Construction of LRR-Ab Library Based on Leucine-rich Repeats by Module Engineering

Haruka Sezaki[1], Keisuke Yoshinaga[2]
[1]Production Systems Engineering course, Advances Course
[2]Department of Biological and Chemical Systems Engineering
National Institute of Technology (KOSEN), Kumamoto College
Email: yoshinaga@kumamoto-nct.ac.jp

ABSTRACT

Immunoglobulins (Ig), also known as antibodies in jawed vertebrates, have been widely used as antibody pharmaceuticals and other research applications. Jawless vertebrates including lampreys and hagfishes use VLR for their antibody. VLR consist of several Leucine-rich repeat (LRR) motifs which have helical secondary structure. VLR protein recognizes an antigen on the flat surface consist of a set of several LRR motif β-sheets. On the other hand, an Ig type antibody recognizes an antigen by a set of six CDR loops. Because of highly modular structure and antigen binding manner distinct from conventional Ig antibodies, VLR is considered suitable molecule for protein engineering of binders.

In our group, the LRR Antibody (LRR-Ab) library was designed based on human TLR3 its LRR structure is similar to that of VLR. Five residues in each of the three LRR motifs can be randomized to generate molecular binders for various targets. LRR-Ab library was constructed by EASeL method. Although LRR-antibodies were expressed in phage display system, their expression levels on phage particles were insufficient for following screenings. Therefore, we attempted to substitute the N-terminal region of LRR-Ab in order to improve protein folding and expression levels.

Keywords *non-Ig scaffold , repeat protein, modular architecture, antibody alternatives, protein binder, leucine-rich repeat, phage display*

INTRODUCTION

Antibodies have been widely used as binding molecules in researches and therapeutics. However, they have a number of disadvantages. They have large molecular weight and tendency to

aggregate, require manufacturing in expensive mammalian expression system. To overcome these disadvantages, much works have been developed alternative protein scaffolds, such as ankyrin repeat, fibronectins, anticalins, and affibodies [1].

Jawed vertebrates used immunoglobulins (Ig) as antibody. Meanwhile, jawless vertebrates including lampreys and hagfishes use variable lymphocyte receptors (VLRs) for their antibody. VLR is made up of numerous helical secondary structure Leucine-rich repeat (LRR) motifs. The VLR protein recognizes an antigen on a flat surface consist of a series of LRR motif β-sheets. By contrast, the Ig type antibody recognizes an antigen thorough a series of six CDR loops. VLR is attractive candidate for protein engineering of binders because of its highly modular structure and paratope structure differ from traditional Ig antibodies. The VLR based binder Repebody has been developed [2], however VLR has antigenicity in humans.

Here, we report the construction of LRR antibody (LRR-Ab) library based on human Toll like receptor 3 (TLR3), its structure similar to VLR. The Extension Assembly and Self-Ligation (EASeL) method [3] was used to construct the LRR-Ab library. Three rounds of LRR-Ab library screening against human epidermal growth factor (hEGF) were performed. Several clones were isolated, whereas those expression level on the phage particle were insufficient for additional experiments. Therefore, we tried replacing the N-terminal region of LRR-Ab with internalin-B.

METHODOLOGY

Construction of LRR library by EASeL method

To construct the LRR library, a LRR coding gene was synthesized and recombined into pSEX81 phagemid vector by EASeL method [3]. The codon usage of LRR coding gene was optimized to increase its expression in *E. coli* system. The LRR-Ab gene expression was controlled by Lactose promoter (P_{lac}), *i.e.* LRR expression could be positively or negatively regulated using IPTG or glucose, respectively. The signal peptide of pectate lyase (pel B) is located on the N-terminal of LRR-Ab for extracellular secretion. Between the NT and CT domains of the LRR-Ab gene, there are three LRR motifs (LRR21-23) that serve as antigen binding sites. The amino-acid sequences and numeration of LRR motifs were defined by human TLR3 LRR motifs. Five residues in each of the three LRR motifs can be randomized to create molecular binders for varied targets.

The DNA fragment of internalin-B N-terminal (intNT) was synthesized and amplified by PCR using intNT forward and intNT reverse primers. Original NT region (derived hTLR3) was substituted with intNT by EASeL method. For LRR antibody library construction, the backbone of the linear vector was amplified by PCR using the NT reverse and Jab LRR CT-forward primers. LRR21, LRR22, and LRR23 oligo DNA correspond to each LRR motifs were synthesized. For randomized residues, the NNK (N=A, G, C or T; K=G or T) codon was using in oligo DNA synthesis. LRR22-23 fragments were prepared by assembly of LRR22 and LRR23 oligos by PCR. Further LRR21-22-23 fragments were prepared by assembly of LRR22-23 fragments and LRR21 oligos by PCR. Then LRR21-22-23 fragments were assembled with linear vector backbone by PCR using 5'-phosphorylated LRR21 oligos and intNT reverse primer. Finally, assembled LRR21-22-23-vector was circularized by self-ligation using T4 DNA ligase for 4hr at 16°C. The ligation products were purified by spin column and transformed into electro-competent XL1-Blue cells (Stratagene) by electroporation using Micropulser (Bio-Rad; 1.5kV). Transformants were incubated in SOC medium at 37°C for 1hr, then amplified onto 2TY medium plates supplemented 100μg/ml

Ampicillin and 2% glucose (2TYAG) at 30° overnight. Amplified colonies were collected using cell scraper, add final 16% of glycerol and store at -80°C (Library stock). The diversity of library was defined as the number of single ampicillin-resistant clones obtained from electroporation prior to any amplification process of clones.

Phage preparation and western blotting

The *E. coli* suppressor strain XL1-Blue (*supE44*) harboring phagemid pLRR-Ab/SEX81 were overinfected with the M13KO7 helper phage (NEB, 1×10^{11} pfu/ml) at 37°C for 30min and additionally incubate with shaking for 30min. After centrifugation 2,500×g at room temperature for 5 min, precipitations were suspended in 2TY medium supplemented 100µg/ml Ampicillin, 50µg/ml Kanamycin and 0.1mM Isopropyl β-D-1-thiogalactopyranoside(IPTG) (2TYAKI). The culture was incubate with shaking at 30°C over night (16hr). The phage particles were PEG-precipitated. Relative LRR-Ab expression levels on the phage were determined by Western blotting using HRP conjugated anti-His6 tag mAb (Wako 9C11). The signals were detected using chemiluminescent substrate (Chemi-Lumi One; Nacalai tesque, Kyoto, Japan).

Panning

Panning was performed as described by Yoshinaga et al. [4]. Briefly, 10 µg/ml of recombinant human EGF (FUJIFILM Wako, Japan) in 0.1M $NaHCO_3$ (pH8.6) was incubated at 4°C overnight in an immuno-tube. The immuno-tube was blocked with blocking solution. As the blocking solution, 0.5 % gelatine in the first round, 1 % BSA in the second round, and 1 % skimmed milk in the third round was used, respectively. A LRR-Ab library (1.2×10^7 cfu/ml) was incubated in an EGF-coated immuno-tube at room temperature for 1hr. The tube was washed 10 times with PBS containing 0.05% tween-20 (PBST). The bound phage were eluted with 1ml 0.1M glycine-HCl (pH 2.2), immediately neutralized with 0.1 volume of 1M Tris-HCl (pH 9.1), and infected to early log-phase *E. coli* XL1-Blue. *E. coli* infected with phage clones were amplified onto 2TYAG plates at 30°C overnight. Amplified colonies were collected using cell scraper and prepared phage particles with over infection of the M13KO7 helper phage. The amplified phages were used in the following round of panning. This procedure was performed three times in order to obtain clones that strongly bind to hEGF.

RESULT

The LRR-Ab phage clones were selected from LRR-Ab displaying phage library by three rounds of panning with recombinant human EGF (rhEGF). We isolated several EGF binding clones and DNA sequenced (Figure 1). The hydrophobic or positively charged residues were observed at putative antigen binding sites. However, the expression levels on the phage were insufficient for additional screenings. Therefore, we attempted to replace the N-terminal region of LRR-Ab (NT derived hTLR3) with internalin-B (intB) NT region. LRR-Ab (NT derived intB) was reconstructed by EASeL method. This library was estimated to contain 5×106 independent clones. Before panning, randomly selected clones were examined LRR-Ab expression level by western blotting analysis using anti-His tag antibody (Figure 2). LRR-Ab expression levels were somewhat improved by substitute NT region.

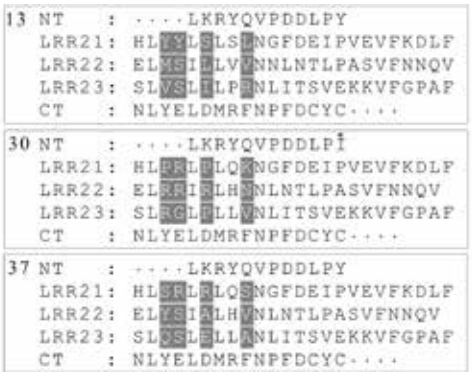

Figure 1 The amino acid sequences of isolated clones. The amino acid sequences of isolated LRR-Ab clones were deduced from the DNA sequences. Asterisk in the clone #30 indicate amino acid differ from originally designed, maybe it caused by spontaneous mutation. Filled boxes indicate the amino acid sequences of randomized position in library design

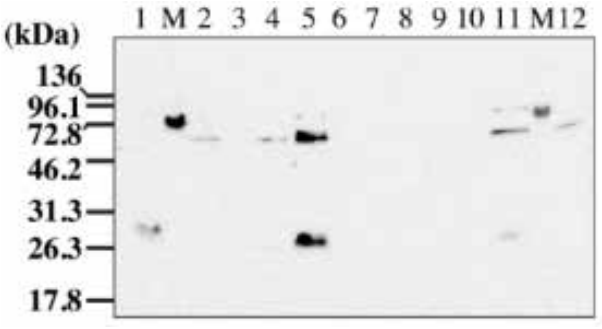

Figure 2 Expression of LRR-Ab protein on the phage. Western blotting was performed with HRP-conjugated anti-His tag mAb. The numbers above lanes show the clone numbers. "M" indicate molecular weight protein marker. The 70kDa bands corresponded to LRR-Ab-g3p fusion protein. The 30kDa bands are considered soluble LRR-Ab protein co-precipitated with phage. The 72.8kDa bands in marker lanes are considered the result of anti-His tag mAb cross-reacted with BSA protein in marker

The LRR-Ab phage clones were selected from LRR-Ab (NT derived intB) displaying phage library by three rounds of panning with rhEGF. Nineteen six of isolated clones were screened by EGF-binding activity using ELISA. Several clones show EGF-binding activity. DNA sequencing and binding specificity analyses will be performed.

DISCUSSION

In the present study, we successfully constructed phage displayed LRR-Ab library with a size of 5×10^6. To construct antibody (or protein binder) phage display libraries, widely used methods consist of PCR amplification of antibody fragments, followed by enzymatic digestion and ligation with the vector. This method is time consuming and has a low efficiency in the digestion, purification, ligation and transformation step. In this study, we used the EASeL method to construct an LRR-Ab phage library. It only took a few weeks to construct the LRR-Ab library while conventional approaches would require several months. The ligation reaction by T4 DNA ligases require 5' termini phosphate of DNA fragments. To circularize successfully assembled LRR21-22-23-vector construct selectively, we used 5'-phosphorylated forward primer in only PCR steps just before self-ligation step. After circularization by self-ligation, a nick was remaining in a ligation site, however the nick was repaired in *E. coli* cell after transformation.

In isolated EGF binding clones, several hydrophobic or positively charged residues were observed in the putative antigen binding sites. These are suggested that LRR-Ab clones bind to EGF through hydrophobic or positively charged residues. In fact, hEGF molecule has low PI value and negatively charged in a physiological environment. Remarkably, the clone #30 (Figure 1) has

8 of positively charged residue in a putative antigen binding sites. It shows that the EGF binding clones were successfully selected in the three rounds of panning procedures.

Although the EGF binding clones isolated, its expression levels on the phage particles were too low for additional screenings and subsequent analyses, presumably by insufficient protein folding and formation of inclusion bodies in the *E. coli* cells. The folding of LRR proteins proceeds through an N-terminal transition state ensemble and that the α-helical cap may polarize better the folding pathway by acting as a fast-growing nucleus. In accord with this idea, Lee *et al.* substituted NT region of VLR with NT region of intB, and thereby protein expression level was significantly increased [2]. Similarly, we attempted to substitute NT region of LRR-Ab (derived hTLR3) with NT region of intB. As a result, LRR-Ab expression level was improved.

We reconstruct the LRR-Ab (intB NT) phage display library using NT replaced template. Three rounds of panning against rhEGF were performed and several EGF-binding clones were isolated. Now, we are analyzing DNA sequence and binding specificity of these clones. An LRR-Ab with high affinity and specificity for a target will be applied to therapies, diagnostics, and affinity purifications.

CONCLUSION

The human TLR3 derived LRR-Ab displaying phage library was constructed by novel EASeL method. However, LRR-Ab expression levels on the phages were insufficient for additional screenings. We replace the N-terminal region of LRR-Ab with intB NT region. LRR-Ab expression levels were improved by substitution of NT region.

LRR-Ab (NT derived intB) was reconstructed and panning against rhEGF was performed. We isolated several clones show EGF-binding activity. These clones will be DNA sequenced and analyzed for antigen specificity.

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