Mdm2 binding to a conformationally sensitive domain on p53 can be modulated by RNA

Mdm2 binding to a conformationally sensitive domain on p53 can be modulated by RNA

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Abstract Biochemical characterisation of the interaction of mdm2 protein with p53 protein has demonstrated that full-length mdm2 does not bind stably to p53–DNA complexes, contrasting with C-terminal truncations of mdm2 which do bind stably to p53–DNA complexes. In addition, tetrameric forms of the p53His175 mutant protein in the PAb1620+ conformation are reduced in binding to mdm2 protein. These data suggest that the mdm2 binding site in the BOX-I domain of p53 becomes concealed when either p53 binds to DNA or when the core domain of p53 is unfolded by missense mutation. This further suggests that the C-terminus of mdm2 protein contains a negative regulatory domain that affects mdm2 protein binding to a second, conformationally sensitive interaction site in the core domain of p53. We investigated whether there was a second docking site on p53 for mdm2 protein by examining the interaction of full-length mdm2 with p53 lacking the BOX-I domain. Although mdm2 protein did bind very weakly to p53 protein lacking the BOX-I domain, addition of RNA activated mdm2 protein binding to this truncated form of p53. These data provide evidence for three previously undefined regulatory stages in the p53–mdm2 binding reaction: (1) conformational changes in p53 protein due to DNA binding or point mutation conceals a secondary docking site of mdm2 protein; (2) the C-terminus of mdm2 is the primary determinant which confers this property upon mdm2 protein; and (3) mdm2 protein binding to this secondary interaction site within p53 can be stabilised by RNA.

Key words: p53 conformation; Mdm2; RNA; Deletion mutation

1. Introduction

The tumour suppressor function of p53 is linked to its activity as a stress-activated sequence-specific DNA binding protein and transcription factor. The modulation of this function of p53 can be controlled by both amino- and carboxy-terminal regulatory domains, which identify at least two distinct pathways playing rate-limiting roles in controlling p53. The first pathway operates in proliferating, undamaged cells and involves regulation of p53 protein degradation. In normal cells, p53 is regulated by cycles of synthesis and degradation through the mdm2-dependent proteosome pathway. Mdm2 protein has a ubiquitin ligase-associated function [1] and its binding within the N-terminal BOX-I domain of p53 directs p53 nuclear export and degradation in the cytoplasm. The inhibition of mdm2 protein function by low-molecular weight peptides [2] or by monoclonal antibody (mAb) injection [3] activates p53 protein function as a transcription factor in the absence of DNA damage and pinpoints one rate-limiting pathway that negatively regulates p53 function in normal cells. A second pathway that regulates p53 function is a C-terminal negative regulatory domain, which ensures p53 is maintained in a low-affinity DNA binding or latent state. Modification of the C-terminal regulatory domain by peptides [4–6] or by the intracellular synthesis of activating mAbs [7] can induce p53-dependent gene expression and apoptosis. That both the N-terminal and C-terminal regulatory domains of p53 can be modified by peptides or antibodies that alter p53's specific activity places emphasis on identifying cellular enzymes that target these domains and modulate the function of p53.

Studies on identifying the cellular factors that target these two regulatory domains of p53 have identified two key phosphorylation site domains whose modification changes in response to DNA damage. Following low levels of irradiation injury to a cell, p53 protein can be activated as a transcription factor without protein stabilisation [4] that is modulated in part by increased phosphorylation at the C-terminal CK2 site at serine-392. The unusual feature of this upstream signaling pathway is that it is resistant to an inhibitor of CK2, identifying a CK2-independent pathway targets serine-392 in response to DNA damage [8]. Higher levels of irradiation damage to a cell can both stabilise p53 protein and activate its function as a transcription factor and this stabilised form of p53 protein has an elevated steady-state phosphorylation at serine-15, the ATM/ATR/DNA-PK site [9]. The stabilisation of p53 protein has a striking requirement for p300 protein [10] and phosphorylation within the BOX-I domain at serine-15 can stimulate p300 binding to and acetylation of p53 protein, suggesting that the primary role of serine-15 phosphorylation may be to stabilise p53 via p300 binding. However, serine-15 phosphorylation can also reduce the binding of mdm2 protein to p53 [9] and may play a role in reducing the rate of p53 degradation after high levels of irradiation damage. Thus, at least two upstream kinase signaling cascades can modulate the biochemical function of p53 as a transcription factor; one pathway targets the C-terminus of p53 and regulates specific DNA binding and a second pathway targets the N-terminal BOX-I domain of p53 and stimulates p300 protein binding while reducing mdm2 protein binding to p53.

Given the central role of mdm2 protein in the control of p53 function, studying the regulation of mdm2–p53 binding in

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vitro is therefore important for understanding the maintenance of p53 protein stability following activation; particularly relevant in view of the complexity of conformations that p53 can adopt (including latent, active and inactive isoforms [11,12]) and the multiple isoforms of mdm2 protein that can be synthesised. Binding studies using p53 truncation mutants [13] have shown that the minimum p53 binding site for mdm2 can be mapped to the first 50 amino acids of p53 and peptide binding studies refined this to between amino acids 18 and 23 [14]. Specific contact sites on p53 important for mdm2 binding and for transactivation were identified as Phe19, Trp23 and Leu26 by crystal structure analysis [15]. Recent studies have also identified two novel in vivo BOX-I phosphorylation sites on p53 at serine-20 and threonine-18 that can disrupt mdm2 binding [12,16] and highlight the existence of a clustering of regulatory phosphorylation sites within the BOX-I domain that can modulate p53 protein–protein interactions. However, there is a C-terminal element within p53 that controls p53 binding to mdm2, as C-terminal truncation mutants of p53, with an intact N-terminal docking site, decrease association with mdm2 [17–19].

Although the primary docking site for p53 on mdm2 is contained within the N-terminal domain of mdm2 [15], an independent function for the C-terminal RING-finger domain on mdm2 was identified after showing that polyguanylic acid (poly-G) binds specifically to this domain on mdm2 [20], and was suggested to participate in protein–protein interactions or DNA binding [21]. However, little is known about the effect of RNA binding to this domain of mdm2 on subsequent interactions between mdm2 and p53, nor the effect of full-length mdm2 protein binding to p53 protein. In an effort to identify novel regulatory interactions in the assembly of an mdm2 protein–p53 protein complex, quantitative BIAcore and enzyme-linked immunosorbent assay (ELISA) analyses of p53–mdm2 interactions were used to determine the nature and extent to which structural and conformational changes in p53 control binding to mdm2, and to look at the effect of other factors that regulate conformationally sensitive associations between the two proteins.

2. Materials and methods

2.1. Construction of expression vectors for the production of p53 and p53 deletion mutant proteins

Deletion mutagenesis of the human p53 gene and subcloning into a T7.7 expression vector was carried out as described from a single-stranded pBSK vector DNA containing the wild-type p53 gene [22]. Wild-type and a His175-substituted p53 were subcloned into pVL1393 baculovirus expression vector as described [11]. Amino- and carboxy-terminal deletion mutants of p53 and full-length mdm2 were expressed in *Escherichia coli* BL21 cells (0.5 l culture volumes) and p53 was induced by the addition of IPTG (1 mM final concentration). Cells were centrifuged at 10 000 rpm and the pellets were lysed on ice by sonication, after being resuspended in 4 ml of buffer A (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA) containing 0.8 ml of a protease inhibitor mix (10 mM pefabloc, 0.1 mg/ml leupeptin, 5 mg/ml aprotinin, 10 mg/ml pepstatin, 8 mM EGTA, 5 mM EDTA, 50 mg/ml trypsin inhibitor and 5 mM benzamidine; all inhibitors were obtained from Boehringer Mannheim). Insoluble material was pelleted by centrifugation and the supernatant was initially probed for the presence of p53 protein by Western blotting using PAb240 (Fig. 1) and DNA binding assays (as in [23]). The soluble proteins were further fractionated on a 5 ml HiTrap-Heparin column (Pharmacia) equilibrated with buffer A, and eluted in a gradient of buffer A from 0.15 M to 1.0 M NaCl. Fractions from the HiTrap-Heparin gradient were assayed for the presence of p53 by Western blotting probed with anti-p53 mAb 240, and for mdm2 with mAb 4B2. Fractions containing p53 or mdm2 were stored at −70°C after the addition of glycerol to 10% (v/v). Full-length p53 was expressed in Sf9 cells as described [11].

2.2. Immunological methods and reagents

All reagents were from Sigma unless indicated otherwise. Interaction of the C-terminal deletion mutants of p53 with mdm2 protein was carried out using a two-site ELISA by first coating wells with CM5 polyclonal antibody (pAb) at 2 μg/ml in carbonate buffer (pH 9.0) at 4°C for 16 h. Wells were blocked with 3% bovine serum albumin in phosphate-buffered saline plus 0.1% Tween-20. This was followed by titrating increasing amounts of deletion mutants p53CΔ30, CΔ44, CΔ67 and CΔ79. Wells were then loaded with mdm2 protein followed by mAb 4B2 and detected by the appropriate secondary antibody linked to peroxidase (rabbit anti-mouse horseradish peroxidase (HRP) from Dako).

In experiments to look at the interaction of mdm2 protein with the p53 deletion mutant proteins, in the presence of poly-G, the ELISA wells were first coated with mAb 4B2 (anti-mdm2) and treated essentially as described above followed by mdm2 and then p53. In one set of experiments, mdm2 protein was incubated with poly-G (10 mg/well), followed by increasing amounts of p53 protein. In a second set of experiments, increasing amounts of p53 protein were associated with poly-G (1 mg/ml p53), before being added to wells containing mdm2. Wells were then probed with CM5 pAb followed by the appropriate secondary antibody linked to peroxidase (goat anti-rabbit HRP).

2.3. BIAcore

Surface plasmon resonance (SPR) measurements were performed using BIAcore (Pharmacia). The N-terminal anti-mdm2 mAb 4B2 was captured on a CM5 sensor chip surface by amine coupling as described in the manufacturer's instructions (Pharmacia Biosensor AB) and used in subsequent experiments to reversibly bind mdm2 and mdm2 plus poly-G. p53 was passed across this surface and the binding characteristics of different forms of p53 protein were determined. SPR response was measured in resonance units (RU) (for most proteins, 1000 RU corresponds to a surface concentration of about 1 ng/mm^2). HBS (10 mM HEPES, pH 7.6, 150 mM NaCl, 3.4 mM EDTA and 0.05% BIAcore surfactant P20) was used as a buffer in this system at a flow rate of 5 ml/min.

3. Results

3.1. Conformational changes within p53 protein induced upon DNA binding or by missense point mutation conceal the mdm2 binding site on p53 protein

p53 protein truncation mutants and full-length, untagged mdm2 protein were purified as described above. The activity

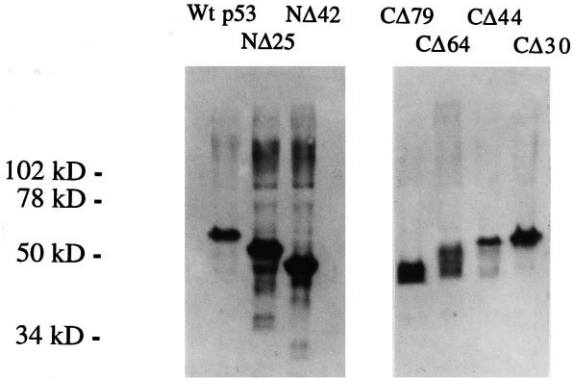

Fig. 1. Western blot of *E. coli* cell lysates expressing wild-type p53, a 25 and 42 amino acid N-terminal truncated p53, and a 30, 44, 64 and 79 amino acid C-terminal truncation of p53. The Western blot was probed with a p53-specific mAb 241, followed by a HRP-tagged rabbit anti-mouse secondary antibody.

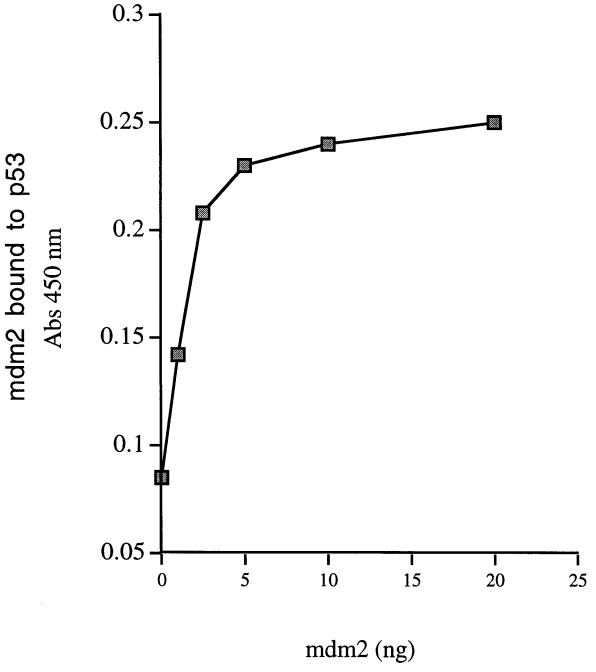

Fig. 2. Binding of full-length recombinant mdm2 expressed in *E. coli* cells to wild-type p53 expressed in Sf9 cells determined by two-site ELISA. Both mdm2 and p53 were partially purified by heparin-Sepharose chromatography as outlined in Section 2. A fixed amount of p53 was captured in ELISA wells by Ab CM5 (10 ng) followed by addition of increasing amounts of mdm2 (0–20 ng). Mdm2 bound to p53 was detected by an mdm2-specific antibody 4B2, followed by a HRP-tagged rabbit anti-mouse secondary antibody.

of full-length mdm2 protein in the p53 binding assays was established using ELISA, where an increase in the levels of mdm2 protein gave a corresponding increase in p53–mdm2 complex formation (Fig. 2). The stoichiometry of binding (25 ng of mdm2 protein gives the maximal signal with 50 ng of p53) suggests that a large majority of the mdm2 protein from the bacterial preparation is active in binding to p53 protein. Surprisingly, however, this active fraction of mdm2 was unable to supershift p53–DNA complexes using an in vitro DNA binding assay. Previous experiments have already shown that tetrameric forms of p53 protein undergo a conformational change upon binding to DNA [23], so it is possible that the N-terminal BOX-I domain of p53 becomes concealed upon DNA binding, thus precluding the binding of any protein to the BOX-I domain of p53. However, the antibody DO1, which shares an overlapping, but distinct, specificity with mdm2 protein in the BOX-I domain [24,12], was able to supershift the p53–DNA complex (data not shown).

The inability of full-length mdm2 protein to bind to p53–DNA complexes contrasts with that previously published using GST-mdm2/C-terminal truncation mutants, where the GST-mdm2ΔC-terminal mutant was able to bind to and supershift p53 protein–DNA [2]. Nevertheless, the inability of full-length, untagged mdm2 protein to supershift p53–DNA complexes suggested to us one hypothesis whereby conformational changes within the p53 tetramer, induced upon binding to DNA, concealed a secondary interaction site for full-length mdm2 protein.

Quantitative ELISA and BIAcore were subsequently used to determine whether conformational changes in p53 tetramers could reduce the specific activity of mdm2 protein in a p53 protein binding assay. Firstly, in the ELISA format, where mdm2 can stably bind to p53 protein, the addition of oligonucleotide DNA can reduce the amount of mdm2 protein bound to p53 protein (Fig. 3). Although the reduction in mdm2 protein binding to p53–DNA complexes data is not as pronounced as that observed using the band shift assay where there is no detectable mdm2–p53–DNA complex, it is well established that the band shift assays can better stabilise protein–DNA complexes than that which can be observed in solution. This is the predominant reason for the widespread use of gel electrophoresis as a 'ligand binding assay' for sequence-specific eukaryotic transcription factors. Second, increasing amounts of DNA also reduced the binding constant of mdm2 protein for p53 protein in a BIAcore (Fig. 4) and established the use of BIAcore as a very sensitive and quantitative, 'real-time', assay to manipulate and define the specific activity of mdm2 protein. Together, these data are consistent with the DNA band shift assay in establishing that p53–DNA complexes are refractory to interaction with mdm2 protein.

As previous data have clearly established that mdm2 protein can interact stably with small peptides from the BOX-I domain of p53 protein [14,15,2], it has been generally thought that this is the primary, if not sole, determinant of the mdm2 protein–p53 protein interface. An independent assay was therefore developed in attempts to test the hypothesis that the conformation of the p53 tetramer is a critical determinant in its interaction with mdm2 protein. The naturally occurring mutant form of p53 protein (Arg175 to His175) is inactive for sequence-specific DNA binding despite being in the tetrameric and PAb1620+ conformation [25]. These data indicate that this mutant form of p53 is not 'denatured' as defined by oligomeric status and PAb1620 reactivity, but it has some undefined structural defect, as it remains inactive for sequence-specific DNA binding. Using the ELISA, mdm2 pro-

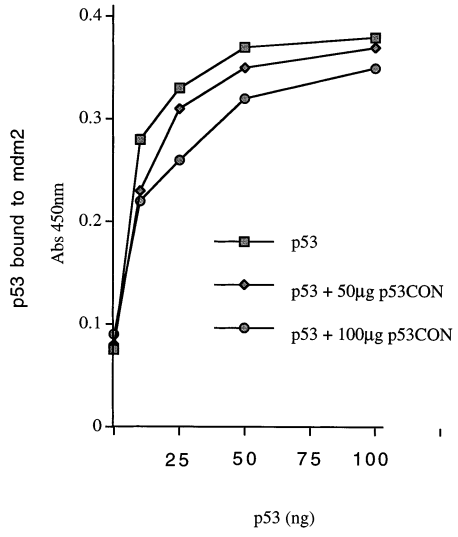

Fig. 3. Effect of increasing amounts of p53-specific oligonucleotide (p53CON) on p53 binding to mdm2 detected in a two-site ELISA. A fixed concentration of oligonucleotide (50 and 100 μg) was added to increasing amounts of wild-type p53 (0–100 ng) expressed in and partially purified from Sf9 cells. These aliquots of p53 were added to ELISA wells containing fixed amounts of mdm2 captured by a specific anti-mdm2 mAb, 4B2, and p53 was detected by pAb CM5 and HRP-tagged goat anti-rabbit secondary antibody.

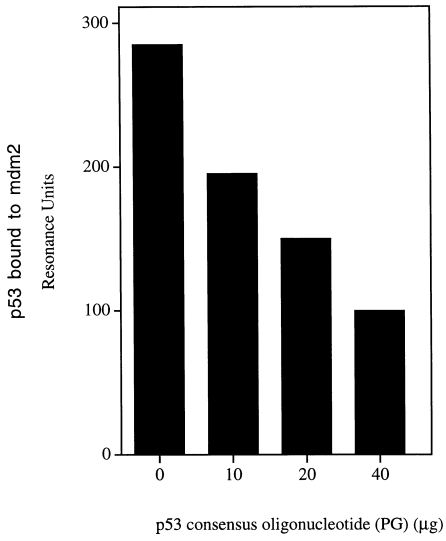

Fig. 4. p53 sequence-specific DNA (PG) binding to wild-type p53 inhibits the subsequent binding of p53 to mdm2 in BIAcore. Increasing amounts of oligonucleotide (PG) (0, 10, 20, 40 μg) bound to p53 and blocked subsequent rapid binding of fixed amounts of p53 (100 ng) to mdm2 in a dose-dependent manner. Mdm2 was initially bound to mAb 4B2 which had been linked to the surface of a CM5 Biosensor chip. The histogram represents the maximum RU for the association phase of p53 binding to mdm2, dissociation rates of p53 from mdm2 were unaffected by DNA.

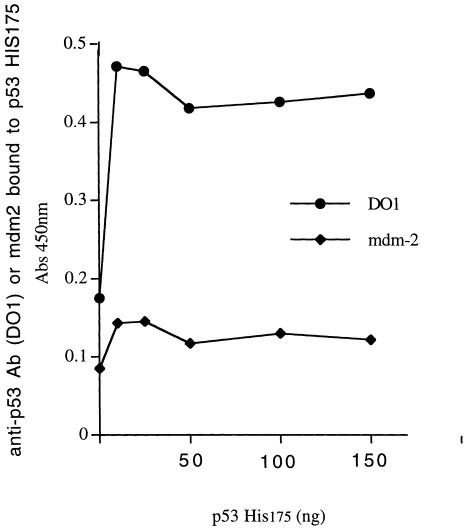

Fig. 5. A p53Arg–His175 substitution mutant was recognised by Ab DO1 but did not bind mdm2 in a two-site ELISA. p53His175 was expressed in Sf9 cells and partially purified by heparin-Sepharose chromatography as outlined in Section 2. Increasing amounts of p53 (0–150 ng) were captured in ELISA wells by Ab CM5 (10 ng) and probed with either p53-specific mAb DO1 or fixed amounts of mdm2 (20 ng), followed by mAb 4B2 and HRP-tagged secondary antibody. This p53 mutant is refractory to sequence-specific DNA binding and represents an as yet undetermined structural mutation of p53, significant in that this core domain mutation also appears to affect the ability of p53 to bind mdm2.

tein binding interacted inefficiently with this mutant protein (Fig. 5), consistent with there being a secondary interaction site on p53 protein for mdm2. Consistent with these ELISA data, a recent report has been published showing that the mutant form of p53 encoded by the His273 allele is defective in mdm2-dependent ubiquitination in vitro and in vivo, further confirming that mdm2 binding to p53 is conformationally regulated by alterations in the structure of the p53 tetramer.

3.2. *Deletion of the C-terminal tetramerisation domain of p53 destroys the mdm2 interaction site on p53*

The previous data suggested the existence of a second, conformationally sensitive interaction site of mdm2 in the core domain of p53. As such, we investigated whether perturbation of the quaternary structure of p53 by progressive deletion of p53's C-terminus would also prevent mdm2 from forming a stable complex with p53. Using ELISA, although deleting the C-terminal 30 amino acids of p53 had little effect on mdm2 binding (Fig. 6), deletion of 44, 64 and 74 amino acids resulted in progressively reduced binding of mdm2 to p53 (Fig. 6) similar to that previously observed in immunoprecipitates [17]. These data are supported by BIAcore (Fig. 7A).

3.3. *Poly-G RNA enhances the binding of p53 truncation mutations*

The mdm2 protein contains a number of domains that are likely to be involved in control of protein binding, with the N-terminus known to contain the primary docking site for p53, and an internal docking site present for the binding of ribosomal protein L5. However, the C-terminal RING-finger domain on mdm2 is also likely to be involved in the control of the mdm2–p53 binding interface since homologous regions in other proteins appear also to have roles in protein–protein interaction or DNA binding [21]. Poly-G, a homopolyribonucleotide RNA of about 400 bases, binds specifically to the RING-finger domain of mdm2 and deletion of this region abrogates polynucleotide binding by mdm2 [20]. Data are presented that show that RNA binding at the C-terminus of

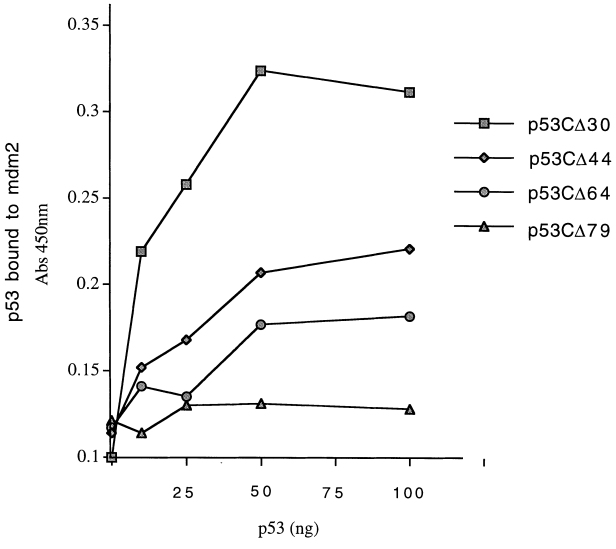

Fig. 6. Decreasing levels of binding of C-terminal p53 deletion mutants (CΔ30, CΔ44, CΔ64 and CΔ79) to mdm2 by two-site ELISA. A fixed amount of mdm2 (20 ng added) was captured in ELISA wells by mAb 4B2 (10 ng) before the addition of increasing amounts of each p53 C-terminal deleted protein. p53 was detected by pAb CM5 and HRP-tagged goat anti-rabbit secondary antibody. Even with an intact N-terminus, successive deletions at the C-terminus of p53 abrogate mdm2 binding, indicating the importance of this region of p53 in protein binding.

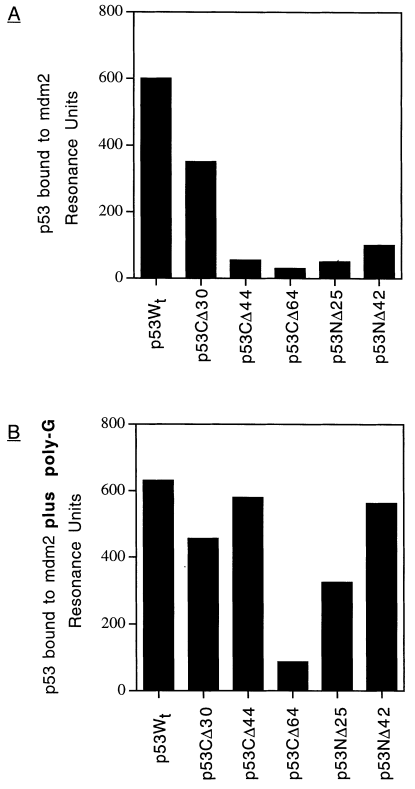

Fig. 7. Poly-G RNA enhances the binding of p53 truncation mutations to mdm2. (A) The p53 truncation mutants, CΔ44, CΔ64, NΔ25, NΔ42 (100 ng), had minimal rapid binding to mdm2 in BIAcore and compared to wild-type p53, the p53CΔ30 mutation also had decreased binding to mdm2 in the same assay. (B) Poly-G (5 μg) precaptured by mdm2 bound to mAb 4B2 linked to the surface of a CM5 Biosensor chip, enhanced the binding of all the p53 mutations apart from p53CΔ64, showing that a tetramerisation domain is an absolute requirement for rapid binding of p53 to mdm2. The histogram represents the maximum RU for the association phase of p53 binding to mdm2.

mdm2 may affect the ability of mdm2 to associate with truncated forms of p53.

Deletion of both the BOX-1 domain (p53NΔ25) and transactivation domain (p53NΔ42) almost entirely abrogates p53 binding to mdm2 in BIAcore (Fig. 7A), however, mdm2 with captured poly-G resulted in a substantial increase in the rapid binding of both N-terminal p53 mutant proteins to mdm2 (Fig. 7B), the binding of the transactivation domain truncated p53 approaching that of wild-type p53 controls.

3.4. A p53 tetramerisation domain is required for rapid binding to mdm2

The necessity for an intact p53 tetramerisation domain for rapid binding to mdm2 was indicated in BIAcore where deletion of 44 or 64 amino acids from the C-terminus of p53 almost completely abrogates binding to mdm2 (Fig. 7A). However, in the presence of an mdm2–poly-G complex, the p53CΔ44 deletion bound to mdm2 at rates indistinguishable from that of the full-length wild-type p53 (Fig. 7B), although deletion of the tetramerisation domain in the p53CΔ64 mutant protein completely abolished binding to mdm2 in BIAcore (Fig. 7A) even in the presence of an mdm2–poly-G complex (Fig. 7B).

4. Discussion

There is detailed information about the mdm2 binding site at the N-terminus of p53 which has shown that three amino acid residues (Phe19, Trp23 and Leu26) are necessary for binding at the BOX-1 region and that phosphorylation at critical residues in this region can block this interaction. In contrast, the present study shows that there is also a requirement for an intact tetramerisation domain (or association with RNA) for mdm2 binding to p53 C-terminal truncation mutants and describes the binding of mdm2 to p53 lacking a BOX-1 domain. In broader terms, phosphorylation at the C-terminus of p53 may also function not only to affect the conformational state of p53, from latent to an active conformation, but also to control stability through the ability to block the binding of mdm2, in a similar fashion as that known to take place at the N-terminus. The C-terminal regulatory site of p53 has been shown to control its stability and DNA binding function through phosphorylation by casein kinase II and protein kinase C [11,4] and this may also affect the avidity of mdm2 binding in a similar way. In this study, binding of either DNA or RNA by p53 has been shown to inhibit the subsequent attachment of mdm2 in vitro, indicating that polynucleotide binding also involves a conformational shift in p53 that blocks subsequent binding of mdm2. The importance of the core domain in conformational regulation of mdm2 binding was also shown by a mutant p53 with a single histidine-175 substitution resulting in a disrupted core domain that blocked mdm2 binding.

What is particularly interesting about this present work is that not only does polynucleotide binding by p53 block mdm2 binding but that specific RNA binding by mdm2 actually enhances the association of mdm2 to a mutant form of p53 lacking seven amino acids of a complete tetramerisation domain (CΔ44). This form of p53 is mAb 1620 negative in ELISA, but may still have enough structural integrity to form a dimer rather than a tetramer, and dimer formation has been proposed to be sufficient for in vivo p53 activity [26]. However, deletion of the entire tetramerisation domain (CΔ64) to produce a monomeric form of p53 completely abrogates binding of mdm2 to p53. An in vivo function of mdm2-enhanced binding to p53 by RNA may therefore be to target truncated or mutant/misfolded forms of p53 for degradation.

Although there is no definite function known for the C-terminal RNA binding of the RING-finger domain of mdm2, one can speculate from the functions proposed for other proteins containing a similar domain. The *Drosophila* inhibitor of apoptosis protein DIAP1 also contains a C-terminal RING-finger domain, similar to that of mdm2, which may negatively regulate its activity [27]. The mdm2 RING-finger domain might also act as a regulator of some target involved in apoptosis [28]. There is a precedence for this in that the promyelocytic leukemia oncoprotein PML has been shown to have a pro-apoptotic activity which is mediated through its RING-finger domain [29]. Single-stranded RNA viruses cause relocation of PML bodies to the cytoplasm which results in resistance to serum starvation in infected cells and decreased apoptosis. Viral RING-finger proteins (Z protein) also bind to PML and cause relocation of these nuclear bodies to the cytoplasm [30], although Z protein binding to PML is at the N-terminal region and does not require the PML RING-domain for colocalisation.

This work demonstrates the level of complexity involved in the binding of mdm2 to p53. Not only can this be regulated by phosphorylation at the N-terminus, but it is also dependent on the conformational state of the protein, the presence of an intact tetramerisation domain, the structure of the core domain and polynucleotide binding; all of which can act as negative regulators of mdm2 binding and subsequent targeting for degradation.

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