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Engineering a growth sensor to select intracellular antibodies in the cytosol of mammalian cells

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Intracellular antibodies (intrabodies) are expected to function as therapeutics as well as tools for elucidating *in vivo* function of proteins. In this study, we propose a novel intrabody selection method in the cytosol of mammalian cells by utilizing a growth signal, induced by the interaction of the target antigen and an scFv-c-kit growth sensor. Here, we challenge this method to select specific intrabodies against rabies virus nucleoprotein (RV-N) for the first time. As a result, we successfully select antigen-specific intrabodies from a naïve synthetic library using phage panning followed by our growth sensor-based intracellular selection method, demonstrating the feasibility of the method. Additionally, we succeed in improving the response of the growth sensor by re-engineering the linker region of its construction. Collectively, the described selection method utilizing a growth sensor may become a highly efficient platform for selection of functional intrabodies in the future.

[**Key words:** Intracellular antibody; Library screening; Growth signal; Receptor engineering; Rabies virus; Mammalian cell; c-Kit]

Antibodies combine target molecules or structures with potent binding capacities that have important roles in the immune system as well as in therapeutics, and are therefore expected to be valuable materials for modern medicine. However, their large sizes make antibodies arduous to apply to target cytoplasmic proteins because of poor permeability across the cell membrane. Accordingly, researchers have made recombinant antibody fragments, such as single chain variable fragments (scFvs) and single domain antibodies which can function inside the cells, named as intracellular antibodies (intrabodies) (1). These fragments retain the antigen-binding domain of antibodies but have comparatively small sizes, which allow easy expression from single genes. Intrabodies can be stably expressed and bind to the target proteins expressed inside of the same living cell. Thus, intrabodies can selectively inhibit the specific function of proteins and neutralize disease-related intracellular proteins (2,3). Hence, the intrabody technique is an attractive alternative to the current knockdown techniques, such as RNAi and small-molecule inhibitors, creating a promising tool for analyzing the function of proteins and for therapeutic use (4–8). However, due to the low stability of antibodies in the reducing cytoplasmic environment (9), it is challenging to select effective intrabodies against a target protein.

Phage display is one of the most-used methods for selecting functional intrabodies because of its robustness and high throughput. However, antigen-binding scFvs selected from phage display have to be validated in terms of their intracellular stabilities and functionalities. In this step, most of the scFv candidates fail to meet the requirements due to their misfolding in the cytoplasm. This is attributed to loss of disulfide bonds in the reducing environment (9–11), which is different from the oxidizing environment for selecting antigen-binding scFv candidates, leading to the poor efficiency of phage display in selecting intrabodies. Hence, an alternative method which selects antigen-binding intrabodies directly in the intracellular environment, where these intrabodies would perform their functions, is highly desirable.

In order to circumvent problems of the conventional selection methods, we propose an inventive intrabody selection method in the cytoplasm of mammalian cells. In this method, an scFv fusion protein termed as a growth sensor is constructed by linking an scFv fragment and the cytoplasmic domain of c-kit as a receptor tyrosine kinase which is activated by dimerization. Upon the interaction between the scFv and a homo-oligomeric target antigen, the growth sensor forms a homo-oligomer, allows c-kit activation, and then results in transducing a growth signal (Fig. 1A). Therefore, by collecting the cells which proliferate with the growth signals, we can gather all potential scFvs that possibly bind to the target antigen.

Recently, we have demonstrated the feasibility of this selection method by utilizing a growth sensor to select intrabodies against rabies virus phosphoprotein (RV-P) (12). We showed that the method was able to select the same or similar RV-P-specific intrabodies, which had also been obtained by phage display in another previous study (13), from the same library. However, it has yet to be investigated whether the growth sensor could be applied to select

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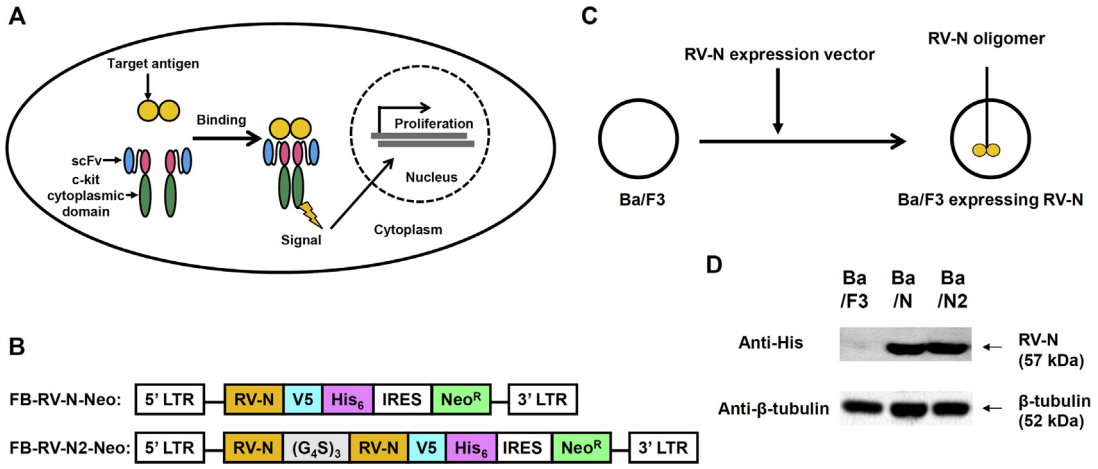

FIG. 1. Illustration of the growth sensor activation and the establishment of cells expressing RV-N. (A) Mechanism of the growth sensor activation. When the growth sensor composed of scFv and the c-kit cytoplasmic domain binds to the homo-oligomeric target antigen, it forms oligomers and transduces a growth signal. (B) Construction of antigen expression vectors. FB-RV-N-Neo^R encodes V5- and His_6-tagged RV-N, whereas FB-RV-N2-Neo^R encodes a dimer of the tagged RV-N connected by a flexible linker $(G_4S)_3$. Both vectors have the neomycin resistance gene. (C) Illustrating the generation of antigen-expressing cells. (D) Western blot analysis to confirm antigen expression. Anti-β-tubulin blots are the loading control.

intrabodies against different antigens. In this study, we challenge the proposed method to select specific intrabodies against rabies virus nucleoprotein (RV-N), because anti-RV-N intrabodies have not been reported to our best knowledge. As a result, we successfully select antigen-specific intrabodies from a naïve synthetic library using phage panning followed by the growth sensor-based intracellular selection method, indicating the powerful capabilities of our method. Additionally, we ameliorate the response of the growth sensor by inserting a flexible linker between the scFv and c-kit domains. These results demonstrate that our proposed method can be used as a promising intrabody selection method and is expected to contribute substantially to drug discovery and academic research.

MATERIALS AND METHODS

Vector construction The DNA fragment encoding the nucleoprotein of a rabies virus fixed strain CVS-11 (RV-N) was cloned between the *Kpn*I/*Xho*I sites of pcDNA3.1/V5-His (Thermo Fisher Scientific, Waltham, MA, USA) with C-terminal V5 and His_6 tags. Then, the RV-N sequence was amplified by PCR using the following primers (BamHI For, 5′-gggggatccggctagttaagcttggtacc-3′; NotI Rev, 5′-gggcggccgcaaactcaatggtgatgg-3′) and cloned between the *Bam*HI/*Not*I sites of pFB-Neo plasmid (Agilent Technologies, Santa Clara, CA, USA) to create pFB-RV-N-Neo^R.

The pFB-RV-N2-Neo^R was constructed by 4 steps. (i) Using pFB-RV-N-Neo^R as a template, PCR was conducted with 2 primers (for1-BamHI-N, 5′-ggatccaccatg-gatgccgacaagattg-3′; rev1-NotI-ClaI-(G4S)3linker-N, 5′-gcggccgcatcgatcgacccgc-caccgccgctgccacctccgcctgaaccgcctccacctt cgaagggccctctagac-3′) and 2 primers (for2-BamHI-ClaI-N, 5′-ggatccatcgatatggatgccgacaagattg-3′; rev2-NotI-stop-His-V5-N, 5′-gcggccgctcaatggtgatggtgatg-3′), to obtain BamHI-RV-N-$(G_4S)_3$-ClaI-NotI and BamHI-ClaI-RV-N-V5-His_6-stop-NotI fragments, respectively. On the other hand, the host plasmid was modified by PCR with 2 primers (host-for-NotI, 5′-ccgcggccgctcgtgactgggaaaaccc-3′ and host-rev-BamHI 5′-gcaggtcgactctagagg-3′) using pHSG398 (Clontech, Mountain View, CA, USA) as a template to obtain pHSG398 with an additional *Not*I site (pHSG398/NotI). (ii) This pHSG398/NotI was digested with *Not*I/*Bam*HI and ligated with the inserts BamHI-RV-N-$(G_4S)_3$-ClaI-NotI and BamHI-ClaI-RV-N-V5-His_6-stop-NotI, which were also digested with the same restriction enzymes, to obtain pHSG398-BamHI-RV-N-$(G_4S)_3$-ClaI-NotI and pHSG398-BamHI-ClaI-RV-N-V5-His_6-stop-NotI, respectively. (iii) These two resulting plasmids were digested with *Not*I/*Cla*I and ligated to obtain pHSG398-BamHI-RV-N-$(G_4S)_3$-ClaI-RV-N-V5-His_6-stop-NotI (in short, pHSG398-RV-N2). (iv) Finally, the RV-N2 fragment was obtained by digesting pHSG398-RV-N2 with *Not*I/*Bam*HI, and ligated with the host vector pFB-Neo digested with the same enzymes to obtain the final vector pFB-RV-N2-Neo^R.

A phage-displayed synthetic human scFv library Tomlinson I (14) was applied for one-cycle bio-panning against the RV-N protein by the protocol described previously (13). The eluted phages were used for transforming an *Escherichia coli* TG-1 strain, and the transformed cells (library size: 1.5×10^5) were harvested to extract the plasmid library with Plasmid Midi Kit (Qiagen, Valencia, CA, USA).

For stable expression of the scFv-c-kit library, the library of scFv fragments was amplified with 2 primers (pIT2 SfiI for, 5′-gcaaattctatttcaaggagac-3′; pIT2 NotI rev, 5′-gccccattcagatcctcttc-3′) using the phage-selected scFv plasmid library as the template, and digested with *Sfi*I and *Not*I. These digested fragments were inserted into pMK-stuffer-c-kit-IRES-GFP, which was originally denoted as pMK-ΔL-stuffer-ΔTM-Kit-Flag-IG (12), digested with the same enzymes to obtain pMK-scFvlib-c-kit-IRES-GFP. Then, MegaX DH10BT1^{R}Electrocomp Cells (Thermo Fisher Scientific) were transformed with pMK-scFvlib-c-kit-IRES-GFP by electroporation using GenePulser Xcell (Bio-Rad, Hercules, CA, USA), and the transformed colonies were harvested to extract the plasmid library with Plasmid Midi Kit. The colony number was 6.6×10^5, thus covering the original library size of 1.5×10^5.

For stable expression of each scFv-c-kit clone, scFv fragments were amplified with 2 primers (EcoRI scFv for, 5′-cggatctgatcagtaactagc-3′; scFv NotI c-kit rev, 5′-ccactgtacttcatacatggg-3′) using the genomic DNA extracted from growing cells as the templates. After digesting with *Sfi*I and *Not*I these fragments were subcloned into pMK-stuffer-c-kit-IRES-Puro^R, which was originally denoted as pMK-ΔL-stuffer-ΔTM-Kit-Flag-IP (12), with the same enzymes to obtain pMK-scFv-c-kit-IRES-Puro^R.

The $(G_4S)_1$, $(G_4S)_2$, and $(G_4S)_3$ linker fragments (named F1, F2, and F3) with cohesive *Not*I and *Bsi*WI sites in the 5′ and 3′ termini, respectively were made by annealing the corresponding oligo-DNAs (F1f, 5′-ggccgcaggtggaggcggttcac-3′; F1r, 5′-gtacgtgaaccgcctccacctgc-3′; F2f, 5′-ggccgcaggtggaggcggttcaggcggaggtggcagcc-3′; F2r, 5′-gtacggctgccacctccgcctgaaccgcctccacctgc-3′; F3f, 5′-ggccgcaggtggaggcggttcaggcggaggtggcagcggcggtggcgggtcgc-3′; F3r, 5′-gtacgcgacccgccaccgccgctgccacctccgcctgaaccgcctccacctgc-3′). Then these linker fragments were subcloned into pMK-stuffer-c-kit-IRES-GFP digested with the *Not*I and *Bsi*WI enzymes to obtain pMK-stuffer-linker(F1, F2, or F3)-c-kit-IRES-GFP.

pMK-stuffer-linker(F1, F2, or F3)-c-kit-IRES-GFP was digested with *Not*I and *Bam*HI to obtain the linker(F1, F2, or F3)-c-kit fragment. This digested fragment was subcloned into pMK-stuffer-c-kit-IRES-Puro^R digested with the same enzymes to obtain pMK-stuffer-linker(F1, F2, or F3)-c-kit-IRES-Puro^R.

pMK-scFv-c-kit-IRES-Puro^R was digested with *Eco*RI and *Not*I to obtain the scFv fragment. This digested fragment was subcloned into pMK-stuffer-linker(F1, F2, or F3)-c-kit-IRES-Puro^R digested with the same enzymes to obtain pMK-scFv-linker(F1, F2, or F3)-c-kit-IRES-Puro^R.

A linear backbone (pMK-RV-N-IRES-Blast^R) was amplified with 2 primers (IFblast-For, 5′-atccgccctctcc-3′; IFNF1-b, 5′-cctccacctgcggccgcttcgaagggccctctag-3′) using pMK-RV-N-IRES-Blast^R as the template. Then F3-c-kit fragments were amplified with the primers (F1f, 5′-ggccgcaggtggaggcggttcac-3′; IFNck-b, 5′-ggga-gaggggcggatc-3′) using pMK-stuffer-F3-c-kit-IRES-Puro^R as the template. The F3-c-kit-flag fragments were infused to the linear backbone to get pMK-RV-N-c-kit-IRES-Blast^R.

Cell culture Ba/F3 cells, which were originally obtained from RIKEN Cell Bank (Tsukuba, Japan; RCB0805), were maintained in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 1 ng/mL murine IL-3 (Thermo Fisher Scientific) and 10% fetal bovine serum (FBS) (Biowest, Paris, France). Plat-E cells, which are retroviral packaging cells (15), were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical) containing 10 μg/mL blasticidin (Kaken Pharmaceutical, Tokyo, Japan), 1 μg/mL puromycin (Sigma, St. Louis, MO, USA), and 10% FBS.

Non-library vector transduction Retroviral packaging Plat-E cells were transfected with the corresponding plasmid using Lipofectamine^R LTX reagent