Takashi Nagata1,2*, Kie Shirakawa3, Naohiro Kobayashi4, Hirokazu Shibeido5, Noriko Tabata3, Yuko Sakuma-Yonemura3, Kenichi Horisawa3, Masato Kato1,2, Nobuhide Doi3, Hiroshi Yanagawa3*

1 Institute of Advanced Energy, Kyoto University, Gokasho, Uji, Kyoto, Japan, 2 Graduate School of Energy Science, Kyoto University, Gokasho, Uji, Kyoto, Japan, 3 Department of Biosciences and Informatics, Keio University, Yokohama, Kanagawa, Japan, 4 Institute for Protein Research, Osaka University, Suita, Osaka, Japan

Abstract

The oncoprotein MDM2 binds to tumor suppressor protein p53 and inhibits its anticancer activity, which leads to promotion of tumor cell growth and tumor survival. Abrogation of the p53:MDM2 interaction reportedly results in reactivation of the p53 pathway and inhibition of tumor cell proliferation. We recently performed rigorous selection of MDM2-binding peptides by means of mRNA display and identified an optimal 12-mer peptide (PRFWEYWLRLME), named MDM2 Inhibitory Peptide (MIP), which shows higher affinity for MDM2 (and also its homolog, MDMX) and higher tumor cell proliferation suppression activity than known peptides. Here we determined the NMR solution structure of a MIP-MDM2 fusion protein to elucidate the structural basis of the tight binding of MIP to MDM2. A region spanning from Phe3 to Met10 of MIP forms a single z-helix, which is longer than those of the other MDM2-binding peptides. MIP shares a conserved Phe3-Trp7-Leu10 triad, whose side chains are oriented towards and fit into the hydrophobic pockets of MDM2. Additionally, hydrophobic surface patches that surround the hydrophobic pockets of MDM2 are covered by solvent-exposed MIP residues, Trp4, Tyr6, and Met10. Their hydrophobic interactions extend the interface of the two molecules and contribute to the strong binding. The potential MDM2 inhibition activity observed for MIP turned out to originate from its enlarged binding interface. The structural information obtained in the present study provides a road map for the rational design of strong inhibitors of MDM2:p53 binding.

Introduction

Tumor suppressor protein p53 plays a crucial role in maintaining genetic stability and preventing cancer formation [1], p53, a transcription factor whose expression level increases in response to cellular stress such as DNA damage, transactivates various target genes that are involved in antitumor activities, as exemplified by p21WAF1/CIP1 (cell-cycle arrest), and Bax and Puma (induction of apoptosis) [2–4]. Thus, inactivation of p53 leads to loss of the p53 pathway and inhibition of tumor cell proliferation [15,16]. The oncoprotein MDM2 binds to tumor suppressor protein p53 and inhibits its anticancer activity, which leads to promotion of tumor cell growth and tumor survival. Abrogation of the p53:MDM2 interaction reportedly results in reactivation of the p53 pathway and inhibition of tumor cell proliferation [14,17–19]. The crystal structure of MDM2:p53 complex revealed that the region spanning amino acid residues 15–29 of p53 (p5315–29) is important in binding to MDM2, and residues F19 to L26 form an amphiphilic z-helix in the transactivation domain. MDM2 promotes nuclear export of p53, by which the expression of p53-regulated genes is suppressed [9,10]. In other cases, MDM2 recruits E2 ubiquitin-conjugating enzymes to ubiquitinate p53, resulting in proteasomal degradation of p53 [7,11–13]. MDMX, a homolog of MDM2 that lacks E3 ubiquitin ligase activity, binds to the same region of p53 as MDM2 and thereby negatively regulates p53 [14]. It has been shown that abrogation of the MDM2:p53 interaction leads to reactivation of the p53 pathway and inhibition of tumor cell proliferation [15,16]. Several small-molecular compounds and peptides mimicking the MDM2 binding site of p53 have been reported to inhibit the MDM2:p53 interaction, antagonizing MDM2 and activating the p53 pathway in cancer cells [14,17–19]. The crystal structure of the MDM2:p53 complex revealed that the region spanning amino acid residues 15–29 of p53 (p5315–29) is important in binding to MDM2, and residues F19 to L26 form an amphiphilic z-helix in...
the complex, in which the side chains of F19, W23, and L26 (Phe-Trp-Leu triad) dock inside the hydrophobic pockets of MDM2 [20]. The crystal structures of peptide antagonists against MDM2 in complexes with MDM2 showed that this docking of the Phe-Trp-Leu triad is conserved [21]. The crystal structures of small-molecule antagonists in complexes with MDM2 showed that the Phe-Trp-Leu triad is replaced by simple hydrophobic functionalities, which fill the hydrophobic pockets of MDM2 [22]. Therefore, one possible approach for the discovery of better MDM2 binders would be the exploration of additional possible interactions.

Generally, peptides are more robust tools for disrupting protein-protein interactions compared to small-molecules since their large interacting surfaces confer higher specificity and affinity, resulting in fewer adverse side effects when applied as pharmaceutical agents. We recently performed in vitro selection of MDM2-binding peptides [23] from random peptide libraries using the in vitro virus (mRNA display) method [24,25]. This system, based on cell-free translation, is a potent method for the screening of functional peptides [26,27] and proteins [28–30] from large-sized libraries (~10^{13} unique members), which exceed the sizes of libraries covered by phage display. We divided the mRNA display screening procedure into two stages, the size of the search space being reduced in the second stage according to the solution of the first stage, to perform a complete search efficiently. As a result, we identified an optimal 12-mer peptide (PRFWEYWLRLME), which was named MIP [23].

We recently showed that (i) MIP inhibits the MDM2:p53 interaction in living cells and thereby blocks tumor cell growth, and (ii) MIP exhibits a higher affinity for MDM2 (and MDMX) and higher tumor cell proliferation suppression activity than known peptides, such as DI [14]. Here, we report investigation of the MIP:MDM2 interaction through NMR structure determination to better understand the origin of the MIP’s optimized binding and functional characteristics.

Materials and Methods

Construction of expression vectors

First, a DNA fragment encoding a HAT-GB1-MIP-TEV cleavage site was generated as follows. Two oligonucleotides, 5' TTTC-3' and 5' TCATGCTAGCCATCATGGTATATATTTTCCTGTGCTC-3', were phosphorylated with T4 polynucleotide kinase (Takara), and the PCR was performed using the primers: 5' TTTC-3' and 5' TCATGCTAGCCATCATGGTATATATTTTCCTGTGCTC-3'. Finally, the PCR product was subcloned into the NcoI/Xhol-digested pET15b plasmid to obtain the pMIP-MDM2 plasmid, which produces the HAT-GB1-MIP-MDM2-T7tag fusion protein (Figure 1A).

Protein expression and purification

Escherichia coli strain BL21 (DE3) codon-plus was transformed with pMIP-MDM2. Cells were grown in LB containing 100 µg/mL ampicillin at 37°C to an optical density (OD_{600}) of 0.6. After centrifugation at 2,500 g for 5 min at 4°C, the pellets were washed with a 140 mM NaCl aqueous solution. The washed pellets were resuspended in M9 minimal medium containing either 0.2 g/L 15N-NH₄Cl (ISOTEC) or 1 g/L 13C-NaCl and 5 g/L 13C-glucose (ISOTEC) as nitrogen and carbon sources. The former and latter conditions were used to obtain 15N-single labeled and 15N/13C-double labeled proteins, respectively. The cells were grown at 30°C overnight to an OD_{600} of 0.7, and then protein expression was induced with 0.4 mM IPTG, followed by further incubation at 37°C for 4 h. The harvested cells were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.6, 300 mM NaCl). After the addition of a protease inhibitor cocktail (Sigma), sonication (15 min×4 cycles) and centrifugation (2,500 g at 4°C for 20 min) were performed. The collected supernatant was loaded onto a TALON Metal Affinity Resin column (Clontech). After washing with 100 column volumes of lysis buffer, protein was eluted with lysis buffer containing 250 mM imidazole. The obtained protein was dialyzed against the thrombin cleavage buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 250 mM CaCl₂) and then treated with 2 units/mg protein of thrombin at room temperature for 16 h. The protein solution was passed through the TALON Metal Affinity Resin column for the second time. The HAT-GB1 tag was retained on the column. Finally, the MIP-MDM2 fusion protein, which was collected in the flow through fraction, was further purified by size-exclusion chromatography on a Superdex 75 10/300 GL column (GE Healthcare) in lysis buffer. The fractions containing the fusion protein were pooled and concentrated to about 10 mg/mL using an Amicon Ultra-4 (Millipore). Finally, three NMR samples: each containing 150 µM 13C-labeled MIP-MDM2 fusion, 15N-labeled 150 µM MIP-MDM2 complex, and 650 µM 13C,15N-labeled MIP-MDM2 fusion; all dissolved in 20 mM Tris-HCl, pH 7.6, 300 mM NaCl, and 5% 2H₂O, were prepared.

NMR spectroscopy

All NMR data were collected at 298 K on a Bruker AVANCE 600 MHz NMR spectrometer equipped with a cryogenic probe. NMR spectra were processed with NMRPipe/NMRDraw [32]. Spectral analysis was performed with Kujiira 0.984 [33], a program suite for interactive NMR analysis working with NMRView [34], according to the method described previously [35]. The backbone and side chain $^1$H, $^15$N, and $^{13}$C resonances of MIP-MDM2-T7tag were assigned by means of standard double- and triple-resonance NMR experiments [36,37], and were deposited in BioMagResDB (BMRB ID: 11569). Distance restraints were derived from three-dimensional (3D) $^{15}$N-edited and $^{13}$C-edited nuclear Overhauser effect spectroscopy (NOESY)-HSQC spectra, each being measured with a mixing time of 80 msec. To determine the steady-state $^1$H-$^{15}$N NOE value of MIP-MDM2-T7tag, an enhanced-sensitivity experiment was performed using the standard method with the parameters described previously [35,38]. The spectra were analyzed with Sparky [39] as described previously [35].
Structure calculations for MIP-MDM2-T7tag were performed using CYANA 2.1 [40–42], with the standard CYANA simulated annealing schedule and 40,000 torsion angle dynamics steps per conformer, starting with 200 randomized conformers. The 40 conformers exhibiting the lowest final CYANA target function were selected for analysis.

Figure 1. NMR analysis of the MIP-MDM2 fusion and MIP:MDM2 complex. (A) Schematic diagram of the HAT-GB1 fused MIP-MDM2-T7tag protein expression plasmid. The T7 promoter, ribosome binding site (RBS), restriction enzyme sites, and protease cleavage sites are also indicated. (B, left) Shown in orange is the 2D ¹H-¹⁵N HSQC spectrum of the MIP-MDM2-T7tag linked protein (MIP-MDM2) and in cyan the MIP-MDM2-T7tag complex (MIP:MDM2 complex). (B, right) 2D ¹H-¹⁵N HSQC spectrum of MIP-MDM2. Signals are labeled with the residue number and a one-letter amino acid code: The MIP, TEV cleavage site, and MDM2-T7tag portions are colored brown, gray, and blue, respectively. (C, left) Chemical shift differences of the corresponding signals in Fig. 1 (B, left). The chemical shift difference, Δδ, was determined as Δδ = [(ΔδH)² + (ΔδN/6.5)²]¹/², where ΔδH and ΔδN are the chemical shift differences for HN and ¹⁵N, respectively. The mean value and the mean value ±1SD are shown by solid and dashed lines, respectively. (C, right) Steady-state ¹H-¹⁵N NOE values are shown for MIP-MDM2. ‘P’ s indicate proline residues and asterisks indicate residues whose ¹H-¹⁵N resonance was not assigned.

doi:10.1371/journal.pone.0109163.g001

Structure calculations

Structure calculations for MIP-MDM2-T7tag were performed using CYANA 2.1 [40–42], with the standard CYANA simulated annealing schedule and 40,000 torsion angle dynamics steps per conformer, starting with 200 randomized conformers. The 40 conformers exhibiting the lowest final CYANA target function...
values were further refined with AMBER12 [43], using the AMBER 2003 force field and a generalized Born model, as described previously [35]. The force constants for distance, torsion angle, and \( \phi \) angle restraints were set to 32 kcal mol\(^{-1}\) \( \text{Å}^{-2} \), 60 kcal mol\(^{-1}\) rad\(^{-1} \), and 50 kcal mol\(^{-1}\) rad\(^{-2} \), respectively. The 20 conformers that were most consistent with the experimental restraints were then used for further analyses. The final structures were validated and visualized by using the Ramachandran plot web server [44] and software CHIMERA [45,46]. Detailed experimental data and structural statistics are summarized in Table 1. The final ensembles of 20 conformers were deposited in the Protein Data Bank (PDB ID: 2RUH).

**Surface plasmon resonance (SPR) analysis**

Binding kinetics were determined by SPR with a Biacore 3000. All experiments were performed at 25°C using HBS-EP buffer (10 mM HEPES–NaOH, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Tween-20). Biotinylated LC-biotin-MIP (PRFWEYWLR LME, 2,065 Da), LC-Biotin-DI (LTFeHYWAQLTS, 1,835 Da), and LC-Biotin-p53 (17-28 amino acid residues, EFSDMIWL LPE, 1,817 Da) were chemically synthesized and immobilized on a streptavidin sensor chips, respectively. The measurements were performed with resonance units of 50.4 (MIP), 57.1 (DI) and 89.5 (p53\(^{17-28}\), and at a flow rate of 20 \( \mu \)l/min. The MDM2\(^{7-300} \) (7-300 amino acid residues) gene was amplified from the pDrive-MDM2 plasmid [23] by PCR using the primers: 5’-CACCA-TGTGCAATACCAACATGTCTG-3’ and 5’-CTTGAGCTC-GAGATCTTCTTCAATGAATCTGTATC-3’. The PCR product was subcloned into the pENTR/D-TOPO vector (Invitrogen). The resulting plasmid was recombined with the pDEST15 vector to generate a GST-MDM2 expression construct (pDEST15-MDM2). pDEST15-MDM2 was used for transformation of E. coli strain BL21 (DE3) codon-plus. The cells were

| Table 1. Structural Statistics for MIP-MDM2. |
|---------------------------------------------|
| **NMR restraints**                          |
| Distance restraints                        |
| Total NOE                                   | 1823 |
| Intra-residue                               | 568  |
| Inter-residue                               |      |
| Sequential (|i–j| =1)                               | 387  |
| Medium-range (1<|i–j| <5)                 | 365  |
| Long-range (|i–j| ≥5)                  | 503  |
| Hydrogen bonds restraints a                 | 34   |
| Dihedral angle restraints a                 | 3/3  |
| \( \phi \) and \( \psi \)                 |      |
| \( \chi_1 \) and \( \chi_2 \)             | 21/16|
| **Structure statistics (20 conformers)**   |
| CYANA target function (\( \text{Å}^2 \))    | 0.29 |
| Residual NOE violations                     |      |
| Number >0.1 Å                              | 3    |
| Maximum (Å)                                | 0.37 |
| Residual dihedral angle violations          |      |
| Number >5\(^{-} \)                         | 0    |
| Maximum (\(^{-} \))                        | 0.83 |
| AMBER energies (kcal/mol)                  |      |
| Mean AMBER energy                          | −3612|
| Mean restraints violation energy            | 5.44 |
| Ramachandran plot statistics (%) b         |      |
| Residues in most favored regions            | 82.3 |
| Residues in additionally allowed regions    | 15.7 |
| Residues in generously allowed regions      | 1.8  |
| Residues in disallowed regions              | 0.2  |
| Average R.M.S.D. to mean structure (Å) c    | 0.53 |
| Protein backbone                            | 1.28 |
| Protein heavy atoms                         |      |

*a*Used only in CYANA calculations.

*b*Calculated with the Ramachandran plot server at the Indian Institute of Science.

*c*For residues Phe3-Glu12 of MIP and Leu33-Gly114 of MDM2.

doi:10.1371/journal.pone.0109163.t001
grown in LB with 100 μg/ml ampicillin at 37°C until OD₆₀₀ reached 0.7, induced with 1 mM IPTG for 5 h at 30°C, and then harvested by centrifugation. The pellets were resuspended in PBS supplemented with a protease inhibitor cocktail (Sigma), sonicated, and then centrifuged. The resulting supernatants were added to glutathione-Sepharose 4B (GE Healthcare), and then mixed on a rotator for 2 h at 4°C. The beads were washed with PBS and eluted with 50 mM Tris-Cl, pH 8.0, containing 5 mM glutathione, followed by dialysis against PBS using Slide-A-Lyzer dialysis cassettes (Thermo Scientific) to obtain purified GST-MDM27–300. To determine dissociation constants, two different concentrations (100 nM and 200 nM) of the purified GST-MDM27–300 were injected. The injection periods for association and dissociation were 30 and 180 s, respectively. After each measurement, the chip surface was regenerated with 10 mM glycine 2.0 (Biacore). The binding data were analyzed with the 1:1 Langmuir binding model in the BIAevaluation software ver. 4.1 (Biacore). The binding binding was cleaved and eliminated during the purification steps, while the T7tag was left attached. The obtained MIP-MDM2-T7tag fusion protein (hereinafter referred to as the MIP-MDM2 fusion) was further treated with a protease and thereby cleaved at the C-terminal end of MIP, which resulted in generation of the MIP-MDM2-T7tag complex (referred to as the MIP-MDM2 complex). Comparison of the 1H-15N HSQC spectra of the MIP-MDM2 fusion and MIP-MDM2 complex showed the considerable similarity in their signal patterns (Figure 1B, left). Signal assignments of the 1H-15N HSQC spectra were further analyzed, it being found that the Δδ values of all the residues are very small (Figure 1C, left). Despite the small Δδ values (all the values are less than 0.1 ppm), the residues in the flexible regions such as the linker portion attached to the C-terminus of MIP and the N-terminal region of MDM2 (see steady-state 1H-15N NOE values in Figure 1C, right) and some residues in the less flexible regions of MDM2 (T33, Y54, M56, H79, K104, Y106, and T107, which will be discussed later in this section) showed larger Δδ values. Thus, we concluded that the structures of the MIP-MDM2 fusion and MIP-MDM2 complex are similar if not the same, and decided to carry out the structural study using the MIP-MDM2 fusion.

To determine the structure of the MIP-MDM2 fusion, NMR experiments, spectral analysis, and structural calculation were performed following the methods described previously [35]. The experimental restraints and structural statistics for the 20 lowest energy structures are summarized in Table 1, it being indicated that residues F3-E12 of MIP and L33-G114 of MDM2 adopt a well-defined structure, with an RMSD of 0.53 Å for the backbone atoms (Figure 2A). Although, some residues in the linker portion (G13, G14, G15, Y19, and Q21) showed higher steady-state 1H-15N NOE values (>0.5) in Figure 1C (right), we were not able to identify their inter-residue NOE signals in NOESY spectra. Thus, it is assumed that the mobility of some part of the linker portion might have been restricted by such as steric hindrance (Figure 2A, left). The MIP portion is composed of a single α-helix, F3-E12, while the MDM2-T7 portion comprises four α-helices: αA (P38-V47), αB (M56-K70), αC (L87-F92), and αD (H102-M112); and two short β-strands: β1 (I80-Y82) and β2 (S96-S98). It can be seen that the last one-third of αD, which corresponds to the N-terminal region of the T7tag, appears as less flexible (Figure 1C, right), but distant from the MIP binding site.

Among the aforementioned residues of MIP that showed larger Δδ values in Figure 1C (left), the residues T33, Y54, M56, H79, K104, Y106, and T107 are located in the structured regions, which is consistent with the steady-state 1H-15N NOE values (Figure 1C, right). As expected, the locations of the residues T33, Y54, M56, K104, Y106, and T107 are close to the linker portion. On the other hand, the location of H79 seemed far from the linker portion at a first glance. However, it turned out that the location of H79 is close to those of the residues K104, Y106, and T107 on the same surface of MDM2. Although, it is not possible to determine the position of the linker portion because it is flexible, all the residues that showed larger Δδ values in Figure 1C (left), including H79, are indeed seem to locate close to the linker portion.

An opened-up view of the MIP and MDM2 interface shows that the residues in close contact (intermolecular distances of up to 3 Å) are mostly hydrophobic (Figure 2B). MIP fits into the large concavity on the MDM2 surface (Figure 2C, left). The Phe-Trp-Leu triad of MIP, which is completely conserved among the MDM2-binding peptides, orients the side chains deep inside the hydrophobic pockets located at the center of the large concavity (Figure 2C, right). Additionally, three rather large hydrophobic...
residues, W4, Y6, and M11, of MIP fit on the hydrophobic patches along the rim of the large concavity of MDM2 (Figure 2C, left). These three residues are unique to MIP and the most probable candidates increasing the MIP’s affinity towards MDM2.

|          | $k_a$ (1/Ms)$^a$ | $k_d$ (1/s)$^a$ | $K_a$ (1/M) | $K_d$ (M) |
|----------|-----------------|-----------------|-------------|-----------|
| MIP      | $1.20 \times 10^5$ | $2.21 \times 10^{-2}$ | $5.42 \times 10^{7}$ | $1.84 \times 10^{-8}$ |
| DI       | $1.95 \times 10^4$ | $4.09 \times 10^{-3}$ | $4.77 \times 10^{6}$ | $2.10 \times 10^{-7}$ |
| p53 peptide | 411            | $5.98 \times 10^{-3}$ | $6.88 \times 10^{4}$ | $1.45 \times 10^{-5}$ |

$^a$ the standard error for the kinetic parameters in each global fit was $\pm 1\%$.

doi:10.1371/journal.pone.0109163.t002

### Binding mode of MIP to MDM2

The solution structure of the MIP-MDM2 fusion is very similar to the crystal structures of the p53$^{15-29}$:MDM2 complex (RMSD of 0.74 Å between superimposed 80 C$_\alpha$ atoms), DEDMD2 complex (RMSD of 0.95 Å between superimposed 81 C$_\alpha$ atoms), and PMI:MDM2 complex (RMSD of 0.79 Å between superimposed 79 C$_\alpha$ atoms) (Figure 3A). As expected, the side chains of the conserved Phe-Trp-Leu triad of MIP superimpose very well with those of DI, PMI, and the p53 peptide (Figure 3B). On the other hand, the lengths of the $\alpha$-helices are in the order of MIP $\leq$ DI $\leq$ PMI $> p53$ peptide, where the $\alpha$-helix of MIP is either the same length as or one residue longer than those of DI and PMI, and two residues longer than that of the p53 peptide. Extension of the $\alpha$-helix in MIP is achieved by M11. In the case of the p53 peptide, this position is occupied by proline, which is known as a breaker of an $\alpha$-helical structure. Substitution of this proline to serine was shown to endow the p53 peptide with its $\alpha$-helical nature [48]. The corresponding positions in DI and PMI are occupied by threonine (T27) and serine (S11), respectively. T27 of DI was seen to continue the helical turn and H-bonding pattern loosely [49]. It was briefly mentioned in the previous section that M11 of MIP covers a hydrophobic patch on the surface of MDM2. In detail, its side chain forms hydrophobic contacts with K57 and F61 of MDM2, which is supported by the observation of NOEs between the methyl H$_C$ of M11 and the side chains of K57 and F61. Thus, it seems that these hydrophobic contacts together with the backbone and side chain conformations of M11 are mutually stabilized.

The positions of the other aforementioned large hydrophobic residues, W4 and Y6, of MIP, which form hydrophobic contacts with MDM2, were also compared (Figure 3B). Interestingly, the position of Y6 in MIP is also occupied by tyrosine in DI (Y22) and PMI (Y6), but by leucine in P53 peptide (L22). Since tyrosine has a much larger side chain than leucine, MIP, DI, and PMI are able to fill the hydrophobic concavity on the MDM2 surface, while the corresponding space remains solvent-accessible in the p53 peptide:MDM2 complex. This additional interaction by tyrosine at this position and the fact that its side chain forms extensive hydrophobic contacts with H73 and K94 of MDM2 were also compared in the respective structures of DEDMD2 and PMI:MDM2 complexes [47,49]. Thus, tyrosine at this position is highly preferred by MDM2-binding peptides to achieve strong binding.

The role of the position of W4 in MIP exhibits distinct differences with those of the equivalent positions in DI, PMI, and p53 (Figure 3B). The side chain ring of W4 is sandwiched by the hydrophobic groups of L8 of MIP and M68 of MDM2 in the so called CH-$\pi$ interaction manner (Figure 3C). Additionally, the backbone of G64 and the plane surface of the side chain ring of F61 in MDM2, and the methyl group of MIP M11 surround the side chain ring of W4 (Figure 3C). The presence of these interactions is fully supported by NOEs. The residues in DI, PMI, and p53 at the
equivalent position to W4 in MIP are E20, A4, and S20, respectively (Figure 3B). These residues seem not to undergo direct intermolecular interactions in their respective complex structures. Hence, W4 in MIP adds an extra intermolecular interaction on the surface of MDM2 and thereby contributes to stronger binding to MDM2.

MIP preferentially forms \( \alpha \)-helical structure in a hydrophobic environment

A large number of intrinsically disordered regions (or intrinsically unstructured domains), which become structured only during binding to the target (i.e., coupled folding and binding), have already been identified in nature \[50\]. The N-terminal region of p53 is intrinsically unstructured in solution \[50\] but, however, it folds into an amphipathic helical structure upon binding to its target protein, MDM2 \[20\]. As described above, the NMR data analysis suggested that hydrophobic contacts between MIP and MDM2 play critical role to their strong binding. A hydrophobic environment generally stabilizes the formation of secondary structures of peptides and proteins \[51–53\]. Therefore, we examined the influence of a hydrophobic environment on the structure formation of MIP. Circular dichroism (CD) spectra of MIP, DI, and the p53 peptide were measured in the presence of different concentrations of a 2,2,2-trifluoroethanol (TFE), which mimics a partial hydrophobic environment (Figure 4).

The CD spectrum of the p53 peptide in the absence of TFE showed a characteristic spectral pattern for an unstructured peptide (Figure 4C). The presence of TFE hardly affected the spectral pattern of the p53 peptide, which indicates that a hydrophobic environment is not sufficient to promote the structure formation of the p53 peptide. On the other hand, TFE caused distinct changes in the CD spectral patterns of MIP and DI, respectively (Figure 4A, B). In particular, the CD spectrum of MIP in the presence of TFE exhibited a characteristic pattern of \( \alpha \)-helical structure. A characteristic minimum at 227 nm in the CD spectrum of MIP in the absence of TFE may be due to W4 and/or W7 of MIP \[54,55\]. The CD spectrum of DI also showed a characteristic pattern of an unstructured peptide in the absence of TFE, however, the presence of TFE converted it to a pattern of a mixture of secondary structure (Figure 4B). Thus, strong binding of MIP with MDM2 is not only due to the enlarged binding interface, but supposedly also due to the preferred formation and stabilization of the \( \alpha \)-helical structure in MIP in the hydrophobic environment.

Conclusions

In this study, we investigated the interaction between MDM2 and MIP, the optimal 12-mer peptide that we had screened and identified from random peptide libraries using the \textit{in vitro} virus (mRNA display) method, through NMR structure determination. MIP utilized not only the sequentially and functionally conserved Phe-Trp-Leu triad to fill the hydrophobic pockets of MDM2 but also the solvent-exposed W4, Y6, and M11 to enlarge the binding interface and to cover the hydrophobic surface patches that surround the hydrophobic pockets of MDM2. The first case of involvement of W4 and M11 in binding with MDM2 was confirmed structurally in this study. Significance of Y6 and M11 in binding with MDM2 was also supported by previous mutation experiments \[23\]. The structural information we obtained here provides important clues for designing small molecule inhibitors of the MDM2:p53 binding. Therefore, we are going to approach design of novel small molecule inhibitors of the MDM2:p53
binding through docking simulation based on this structural information. Currently known small molecule inhibitors, Nutlin-3 for example [16], have functional groups that only fit into the hydrophobic pockets, however, our results suggest that the hydrophobic surface patches surrounding the hydrophobic pockets should also be considered. Thus, the structure of MIP in the complex should be suitable as a template for designing a new small molecular inhibitor. Furthermore, for development of drugs utilizing MIP itself, we are constructing novel fusion proteins in which MIP was connected with transmembrane amino acid sequences and exploring an efficient delivery system of MIP to inside the cell.

**Author Contributions**

Conceived and designed the experiments: TN HY. Performed the experiments: TN KS NT. Analyzed the data: TN KS NK HS NT. Contributed reagents/materials/analysis tools: TN NK YSY KH MK ND HY. Wrote the paper: TN KS HY. Designed the software used in analysis: NK.

**References**

1. Vousden KH, Lane DP (2007) p53 in health and disease. Nat Rev Mol Cell Biol 8: 275–283.
2. Nakano K, Vousden KH (2001) PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell 7: 683–694.
3. Shangary S, Wang S (2008) Targeting the MDM2-p53 interaction for cancer therapy. Clin Cancer Res 14: 5318–5324.
4. Wasylyk C, Salvi R, Argentini M, Dureuil C, Delumeau I, et al. (1999) p53 mediated death of cells overexpressing MDM2 by an inhibitor of MDM2 interaction with p53. Oncogene 18: 1921–1934.
5. Chene P (2003) Inhibiting the p53-MDM2 interaction: an important target for cancer therapy. Nat Rev Cancer 3: 102–109.
6. Harris CC (1993) p53: at the crossroads of molecular carcinogenesis and risk assessment. Science 262: 1980–1981.
7. Haupt Y, Maya R, Kazaz A, Oren M (1997) Mdm2 promotes the rapid degradation of p53. Nature 387: 296–299.
8. Holstein M, Sidransky D, Vogelstein B, Harris CC (1991) p53 mutations in human cancers. Science 253: 49–53.
9. Boyd SD, Tsai KY, Jacks T (2000) An intact HDM2 RING-finger domain is required for nuclear exclusion of p53. Nat Cell Biol 2: 563–568.
10. Geyer RK, Yu ZK, Maki CG (2000) The MDM2 RING-finger domain is required to promote p53 nuclear export. Nat Cell Biol 2: 569–573.

Figure 4. MIP preferentially forms α-helical structure in a hydrophobic environment. CD spectra of MIP (A), DI (B), and the p53 peptide (C) with different concentrations: 0% (solid line), 30% (dashed line), and 50% (dotted line) of TFE. doi:10.1371/journal.pone.0109163.g004
11. Honda R, Tanaka H, Yasuda H (1997) Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett 420: 25–27.

12. Kubbath MH, Jones SN, Vousden KH (1997) Regulation of p53 stability by Mdm2. Nature 387: 299–303.

13. Mornad J, Zambetti GP, Olson DC, George D, Levine AJ (1992) The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 69: 1237–1245.

14. Hu B, Gilbert DM, Chen J (2007) Efficient p53 activation and apoptosis by simultaneous disruption of binding to MDM2 and MDMX. Cancer Res 67: 8810–8817.

15. Chene P, Fuchs J, Carena I, Furet P, Garcia-Echeverria C (2002) Study of the interaction of the amino-terminal domain MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science 274: 948–953.

16. Li C, Piazza M, Li CQ, Yuan WR, Liu M, et al. (2010) Systematic Mutational Analysis of Peptide Inhibition of the p53-MDM2/MDMX Interactions. Journal of Molecular Biology 398: 200–213.

17. Popowicz GM, Domling A, Holak TA (2011) The Structure-Based Design of Mdm2/Mdmx-p53 Inhibitors Gets Serious. Angewandte Chemie-International Edition 50: 2680–2688.

18. Kobayashi N, Iwahara J, Yoshida K, Satoh A, Kohno T, et al. (1999) Determinants of specificity of MDM2 for the activation domains of p53 and p65: proline27 disrupts the MDM2-binding motif of p53. Biochemistry 45: 11945–11957.

19. Curtiss RF, Drabkin DL, Kahan BD, Cossman J, Thomas CW, et al. (1978) The structure of the MIP bound to MDM2. J Mol Biol 121: 281–298.

20. Wright PE, Dyson HJ (1999) Intrinsically unstructured proteins: re-assessing the protein structure-function paradigm. J Mol Biol 285: 2174–2183.