt p53
p53273
2% DNA Input
Protein Con
wt p53
p53273
Sf9
Yeast
Con DNA
C3 DNA
3C3 DNA

Figure 2:

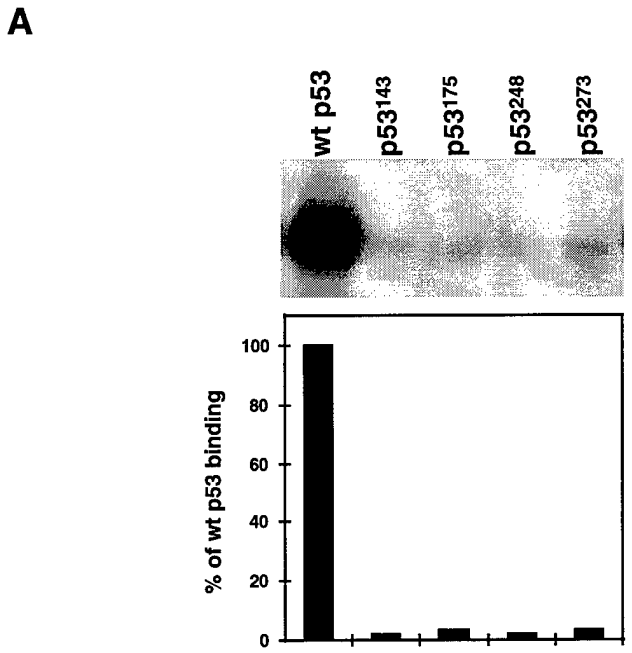

A
wt p53
p53143
p53175
p53248
p53273
% of wt p53 binding
100
80
60
40
20
0
B
wt p53
p53143
p53175
p53248
p53273
68 kD
48 kD

Figure 3:

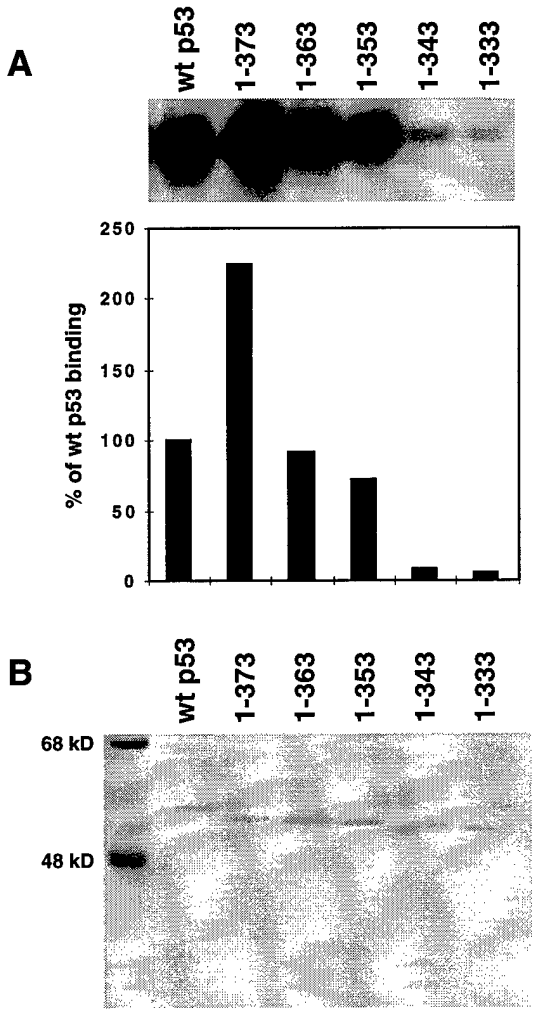

A
wt p53
1-373
1-363
1-353
1-343
1-333
% of wt p53 binding
250
200
150
100
50
0
B
wt p53
1-373
1-363
1-353
1-343
1-333
68 kD
48 kD

Figure 4:

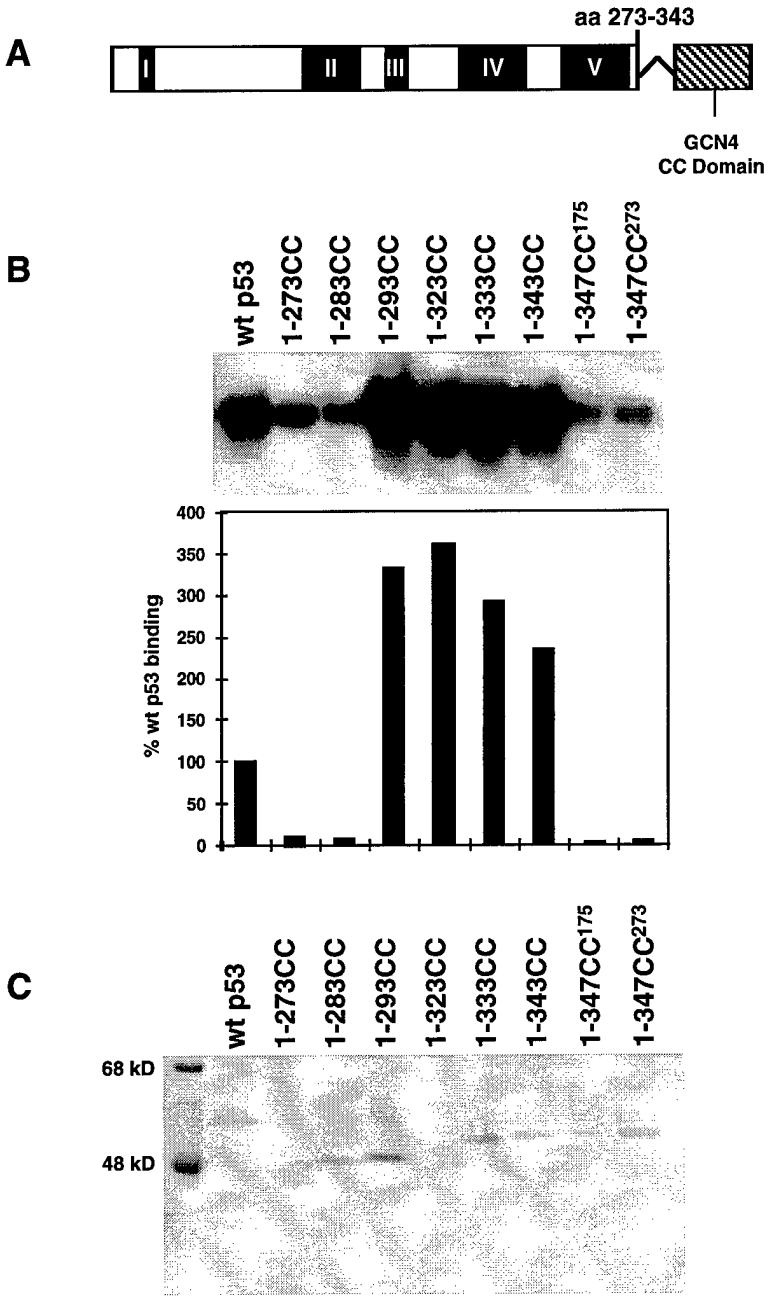

A
aa 273-343
I
II
III
IV
V
GCN4
CC Domain
B
wt p53
1-273CC
1-283CC
1-293CC
1-323CC
1-333CC
1-343CC
1-347CC175
1-347CC273
% wt p53 binding
400
350
300
250
200
150
100
50
0
C
wt p53
1-273CC
1-283CC
1-293CC
1-323CC
1-333CC
1-343CC
1-347CC175
1-347CC273
68 kD
48 kD

Figure 5:

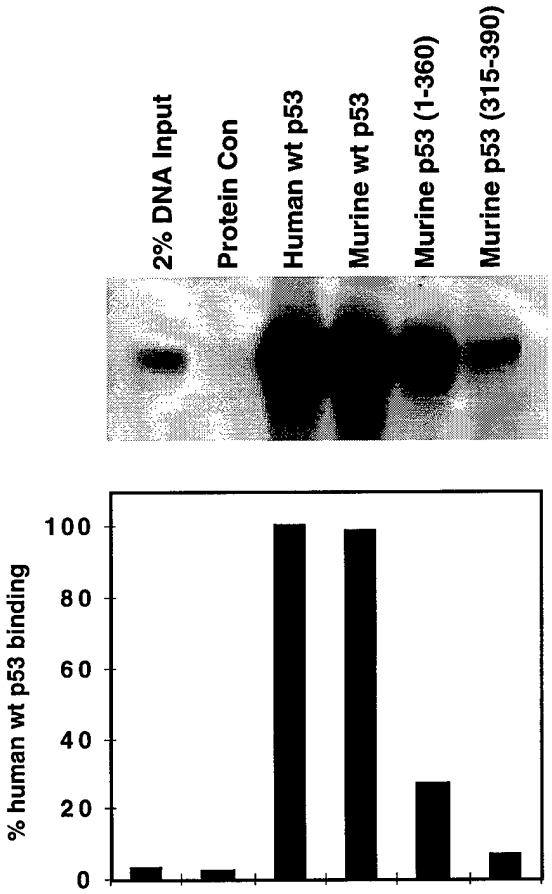

2% DNA Input
Protein Con
Human wt p53
Murine wt p53
Murine p53 (1-360)
Murine p53 (315-390)
% human wt p53 binding
100
80
60
40
20
0

Figure 6:

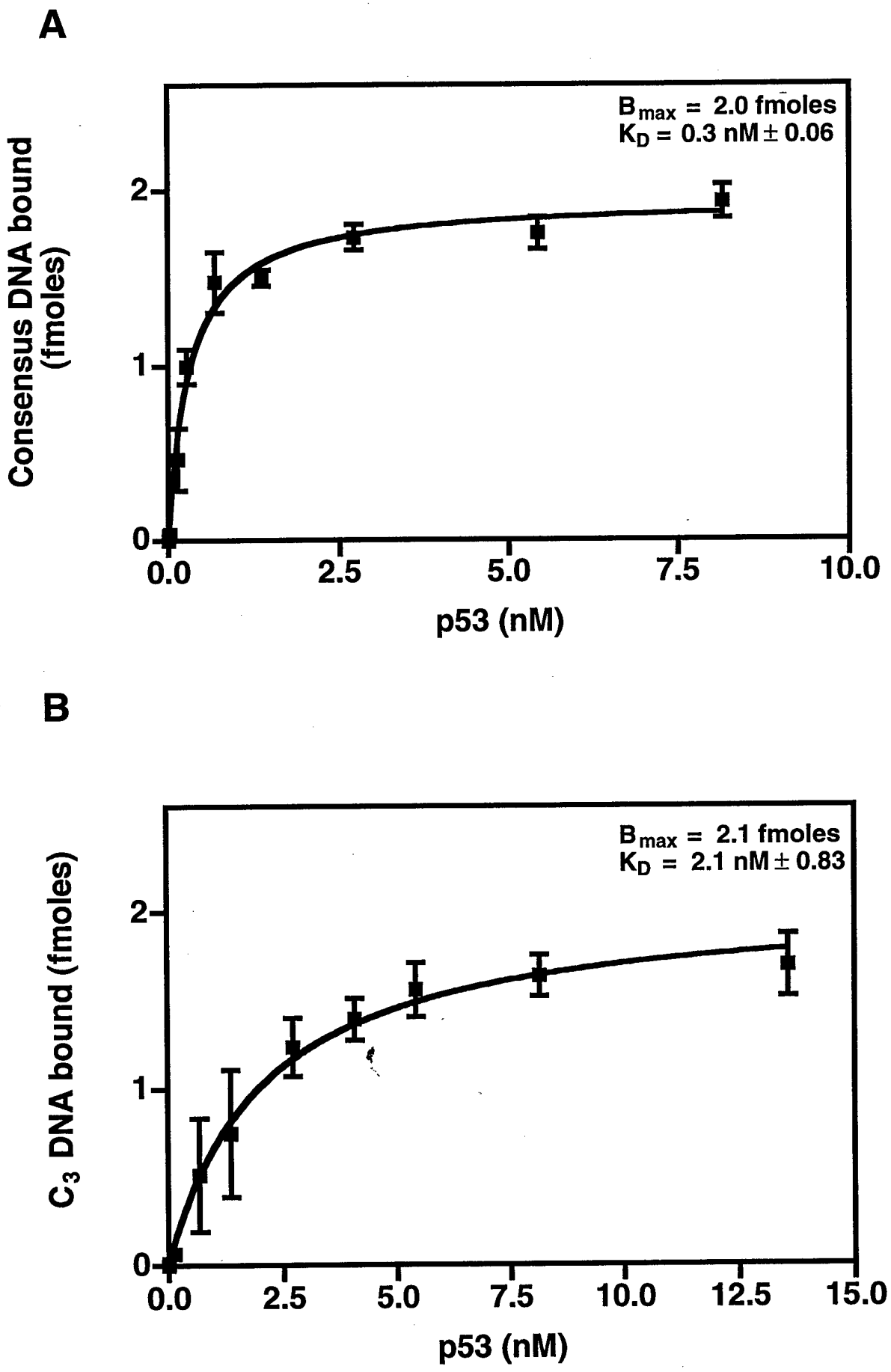
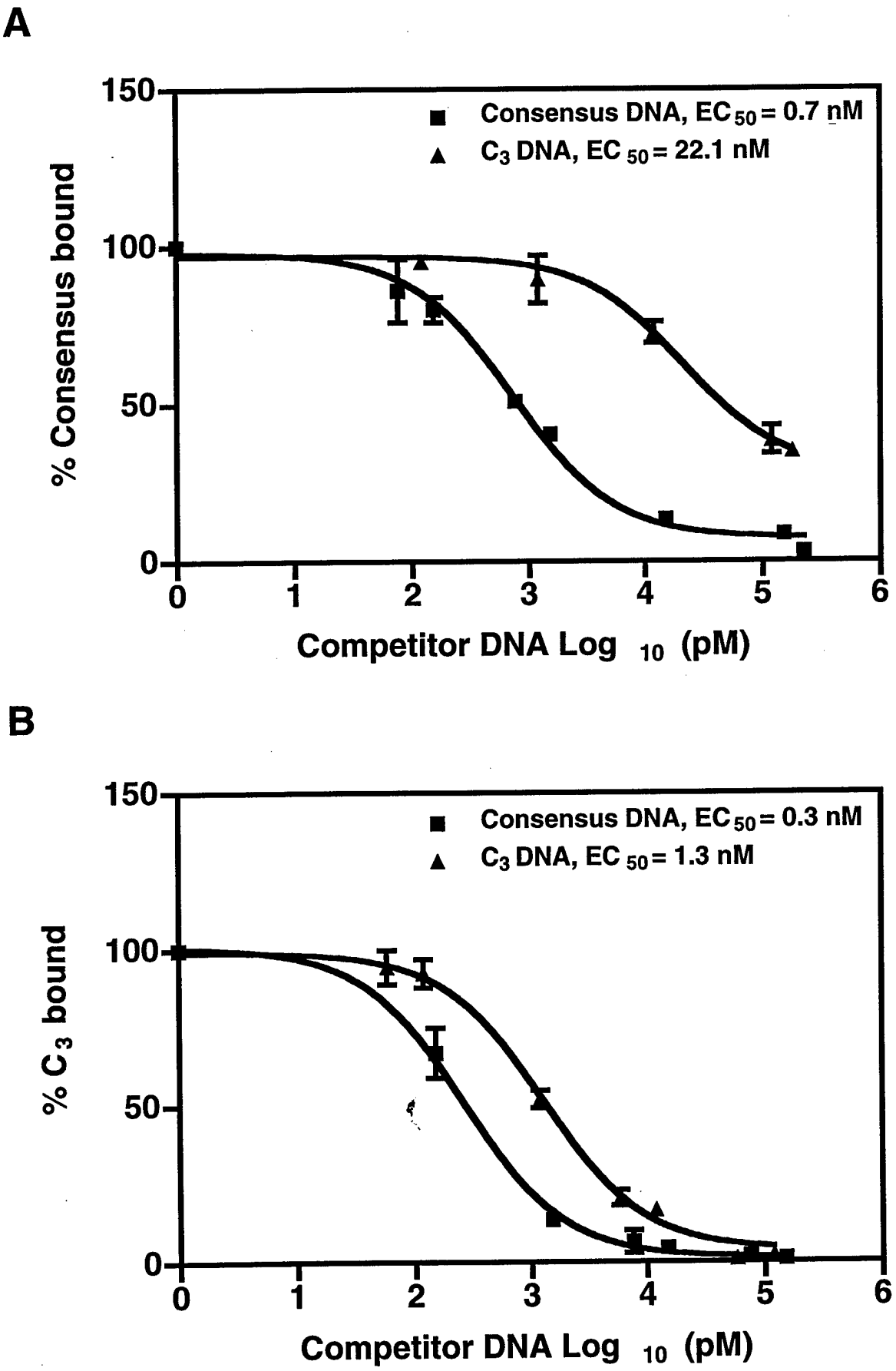

A
Consensus DNA bound (fmoles)
B_{max} = 2.0 fmoles
K_D = 0.3 nM ± 0.06
0
1
2
0.0
2.5
5.0
7.5
10.0
p53 (nM)
B
C_3 DNA bound (fmoles)
B_{max} = 2.1 fmoles
K_D = 2.1 nM ± 0.83
0
1
2
0.0
2.5
5.0
7.5
10.0
12.5
15.0
p53 (nM)

Figure 7:


A
150
100
50
0
% Consensus bound
Consensus DNA, EC50 = 0.7 nM
C3 DNA, EC 50 = 22.1 nM
0
1
2
3
4
5
6
Competitor DNA Log 10 (pM)
B
150
100
50
0
% C3 bound
Consensus DNA, EC50 = 0.3 nM
C3 DNA, EC 50 = 1.3 nM
0
1
2
3
4
5
6
Competitor DNA Log 10 (pM)