Published in final edited form as:

Biotechnol Bioeng. 2010 April 1; 105(5): 973–981. doi:10.1002/bit.22607.

Flow Cytometry-Based Methods for Assessing Soluble scFv Activities and Detecting Antigens in Solution

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Abstract

Novel methods are reported for evaluating and utilizing single chain fragment variable (scFv) antibodies derived from yeast-display libraries. Yeast-display was used to select scFv specific to invariant surface glycoproteins (ISG) of *Trypanosoma brucei*. A limiting step in the isolation of scFv from non-immune libraries is the conversion of highly active yeast-displayed scFv into soluble antibodies that can be used in standard immunoassays. Challenges include limited solubility or activity following secretion and purification of scFv. For this reason, few scFv derived from yeast-display platforms have moved into development and implementation as diagnostic reagents. To address this problem, assays were developed that employ both yeast-displayed and -secreted scFv as analytical reagents. The first is a competitive inhibition flow cytometry (CIFIC) assay that detects secreted scFv by virtue of their ability to competitively inhibit the binding of biotinylated antigen to yeast-displayed scFv. The second is an epitope binning assay that uses secreted scFv to identify additional yeast-displayed scFv that bind non-overlapping or non-competing epitopes on an antigen. The epitope binning assay was used not only to identify sandwich assay pairs with yeast-displayed scFv, but also to identify active soluble scFv present in low concentration in a crude expression extract. Finally, a CIFIC assay was developed that bypasses entirely the need for soluble scFv expression, by using yeast-displayed scFv to detect unlabeled antigen in samples. These methods will facilitate the continued development and practical implementation of scFv derived from yeast-display libraries.

Keywords

Trypanosoma; African trypanosomiasis; antibodies; scFv; yeast-display; *S. cerevisiae*; invariant surface glycoproteins; ISG; flow cytometry; yeast-display; competitive inhibition

Introduction

Sleeping sickness, also called human African trypanosomiasis (HAT), is caused by the protozoan parasites *Trypanosoma brucei gambiense* and *T. brucei rhodesiense*. Following insect-borne transmission, systemic disease is characterized by parasites spreading through the

lymph, bloodstream, and other organs (Checchi et al., 2008; Fevre et al., 2008). Eventually, the parasite infects the central nervous system resulting in severe neurological symptoms. Left untreated, the infection is nearly always fatal (Burri and Brun, 2009; Checchi et al., 2008).

Diagnosis of HAT is problematic and is typically based on ruling out of malaria, microscopic visualization of parasites, and, for *T. brucei gambiense*, a positive card agglutination test (Simarro et al., 2008). Patients who receive a correct diagnosis can be effectively treated, however, drugs for late-stage disease are highly toxic. To reduce life-long disabilities and the need for toxic late-stage treatments, diagnostic tests are needed to detect the infection early. Ideally, new tests should detect parasites or parasite components, because tests for immune responses cannot discriminate between active and past infections (Buscher and Lejon, 2004).

The surfaces of bloodstream form *T. brucei* trypomastigotes are covered with highly immunogenic variable surface glycoproteins (VSG) (Kennedy, 2008; Stockdale et al., 2008; Van Meirvenne et al., 1977). Frequent variation of the antigenic type of VSG complicates detection of parasites by immunoassays (Buscher and Lejon, 2004). Within the VSG layer are invariant surface glycoproteins (ISG) that do not antigenically shift and are present in the bloodstream form of all *T. brucei* isolates examined (Burgess and Jerrells, 1985; Tran et al., 2006, 2008; Ziegelbauer and Overath, 1992; Ziegelbauer et al., 1995). For this reason, ISG have been targeted as markers for diagnosis of HAT (Tran et al., 2008; Ziegelbauer and Overath, 1992).

The present study targeted three ISG, namely ISG64, ISG65, and ISG75, with the goal of generating panels of single chain fragment variable (scFv) that could be further used to develop an immunoassay for parasite detection. IgG or IgM antibodies specific to ISG have not been useful for detection of *T. brucei* cells in patient samples, in part because of their hypothetical inability to penetrate the densely packed VSG shield on living trypanosomes. To this end, efforts are aiming to generate the scFv antibodies specific to ISG64, ISG65, and ISG75. Because scFv antibodies are much smaller in size (~32–38 kDa) than immunoglobulin (commonly IgG or IgM) antibodies, they are hypothesized to be capable of penetrating the VSG layer and binding to ISG.

ScFv antibodies are fragments derived from IgG or IgM antibodies containing a single variable heavy (V_H) domain and a single variable light or kappa (V_L or V_K) domain connected via a flexible amino acid linker. A library of 10^9 non-immune human scFv antibodies displayed on the surface of the yeast *Saccharomyces cerevisiae* has been used as a source of antigen-specific scFv (Feldhaus et al., 2003). For use in functional assays, scFv isolated from the library can be cloned into expression vectors for production in heterologous hosts such as *Escherichia coli* or yeast and the soluble scFv purified for use in biological assays (Boder and Wittrup, 2000; Miller et al., 2005; Siegel et al., 2004).

A limitation of yeast-displayed scFv libraries has been the difficulty of generating soluble scFv antibodies as translational reagents. To help address this problem, researchers have compared protein secretion systems (Miller et al., 2005; Shusta et al., 1998) to improve the yield and activity of secreted scFv. For example, thioredoxin A–scFv fusion proteins (TrxA–scFv) have been shown to enhance solubility and folding in the cytoplasm of *E. coli* (Jurado et al., 2002, 2006). More recently, scFv were purified and used as detection reagents by microarray (Seurynck-Servoss et al., 2008).

Despite these improvements, few scFv antibodies derived from yeast-display have been utilized in diagnostic tests. A critical challenge has been the lack of convenient methods for assessing their activities in solution. To accomplish this, soluble scFv are typically paired with pre-existing full-size IgG antibodies in sandwich ELISA assays. The requirement for IgG antibodies generated by immunization of animals partly defeats the purpose of in vitro antibody

selection. To address these shortcomings, assays were developed utilizing yeast-displayed scFv as reagents for both characterizing soluble scFv activities and detecting antigens in the absence of soluble antibodies. These assays were applied for the development of scFv antibodies specific for ISG proteins of *T. brucei*.

Materials and Methods

Materials

Recombinant ISG75 from *T. brucei gambiense* LiTat1.3 was cloned and expressed as described previously (Tran et al., 2006, 2008). Additional recombinant ISG75 as well as recombinant ISG65 and ISG64 from *T. brucei gambiense* were provided by Dr. Mark Carrington (University of Cambridge, UK). These ISG were biotinylated by using the Pierce EZ-Link Sulfo-NHS-LC-Biotin biotinylation kit (Thermo Scientific, Rockford, IL) and biotinylation was quantified by using the Pierce Biotin Quantitation (HABA) Assay (Thermo Scientific) with each ISG antigen containing between 3 and 5 biotins per molecule. Miltenyi Macs Streptavidin microbeads and anti-biotin microbeads were obtained from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Streptavidin-phycoerythrin (SA-PE) and goat-anti-mouse conjugated to fluorescein isothiocyanate (FITC) were obtained from Molecular Probes (Invitrogen, Carlsbad, CA). A 10^9 diverse human non-immune yeast-display library (Feldhaus et al., 2003; Rakestraw et al., 2006; Wang and Shusta, 2005) was a kind gift of Dr. K. Dane Wittrup (Massachusetts Institute of Technology).

Selections From the Yeast-Displayed Human Non-Immune scFv Library

Selections were performed as described previously (Chao et al., 2006; Feldhaus and Siegel, 2004; Feldhaus et al., 2003; Siegel et al., 2004). The first two rounds of magnetic sorting were conducted with a cocktail containing all three biotinylated ISG antigens at 100 nM each. For round 3, the round 2 output was incubated with each biotinylated antigen separately at 100 nM. Antigen-binding yeast cells were detected by incubating with SA-PE, followed by flow cytometric analysis and sorting of the top 1% of PE positive yeast. The sorted yeast cells, constituting the round 3 outputs, were grown on synthetic dextrose casamino acid (SDCAA) minus-His/Ura/Trp agar plates supplemented with penicillin and streptomycin (both at 50 $\mu\text{g mL}^{-1}$). For ISG75, aliquots of yeast cells were incubated separately with 1, 10, and 100 nM biotinylated antigen, sorted, and grown on plates as described above.

scFv Production and Purification

Plasmids were isolated from 21 antigen-selected, FACS-sorted yeast clones, and scFv inserts were PCR-amplified from 10-fold diluted plasmid template as described by Feldhaus et al. (2003) using Phusion Taq DNA Polymerase. Each scFv amplicon was ligated into four vectors: pET28a, pET27b, pET32b (EMD Biosciences, Darmstadt, Germany), and pET102d (Invitrogen). Single transformant colonies were selected and inserts were confirmed for size and directionality by PCR and sequencing. The plasmids were transformed into *E. coli* expression hosts Rosetta BL21 (IDE3) (for scFv in pET27b and pET28a) or Origami BL21 (IDE3) (for scFv in pET32b and pET102d).

ScFv expressed from pET27b were purified as described by Miller et al. (2005). Expressed scFv from pET28a, pET32b, and pET102d were purified using the MagnaHis method as follows: *E. coli* transformants were picked into 2 mL deep well plates and grown in 2 mL LB supplemented with 50 $\mu\text{g mL}^{-1}$ ampicillin. BL21 cultures were induced by adding 0.1–0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) to LB cultures at 0.5–0.75 OD₆₀₀. Cultures were grown for 15–20 h at 188C. Cells were centrifuged and pellets resuspended in 1:100 culture volume of 1 $\times$ Fast Break lysis reagent (Promega, Madison, WI) supplemented with 2 mg mL⁻¹ lysozyme. After 20-min incubation at RT, the lysates were cooled on ice, and DNaseI

and RNaseA were added to 10 $\mu\text{g mL}^{-1}$. Following an additional 10-min incubation on ice, samples were centrifuged for 10 min at 10,000g, and supernatants were decanted. Thirty microliters of MagneHis beads (Promega) were added to each lysate and incubated for 10 min on ice. The Eppendorf tubes were fitted into the MagneHis magnetic rack, and the lysates were removed by aspiration. The particles were washed two times with wash buffer (0.5 M NaCl, 0.05 M NaPO_4 , 0.02 M Imidazole, pH 8.0) before eluting the TrxA–scFv fusion proteins with 50 μL of elution buffer (0.3 M NaCl, 0.05 M NaPO_4 , 0.3M Imidazole, pH 8.0).

Activity of Secreted scFv by Biacore

Biacore assays were performed with the purified TrxA–scFv using a Biacore 3000 instrument, and data were fit using BIAevaluation 3.1 software (GE Healthcare, Piscataway, NJ). Approximately 12,000 RU of mouse anti-TrxA monoclonal antibodies (MAb) was covalently linked to a Biacore CM5 chip using EDC/NHS amine coupling chemistry. TrxA–scFv were diluted 1/10 in Hepes buffered saline containing 0.005% Tween-20 and 0.05 μM EDTA (HBS-EP), and injected onto the chip at a flow rate of 5 $\mu\text{L min}^{-1}$ until 400–1,000 RU of scFv was captured. Cognate unlabeled ISG at a concentration of 500 nM was then injected for 3 min at a flow rate of 40 $\mu\text{L min}^{-1}$.

Epitope Binning Assay for Identifying ELISA-Compatible scFv Pairs

Yeast-displayed scFv were incubated with 100 μL of 200 nM unlabeled ISG75 for 1 h. Unbound ISG75 was then washed away, and yeast incubated with 50 μL soluble W75D4 scFv (0.01 mg mL^{-1} ~220 nM) for an additional hour at RT. Bound W75D4 scFv was detected by adding 100 μL of a 1/1,000 dilution of mouse anti-TrxA MAb (Invitrogen) and incubating for 1 h. This MAb binds to the TrxA domain in the recombinant W75D4 scFv. Following washing, yeast were incubated with goat-anti-mouse FITC MAb (Invitrogen) for 1 h at RT. Yeast were washed again and FITC labeling was measured by flow cytometry. If the secreted scFv bound to sites on ISG75 that were not occupied by the yeast-displayed scFv, then a FITC signal was observed.

Competitive Inhibition Flow Cytometry Antibody (CIFC-Ab) Assay

Activity of soluble scFv were measured by their ability to competitively inhibit binding of cognate antigen to yeast displaying the identical scFv. Ten microlitres of TrxA–scFv (0.01 mg mL^{-1}) was incubated with 100 μL of 20 nM biotinylated cognate antigen for 1 h at RT. The scFv-antigen complex was added to a suspension of yeast expressing the same scFv and incubated for another hour at RT. Unbound antigen was removed by two washes with phosphate buffered saline (pH 7.4) supplemented with 0.5% bovine serum albumin (PBSB). After washing, bound antigen was detected by adding 100 μL of a 1:1,000 dilution of SA–PE, and analyzing PE fluorescence by flow cytometry. A positive CIFC-Ab assay was visible as reduced binding of the biotinylated antigen to the yeast-displayed scFv. Results were expressed as percent inhibition relative to control yeast that were incubated with biotinylated antigen that had not been pre-incubated with soluble scFv. An arbitrary cutoff of threefold inhibition was used to prioritize scFv for future development.

Competitive Inhibition Flow Cytometry Antigen (CIFC-Ag) Assay

Yeast-display scFv clone W75C6 was induced for scFv expression and cell numbers estimated by OD_{600} (1.0 $\text{OD}_{600} = 2 \times 10^7$ yeast per mL). Approximately 10^5 induced yeast cells were incubated for 1 h with 100 μL of 0.23–500 nM unlabeled ISG75 in threefold serial dilutions. Cells were washed two times with PBSB to remove unbound ISG75, and then were incubated with 100 nM of biotinylated ISG75 for 1 h on ice. Following washing to remove unbound biotinylated ISG75, cells were incubated with a 1:1,000 dilution of SA–PE, and PE

fluorescence quantified by flow cytometry. The assay was performed in triplicate using three independent cultures of W75C6 cells.

To calculate percent inhibition, the equation $[\% \text{ inhibition} = 100 (\text{y-mean fluorescence at } X \text{ nM inhibitor} / \text{y-mean fluorescence at } 0 \text{ nM inhibitor}) \times 100]$ was applied. Data were plotted, and y-mean fluorescence calculated using the FlowJo flow cytometry software analysis program (Tree Star, Inc., Ashland, OR). To confirm specificity of the assay, triplicate measurements were made with the same antigen concentration series as above, except that the antigen was an irrelevant protein, PhoS1 from *Mycobacterium tuberculosis* (Braibant et al., 1996). Robustness was assessed by conducting replicate reactions with varying numbers of W75C6 yeast cells or with varying concentrations of biotinylated ISG75.

Results

Selection of yeast clones with antigen-specific binding activity began with two rounds of magnetic particle enrichment followed by one to two rounds of flow cytometric cell sorting. Incubations of the round 3 output with antigen showed significant reactivity to ISG75 (~5% ISG75-positive at 100 nM antigen), slightly less reactivity to ISG65 (6.0% at 200 nM), and slight reactivity to ISG64 (0.12% at 100 nM). Selected yeast clones were tested for binding to cognate antigen at 100 nM. For ISG75, 19 of 24 selected clones were positive for antigen binding. For ISG65, 8 of 24 selected clones were positive. Forty-eight additional clones were subsequently screened for ISG65 reactivity, from which another 16 antigen-binding clones were isolated. Twenty out of 24 ISG64 clones were positive for binding, but in contrast to ISG65- and ISG75-binding clones, most ISG64-binding clones exhibited weak binding or lacked specificity upon confirmatory analysis.

Specificity was assessed by incubating each antigen-binding clone with all three ISG antigens each at 100 nM. Only 3 out of 20 ISG64 clones were specific only to ISG64. Two others bound both ISG64 and ISG65, but not ISG75. For ISG65, 15 out of 24 clones were specific to ISG65, 3 were specific to ISG65 and ISG75, and 5 bound all three ISG. For ISG75, 13 of 19 clones were specific to ISG75, 1 was slightly reactive to ISG65 and ISG75, and 5 were not confirmed to bind any ISG antigen. No ISG75 scFv clones bound all 3 ISG antigens.

Nucleotide sequence analysis of recombinant genes encoding scFv from each antigen-binding clone identified 3 scFv clones that were isolated multiple times from both the ISG64 and ISG65 selections. These clones may bind to common or overlapping epitopes on both antigens. From the sequence data, 8 unique ISG64 clones, 5 unique ISG65 clones, and 8 unique ISG75 clones were prioritized for further analysis. Supplemental Table I (S1) describes all 21 unique antigen-binding clones including their variable heavy and variable light chain gene families, their complementarity determining regions (CDR), and their antigen-binding profiles.

Because some scFv bound multiple ISG antigens, amino acid alignments of ISG64, ISG65, and ISG75 were analyzed to assess their similarity. Amino acid sequences of ISG64 and ISG65 are 59% conserved and 37% identical (data not shown). ISG75 exhibited lower conservation (30% and 32%, respectively) and identity (17% and 16%, respectively) with ISG64 and ISG65 (not shown). It is possible that some of the cross reactivity exhibited between ISG64 and ISG65 clones may be due to epitopes common to both antigens.

ScFv genes from all 21 ISG-binding yeast clones were sub-cloned into four *E. coli* expression vectors: pET28a, pET27b, pET32b, and pET102d. The plasmid pET27b adds a N-terminal PelB leader sequence which localizes scFv to the periplasm, thereby facilitating purification (Miller et al., 2005). The plasmids pET32b and pET102d encode an N-terminal TrxA to the expressed proteins. The TrxA fusion enhances expression and cytoplasmic folding of scFv (Jurado et al., 2002, 2006; Seuryneck-Servoss et al., 2008). The vector pET102d was chosen,

in addition to pET32b, to take advantage of additional cloning and expression features. Small-scale expression cultures were performed followed by MagneHis purification. All clones yielded proteins of the predicted molecular weight as determined by SDS-PAGE (data not shown). Many of the scFv formed inclusion bodies in the cytoplasm of *E. coli*, however, only the soluble fractions were tested for activity. SDS-PAGE and anti-6X-His Western analysis showed that many scFv formed multimers, most typically dimers.

The TrxA-scFv were tested by Biacore following capture to an immobilized anti-TrxA MAb. Only one of the 21 TrxA-scFv (W75D4) exhibited cognate antigen binding that was detectable by Biacore analysis. Biacore analysis performed on a concentration series of ISG75 revealed an affinity of 18.5 nM and 33–35% activity (data not shown). The small number (1/21) of active scFv identified by Biacore may reflect the selection process that favors scFv that function well on the surface of yeast cells, but do not necessarily function well in solution. However, in some cases, it may also have been an artifact of the analytical methods used to assess soluble scFv activity. For example, if a high percentage of expressed scFv molecules were inactive, then antigen binding by small numbers of active molecules present in the expressed scFv populations would have been difficult to detect following capture onto Biacore chip surfaces by using anti-TrxA MAb. The standard alternative method for making such assessments, namely ELISA assays with paired IgG antibodies, was not available because preexisting IgG antibodies were unavailable. Therefore, new methods were developed to assess the activities of novel scFv.

A CIFIC antibody (CIFIC-Ab) assay, which is diagrammed in Figure 1 (far left), was developed to assess the activities in solution of scFv expressed in *E. coli*. The assay was performed by incubating purified soluble TrxA-scFv with 20 nM of biotinylated cognate antigen and allowing them to bind to equilibrium. The scFv-antigen mix was then added to a suspension of yeast cells displaying the same scFv on their surfaces. If the soluble scFv is active, then it is expected to bind and occupy the epitope and thereby prevent the yeast-displayed scFv from binding. Flow cytometry was used to quantify this competitive activity as reduced PE-fluorescence relative to a baseline control in which biotinylated antigen was incubated with cognate yeast cells without pre-incubation with purified scFv. Analysis of all 21 scFv in the CIFIC-Ab assay identified 6 scFv that exhibited competitive inhibition above a threshold value of threefold, and 15 that did not (Table I). Although the threshold value was chosen arbitrarily, the quantitative results facilitate the prioritization of relatively active scFv for future development.

To test the specificity of the CIFIC assay, a negative control was performed in which two soluble scFv, W65B3, and W64C4, were incubated with the non-cognate biotinylated antigen ISG75 in solution. Although both of these scFv exhibited CIFIC activity when incubated with their cognate antigens, they failed to inhibit binding of the non-cognate antigen ISG75 to W75D4 yeast (Table I). This result was expected if the inhibition observed with ISG75-specific Abs was antigen specific. Positive controls used W75D4 scFv purified using large-scale HPLC methods and confirmed to be active by Biacore analysis. These scFv exhibited somewhat greater overall activity versus the same scFv in the crude MagneHis purifications (results of two separate HPLC purifications are shown in Table I).

In order to further characterize scFv activities without requiring ELISA or Biacore analyzes, a flow cytometry-based epitope binning assay was developed to rapidly identify scFv pairs that bind to non-overlapping epitopes on an antigen. Figure 1 (center) depicts an epitope binning assay in which unlabeled antigen is captured onto antigen-specific yeast-displayed scFv. Captured antigen is detected by binding a purified TrxA-scFv. The purified scFv can bind the antigen only if it binds a site that is not occupied by the yeast surface displayed-scFv. Bound antigen is then detected by adding mouse anti-TrxA MAb conjugated to FITC.

Figure 2A shows an epitope binning assay in which ISG75 was incubated with three yeast-displayed ISG75-specific clones and then detected by using soluble TrxA–scFv W75D4. Positive results were observed when scFv W75C2 or scFv W75C6 was used as soluble detection probe. Therefore, either of these scFv can be paired with W75D4 in sandwich assays. In contrast, ISG75 bound to yeast W75D4 was not detected using TrxA–scFv W75D4 as a detection probe, as expected given that this antigen typically exists primarily in a monomeric state (Tran et al., 2008). To further confirm that the assay reflects specific interactions, it was repeated using yeast-displayed W75C6 and soluble W75D4, except that three non-cognate antigens, each at 200 nM, were used (Fig. 2B). The results demonstrated specificity to the cognate antigen, ISG75.

The CIFC-Ab and epitope binning assays use flow cytometry to rapidly assess the activities of novel scFv antibodies in solution. An alternative to generating and assessing soluble scFv antibodies is to avoid their use altogether, and rely instead on yeast-displayed scFv to detect antigens in samples. An example is the assay described by Cho et al. (2009) which uses whole yeast immunoprecipitation followed by tandem mass spectrometry. As a simpler alternative to the use of mass spectrometry, a reverse CIFC assay (CIFC-Ag) was developed in which the binding of unlabeled antigen to yeast-displayed scFv was measured by virtue of its competitive inhibition of binding of biotinylated antigen (Fig. 1, right side). As in the CIFC-Ab assay, activity was conveniently measured by flow cytometry. As an example of this method, a CIFC-Ag assay was assembled that utilized yeast-displayed scFv W75C6, which binds specifically to ISG75 with an affinity of 52 nM (estimated using a flow cytometric assay for affinity; Boder and Wittrup, 2000; Siegel et al., 2004). Yeast cells displaying W75C6 were incubated with unlabeled ISG75 at concentrations ranging from 500 nM down to 0.23 nM in threefold serial dilutions. Following binding of unlabeled ISG75, the yeast cells were then incubated with excess biotinylated ISG75. Yeast-bound biotinylated ISG75 was quantified by incubation with SA–PE. As shown in Figure 3, unlabeled ISG75 bound competitively to the yeast-displayed scFv and inhibited binding of biotinylated ISG75 in a concentration-dependent manner. Competitive inhibition was consistently detected at concentrations down to ~2 nM (15 ng/100 μ L) unlabeled ISG75. In contrast, the non-cognate antigen PhoS1 did not inhibit binding of biotinylated ISG75 at any concentration, consistent with antigen specificity (Fig. 3, and raw data shown in Supplemental Fig. 1).

The experiment in Figure 3 used biotinylated ISG75 at a concentration of 100 nM and 100,000 yeast cells per incubation. Because competitive inhibition assays can be sensitive to the stoichiometry of antibody probe concentrations, the robustness of the assay was evaluated by varying the number of yeast cells used per antigen incubation. The CIFC-Ag assay exhibited similar performance using 50,000, 100,000, 200,000, or 400,000 yeast cells per incubation (Fig. 4A). All four curves exhibited lower limits of detection (LLD) of at least 2 nM. Similarly, variations in the concentration of biotinylated ISG75 (2, 20, or 100 nM) did not strongly perturb the assay (Fig. 4B).

Discussion

Trypanosoma brucei has a surface layer of VSG that mask invariant proteins, including ISG, from detection reagents and from host immune responses. Structural and density studies have found the VSG proteins to be densely packed (Homans et al., 1989). Researchers are working to develop lower molecular weight affinity reagents that will have a better chance of penetrating the VSG shield and binding conserved VSG or ISG epitopes. Examples include nanobodies (Baral et al., 2006; Saerens et al., 2008; Stijlemans et al., 2004) and RNA aptamers (Goringer et al., 2006; Homann et al., 2006; Menger et al., 2006). In the present study, we generated a panel of scFv antibodies and specific assays to assess scFv activity and identify candidate scFv that can be further developed for diagnostic assays.

Selections were performed on a yeast-displayed human non-immune scFv library to isolate scFv clones that bound specifically to ISG64, ISG65, and ISG75. ISG75 generated the most significant binding response and greatest number of antigen-specific scFv clones. Consistent with the partial homology between ISG64 and ISG65, several clones bound both antigens. Although most (six out of eight) ISG64 clones bound to ISG65 as well, fewer ISG65 clones bound to ISG64. While ISG64 and ISG65 may share common epitopes, ISG65 may have unique dominant epitopes that are not present on ISG64. Epitope mapping would resolve these questions.

A limitation of synthetic scFv antibody libraries has been the generation of translational reagents, specifically scFv that can be isolated in soluble, secreted, and active form for direct application in diagnostic assays. Genes encoding 21 scFv were subcloned into four *E. coli* expression vectors. *Escherichia coli* was chosen as the expression system over *S. cerevisiae* and *Pichia pastoris* expression platforms because *E. coli* was shown in numerous comparisons to be superior for scFv expression (Jurado et al., 2006; Miller et al., 2005; Seurynck-Servoss et al., 2008). Despite these advantages of the *E. coli* system, many scFv predominately form inclusion bodies. There are several protocols for solubilization and refolding of scFv antibodies, however, these are laborious and a unique protocol must be developed for each scFv. The CIFIC-Ab assay may offer a more rapid and economical strategy, in which large numbers of candidate scFv are screened for activity without the requirement for refolding.

Crude scFv purifications were tested for antigen-binding activity in solution by Biacore analysis. This approach identified only 1 active scFv out of 21 scFv tested. Not knowing whether this low frequency of activity was a characteristic of ISG selections or a technical limitation of scFv analysis in the absence of pre-existing ISG-specific antibodies, novel assays were developed which do not require pre-existing antibodies to detect soluble scFv activity. One of these was CIFIC-Ab, a competitive inhibition assay that measures activity of scFv in crude protein purifications. CIFIC-Ab may be able to detect scFv with low percent activity in solutions containing excess inactive scFv due to misfolding or denaturation. In contrast, Biacore analysis, as conducted in this study, used an anti-TrxA MAb to tether scFv to a solid support. If most of the tethered scFv was inactive and the fraction of active scFv on the chip was very low, then there may have been insufficient activity for detectable antigen binding. Similar problems may occur in other assay systems such as ELISA, where scFv is directly or indirectly tethered to solid supports. In CIFIC-Ab, inactive scFv in solution may not interfere with the binding of soluble antigen to yeast-displayed scFv. This would enable the detection of active scFv even when percent activity is low. Such scFv, which might otherwise be rejected following negative Biacore or ELISA results, can be prioritized for further development to improve expression and stability.

Of 21 scFv examined by CIFIC-Ab, 6 exhibited activity that exceeded the threshold value of threefold inhibition relative to the no scFv control. Several other scFv exhibited activity ranging from 1.5- to 2.8-fold inhibition. These may have had activity but they were not prioritized for further development.

A second assay, termed epitope binning, was developed to identify compatible scFv sandwich assay pairs and to further prioritize scFv candidate antibodies. In this assay, a panel of yeast, each displaying a scFv to a given antigen, were bound to unlabeled cognate antigen. The bound antigen was then detected using a soluble purified scFv specific to the cognate antigen. Binding of the soluble scFv was detected by a fluorophore or antibody specific to an epitope tag that exists only on the soluble scFv, and not on the displayed scFv. The antigen was detected only if the soluble scFv binds to a non-competing and non-overlapping epitope from the yeast-displayed scFv. An advantage of the epitope binning assay is that only one soluble antibody is required to perform the assay while the remaining scFv can be yeast-displayed. By

identifying compatible scFv pairs for sandwich assays and other diagnostic test formats, the epitope binning assay can further prioritize scFv for cloning, secretion, and purification. The method can also serve as an alternative to C1FC-Ab, ELISA, and Biacore as a means to identify novel scFv activities in solution.

For some applications, it would be useful to eliminate altogether the need for the extracellular expression and purification of active scFv. This is possible if scFv-displaying yeast cells are used directly as detection reagents. For example, intact yeast have been used to immunoprecipitate antigens from solution for subsequent detection by mass spectrometry (Cho et al., 2009). The C1FC-Ag assay reported here follows a similar strategy but eliminates the need for separate detection methods such as mass spectrometry. Using the C1FC-Ag assay, yeast cells which displayed a scFv with an estimated affinity of 52 nM detected unlabeled antigen in solution at concentrations down to 2 nM. This level of sensitivity is comparable to that which might be expected of an ELISA that utilizes an antibody with similar affinity. The C1FC-Ag assay was robust, in that probe concentrations could be varied without strongly affecting assay performance.

All three flow cytometry-based methods described in this report were easy to set up and inexpensive to perform. They required no pre-existing soluble antibodies and little in the way of optimization. They were fast, requiring less than 2 h to complete. These methods may facilitate the development and utilization of scFv antibodies derived from yeast-display and other in vitro selection methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr. Mark Carrington for providing recombinant ISG64, ISG65, and ISG75 antigen for this study. We also thank Magdalena Radwanska for helpful input throughout the project. This work was supported by a grant from the Foundation for Innovative New Diagnostics (FIND, Geneva, Switzerland), by NIAID grant R21 AI0744571 to S.A.G., and by NIAID grant U01AI082186 to G.A.C.

Contract grant sponsor: Foundation for Innovative New Diagnostics (FIND, Geneva, Switzerland)

Contract grant sponsor: NIAID

Contract grant numbers: R21 AI0744571, U01AI082186

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Figure 1. Schematic diagram of assays employed in this study. The three flow cytometry-based methods for assessing soluble scFv activities and detecting antigens in solution are diagrammed. Positive results are depicted in each case. In CIFIC-Ab and CIFIC-Ag assays, positive results occur when unlabeled soluble scFv antibodies or unlabeled antigen, respectively, competitively inhibit binding of biotinylated antigen to yeast cells. In the epitope binning assay, a positive result is detected when distinct epitopes are bound by distinct yeast-bound and soluble scFv, resulting in a scFv antibody “sandwich” detectable by appropriate monoclonal antibodies (MAb). SA-PE, streptavidin–phycoerythrin.

Figure 2. Epitope binning assay. **Panel A:** Epitope binning assays using three different yeast-displayed ISG75-specific scFv clones. Bound ISG75 antigen was detected by adding soluble TrxA-fused W75D4 scFv. The TrxA-W75D4 scFv was detected by adding FITC-tagged anti-TrxA monoclonal IgG antibody. The no-antigen control used yeast-displayed W75C6 and soluble TrxA-W75D4. Means and standard deviations of two experiments are shown. **Panel B:** Specificity of the assay for ISG75. Yeast-displayed W75C6 scFv was used as the capture scFv and soluble W75D4 TrxA-scFv was the detection scFv. Yeast were incubated with 200 nM concentrations of the cognate antigen, ISG75, and three non-cognate antigens.

Figure 3. Competitive inhibition flow cytometry antigen assay (CIFC-Ag). Yeast cells displaying W75C6 scFv were incubated with a concentration series of unlabeled ISG75, washed, and then incubated with excess biotinylated ISG75 followed by SA-PE. To evaluate specificity, the assay was also conducted using a non-specific potential inhibitor, PhoS1. Each line represents the mean and standard deviation of three independent trials.

Figure 4.

Robustness of the CIFIC-Ag assay. **Panel A:** Four CIFIC-Ag assays using varying numbers of yeast cells per antigen incubation. In this assay, a concentration of 100 nM biotinylated ISG75 was used. **Panel B:** Using concentrations of biotinylated antigen from 2 to 100 nM result in very similar graphs with identical lower limits of detection. All of these assays were performed with the ISG75-specific yeast clone W75C6.

Table 1

Competitive inhibition flow cytometry antibody activity (CIFC-Ab) assay.

Yeast scFv	Soluble scFv	Antigen	Fold inhibition	Vector	Interpretation
W75D4	No scFv	ISG75	0.0	n/a	Negative control (no scFv)
W75D4	solW65B3	ISG75	0.9	pET27b	Negative control (non-cognate scFv)
W75D4	solW64C4	ISG75	1.0	pET32b	Negative control (non-cognate scFv)
W75D4	solW75D4	ISG75	4.8	pET32b	Positive control (cognate scFv); purif. 1
W75D4	solW75D4	ISG75	6.4	pET32b	Positive control (cognate scFv); purif. 2
W64C1	Not tested	n/a	n/a	n/a	n/a
W64C4	solW64C4	ISG64	3.1	pET32b	Active scFv
W64C7	solW64C7	ISG64	3.1	pET102d	Active scFv
W64D1	solW64D1	ISG64	1.3	pET102d	No active scFv
W64D2	solW64D2	ISG64	1.0	pET102d	No active scFv
W64D9	solW64D9	ISG64	1.4	pET102d	No active scFv
W64D10	solW64D10	ISG64	0.2	pET102d	No active scFv
W64D11	solW64D11	ISG64	1.6	pET102d	No active scFv
W65A4	solW65A4	ISG65	1.8	pET32b	No active scFv
W65p2D4	solW65p2D4	ISG65	2.4	pET27b	No active scFv
W65B3	solW65B3	ISG65	4.8	pET27b	Active scFv
W65p2A3	solW65p2A3	ISG65	1.7	pET32b	No active scFv
W65B12	solW65B12	ISG65	3.3	pET102d	Active scFv
W75C2	solW75C2	ISG75	3.7	pET27b	Active scFv
W75C3	solW75C3	ISG75	2.6	pET27b	No active scFv
W75C5	solW75C5	ISG75	1.3	pET32b	No active scFv
W75C6	solW75C6	ISG75	1.1	pET32b	No active scFv
W75C7	solW75C7	ISG75	1.5	pET102d	No active scFv
W75C12	solW75C12	ISG75	1.1	pET32b	No active scFv
W75D4	solW75D5	ISG75	3.5	pET32b	Active scFv
W75D6	solW75D6	ISG75	1.7	pET32b	No active scFv