THE PROTEIN SOCIETY

Selection of recombinant anti-SH3 domain antibodies by high-throughput phage display

Haiming Huang,[1,2†] Nicolas O. Economopoulos,[1,2†] Bernard A. Liu,[3,4] Andrea Uetrecht,[1,4] Jun Gu,[1,2] Nick Jarvik,[1] Vincent Nadeem,[1,2] Tony Pawson,[3,4] Jason Moffat,[1,4] Shane Miersch,[1,2*] and Sachdev S. Sidhu[1,2,4†*]

[1]The Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, Ontario, Canada M5S 3E1
[2]Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario, Canada M5S 3E1
[3]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, University of Toronto, Toronto, Ontario, Canada M5S 3E1
[4]Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada M5S 3E1

Received 3 July 2015; Accepted 26 August 2015
DOI: 10.1002/pro.2799
Published online 1 September 2015 proteinscience.org

Sachdev Sidhu is the recipient of the Protein Society 2015 Christian B. Anfinsen Award.

Abstract: Antibodies are indispensable tools in biochemical research and play an expanding role as therapeutics. While hybridoma technology is the dominant method for antibody production, phage display is an emerging technology. Here, we developed and employed a high-throughput pipeline that enables selection of antibodies against hundreds of antigens in parallel. Binding selections using a phage-displayed synthetic antigen-binding fragment (Fab) library against 110 human SH3 domains yielded hundreds of Fabs targeting 58 antigens. Affinity assays demonstrated that representative Fabs bind tightly and specifically to their targets. Furthermore, we developed an efficient affinity maturation strategy adaptable to high-throughput, which increased affinity dramatically but did not compromise specificity. Finally, we tested Fabs in common cell biology applications and confirmed recognition of the full-length antigen in immunoprecipitation, immunoblotting and immunofluorescence assays. In summary, we have established a rapid and robust high-throughput methodology that can be applied to generate highly functional and renewable antibodies targeting protein domains on a proteome-wide scale.

Keywords: phage display; antibodies; SH3 domain; high throughput method

Introduction

Since its inception almost 40 years ago, hybridoma technology has been the dominant method for antibody production.[1] However, the technology suffers from fundamental drawbacks that have limited antibody-based reagents from reaching their full potential, especially in the post-genomic era.[2–4] First, hybridomas are derived from immunization, which requires animal manipulation and facilities that are cumbersome and cost-prohibitive on a large scale. Second, hybridoma methods do not facilitate facile genetic manipulations and antibody engineering, because they provide the antibody protein but not the

Additional Supporting Information may be found in the online version of this article.

†The authors wish it to be known that, in their opinion, the first 2 authors should be regarded as joint First Authors

Grant sponsor: Canadian Institutes for Health Research; Grant number: MOP-93684.

*Correspondence to: Shane Miersch, The Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, Ontario, Canada M5S 3E1. E-mail: shane.miersch@utoronto.ca and Sachdev Sidhu, The Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, Ontario, Canada M5S 3E1. E-mail: sachdev.sidhu@utoronto.ca

encoding DNA. This complicates the alteration or improvement of antibody properties by requiring prior conversion of the genetic material encoding the antibody molecule to a recombinant form. Third, hybridoma throughput is limited by both the immunization process and the significant investment required for automating the various aspects of clone development.[2] Overall, antibody production by hybridoma technologies is constrained by these inherent limitations and fails to meet the massive and urgent demand for high quality and cost-effective antibody reagents in the genomics era. Consequently, a cost-effective, high-throughput (HTP) method to rapidly generate renewable antibodies with quality comparable to or better than those generated from hybridomas would be invaluable to the life science community.

To bypass animal immunization and hybridoma generation, man-made, synthetic antibody libraries were first proposed over 20 years ago.[5–7] Uniting combinatorial DNA and phage display technologies, it was shown that synthetic antibody libraries can be generated in bacteria and displayed on bacteriophage for use in binding selections *in vitro*.[5] *In vitro* selection enables tailored binding conditions to derive antibodies with properties that could not be obtained by conventional techniques.[4,8] Further, because the stringency of selection can be controlled during phage selection, the affinities of synthetic antibodies from modern repertoires are comparable to, or even higher than, those from secondary immune responses.[8]

Since their first application,[9] tight and specific synthetic antibodies have been generated against proteins,[10] haptens,[11] lipids,[12] and nucleic acids[13] for the purposes of detection, diagnosis and potential clinical applications. Because phage display technology can be adapted to a 96-well format, hundreds of antigens can be handled in parallel,[14] and the entire selection process can take as little as two weeks. This approach promises to dramatically increase throughput and studies with full-length protein antigens have demonstrated that generating synthetic antibodies on a proteome-wide scale is an achievable goal.[15] Full-length antigens are not, however, the only route to antibodies that recognize full-length protein. Previous studies have shown that antigens can also be obtained from the cloning and expression of either expressed sequence tag-encoded polypeptides[16] or small modular domains.[17,18] Encoding the modular subunits of proteins as domains fused to a common tag can enhance solubility and stability[19,20] and can also facilitate the parallelization of antigen purification and antibody selection. Modular domain antigens are suitable for selections using phage-displayed antibody libraries and can be used to isolate antibodies that specifically interact with not only the target antigen, but also, the native full-length protein.

To realize the full potential of antibody generation by phage display, we developed a high-throughput pipeline that allows all steps from antigen production through antibody characterization to be performed in a 96-well format. We used the pipeline to generate hundreds of synthetic antigen-binding fragments (Fabs) targeting a large set of human Src Homology 3 (SH3) domains - members of a family of polyproline-recognition domains that play critical roles in cytoskeletal organization, endocytosis, and cell signaling.[21,22] Representative Fabs were shown to recognize full-length proteins containing SH3 domains with affinities and specificities comparable to those of commercial hybridoma antibodies. Most importantly, the pipeline can be applied to any family of modular, folded domains and can thus be scaled to generate highly functional and renewable antibodies to broad segments of the proteome.

Results

HTP selection of anti-SH3 fabs

To test our high-throughput synthetic antibody generation system, we picked 110 diverse human SH3 domains (Supporting Information Table S1) as antigens. We employed our HTP protein expression and purification pipeline to purify the antigens as hexa-His-tagged GST-SH3 (His6-GST-SH3) fusions and resolved the purified proteins by SDS-PAGE to verify correct size, purity and yield (Supporting Information Figure S1). In total, 84 of the 110 His6-GST-SH3 proteins were purified in a form suitable for use as antigens in phage display selections. The remaining 26 proteins either underwent partial proteolysis or could not be purified due to poor expression or insolubility. The yields of the purified proteins ranged from 20-200 μg from 3 mL of bacterial culture, as estimated by Bradford assay. The purified proteins were employed directly as antigens for phage display selections.

Selections were performed in a 96-well format using Library F, a highly diverse (3×10^{10} unique members) synthetic Fab-phage repertoire.[23] The library phage pool was first incubated with wells coated with GST to remove non-specific Fab-phage and subsequently was transferred to the selection plate, in which each well was coated with a different His6-GST-SH3 protein. Non-binding Fab-phage particles were removed by washing and the remaining bound clones were eluted and amplified by direct incubation with *E. coli* XL1-Blue, allowing for further rounds of screening to enrich for Fab-phage with specificities of interest. Population enrichment was determined by performing ELISAs after round 4 and successful enrichment was defined as an ELISA absorbance (OD_{450}) ratio greater than 3 for Fab-phage binding to target protein relative to the GST negative control. Based on these criteria, enrichment was observed for binding selections against 58 of the 84 (70%) successfully purified antigens.

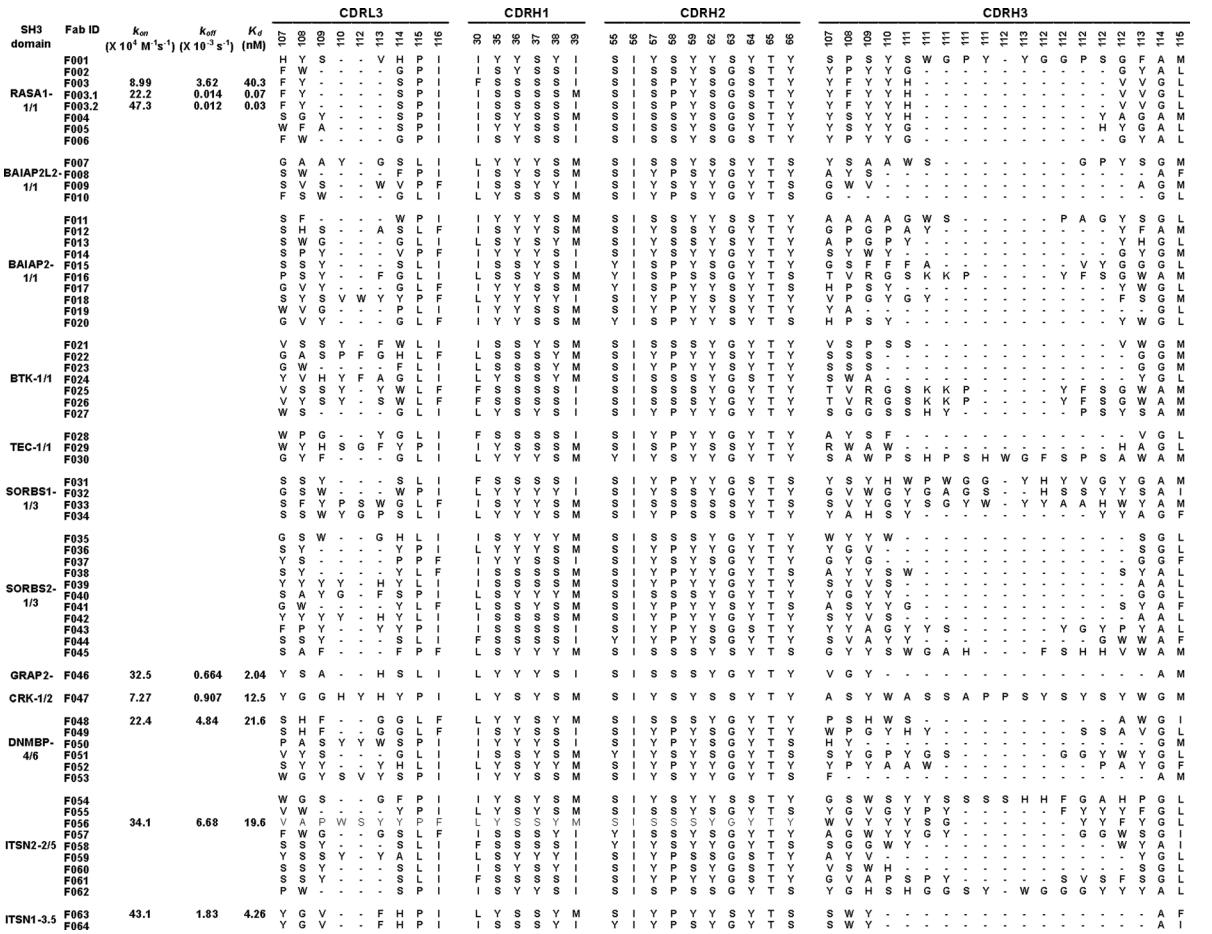

Figure 1. CDR sequences and binding kinetics of Fabs. Positions randomized within each of the CDRS are shown at the top of each column numbered according to IMGT standards.[24] Each Fab is identified with a unique identifier (Fab ID #) used throughout the manuscript. For each SH3 domain antigen, abbreviated protein codes corresponding to the Uniprot recommended gene codes are provided to unambiguously identify the parent protein, and its position relative to the total number of SH3 domains in the parent protein is denoted numerically (e.g., ITSN2-2/5 represents the second of five SH3 domains in the protein ITSN2). Binding parameters (k_{on}, k_{off}, K_d) were determined from kinetic analysis of Fabs binding to immobilized antigens measured by surface plasmon resonance. Sequences are shown for Fabs raised against 12 antigens discussed in detail in the text and other Fab sequences are shown in Supporting Information Figure S2.

For each of the 58 positive selections, we analyzed 12-24 clones by phage ELISA to assess specific binding to antigen at the clonal level. In total, 1236 clones were assayed for binding to their cognate antigens and for non-specific binding to three irrelevant GST fusion proteins. Positive clones were defined as having a cognate antigen ELISA signal at least 10-fold greater than the signal against any of the three negative controls, and by this criteria, we identified 582 positive clones. DNA sequence analysis revealed 216 unique Fab-phage clones (Fig. 1, and Supporting Information Table S1 and Figure S2), each of which bound specifically to one of the 58 SH3 domains.

Synthetic fabs are highly specific

To further assess specificity, we asked whether isolated synthetic clones could distinguish between homologs within the same family amongst our set of human SH3 domains. ELISAs with 35 Fab-phage clones (Fig. 2) were used to assess binding to three pairs of homologous SH3 domains (BAIAP2-1/1 and BAIAP2L2-1/1, 71% sequence similarity; BTK-1/1 and TEC-1/1, 79% sequence similarity; SORBS1-1/3 and SORBS2-1/3, 85% sequence similarity). Only one of four Fabs selected for binding to BAIAP2L2-1/1, also recognized BAIAP2-1/1 [Fig. 2(A)]. Conversely, nine of the ten clones selected for binding to BAIAP2-1/1 did not recognize BAIAP2L2-1/1 while one exhibited comparable binding to both antigens [Fig. 2(B)]. We obtained three clones each for BTK-1/1 and TEC-1/1, and all six were highly specific for their cognate antigen in comparison with the respective homolog [Fig. 2(C,D)]. Three of four clones selected for binding to SORBS1-1/3 did not recognize SORBS2-1/3 while one exhibited some cross-reactivity [Fig. 2(E)]. Finally, seven of 11 clones selected for binding to SORBS2-1/3 were highly specific while three exhibited comparable binding to both homologs and one exhibited mild cross-reactivity with SORBS1-1/3 [Fig. 2(F)]. In summary, 28 of 35 clones

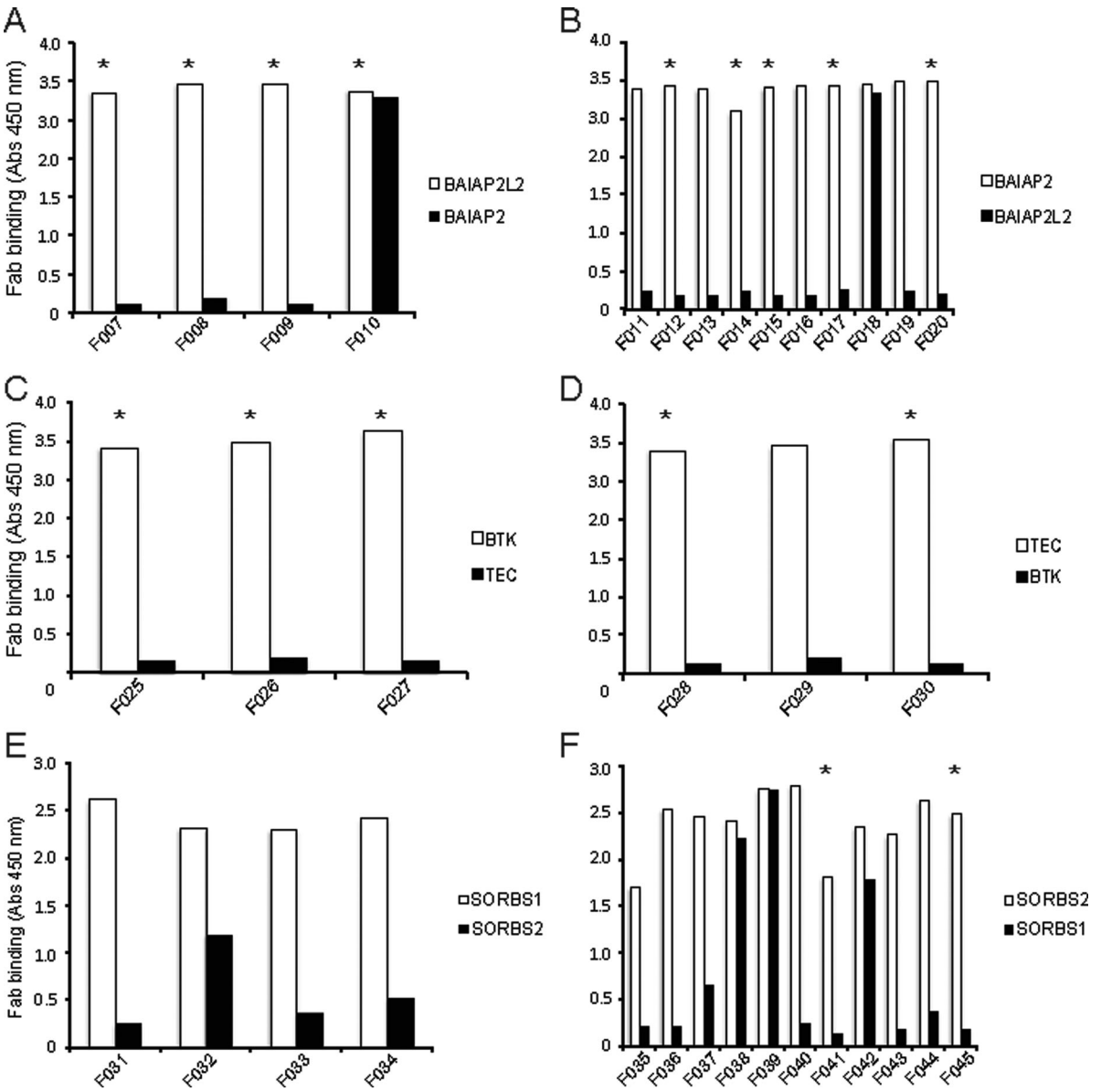

Figure 2. Evaluation of Fab specificities. The binding specificities of Fab-phage clones were evaluated by phage ELISAs. Signals were measured for Fab-phage binding to cognate SH3 domains (*white bars*) or homologous SH3 domains (*black bars*). Asterisks denote Fab-phage exhibiting high affinities by competitive phage ELISAs ($IC_{50} < 50$ n*M*).

exhibited high specificity when assayed for binding to close homologs.

Synthetic fabs exhibit high affinities

To assess affinities, we focused on Fabs raised against SH3 domains of biological interest (GRAP2-2/2, CRK-1/3, DNMBP-4/6, RASA1-1/1, ITSN2-2/5 and ITSN1-3/5). We first used competitive phage ELISAs as a rapid means of estimating affinities without the need for Fab purification.[25] The binding of Fab-phage to immobilized antigen was measured in either the absence or presence of 50 n*M* solution-phase antigen. In this assay format, a greater reduction of signal in the presence of solution-phase antigen is indicative of higher affinity. We used the competitive phage ELISA data to rank order Fab-phage against each antigen on the basis of estimated affinities (data not shown).

For each antigen, we chose the Fab with the highest estimated affinity by competitive phage ELISA for further characterization as a purified protein. To expedite the protein expression process, we used single-stranded site-directed mutagenesis[26] to convert the phage display vector into a Fab expression vector by inserting a stop codon between the heavy chain and the gene-3 minor coat protein, thus avoiding the need for sub-cloning into a new expression vector. In addition, Fab purification was facilitated by using Protein A resin, which binds to the heavy-chain variable domain framework used for the construction of our Fab-phage library.[23] Thus, we were able to rapidly

purify milligram quantities of high affinity Fabs for detailed analysis of binding kinetics by surface plasmon resonance (SPR). The six Fabs were assayed for binding to all six antigens and each Fab only exhibited detectable SPR signals against its cognate antigen, confirming high specificity binding. Moreover, the SPR analysis showed that the Fabs all bound with high affinities in the low double-digit to single-digit nanomolar range (Fig. 1). In our experience, conversion of monovalent Fabs to bivalent IgGs typically results in at least ten-fold enhancement of affinities due to avidity effects, and thus, these Fabs should yield IgGs with affinities in the single-digit or sub-nanomolar range.

Affinity maturation

We also explored the use of a potential HTP method for affinity maturation of Fabs to enable affinity optimization in a rapid manner. We applied the method to the highest affinity anti-RASA1-1/1 Fab derived from the naïve library, but importantly, the method can be applied to any Fab of interest or even to a pool of binding Fab-phage without knowledge of the Fab sequence. The method uses a mutagenesis strategy in which single-stranded DNA purified from phage particles can be used directly as the template for library selection. Moreover, the mutagenic oligonucleotides used to introduce diversity into the affinity maturation library are generic sequences that can be used with any Fab-phage template or pool from the naïve library. Specifically, we diversified positions in CDR-H1 and CDR-H2 using the same design principles that were applied to the initial naïve library,[23] because these CDRs often act as auxiliary binding elements that enhance the binding of Fabs in which CDR-H3 and CDR-L3 provide the major antigen contacts. We reasoned that targeting these regions would be useful for the optimization of existing antigen-binding sites.

Following four rounds of binding selections, 96 positive clones were subjected to competitive phage ELISAs in the presence of 10 n*M* solution-phase antigen and two clones (F003.1 and F003.2) that exhibited complete inhibition of binding were subjected to DNA sequence analysis. The two Fabs contained one or two changes relative to the parental sequence of CDR-H1, including a common Phe to Ile mutation at position 30 (Fig. 1). Remarkably, both Fabs exhibited affinities in the low picomolar range and the Fab F003.2 with the single F30I mutation exhibited the highest affinity ($K_D = 0.026$ n*M*), which represented an approximately 1500-fold improvement over the affinity of the parental Fab ($K_D = 40$ n*M*). Thus, it is possible to further optimize the affinities of our synthetic Fabs using a method that could be potentially adapted to a HTP format, because it does not require knowledge of the Fab sequence, it can be applied to either single Fab-phage clones or Fab-phage pools, and it uses phage-derived DNA and generic mutagenic oligonucleotides for library construction.

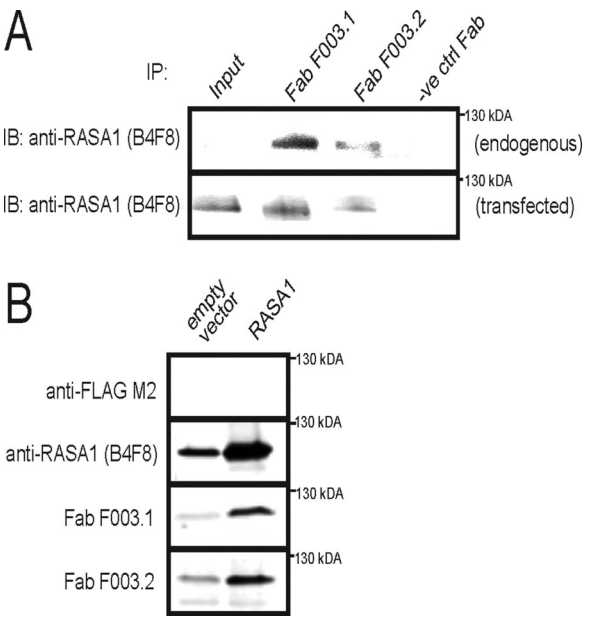

Figure 3. Evaluation of anti-RASA1 fabs in IP and western blot applications. (A) Fabs F003.1 and F003.2 (but not a negative control Fab) immunoprecipitated full-length RASA1 protein from lysates of both untransfected HEK293T cell lysates and from lysates of HEK293T cells transfected with a vector designed to over-express RASA1. Immunoprecipitated RASA1 (MW ~116 kDa) was visualized using a commercial anti-RASA1 antibody (B4F8). (B) Fabs F003.1 and F003.2, and the commercial anti-RASA1 antibody B4F8, recognize a common 116 kDa species corresponding to the molecular weight of full-length RASA1 in western blot experiments with SDS-PAGE of cell lysates from untransfected HEK293T cells or HEK293T cells transfected with a vector designed to over-express RASA1.

Application of synthetic fabs in cell-based assays

We assessed the utility of our high affinity anti-RASA1-1/1 Fabs (Fig. 1, F003.1 and F003.2) in three major cell-based assays: immunoprecipitation, western blotting and immunofluorescence. For detection of the Fab protein, we took advantage of a FLAG epitope tag that was fused to the C terminus of the light chain. However, it should be noted that any tag of interest can be readily added to the Fabs by site-directed mutagenesis, or alternatively, the Fabs can be converted rapidly to full-length IgGs by sub-cloning into appropriate mammalian expression vectors.

Both Fabs were able to immunoprecipitate full-length RASA1 from either untreated endogenously expressing HEK293T cells as well as from HEK293T cells transiently transfected with a vector designed for over-expression of exogenous full-length RASA1 [Fig. 3(A)]. The Fabs were also effective in western blotting experiments, as they detected both over-expressed and endogenous RASA1 in lysates from HEK293T cells

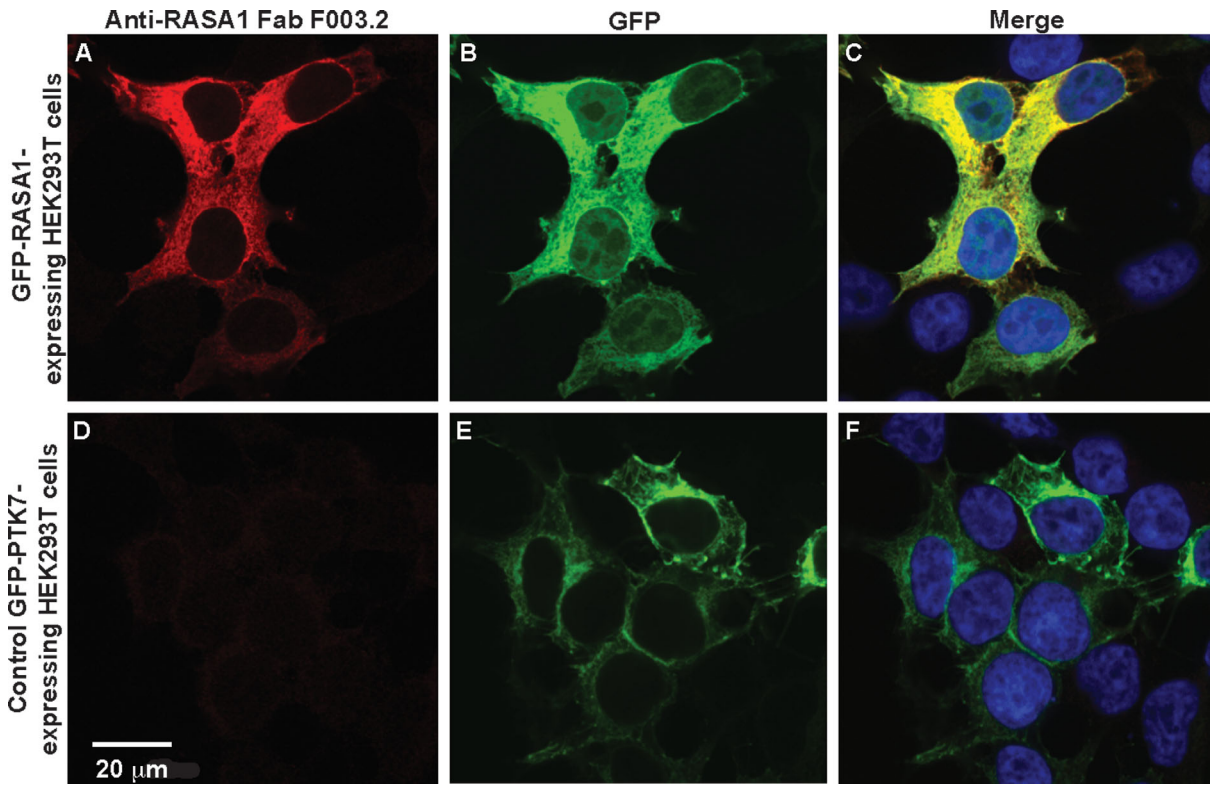

Figure 4. Evaluation of anti-RASA1 Fab F003.2 in immunofluorescence applications. HEK-293T cells transfected with a vector for expression of a GFP-tagged full length RASA1 protein were stained with Fab F003.2 and an anti-FLAG Alexa Fluor 647 secondary (recognizing the FLAG tag on the C-terminus of the Fab light chain). Fluorochrome emission from Fab F003.2 staining of cells transfected with the RASA1 expression construct is shown in "A" (red), emission from the GFP tag shown in "B" (green) and an overlay of the two images shown in "C." The resultant yellow staining pattern observed upon merger of the two images serves as evidence of co-localization between the GFP fusion tag on RASA1 and Fab F003.2 bound to RASA1, confirming target specificity. HEK293T cells transfected with a vector for expression of a negative control GFP-tagged protein (PTK7) were stained as above. In contrast to the staining observed in cells expressing RASA1, no red fluorochrome emission was observed in cells incubated with Fab F003.2 (panel D), despite that GFP emission confirms robust expression of the control PTK7 fusion protein as observed in "E." Further, upon merger of the two images, only green pseudo-coloured GFP emission is observed in "F," further attesting to the specificity of the anti-RASA1 Fab F003.2 for it's target only. For contrast, cells were counter-stained with DAPI and shown in the overlays (C and F).

transfected with a vector designed to express full-length RASA1 or an empty vector, respectively [Fig. 3(B)]. Although a commercial anti-RASA1 (B4F8) antibody exhibited more intense bands, the intensities are not directly comparable because detection of the Fabs necessitated the use of an additional anti-FLAG antibody prior to detection with a labeled goat-anti-mouse secondary antibody, while the commercial IgG could be detected directly with the secondary antibody (see Materials and Methods). Furthermore, since Fab-phage are selected on folded domains, rather than peptides, the Fabs are likely to recognize folded epitopes that may only be partially available when immobilized on western blots. Finally, we performed immunofluorescence experiments with Fab F003.2 with transiently transfected HEK293T cells expressing GFP-tagged RASA1. Strong co-localization was observed for the Fab and GFP in cells expressing GFP-tagged RASA1 [Fig. 4(A)] but no staining with Fab was observed in cells expressing an irrelevant GFP-tagged protein [Fig. 4(B)]. Moreover, co-localization experiments confirm an apparent cytosolic localization of RASA1 in accord with previous reports.[27,28] Taken together, these results confirm that synthetic Fabs are tight and specific enough to enable sensitive detection of full-length native proteins in major cell biology applications, including immunoprecipitation, western blotting and immunofluorescence.

Discussion

In an attempt to approximate the number of modular building blocks from which the proteome is composed, Heger *et al*. used an automatic algorithm for domain decomposition.[29] These results, validated against the manually curated PFAM and SCOP databases, can be filtered to limit exclusively to eukaryotic modules for which structural data exists and that exhibit mobility (i.e. found in alternate protein family contexts with variable domain architecture), reducing the number to 327 domain families (in rough agreement with estimates obtained by alternate means[30] and on the order of magnitude

asserted by others).[31] Although this may underestimate the total number of classes of human domains, it does represent a high-confidence, lower limit on the number of autonomously folding domain families within the proteome and, with many families possessing ten to hundreds of unique domains,[32] this represents a sizable portion of the proteome made accessible. To the extent that phage selections are dependent on the display of well-folded protein, and assuming that most proteins bear at least one modular domain, this provides a rough estimate of potentially tens of thousands of full-length proteins made amenable to selections by using domains for isolation of specific antigen binding fragments.

Toward to the aim of developing defined renewable antibodies against all accessible elements of the proteome, we have developed a HTP method for conducting phage-displayed antibody selections completely in a 96-well format, starting with antigen production and culminating with sequencing of individual binding clones. Our method provides a novel option, distinct from previously published methods,[15,17,18] for antigen generation from synthesized expression constructs that obviates the need for access to clone libraries and laborious sub-cloning, thus relieving what is considered a major bottleneck of high-throughput antibody selection. Further, demonstration of the viability of the GST affinity tag in our selection platform broadens the scope of domains amenable for use in selections due to the documented ability of affinity tags to solubilize and stabilize tagged proteins and protein domains[19,20] while maintaining a similar success rate of antibody generation against bacterially expressed antigens.[15,17,18] Although mammalian expression of antigens appears to enhance the selection success rate, [15] the substantial costs associated with obtaining full-length expression constructs and generating antigen in this manner can make this approach prohibitive. Though our methods offer a means of alleviating the bottleneck that antigen generation can present, it becomes clear that, with the rapid rate of antibody selection and necessity of stratifying generated antibodies for functional characteristics, the ability to validate large numbers of antibody clones becomes the rate-limiting step in obtaining high-quality antibodies.

To confirm the utility of isolated Fabs as viable research reagents, the performance of select binders was evaluated in standard immunoassays including western blot, immunoprecipitation and immunofluorescence. Our results show that synthetic antibodies generated for binding to isolated SH3 domains are capable of recognizing the native, full-length SH3-containing proteins with high affinity and specificity.

Due to the accessibility of CDR-encoding genes from phage-displayed Fabs and the consequent portability, ease of modification and versatility, recombinant antibodies have enormous potential to standardize immunochemical inquiry. Despite the description of an increasing number of highly functional recombinant antibodies derived from synthetic libraries,[13,33,34] there is an apparent reluctance toward widespread adoption and use. This reluctance likely stems from the historical reliance upon and familiarity with monoclonal antibodies despite an awareness of the sub-standard performance and consistency of many commercially available antibodies.[35–39]

Nevertheless, recombinant antibody technologies are gradually receiving recognition as essential to overcoming some of the limitations that researchers currently face.[40] With the accessibility of antigen and antibody sequences and transparency of validation methods and associated data as described herein, it is hoped that the community will move towards a set of proven, renewable, open-source reagents that will enhance scientific rigour, consistency and accountability.

Materials and Methods

Strains, vectors, and antibodies

Escherichia coli SS320 was used for electroporation during library construction[41] and for SH3 domain production. *E. coli* XL1-blue (Stratagene, La Jolla, CA) was used for phage elution and amplification during binding selections. *E. coli* 55244 (ATCC, Manassas, VA) was used for Fab expression. *E. coli* CJ236 (New England Biolabs, Ipswich, MA) was used for production of dU-ssDNA for site-directed mutagenesis. The vector used for expression of human RASA1 in HEK293T cells was pcDNA3 (Invitrogen). Anti-RASA1 (B4F8) and anti-Myc (9E10) monoclonal antibodies were from Santa Cruz Biotech (Dallas, TX). Anti-CRK monoclonal antibody (610035) was from BD Biosciences (Mississauga, ON). Polyclonal Alexa Fluor 647-conjugated anti-DYKDDDDK antibody (#3916) was from Cell Signaling Technology (Danvers, MA).

Antigen identification and cDNA sub-cloning

All human SH3 domain sequences predicted by InterProScan[42] using profiles from the PROSITE,[43] Superfamily,[44] Pfam,[45] and SMART[46] databases were downloaded from the Ensembl 6 database (version 62).[47] Domain definitions for the same protein predicted by different domain profile sources may not have identical boundaries. Therefore, SH3 domain definitions from all four SH3 profile sources were merged using a generalized suffix tree based algorithm. Pairs of SH3 domain definitions that overlap at least 80% of the length of the shorter sequence are considered to be the same SH3 domain. We identified 320 SH3 domains from 219 human proteins.

DNA fragments encoding 241 SH3 domains were amplified from a human SH3 cDNA library (GeneArt, Inc., Etobicoke, ON) by using the polymerase chain reaction (PCR). The fragments were ligated between SfiI and NotI sites into a custom isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible vector designed

for expression and purification of SH3 domains fused to the C-terminus of glutathione S-transferase (GST) with a hexa-histidine tag (His6 tag) at the N-terminus. DNA fragments encoding for the remaining 79 SH3 domains were synthesized and cloned into the same vector by Genscript, Inc. (Piscataway, NJ). We chose 110 diverse SH3 domains (Supporting Information Table S1) to be used as antigens for antibody production. For ease of reference and economy of space, protein codes for antigens described throughout the manuscript were derived from the standard abbreviated gene code listed in Uniprot and reported in the non-italicized form, as is customary for proteins (e.g. RASA1 (Ras GTPase-activating protein 1) represents the protein expressed from the *RASA1* gene).

HTP antigen production

E. coli SS320 cells were transformed in a 96-well format with individual His6-GST-SH3 expression plasmids and 10 μL of transformed cells in liquid culture were used to inoculate 0.4 mL 2YT media supplemented with 50 μg/ml carbenicillin. The cultures were grown overnight at 37°C with shaking at 200 rpm. A 50 μL aliquot of overnight culture was used to inoculate each well of a 96-deep-well block (Whatman GE Healthcare) containing 1.5 mL 2YT media supplemented with 50 μg/ml carbenicillin. The cultures were grown at 37°C with shaking at 200 rpm. Protein production was induced by the addition of IPTG to a final concentration of 1 m*M* to cultures with an OD_{600} of 0.6-0.8. The cultures were incubated for 24 h at 18°C with shaking at 200 rpm. Bacterial pellets were harvested by centrifugation at 4000*g* and stored at −20°C.

Protein purification was initiated by thawing the frozen bacterial pellets for 5 min at 20°C and resuspending in 250 μL suspension buffer (50 m*M* HEPES pH 7.5, 500 m*M* NaCl, 5 m*M* imidazole, 5% Glycerol, 1 m*M* phenylmethanesulfonyl fluoride (PMSF), 1 m*M* benzamidine). Cells were lysed by adding 750 μL lysis buffer (50 m*M* HEPES pH 7.5, 500 m*M* NaCl, 5 m*M* imidazole, 5% glycerol, 1% TritonX-100, 0.5 mg/mL lysozyme, 40 units benzonase, 1 m*M* PMSF, 1 m*M* benzamidine) and incubating on ice for 15 min. The lysates were centrifuged for 20 min at 4000*g* and 4°C. Supernatants were transferred to a 96-well filter plate (Seahorse Bioscience, North Billerica, MA) containing 150 μL Ni-NTA resin (Qiagen, Valencia, CA) pre-equilibrated with suspension buffer. The plate was shaken at 200 rpm for 1 h at 25°C. The resin in each well was washed three times by adding wash buffer (50 m*M* HEPES pH 7.5, 500 m*M* NaCl, 30 m*M* imidazole, 5% glycerol) and centrifuging in a swing-bucket centrifuge at 1000 rpm for 5 min. The washed columns were incubated with 100 μL elution buffer (50 m*M* HEPES pH 7.5, 500 m*M* NaCl, 250 m*M* imidazole, 5% glycerol) for 5 min at 20°C and proteins were eluted in to a 96-well storage plate by centrifuging at 2000*g* for 2 min at 4°C. Purified proteins were resolved on an SDS-PAGE gel to assess the protein purity, yield and size.

HTP phage display selections

Phage selections and overnight amplifications were performed in 96-well format. Prior to each round, 96-well Maxisorp immunoplates (NUNC, Rochester, NY) were coated with 100 μL (5 μg/mL) of the purified His6-GST-SH3 antigens (selection plate) and incubated overnight with shaking at 4°C to allow for antigen adsorption. In addition to the plates containing immobilized antigens, negative selection plates (mirror plates) were coated with 100 μL (5 μg/mL) GST for rounds 1 and 2 to remove GST binders and non-specific binders.

The selection and mirror plates were blocked at 20°C for 1 h with 200 μL 0.5% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.4. 100 μL of naïve Fab-phage library (3×10^{11} phage particles, round 1) or pH-adjusted phage supernatant (pH 7.6, rounds 2-4) were added to each well of the mirror plate and incubated for 1 h at 20°C. The supernatants were then transferred to the selection plate and incubated for 2 h at 4°C. Unbound Fab-phage were removed by washing the selection plate eight times with PBS, 0.05% Tween-20 (PT) buffer and the remaining clones were eluted by adding 100 μL of actively growing *E. coli* XL1-Blue ($OD_{600} = 0.5$) followed by incubation at 37°C for 30 min with shaking at 200 rpm. M13KO7 helper phage were added to each well at a final concentration of 1×10^{10} pfu/mL and the plates were incubated for 45 min at 37°C with shaking at 200 rpm. Phage were amplified by transferring the contents of each well to 2YT media supplemented with carbenicillin (50 μg/mL) and kanamycin (25 μg/mL) and incubating overnight at 37°C with shaking at 200 rpm. Bacteria were removed by centrifugation at 4000*g* and phage-containing supernatants were pH-adjusted by adding one-tenth volume of 10× PBS and used for the next round of selection. Enrichment of Fab-phage for each target was examined by pooled phage ELISA described previously,[25] using GST as negative control. Positive selections were defined by an ELISA signal ratio greater than 3 for binding to His6-GST-SH3 versus binding to GST.

Phage ELISA screens

Single clones from positive selection pools were isolated to confirm binding to their cognate SH3 antigens by Fab-phage ELISA.[25] Phage-containing supernatant (10 μL) was used to infect 90 μl actively growing *E. coli* XL1-Blue cultures. Infected cultures were plated on LB agar plates supplemented with carbenicillin (50 μg/mL). Single bacterial colonies

were used to inoculate 2YT media supplemented with carbenicillin (50 μg/mL), kanamycin (25 μg/mL) and M13KO7 helper phage (10^{10} pfu/mL). Specificity was assessed by measuring binding of Fab-phage to the cognate His6-GST-SH3 fusion protein and three irrelevant GST fusion proteins as negative controls. For phage ELISA, 50 μL phage solution was added to wells of a 96-well Maxisorp immunoplate (NUNC) coated with either target or negative control protein. After incubation for 1 h at room temperature, plates were washed eight times with PT buffer, incubated with horseradish peroxidase/anti-M13 antibody conjugate (GE Healthcare, 1:5000 dilution) for 30 min at 20°C and washed six times with PT buffer and twice with PBS. Plates were developed with 50 μL 3,3,5,5′ tetramethylbenzidine (TMB) peroxidase substrate (Kierkegaard and Perry Labs, Inc. - Gaithersburg, MD) for 5 min, the reaction was quenched with 50 μL 1.0*M* H_3PO_4 and absorbance was measured spectrophotometrically at 450 nm. Positive binding clones were defined as those exhibiting at least 10-fold greater signal for the cognate antigen in comparison with each of the three negative control proteins. Positive clones were subjected to DNA sequencing to determine the sequences of the variable CDRs of each displayed Fab, as described.[43]

Competitive phage ELISAs

Fab-phage affinities were estimated by competitive phage ELISAs performed in 96-well Maxisorp immunosorbent plates. Wells were coated overnight at 4°C with 50 μL antigen (5 μg/mL) and blocked with PBS, 0.5% BSA for 1 h at room temperature with shaking at 200 rpm. During the blocking step, pH-adjusted phage supernatants (pH 7.6) were diluted five-fold in PBS, 0.2% BSA, 0.05% Tween (PBT) buffer and were incubated without or with antigen (50 n*M* in PBT) for 1 h in 96-well non-binding microtiter plates (Corning). Coated and blocked plates were washed four times with PT buffer and the diluted phage solutions without or with antigen were transferred to the coated wells. After incubation for 20 min at 20°C, plates were washed and signals were developed as described above. The inhibition of binding to coated antigen by 50 n*M* solution-phase antigen was calculated as the ratio of the signal from the well with phage incubated with solution-phase antigen divided by the signal from the well with phage incubated without solution-phase antigen.

Library construction

The library for affinity maturation of the anti-RASA1-1/1 Fab was constructed as described.[23,48] Briefly, the phagemid for phage display of the Fab was used as the template in a mutagenesis reaction with oligonucleotides designed to introduce diversity into the regions encoding for CDR-H1 and CDR-H2. The mutagenic oligonucleotides were designed, as described.[23]

Fab purification and affinity analysis

Phage display phagemids were converted into Fab expression vectors by site-directed mutagenesis to introduce a hexa-His encoding sequence followed by a stop codon between the regions encoding the heavy chain constant domain and P3. Fab proteins were then purified from *E. coli* 55244 cells, as described.[49] Purified Fabs were resolved by SDS-PAGE to verify the correct molecular weight, and protein concentrations were determined by Bradford assay (Bio-Rad) using bovine γ-globulin as a standard. Purified Fabs were used to determine the kinetics of binding to their cognate antigens by surface plasmon resonance (SPR) using the ProteOn XPR36 protein interaction array system (Bio-Rad Laboratories, Inc.), as described.[50]

Mammalian cell culture and transfection

HEK293T cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal calf serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin. For expression of full-length SH3 domain-containing proteins, cells were transiently transfected with appropriate expression vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In brief, 24 h before transfection, the cells were seeded at 6×10^5 cells/mL to be 80–90% confluent at the time of transfection. A mixture of 15 μL Lipofectamine and 10 μg DNA in serum-free media were added to individual wells of a 6-well plate for transfection, and allowed to incubate for 12 h, after which cells were replenished with normal media conditions.

Immunoprecipitation of SH3 domain-containing proteins

Cell lysates were generated 36–48 h post-transfection by washing adherent cells twice with ice-cold PBS followed by the addition of lysis buffer (1% NP-40, 50 m*M* Tris-HCl, 150 m*M* NaCl, 2 m*M* EDTA, 50 m*M* NaF, 10% glycerol). Cells in lysis buffer were scraped and collected from one well of a 6-well plate, sonicated twice for 2 sec, cleared of debris by centrifugation at 15,000*g* for 5 min, precleared with 50 μL of Protein A sepharose resin (GE Healthcare), then divided into three equal volumes for immunoprecipitation. For each immunoprecipitation, 2 μg Fab were added to the lysate and incubated at 4°C for 2 h. A 30 μl aliquot of washed protein A sepharose was added to immunoprecipitate antibody-protein complexes, incubated for 1 h at 4°C, then washed three times with 500 μl lysis buffer. Precipitated proteins (or whole cell lysate controls) were solubilized by the addition of 20 μL SDS-PAGE loading buffer (50 m*M* Tris-HCl pH 6.8, 100mM DTT, 2% SDS, 0.1% Bromophenol Blue, 10%

glycerol) and subsequently resolved on a 12% SDS-PAGE gel in preparation for transfer to a nitrocellulose membrane for western blot analysis.

Western blot analysis

Following SDS-PAGE, proteins were transferred to nitrocellulose membrane by electrophoresis at 100 V and 400 mA for 1 h in transfer buffer (25 m*M* Tris-Base, 192 m*M* glycine, 20% MeOH, pH 8.3). Membranes were blocked overnight at 4°C in 5% non-fat milk in Tris-buffered saline (50mM Tris, 150 m*M* NaCl, pH 7.6). Fabs were added at 1 μg/mL to the membrane and incubated for 1.5 h at room temperature. The membranes were washed three times with Tris-buffered saline, 0.1% Tween-20 and bound Fabs were visualized by detection of a FLAG tag fused to the C-terminus of the light chain by incubation for 1.5 h with anti-FLAG antibody M2 (Sigma, 1 μg/mL). For detection of commercial IgGs, incubation with anti-FLAG antibody M2 was omitted. Washed membranes were incubated with IR-Dye-800 goat-anti-mouse secondary antibodies for 1 h, washed and scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Immunofluorescence

HEK293T cells were plated on glass coverslips and allowed to adhere overnight. Transfections were carried out with Fugene 9 at a ratio of 1:3 (DNA:transfectant) according to the manufacturer's directions and allowed to grow 48 h before fixation. Cells were fixed for 10 min in ice-cold 4% paraformaldehyde, washed twice with PBS, permeabilized for 5 min in PBS, 0.1% Triton X-100 and washed twice with PBS. Cells were blocked with PBS, 5% BSA for 20 min, washed once with PBS and incubated with Fab diluted with PBS, 1% BSA to the indicated concentration for 1 h at room temperature. Stained cells were then washed twice with PBS and incubated with Alexa Fluor 647-conjugated anti-DYKDDDDK antibody (Cell Signaling Technology) (diluted 1:50 in PBS, 1% BSA) for 1 h. Coverslips were washed twice with PBS and mounted on glass slides using ProLong Gold with DAPI (Invitrogen). Coverslips were imaged on a Leica inverted microscope equipped with Quorum WaveFX spinning disc confocal system.

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