論 文 の 内 容 の 要 旨

論文題目　Identification of macrocyclic peptide-based drug candidates for COVID-19 treatment and development of peptide-based fluorescent tags for specific protein labeling(COVID-19感染症に資する環状ペプチド薬剤候補およびタンパク質の特異的蛍光化に資するペプチドの開発)

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General introduction

Peptide which has medium molecular size (2-3 kDa) shows both properties of small molecule and protein. With larger interaction surface compared with small molecule, peptide, especially macrocyclic peptide can have high affinity and selectivity to its target and can aim for protein-protein interaction (PPI) in spite of its smaller size than protein. With these advantages, peptide is regarded as an attractive modality for drug discovery[1]. We have developed *de novo* macrocyclic peptide selection method called Random non-standard Peptides Integrated Discovery (RaPID) by the combination of *in vitro* translation system and codon reprograming (Fig. 1), and numbers of target binders have been identified[2].

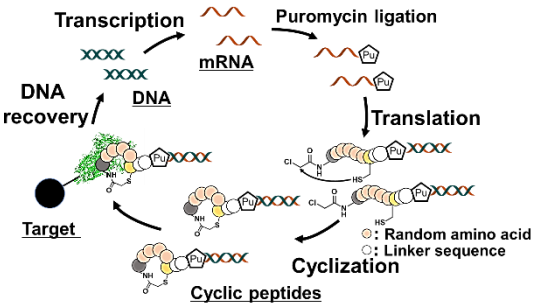

Fig.1 Scheme of RaPID selection.

Here, we report ①Discovery of peptide inhibitors for infectious disease, ②Development of a peptide-based imaging tool, by means of RaPID selection.

Topic ①: Identification of macrocyclic peptide-based drug candidates for COVID-19 treatment

Introduction

Coronavirus disease 2019 (COVID-19), induced by severe acute respiratory syndrome related coronavirus-2 (SARS-CoV-2) is highly infectious and fatal, which is a pandemic all over the world. Vaccines for SARS-CoV-2 are effective ways to contain the pandemic, but the effect is limited for infection

prevention. Therefore, establishment of therapeutics for COVID-19 is of great interest. **The interaction between SARS-CoV-2 spike protein (S-pro) and angiotensin-converting enzyme 2 (ACE2) on the surface of human cells is essential for the virus infection**, thus making it a promising target for drug discovery[3].

The goal of this research is **the identification of macrocyclic peptides that inhibit infection of SARS-CoV-2** by targeting the S-pro by means of RaPID selection. In addition, by comparing the peptides' activities from selections targeting different partial proteins of S-pro (Ectodomain vs receptor binding domain (RBD)), we tried to **obtain insights for the suitable target proteins for peptide drug discovery targeting S-pro**.

Result 1: RaPID selection toward SARS-CoV-2 S-pro

The scheme of RaPID selection is shown in Fig. 1. After 5 cycles of RaPID selection targeting ectodomain of SARS-CoV-2 S-pro, **14 peptides were identified, showing potent binding affinity to the S-pro (dissociation constant: K_D is 0.06–15 nM)**. Macrocyclic peptide with the strongest affinity named STL5-1(Fig. 2a) was estimated to bind across domains of the S-pro and to recognize RBD-up state of S-pro by surface plasmon resonance measurement with partial proteins of the S-pro. This result indicates that STL5-1 has a unique binding mode.

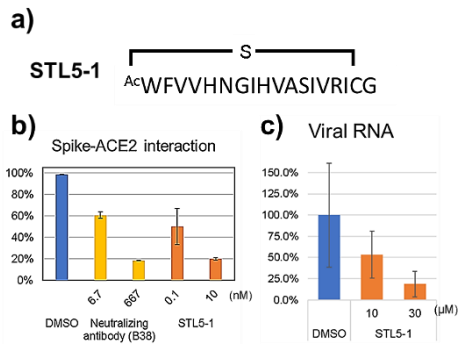

Fig.2 a) Sequence of STL5-1.
b) PPI inhibitory activity of STL5-1.
c) Infection inhibition by STL5-1.

Result 2: PPI inhibitory activity and infection inhibitory activity of STL5-1

Next, inhibitory activity of STL5-1 for PPI between the S-pro and ACE2 was evaluated. Fluorescent labeled S-pro was incubated with ACE2 expressing Human embryonic kidney cells in the presence of STL5-1. Fluorescent intensity of each cell was measured by flow cytometry to calculate the affinity between the S-pro and ACE2. **STL5-1, upon addition of 10 nM, decreased the fluorescent intensity by 80% comparing with non-treated condition** (Fig. 2b). This result indicates that STL5-1 strongly inhibits the PPI.

Finally, we evaluated virus infection inhibitory activity of STL5-1. Monkey kidney cells were incubated with SARS-CoV-2 isolated from a clinical patient in the presence of STL5-1. The viral RNA in the culture medium was quantified by qRCR to detect the replication of the virus at 24 hours after infection**. STL5-1 decreased Viral RNA by 20% at 30 μM compared with that of no treatment.** This result indicated that STL5-1 has inhibitory activity of SARS-CoV-2 infection *in vitro* although the effective concentration is much higher than K_D value.

Result 3: Consideration of suitable target proteins for the peptide selection

Although the identification of bioactive peptide by targeting ectodomain of S-pro, no bioactive peptides were identified by the selection targeting RBD of the S-pro. This result indicates that **targeting ectodomain is a better strategy to identify peptide with the virus infection**

inhibitory activity.

Conclusion

In this research, we identified a potent macrocyclic peptide binder of SARS-CoV-2 S-pro, STL5-1, showing efficient *in vitro* inhibition of S-pro-ACE2 PPI at 10 nM. Moreover, inhibition of virus infection was observed upon addition of STL5-1 to SARS-CoV-2 virus isolated from a clinical patient. **The development of STL5-1 reveals a promising drug candidate for COVID-19, whereas the unique binding mode of STL5-1 gains a new insight into the inhibition mechanism of SARS-CoV-2 infection.**

In addition, our result gave an insight about the suitable target for peptide selection to identify virus infection inhibitors. This knowledge would contribute to a quick response for the coming infectious diseases.

Topic ②: development of peptide-based fluorescent tags for specific protein labeling

Introduction

Protein imaging is a powerful method to directly observe behaviors and functions of proteins in living cells and elucidate the life phenomenon at molecule level. Common protein labeling methods can be mainly divided into two categories: fluorescent protein fusion and fluorescent tag application[4]. Although fluorescent proteins can be easily fused with other proteins by genetical operation, relatively large size (*e.g.* GFP is 27 kDa) might influent to the behavior of protein of interest. Compared with fluorescent proteins, fluorescent tags suffer less from this issue because of their relatively smaller size than fluorescent proteins. However, washout of excess fluorescent molecules for labeling is necessary and background might be significant because of low affinity of the fluorescent tag.

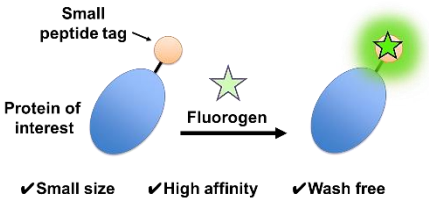

Fig.3 Concept of this research.

To overcome the problems, we envisioned that peptide binder of a fluorescent molecule can be applied for a novel fluorescent tag. With much smaller size than fluorescent protein (~3 kDa), peptide can be easily fused with protein without changing the protein's properties. Moreover, high affinity of peptide to its target could effectively reduce the background fluorescence In addition, to further decrease the background, **we utilized fluorogenic small molecules (fluorogens) which** show fluorescence only when fixed to a specific structure[7]. Peptides that bind and activate fluorogens can be applied for wash-free fluorescent tags.

Here, we identified a peptide that activate a fluorogen, HBC by RaPID selection. We also checked the potency of the peptide as a fluorescent tag.

Result1: RaPID selection and properties of the hit peptide

The same method described in fig. 1 was applied for the selection against HBC[5] (Fig. 4a). 7 peptides were identified from the selection, among which HL2 showed the highest fluorescence when incubated with HBC. **HL2 enhanced fluorescence of HBC by 11 times** at the concentration of 50 μM. We synthesized a linear variant of HL2 (HL2 linear), that lacks thioether bond (Fig. 4b), and it showed comparable activity

with HL2 (Fig. 4c). Since the linear structure is easier to handle for tag development, we focused on HL2 linear for further development. K_D of the HL2 linear with HBC was measured as 6.3 μM.

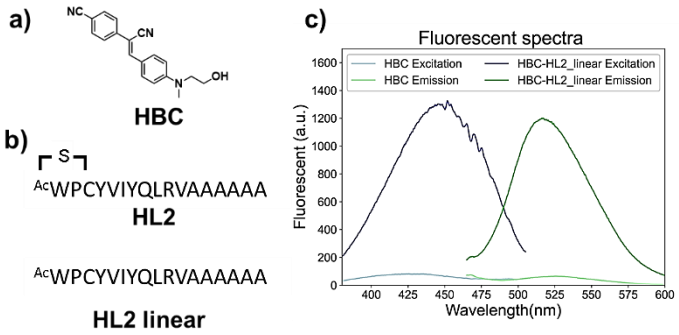

Fig.4 a) HBC. b) Sequence of active peptides. c) Fluorescent spectra of HBC-HL2 linear complex.

Result 2: Application of HL2 linear as a fluorescent tag

To apply HL2 linear peptide for fluorescent labeling, fusion protein of maltose-binding protein and MS2 coat protein (MBP-MS2 ,**1**) was selected as a scaffold, and HL2 sequence was fused to its C-terminus. **The purified fusion protein MBP-MS2-HL2 (2) showed 3 times higher fluorescence** when incubate with HBC comparing to that of the non-fused protein **1** (Fig. 5). The result indicates the potency of H2 linear sequence for a fluorescent tag.

Conclusion

Here, we identified HL2 peptide that activate fluorogenic HBC by RaPID selection. The linear form of HL2 enhanced fluorescence of a protein when fused with the protein as a fluorescent tag. Although the fluorescent enhancement activity should be improved, the **HL2 tag has the potency for a fluorescent tag that enable accurate and clear protein imaging**.

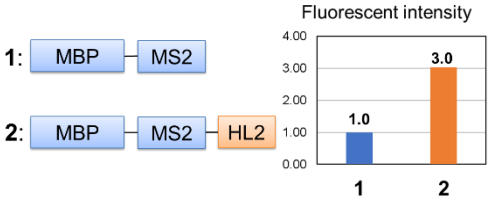

Fig.5 HL2 tag fused protein and relative fluorescent intensity of HBC-protein.

References

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