Asparaginyl Endopeptidase-Mediated Peptide Ligation and Cyclization for Phage Display

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Abstract: Genetically encoded cyclic peptide libraries are invaluable for peptide drug discovery. Here we report an enzymatic strategy for asparaginyl endopeptidase-mediated peptide ligation and cyclization, and its application in the construction of phage-displayed cyclic peptide libraries. Introduction of a low-reactive chloroacetyl group into the tripeptide recognition sequence of *Oa*AEP1 allows intramolecular cyclization with Cys residues to generate macrocyclic peptides. By optimzing *Oa*AEP1 activation conditions and *Oa*AEP1-catalyzed peptide ligation, we establish an efficient *Oa*AEP1-based enzymatic peptide ligation under acidic conditions. The *Oa*AEP1-based enzymatic ligation is fully compatible with phage display and enables the construction of genetically encoded monocyclic and bicyclic peptide libraries. By using *Oa*AEP1-based phage display, we identify macrocyclic peptide ligands targeting TEAD4 at the nanomolar level. One of the bicyclic peptides binds to TEAD4 with a K_D value of 139 nM, 16-fold lower than its linear analogue, indicating the contribution of the bicyclic scaffold to its biological activity and demonstrating the utility of the technology platform in the discovery of high-affinity cyclic peptide ligands.

Introduction

Macrocyclic peptides are invaluable scaffolds for the discovery and development of peptide drugs.[1-3] Compared with linear peptides, cyclization modification imparts structural rigidity to macrocyclic peptides, making them more suitable for drug development in terms of enzymatic stability, target affinity, target specificity, and cell membrane permeability.[4-7] Macrocyclic peptides have larger molecular surfaces than traditional small molecules and are perceived to be well suited for blocking protein-protein interactions.[8] Many natural products belonging to macrocyclic peptides, such as cyclosporin, somatostatin, and ziconotide, have been approved as clinical drugs.[9-10] Two leading companies in the field of peptide drug discovery including Bicycle Therapeutics and Peptidream have advanced several macrocyclic peptides into clinical trials. Considering the increasingly important role of macrocyclic peptides in drug discovery, new technologies for large-scale combinatorial cyclic peptide libraries will facilitate the development of peptide drugs.[11,12]

Two important parameters for a combinatorial cyclic peptide library are library size in terms of the number of the molecules and library diversity in terms of molecular structure.[13-14] Compared with synthetic combinatorial chemical libraries, genetically encoded combinatorial libraries, such as phage display and mRNA display, are easier to provide large-scale libraries and are more amenable to multiple rounds of iterative screening.[15] Phage display is widely used for genetically encoded peptide libraries due to its relatively low cost and ease of operation.[16] The core of constructing a phage-displayed cyclic peptide library is to introduce a structurally rigid scaffold into phage library without affecting the endogenous nucleic acid coding system.

Traditional phage-displayed cyclic peptide libraries utilize air oxidation of cysteine residues to form disulfide-tethered cyclic peptides.[17-19] The representative of this direction is the disulfide bond-directing motifs invented by Wu *et al*., which can be used to construct multicyclic peptide libraries on the phage surface.[20-23] Mild air oxidation conditions have little effect on phage infectivity, but the disulfide bond has inherent drawbacks in terms of reductive instability.[24,25] Chemical macrocyclization of specific amino acids on the phage surface is another attractive strategy for the constructing cyclic peptide libraries, as illustrated by the pioneering phage-displayed bicyclic peptides invented by Heinis *et al*.[26-28] Importantly, Derda *et al*. recently modified two cysteine residues of a phage library with dichloroacetone and then perform Knorr pyrazole reaction to incorporate various unnatural pharmacophores.[29-31] However, alkylation of cysteine residues under weakly alkaline conditions sometimes induces side reactions and phage toxicity.[32-36]

Enzyme-based peptide macrocyclization has emerged as another option for constructing genetically encoded cyclic peptide libraries.[37] Through introducing the pyrrolysyl-tRNA synthetase/tRNA pairing system, orthogonal reactive unnatural amino acids can be incorporated into the phage library to establish a cyclic peptide library.[38-42] However, the yields of phages containing unnatural amino acids were lower than those of phages containing natural amino acids.[43] Another enzyme-based strategy is to use peptide-modifying enzymes, such as the lanthiopeptide synthetase and tyrosinase, to macrocyclize phage-displayed peptide libraries.[44-49] Compared to chemical macrocyclization, enzyme-based approaches do not involve highly reactive electrophiles and have little impact on the phage infectivity. Asparaginyl endopeptidases, such as OaAEP1 and butelasel, can act as ligases to catalyze the transpeptidylation of a short tripeptide motif Asn-Xaa_1-Xaa_2 with another N-terminal Gly-Leu peptide.[50-56] To date, asparagine endopeptidases have been used for protein semisynthesis and surface modification of living bacterial cells, but have not been used to construct genetically encoded cyclic peptide libraries such as phage display and mRNA display.[57,58]

Herein we report an asparaginyl endopeptidase-mediated peptide ligation and cyclization strategy and its application to phage display. *Oa*AEP1 cleaves the tripeptidic Asn-Gly-Leu motif at the Asn-Gly junction to form a thioester intermediate that can connect with another N-terminal dipeptidic Gly-Leu motif. In our *Oa*AEP1-based strategy, a low-reactive electrophilic

chloroacetyl group is introduced onto the N-terminal amino group of the tripeptidic Asn-Gly-Leu motif. After peptide ligation catalyzed by *Oa*AEP1, the chloroacetyl group undergoes intramolecular cyclization with cysteine residue to form a macrocyclic peptide. By the combination of the *Oa*AEP1-based strategy and phage display, we establish phage-displayed cyclic peptide libraries to screen cyclic peptide ligands targeting TEAD4 and identify several macrocyclic peptides with nanomolar K_D values. The *Oa*AEP1-based phage-displayed cyclic peptide libraries will provide a powerful platform for the discovery of high-affinity cyclic peptide ligands.

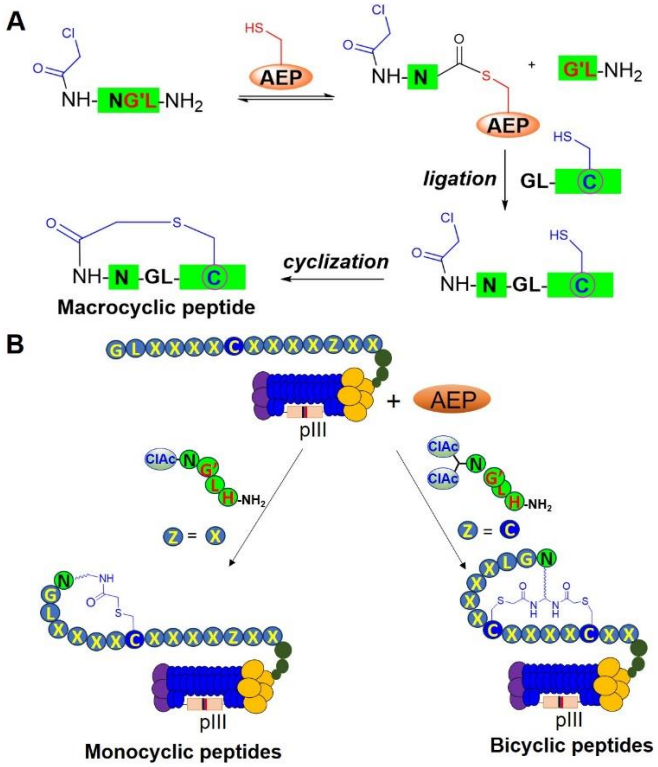

Fig. 1 Asparaginyl endopeptidase-mediated peptide cyclization for phage display. (A) Schematic representation of *Oa*AEP1-mediated peptide ligation and cyclization. (B) OaAEP1-based phage display cyclic peptide libraries (G': glycolic acid, ClAc: chloroacetyl group).

Table 1 *Oa*AEP1[C247A] activation under different conditions

Entry	pH	Buffer	Activate[f]	Precipitate[g]
1	6.5	NaOAc/EDTA/TCEP[a]	-	-
2	6.0	NaOAc/EDTA/TCEP	-	+
3	5.5	NaOAc/EDTA/TCEP	-	+
4	5.0	NaOAc/EDTA/TCEP	-	+
5	4.5	NaOAc/EDTA/TCEP	+	+
6	4.0	NaOAc/EDTA/TCEP	+	+
7	4.0	Tris/NaCl/AcOH[b]	+	+
8	4.0	AcOH/TCEP[c]	+	-
9	4.0	NaOAc[d]	+	-
10	4.0	NH_4OAc[e]	+	-

[a]50 mM NaOAc, 0.5 mM TCEP, 1.0 mM EDTA, pH adjusted by HCl. [b]20 mM Tris, 500 mM NaCl, pH adjusted by AcOH. [c]0.5 mM TCEP, pH adjusted by AcOH. [d]50 mM NaOAc, pH adjusted by HCl. [e]50 mM NH_4OAc, pH adjusted by HCl. [f] "–" indicates the presence of a large amount of unactivated *Oa*AEP1[C247A] in SDS-PAGE analysis; "+" indicates that *Oa*AEP1[C247A] was completely activated. [g]"–" indicates no precipitation of *Oa*AEP1[C247A]; "+" indicates the precipitation of *Oa*AEP1[C247A].

Results and Discussion

Optimization of the conditions for *Oa*AEP1 activation

Our study began with the activation of asparaginyl endopeptidase. We chose the engineered AEP *Oa*AEP1[C247A] invented by Wu *et al.*,[52] which can be expressed in *E. coil* and is better than the parent WT *Oa*AEP1 in enzymatic ligation.

*Oa*AEP1[C247A] was incubated in 100 mM sodium acetate buffer (0.5 mM TCEP, 1.0 mM EDTA, pH4) for 5 h at 37℃. As reported previously, we encountered protein precipitation but with little activated *Oa*AEP1[C247A] in the supernatant. Through SDS-PAGE analysis, we found a large amount of *Oa*AEP1[C247A] in the precipitate, indicating that asparaginyl endopeptidase has been denatured. The amount of activated *Oa*AEP1[C247A] under these conditions is insufficient for subsequent enzymatic peptide ligation. To this end, we optimized the activation conditions to avoid the precipitation of *Oa*AEP1[C247A]. Fortunately, we found that *Oa*AEP1[C247A] did not precipitate in the sodium acetate buffer when EDTA was not added (**Table 1**, Entry 9). SDS-PAGE analysis confirmed the presence of a large amount of activated *Oa*AEP1[C247A] in the supernatant (Fig. S3). The EDTA-free activation condition will provide a practical solution for the use of *Oa*AEP1[C247A].

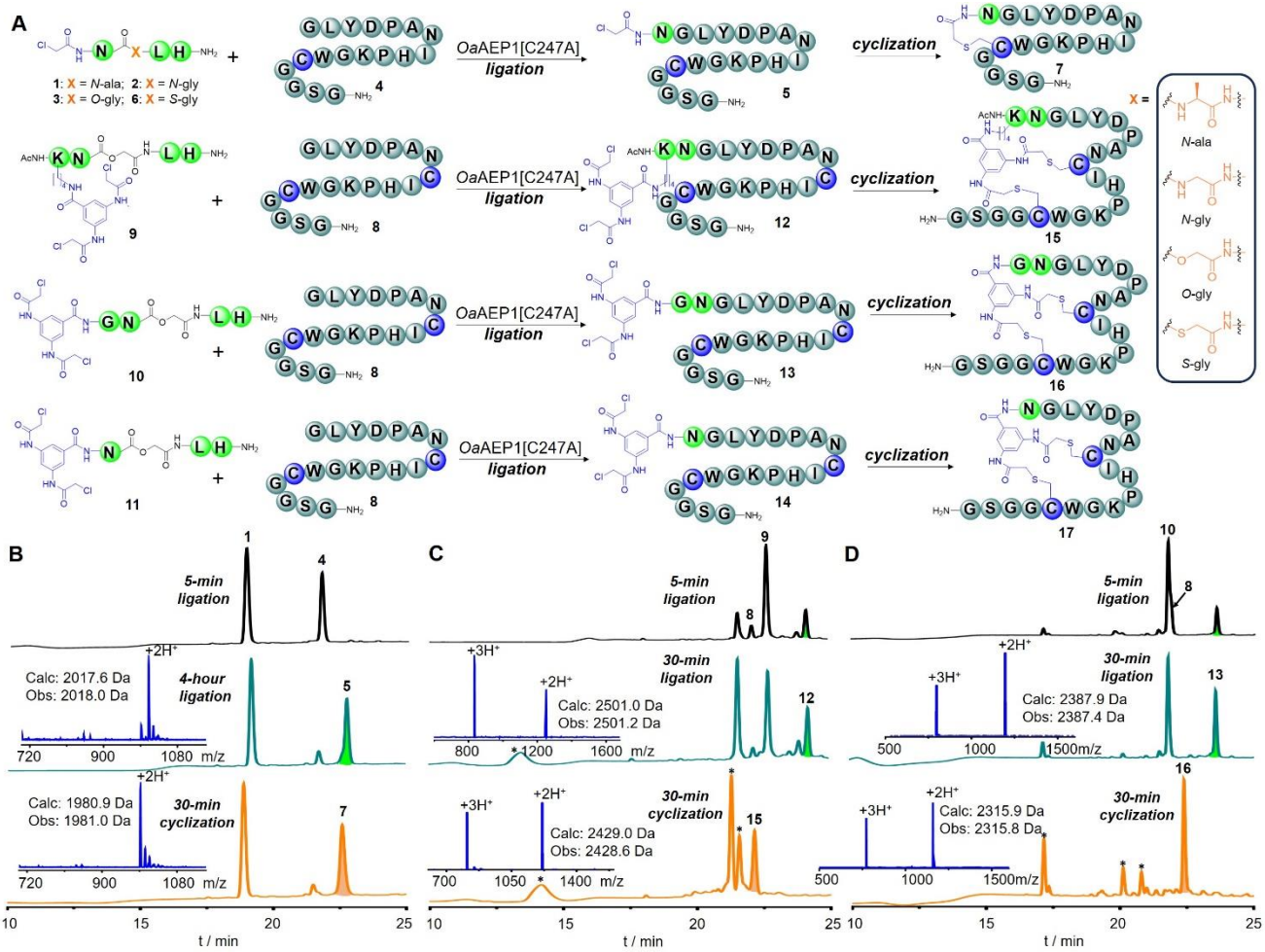

Fig. 2 *Oa*AEP1[C247A]-mediated peptide ligation and cyclization. (A) Generation of monocyclic and bicyclic peptides through *Oa*AEP1[C247A]-mediated peptide ligation and cyclization. (B) HPLC chromatograms (210 nm) of *Oa*AEP1[C247A]-mediated ligation and cyclization of tetrapeptide **1** (500 μM) and **4** (50 μM). (C) HPLC chromatograms (210 nm) of *Oa*AEP1[C247A]-mediated ligation and cyclization of pentapeptide **9** (500 μM) and **8** (50 μM). (D) HPLC chromatograms (210 nm) of *Oa*AEP1[C247A]-mediated ligation and cyclization of pentapeptide **10** (500 μM) and **8** (50 μM). Conditions for *Oa*AEP1[C247A] activation: 50 mM NaOAc, pH 4.0, 0.43 μg/μL of *Oa*AEP1[C247A], 37℃, 5 h. Conditions for enzymatic ligation: 50 mM NaOAc, pH 5.0, 4℃, 30 min or 4 h. Conditions for peptide cyclization: 50 mM NaOAc, 1.0 mM TCEP, pH 8.0, 37℃, 30 min. The peaks labeled by "*" are produced by hydrolysis of the chloroacetyl-containing depsipeptide at pH8 (Fig. S38 and Fig. S41).

*Oa*AEP1-catalyzed peptide ligation

We synthesized three tetrapeptides (**1**, **2** and **3**) bearing a chloroacetyl group at the N terminus (Fig. 2A), all of which contain the Asn-Gly-Leu motif or its analogues that can be recognized by *Oa*AEP1[C247A]. Peptide **4** contains an N-terminal dipeptidic Gly-Leu motif for *Oa*AEP1-mediated peptide ligation and an internal cysteine residue for intramolecular cyclization with the chloroacetyl moiety. To simplify the operation, the crude activated *Oa*AEP1[C247A] was used directly as the catalyst for peptide ligation. Peptide **1** (500 μM) and peptide **4** (50 μM) were co-incubated in 50 mM HEPES buffer (50 mM HEPES, 100 mM NaCl, 2.0 mM $CaCl_2$, 1.0 mM TCEP, 5.0 μM of activated *Oa*AEP1[C247A], pH7.7). After 2 h, we observed the desired ligation product **5**, but the HPLC yield was less than 20% (Fig. S16). When pH value of the reaction mixture was adjusted to 6.6, the ligation yield increased to 38% (Fig. S17). Note that the activated *Oa*AEP1[C247A] precipitates under the two conditions, which may be responsible for the low ligation yield.

Then, we optimized the enzymatic ligation conditions for *Oa*AEP1[C247A]. First, *Oa*AEP1[C247A] activation and the subsequent peptide ligation were performed in sodium acetate buffer with the same pH value. Although *Oa*AEP1[C247A] did not precipitate at pH 3.5, no ligation product was observed (Fig. S18). At pH4.5, approximately 46% of **4** was converted to the ligation product, but *Oa*AEP1[C247A] remained precipitated (Fig. S19). To accelerate the enzymatic ligation, *Oa*AEP1[C247A] was activated at pH4, but the peptide ligation reaction was performed at pH5. Gratifyingly, approximately 87% of **4** reacts with **1** to afford the desired ligation product **5** (Fig. 2B) in four hours, although *Oa*AEP1[C247A] still precipitated at pH5. Under the conditions, the reaction of **2** (the Gly mutant of **1**) and **4** gives a HPLC yield of 67% in 1 hour (Fig. S24).

Next, we investigated the enzymatic ligation between the depsipeptide **3** (500 μM) and **4** (50 μM).[59,60] To our delight, **4** was completely converted to the ligation product **5** in 30 minutes (Fig. S25). Inspired by the efficient ligation of the depsipeptide substrate, we tested the ligation of the thioester peptide **6** and **4**. Similar to **3**, the reaction for **6** and **4** was completed within 30 minutes (Fig. S30). Note that the chemical synthesis of **3** is much easier than that of **6**, so we chose the depsipepetide **3** as the substrate for the following test. Peptide ligation between **3** and **4** is quite fast, but the activated *Oa*AEP1[C247A] still precipitated after 15 minutes, which may reduce the yield of enzymatic modification of phage-displayed peptide libraries. To solve this problem, the temperature of ligation buffer (50 mM sodium acetate, pH5) was lowered from 37℃ to 25℃ or 4℃. To our delight, *Oa*AEP1[C247A] did not precipitate even after 3 hours at 4℃. At 4℃, the ligation of **4** and **3** was still completed in 30 minutes (Fig. S27). When the sodium acetate buffer was replaced by ammonium acetate buffer, the activation of *Oa*AEP1[C247A] and the peptide ligation between **3** and **4** also proceeded smoothly (Fig. S29).

We further studied the substrate scope of the *Oa*AEP1-catalyzed peptide ligation for the N-terminal Gly peptide. We replaced the N-terminal dipeptidic Gly-Leu motif of **4** with the dipeptidic Gly-Xaa motif (Xaa = Glu, Lys, His, Asp, Phe, Gly, Met, Pro, Ala, Arg, Trp, Thr, Val, Ile). Of these mutants, 12 peptides (except Val and Ile) did not react with **3** in the presence of the activated *Oa*AEP1[C247A]. After incubation for 2 hours, **4[Leu2Val]** and **4[Leu2Ile]**, provided ligation yields of 37% and 68% respectively (Fig. S31-S32), much lower than that of **4**, indicating that *Oa*AEP1[C247A] prefers the N-terminal dipeptidic Gly-Leu motif.

*Oa*AEP1-mediated peptide cyclization

Macrocyclization of the ligation product was performed in pH8 buffer. TCEP (Tris(2-carboxyethyl)phosphine, 1 mM) was added to the ligation reaction mixture containing the ligation product **5**, and then the pH value was adjusted to 8. After 30-min incubation at 37℃, HPLC analysis confirmed the complete conversion of **5** to the desired cyclized peptide **7** (Fig. 2B).

We then explored the *Oa*AEP1[C247A]-mediated peptide ligation and bicyclization. Three depsipeptides containing two chloroacetyl groups were used to react with **8** under weakly acidic conditions. In the presence of *Oa*AEP1[C247A], **9**, **10**, and **11** react smoothly with **8** to produce the ligation products, **12**, **13**, and **14**, respectively (Fig. 2C-2D, Fig. S42). It is noteworthy that the depsipeptide **9** does not react with **8** in the absence of *Oa*AEP1[C247A] (Fig. S36), indicating that under these conditions the chloroacetyl group does not react with endogenous Cys residues of the pIII protein of the M13KE phage.

Next, we adjusted the pH of the reaction solution to 8 to perform bicyclization of the ligation products. To our delight, **12**, **13**, and **14** were quantitatively converted to the desired bicyclic peptide products (Fig. 2C-2D, Fig. S43). In pH8 buffer, the remaining depsipeptide was hydrolyzed, but had little effect on the macrocyclization of ligation product (Fig. S38, Fig. S41). During enzymatic modification of phage-displayed library, excessive unreacted depsipeptide can be readily removed from the phages by PEG8000 precipitation, avoiding side reactions with the phage-displayed peptide library at pH8. Note that, the two-fold symmetry of the two chloroacetyl moieties in the ligation products ensures that only one topological structure of the bicyclic peptide is formed, which will facilitate the identification of the bioactive bicyclic peptide ligands in phage display. *Oa*AEP1[C247A]-mediated peptide ligation and bicyclization will benefit the discovery of highly potent peptide ligands via phage display.

*Oa*AEP1-mediated modification of phage library

We evaluated the effect of the enzymatic ligation conditions on phage infectivity. Gratifyingly, we observed little difference in titers between phage libraries treated with sodium acetate solution (pH5) and those treated with PBS buffer (Fig. S46). The yield of *Oa*AEP1[C247A]-mediated peptide ligation on phage library was then determined by a biotin-avidin capture assay. Starting from M13KE-GX_{12} phage library (a diversity of 10^6), a peptide sequence of GLSFYSHS was engineered into the N-terminus of the phage to afford the M13KE-GLSFYSHSGX_{12} phage library. The phage library was treated with **Biotin-1**, a chloroacetyl and biotin modified depsipeptide that can react with **4** under the catalysis of *Oa*AEP1[C247A] (Fig. S44). According to the protocol reported by Derda et al., the ligation yield of *Oa*AEP1-mediated phage library modification was determined to be 68% (Fig. S45).[29]

Selection of macrocyclic peptide ligands for TEAD4

We selected TEAD4 as a model target for phage screening. TEAD4 is a transcription factor that regulates cell proliferation and organ development by interacting with YAP/TAZ.[61] In order to obtain a large-scale phage library, we decided to use the phagemid system consisting of pCantab5E vector and M13KO7 helper phage.[62] To establish phage-displayed monocyclic peptide library, a peptide library of $GLX_{12}C$ (X is any natural amino acid) was inserted into the N-terminus of pIII protein to give a diversity of $1.5x10^9$. To establish phage-displayed bicyclic peptide library, a peptide library of GLX_6CX_6C (X is any natural amino acid) was inserted into the N-terminus of pIII protein to give a diversity of $1x10^9$.

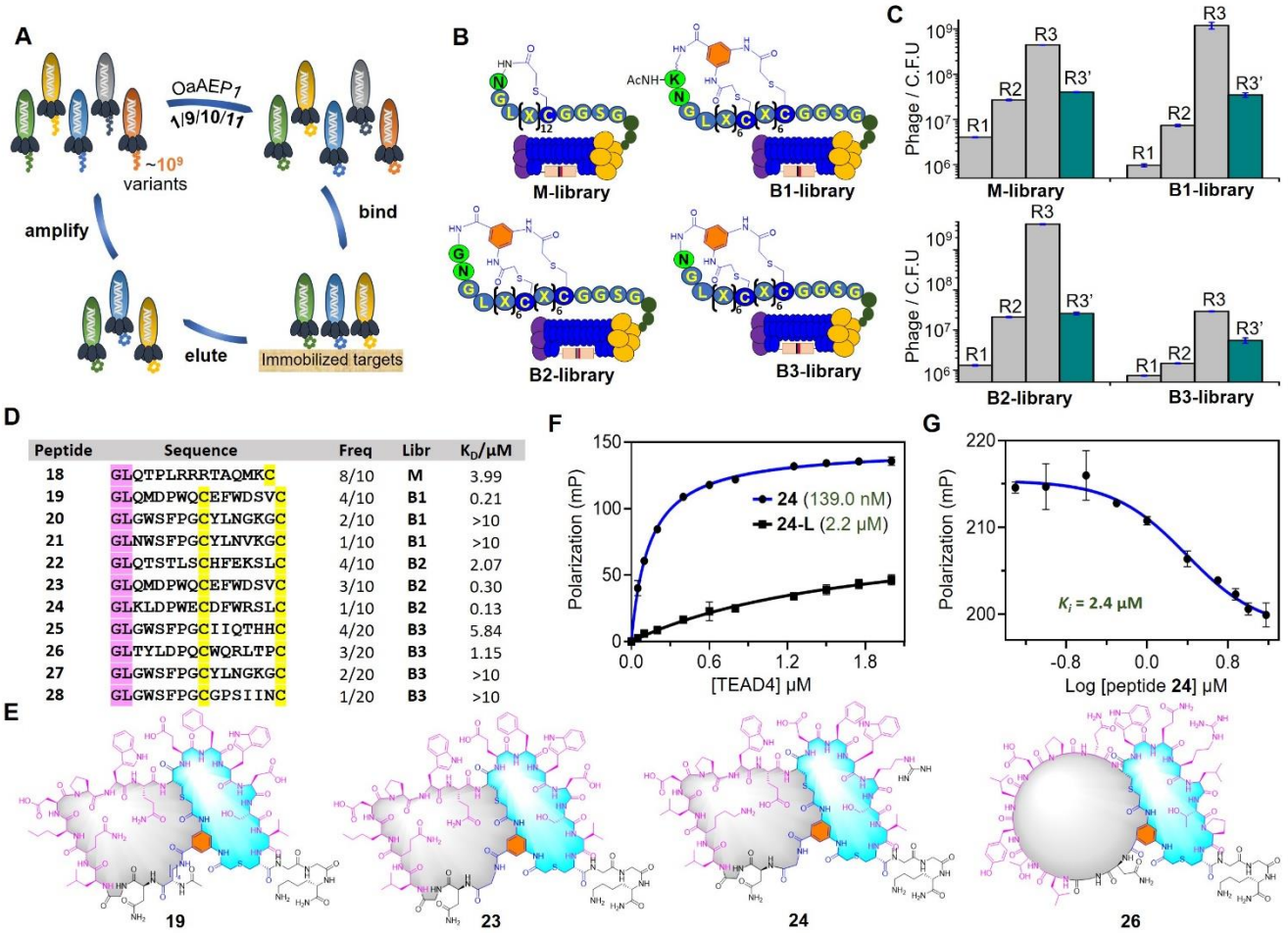

Fig. 3 *Oa*AEP1[C247A]-mediated phage display of cyclic peptides against TEAD4. (A) General procedure for the *Oa*AEP1-based phage display. (B) Four phage displayed cyclic peptide libraried constructed by *Oa*AEP1[C247A]-mediated peptide ligation and cyclization (**M-library** constructed by enzymatic modification of phage library with **3**; **B1-library** constructed by enzymatic modification of phage library with **9**; **B2-library** constructed by enzymatic modification of phage library with **10**, **B3-library** constructed by enzymatic modification of phage library with **11**). (C) Phage tilters after 3 rounds of selection against immobilized TEAD4 (R3' is negative selection using solely avidin-coated beads as the bait). (D) Enriched peptide sequences from phage biopanning (Freq: Frequency, Libr: Library). (E) Chemical structure of four representative macrocyclic peptides (the C-terminal GGK is used for the conjugation of FITC for fluorescence polarization assay) from the phage biopanning. (F) Binding affinity of **19** to TEAD4 determined by fluorescence polarization (**24-L** is the linear version of **24**). (G) Inhibition of TEAD4·YAP by **24** based on fluorescence polarization method.

We then conducted phage display to screen for monocyclic peptides targeting TEAD4. Phage-displayed $GLX_{12}C$ library was treated with peptide **3** and *Oa*AEP1 [C247A] in pH 5 buffer for 1 h at 4℃, followed by PEG8000 precipitation to remove the remaining **3**. Then, the obtained phage pellet was resuspended in 50.0 mM NaOAc buffer (pH8, 1.0 mM TCEP) and incubated at 37℃ for 30 min for peptide macrocyclization. The resulting phage-displayed monocyclic peptides were biopanned against immobilized TEAD4. After 3 rounds of selection, the amount of recovered phages increased 111-fold compared to the first round (Fig. 3C). Notably, the amount of phage recovered from TEAD4-coated beads was 10-fold higher than that recovered from TEAD4-unbound beads, indicating enrichment of phages targeting TEAD4. Sequencing of 10 randomly selected phage clones revealed one highly enriched peptide sequence (Fig. 3D).

Next, we performed phage display to screen for bicyclic peptides targeting TEAD4. We treated the phage library containing the GLX_6CX_6C peptide library with **9**, **10** and **11** respectively, and obtained three phage-displayed bicyclic peptide libraries of different topologies, which can increase the likelihood of screening high-affinity peptides against TEAD4. To our delight, after three rounds of selection, the recovery of the three phage libraries increased significantly compared with the first round (Fig. 3C). Of these, **10**-treated phage library provides up to 3111-fold enrichment compared to the first round and 168-fold enrichment compared to the phages captured by TEAD4-unbound beads. After sequencing randomly selected phage clones, we obtained ten enriched peptide sequences.

Finally, we chemically synthesized the enriched monocyclic and bicyclic peptides. The binding affinity of cyclic peptides to TEAD4 was determined by fluorescence polarization. As shown in Fig. 3D-3E, the K_D values of **19**, **23**, and **24** were measured to be 210 nM, 300 nM and 139 nM, respectively. Note that the binding affinity of the cyclic peptides is higher than that of their corresponding linear peptides. For example, the K_D value of **24** is 16 times lower than its linear version (Fig. 3F). In the competition assay with YAP, these macrocyclic peptides provided K_i values in the range of 1~2.4 μM (Fig.3G, Fig.

S76). Collectively, the discovery of the nanomolar peptide ligands for TEAD4 demonstrates the utility of *Oa*AEP1[C247A]-mediated peptide ligation and cyclization in the construction of phage-displayed cyclic peptide library.

Conclusions

In summary, we report here an enzymatic strategy for *Oa*AEP1[C247A]-mediated peptide ligation and cyclization to generate macrocyclic peptide library. The combination of the *Oa*AEP1[C247A]-based strategy with phage display establishes a new method for generating genetically encoded cyclic peptide libraries. In this platform, the low-reactive chloroacetyl group is introduced into the N-terminal amino group of the tripeptidic Asn-Gly-Leu motif, which allows for intramolecular cyclization with Cys residues after enzymatic ligation with an N-terminal Gly-Leu peptide. From a technical perspective, we first solved the problem of protein precipitation during the *Oa*AEP1[C247A] activation. Then, we utilized the depsipeptide as the substrate for *Oa*AEP1[C247A] and established an efficient *Oa*AEP1[C247A] -catalyzed peptide ligation system under acidic conditions. The weakly acidic reaction conditions have little effect on phage infectivity, completely avoiding non-specific side reactions between the chloroacetyl group and the Cys residues of pIII protein. The utility of *Oa*AEP1[C247A]-based phage display has been demonstrated by the discovery of nanomolar cyclic peptide ligands targeting TEAD4.

Author Contributions

The idea of the study was designed by G.-M. Fang. The expression and activation of *Oa*AEP1[C247A] was performed by X.-C. Wan. The *Oa*AEP1[C247A]-mediated peptide ligation and cyclization was performed by X.-C. Wan. The procedure for phage display were established by X.-C. Wan and Y.-N. Zhang. TEAD4-targeting cyclic peptides were synthesized and analysed by X.-C. Wan, Z.-H. Cui, Y. Chen, H. Zhang, W.-J. Zhu. The manuscript was written and checked by G.-M. Fang and X.-C. Wan.

Conflicts of interest

The authors declare there to be no conflicts of interest.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 22077002, 21807001). We thank Dr. Maximilian Fottner (ETH Zurich), Prof. Kathrin Lang (ETH Zurich), and Prof. Peng Zheng (Nanjing University) for their constructive suggestions on the activation of *Oa*AEP1[C247A]. We thank the staff for providing technical support with using the facility of Institute of Health Sciences & Technology, Anhui University.

Keywords: phage display • cyclic peptide library • enzyme-mediated cyclization • bicyclic peptide

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