RNA−p53 Interactions in Vitro†

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ABSTRACT: The tumor suppressor protein p53 is mutated in over half of human cancers. Despite 25 years of study, the complex regulation of this protein remains unclear. After serendipitously detecting RNA binding by p53 in the yeast three-hybrid system (Y3H), we are exploring the specificity and function of this interaction. Electrophoretic mobility shift assays show that full-length p53 binds equally to RNAs that are strongly distinguished in the Y3H. RNA binding blocks sequence-specific DNA binding by p53. The C-terminus of p53 is necessary and sufficient for strong RNA interaction in vitro. Mouse and human C-terminal p53 peptides have different affinities for RNA, and an acetylated human p53 C-terminal peptide does not bind RNA. Circular dichroism spectroscopy of p53 peptides shows that RNA binding does not induce a structural change in the p53 C-terminal peptide, and C-terminal peptides do not detectably affect the structure of RNA. These results demonstrate that p53 binds RNA with little sequence specificity, RNA binding has the potential to regulate DNA binding, and RNA−p53 interactions can be regulated by acetylation of the p53 C-terminus.

Sequence-nonspecific RNA binding proteins play crucial cellular roles. For example, the largest class of sequence-nonspecific RNA binding proteins, hnRNP proteins, coat nascent RNAs during and after transcription to protect the transcripts from degradation and guide processing (*1*). A second large class of sequence-nonspecific RNA binding proteins has been termed "RNA chaperones". These proteins are proposed to function in a manner analogous to protein chaperones in facilitating proper RNA folding by preferential binding to incorrectly folded RNAs (*2*).

The p53 tumor suppressor protein has been extensively studied and is the subject of nearly 40 000 publications to date. Although considered to be a sequence-specific DNA binding transcription factor, many mysteries still challenge the field, and new interacting partners are continually proposed. Full-length human p53 is a 393 amino acid, homotetrameric transcription factor that binds DNA sequence-specifically via its core domain (residues 100−300 of human p53) (*3*). p53 has the potential to activate transcription of at least 122 target genes involved in cell cycle arrest, apoptosis, and other regulatory functions (*4*). Human and mouse p53 contain two unique nucleic acid binding domains. In addition to the sequence-specific DNA binding core domain, p53 also contains a sequence-nonspecific nucleic acid binding domain at its C-terminus (364−393 of human p53), which is thought to be involved in regulation of p53 activity (*3*). Other major domains include an activation domain, a proline-rich domain, and a tetramerization domain (*5*).

The potent regulatory functions of p53 are usually under tight control. In normal cells, p53 is present in almost undetectable levels due to constitutive ubiquitination by MDM2, an E3 ligase, which leads to proteasomal degradation. Various cellular stressors signal to proteins that abrogate the p53−MDM2 interaction, and p53 levels quickly rise (*6*). The p53 protein is thought to be heavily post-translationally modified, but these modifications are not well understood (*5*).

Reports of p53 involvement in RNA metabolism began to surface over a decade ago when p53 facilitation of sequence-nonspecific, RNA−RNA annealing was described (*7*). The annealing activity was mapped to human p53 C-terminal amino acids 311−393 (*8*). Another early report suggested that the C-terminus of mouse p53 protein is covalently attached to 5.8S rRNA (*9*, *10*), though this report has been questioned (*11*). We consider p53 to be a part of an interesting family of DNA binding proteins that may also have RNA partners (*12*).

The sequence specificity of RNA−p53 interactions is controversial. We serendipitously discovered and explored RNA binding by p53 in the context of the yeast three-hybrid system (*13*). In contrast with the implications of published reports (*14*−*16*), we found that the RNA−p53 interaction was largely sequence-nonspecific (*13*).

In the current study, we sought to expand our understanding of the sequence-nonspecific RNA binding characteristics of p53 in vitro. Circular dichroism spectroscopy of p53 peptides shows that RNA binding does not induce a structural change in the p53 C-terminal peptide, and C-terminal peptides do not detectably affect the structure of RNA. We have used electrophoretic mobility shift assays (EMSAs) to explore in detail the relative contributions to RNA binding of different portions of p53. We demonstrate that unmodified p53 binds RNA with little sequence specificity, RNA binding has the potential to regulate DNA binding, and RNA−p53

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interactions can be regulated by acetylation of the p53 C-terminus.

MATERIALS AND METHODS

Recombinant p53 Proteins and Synthetic Peptides. Recombinant, HA-tagged, purified human full-length (1−393), and C-terminally truncated (1−363) p53 were graciously provided by C. Prives (*17*). Peptides were synthesized by the MPRC Peptide Synthesis Facility at Mayo Clinic. Peptide stocks (∼700 μM) were prepared by dissolving lyophilized peptide in water. Peptide concentrations were determined by UV absorbance at 224 and 233 nm using the difference between absorbances to generate a bovine serum albumin (BSA) standard curve based on amide bond absorbance.

RNA. RNAs were transcribed in vitro using the AmpliScribe T7 High Yield Transcription Kit (Epicentre). Transcripts were gel purified, resuspended in water, and quantitated by UV absorbance at 260 nm.

Electrophoretic Mobility Shift Assays. Where indicated, a 66 bp duplex DNA with multiple, strong p53 binding sites was used (*39*). RNA probes were labeled using T4 RNA ligase (New England Biolabs) and [^{32}P]-pCp (Amersham). Reaction mixtures contained EMSA buffer: 20 mM HEPES (pH 7.9), 25 mM KCl, 0.1 mM EDTA, 10% glycerol, 2 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 0.025% NP-40, 2 mM spermidine, 0.5 mM DTT, 8.5 ng/μL heat-denatured salmon sperm DNA (Invitrogen) as noted, ∼3 nM or 30 nM probe RNA or DNA, and proteins as indicated, in a total volume of 20 μL. Reaction mixtures were incubated at room temperature for 15 min before loading them onto native 4% polyacrylamide (29:1 acrylamide−bisacrylamide) gels containing 0.5× TBE buffer for room temperature electrophoresis at 165 V for 1.5 h. Radioactivity was detected and analyzed by storage phosphor technology.

Cross-Linking Analysis. Proteins were incubated in the presence of 0, 0.01, 0.025, or 0.05% glutaraldehyde for 30 min at room temperature in modified EMSA buffer lacking BSA and salmon sperm DNA. Where indicated, p53 and 2 nM RNA were incubated for 15 min prior to glutaraldehyde treatment. Cross-linking reactions containing RNA were then quenched with 0.25 M glycine for 10 min and treated with 20 U RNaseA (USB) for 1 h at room temperature. All samples were heat denatured in 2% SDS and analyzed by electrophoresis through 4−12% SDS−polyacrylamide gels. Proteins were stained with the SilverQuest silver stain kit (Invitrogen).

Circular Dichroism Spectroscopy. Spectra were recorded using a Jasco 810 spectropolarimeter. Far-UV CD spectra were obtained in the continuous mode, collecting measurements every 1 nm with an averaging time of 5 s at 295 K between 200 and 340 nm. RNA samples were 5 μM, and peptide samples were 5, 10, or 100 μM in 0.2 cm path length cells. The sample buffer was 10 mM sodium phosphate (pH 7.0), 20 mM NaCl, and 0.5 mM EDTA with either 5 μM unbound peptide or peptide/RNA complexes titrated to final molar ratios of 1:1 or 1:20 as noted. All baseline buffer and nucleic acid contributions to ellipticity for each sample were subtracted mathematically after collection. Mean residual ellipticity (Θ_{MRE}, expressed as deg cm^2/dmol) was calculated for peptide spectra according to

$$\Theta_{MRE} = \frac{\Theta_{mdeg}}{10lcn}$$

where Θ_{mdeg} is the ellipticity in mdeg, l is the path length in cm, c is the molar peptide concentration, and n is the number of residues in the peptide. RNA spectra are reported in raw ellipticity.

RESULTS

p53 Binds Sequence-Nonspecifically to RNA. The RNA−p53 interaction appears to be largely RNA sequence-nonspecific (*13*). To confirm this conclusion in vitro, a panel of seven unique, ∼300 nt RNAs was first tested for p53 interaction in EMSAs, and all RNAs displayed comparable affinity (data not shown). Two of these RNA molecules differ by only three nucleotides and had been strongly distinguished by p53 in the yeast three-hybrid system (Figure 1A) (*13*). These RNAs were chosen for further study in vitro. Recombinant, full-length, HA-tagged, human p53 protein bound equally to both RNAs in a protein-concentration-dependent manner (Figure 1). As the amount of p53 was increased relative to the constant amount of RNA, multiple, discrete shifted species appeared at lower mobilities (labeled A, B, and C in Figure 1B). It is known that p53 can undergo a monomer−dimer−tetramer equilibrium in solution (*18*), so our results could indicate (i) multimerization of p53 monomers on individual RNAs (e.g., species C, B, and A represent monomer, dimer, and tetramer forms, respectively) or (ii) loading of multiple p53 proteins (e.g., tetramers) onto individual RNAs (e.g., C, B, and A represent one, two, or three bound p53 tetramers, respectively). The former model would be expected for sequence-specific RNA binding, while the latter would be expected for sequence-nonspecific RNA binding. The two models predict different concentration dependences for the components of the complexes. We took two approaches to determine if multiple p53 tetramers bind nonspecifically to RNA.

First, we performed an electrophoretic mobility shift assay with a fixed concentration of p53 while increasing the concentration of labeled RNA (Figure 1C). The single protein concentration should preserve a single multimer state of p53. We observed that increasing RNA concentration in the presence of a fixed p53 concentration depleted slower species (Figure 1C, compare lanes 2 and 5). This result strongly suggests that multiple p53 molecules bind to individual RNAs in a sequence-nonspecific mode.

Second, we used glutaraldehye cross-linking (*19*) to determine which p53 form (monomer, dimer, or tetramer) binds RNA. Full-length human p53 (17 nM) was incubated with increasing glutaraldehyde concentrations and subjected to electrophoresis and silver staining (Figure 1D). Consistent with previous results (*19*), un-cross-linked p53 migrates at ∼53 kDa (Figure 1D, lane 1), while increasing glutaraldehyde concentrations yield a mixture of monomers and cross-linked dimers, trimers, and tetramers at the expected molecular weights (Figure 1D, lane 2). Tetrameric p53 is a dimer of dimers, so the ∼160 kDa band reflects incomplete cross-linking of tetramers rather than the existence of trimers. At 0.025% glutaraldehyde, p53 cross-links as a tetramer (Figure 1D, lane 3). Higher concentrations of glutaraldehyde do not reveal additional multimeric states, and cross-linking is not observed for bovine serum albumin (data not shown).

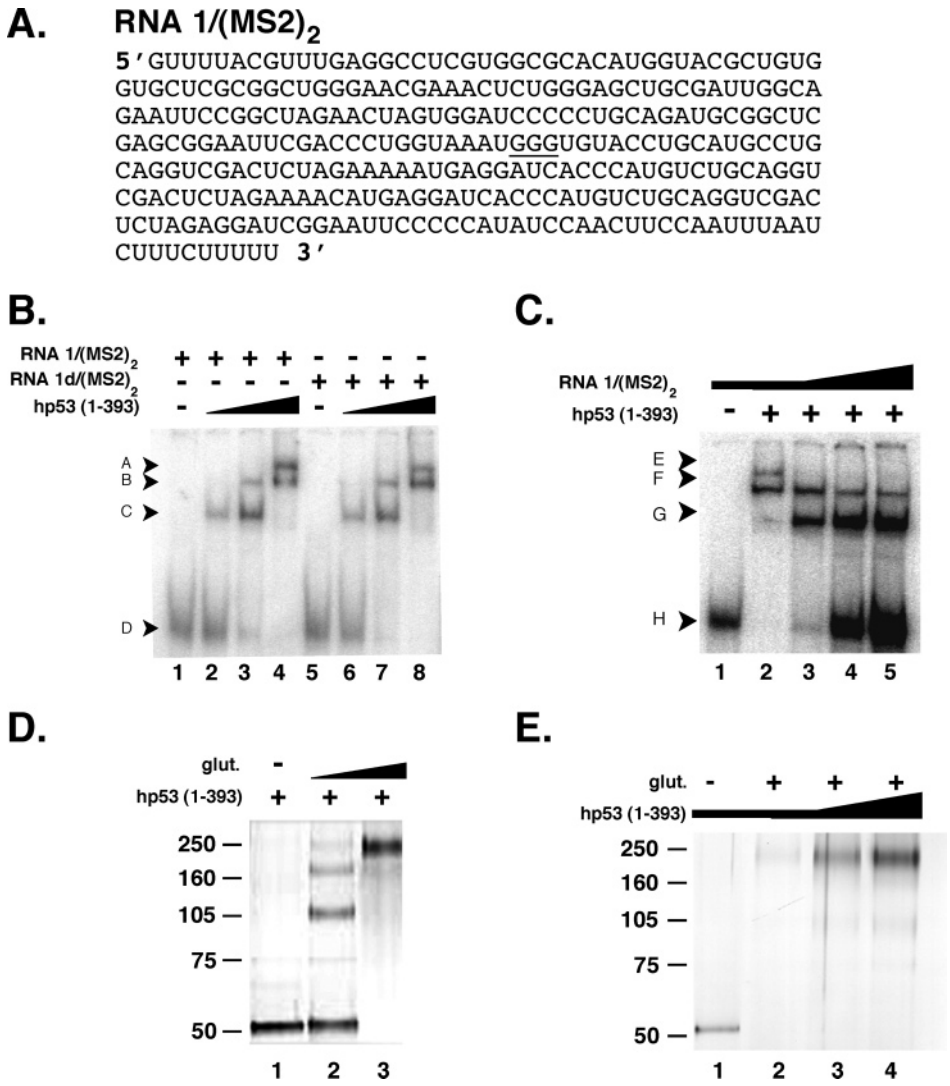

FIGURE 1: p53 binds sequence-nonspecifically to RNA in the absence of competitor DNA. (A) Sequence of RNA 1/$(MS2)_2$. RNA 1d/$(MS2)_2$ lacks the underlined nucleotides. (B) p53 exhibits complex shifts with RNA. Varying amounts of full-length human p53 (1−393) were incubated with 3 nM ^{32}P-labeled RNA 1/$(MS2)_2$ (lanes 1−4) or 1d/$(MS2)_2$ (lanes 5−8). Protein concentrations were 0, 2.75, 13.7, and 34.4 nM. No competitor nucleic acid was used in these reactions. (C) Multiple p53 tetramers load onto a single RNA. Increasing concentrations of ^{32}P-labeled RNA 1/$(MS2)_2$ were incubated with a constant concentration (17 nM) of full-length human p53 (1−393). RNA concentrations were 2 nM (lanes 1 and 2), 4 nM (lane 3), 8 nM (lane 4), and 16 nM (lane 5). Arrowheads indicate shifted species. No competitor nucleic acid was present in these reactions. (D) Optimization of glutaraldehyde (glut.) cross-linking to characterize p53 multimerization state. Full-length human p53 (1−393; 17 nM) was incubated with 0, 0.01, 0.025, and 0.05% glutaradehyde (lanes 1−4, respectively). (E) p53 is tetrameric at the protein and RNA concentrations tested. Varying concentrations of full-length human p53 (1−393) were incubated with 0 (lane 1) or 0.025% (lanes 2−4) glutaraldehyde and 2 nM RNA 1/$(MS2)_2$. Protein concentrations were identical to those used in lanes 2−4 of part B.

To test the multimerization status of p53 bound to RNA (Figure 1B, lanes 1−4), we cross-linked p53 in the presence of 2 nM RNA (Figure 1E). The tetrameric state of p53 was preserved under all conditions. This result is consistent with sequence-nonspecific binding of multiple p53 tetramers onto individual RNA molecules.

Effect of Competitor DNA on RNA−p53 Interactions. Our previous study suggested that RNA binding largely reflects electrostatic interactions with basic residues among the C-terminal 30 amino acids of p53 (*13*). To distinguish the relative contributions to RNA binding made by this C-terminal region versus other portions of p53, we studied RNA binding of full-length and C-terminally truncated p53 in the absence (Figure 2A) and presence [Figure 2B; (*13*)] of competitor salmon sperm DNA (mean length ~2000 bp). We performed gel mobility shift assays with radiolabeled DNA containing p53 binding sites to establish concentrations of full-length and truncated p53 that had equal sequence-specific DNA binding activities [(*20*); data not shown]. Equal activities of full-length and truncated p53 were analyzed for RNA binding by mobility shift assay (Figure 2). We observed discrete complexes for both full-length and truncated p53, but a much larger proportion of RNA was bound by full-length p53 relative to truncated p53 (Figure 2A, compare unbound RNA in lanes 3 and 9). RNA binding to full-length p53 is strong and persists in the presence of double-stranded genomic DNA [(*17*); data not shown] or single-stranded salmon sperm DNA (Figure 2, compare lanes 1−6 in panels A and B). In contrast, RNA binding sites outside of the C-terminus are weaker, so RNA binding does not persist for C-terminally truncated p53 in the presence of nonspecific DNA (Figure 2, compare lanes 7−12 of panels A and B).

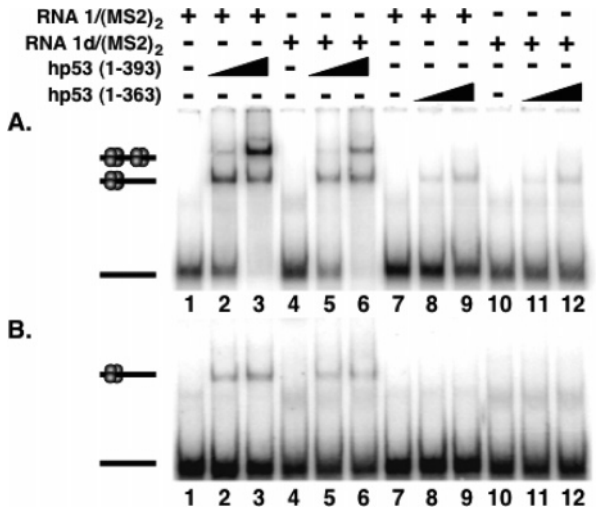

FIGURE 2: RNA binding by full-length and truncated human p53 in the absence (A) or presence (B) of competitor salmon sperm DNA. (A) Equal DNA binding activities of full-length or truncated p53 (0, 6, or 17 nM full-length; 0, 2, or 6 nM truncated) were incubated with 3 nM ^{32}P-labeled RNA, as indicated before analysis by EMSA. (B) Reactions were identical to part A but with the addition of 8.5 ng/μL heat-denatured salmon sperm DNA in all reactions (see also ref *13*). The diagrams to the left propose protein/RNA complexes (protein monomers shown as spheres, RNA shown as a line).

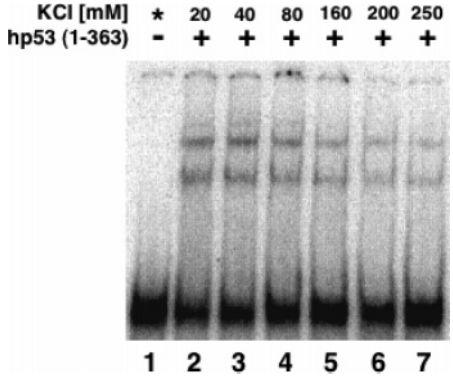

FIGURE 3: C-terminally truncated p53 interaction with RNA 1/(MS2)$_2$ is relatively salt-resistant. Where indicated, 19 nM truncated human p53 (1−363) was incubated with 3 nM ^{32}P-labeled RNA 1/(MS2)$_2$ in the presence of the indicated salt concentrations.

Strength of RNA−p53 Interactions outside of the C-Terminal Domain. We wished to specifically explore the strength of the RNA interactions outside of the C-terminal domain (Figure 3). A high concentration of truncated human p53 (1−363; 19 nM) was tested for interaction with RNA. Increasing concentrations of KCl (up to 250 mM) weakened, but did not completely disrupt, the interaction of RNA with truncated p53. The gradual weakening of the interaction with the addition of salt supports a sequence-nonspecific, electrostatic interaction model rather than an interaction mediated primarily by hydrogen bonding or hydrophobic interactions. Also, this result confirms that, although the p53 C-terminal 30 amino acids provide a strong RNA binding domain, an RNA binding contribution is made outside of this region, which can be demonstrated only in the absence of competitor DNA.

Mouse and Human C-Terminal Peptides Bind RNA. Since much of the affinity of p53 for RNA appeared to be due to binding to the C-terminal 30 amino acids, we explored the

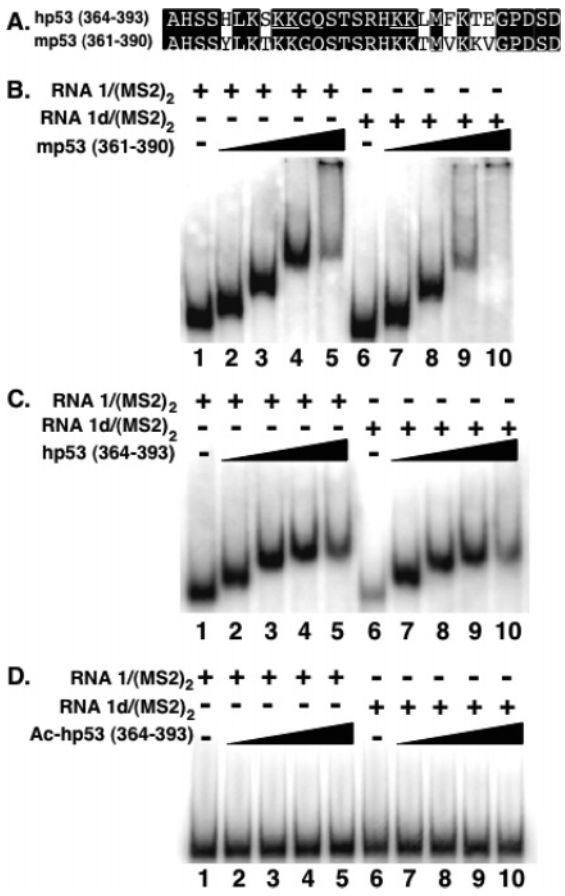

FIGURE 4: Mouse and human p53 C-terminal peptides bind RNA 1/(MS2)$_2$ and 1d/(MS2)$_2$. (A) Sequences of the human [hp53 (364−393)] and mouse [mp53 (361−390)] peptides used in this study. White residues reflect identical amino acids, and underlined residues indicate sites of acetyl group attachment in the Ac−hp53 peptide at positions 372, 373, 381, and 382 (numbering based upon full-length human p53 protein). (B) Increasing concentrations of mouse p53 C-terminal peptide [mp53 (361−390)] were incubated with 30 nM ^{32}P-labeled RNA 1/(MS2)$_2$ or 1d/(MS2)$_2$. Peptide concentrations were 14, 41, 123, and 369 μM. (C) Interaction between ^{32}P-labeled RNA 1/(MS2)$_2$ or 1d/(MS2)$_2$ and human p53 C-terminal peptide [hp53 (364−393)] Nucleic acid and peptide concentrations as indicated in part B. (D) Interaction between ^{32}P-labeled RNA 1/(MS2)$_2$ or 1d/(MS2)$_2$ and acetylated human p53 C-terminal peptide [Ac−hp53 (364−393)]. Nucleic acid and peptide concentrations as indicated in part B.

interaction between C-terminal p53 peptides and RNA. Mouse and human p53 proteins are commonly used interchangeably in functional studies [e.g., (*21*)]. Although the two proteins are similar, they are not identical. A sequence comparison between mouse p53 (361−390) and human p53 (364−393) showed that the two C-terminal peptides differ by a net charge of +2 (Figure 4A). Both peptides interacted strongly with RNA (Figure 4B and C), giving rise to complexes whose mobilities decreased with increasing peptide concentration until complexes were retained in the wells for mouse p53 peptide (Figure 4B). As predicted, the mouse p53 peptide (+6 net charge) bound more strongly to RNA than the human p53 peptide (+4 net charge; compare parts B and C of Figure 4).

Though micromolar peptide concentrations were used in this experiment to demonstrate shifted complexes, nanomolar p53 peptide concentrations are sufficient for RNA binding (data not shown). The high affinity of the p53 C-terminal domain for RNA is provocative because p53 is known to

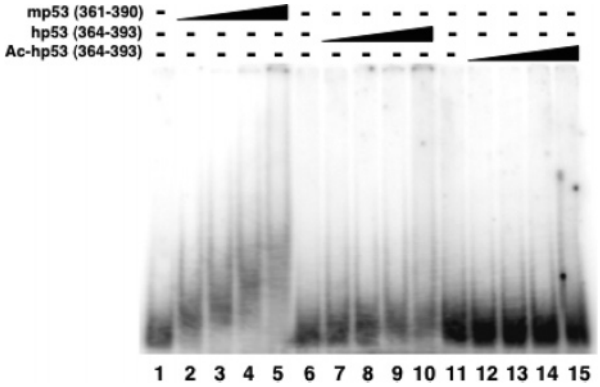

FIGURE 5: p53 C-terminal peptide binds tRNA weakly. Varying concentrations of mouse p53 C-terminal peptide [mp53 (361−390)], human p53 C-terminal peptide [hp53 (364−393)], or acetylated human p53 C-terminal peptide [Ac−hp53 (364−393)] were incubated with 30 nM ^{32}P-labeled tRNA. Peptide concentrations were 14, 41, 123, and 369 μM for each peptide.

undergo a number of post-translational modifications in this region, and little is currently known about their significance and function (*22*). There are four established lysine acetylation sites near the human p53 C-terminus (Figure 4A), and simultaneous acetylation of all four lysine residues blocks sequence-nonspecific association of p53 with DNA (*23*). We found that acetylation of human C-terminal peptides at these residues completely prevented binding to RNA (Figure 4, compare panels C and D).

Mouse p53 Peptide Binds Weakly to tRNA. Given the general promiscuity of p53 for RNA binding (*13*), we were curious if p53 could interact with highly compact, structured RNAs such as tRNA. The mouse p53 C-terminal peptide bound only weakly to radiolabeled yeast total tRNA (Figure 5, lanes 1−5), and the interaction between human p53 C-terminal peptide and tRNA was even weaker (Figure 5, lanes 6−10), as reflected in both lesser mobility shifts and a more diffuse banding pattern, indicative of complex instability. Consistent with the results in Figure 4, lanes 11−15 of Figure 5 demonstrate that the acetylated human p53 C-terminal peptide shows no affinity for tRNA. These results suggest that p53 binds poorly to highly structured RNAs.

RNA Binding Does Not Induce Folding of p53 C-Terminal Peptides. Many transcription factors undergo conformational changes upon nucleic acid binding (*24*). The p53 C-terminus is predicted to be largely unstructured, and NMR studies of the sequence-nonspecific interaction between the p53 C-terminus and DNA suggest that little or no peptide folding occurs in the presence of bound DNA (*23*). To test the case of RNA−p53 interaction, we used circular dichroism (CD) spectroscopy to monitor peptide structural changes in the absence and presence of RNA at peptide concentrations shown to support peptide/RNA complexes (Figure 6A). RNA (5 μM) was incubated with three different p53 C-terminal peptide preparations at concentrations of 5, 10, and 100 μM. Even under conditions sufficient for strong RNA−peptide interaction (5 μM RNA and 100 μM mouse C-terminal p53 peptide), C-terminal peptides retained CD spectra consistent with disordered polypeptide conformations [Figure 6A; (*25*)].

p53 C-Terminal Peptides Do Not Induce Structural Changes in RNA. It has been suggested that p53 can promote nucleic acid strand annealing (*7*). It seemed possible that binding of p53 C-terminal peptides to RNA might be

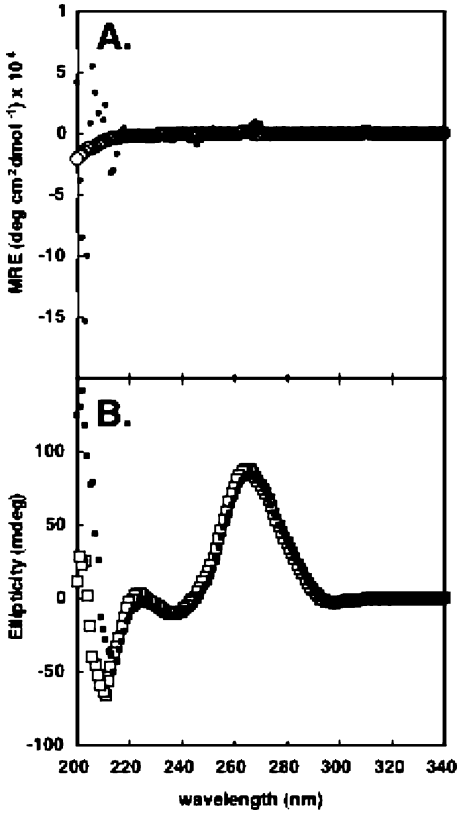

FIGURE 6: (A) Far-UV CD spectra of the mouse p53 C-terminal peptide [mp53 (361−390); 5 μM] alone (open circles) or in the presence of 5 μM RNA 1/(MS2)$_2$ (filled circles). (B) Near-UV CD spectra of 5 μM RNA 1/(MS2)$_2$ alone (open squares) in the presence of 100 μM mouse p53 C-terminal peptide (filled squares).

accompanied by changes in RNA conformation such as stabilization of double-helical regions. We acquired CD spectra for RNA/peptide complexes and monitored the RNA contribution to the spectra (Figure 6B). The CD spectrum of the isolated RNA confirms the presence of some double-stranded, A-form RNA helices in the folded molecules. However, CD spectroscopy provided little evidence for change in RNA structure after addition of any of the three tested peptides in concentrations known to support RNA complexes. It should be noted that some signal scattering occurs in the 200−210 nm range. This might be due to concentration effects, UV absorption of air, or a small change in amide bond structure, but it is unclear under these conditions.

RNA Blocks Sequence-Specific DNA Binding by p53. There is significant debate concerning the mechanism of p53 latency. The high affinity of p53 for RNA raises the possibility that nonspecific RNA binding could obscure the DNA binding domain of p53, a model that has been proposed for sequence-nonspecific DNA binding by p53 (*23*, *26*, *27*). We tested this concept in competition EMSA experiments where p53 proteins were incubated with DNA duplexes containing strong p53 consensus binding sites in the absence or presence of unlabeled, competitor nucleic acids (Figure 7). A 60-fold molar excess of competitor nucleic acid was added to the indicated reactions. In the absence of competitor, full-length p53 binds strongly to the p53 sites (Figure 7, lane 2), and this interaction is destroyed by excess sequence-specific competition (Figure 7, lane 3) but not by a sequence-nonspecific 38 bp DNA duplex (Figure 7, lane 4). Strikingly,

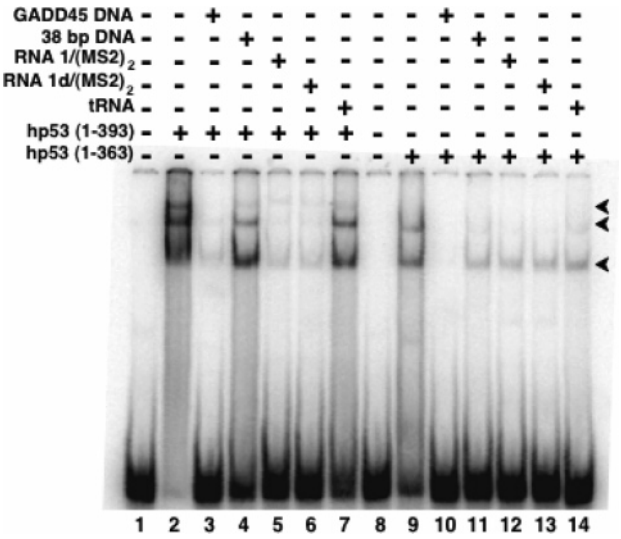

FIGURE 7: RNA blocks sequence-specific DNA binding by p53. Full-length (17 nM) or truncated (19 nM) p53 were incubated with ^{32}P-labeled GADD45 DNA (3.4 nM) in the presence of the indicated unlabeled competitor nucleic acids (60-fold molar excess). Arrows indicate the shifted species as in Figure 1B.

both RNA 1/$(MS2)_2$ and RNA 1d/$(MS2)_2$ inhibited p53 binding to specific duplex DNA binding as effectively as the unlabeled specific DNA duplex (Figure 7, compare lane 3 to lanes 5 and 6). Unlabeled tRNA was a poor competitor (Figure 7, lane 7), consistent with its poor affinity for the p53 C-terminal domain (Figure 5). In contrast to full-length p53, the C-terminally truncated protein formed complexes that were relatively resistant to competition by RNA (Figure 7, lanes 9−14). These results suggest that the C-terminal domain of p53 confers RNA inhibition of specific binding to duplex DNA.

DISCUSSION

After observing strong RNA−p53 interactions in the yeast three-hybrid system, we have now explored in more detail the interaction between p53 and RNA in vitro. Using two ∼300 nt RNAs strongly distinguished by p53 in vivo, we demonstrated that p53 tetramers equally bind these molecules in vitro. This and other observations (*13*) lead us to conclude that p53 binds RNA with little sequence specificity but that differing efficiencies of yeast display can give the appearance of RNA sequence preference. In the present work, we found that multiple RNA−p53 species appeared upon addition of protein, and an EMSA with a constant protein concentration demonstrated that these species correspond to multiple p53 tetramers loading on a single RNA. Both full-length and C-terminally truncated p53 bound to RNA in vitro in the absence of competitor nucleic acid. The addition of competitor genomic DNA (single- or double-stranded) demonstrated that the majority of the RNA−p53 interactions occur in the last 30 amino acids of the protein. Together, these results suggest a strong and sequence-nonspecific interaction between RNA and the p53 C-terminus. We also found that the p53 C-terminal domain is sufficient for RNA binding using EMSAs with both mouse and human p53 C-terminal peptides. These peptides bound weakly to tRNA, and an acetylated human peptide did not bind RNA. RNA binding did not induce detectable structure in the p53 peptides, and conversely, the peptides did not seem to influence RNA structure. Using full-length and C-terminally truncated p53, we demonstrated that C-terminal p53 binding to the ∼300 nt RNAs, but not tRNA, prevented sequence-specific DNA binding by full-length p53.

One goal of this work was to establish whether p53 binds to RNA in a sequence-specific or sequence-nonspecific manner. The sequence specificity of RNA−p53 interactions has been controversial. Three reports have suggested that p53 binds sequence-specifically to mRNAs from fibroblast growth factor 2 (*14*), Cdk4 (*15*), or p53 itself (*16*). All three reports point to an important function of p53 in sequence-specific regulation of mRNA function. However, our results challenge these interpretations, as we show that p53 does not recognize specific RNA sequences or obvious structures in vivo or in vitro, and p53 binds most tested RNAs with high affinity [low nanomolar range; (*13*)]. Our results are not surprising given similar observations regarding the interaction between nonspecific DNA and the C-terminus of p53 (*7*, *23*).

Our work raises the key issue of possible functions for the strong and nonspecific RNA−p53 interaction. We suggest several plausible models. The original proposed function for p53 C-terminal nonspecific nucleic acid binding involved catalysis of complementary strand annealing (*7*). It is possible that the RNA−p53 interaction observed here can indeed mediate RNA−RNA annealing. Since p53 is a natively disordered protein (*28*), it might have been expected that RNA binding would influence the protein structure and that protein binding would increase A-form, duplex RNA content. We did not detect changes in either peptide or RNA structure upon their interaction (Figure 6), measuring only a persistent random coil structure for the p53 peptide, as predicted by prior work (*23*). These results do not rule out the possibility that RNA or p53 participates in more highly structured complexes in the tetrameric form of p53. If RNA strand annealing is a physiological function of p53, it apparently occurs without major structural changes by either partner under these conditions.

A novel function for RNA−p53 interactions has been recently proposed (*29*). Nuclear export signal (NES)-mediated export of proteins has been linked to mRNA export (*30*). Because p53 appears to possess multiple NES sequences (including tetramerization domain amino acids 340−351) (*31*, *32*), it is a possible client for mRNA-linked nuclear export. Recent observations have shown that antibody inhibition of RNA polymerase II, inhibition of mRNA export component TAP, or expression of dominant negative NUP160 all lead to significant nuclear accumulation of p53 (*29*). It is thus possible that the sequence-nonspecific RNA binding observed here could function in exporting non-DNA-binding forms of p53 from the nucleus under certain cellular circumstances. This would be easily reversible by post-translational modification of the C-terminus. Future studies could test nuclear localization and export of a C-terminally truncated form of p53 deficient in RNA binding but retaining other NES signals. RNA binding by p53 also has the potential to competitively inhibit interaction with C-terminal binding proteins. It might also be interesting to examine whether RNA inhibits the co-immunoprecipitation of p53 with C-terminal binding partners such as S100A2 in vitro (*33*).

Perhaps the most intriguing possible role of RNA−p53 interactions involves activation of latent p53. The mechanism

of p53 activation has long been debated, mostly due to inconsistencies in the assays used to study the latent and active forms of the protein (*26*, *27*, *34*−*39*). For example, *E. coli*-produced, full-length p53 is reportedly not competent for sequence-specific DNA binding (*40*), but insect-cell expressed full-length p53, which may be post-translationally modified, binds to DNA target promoters in vitro (Figure 7). Acetylation by p300 on p53 C-terminal lysines is known to activate sequence-specific DNA binding in an additive manner (*23*, *41*). It was initially proposed that acetylation caused an activating conformational change allowing p53 to bind DNA (*41*). This allosteric mechanism was later ruled out by the observation that the NMR structures for full-length (latent) and C-terminally truncated (active) p53 are indistinguishable, and the C-terminus does not interact with any other domain of the protein, leaving open for debate the actual mechanism for activation of p53 by acetylation (*37*, *42*). We show that an RNA-bound form of p53 is unable to bind promoter sequences and that acetylation can prevent the RNA−p53 interaction, releasing the p53 core domain for DNA binding. It is likely that the same residues previously invoked in single-stranded DNA contacts are also involved in RNA binding (*43*).

The C-terminus of p53 is known to be heavily post-translationally modified, and it is still unclear what role the modifications play in p53 regulation. We tested the effect of acetylation on RNA binding, but methylation, ubiquitination, neddylation, sumolyation, and phosphorylation might differently affect RNA−p53 interactions (*5*, *44*). Two groups have recently developed mouse models to explore the role of C-terminal post-translational modifications (*21*, *45*). Both groups concluded that C-terminal post-translational modifications most likely contribute to fine-tuning p53 function. We caution that though mouse and human p53 share high sequence similarity, they interact with RNA differently (Figure 4,5)

Other obvious questions also arise from this work: is RNA bound by p53 in vivo? Are certain RNA partners favored? Might the RNA−p53 interactions be regulated? A recent report suggests that p53 can be co-immunoprecipitated with RNA in MCF-7 cells (*34*). Though our work shows no evidence for RNA sequence specificity by p53, the possibility of specific biological partners must be addressed by identifying p53 binding RNAs in vivo.

Is it possible that the RNA binding function of p53 might be deregulated in cancers? Over 80% of p53 mutations result in full-length protein with missense mutations, and although a majority of p53 mutations are in "hot spot" regions within the DNA binding domain (*3*), nearly 30% of p53 mutations occur at other, more unique locations distributed throughout the protein (*46*). Indeed, C-terminal p53 mutations have been observed in at least 11 tumors, and a mechanism for these mutations is not yet established (*47*−*52*).

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