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Topical Perspectives

Rational design of cyclic peptides to disrupt TGF-B/SMAD7 signaling in heterotopic ossification

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ABSTRACT

The human TGF-β/SMAD7 signaling has been recognized as an attractive target of heterotopic ossification (HO). Here, we report a successful rational design of cyclic peptides to disrupt the signaling pathway by targeting TGF-β–receptor complex. The intermolecular interaction between TGF-β and its cognate receptor is characterized in detail using molecular dynamics simulation, binding energetic analysis, and alanine scanning. With the computational analysis a binding loop of receptor protein is identified that plays an essential role in the peptide-mediated TGF-β–receptor interaction. Subsequently, the loop is stripped from the protein context to generate a linear peptide segment, which possesses considerable flexibility and intrinsic disorder, and thus would incur a large entropy penalty upon binding to TGF-β. In order to minimize the unfavorable entropic effect, the linear peptide is cyclized by adding a disulfide bond between the N- and C-terminal cysteine residues of the peptide, resulting in a cyclic peptide. *In vitro* fluorescence anisotropy assays substantiate that the cyclic peptide can bind tightly to TGF-β with determined K_d value of 54 μM. We also demonstrated that structural optimization can further improve the peptide affinity by site-directed mutagenesis of selected residues based on the computationally modeled complex structure of TGF-β with the cyclic peptide.

1. Introduction

Heterotopic ossification (HO) is the presence of bone in soft tissue where bone normally does not exist, which frequently occurs in three distinct contexts-trauma, neurologic injury and genetic abnormalities [1,2]. Over the past decades, *in vitro* evidences and animal models have shown that transforming growth factor β/mothers against decapentaplegic homolog 7 (TGF-β/SMAD7) signaling plays a critical role in the development and progress of HO [3]. The TGF-β/SMAD7 is involved in a vast majority of cellular processes and is fundamentally important throughout life, which has widely recognized roles in bone formation during mammalian development and exhibits versatile regulatory functions in the body. Signaling transduction by TGF-β is specifically through both canonical SMAD-dependent pathway and non-canonical SMAD-independent signaling pathway [4]; the former has been thought to closely relate to the pathological process of HO, that is, HO is initiated with ligand-induced oligomerization of serine/threonine receptor kinases and phosphorylation of SMAD protein. Recently, we also found that SMAD7 can prevent HO in a rat Achilles tendon injury model *via* regulation of endothelial-mesenchymal transition [5].

The pathological condition of HO is characterized by fibrosis and inflammation, which is associated with several inflammatory cytokines and growth factors affecting fibroblast activity. The TGF-β plays a central role in the TGF-β/SMAD7 signaling pathway and has strong potency to activate fibroblast by stimulating its differentiation to myofibroblast, which was found to play a crucial role in the formation of HO [6]. By modulating level of the protein, fibroblast activation is attenuated considerably, then effectively preventing the resulting HO. Thus, targeting TGF-β/SMAD7 pathway has been recognized as an attractive strategy to combat HO at molecular level. Previously, Chen et al. have successfully derived a short proline-rich peptide from the SMAD protein to suppress the pathway activation by targeting Yes-associated protein (YAP), a transcription coactivator of SMAD family [7]. However, this indirect approach can only exert moderate effect and may cause unexpected outcomes due to multiple bypasses associated with the YAP protein. Thus, we herein attempted to target TGF-β directly. The TGF-β is a disulfide-bonded homodimer consisting of two identical subunits linked by four internal disulfide bonds and a single cross-linking disulfide bond, which is further stabilized by hydrophobic forces [8]. TGF-β signaling is initiated through highly specific binding and

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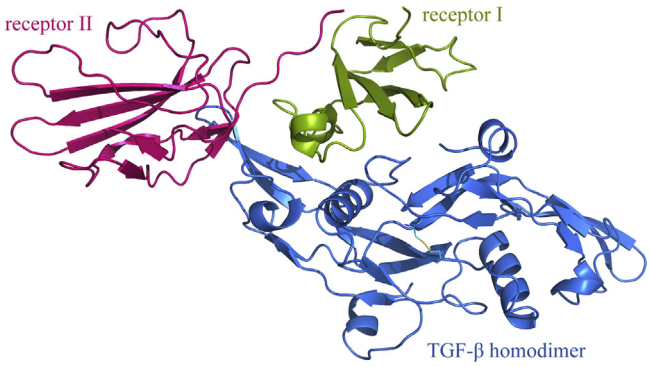

Fig. 1. Stereoview of the crystal structure of TGF-β homodimer in complex with its cognate receptors I and II [9].

complex formation between the active TGF-β ligand and its cognate receptors, the TGF-β receptor types I and II (Fig. 1) [9], which are serine/threonine kinases and are brought into proximity such that the receptor II phosphorylates the receptor I kinase domain and then phosphorylates SMAD proteins [10].

As a small protein factor, TGF-β is not a good target of chemical agents owing to its small size and flat profile. Instead, biologic peptides were found to have potential capability to interact and inhibit a variety of protein factors with a high potency [11]. Here, we attempted to extract peptide segments from the complex interface of TGF-β with its cognate receptors to disrupt intermolecular interaction of the complex and to suppress the receptor-mediated TGF-β/SMAD7 signaling. The method employed a variety of molecular modeling techniques such as molecular dynamics simulations, binding energy calculations and computational alanine scanning to characterize the structural basis and energetic property of TGF-β–receptor complex and to extend and cyclize peptide segments deriving from the complex interface. We also performed *in vitro* affinity assays to determine the binding capability of designed cyclic peptides to TGF-β. The nonbonded interactions that confer stability and specificity to the TGF-β–cyclic peptide adducts were examined in detail to elucidate the molecular mechanism and biological implication underlying the adduct binding.

2. Materials and methods

2.1. Setup of TGF-β–receptor complex structure

The high-resolution crystal structure of TGF-β in complex with its cognate receptors I and II was retrieved from the protein data bank (PDB) database (2PJY) [9]. The X-ray crystallography only presents heavy atoms such as carbon, nitrogen, oxygen and sulfur, whereas the small hydrogen atoms have not been solved in the structure. In general, protein hydrogen atoms can be classified into three categories, *i.e.* polar, nonpolar and charged. Here, the polar and nonpolar hydrogens were added using *reduce* program for the structure [12]; the method predicts hydrogen's positions by optimizing stereochemistry and hydrogen-bonding networks in protein context. For those titratable residues such as Asp, Glu, His, Lys and Arg, the hydrogen protonation states of charged groups were assigned with online *h++* server [13] in terms of their surrounding chemical microenvironments. Consequently, the complex system was identified to have 63 titratable residues; the pK_a value of each residue was computed in *h++* to determine their protonation state.

In addition, we also employed the *falc-loop* tool [14] to computationally model missing loop regions in the structure. The *falc-loop* is a knowledge-based loop modeling method that searches against more than 4000 candidate loop structures and to derive loop fragment for the target from a fragment assembly that includes a number of best matched candidates. Thus, the *falc-loop* does not employ a specific template to model the loops. Instead, a cluster of template candidates derived from its in-house loop structure database is utilized to generate the target loops.

2.2. Molecular dynamics simulation

2.2.1. Routine molecular dynamics (MD) simulation

A total of six systems, including a TGF-β–receptor complex and five TGF-β–peptide adducts, were subjected to MD simulations using AMBER ff03 force field [15] implemented in *amber11* suite of programs [16]. The system was dissolved in a TIP3P water box [17] with at least 12 Å buffer wall and neutralized by counter ions of Na^+. The solvated system was pre-minimized in two steps: First, only water molecules were subjected to 200 cycles of the steepest descent followed by 1000 cycles of conjugate gradient. Second, the entire system was minimized with 500 cycles of the steepest descent followed by 3000 cycles of conjugated gradient. Subsequently, the system was heated from 0 to 300 K over 500 ps and equilibrated at 300 K for 1000 ps. Finally, MD production simulations were performed in an isothermal isobaric ensemble with periodic boundary conditions. A time step of 2 fs was set [18,19]. The SHAKE algorithm [20] and particle mesh Ewald (PME) method [21] were employed to constrain chemical bonds involving hydrogen atoms and to treat long-range electrostatic interactions, respectively. A cut-off distance of 10 Å was applied for the short-range electrostatics and van der Waals interactions. The parameter settings for the MD simulations of different systems are tabulated in Table 1.

2.2.2. Force-directed MD (FDMD) simulation

FDMD simulations were performed on the extended linear peptide (EL-peptide) dissolved in a periodic box full of TIP3P water molecule [17]. The method was employed to restrain the distance between the two sulfur atoms of N- and C-terminal cysteine residues of the peptide into the standard length of a covalent disulfide bond [22]. In the simulation procedure, a force was added between the two sulfur atoms; the force was described by a harmonic oscillator model with equilibrium distance = 2.05 Å [23] and force constant = 10 kcal/mol/Å. By about 30-ns simulations the distance between the two sulfur atoms reaches at the equilibrium distance; the simulation was continued to 60 ns to ensure the entire system (peptide + water molecules) relaxed. After that, a disulfide bond was artificially added between the two sulfur atoms to cyclize the peptide, and then an additional 60-ns routine MD simulation was performed to equilibrate the artificial cyclic peptide.

2.3. Binding energetic analysis

2.3.1. Molecular mechanics/Poisson-Boltzmann surface area (MM/PBSA)

MM/PBSA analysis [23] was performed over a total of 500 snapshots extracted evenly from the MD trajectory file during the sampling interval of 50–120 ns. In the procedure, the intermolecular interaction energy ΔE_{int} between two members (TGF-β and receptor or peptide) of a complex system was calculated *via* forcefield-based molecular mechanics (MM) approach, while the desolvation free energy ΔG_{dslv} upon the interaction was estimated by finite difference solution of Poisson-Boltzmann (PB) (for polar contribution) and surface model (SA) (for nonpolar contribution). The grid size used to solve the PB equation was set to 0.5 Å, and the interior and exterior dielectric constants were 1 and 80, respectively. The SA was defined as $a + b{\cdot}S$, where S is solvent accessible surface area computed with a solvent-probe radius of 1.4 Å and the protein atomic radii taken from PARSE parameter set [24], and

Table 1
A summery of parameter settings in MD simulations of different systems.

System	Total simulation time (ns)	Sampling interval (ns)	Number		Number of snapshots	
			Water molecule	Counter ion	MM/PBSA	NMA
TGF-β–receptor	120	50–120	~6500	16	500	50
TGF-β–L-peptide	120	50–120	~4700	9	500	50
TGF-β–EL-peptide	120	50–120	~4900	10	500	50
TGF-β–C-peptide	120	50–120	~4800	10	500	50
TGF-β–CM1-peptide	120	50–120	~4800	10	500	50
TGF-β–CM2-peptide	120	50–120	~4800	10	500	50

a and *b* are coefficients that were assigned to 0.92 kcal/mol and 0.00542 kcal/mol/Å^2, respectively [25]. The MM/PBSA was implemented with the *mm/pbsa* module of *amber11* suite.

2.3.2. Normal mode analysis (NMA)

NMA was carried out to estimate the vibrational component of entropy. Since the analysis is computationally expensive, only 50 evenly out of the totally 500 snapshots were used to estimate the entropy penalty $-T\Delta S$. Frequencies of the vibrational modes were computed at 300 K for these snapshots and using a harmonic approximation of the energies [26]. The NMA was implemented with the *nmode* module of *amber11* suite.

The binding free energy of a complex system can be written as follows:

$$\Delta G = \Delta E_{\text{int}} + \Delta G_{\text{dslv}} - T\Delta S \quad (1)$$

2.3.3. Computational alanine scanning (CAS)

The CAS scanning of residue contribution to TGF-β–receptor binding was conducted based on the MM/PBSA binding energy calculations [27]. The method virtually mutated a receptor residue of complex system to alanine (Ala) and calculated the change in binding free energy ($\Delta\Delta G$) of the complex upon the mutation; this was repeated over all residues of receptor protein. Alanine is the substitution residue of choice since it eliminates the side chain beyond the C_β carbon and yet does not alter the main-chain conformation (as can glycine and proline) nor does it impose extreme electrostatic or steric effects (as can arginine and tryptophan) [28]. The CAS scanning was implemented with the *mm/pbsa* module of *amber11* suite.

2.4. Fluorescence anisotropy assay

Fluorescence anisotropy assays were performed to determine the binding affinity (K_d) for designed peptides to TGF-β following a previously described protocol [29]. The assay buffer is 30 mM HEPES pH 7.5, 100 mM NaCl and 5 mM DTT. The reaction mixtures contain fluorescein-labeled peptides (100 nM) and proteins with concentration ranging from 0 to 500 mM. The reactions were carried out in a 384-well plate and measured after 30 min using a plate reader. The anisotropy values were normalized and the K_d values were determined using nonlinear fitting of the measured anisotropy points. Linear peptides were synthesized with standard 9-fluorenyl methoxycarbonyl (Fmoc) solid phase chemistry. The PAM-Gly-Boc resin was used with an *S*-tritylmercaptopropionic acid linker to cyclize linear peptides [30]. The synthetic peptides were purified by RP-HPLC columns and then confirmed using MS and component analysis. All the peptide synthesis, purify and confirmation were conducted by GBiochem Ltd.

3. Results and discussion

The complex system of TGF-β with receptor was subjected to 120-ns MD simulations; the simulations were run in duplicate and resulting atomic root-mean-square deviation (*rmsd*) fluctuation is shown in Fig. 2. As can be seen, in the first 50-ns simulations the trajectory changes dramatically, indicating that the system has undergone a large conformational adjustment during the period, whereas the trajectory becomes stable and no obvious *rmsd* fluctuation can be observed after ~50-ns simulations, suggesting that the system maintains in an equilibration state during the 50–120-ns simulations and thus we will used this sampling interval to perform subsequent energetics analysis. In addition, the *rmsd* profiles of two independent simulations are basically consistent and no obvious difference can be observed between the first and second runs, suggesting that the MD simulations performed in this study are reliable and repeatable.

Computational alanine scanning was employed to analyze the residue contribution to the complex interaction. In the procedure, each residue of protein receptor II was mutated to the neutral alanine (Ala) and then calculated the change in complex binding free energy ($\Delta\Delta G$) upon the mutation; the $\Delta\Delta G > 0$ and <0 indicate the favorable and unfavorable contribution of a residue to complex binding, respectively. It can be seen from Fig. 3A that most receptor residues possess a positive value of $\Delta\Delta G$, suggesting that they are favorably responsible for the binding, since the receptor protein has been evolutionarily optimized to well match its cognate partner TGF-β in geometric shape and physiochemical property. Therefore, the alternation of most receptor residues to the neutral Ala would undermine the compatibility between the TGF-β and its receptor and cause unfavorable effect to the TGF-β–receptor interaction. Most unfavorable mutations can only change the total binding energy slightly ($\Delta\Delta G < 0.5$ kcal/mol). This is expected since a majority of receptor residues are not present at the complex interface that would influence the complex interaction limitedly. However, a peptide segment that corresponds to the residues 50–56 seems to play an important role in the complex binding; substitution of these residues would cause a large free energy loss ($\Delta\Delta G > 1$ kcal/mol). This segment can be considered as a core binding region of the complex. In addition, two extended regions (residues 44–49 and 57–61) flanking the core binding region are also responsible for the binding, although their contributions are not very significant as compared to rest of the protein (Fig. 3A).

The core binding region and the two extended regions come together to define a binding loop of receptor protein to TGF-β (Fig. 3B). This loop can be identified at the complex interface in terms of the residue contribution profile, which is thought to play a central role in peptide-mediated interaction [31] of the TGF-β–receptor complex. Thus, we considered that the loop segment can be used as a self-inhibitory peptide [32] to disrupt the interaction. The binding loop region as well as the core binding region were extracted from the TGF-β–receptor complex crystal structure, resulting in two linear peptide segments EL-peptide (^{44}CMSNLSITSIVEKPQEVC^{61}) and L-peptide (^{50}ITSIVEK^{56}), respectively (Fig. 4A). The two peptides were then subjected to 60-ns MD simulations, during which a large conformational variation can be observed and the peptides cannot fold into a structured confor-

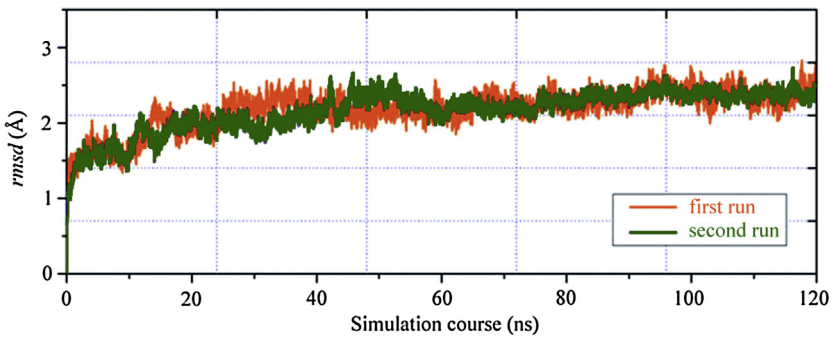

Fig. 2. The *rmsd* fluctuation during 120-ns MD simulations of TGF-β–receptor complex (run in duplicate).

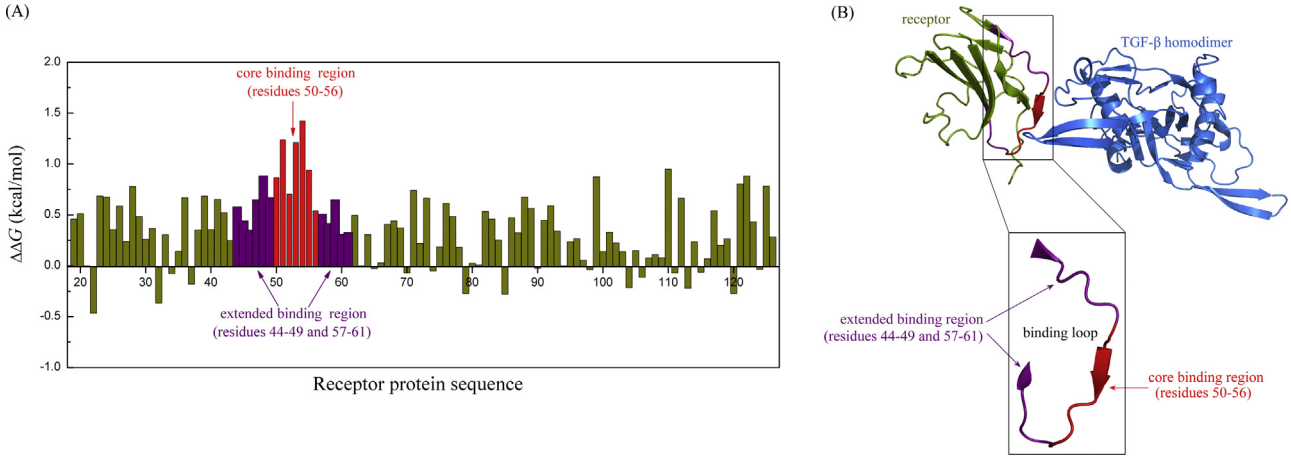

Fig. 3. (A) Histogram of the receptor residue contribution profile to TGF-β–receptor complex binding. The profile was generated by computational alanine scanning over all receptor residues (residues 19–126); i^{h} bar represents the change in complex binding free energy ($\Delta\Delta G$) upon mutation of the i^{h} receptor residue to alanine ($\Delta\Delta G > 0$ and <0 indicate the favorable and unfavorable contribution of a residue to the binding, respectively). (B) The receptor binding loop extracted from the complex interface in terms of the residue contribution profile; the loop consists of a core binding region (residues 50–56) and two extended binding regions (residues 44–49 and 57–61).

Table 2
The binding energy and affinity of TGF-β complex systems.

System	Peptide sequence	Binding energy decomposition (kcal/mol)				Dissociation constant K_d (μM)
		ΔE_{int}	ΔG_{dslv}	$-T\Delta S$	ΔG	
TGF-β–receptor	/	−164.0	115.4	25.2	−23.4	N.A.[a]
TGF-β–L-peptide	^{50}ITSIVEK^{56} (linear)	−67.3	38.9	25.7	−2.7	N.D.[b]
TGF-β–EL-peptide	^{44}CMSNLSITSIVEKPQEVC^{61} (linear)	−124.9	71.0	52.1	−1.8	N.D.[b]
TGF-β–C-peptide	CMSNLSITSIVEKPQEVC (cyclic)	−117.2	85.1	18.5	−13.6	54 ± 8
TGF-β–CM1-peptide	CMSNLSFTSIVEKPQEVC (cyclic)	−122.6	86.3	20.1	−16.2	26 ± 3
TGF-β–CM2-peptide	CMSNLSITSIVLKPQEVC (cyclic)	−125.1	88.2	19.5	−17.4	7 ± 1

[a] N.A., not assayed.
[b] N.D., not detectable.

mation even the simulations were prolonged to 200 ns, exhibiting considerable intrinsic disorder (Fig. 4B). The total binding behavior of L-peptide, EL-peptide and, for comparison purpose, receptor to TGF-β was examined using MM/PBSA and NMA, and the resulting binding free energy and decomposed energetic terms are listed in Table 2. It is seen that the two peptides can only bind to TGF-β with a very low energy ($\Delta G = -2.7$ and -1.8 kcal/mol, respectively), indicating a weak interaction potency between TGF-β and the two linear peptides. In contrast, the intact receptor protein can bind tightly to TGF-β with $\Delta G = -23.4$ kcal/mol. This is expected if considering that the contextual specificity and global evolutionary mechanism have been reported to have a significant effect on the binding stability and specificity of peptide-mediated interactions [33].

The total binding energy was decomposed to intermolecular interaction potential ΔE_{int}, desolvation effect ΔG_{dslv} and entropy penalty $-T\Delta S$. It is found that the direct interaction contributes very favorably to the binding of L-peptide and EL-peptide ($\Delta E_{int} = -67.3$ and -124.9 kcal/mol, respectively), whereas the desolvation and entropy exhibit large favorable effect on the binding ($\Delta G_{dslv} = 38.9$ and 71.0 kcal/mol, $-T\Delta S = 25.7$ and 52.1 kcal/mol, respectively). In particular, the entropy penalty increases consistently with the length extension from L-peptide to EL-peptide ($-T\Delta S$ changes from 25.7 and 52.1 kcal/mol), suggesting that the

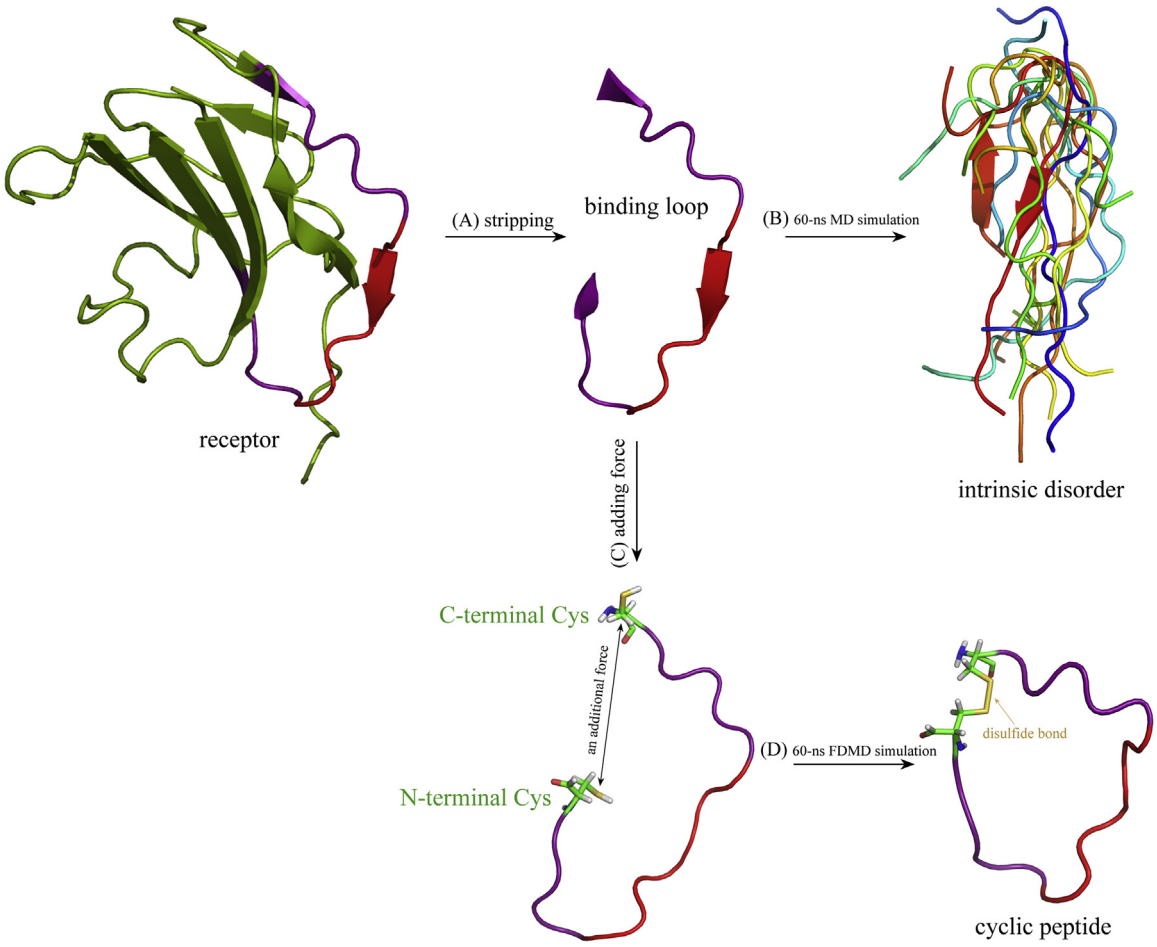

Fig. 4. The binding loop region was stripped from the crystal structure of receptor protein in complex with TGF-β (A), which was then subjected to 60-ns MD simulations, exhibiting a considerable intrinsic disorder (B). Instead, a force was added between the two sulfur atoms of N- and C-terminal cysteine (Cys) residues of the binding loop (C), and then subjected to 60-ns FDMD simulations, resulting in a cyclic peptide (D).

linear peptide ligands are highly flexible that would incur a large penalty from freezing peptide conformation upon binding to TGF-β. Subsequently, the L-peptide to EL-peptide was synthesized, purified and confirmed, and their dissociation constants (K_d values) with TGF-β were determined using fluorescence anisotropy assays. As might be expected, we did not observe detectable affinity between the TGF-β and the two peptides (K_d = N.D.), suggesting that the linear peptide ligands cannot bind effectively to TGF-β in their free state due to the considerable difficulty of peptide conformational selection in the binding [34]. Recently, the entropy penalty has been revealed to play a central role in TGF-β–peptide recognition [35].

Several chemical strategies have been used to constrain the conformational degree of freedom of peptide ligands in free state and thus minimize the entropy penalty of peptide binding, including hydrocarbon stapling [36] and cyclization [37]. Since the hydrocarbon stapling technique can only be applied for helical peptides, which is not the current case, we herein considered to constrain the EL-peptide conformation by cyclizing the peptide. Theoretically, this is feasible because (i) the EL-peptide is sufficiently long to form a closed cycle without additional conformational distortion and the N- and C-terminal residues of the peptide both are cysteine (Cys), which can readily form a disulfide bond between them to cyclize the peptide. The standard length of disulfide bond is 2.05 Å, and thus we need first to force the distance between the sulfur atoms of the two Cys residues to this length. Here, we employed a force-directed MD simulation to fulfill this. As seen in Fig. 5, the EL-peptide segment stripped from its parent protein stands in a conformation as that in protein context; the distance between the two sulfur atoms of its terminal Cys residues is to larger to form a covalent disulfide bond and thus we performed FDMD simulations to shorten this distance. In the simulation procedure a force was added between the two sulfur atoms; the force is described by a harmonic oscillator model with equilibrium distance = 2.05 Å and force constant = 10 kcal/mol/Å. By ~30-ns simulation the distance between the two sulfur atoms reaches at the equilibrium distance; the simulation was continued to 60 ns to ensure the entire system (peptide + water molecules) equilibrated. After that, a disulfide bond was artificially added between the two sulfur atoms to cyclize the peptide, and then an additional 60-ns routine MD simulation was performed to equilibrate the artificial cyclic peptide.

The cyclic peptide (C-peptide) possesses a low flexibly as compared to the linear EL-peptide. As can be seen in Table 2, the binding free energy ΔG of C-peptide was calculated to −13.6 kcal/mol; this value is significantly favorable as compared to linear EL-peptide (ΔG = −1.8 kcal/mol), suggesting that the cyclization can effectively promote peptide binding to TGF-β. Energetic decomposition revealed that EL-peptide can interact tightly with TGF-β (ΔE_{int} = −124.9 kcal/mol). However, predominant entropy penalty ($-T\Delta S$ = 52.1 kcal/mol) and desolvation free energy (ΔG_{dslv} = 71.0 kcal/mol) can largely offset the favorable interaction, resulting in a very low binding energy (ΔG = −1.8 kcal/mol). Although the cyclized C-peptide has only a relatively low interaction potency (ΔE_{int} changes from −124.9 to −117.2 kcal/mol) and

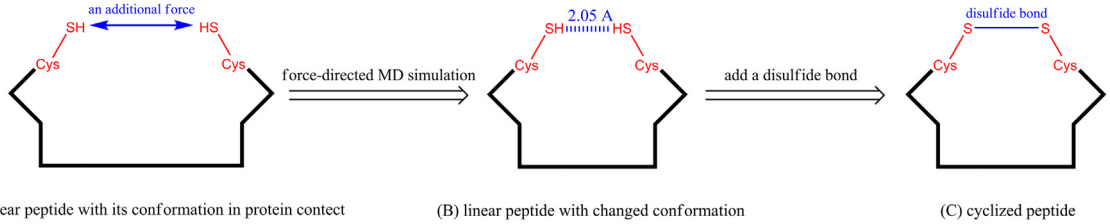

Fig. 5. Schematic representation of the force-directed MD simulations of a linear peptide as well as cyclization of the peptide by adding a disulfide bond between two sulfur atoms of its terminal cysteine (Cys) residues.

exhibits a more unfavorable desolvation as compared to EL-peptide (ΔG_{dslv} increases from 71.0 to 85.1 kcal/mol), entropy penalty is indeed minimized largely upon the cyclization ($-T\Delta S$ decreases from 52.1 to 18.5 kcal/mol). Consequently, the total binding free energy is improved for C-peptide relative to EL-peptide. This can be substantiated by *in vitro* binding assays; the cyclization alters the peptide from a nonbinder EL-peptide (K_d = N.D.) to a moderate binder C-peptide (K_d = 54 μM) of TGF-β, confirming that the minimizing entropy penalty is feasible strategy to design potent peptide ligands.

Next, by visually examining the intermolecular interaction between TGF-β and C-peptide based on their complex structure modeled computationally, we intuitively identified a number of residues that could be changed by other amino acids to further improve the binding capability of C-peptide. As a trial, we separately replaced the hydrophobic residue Ile50 by an aromatic amino acid Phe (I50F mutation) and the negatively charged residue Glu55 to hydrophobic amino acid Leu (E55L mutation), resulting in two mutants of C-peptide, namely, CM1-peptide and CM2-peptide, respectively. The former is expected to constitute an additional π-π stacking and a number of van der Waals contacts between TGF-β and the mutated residue Phe50 of CM1-peptide, while the latter is thought to reduce the unfavorable desolvation and to enhance hydrophobic interaction between TGF-β and the mutated residue Leu55 of CM2-peptide. As tabulated in Table 2, the total binding free energies ΔG of CM1-peptide and CM2-peptide were estimated to −16.2 and −17.4 kcal/mol, suggesting that the two peptide mutants can bind more tightly than the native C-peptide (ΔG = −13.6 kcal/mol). This is in line with the findings of fluorescence anisotropy assays, with K_d values improved from 54 to 26 and 7 μM, respectively.

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References

[1] D. Shehab, A.H. Elgazzar, B.D. Collier, Heterotopic ossification, J. Nucl. Med. 43 (2002) 346–353.
[2] T.A. Balboni, R. Gobezie, H.J. Mamon, Heterotopic ossification: pathophysiology, clinical features, and the role of radiotherapy for prophylaxis, Int. J. Radiat. Oncol. Biol. Phys. 65 (2006) 1289–1299.
[3] X. Yan, Z. Liu, Y. Chen, Regulation of TGF-β signaling by smad7, Acta Biochim. Biophys. Sin. 41 (2009) 263–272.
[4] G. Chen, C. Deng, Y.P. Li, TGF-β signaling in osteoblast differentiation and bone formation, Int. J. Biol. Sci. 8 (2012) 272–288.
[5] C. Zhang, Y. Zhang, B. Zhong, C.F. Luo, SMAD7 prevents heterotopic ossification in a rat Achilles tendon injury model *via* regulation of endothelial-mesenchymal transition, FEBS J. 283 (2016) 1275–1285.
[6] S. Wang, X.M. Meng, Y.Y. Ng, F.Y. Ma, S. Zhou, Y. Zhang, C. Yang, X.R. Huang, J. Xiao, Y.Y. Wang, S.M. Ka, Y.J. Tang, A.C. Chung, K.F. To, D.J. Nikolic-Paterson, H.Y. Lan, TGF-β/Smad3 signalling regulates the transition of bone marrow-derived macrophages into myofibroblasts during tissue fibrosis, Oncotarget 7 (2016) 8809–8822.
[7] D. Chen, S. Liu, W. Zhang, L. Sun, Rational design of YAP WW1 domain-binding peptides to target TGFβ/BMP/Smad-YAP interaction in heterotopic ossification, J. Pept. Sci. 21 (2015) 826–832.
[8] M. Shi, J. Zhu, R. Wang, X. Chen, L. Mi, T. Walz, T.A. Springer, Latent TGF-β structure and activation, Nature 474 (2011) 343–349.
[9] J. Groppe, C.S. Hinck, P. Samavarchi-Tehrani, C. Zubieta, J.P. Schuermann, A.B. Taylor, P.M. Schwarz, J.L. Wrana, A.P. Hinck, Cooperative assembly of TGF-beta superfamily signaling complexes is mediated by two disparate mechanisms and distinct modes of receptor binding, Mol. Cell 29 (2008) 157–168.
[10] J. Massagué, J. Andres, L. Attisano, S. Cheifetz, F. López-Casillas, M. Ohtsuki, J.L. Wrana, TGF-beta receptors, Mol. Reprod. Dev. 32 (1992) 99–104.
[11] K. Fosgerau, T. Hoffmann, Peptide therapeutics: current status and future directions, Drug Discov. Today 20 (2015) 122–128.
[12] J.M. Word, S.C. Lovell, J.S. Richardson, D.C. Richardson, Asparagine and glutamine: using hydrogen atom contacts in the choice of side-chain amide orientation, J. Mol. Biol. 285 (1999) 1735–1747.
[13] J.C. Gordon, J.B. Myers, T. Folta, V. Shoja, L.S. Heath, A. Onufriev, H++: a server for estimating pKa and adding missing hydrogens to macromolecules, Nucleic Acids Res. 33 (2005) W368–W371.
[14] J. Ko, D. Lee, H. Park, E.A. Coutsias, J. Lee, C. Seok, The FALC-loop web server for protein loop modeling, Nucleic Acids Res. 39 (2011) W210–W214.
[15] Y. Duan, C. Wu, S. Chowdhury, M.C. Lee, G.M. Xiong, W. Zhang, A point-charge force field for molecular mechanics simulations of proteins based on condensed-phase quantum mechanical calculations, J. Comput. Chem. 24 (2003) 1999–2012.
[16] D.A. Case, T.E. Cheatham, T. Darden, H. Gohlke, R. Luo, K.M. Merz, A. Onufriev, C. Simmerling, B. Wang, R.J. Woods, The amber biomolecular simulation programs, J. Comput. Chem. 26 (2005) 1668–1688.
[17] W.L. Jorgensen, J. Chandrasekhar, J.D. Madura, R.W. Impey, M.L. Klein, Comparison of simple potential functions for simulating liquid water, J. Phys. Chem. 79 (1983) 926–935.
[18] C. Yang, S. Zhang, P. He, C. Wang, J. Huang, P. Zhou, Self-binding peptides: folding or binding, J. Chem. Inf. Model. 55 (2015) 329–342.
[19] P. Zhou, S. Zhang, Y. Wang, C. Yang, J. Huang, Structural modeling of HLA-B*1502/peptide/carbamazepine/T-cell receptor complex architecture: implication for the molecular mechanism of carbamazepine-induced Stevens-Johnson syndrome/toxic epidermal necrolysis, J. Biomol. Struct. Dyn. 34 (2016) 1806–1817.
[20] J.P. Ryckaert, G. Ciccotti, H.J.C. Berendsen, Numerical integration of the cartesian equations of motion of a system with constraints: molecular dynamics of *n*-alkanes, J. Comput. Phys. 23 (1997) 327–341.
[21] T. Darden, D. York, L. Pedersen, Particale mesh Ewald and N.log(N) method for Ewald sums in large systems, J. Chem. Phys. 98 (1993) 10089–10092.
[22] I. Bošnjak, V. Bojović, T. Šegvić-Bubić, A. Bielen, Occurrence of protein disulfide bonds in different domains of life: a comparison of proteins from the protein data bank, Protein Eng. Des. Sel. 27 (2014) 65–72.
[23] N. Homeyer, H. Gohlke, Free energy calculations by the molecular mechanics poisson-boltzmann surface area method, Mol. Inf. 31 (2012) 114–122.
[24] D. Sitkoff, K.A. Sharp, B. Honig, Accurate calculation of hydration free energies using macroscopic solvent models, J. Phys. Chem. 98 (1994) 1978–1988.
[25] P.A. Kollman, I. Massova, C. Reyes, B. Kuhn, S. Huo, L. Chong, M. Lee, T. Lee, Y. Duan, W. Wang, O. Donini, P. Cieplak, J. Srinivasan, D.A. Case, T.E. Cheatham, Calculating structures and free energies of complex molecules: combining molecular mechanics and continuum models, Acc. Chem. Res. 33 (2000) 889–897.
[26] T. Hou, K. Chen, W.A. McLaughlin, B. Lu, W. Wang, Computational analysis and prediction of the binding motif and protein interacting partners of the Abl SH3 domain, PLoS Comput. Biol. 2 (2006) e1.
[27] M. Peräkylä, N. Nordman, Energetic analysis of binding of progesterone and 5 beta-androstane-3,17-dione to anti-progesterone antibody DB3 using molecular dynamics and free energy calculations, Protein Eng. 14 (2001) 753–758.
[28] F. Lefèvre, M.H. Rémy, J.M. Masson, Alanine-stretch scanning mutagenesis: a simple and efficient method to probe protein structure and function, Nucleic Acids Res. 25 (1997) 447–448.
[29] M.E. Ivanova, G.C. Fletcher, N. O'Reilly, A.G. Purkiss, B.J. Thompson, N.Q. McDonald, Structures of the human Pals1 PDZ domain with and without ligand suggest gated access of Crb to the PDZ peptide-binding groove, Acta Crystallogr. D Biol. Crystallogr. 71 (2015) 555–564.

[30] L.Y. Chan, S. Gunasekera, S.T. Henriques, N.F. Worth, S.J. Le, R.J. Clark, J.H. Campbell, D.J. Craik, N.L. Daly, Engineering pro-angiogenic peptides using stable, disulfide-rich cyclic scaffolds, Blood 118 (2011) 6709–6717.
[31] E. Petsalaki, R.B. Russell, Peptide-mediated interactions in biological systems: new discoveries and applications, Curr. Opin. Biotechnol. 19 (2008) 344–350.
[32] N. London, B. Raveh, D. Movshovitz-Attias, O. Schueler-Furman, Can self-inhibitory peptides be derived from the interfaces of globular protein-protein interactions, Proteins 78 (2010) 3140–3149.
[33] A. Stein, P. Aloy, Contextual specificity in peptide-mediated protein interactions, PLoS One 3 (2008) e2524.
[34] S. Gianni, J. Dogan, P. Jemth, Distinguishing induced fit from conformational selection, Biophys. Chem. 189 (2014) 33–39.
[35] Z. Zhu, C. Zhang, W. Song, Self-inhibitory peptides to target TGF-β in ONFH, Amino Acids (2016).
[36] P.M. Cromm, J. Spiegel, T.N. Grossmann, Hydrocarbon stapled peptides as modulators of biological function, ACS Chem. Biol. 10 (2015) 1362–1375.
[37] J.S. Davies, The cyclization of peptides and depsipeptides, J. Pept. Sci. 9 (2003) 471–501.