p53–RNA interactions: New clues in an old mystery

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ABSTRACT

The p53 tumor suppressor protein is typically considered to be a sequence-specific DNA-binding transcription factor. However, reports over the last 15 years have described RNA binding by p53 in a variety of contexts, suggesting the possibility of new p53 functions. It is clear that p53–RNA interactions are mediated by a nucleic acid-binding domain of p53 independent of the sequence-specific core domain responsible for DNA recognition. Reports disagree on several aspects of the putative RNA interaction, including sequence specificity and biological relevance. Here we review the history and recent advances in the study of p53–RNA interactions. We argue that p53–RNA interactions are sequence nonspecific and depend on incomplete post-translational modification of the p53 C-terminal domain when the protein is expressed in heterologous systems. It is unknown what fraction of p53 protein exists in a state competent for RNA binding *in vivo*. Thus, potential physiological roles of p53–RNA interactions remain mysterious.

Keywords: p53; RNA; sequence specificity; post-translational modifications

BACKGROUND

The sequence-specific DNA-binding transcription factor p53 is one of the most thoroughly investigated proteins in biomedical science. It has been proposed that p53 evolved in higher organisms due to natural selection for its role as a tumor suppressor (Vousden and Lu 2002). The human p53 gene, *TP53*, is mutated in over half of cancers, making cancer-associated mutation of *TP53* more common than that of any other gene (Braithwaite and Prives 2006). In the ~50% of tumor cells that retain wild-type (WT) p53, there are typically mutations that abrogate p53 functions either upstream or downstream in the p53 signaling cascade (Vousden and Lu 2002). Thus, p53 is generally considered to be a central protein in human carcinogenesis. The p53 protein is both modified and a modifier with roles that integrate cellular stress signals and orchestrate an appropriate cellular response.

The potent p53 protein is held in check under normal circumstances, yet must be rapidly activated in response to cell signaling (Sionov and Haupt 1999). Upon stabilization and activation, p53 elicits three major outcomes: apoptosis, cell cycle arrest/senescence, and DNA repair/recombina-

tion, largely by the action of p53 transcriptional targets (Braithwaite and Prives 2006).

p53 structure

The p53 protein is modular, enabling the functional and structural study of individual domains. Each of the five major p53 domains has been associated with distinct, yet interdependent functions (Fig. 1; Prives and Hall 1999; Rajagopal 2007). Full-length human p53 consists of 393 amino acids. Like many transcription factors, p53 contains an acidic, N-terminal activation domain (amino acids 1–44). Amino acids 58–101 form a proline-rich (P-X-X-P) domain thought to mediate interactions with apoptotic modulators (Baptiste et al. 2002). A sequence-specific DNA-binding domain comprises the central core of p53 (amino acids 102–292). This core domain is responsible for recognition of the p53 duplex DNA consensus sequence in promoters of target genes (Kern et al. 1991). More than 97% of tumor-derived missense mutations occur in the core domain (Olivier et al. 2002). Oligomerization interactions near the C terminus (amino acids 325–356) enable p53 to function in its most transcriptionally active form, a tetramer (Clore et al. 1995). As a nuclear protein, the amino acid sequence of p53 encodes both nuclear localization signals (NLS) and nuclear export signals (NES), and p53 is thus subject to complex nuclear-cytoplasmic shuttling (Middeler et al. 1997). Two NES (amino acids 11–24 and 341–350) (Fig. 1) and three NLS (within amino acids 316–386) (Fig. 1) have been proposed (Rajagopal 2007).

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FIGURE 1. Human p53 domain structure (Rajagopal 2007). The 393-amino acid p53 protein can be divided into five functional domains. Two transactivation regions comprise the N-terminal transactivation domain (residues 1–44). The proline-rich (P-X-X-P domain; residues 58–101) domain mediates interactions with p53 binding partners. The bulk of the p53 protein forms the sequence-specific DNA-binding “core” domain (residues 102–292). The C terminus consists of an oligomerization domain (residues 325–356) and the C-terminal regulatory domain (residues 364–393). p53 has three nuclear export signals (NES, red; residues 11–24, 340–351) and three nuclear localization signals (NLS, green; residues 316–324, 370–376, 380–386) (Rajagopal 2007).

A unique feature of p53 is its two distinct nucleic acid-binding domains (Liu and Kulesz-Martin 2006). In addition to the DNA-binding core domain, a second nucleic acid-binding domain, dependent upon the C-terminal 30 amino acids of p53 (364–393; here termed the C-terminal domain [CTD]) is of particular interest. This highly basic domain has been described as exhibiting little nucleic acid sequence preference, and reportedly binds with high affinity to single- and double-stranded DNA and RNA, irradiated DNA, and four-way DNA junctions (Ahn and Prives 2001). The C-terminal 27 residues are encoded on a separate exon (Soussi and May 1996) that is missing in certain human (Bourdon et al. 2005) and mouse (Kulesz-Martin et al. 1994) p53 isoforms, suggesting the possibility of a special function for this domain. The nucleic acid-binding characteristics of the CTD are considered in this perspective.

p53 post-translational modifications

The function of p53 is exquisitely controlled by combinations of at least 10 different kinds of post-translational modification. The most frequently described modifications include phosphorylation, ubiquitination, acetylation, and sumoylation (Bode and Dong 2004). In addition, glycosylation (Shaw et al. 1996), ribosylation (Wesierska-Gadek et al. 1996), farnesylation (Couderc et al. 2006), neddylation (Xirodimas et al. 2004), and methylation (Chuikov et al. 2004) have been reported. Most recently, the addition of O-linked N-acetylglucosamine on p53 S149 was shown to stabilize the protein (Yang et al. 2006). There have been reports of six different modifications in the CTD (Fig. 2). A consequence of multiple alternative post-translational modifications is the probability of p53

heterogeneity in vivo. The p53 protein undoubtedly exists in different modified forms at different times in the cell cycle, under different cellular stressors, and in different subcellular localizations.

The role of p53 acetylation is particularly controversial and important in the consideration of nucleic acid interactions. Histone acetyltransferases (HATs) were originally thought to modify only histone substrates (Gu and Roeder 1997), coactivating transcription in partnership with many different transcription factors (Xu 2003). p53 was the founding member of a group of nonhistone targets of HAT acetylation (Glozak et al. 2005). As might be expected, p53 is also the target of at least one histone deacetylase enzyme (HDAC) (Juan et al. 2000; Luo et al. 2000). p53 acetylation requires prior p53 binding to consensus site DNA in vitro and in vivo. This p53 acetylation at promoters can subsequently recruit transcriptional coactivator complexes (Barlev et al. 2001). All five proposed C-terminal acetylation sites overlap with ubiquitination sites (Fig. 2; Xu 2003), making their analysis particularly challenging. It is likely that acetylation reactions are triggered by upstream phosphorylation events elsewhere on p53, further complicating the process (Warnock et al. 2005). It is thought that acetylation may be responsible for the activation of p53 protein for sequence-specific DNA binding and/or transcription activation (Sakaguchi et al. 1998; Juan et al. 2000; Appella and Anderson 2001; Barlev et al. 2001; Prives and Manley 2001; Luo et al. 2004). Friedler et al. (2005) have carefully demonstrated the effects of acetylation on sequence-nonspecific DNA interactions with the p53 CTD in biochemical assays. Interestingly, in cells expressing p53 variants that cannot be acetylated, it is transactivation rather than p53 degradation that is affected (Feng et al. 2005). This result suggests that lysine acetylation is more important for p53 activity than for determining stability. The effect of acetylation on RNA binding is discussed in greater detail below.

Post-translational modifications of the p53 CTD have so far proven to be functionally enigmatic. It is accepted that

FIGURE 2. Examples of p53 C-terminal post-translational modifications (Lavin and Gueven 2006). Human p53 modifications include acetylation (Ac: green), methylation (Me: blue), neddylation (Ne: gray), phosphorylation (P: red), sumoylation (S: purple), and ubiquitination (Ub: yellow), of the indicated residues. Modifications were proposed and/or documented as follows: 386-S (Rodriguez et al. 1999), 370-N/372-N/373-N (Xirodimas et al. 2004), 372-Me (Chuikov et al. 2004), 370-Me (Huang et al. 2006), 378-P/392-P (Soussi and May 1996), 382-Ac/366-P/371-P/376-P/377-P/378-P/387-P (Ou et al. 2005), 376-P/378-P (Waterman et al. 1998), 370-Ac/372-Ac/373-Ac/381-Ac/382-Ac (Gu and Roeder 1997), 382-Ac (Sakaguchi et al. 1998), 373-Ac/381-Ac/382-Ac (Liu et al. 1999), 370-Ub/372-Ub/373-Ub/381-Ub/382-Ub/386-Ub (Honda et al. 1997).

many sites within the CTD can be post-translationally modified under certain conditions, but the presence, significance, and timing of such modifications *in vivo* remain elusive (Prives and Manley 2001; Xu 2003; Bode and Dong 2004). Limited attempts have been made to identify specific p53 post-translational modifications under certain conditions using site-specific antibodies (Xu 2003; Warnock et al. 2005), but functional implications remain unclear. It has been reported that phosphorylation of C-terminal S392 can stimulate DNA binding by p53 (Hupp et al. 1992), can alter tetramerization (Sakaguchi et al. 1997), and that mutation of this residue can enhance cancer development in transgenic animals (Bruins et al. 2004). Phosphorylation of p53 at S315 is induced by DNA damage (Blaydes et al. 2001), can mediate p53–E2F1 cooperation (Fogal et al. 2005), or, paradoxically, can mediate p53 nuclear export (Qu et al. 2004). Phosphorylation of p53 S20 has been reported to promote p300 binding (Dornan and Hupp 2001), and mutation of this residue can stimulate spontaneous tumorigenesis in some transgenic mice (MacPherson et al. 2004). Given the generally uncertain degree of p53 post-translational modifications upon isolation from different cell lines, tissues, and tumors, little progress has been made in clearly defining the functions of the CTD. Furthermore, studies of p53 CTD modifications are often difficult to interpret due to differences in experimental design. This is particularly true for studies involving p53 protein expressed in heterologous systems such as bacteria, yeast, or insect cells, where the extent of post-translational modification of p53 is poorly characterized and most likely different from that in mammalian cells.

It is unclear if p53 is truly unique in its degree of post-translational modification or if such modifications have been detected for p53 simply due to keen scrutiny (more than 40,000 publications since 1979). Indeed, we are just beginning to learn about the general significance of protein modifications such as methylation, originally considered to be rare (Morgunkova and Barlev 2006). The timing, cell specificity, and localization of p53 post-translational modifications remain of key interest in understanding p53 regulation and, particularly, the ability of the p53 CTD to interact with nucleic acids.

The mystery of p53–RNA interactions

This review focuses on p53–RNA interactions, a perplexing topic. As summarized in Figure 3, p53–RNA interactions have previously been described in at least four contexts, including covalent complexes, sequence-nonspecific RNA annealing, sequence-specific mRNA binding/translational control, and sequence-nonspecific RNA binding. We will argue that sequence-nonspecific RNA binding provides the best interpretation for experimental p53–RNA interactions. However, whether interactions between p53 and RNA are physiologically relevant has been unclear, and there is

FIGURE 3. p53–RNA interactions previously proposed in the literature. Counter-clockwise from *right*: 5.8S rRNA covalent linkage to p53 via S389 (Samad and Carroll 1991; Fontoura et al. 1992); RNA–RNA annealing catalyzed by p53 (Oberosler et al. 1993); 5′-UTR p53–RNA binding proposed for p53 (Mosner et al. 1995), Cdk4 (Miller et al. 2000), and FGF-2 (Galy et al. 2001) mRNA transcripts; sequence-nonspecific RNA binding *in vitro* (Riley et al. 2007) and in the yeast three-hybrid system (Riley et al. 2006).

evidence that p53–RNA interactions are prevented *in vivo* by post-translational modification of the p53 CTD.

p53–RNA: Covalent interactions?

Perhaps the most unusual p53 CTD post-translational modification reported to date, and the first mention of p53–RNA interactions in the literature, suggested the covalent attachment of 5.8S rRNA to the C-terminal S389 residue of p53 (Fig. 3, right; Samad and Carroll 1991). Preparations from five transformed cell lines (one human) were purified by HPLC after p53 immunoprecipitation and tryptic digestion. Additionally, undigested, labeled, immunoprecipitated p53 was rigorously purified by SDS-PAGE and chromatography. The presence of RNA was confirmed by alkaline and enzymatic hydrolyses (Samad and Carroll 1991). This report was met with understandable skepticism. One concern with the purification protocol was the reliance on immunopurification, which can yield copurifying contaminants (Turpeinen et al. 2002). Nevertheless, follow-up experiments by the same group confirmed that the RNA was not purified from cells lacking p53 (Fontoura et al. 1992).

In contrast, Oberosler et al. (1993) prepared immunopurified p53 expressed from insect cells infected with a recombinant baculovirus and then compared the samples before and after treatment with RNase. These investigators observed no change in the resulting sedimentation profile, which they interpreted as ruling out the covalent attachment of RNA. However, RNase inhibition was a part of the initial purification (Fontoura et al. 1992), and might have

been necessary to preserve the nucleic acid through processing. It appears that the Oberosler et al. (1993) purification protocol did not include an RNase inhibitor. It is possible that insect cells lack the implied machinery necessary for covalent attachment of RNA to p53.

It is known that the SV40-transformed cells used to identify the covalent RNA modification behave uniquely. For example, drastic differences in p53 phosphorylation profiles are observed between transformed and untransformed cells both within and between species (Patschinsky et al. 1992). S389 is phosphorylated in response to UV treatment, which correlates with partial p53 activation (Huang et al. 1999). This raises the possibility that an apparent p53–RNA covalent complex might have been unique to the cell lines in which it was discovered. In other studies, p53 was found to copurify with polysomes (Fontoura et al. 1997), but no new data have been presented to further substantiate the concept of p53 covalent attachment to 5.8S rRNA, establish such an interaction in normal human cells, or suggest a function for this peculiar modification.

p53–RNA interactions: Sequence-nonspecific “anti-helicase” activity?

A property commonly attributed to the p53 CTD is single-stranded nucleic acid binding (Fig. 3, top; McKinney et al. 2004). The first observation of this high-affinity interaction was serendipitous: p53 was reported to inhibit the DNA helicase activity intrinsic to SV40 large T antigen (Sturzbecher et al. 1988). However, the helicase inhibition was found to be more general, as p53 reportedly affected the function of several other DNA and RNA helicases (Oberosler et al. 1993). Rather than inhibiting helicase activity by protein–protein interactions, tetrameric p53 purified from insect cells was reported to reverse-strand separation by binding directly to single-stranded DNA or RNA products of helicase activity, enhancing the reannealing rate for both DNA–DNA and RNA–RNA duplexes. Filter-binding assays with radiolabeled oligonucleotides demonstrated a 10-fold higher affinity of p53 for ssRNA as compared with ssDNA (Oberosler et al. 1993). The p53 CTD was also capable of binding to dsDNA, but with a weaker affinity (Oberosler et al. 1993). Unlabeled test RNA sequences and total cytoplasmic RNA isolated from HeLa cells competed equally for p53 binding, leading the investigators to conclude that the interaction was sequence nonspecific (Oberosler et al. 1993). Based on these observations, p53 would be classified among “RNA chaperones,” proteins that facilitate RNA folding by stabilizing certain RNA structures (Hargittai et al. 2004; Levin et al. 2005).

The detection of “anti-helicase” activities for p53 led researchers in two different directions. Several groups began to study p53 CTD–ssDNA interactions because of the potential of such interactions to mediate the DNA damage response attributed to p53 (Bayle et al. 1995; Jayaraman and Prives 1995; Reed et al. 1995). Other groups reported

specific p53–RNA interactions, implicating RNA binding and strand reannealing in the translational control of certain mRNA transcripts (Mosner et al. 1995; Miller et al. 2000; Galy et al. 2001), although studies of nucleic acid binding by the p53 CTD tended to consider binding to either DNA or RNA, Oberosler et al. (1993) clearly demonstrating that RNA and DNA can compete for CTD binding.

Noncovalent p53–RNA interactions: Sequence-specific mRNA regulation?

Three groups asserted that p53 binds specifically to mRNA targets (Fig. 3, left; Mosner et al. 1995; Miller et al. 2000; Galy et al. 2001). In all cases, the 5′-UTR of an RNA transcript was implicated in direct p53 binding. Each observation stemmed from independent evidence that the translational modulation of certain target proteins correlated with p53 expression, leaving open a number of possible mechanisms. Each research group then examined the potential of p53 to bind particular mRNA subsequences based upon the reports of p53 nucleic acid annealing activity (Oberosler et al. 1993) and p53 copurification with polysomal fractions (Fontoura et al. 1997).

p53 was suggested to negatively regulate translation of its own mRNA by direct binding of the p53 protein to the 5′-UTR of p53 mRNA (Mosner et al. 1995). Mosner et al. (1995) used biotinylated, in vitro-transcribed fragments of mouse p53 mRNA and a negative control SV40 mRNA to measure the binding of p53 affinity purified from insect cells. Western blot data suggested a preferential affinity of the p53 protein for p53 mRNA. Subsequently, the investigators observed an inhibition of p53 translation in cells, reportedly due to p53 protein binding to p53 mRNA (Mosner et al. 1995). These studies of p53–RNA interactions did not directly assay sequence requirements for p53 binding to p53 mRNA, or the stoichiometry of the interaction, both of which could be important to confirm sequence specificity. Moreover, the investigators did not test a wide or varied panel of RNAs for p53 binding. In addition, the nonspecific competitor RNA in the binding reactions was yeast tRNA, which may not be ideal (see below; Riley et al. 2007).

It should be noted that established RNA-binding proteins have also been reported to modulate p53 translation by binding to p53 mRNA. The thymidylate synthase enzyme has been reported to suppress p53 translation by binding to the coding sequence of p53 mRNA (Chu et al. 1999; Ju et al. 1999). In separate work, Hu antigen R was reported to enhance the translation efficiency of p53 mRNA by binding to an AU-rich sequence at the 3′-UTR after UV irradiation (Mazan-Mamczarz et al. 2003). An unbiased yeast three-hybrid screen for proteins that specifically bind to the 5′-UTR of p53 mRNA identified RNA sequence-dependent interactions with ribosomal protein L26 (RPL26) and nucleolin (Takagi et al. 2005). Despite the observations of Mosner et al. (1995), the screen did not identify p53. The RNA affinities of RPL26 and nucleolin

were confirmed *in vivo* by both overexpression and knock-down of these proteins in mammalian cell lines (Takagi et al. 2005). These results demonstrated that L26 and nucleolin have the potential to modulate p53 levels and affect p53 induction after DNA damage with the expected consequences on growth arrest and apoptosis (Takagi et al. 2005). The absence of p53 among *bona fide*, yeast three-hybrid partners does not support claims of sequence-specific RNA binding by p53.

In a limited set of low-resolution electrophoretic gel mobility-shift experiments, Miller et al. tested the ability of p53 (purified from insect cells) to bind 230 nucleotides (nt) of the Cdk4 mRNA 5'-UTR (Miller et al. 2000). In an experimental design similar to Mosner et al. (1995), these investigators employed a tRNA competitor and argued that two other RNA fragments were unable to compete with Cdk4 mRNA (Miller et al. 2000). The overall conclusion from this study was similar to that of Mosner et al. (2005): p53 binds to and specifically regulates the translation of Cdk4 mRNA.

Finally, p53 has been reported to specifically control translation of the FGF-2 mRNA. Galy et al. reported that recombinant wild-type p53 showed dose-dependent repression of FGF-2 mRNA translation in rabbit reticulocyte lysate (Galy et al. 2001). Surprisingly, although RNA binding was mapped to the p53 C terminus (Riley et al. 2006), a point mutation in the p53 core domain (V143A) was reported to totally abrogate repression of FGF-2 mRNA translation (Galy et al. 2001). Further, the mechanism of p53 binding to the FGF-2 transcript was unclear since RNA affinity in a filter-binding assay did not appear to correlate with translational inhibition for several RNA species (Galy et al. 2001).

In the three cases discussed above, the investigators suggested that the mechanism of sequence-specific translational repression by p53 was related to the putative nucleic acid annealing activity of p53, rather than RNA binding alone (Mosner et al. 1995; Miller et al. 2000; Galy et al. 2001). However, the groups that initially reported RNA annealing activity considered the required p53–RNA interaction to be entirely sequence nonspecific (Oberosler et al. 1993). All three publications reported translational inhibition during transfection experiments and *in vitro* transcription reactions, but data relevant to these interactions in normal cells or animal models are lacking (Mosner et al. 1995; Miller et al. 2000; Galy et al. 2001). Despite early evidence suggesting that p53 may exhibit specific binding to a subset of mRNA transcripts, RNA sequences and/or secondary structures preferentially recognized by p53 have not been resolved. Finally, studies proposing translational regulation by p53 utilized p53 protein with uncharacterized post-translational modifications as obtained from heterologous expression systems.

Sequence-nonspecific RNA binding by p53?

The most recent studies of p53–RNA binding focus on sequence-nonspecific interactions (Fig. 3, bottom). For

example, it has been proposed that the C terminus of p53 regulates DNA binding through sequence-nonspecific interactions with RNA *in vivo* (Yoshida et al. 2004). RNase A treatment, phosphorylation, or truncation of the p53 C terminus all were expected to activate independently DNA binding by full-length GST–p53 or FLAG–p53 *in vitro*. RNase A treatment also stabilized oligomerization of the protein (Yoshida et al. 2004). These investigators proposed a model in which cytoplasmic RNA binds p53, maintaining the protein in a latent form that is quickly degraded. This model suggests that, upon phosphorylation, such RNAs are displaced and p53 forms tetramers that bind to target DNA (Yoshida et al. 2004). This study proposes a potentially important role for RNA in p53 activation, but contradicts both observations of tetrameric p53–RNA interactions (Oberosler et al. 1993) and claims of sequence-specific mRNA binding (Mosner et al. 1995; Miller et al. 2000; Galy et al. 2001).

Having inadvertently detected RNA binding by p53 in the yeast three-hybrid system (SenGupta et al. 1996; Riley et al. 2006), we sought to shed light on the issue of RNA sequence preference using the yeast three-hybrid (Y3H) system (Fig. 3, bottom). We examined hundreds of tight-binding RNAs selected from a random sequence library (Fig. 3, bottom; Riley et al. 2006). Examples of detected interactions are shown in Figure 4. Computer-based sequence and structural analyses suggested that p53 has no preference for either RNA primary sequence or secondary structures (Riley et al. 2006), so we concluded that p53 binds RNA without sequence preference, and it is the quality of RNA display in the yeast three-hybrid system that dictates the reporter gene assay result. This interpretation was supported by the observation that the RNA-binding preferences of p53 for a panel of test RNAs in the yeast three-hybrid system largely mirrored that observed for the sequence-nonspecific HIV-1 nucleocapsid protein (Levin et al. 2005; Riley et al. 2006).

In our subsequent *in vitro* analysis of p53–RNA interactions we used p53 protein produced in insect cells. These studies further confirmed strong, sequence-nonspecific p53–RNA interactions (Fig. 3, bottom; Figs. 5, 6; Riley et al. 2007). When studied *in vitro*, RNA 1, a 310-nt RNA selected for strong p53 interaction in yeast, displayed multiple p53 complexes in the presence of small amounts of full-length human p53 (Fig. 5A; Riley et al. 2007). Several publications have shown that the p53 CTD binds sequence nonspecifically to DNA, but when unlabeled competitor salmon sperm DNA (either single or double stranded) was included with p53 in RNA-binding reactions, RNA binding persisted (Fig. 5B). Electrophoretic gel mobility-shift competition experiments were enlightening (Riley et al. 2007): several RNAs competed with RNA 1 for CTD binding (Fig. 5C, lanes 3–5), but tRNA did not bind to the CTD (Fig. 5C, lane 6). To test whether CTD RNA binding affected sequence-specific DNA binding by the

FIGURE 4. p53 binds RNA sequence nonspecifically in the yeast three-hybrid system. Data shown here recapitulate our published report (Riley et al. 2006). RNA sequences (~60 nucleotides unique sequence each) were selected in a yeast three-hybrid screen for ability to bind to p53. (Left) Growth assay for *HIS3* reporter gene expression on selective medium (-His/-Leu/-Ura) containing a gradient of 0–35 mM 3-aminotriazole. (Right) Liquid and colony filter lift β-galactosidase assays for *lacZ* reporter gene expression showing that gene activation is p53 dependent.

core domain, we monitored RNA-binding competition with a specific DNA sequence from the GADD45 promoter (Fig. 5C, lanes 7–8; Riley et al. 2007). Regardless of whether DNA or RNA was preincubated with p53, RNA binding was preferred to sequence-specific DNA binding (Fig. 5C, cf. lanes 7 and 8) or nonspecific dsDNA binding (Fig. 5C, lane 9; Riley et al. 2007). We concluded that human p53 protein purified from insect cells binds RNA without sequence preference, and RNA binding inhibits sequence-specific DNA binding by p53 (Riley et al. 2007).

We further demonstrated that the p53 CTD was necessary for strong RNA interaction in vitro and in yeast (Riley et al. 2006, 2007) and showed that the CTD was sufficient for RNA binding (Fig. 6; Riley et al. 2007). Using a human p53 CTD peptide (Fig. 6A), we found that the p53 CTD is sufficient for RNA interaction (Fig. 6B, lanes 1–5; Riley et al. 2007). To explore the effect of acetylation on this interaction, we compared RNA binding by a tetra-acetylated peptide (Fig. 6, lanes 6–10; Riley et al. 2007), and demonstrated that the acetylation of four lysine residues of the p53 CTD eliminates RNA interaction. Thus, our yeast and in vitro experiments led us to conclude that RNA binding by the p53 CTD is strong and sequence nonspecific (Riley et al. 2007).

Interestingly, the Prives laboratory recently reported gel mobility-shift assays with p53 protein (both WT and a mutant lacking the C-terminal 30 residues, purified from bacteria and insect cells) and chromatin immunoprecipitation assays with p53 expressed from tetracycline-responsive promoters (McKinney et al. 2004). These studies suggested that the p53 C terminus facilitates diffusion on double-stranded DNA and is required for efficient transactivation. Given that acetylated p53 does not interact with nonspecific double-stranded DNA (Friedler et al. 2005), it would be interesting to determine the modification status

of endogenous p53 present on DNA. Such studies could be undertaken by adapting chromatin immunoprecipitation with anti-p53 antibodies specific for different post-translational modifications.

RNA binding by p53 in vivo?

It remains unclear whether p53 protein with incomplete CTD modification is present in vivo, and if so, if it is associated with certain RNAs. To explore this possibility, several different approaches to detect p53–RNA interactions have been applied in cultured cells. These methods have included p53 coimmunoprecipitation with or without prior cross-linking using formaldehyde or UV light.

Yoshida et al. applied low-stringency conditions to coimmunoprecipitate p53 with RNA (Yoshida et al. 2004). Coimmunoprecipitated RNAs were detected by radiolabeling, but were not cloned or sequenced (Yoshida et al. 2004). A challenge in RNA coimmunoprecipitations is the potential for redistribution of macromolecules within complexes after cell lysis (Mili and Steitz 2004). Contamination of immunoprecipitation reactions by nonspecific RNAs is also a concern. High-salt washes have the potential to increase stringency but may disrupt both specific and nonspecific RNA–protein interactions (Riley et al. 2007).

FIGURE 5. RNA binding by full-length human p53 purified from insect cells. Data shown here recapitulate our published report (Riley et al. 2007). (A) Full-length human p53 (hp53 1–393; 0, 5.5, or 17 nM) was incubated with 3 nM ³²P-labeled RNA 1 before electrophoresis through a native polyacrylamide gel. (B) Full-length human p53 (0, 7, 14, 21, 28 nM) was incubated with 30 nM ³²P-labeled RNA 1 and a twofold molar excess (per nucleotide) of unlabeled salmon sperm DNA before electrophoresis. (C) Full-length human p53 (14 nM) was incubated with 3 nM ³²P-labeled RNA 1 and 30-fold molar excess (90 nM) unlabeled competitor nucleic acids before electrophoresis. (Lanes 1,2) No competitor; (lane 3) RNA 1; (lane 4) RNA d, (lane 5) RNA –/–, (lane 6) tRNA, (lanes 7,8) GADD45 DNA, (lane 9) nonspecific 38 bp DNA duplex. (Lane 7) p53 incubated with DNA competitor GADD45 before RNA 1. (Lane 8) p53 incubated with RNA 1 before GADD45 DNA competitor.

FIGURE 6. Acetylated p53 CTD does not bind RNA. Data shown here recapitulate our published report (Riley et al. 2007). (A) Human p53 CTD peptide (364–393) sequence. Acetylated lysines are highlighted. (B) Increasing amounts of unmodified or acetylated human p53 CTD (0, 14, 41, 123, and 369 μ M) were incubated with 30 nM 32 P-labeled RNA 1 before analysis by electrophoresis.

Studies of p53–RNA interactions within cells might be undertaken using several additional approaches. A common strategy to allow increased purification stringency involves *in vivo* covalent cross-linking with formaldehyde to capture *in vivo* protein–RNA interactions rapidly and reversibly (Niranjankumari et al. 2002). Nonetheless, the nonspecificity of chemical cross-linking reactions can complicate interpretation of such experiments (Ule et al. 2005). The use of partial nuclease treatment to enrich specific complexes requires that the RNA in such complexes is at least partially protected from digestion.

A technique was recently reported to detect and clone protein-bound RNAs *in vivo* (Ule et al. 2005). The UV-cross-linking and immunoprecipitation protocol (CLIP) reportedly addresses concerns such as low signal-to-noise ratio, difficulty in differentiating direct versus indirect protein–RNA interactions, and the inability to map a protein–RNA interaction at nucleotide resolution (Ule et al. 2005). CLIP involves the brief UV irradiation of live cells followed by extract preparation for immunoprecipitation. Selection among the many commercial antibodies specific for p53 might improve the stringency and specificity of this method. The lysate used for immunoprecipitation is treated with nucleases to eliminate DNA contamination, generating protein-bound RNA sequence tags for cloning. Linker oligonucleotides are ligated to radiolabeled RNAs, and the protein–RNA complexes are separated by SDS-PAGE. RNA tags associated with the protein of interest are purified, amplified, cloned, and sequenced. Application of CLIP to studies of p53–RNA interactions appears attractive. Concerns include the possibility that RNA binding by p53 is sequence nonspecific and does not juxtapose nucleotide bases and protein chromophores to allow UV cross-linking. An additional concern is that even the brief UV treatment required for CLIP may induce p53 conformational changes that prevent RNA binding.

To date, our own application of these methods has failed to detect specific p53–RNA interactions in either MCF-7 or

HCT116 cell lines (Riley and Maher 2007). One possible explanation is that the vast majority of p53 protein in these cells is acetylated or otherwise modified on the CTD, and thus unable to bind to RNA. In addition to casting doubt on the physiological significance of RNA binding by p53, this work revealed that sequence-nonspecific binding of RNA to antibodies can seriously complicate experimental interpretations.

CONCLUSION

Early evidence suggested a wide variety of possible p53–RNA interactions including specific binding to a subset of mRNA transcripts and/or to nonspecific RNAs. However, RNA sequences and/or secondary structures preferentially recognized by p53 were not conclusively resolved, and all data were obtained *in vitro* with p53 bearing nonphysiological post-translational modifications (Delphin et al. 1994). Recent studies of p53–RNA interactions both *in vitro* and *in vivo* lead us to suggest that RNA binding by p53 is sequence nonspecific, and the *in vivo* occurrence and significance of p53–RNA interactions remain debatable. Indeed, p53 highly modified in its CTD—the major *in vivo* form of the protein—appears unable to bind RNA. Thus, careful interpretation must be applied when analyzing p53–RNA interactions and translating the observed results to the context of living cells.

Despite the absence of evidence for p53–RNA interaction *in vivo*, *in vitro* data for partially modified forms of p53 suggest tempting speculation and directions for possible future study. *In vitro* studies have suggested that RNA binding to unmodified p53 can occur at both the C terminus and the core DNA-binding domain, although the former is much stronger (Riley et al. 2007). It therefore cannot be ruled out that p53 interacts with RNA via the core DNA-binding domain even when RNA binding at the C terminus is inhibited by p53 post-translational modifications. The fact that RNA binding may compete with C-terminal acetylation of p53 suggests the possibility that even sequence-nonspecific RNA binding could antagonize gene activation by p53 (Barlev et al. 2001). Thus, polysomes, microRNAs, or other RNA species could conceivably block DNA binding or gene activation by nonacetylated forms of p53. Another possible model is RNA anchoring of p53 at polysomes. Because HDM2, the human homolog of mouse MDM2, has also been reported to interact with RNA, and possibly with ribosomal components (Lai et al. 1998), it is conceivable that the presence of RNA could stabilize ternary RNA:p53:HDM2 complexes.

A key future challenge will be determining the best cell culture or animal model systems for investigating p53–RNA interactions, especially if the interactions are rare or occur only under special circumstances. Long the subject of speculation, we must conclude that the physiological

significance of RNA binding by tumor suppressor p53 remains an intriguing mystery.

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