学位論文（要約）

Development of macrocyclic peptide for the control of VP24-KPNA interaction and conformation dynamic of channelrhodopsin

（VP24-KPNA 相互作用とチャネルロドプシン構造を制御する大環状ペプチドの開発）

平成２８年１２月　博士（理学）申請

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Abstract (abridged)

In this study, I will describe my work toward discovering macrocyclic peptides against two proteins, which have their own intrinsic structural difficulty as a target.
One protein is involved in the protein-protein interaction (PPI)，which is considered to be difficult to target due to the large binding interface. The other protein is an open state of channelrhodopsin. At this state, the protein conformation is unstable and transient. To stabilize such protein, a molecule that is capable of binding to a large interface or recognizing a specific conformation of protein is required. Peptides are slightly larger in size and have certain flexibility, which allows them to have a good shape complementary to target protein, therefore have the potential to cover a large PPI interface as well as recognize specific conformation. The RaPID (Random non-standard Peptide Integrated Discovery) system enabled a rapid selection of binders with high-affinity and selectivity to target proteins. I used ed the RaPID system to generate macrocyclic peptides binders against eVP24 and channelrhodopsin to see whether these peptides are capable of inhibiting PPI or stabilizing the open state structure of channelrhodopsin.

In chapter 1, I introduce the superior features of peptides as well as the advantages of the RaPID system as a peptide discovery platform. At the same time, I also introduce the binding mode of macrocyclic to their targets and give a brief introduction about the reason to initialize the studies of thesis.

In chapter 2, I discuss the current state of targeting protein-protein interaction using conventional small molecules. I introduce the role of eVP24-KPNA protein-protein interaction in the pathogenesis of Ebola virus disease and the potential of eVP24 as a therapeutic target. Development of evp24-binding peptides as well as the evaluation of their biological activity is described. Three macrocyclic peptides inhibitors were

developed.

In chapter 3, I introduce the biological function of channelrhodopsin as well as the significance of stabilizing the open state structure. I also described a selection strategy for the development of such peptides. Several channelrhodopsin binders were developed and their selectivity against the open state was evaluated. I also report the attempt to co-crystallize one of selected peptides with channelrhodopsin to see whether the peptide assists with structure determination of open state structure.

In the chapter 4, the conclusions of the thesis are discussed. Novel findings as well as the achievements are discussed.

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Chapter 1

General introduction

Peptides as therapeutic agents

Peptides have emerged as attractive therapeutic agents in drug discovery due to their advantages over traditional oral available small molecule drugs and biologics (antibodies etc.) (Table 1-1). Similarly to biologics (antibodies etc.), peptides are capable of binding to their targets with high complementary in shape and size, resulting in exhibiting high-specificity for their targets with minimal side-effects comparing with traditional oral available small molecule drugs. [1-3] On the other hand, biologics, which required many cost and time-consuming steps for production, for example the use of expensive reactor for production or intensive personnel labor to assist the process in order to meet stringent quality control guidelines.[4,5] The production of peptides are mainly relied on established chemistry, particularly the solid-phase procedures based on Fmoc-chemistry, which requires less process development, and less personnel intensive regarding to production process, quality control and regulatory affairs.[6,7] Therefore peptide could be more accessible and cheaper for manufacture to produce use chemical methods than biologics.

Beside the economical efficiency in production, the advancement in screening methods[8-9] also drives peptides into therapeutic agents. *In vitro* display, such as mRNA display or phage display, has a large capacity of library, which is consisted of millions or trillions numbers of unique peptides, resulting in an improved hit rate comparing with high-throughput screening. Recently, the chemical space of these libraries has been expanded and it is possible to select peptides with various nonproteinogenic amino acids,[10,11] as well as unique scaffold such as macrocyclic scaffold,[12] bicyclic scaffold,[13,14] and lanthionine bridge.[15-17]

Table 1-1. Comparison of peptide, small molecule and biologics-based therapeutics.

Entry	Molecular weight	Selectivity	Affinity	Membrane permeability	Oral availability	Production Cost and method
Small molecules	< 500 Da	Low	⩾ nM	High	High	Low (Chemical synthesis)
Peptides	In between 500 and 5000 Da	High	pM ~ uM	Potential (e.g. cyclosporine)	Potential (e.g. HIV-1 Tat)	Low (SPPS method)
Biologics	> 5000 Da	High	fM ~ μM	None	None	High (Cell cultural process)

Non-standard macrocyclic peptides and the RaPID system

There is a growing interest in introducing modifications (macrocyclization or nonproteinogenic amino acids) into peptides since the modifications could strongly enhance the affinity, selectivity, proteolytic stability and even membrane permeability[18-20]

The RaPID (Random non-standard Peptide Integrated Discovery) system is a drug discovery platform developed by Suga's group and enables a selection of genetic code reprogrammed libraries that contain trillion members of unique non-standard macrocyclic peptides.[9,21] The RaPID system combines of two technologies, Flexible *in vitro* translation (FIT) system[22,23] and the mRNA display.[24] The FIT system is the combination of flexible tRNA-acylation ribozyme (Flexizyme) and a custom-made reconstituted translation system. Flexizyme enables the aminoacylation of tRNA with a wide variety of non-proteinogenic amino acids. By the use of FIT system, it is possible to ribosomally synthesize peptides with various non-proteinogenic amino acid[11], e.g. D-amino acids, N-methylated amino acids, and introduce exotic features e.g. macrocyclization to improve drug-like properties of peptides (Figure 1-1).

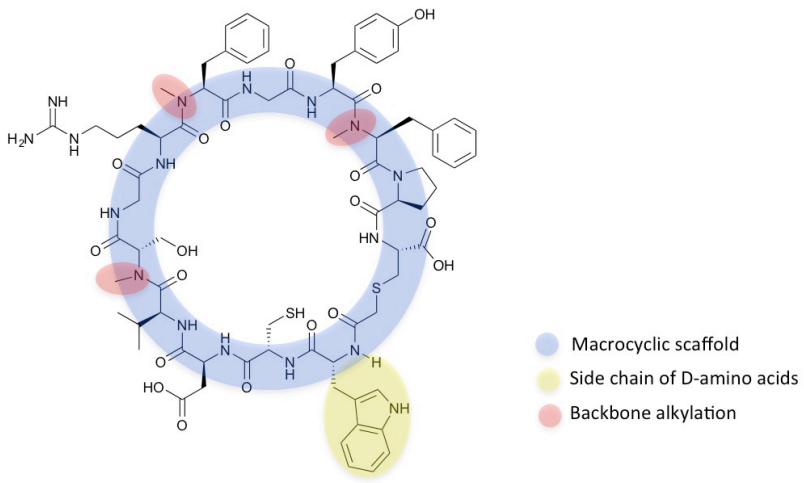

Figure 1-1. An example of non-standard macrocyclic peptide that prepared by the FIT system. Non-standard features of the peptide were shown in blue (macrocyclic scaffold), yellow (D-amino acids), red (backbone alkylation).

Currently, the RaPID system has been employed to target various disease-relevant proteins, such as kinase,[25] ubiquitin ligases,[26] deacetylases,[27] transporters[28,29] and receptors (Table 1-2).[30] Several peptides with remarkable high-affinity as well as bioactivities such as inhibitory activity and isoform selectivity have been discovered. Interestingly, a macrocyclic peptide PB1m6 formed an antiparallel β-sheet in the complex with its target protein PlxnB1 and interacted extensively across a wide groove of PlxnB1 (Figure 1-2A), which resulted in the allosteric inhibition of Semophore 4D-PlexinB1 protein-protein interaction, demonstrating that the RaPID system has the potential for the development of protein-protein interaction inhibitor. This encouraged me to employ the RaPID selection against eVP24, aiming at developing inhibitors against eVP24-KPNA5 protein-protein interaction, who is responsible for the deadly illness of Ebola virus disease (Chapter 2).

On the other hand, macrocyclic peptides MaD5 and aCAP were capable of improve the resolution of crystal structure by stabilization a specific protein conformation, suggesting the potential of macrocyclic peptides as co-crystallization ligands. Additionally, MaD5 buried in the cavity of PfMate (Figure 1-2B), and aCAP bound to

a shallow surface of transporter (Figure 2C), demonstrating that the RaPID system has potential to develop macrocyclic peptides against various membrane proteins. In **chapter 3**, I employed the RaPID system against a membrane channel, aiming at identifying macrocyclic peptides to assist with the crystallization of a specific protein conformation.

Table 1-2. Summary the bioactive macrocyclic peptides developed by the RaPID system.

Name of peptide	Protein target	Class	Affinity (K_D)	Activity
Pakti-L1	Akt2	Kinase	n.d.	Inhibition (IC_{50} = 110 nM)
S2iL8 S2iD7	Sirt2	Deacetylases	3.7-3.8 nM	Inhibition (IC_{50} = 3.2 -3.7nM)
CM_{11}-1	E6AP	Ubiquitin ligases	0.6 nM	Inhibition
MaD5, MaD3S, MaL6	pfMate	Transporter	n.d.	inhibition
aML5, aMD4 aMD5	cMet	Kinase	2-19 nM	inhibition
Hip-8	hHGF	Receptor	0.41 nM	Inhibition (IC_{50} = 0.24 nM)
PB1m6	PlexinB1	Receptor	3.5 nM	Inhibition (IC_{50} = 100 nM)
aCAP	CmABCB1	Transporter	n.d.	Inhibition (K_i = 65 nM)

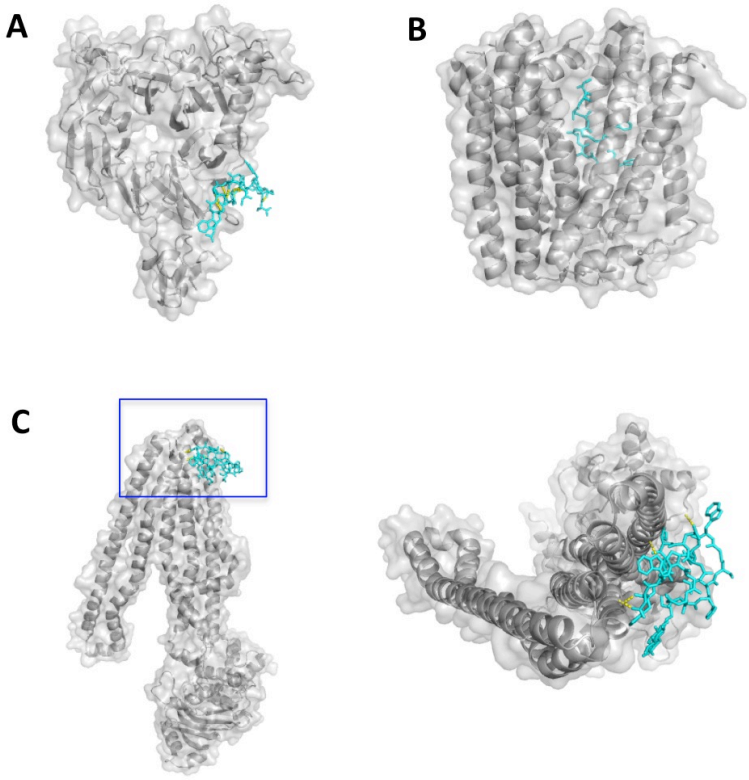

Figure 1-2. Co-crystal structure of macrocyclic peptide in complex with their target protein. (A) Macrocyclic peptide PB1m6 in complex with PlexinB, (PDB: 5B4W) (B) MaD5 in complex with PfMate (PDB: 3VVR) and (C) peptide aCAP in complex with CmABCB1. Interface of aCAP/CmABCB1 (boxed) is shown in right panel. (PDB: 3WMG)

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Chapter 2

Macrocyclic peptides inhibitors for the protein-protein interaction of Ebola virus protein VP24 and KPNA5

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Chapter 3

Development of peptides that trap the open-state structure of Channelrhodopsin

Introduction

Channelrhodopsin is a light-gated ion channel isolated from green alga. Under the stimulus of light, it induces the influx of cations from the extracellular to the intracellular of the plasma membrane (Figure 3-1), and enables the modulation of membrane potential in a precise and reliable manner.[1] Decades ago, Deisseroth group first adapted the channelrhodopsin for manipulation of neurons, and named this technology as "optigenetics". To date, channelrhodopsin was emerged as a crucial tool in the field of optigenetics and enabled us to study functions and dysfunctions of large neuron circuits in various animal models.[2-4]

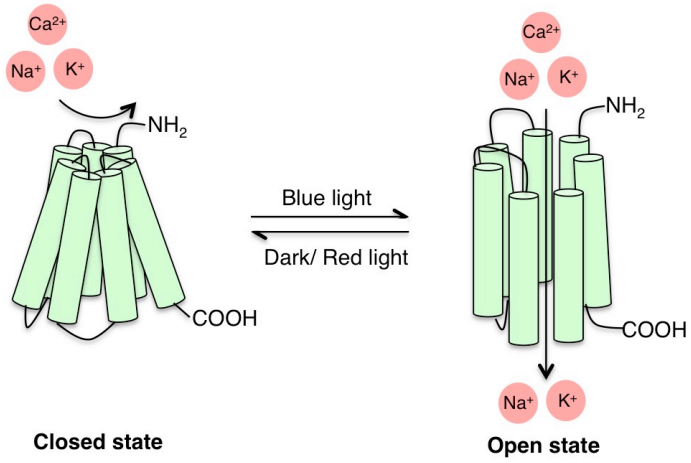


Figure 3-1. Biological function of channelrhodopsin (ChR) upon the stimulation of light. The ChR has two states, a closed stated and an open states. Blue light shifts the closed state into an open state and the red light (in dark) closes the channel.

Despite the widely use of channelrhodopsin as a tool for optogenetics, the mode of action remains elusive. Therefore revealing the structure of channelrhodopsin would facilitate our understanding towards it. In 2002, the structure of a chimera of channelrhodopsin1 and channelrhodopsin 2 (C1C2) has been solved,[5] which provided the closed state of channelrhodopsin for the first time. The open state, however, has not been well understood. So far, DNP solid-state NMR, visible, IR, resonance Raman spectroscopy and Cyro-electron microscopy has been used to study the open state structure.[6-10] These studies enabled us to know partially about mode of action of

channelrhodopsin, e.g. critical transmembrane helix for channel opening, details of photoactive site, however none of them provided an open state structure at atomic level.

X-crystallography is a powerful tool to visualize protein structure at atomic level, however owing to the short lifetime (the half-life of open state is usually less than a minute) of the open state, it could be difficult to "capture" the open state by X-crystallography. In the previous study of the open state structure, a freezing step right after the illumination step enables the trap and analysis of the open state,[8-9] however this methods is not suitable for X-crystallography as the crystallization of protein often requires a temperature at 4°C or 25°C.[11]

There are successful examples of which utilize high-affinity conformation specific molecular chaperon (nanobodies etc.) to trap the active state of receptors.[12] Since the macrocyclic peptides discovered by the RaPID system often exhibited selectivity and high-affinity to their targets, especially having a potential to constrain the movement of transmembrane helix of which is critical for trapping the open state. Therefore in this study, I attempt to use macrocyclic peptides to stabilize the open state structure of channelrhodopsin (Figure 3-2).

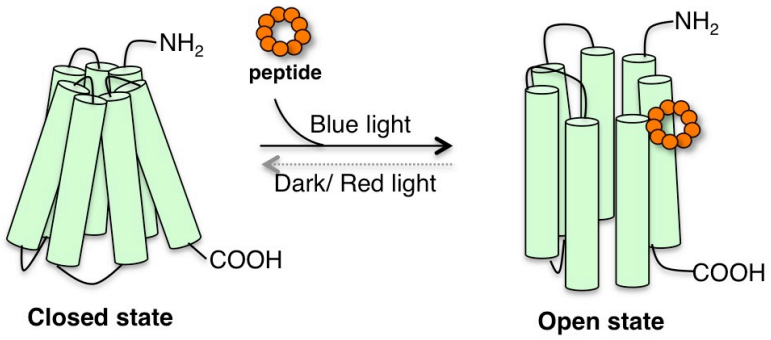


Figure 3-2. Schematic presentation of the aim of the study. The aim of study is using peptide to trap the open state of channelrhodopsin (ChR).

Results and discussion

Selection of channelrhodopsin binding peptides

In order to stabilize the open state of channelrhodopsin (ChR), we use a mutant (C167A) of channelrhodopsin C1C2 of which the structure has been solved. The mutant C167A is able to maintain the open state structure as long as the blue light keeps illuminating the protein. We first designed a selection procedure for identification of binders that specifically bind to open state. It is suggested that a linear peptides has more structural flexibility comparing with the macrocyclized counterparts, which might enable them to bind to a site of which could not be accessed by a macrocyclic peptide. Therefore in this selection, a linear peptide library with an initiator amino acid of N-acetylated phenylalanine (Ac-D-Phe) along with a macrocyclic peptide library which has the initiator amino acid of N-choroacetylated-D-phenylalanine (ClAc-D-Phe) was employed (Figure 3-3A). Following the initiator amino acid, both of the libraries contain a variable region of 4-12 amino acids in length and a flexible linker consisted of three repeats of glycine serine (GS). In order to obtain peptide that specifically bind to the open state of ChR, two independent selections have been performed, in which the peptide library was subjected to ChR-immobilized magnetic beads upon the illumination with blue light or red light. The ChR binders were recovered and subjected to sequencing. By comparing the sequences of the peptide binders, those sequences with different frequency between two selection conditions would be regarded as potent specific binders (Figure 3-3B).

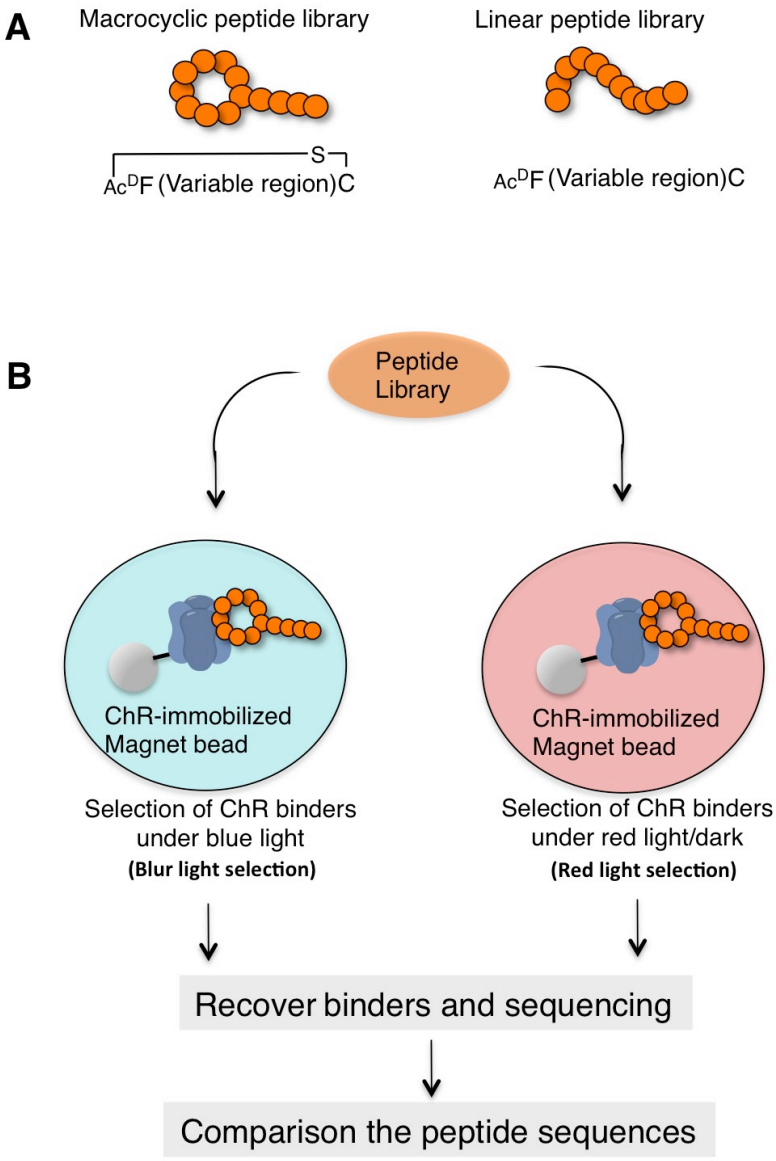


Figure 3-3. (A) Peptide libraries that were used in the study. (B) Flowchart for the selection of potential open state binders. The initial library for blue light and red light selection was the same. Prior to the aforementioned selections, the peptide library was incubated with magnetic beads to remove any beads binding peptides.

After continuative round of selection, I observed an elevation of the recovery rate of cDNAs in the selections that were performed either upon blue light or red light illumination (Figure 3-4). At the same time, I did not observed a significant elevation of cDNAs recovered from beads, indicating channelrhodopsin binders (conformation-selective binders and those binders lose selectivity over conformational changes) were enriched. Interestingly, the recovery rate observed in the selection using blue light was 6-magnitude higher than those selections using red light, indicating the open state is more favorable for linear peptide to bind with.

The recovered cDNA from the last round of all selections were cloned by TA cloning tenique and sequenced to obtain sequence information. In the selection using macrocyclic peptide library, peptide sequences of 15 clones were determined for both of the blue light selection and red light selection. The alignment of peptide sequences revealed a high similarity between the sequence recovered from blue light and red light selection, however the dominated peptide ChRc1 in the blue light selection became less abundance in the red light selection, indicating that ChRc1 could be a potent open state binder. Similarly, we also considered ChRc2 as a potential open state binder as it only appeared in the pool, which was recovered from blue selection. On the other hand, there was a large difference between the peptide sequences obtained from the blue and red light selection. A group of peptides (GL) of which contained multiple arginine (R), isoleucine (I) and Leucine (Leu) resides were only identified in the blue light selection. Therefore we choose ChRc1, ChRc2, ChRl1, and ChRl2 (Note that ChRl1 and ChRl2 are randomly choosen from GL group) for further study.

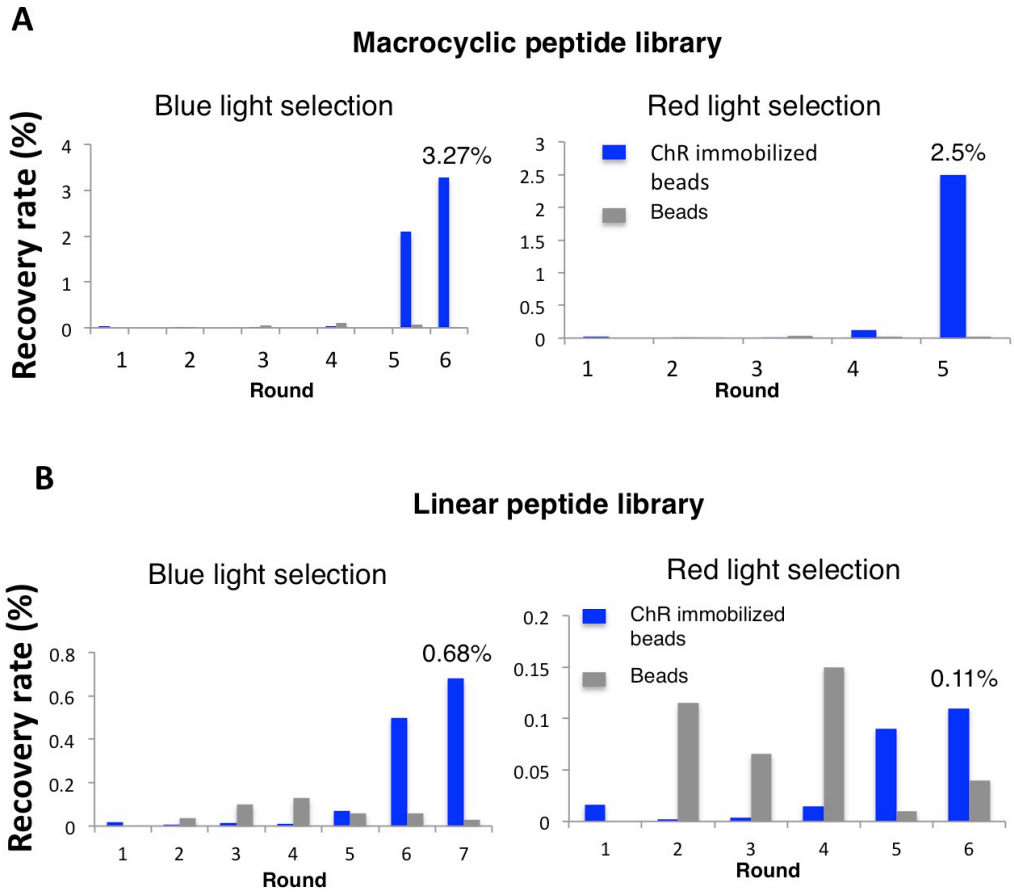


Figure 3-4. Selection progress of (A) macrocyclic peptide library and (B) linear peptide library. Selections were performed under the blue light (left panel) or red light (right panel).

Table 3-1. Peptide sequences recovered from the selections. Conserved residues are colored in grey.

Macrocyclic peptide library $Ac^{D}F$(Variable region)C (S–S)

Clone	Variable region	Frequency (Blue light)	Frequency (Red light)
ChRc1 ChRc2	IVLWWAKRYS	(14/15)	(4/15)
	IILKWAKRFN	(1/15)	–
	IILKWAKRFK	–	(2/15)
	ILISWAKRKQ	–	(6/15)
	ILIKWAKKQN	–	(1/15)
	IVLWWTKRHP	–	(1/15)
	IVLWWTKRYA	–	(1/15)

Linear peptide library $Ac^{D}F$(Variable region)C

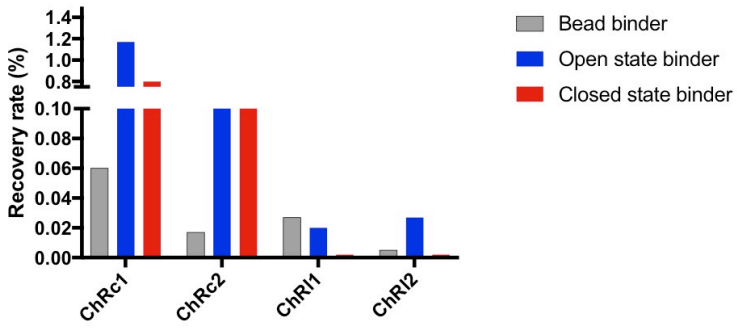
Clone	Variable region	Frequency (Blue light)	Frequency (Red light)
	R----LRIRIVV*	(1/15)	–
ChRl1	PRRI-IRIRIRV*	(1/15)	–
	-----FLIFLDL*	(1/15)	–
ChRl2	-KQRIRVRIRIRL*	(2/15)	–
	IRRRIIIRIRFR*	(1/15)	–
	IRRIIMRIRFR*	(1/15)	–
	---KIILRIRIRRP*	(1/15)	–
	AHSHVLLLILL*	(1/15)	–
	RRRIRVRIRIRL*	(1/15)	–
	IVLWWAKRYS	(3/15)	–
	LIVLYAC	(1/15)	–
	TLKFFTVPCT	–	(3/16)
	AIGPILIFRCT	(1/15)	(2/16)
	VTWYLANLCT	–	(2/16)
	WWYIGPCP	–	(1/16)
	VFFVYTGPCK	–	(1/16)
	IILKWAKRFK	–	(1/16)
	T---YIPQHP	–	(1/16)
	TTPWFRLYKCD	–	(1/16)
	ITPWFRLYKCD	–	(1/16)
	IRYGPLILWTCS	–	(1/16)
	AIWIGRCS	–	(1/16)
	TVRCI	–	(1/16)

* denotes a occurrence of a frame-shift mutation.

Evaluation of the selectivity of selected clone

To identify potential clones for co-crystallization, I performed a single clone assay to evaluate whether these clones are capable of bind to the open state selectively. In the assay, each clones was ribosomally expressed, and subsequently added to the

magnetic beads. The unbound fraction was then added to ChR-immobilized beads under the illumination of blue light or red light, the cDNA that was recovered from ChR binding peptides were qualitatively determined (Figure 3-5).



Clone	Selectivity	
	(Open/bead)	(Open/closed)
ChRc1	19.5	1.5
ChRc2	8	0.6
ChRl1	1	10
ChRl2	5.4	13.5

Figure 3-5. A single clone assay that used to evaluate the specificity of peptides. The recovery rate of bead binders (grey bar), open state binders (blue bar) and closed state binders (red bar). The selectivity was calculated from the ratio between the recovery rate of open state binders and bead binders or the ratio between the recovery rate of open state binders and closed state binders was shown below.

Among these clones, ChRl1 and ChRl2 displayed a selectivity of ~ 13.5-fold in the binding to open state over closed state, however ChRl1 and ChR12 has nearly no selectivity, implying that structural flexibility might be critical for conformational selectivity.

Crystal structure of macrocyclic peptides with channelrhodopsin

(Removed due to the reason that the part contains an unpublished crystal structure).

Conclusions

In this study, I reported the attempt of discovery of specific open state binders of ChR using the RaPID system. Several macrocyclic or linear ChR-binding peptides were developed. By screening against linear peptide library against ChR. a promising open state binder was developed.

The co-crystallization of ChR with macrocyclic peptide ChRc1 did not provide an open-state structure. As there were no data of the binding affinity of these peptides, it is difficult to have a clear explanation for this negative result. The failure might arise from the weak binding of peptides, or might come from the inappropriate of the co-crystallization condition. In the future, after analyze the binding affinity of these peptides, it might be possible to know the reason for the failure.

Although in this study I failed to obtain an open state structure of ChR, this work provided precious information. When the selection was performed under the stimulation of blue light, by using linear peptide library, unique peptide sequences were enriched. By using a binding assay, I also confirmed one of the clone has a preference in binding to the open state. It implies that structural flexibility might be important for the peptide to specifically bind to the open state. As the flexibility of peptide might have a reduced binding affinity, therefore it might be important to achieve a good balance between affinity, and structural flexibility in order to obtain conformation selectivity as well as being a good co-crystallization ligand. In the future, design a linear peptide of which could covalently bind to ChR could be one of the choices to achieve such balance.

Methods

Chemical synthesis of peptides

All peptides were synthesized using Fmoc-based SPPS via Syro Wave automated peptide synthesizer (Biotage) in the same manner as described in Chapter 2. All peptides were purified by reverse-phase HPLC (RP-HPLC), The molecular mass of synthetic peptide was verified by MALDI-TOF mass spectrometry, using a microflex or ultraflex instrument (Bruker Daltonics).

Aminoacylation of tRNA by eFx

eFx was mixed with $tRNA^{fMet}_{CAU}$ to a final concentration of 25μM in 50 mM HEPES-KOH (pH 7.5) with 600 mM $MgCl_2$. This mixture was heated at 95 °C for 2 min, stand at room temperature for 5 min and incubated on ice for another 5 min. Then custom-made N-chloroacetyl D-Phenylalanine cyanomethyl ester (ClAc-D-Phe-CME) or N-acetyl D-Phenylalanine cyanomethyl ester (Ac-D-Phe-CME) dissolved in DMSO was added to a final concentration of 5 mM and incubated for an hour. Then 0.3M sodium acetate (pH5.2) was added to quench the acylation reaction. The acylated tRNA product was participated and purified by ethanol. the final pellet was dried and dissolved in 1.0 μL of 0.1 mM sodium acetate.

Selection procedures

The preparation of peptide library by the FIT system was followed a procedures described in chapter 2. The peptide library was subjected to Ni-NTA magnetic beads and incubated at 30 min and repeated for 3 times. 50 pmol ChR was immobilized on the NTA magnetic beads and under the illumination or incubation in dark for 5 min before mixed with unbound translation mixture. After applied unbound fractions to ChR (C167S/D195A), the mixture solution was incubated at 4°C for 30 min with rotation. At this step, the mixture solution was either illuminated by a continuous illumination of blue light (475 nm, 0.05 mW/mm^2, the distance between the sample

and light is less than 5 cm) or incubated in the dark. After removing the supernatant, the beads were wash three times with 300 μL of cold selection buffer (Tris-HCl pH8.0, 150 mM NaCl, 2.5% (w/v) DDM and 0.5% (w/v) CHS) under the blue light or red light. The bound fraction was reverse transcript followed a protocol described in chapter 2. The cDNAs were recovered, amplified and subjected to next round of selection.

From the second round of selection, the translation volume was reduced to 10 μL and the amount of ChR immobilized on beads was reduced to 6 pmol.

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Chapter 4

General conclusion

In this thesis, I reported the results of my goals of the development of macrocyclic peptides to manipulate the function of eVP24 and channelrhodopsin. In both of the cases, peptide binders were successfully developed.

In the study of development of inhibitor against eVP24-KPNA5 protein-protein interaction, I developed three macrocyclic peptides with a remarkably high affinity to eVP24 by performing a RaPID selection (chapter 2). Furthermore, these macrocyclic peptides inhibited the protein-protein interaction (PPI) of eVP24-KPNA5 in a micromolar range. To the best of our knowledge, only several small molecules that target PPI interface have been developed by *in silico* screening, however their binding affinity or inhibitory activity has never been evaluated by actual experiment.[1,2] The macrocyclic peptides developed in this study provides a chemical probe for the first time that a molecule is capable of modulating the eVP24-KPNA5 protein-protein interaction. Moreover, the success in the inhibition of this PPI using macrocyclic peptides demonstrates that the PPI is druggable. Macrocyclic peptide scaffold developed in the study could be a starting point for the development of highly potent inhibitor as well as a new antiviral agent against Ebola virus disease.

In the study of development of co-crystallization ligand to stabilize the open state of channelrhodopsin, I designed a light-controlling selection strategy (chapter 3) to identify specific open state binders. By screening a linear and macrocyclic peptide library against channelrhodopsin. Several channelrhodopsin-binding peptides have been developed. Interestingly, a linear peptide rather than a macrocyclic peptide showed conformational selectivity, which demonstrated that the selection strategy was partially succeeded. At the same time, it would also provide us a new insight between structural flexibility and the capability to discriminate protein with a subtle conformation changes, like channelrhodopsin.[3] In future, the linear peptide library might also be applied for the selection of peptide with conformational selectivity against other proteins, e.g. ion channel, receptors.

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List of accomplishment

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