공학석사학위논문

Development of antibody drug discovery platform using GPCR-embedded nanodisc as an antigen

GPCR 내재 나노디스크를 항원으로 사용한 항체신약 발굴 플랫폼 개발

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서울대학교 대학원
화학생물공학부
박 현 영

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이 논문을 공학석사 학위논문으로 제출함

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Abstract

Development of antibody drug discovery platform using GPCR-embedded nanodisc as an antigen

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G protein-coupled receptor (GPCR) is one of promising targets for drug discovery. There have been lots of trials to produce recombinant GPCRs for the use as antigens. However, it was difficult to produce the whole GPCR in native form *in vitro* because it is a membrane protein. Insufficient production of GPCR was also problem because of low expression of GPCR. These have been major obstacles in development of novel antibody drugs against GPCR. Only two therapeutic antibodies have been approved by FDA.

Herein, we tried to solve the abovementioned problem with a nanodisc technology, a membrane-mimicking system. Our trial to use a GPCR-embedded nanodisc as antigen for drug discovery has 2 strong advantages. First, it can be mass-produced. To overcome the low expression of GPCR in various expression systems, *E. coli* system was used. GPCR was even co-expressed with effector gene for higher expression. Second, whole GPCR protein could be in native form *in*

vitro. GPCR embedded in nanodisc was reported to show almost native structure and similar functionality as in the cell membrane.

C-X-C Motif Chemokine Receptor 2 (CXCR2) was selected as a target GPCR because chemokine receptors are related with many inflammatory diseases and various types of cancers. As CXCR2 recruits the immunosuppressive cells into the tumor microenvironment, blocking CXCR2 or connecting immune cells with its novel antibody could be a therapy against the cancer.

CXCR2 reconstituted in nanodisc was characterized by several assays, and showed the binding affinity with its ligands. CXCR2 nanodisc could specifically capture its cognate antibody in the mixture of antibodies, implying the potential of nanodisc for high throughput antibody screening.

It is expected that GPCR-embedded nanodisc could be used as an appropriate antigen for drug discovery platform. We hope to find a novel antibody drug candidate with nanodisc as an antigen for phage display biopanning.

Keyword : Nanodisc, GPCR, CXCR2, Antibody screening
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1. Introduction

1.1. Therapeutic antibody discovery for G protein-coupled receptor

G protein-coupled receptors (GPCRs) are membrane proteins consisting of 7 transmembrane domains, extracellular domain, and intracellular domain. GPCRs regulate various physiological functions, including immune response, sensory function, neurotransmission, and hormone release.

GPCRs have been major pharmacological targets [1]. Most of the FDA-approved drugs for GPCRs are small molecules. Only 2 therapeutic antibodies, Erenumab (Amgen & Novartis) and Mogamulizumab (Kyowa Hakko Kirin Co.), were approved by FDA. Difficulties in making suitable antigens are obstacles to antibody development. Antigens commonly used for the antibody development were extracellular region peptide fused with a carrier protein [2, 3] and GPCR-expressing cells [4, 5]. However, these have limitations: limited extracellular domains, low expression of GPCR on cell membranes, and difficulties in the purification of natural functional protein.

There have been developed various antigen formats using whole GPCR such as proteoliposomes, virus-like particles, SMALP, and nanodisc to overcome those limitations [6]. Among these formats, nanodiscs can overcome the limitations of common antigen formats, and are appropriate for antibody screening with phage display.

Here, we produced GPCR in *E. coli* system and reconstituted it into nanodisc. This antigen format has 2 advantages. First, whole GPCR could be used as antigen. Second, the high productivity of GPCRs in *E. coli* enables mass production of GPCR nanodisc, allowing high-throughput antibody screening.

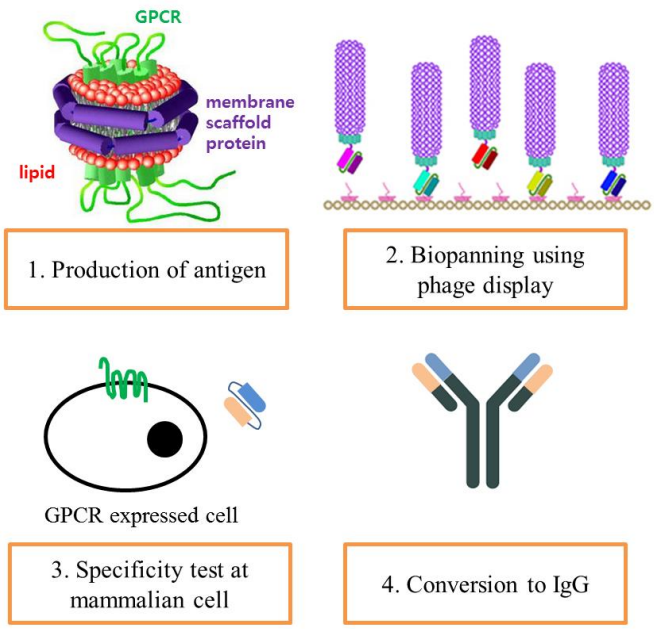


Figure 1. Therapeutic antibody discovery using GPCR-embedded nanodisc as an antigen

1.2. Expression of recombinant protein in *E. coli*

Production of recombinant proteins requires the use of various expression systems. The commonly used expression systems are *E. coli*, yeast, insect cell, and mammalian cell. Selecting the adequate system according to the properties of the protein will increase the yields and quality of proteins [7].

Insect and mammalian cells are the most widely used systems because of posttranslational modification and high folding capacity. However, it is difficult to produce the large amounts of protein due to low biomass density and expensive media [8].

E. coli is a simple and convenient host for recombinant protein expression. It is easy, fast, and cheap to culture, so high protein productivity can be obtained. But there is almost no posttranslational modification. As many proteins tend to be expressed in an insoluble and unfolded form, additional steps such as proper folding or protein secretion are required [9-11].

Table 1. Advantages and disadvantages of expression systems

Expression systems	*E. coli*	Mammalian cells
Advantages	- Rapid growth (days) - Inexpensive media and high biomass density - Simple scale-up - Well-established genetics	- All posttranslational modifications and high folding capacity
Disadvantages	- Limited posttranslational modification - Difficult to produce some proteins in a soluble, properly folded state	- Expensive media and low biomass density - Difficult to scale-up - Moderate to lengthy growth (weeks to months)

1.2.1. Expression of GPCR in *E. coli*

GPCRs are reported to be very difficult to be over-expressed, solubilized, and purified due to their strong hydrophobicity and complex structures. Eukaryotic systems produce GPCRs as folded in the cell membranes. On the other hand, the expression of human GPCR in bacterial cell membrane is considered to be difficult because of different charge distribution between eukaryotic and prokaryotic cells [12]. Therefore, GPCRs are generally produced as insoluble inclusion bodies in the cytoplasm of *E. coli* [13]. Inclusion body formation has advantages. The proteins toxic to host cells could be produced generally in high expression level. Moreover, they can be easily separated from bacterial cytoplasmic proteins through centrifugation [14].

Solubilization and stabilization of GPCR is needed before purification and reconstitution. Detergents, such as sodium cholate, CHAPS, deoxy-BiGCHAP, digitonin, zwittergent 3-14, octyl glucoside, triton X-100, and lubrol PX, have been reported to solubilize the membrane proteins properly [15]. Selection of suitable detergents is critical in correct refolding of GPCR because detergents are sometimes harmful on the stability of GPCR.

1.3. GPCR-embedded nanodisc

Nanodiscs are soluble phospholipid bilayers in which membrane proteins were self-assembled, with amphipathic membrane scaffold proteins wrapped around like a belt. They are composed of membrane protein, 2 copies of membrane scaffold proteins, and phospholipids. Membrane scaffold protein and phospholipids stabilize membrane proteins in aqueous solutions by mimicking the native membrane.

Nanodisc has advantages over other reconstitution forms like liposomes and detergent micelles. First, nanodisc showed good stability over others. Also, GPCR reconstituted in nanodisc showed the increased solubility [16]. The lipid composition affects GPCR structure as well as gives the environment similar with membrane. The size of nanodisc could be handled by using different type of membrane scaffold proteins, followed by production of the uniform size of nanodisc [17].

1.4. CXC motif chemokine receptor 2 (CXCR2)

CXC motif chemokine receptor 2 (CXCR2) is a class A GPCR and one of chemokine receptors. It is expressed on many different cell types, such as endothelial cells, neutrophils, macrophages, and neurons. Its ligands are proinflammatory chemokines regulating the migration and activation of neutrophils, and promote angiogenesis [18]. Overexpression of CXCR2 and its ligands has been associated with inflammatory diseases including chronic obstructive pulmonary disorder and rheumatoid arthritis, and multiple tumor types [19].

A number of small molecule drugs are currently in clinical trials [20, 21]. There are trials for developing the antibodies for CXCR2 but there are no therapeutic antibodies in clinical trials.

2. Materials and methods

2.1. Gene cloning

CXCR2 gene was optimized for expression in *E. coli* and cloned into pET-DEST42 vector. MSP1E3D1 gene was cloned into pET-28a vector. These proteins were expressed as recombinant proteins containing histidine tag (HHHHHH) for purification via immobilized metal affinity chromatography. RraA and DjlA gene were cloned into pBAD33.1 vector. (Fig. 2) Target DNA inserted into the vector was confirmed by Sanger DNA sequencing.

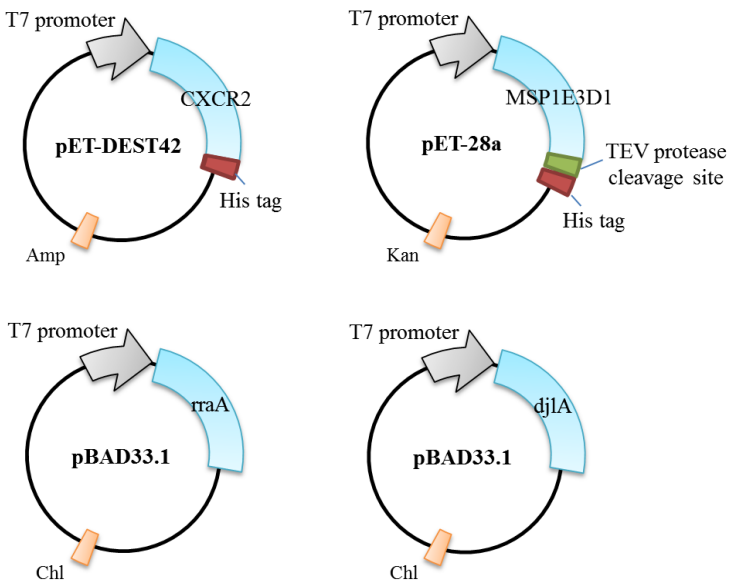


Figure 2. Plasmid construct

Amp : ampicillin resistance gene, Kan : kanamycin resistance gene,

Chl : chloramphenicol resistance gene

2.2. Production of CXCR2-embedded nanodisc

2.2.1. Protein expression and purification of CXCR2

pET-DEST42 vector carrying CXCR2 and pBAD 33.1 vector carrying RraA were transformed into Rosetta competent cells. Transformed *E. coli* was grown in LB broth medium supplemented with 100 μg/ml ampicillin and 40 μg/ml chloramphenicol at 37℃, 180 rpm overnight. The inoculated medium was transferred to 1L LB broth medium containing 0.2% (w/v) l-arabinose, and cultured at 30℃, 150 rpm. When an OD_{600} reached between 0.4 and 0.5, protein expression was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Expression was continued at 25℃, 120 rpm for 16 hours. Cells were harvested by centrifugation at 7000 rpm for 10 minutes at 4℃. After the supernatants were removed, cell pellets were resuspended in buffer (PBS, 2 mM EDTA) and sonicated for 10 minutes at 35% amplitude, 5 s on and 5 s off pulse. After centrifugation at 4℃, 12000 rpm for 30 minutes, the pellets were resuspended in solubilization buffer (0.1 M Tris, 20 mM sodium dodecyl sulfate (SDS), 1 mM EDTA, 100 mM dithiothreitol (DTT), pH 7.4), and incubated at 30℃ overnight to fully solubilize the pellets. After centrifugation at 25℃, 12000 rpm for 30 minutes, the supernatants were packed on a 10K MWCO dialysis cassette to exchange the buffer to the binding buffer (0.1 M sodium phosphate, 10 mM SDS, pH 8.0) through dialysis for 1 day. After centrifugation at 25℃, 12000 rpm for 30 minutes, the supernatants were filtered with 0.45 μm bottle top filter. The recombinant CXCR2 proteins were purified by fast protein liquid chromatography (FPLC, GE Healthcare). Filtered proteins were loaded on a HisTrap HP column (Cytiva) after column equilibration with the binding buffer. The unbound proteins were washed out through a pH gradient from pH 8.0 to 7.0. Remaining proteins were eluted using pH 6.0 buffer. The buffer of purified proteins was changed to the HEPES Ⅰ buffer (20 mM HEPES, 100 mM NaCl, 25 mM sodium cholate, pH 8.0) by passing through HiTrap Desalting column (Cytiva).

2.2.2. Protein expression and purification of MSP1E3D1

pET-28a vector carrying MSP1E3D1 was transformed into Rosetta competent cells. Transformed *E. coli* was grown in LB broth medium supplemented with 50 μg/ml kanamycin at 37℃, 180 rpm overnight. The inoculated medium was transferred to 1L LB broth medium and cultured at 37℃, 180 rpm. When an OD_{600} reached between 0.4 and 0.5, protein expression was induced by addition of 1 mM IPTG. After incubation for an additional 4 hours, cells were harvested by centrifugation at 7000 rpm for 10 minutes at 4℃. After discarding the supernatant, cell pellets were resuspended in His-binding buffer (20 mM Tris, 0.5 M NaCl, 20 mM imidazole, pH 8.0) and sonicated for 10 minutes at 30% amplitude, 5 s on and 5 s off pulse. After centrifugation at 4℃, 12000 rpm for 30 minutes, the supernatants were filtered with 0.45 μm bottle top filter. The recombinant MSP1E3D1 proteins were purified by FPLC. Filtered proteins were loaded on a HisTrap HP column (Cytiva) after column equilibration with the His-binding buffer. The unbound proteins were washed out with His-washing buffer (20 mM Tris, 0.5 M NaCl, 50 mM imidazole, pH 8.0) through increasing the imidazole concentration. Remaining target proteins were eluted with flow of the His-elution buffer (20 mM Tris, 0.5 M NaCl, 350 mM imidazole, pH 8.0).

Finally, the buffer of purified proteins was changed to the HEPES Ⅱ buffer (20 mM HEPES, 100 mM NaCl, pH 8.0) using HiTrap Desalting column (Cytiva). Then, MSP1E3D1 was incubated with TEV protease at a ratio of 1:100 (TEV:MSP) (w/w) at 30℃ for 4 hours. His-tag cleaved MSP1E3D1 were collected by taking the flow-through which did not bind to the HisTrap HP column.

2.2.3. Preparation of lipid (DMPC)

Liquid 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (Avanti Polar Lipids) in a round bottom flask were dried with nitrogen gas, and placed in a vacuum overnight. Fully-dried DMPC was dissolved in HEPES buffer (20 mM HEPES, 100 mM NaCl, 50 mM sodium cholate, pH 8.0) at over 70℃.

2.2.4. Reconstitution of CXCR2 in nanodisc

MSP1E3D1 and DMPC were mixed and incubated for 10 minutes at 25℃, which is the phase transition temperature of DMPC. CXCR2 was added to this mixture and incubated with gentle agitation at 25℃ for 2 hours. Bio-beads (Bio-Rad) were added for 4 times by 1 hour to remove the detergent. After the incubation overnight, the mixture was loaded on HisTrap HP column for the purification of proteins with his-tag. Eluted fractions were loaded through a loop into the size exclusion chromatography (Superdex 200 Increase 10/300 GL, Cytiva) to separate nanodisc from larger protein aggregates and smaller proteins. Fractionated CXCR2-embedded nanodiscs were stored at either -80℃ or 4℃.

2.3. SDS-PAGE and western blot analysis

Purification of recombinant proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. The gel was stained with Coomassie blue solution for Coomassie blue staining. Besides, the proteins on the gel were transferred on a polyvinylidene difluoride (PVDF) membrane for western blot. His-tag on the recombinant proteins was detected by anti-his tag antibody (Santa Cruz, USA). Secondary antibody was goat anti-mouse IgG HRP (AbFrontier, Korea). HRP on secondary antibody was detected by TOPview ECL Pico Plus Western Substrate.

2.4. Dynamic light scattering

After equilibrating the nanodisc to 25℃, 1ml of nanodisc was added to a clean cuvette followed by measurement of hydrodynamic radius with the Malvern Zetasizer Ultra (Malvern Panalytical). The measurement was repeated 5 times to derive the average diameter and number distribution of CXCR2 nanodisc.

2.5. Tryptophan fluorescence quenching assay

It was performed using Perkin Elmer fluorescence spectrometer LS 55 (Perkin Elmer) and a 200 μl quartz cuvette. The excitation wavelength was 280 nm and the emission signal was collected at 340 nm. To measure the interaction between nanodisc and CXCL8, all materials were equilibrated in HEPES Ⅱ buffer at room temperature. Empty nanodisc without CXCR2 and CXCR2 nanodisc were analyzed with CXCL8 ranging from 0 to 1 ng/ml.

2.6. Surface Plasmon Resonance (SPR)

CXCR2 nanodisc containing his-tag on the C terminus was loaded on the nitrilotriacetic acid (NTA) coated surface of a Sensor Chip NTA (Cytiva). The binding interaction between CXCR2 and its ligands (CXCL5 and CXCL8) were measured on Biacore 3000 (Cytiva). CXCR2 nanodisc and ligands were diluted in the HEPES Ⅱ buffer.

2.7. Enzyme-linked immunosorbent assay (ELISA)

CXCR2 nanodiscs were immobilized on the nickel coated clear plates (Thermo Fisher Scientific) at room temperature for 2 hours. Wells were washed 3 times with 200 μl of PBST buffer (0.05% TWEEN20 in PBS, pH 7.4) per well, and blocked with 200 μl of blocking buffer (0.05% TWEEN20, 5% BSA in PBS) per well for 1 hour. Wells were washed 3 times with PBST buffer. 1 μg/ml anti-CXCR2 antibody (Santa Cruz Biotechnology) was serially diluted in 1% BSA PBST buffer, added into each well, and incubated for 1 hour. Wells were washed 3 times with PBST buffer. Goat anti-mouse IgG HRP (1:4000 dilution; AbFrontier) in 5% BSA PBST buffer was added to each well, and incubated for 1 hour. Wells were washed 5 times with PBST buffer. 100 μl of 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific) was added to each well. After incubation in the absence of light for 10 minutes, 100 μl of 2M sulfuric acid was added to each well. Absorbance at 450 nm was measured using Spark 10M multimode microplate reader (TECAN).

2.8. Small-scale antibody screening test

CXCR2 nanodiscs were immobilized on the nickel coated clear plates (Thermo Fisher Scientific) at room temperature for 2 hours. Wells were washed 3 times with 200 μl of PBST buffer (0.05% TWEEN20 in PBS, pH 7.4) per well, and blocked with 200 μl of blocking buffer (0.05% TWEEN20, 5% BSA in PBS) per well for 1 hour. Wells were washed 3 times with PBST buffer. Mixture of antibodies containing anti-CXCR2, CCR5, CCR6, and CCR7 antibody (0.5 μg/ml each) was added to each well, and followed by incubation for 1 hour. Wells were washed 3 times with PBST buffer. Anti-mouse IgG HRP (1:4000 dilution; AbFrontier) or anti-goat IgG HRP (1:4000 dilution; AbFrontier) was added to each well, and incubated for 1 hour. Wells were washed 5 times with PBST buffer. 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific) was added to each well. After incubation for 10 minutes, 2M sulfuric acid was added to each well. Absorbance at 450 nm was measured using Spark 10M multimode microplate reader (TECAN).

3. Results

3.1. Expression and purification of CXCR2

3.1.1 Expression of CXCR2

CXCR2 gene was codon-optimized to increase the expression in *E. coli*. Moreover, co-expression with effector genes, rraA or djlA, could improve the expression of recombinant membrane protein in *E. coli* [22]. RraA (Regulator of ribonuclease activity A) is a protein inhibitor of RNase E which mediates mRNA degradation in *E. coli* [23]. DjlA is a co-chaperone protein [22].

The expression level of CXCR2 was increased with the impact of effector gene (Fig. 3). CXCR2 co-expressed with rraA showed nearly 3-fold higher expression compared to CXCR2 without any effector genes.

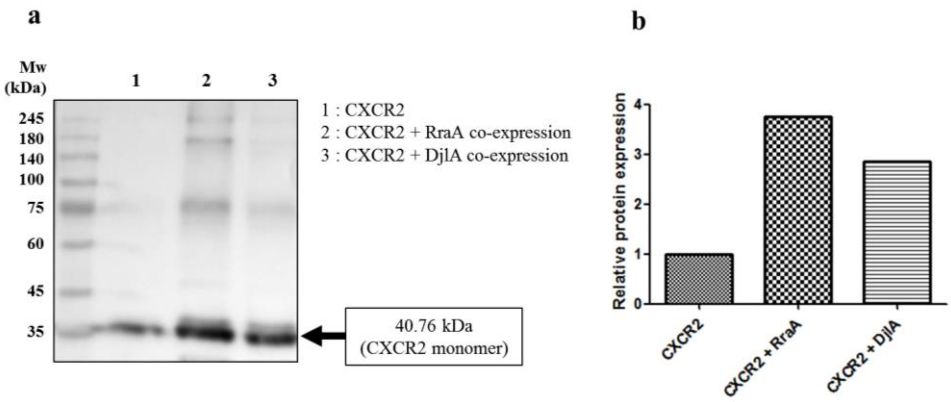


Figure 3. Comparison of CXCR2 expression levels with co-expression of effector genes

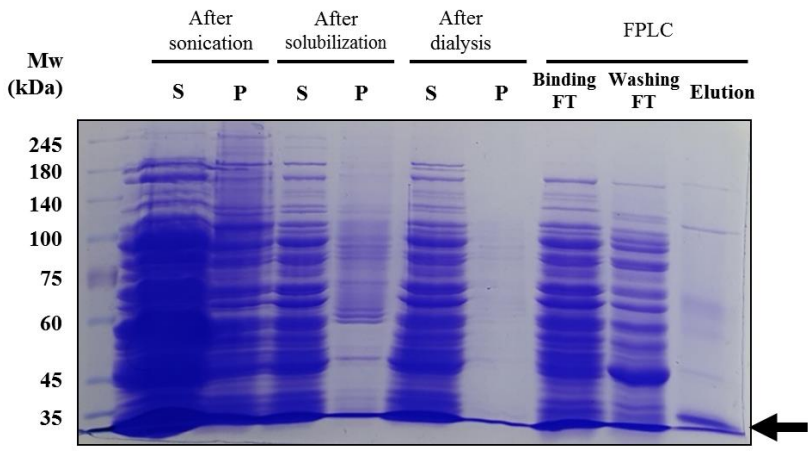
(a) Western blot analysis of CXCR2 expression with or without effector genes using anti-his tag antibody. (b) Relative protein expression calculated using Image J software.

3.1.2 Production of CXCR2

CXCR2 was produced as an inclusion body in *E. coli*. Therefore, proper refolding step should be added.

Lysed cell pellets were solubilized with buffer containing SDS, DTT, and EDTA. EDTA, which is widely used for inhibition of proteases during preparation of cell extracts [24], and DTT were removed by dialysis. SDS-denatured proteins were purified through immobilized metal affinity chromatography, and desalted into HEPES buffer with sodium cholate. Purified CXCR2 is transiently stabilized in the presence of sodium cholate before reconstitution in nanodisc.

Purification process of CXCR2 was identified by SDS-PAGE analysis (Fig. 4). CXCR2 was produced in high purity and amount (Fig. 5). GPCRs have been known as having a tendency to form dimer under the denaturing condition for SDS-PAGE [25].



S : Supernatant after centrifugation
P : Pellet after centrifugation
FT : Flow through
FPLC : Fast protein liquid chromatography

Figure 4. SDS-PAGE analysis of CXCR2 produced in *E.coli*

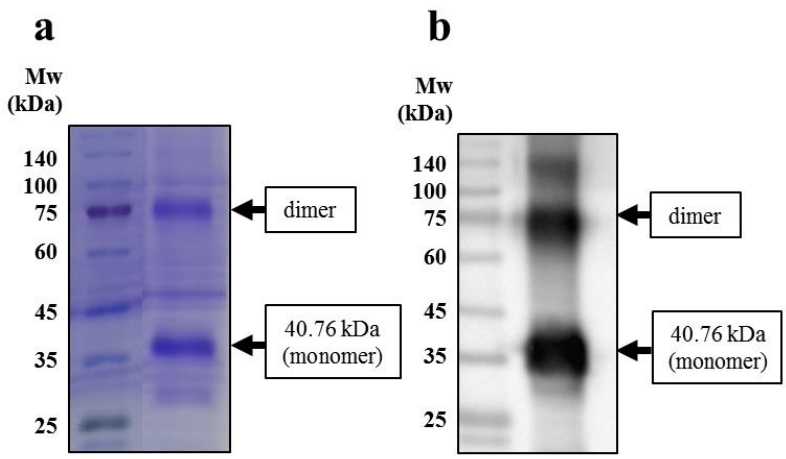


Figure 5. SDS-PAGE (a) and western blot (b) analysis of purified CXCR2 using anti-his tag antibody

3.2. Expression and purification of MSP1E3D1

Membrane scaffold protein 1E3D1 (MSP1E3D1) is a soluble protein. It was expressed sufficiently without any additional steps. His-tag of MSP1E3D1 was used for the purification of MSP1E3D1, and cleaved by TEV protease for further experiments. MSP1E3D1 was produced in high purity and amount (Fig. 6).

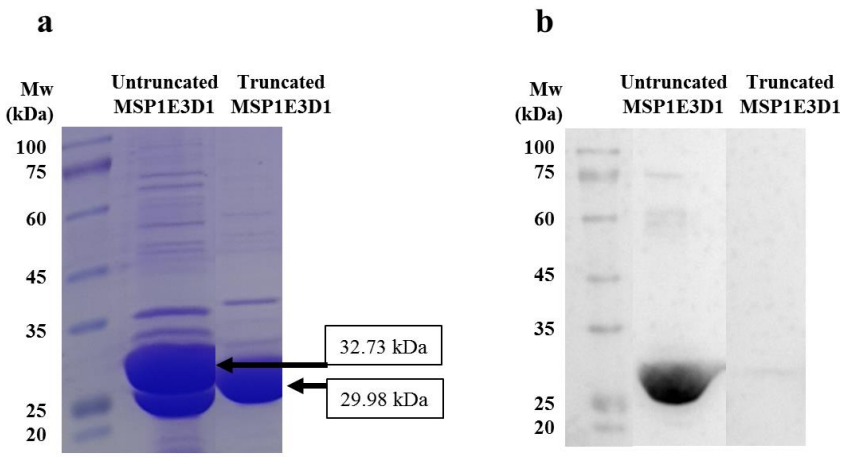


Figure 6. SDS-PAGE (a) and western blot (b) analysis of purified MSP1E3D1 in *E. coli* using anti-his tag antibody

Untruncated MSP1E3D1 (32.72 kDa) is MSP1E3D1 purified with chromatography. Truncated MSP1E3D1 (29.98 kDa) is MSP1E3D1 without his tag.

3.3. Reconstitution of CXCR2 using nanodisc

CXCR2 was further stabilized by incorporation in nanodisc. CXCR2, which was transiently solubilized with sodium cholate, were mixed in the presence of MSP1E3D1 and DMPC. As sodium cholate was removed by the adsorption to hydrophobic beads, CXCR2 simultaneously assembled with phospholipids.

Individual CXCR2 nanodisc was sequentially isolated by affinity chromatography and size exclusion chromatography. The individual nanodisc was eluted approximately 10.8 ml after sample injection, while the soluble aggregates were at 8 ml (Fig. 7b).

Optimization of the process was necessary to separate the peak of nanodisc and the peak of aggregates and improve the purity and productivity of nanodisc. Process of production of nanodisc can be summarized in 3 steps: (1) mixing CXCR2, MSP1E3D1, and DMPC, (2) removal of detergent by adding bio-beads, (3) purification through chromatography (Fig. 7a). Optimization was performed for each step, and the selection criteria were the yield and homogeneity of nanodisc.

First, the molar ratio between CXCR2, MSP1E3D1, and DMPC was the important parameter. Production of high-purity CXCR2 and MSP1E3D1 should be preceded to mix them in exact molar ratio. They were mixed in 3 different cases, 1:5:575, 1:5:600, and 1:20:2300 (Fig. 8). The yield of individual nanodisc compared with the aggregates changed according to the different molar ratio. A molar ratio of 1:5:575 (CXCR2:MSP1E3D1:DMPC) was the optimal.

Second, the removal rate of detergent was modulated by addition of bio-beads. Slow elimination of detergent increased the nanodisc yield by reducing the aggregates, compared with detergent removal at once. The best option was adding the bio-beads in 4 times by 1 hour (Fig. 9).

Last, the number of chromatography steps also gives an influence on the nanodisc production. Two-step chromatography, including his-tag affinity chromatography and size exclusion chromatography, was performed to isolate the nanodisc containing his-tag in high purity. The differences in the amount of purified nanodisc with and without his-tag affinity chromatography were observed (Fig. 10).

Nanodisc samples from each optimization step were characterized by DLS and SEM to confirm and compare the purity and homogeneity of the samples.

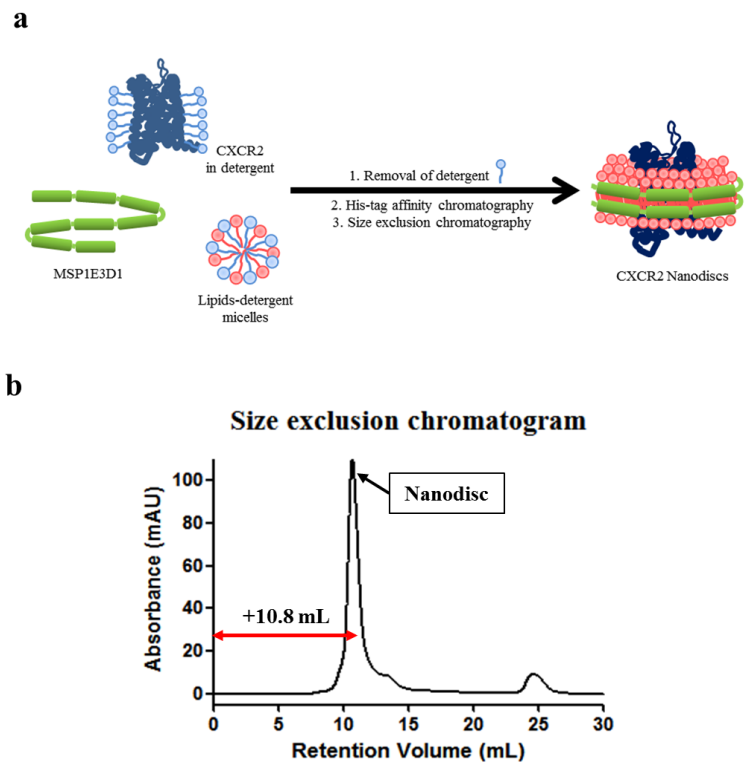


Figure 7. Size exclusion chromatography for the purification of nanodisc

(a) Schematic diagram for the production of nanodisc (b) Size exclusion chromatogram in the optimized purification process

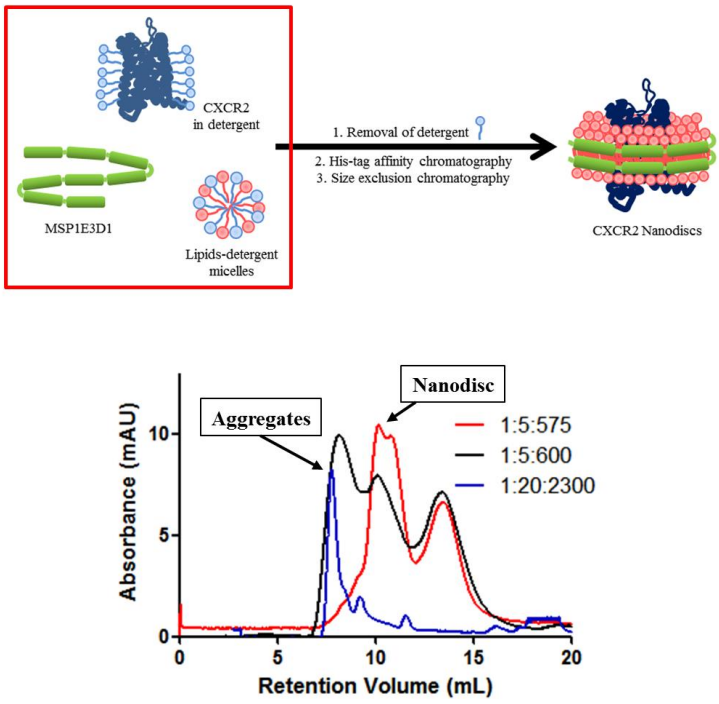
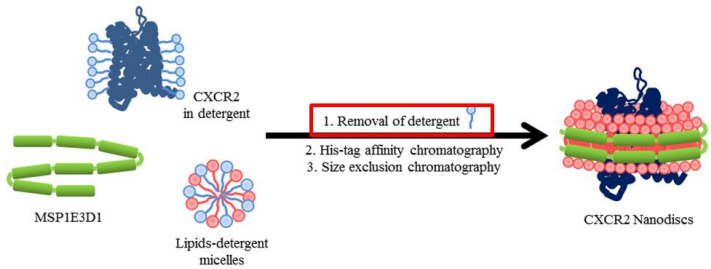


Figure 8. Size exclusion chromatogram for the process optimization (Molar ratio between CXCR2, MSP1E3D1, and lipid)



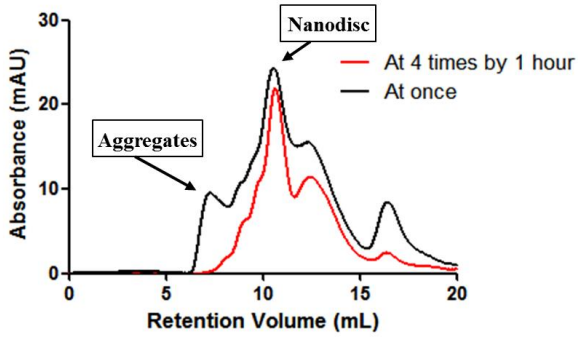


Figure 9. Size exclusion chromatogram for the process optimization (Detergent removal rate)

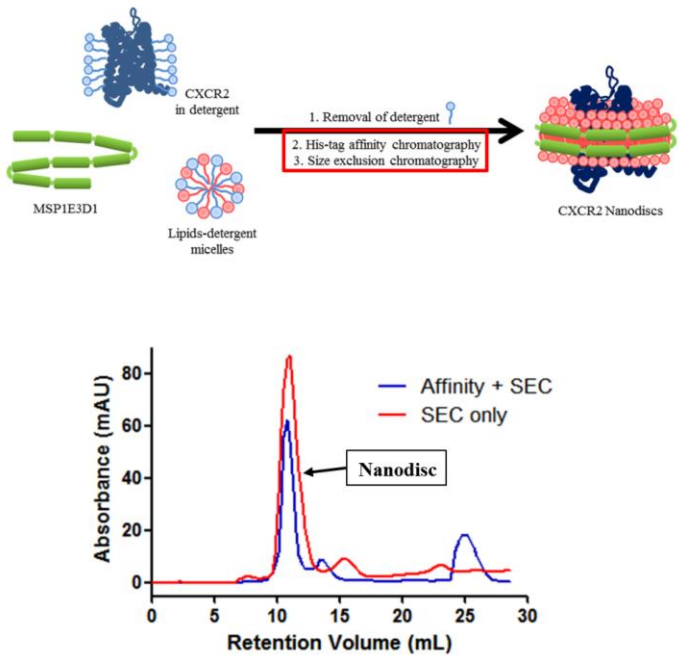


Figure 10. Size exclusion chromatogram for the process optimization (Number of chromatography steps)

3.4. Characterization of CXCR2-embedded nanodisc

Purified CXCR2 nanodisc was characterized and functionally analyzed.

3.4.1. Size analysis of CXCR2 nanodisc with dynamic light scattering (DLS)

The hydrodynamic radius was calculated from the diffusion behavior of molecules in solution [26]. The size of nanodisc made up with MSP1E3D1 was reported as up to 12.8 nm, and the thickness was typically 4.6~5.5 nm depending on the type of phospholipid used [27].

The size of CXCR2 nanodisc was analyzed by dynamic light scattering (DLS). The average diameter was 9.19 nm (Fig. 11). The polydispersity index (PI), which shows the homogeneity, was 0.12.

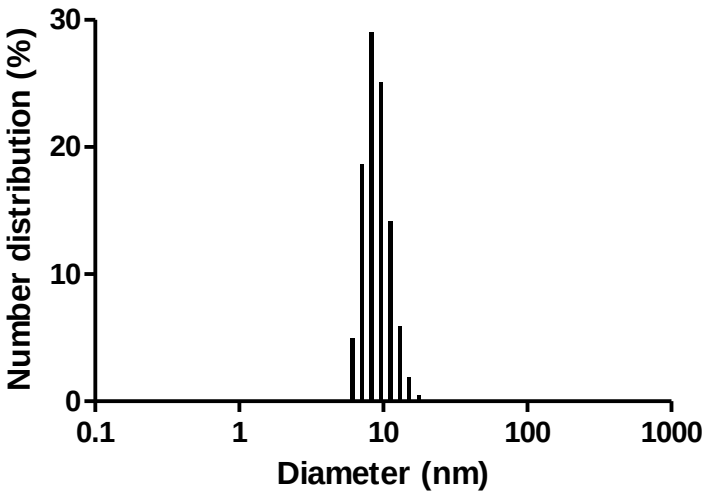


Figure 11. Number distribution of CXCR2 nanodisc

3.4.2. SDS-PAGE and western blot analysis for CXCR2 nanodisc

Purified CXCR2 nanodisc was characterized with SDS-PAGE and western blot (Fig. 12). It was confirmed that it was composed of CXCR2 and MSP1E3D1. Both CXCR2 monomer and dimer were observed in western blot. GPCRs have been reported to form dimer and oligomer under ad conditions like on SDS-PAGE [25].

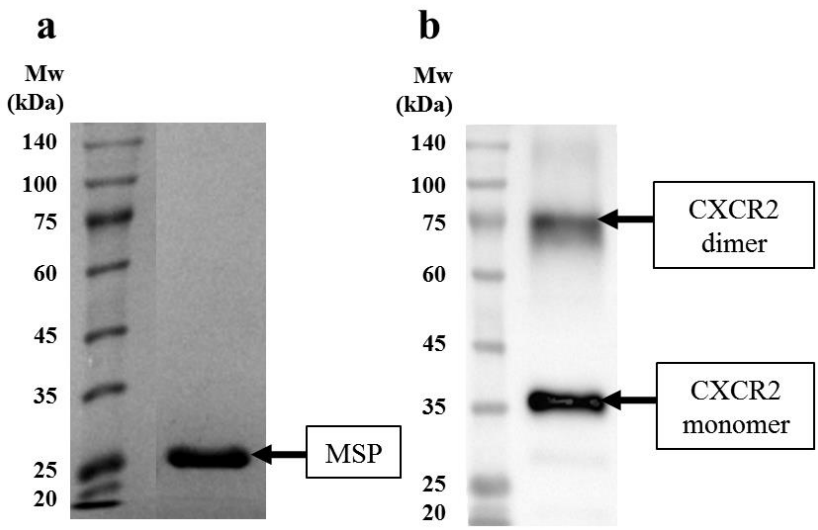


Figure 12. SDS-PAGE and western blot analysis of CXCR2 nanodisc

(a) SDS-PAGE of the purified CXCR2 nanodisc. (b) Western blot of the purified CXCR2 nanodisc using anti-his tag antibody

3.4.3. SEM image of CXCR2 nanodisc

The size of CXCR2 nanodisc was approximately 10 nm in the scanning electron microscope (SEM) images (Fig. 13), consistent with the DLS data.

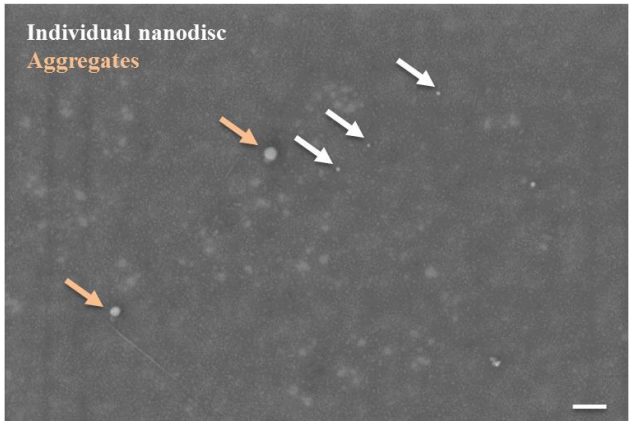


Figure 13. SEM image of CXCR2 nanodisc

CXCR2 nanodisc was immobilized on graphene. (Scale bar : 200 nm)

3.4.4. Tryptophan fluorescence quenching assay

Tryptophan and tyrosine are natural fluorophores in most proteins [28]. As receptor binds to its ligand, the receptor undergoes the conformational change, and the intrinsic fluorescence of tryptophan residues in receptor become quenched [29, 30].

The functionality of CXCR2 in nanodisc was identified by tryptophan fluorescence quenching assay.

Fluorescence intensity was decreased during the interaction of CXCR2 nanodisc and CXCL8, dependent to the concentration of the ligand of CXCR2 (CXCL8). In contrast, empty nanodisc which only consists of MSP1E3D1 and DMPC did not show the significant changes in the intensity, implying there was no interaction between them (Fig. 14).

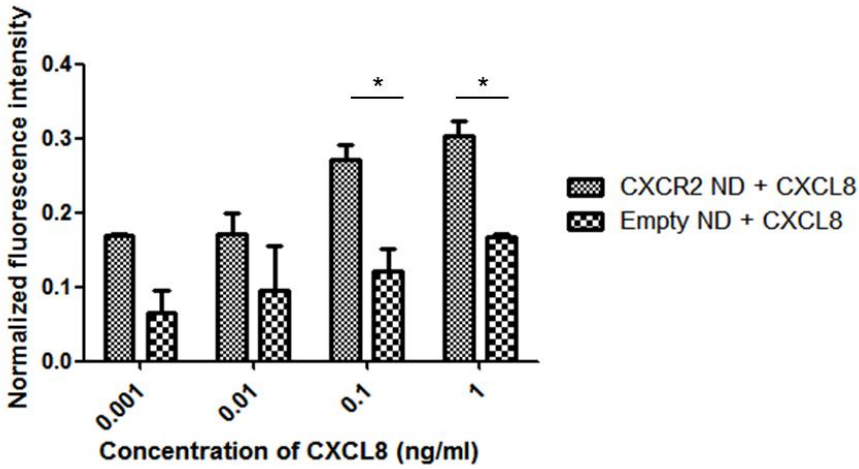


Figure 14. Binding ability of CXCR2 by tryptophan fluorescence quenching assay

3.4.5. Surface plasmon resonance (SPR)

Extracellular domain of CXCR2 nanodisc was oriented upside, as CXCR2 nanodisc was immobilized on NTA chip with his tag. As the ligand associated with the immobilized CXCR2 nanodisc, the SPR signal increased. On the other hand, the signal decreased in the dissociation.

Binding curve of CXCR2 nanodisc with different ligands showed the different form. CXCR2 nanodisc could identify the type of its ligand (Fig. 15a).

Binding curve of CXCR2 nanodisc with same ligand displayed the same form but different binding affinity level. The binding affinity was improved with increasing concentration of ligand (Fig. 15b).

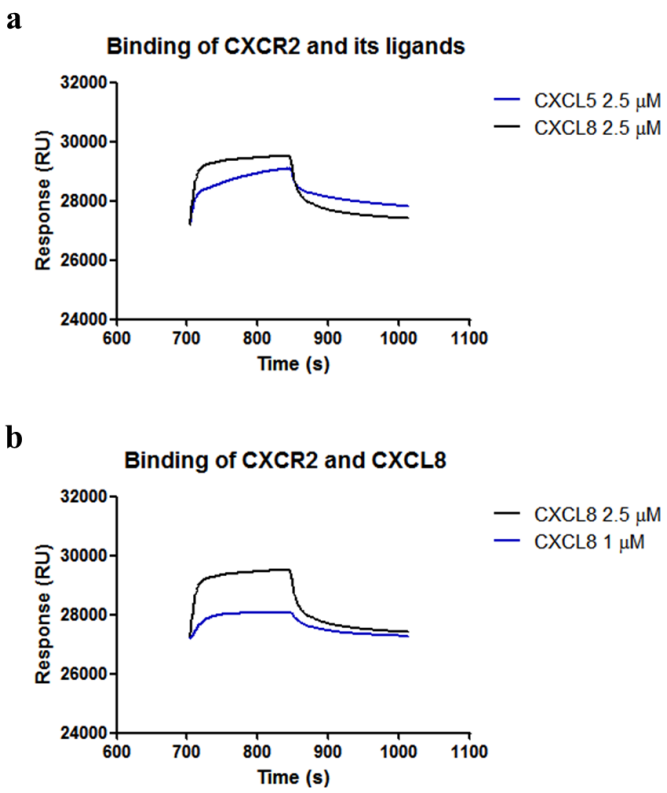


Figure 15. SPR analysis of binding of immobilized CXCR2 nanodisc to its ligand

(a) Overlay plot showing the binding of different ligands at same concentration (2.5 μM)
(b) Overlay plot showing the binding of CXCL8 at different concentrations (1 and 2.5 μM)

3.5. Feasibility of CXCR2 nanodiscs as appropriate antigens for antibody screening

Before using CXCR2 nanodisc as an antigen for antibody screening, the antigenic potential of CXCR2 nanodisc should be confirmed with commercially available antibodies.

First of all, it was necessary to confirm whether the CXCR2 nanodisc could capture anti-CXCR2 antibody. The immobilization of CXCR2 nanodisc on Ni-coated plate has orientation due to his-tag at the C terminus of recombinant CXCR2. The absorbance increased in dose-dependent response with increasing concentration of anti-CXCR2 primary antibody (Fig. 16). It was confirmed that CXCR2 nanodisc could capture its antibody well.

Small-scale antibody screening test was done by ELISA to check the potential of nanodisc as an antigen for antibody screening. Antibody mixture that consists of anti-CXCR2, anti-CCR5, anti-CCR6, and anti-CCR7 antibodies was used as primary antibody. Anti-CXCR2 antibody could be screened in a mixture of antibodies with the differences in the hosts of primary antibodies. The host of anti-CXCR2 antibody was mouse, while the host of the other antibodies was goat. By comparing the HRP signal of the secondary antibodies, it was shown that CXCR2 nanodisc can capture the anti-CXCR2 antibody well where other antibodies are mixed (Fig. 17).

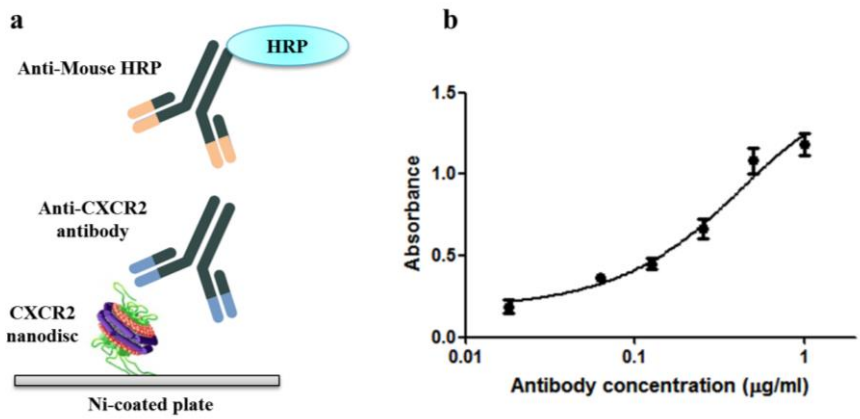


Figure 16. ELISA for CXCR2 nanodisc

(a) Schematic diagram of ELISA for CXCR2 nanodisc (b) Measurement of capturing ability of nanodisc to anti-CXCR2 antibody with increasing concentration of anti-CXCR2 antibody. Curves represent a range of antibody concentrations: 0.031, 0.063, 0.125, 0.25, 0.5, and 1 μg/ml.

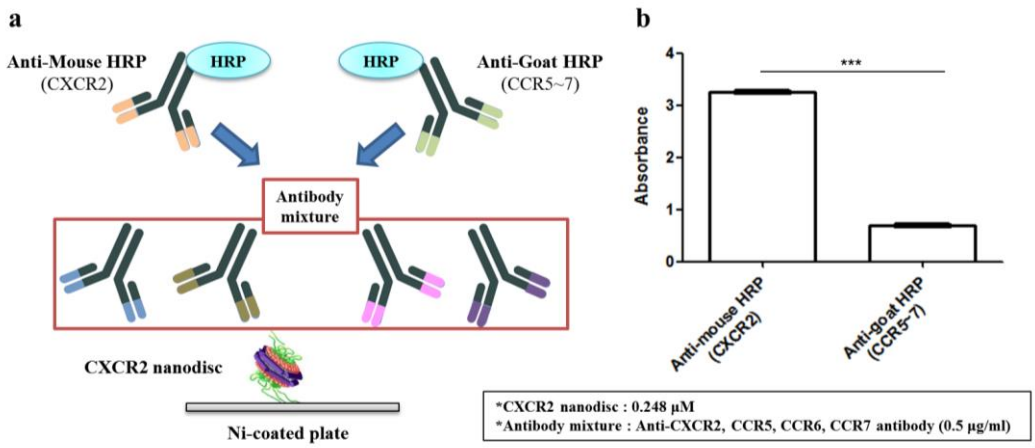


Figure 17. Feasibility of CXCR2 nanodiscs as appropriate antigens for antibody screening

(a) Schematic diagram of small-scale antibody screening test (b) Comparison of absorbance signal of anti-CXCR2 antibody with other antibodies using host differences in antibodies. Concentration of each antibody in antibody mixture is 0.5 μg/ml.

4. Discussion

GPCR expressed in *E. coli* should be further characterized to identify the similarities as in native membrane.

Due to the limitation of post-translational modification in *E. coli* expression, we tried to analyze the functionality of CXCR2 nanodisc with 2 different assays. CXCR2 nanodisc was identified to bind with its ligands. To compare the similarities of CXCR2 in nanodisc with in native cell membrane, further studies are required. Various assays could be set up with nanodisc, as it is accessible from both extracellular domain side and intracellular domain side.

Control of the number of receptor in nanodisc can attribute to make more delicate antigen format.

Reconstitution of GPCR in nanodisc is spontaneous process. It is quite hard to regulate the number of GPCRs in individual nanodisc. But control in the number of receptor in each nanodisc leads to make more delicate antigen, followed by developing antibody with high specificity.

There are difficulties in identifying the number of receptor in nanodisc with SEM image of nanodisc and SDS-PAGE and western blot analysis.

It is reported that CCR5 was reconstituted in proteoliposomes using nonporous paramagnetic beads covalently conjugated with an antibody that recognizes the C-terminal of GPCR [31]. This format enables to control the number of receptor by using the interaction of receptor and antibody. Also, GPCR fused with G protein was used to visualize the structure of individual GPCR [32]. It could be with interacting GPCR-embedded nanodisc with addition of its ligands, except the construction of fusion protein.

Investigating the homogeneity of assembled nanodisc will attribute to make better antigen.

5. Conclusions

GPCRs are major target of drug discovery. Due to low expression of GPCR and difficulties in production of proper GPCR antigen, the development of antibodies against GPCR is still difficult.

This study suggests antibody drug discovery platform using GPCR-embedded nanodisc as an antigen. For mass production of antigen, CXCR2 was expressed using E. coli system and reconstituted into nanodisc to use whole protein for an antigen. CXCR2 was overexpressed in E. coli by the co-expression of rraA. CXCR2 and MSP1E3D1 were purified in high purity. Purified CXCR2 was denatured by SDS, transiently stabilized with sodium cholate, and reconstituted into nanodisc by self-assembly. Nanodisc could be well purified in high homogeneity and separated from soluble aggregates and smaller protein residues through process optimization.

The diameter of CXCR2 nanodisc was approximately 9~10 nm and it was formed uniformly, according to both DLS and SEM data. CXCR2 nanodisc was confirmed its functionality by binding with its cognate ligands well. Additionally, CXCR2 nanodisc was shown to be promising platform to stabilize the membrane protein in vitro.

Feasibility of CXCR2 nanodiscs as proper antigens for antibody screening was confirmed by small-scale antibody screening test. CXCR2 nanodisc could capture the specific antibody among the antibody mixtures. Moreover, immobilized CXCR2 nanodisc on Ni-coated plate would be possible platform for further antibody screening. These implied the possibility of the platform for high-throughput antibody screening.

Furthermore, this platform can also be applied when each side of the bilayer is needed like ion channels. As nanodisc is accessible from both side of the bilayer, it would be convenient to set up an assay for various purposes, such as comparing with native GPCR in mammalian cell or other antigens. This platform will contribute to antibody drug screening, and establishment of antigen-based biosensors.

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국 문 초 록

GPCR 내재 나노디스크를 항원으로 사용한 항체신약 발굴 플랫폼 개발

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G protein-coupled receptor (GPCR)은 신약개발에 있어 유망한 타깃이다. GPCR을 항원으로 사용하기 위해 재조합 단백질로서 GPCR을 생산하기 위한 많은 시도가 있었다. 그러나 막 단백질인 GPCR을 in vitro에서 고유의 상태로 생산하는 것은 어려웠다. GPCR의 낮은 발현량으로 인해 생산량이 충분하지 않다는 것도 문제였다. 이러한 부분들은 GPCR에 대한 새로운 항체의약품 개발에 있어 주요한 장애물이었다. 현재까지 오직 2개의 항체의약품만이 FDA의 승인을 받았다.

본 연구에서 우리는 세포막을 모방하는 시스템인 나노디스크를 활용하여 위에서 언급한 문제들을 해결하고자 했다. 신약개발 과정에서 GPCR 내재 나노디스크를 항원으로 사용하는 것은 2가지 강점을 갖는다. 먼저, 항원의 대량생산이 가능하다는 점이다. 다양한 발현 시스템에서 GPCR의 발현량이 낮은 것을 극복하기 위해 GPCR을 대장균에서 발현시켰다. 더 높은 발현량을 얻기 위해 effector gene을 함께 발현시키기도 했다. 두 번째로, 전체 GPCR 단백질을 체외에서 고유한

구조를 가진 형태로 얻을 수 있다는 점이다. 나노디스크에 내재된 GPCR은 세포막에서와 거의 고유한 구조와 유사한 기능을 나타내는 것으로 알려져 있다.

Chemokine receptor는 많은 염증성 질환과 암과 관련되어 있기에, C-X-C Motif Chemokine Receptor 2 (CXCR2)를 항체의약품의 타깃으로 설정했다. CXCR2가 종양미세환경에서 면역억제세포를 끌어들이기 때문에, 새로운 항체의약품으로 CXCR2를 차단하거나 면역세포를 연결시킨다면 암에 대한 치료가 가능할 것으로 예상된다.

CXCR2 나노디스크는 여러 방법으로 분석되었고 리간드와의 결합 친화도를 보여주었다. CXCR2 나노디스크는 항체 혼합물에서 항 CXCR2 항체를 특이적으로 인식할 수 있어, 고속대량 항체스크리닝에 사용할 수 있는 가능성을 시사한다.

GPCR 내재 나노디스크는 신약개발 플랫폼을 위한 적절한 항원으로 활용될 수 있을 것으로 기대된다. 우리는 나노디스크를 파지 디스플레이 바이오패닝의 항원으로 사용하여 새로운 항체 의약품 후보물질을 찾을 수 있기를 기대한다.

주요단어 : 나노디스크, GPCR, CXCR2, 항체 스크리닝

학번 : 2021-29897