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Design and Vesting of Di/functional, P-loop Vargeted MF O 2 Khibitors

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14. ABSTRACT Our proposal is to design and evaluate a novel class of bifunctional MDM2 inhibitors, based on the discovery that nucleotides can bind to the P-loop of MDM2 and cause its relocalization to the nucleolus. Such bifunctional compounds will be designed to target MDM2, but not other P-loop-containing proteins. This approach represents a new strategy for the inhibition of MDM2 function and the treatment of breast cancer. During the second year of this grant we have used the cloned, expressed, and purified GST-fused Mdm2 wild-type and C-terminally deleted RING domain protein to continue to test all commercially and privately available ATP analogs (including fluorescent analogs) for binding to Mdm2 and have further insight into the features of ATP required for binding to Mdm2. We have also (1) determined that crosslinking to 8-azido ATP inhibits ATP binding and E3 ligase activity; (2) developed high-throughput auto-ubiquitination assay to discover Mdm2 ligase inhibitors (3) developed a high-throughput docking assay based on Mdm2's RING domain structure and (4) developed a high-throughput compatible luciferase-based competition assay for compounds that bind to Mdm2.					
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Introduction

The Mdm2 protein plays essential roles in negatively regulating the p53 tumor suppressor protein. Mdm2 has been found to be upregulated in a significant number of breast cancers. The Mdm2 RING domain possesses a cryptic nucleolar localization signal sequence and a nucleotide-binding P-loop motif. These two motifs are unique to the Mdm2 RING and are not present in the myriad other RING domains encoded in the human genome. Nucleolar relocation of Mdm2 causes p53 stabilization and activation, and nucleotide binding to the Mdm2 RING domain facilitates Mdm2 nucleolar localization.

Mdm2 has been found to play an essential role in negatively regulating the p53. In turn, the p53 protein binds to the promoter of Mdm2 and activates Mdm2 transcription. The importance of Mdm2 in p53 down-regulation has also been shown by genetic analysis: loss of *mdm2* in mice results in early embryonic lethality, but deletion of *p53* with *mdm2* rescues the lethal phenotype.

There is mounting evidence for a pro-oncogenic role for Mdm2 that is independent of p53 in both murine models and in human tumor cohort studies. Therefore the novel inhibitors that we plan to develop may have broader impact than activation of p53.

The design of genetically-targeted anti-tumor agents is an important new strategy in cancer drug discovery. However, it is often difficult to identify the specific proteins that should be targeted for maximal clinical benefit and to develop small molecules that target these proteins. We have identified an opportunity to develop a potentially powerful new class of compounds targeted against breast cancers with a specific genetic modification. We propose to develop genetically targeted small molecules that will selectively eradicate breast cancer cells containing amplified or up-regulated Mdm2.

Project Progress - Body

Previously, we have cloned, bacterially expressed, and affinity purified GST-fused Mdm2 wild-type RING domain as well as a C-terminally deleted Mdm2 RING domain protein that does not oligomerize in solution and exhibits dramatically increased solubility when compared to similarly expressed and purified wild-type Mdm2 RING. This protein is ideal for development of a high-throughput assay due to its high bacterial yield and excellent solubility.

Finally, we have successfully characterized the ATP binding site of the Mdm2 RING domain, identifying the ribose hydroxyls as well as the C6 amino group as being required for binding. We have also determined that the nucleotide binding site of Mdm2 lies between residues 429-484, within the core Mdm2 RING domain, and determined that residues outside of the Mdm2 P-loop are involved in coordinating nucleotides. MdmX, a family member and binding partner of Mdm2, also has a homologous nucleotide binding site, making it another excellent target for a small molecule screen, as this protein is also involved in human tumorigenesis. Please see the attached manuscript in submission, "Deconstructing nucleotide binding activity of the Mdm2 RING domain, for details and figures."

8-azido ATP, an ATP analog that binds to the Mdm2 RING domain with similar affinity as unlabeled ATP *in vitro* (Figure 1,a), can be crosslinked to Mdm2 in a UV-dependent manner. Mdm2 RING domain that is crosslinked to 8-azido ATP can no longer bind ^{32}P γ ATP, suggesting that the crosslinked ATP is specifically occupying the correct ATP binding site of the protein (Figure 1,b). Additionally, Mdm2 RING that has been crosslinked to 8-azido ATP shows decreased ability to polymerize ^{32}P -ubiquitin *in vitro*, suggesting that ATP binding directly inhibits the E3 ligase activity of Mdm2 RING (Figure 1,c). This data suggests that ATP-like compounds that bind selectively to the Mdm2 RING would be possibly inhibitors of E3 ligase activity.

We have also made progress developing a high-throughput screen for compounds that bind to Mdm2. We first attempted to optimize a commercial FRET assay (Lance, Perkin Elmer) that would detect ATP binding to Mdm2 in a high-throughput compatible format (Figure 2,a). In this assay, biotinylated ATP, bound to a streptavidin molecule with a acceptor fluorophore binds to GST-Mdm2 that has been labeled with an anti-GST antibody tagged with the donor fluorophore. Fluorescence output is disrupted if a test compound displaces the biotinylated ATP from the Mdm2 binding site, signaling a compound that binds to Mdm2. We determined that biotinylated ATP binds to the Mdm2 RING with similar affinity as unlabeled ATP (Figure 2,b) and that this binding is not disrupted in the presence of streptavidin-APC (Figure 2,c). However, we were unable to detect any fluorescence output with GST-Mdm2, although our positive and negative controls were successful (Figure 2,d). In order to optimize our reaction conditions, we directly biotinylated GST and GST-Mdm2 RING, confirming labeling using an anti-biotin immunoblot (Figure 2,e). We were able to detect fluorescence output from biotinylated GST, but not GST-Mdm2 RING (Figure 2,f), suggesting that the Mdm2 RING obscures the epitope of the fluorescently labeled anti-GST antibody.

In an effort to find compounds that inhibit Mdm2 ligase activity, we have succeeded in developing a high throughput *in vitro* ubiquitination assay which makes use

of the Mdm2 RING domain's ability to auto-ubiquitinate (Figure 3). The assay involves the reconstitution of the ubiquitination pathway using biotin-labeled ubiquitin. In the assay, E1 enzyme is used to activate the labeled substrate and transfer it to an ubiquitin carrier protein, UbcH5c. The substrate bound E2 is then used by GST-tagged Mdm2 RING domain to auto-ubiquitinate. Detection of ubiquitinated Mdm2 is done by transferring the reaction to a streptavidin-coated plate, washing and detecting bound Mdm2 by incubating with HRP-linked α -GST antibodies then detecting luminescence by adding ECL reagent.

Also, as a new approach to find hit compounds, we have developed a virtual docking assay, which was made possible by the recent publication of the structure of the MDM-2 RING domain. This computational approach to drug discovery makes use of the existence of an available crystallographic or NMR structure of the target by docking small molecules into the ligand binding site of the protein. We used the monomeric form of the published RING domain structure (Kostic et al. 2006.) as a docking target. Based on previous mutational studies on the RING domain (Poyurovsky et al. 2003.) and molecular dynamics simulations we predicted the ATP binding site to be in an exposed surface with K454 of the RING domain coordinating the beta- and gamma-phosphates of ATP (Figure 4).

For this study, we screened 239,199 compounds available from commercial vendors using FRED2.1 (Openeye Scientific) for docking and OMEGA 1.8 (Openeye Scientific) for conformer generation. Each compound was represented by 200 conformers in the screening dataset to properly explore conformational space when docking. The top scoring 500 compounds will be ordered and screened in an assay yet to be developed.

Key Research Accomplishments

- Cloned, expressed, and purified GST-fused Mdm2 wild-type and C-terminally deleted RING domain protein
- Tested all commercially available ATP analogs (including fluorescent analogs) for binding to Mdm2
- Identified features of ATP required for binding to Mdm2
- Localized Mdm2 nucleotide binding site to residues 429-484 within the RING domain
- Determined that residues outside Mdm2 P-loop motif are involved in nucleotide coordination
- Identified the Mdm2 homologue, MdmX, as having a homologous and specific adenine nucleotide binding site to Mdm2
- Determined that crosslinking to 8-azido ATP inhibits ATP binding and E3 ligase activity
- Developed high-throughput docking assay based on Mdm2-s RING domain structure

Reportable Outcomes

Posters

13th Annual p53 Workshop, May 20-24, 2006. Columbia University, New York, NY, USA.

Deconstructing Nucleotide Binding Activity of the Mdm2 RING Domain

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The RING domain of Mdm2 contains a conserved Walker A or P-loop motif characteristic of nucleotide binding proteins. As has been previously shown, Mdm2 preferentially binds adenine base nucleotides and that such binding leads to a conformational change in the Mdm2 C-terminus. (Poyurovsky et al. Mol Cell. 12: 875-87, 2003). Nucleotide binding defective Mdm2 mutants are impaired in p14/ARF-independent nucleolar localization both *in vivo* and *in vitro*, and ATP-bound Mdm2 is preferentially localized to the nucleolus.

Here we present further biochemical analysis of the nucleotide-Mdm2 interaction. We confirmed the original ATP binding and specificity results using Isothermal Titration Calorimetry (ITC). Further investigation of the interaction using a series of ATP derivatives identified 2' and 3' hydroxyls of the ribose as well as the C6 amino group of the adenine base moiety as being essential for the interaction. These results further support our previous data on ATP specificity, as the C6 amino group is a unique feature of adenine. MdmX, an Mdm2 family protein with high sequence homology, similarly binds adenine nucleotides preferentially. In order to further elucidate the structural features of Mdm2 necessary for ATP interaction, we have created a series of substitution mutations in residues within the Mdm2 RING domain that were predicted to be involved in base recognition. We assayed the resulting mutant proteins for nucleotide binding, nucleotide specificity, and E3 ligase activity. Our results highlight an intriguing separability between nucleotide binding and E3 functions of the Mdm2 RING domain, indicating that this domain may be involved in several unrelated biochemical processes. (Supported by DOD proposal #BC044468)

Journal Articles

Priest, C., Prives, C., and Poyurovsky, M. V. (2010) **Deconstructing nucleotide binding activity of the Mdm2 RING domain**. Nucleic Acids Research. (*provisionally accepted, please see attached .pdf*)

Abstract

Mdm2, a central negative regulator of the p53 tumor suppressor, possesses a Really Interesting New Gene (RING) domain within its C-terminus. In addition to E3 ubiquitin ligase activity, the Mdm2 RING preferentially binds adenine base nucleotides,

and such binding leads to a conformational change in the Mdm2 C-terminus. Here we present further biochemical analysis of the nucleotide–Mdm2 interaction. We have found that MdmX, an Mdm2 family member with high sequence homology, binds adenine nucleotides with similar affinity and specificity as Mdm2, suggesting that residues involved in nucleotide binding may be conserved between the two proteins and ATP binding may have similar functional consequences for both Mdm family members. By generating and testing a series of proteins with deletions and substitution mutations within the Mdm2 RING, we mapped the specific adenine nucleotide binding region of Mdm2 to residues 429–484, encompassing the minimal RING domain. Using a series of ATP derivatives, we demonstrate that phosphate coordination by the Mdm2 P-loop contributes to, but is not primarily responsible for, ATP binding. Additionally, we have identified the 2' and 3' hydroxyls of the ribose and the C6 amino group of the adenine base moiety as being essential for binding.

Conclusions

In summary, although we do not have a high-throughput assay to find small molecule inhibitors of Mdm2, we have made significant progress in determining the features of ATP required for interaction with Mdm2. We have also identified the area of the Mdm2 RING domain that contains a nucleotide binding site. Additionally, we have identified MdmX as another potential target for a nucleotide analog inhibitor. We have also developed and performed an *in silico* screen for small molecules that may bind to the nucleotide binding site of Mdm2. These studies may lead to the discovery of novel, selective Mdm2 and MdmX inhibitors that are effective treatments for breast cancers.

References

Kostic, M., Matt, T. Martinez-Yamout, M.A., Dyson, H.J. Wright, P.E., (2006) Solution structure of the Hdm2 C2H2C4 RING, a domain critical for ubiquitination of p53. *J Mol Biol.* 2, 433-50.

Openeye Scientific, Santa Fe

Poyurovsky, M. V., Jacq, X., Ma, C., Karni-Schmidt, O., Parker, P. J., Chalfie, M., Manley, J. L., and Prives, C. (2003). Nucleotide binding by the Mdm2 RING domain facilitates Arf-independent Mdm2 nucleolar localization. *Mol Cell* 12, 875-887.

Appendix/Supporting Data

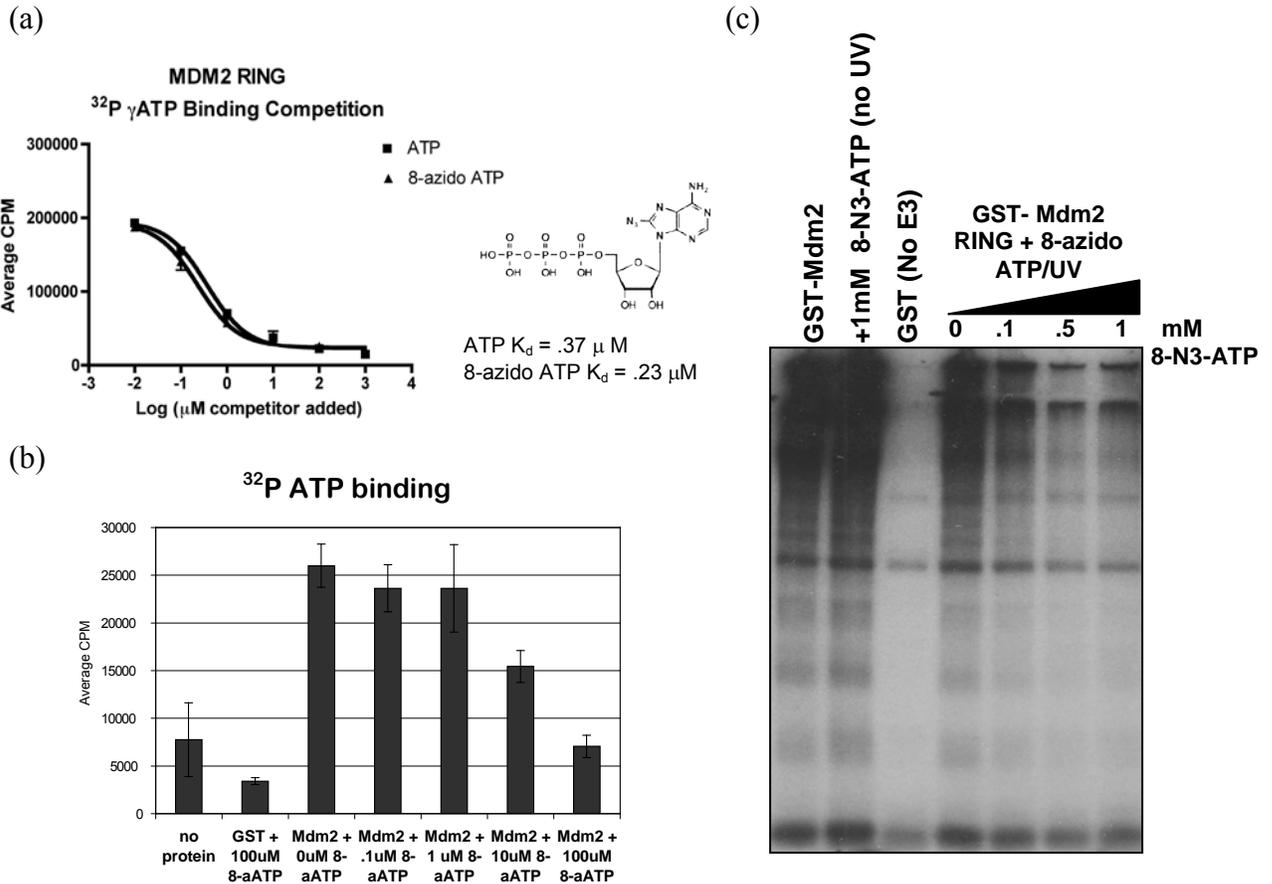


Figure 1. Crosslinking 8-azido ATP to the Mdm2 RING domain inhibits ATP binding and E3 ligase activity (a) 8-azido ATP binds Mdm2 with similar affinity as ATP. (b) Crosslinking to 8-azido ATP significantly reduces ATP binding by Mdm2. (c) Crosslinking to 8-azido ATP inhibits Mdm2 *in vitro* ubiquitin polymerization activity. All error bars represent the standard deviation of 2 replicates.

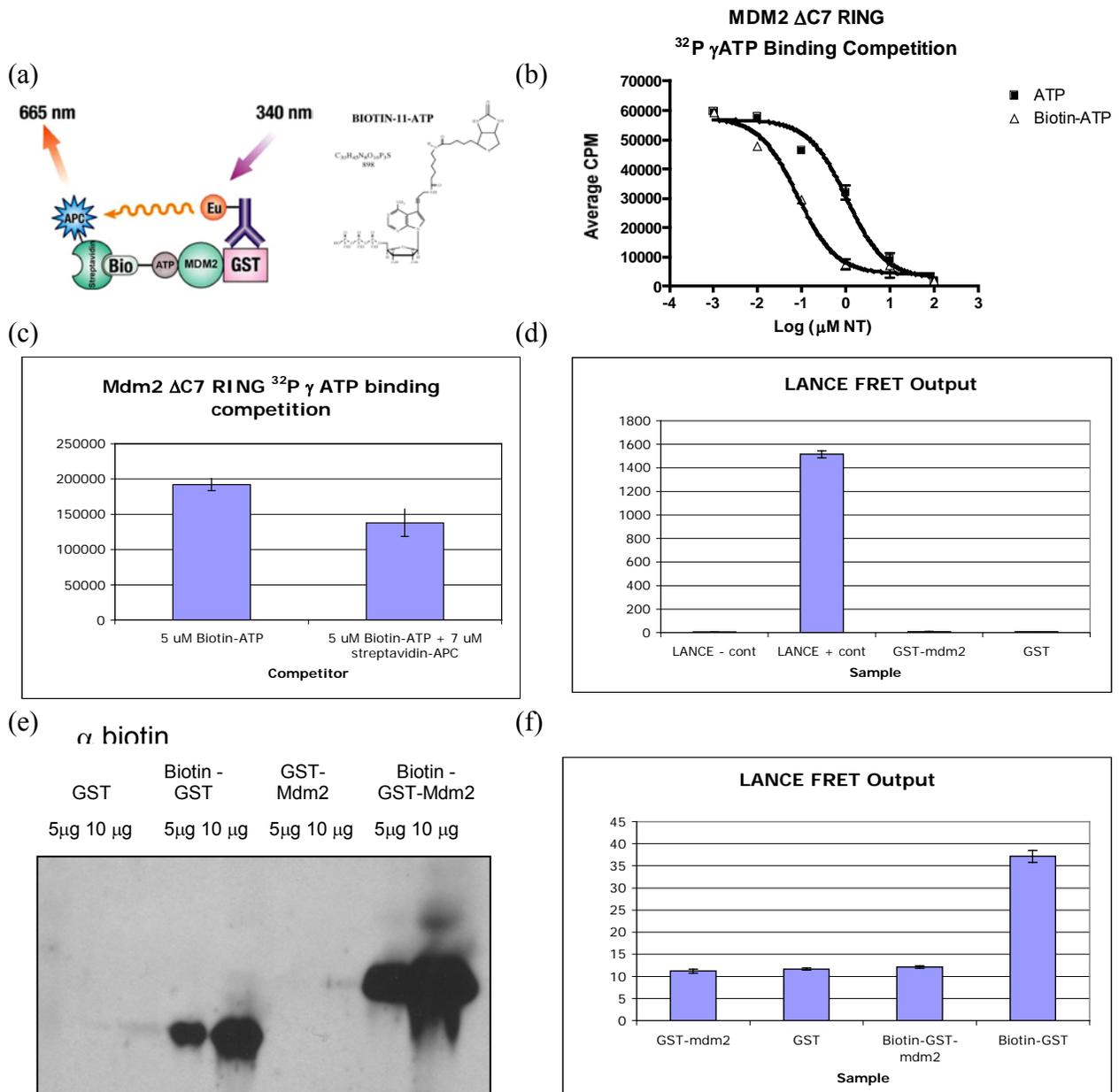


Figure 2. Design and optimization of Lance TR-FRET assay. (a) Assay design and structure of biotin-labeled ATP. (b) Competition of unlabeled ATP and Biotin-11-ATP with ^{32}P γ -ATP for binding to GST-Mdm2 Δ 7 RING. (c) Competition of Biotin-11-ATP with ^{32}P γ -ATP for binding to GST-Mdm2 Δ 7 RING is unaltered in the presence of streptavidin-APC. (d) Lance FRET assay output in the presence of GST (negative control) or GST-Mdm2 Δ 7 RING. (e) α -biotin immunoblot after biotinylation of GST and GST-Mdm2 Δ 7 RING. (f) Lance FRET output in the presence of untreated or biotinylated GST or GST-Mdm2 Δ 7 RING. All error bars represent the standard deviation of two replicates.

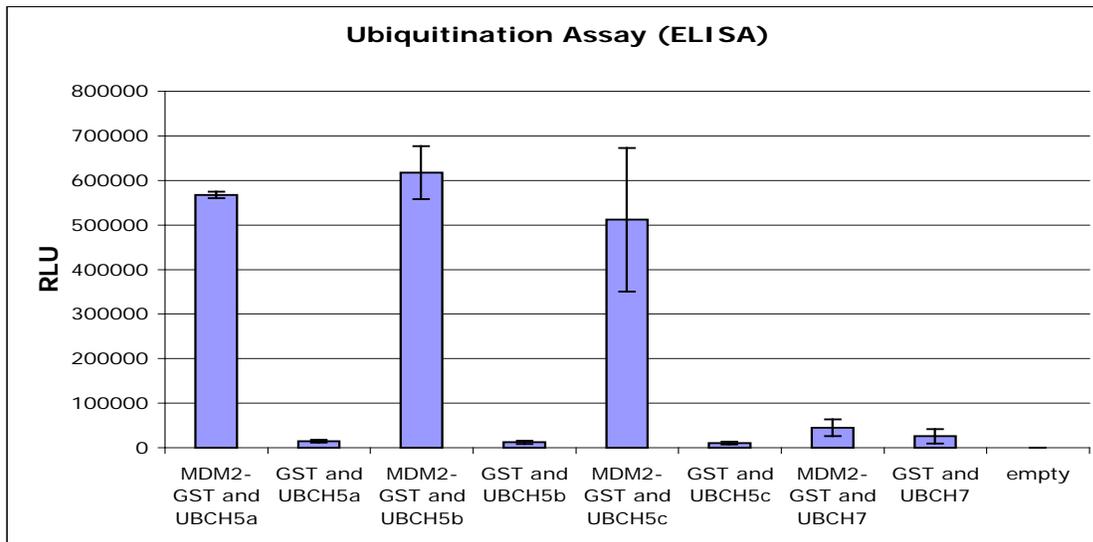


Figure 3. Mdm2 auto-ubiquitination assay. The assay works with any of the UBCH5 proteins, which are known to function with MDM2 (400-491)-GST. However, UBCH7 which has not been shown to function as an E2 with MDM2 does not work in this assay. Additionally the ubiquitination reaction was verified on a Western Blot analysis (data not shown). This assay can be used to screen large numbers of small molecules in a 384-well high-throughput format to find MDM2 E3 ligase inhibitors.

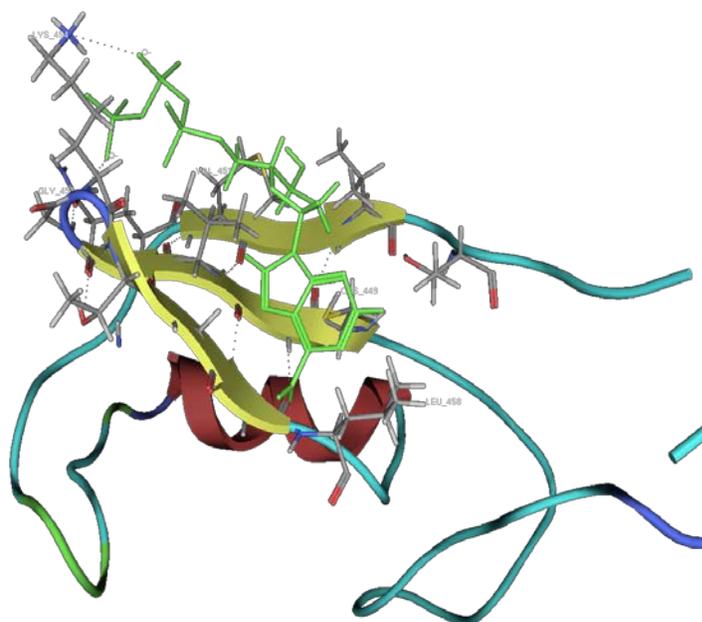


Figure 4. Proposed binding site on Mdm2 RING domain, showing K454 coordinating phosphate groups of ATP.

Deconstructing nucleotide binding activity of the Mdm2 RING domain

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ABSTRACT

Mdm2, a central negative regulator of the p53 tumor suppressor, possesses a Really Interesting New Gene (RING) domain within its C-terminus. In addition to E3 ubiquitin ligase activity, the Mdm2 RING preferentially binds adenine base nucleotides, and such binding leads to a conformational change in the Mdm2 C-terminus. Here, we present further biochemical analysis of the nucleotide–Mdm2 interaction. We have found that MdmX, an Mdm2 family member with high sequence homology, binds adenine nucleotides with similar affinity and specificity as Mdm2, suggesting that residues involved in nucleotide binding may be conserved between the two proteins and adenosine triphosphate (ATP) binding may have similar functional consequences for both Mdm family members. By generating and testing a series of proteins with deletions and substitution mutations within the Mdm2 RING, we mapped the specific adenine nucleotide binding region of Mdm2 to residues 429–484, encompassing the minimal RING domain. Using a series of ATP derivatives, we demonstrate that phosphate coordination by the Mdm2 P-loop contributes to, but is not primarily responsible for, ATP binding. Additionally, we have identified the 2' and 3' hydroxyls of the ribose and the C6 amino group of the adenine base moiety as being essential for binding.

INTRODUCTION

Murine double-minute 2 (Mdm2) oncoprotein is a critical negative regulator of the transcriptional activity and stability of the p53 tumor suppressor (1,2). As such, Mdm2 has been the focus of numerous and diverse studies aimed at describing the structural and functional aspects of Mdm2 as well as Mdm2's ability to interact with and regulate p53 and other proteins (3,4).

Mdm2 possesses a number of functionally distinct regions. The N-terminal p53-binding domain (amino acids 26–108) is primarily responsible for inhibition of p53 transcriptional activity (5,6). The central acidic portion of Mdm2 (amino acids 230–274) is the site of multiple posttranslational modifications and is essential for p53 degradation (7,8). Mdm2 also has a zinc-finger domain (amino acids 289–331) whose function has been correlated with oncogenic properties of Mdm2 (9). At the extreme C-terminus of Mdm2 (amino acids 437–491) is a C2H2C4 RING domain (Figure 1A) (10). C2H2C4 refers to the order of the cysteine and histidine residues that coordinate two molecules of zinc in a characteristic 'crossbrace' fold necessary for structural integrity (11). The RING domains of Mdm2 and its closely related family member, MdmX, are highly conserved structurally although only Mdm2 has demonstrable E3 ligase activity (12,13).

Like many other RING containing proteins, the Mdm2 E3 ubiquitin ligase stimulates the transfer of ubiquitin from the E2 (ubiquitin conjugating enzyme) to target proteins (14). Mdm2 itself, p53, and MdmX are among the best-described targets of Mdm2 E3 activity (6,15,16). MdmX is also able to bind to and transcriptionally inhibit p53 and is an essential negative regulator of p53 activity (17,18).

Some RING-containing proteins form higher order oligomeric complexes that are hypothesized to act as staging platforms for enhancement of biochemical reactions (19,20). The formation of higher-order oligomers by Mdm2 requires the extreme C-terminus and is necessary for its ubiquitin ligase activity. Mdm2 is able to form homo-oligomers with itself and hetero-oligomers with MdmX (21–23). Consequently, the E3 activity of Mdm2 is altered by the composition of these complexes. Mdm2 homo-oligomers are thought to function primarily in auto-ubiquitination of Mdm2, while the Mdm2/MdmX complex seems to be the primary ligase for p53 (24–26).

In addition to mediating ubiquitin ligase activity and oligomerization, the RING domain of Mdm2 contains a functional albeit cryptic nucleolar localization signal

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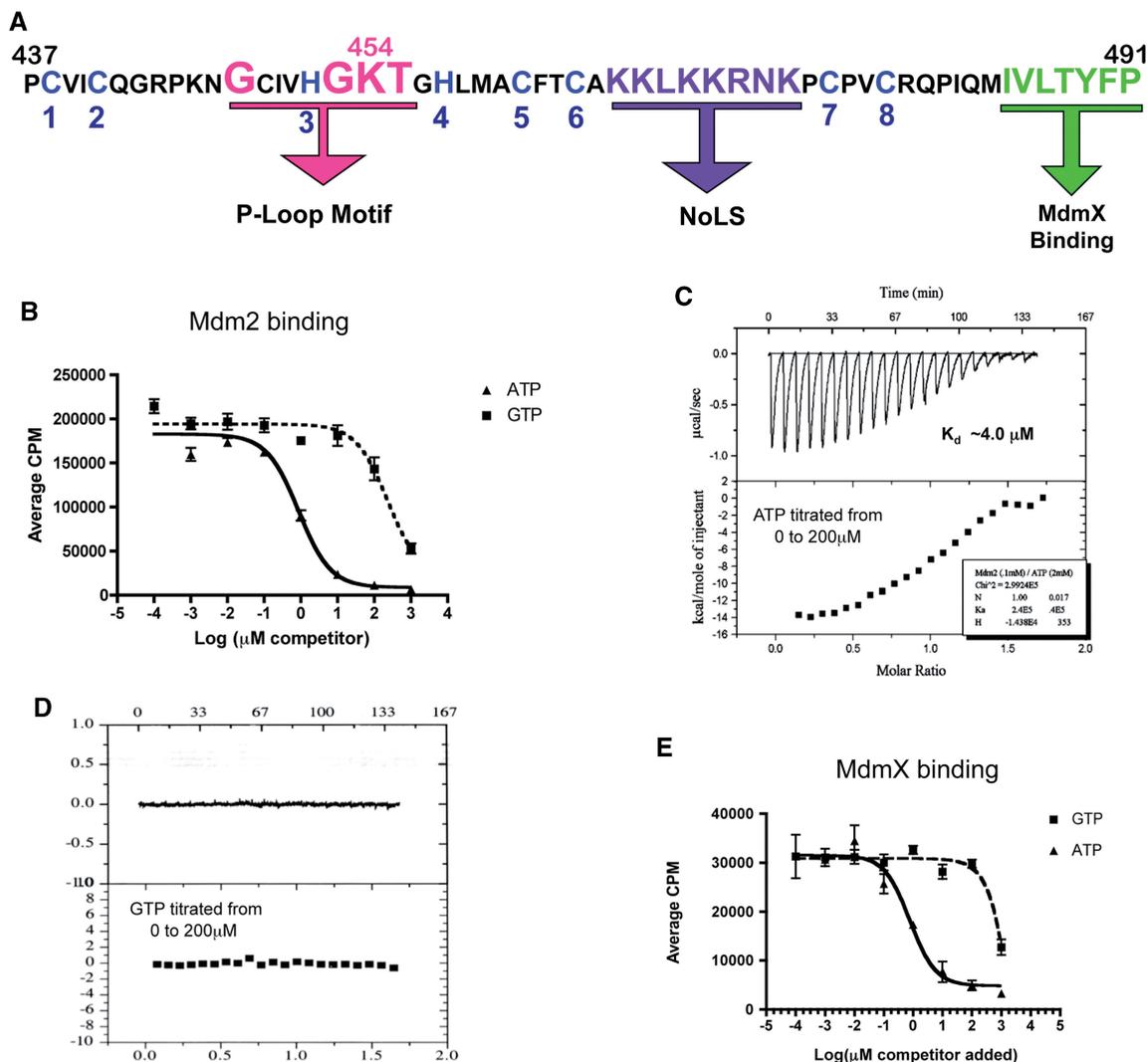


Figure 1. Mdm2 and MdmX bind ATP specifically. (A) Diagram of the Mdm2 RING domain. Zinc-coordinating residues (blue) are numbered, and P-loop motif (pink), nucleolar localization motif (NoLS, purple), and region necessary for Mdm2/X oligomerization (green) are indicated. (B) GST-Mdm2(400–491) protein binds ATP selectively. Following incubation of Mdm2 with ATP, increasing concentrations of the competitor nucleotides (as indicated) were added to the reaction mixtures. The γ - ^{32}P ATP-bound fraction was analyzed by liquid scintillation. (C) Mdm2–ATP interaction characterized by isothermal titration calorimetry (ITC). Original raw data (upper panel), fit after integration (lower panel). Two millimoles of ATP was titrated into 100 nM GST-Mdm2(400–491). The binding data was fitted to a single-site binding isotherm after subtracting the heat of dilution generated by injecting ATP into buffer alone. The extracted K_d was $\approx 4.0 \mu\text{M}$. (D) Binding of Mdm2 to GTP assessed by ITC. ITC experiments performed as in (B), 100 nM GST-Mdm2(400–491) titrated with 2 mM GTP. (E) GST-MdmX(403–490) protein binds ATP selectively. Competition experiments were performed as in (A) with GST-MdmX(403–490) proteins and a titration of the indicated competitor nucleotides.

spanning residues 466–473 (Figure 1A) (27). This basic patch is able to facilitate association of Mdm2 with the nucleolus following some forms of DNA damage (27). Perhaps related to Mdm2's ability to localize to the nucleolus is the fact that the RING of Mdm2 interacts with RNA (28). Additionally, this domain is implicated in allosteric control of Mdm2 structure and function (29).

Adding further complexity, the Mdm2 RING domain contains a Walker A or P-loop motif, characteristic of ATP/GTP binding proteins (Figure 1A). P-loop residues are involved in the coordination of the β - and γ -phosphates of nucleotides (30,31). A Walker A consensus sequence is present in all Mdm2 orthologues as well as

MdmX. In a previous study, we determined that Mdm2 is indeed able to bind nucleotides and that mutations of the P-loop residues diminish nucleotide-binding activity (32). Treatment of cells with actinomycin D induces nucleolar localization of Mdm2, (33) and point mutations in the P-loop lead to reduced nucleolar localization of Mdm2 following actinomycin D treatment (32). Thus, at least one of the likely functions of ATP binding by Mdm2 is the regulation of sub-nuclear compartmentalization. ATP binding has also been linked to an activity of Mdm2 as a molecular chaperone for p53 (34), as well as the ability of Mdm2 to inhibit the DNA-binding activity of the E2F1 transcription factor (35). These studies suggest diverse

roles for ATP in the activity of Mdm2 and provide the basis for a more comprehensive investigation of Mdm2-ATP interaction.

In this study, we interrogate the ATP binding features of the Mdm2 RING domain, characterize the aspects of the ATP molecule that are important for the interaction, and narrow down the region of Mdm2 where ATP binding occurs. We also show that, like Mdm2, MdmX is able to bind adenine nucleotides preferentially, suggesting a conserved functional role for ATP binding between the Mdm2 and MdmX proteins.

MATERIALS AND METHODS

Protein purification

Glutathione S-Transferase (GST) fusion human Mdm2 RING and MdmX C-terminal domain constructs were cloned unidirectionally into the pGEX4T1 vector. Point mutant constructs were created using these GST fusion constructs as a backbone using the Quickchange Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. Constructs were expressed in *Escherichia coli* BL21 cells. After induction at 25°C for 16 h with 0.1 M IPTG, soluble proteins were extracted by sonication in lysis buffer (50 mM Tris-HCl pH 7.0, 300 mM Li₂SO₄, 1% NP-40, 0.1% aprotinin, 1 mM DTT, 0.5 mM PMSF). The soluble protein fraction, isolated by ultracentrifugation for 1 h at 35 k r.p.m., was incubated with glutathione-Sepharose beads at 4°C for 1 h, washed extensively with wash buffer (50 mM Tris-HCl pH 7.0, 500 mM Li₂SO₄, 1 mM DTT), and eluted with reduced glutathione in elution buffer (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 1 mM DTT, 15 mM glutathione).

PK-Ubiquitin, a His-tagged ubiquitin protein that contains a Protein Kinase A site at the N-terminus, and His-UbcH5 were prepared as previously described (21).

ATP filter binding and competition assays

Indicated amounts of purified proteins were incubated with 5 μCi γ-³²P ATP (Perkin Elmer) and 300 pM unlabeled ATP in 50 μl binding buffer (0.2 mg/ml BSA, 0.5 mM DTT, 7 mM MgCl₂, 15 mM NaCl, 10 mM Tris-HCl, pH 7.0) or magnesium free buffer (20 mM Tris-HCl, 250 mM NaCl, 250 mM L-arginine, 0.5 mM TCEP, pH 7.0) with or without added magnesium (7 mM MgCl₂) for 10 min at room temperature. Reaction mixtures were passed through 0.45-μm pore membranes (Whatman) under vacuum and washed extensively with 25 mM HEPES buffer, pH 8.0. Filters were air-dried and radioactivity measured by liquid scintillation. Data were analyzed with Graphpad Prism software (version 4.0c). Curves were fit using a sigmoidal dose-response equation with variable slope $[Y = \text{Bottom} + (\text{Top}-\text{Bottom}) / (1 + 10^{((\log EC_{50}-X) * \text{Hillslope}))}]$. Error bars represent the standard deviation of two replicates.

For ATP binding competition assays, purified proteins (7 μg/reaction) were incubated with 5 μCi γ-³²P ATP and 300 pM unlabeled ATP in 45 μl binding buffer (0.2 mg/ml BSA, 0.5 mM DTT, 7 mM MgCl₂, 15 mM NaCl, 10 mM

Tris-HCl, pH 7.0) for 10 min at room temperature. Increasing concentrations of unlabeled competitor nucleotides were added and reaction mixtures were incubated an additional 10 min at room temperature. Reaction mixtures were processed as described above. Data were analyzed as described above and curves were fit with a one-site competition equation $[Y = \text{Bottom} + (\text{Top}-\text{Bottom}) / (1 + 10^{(X-\log EC_{50})})]$. ATP, GTP, AMP, Ribavirin, Nebularine, adenosine, adenine, 3' deoxyadenosine (Cordycepin), 2'deoxyATP and 2'deoxyadenosine were purchased from Sigma. Ara-A (Vidarabine), F-Ara-A (Fludarabine), 8-Cl-ATP, and 8-amino-ATP were kind gifts of Dr Varsha Ghandi.

Isothermal titration calorimetry

Isothermal calorimetry experiments were performed with a Micro Calorimetry System (Microcal Inc.). 2 mM ATP and GTP in assay buffer (100 mM Tris-HCl pH 7.0, 100 mM NaCl, 10 mM MgCl₂, 2 mM DTT) were injected into 100 nM GST-Mdm2(400–491). Twenty injections were performed. Reactions were normalized using buffer alone titration data. Titration data were analyzed using MicroCal Origin software, and the reported binding constant was derived from four independent measurements.

In vitro ubiquitination assay

[³²P]-labeled PK-ubiquitin was prepared by incubating 50 μg of purified PK-ubiquitin in 50 μl labeling buffer (20 mM Tris-HCl, 12 mM MgCl₂, 2 mM NaF, 50 mM NaCl, 25 μM ATP, 0.1 mg/ml BSA) with 20 μCi γ-³²P ATP and 500 ng purified PKA catalytic subunit β (Sigma) for 1 h at 37°C. The kinase was then heat inactivated for 5 min at 65°C. To perform *in vitro* ubiquitination reactions, 0.5–2 μg of purified GST-Mdm2 proteins or GST were incubated with 150 ng rabbit E1 (Boston Biochem), 50 ng E2 (His-UbcH5c), Phosphatase Inhibitor Cocktail (Calbiochem) and 2 μg [³²P]-labeled PK-ubiquitin in 30 μl of reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 0.5 mM DTT, 2 mM ATP, 0.1 mg/ml BSA) for 1 h at 37°C. Aliquots of reactions were resolved using 8% SDS-PAGE and analyzed by autoradiography.

RESULTS

Characterization of ATP binding by Mdm2 and MdmX

To begin our study of the Mdm2–nucleotide interaction, we first confirmed the binding and specificity of Mdm2 for ATP. To this end, we performed an *in vitro* competition assay measuring the fraction of γ-³²P ATP bound to GST-Mdm2(400–491) in the presence of increasing concentration of a nucleotide competitor. As all the proteins in this study are fused to GST at the N-terminus, hereto forth GST will be omitted. We assume that the affinity of the competitor nucleotide for Mdm2 is directly proportional to the extent of competition. Using this assay, we determined that Mdm2(400–491) binds adenine nucleotides preferentially with a dissociation constant (*K_d*)

in the low micromolar range (Figure 1B). Furthermore, when GTP was titrated into the binding reaction, we observed a competition of at least two orders of magnitude weaker (in excess of 200 μM) than that detected in the presence of ATP, confirming the ability of Mdm2 to discriminate between the purine bases. Additional confirmation of affinity and specificity was obtained from isothermal titration calorimetry (ITC) experiments. Titration of increasing amounts of ATP into purified Mdm2(400–491) provided a K_d of 4 μM , a value consistent with our competition experiment data (Figure 1C). ITC also confirmed both adenine base specificity, as GTP binding to Mdm2 was not detected in this assay, as well as a lack of hydrolysis that we have previously reported (Figure 1D) (32). These data also validate our competition assays as an accurate measurement of affinity.

As MdmX has a highly homologous RING domain and a P-loop motif, we also examined the ability of the MdmX RING domain to coordinate nucleotides. Using the competition assay described above, we found that MdmX binds ATP with markedly greater affinity than GTP (Figure 1E). Furthermore, both Mdm family members bind ATP with a K_d in the low micromolar range.

ATP binding is structure dependent and magnesium independent

Magnesium coordination is often a requirement for P-loop containing proteins to interact with a nucleotide (36,37). To establish the dependence on magnesium of the Mdm2–ATP complex, we tested ATP binding of Mdm2(400–484) in buffers varying in their magnesium composition. The buffers used in this assay, except for varying in their magnesium content, are identical to those used to obtain the NMR solution structure of the Mdm2 RING domain (10). Interestingly, we found that Mdm2 is able to bind ATP both in the presence and absence of magnesium (Figure 2A). The addition of magnesium to the binding buffer somewhat stimulated the Mdm2–ATP interaction; however, we also observed a robust magnesium-independent ATP binding. Thus, while it is likely that magnesium contributes to the most optimal binding conditions, the magnesium-independent binding of ATP by Mdm2 is notable, in light of the fact that many P-loop-containing proteins require magnesium (36,37).

We next tested the dependence of ATP binding on the structural integrity of the Mdm2 RING domain. After treatment of Mdm2(400–491) with three different denaturing conditions, the protein was no longer able to bind γ - ^{32}P ATP (Figure 2B). Performing a similar experiment with the RING domain of MdmX, we determined that, likewise, ATP binding by MdmX occurred in a structure-dependent manner (Figure 2C).

Mdm2-ATP interaction is specific and requires residues outside the P-loop

ATP is highly negatively charged and the RING domain of Mdm2 contains a cluster of basic amino acids composing the nucleolar localization signal (Figure 1A) (27). To rule out non-specific electrostatic interactions of

the phosphate groups of ATP with this region of the protein, we tested a mutant Mdm2 in which the eight basic residues of the nucleolar localization signal (466–473) (Mdm2 NoLS) have been mutated to alanine. Mutation of these residues neither decreased nucleotide binding nor affected the specificity (Figure 3A), suggesting that these residues are not involved in coordination of nucleotide and excluding the contribution of non-specific electrostatic interactions as a major component of the overall binding.

As the Walker A sequence is involved specifically in the coordination of the β - and γ -phosphates of the bound nucleotide (31) mutations of the conserved residues of the P-loop motif should decrease the affinity for ATP. We previously reported that a lysine to alanine (K454A) substitution in the P-loop causes a reduction of Mdm2 RING ATP binding (32). To expand on this observation we generated an additional mutation of lysine 454 to aspartic acid (K454D) and a similar substitution in the P-loop of MdmX (R453D). Human MdmX has a conservative substitution of arginine for lysine in the P-loop, however this is not the case for mouse MdmX. Consistent with previous data, Mdm2(K454A) showed an impairment in ATP binding. Both Mdm2(K454D) and MdmX(R453D) were defective in binding ATP, albeit to a lesser extent than the alanine substitution in the case of Mdm2 (Figure 3B and C). Also consistent with previous data is the fact that these mutant Mdm2 proteins plateau at a lower level of bound ATP when compared to wild-type protein. Because none of these P-loop mutants completely lost the ability to bind ATP, it is highly likely that other residues outside the P-loop region of the RING domain are also involved in nucleotide binding.

We confirmed that more than P-loop mediated phosphate binding is required for the full extent of the interaction with nucleotide by showing that Mdm2 bound AMP, although with reduced affinity. The apparent K_d for AMP was ~ 10 -fold greater than that of ATP, confirming the contribution of the P-loop to the overall binding. However, neither removal of the phosphates nor mutation of the P-loop residues could disrupt the binding completely (Figure 3B–D). This, together with the fact that Mdm2 very efficiently discriminates between adenine and guanine bases, a function independent of the P-loop, we conclude that other regions within the RING domain are involved in ATP binding.

Identification of the minimal region of Mdm2 required for ATP binding

Having established that residues outside the P-loop are involved in ATP binding by Mdm2, we set out to map the location of the ATP binding site within the Mdm2 RING domain. This proved challenging, as the structural integrity of the domain is dependent on eight widely spaced zinc-coordinating residues, and thus the structure of the RING will not tolerate significant deletions.

In a previous study, we established that deletion of the last 7 amino acids of Mdm2 [Mdm2(ΔC7)] had no effect on ATP binding, while completely disrupting RING oligomerization (21). To extend these results, we generated

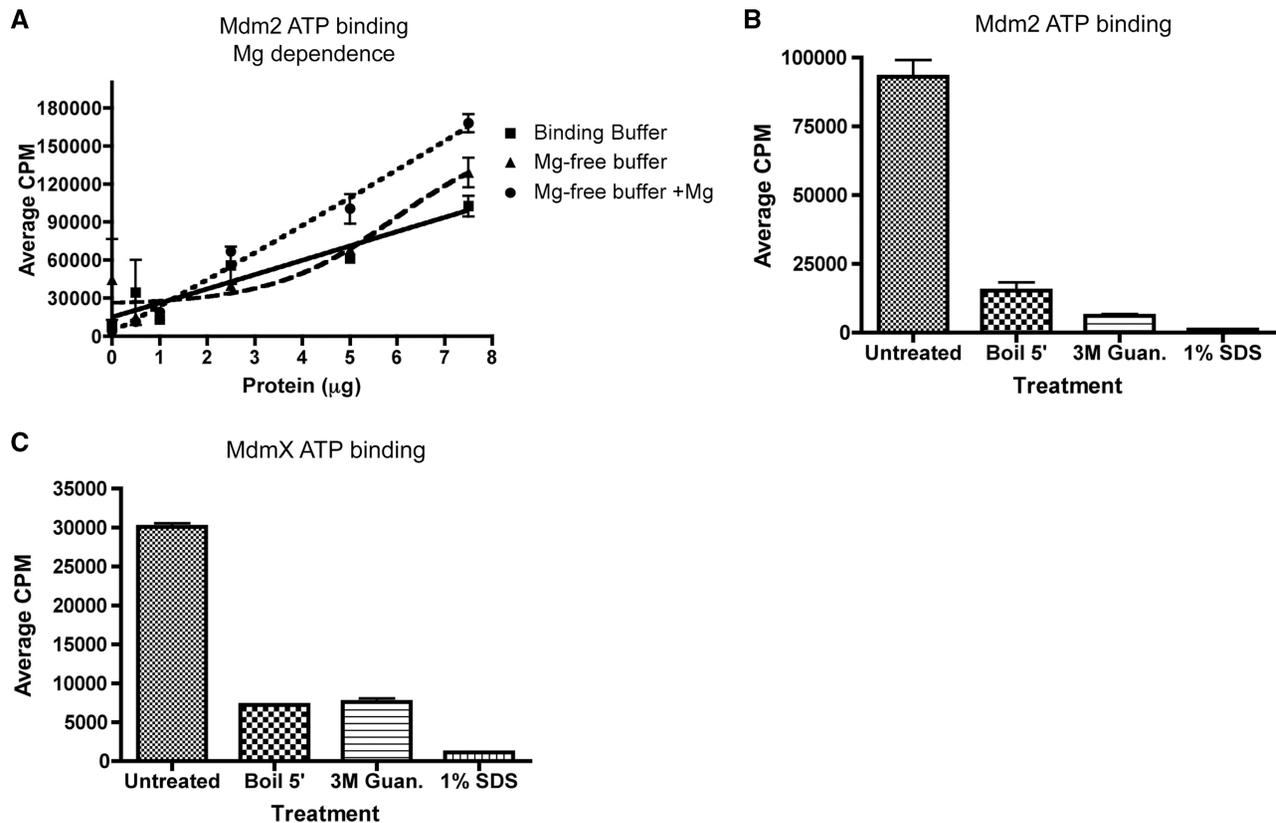


Figure 2. Binding of nucleotide by Mdm2 does not require magnesium but is structure-dependent. (A) Mdm2 binds ATP in the absence of magnesium. Increasing amounts of GST-Mdm2(400–484) protein were incubated with ATP in binding buffer (7mM MgCl_2 , 15mM NaCl, pH 7.5), magnesium-free buffer (20mM Tris-HCl, 250mM NaCl, 250mM L-arginine, 0.5mM TCEP, pH 7.0) or magnesium-free buffer with added magnesium (7mM MgCl_2). Complexes were filtered through nitrocellulose and counted by liquid scintillation. (B) Mdm2 fails to bind ATP following denaturation. GST-Mdm2(400–491) (5 μg) was incubated with ATP in binding buffer or in the same buffer supplemented with indicated denaturing treatments for 10min. Complexes were filtered through nitrocellulose and measured by liquid scintillation. (C) MdmX fails to bind ATP following denaturation. ATP binding experiments were performed as in (B) using GST-MdmX(403–490) (5 μg).

two N-terminally deleted GST-Mdm2 RING proteins Mdm2(415–491) and Mdm2(429–491). These proteins bound ATP to the same extent as Mdm2(400–491) in the *in vitro* competition assays (Figure 4A). We also tested the integrity of the RING by comparing the truncated proteins to Mdm2(400–491) in *in vitro* ubiquitin polymerization reactions. Because enzymatic activity of the truncated proteins did not differ from Mdm2(400–491), we expect that these proteins are properly folded (Supplementary Figure S1). Additionally, we determined that both Mdm2(429–491) and Mdm2(400–484) retained specificity for adenine, as evidenced by the fact that their affinity for GTP is 200-fold reduced compared to that of ATP (Figure 4B and C). From the above data, we conclude that ATP binding region falls within amino acids 429–484 of Mdm2. We have made a number of targeted point mutations within this region; however, all resulting proteins retained both nucleotide binding and specificity (Supplementary Figure S2). Due to the lack of difference in activity and specificity we used Mdm2(400–484) in all subsequent binding experiments in this study, although some of the experiments were also reproduced with Mdm2(400–491) (Supplementary Figure S3).

Removal of the C6 amino group of the adenine base prevents binding to Mdm2 but modification of C8 is tolerated

To further characterize Mdm2–nucleotide binding we determined features of the ATP molecule that are either required or dispensable for the interaction. In order to do this, and to potentially elucidate the environment of the ATP-binding pocket of Mdm2, we used a number of adenosine analogs. We tested their ability to displace bound γ - ^{32}P ATP from Mdm2(400–484) in the *in vitro* competition assays as a measurement of affinity. ATP analogs modified at the C8 position of the adenine base with either a chlorine or an amine group bound with the same affinity as unmodified ATP to Mdm2(400–484) (Figure 5A and B). Based on these data, we concluded this region of adenine is unlikely to be making contacts with the RING.

Trying to address the rather remarkable difference in binding between adenine and guanine base nucleotides, we also looked at the ability of two other base-modified ATP analogs to bind to Mdm2. We first examined Ribavirin, a molecule used as an antiviral drug in humans, which has a base consisting of a single five-member ring attached to an amide group (38).

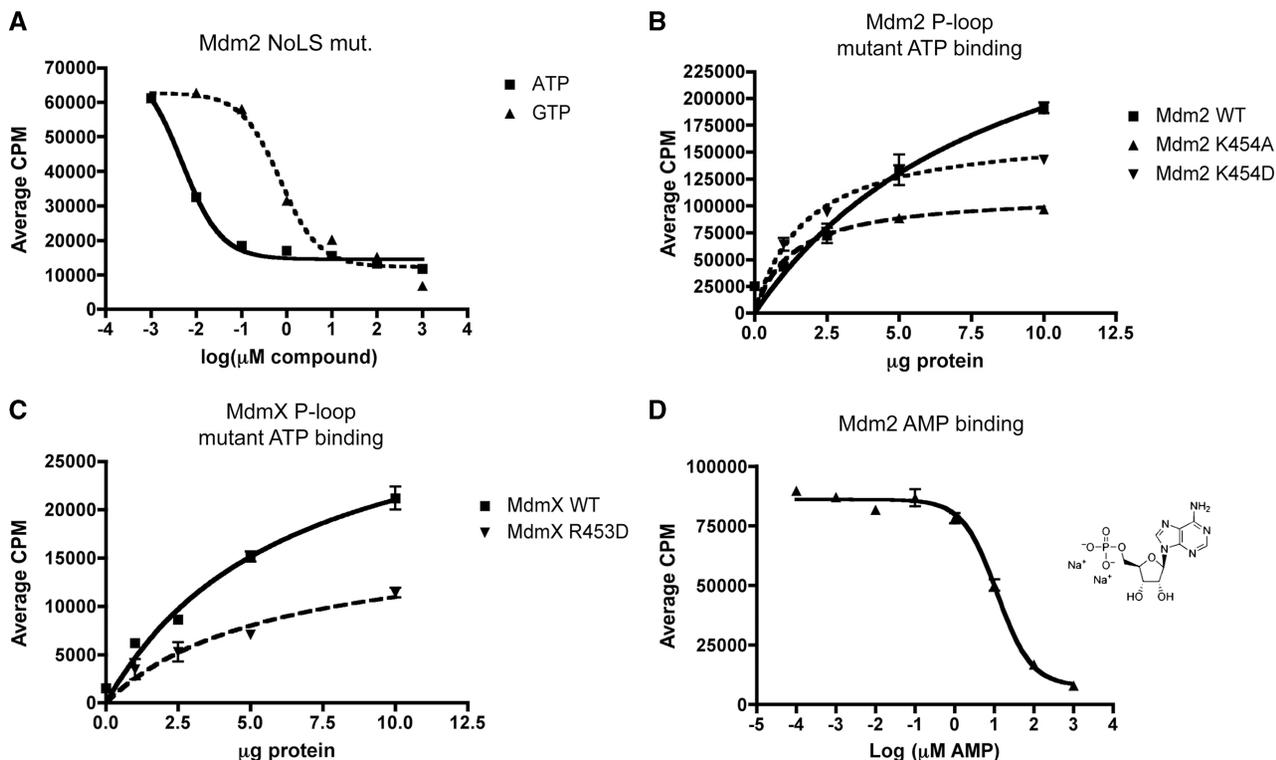


Figure 3. ATP interaction is specific and requires residues outside the P-loop of Mdm2. (A) Multiple lysine substitution mutation does not effect ATP binding. Competition assay was performed with indicated nucleotides using an Mdm2(410–491) protein that has eight lysines mutated to alanine (8KA, Mdm2-NoLS). Mdm2-NoLS bound ATP with a K_d in the low micromolar range, and showed specificity for ATP over GTP. (B) Mutation of P-loop lysine 454 disrupts Mdm2–ATP binding. Increasing amounts of wild-type GST-Mdm2(400–491) and mutant (K454A and K454D) proteins were incubated with ATP in binding buffer. ATP binding was detected as in (2A). (C) P-loop mutation decreases ATP binding of MdmX. Increasing amounts of wild-type GST-MdmX(403–490) and mutant (R453D) proteins were incubated with ATP in binding buffer. ATP binding was detected as in (2A). (D) Removal of β - and γ -phosphates of ATP reduces binding. Pre-formed Mdm2(400–484)–ATP complex was incubated with increasing amounts of AMP as competitor. Competition assay was performed as in (1A).

Testing the affinity of this compound for Mdm2 allowed us to determine the relative involvement of the two ring portions of the adenine base, since Ribavirin lacks the second, six-member ring of adenine. Ribavirin had a K_d \sim 20-fold greater than adenosine (Figure 5C), indicating a requirement for the missing portion of the adenine base. We also tested Nebularine, a toxic nucleoside initially isolated from fungi, which contains an adenine-like base that has both ring structures but lacks the C6 amine group (39). This compound showed no binding to Mdm2 (400–484) in the concentration range up to 1 mM, suggesting that the C6 amino group is critical for interaction with the Mdm2 RING (Figure 5D). Ribavirin may bind with higher affinity than Nebularine because the amide group of Ribavirin may substitute for the absent C6 amino group. The requirement for the C6 amino group for binding to Mdm2 is both consistent with and lends further support to our data demonstrating that the Mdm2 RING binds adenine nucleotides specifically.

The Mdm2 RING domain requires 2' and 3' ribose hydroxyls for binding

Having addressed the features of the nucleotide base required for interaction with Mdm2, we next wanted

to interrogate the contribution of the ribose to the overall binding. To this end, we examined several ATP analogs that contain a modified ribose in the competition assay.

Adenosine, an analog of ATP lacking all phosphate groups but which retains the ribose and the adenine base bound Mdm2 with affinity approximately a factor of 10 less than ATP (Figure 6A). However, removal of the ribose (leaving only the adenine base) decreased binding to the Mdm2 RING by \sim 1000-fold compared to ATP (Figure 6B). Thus, the ribose part of the nucleotide is essential for ATP–Mdm2 binding.

Exploring the contribution of the sugar hydroxyls, we found that 3' deoxyadenosine bound Mdm2 very poorly (Figure 6C) (this compound did not completely compete off bound ATP within the concentrations used in this assay so a K_d can not be accurately determined). 2' deoxyATP bound the Mdm2 RING 100-fold less well than ATP and further removal of the phosphate groups (2' deoxyadenosine) completely abrogated the binding (Figure 6D and E). These data suggest that the ribose portion of the nucleotide, specifically the 2' and 3' hydroxyl groups, are the primary energetic contributors to the interaction with Mdm2.

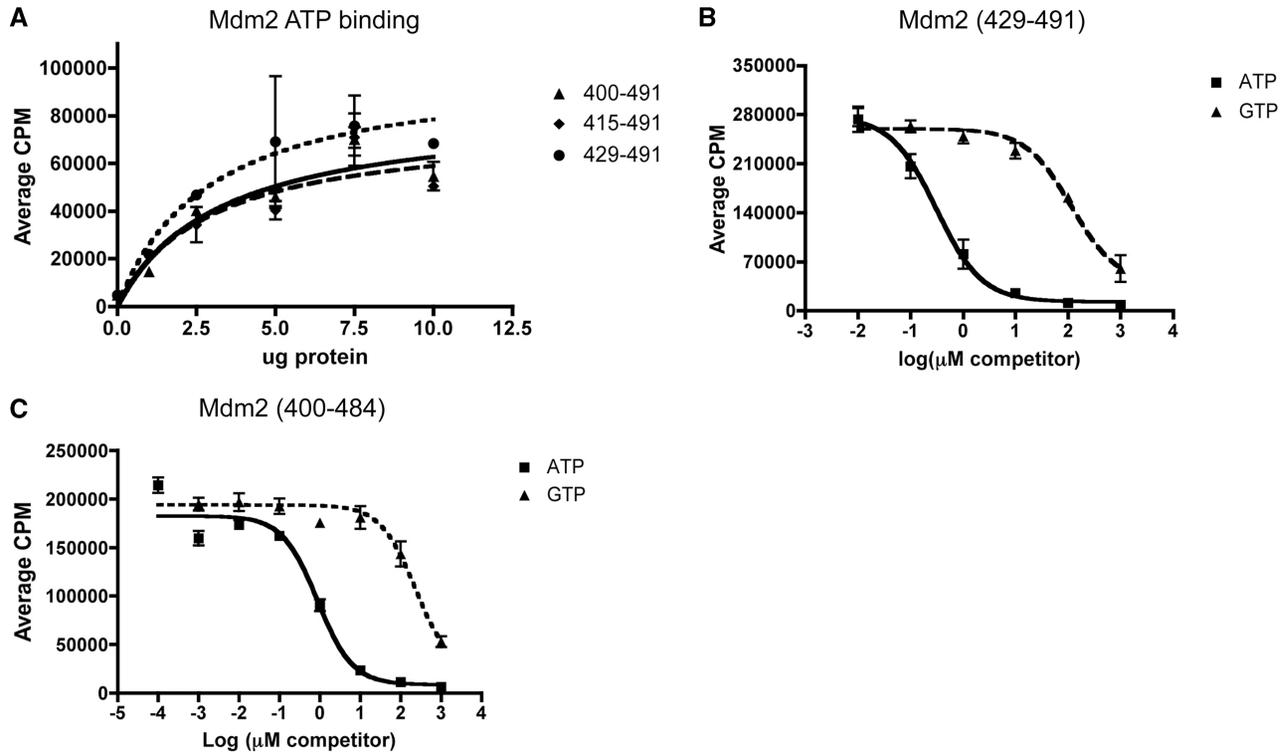


Figure 4. Shorter constructs of Mdm2 retain the ability to bind ATP. (A) N-terminally deleted Mdm2 constructs (415–491 or 429–491) bind ATP similarly to Mdm2(400–491). Increasing amounts of GST-tagged Mdm2 proteins (as indicated) were incubated with ATP. Mdm2–ATP complexes were measured as in (2A). (B and C) Shorter Mdm2 RING domain constructs retain specificity for ATP. (B) GST-Mdm2(429–491) and (C) GST-Mdm2(400–484) were subjected to competition experiments with ATP and GTP as competitor nucleotides. Experiments were performed as in (1A).

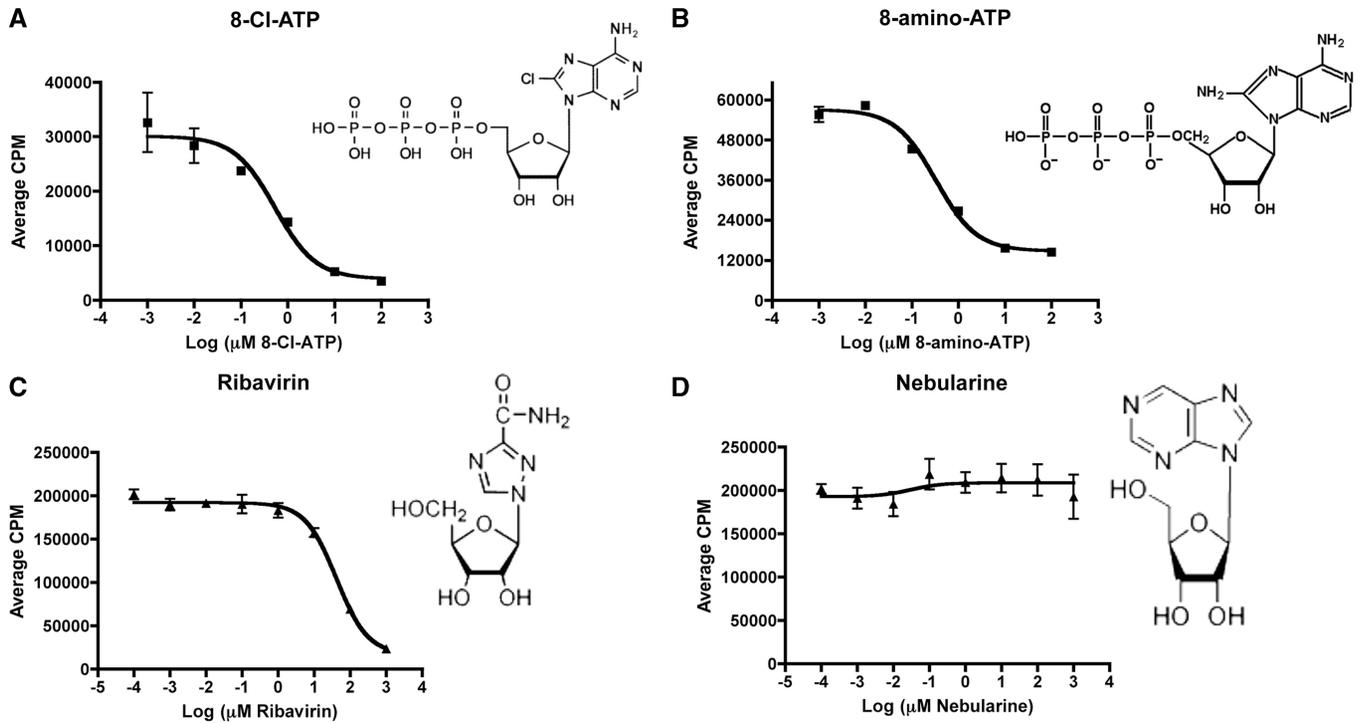


Figure 5. Mdm2 requires the adenine base for binding to ATP. (A–D) GST-Mdm2(400–484)–ATP complexes were subjected to competition by base modified adenine nucleotide analogs [(A) 8-Cl-ATP, (B) 8-amino ATP, (C) Ribavirin, (D) Nebularine]. Competition assays were performed as in (1A). Structures of competitor nucleotides are shown next to their respective binding curves.

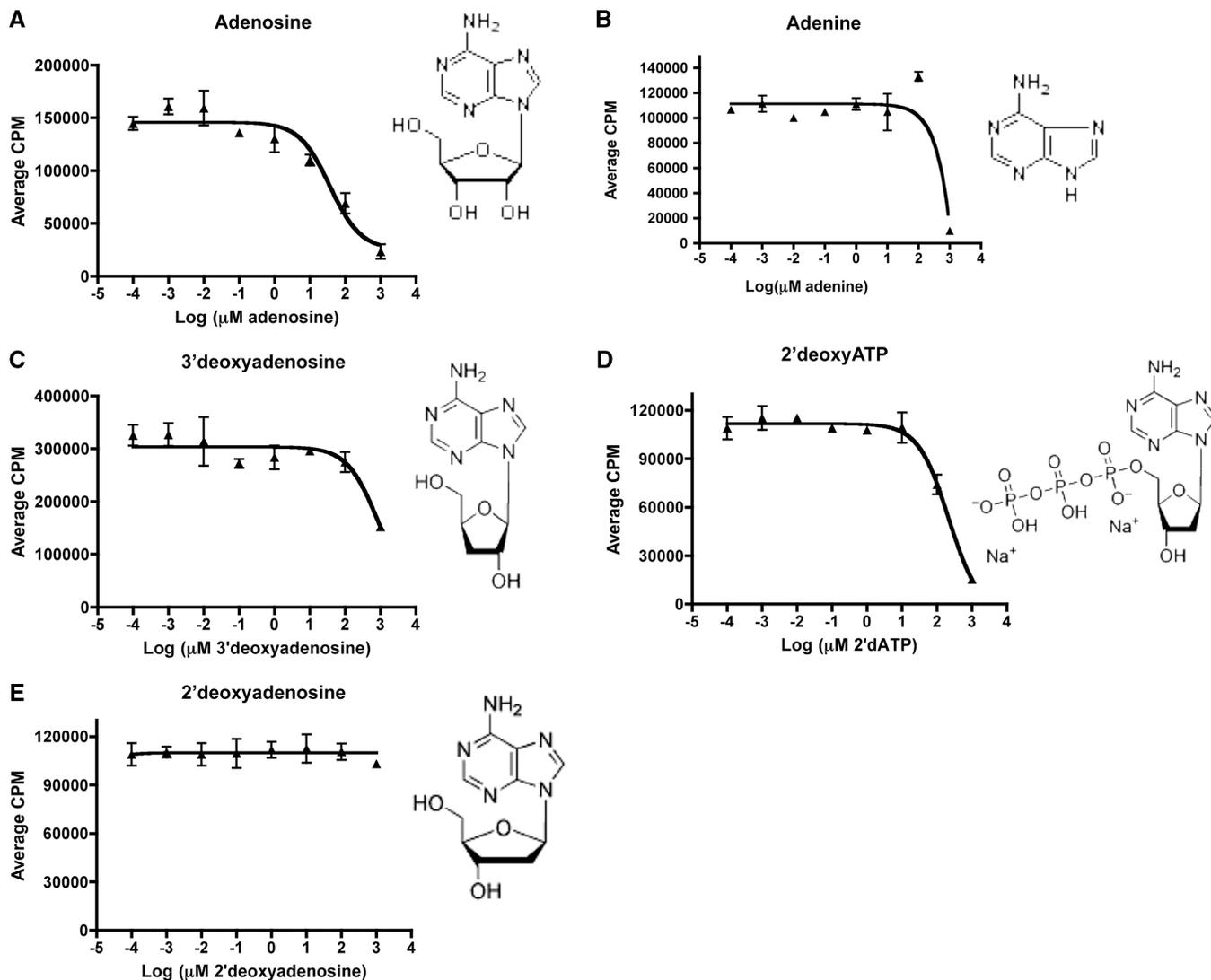


Figure 6. Mdm2 RING domain requires 2' and 3' ribose hydroxyls for binding. (A–E) GST-Mdm2(400–484)–ATP complexes were subjected to competition by sugar-modified adenine nucleotide analogs [(A) Adenosine, (B) Adenine, (C) 3'deoxyadenosine, (D) 2' deoxyATP, (E) 2' deoxyadenosine]. Competition assays were performed as in (1A). Structures of competitor nucleotides are shown next to their respective binding curves.

Mdm2 ATP binding requires *cis*-orientation of the ribose hydroxyls

Based on the fact that ribose appeared to be essential for binding to Mdm2, we tested the binding of an arabinose (an identical monosaccharide to ribose except for a *trans*-orientation of the 2' and 3' hydroxyl groups) containing adenosine analog, Ara-A. This compound, also known as Vidarabine, is used in humans as an anti-viral drug (40–42). Ara-A bound to Mdm2(400–484) with significantly lower affinity than adenosine (Figure 7A). The K_d for this compound could not be accurately calculated due to incomplete competition of ATP within the concentration range used in the experiment. We also tested the binding of an analog of Ara-A in which the C2 position is modified with a fluorine atom. This compound, F-Ara-A or Fludarabine, is used as a chemotherapeutic agent against hematologic

malignancies and functions by interfering with ribonucleotide reductase and DNA polymerase (43–45). Similarly to Ara-A, F-Ara-A did not bind to the Mdm2 RING within the concentrations tested in our assay (Figure 7B). These data suggest that Mdm2–ATP interaction requires the *cis*-orientation of hydroxyl groups found in ribose. We also observed that *cis*-orientation of the sugar hydroxyls combined with modification of the adenosine base is completely prohibitive of binding to Mdm2 (Figure 7B).

Using the binding data from our competition studies with nucleotide analogues, we have constructed a map of the features of the ATP molecule that are required for interaction with Mdm2 (Figure 8). This map identifies both molecular moieties that are required for interaction with Mdm2 as well as modifications that interfere with binding.

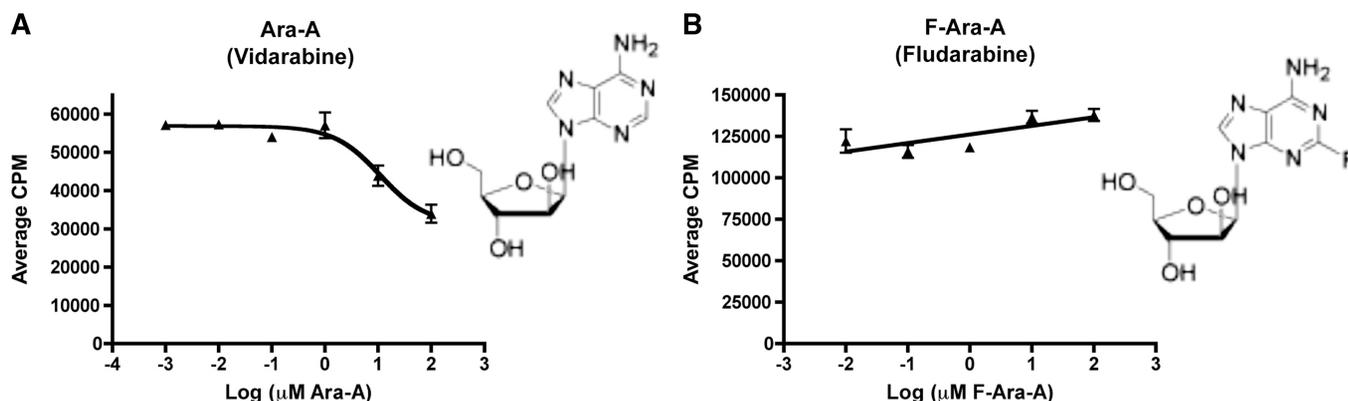


Figure 7. Mdm2 requires *cis*-orientation of 2' and 3' sugar hydroxyl for nucleotide binding. (A and B) GST-Mdm2(400–484)–ATP complexes were subjected to competition by arabinose-containing ATP analogs. [(A) Ara-A, (B) F-Ara-A]. Competition assays were performed as in (1A). Structures of competitor nucleotides are shown next to their respective binding curves.

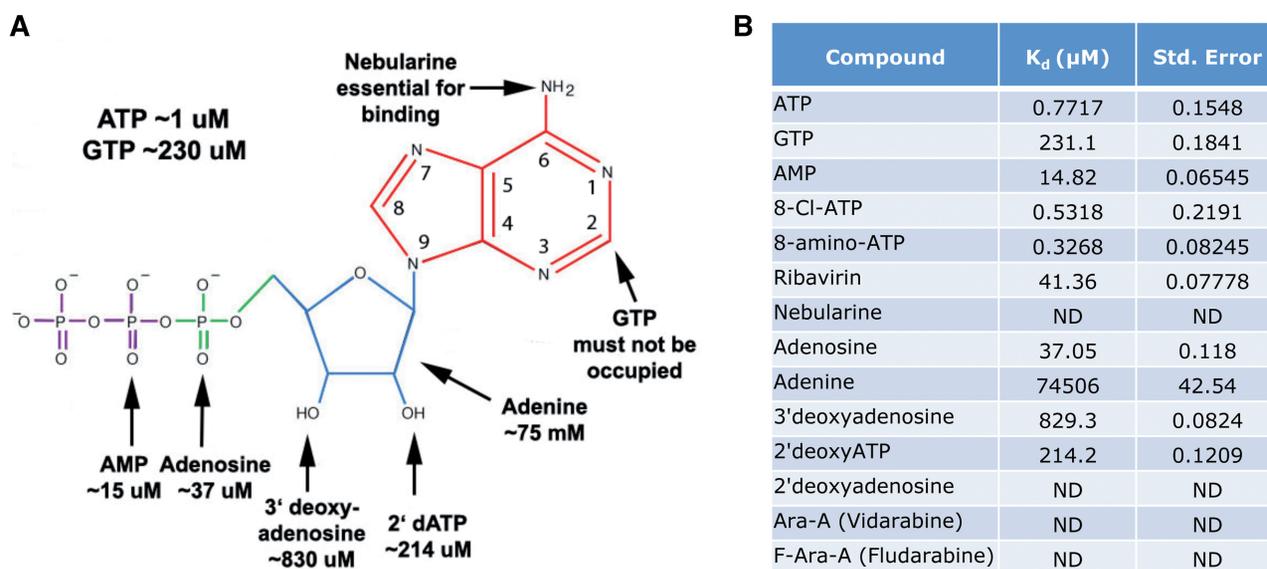


Figure 8. Features of ATP required for binding to Mdm2 RING domain. (A) Structure of ATP summarizing the relative requirements of its structural features for binding to the Mdm2 RING domain. These requirements are represented by dissociation constants (K_d) of adenine nucleotide analogs calculated from binding experiments shown above. Atoms of the adenine base are numbered for reference. (B) Table of K_d values for nucleotide analogs binding to the Mdm2 RING domain calculated from binding experiments above. ND (not determined) indicates that a K_d was not calculable from the measurements obtained from the competition assay. Standard error is indicated.

DISCUSSION

Overall, our data describe the unique characteristics of nucleotide binding by Mdm2 and MdmX. We have confirmed that the Mdm2 RING domain binds to adenine nucleotides specifically. We showed that Mdm2 and MdmX each contain a functional P-loop motif both by mutagenesis of the P-loop lysine and via binding studies with nucleotide analogues. Through the use of deletion mutagenesis, we found that the ATP binding region lies between residues 429–484 within the zinc-coordinating region of the Mdm2 RING domain.

Our inability to identify residues outside the P-loop involved in nucleotide binding could stem from the fact that ATP interaction may involve the peptide backbone of Mdm2. Several instances of at least a portion of the ATP

binding pocket involving the peptide backbone have been described (46,47). Backbone interactions may be uniquely required or might function in concert with a number of R-groups in ATP coordination by Mdm2. Once identified, such interactions could increase specificity of designed inhibitors of ATP binding (48).

While the phosphate groups of ATP contribute to binding, the removal of the β - and γ -phosphates only reduced affinity by approximately a factor of 10. It has been previously established that removal of the γ phosphate of ATP has little effect on Mdm2 binding, as ADP binds with similar affinity as ATP to the Mdm2 RING (32). This fact as well as the fact that the addition of magnesium to the binding buffer only modestly increases ATP binding by Mdm2 supports a

limited role for the P-loop residues in ATP binding. Our study established that the largest contributors to the binding are both of the ribose hydroxyls as well as the amine group on the nucleotide base. Based on these data, we conclude that residues outside of the Mdm2 P-loop are involved in the interaction with nucleotide. Furthermore, the decrease in affinity following the removal of the C6 amine group is consistent with Mdm2 binding adenine nucleotides specifically, showing a requirement for adenine-specific aspects of ATP and an intolerance for modifications of the ATP molecule that make it more similar to GTP. Consistently, we observed that the C8, but not the C2 carbon of the adenine base might be modified without disruption of the binding.

We identified a striking requirement for *cis*-orientation of the ribose hydroxyls for full Mdm2–nucleotide binding. Adenosine and deoxyadenosine analogs that contain arabinose are well established as inhibitors of DNA synthesis and repair enzymes and have been used as chemotherapeutic agents (49–51). Although these analogs have been shown to bind several ATP-dependent enzymes, they will not bind efficiently to Mdm2 or MdmX, presumably because of a more specific binding pocket. Such specificity could aid in identification of the residues involved as well as aid in design of potential inhibitors.

As mentioned above, Mdm2 shows a special specificity for nucleotides, including specificity for aspects of the sugar and base moieties of ATP that many other nucleotide-binding proteins lack. For example, many P-loop containing proteins are well-characterized GTPases and can bind both ATP and GTP (31). Extending this study will hopefully provide additional insight into the ATP-regulated functions of the Mdm2 RING domain, which may be diverse and important (29,35).

In our experiments, Mdm2 homolog MdmX also bound ATP specifically and with the same affinity as Mdm2 and mutation of the P-loop of MdmX disrupted but did not abrogate ATP binding, suggesting a binding site that is very similar to that contained within the Mdm2 RING. Some but not all functions of Mdm2 are shared by MdmX; our data indicate that ATP binding and its subsequent effects on MdmX function may be related to that of Mdm2.

Several studies have focused on the functional implications of the Mdm2–ATP interaction. Our original study of ATP binding revealed that ATP-bound Mdm2 is preferentially localized to the nucleolus (32). Nucleolar localization of Mdm2 is one of the mechanisms by which Mdm2 function is downregulated following multiple forms of DNA damage. This is particularly interesting in light of emerging evidence for the role of Mdm2 in monitoring ribosomal biogenesis. Mdm2 binds several ribosomal proteins, including RPL5, RPL11, RPL23 and RPS7. Binding to these proteins inhibits the ubiquitin ligase function of Mdm2 and promotes stabilization and activation of p53. This provides a mechanism for signaling to p53 after disruption of the nucleolus or protein synthesis (52,53). Perhaps ATP-bound Mdm2 is more likely to interact with the above-mentioned subset of ribosomal

proteins than the unbound form, establishing an additional level of regulation. What is further intriguing is that while ATP binding is conserved between Mdm2 and MdmX, the latter lacks an NoLS signal and thus it is possible that ATP binding of MdmX could lead to different changes in localization and have additional effects on the activity of MdmX.

Mdm2 also possesses ATP-dependent chaperone activity and can substitute for Hsp-90 in promoting sequence specific binding of p53 at 37°C. In a manner similar to other chaperones, Mdm2 binds partially unfolded p53 and, upon ATP-binding, releases p53, which then assumes the most energetically favorable conformation (34). In this light, our data may suggest that the features of ATP that do not play a direct role in binding to Mdm2 may actually be essential for the dissociation and subsequent proper folding of p53 and for regulation of transcriptional repression of p53. The ability of MdmX to bind ATP is also interesting here, as one would predict that both family members would have similar chaperone activity toward p53.

Additionally, ATP binding has been implicated in allosteric regulation of the Mdm2 protein (29,34). In this model, binding of different ligands (such as ATP or its variants) to the RING domain of Mdm2 induces long-range conformational changes in the N-terminal hydrophobic pocket region of Mdm2, leading to alterations in binding to p53 and other N-terminal interactors. If independent structural domains of Mdm2 and, presumably, MdmX are indeed allosterically connected, it would seem likely that the features of the nucleotide bound to the RING domain could modulate the range and amplitude of the induced structural changes.

We have previously shown that the ability of Mdm2 to bind ATP and its ability to ubiquitinate targets are separable biochemical activities (32). Mdm2(Δ C7) is able to bind nucleotide to the same extent as wild-type protein but is not a competent E3 ligase as it is unable to oligomerize (21,22). Conversely, Mdm2 K454A is a good E3 ligase but a poor ATP binder. The notion that ATP binding is a function unrelated to Mdm2 E3 activity is further supported by our data that MdmX, which is not a ubiquitin ligase, is also able to bind ATP (13). As more evidence within the Mdm2 field accumulates to show that Mdm2 and MdmX work as a complex to regulate their major target, p53, it will be interesting to see how their shared ability to bind nucleotide contributes to this function. As binding to ATP causes a conformational change in the Mdm2 RING domain (32), and the Mdm2–X complex is formed by the interaction of these domains (21–23), it is possible that ATP binding is involved in the modulation of the formation of this complex and the regulation of its activity.

Disruption of Mdm2 activity is one of several methods currently being pursued as a mechanism of reactivation of the tumor suppressor functions of p53 in malignancies (16,54). The existence of a unique ATP binding pocket in Mdm2 could provide a rare opportunity to design small compounds that interact with and inhibit Mdm2 with high level of specificity *in vivo*. Such compounds

may have therapeutic value and serve as invaluable investigative tool in the laboratory setting.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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