In vitro selection and affinity maturation of CTLA-4 binding cyclic peptide with PURE*frexRD*, and conversion of the peptide to small molecules.

PURE*frexRD*によるCTLA-4結合環状ペプチドの試験管内選択と親和性向上および環状ペプチドの低分子化合物への変換

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Abstract

[Background]

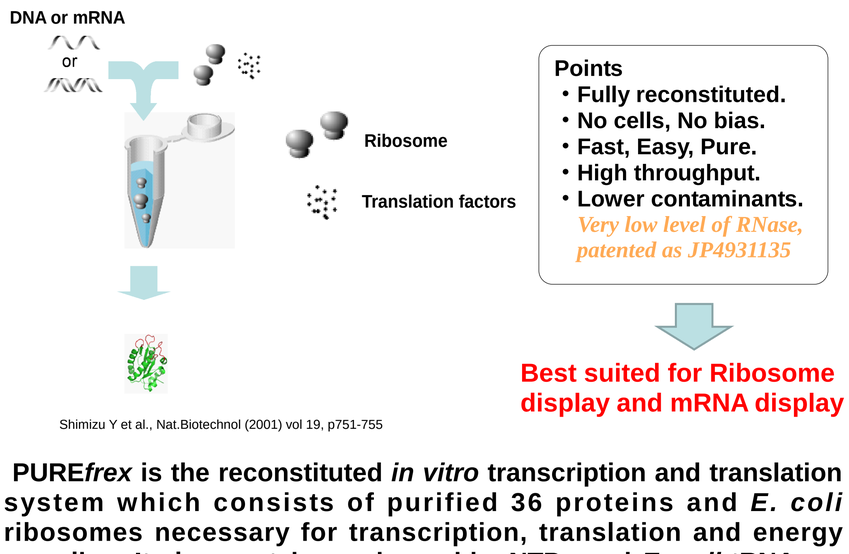
In the drug development, small molecule drugs have a lot of advantage over peptides in terms of oral administration and intracellular migration. However, since diversity of small molecule library and selection methods are limited, it is difficult to get the promising drugs that has the inhibitory activity for protein-protein interactions. On the other hands, we have reported that the functional cyclic peptides can be easily selected / screened by the ribosome display method (PURE*frexRD*) using PURE*frex*. Here, we report selection and affinity maturation of functional cyclic peptide that inhibited the binding between CD80 and CTLA-4, and conversion of the cyclic peptide to small molecules.

[Method and Results]

A lot of cyclic peptides that specifically binds to CTLA-4 was obtained using PURE*frexRD* (3 rounds selection) from cyclic peptide library consisting of a random sequence of 12 residues. Next, a cyclic peptide having inhibitory activity was selected and applied for affinity maturation (off-rate selection) by PURE*frexRD*. As a result, a high affinity cyclic peptide having an improved affinity of about 630 times was obtained. Furthermore, the comprehensive information related to the correlation between amino acid sequence and binding activity was obtained from a series of experiments such as identification of essential amino acids for binding by alanine scanning, exhaustive expression of recombinant mutants and ELISA. Based on these data, the 3D-structure of the cyclic peptide was predicted in silico, and about 350 small molecules similar to the simulated cyclic peptide structure were picked out from PRISM BioLab 's small molecule library, and binding inhibition activity were estimated by ELISA. As a result, a several small molecules having inhibitory activity could be obtained.

It indicates that PURE*frexRD* is an effective way for obtaining functional cyclic peptides and their structure-activity relationship information, and conversion from cyclic peptides to small molecules.

①Best reconstituted cell-free protein synthesis system for *in vitro* selection having the lowest level of RNase contamination: PURE*frex*



Points
- Fully reconstituted.
- No cells, No bias.
- Fast, Easy, Pure.
- High throughput.
- Lower contaminants.

Very low level of RNase, patented as JP4931135

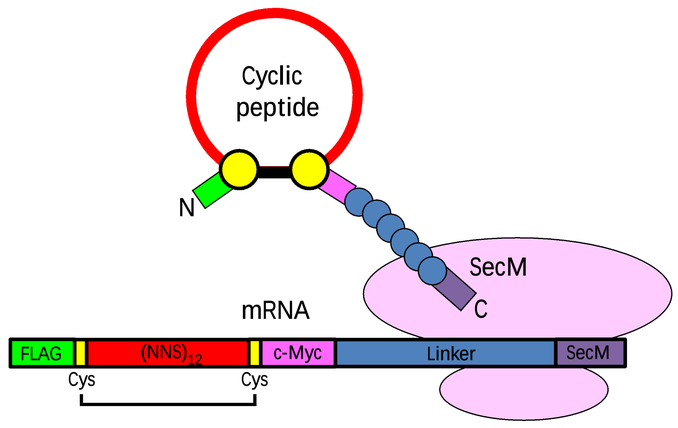
Best suited for Ribosome display and mRNA display

Shimizu Y et al., Nat.Biotechnol (2001) vol 19, p751-755

PURE*frex* is the reconstituted *in vitro* transcription and translation system which consists of purified 36 proteins and *E. coli* ribosomes necessary for transcription, translation and energy recycling. It also contains amino acids, NTPs and *E. coli* tRNA, so the target protein can be synthesized just by addition of the template DNA to the reaction mixture.

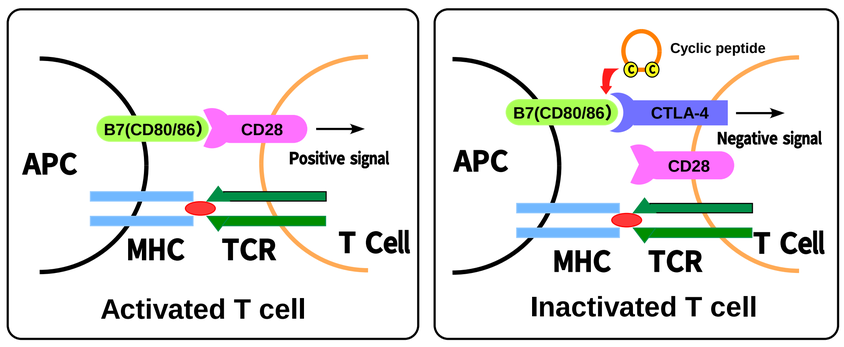
②Simple selection with PURE*frexRD* for cyclic peptide

Ribosome Display complex with cyclic peptide



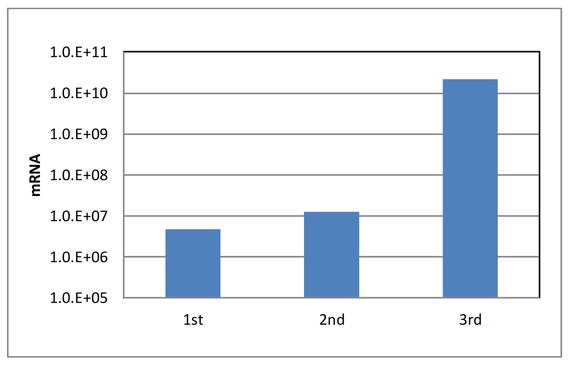
When the arrest sequence of SecM at 3'terminus is translated in RURE*frex*, a ribosome can be fixed strongly on mRNA. Also, release factors (RF1, RF2, RF3, RRF) are removed from PURE*frex*, and oxidized glutathione (GSSG) and disulfide isomerase from *E.coli* (DsbC) in optimized concentration to form disulfide bond are added into PURE*frex*. As a result, RD complex become highly stable, and cyclized peptide is displayed on ribosome with high efficiency.

③Immune checkpoint via CTLA-4



When CTLA-4 bind to B7, T-cells are led to anergy. Anergic T-cells have limited effector function. Anti-CTLA-4 neutralizing cyclic peptides inhibit CTLA-4 binding to B7 and promote T-cell activation.

④*In vitro* selection by PURE*frexRD*



Cyclic peptide library having 12mer random sequences (Cys - X12 - Cys) was screened against biotinylated CTLA-4-Fc protein. Recovered mRNA increased along with the progress of selection round.

⑤Sequencing after 3rd round selection

		Squence													
Number of conc.	clone name	Cys	1	2	3	4	5	6	7	8	9	10	11	12	Cys
51	3-02														
6	3-51														
4	3-33														
3	3-80														
2	2-23														
2	3-90														
2	3-38														

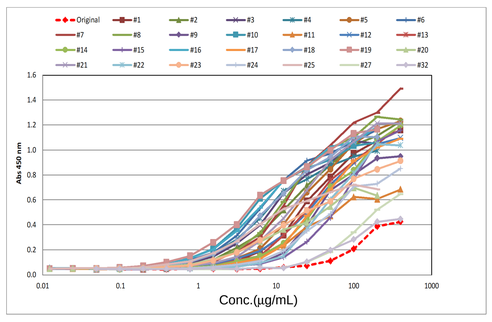
Some of enrichment were observed from the sequencing (94 clones). Each color represents specific amino acid.

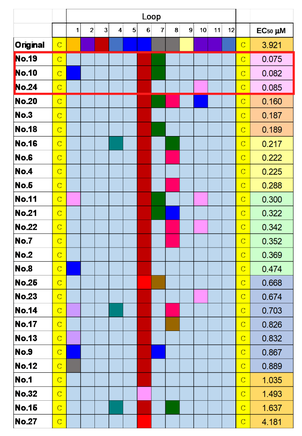
⑥Measurement of affinity (EC_{50}) as MBP-fusion peptide after affinity maturation (off-rate selection) by PURE*frexRD*

(Off-rate slection)
Library:Error prone mutants
Wash time:1st-2H, 2nd-19H, 3rd-67H

(Expression)
E.coli host : BL21(DE3)
Culture condition : 30℃

(Purification)
His tag purification

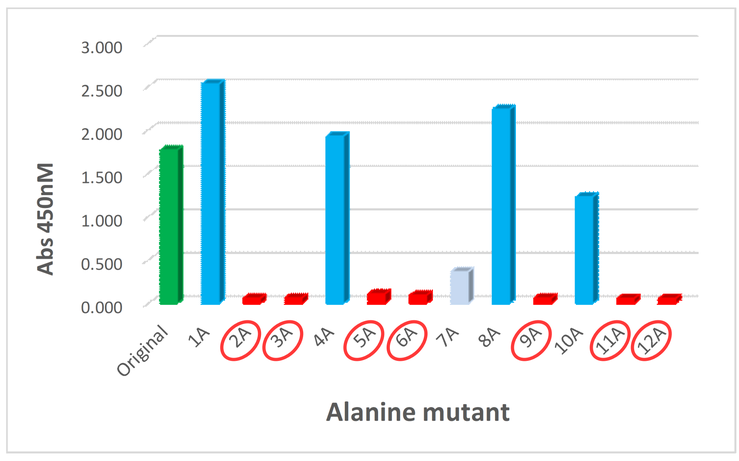




The high affinity clones (27 clones) from ELISA were purified, and EC_{50} of the each were measured. As a result, all clones except No.27 showed higher EC_{50} than the original (3-02). In particular, No.19, 10, 24 (red square) showed about 50 times higher affinities than the original.

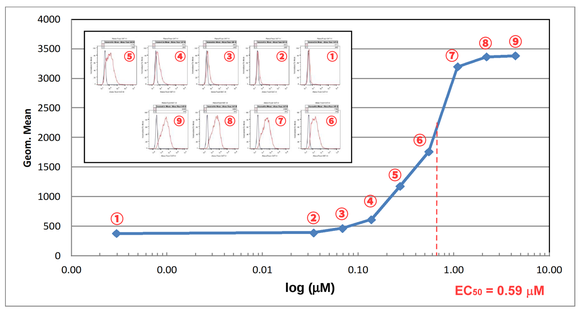
⑦Identification of amino acids required for binding by alanine scanning

(Construct)
MBP-fusion peptide



Amino acids of position 2, 3, 5, 6, 9, 11, 12 (red circle) were necessary for binding to CTLA-4.

⑧Cell based binding assay with the synthetic peptide (FACS)



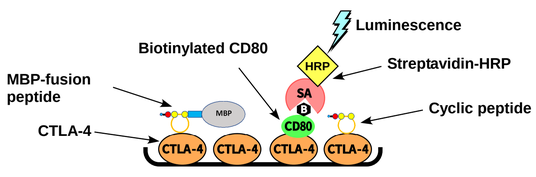
CTLA-4 Cell line:
1X 10^5 cells/reaction, 100 μL

Synthetic peptide:
N-term Biotinylated peptide

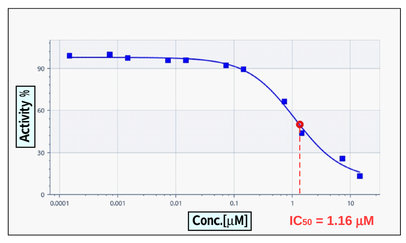
2nd antibody:
Streptavidin-iFluor 647

No.19 binder as synthetic peptide (N-term biotinylated) bound to CTLA-4 on the cells.

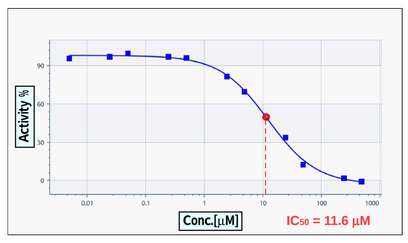
⑨Measurement of inhibition (IC_{50}) by inhibitory ELISA



MBP-fusion

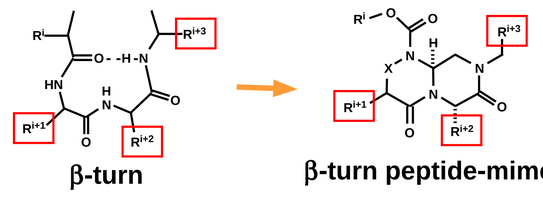


Peptide

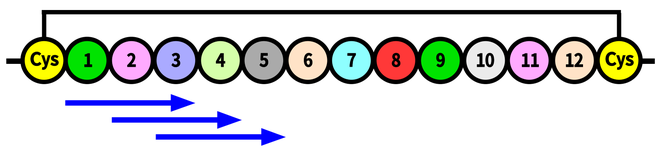


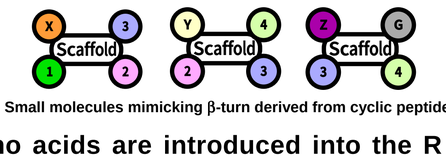
No.19 binder showed the inhibitory activity against interaction between CD80 and CTLA-4 in both MBP-fusion and fully synthetic peptide format. The inhibitory activity of the synthetic peptide was lower than MBP-fusion.

⑩Designing small molecules based on cyclic peptides



J.Med.Chem., 45, 1396 (2002)

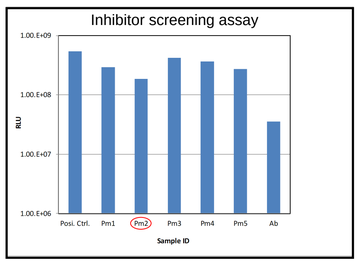


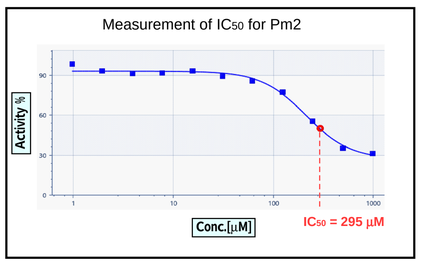


Small molecules mimicking β-turn derived from cyclic peptides

Three amino acids are introduced into the R position (red square) of the β-turn peptide-mimetic scaffold in order from the N-terminus of the cyclic peptide.

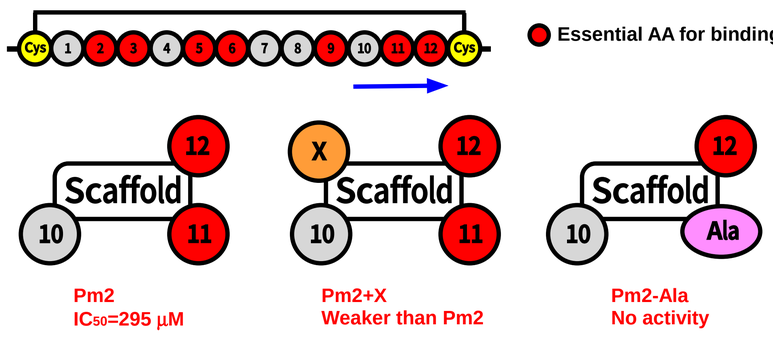
⑪Inhibitor Screening assay and Measurement of IC_{50}





Several small molecules with inhibition activity were obtained. IC_{50} of Pm2 with the highest activity showed about 1/20 of the synthetic peptide.

⑫Comparison of affinity among Pm2 analogs



Pm2
IC_{50}=295 μM

Pm2+X
Weaker than Pm2

Pm2-Ala
No activity

Pm2 was designed from 3 amino acids including essential 2 amino acids for binding at C-terminal of peptide. Two analogs with adduct or substitution of 1 amino acid showed the decrease in IC_{50}.

Summary

1, Functional cyclic peptides against CTLA-4 were selected easily and rapidly by PURE*frexRD*.

2, In affinity maturation of CTLA-4 binder, we succeeded to select lead cyclic peptide with good EC_{50}/IC_{50}.

3, Essential amino acids for binding were indentified by alanine scanning.

4, Based on structure-activity relationship information, peptide-mimetic small molecules were designed, and successfully selected with good inhibitory activity.

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