MDMX contains an autoinhibitory sequence element

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MDM2 and MDMX are homologous proteins that bind to p53 and regulate its activity. Both contain three folded domains and ~70% intrinsically disordered regions. Previous detailed structural and biophysical studies have concentrated on the isolated folded domains. The N-terminal domains of both exhibit high affinity for the disordered N-terminal of p53 (p53TAD) and inhibit its transactivation function. Here, we have studied full-length MDMX and found a ~100-fold weaker affinity for p53TAD than does its isolated N-terminal domain. We found from NMR spectroscopy and binding studies that MDMX (but not MDM2) contains a conserved, disordered self-inhibitory element that competes intramolecularly for binding with p53TAD. This motif, which we call the WWW element, is centered around residues Trp200 and Trp201. Deletion or mutation of the element increased binding affinity of MDMX to that of the isolated N-terminal domain level. The self-inhibition of MDMX implies a regulatory, allosteric mechanism of its activity. MDMX rests in a latent state in which its binding activity with p53TAD is masked by autoinhibition. Activation of MDMX would require binding to a regulatory protein. The inhibitory function of the WWW element may explain the oncogenic effects of an alternative splicing variant of MDMX that does not contain the WWW element and is found in some aggressive cancers.

Results

MDMX Reversibly Dimerizes. MDMX was expressed in Escherichia coli and purified to homogeneity using standard methods (12, 21). We found by size-exclusion chromatography (SEC) that the protein eluted at volumes typical for ~400-kDa globular proteins, much earlier than expected from a monomeric 55-kDa molecule (Fig. L4). The elution volume was concentration dependent, suggesting that the protein was undergoing a reversible oligomerization. Multitlight scattering (MALS) analysis yielded molecular weight of the eluted protein fraction to be ~100–110 kDa, in agreement with a dimer (110 kDa)–monomer (55 kDa) equilibrium. We used analytical ultracentrifugation to find the dimerization Kd to be 1.12 ± 0.18 μM (Fig. S2). C-terminally truncated mutants of MDMX were exclusively monomeric, showing that the RING domain was essential for dimerization.

MDMXbinds to p53. We mixed MDMX with tetrameric p53 and subjected the complexes to SEC-MALS analysis. Their sizes were dependent on the stoichiometry, reaching maximal values of ~400 kDa for equimolar mixtures, which corresponded to octamers built of four MDMX and four p53 subunits (Fig. L4). Interestingly, even though interaction of flexible, multivalent receptor–ligand pairs (like MDMX and p53) could potentially lead to formation of high–molecular-weight polymers (22), we found a size cap of 400 kDa that was preserved even at high ratios of MDMX to p53 and vice versa.

MDMX Stimulates Ubiquitination. SEC-MALS analysis confirmed that the recombinant MDMX contained functional NTD (responsible for p53 binding) and C-terminal RING domain capable of homodimerization. To confirm the biochemical activity of recombinant proteins, we performed an in vitro ubiquitination assay. MDMX did not exhibit intrinsic E3 ligase activity, and MDM2 was only weakly active. Mixing the two recombinant proteins yielded a highly active complex capable of performing

Significance

The protein MDMX is an important negative regulator of the tumor suppressor p53 in normal cells and can become a powerful oncogene. Previous studies on isolated protein fragments established that the N-terminal domain of MDMX binds tightly to the N-terminal domain of p53 and inhibits it. We now find that full-length MDMX contains a regulatory element (the “WWW element”) that binds to its own N-terminal domain and prevents MDMX from binding to p53. This autoinhibition introduces a new level of regulation of MDMX, whereby accessory proteins may sequester the WWW sequence and reactivate MDMX, and may explain why a variant of MDMX that lacks the WWW sequence, which is found in some cancer cells, might be oncogenic.

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polyubiquitination (Fig. 1B). The results of the in vitro assay performed with recombinant proteins were in good agreement with the previously reported activities of endogenous proteins in cellular context (6); we found that MDMX stimulated both autoubiquitination of MDM2–MDMX complex and p53 ubiquitination (Fig. S3) (23).

NMR Spectra of MDMX. We labeled the full-length MDMX with $^{15}$N and recorded a transverse relaxation-optimized spectroscopy (TROSY) spectrum. The spectrum shown in Fig. 2A was dominated by signals from highly flexible, intrinsically disordered linkers. Such appearance, with majority of resonances occupying the central part of the spectrum is typical of intrinsically disordered proteins. In addition to these signals from disordered linkers, we could also identify a range of broader and more disperse resonances originating from the folded NTD and RanBP2 domains. The absence of RING signals from the spectrum was anticipated because in multidomain proteins with intrinsically disordered linkers, central domains are frequently more constrained rotationally than structures located distally (24, 25).

There were unexpected significant differences between the spectra of the NTD in full-length protein and the isolated domains, implying that MDMX–NTD interacts with another part of the protein (Fig. 2A). Addition of the p53TAD peptide to MDMX gave an NMR spectrum that corresponded to that from the isolated p53TAD–NTD complex (Fig. 2B). Surprisingly, besides anticipated chemical shift perturbations in the canonical p53-binding pocket within the NTD, several sharp resonances characteristic for unstructured linkers appeared on addition of p53TAD to MDMX. Particularly noticeable were three downfield shifted signals that appeared at frequencies typical for HN groups of Trp side chains.

The TROSY spectrum of a $^{15}$N-MDMX–$^{14}$N-p53 complex (Fig. 2C) was also dominated by signals of flexible linkers, although few residual signals of RanBP2 domain (but not NTD) were also identifiable. Notably, the detectable resonances aligned well with the MDMX–p53TAD complex, and similar patterns were observed in the random coil and Trp regions of the spectrum. The absence of NTD signals is most likely explained by exchange between binding of TAD (p53 19–26) and a further transactivation fragment (p53 49–54) (26) and possibly also rotational restriction of the domain in the complex.

**NTD Interacts Intramolecularly with a Conserved “WWW Element.”** The spectral changes in the NTD and flexible linkers suggested that the p53TAD displaces an intramolecular interaction of the NTD with a segment of MDMX, releasing Trp residues. Basing on observing that three tryptophans were involved and primary sequence analysis, we hypothesized that the interaction might involve a tryptophan-rich segment within residues 190–210 (190FEEDVAGLPPWFLGNLRSNY210, which we call the WWW element). Notably, the element is specific to MDMX, as the corresponding region of MDM2 contains an unrelated sequence (Fig. 3A).

To verify binding of the segment experimentally, we $^{15}$N-labeled the WWW-containing peptide (181–209) and titrated it with the isolated NTD. The NTD–WWW interaction was strong enough to occur between isolated domains that were not covalently linked. Numerous resonances of the WWW were broadened on binding. We assigned the heteronuclear single-quantum coherence (HSQC) spectrum of the WWW construct and mapped the interaction site to amino acids 194–206 (Fig. 3B and C). Interestingly, the WWW element remained in the intermediate timescale of chemical exchange in excess of MDMX–NTD. This suggested that, even in the bound state, the WWW was not stabilized in a single conformation. The unbound, isolated WWW seemed to lack stable structure, as its backbone chemical shifts were close to the random coil values, $^{1}H$–$^{15}$N heteronuclear nuclear Overhauser effect (NOE) enhancements were low and nuclear Overhauser effect spectroscopy (NOESY) spectrum revealed little interresidual contacts (Fig. S4). Notably, we observed that p53TAD and to a lesser extent also nutlin-3 were capable of dissociating the preformed NTD–WWW complex (Fig. S5 A–E).

Addition of $^{14}$N-WWW to $^{15}$N-NTD (Fig. 4A) caused numerous NTD cross-peaks to become broadened or perturbed. The chemical shift perturbations in the NTD were analogous to those induced by p53TAD; using the NTD backbone assignment, we confirmed that the WWW and p53 TAD binding sites coincided (Fig. 4C). The resulting spectrum of the NTD–WWW complex reproduced that of full-length MDMX (Fig. 4B).

The TROSY spectrum of a monomeric MDMX variant (1–340) matched both the spectrum of the NTD–WWW complex and that of full-length MDMX, suggesting that the NTD domain in MDMX interacts with the WWW element in its own chain (Fig. S5F).
WWW Element Inhibits p53 Binding. We used isothermal titration calorimetry to measure the thermodynamics of MDMX–p53 interactions and the contribution of the WWW element (Table 1). p53TAD was tightly bound by isolated NTD (K_d = 30 nM). In contrast, MDMX bound p53TAD 100-fold less tightly (K_d = 2900 nM). Titrations with truncated MDMX variants (Table 1, with p53TAD) revealed that as long as the WWW element was present, p53 binding was inhibited. The K_d of NTD and WWW was 1,300 nM.

The K_d of MDMX with full-length p53 (1,110 nM) was similar to that with p53TAD (2,870 nM), implying that association of the NTDs is the main energetic component of binding.

Deletion or Mutation of the WWW Element Enhances Binding. We further corroborated the WWW–NTD interaction in the full-length protein, by introducing mutations in WWW aiming to disrupt the WWW–NTD interaction. As a key residue for p53–NTD interaction is tryptophan (9, 10) and the NMR spectrum of the NTD–WWW complex resembles that of the NTD–p53TAD complex, we focused on mutating tryptophans that were implied by NMR to be directly involved in the interaction (W200 and W201). Although mutation of a single Trp to Ala did not increase the p53 affinity markedly (Table 1), double mutation to Asp had more pronounced effect and complete removal of the WWW element resulted in MDMX binding p53 almost as tightly as the isolated NTD (90 nM).

Fig. 2. Overlays of 15N-TROSY spectra of MDMX. (A) 15N-MDMX (red) and isolated 15N-NTD (16–116; gray). (B) 15N-MDMX–14N-p53TAD (17–32) complex (blue) and 15N-NTD (16–116)–14N-p53TAD (17–32) complex (yellow). (C) 15N-MDMX–14N-p53 complex (green). Insets show fragments overlaid with spectrum (A).

WWW Element interacts with NTD. We used isothermal titration calorimetry to measure the thermodynamics of MDMX–p53 interactions and the contribution of the WWW element (Table 1). p53TAD was tightly bound by isolated NTD (K_d = 30 nM). In contrast, MDMX bound p53TAD 100-fold less tightly (K_d = 2900 nM). Titrations with truncated MDMX variants (Table 1, with p53TAD) revealed that as long as the WWW element was present,
MDMX-S Splicing Variant. MDMX is regulated at the posttranscriptional level by alternative splicing. One of the main splicing forms, MDMX-S, is a product of exon 6 skipping, producing a 127-aa protein sequence containing the NTD and 17 additional amino acids (27). Recombinant MDMX-S binds p53 with high affinity similar to the isolated NTD and by two orders of magnitude stronger than full-length MDMX. Owing to their large difference in p53 binding affinities, even low levels of MDMX-S relative to MDMX may thus have a significant effect on p53 activity.

Discussion

We noted that the NMR spectrum of the N-terminal domain of MDMX resembled that of the isolated domain when bound to a peptide rather than the unliganded domain. Furthermore, we noted that three tryptophan side chains in a disordered region of the protein had perturbed signals. We surmised that an internal sequence of the protein was binding to its N-terminal domain. We located the relevant sequence element by deletion and point mutation analysis, and showed that the isolated sequence element bound tightly to MDMX-NTD. Furthermore, we found that MDMX lacking the sequence element bound 32-fold more tightly to p53.

Consequently, there is an important sequence element in the central region of MDMX (but not MDM2), the existence of which implies a mechanism for its regulation. Residues 190–210 in that intrinsically disordered region constitute an inhibitory module, which we term the WWW element. It competes with the p53TAD for binding to the NTD of MDMX, thus lowering the affinity of MDMX for p53. Efficient binding of MDMX to p53 requires a mechanism to relieve the allosteric self-inhibition.

The WWW element does not contain known posttranslational modifications sites, implying that activation of MDMX for p53 binding must be achieved by another mechanism. There is some evidence for two processes: alternative splicing; or by accessory proteins that can bind the WWW element (Fig. 5).

Alternative splicing is a very common regulatory mechanism in proteins containing inhibitory modules (28), and indeed, a splicing variant MDMX-S, containing only the NTD is overexpressed in some cancers and strongly associated with a negative outcome (29, 30). The affinity of MDMX-S toward p53 was two orders of magnitude higher than for the standard variant, which may be a possible explanation of the high oncogenic potential of the short protein.

Table 1. ITC results

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<tr>
<th>Protein</th>
<th>( K_{D}, \text{nM} )</th>
<th>( \Delta H, \text{kcal/mol} )</th>
<th>( \Delta S, \text{cal mol}^{-1} \text{K}^{-1} )</th>
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<td>With p53TAD*</td>
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<tr>
<td>MDMX</td>
<td>2,870 ± 558</td>
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<td>MDMX 1–340</td>
<td>2,700 ± 531</td>
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<tr>
<td>MDMX 1–303†</td>
<td>8,200 ± 626</td>
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<tr>
<td>MDMX 1–238‡</td>
<td>5,560 ± 491</td>
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<td>-31.5</td>
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<tr>
<td>MDMX 1–111</td>
<td>30.3 ± 3.9</td>
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<tr>
<td>MDMX 5WWW‡</td>
<td>92.6 ± 11.7</td>
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<tr>
<td>MDMX W200D/201D</td>
<td>385 ± 43.5</td>
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<td>With p53</td>
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<tr>
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<td>1,110 ± 101</td>
<td>-8.3 ± 0.2</td>
<td>-1.2</td>
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<tr>
<td>With WWW element</td>
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<tr>
<td>MDMX 1–111§</td>
<td>1,290 ± 641</td>
<td>-3.4 ± 0.4</td>
<td>15.3</td>
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<td>Mutants with p4§</td>
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<tr>
<td>MDMX</td>
<td>508 ± 36.8</td>
<td>-14.4 ± 0.1</td>
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<tr>
<td>MDMX W200A</td>
<td>209 ± 27.6</td>
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<tr>
<td>MDMX W201A</td>
<td>485 ± 50</td>
<td>-14.3 ± 0.2</td>
<td>-19.9</td>
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<td>Splicing variant**</td>
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<tr>
<td>MDMX-S + p53TAD</td>
<td>40.3 ± 6.3</td>
<td>-15.2 ± 0.2</td>
<td>-18.0</td>
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*with p53TAD, residues 17–32.
†Protein has C-term His-tag, which minimizes degradation.
‡Residues 193–210 deleted.
§Independent association of p53 and MDMX monomers assumed; values calculated for monomers concentrations.
§*Sequence 181–211 of MDMX.
¶Protein high-affinity p53-derived peptide (sequence (5,6-FAM)-LTFEHYWQLTS) (37).
**MDMX alternative splicing variant (27).
The sequence of the WWW has been strongly preserved during evolution (Fig. 3A) (31). High abundance of hydrophobic amino acids and its intrinsically unstructured character, suggested that the motif is likely to be a promiscuous binding site for other partners besides the NTD of MDMX. Indeed, Chen and coworkers (32, 33) have recently found that the WWW element provides a docking site for casein kinase 1α, which in turn activates MDMX for p53 binding and phosphorylation on S289. DNA damage relieves the CK1α–MDMX interaction, resulting in release of p53 from the complex. Accordingly, it is likely that other p3STAD binding partners and other proteins might activate MDMX by binding to the WWW segment.

Materials and Methods

Protein Production. Full-length MDMX, MDM2, as well as truncated constructs (MDMX 1–111, MDMX-S, MDMX 16–116, MDMX 1–238 C-terminal 6xHis, MDMX 1–303 C-terminal His, MDMX 1–340, MDMX W200,201D, MDMX ΔWWW (lacking residues 193–210), MDMX-WWW 181–209, MDMX zinc finger 292–340) were cloned into pETM plasmids (34) (obtained from the obi). Recombinant MDMX by binding to the WWW segment.


Supporting Information

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Fig. S1. Overview of domain organization of MDMX. (A) Primary sequence and domain organization. (B–D) Ternary structures of isolated MDMX domains: (B) NTD–p53TAD complex (1); (C) RanBP2 zinc finger domain (PDB ID code 2CR8); (D) RING domain MDMX–MDM2 heterodimer (2).

Fig. S2. A representative sedimentation equilibrium scan collected at 18,000 rpm. Absorbance data measured at 280 nm fitted well to a double-exponential model to deduce apparent molecular weights of 44,844 ± 10,703 Da (monomer) and 108,896 ± 8,100 Da (dimer), respectively, in agreement with theoretical masses of 54,864 Da (monomer) and 109,728 Da (dimer). The equilibrium constant between monomer and dimer ($K_{M-D}$) was calculated from the absorbance data at the bottom of the cell from the equation $K_{M-D} = [M]^2/[D] = [A_M(l)]^2/[A_D(2l)] = 1.12 ± 0.18$, where M and D denote monomers and dimers, respectively; $l$ is the optical path length of the cell; and $\varepsilon$ is the molar extinction coefficient of the MDMX monomer. Residuals were randomly distributed deviating below ±0.008 from baseline, fitting well to a double-exponential model (and not fitting to a single-exponential model).

Fig. S3. Enzymatic activity of recombinant proteins. In vitro ubiquitination reactions containing p53, MDM2, and MDM2+MDMX were resolved by denaturing polyacrylamide gel electrophoresis and immunoblotted with anti-p53, anti-MDM2, and anti-MDMX antibodies. Addition of MDMX stimulated both autoubiquitination and p53-ubiquitination. Immunodetection of ubiquitin acceptors emphasizes monoubiquitinated forms.
Fig. S4. Isolated WWW element (181–209) is intrinsically disordered. (A–C) Secondary chemical shifts of backbone atoms (reference values of neighbor corrected chemical shifts in random coil were calculated according to ref. 1). (D) $^1$H-$^{15}$N nuclear Overhauser effect (NOE) enhancement; uncertainties were calculated assuming standard propagation of errors, which were estimated from peak intensities and noise level of the spectra. (E) $^1$H-$^1$H projection of $^{15}$N–nuclear Overhauser effect spectroscopy (NOESY)-heteronuclear single-quantum coherence (HSQC) (mixing time, 120 ms), recorded at $^1$H Larmor frequency of 500 MHz.

Fig. S5. Supplementary transverse relaxation-optimized spectroscopy (TROSY)/HSQC spectra. (A) $^{15}$N-MDMX (1–211); WWW element in bound state (B) $^{15}$N-MDMX (1–211)–nutlin-3a complex; (C) $^{15}$N-MDMX (1–211)–$^{15}$N-p53TAD; WWW element fully released by p53TAD competition; (D) 40 µM $^{15}$N-WWW (181–211)–29 µM $^{14}$N-NTD (1–111) mixture; many signals of WWW broadened by intermediate timescale chemical exchange; (E) sample (D) titrated with 50 µM nutlin-3a; addition of inhibitor partially inhibits WWW binding and results in recovery of its HSQC signals; (F) overlay of $^{15}$N-MDMX (1–340) (pink) and full-length $^{15}$N-MDMX spectra (gray).