Methods xxx (2011) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Rapid identification of recombinant Fabs that bind to membrane proteins

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ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Phage display
Recombinant Fabs
Membrane proteins
ELISA

ABSTRACT

Crystallographic studies of membrane proteins have been steadily increasing despite their unique physical properties that hinder crystal formation. Co-crystallization with antibody fragments has emerged as a promising solution to obtain diffraction quality crystals. Antibody binding to the target membrane protein can yield a homogenous population of the protein. Interantibody interactions can also provide additional crystal contacts, which are minimized in membrane proteins due to micelle formation around the transmembrane segments. Rapid identification of antibody fragments that can recognize native protein structure makes phage display a valuable method for crystallographic studies of membrane proteins. Methods that speed the reliable characterization of phage display selected antibody fragments are needed to make the technology more generally applicable. In this report, a phage display biopanning procedure is described to identify Fragments antigen binding (Fabs) for membrane proteins. It is also demonstrated that Fabs can be rapidly grouped based on relative affinities using enzyme linked immunosorbent assay (ELISA) and unpurified Fabs. This procedure greatly speeds the prioritization of candidate binders to membrane proteins and will aid in subsequent structure determinations.

1. Introduction

Membrane proteins are notoriously difficult to crystallize. It is often challenging to obtain structurally homogeneous proteins due to their conformationally heterogeneous nature. They need to be incorporated into artificial membranes that mimic their native state [1], and the detergents used to solubilize the membrane proteins often lead to inactivity and aggregation [2]. Moreover, membrane proteins have less surface area for crystal contacts due to micelle formation around the transmembrane segments. To make membrane proteins more amenable to crystallographic studies, these properties must be improved. Stable binding proteins can be used to improve some of these properties and have been shown to assist crystallization of membrane proteins [3–8].

Binding proteins can be either antibodies/antibody fragments, or non-antibody proteins. Non-antibody binding proteins include avimers, which are artificial multidomain proteins derived from human extracellular receptor domains [9], and DARPINs, which are derived from the ankyrin repeat motif present in numerous naturally occurring proteins [3]. These non-antibody binding proteins were engineered to create antibody like molecules with improved stability and production yield [3,4,9]. Nevertheless, the most commonly used binding proteins in co-crystallization experiments are antibody fragments. They have a tendency to bind flexible regions of macromolecules [10,11], which can result in a more rigid and structurally homogeneous population of the complex. Antibody fragments can also aid in obtaining a conformationally homogeneous population by recognizing a single conformation and locking the protein in that conformation [3]. Furthermore, antibody fragments can provide additional crystal contacts of complexes. Such contacts are well depicted in the structures of the antibody fragment–membrane protein complexes such as the KscA potassium channel [5,6] as well as soluble protein complexes [3,12].

Antibody fragments exist in various forms, including nanobodies that are single variable domains, Fvs that are composed of variable domains of light and heavy chains, and Fragments antigen binding (Fabs) that are composed of variable domains and single constant domains of light and heavy chains. Despite that all three forms have been co-crystallized with proteins, Fabs are the most commonly used form. This may be explained by their physical properties. Fabs are more likely to provide sufficient additional crystal contacts especially compared to nanobodies (15 kDa), which are a quarter the size of Fabs (55 kDa). They are typically more stable than Fv fragments [13]. Moreover, single chain Fvs, the most common form of Fv, can exist in a monomer–dimer equilibrium, which is not ideal for crystallization [14]. Using standard

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doi:10.1016/j.ymeth.2011.09.012

procedures for generating a phage Fab library [15], a fully human naïve B cell Fab library was produced as a rich source of potential binding partners to proteins of interest [16].

Phage display is a biopanning technique, which uses affinity selection to generate recombinant antibodies against a variety of antigens [17]. In M13 filamentous phage display, which is the most commonly used form, the DNA encoding antibody fragment library is fused into the minor coat protein gene, g3 in a cloning vector called a phagemid. The fusion protein is expressed and incorporated into phage particles assembled in *Escherichia coli* when the cells are infected with helper phage, which harbors the full phage genome necessary for completing the phage life cycle. Thus the antibody fragment is presented on the phage surface while its encoding gene resides within the phage particle. Antibody presenting phage particles are subject to a procedure called biopanning or panning, which refers to repeated cycles of binding between phage and immobilized antigen, washing away unbound phage, eluting bound phage, infecting *E. coli* with the eluted phage and propagating the eluted phage (Fig.1). Thus, the phage particles that display antigen-binding antibody fragments become enriched. Relevant phagemids can be extracted from the *E. coli* cells infected with the eluted phage.

In comparison to hybridoma antibody production, which can take several months, phage display antibodies can be obtained in a few weeks and provide a stable, renewable source of antibodies. *In vitro* selection of phage display does not rely on antigen immunogenicity, lack of which is a major limitation of hybridoma antibody production, and thus extends a range of target antigens [17]. The antigens are presented in their native state, allowing for the recognition of three-dimensional epitopes by the antibodies. Manipulation of selection conditions also facilitates generation of phage display antibodies with desired specificity. Additionally, phage display is a relatively straightforward and cost effective technique to set up. Selected antibody fragments can be easily produced in large quanti-

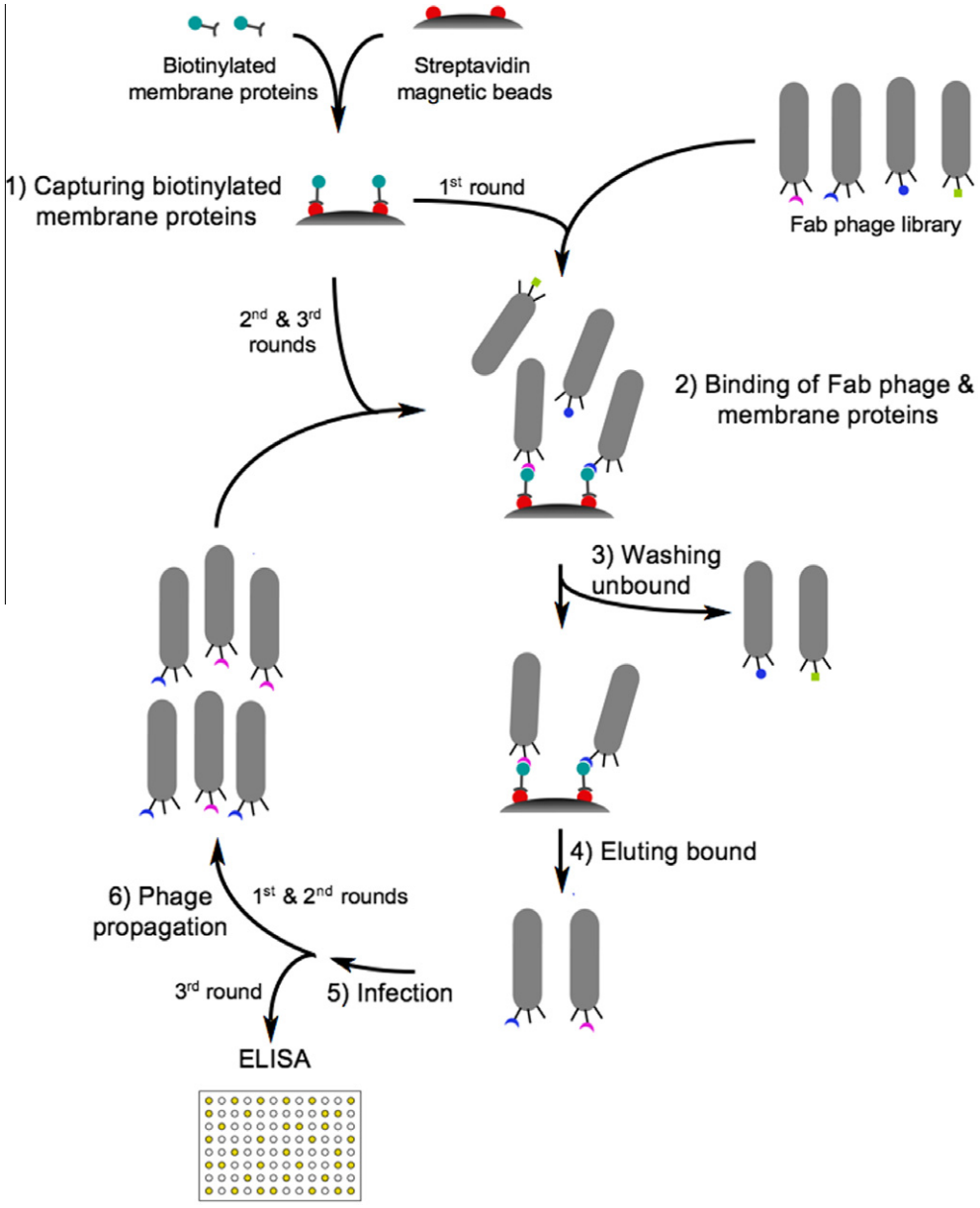

Fig. 1. A panning scheme for membrane proteins. Indirect immobilization is applied where biotinylated membrane proteins are captured by streptavidin magnetic beads. Panning consists of (1) capturing membrane proteins, (2) binding of Fab phage and captured membrane proteins, (3) washing away unbound phage, (4) eluting bound phage, (5) infecting Tg1 cells with the eluted phage and (6) propagating phage for the subsequent round of panning. Three rounds of panning are performed. The 3rd round selected Fabs are subject to ELISA analysis to identify Fabs that bind to target membrane proteins.

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ties. Thus, phage display biopanning is a powerful method for identifying antibody fragments used for crystallographic studies of membrane proteins.

Despite these advantages, biopanning against membrane proteins brings forth some challenges. Antigens need to be soluble throughout the panning process to ensure selection of antibodies recognizing native forms. This can be problematic for some membrane proteins because they are typically less stable than soluble proteins. Nonetheless, membrane protein aggregation can be minimized by proper stabilizing additives [2]. Even well solubilized and stable membrane proteins have reduced solvent exposed surface area compared to soluble proteins of equal size due to micelle formation around the transmembrane segment. This reduces the potential antibody binding surface, reducing the chance of selecting binding partners for highly compact membrane proteins with little solvent exposed surface area. Nevertheless, numerous antibodies against membrane proteins have been successfully generated by phage display biopanning [6,8,14].

Successful identification of a Fab that stabilizes a membrane protein requires a strategy to rank the numerous candidates resulting from a biopanning experiment. A precise determination of the binding energy for each candidate is preferred, but quickly becomes untenable when working with hundreds of putative binders using current technologies. For crystallographic studies, determining the binding energy for each antibody interaction may not be required because any interaction that leads to a rigid and stable complex structure and/or provides an additional crystal contact could aid crystal formation. In addition, protein crystals grow at concentrations typically orders of magnitude higher than K_d values. Therefore, subjecting all selected Fabs for analytical biophysical assays, though preferred, is not necessary. Grouping selected antibodies based on their relative affinities should be sufficient to expedite the identification of higher affinity binders. Rapid and reliable characterization of selected antibodies is an essential step for phage display becoming a more generally applicable procedure.

We have successfully identified Fabs for multiple membrane proteins, including transporters and a cation channel, by phage display panning of a human naïve B cell Fab phage library [16]. Here we describe our general panning procedure for membrane proteins and our procedure to rapidly group the selected Fabs based on their relative affinities, using enzyme linked immunosorbent assay (ELISA) and small quantities of the unpurified Fabs. This procedure greatly speeds the prioritization of candidate binders to membrane proteins and will aid in structure determinations. In addition, these Fabs can prove useful for functional characterization since they can serve as agonists or antagonists of the target protein.

2. Materials and methods

2.1. Fab phage library

The construction of a human naïve B cell Fab phage library (diversity of 4.1×10^{10}) has been of previously described [15,16]. A schematic our phage library construct is shown in Fig. 2. A single lac promoter drives the synthesis of both genes encoding light chain and heavy chain fragments. Two ribosome binding sites initiate the translation of both fragments. Heavy chain is fused to a his tag and a myc tag. The amber codon between the heavy chain gene and phage g3 allows production of heavy chain-g3p fusion protein in an amber suppressor strain such as Tg1 (Lucigen). Through the signal sequences, both chains are transported to the periplasm of *E. coli* where Fabs are formed via disulfide bond formation between light and heavy chains.

2.2. Biotinylation of membrane proteins

Membrane proteins were biotinylated using EZ-Link® NHS-Chromogenic-Biotin (Pierce). Specific buffers were used for each target membrane protein and are referred to as *protein buffer*. Biotinylated membrane proteins were subjected to size exclusion chromatography to remove excess unincorporated chromogenic biotin, reaction byproducts and to ensure that the biotinylated proteins elute at the same fraction as the non-biotinylated proteins. The average extent of biotinylation was calculated by comparing total protein and incorporated chromogenic-biotin concentrations as described in the EZ-Link® NHS-Chromogenic-Biotin instructions. Typically an average of two biotin molecules per protein or protein complex were incorporated. Streptavidin binding capacity was determined empirically for each biotinylated membrane protein by mixing with different volumes of streptavidin magnetic beads (Dynabeads® M-280 Streptavidin, Invitrogen) and analyzing the respective protein content in the supernatant by SDS–PAGE. Throughout our study, all experiments using magnetic beads were carried out in microcentrifuge tubes and the supernatant was separated from the beads, using a magnet.

2.3. Phage display panning

Our overall panning scheme is shown in Fig. 1, which comprises 3 rounds of capturing biotinylated membrane proteins, binding of Fab phage and membrane proteins, washing unbound phage, eluting bound phage, infecting cells with the eluted phage and propagating the eluted phage.

2.3.1. 1st round

(1) *Capturing biotinylated membrane proteins:* Streptavidin magnetic beads were blocked with 2% BSA PBS at room temperature for 1 h. The blocked beads were washed twice with 1 ml of *protein buffer*. Biotinylated membrane proteins (1 ml of 50–100 ng/μl in the 1% BSA *protein buffer*) were incubated with the blocked streptavidin magnetic beads at room temperature for 30 min by constant inversion. The protein–bead mixture was washed 3 times by resuspending the protein immobilized beads in 1 ml of *protein buffer*.

(2) *Binding of Fab phage and membrane proteins:* The Fab phage library (1 ml of 10^{12}–10^{13} phage particles) and the captured membrane proteins were incubated at room temperature by constant inversion for 1 h.

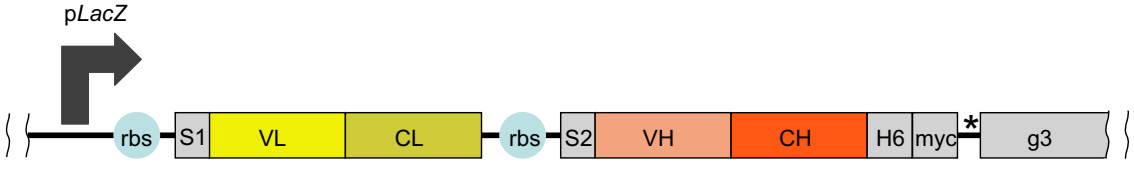

Fig. 2. A schematic of the Fab phage library construct used in this study. A single lac promoter (p*LacZ*) drives the synthesis of both genes encoding light chain (VLCL) and heavy chain (VHCH) fragments. Two ribosome binding sites (rbs) initiate the translation of both fragments. Heavy chain is fused to a his tag (H6) and a myc tag (myc). The amber codon (*) between the heavy chain gene and phage g3 allows production of heavy chain-g3p fusion protein in an amber suppressor strain. Through the signal sequences (S1, pelB and S2, g3 leader sequence), both chains are transported to the periplasm of *E. coli* where Fabs are formed via disulfide bond formation between light and heavy chains.

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(3) *Washing unbound phage:* The phage-bead captured protein mixture was washed 12 times with 1 ml of protein buffer.

(4) *Eluting bound phage:* The bound phage was eluted with 1 ml of 100 mM triethylamine after constant inversion at room temperature for 10 min. The eluted phage was neutralized with 0.5 ml of 1 M Tris HCl, pH 7.4.

(5) *Infection*: The neutralized phage solution was added to 8.5 ml of *E. coli* Tg1 cells (OD_{600} = 0.5–0.8). Infection was done at 37 °C without shaking for 30 min, followed by shaking at 200 rpm for 15 min.

A series of small volumes of the infected cells (0.1–100 μl) were plated on 10 cm 2YT agar plates containing 100 μg/ml ampicillin and 2% glucose in order to determine the output phage titer. The rest of the cell culture was plated on several 15 cm plates to grow cells harboring phagemids. The plates were incubated at 30 °C overnight. To determine the input phage titer, 1 μl of the Fab phage library was diluted by 10^8 fold and used to infect Tg1 cells.

(6) *Phage propagation*: The cells grown on the plates were collected in 36 ml of 2YT containing 100 μg/ml ampicillin and 2% glucose. Density of the collected cells was determined by measuring absorption at 600 nm. The collected cells were inoculated into 50 ml of 2YT containing 100 μg/ml ampicillin and 2% glucose such that the inoculated cell density represented 1500× output phage titer. The volume to be inoculated was calculated by using the following formula,

$$\text{output phage titer} \times 1500/(OD_{600} \times 3 \times 10^8)$$

where 1 of $OD_{600} = 3 \times 10^8$ cells/ml. The culture was grown at 37 °C with shaking at 250 rpm until OD_{600} = 0.5–0.8. M13K07 helper phage was added to 10 ml of the culture at 50-fold excess to the number of cells. Infection was carried out at 37 °C without shaking for 30 min followed by shaking at 200 rpm for 15 min. The cells were harvested by centrifugation at 4000*g* for 10 min. The cell pellet was resuspended in 50 ml of 2YT containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. The resuspended culture was grown at 30 °C with shaking at 250 rpm for 17 h for phage propagation. The phage was precipitated from the 50 ml overnight culture with 10 ml polyethylene glycol (PEG) solution (20% PEG 6000, 2.5 M NaCl). The mixture was incubated on ice for 1 h and the phage pellet was collected by centrifugation at 10,000*g* for 10 min. The phage pellet was resuspended in 1 ml PBS and precipitated once more.

2.3.2. 2nd and 3rd rounds

Panning followed a similar procedure as the 1st round with following changes.

(1) Propagated phage from the 1st and 2nd rounds was pre-incubated with streptavidin beads to eliminate streptavidin binding phage.
(2) Amounts of membrane proteins were reduced to 0.5 ml at a half and a quarter of the 1st round protein concentration in the 2nd and 3rd rounds, respectively.
(3) Fractions of the propagated phage from the previous round were used (100 μl and 10 μl of the 1 ml phage solution from the 1st round for the 2nd round panning and from 2nd round for the 3rd round panning, respectively). The input phage titers typically ranged between 1012 and 1013 phage particles.
(4) The phage–protein mixtures were washed 15 times with 0.5 ml of protein buffer and 20 times with 0.25 ml of protein buffer in the 2nd and 3rd rounds, respectively.
(5) In the 3rd round, the cells infected with the eluted phage were further diluted by 103-fold and various culture volumes (1–100 μl) were plated to obtain well separated colonies on the plates for ELISA analysis.

2.4. Fab preparation for ELISA

ELISA analysis was done with phage free Fabs that leaked into the cell culture media. Colonies (384) of infected cells from the 3rd round were picked to inoculate 150 μl of 2YT containing 100 μg/ml ampicillin and 2% glucose in 96 well plates. The plates were incubated at 37 °C with shaking at 250 rpm overnight. Each overnight culture was used to inoculate 120 μl of 2YT containing 100 μg/ml ampicillin and 0.1% glucose in 96 well plates, using 96 pin replicators. The plates were incubated at 37 °C with shaking at 250 rpm until OD_{600} = 0.6, at which the expression of Fabs were induced with 30 μl of 2YT containing 100 μg/ml ampicillin and 5 mM IPTG. The plates were incubated at 30 °C with shaking at 250 rpm overnight. The plates were centrifuged at 2000*g* for 10 min. Supernatant was used for ELISA.

2.5. ELISA analysis with a single Fab concentration

Wells of Maxisorp plates (Nunc) were coated with 50 μl of streptavidin (5 μg/ml in PBS, Promega) overnight at 4 °C. Wells were washed twice with PBS and blocked with 370 μl of PBS containing 2% BSA for 1 h. Wells were washed 3 times with *protein buffer*. Target membrane protein (50 μl of 10–20 μg/ml in *protein buffer* containing 1% BSA) or *protein buffer* for control experiments was added to each well. Plates were shaken at room temperature for 30 min. Wells were washed 3 times with *protein buffer*. *Protein buffer* (10 μl) containing 5% BSA and excess protein stabilizing components such as a detergent (the final concentration in 50 μl equal to that of *protein buffer*) was added to each well, followed by the addition of 40 μl of Fab supernatant prepared as described in Section 2.4. Plates were shaken at room temperature for 1 h. Wells were washed 3 times with *protein buffer*, followed by the addition of 50 μl of anti-myc antibody conjugated to peroxidase (Roche) in *protein buffer* containing 1% BSA. Plates were shaken at room temperature for 1 h, followed by 3 washes with *protein buffer*. Turbo TMB (50 μl, Pierce) was added to each well. Plates were incubated without shaking for 15–30 min until blue color appears. Reactions were stopped with 15 μl of 2.5 M H_2SO_4. Absorbance was read at 450 nm.

2.6. ELISA analysis with relative Fab concentrations

The procedure was the same as Section 2.5 except serial dilutions of supernatant from Section 2.4 were assayed. The dilutions were made with 2YT media and the undiluted supernatant was considered a relative Fab concentration of 1.

3. Results and discussion

3.1. Indirect immobilization of target membrane proteins improved panning efficiency against membrane proteins

Initially membrane proteins were directly immobilized on a Maxisorp plate for panning trials. Despite previous success when panning with soluble proteins [16,18,19], using direct immobilization, Fabs against our target membrane proteins were not enriched. Potentially direct immobilization may cause a high percentage of protein molecules to denature as a consequence of passive protein adsorption to the plastic surface [17,20]. Such denatured protein molecules may exist in several different conformations and adsorb in non-uniform depositions over the surface of the plate [21]. In any event, the chance of selecting Fabs for the native conformation was reduced by direct immobilization. Because no binding Fabs were identified, we suspect that multiple conformations of the denatured protein molecules and the non-uniform depositions on the plate reduced availability of each potential epitope. This could

have significantly affected panning efficiency against our target membrane protein because it already has reduced potential antibody binding surface. Thus, indirect immobilization was applied, using biotinylated membrane proteins and streptavidin magnetic beads.

Indirect immobilization that uses biotinylated target proteins and subsequent streptavidin capture can maintain protein molecules in their native conformation [21]. This is largely due to the selective labeling of exposed lysines by the biotinylation reaction and the highly specific biotin–streptavidin interaction. All our biotinylated membrane proteins eluted at the same fractions as their respective non-biotinylated proteins in size exclusion chromatography, ensuring biotinylation did not affect protein integrity. In addition, streptavidin is evenly distributed on the beads, ensuring an even distribution of the protein molecules on the beads. Biotin is linked to the protein via a polyethylene glycol spacer, which provides space between the protein molecules and beads and allows the protein molecules to move in solution to a certain extent. All these above factors should increase epitope availability compared to static adsorption to the plastic surface. Consequently, using indirect immobilization and the procedure described in Section 2.3 and Fig. 1, we have successfully identified Fabs for membrane proteins by panning of a human naïve B cell phage library. Our success with indirect immobilization for panning against membrane proteins suggests that panning efficiency for membrane proteins is highly sensitive with regard to how antigens are immobilized and thus how epitopes are displayed.

3.2. Selected Fabs specifically recognized their target protein, not streptavidin

In our ELISA analyses, unpurified Fabs in culture supernatant are assayed for binding, as opposed to in phage-attached formats. This is possible without further modification of the Fab library constructs because the amber codon between the heavy chain and phage gene 3 (g3) allows production of both heavy chain and heavy chain-g3p fusion protein in an amber suppressor strain such as Tg1 (Fig. 2), both of which are transported to the periplasmic space and form Fabs with light chains. A low percentage of these Fabs leaks into media during phage propagation. Because Fab-g3p fusion proteins are anchored in the cell membrane through g3p, only Fabs free of g3p leak into media. The leaked Fabs are relatively pure at this stage and thus could be used directly in our ELISA analyses with no further purification.

ELISA analysis after the 3rd round of panning identifies Fabs that putatively bind to the target protein. Such an analysis does not differentiate among non-specific binding to the well, specific binding to streptavidin and specific binding to the target protein. Hence, Fabs identified by the initial ELISA were subject to the secondary ELISA to ensure that they were not binding to streptavidin or binding non-specifically to the well. Representative data of Fabs against an ABC transporter, AD12, AH11, BC10 and BG12 are shown in Fig. 3. All four Fabs displayed significantly higher ELISA signals in the ABC transporter coated wells than streptavidin coated wells (by more than 5-fold, Fig. 3), indicating specific binding for the ABC transporter.

3.3. Fabs can be grouped based on relative affinities, using ELISA analysis and unpurified Fabs

Recombinant Fabs can be produced easily in *E. coli*, which is one of the benefits of using phage display antibodies. However, expressing and purifying multiple Fabs for each target protein can be laborious. More importantly, producing all Fabs identified by the ELISA analysis with a single concentration is wasteful because some Fabs may display much weaker binding affinity than others. Fabs with higher affinity for their targets are typically more desirable for crystallographic studies although determining the binding energy is not necessary. Moreover, despite sharing sequence similarity [15], each Fab has different physical properties including variable protein expression levels in *E. coli*. For some Fabs that express poorly, much effort is needed to optimize expression to get enough material for biophysical analysis. Therefore it is necessary to prioritize Fabs for production to eliminate unnecessary efforts.

To group Fabs into categories of either higher or lower affinity, ELISA with relative Fab concentrations was performed for Fabs identified initially by ELISA with a single concentration. Various Fab concentrations were prepared by making serial dilutions of supernatant of Fab induced cultures as described in Section 2.4. In this assay, because Fab concentrations were unknown, serial dilutions yielded relative concentrations for each Fab where the undiluted supernatant was considered a relative concentration of 1. Representative data of ABC transporter Fabs, AD12, AH11, BC10 and BG12 are shown in Fig. 4. ELISA signals reached a plateau, hereafter referred to as saturating ELISA signals, as Fab concentrations increased for AD12 or AH11, but not for BC10 or BG12, This suggests the binding interaction between AD12 or AH11 and the ABC transporter saturates. Expression levels of the Fabs that leaked into media and were used in our ELISA analysis were also assessed by immunoblotting. All Fabs were expressed at comparative levels (Fig. 4). These results suggest AD12 and AH11 are tighter binders than BC10 and BG12. This was confirmed by size exclusion chromatography where AD12 and AH11 co-eluted with the ABC transporter, but BC10 and BG12 did not, indicating stable complexes only formed with AD12 and AH11. The correlation between the results of the two assays further supports that ELISA with relative Fab concentrations can separate tight binders from weak binders.

The relative Fab concentration analysis provides information that the single Fab concentration analysis cannot. For example, a lower maximum (saturating) ELISA signal was observed for AD12 than AH11 (Fig. 4) although the ELISA signals reached plateaus for both Fabs. This could be due to reduced availability of the AD12 epitopes among the immobilized ABC transporter molecules.

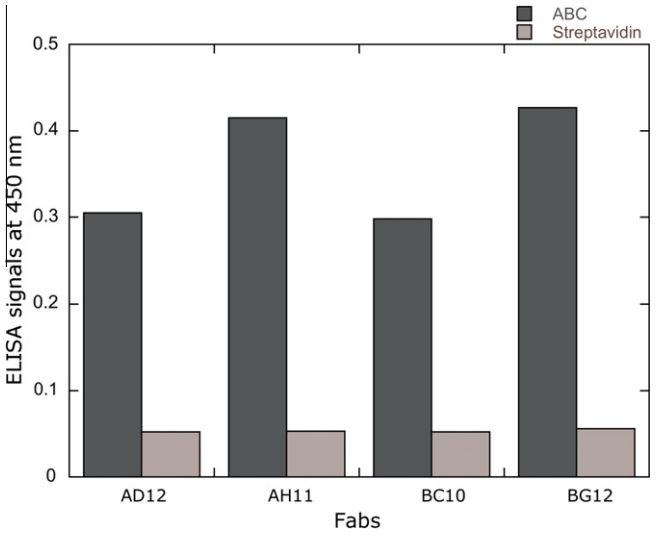

Fig. 3. ELISA with a single Fab concentration against the ABC transporter or streptavidin. Wells of Maxisorp plates were coated with streptavidin, followed by adding either the biotinylated ABC transporter (ABC) or buffer (streptavidin). Supernatant of spun cell cultures, which contains free Fab fragments leaked into the media, was added to the wells for binding. All four Fabs displayed significantly higher ELISA signals for the ABC transporter than streptavidin.

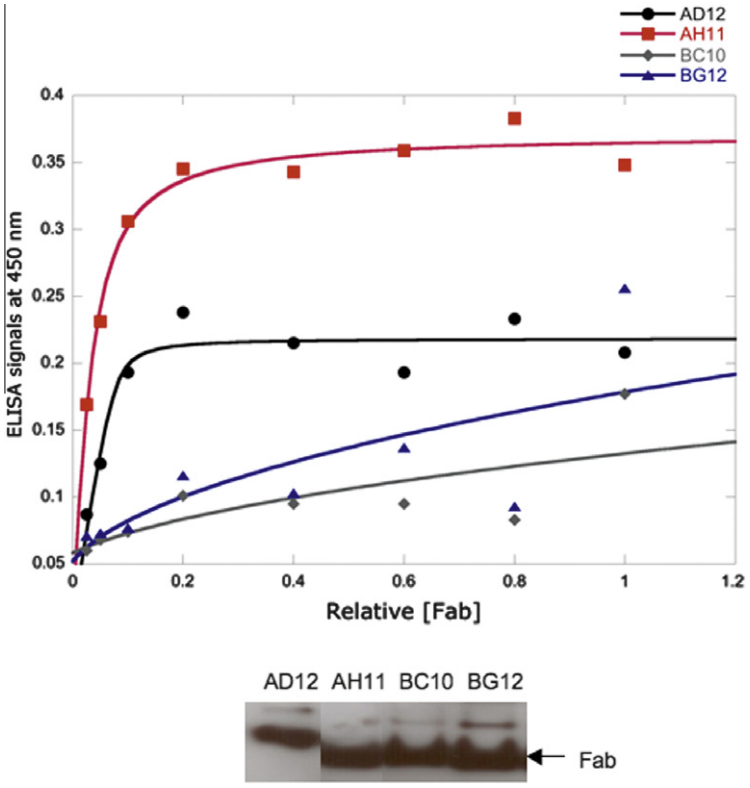

Fig. 4. ELISA with relative Fab concentrations against the ABC transporter. Serial dilutions of supernatant of spun cell cultures were prepared. The undiluted supernatant was considered a relative Fab concentration of 1. AD12 and AH11 displayed saturating signals while BC10 and BG12 did not. Presence of Fabs in the supernatant was assessed by immunoblotting with myc antibody.

The epitope for AD12 may overlap with a biotinylation site or may not be accessible after the ABC transporter is immobilized. Because biotinylation is random, some molecules would have their epitope blocked, resulting in an overall reduction of binding sites within the assay and thus lowering the maximum ELISA signal. Conducting ELISA with a single Fab concentration would not allow differentiation between a saturating signal with a reduced maximum and a non-saturating signal with a higher maximum. The difference between these two cases is clearly shown in this study where signals at the relative concentration of 1 were similar among AD12, BC10 and BG12 despite the fact that ELISA signals saturated for AD12, not for the other two (Fig. 4).

The relative Fab concentration analysis does not ensure the binding interaction between Fabs and native forms of the target protein reaches saturation because Fabs are not purified and the protein is biotinylated and immobilized. However, false positives for binding saturation have not yet been detected. We suspect that a high number of false positives indicate poor panning conditions. For example, a selected Fab that recognizes an epitope only present in a denatured form of the target protein may display saturating binding in ELISA by selectively binding the denatured form, but will not bind a native form. It is also possible that an unpurified Fab sample may contain proteins or other substances that negatively affect Fab binding, resulting in apparent poor binding in ELISA. However, no evidence of this was observed in cases where ELISA with unpurified Fabs and size exclusion chromatography analysis of purified Fabs were compared. Taken together, signal saturation in ELISA with relative Fab concentrations, not raw signal values, is a reliable indicator of Fab binding affinity. This method helps detect false positives in the early stages of a project, and provides an efficient way to characterize Fab binding using small amounts of unpurified Fabs.

4. Conclusion

Despite great advances in structural studies of membrane proteins, many challenges including expression, purification and crystallization still remain. There is no single solution to these challenges, which encompasses all membrane protein studies. However, antibody binding can provide useful reagents to address some of these challenges. Here we show that phage display biopanning followed by ELISA analysis with relative Fab concentrations is a useful method for easy, inexpensive and quick Fab production for membrane proteins. The characterization of these binding partners as agonists, antagonists and facilitators of structural determination are currently underway.

Acknowledgments

We thank Robert Edwards, Robert Tampé, and David Julius for supplying proteins. We also thank Duane Smith for critical reading of the manuscript and Cheng-I Wang for helpful discussions. This work was supported by NIH/NIGMS grant P50 GM73210.

References

[1] R. Grisshammer, C.G. Tate, Q. Rev. Biophys. 28 (1995) 315–422.
[2] J.U. Bowie, Curr. Opin. Struct. Biol. 11 (2001) 397–402.
[3] G. Sennhauser, M.G. Grutter, Structure 16 (2008) 1443–1453.
[4] S. Koide, Curr. Opin. Struct. Biol. 19 (2009) 449–457.
[5] Y. Zhou, J.H. Morais-Cabral, A. Kaufman, R. MacKinnon, Nature 414 (2001) 43–48.
[6] S. Uysal, V. Vasquez, V. Tereshko, K. Esaki, F.A. Fellouse, S.S. Sidhu, S. Koide, E. Perozo, A. Kossiakoff, Proc. Natl. Acad. Sci. USA 106 (2009) 6644–6649.
[7] Y. Fang, H. Jayaram, T. Shane, L. Kolmakova-Partensky, F. Wu, C. Williams, Y. Xiong, C. Miller, Nature 460 (2009) 1040–1043.
[8] S.G. Rasmussen, H.J. Choi, J.J. Fung, E. Pardon, P. Casarosa, P.S. Chae, B.T. Devree, D.M. Rosenbaum, F.S. Thian, T.S. Kobilka, A. Schnapp, I. Konetzki, R.K. Sunahara,

S.H. Gellman, A. Pautsch, J. Steyaert, W.I. Weis, B.K. Kobilka, Nature 469 (2011) 175–180.
[9] J. Silverman, Q. Liu, A. Bakker, W. To, A. Duguay, B.M. Alba, R. Smith, A. Rivas, P. Li, H. Le, E. Whitehorn, K.W. Moore, C. Swimmer, V. Perlroth, M. Vogt, J. Kolkman, W.P. Stemmer, Nat. Biotechnol. 23 (2005) 1556–1561.
[10] H.M. Geysen, J.A. Tainer, S.J. Rodda, T.J. Mason, H. Alexander, E.D. Getzoff, R.A. Lerner, Science 235 (1987) 1184–1190.
[11] J.A. Tainer, E.D. Getzoff, H. Alexander, R.A. Houghten, A.J. Olson, R.A. Lerner, W.A. Hendrickson, Nature 312 (1984) 127–134.
[12] C.J. Farady, P.F. Egea, E.L. Schneider, M.R. Darragh, C.S. Craik, J. Mol. Biol. 380 (2008) 351–360.
[13] D. Rothlisberger, A. Honegger, A. Pluckthun, J. Mol. Biol. 347 (2005) 773–789.
[14] D. Rothlisberger, K.M. Pos, A. Pluckthun, FEBS Lett. 564 (2004) 340–348.
[15] H.J. de Haard, N. van Neer, A. Reurs, S.E. Hufton, R.C. Roovers, P. Henderikx, A.P. de Bruine, J.W. Arends, H.R. Hoogenboom, J. Biol. Chem. 274 (1999) 18218–18230.
[16] S. Duriseti, D.H. Goetz, D.R. Hostetter, A.M. LeBeau, Y. Wei, C.S. Craik, J. Biol. Chem. 285 (2010) 26878–26888.
[17] W.G. Willats, Plant Mol. Biol. 50 (2002) 837–854.
[18] J. Sun, J. Pons, C.S. Craik, Biochemistry 42 (2003) 892–900.
[19] G. Winter, A.D. Griffiths, R.E. Hawkins, H.R. Hoogenboom, Annu. Rev. Immunol. 12 (1994) 433–455.
[20] G. Houen, C. Koch, J. Immunol. Meth. 200 (1997) 99–105.
[21] J. Davies, A.C. Dawkes, A.G. Haymes, C.J. Roberts, R.F. Sunderland, M.J. Wilkins, M.C. Davies, S.J. Tendler, D.E. Jackson, J.C. Edwards, J. Immunol. Meth. 167 (1994) 263–269.

Please cite this article in press as: J. Kim et al., Methods (2011), doi:10.1016/j.ymeth.2011.09.012