Latent and active p53 are identical in conformation

Ayeda Ayed[1], Frans A.A. Mulder[2], Gwan-Su Yi[1], Ying Lu[1], Lewis E. Kay[2] and Cheryl H. Arrowsmith[1]

[1]*Ontario Cancer Institute, and Department of Medical Biophysics, University of Toronto, 610 University Ave., Toronto, Ontario M5G 2M9, Canada.* [2]*Protein Engineering Network of Centres of Excellence and Departments of Medical Genetics and Microbiology, Biochemistry and Chemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada.*

p53 is a nuclear phosphoprotein that regulates cellular fate after genotoxic stress through its role as a transcriptional regulator of genes involved in cell cycle control and apoptosis. The C-terminal region of p53 is known to negatively regulate sequence specific DNA-binding of p53; modifications to the C-terminus relieve this inhibition. Two models have been proposed to explain this latency: (i) an allosteric model in which the C-terminal domain interacts with another domain of p53 or (ii) a competitive model in which the C-terminal and the core domains compete for DNA binding. We have characterized latent and active forms of dimeric p53 using gel mobility shift assays and NMR spectroscopy. We show on the basis of chemical shifts that dimeric p53 both containing and lacking the C-terminal domain are identical in conformation and that the C-terminus does not interact with other p53 domains. Similarly, NMR spectra of isolated core and tetramerization domains confirm a modular p53 architecture. The data presented here rule out an allosteric model for the regulation of p53.

Inactivation of p53 through either deletion, mutation or interaction with cellular or viral proteins is a key step in over half of human cancers[1,2]. The main role of p53 in normal cells is the induction of cell cycle arrest or apoptosis in response to cellular stress, particularly DNA damage[3]. The central role that p53 plays in determining the fate of a cell mandates that its functions be tightly regulated (reviewed in refs 4,5). At the DNA binding level, several aspects of the regulation of p53 activity remain ellusive, most notably the mechanism by which the C-terminal domain inhibits specific DNA binding[6,7].

p53 binds sequence-specifically as a tetramer to DNA targets with a consensus sequence consisting of either a two 10-base pair repeat of 5′-PuPuPu-C(A/T)(T/A)GPyPyPy-3′ (where Pu is a purine and Py is a pyrimidine) or a palindromic site comprised of four five-base pair inverted repeats with a similar sequence[8,9]. Full length p53 has been shown to be inactive for specific DNA binding; activation can be achieved by covalent and noncovalent modifiers of the basic C-terminal domain[5]. p53 constructs lacking the C-terminal domain exhibit enhanced specific DNA binding activity, confirming the inhibitory effect of this domain on p53 activity[10]. Murine p53 has an alternatively spliced form, which lacks the basic C-terminal domain and exhibits enhanced DNA binding, strengthening the case for a regulatory role for the C-terminal domain *in vivo*[11,12].

The p53 protein can be divided functionally and structurally into five regions: an acidic, N-terminal transactivation domain (residues 1–70); a proline-rich stretch containing five copies of the sequence PXXP (residues 60–97); a hydrophobic DNA binding domain highly conserved among p53 from different species and within the p53 family (100–300); an oligomerization domain (320–360); and a basic C-terminal domain (360–393).

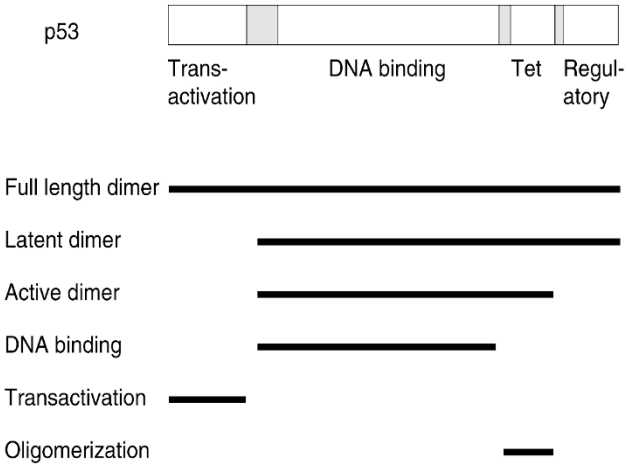

Fig. 1 Linear sequence and domain structure of p53. The constructs used in this work include stabilized (N268D) and dimeric (M340Q, L344R) full length (1–393), latent (82–393) and active (82–360) forms; the dimeric oligomerization domain (310–360); the core domain (82–312) and the N-terminal domain (1–70) constructs of p53.

Several three-dimensional structures of individual domains of p53 have revealed important determinants of p53–DNA and p53–protein interactions, and the consequences of oncogenic mutations and posttranslational modifications on these interactions[13–20]. However, these studies have not provided any insight into the overall architecture of oligomeric p53, especially whether the different domains of p53 interact with one another. Of particular interest is whether or not the C-terminal regulatory domain interacts with any of the other p53 domains, as proposed as a mechanism for inhibition of the specific DNA binding of p53 (refs 6,7,21). In this study we used NMR spectroscopy to detect possible conformational differences between the latent and active forms of p53.

Selection of p53 constructs

The structural consequences of the basic regulatory C-terminal domain on the conformation of p53 can be addressed by the analysis of carefully chosen p53 constructs (Fig. 1). Removal of the C-terminal domain has been shown to activate p53 for DNA binding *in vitro*[10], and this was used as the basis for constructing p53 molecules containing and lacking this domain to represent the latent and active forms, respectively. In order to increase the sensitivity of the NMR experiments described below, we chose to study dimeric forms of p53 constructs with a stabilized DNA binding domain (Fig. 1). The M340Q, L344R double mutant (MQLR) is a half-tetramer, in which the substituted hydrophilic residues, Gln 340 and Arg 344, prevent two p53 dimers from associating *via* the normally hydrophobic dimer–dimer interface of the oligomerization domain[22]. The stabilizing substitution N268D in the DNA binding domain has been shown to produce an active p53 with enhanced stability but with DNA-binding activity similar to that of WT p53 (ref. 23).

Dimeric p53 latency is mediated by the C-terminus

We first confirmed that dimeric p53 containing the regulatory C-terminal domain exists in a DNA binding latent state similar to that of wild type tetrameric p53. To this end, we performed electrophoretic-mobility shift assays (EMSA) using the consensus p53 DNA sequence as a probe. Both in the presence and absence of the competitor DNA poly(dI-dC), MQLR(82–393) and MQLR

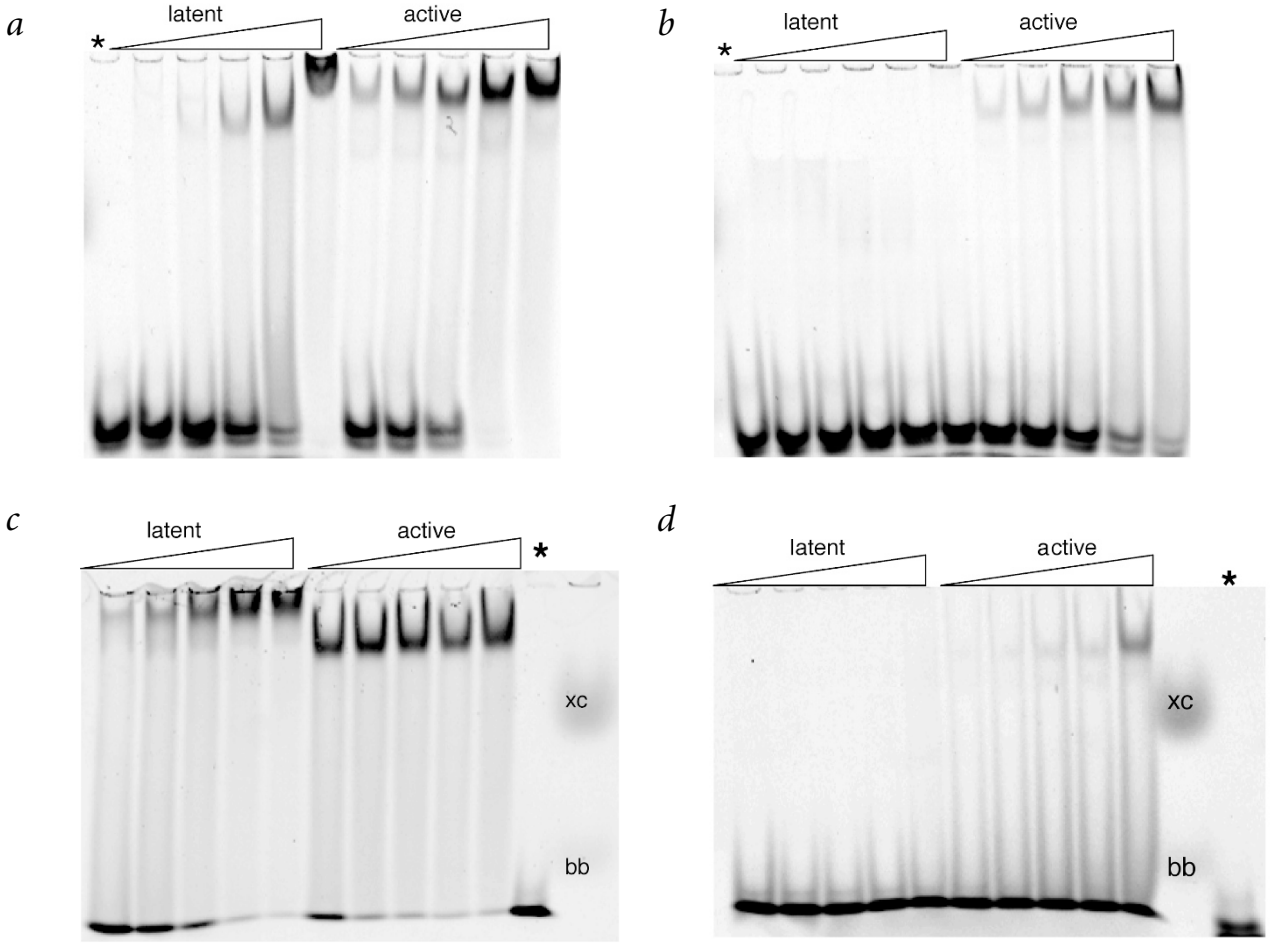

Fig. 2 DNA binding properties of dimeric p53. For all gels, p53 concentrations increase by two-fold increments from left to right and are identical for the active and latent p53s in each experiment. The asterisk indicates the lane with free DNA only. ***a***, Assay of dimeric latent and active p53 against a full p53 consensus DNA sequence with protein concentrations of 0.0625, 0.125, 0.25, 0.5 and 1 mM for both dimeric p53 forms. ***b***, Same as in (*a*) but in the presence of 1 mg poly(dI-dC). ***c***, Same as (*a*) using the half p53 consensus DNA sequence. ***d***, Same as (*c*) but in the presence of 0.25 mg poly(dI-dC). XC and BB are the dyes xylene cyanol and bromophenol blue, respectively.

(82–360) do indeed exhibit significant differences in binding to the p53 consensus DNA (Fig. 2*a*,*b*), as do the tetrameric proteins (data not shown). The active dimer forms well-defined bands corresponding to p53–DNA complexes with electrophoretic migrations that are identical at all protein concentrations, with a K_d ~0.19 μM, whereas the latent protein gives rise to diffuse bands indicative of heterogeneous stoichiometric populations typical of nonspecific complexes with DNA (Fig. 2*a*). In the presence of unlabeled competitor DNA (Fig. 2*b*), DNA–protein complexes are not observed for MQLR(82–393), but those of MQLR(82–360) persist under these conditions. These results demonstrate that the presence of the C-terminal regulatory domain inhibits specific DNA binding of dimeric p53 to the consensus DNA, as was reported for the full length tetrameric p53 (ref. 24). These observations show that dimeric MQLR(82–360) and MQLR(82–393) are suitable samples for structural investigations of the putative latent and active conformations.

The latency observed for MQLR(82–393), however, may be due to tetramerization of the p53 dimers when bound to the consensus site containing two half-sites (or four individual sites for the core p53 DNA-binding domain); dimeric p53 has been shown to assemble into tetramers when bound to the full consensus sequence[25]. In order to rule out this possibility we performed the EMSA of MQLR(82–360) and MQLR(82–393) with a half-consensus site also in the presence and absence of competitor DNA (Fig. 2*c*,*d*). Although binding affinities are weaker due to reduced cooperativity — high affinity DNA binding has been shown to be dependent on dimer–dimer interaction[25] — the results parallel those observed for the full consensus site, confirming that tetramerization is not needed for the inhibitory effect of the C-terminal domain on sequence-specific DNA binding.

NMR spectra of active and latent p53 are similar

To examine the potential differences in conformation between latent and active forms of p53, we compared NMR spectra of MQLR(82–360) and MQLR(82–393) (Fig. 3*a*,*b*). Both proteins exhibit nearly identical spectra, suggesting that the C-terminal regulatory domain does not affect the conformation of other regions of p53. The lack of conformational differences between the two p53 constructs was confirmed by a more detailed comparison of the chemical shift changes obtained for MQLR (82–360) and MQLR(82–393) (Fig. 3*c*). Significant conformational differences between the two constructs would be expected

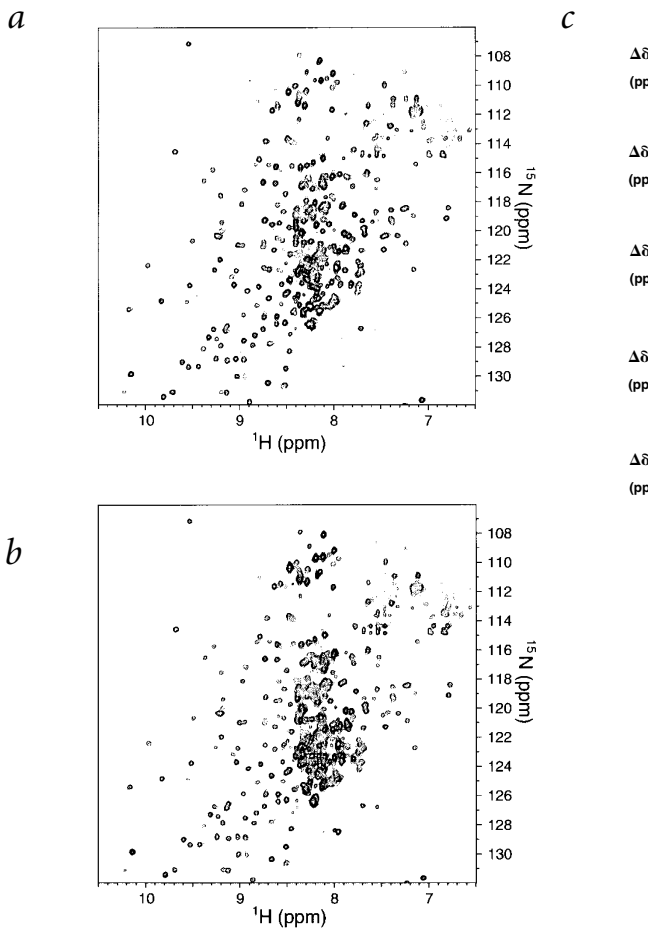

Fig. 3 Comparison of NMR spectra of active and latent dimeric p53. Backbone resonance assignments were completed using four-dimensional TROSY-based experiments[32–34], the details of which are reported elsewhere[35]. ***a***, MQLR(82–360) represents the active form. ***b***, MQLR(82–393) represents the latent form. Samples were 0.8 and 0.6 mM (monomer concentration) in (*a*,*b*), respectively, in 25 mM phosphate, pH 6.5, 250 mM NaCl, 5 mM $MgCl_2$ and 10 mM DTT in 10% (v/v) D_2O. Spectra were recorded at 600 Mhz at 25 °C. ***c***, Comparison of the backbone resonances (HN, N, CO_{i-1} and Cα). The combined chemical shift difference $\Delta\delta_{tot}$ on a per residue basis of residues in MQLR(82–360) and MQLR(82–393) was calculated according to Eq. 1. The vertical bars correspond to the errors in measuring chemical shifts given by the spectral resolution of the data sets in the appropriate frequency domain. The backbone chemical shifts were compared for 95% of nonprolines between residues 82 and 360.

to give rise to substantial changes in $\Delta\delta_{tot}$ for a given residue, calculated using the equation:

$$\Delta\delta_{tot} = ((\delta_{HN}W_{HN})^2 + (\delta_N W_N)^2 + (\delta_{CO}W_{CO})^2 + (\delta_{C\alpha}W_{C\alpha})^2)^{1/2} \quad (1)$$

where δ_i is the chemical shift of nucleus i, and W_i denotes its weight factor. The weight factors were determined from the ratio of the average variances[26] of the ^{1}HN shifts and the chemical shifts of nucleus type i as observed for the 20 common amino acids in proteins using the BioMagResBank chemical shift database, where $W_{HN} = 1$, $W_N = 0.154$, $W_{C\alpha} = 0.276$ and $W_{CO} = 0.341$ (ref. 27; *http://www.bmrb.wisc.edu/*). With the exception of the expected large C-terminal change of MQLR(82–360) due to the negative charge introduced at the new C-terminus, we note that the perturbations of the combined HN, N, CO(i–1) and Cα backbone chemical shifts for each residue were well below 0.1 p.p.m., indicating that the C-terminal domain does not interact with other parts of the p53 molecule and that it does not cause a conformational change. In addition, all of the new resonance peaks observed in the p53 construct containing the C-terminal domain (Fig. 3*b*) appear at frequencies typical of unstructured amides (~8 p.p.m. in the proton dimension). This suggests that the C-terminal domain is not likely to be folded into regular secondary structure.

NMR spectra confirm the modular nature of p53

The NMR data presented here also underscore the modular architecture of p53 as revealed, for example, by proteolytic frag-

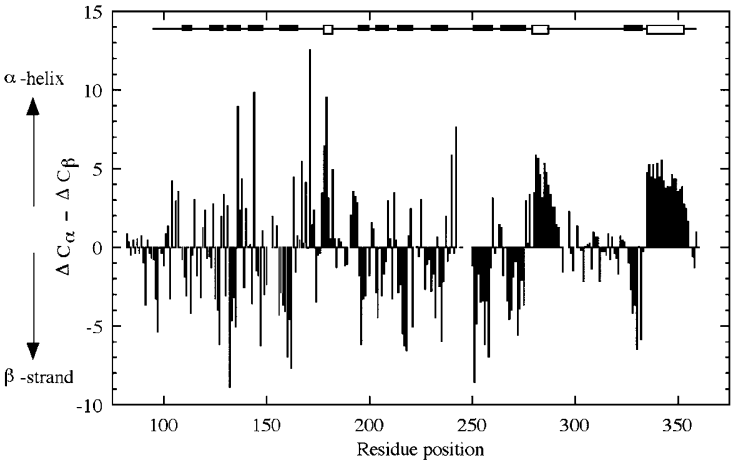

Fig. 4 Solution secondary structure of MQLR(82–360). Based on deviations of ^{13}Cα and ^{13}Cβ chemical shifts[29] ($\Delta^{C\alpha}$ and $\Delta^{C\beta}$) from random coil values[30] for each residue in the sequence, positive and negative values of $\Delta^{C\alpha} - \Delta^{C\beta}$ are reflective of α-helical and β-strand structure, respectively. The secondary structure defined by the crystal structure of the DNA binding domain of p53 bound to DNA and that of the tetramerization domain determined by NMR are shown as solid rectangles for strands and open rectangles for helices, with loops or undefined regions represented as a line. The chemical shift values were corrected for the TROSY shift and for the effect of deuteration.

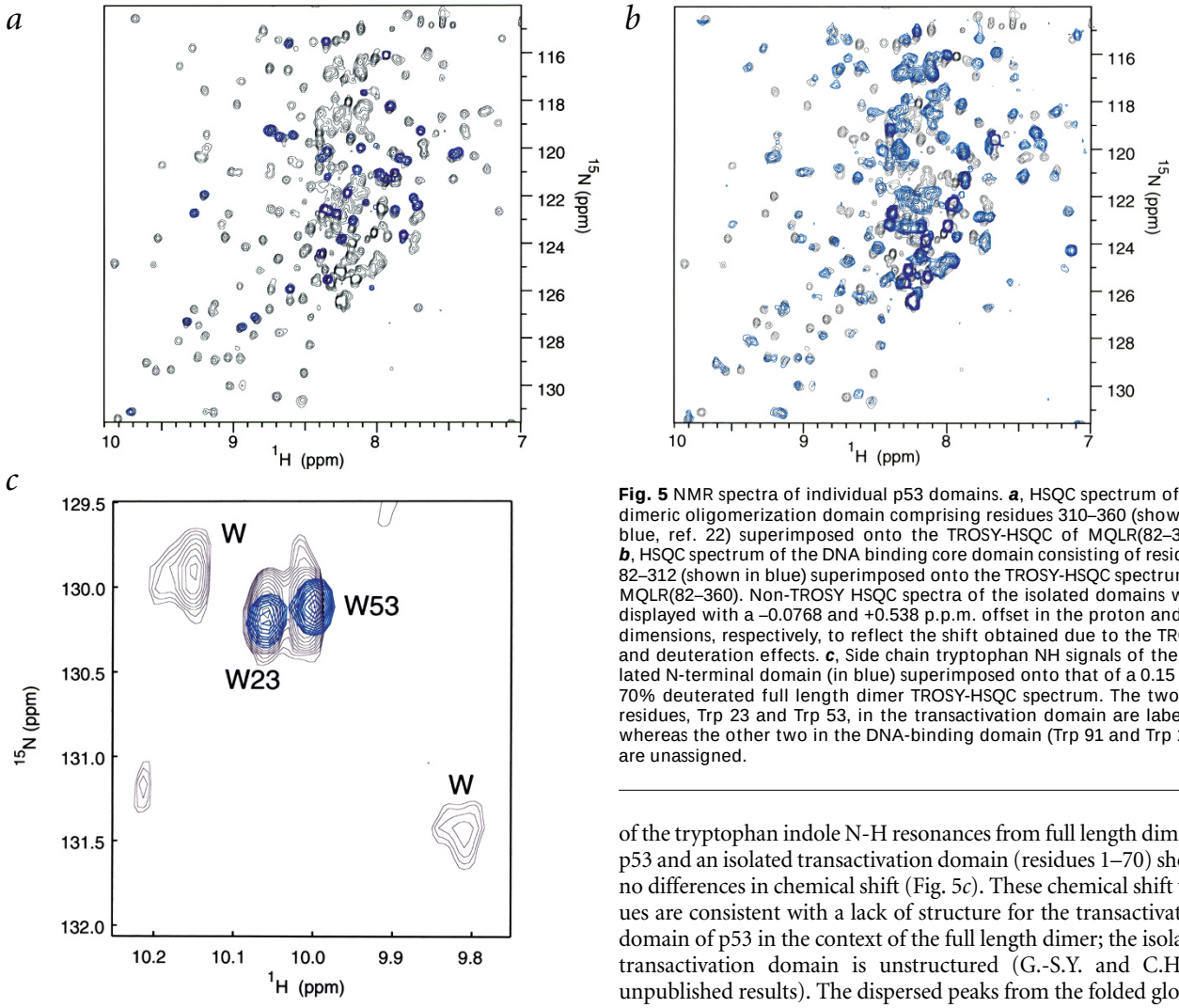

Fig. 5 NMR spectra of individual p53 domains. ***a***, HSQC spectrum of the dimeric oligomerization domain comprising residues 310–360 (shown in blue, ref. 22) superimposed onto the TROSY-HSQC of MQLR(82–360). ***b***, HSQC spectrum of the DNA binding core domain consisting of residues 82–312 (shown in blue) superimposed onto the TROSY-HSQC spectrum of MQLR(82–360). Non-TROSY HSQC spectra of the isolated domains were displayed with a –0.0768 and +0.538 p.p.m. offset in the proton and ^{15}N dimensions, respectively, to reflect the shift obtained due to the TROSY and deuteration effects. ***c***, Side chain tryptophan NH signals of the isolated N-terminal domain (in blue) superimposed onto that of a 0.15 mM 70% deuterated full length dimer TROSY-HSQC spectrum. The two Trp residues, Trp 23 and Trp 53, in the transactivation domain are labeled, whereas the other two in the DNA-binding domain (Trp 91 and Trp 146) are unassigned.

mentation[28]. The NMR resonance frequencies of the backbone nuclei reflect the conformation and local environment of the backbone and, therefore, the secondary and tertiary structure of a protein. The difference in Cα and Cβ chemical shifts[29] of assigned residues of MQLR(82–360) relative to residue-specific random coil values[30] were analyzed along with the secondary structural elements of the DNA binding domain and the tetramerization domain, as determined by X-ray crystallography and NMR, respectively[14,16] (Fig. 4). The plot shows that the secondary structure determined for the isolated domains agrees well with that obtained here for MQLR(82–360).

To further confirm the modular nature of p53, NMR spectra of shorter constructs corresponding to separate domains of p53 were compared to that of MQLR(82–360). The resonance positions observed for the residues in the isolated oligomerization domain[22] (residues 310–360) or those in the monomeric DNA binding domain (corresponding to residues 82–312) were readily identifiable in the NMR spectra of dimeric MQLR(82–360) (Fig. 5*a*,*b*). The DNA-binding and oligomerization domains do not interact, and oligomerization does not introduce contacts between the DNA binding domains in the dimer. To see whether the N-terminal region of p53 has any effect on the conformation of other domains, we recorded a TROSY-HSQC spectrum of full length dimeric ^{15}N, ^{2}H-labeled p53 MQLR(1–393) (Fig. 5*c*). Comparison of the tryptophan indole N-H resonances from full length dimeric p53 and an isolated transactivation domain (residues 1–70) shows no differences in chemical shift (Fig. 5*c*). These chemical shift values are consistent with a lack of structure for the transactivation domain of p53 in the context of the full length dimer; the isolated transactivation domain is unstructured (G.-S.Y. and C.H.A., unpublished results). The dispersed peaks from the folded globular regions of MQLR(1–393) and the shorter constructs described above (MQLR(82–360) and MQLR(82–393)) are superimposable (data not shown), strongly supporting the idea that no additional interactions are mediated by the presence of the highly acidic N-terminal domain. These data also indicate that interactions between the N-terminus and other parts of p53, including the basic C-terminal domain, are unlikely.

Models for C-terminal regulation of DNA binding

Two main models have been proposed to explain the involvement of the C-terminal domain in the negative regulation of sequence-specific DNA binding by p53. First, in an allosteric model, the C-terminal domain is believed to interact with another domain of p53 and induce a conformation that is incapable of binding DNA. Through this mechanism, the C-terminal domain is hypothesized to maintain the latency of p53 until the appropriate signals release the C-terminal domain from its *cis*-interaction and allow DNA binding to take place. Evidence supporting this model includes the demonstration of enhanced DNA binding when the C-terminal domain is either post-translationally modified, removed or bound by a monoclonal antibody[4]. However, the same lines of evidence that support this mechanism may also be explained by a second proposal: a competitive model in which the C-terminal domain vies with the DNA binding domain for interactions with DNA. In the presence of a large excess of nonspecific DNA, such

as poly dI-dC *in vitro* or genomic DNA *in vivo*, this would result in sequestering the protein at nonspecific sites. Supporting this latter model, the C-terminal domain interacts with a variety of nucleic acid forms, including single stranded, double stranded, small short nucleic acid oligonucleotides and broken strands[4].

In this work, we have sought evidence supporting or disputing the hypothesis that a conformational difference exists between the latent and active forms of p53. The NMR data reported here demonstrates that there are no conformational differences between the active and inactive dimeric p53 constructs and that the C-terminal region does not interact with other p53 domains. Nevertheless, the two forms of p53 (lacking and possessing the C-terminal domain) differ in their DNA binding properties and, therefore, a mechanistic explanation is still needed. We suggest that some form of a competitive binding model may be in operation, although a simple two-site competitive binding model may not be sufficient to fully explain all the biochemical data. For example, because an excess of nonspecific DNA is not required for p53 latency, additional mechansism(s), such as steric interference[24], may be involved. Although other models may explain the difference in DNA binding between the active and latent forms of p53, our findings here do not support the allosteric model.

Methods

p53 constructs and purification. Human p53 residues 82–360 and 82–393, which contain the stabilizing mutation N268D, and the dimeric p53 mutant M340Q/L344R were overexpressed *via* a pET15b expression vector (Novagen Inc.) encoding a fusion protein with a 21-residue tag, containing His_6 and a thrombin cutting site, in *E. coli* host strain BL21 (DE3)-[pLys-S]. p53 was typically produced by induction with 0.5 mM IPTG in 2 l of LB-grown culture supplemented with 100 μg ml^{-1} ampicillin and 34 μg ml^{-1} chloramphenicol. Cells were harvested 5–12 h post induction, resuspended in lysis buffer (25 mM Tris, 500 mM NaCl, 1% Triton X-100, 5 mM imidazole and a cocktail of protease inhibitors) and frozen at –70 °C. p53 was purified from clarified cell lysates by metal affinity and size exclusion chromatographies.

Preparation of isotopically-labeled p53. Freshly transformed *E. coli* strain BL21(DE3)-[pLys-S] with the appropriate expression vector was cultured in 99.9% (v/v) D_2O M9 medium, supplemented with ^{15}N ammonium chloride (1 g l^{-1}) and ^{13}C, ^{2}H glucose (2 g l^{-1}), and cultured as described for the perdeuteration of proteins for NMR[31]. Induction and purification were carried out as described above. For NMR spectroscopy, dimeric p53 samples were concentrated to 0.5–1 mM in 25 mM phosphate, pH 6.5, 250 mM NaCl, 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT) and a cocktail of protease inhibitors.

NMR Spectroscopy. NMR spectra were acquired at 600 MHz at 25 °C and processed with the NMRPipe software suite[32]. 2D, 3D and 4D TROSY-based[33] triple-resonance experiments[34,35] were recorded, and the backbone resonances were assigned as described[36].

Electrophoretic mobility shift assays. p53 (at final concentrations of 0.05–10 μM) was incubated with double stranded DNA probe (1–2 pmol) tagged with the monoreactive fluorescent reagent Cy5-Dye (Amersham Pharmacia Biotech) in EMSA buffer (25 mM TRIS, 100 mM KCl, 6 mM $MgCl_2$, 0.5 mM EDTA, 10% (v/v) glycerol and 5 mM DTT) in the presence or absence of excess (0.001–0.1 μg) poly-(dI-dC) (Pharamcia Biotech). The probes used contained either the full (5′-GGACATGCCCGGGCATGTCC-3′) or half (5′-GGACATGCCC-3′) p53 consensus sequence. Complexes were resolved by electrophoresis using a prerun 5% polyacrylamide gel containing TRIS-Borate-EDTA buffer and then visualized by red fluorescence emission measured at 635 nm from the unprocessed gels using a STORM860 scanner (Molecular Dynamics).

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Correspondence should be addressed to A.A. *email: Ayeda@uhnres.utoronto.ca*

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