Efficient production of antibodies against a mammalian integral membrane protein by phage display

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The application of phage display technology to mammalian proteins with multiple transmembrane regions has had limited success due to the difficulty in generating these proteins in sufficient amounts and purity. We report here a method that can be easily and generally applied to sorting of phage display libraries with multispan protein targets solubilized in detergent. A key feature of this approach is the production of biotinylated multispan proteins in virions of a baculovirus vector that allows library panning without prior purification of the target protein. We obtained Fab fragments from a naïve synthetic antibody phage library that, when engineered into full-length immunoglobulin (Ig)G, specifically bind cells expressing claudin-1, a protein with four transmembrane regions that is used as an entry co-receptor by the hepatitis C virus (HCV). Affinity-matured variants of one of these antibodies efficiently inhibited HCV infection. The use of baculovirus particles as a source of mammalian multispan protein facilitates the application of phage display to this difficult class of proteins.

Keywords: phage display/membrane protein/antibody/ claudin/baculovirus

Introduction

Phage display is widely used to obtain ligands for applications ranging from structural biology to therapeutic use and is particularly well-suited when there is a need for high-throughput or the ability to obtain non-antibody ligands. However, phage display has had limited success in obtaining ligands to mammalian proteins with multiple transmembrane regions, a major class of current and potential drug targets (Hopkins and Groom, 2002; Russ and Lampel, 2005). The two main approaches that have been used for application of phage display to membrane proteins are cell-based selection and selection with purified membrane proteins either in solution or in reconstituted lipid bilayers. Selection of binders to multispan membrane protein targets overexpressed on mammalian cells often requires complex steps of negative selection to avoid the unwanted specificities that are usually dominant (Sawyer et al., 1997; Giordano et al., 2001; Souriau et al., 2004). The use of purified mammalian multispan protein for library sorting is more effective at obtaining antibodies from phage display libraries than panning on whole cells but it is usually hampered by the need for time-consuming optimization of conditions for expression, purification, and sometimes reconstitution of these proteins in lipid bilayers (Jespersen et al., 2000; Mirzabekov et al., 2000; Milovnik et al., 2009). As a consequence, reports of application of phage display with solubilized multispan proteins are limited to those that have been optimized for expression and stability for structural studies (Milovnik et al., 2009; Uysal et al., 2009). A rapid and simple method enabling phage selection against multispan transmembrane proteins would facilitate the identification of novel antibody fragments for structural studies or therapeutic development.

The selection of phage display libraries with a variety of multispan proteins that have not been extensively characterized would ideally require limited amounts of protein and little if any protein purification to avoid protein loss and stability issues. The baculovirus expression system is widely used for the expression of mammalian membrane proteins and requires little time from cloning the gene of interest in expression vectors to protein expression. Membrane proteins are traditionally harvested with cells, often requiring purification of the active recombinant multispan protein in several steps. However, a significant portion of the expressed membrane proteins is also incorporated into virions from the baculovirus vector during budding at the cell surface, where the virus acquires its envelope membrane from the host cell membrane (Loisel et al., 1997; Masuda et al., 2003; Hayashi et al., 2004). We reasoned that incorporation of membrane proteins by virions could be used to enrich membrane proteins to levels sufficient for sorting of phage display libraries without the need for protein purification for three main reasons. First, a major fraction of contaminants is associated with the capsids of viral particles that can be easily and rapidly removed from detergent-solubilized samples by a simple centrifugation step. Second, virions could be used to separate membrane proteins biotinylated in vivo from other physiologically biotinylated proteins without the need for purification of membrane and organelle fractions, allowing immediate use of protein without prior chemical or enzymatic biotinylation and free biotin removal steps.

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multispan membrane protein in the virion fraction is enriched with fully processed membrane proteins relative to cell membrane fractions which may contain relatively large amounts of incompletely processed and inactive recombinant membrane protein (Shorr et al., 1982; Loisel et al., 1997; Hayashi et al., 2004).

Based on the principles outlined above, we devised a method for selecting antibody fragments specific for mammalian multispan membrane proteins from phage display libraries using recombinant multispan protein incorporated in baculovirus virions. We tested our approach by generating and affinity maturing Fab fragments to claudin-1 (CLDN1), a tight junction protein that is used by the hepatitis C virus (HCV) as an entry co-receptor (Evans et al., 2007). Antibodies based on the Fab fragments that we selected and affinity matured using unpurified CLDN1 from baculovirus particles bind native CLDN1 and inhibit HCV infection of human cells.

Materials and methods

Production of baculovirus particles incorporating membrane proteins

A baculovirus transfer vector based on the pFastbac Dual vector (Invitrogen) was constructed for expression of biotinylated membrane protein. The vector includes a cassette with the baculovirus gp64 promoter followed by a multiple cloning site and a baculovirus vp39 promoter driving transcription from the Escherichia coli birA gene in opposite direction from the gp64 promoter. A cytomegalovirus promoter driving low-level expression of the green fluorescent protein (GFP) was located after the birA sequence to facilitate virus titration. The claudin genes were cloned in the multiple cloning site fused to a sequence encoding a linker and AviTag sequence (GSGGLNDIFEAQKIEWH) (Beckett et al., 1999) at the 3' end. Baculovirus was produced and propagated up to second passage following the protocols used for the bac-to-bac system (Invitrogen). Viruses incorporating claudins were produced by infecting 1.8 × 10^9 SF9 cells in 600 ml of ESF media (Expression Systems) with enough baculovirus to infect >95% of cells by 24 h as assessed by GFP expression. Cultures were harvested 40 h post-infection, cells were pelleted by centrifugation at 5000 × g for 10 min, and virus in the supernatant was pelleted by centrifugation at 25 000 × g for 4 h at 4°C. Pellets with virus were resuspended in 8 ml of PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.4), layered on top of a 4 ml 35% (w/v) sucrose cushion and centrifuged in an SW40Ti rotor (Beckman) at 30 000 rpm for 1 h at 4°C. The supernatant with debris was discarded, the virus pellet gently rinsed once with PBS, resuspended in 1.2 ml of PBS with protease inhibitor cocktail (Roche) and stored at 4°C for up to 2 weeks. Expression of protein was monitored by western blotting of whole baculovirus particles with a streptavidin-horseradish peroxidase conjugate (Pierce). Yield of CLDN1 in baculovirus particles was estimated by purification of a His-tagged form of the protein from baculovirus particles. Briefly, purified baculovirus particles containing His-tagged CLDN1 were lysed with 1% dodecylmaltoside (DDM) and CLDN1 was purified by nickel-nitriloacetic acid affinity chromatography in the presence of DDM. Eluted protein in imidazole containing 0.1% DDM was dialyzed against PBS containing 0.1% DDM. Concentration of CLDN1, estimated to be >90% pure by coomassie blue staining, was determined spectrophotometrically using an extinction coefficient at 280 nm of 40 170 M^-1 cm^-1.

Preparation of solubilized membrane protein for sorting

For the first round of sorting 200 μl of baculovirus suspension containing CLDN1 were mixed with 200 μl of PBS containing 0.5% BSA (PBS-B) and 100 μl of 5% DDM (Anatrace) in PBS. For the second through fifth rounds of sorting 80 μl of baculovirus suspension was mixed with 80 μl of PBS-B and 40 μl of 5% DDM in PBS. The baculovirus lysates were incubated on ice for 30 min and centrifuged at 16 000 × g for 30 min at 4°C to remove virus capsids and other insoluble debris. The clear supernatants containing solubilized protein were transferred to a new tube and used for sorting immediately.

Phage display library

The synthetic Fab phage display library (Library D, 5 × 10^10 colony forming units, 1.2 × 10^9 independent clones) used for sorting with CLDN1 was previously described (Fellouse et al., 2007). Briefly, this library has surface positions in the heavy chain CDR 1 and 2 and light chain CDR 3 randomized as Tyr or Ser and buried positions randomized as small or large hydrophobic residues. The heavy chain CDR 3 is randomized with a codon mixture enriched in Tyr, Ser and Gly but allowing any amino acid except Cys. The light chain CDR 3 can have one or two Tyr/Ser randomized codons inserted after Kabat position 94 (Kabat et al., 1991), resulting in three different length variations. The light chain CDR 3 can also have the non-randomized vector sequence HYTTTP between Kabat positions 91 and 96. The heavy chain CDR 3 varies in length from 10 to 21 residues between Kabat positions 95 and 102, included.

First round of phage library sorting

The phage display library in 500 μl of PBS-B and 1% DDM was mixed with 500 μl of cleared baculovirus lysate and incubated for 2 h at 4°C. The mixture was added to 1.5 mg of Dynabeads MyOne Streptavidin T1 (Invitrogen) and incubated for 10 min at room temperature under constant gentle rotation. The beads were washed six times with 1 ml of PBS containing 0.1% DDM using a Dynal MPC-S magnet (Invitrogen). Phage was eluted by resuspending the beads in 400 μl of 0.1 N HCl for 10 min under constant gentle rotation. The beads were pelleted with a magnet and the eluted phage was transferred to a new tube and neutralized with 100 μl of 1 M Tris, pH 11. Escherichia coli XL1-Blue (Stratagene) was infected with the eluted phage and rescued overnight with M13KO7 helper phage (New England Biolabs). The rescued phage was precipitated with polyethylene glycol, resuspended in 1 ml of PBS-0.5% BSA and used in the next round of sorting.

Second to fourth rounds of library sorting

Ten wells of a 96-well Maxisorp plate were coated overnight with 100 μl of 5 μg ml^-1 neutravidin (Pierce) for the second and fourth round of sorting or streptavidin (Pierce) for the third round of sorting, and blocked with PBS-B. Phage amplified from the first through third rounds of sorting in

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200 μl of PBS-B containing 1% DDM were mixed with 200 μl of baculovirus lysate and incubated for 2 h at 4°C. The mixture was brought up to 1 ml of PBS-B containing 1% DDM, 100 μl added to each of the coated wells and incubated under agitation for 15 min at room temperature. The wells were washed 10 times with PBS containing 0.1% DDM and bound phages in each well were eluted with 100 μl of 0.1 N HCl for 10 min under agitation, pooled and neutralized with 250 μl of 1 M Tris, pH 11. Eluted phages were amplified as described above. Phage clones were screened by a ‘phage-ELISA (enzyme-linked immunosorbent assay)’ procedure as previously described (Deshayes et al., 2002) with the following modifications: culture supernatants containing phage were diluted 1:5 in PBS-0.1% DDM before being mixed at a 1:1 ratio with solubilized multispan protein (equivalent to 1 μl of concentrated baculovirus stock), incubated for 1 h at 4°C and captured on neutravidin-coated plates for 10 min under agitation. Bound phage was detected with a horseradish peroxidase/anti-M13 antibody conjugate (Pharmacia) diluted in PBS-0.1% DDM. All washes were done with PBS-0.05% DDM.

**Affinity maturation of Fab 5.16**

Initial affinity maturation of clone 5.16 was achieved by randomization of heavy chain CDR 1 and 2 and heavy and light chain CDR 3 in two different libraries as previously described (Bostrom et al., 2009). A ‘stop template’ was produced by introducing stop codons in the CDRs to be randomized by oligonucleotide-directed mutagenesis. Positions predicted to be exposed and most buried in heavy chain CDR 1 and 2 and light chain CDR 3 were randomized by oligonucleotide-directed mutagenesis allowing either the amino acid occurring in clone 5.16 or amino acids common in natural human antibodies for each position. Two oligonucleotides were combined to randomize each of the CDR regions: 5’-TATTACTGTACAAATMTDMCDVTNHTCTT (TWT/YKG)ACGTTCGACAGGGTAC-3’ (light chain CDR 3, variable positions in the two oligonucleotides in parenthesis), 5’-GCACTCTTGGCTTCAACWTWTTVDWM WMC(KMT/KGG)ATCCACTGGGGTCGTCAG-3’ (heavy chain CDR 1) and 5’-GGCTTGAAGGGGTTCAGA(DGG/DHT)ATCDMTYCTDMTRRCGTDMTCCDMTATGC CGATAGGCTCA (heavy chain CDR 2). Kabat positions 95 to 101b in heavy chain CDR 3 were ‘soft randomized’ by allowing 80% of the wild-type nucleotide and 10% of each of the other three nucleotides for position. This results in each position encoding about 50% of the 5.16 clone amino acid and 50% of all other amino acids in each position. Heavy chain CDR 3 residues 100c and 101d were randomized as Gly/Ala and Met/Ile/Ph/Leu, respectively. The oligonucleotide used to randomize this region was 5’-GTCTATATTGTGCCTCGCGCCATGAATAGGTGGTTGCA(DGG/DHT)ATCDMTYCTDMTRRCGTDMTCCDMTATGC CGATAGGCTCA (heavy chain CDR 2). Kabat positions 95 to 101b in heavy chain CDR 3 were ‘soft randomized’ by allowing 80% of the wild-type nucleotide and 10% of each of the other three nucleotides for position. This results in each position encoding about 50% of the 5.16 clone amino acid and 50% of all other amino acids in each position. Heavy chain CDR 3 residues 100c and 101d were randomized as Gly/Ala and Met/Ile/Ph/Leu, respectively. The oligonucleotide used to randomize this region was 5’-GTCTATATTGTGCCTCGCGCGCAGGA-3’ (‘soft randomized’ positions shown in lower case). The heavy chain CDR 3 of clone 5.16v5 was randomized in a similar manner, using its sequence to determine the ‘soft randomization’. Library construction was done as previously described (Sidhu et al., 2000). Sorting of affinity-maturation phage display libraries was done as described above for the second to fourth rounds of panning except that phage was incubated with antigen for only 10 min at room temperature before capturing with immobilized neutravidin.

**Production of immunoglobulin G**

The regions encoding antibody variable regions in phagemid clones were subcloned into mammalian immunoglobulin (Ig)G1 and kappa chain expression vectors. Immunoglobulin G was obtained by cotransfection of plasmids encoding the heavy and light chains into 293T cells with Lipofectamine2000 (Invitrogen). Transfection medium was replaced with DMEM containing 5% ultra-low IgG fetal bovine serum (Gibco) 16 h post-transfection and supernatants were harvested 3 days post-transfection. Human IgG was purified from supernatants by protein A chromatography.

**Production and purification of Fab fragments**

Antibody Fab fragments were produced by introducing a stop codon immediately after the codon encoding threonine in Kabat position number 225 of CH1 into phage-display phagemids by oligonucleotide-directed mutagenesis. Escherichia coli 62A7 cells were transformed and inoculated in C.R.A.P. phosphate-limiting media (Simmons et al., 2002) containing 50 μg ml⁻¹ carbenicillin and grown for 24 h at 30°C with shaking. Fabs were purified directly from culture supernatants by Protein A affinity chromatography on ProSepA resin (Millipore). The eluted Fabs were dialyzed overnight against PBS and their monomeric state confirmed by size-exclusion chromatography. Protein concentrations were determined spectrophotometrically using an extinction coefficient at 280 nm of 66 800 M⁻¹ cm⁻¹.

**Binding kinetics measurements**

Kinetic binding measurements were performed on an Octet Red instrument (FortBio, Menlo Park, CA, USA). All washes, dilutions and measurements were performed in Octet buffer (0.2% DDM–PBS) with the plate shaking at 1000 rpm. Streptavidin biosensors were equilibrated in Octet buffer for 10 min and then loaded with biotinylated CLDN1 (from viral lysate, diluted 1:8 in Octet Buffer) for 5 min and washed for 10 min. For the association phase, the ligand-coated streptavidin tips were immersed in anti-CLDN1 Fab fragments for 10 min (eight serial two-fold dilutions, starting at 500 or 50 nM). Dissociation of the Fab-CLDN1 complex was measured in wells containing Octet buffer alone for 600 s. $K_D$, $K_s$ and $K_d$ were determined with Octet evaluation software v6.3 using a 1:1 binding model with global fitting.

**Expression of CLDN1 and CD20 in human cells**

Human 293 cells stably expressing human CLDN1 and CD20 were obtained by subcloning cDNAs into the pcDNA5/FRT (Invitrogen) expression vector and co-transfecting 293 Flp-In cells (Invitrogen) with the expression vectors and plasmid pOG44 (Invitrogen). Stably transfected cells were selected and maintained with Zeocin and used uncloned. Transient expression of human and mouse CLDN1 and mouse CD20 was achieved by transfecting mammalian expression vectors expressing cDNAs into 293T cells with Lipofectamine2000. Transfected cells were harvested 24 h post-transfection for fluorescence-activated cell sorting (FACS).

**FACS analysis**

HEK293 cells expressing CLDN1 or CD20 were resuspended in DMEM-10% fetal bovine serum at $5 \times 10^6$ cells ml⁻¹ and dispensed in U-bottom 96-well plates at 100 μl/well. An
equal volume of purified IgG diluted in PBS (150 mM NaCl, 10 mM sodium phosphate pH 7.4) was added to the cells and incubated for 1 h at 4°C. Cells were then washed three times with ice-cold PBS, incubated with an anti-human IgG Fc-R-Phycocerythin conjugate (Jackson Immunoresearch) or an anti-human IgG-Alexa488 (Molecular Probes) conjugate for 30 min at 4°C, washed twice in ice-cold PBS and fixed in PBS with 0.1% paraformaldehyde. Cells were analyzed in a FACSCalibur flow cytometer (BD Biosciences) with a high-throughput sampler.

**Generation of HCVcc stocks**

Jc1 virus stocks were generated by infecting Huh-7.5 cells as described previously (Zhong et al., 2005). Huh-7.5 cells were obtained from Apath LLC under license and cultured in complete Dulbecco’s modified Eagle’s medium (cDMEM; supplemented with 10% fetal calf serum, 100 U ml⁻¹ penicillin, 100 mg l⁻¹ streptomycin, 2 mM L-glutamine and 0.1 mM non-essential amino acids). Genotype 2a Jc1 was chemically synthesized using sequences from the Pubmed database database (genotype 2a JFH-1—GenBank accession number AB047639; genotype 2a J6CF—GenBank accession number AF177036) based on the chimeric strategy published previously (Pietschmann et al., 2006) and cloned into pUC19 plasmid (pUC Jc1). Plasmids were linearized with Xbal and in vitro transcribed RNA was generated using Megascript kit (Ambion) as per the manufacturer’s instructions. Hepatitis C virus RNA was transfected into Huh-7.5 cells by electroporation as described previously (Zhong et al., 2005; Kapadia et al., 2007). Supernatants from Jc1 RNA-transfected cells were harvested starting from Day 3, tittered and pooled to generate laboratory stocks.

**Generation of HCVpp stocks**

HCVpp stocks were generated as previously described previously (Zhong et al., 2005) with changes. Briefly, HEK 293T cells were co-transfected with the Delta 8.9 plasmid containing gag-pol, the CMV-Luc-DsRed lentiviral transfer construct, and a plasmid expressing HCV E1E2 sequences from genotype 2a (J6CF) using Lipofectamine 2000, as per the manufacturer’s instructions. Forty-eight hours following transfection, the medium containing HCVpp was collected, clarified, filtered through 0.45-μm pore-sized membranes and used for infection of Huh-7.5 cells.

**Neutralization assays**

Multiplicity of infection for Jc1 HCVcc was calculated based on virus titer as measured by TCID₅₀ calculations (Lindenbach et al., 2005). HCVpp stocks were normalized for luciferase activity before infecting cells. Neutralization assays using HCVpp or HCV infectious cell culture (HCVcc) viruses were performed in 96-well plates (Costar). Briefly, 5 × 10⁵ Huh-7.5 cells were seeded in a 96-well plate 16 h prior to neutralization assay. The following day, three-fold dilutions of antibody were incubated with naïve Huh-7.5 cells for 1 h at 37°C, followed by the addition of HCVcc or HCVpp for an additional 4 h (Zhong et al., 2005). Unbound virus was removed by replacing with fresh cDMEM and the cells were maintained in culture for 3 days before measuring HCV infection. HCVpp infection was determined using the Luciferase Assay System (Promega), as per the manufacturer’s instructions. In the initial screen of anti-CLDN1 antibodies, HCVcc infection was measured by staining of fixed cells with an anti-HCV core antibody (clone C7–50, Thermo Scientific) followed by an anti-mouse IgG-Alexa 555 conjugate (Invitrogen) and quantitating infected cells using high-throughput fluorescence microscopy. The effect of subsequent affinity-matured variants on HCVcc infection was determined by a more sensitive real-time quantitative PCR assay. Briefly, total RNA was harvested using the SV 96 Total RNA Isolation System (Promega), as per the manufacturer’s instructions. cDNA synthesis and real-time PCR was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems), respectively. Hepatitis C virus infection was measured by real-time PCR to detect HCV and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels using multiplex PCR. The probe sequence for HCV was 6-FAM-TATGAGTGTCGTACAGCCTC-MG BNFQ. Hepatitis C virus primer sequences were: (sense) 5’-CTTCACGCAGAAACGCCTA-3’ and (antisense) 5’-CA AGGGCCCTATACGGCAGT-3’. The probe sequence for human GAPDH was VIC-ATGACCCCTTTCTTGACC TC-MGBNFQ and primer sequences were: (sense) 5’-GAA GGTTAAGGTTGGAGTC-3’ and (antisense) 5’-GAAGA TGTTGATGGGATTT-3’.

**Results**

Budded baculovirus particles are produced naturally during expression of recombinant proteins in insect cells infected with recombinant baculoviruses. To obtain baculovirus particles displaying biotinylated multispan protein, the membrane proteins are expressed in Sf9 insect cells using a baculovirus vector expressing the membrane protein fused to an Avitag sequence in the intracellular amino- or carboxy-terminal regions of the protein (Fig. 1). The Avitag is biotinylated in the Sf9 cells by the E.coli biotin ligase BirA, expressed from the same baculovirus vector. Baculovirus particles displaying membrane protein on the envelope membrane are semi-purified and concentrated by differential centrifugation and ultracentrifugation through a sucrose cushion.

For phage selection, the multispan protein in the concentrated viral particles is solubilized in detergent immediately prior to use in selection and viral capsids and insoluble debris are removed by a rapid centrifugation step. The solubilized membrane protein is directly mixed and incubated with the phage display library and then captured with streptavidin or neutravidin and washed in the presence of detergent. Thus, purification of protein from the enriched crude sample occurs in one step simultaneously with phage library sorting (Fig. 1). Furthermore, the limited time that the protein is in the presence of detergent during lysis and incubation with the library should minimize the impact of instability of multispan proteins solubilized in detergent.

**Selection and screening of phage antibodies binding CLDN1**

The conditions to purify CLDN1 solubilized in detergent in a native conformation have not been established. Furthermore, there are no biochemical assays to estimate the activity of CLDN1 in solution or upon reconstitution into lipid bilayers that would help determine the optimal detergent for solubilization. Therefore, we solublized CLDN1 with the detergent DDM, which is widely used to solubilize
multispan proteins in their native conformation. All steps of phage selection except phage elution were performed in the presence of DDM.

An important parameter in phage display is the concentration of protein used for selection. The yield of CLDN1 in the virion fraction, assessed by protein purification experiments, was 0.1 μg (4 pmol) per ml of culture, similar to the yield of active β2-adrenergic receptor in baculovirus particles (Loisel et al., 1997). Based on these experiments, the concentration of freshly solubilized CLDN1 used in phage selection was estimated to be about 400 nM. The Fab phage display library was selected in one round of solution sorting followed by three rounds of panning, using freshly solubilized CLDN1 for each round. Enrichment of phage binding CLDN1, as assessed by titration of output phage from wells containing CLDN1 or a control multispan protein, was 1.7, 50 and 800-fold in Rounds 2–4. A set of 95 phage clones eluted from Round 4 was screened by a phage ELISA assay. All 95 clones tested bound specifically to solubilized CLDN1 in a phage ELISA assay. Sequencing of these 95 specific binders showed 18 unique clones, with individual clones repeated between 1 and 23 times in the set (Fig. 2). Robustness of selection was determined by sequencing 96 random clones from Rounds 2 and 3 of panning. Sixteen of the positive clones from Round 4 were already present in the 96 clones from the early rounds of panning indicating a rapid enrichment for specific clones with the selection method (not shown).

Inserts of phage clones were subcloned into human IgG1 expression vectors and transiently expressed in 293T cells to produce full-length antibodies derived from the selected Fab fragments. Seventeen of the 18 antibodies in transfected cell supernatants specifically bound to DDM-solubilized CLDN1 in ELISA (not shown). Binding of antibodies to native CLDN1 was tested by flow cytometry using purified IgG from 10 of the clones with the strongest ELISA signal. Six of these antibodies specifically bound CLDN1-expressing cells (Fig. 3A), with antibodies 5.13 and 5.16 showing the strongest binding to native CLDN1 in titration experiments (Fig. 3B). Antibodies 5.3, 5.13 and 5.16 also bound cells transiently expressing mouse CLDN1 but not to cells expressing mouse CD20 used as negative control (Fig. 4).

To determine if any of these antibodies inhibit HCV entry, the antibodies were screened for inhibition of HCV infection using cell culture HCV (HCVcc). Huh-7.5 human hepatocarcinoma cells were incubated with serial dilutions of the

Fig. 1. Outline of the use of multispan membrane proteins from baculovirus (BV) particles to sort phage display libraries. See the main text and Methods section for full description. This figure shows the procedure up to the first round of sorting where streptavidin-coated paramagnetic beads (SA) are used to capture the biotinylated multispan protein. In rounds two and above of sorting either beads or ELISA plates coated with streptavidin or neutravidin are used for capture of biotinylated multispan protein.
Antibodies followed by inoculation with HCVcc derived from genotype 2a. Antibody 5.16 inhibited HCV infection with an IC50 of 120 nM and almost completely inhibited virus infection at an antibody concentration of 1 μM. No significant inhibition of HCV infection was observed with the other antibodies. Since CLDN6 and CLDN9 have also recently been demonstrated to act as co-receptors for HCV entry (Zheng et al., 2007; Meertens et al., 2008), we tested cross-reactivity of antibody 5.16 to a panel of other claudins in ELISA using solubilized protein from baculovirus particles. Antibody 5.16 bound to CLDN1 but did not cross-react with other claudins we tested, including CLDN6 and CLDN9 (Fig. 5), indicating that HCV neutralization is due to specific blocking of CLDN1.

Affinity maturation of antibody 5.16

Affinity maturation of antibodies against multispan proteins is also hampered by the lack of a platform allowing the straightforward application of phage display to these proteins. We tested whether the phage display approach using a multispan protein derived from baculovirus particles could also be applied to affinity maturation of antibodies against these proteins. Antibody 5.16 was affinity matured by re-randomizing the heavy chain CDR1 and 2 or the light and heavy chain CDR3 in two separate libraries and selecting binders using the same method that was used for selection of naïve phage libraries. Selected clones were purified as Fab fragments from E.coli and kinetics of binding to CLDN1 determined by reflectometric interference spectroscopy (RIS) in an Octet Red apparatus using freshly solubilized biotinylated CLDN1 captured on streptavidin-coated sensor tips. Improvements in the affinity of binding of selected Fabs to solubilized CLDN1 were confirmed for three of the purified Fab fragments (Table I, Figs. 2 and 6). Most of the improvements were due to increased on-rates of binding, with an overall affinity improvement of up to 10-fold. Antibody 5.16v3, which had the best affinity of the three characterized affinity-matured variants, acquired a potential N-linked glycosylation site in heavy chain CDR1. Although the potential glycosylation site does not seem to impact the affinity of this antibody when expressed in mammalian cells (Table I) we did not pursue further affinity improvements based on 5.16v3. Instead, further affinity maturation was attempted by combining the mutations from the other two clones, 5.16v2 and 5.16v4, to obtain clone 5.16v5 and performing one additional round of affinity maturation by re-randomizing the heavy chain CDR3. Clone 5.16v6 was identified which had an affinity improvement of about 20-fold relative to the
parental antibody 5.16 with most of the improvement due to an increase in on-rate of binding. All the affinity-matured variants bound to human CLDN1 on the surface of cells (Fig. 4). A bimodal profile was observed for binding of antibody 5.16 and its affinity variants to cells transiently expressing human CLDN1. The main reason for this bimodal profile is likely a heterogeneous CLDN1 expression in the transiently transfected cell population as all the 5.16 variants bound cells expressing CLDN1 stably with a single-peak profile (Fig. 3 and not shown). In addition, all the affinity-matured variants had an increased binding intensity to cells expressing mouse CLDN1 relative to the parental antibody 5.16 in FACS experiments (Fig. 4) indicating an improved affinity of antibodies for the murine protein. All the affinity-matured antibody variants retained specificity for CLDN1 in ELISA assays with solubilized protein except 5.16v6, which bound to CLDN6 and CLDN9 very weakly in some experiments.

CLDN1 expressed in insect cells and solubilized in DDM may have subtle but significant conformational differences relative to the native protein. Therefore, it is important to determine whether the improved binding of phage-derived clones is observed with native CLDN1 and not solely with the solubilized protein. The improved affinity of matured variants to native CLDN1 expressed in a stably transfected 293 cell line was tested in competition binding assays with radiolabeled IgG or Fab and homologous or heterologous IgG or Fab. The improvement in apparent affinity of the five affinity-matured variants against native CLDN1 were similar to the affinity improvements against solubilized CLDN1 as determined by kinetic analysis in RIS assays (Table I), suggesting that CLDN1 solubilized in DDM has a conformation similar to the native protein on the surface of cells.

**Blocking of HCV infection of human cells by affinity-matured variants of antibody 5.16**

The potency of inhibition of HCV infection by the affinity-matured antibodies in IgG format was measured in infection inhibition assays using genotype 2a HCVcc and HCV pseudotyped particles (HCVpp). The five selected affinity-matured variants of antibody 5.16 inhibited
HCVpp and HCVcc infection about 300-fold more potently than the parental antibody 5.16 (Fig. 7A and B), reflecting the affinity improvements of antibody 5.16 variants against solubilized and native CLDN1. No neutralization was observed with control antibody trastuzumab, which binds Her2 on the surface of 293 cells, even at concentrations as high as 1 μM.

**Table I.** Kinetic and cell-based affinity analysis of binding of anti-CLDN1 Fab fragments and IgG

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<th>Kinetic analysis with soluble CLDN1</th>
<th>Cell-based affinity analysis</th>
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*Values represent $K_D$ of inhibition of binding of each radiolabeled Fab by homologous unlabeled Fab to 293 cells expressing CLDN1 measured in duplicate.

**Discussion**

Here we show that antibodies against a multispan membrane protein can be efficiently generated by phage display using solubilized protein derived from baculovirus particles as antigen. Using this approach we obtained Fab fragments
from a synthetic phage display library that bind surface epitopes of CLDN1 when reformatted as IgG. One of these reformatted antibodies bound both human and mouse CLDN1 and blocked infection of hepatocarcinoma cells by HCV. Previous attempts to induce antibodies against human CLDN1 by immunizing with the purified large extracellular loop in isolation were unsuccessful (Evans et al., 2007), suggesting that the transmembrane regions or other parts of the protein are necessary to induce antibodies against the native protein. In fact, polyclonal and monoclonal antibodies against native surface epitopes of CLDN1 have been obtained only by DNA immunization procedures that present the protein to the immune system in its native conformation (Fofana et al., 2010; Krieger et al., 2010). Therefore, the use of freshly solubilized biotinylated multispan protein from baculovirus particles for selection of phage display libraries allows the discovery of antibody fragments against a multispan target with stringent requirements for structural integrity.

While phage display has been applied to detergent-solubilized multispan proteins, these protein targets tend to be either relatively more stable bacterial multispan proteins or mammalian multispan proteins that have been optimized for expression and purification for structural studies (Rothlisberger et al., 2004; Milovnik et al., 2009; Uysal et al., 2009). The method shown here should be applicable to other mammalian membrane proteins with little or no prior biochemical characterization such as CLDN1. In fact, we have extended the application of this panning strategy to other poorly characterized multispan membrane proteins using essentially the same methodology described here (Supplementary data, Table I). However, detergent solubilization conditions may require optimization for some proteins and this could be achieved by simple ligand-binding assays, if a known ligand is available, or activity assays. The facile expression of protein from baculovirus particles facilitates the design of small scale experiments to optimize solubilization conditions by biosensor or other assays (Navratilova et al., 2005). Alternatively, if no assay is available to confirm that the protein retains its native conformation in solution, as in the case of CLDN1, the ease with which soluble multispan protein can be generated from baculovirus particles also allows several detergent conditions to be attempted simultaneously for one protein target to increase the chances of obtaining antibodies against the native protein.

The method for selection of phage display libraries we describe requires the use of the baculovirus expression system for antigen production. This has an impact on the type of post-translational modifications to the multispan protein used for phage selection. The post-translational modification most relevant for antibody discovery is protein glycosylation. Insect cells can add N-linked glycans to secreted and membrane proteins but the end products differ...
considerably from the ones added by mammalian cells (Kost et al., 2005). Therefore, glycosylation of the multispan target expressed in insect cells is expected to differ from the native mammalian protein. Human CLDN1 has one glycosylation site in its relatively small extracellular loop 1 but this does not seem to have a major impact on selection of binders to the native protein as most of the antibodies tested bind CLDN1 expressed on human cells. However, this may be a significant limitation for other glycosylated multispan protein targets, especially those that have very small extracellular regions. This limitation could be bypassed by using commercially available engineered insect cells that add N-linked glycans of the type added by mammalian cells to produce baculovirus with multispan protein for phage selection.

Antibodies binding surface epitopes are useful for detection of the native protein on cells or for therapeutic applications. Antibodies and antibody fragments binding intracellular epitopes are useful as diagnostic tools or as chaperones for structural studies of multispan proteins (Rothlisberger et al., 2004; Day et al., 2007; Milovnik et al., 2009; Uysal et al., 2009). The method described here should allow selection of antibody fragments binding extracellular and intracellular epitopes. Six of 10 tested antibodies made from the Fab fragments obtained by phage display bind cell surface epitopes of CLDN1, indicating that a significant fraction of the selected clones bind extracellular epitopes. Reconstituted liposomes with membrane protein constrained to expose the extracellular region on the liposome surface have been used to selectively obtain antibody fragments that bind extracellular epitopes of multispan proteins (Mirzabekov et al., 2000). Our data indicate that processing of multispan proteins to bias selection against intracellular binders is not necessary to allow selection of surface binders by phage display. In addition, the use of liposomes limits the choice of detergents to those with high critical micelle concentrations that are required for reconstitution of multispan proteins in lipid bilayers. Selection of phage display libraries with protein in solution has the advantage of allowing the use of detergents with low critical micelle concentration for protein solubilization. The other four clones tested by flow cytometry that did not bind CLDN1 surface epitopes could bind intracellular or non-native CLDN1 epitopes.

A limitation of phage and other antibody display methods with multispan proteins has also been the lack of methods for affinity maturation of antibody leads. Here we show the affinity maturation of antibody 5.16 to sub-nanomolar affinities using methods similar to those used to affinity-mature antibodies against soluble targets. This extends a traditional application of phage display to the engineering of antibodies against multispan proteins. The selection of detergent to solubilize the multispan protein target for affinity maturation of antibodies that were obtained by other methods such as hybridoma technology is facilitated by the ease of antigen production in baculovirus particles. Detergents can be easily and rapidly screened by small-scale solubilization trials in ELISA assays using binding of the antibody to be affinity matured as a readout for epitope integrity without the need for labor intensive large-scale purification trials with different detergents.

One advantage of phage display over immunization to obtain therapeutic antibodies is that the former is more likely to yield antibodies that cross-react between the human and mouse forms of a protein target. For instance, only antibodies specific for human CLDN1 were obtained by immunization of mice with human CLDN1-expressing plasmids (Fofana et al., 2010). In this case, immunization of CLDN1-deficient mice to overcome tolerance is not possible because these mice die perinatally (Furuse et al., 2002). In contrast, three of the six antibodies obtained by phage display that bind extracellular epitopes of CLDN1 cross-react between the human and mouse proteins, including antibody 5.16 and its high affinity derivatives that block HCV infection. This will enable toxicity studies with antibodies directed against CLDN1 in small animal models that are necessary to determine the viability of this HCV co-receptor as a therapeutic target.

Supplementary data

Supplementary data are available at PEDS online.

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References


