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A Yeast Display Immunoprecipitation Method for Efficient Isolation and Characterization of Antigens

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Abstract

Yeast antibody display has found a wide variety of applications including antibody affinity maturation, epitope mapping, and library screening. Here we report a yeast display immunoprecipitation (YDIP) technique that employs yeast cells displaying single-chain antibody fragments (scFv) on their surface as affinity capture reagents to isolate and characterize antigens. We show that displayed single-chain antibody fragments are active in a variety of detergent solutions commonly used for immunoprecipitation and that the antigen-antibody interaction can be accurately quantified by YDIP coupled with flow cytometry. The YDIP method has also been optimized so that it is compatible with commonly used protein characterization tools such as Western blotting, silver staining, and mass spectrometry. From complex protein mixtures, we have used YDIP to isolate, analyze and sequence both soluble and plasma membrane antigens using tandem mass spectrometry. In the case of the membrane antigen, YDIP coupled with tandem mass spectrometry was successful in identifying neural cell adhesion molecule (NCAM) as the antigen for an antibody previously selected as binding to the plasma membranes of endothelial cells. The presented method therefore has potential to facilitate antibody-antigen characterization.

Keywords

Immunoprecipitation; yeast display; antigen identification

Introduction

Antibody library screening technologies have been developed to allow rapid selection of antibodies against a wide variety of targets (Hoogenboom, 2005). For example, antibody libraries have been used to select antibodies that bind haptens, immobilized antigens, and cell-surface antigens. Among these targets, antibody selections against cell-surface antigens are of particular interest given the potential for downstream use of such antibodies as biomarker diagnostics and therapeutics (Brekke and Sandlie, 2003; Schrama et al., 2006). For most cell targeting applications, it is important to characterize and identify the antigen of the selected antibodies, since the antibodies selected could in principle bind to any of a large number of accessible cell surface targets. Therefore, an efficient antigen characterization and identification method would be an important accompaniment to any high throughput antibody selection/evaluation process.

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To date, several different methods for antigen characterization have been employed, mainly following antibody library screening using phage display. For example, cross-linked phage has been used as an affinity matrix (Smith et al., 1998) and single-chain antibody fragments (scFvs) cloned from phage have been immobilized on a Ni-NTA column for antigen purification (Poul et al., 2000). In other cases, scFvs selected from a phage display library has been cloned into IgG (Geuijen et al., 2005) or scFv-Fc fusion protein constructs (Valadon et al., 2006) and used for immunoprecipitation of the antigen. In each case, antibodies selected from libraries had to be modified either by being immobilized on a substrate or more frequently, subcloned produced and purified as soluble proteins, making antigen characterization laborious.

Antigen characterization could be facilitated by an antibody selection platform which allows highly multivalent antibody display, such as in yeast surface display (Gai and Wittrup, 2007; Pepper et al., 2008). As a result of each yeast cell expressing 50-100,000 copies of functional scFv on its surface, scFv-displaying yeast could be viewed as an affinity capture reagent (Feldhaus and Siegel, 2004). In such a way, the same scFv display construct and displaying yeast used for antibody selections could be used directly for antigen purification and subsequent analysis without any scFv manipulation or soluble expression. Recently, yeast library screening methods have been expanded to cell-surface selections, allowing identification of antibodies (Wang et al., 2007) and T cell receptors (Richman et al., 2006) against mammalian cell-surface antigens, and we have demonstrated that yeast cells displaying scFvs can be used for immunoprecipitation and Western blotting of target cell surface antigens (Wang et al., 2007). Here, we examined the full capability of the yeast display immunoprecipitation (YDIP) technique is examined for recovery and analysis of both soluble and plasma membrane antigens, including demonstration of YDIP compatibility with tandem mass spectrometry for facile antigen identification.

Materials and Methods

Cells, media and plasmids

Saccharomyces cerevisiae strain EBY100 (Boder and Wittrup, 1997) was used for surface display of scFvs. Surface display plasmid pCT201-D1.3 (VanAntwerp and Wittrup, 2000) and pCT302 (Boder and Wittrup, 1997) were used for the display of anti-hen egg lysozyme D1.3 scFv and anti-fluorescein 4420 scFv, respectively. All human scFvs (scFvA, scFvD, scFvJ, and scFvK) in the surface display format were selected in a previous study (Wang et al., 2007). Yeast cells were grown in SD-CAA medium (20.0 g/L dextrose, 6.7 g/L yeast nitrogen base, 5.0 g/L casamino acids, 10.19 g/L Na₂HPO₄·7H₂O, 8.56 g/L NaH₂PO₄·H₂O) at 30°C to reach an OD_{600nm} of approximately 1.0 and induced in the same volume of SG-CAA medium (dextrose replaced by galactose in SD-CAA) for 16-18 hrs at 20°C for scFv display. The rat brain endothelial cell line (RBE4) was a kind gift from Dr. Françoise Roux (Roux 1994). RBE4 cells were grown at 37°C in 5% CO₂, in 45% Alpha Minimum Essential Medium, 45% Ham's F10 medium, and 10% heat inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) supplemented with 100 µg/mL streptomycin, 100 units/mL penicillin G (Invitrogen, Carlsbad, CA), 0.3 mg/mL geneticin (Fisher Scientific, Pittsburgh, PA) and 1 µg/L basic Fibroblast Growth Factor (bFGF) (Roche Diagnostics, Indianapolis, IN).

Preparation of biotinylated RBE4 cell lysates

Plasma membrane proteins of RBE4 cells were biotinylated by incubating live RBE4 cells with 0.5 mg/mL sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) in 10 mM phosphate buffered saline (PBS, pH 7.4) supplemented with 1 mM CaCl₂, 0.5 mM Mg₂SO₄ (PBSCM) for 30 min with rocking at room temperature. The charged sulfoxide group of the biotinylating reagent prevents the biotinylation of cytosolic proteins by hindering the diffusion through cell membranes. After the biotinylation, approximately 5×10⁶ biotinylated RBE4 cells were lysed at 4°C by scraping

the cells into 1 mL of PBS supplemented with a protease inhibitor cocktail (Calbiochem, Gibbstown, NJ), 2 mM EDTA and containing one of the following detergents: 0.1 % (w/v) Triton X-100 (TX), 1% (w/v) *n*-octyl- β -D-glucopyranoside (OG) (Anatrace, Maumee, OH), 0.5% (w/v) CHAPS (Fisher Scientific), or radio-immunoprecipitation assay (RIPA) buffer (0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate (DOC), and 1% (w/v) Triton X-100 in PBS). The initial cell lysates were then centrifuged at 18,000 $\times$ g for 15 min at 4°C to remove insoluble debris. The total protein concentration remaining in the supernatant was determined using the BCA assay per manufacturer's instructions (Pierce, Rockford, IL).

Detection of scFv activity in detergent solutions

To assess the effects of detergents on scFv affinity, an anti-hen egg lysozyme antibody D1.3 scFv (VanAntwerp and Wittrup, 2000) and hen-egg lysozyme (HEL) from chicken egg white (Sigma, St. Louis, MO) were used. Yeast cells were fixed with 3% v/v formaldehyde in PBS for 1 hr at room temperature when indicated. The HEL was biotinylated using sulfo-NHS-LC-biotin (Pierce, Rockford, IL) and the degree of biotinylation was determined to be 2.1 biotins per HEL using the 4-Hydroxyazobenzene-2-carboxylic acid (HABA) assay (Sigma, St. Louis, MO). Serial dilutions of HEL were prepared in the various lysate buffers described above minus protease inhibitors and incubated with yeast cells displaying anti-HEL D1.3 scFv for 2 hrs at 20°C. The incubated yeast cells were washed once with the corresponding detergent solution, once with PBS containing 0.1% (w/v) bovine serum albumin (PBS-BSA) and subsequently incubated with a mouse anti-*c-myc* antibody (9E10, 30 μ g/mL, Covance, Berkeley, CA) to selectively analyze the antibody-displaying yeast population. Next, goat anti-mouse IgG-Alexa488 conjugate (α M488, 1:1000 dilution, Invitrogen, Carlsbad, CA) and streptavidin-phycoerythrin conjugate (SAPE, 1:80 dilution, Sigma) were added to quantify ligand binding. All of the washing and labeling steps were performed at 4°C. The fluorescence intensities were quantified using the FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and fitted to a bimolecular equilibrium binding model to determine the dissociation constants (K_d). To test the binding activity of scFvA, scFvK, scFvD, and scFvJ, yeast displaying scFv were incubated with biotinylated RBE4 cell lysate (1% OG, 200 μ g total protein) for 2 hrs at 4°C. Yeast were then washed twice with PBS containing 1% OG. Labeling with 9E10, SAPE, and α M488 was identical to that described above, except for scFvJ, for which mouse anti-HA antibody (12CA5, Roche Diagnostics, Indianapolis, IN) was used instead of 9E10, since scFvJ is actually a single domain Fv consisting of only the variable heavy chain and lacking the C-terminal *c-myc* epitope (Wang et al., 2007). To measure the relative affinity of scFvA and scFvK, all procedures were identical as in measuring affinity of D1.3 scFv, except that serially diluted RBE4 cell lysate (biotinylated, 1% OG) was used as a ligand source.

Yeast display immunoprecipitation

Immunoprecipitation of HEL from complex mixtures—Approximately 5×10^7 of yeast cells displaying D1.3 scFvs were used for YDIP. Yeast cells were fixed with 3% v/v formaldehyde in PBS for 1 hr at room temperature when indicated. Yeast were incubated 2 hrs at 4°C with 34 nM HEL diluted into RBE4 cell lysate prepared with 1% (w/v) TX and washed twice with and eluted with 50 μ L of 0.2 M glycine-HCl solution (pH 2.0). Yeast displaying the anti-fluorescein 4-4-20 scFv were used as a negative control.

Elution methods for antigen recovery—To monitor the amount of antigen obtained by various elution methods, D1.3 scFv and biotinylated HEL were used. A batch of 3×10^7 yeast cells displaying D1.3 scFv were incubated with 10 nM HEL in PBS-BSA for 2 hr at 4°C. The yeast cells were washed twice with PBS at 4°C prior to elution. Elution of immunoprecipitated product was performed by resuspending yeast cells in 30 μ L of 0.2 M glycine-HCl solution (pH 2.0), 9 M urea, 0.2 M NaOH, 3 M NaCl, or 0.2 % (w/v) SDS for 10 minutes at 4 °C. Where indicated, yeast cells were fixed with 3% v/v formaldehyde in PBS for 1 hr at room temperature

before incubation with the antigen, to reduce the co-elution of yeast proteins. The eluates were separated with SDS-PAGE (15% separating gel) and either probed with streptavidin-HRP conjugate (1:2000 dilution, GE healthcare, Piscataway, NJ) or silver-stained following standard protocols (Blum et al., 1987). The HEL bands from Western blot films or silver stained gels were quantified using densitometry with ImageJ (US National Institutes of Health, Bethesda, MD) to compare the relative amount of biotinylated HEL eluted under each condition.

Immunoprecipitation of cell-surface antigens and Western blotting—For Western blotting analysis of immunoprecipitated antigens, 3×10^7 yeast cells were used. Induced yeast cells were collected by centrifugation and washed with PBS-BSA. Biotinylated RBE4 cell lysate prepared using the various detergents containing approximately 400 μ g of total protein (corresponding to approximately 2×10^6 RBE4 cells) was mixed with yeast cells and incubated overnight at 4°C in a total volume of 200 μ L. After the incubation, yeast cells were washed three times by resuspension in 1 mL of the corresponding lysis buffer (without protease inhibitor cocktail) and incubated for 15 minutes at 4°C. Elution of immunoprecipitated product was performed by resuspending yeast cells in 30 μ L of 0.2 M glycine-HCl solution (pH 2.0) for 10 minutes. The mixtures were briefly centrifuged and the eluted supernatants were separated with non-reducing SDS-PAGE (8% separating gel), and blotted onto a nitrocellulose membrane (BioRad, Hercules, CA). Yeast displaying D1.3 scFv were used as a negative control for these experiments. Western blotting was subsequently performed with an anti-biotin monoclonal antibody (0.5 μ g/mL, clone BTN.4, Labvision, Fremont, CA).

To test the serial elution process, scFvJ was used. The IP procedure is identical to that above except the eluted supernatant was serially applied for the indicated number of times to elute additional batches of yeast that have undergone the immunoprecipitation process. To confirm that the scFvJ antigen was neural cell adhesion molecule (NCAM), a monoclonal anti-NCAM antibody (clone 5B8, Developmental studies Hybridoma Bank, Iowa Cities, IA) was used for Western blotting.

Identification of immunoprecipitated proteins using tandem mass spectrometry

To sequence hen-egg lysozyme using tandem mass spectrometry (MS/MS), approximately 3×10^8 yeast displaying the D1.3 scFv were incubated for 2 hrs at 4 °C with 30 nM HEL in PBSTX, washed twice with PBSTX and eluted with 50 mL of 0.2 M glycine-HCl solution (pH 2.0). The elution product was precipitated for 2 hrs by adding 250 μ L of ice-cold acetone at -20 °C. The protein pellet was dried and reconstituted in 5 μ L of 8 M urea and then diluted with 40 μ L of 50 mM ammonium bicarbonate buffer with 10 mM DTT. Approximately 100 ng of sequencing grade modified trypsin (Promega, Madison, WI) was added and digestion was allowed to proceed for 16 hrs at 37°C. Peptide fractions were individually analyzed by nanoLC-MS/MS using 1100 series LC/MSD Trap SL spectrometer (Agilent, Palo Alto, CA). Chromatography of peptides prior to mass spectral analysis was accomplished using C18 reverse phase HPLC trap column (Zorbax 300SB-C18, 5 μ M, 5×0.3 mm, Agilent) and separation column (Zorbax 300SB-C18, 3.5 μ m, 0.075×150 mm, Agilent) onto which 40 μ L of each extracted peptide fraction was automatically loaded. An Agilent 1100 series HPLC delivered solvents A: 0.1% (v/v) formic acid in water, and B: 95% (v/v) acetonitrile, 0.1% (v/v) formic acid at either 10 μ L/min, to load sample, or 0.28 μ L/min, to elute peptides directly into the nanoelectrospray source over a 60 minute 20% (v/v) B to 80% (v/v) B gradient. As peptides eluted from the HPLC-column/electrospray source, MS/MS spectra were collected over 5 channels from 300 to 2200 m/z; redundancy was limited by dynamic exclusion (120s). MS/MS data were converted to mgf file format using Data Analysis Software (Agilent). Resulting mgf files were used to search the non-redundant NCBI database with an in-house licensed Mascot search engine (Matrix Science, London, UK) with methionine oxidation,

cysteine carbamidomethylation and glutamine and asparagine deamidation as variable modifications.

For MS/MS sequencing of the RBE4 cell-surface antigen recognized by scFvJ, 3×10^9 formaldehyde fixed yeast cells displaying scFvJ were divided into eight microfuge tubes and RBE4 cell lysate containing 3 mg of total protein (both biotinylated and non-biotinylated ID was successful) in 1 mL of 1% OG with protease inhibitors was added to each tube. The yeast cells were incubated overnight at 4°C in the cell lysate, and then 4 product pools created each by serial elution of two tubes with 70 µL of 0.2 M glycine-HCl solution (pH 2.0). To concentrate the antigen, the four elution products were combined and precipitated at 4°C for 2 hrs by the addition of trichloroacetic acid (Fisher Scientific) to a final concentration of 13% (w/v). Protein was pelleted by centrifuging at $12,000 \times g$ for 15 min at 4°C. The pelleted protein was then dissolved in 2X Laemmli sample buffer (Laemmli, 1970) for separation by SDS-PAGE (8%). Gels were stained using colloidal coomassie (Candiano et al., 2004) to visualize the eluted proteins. In-gel digestion was performed using standard protocols (Shevchenko et al., 1996). In short, gel pieces were excised, dehydrated, and reduced in 50 µL 25 mM DTT in 25 mM ammonium bicarbonate for 20 min at 56 °C, and alkylated with 50 µL of 55 mM iodoacetamide (Sigma, St. Louis, MO) in 100 mM ammonium bicarbonate for 20 min at room temperature. Then the gel pieces were washed, dried and digested for 18 hrs at 37°C with 400 ng of sequencing grade modified trypsin in 25 mM ammonium bicarbonate with 5% (v/v) acetonitrile. Digested peptides were eluted off from a C18 micro ZipTip (Millipore, Billerica, MA) with acetonitrile/H₂O/TFA (60%:40%:0.2%) directly onto the Opti-TOF™ 384 Well plate (Applied Biosystems, Foster City, CA) and recrystallized with 0.75 µL of matrix (10mg/ml α-Cyano-4-hydroxycinnamic acid in acetonitrile/H₂O/TFA (70%:30%:0.2%)). Peptide identification via result-dependent MS/MS analysis was performed on a 4800 Matrix-Assisted Laser Desorption/Ionization-Time of Flight-Time of Flight (MALDI TOF-TOF) mass spectrometer (Applied Biosystems, Foster City, CA). In short, a peptide fingerprint was generated by scanning the 700-4,000 Da mass range using 1500 shots acquired from 20 randomized regions of the sample spot using an OptiBeam™ on-axis Nd:YAG laser (4200 intensity, 200 Hz firing rate, 3-7 ns pulses). The fifteen most abundant precursors, excluding trypsin autolysis peptides and sodium/potassium adducts, were selected for subsequent tandem MS analysis where 2000 total shots were taken with 4700 laser intensity and 2 kV collision induced activation (CID) using air. Post-source decay (PSD) fragments from the precursors of interest were isolated by timed-ion selection and reaccelerated into the reflectron to generate the MS/MS spectrum. Raw data was deconvoluted using GPS Explorer™ software and submitted for peptide mapping and MS/MS ion search analysis against non-redundant NCBI database with an in-house licensed Mascot search engine (Matrix Science, London, UK) with fixed modification of carboxyamidomethylation of cysteines and parent/fragment ion mass tolerance of 0.3 Da.

Quantitation of scFv and NCAM expression levels using flow cytometry

To quantitate the expression level of scFvs on the yeast cell surface, Quantum simply cellular anti-mouse IgG microbeads (Banglabs, Fishers, IN), which contain four populations of beads with known mouse IgG binding sites were used. Yeast cells displaying either D1.3 scFv or scFvA were incubated with a mouse anti-c-myc antibody (9E10, 30 µg/mL, Covance, Berkeley, CA) in PBS-BSA for 1 hr. For scFvJ quantitation, a mouse anti-HA antibody (12CA5, Roche Diagnostics, Indianapolis, IN, 1:100 dilution) was used instead. The microbeads were also incubated under the same conditions. Next, the yeast cells and the microbeads were washed, incubated with a goat anti-mouse IgG-Alexa555 conjugate (1:500 dilution, Invitrogen, Carlsbad, CA) for 30 min. and analyzed using the FACSCalibur flow cytometer.

To quantitate the expression level of NCAM in RBE4 cells, RBE4 cells were permeabilized with 0.5% (w/v) saponin in PBSCM with 40 % (v/v) goat serum (PBSCMG, Sigma, St. Louis, MO) and incubated with a mouse anti-NCAM antibody (clone 5B8, diluted to 50 µg/mL) in PBSCMG for 1hr. The microbeads were also incubated under the same conditions. The secondary reagents used were identical to the scFv quantitation.

Results

Effect of detergents on yeast-displayed scFv binding activity

Yeast display immunoprecipitation (YDIP) takes advantage of scFv expressed as fusions to the yeast cell wall for capture of antigens from complex mixtures such as cell lysates (Figure 1). Since creation of solubilized protein mixtures in the form of lysates requires the use of detergents (Figure 1 step 1), particularly for the solubilization of membrane proteins (Silvius, 1992), the scFv used in YDIP must retain their binding activities in the presence of detergents. Thus, as a model system, the affinity of yeast surface-displayed anti-hen egg white lysozyme (HEL) scFv (D1.3) for its HEL ligand was assessed in various detergent solutions. Detergents tested included 1% (w/v) Triton X-100 (TX), 1% (w/v) *n*-octyl-β-D-glucopyranoside (OG), 0.5% (w/v) CHAPS, and 0.1% (w/v) SDS as well as a mixture of detergents (0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate (DOC), and 1% (w/v) Triton X-100), which composes the radio-immunoprecipitation assay (RIPA) buffer. The concentrations of detergents were chosen to be higher than the critical micelle concentration (CMC) of each detergent, since complete solubilization of cell membranes generally occurs above the CMC (Garavito and Ferguson-Miller, 2001). The D1.3 scFv remained active with comparable affinity for HEL to that found in PBS-BSA control buffer, varying by at most 3-fold (Figure 2A, Table 1). The lone exception was SDS, which resulted in a complete inhibition of the antigen-antibody interaction. Interestingly however, when 0.1% SDS was mixed with 1% TX and 0.5% DOC as in the RIPA buffer, the scFv remained active (Figure 2A). In addition to maintaining the nanomolar binding affinity, it was important to determine the amount of antigen that can be immunoprecipitated under each detergent condition, or in other words the percentage of scFvs that remain active. The levels of antigen-antibody interaction were compared at saturating concentrations of HEL (142 nM) in each detergent solution. Compared to the displayed scFvs binding to HEL in PBS-BSA, with the aforementioned exception of SDS, more than 75% of the scFvs remained active in terms of the amount of antigen that can be immunoprecipitated (Figure 2B).

ScFvs (scFvA, scFvK, scFvD, and scFvJ) that were previously selected as capable of binding to rat brain endothelial cell line (RBE4) plasma membrane proteins (Wang et al., 2007) were used to assess the general compatibility of yeast displayed scFv for immunoprecipitation both from the standpoint of detergent tolerance and applicability to membrane protein targets. Yeast cells displaying these scFvs were used to immunoprecipitate antigens from RBE4 cell lysates generated by TX detergent-solubilization of biotinylated plasma membranes (Figure 1, steps 1-3). Using flow cytometry, significant antigen-antibody interaction was detected for yeast cells displaying scFvA, scFvK, scFvD, and scFvJ (Figure 1, step 4) while no interaction was detected for yeast cells displaying an irrelevant scFv (Figure 2C). Similar results were obtained by using cell lysates generated by OG-mediated solubilization (data not shown and Figure 2C inset). Moreover, Western blotting using an anti-biotin antibody indicates the presence of specific immunoprecipitation products and since biotinylated rat proteins are not evident in the immunoprecipitation product using yeast displaying irrelevant antibody, the preparations are quite clean (Figure 2C inset). In addition, since antigen-antibody interactions can be accurately quantified on the yeast surface (Chao et al., 2006), important antibody characteristics such as relative affinity could be rapidly determined using YDIP (Figure 1, step 4). As an example, scFvA and scFvK yield the same immunoprecipitation product (Figure 2C inset) and these antibodies differ by only six amino acids in the light-chain of CDR3 (Figure 2D). To rapidly

compare these two antibody clones, an affinity titration on the surface of yeast using biotinylated cell lysates was performed and it was determined that scFvA has a two-fold improved affinity compared with scFvK under these conditions (Figure 2D). Notably, the relative affinity was determined by direct titration of the cell lysate, thereby eliminating the process of antigen purification, which is often a challenge for cell-surface antigens. These results suggest that yeast displayed scFvs retain their binding activities in various detergent solutions and that YDIP is effective in antigen isolation and characterization of antigen-antibody interactions.

Elution, antigen purity and quantity by YDIP

Next, we further assessed the purity and quantity of antigens isolated using YDIP under a variety of elution conditions. The anti-HEL D1.3 scFv was again tested as a model system representing an antibody-soluble antigen combination. We first sought to identify the most efficient method for dissociation of bound antigens using YDIP of biotinylated HEL from a buffer solution (Figure 1 step 5). Since it is known that various yeast cell wall proteins are also extracted from yeast under the elution conditions tested here (De Groot et al., 2005), we assessed each elution condition not only in terms of recovered antigen quantity but also antigen purity relative to coextracted yeast proteins. Tested elution conditions included low pH (0.2 M glycine-HCl, pH 2.0), high pH (0.2 M NaOH), 9 M urea, high salt (3 M NaCl), and 0.2 % (w/v) SDS. Each method resulted in elution of antigen; however, it was immediately apparent that the amount of coextracted yeast protein was substantial (data not shown). Thus, to reduce the amount of coextracted yeast protein, yeast cells were first fixed/crosslinked with a 3% formaldehyde solution prior to YDIP (Figure 1, step 2). Using fixed yeast cells for YDIP, there was no significant effect on D1.3 scFv binding affinity and a small 10-20% decrease in HEL capture (Figure 2A and B). For elution of immunoprecipitated HEL from fixed yeast, the low pH, SDS and urea elution methods had comparable efficiency, while high pH and high salt were less effective (Figure 3A). In addition, the amount of coextracted yeast protein was significantly diminished by yeast fixation, with low pH elution having the least amount of coextracted protein (data not shown). Since low pH elution gave excellent yields and minimal yeast protein background, it was generally used as the elution method.

In addition to coextracted yeast proteins, another confounding factor for antigen analysis is the non-specific binding of mammalian cell lysate proteins to yeast. Lysate-derived impurities can be more detrimental than the coextracted yeast proteins in applications such as antigen identification, since yeast proteins can be distinguished from mammalian proteins by sequence in many cases, while contaminants from same species cannot. To evaluate lysate-derived impurities, YDIP was performed to recover HEL that had been doped into RBE4 cell lysates. First, using unfixed yeast, several bands other than HEL were detected on a silver-stained gel, especially in the lower molecular weight range (Figure 3B lanes 1 and 2), yielding an HEL purity of approximately 50-60%. For the most part, these proteins did not appear to be lysate-derived as many of the same contaminants were detected in the YDIP of HEL in the experiments described above where lysate was absent (data not shown). Upon yeast fixation, the purity increased to 80% with both yeast and lysate contaminants taken into account (Figure 3B lane 4). Combined with the anti-biotin Western blotting results for scFvA, scFvK, scFvD, and scFvJ (Figure 2C inset), these results show that proteins in RBE4 cell lysate have minimal non-specific binding to yeast. Finally, the immunoprecipitation of HEL from solution was specific to the yeast displaying D1.3 scFv and was not detected with yeast displaying an irrelevant scFv (Figure 3B).

Another important aspect to consider is the amount of antigen isolated, because 50-500 nanograms are desired for characterization by peptide sequencing using MS/MS. We first estimated the quantity of antigen that could be reasonably isolated using YDIP. Using the HEL-

D1.3 pair that has an affinity of $K_d = 0.488$ nM in TX lysate (Table 1) with 31,000 scFv per yeast cell (see Methods for scFv quantification details) and ten nanomolar or higher antigen concentration, approximately 150 ng of antigen can be isolated with 3×10^8 D1.3-displaying yeast cells (15 mL overnight culture with density of 2 OD_{600nm}). Using 5×10^7 yeast cells (~3 mL overnight culture), as was the case in Figure 3B, it was thus predicted that 25 ng of HEL could be immunoprecipitated from an RBE4 cell lysate containing a 34 nM concentration of HEL. In practice, approximately 18 ng of HEL was immunoprecipitated (Figure 3B lane 2) representing 70% of the theoretical yield. Therefore, using 10-100 mL of overnight yeast display cultures for YDIP is sufficient to isolate antigens in amounts suitable for downstream characterization.

YDIP of mammalian cell-surface antigens

Since YDIP exhibited good recovery and purity for the soluble HEL antigen, the optimized YDIP protocol was applied to analyze plasma membrane antigens. We have shown that yeast cells displaying scFvs selected from a non-immune human scFv library can be used to immunoprecipitate mammalian cell-surface antigens from cell lysates for Western blot analysis using various detergents and elution conditions (Figure 2C and (Wang et al., 2007)). However, unlike the case of a soluble antigen like HEL, the plasma membrane antigen was being isolated from cell lysates in low nanogram quantities that could not be detected by either silver/coomassie staining or MS/MS. The reduced recovery of antigen was likely a combined result of both antigen expression levels and reduced antibody affinities (80-1000 nM, (Wang et al., 2007)) compared with the high affinity D1.3 scFv. Therefore, to increase the YDIP yield as well as antigen concentration for downstream applications, we adapted a 'serial elution' method in which a single small volume of elution buffer is used to serially elute multiple batches of yeast cells each used to immunoprecipitate antigen from fresh cell lysate. Using scFvJ displaying yeast with OG-solubilized RBE4 cell lysate, YDIP was performed. Here, OG was used for the lysis due to its compatibility with MS/MS analysis (Zhang and Li, 2004). ScFvJ immunoprecipitates an antigen of approximately 130 kD (Figure 2C), and serial elution effectively increased the antigen amount and concentration nearly 20-fold over a single elution approach (Figure 4A and B). After 4 elutions, little increase in eluted antigen is seen since the pH buffering capacity of 0.2 M glycine-HCl becomes limiting. While antigen concentration increases dramatically, selectivity of the YDIP process is maintained as the level of irrelevant biotinylated lysate proteins isolated during the procedure remains very low (Figure 4).

Identification of immunoprecipitated proteins using tandem mass spectrometry

To assess the compatibility of YDIP with mass spectrometry for peptide sequencing and antigen identification, we first tested the D1.3-HEL system. To this end, HEL was immunoprecipitated from a TX containing buffer using 3×10^8 formaldehyde-fixed yeast cells. HEL was subsequently eluted with low pH buffer, acetone precipitated and the entire elution product was trypsinized in solution. The trypsinized elution product was directly subjected to LC-MS/MS to attempt detection of HEL and to monitor the effects of yeast protein contaminants in the MS/MS analysis. As expected by the fact that HEL was isolated in high purity (Figure 3, lane 4) and in relatively large amounts, the HEL antigen was unequivocally identified by the sequencing of four peptides. In addition, three other yeast proteins were identified including some scFv that was leached from the yeast surface during the YDIP procedure (Table 2).

Next, YDIP was applied for *de novo* identification of the RBE4 plasma membrane antigen immunoprecipitated by scFvJ. Although the molecular weight of the antigen was previously determined by anti-biotin Western blotting (Figure 2C inset and (Wang et al., 2007)), the identity of the antigen is unknown and thus provides a true test of the full YDIP method. The in-solution digestion approach used for HEL was not effective for this particular antigen since

the amount of isolated antigen was smaller than that of HEL. In addition, 10-fold more yeast cells were required to isolate enough antigen, which greatly increased the relative concentration of contaminating yeast proteins in the sample, thereby dominating the MS/MS signal. Thus, after concentrating the immunoprecipitated antigens by serial elution followed by trichloroacetic acid precipitation and resuspension (see methods for details), the product was first resolved by SDS-PAGE to separate the antigen from other proteins in the elution mixture (Figure 5A). The band at the size identified by Western blotting and not present in a mock YDIP control sample, was excised and in-gel digested. Using tandem mass spectrometry (MS/MS), two peptide sequences were obtained that identified rat neural cell adhesion molecule (NCAM) as the immunoprecipitated product (Figure 5B). The MS/MS sequencing result was further confirmed by Western blotting with an anti-NCAM antibody (Figure 5C). The 130 kDa band corresponding to the biotinylated immunoprecipitation product of scFvJ was also recognized by an anti-NCAM antibody. To get a feel for the antibody and antigen densities that allowed successful YDIP-coupled MS/MS identification, total cellular NCAM protein and the number of scFvJ molecules per yeast cell were quantified and found to be approximately 72,000 NCAM molecules per cell (2 nM lysate concentration) and 47,000 scFvJ molecules per yeast, respectively (see Methods for details).

Discussion

This study demonstrates that scFv-displaying yeast cells are effective affinity reagents allowing the immunoprecipitation and identification of antigens from detergent solubilized cell lysates. We have shown that yeast displayed scFvs retain their activities in detergent solutions by quantitative assessment of antigen-antibody interactions and two-fold differences in antibody affinity could be accurately determined using cell lysate as an antigen source. Furthermore, the immunoprecipitated proteins could be readily characterized by well-established downstream procedures such as Western blotting, silver/coomassie staining, and mass spectrometry. Optimized YDIP conditions that combine fixed yeast, a low pH serial elution strategy and concentration by TCA precipitation allowed isolation of enough cell-surface receptor for MS/MS antigen identification.

To date, the activity of yeast-displayed scFvs in detergent solutions has not been assessed in detail. Although it is known that various non-ionic detergents such as Triton X-100, Tween 20, and Brij do not affect the activity of antibodies (Dimitriadis, 1979), this generalization may not apply for antibody fragments such as scFvs that could be less stable than the whole immunoglobulin molecule (Wörn and Plückthun, 2001). Here we have shown that scFvs expressed on yeast surface are active in non-ionic detergents (TX, OG), zwitterionic detergent (CHAPS), and a mixture of ionic and non-ionic detergents (RIPA buffer). It was important to test a wide variety of detergents, since antigens have varying solubility depending on the detergent (Schuck et al., 2003). Interestingly, while scFvs were inactive in the highly denaturing ionic detergent, SDS, as previously seen for antibodies (Crumpton and Parkhouse, 1972), when SDS was in a mixture with TX and DOC (RIPA buffer), the scFvs remained active. This might be explained by the fact that proteins denatured in SDS can be renatured by the addition of TX (Clarke, 1981).

For the elution of antigens, we have applied conditions classically used in immunoprecipitations (Figure 3A). In the case of the HEL-D1.3 scFv pair, the low pH and Urea were most effective in dissociating the interaction. However, the optimal elution may vary depending on the nature of antigen-antibody interaction. For example, it is thought that hydrophobic interactions are poorly disrupted by low pH conditions (Yarmush et al., 1992). Therefore, although the low pH elution conditions have been widely used with success (Yarmush et al., 1992), the elution method may need to be optimized for a given antigen-antibody pair.

Another important consideration is the quantity of antigen isolated with YDIP. When the antigen concentration is in the nanomolar range, >200 ng of antigens could be easily isolated. However, as an example, this antigen concentration corresponds to a protein expressed at approximately 100,000 copies per mammalian cell for the case of a 14 kDa protein, with 5×10^7 mammalian cells in 1 mL of lysis buffer. This concentration of antigen may not be achievable in every situation, since normal protein expression levels may vary between 100 to 100,000 copies per cell (Greenbaum et al., 2003), averaging about 50,000 copies per cell (Gygi et al., 2000), with plasma membrane proteins being on the low end of this spectrum (Santoni et al., 2000). In the case of scFvJ-NCAM pair that was evaluated in this study, quantification of NCAM expression level in RBE4 cells showed that there are 72,000 copies of NCAM per RBE4 cell corresponding to a 2 nM antigen concentration in the cell lysate. This result suggests that for antigens with average expression level, YDIP can be applied for *de novo* identification. On the yeast side of the equation, when the antibodies are isolated from a nonimmune or synthetic library, K_d values can be in the low micromolar range (Feldhaus et al., 2003) limiting recovery from dilute cell lysates. Theoretical calculations based on bimolecular equilibrium show that even with antibodies with micromolar affinities, it is estimated that >100 ng of antigens can be isolated using 10^9 yeast cells. Indeed this was the case for the scFvJ-NCAM system where 3×10^9 yeast cells from 150 mL of overnight culture were sufficient. In terms of scFv expression levels which can also affect YDIP yield, quantification of displayed D1.3, scFvA and scFvJ showed expression levels ranging from 31,000-68,000 scFv per yeast cell, differences that can be accommodated for simply by scaling the number of yeast used in the YDIP procedure.

The major immunoprecipitation product that appears in anti-biotin Western blotting of the scFvJ elution product was identified to be the neural cell adhesion molecule (NCAM) (Figure 5). NCAM is a transmembrane protein with immunoglobulin-like domains that has three alternatively spliced isoforms of varying predicted molecular weights (120, 140, 180 kDa). Since the antibody that was used to confirm the YDIP result is specific to the cytosolic domain of NCAM (Dodd et al., 1988), which is absent in the 120 kDa isoform, it is likely that scFvJ immunoprecipitates the 140 kDa isoform. NCAM is known to be involved in cell-cell adhesion and neurite outgrowth (Cunningham et al., 1987). While NCAM is mainly found in early embryonic cells, neural cells, and natural killer cells (Cunningham et al., 1987), it has previously been detected in gene profiling studies as being expressed at the blood-brain barrier (Enerson and Drewes, 2006). Our finding that the actual protein is expressed in a rat brain endothelial cell line (RBE4) model of the blood-brain barrier confirms the gene profiling study and suggests an endothelial role for NCAM in brain function. Since scFvJ was raised against intact cells, a cell surface biotinylation protocol was used, there was a single biotinylated immunoprecipitation product and the sequenced product is a membrane protein with an extracellular epitope, NCAM is most probably the actual antigen recognized by scFvJ. However, we cannot rule out the possibility that scFvJ interacts with another member of an immunoprecipitated protein complex of which NCAM is a biotinylated member. ScFvJ was selected under native, live cell conditions (Wang et al., 2007) and does not recognize its antigen under the denaturing conditions of Western blotting, and thus we could not show a direct interaction between scFvJ and NCAM. This level of confirmation is dictated by the scFv's properties and is therefore not a challenge unique to YDIP. Nonetheless, our results suggest that YDIP can be generally applied for antigen/antigen-complex identification for antibodies originating from cell surface screens.

In conclusion, the results show that YDIP provides an efficient and facile method for antigen identification compared to conventional immunoprecipitation approaches since it eliminates additional cloning and production steps needed to obtain affinity reagents. Therefore, we anticipate that YDIP will enable a wide range of applications in combination with yeast antibody library screening technology.

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Figure 1.

Schematics of the relevant steps of the YDIP procedure. In step (1), target cells containing the antigen are lysed using detergents. Cell surface antigens such as membrane proteins are solubilized by the detergent. Yeast cells that have been induced for surface display of scFvs in step (2) are combined with the cell lysate from (1). In step (3), components in the cell lysate that are not bound to scFvs are washed away, and the yeast cells are collected by centrifugation. At this point, antibody-antigen characteristics can be quantified by flow cytometry (step 4). In step (5), denaturing conditions such as lowering the pH are used to dissociate the antigen-antibody interaction. The collected antigen can then be analyzed for antigen identification and characterization in step (6).

Figure 2.

ScFv binding activity in detergent solutions. (A) Equilibrium binding titration curves of yeast-displayed D1.3 scFv with HEL in PBS and containing the indicated detergents. The lines represent fitted solutions using least-squares regression with a standard bimolecular equilibrium binding model. BSA Fixed indicates binding data using formaldehyde-fixed yeast. (B) Comparison of absolute levels of biotinylated HEL binding in various detergent solutions using unfixed (open bars) or formaldehyde-fixed (grey bars) yeast. HEL binding to D1.3 on the surface of yeast was detected by using a fluorescent streptavidin-phycoerythrin conjugate and quantitated by flow cytometry as the mean fluorescence (HEL binding) per cell. Data were normalized to the case of PBS-BSA. (C) Detection of antigen-antibody interaction for scFvA, scFvK, scFvD, and scFvJ. Yeast cells displaying each scFv were labeled with biotinylated RBE4 cell lysate generated by 1% TX. Yeast displaying an irrelevant antibody were used as a negative control (NC). The inset shows an anti-biotin Western blot of antigens isolated using YDIP from biotinylated RBE4 cell lysate generated using OG. Lane 1: irrelevant scFv, lane 2: scFvA, lane 3: scFvK, lane 4: scFvD, and lane 5: scFvJ. (D) Measurement of relative affinities of scFvA and scFvK by titrating RBE4 cell lysate generated with 1% OG. X-axis indicates the concentration of RBE4 lysate in terms of percent of a cell lysate containing approximately 2 mg of total protein. The light-chain CDR3 amino acid sequence of scFvA and scFvK and the relative affinity (K_d) calculated as described above are indicated in the inset. Error bars represent standard deviations from two (A and D) or three (B and C) independent measurements.

Figure 3.

Recovered antigen purity and quantity by YDIP. (A) Comparison of elution methods from formaldehyde-fixed yeast cells displaying D1.3 scFv. The amount of biotinylated HEL recovered after each elution was quantified by Western blot. Elutions were performed using 3M NaCl (High Salt), 0.2M glycine-HCl pH 2.4 (Low pH), 0.2M NaOH (High pH), 9M urea (Urea), or 0.2% SDS in 0.1M Tris (SDS). Elution with PBS-BSA was used as a negative control. The data represent mean $\pm$ S.D. from triplicate measurements expressed as the percentage of maximum theoretical immunoprecipitation yield. (B) Yeast immunoprecipitation of HEL from RBE4 cell lysate with low pH elution. HEL was added to RBE4 cell lysates at a concentration of 34 nM and YDIP was performed. Immunoprecipitation products were separated using SDS-PAGE and subsequently silver-stained. YDIP was performed using either D1.3-displaying yeast (lanes 2 and 4) or an irrelevant 4420 scFv displaying yeast (lanes 1 and 3). Lanes 1 and 2 are YDIP products from unfixed yeast, and lanes 3 and 4 are from formaldehyde-fixed yeast. Lane 5 contains 25 ng of purified HEL for comparison. The gel was quantified by densitometric analysis.

Figure 4. Serial elution method of antigen concentration. (A) The scFvJ antigen was immunoprecipitated from biotinylated RBE4 cell lysate using fixed yeast. Elution was performed by adding 50 μ L of 0.2M glycine-HCl buffer (pH 2.0) to serially elute batches of 6×10^7 immunoprecipitated yeast. Each lane number corresponds to the number of yeast batches eluted. (B) Densitometric quantitation of (A).

Figure 5.

YDIP coupled with MS/MS for antigen identification. (A) Silver-stained gel showing the scFvJ YDIP product separated by SDS-PAGE. Lane 1: Raw cell lysate, Lane 2: YDIP using yeast displaying irrelevant scFv, Lane 3: YDIP using yeast displaying scFvJ. Although coomassie-stained gels were used for MS/MS experiments, a silver-stained gel is shown for clarity. The scFvJ antigen band that corresponds to the band identified by anti-biotin Western blotting is indicated by an arrowhead. (B) MS/MS results showing the two peptides sequenced that correspond to NCAM. All the y-ions identified are indicated on the figure along with the m/z values. The Mascot score of each ion is also indicated and as defined in table 2, the NCAM identification has a confidence level of $p < 0.05$. (C) Confirmation of antigen identification by Western blotting. The left panel (lanes 1-3) was probed with an anti-biotin antibody and the right panel (lanes 4-6) was probed with an anti-NCAM antibody. Lanes 1 and 4: raw biotinylated RBE4 cell lysate, Lanes 2 and 5: YDIP product from irrelevant scFv, Lanes 3 and 6: YDIP product from scFvJ.

Table 1

Affinity of D1.3-HEL interaction in detergent solutions

PBS additives	K_d (nM)
BSA	1.07 ± 0.03
TX	0.488 ± 0.03
OG	2.61 ± 0.54
CHAPS	1.80 ± 0.16
RIPA	3.43 ± 0.15

Reported $K_d = 1.37 \pm 0.14$ nM (VanAntwerp and Wittrup, 2000)

Table 2

List of peptides sequenced by LC-MS/MS

Protein	Host	Unique peptides sequenced	Ion score ^a
Hen egg white lysozyme	Chicken	GTDVQAWIR	33
		GYS LGNWVCAAK	43
		IVSNGNGMNAWVAWR	104
		NTDGSTDYGILQINSR	83
single chain antibody scFv	Mouse	AAAEQKLISEEDLN	64
ZPS1 (YOL154W)	Yeast	HYAGIDMLHR	38
		LLNYGVDDVYYK	23
		KPLSTICFEGTIVDVGPK	31
		QSAPAETVICDYFYTSK	46
		CDDIDGLCAANPNYYAGHHR	30
glyceraldehyde-3-phosphate dehydrogenase	Yeast	IVSNASCTTNCLAPIAK	56
		TASGNIIPSSTGAAKAVGK	40

^aProteins that have a summed peptide ion score greater than 67 carry a significance of $p < 0.05$.