論文の内容の要旨

The construction of artificial macrocyclic non-standard peptide libraries and discovery of drug leads against proteins related to intracellular proteolysis pathways

(人工特殊ペプチドライブラリーの構築と
細胞内タンパク質分解経路関連タンパク質に対する薬剤候補探索)

氏名　　山口　淳

Introduction

Peptides exhibiting macrocyclic structures often show properties such as high bioactivity, structural rigidity, membrane permeability, and protease resistance. Peptides containing *N*-alkyl amino acids also show membrane permeability, and protease resistance. Due to these characteristics, peptides exhibiting macrocyclic structures and *N*-alkyl backbone modifications, referred to as macrocyclic non-standard peptides, have attracted recent attention as candidate therapeutic agents.

In our laboratory, the Random non-standard Peptide Integrated Display (RaPID) system was developed as a tool to discover bioactive macrocyclic non-standard peptides using an intramolecular thioether bridge formation reaction between a *N*-chloroacetoamide moiety and a sulfhydryl group of cysteine (Cys) residue as a peptide macrocyclization method. However, in many cases RaPID screening leads to isolation of peptides carrying small cyclic structures with linear peptide tails rather than macrocyclic peptides because of the unintentional thioether formation could occurred in peptides containing several cysteines (**Fig. 1**). In these cases, the peptide tail may be easily degraded by exopeptidases; hence

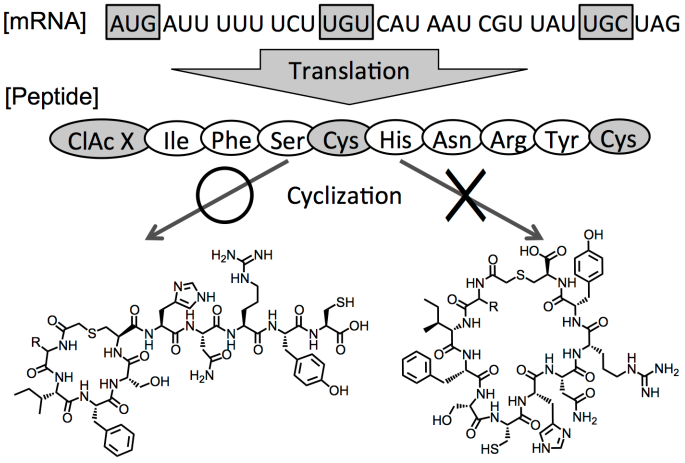

Fig. 1　Peptides carrying several cysteines are usually converted into "lariat" shape structures rather than macrocyclic structures.

even though thee "lariat-shaped" peptides are interesting for functional molecules, they may require reengineering of the tail region to install protease resistance after the selection. To avoid the appearance of such lariat peptides in the library, we need to diverse a method to exclude Cys residues in the random region, *i.e.* exclusively express desired macrocyclic peptides. Moreover, installing the *N*-methyl backbone modification and/or proline-like secondary amino acids into peptide backbone also makes them resistant against proteases.

In this study, I constructed macrocyclic peptide libraries composed exclusively of thioether-macrocyclized peptides with multiple *N*-methyl and proline-like backbone modifications, I then used these libraries for RaPID screening against two target proteins, hAtg3 and Smurf2, related to protein degradation systems in cells, leading to the identification of binders and/or inhibitors of those proteins.

Construction of artificial macrocyclic non-standard peptide library (Chapter 2)

Messenger RNA (mRNA) templates used for the construction of peptide libraries for RaPID screening generally have several randomized codons (*e.g.* NNU codons where N = U, C, A, or G) and since 15 amino acids occur randomly at each NNU codon that allows the synthesis of diverse peptide sequences. However, the cysteine UGU codon could appear in an NNU randomized library and incorporation of cysteine in any of these codons leads to the formation of lariat shaped peptides rather than macrocycles through reaction of the *N*-chloroacetoamide group with the more proximal cysteine in the random region.

In this chapter, I describe my work in developing the method for the synthesis of exclusively cyclized peptides through ribosomal synthesis using the reaction of the *N*-chloroacetoamide group with cysteine and artificially constructing natural product-like peptide libraries consisting of approximately 10^{12} kinds of macrocyclic peptides.

***In vitro* selection of macrocyclic non-standard peptides against hAtg3 (Chapter 3)**

Autophagy is a protein degradation systems conserved widely in eukaryotic cells and through promiscuous degradation of cytoplasmic constituents such as organelles and damaged proteins, plays the important roles in cell homeostasis and programmed cell death. The protein product of human autophagy related gene 3 (hAtg3) regulates the phospholipidation of LC3, another autophagy related protein and this reaction appears necessary for autophagy. However, the possible other functions of hAtg3 are not known and the localization of hAtg3 in cells are also unclear. Therefore, compounds that bind to and/or inhibit hAtg3 in cells (none of which are known) could be valuable tools for hAtg3 research.

In this chapter, I describe my work in selecting for macrocyclic non-standard peptides that bind hAtg3 and analyzing the functions of selected peptides.

***In vitro* selection of macrocyclic non-standard peptides against HECT domain of Smurf2 (Chapter 4)**

The ubiquitin-proteasome system is another protein degradation system. In contrast to autophagy, poly-ubiquitinated proteins are selectively degraded in the ubiquitin-proteasome system, through the activity of three types of enzyme (E1, E2, and E3 enzymes). SMAD ubiquitination regulatory factor 2 (Smurf2) is an E3 ubiquitin ligase and down-regulates the transforming growth factor-β (TGF-β) signaling pathway through ubiquitination of receptor-regulated SMAD protein positive-modulators of TGF-β signaling. This pathway regulates cell differentiation and proliferation, thus the overexpression of Smurf2 induces tumor formation, for example in breast cancer and esophageal squamous carcinoma. The C-terminus domain of Smurf2, termed the HECT domain, plays a critical role in the ubiquitination function of Smurf2, and thus, inhibitors of the HECT domain of Smurf2 may be new candidate therapeutic agents against cancers.

In this chapter, I describe my work in selecting for macrocyclic non-standard peptides that bind Smurf2 and analyzing the functions of selected peptides. The contribution of macrocyclic structures and *N*-alkyl backbone modifications of peptides for target binding affinities is also discussed.

General conclusion (Chapter 5)

In this study, an improved method for the synthesis of exclusively cyclized peptides through ribosomal synthesis through the reaction of the *N*-chloroacetoamide group with cysteine was successfully developed. The construction of highly diverse macrocyclic non-standard peptide libraries was achieved using this method. Selections against hAtg3 and Smurf2 from these peptide libraries were performed, and I successfully isolated target binding macrocyclic non-standard peptides. These peptides could be powerful tools for Autophagy researches or good therapeutic agents against cancers.