学位論文　（要約）

Robotic *in vitro* selection of functional cyclic peptides for diverse target proteins

（ラボオートメーションの環状ペプチド創薬への展開）

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Abstract

Macrocyclic peptides are an attractive therapeutic modality due to their favorable features and have the potential to target classically 'undruggable' biological processes. The RaPID (Random non-standard Peptide Integrated Discovery) system has previously been shown to be a reliable platform for the de novo discovery of macrocyclic peptides that bind to a wide variety of proteins with high affinities and specificities. As it is typical for other *in vitro* selection systems, the RaPID system workflow requires researchers to perform laborious repetitive tasks. In order to streamline peptide drug discovery allowing researchers to focus on intellectual work, I have semi-automated the RaPID system using a humanoid robot, named Senta-kun. As benchmark experiments, in this thesis, four RaPID selections were performed against therapeutically relevant proteins namely TET1, cMET, Smurf2 and Akt2 to demonstrate the feasibility of performing automated RaPID system using the robot.

In chapter 1, I discuss the advantages and disavdantages of macrocyclic peptides over other modalities such as small molecules and biologics in terms of properties suitable for drugs as well as other comprehensive drug discovery technologies focusing on macrocyclic peptides. Through in depth comparisons, the superior features of the RaPID system amongst these drug discovery technologies highlight the past successes of macrocyclic peptides discovered with the RaPID system. Moreover, the general significance, current situation, and challenges for the future of laboratory automation are discussed.

In chapter 2, the specifications of the robot used in this study and its compatibilities with the RaPID system are described. The setup process of the robot and optimization process for performing each step of the RaPID system are discussed in detail. Notably, experimental considerations, pitfalls and the know-how gained from the successful setup of the robot for the RaPID system are illustrated.

Chapter 3 describes how the RaPID system was performed against two proteins, TET1 and cMET. Manual selection and automated selection are compared as a benchmark experiment to demonstrate the viability of the RaPID system using the robot. In addition, to reveal the effects of experimental conditions on the RaPID system outcomes, the same conditions between manual and robotic selections were used for TET1 and different conditions were used for cMET. As a result, similar peptide sequences were identified between the manual and robotic selection against TET1, whereas the majority of the peptide sequences discovered from the cMET selections was strongly varied from each other. All peptides

tested in this study exhibited binding activity for the target protein. Moreover, three peptides, TiP1, 2, 3, that were confirmed to bind TET1 also exhibited TET1 inhibitory activity.

In chapter 4, the RaPID system was solely performed by the robot against a new target, Smurf2, to show that it is possible to conduct the RaPID system with only using the robot, resulting in the successful discovery of four peptides that bind Smurf2. Furthermore, the RaPID system workflow was applied to the affinity maturation of a known peptide inhibitor as a new application of the RaPID system. This experiment was conducted using the protein target, Akt2, and its peptide inhibitor, Pakti-L1. The result suggested that Pakti-L1 is a local maximum inhibitor, having the strongest binding affinity compared to other peptides binding via the same motif. It also resulted in the identification of a mutant with comparable activity as Pakti-L1.

Chapter 5 summarizes the results and provides an overall conclusion to the aims described in chapter 1. In this study, the RaPID system, which consists complicated multi-steps and requires precision, has been semi-automated using a humanoid robot and this system has successfully been used to discover new functional macrocyclic peptides for protein targets. The combination of laboratory automation and the RaPID system will enable us to more efficiently and productively conduct macrocyclic peptide drug discovery. In addition to the conclusions of my work, future prospects for laboratory automation and macrocyclic peptide drug discovery are also presented.

Table of Contents

Chapter 1

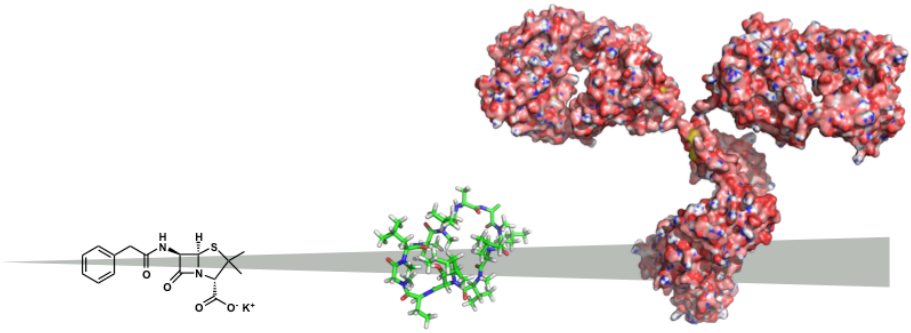
General introduction

1.1 Macrocyclic peptides as a therapeutic modality

The majority of therapeutics in clinical use is either small molecules or biologics like antibodies [1,2,3]. These molecules possess characteristic features respectively; small molecules can easily access to intracellular targets and their production cost is very low due to their sizes and simplistic structures. The advantages of biologics, on the other hand, are low toxicity and fewer side effects since they have high activity and specificity against targets and their composition is mainly amino acids. Moreover, biologics have possibility to target protein-protein interaction (PPI) that is difficult for small molecules to regulate. Though, these molecules have both merits and demerits, and there are still problems for drug discovery [4] [5]. For example, small molecules have adverse effects or toxicity due to their poor selectivity. Biologics can't target intracellular proteins and cost a lot to produce.

As another therapeutic modality, macrocyclic peptides have been getting successful [6,7]. Leading examples of macrocyclic peptide drugs are the hormones oxytocin and vasopressin, the antibiotics vancomycin and polymyxin B, and the immunosuppressant cyclosporines [8,9]. The success of macrocyclic peptides as a therapeutic modality can be attributed to their adorable properties as follows: First, macrocyclic peptides display a large surface area providing a strong binding and selectivity against target proteins. The constrained structures of their macrocyclic backbone improve their attractive binding properties as well by reducing the entropic penalty upon the binding. The large surface areas also allow macrocyclic peptides to bind to "hot spots" with binding sites generally in the range of 800-1200Å^2 because of their large size [10] [11] [12]. Second, the macrocyclic structure improves their stability against peptidases or proteases [13]. Third, macrocyclic peptides or their metabolites have few toxicity and immunogenicity as their compositions are amino acids and they are small. Fourth, macrocyclic peptides can be chemically synthesized, so they are easy to handle, modify and characterize in addition with their low production cost[14]. Furthermore, several macrocyclic peptides that pass through cell membrane have been observed, but membrane permeability is still challenging for peptides [15] [16]. Macrocyclic peptides are expected to show better profiles compared to existing drugs and could target mechanisms of action such as intracellular PPIs due to their good points as drugs (Table 1) [17].

Macrocyclic peptides have been a minor modality as therapeutics in spite of their adorable properties. The main reason for this was that there had been few methodologies to discover hit peptides and traditional peptide drugs have depended heavily on the discovery from natural products. Therefore, target-directed peptide discovery methodologies are necessary, instead of the serendipitous discovery, to accelerate macrocyclic peptide drug generation.



	Small molecules	Peptides	Biologics
Molecular weight	< 1,000	1,500 - 5,000	> 150,000
Affinity	≥ nM	pM - μM	fM - μM
Specificity	Low to High	High	High
Intracellular access	High	Possible but still challenging	Impossible
PPI regulation	Difficult	Possible	Possible
Oral delivery	Possible	Potential	Impossible
Toxicity	High	Low	Low
Production cost	Low	Low - Medium	High

Table 1. Comparison of various pharmacological parameters between three modalityes: small molecules, peptides and biologics.

1.2 Technologies for the discovery of macrocyclic peptide therapeutics

Recently, a number of technologies have been developed to overcome the problem for the macrocyclic peptide drug discovery, and macrocyclic peptide drug discovery has been gaining great attention and activated [18] [19]. Current technologies for macrocyclic peptide drug discovery and either pharmaceutical/biotech companies or organizations that are working on these technologies are shown in table 2 [9] [20] [21] [22] [23] [24] [25] [26] [27] [28] [29] [30] [31]. From this table, it is obvious that during recent years there are a lot of company or research groups working on the development of new technologies or macrocyclic peptide drug discovery with enthusiasm.

These technologies can be categorized into three groups, chemically synthesized peptide libraries, gene-encoded peptide libraries, and computational designed peptides. There are two strategies to make the peptide libraries: one is fully randomized sequences and the other is nature inspired designs, e.g. protein epitope or alpha-helixe. The advantages of chemically synthesized peptide libraries are structural diversities of peptide backbone or

side chain, e.g. noncanonical amino acids such as D-conformers, peptide mimetics, or macrocyclization. By contrast, the disadvantage of them is poor diversity of the molecules in libraries. The number of peptides to be handle at a time is usually several hundreds to ten thousands. To identify the sequences of hit peptides after screening, peptide array, partial Edman degradation and mass spectrometry (PED-MS), or MS alone is typically used [32] [33]. The gene-encoded peptide libraries are typically generated by in vitro translation system [34] [35], phage [36], bacteria [37], or mammalian cells [38] [39] and screened by affinity based or activity based selection. The diversity of gene-encoded peptide libraries is much higher than the chemical libraries, but only proteogenic amino acids can be generally used as building blocks since peptides are synthesized by ribosome and translation machineries. Thus, chemical diversity of gene-encoded peptide libraries is limited. Computational design is minor and the number of target proteins is limited compared with others because it requires a crystal structure of target proteins in addition to a reliable and validated program [40].

These technologies are already suitable for practical use. To my knowledge, at least, eight peptide drugs have been approved in the last 10 years and nine peptides are currently under clinical development [18] [41].

Table 2. List of current peptide discovery technologies

Approach	Technology	Company/Organization
Chemically synthesized peptide	PEM	Polyphor
	MATCH (Macrocyclic Template Chemistry)	Tranzyme Pharma (now Ocera Therapeutics)
	Stapled peptides	Aileron Therapeutics
	Epitope mapping	Pepscan
	Nacellins	Encycle Therapeutics
	Combinatrial chemistry	Tranzyme Pharma (now Ocera Therapeutics)
	Stapled peptides	Dehna Pei group (The Ohio State University)
	One-Bead-One-Peptide	Kit Lam group (UC Davis)
Gene-encoded peptide	mRNA display	Ra Pharma, PeptiDream
	Phage display	Bicycle Therapeutics, Polyphor
	SICLOPS	University of Southampton
	Phylomer	Phylogia
Computer design	Structure-based design (Virtual library)	Circle Pharma
	de novo selection of peptide scafold	Protagonist

1.3 RaPID system

The RaPID (Random non-standard Peptide Integrated Discovery) system developed by Suga et al is one of most successful technologies amongst descried above[42 43 44]. The RaPID system is a mRNA display-based platform enhancing its chemical diversity by substitution of in vitro translation system with the FIT (Flexizyme-assisted In vitro Translation) system. The FIT system is an in vitro translation system that allows one to reprogram the natural genetic code by adding any preaminoacylated tRNA with a wide variety of amino acids using aminoacylating ribozyme termed, flexizyme[45,46 43 42 47]. This technology allows mRNA display platform to overcome the limitation of the peptide building blocks and expand the chemical diversity such as D-conformers, *N*-methyl amino acids, macrocyclization, in addition to tri-cyclization with post-translational modifications[47 48]. Herewith, the RaPID system enables one to discover macrocyclic peptides with high affinity and selectivity for a multiple of proteins.

To date, an extremely wide variety of proteins such as kinases, glycosylases, ubiquitin ligases, histone deacetylases, transporters, and receptors have been targeted by the RaPID system and macrocyclic peptides with biological activities have been successfully discovered[49 50 51 52 53 54 55 56 57]. The macrocyclic peptides selected by the RaPID system, in general, exhibit high affinities with micro molar order of dissociation constant and selectivity even against isoforms. Moreover, the selected macrocyclic peptides show biological activity though the RaPID system is an affinity based selection platform. For example, a macrocyclic peptide selected against Akt2 showed inhibitory activity as well as binding affinity and high selectivity against its isoforms, Akt2 and Akt3. In another study, macrocyclic peptides selected against the hepatocyte growth factor (HGF) receptor, also known as cMET, exhibited no biological activity, but when homodimerized with a chemical linker, they could agonize its biological activity by dimerizing two cMET molecules and transduce downstream cellular signal. Furthermore, other applications in addition to regulation of target protein's activity have been reported. Some macrocyclic peptides were used as crystallization chaperons, which assist protein crystallization or improve the resolution by stabilizing the protein structure[58]. Another application is molecular probe. Macrocyclic peptide conjugated with a fluorophore can be used for the imaging of the target protein toward a research tool or diagnostic in place of antibodies[59 60].

There is still a problem for the RaPID system although it is a potent and robust technology as mentioned above. The RaPID system consists of simple and repetitive experiments, that means researchers are forced to spend a lot of time for no brain work and huge man power and time is necessary to develop macrocyclic peptides for numerous proteins using the

RaPID system. Besides, the repertoires of peptide libraries available are increasing. Therefore, more effective operation of the RaPID system with different kinds of libraries is needed to develop macrocyclic peptides for an extremely wide variety of proteins. Automation of the RaPID system could be a possible solution for the problem.

1.4 Current situation of laboratory automation

At least, the first laboratory automation robot has been reported in 1875, and then a number of machines have been developed to automate research experiments[61-63]. There are numerous advantages for the automation of experiments. First, the automation lets researchers focus on creative and brain work that is what researchers really have to do and unsubstitutable with robots, which results in high productivity. Second, researcher's safety can be improved by avoiding them from exposures to hazardous chemicals, radioisotopes, or viruses. Third, the data generated by robots is uniformed and reproducible. The reproducibility of experimental data is a big problem, especially in the field of biology, the automation has a possibility to overcome this problem[64]. Additionally, for simple experiments, robots can treat a number of samples in parallel, for example plate readers enable to measure a 96-well or 384-well plate at a time.

Conventional automation robots or machines have been mainly developed for a single and simple task. Excellent examples that are widely seen in laboratories today are liquid handlers, plate readers, autosamplers, DNA or peptide synthesizers, High throughput screening machines, and so on. These machines make researches effective by automate experiments that require repetitive work and treat numerous samples ill-suited for manual handling. Likewise, an artificial intelligent and automation machines have been combined to automate not only experiments but also researches by repeating the cycle of experiments, data analysis, and design of next experiments. This system was named as the robot scientist and two researches, identification of gene function and drug screening, have been succeeded so far[65-67]. However, kinds of experimental steps that can be conducted by automation machines are still limited and it is mainly simple experimental steps such as liquid handling. Thus, to expand the repertoires of experiments to be automated, there are some companies offering containerized robotic workcells that contains many a series of automation machines[68]. They also provide general protocols for a wide variety of experiments using the workcells and enable us to generate reliable and reproducible data by the automated experimental procedures and the homogenized protocols but still have problems. The workcells occupy large spaces and complicated experiments can't be automate because they are just combinations of existing machines. Additionally, it is difficult to change experiment

descriptions since different combinations of machines have to be set for different kinds of experiments. To overcome these problems, a humanoid robot has been developed recently[68]. This robot is able to conduct more complicated and precisive experiments by mimicking human procedures and has capability to be set flexibly for different kinds of experiments. Therefore, this robot is expected to automate experiments that are impossible for conventional machines to automate.

1.5 Aim of this study

In this study, I attempted to automate the RaPID system using a newly developed robot toward the effective development of functional macrocyclic peptides for a bunch of proteins. There is no precedent for the automation of experiments such as the RaPID system because the RaPID system consists of numerous experimental steps and requires handling of a tiny amount of samples, that means more complicated and precisive experiments need to be conducted by a robot although the RaPID system is simple and repetitive labor for human researchers.

For that reason, the robot has been setup by optimizing its motion and parameter settings and the protocols have been made in accordance with the capabilities of the robot to automate the RaPID system in chapter 2. In chapter 3, the RaPID system has been performed using the robot against two different target proteins to check the operation of the robot, and the results were compared with ones generated manually to test the reproducibility of the RaPID system and the effects of selection conditions. In chapter 4, a new protein has been targeted by the RaPID system as a first example of practical operations of the robot. Furthermore, an application of the RaPID system is described to show the further utility of the robot.

1.6 References

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Chapter 2

Setup of a humanoid robot toward RaPID system operation

2.1 Introduction

As a track record of functional macrocyclic peptides identified from the RaPID system shows, the RaPID system is a robust technology to discover new peptide ligands for a wide variety of proteins. On the other hand, the RaPID system requires repetitive work of researchers, resulting that researchers spend too long time on no brain work and the RaPID system has a labor shortage to fulfill the demand. Automation of the RaPID system has possibility to solve those problems and makes it more effective and productive. However, conventional automation machines have been generally developed for a simple and specific task, thus it has been difficult to automate experiments that are complicated and consists of multiple works such as the RaPID system. To overcome this problem and automate myriad experiments, a humanoid robot, namely Maholo, has been recently developed and employed in academic and industrial laboratories for the automation of advanced experiments such as cell culture and high throughput screening (HTS)[68]. Since Maholo can be flexibly customized and setup to suit any purpose, it has been installed in our laboratory for the automation of the RaPID system.

In general, experimental equipments and machines need to be setup by optimization of their motions, while conventional ones, e.g. plate readers and peptide/DNA synthesizers, are usually setup by a supplier and ready to use upon installation since they are developed and used for a specific task. By contrast, Maholo has been developed to execute a wide variety of experiments by mimicking human behaviors, so it needs to be setup for the purposes one by one. The first thing for the automation of the RaPID system was setup of Maholo. The RaPID system had difficulties due to its characteristics to be automated using Maholo although Maholo has been validated to execute experiments that are considered as difficult ones to automate by conventional machines. The difficulties for the RaPID system are as follows: i) sample volume is much smaller (2-40 μL), ii) the RaPID system consists of different types of experiments compared with other experiments such as cell culture. Therefore, the automation of the RaPID system required more precise optimization of the settings of Maholo for a series of respective experiments. Moreover, the protocols and motions need to make and adapt to the capabilities of Maholo because the motions and capabilities of Maholo are not completely same as human behaviors and abilities. In this chapter, the setup process of Maholo for the automation of the RaPID system is described.

2.2 Specifications of Maholo and compatibility with RaPID system

Maholo is a dual-arm humanoid robot and conducts experiments in a personal space caged in metal frames and glass windows (Fig.1). The robot uses same equipments as that human researchers use except for electric pipettes and tube stands. The electric pipettes are connected to a computer via Bluetooth, and aspiration volume and speed can be controlled by a program. Same pipettes as human were also used in addition to electric pipettes for experiments that require high-speed discharge of solution, such as magnetic beads suspension. In the cage, -20°C and -80°C freezers (the temperature can be changed), a 4°C refrigerator containing a agitator (the temperature can be changed), four gas-phase incubators (set at 25°C, 37°C, 42°C for enzymatic reactions), a centrifuge, a tabletop centrifuge, and four 4°C heat blocks have been placed and set. The temperature in the cage is controlled by an air conditioner. Maholo in our laboratory was named as Senta-kun for a reason that it was setup and used for *in vitro* selection (選択).

An overview of the RaPID system is shown in Figure 2[44]. The initial DNA library containing random sequences is prepared by chemical synthesis, in vitro extension and PCR amplification. DNA library is transcribed to mRNA library followed by ligation of the puromycin linker (①). This complex is translated by FIT system, and the translated peptide and its corresponding mRNA are covalently linked via puromycin upon translation (②). In general, a *N*-chloroacetyl amino acid is assigned at N-terminal and cysteine is located after the random region to cyclize the peptides. Following reverese transcription, macrocyclic peptide-mRNA-cDNA complexes are formed (③). To remove non-specific spices, counter selection is performed before affinity selection against the targets to recover peptides that bind the targets specifically (④, ⑤). The recovered cDNA is quantified by qPCR and PCR amplified to prepare the cDNA library used for next round (⑥, ⑦). This cycle is repeated until hit species are enriched in the library (normally 4-7 times) followed by sequencing of cDNA of hit peptides. Senta-kun was assigned to Step ①-⑤ and the rest parts of the RaPID system were conducted by myself, because gel electrophoresis, UV-vis measurement, calculation of mRNA concentration, and qPCR analysis that are involved in step ⑥ or ⑦ were incompatible with the utility of Senta-kun. Roughly 60-70% of the RaPID system was assigned to Senta-kun. Senta-kun was setup to perform the RaPID system with four samples in parallel. The number of samples becomes limited by the number of tube stands and four samples are maximum for the current fixings although it can be increased by additional tube stands.

Figure 1. Photographs of Senta-kun. (a-c) Front view. (d-f) Back view. (g) Senta-kun performing the RaPID system.

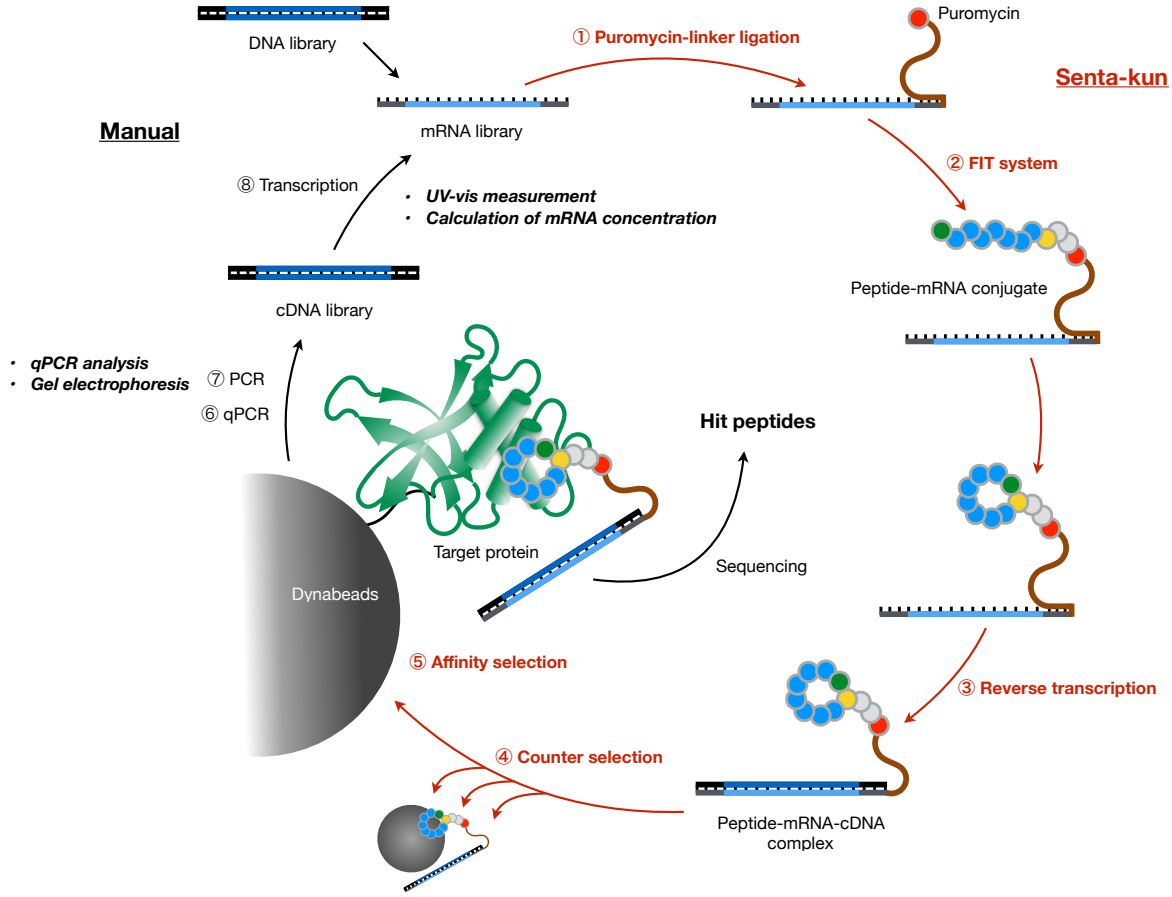

Figure 2. An overview of the RaPID system.

2.3 Optimization of Senta-kun for the respective steps of RaPID system

The protocols for the RaPID system are different between protein tags that are used for immobilization to magnetic beads. Biotin-tag, Fc-tag and histidine-tag are mainly used. First, Senta-kun was setup based on the protocol for the RaPID system using a biotin-tagged protein, because the protocol is simplest and frequency in use is highest among these protein tags. Fc-tagged or histidine-tagged proteins were also used in this thesis. The protocols and Senta-kun's settings for them are described in the latter chapters in each case. The protocol and parameters of Senta-kun was optimized for each experiment step by step. At first, step ③-⑤ was optimized and extended steps one by one. After checking step ②-⑤ followed by optimization of ligation step (①) alone, all steps assigned to Senta-kun were conducted through to check whether or not Senta-kun had been setup to perform the RaPID system with same quality as the human researchers. All optimization experiments was done using a clonal peptide that is validated its binding to the target protein in stead of

peptide library, and the success of experiments was confirmed by comparing the results with the data generated by myself. The results of optimization of respective steps are described in sequence below.

Step ③-⑤ includes an enzymatic reaction and magnetic beads handling. The difficulties in this section were to make sure that Senta-kun added and mixed a tiny amount of solutions (single digit μL) accurately, treated the enzyme (Reverse transcriptase) with retaining activity and suspended magnetic beads uniformly. To overcome these difficulties, physiological position and height of pipette was exactly optimized and normal pipettes were used for suspension of magnetic beads because normal ones could discharge solution with higher speed than electric ones. For optimization of this section, I conducted step ① and ② by myself with five samples. Senta-kun conducted step ③-⑤ using four sample in parallel and I used the last one as a control. After affinity selection, cDNA recovered from

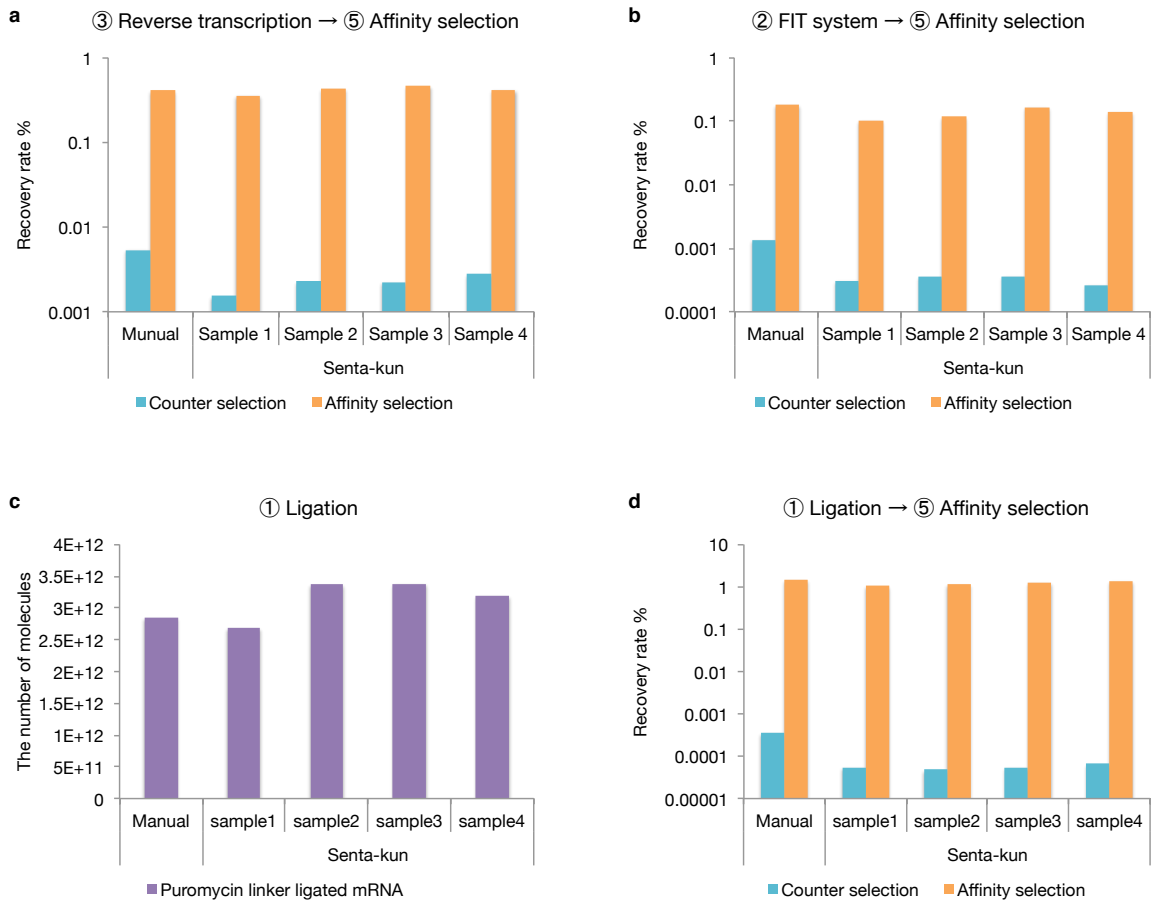

Figure 3. Optimization of Senta-kun. (a) Result of optimization from reverse transcription to selection step. (b) Result of optimization of in vitro translation. (c) Result of optimization ligation. (d) Result of a through experiment.

counter selection and affinity selection were qualified by qPCR analysis. I modified the parameters and repeated the same experiments until the recovery rates of Senta-kun's experiments became comparable as the data of my experiments. After optimization, Senta-kun could be setup to conduct step ③-⑤ with similar quality as my experiment (Figure 3a). Step ②-⑤ was optimized in the same manner as above. Since step ③-⑤ was already optimized, translation with FIT system (②) was only needed to optimized. The difficulty of translation was that a tiny amount of pellet of aminoacyl-tRNA needed to be resuspended in several μL of solution. When human researchers do the same experiment, aminoacyl-tRNA pellet is usually resuspended in 1 μL of solution. However, it is difficult for Senta-kun to do this with same scale or possibly cause poor reproducibility. Therefore, I determined a minimal volume of solution to resuspend the pellet that is compatible with Senta-kun's ability. 3 μL of solution results in stable data. With this condition, Senta-kun succeeded to perform step ②-⑤ with comparable data as my experiment (Figure 3b). Step ① was optimized by itself because this step consists of different types of experiments from the latter steps. Especially, this step requires phenol/chloroform extraction to purify the puromycin linker ligated mRNA after the ligation reaction. The detailed scheme of phenol/chloroform extraction is as follows: the ligation reaction solution is mixed with an equal amount of phenol/chloroform/isoamyl alcohol to insolubilize the proteins contained in the reaction mixture and the aqueous phase is transferred to a new tube. Chloroform/isoamyl alcohol solution is mixed to remove contaminated phenol and then the aqueous phase is transferred to a new tube again followed by ethanol precipitation to collect the puromycin linker ligated mRNA. Commonly, the volume of the reaction mixture is 40 μL. However, Senta-kun can't transfer full volume since Senta-kun can't recognize the interface between the aqueous phase and the organic phase. Thus, the sample volume was increased from 40 μL to 60 μL in accordance with the possible sample recovery volume in two consecutive extraction steps without contamination of organic phase. For the sample transfer, middle size tips are first used to aspirate greater part of samples and then switch to small tips to transfer samples as much as possible in a precise manner. To check this step, the purified mRNA were reverse transcribed and qualified by qPCR by myself. Figure 3c indicates that Senta-kun can recover enough amount of mRNA without contamination of organic solvent after the purification steps since the reverse transcription reaction is inhibited by phenol contamination. With the validation of optimization of each step, the optimization of Senta-kun has been finally done by a through experiment. I set four samples (a monoclonal mRNA) on Senta-kun and performed step ①-⑤. The recovered cDNA of all samples were qualified by qPCR by myself. The recovery rate of both the counter selection or the affinity selection was comparable among all samples generated by Senta-kun and myself (Figure 3d).

2.4 Conclusion

In this chapter, I described about the setup of Senta-kun after installing it in our laboratory. At the early days of the setup, the protocols and programs were just developed to behave similarly to a human researcher and didn't work at all for the automation of the RaPID system. Senta-kun couldn't do even simple things such as adding and mixing solutions using pipettes. I divided the whole procedure of the RaPID system into two parts in accordance with the capabilities of Senta-kun; one was done by Senta-kun and the other was done by myself. The protocol such as experimental procedures (additional steps, vortex, centrifugation) and sample volumes has been adapted to the abilities of the Senta-kun, and the parameters of robotic program have been optimized one by one for respective steps, with the result that Senta-kun can handle four samples in parallel and generate comparable data with my experiments.

In addition to the difference in motions, the biggest difference between Senta-kun and human is visual perception. Human researchers can easily recognize the position of a drop or a pellet in the tube and whether the samples are treated correctly, so human researchers can respond successfully as the situation demands on the other hand Senta-kun can't do so. Thus, Senta-kun has to be optimized precisely by repeating optimizations over and over to conduct experiments with high accuracy and reproducibility. Although Senta-kun has been setup to perform the RaPID system for biotin-tagged proteins in this chapter, these settings can be applied to the protocols for proteins with a different tag with modifications. The RaPID system for the proteins with Fc- or histidine-tag is also described in the latter chapters. The efforts and experiences archived in this chapter are not only useful for the automation of the RaPID system but also transferrable to other experiments that require accurate operations and are formally impossible to automate.

2.5 Materials and methods

Maholo (Senta-kun)

Maholo was purchased from YASKAWA Electric Corporation (now available from Robotic Biology Institute)

Instruments for Senta-kun

Graphic operation terminal GOT1000 (Mitsubishi Electric), A/C (Apiste), pipetty 20 µl, 250 µl, 1,000 µl (electric pipettes, Icomes Lab Co., Ltd.), pipettes (Thermo Scientific), MY BIO VT-78 (Nihon Freezer Co., Ltd.), MX-307 (refrigerated micro centrifuge, TOMY), 2x Incubator A0601-2V (AS ONE), 4x Cool Block Bath EC-40R (AS ONE), Rotary mixer NRC-20D (NISSIN), Vortex Genie-2 (Scientific Industries, Inc.), Capsulefuge PMC-060 (TOMY), Dehumidifier CD-P6316 (CORONA) installed especially for rain and summer seasons

Primers

All primers were purchased from Operon Biotechnology:

Fx-5'.F36:

5'- GTAATACGACTCACTATAGGATCGAAAGATTTCCGC -3'

eFx.R45:

5'- ACCTAACGCTAATCCCCTTTCGGGGCCGCGGAAATCTTTCGATCC -3'

T7ex5.F22:

5'- GGCGTAATACGACTCACTATAG -3'

eFx.R18:

5'- ACCTAACGCTAATCCCCT -3'

Ini1-1G-5'.F49:

5'-GTAATACGACTCACTATAGGCGGGGTGGAGCAGCCTGGTAGCTCGTCGG -3'

Inicat.R44:

5'- GAACCGACGATCTTCGGGTTATGAGCCCGACGAGCTACCAGGCT -3'

Ini-3'.R38:

5'- TGGTTGCGGGGGCCGGATTTGAACCGACGATCTTCGGG -3'

Ini-3'.R20 OMe:

5'- TGGTTGCGGGGGCCGGATTT -3'

T7g10M.F48:

5'-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATG-3'

CGS3an13.R39:

5'-TTTCCGCCCCCCGTCCTAGCTGCCGCTGCCGCTGCCGCA-3'

Puromycin linker:
5'-CTCCCGCCCCCCGTCC(SPC18)$_5$CC(Puromycin)-3'

Methionine(-) Flexizyme assisted In vitro Translation (FIT) system.
All in vitro translations in this thesis were conducted using a methionine-deficient version of the FIT system. The composition of the FIT system without pre-aminoacylated tRNA is as follows: 1.2 μM ribosome, T7 RNA polymerase, 4 μg/mL creatine kinase, 3 μg/mL myokinase, 0.1 μM pyrophosphatase, 0.1 μM nucleotide-diphosphatase kinase, 0.73 μM AlaRS, 0.02 μM CysRS, 0.13 μM AspRS, 0.23 μM GluRS, 0.68 μM PheRS, 0.09 μM GlyRS, 0.02 μM HisRS, 0.4 μM IleRS, 0.11 μM LysRS, 0.04 μM LeuRS, 0.03 μM MetRS, 0.38 μM AsnRS, 0.16 μM ProRS, 0.06 μM GlnRS, 0.03 μM ArgRS, 0.04 μM SerRS, 0.09 μM ThrRS, 0.02 μM ValRS, 0.03 μM TrpRS, 0.02 μM TyrRS, 0.26 μM EF-G, 10 μM EF-Tu, 10 μM EF-Ts, 2.7 μM IF1, 0.4 μM IF2, 1.5 μM IF3, 0.6 μM MTF, 0.25 μM RF2, 0.17 μM RF3, 0.5 μM RRF, 1.5 mg/mL E. coli total tRNA, 2 mM ATP, 1 mM UTP, 2 mM GTP, 1 mM CTP, 2 mM spermidine, 0.5 mM of all 20 proteinogenic amino acids except for methionine, 2 mM DTT, 0.1 mM 10-formyl-5,6,7,8-tetrahydrofolic acid, 20 mM creatine phosphate, 12 mM magnesium acetate, 100 mM potassium acetate, 50 mM HEPES-KOH (pH 7.6).

Prepararion of eFx, tRNA$^{fMet}_{CAU}$
eFx was in vitro transcribed using T7 RNA polymerase from a DNA template made by annealing and extension of primers, Fx-5'.F36 and eFx.R45 at 1 μM concentrations at 1 ml scale using an in-house made Taq polymerase. Following the extension reaction 10 μl of the extension reaction was added to a 2 ml PCR reaction containing 1 μM of both T7ex5.F22 and eFx.R18 and amplified until a band from a 2 μl sample of the PCR reaction was visible on a 3% agarose gel (approximately 12-15 cycles). All 2 ml of the PCR reaction was purified by phenol/chloroform extraction and ethanol precipitation. The pellet was resuspended in milliQ water and added to an in vitro transcription reaction with a total volume of 2 ml. After an overnight incubation at 37ºC, $MnCl_2$ was added to a 2 mM concentration along with DNase I and incubated for 30 min at 37ºC to degrade the DNA template. The transcribed product was pelleted via isopropanol precipitation and run on a denaturing 12% PAGE gel for 90 min at 230V. The slowest migrating band (eFx) was visualized with UV on a TLC plate and cut out. The eFx RNA was extracted from the band by three times treatments with ~7.5 ml 0.3 M NaCl. The 0.3 M NaCl solution containing the eFx RNA was collected by centrifugation, filter sterilized, and ethanol precipitated.

tRNA$^{fMet}_{CAU}$ was prepared in the same manner as eFx with the following exceptions: The

DNA template used for in vitro transcription of $tRNA^{fMet}_{CAU}$ was made by an initial extension reaction of the primers, Ini1-1G-5'.F49 and Inicat.R44. The reaction product was then added to a PCR reaction solution containing the primers, T7ex5.F22 and Ini-3'.R38. This PCR product was further extended and amplified using the primers, T7ex5.F22 and Ini-3'.R20-OMe, until a band from a 2 µl sample of the PCR reaction was visible on a 3% agarose gel (approximately 12-15 cycles). In vitro transcription was performed with an additional 5 mM GMP. After in vitro transcription and DNase I treatment, the sample was purified by denaturing 8% PAGE gel. Following gel extraction, the $tRNA^{fMet}_{CAU}$ RNA was precipitiated and resuspended in milliQ water. The concentration of the $tRNA^{fMet}_{CAU}$ RNA was adjusted to 250 µM (Absorbance at 260 nm, 77 bases).

eFx assisted aminoacylation of $tRNA^{fMet}_{CAU}$ with *N*-chloroacetyl D-tryptophan

eFx and $tRNA^{fMet}_{CAU}$ were mixed to a final concentration of 25 µM each with a $MgCl_2$ concentration of 600 mM in 50 mM HEPES-KOH pH 7.5. To this, an in-house synthesized *N*-chloroacetyl D-tryptophan cyanomethyl ester was added to a final concentration of 5 mM. After a 1 hour incubation on ice, the reaction contents were precipitated using an ethanol/sodium acetate mixture at pH 5.2. Pellets were kept dry and at -80ºC until use.

Puromycin linker ligation to mRNA

The mRNA was ligated to the puromycin linker primer via T4 ligase for 30 min at 25ºC at a 30 µL reaction scale and 30 µl of 0.6 M NaCl/10 mM EDTA was added to the ligation reaction solution and vortexed. The ligation product was extracted with phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol, and ethanol precipitated followed by 70% ethanol wash.

Translation with FIT system

1.5 µl of puromycin-conjugated mRNA was added to 2.5 µl of translation premix. tRNA pellets were resuspended in 3 µl of 1 mM sodium acetate and 1 out of 3 µL was added to the translation reaction followed by pipetting to mix homogeneously. The reaction was done at 37°C for 30 min and left at 25°C for 12 min. 1 µL of 100 mM EDTA was added to the reaction and incubated at 37°C for 30 min.

Reverse transcription

1.2 µl of MMLV RTase H- (Promega) was mixed with 15.3 µL of premix and 3.5 µl of this solution was added to the translation product followed by pipetting. The reaction was carried out at 42°C for 1 hour. After adding 3.5 µL of selection buffer, 0.5 µl of the reverse transcription reaction was removed and added to 500 µL of milliQ water for later

determination of the quantity of inputted mRNA. 13 µl of 2x blocking solution (0.2% acetylated BSA, 0.2 mg/ml yeast tRNA in selection buffer) was added and kept at 4°C.

Selection steps

74.4 µl of M280 Streptavidin Dynabeads was washed with 100 µl of ice-cold selection buffer three times and resuspended with 120 µl of ice-cold selection buffer. This beads was split into two 60 µls, and 3.2 µl of 500 µM biotin was added to one aliquot and incubated at 4°C for 10 min. This beads was washed again with 100 µL of ice-cold selection buffer three times. Following resuspension of washed beads, this beads was recombined with untreated beads. All this beads was split into 12 tubes and used for the counter selection. Positive beads was prepared by mixing of 24.8 µl of washed M280 Streptavidin Dynabeads and 10 µl of TET1CCD. TET1CCD immobilization was carried out at 4°C for 10 min and possible unoccupied streptavidin was blocked by incubation with 25 µl of biotin at 4°C for 10 min. This positive beads was washed with 100 µl of ice-cold selection buffer and split into four tubes. Three counter selections were performed for each sample per one round. All of 25.5 µl of reverse transcription product was added to beads prepared above for the counter selection and incubated at 4°C for 15 min. The sample was transferred to the next counter beads and repeated three times. The supernatant of the last counter selection was added to the positive beads and incubated at 4°C for 30 min. The both counter and positive beads were washed with 100 µL of ice-cold selection buffer while being transferred to new tubes every washing step. 100 µl of PCR mix containing the primers, T7g10M.F48 and CGS3an13.R39, and no Taq DNA polymerase was added to the beads.

qPCR, PCR amplification, and in vitro transcription

The amount of recovered cDNA from counter selection and affinity selection and input was quantified by Real-time PCR (Roche Light Cycler 2.0). The recovered cDNA from affinity selection was PCR amplified until a band from a 2 µl sample of the PCR reaction was visible on a 3% agarose gel and purified by phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol extraction, and ethanol precipitation followed by 70% ethanol wash. This cDNA mixture was in vitro transcribed and purified by phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol extraction, and isopropanol precipitation followed by 70% ethanol wash. The concentration of the mRNA library was measured using NanoDrop 2000c (ThermoFisher Scientific) and diluted to 10 µM.

2.6 References

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Chapter 3

Confirmation of the robotic operation and effects of selection conditions on outcomes

3.1 Introduction

In the previous chapter, we discussed the successfully setup of Senta-kun to carry out respective experiments with high quality and reproducibility. In this chapter, we discuss how our robot, Senta-kun, was tested and examined in a practical setting. To check if Senta-kun can successfully carry out the RaPID system to identify binders, proteins were chosen for which binders have been successfully identified by a human. This comparison was required because the RaPID system sometimes fails due to the quality or stability of the target proteins. Here, Ten-Eleven Translocation 1 (TET1) and cMET were chosen as the targets and the RaPID system was performed against them using the robot Senta-kun.

Additionally, there was another reason why target proteins that were successful in identifying binders during the RaPID system were used. The reason was to examine the reproducibility of the RaPID system and the effect of varying selection conditions on selection results. There are several parameters that are fixed individually by the researchers, for example selection temperature, protein concentration or the target for the counter selection (Table. 1). Those parameters are usually chosen based on previous case of success, and the RaPID system is never performed in several conditions against same target until it fails, in spite of the fact that the selection pressure is thought to be the most important factor for in vitro selection (direct evolution). Macrocyclic peptides with high binding affinity are selected in most of cases, but nobody knows whether or not they are the most potent possible species and how the conditions used during the selection compared to other results. If more potent peptides can be selected when repeating the RaPID system with different conditions, then performing the RaPID system under these different conditions in parallel is worth it.

In this chapter, a robotic selection has been performed against TET1 under the same conditions as a manual selection to investigate the reproducibility of the RaPID system, and a robotic selection has been performed against cMET under different selection conditions to see if there is an effect on the outcomes of the RaPID system.

Table 1. Various parameters of the RaPID system

Condition	General setting
Selection temperature / time	4°C / 30 min
Target protein concentration	200 nM
Peptide concentration	500 - 200 nM
Ratio of peptide and target protein	1 - 2.5
Counter part	Protein tag
Blocking reagent	Acetylated BSA
Wash conditions	100 - 300 μL of selection buffer / three times

3.3 Discovery of macrocylic peptide binders for cMET

3.3.1 cMET and cMET-binding macrocyclic peptides

The tyrosine-protein kinase MET (cMET), also known as the Hepatocyte Growth Factor Receptor (HGFR), is a single transmembrane protein and one of the receptor tyrosine kinases, consisting of a Semaphorin domain, a Plexins Semaphorin Integrins (PSI) domain and four Immunoglobulin-like Plexins Transcriptional factor (IPT) domains[98]. cMET interacts with its ligand, HGF, with stronger affinity between the IPT domain and the alpha subunit of HGF and with a weaker affinity between the Sema domain and the beta subunit of HGF[99]. The cMET/HGF signal pathway is associated with the regulation of many biological phenotypes, for example the Epithelial-Mesenchymal-Transition (EMT), cell proliferation, formation, differentiation, migration and invasion[100-104]. The cMET/HGF signalling pathway is initiated by the binding of HGF to cMET, forming a tetramer of 2:2 complex of cMET and HGF followed by phosphorylation of Y1234, Y1235, Y1349 and Y1356 in the kinase domain of cMET[105]. Subsequently, second messenger proteins such as GAB1 and GRB2 bind to the intracellular region of cMET to transmit downstream signals[106].

The cMET/HGF signalling pathway is also associated with the malignant alteration of cancers while the cMET/HGF signal pathway is essential for the regeneration of tissues[107]. Therefore, regulation of the cMET/HGF signalling pathway either by activation or inhibition, is thought to be a potential drug target for cancers (inhibition) and acute hepatitis, chronic kidney disease, fibroid lung and brain ischemia (activation) [108-112]. To date, many drugs targeting this pathway have been developed and they can be categorized into small molecules, proteins and antibodies. One possible strategy for targeting this pathway is by regulation of the cMET/HGF interaction. Antibodies and proteins such as HGF mimetics target extracellular domains, while small molecules interact with intracellular domains such as kinase domains. Drugs targeting the extracellular domain of cMET are only proteins, HGF mimetics or antibodies, thus small molecules that interact with the extracellular domain of cMET are needed in terms of cost. But at the same time it is difficult for small molecules to successfully regulate PPIs.

Because of this circumstance, macrocyclcic peptides that bind to an extracellular domain of cMET have been developed using the RaPID system in our group aiming at regulation of cMET/HGF signal pathway, and these peptides were reported as a research paper[53]. Although the selected peptides did not inhibit or activate cMET/HGF signal pathway, homodimerized peptides successfully accelerate cMET/HGF binding and downstream

signals. In this study, a selection was performed against the same cMET construct using Senta-kun in different conditions in order to assess effects of selection conditions to selection results (Figure 8a).

3.4 Conclusion

In this chapter, Senta-kun has been applied to perform conventional RaPID system against two targets, one is for TET1 and the other is for cMET, to confirm the utility of Senta-kun on daily operation. In addition, effects of selection conditions on selection results, which have been unclear for long, were examined by performing the RaPID system in different conditions against the same target protein, cMET. In terms of utility, the selected peptides from both robotic selections against TET1 or cMET exhibited binding ability (and inhibitory activity for TET1) for each target protein. Therefore, it is confirmed that Senta-kun is able to successfully perform the RaPID system in place of human researchers.

For TET1 selection, the most abundant sequence in the w-library of the manual selection and its mutants have been highly enriched in both y- and w-library of the robotic selection. This is not new sequence although other sequences were identified in the manual selection. Thus, differences could be observed in the results between the manual and robotic selection but similar. This sequence may be accidentally loss in the manual selection but recovered in the robotic selection in y-library. It is thought that these differences arose from differences of capabilities of human or Senta-kun, even if Senta-kun has been setup to perform the RaPID system in the same conditions as the manual one. These differences possibly take its rise between junior and senior researchers. However, it is unknown that which of smooth or slow operation of the RaPID system results better peptide sequences. TiP1-5 exhibited binding and inhibitory activity against TET1CD even they have been selected against TET1CCD. Furthermore, these peptides are TET1 inhibitors other than iron chelators. Repeating modification-reevaluation cycle of those peptides is a future work to improve their cell membrane permeability for further application, because they did not show cell activity (data not shown).

By contrast, in the cMET selections, different peptides have been enriched in the manual or robotic selection when the RaPID system was performed in the different conditions in an arbitrary manner. However, respective peptides exhibited tight binding to cMET extracellular domain that is used for the selection. A variety of peptides, which are assumed to bind different binding sites of cMET, with range of binding affinities were obtained, indicating that this is a successful strategy to discover lead compounds toward drugs. However, It is impossible to say that how big difference of which condition affects selection results and which condition settings are “best” before performing the RaPID system and evaluate the selected peptides, because it depends on target proteins, which indicate that the RaPID system have to be carried out in multiple different conditions to obtain better peptides. Moreover, there is a psychology barrier to repeat same experiments even if

researchers know performing the RaPID system in different conditions results more potent peptides. Therefore, automation of the RaPID system has a physical and psychological advantage for such application over manual operation.

3.5 Materials and methods

Primers

T7g10M.F48:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATG-3’
CGS3an13.R39:
5’-TTTCCGCCCCCCGTCCTAGCTGCCGCTGCCGCTGCCGCA-3’
RV template NNK:
5’-TGCGGCAGCGGCAGCGGCAGCTAGGACGGGGGGCGGAAA-3’
FW template NNK:
5’-GCTGCCGCTGCCGCTGCCGCA$(MNN)_n$CATATGTATATCTCCTTCTTAAAGTTAA-3’

Reagents for solid phase peptide synthesis (SPPS) and purification using reverse-phase HPLC

Following Fmoc-protected amino acids and reagents purchased from Merck Millipore were used for SPPS of the macrocyclic peptides described in this thesis; Fmoc-L-Ala-OH, Fmoc-L-Cys(Trt)-OH, Fmoc-L-Asp(tBu)-OH, Fmoc-L-Glu(tBu)-OH, Fmoc-L-Phe-OH, Fmoc-Gly-OH, Fmoc-L-His(Trt)-OH, Fmoc-L-Ile-OH, Fmoc-L-Lys(Boc)-OH, Fmoc-L-Leu-OH, Fmoc-L-Asn(Trt)-OH, Fmoc-L-Pro-OH, Fmoc-L-Gln(Trt)-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Ser(tBu)-OH, Fmoc-L-Thr(tBu)-OH, Fmoc-L-Val-OH, Fmoc-L-Trp(Boc)-OH, Fmoc-L-Tyr(tBu)-OH, Fmoc-D-Trp(Boc)-OH, O-(Benzotriazol-1-yl)-N,N,N’,N’,-tetramethyluronium hexafluorophosphate (HBTU), NovaPEG Rink Amide resin. 1-Hydroxy-1H-benzotriazole (HOBt) was purchased from Watanabe Chemical Industries. Dimethylformamide (DMF), N-methyl pyrrolidone (NMP), Dichloromethane (DCM), Acetonitrile, Diethyl Ether, Dimethyl sulfoxide (DMSO) piperidine, N,N-Diisopropylethylamine (DIPEA), Trifluoroacetic acid (TFA), Triethylamine (TEA), 1,2-Ethanedithiol (EDT), and Triisopropylsilane (TIS) were purchased from Wako pure chemical industries.

Expression of TET1 protein constructs

Biotinylated TET1CD (residues 1418-2136) and TET1CCD (residues 1418-2136, deleted 1744-1959) were expressed in insect cells as follows. FLAG-Avitag-TET1CD or FLAG-Avitag-TET1CCD was coexpressed with HA-BirA (an Avitag biotinylation enzyme) in Sf9 cells using the standard insect cell medium supplemented with 20 μM biotin. The biotinylated TET1CCD was purified by the affinity chromatography with anti-DYKDDDDK resin (WAKO) followed by the gel-filtration chromatography with the

Superdex200 16/60 column (GE). His-tagged TET1CD and its deletion mutants were expressed using the E. coli cell-free protein synthesis system[113]. As TET1CD was predicted to contain several Zn-binding sites, ZnCl2 (50 μM of final concentration) was added to the reaction mix to enhance the soluble expression of the proteins. The linear DNA templates for protein synthesis were generated using the two-step PCR method[114]. The expressed proteins were purified by the affinity chromatography with Ni Sepharose resin (GE).

Figure 10. Design of each TET1 construct.

Preparation of mRNA library

The mRNA library used in this thesis was prepared as follows: The mRNA library was transcribed from the DNA templates formed by the extension reaction of RV template NNK with either FW template NNK 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15. The extension reaction product was then PCR amplified using T7g10M.F48 and CGS3an13.R39. Following in vitro transcription, PAGE purification and gel extraction, the mRNAs were each adjusted to a final concentration of 20 μM. The composition of the final mixed library was 1.9×10^{-5}, 6.1×10^{-4}, 0.020, 0.625, 12.5, 12.5, 12.5, 12.5, 12.5, 12.5, 12.5, and 12.5% for

NNK 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15, respectively at a 10 μM concentration of total mRNA.

Aminoacylation of $tRNA^{fMet}_{CAU}$ with *N*-chloroacetyl L-tyrosine, *N*-chloroacetyl D-tyrosine and *N*-chloroacetyl D-tryptophan

Preaminoacylated $tRNA^{fMet}_{CAU}$s used in this thesis were prepared as follows: eFx and $tRNA^{fMet}_{CAU}$ were mixed at a final concentration of 25 μM in each 50 mM HEPES-KOH (pH 7.5) and placed on a heat block at 95°C for 5 min. This was left to cool at room temperature, and then $MgCl_2$ (f.c.: 600 mM) was added and left at room temperature for 5 min followed by cooling on ice. *N*-chloroacetyl L-tyrosine, *N*-chloroacetyl D-tyrosine or *N*-chloroacetyl D-tryptophan was added at a final concentration of 5 mM and incubated on ice for 2 hours. The RNAs contained in the reaction were precipitated by a sodium acetate/ethanol solution at pH 5.2. The obtained pellets were kept dry and at -80°C until use.

Selection of TET1CCD and cMET binding macrocyclic peptides via the RaPID system

The first round of selection was performed only against TET1CCD because the first round for cMET was shared with the previous study. At the first round of selection, the initial mRNA library was ligated to the puromycin linker using T4 ligase for 30 min at 25ºC and extracted with phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol, and ethanol precipitated. A 150 μl translation reaction with the methionine(-) FIT system and a 50 μM concentration of ClAc-D-Trp-$tRNA^{fMet}_{CAU}$ was used to convert the mRNA library into peptide-mRNA fusions. The translation was performed at 37ºC for 30 min followed by incubation at 25ºC for 12 min to enhance the formation of the peptide-mRNA complexes. 30 μl of 100 mM EDTA was added to dissociate the ribosome and peptides were incubated at 37ºC for 30 min to allow the thioether bond formation for peptide cyclization. The mRNA of the peptide-mRNA fusions were reverse transcribed using MMLV RTase H- (Promega) with the CGS3an13.R39 primer. The reaction was done at 42ºC for 1 hr. During reverse transcription, 90 pmol of biotinylated human TET1CCD was bound to 372 μl of M280 streptavidin dynabeads (Life Technologies) at 4ºC for 15 min. Possible unoccupied streptavidin molecules were blocked by the addition of 37.9 μl of 500 μM free biotin (f.c.: 25 μM biotin) for another 15 min at 4ºC. As blocking reagents, 0.1% acetylated BSA and 0.1 mg/ml yeast tRNA at a final concentration were added to the reverse transcribed product. TET1 reaction buffer (50 mM HEPES (pH 7.0), 90 mM NaCl, 2.6 mM DTT, 100 μM $Fe(NH_4)_2(SO_4)_2$, 2 mM L-ascorbic acid, 1 mM 2-oxoglutarate, 1 mM ATP, 0.05% TritonX-100) was used as the selection buffer for TET1 selection. These positive selection beads were then washed with 100 μL of ice-cold selection buffer and left on ice. 0.5 μl of the peptide-mRNA-cDNA complexes was removed and added to 500 μl of H_2O to later

determine the total amount of inputted mRNA. The peptide-mRNA-cDNA complexes were then incubated with the positive selection beads (200 nM of a final concentration) for 30 min at 4ºC. Following incubation, the beads were washed three times with ice-cold selection buffer while being transferred to new tubes every washing step. 400 µl of PCR mix containing the primers, T7g10M.F48 and CGS3an13.R39, and no Taq DNA polymerase was added to the beads. The tube containing the beads were placed on a 95ºC head block for 5 min to recover the cDNA of bound peptide-mRNA-cDNA complexes. The amount of recovered cDNA and input was determined by Real-time PCR (Roche Light Cycler 2.0). The recovered cDNA was PCR amplified until a 2 µl aliquot produced a visible band on a 3% agarose gel. This PCR product was used to transcribe the mRNA library for the next round after purification by Phenol/Chloroform extraction and ethanol precipitation. The subsequent rounds of selection were performed by Senta-kun in the same manner with the following exceptions: The translation scale was 5 µl and all following steps were scaled down accordingly. Three counter selection steps were added preceding the positive selection steps using 6.2 µl each of biotin bound and unbound M280 Streptavidin dynabeads. The counter selection steps were washed and cDNA of bound peptide-mRNA-cDNA complexes were quantified in the same manner as the affinity selection. For the cMET selection, Fc-tagged human cMET extracellular domain (Sino Biological Inc.) was bound to Dynabeads protein G and TBST (0.05% Tween20) was used as the selection buffer. Additionally, 0.1% Acetylated BSA was added to the reverse transcription product as the blocking reagent, and the recombinant human IgG_1 Fc (R&D systems) bound beads and untreated beads were used for the counter selection.

DNA sequencing via MiSeq

The index sequences were attached to both termini of the recovered cDNAs by two consecutive PCR extensions. The PCR reaction was performed until a 2 µl aliquot produced a visible band on a 3% agarose gel (4 cycles for the first PCR and approximately 8-12 cycles for the second PCR). The extension products were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) following the instruction attached. The concentration of the purified DNAs were determined by Qubit and Qubit dsDNA BR Assay kit (Invitrogen) and adjusted to 10 nM (average length: NNK_{10}, 253 bp). Subsequent preparation of samples for MiSeq (Illumina) was done by following the general instruction and MiSeq Reagent Kit v2 was used for the runs.

Solid phase peptide synthesis (SPPS) and purification using a reverse-phase HPLC (RP-HPLC)

All peptides were synthesized using the conventional Fmoc-based SPPS via a Syro Wave automated peptide synthesizer (Biotage). Each coupling step of Fmoc-protected amino acids was performed on DMF engorged NovaPEG Rink Amide resins with 0.5 M Fmoc-protected amino acid, 0.5 M HBTU/HOBt and 0.5 M DIPEA in DMF. After three programmed washes with DMF, Fmoc-deprotection was performed by a 30 min RT treatment with 40% piperidine in DMF. Double coupling was used for all amino acids. After all amino acids were coupled, 0.2 M chloroacetyl-NHS in DMF was used to add a chloroacetyl group to the N-terminus of each peptide. This step was repeated twice, followed by three DMF washes, six DCM washes and dried in vacuo. Dried peptide-conjugated resins were then fully deprotected and cleaved from the resin in a mixture of pre-chilled TFA/H_2O/EDT/TIS (92.5 : 2.5 : 2.5 : 2.5%) for 3 hours at RT. The deprotection solution was precipitated by cold diethyl ether and the pellets were dried in the fume hoods. These pellets were dissolved in DMSO 0.1% TFA followed by the addition of TEA until the solution was basic (confirmed using H_2O and pH indicator paper) to induce cyclization of the peptides. After 30 min at RT in basic conditions, peptide mass and cyclization was confirmed on MALDI-TOF MS analysis (Autoflex II, Bruker Daltonics). Once peptide mass and cyclization was confirmed, the peptide solution was brought back to acidic conditions with TFA (confirmed using H_2O and pH indicator paper) and purified on a Shimadzu Prominence RP-HPLC using a Chromolith ODC column with H_2O 0.1% TFA as solution A and acetonitrile 0.1% TFA as solution B. The acetonitrile from the fractions containing pure peptide samples was centrifugally evaporated followed by lyophilization. Final peptide masses and purity were confirmed by MALDI-TOF MS analysis.

Surface plasmon resonance (SPR)

The dissociation constants of the selected peptides from the RaPID system against TET1CCD, TET1CD and cMET were determined using single cycle kinetics method on a Biacore T200 (GE Healthcare). The same TET1CCD and cMET extracellular domain used in the selection and biotinylated full length TET1 catalytic domain (TET1CD, kindly provided from Prof. Aburatani group in The University of Tokyo) were immobilized to a Biotin Capture-SA sensor chip (GE Healthcare) for TET1 constructs and Series S Sensor Chip Protein G for cMET as ligands. Filter sterilized TET1 reaction buffer used in the selection containing 0.1% DMSO and HBS EP+ containing 0.1% DMSO were used as running buffers for TET1 constructs and cMET respectively. For TET1, two lanes of the sensor chip was regenerated with 6 M guanidine-HCl, 250 mM NaOH for 120 s at 20 μl/min. Oligo-SA was immobilized to two lanes of the sensor chip at 2 μl/min for 300 s.

100 nM TET1CCD or TET1CD in running buffer was immobilized to the second lane at 2 μ l/min for 900 s with 900 s of stabilization step. An initial run was performed with each peptide using single cycle kinetics with five concentrations between 1 and 1000 nM. Dissociation constants from the initial run were used to determine the peptide concentrations used for the second run for cleaner peaks (10 times higher the concentration of K_D as the highest peptide concentration and four 2 times serial dilutions from the highest concentrations). The peptides were run at 30 µl/min with 120 s of association and dissociation time and 30 s of stabilization step. For cMET, two lanes of the sensor chip was regenerated with 10 mM Glycin HCl (pH 1.5) for 120 s at 20 µl/min. 1.93 µM human cMET extracellular domain in running buffer was immobilized to the second lane at 2 µl/min for 600 s with stabilization step for 60 s. An initial run was performed with each peptide using single cycle kinetics method with five concentrations between 1 and 1000 nM. Dissociation constants from the initial run were used to determine the peptide concentrations used for the second run for cleaner peaks (10 times higher the concentration of K_D as the highest peptide concentration and four 2 times serial dilutions from the highest concentrations). The peptides were run at 30 µl/min with 120 s of association and dissociation time and 30 s of stabilization step.

In vitro TET1 inhibitory assay using Alpha technology

Alpha screen technology was employed to assess TET1 inhibitory activity by quantifying 5-hmC containing DNA in TET1 reaction mixture. In vitro TET1 reaction was carried out in 10 µL reaction volume in assay buffer (50 mM HEPES-KOH (pH 6.7), 150 mM NaCl, 0.1% BSA, 0.01% Tween20). The reaction consisted of 10 µM $(NH_4)_2Fe(SO_4)_2$, 10 µM 2OG, 100 µM Ascorbic acid, 10 nM substrate ssDNA, 8 nM human TET1CD (Epigentek) and incubated for 30 min (TET1CD) at 25℃ on a 384-well proxi plate. In this experiment, ssDNA (5'-TCG GAT GTT GTG GGT CAG ^{m}CGC ATG ATA GTG TA-3' with 5' Biotin label) was used as a substrate. Both acceptor and donor beads were combined, and preincubated with anti-5hmC antibody (1:2000, Active Motif) for 30 min at 25℃. TET1 reaction was quenched by adding 20 mM EDTA (5 µL). Anti-5hmC antibody immobilized Alpha screen bead mixture (5 µL) was then added and further incubated for 1 h at 25℃. Alpha screen signals were measured using EnVision plate reader (PerkinElmer). All steps treating with alpha screen beads were carried out in a dark.

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Chapter 4

Practical operation and a novel application

4.1 Introduction

In chapter 3, it was confirmed that Senta-kun could perform the RaPID system and target binders could be identified against secure proteins, TET1 and cMET. In this chapter, a robotic selection has been performed against a new target protein, Smurf2, as the first example toward practical operations of Senta-kun for future.

Furthermore, I attempted to develop and conduct a new strategy toward affinity maturation of a particular peptide using the same protocol as the RaPID system and Senta-kun. As a strong point of the RaPID system, a vast library of peptides can be used. However, a whole diversity of designed peptides (10^{19} order) is not covered by initial library (10^{14} order), and selection results are biased by PCR amplification (Once a less potent peptide is amplified, it will be enriched round by round and suppress better peptides.). Those demerits of the RaPID system can cause a loss of desired peptides. From those reason, there is a possibility that peptides, which are similar to a selected peptide, show stronger binding affinity than the original peptide. I thought that selected peptides from the RaPID system still have room to improve their affinity. If similar peptides are evaluated systematically, more potent peptides could be discovered. To archive this, I prepared partially randomized peptides based on the sequence of a know peptide inhibitor as a library and conducted the same experimental scheme as the RaPID system to select better peptides from the library. Since experimental scheme itself was same as the RaPID system, Senta-kun could conduct the experiment based on the protocol developed in chapter 2 with modifications.

4.2 Discovery of macrocyclic peptides for Smurf2

4.2.1 Mechanism of ubiquitination and biological role of Smurf2

Ubiquitination regulates numerous cellular functions by tagging proteins for proteasome degradation or incorporation into other regulatory complexes, e.g. the removal of misfolded proteins, the regulation of signaling pathways, DNA repair, the cell cycle, and apoptosis [115] [116] [117] [118]. Target-selective ubiquitination is controlled by a series of proteins, termed E1-3. E1 proteins, also know as ubiquitin-activating enzymes, initiate the ubiquitination process. E1 catalyze activation of free ubiquirtin using ATP and attach it onto a cysteine residue on an ubiquitin conjugation protein E2 through thioester bond. The substrate protein, which is needed to be tagged, is recognized by E3 ubiquitin ligases and transferred ubiquitin protein from the E2. The ubiquitin is attached to a lysine residue on the substrate protein through a isopeptide bond (Figure 1). This process repeats until a full ubiquitin chain is attached to the substrate protein[117].

There are two main E3 ubiquitin ligase classes: the RING domain ligases involving several hundred E3 ubiquitin ligases and the HECT domain ligases consisting of around 28 members [119] [120] [121]. Smurf2 is one of the HECT domain ligases and its gene was identified in 2000 by searching sequence database [122] [123]. Smurf2 encodes a C2-WW-HECT domain ubiquitin ligase: C2 is a phospholipid/calcium-binding domain and WW domain binds to proteins containing PPXY or similar motif in substrates, adaptor, regulatory proteins. The HECT domain binds to substrate proteins and contains a catalytic cysteine residue in the HECT domain to intermediate ubiquitin ligation reaction. Smurf2 is involved in degradation of SMADs, TGF-beta receptor, and other substrates. Smurf2 also functions in regulation of neuronal and planar cell polarity, induction of senescence, and tumor suppression [124]. By contrast, it has been reported that high-level expression of Smurf2 is correlated with depth of invasion, lymph node metastasis and esophageal squamous cell carcinoma, indicating that Smurf2 is a potent drug target [125,126] [127].

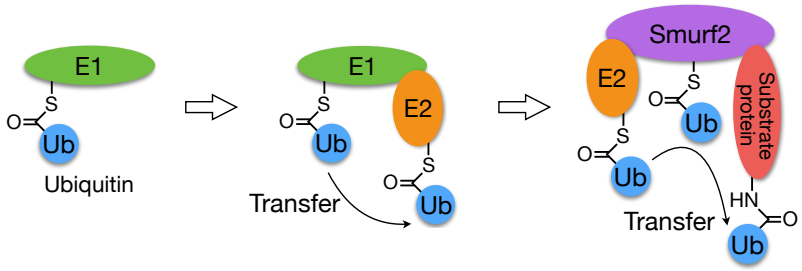

Figure 1. Ubiquitination cascade. Smurf2 is one of E3 ubiquitin ligases.

Here, the RaPID system has been performed against Smurf2 HECT domain that is a new target protein for the RaPID system. By targeting Smurf2, useful macrocyclic peptides as drug leads or molecular tools were discovered, in addition to performing the RaPID system against a new target protein using Senta-kun.

4.3 A novel selection approach against Akt2

4.3.1 Biological role of Akt2 and macrocyclic peptide inhibitor

The Akt family, also known as protein kinase B, belongs to the serine/threonine kinase family, playing crucial roles in regulation of various cellular signal transduction pathways[129]. In human, three isoforms, Akt1, Akt2, and Akt3, are known and all isoforms share similar structures: an N-terminal regulatory domain including a pleckstrin homology (PH) domain, a hinge region connecting the PH domain and a serine/threonine specific kinase domain, and a C-terminal region necessary for the induction and maintenance of its kinase activity. While Akt2 is ubiquitously expressed, Akt2 is highly expressed in insulin sensitive tissues, e.g. liver, skeletal muscle and adipose tissue, and Akt3 is expressed in brain at most high level and in intestinal organs and muscle tissue at lower level[130-132].

Since Akt family was originally discovered as a homolog protein encoded by the viral oncogene *v-akt*, numerous studies have conducted to reveal oncogenic amplifications of *Akt* genes in primary human tumors and cancer cells[132]. Elevated expression of Akt1 has been observed in gastric, breast, prostate, and ovarian cancers, implying that Akt1 functions in the cancer development. Overexpression of Akt2 has been also observed in various carcinomas, suggesting that Akt2 plays similar roles as Akt1 in alteration and development of these cancer cells. However, it has been suggested that Akt2 likely plays a specific role in the insulin receptor signal transduction, implying that Akt2 is a potent drug target for diabetes mellitus[133]. A number of Akt inhibitors have been discovered using four different strategies: i) competitive inhibitors bind to the ATP-binding site, ii) those which bind to the PH domain, iii) allosteric inhibitors, and iv) substrate peptide analogs. Akt family selective inhibitors had been obtained, but it was still a difficult challenge to develop Akt family and isoform-selective inhibitors.

To achieve an Akt2 selective inhibitor, the RaPID system has been previously performed again full length Akt2 in our group, and a selective peptide inhibitor, named as Pakti-L1, has been discovered. Pakti-L1 exhibited a strong inhibitory activity for Akt2 with an IC50 of 110 nM and showing more than 225 fold and 38 fold selectivity against Akt1 and Akt2 respectively[50]. In this study, I attempted to improve the Pakti-L1's activity by performing the RaPID system using a focused library based on the Pakti-L1 sequence since I hypothesized that more active peptides could be obtained by searching similar sequences as Pakti-L1 (Figure 5).

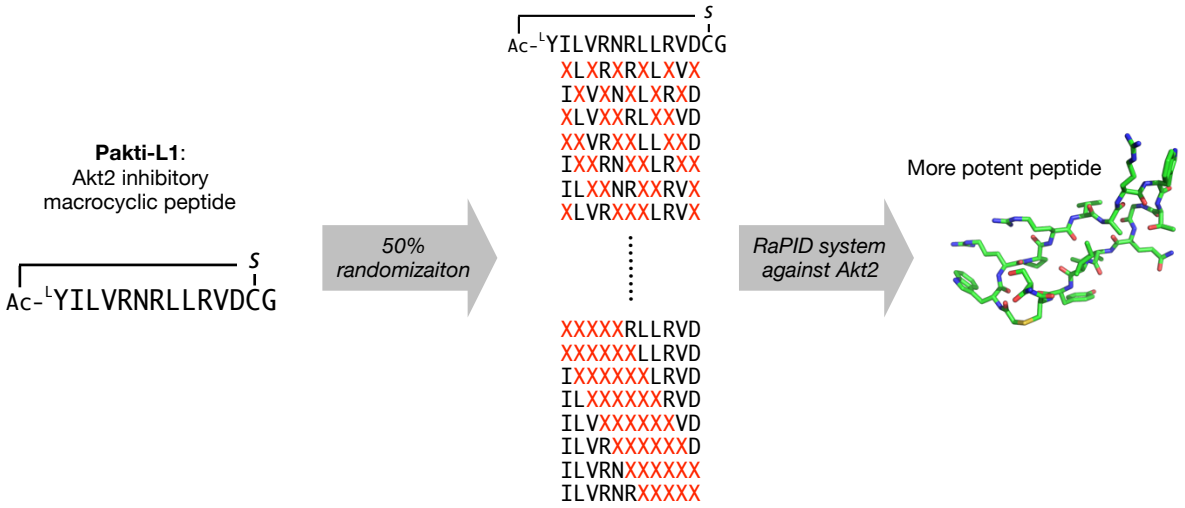

Figure 5. Strategy overview of this section.

4.4 Conclusion

In this chapter, the RaPID system has been performed against two different proteins to show examples for future applications of Senta-kun. Smurf2 was chosen just as one of new targets for the RaPID system and has been successfully targeted thus other proteins that have never been targeted by the RaPID system in spite of their charm as a scientific or therapeutic purpose will be performed the RaPID system against using Senta-kun yet. In another experiment in this chapter, I attempted to show an application of the RaPID system toward affinity maturation of a specific peptide while expected results were not obtained. However, it is worthy to apply this strategy to other peptides. There is still room for the RaPID system to improve its ability or to apply for other purposes. Automation saves time and effort of researchers to conduct experiments and makes it easy to try new ideas or to conduct troublesome experiments by reducing psychological barrier.

4.5 Materials and methods

Primers

Pakti-L1.F94:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTC
TGGTCCGTAATCGCTTATTGCGTGTCGATTGCGGCAGCGGCA-3’
FWPakti-L1-1:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKC
TGNNKCGTNNKCGCNNKTTGNNKGTCNNKTGCGGCAGCGGCA-3’
FWPakti-L1-2:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTN
NKGTCNNKAATNNKTTANNKCGTNNKGATTGCGGCAGCGGCA-3’
FWPakti-L1-3:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKC
TGGTCNNKNNKCGCTTANNKNNKGTCGATTGCGGCAGCGGCA-3’
FWPakti-L1-4:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKN
NKGTCCGTNNKNNKTTATTGNNKNNKGATTGCGGCAGCGGCA-3’
FWPakti-L1-5:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTN
NKNNKCGTAATNNKNNKTTGCGTNNKNNKTGCGGCAGCGGCA-3’
FWPakti-L1-6:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTC
TGNNKNNKAATCGCNNKNNKCGTGTCNNKTGCGGCAGCGGCA-3’
FWPakti-L1-7:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKC
TGGTCCGTNNKNNKNNKTTGCGTGTCNNKTGCGGCAGCGGCA-3’
FWPakti-L1-8:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKN
NKGTCCGTAATNNKNNKNNKCGTGTCGATTGCGGCAGCGGCA-3’
FWPakti-L1-9:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKN
NKNNKCGTAATCGCNNKNNKNNKGTCGATTGCGGCAGCGGCA-3’
FWPakti-L1-10:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTN
NKNNKNNKAATCGCTTANNKNNKNNKGATTGCGGCAGCGGCA-3’

FWPakti-L1-11:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTC
TGNNKNNKNNKCGCTTATTGNNKNNKNNKTGCGGCAGCGGCA-3’
FWPakti-L1-12:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTC
TGGTCNNKNNKNNKTTATTGCGTNNKNNKTGCGGCAGCGGCA-3’
FWPakti-L1-13:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKC
TGGTCCGTAATNNKNNKNNKNNKGTCGATTGCGGCAGCGGCA-3’
FWPakti-L1-14:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKN
NKGTCCGTAATCGCNNKNNKNNKNNKGATTGCGGCAGCGGCA-3’
FWPakti-L1-15:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKN
NKNNKCGTAATCGCTTANNKNNKNNKNNKTGCGGCAGCGGCA-3’
FWPakti-L1-16:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKN
NKNNKNNKAATCGCTTATTGNNKNNKNNKTGCGGCAGCGGCA-3’
FWPakti-L1-17:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTN
NKNNKNNKNNKCGCTTATTGCGTNNKNNKTGCGGCAGCGGCA-3’
FWPakti-L1-18:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTC
TGNNKNNKNNKNNKTTATTGCGTGTCNNKTGCGGCAGCGGCA-3’
FWPakti-L1-19:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTC
TGGTCNNKNNKNNKNNKTTGCGTGTCGATTGCGGCAGCGGCA-3’
FWPakti-L1-20:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTC
TGGTCCGTNNKNNKNNKNNKCGTGTCGATTGCGGCAGCGGCA-3’
FWPakti-L1-21:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKC
TGGTCCGTAATCGCNNKNNKNNKNNKNNKTGCGGCAGCGGCA-3’
FWPakti-L1-22:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKN
NKGTCCGTAATCGCTTANNKNNKNNKNNKTGCGGCAGCGGCA-3’

FWPakti-L1-23:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKN
NKNNKCGTAATCGCTTATTGNNKNNKNNKTGCGGCAGCGGCA-3’
FWPakti-L1-24:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKN
NKNNKNNKAATCGCTTATTGCGTNNKNNKTGCGGCAGCGGCA-3’
FWPakti-L1-25:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKN
NKNNKNNKNNKCGCTTATTGCGTGTCNNKTGCGGCAGCGGCA-3’
FWPakti-L1-26:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTN
NKNNKNNKNNKNNKTTATTGCGTGTCGATTGCGGCAGCGGCA-3’
FWPakti-L1-27:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTC
TGNNKNNKNNKNNKNNKTTGCGTGTCGATTGCGGCAGCGGCA-3’
FWPakti-L1-28:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTC
TGGTCNNKNNKNNKNNKNNKCGTGTCGATTGCGGCAGCGGCA-3’
FWPakti-L1-29:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTC
TGGTCCGTNNKNNKNNKNNKNNKGTCGATTGCGGCAGCGGCA-3’
FWPakti-L1-30:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTC
TGGTCCGTAATNNKNNKNNKNNKNNKGATTGCGGCAGCGGCA-3’
FWPakti-L1-31:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKC
TGGTCCGTAATCGCTTANNKNNKNNKNNKTGCGGCAGCGGCA-3’
FWPakti-L1-32:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKN
NKGTCCGTAATCGCTTATTGNNKNNKNNKTGCGGCAGCGGCA-3’
FWPakti-L1-33:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKN
NKNNKCGTAATCGCTTATTGCGTNNKNNKTGCGGCAGCGGCA-3’
FWPakti-L1-34:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKN
NKNNKNNKAATCGCTTATTGCGTGTCNNKTGCGGCAGCGGCA-3’

FWPakti-L1-35:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKN
NKNNKNNKNNKCGCTTATTGCGTGTCGATTGCGGCAGCGGCA-3’
FWPakti-L1-36:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGNNKN
NKNNKNNKNNKNNKTTATTGCGTGTCGATTGCGGCAGCGGCA-3’
FWPakti-L1-37:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTN
NKNNKNNKNNKNNKNNKTTGCGTGTCGATTGCGGCAGCGGCA-3’
FWPakti-L1-38:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTC
TGNNKNNKNNKNNKNNKNNKCGTGTCGATTGCGGCAGCGGCA-3’
FWPakti-L1-39:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTC
TGGTCNNKNNKNNKNNKNNKNNKGTCGATTGCGGCAGCGGCA-3’
FWPakti-L1-40:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTC
TGGTCCGTNNKNNKNNKNNKNNKNNKGATTGCGGCAGCGGCA-3’
FWPakti-L1-41:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTC
TGGTCCGTAATNNKNNKNNKNNKNNKNNKTGCGGCAGCGGCA-3’
FWPakti-L1-42:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGATTC
TGGTCCGTAATCGCNNKNNKNNKNNKNNKTGCGGCAGCGGCA-3’
T7g10M.F48:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATG-3’
CGS3an13.R39:
5’-TTTCCGCCCCCCGTCCTAGCTGCCGCTGCCGCTGCCGCA-3’
Smurf-y1.F84:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGCTGA
GCTGGCGCTGGATTCTGACCAAACGTGCTCTG-3’
Smurf-y1.R72:
5’-TTTCCGCCCCCCGTCCTAGCTGCCGCTGCCGCTGCCGCAGGTATATTGCAGAG
CACGTTTGGTCAGAATCCA-3’
Smurf-y2.F78:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGAAGG
AGACACCCCCAAAATGCCCGTGGTTC-3’

Smurf-y2.R69:
5’-TTTCCGCCCCCCGTCCTAGCTGCCGCTGCCGCTGCCGCAACGCTGTAAGAACC
ACGGGCATTTTGGGGG-3’
Smurf-w1.F84:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGACCA
TCTGCTTGCATATTGCGTTGCGTAAACAATTA-3’
Smurf-w1.R75:
5’-TTTCCGCCCCCCGTCCTAGCTGCCGCTGCCGCTGCCGCAAGAAAATAATTGTT
TACGCAACGCAATATGCAAGCA-3’
Smurf-w2.F84:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGAGCA
TTTGCCTGCATATTTTGTTATTCTGTCCGAAT-3’
Smurf-w2.R78:
5’-TTTCCGCCCCCCGTCCTAGCTGCCGCTGCCGCTGCCGCAGATTGGCGTATTCG
GACAGAATAACAAAATATGCAGGCA-3’
Smurf-w3.F84:
5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGACAA
TCTGTCTTCACTTTGCCATTTTTAAGGATCAT-3’
Smurf-w3.R78:
5’-TTTCCGCCCCCCGTCCTAGCTGCCGCTGCCGCTGCCGCATTCACGATGATCCT
TAAAAATGGCAAAGTGAAGACAGAT-3’

Selection of Smurf2 binding macrocyclic peptides via the RaPID system

The first round of selection was performed manually. At the first round of selection, the initial mRNA library was ligated to the puromycin linker using T4 ligase for 30 min at 25ºC and extracted with phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol, and ethanol precipitated. A 150 µl translation reaction with the methionine(-) FIT system and a 50 µM concentration of ClAc-D-Trp-tRNA$^{fMet}_{CAU}$ and ClAc-D-Try-tRNA$^{fMet}_{CAU}$ were used to convert the mRNA library into peptide-mRNA fusions for preparation of y- and w-library. The translation was performed at 37ºC for 30 min followed by incubation at 25ºC for 12 min to enhance the formation of the peptide-mRNA complexes. 30 µl of 100 mM EDTA was added to dissociate the ribosome and peptides were incubated at 37ºC for 30 min to allow the thioether bond formation for peptide cyclization. The mRNA of the peptide-mRNA complexes were reverse transcribed using MMLV RTase H- (Promega) with the CGS3an13.R39 primer. The reaction was carried out at 42ºC for 1 hr. During reverse transcription, biotinylated human Smurf2 HECT domain was bound to 27.8 µl of M280 streptavidin dynabeads (Life Technologies) at 4ºC for 15 min. GB1 protein was

immobilized on counter beads since GB1 protein was conjugated to the Smurf2 HECT domain to be solubilized. As blocking reagents, 0.1% Acetylated BSA at a final concentration in TBST was added to the reverse transcribed product. TBST (0.05% Tween20) was used as the selection buffer for Smurf2 selection. These positive selection beads were then washed with 100 µL of ice-cold selection buffer and left on ice. 0.5 µl of the peptide-mRNA-cDNA complexes was removed and added to 500 µl of H_2O to later determine the total amount of inputted mRNA. The peptide-mRNA-cDNA complexes were then incubated with the positive selection beads (200 nM of a final concentration) for 30 min at 4ºC. Following incubation, the beads were washed three times with ice-cold selection buffer while being transferred to new tubes every washing step. 400 µl of PCR mix containing the primers, T7g10M.F48 and CGS3an13.R39, and no Taq DNA polymerase was added to the beads. The tube containing the beads were placed on a 95ºC head block for 5 min to recover the cDNA of bound peptide-mRNA-cDNA complexes. The amount of recovered cDNA and input was determined by Real-time PCR (Roche Light Cycler 2.0). The recovered cDNA was PCR amplified until a 2 µl aliquot produced a visible band on a 3% agarose gel. This PCR product was used to transcribe the mRNA library for the next round after purification by Phenol/Chloroform extraction and ethanol precipitation. The subsequent rounds of selection were performed by Senta-kun in the same manner with the following exceptions: The translation scale was 5 µl and all following steps were scaled down accordingly. Three counter selection steps were added preceding the positive selection steps using 0.93 µl each of biotin bound and unbound M280 Streptavidin dynabeads. The counter selection beads were washed and cDNA of bound peptide-mRNA-cDNA complexes were quantified in the same manner as the affinity selection.

DNA sequencing via MiSeq

The index sequences were attached to both termini of the recovered cDNAs by two consecutive PCR extensions. The PCR reaction was performed until a 2 µl aliquot produced a visible band on a 3% agarose gel (4 cycles for the first PCR and approximately 8-12 cycles for the second PCR). The extension products were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) following the instruction attached. The concentration of the purified DNAs were determined by Qubit and Qubit dsDNA BR Assay kit (Invitrogen) and adjusted to 10 nM (average length: NNK_{10}, 253 bp). Subsequent preparation of samples for MiSeq (Illumina) was done by following the general instruction and MiSeq Reagent Kit v2 was used for the runs.

Surface plasmon resonance (SPR)

The dissociation constants of the selected peptides from the RaPID system against Smurf2 were determined using single cycle kinetics method on a Biacore T200 (GE Healthcare). The same Smurf2 HECT domain used in the selection was immobilized to a Biotin Capture-SA sensor chip (GE Healthcare) as a ligand. Filter sterilized TBST (0.05% Tween20, 0.1% DMSO) was used as running buffer. Two lanes of the sensor chip was regenerated with 6 M guanidine-HCl, 250 mM NaOH for 300 s at 20 µl/min followed by an extra wash with 50% DMSO. Oligo-SA was immobilized to two lanes of the sensor chip at 2 µl/min for 300 s. 5 µM biotinylated Smurf2 HECT domain in running buffer was immobilized to the second lane at 2 µl/min for 300 s with 600 s of stabilization. An initial run was performed with each peptide using single cycle kinetics method with five concentrations between 1 and 1000 nM. Dissociation constants from the initial run were used to determine the peptide concentrations used for the second run for cleaner peaks (10 times higher the concentration of K_D as the highest peptide concentration and four 2 times serial dilutions from the highest concentrations). The peptides were run at 30 µl/min with 120 s of association and dissociation time and the lanes were washed with 50% DMSO followed by 30 s of stabilization step.

Preparation of the partially randomized peptide library used for the Akt2 selection

0.84 µM of mixture of 48 forward primers, FWPakti-L1-1 – FWPakti-L1-48, and the CGS3an13.R39 primer were annealed and extended via Taq DNA polymerase at a 500 µl scale. After checking whether a band from a 2 µl sample of the PCR reaction was visible on a 3% agarose gel, all 500 µl of the extension reaction was purified by phenol/chloroform extraction and ethanol precipitation. Following in vitro transcription at a 1 ml scale, the transcribed product was purified by 8% denaturing PAGE, gel extraction and ethanol precipitation. The pellet of mRNA library was resuspended in milliQ water and adjusted to a final concentration of 20 µM.

Selection of Akt2 binding macrocyclic peptides via the RaPID system

For this selection, every selection round was performed using a 5 µl scale in vitro translation because diversity of the library was small enough to be covered and was done by Senta-kun. The mRNA library was ligated to the puromycin linker using T4 ligase for 30 min at 25ºC and extracted with phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol, and ethanol precipitated. A 5 µl translation reaction with the methionine(-) FIT system and a 50 µM concentration of ClAc-L-Tyr-$tRNA^{fMet}_{CAU}$ was used to convert the mRNA library into peptide-mRNA fusions. The translation was performed at 37ºC for 30 min followed by incubation at 25ºC for 12 min to enhance the formation of the

peptide-mRNA complexes. 1 µl of 100 mM EDTA was added to dissociate the ribosome and peptides were incubated at 37ºC for 30 min to allow the thioether bond formation for peptide cyclization. The mRNA of the peptide-mRNA complexes were reverse transcribed using MMLV RTase H- (Promega) with the CGS3an13.R39 primer. The reaction was conducted at 42ºC for 1 hr. During reverse transcription, human Akt2 (inactive, abcam) was bound to 1 µl of Dynabeads His-Tag Isolation and Pulldown (Life Technologies) at 4ºC for 30 min. As blocking reagents, 0.1% Acetylated BSA at a final concentration in PBST (0.5% Tween20) was added to the reverse transcribed product. PBST (0.5% Tween20) was used as the selection buffer for Akt2 selection. These positive selection beads were then washed with 100 µL of ice-cold selection buffer and left on ice. 0.5 µl of the peptide-mRNA-cDNA complexes was removed and added to 500 µl of H_2O to later determine the total amount of inputted mRNA. Three counter selection steps were performed preceding the positive selection steps using 7 µl each of biotin bound and unbound Dynabeads His-Tag Isolation and Pulldown. The peptide-mRNA-cDNA complexes were then incubated with the positive selection beads (200 nM of a final concentration) for 30 min at 4ºC. Following incubation, the beads of counter and Akt2 selection were washed three times with ice-cold selection buffer while being transferred to new tubes every washing step. 100 µl of PCR mix containing the primers, T7g10M.F48 and CGS3an13.R39, and no Taq DNA polymerase was added to the beads. The tube containing the beads were placed on a 95ºC head block for 5 min to recover the cDNA of bound peptide-mRNA-cDNA complexes. The amount of recovered cDNA and input was determined by Real-time PCR (Roche Light Cycler 2.0). The recovered cDNA was PCR amplified until a 2 µl aliquot produced a visible band on a 3% agarose gel. This PCR product was used to transcribe the mRNA library for the next round after purification by Phenol/Chloroform extraction and ethanol precipitation.

Akt2 inhibitory assay via Time resolved fluorescent resonance energy transfer (TR-FRET)

Akt2 inhibitory activity of the synthesized peptides was evaluated using HTRF KinEASE-STK (cisbio assays) and Akt2 protein (Carna biosciences). The experimental procedures are briefly shown below while following the protocol provided from cisbio assays. 5 µL of the enzymatic buffer (50 mM HEPES (pH 7.0), 0.1 mM Orthovanadate, 0.02% NaN_3, 0.01% BSA) containing the synthesized peptide and 5 µl of 1ng/µl Akt2 was mixed and incubated at room temperature for 15 min. 5 µl of both 1.5 µM biotinylated Akt2 substrate peptide and 10 µM of ATP were added to start kinase reaction. The reaction was conducted at 37°C for 30 min. Following the reaction, 5 µl of both 500 nM streptavidin-XL665 and Cryptate-conjugated anti-Akt2 antibody reconstituted with HTRF detection buffer supplied by cisbio assays were added and incubated at room temperature

for 1 hour to capture the Akt2 substrate. The FRET signal was detected with 317 nm of excitation and 665 nm of emission using a infinite M1000PRO (TECAN), and this experiments were done on a 384-well proxiplate (PerkinElmer). The IC_{50} values were calculated from the fitting curves via GraghPad Prism 7.

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Chapter 5

General conclusion

In this thesis, I reported my work toward acceleration of the development of macrocyclic peptide drugs using the RaPID system. For the goal, I have successful semi-automated the RaPID by employing the dual-armed humanoid robot, termed Senta-kun and succeeded in discovering macrocyclic peptides that bind to the targets, TET1, cMET, Smurf2, and Akt2 respectively using Senta-kun as well as showing a potential strategy for affinity maturation of peptides.

Senta-kun has been successfully setup to conduct the RaPID system and to produce comparable data with manual experiments (Chapter 2). It was also shown that the incompatible part of the RaPID system with Senta-kun could be accommodated by changing the protocols and procedures in accordance to the capabilities of Senta-kun as the behaviors of Senta-kun and human were not completely same even though it had been developed to mimic human procedures. The optimized protocols and settings in this study, and the fact that the RaPID system containing multi-step precise experiments was automated can bring hope to further automation of a extremely wide variety of experiments that require handling of a tiny amount of samples or elaborate steps and have been impossible to be automated. As future task for this system, it is expected that the part performed manually will be automated by Senta-kun. For now, the part that requires measurements and analysis using external devices such as PCR and qPCR has to be performed by a human researcher since those experiments are not compatible with the current capabilities of Senta-kun. However, this is not a technical challenge because those devices and analysis are suitable for computers, thus this will be achieved once the development of the robot is proceeded.

The utility of Senta-kun has been checked by the operation of the RaPID system against two different proteins, TET1 and cMET (Chapter 3), in addition to reproducibility and effects of selection conditions for the RaPID system. For the RaPID system against TET1, similar conditions were used in Senta-kun's and my experiment, resulting in enrichment of almost same peptides although a peptide found in the w-library and its derivatives were most prevalent sequences in y-library of the robotic selection. All peptides tested exhibited strong binding for TET1CD as well as TET1CCD used in the RaPID system and also inhibitory activity in vitro for TET1CD. This case suggested a possible strategy to apply the RaPID system for tough targets due to their properties such as stability or large in size. It can be assumed that the differences in the peptide sequences identified from the RaPID system by Senta-kun and myself came from the slight differences of conditions due to the differences in capabilities of human and robot or accidental loss of active spices in early rounds of selection, while it is still unclear. On the other hand, completely different peptides were

identified from the RaPID system against cMET in different conditions and all peptides tested in this section exhibited high affinity. MP4 and MP5 showed even stronger binding with 4.0 and 5.3 nM of K_D respectively compared to the reported one, aML5. In this investigation, the temperatures and the concentrations of cMET were meaningly changed, whence the difference in results might be attributed to the dynamic structures of cMET or competitive level of peptides during the selection step. The significance performing the RaPID system in multiple conditions to obtain more desirable peptides has been shown although 'best' conditions cannot be determined as it highly depends on the target proteins. To perform the RaPID system in several conditions in any case, huge manpower and tangled work are required. For this reason, it is possible to say that utility and advantage of Senta-kun was further emphasized.

Although the proteins validated as a target for the RaPID system have been targeted in chapter 3, Smurf2 was used as a completely new target for the RaPID system toward practical operation of Senta-kun and four peptides that show binding have been successfully discovered. Evaluation of Smurf2 inhibitory activity is next step for those peptides toward anti-cancer therapeutics while it might be tough to develop an assay as ubiquitin-ligation cascade is very complicate. Further, I attempted to show an application of the RaPID system that aims in affinity maturation of a specific peptide using the procedure of the RaPID system and a partially randomized peptide library. As a result of this strategy using Pakti-L1 that is a known peptide Akt2 inhibitor, N- and C-terminal sequences were highly conserved and significant mutations was observed only at position 5 and 6. There was no peptide with better activity among three mutants tested although R5G mutant exhibited comparable inhibitory activity as Pakti-L1, that means Pakti-L1 could be a local maximum inhibitor and there was no room to improve its activity. However, there is still possibility to apply this strategy for other peptide-target protein pairs.

For future perspectives, if the RaPID system is fully automated, the productivity of research will become much higher. Besides, an intuitive and researcher friendly interface to make or change protocols is desired since professional programing technique is required for now and it is too tough for researchers. Furthermore, the protocols, experience and know-how for optimization of a robot achieved in this study are helpful for further automation of a variety of experiments in future. On the other hand, in terms of the future perspective of macrocyclic peptide drug discovery using the RaPID system, multiple operations of the RaPID system even against a single target will be a major strategy since different conditions possibly yield different type of peptides with a range of binding affinity as shown in chapter 3 in addition to the fact that ample repertoire of peptide libraries with high chemical or

structural diversity will be available, added to which there are numerous proteins to be targeted by the RaPID system. From here onwards, a colossal workload and tangled labor will be required therefore the significance of automation must be highlighted for promotion of streamlining and productivity enhancement.

List of Accomplishments

Papers

O. Shoji, T. Fujishiro, K. Nishio, Y. Kano, H. Kimoto, S. Chien, H. Onoda, A. Muramatsu, S. Tanaka, A. Hori, H. Sugimoto, Y. Shiro and Y. Watanabe, "A Substrate-Binding-State Mimic of H_2O_2-Dependent Cytochrome P450 Produced by One-point Mutagenesis and Peroxygenation of Non-native Substrates ", *Catal. Sci. Tech.* 6, 5806-5811 (2016), ***Selected as the front cover of the issue 15.***

Reviews

Christopher John Hipolito, 西尾 洸祐, 菅 裕明, "*In Vitro* Selected Macrocyclic Peptides: Tools for Regulating the Conformational Freedom of Transmembrane Proteins", YAKUGAKU ZASSHI, 136, 2, 191-196 (2016)

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Oral presentations

西尾洸祐, 木崎 昂裕, 後藤 佑樹, 加藤 敬行, 菅 裕明, "ラボオートメーションによる新規機能性環状ペプチド開発の加速", 第 11 回バイオ関連化学シンポジウム, September 7-9 (2017), 東京

Kosuke Nishio, Takayuki katoh, Hiroaki Suga, "Discovery of TET1-inhibitory macrocyclic peptides", 日本化学会 第 96 春季年会, March 24-27 (2016), 京都

Kosuke Nishio, "RaPID evolution and physiological activity of TET1-inhibitory macrocyclic peptides", The 2nd ALPS Symposium on Photon Science, March 29 (2016), Tokyo

Kosuke Nishio, "Discovery of anti-TET1 macrocyclic peptides ", The 1st ALPS Symposium on Photon Science, December 26 (2013), Tokyo

Poster presentations

Kosuke Nishio, Takahiro Kizaki, Hiroaki Suga, "Semi-automation of the RaPID system: Robotic in vitro selection of functional macrocyclic peptides for diverse target proteins", ETHZ-UTokyo

Strategic Partnership Symposium on Science, Design, Manufacturing, and Information, January 19-20 (2017), Tokyo

Kosuke Nishio, Takahiro Kizaki, Hiroaki Suga, "Semi-automation of the RaPID system: Robotic in vitro selection of functional macrocyclic peptides for diverse target proteins", NTU-SNU-UTokyo chemistry symposium, January 16 (2017), Tokyo

Kosuke Nishio, Takayuki katoh, Hiroaki Suga, "*In vitro* selection of TET1-inhibitory macrocyclic peptides", 日本化学会 第 96 春季年会 ATP セッション, March 24-27 (2016), 京都

西尾洸祐, 米沢理人, 加藤敬行, 油谷浩幸, 菅裕明, "メチルシトシン酸化酵素 Tet1 に対する特殊環状ペプチド阻害剤の試験管内選択、あえてね", 第 26 回若手ペプチド夏の勉強会, August 3-5 (2014), 京都

Kosuke Nishio, Takayuki Katoh, Masato Yonezawa, Hiroyuki Aburatani, Hiroaki Suga, "*In vitro* selection of macrocyclic peptide antagonists against human TET1", 23rd American Peptide Symposium In Conjunction with 6th International Peptide Symposium, June 22-27 (2013), USA

西尾洸祐, 米沢理人, 加藤敬行, 油谷浩幸, 菅裕明, "メチルシトシン酸化酵素 Tet1 に対する特殊環状ペプチド阻害剤の試験管内選択", 日本ケミカルバイオロジー学会 第８回年会, June 19-21 (2013), 東京

Awards

NTU-SNU-UTokyo Chemistry Symposium, Poster Award
CSJ Student Presentation Award 2016
46th Summer Seminar for Young Peptide Scientist, Poster Award
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