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Design and testing of PCR primers for the construction of scFv libraries representing the immunoglobulin repertoire of rats

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Abstract

Rats are widely used as a model in the study of many important diseases, yet their utility in research is limited by the lack of robust technology for the production of rat recombinant antibodies. Here we have identified and compiled putative rat immunoglobulin sequences by bioinformatic analysis of recently available genomic and EST databases, and used this information to design PCR primers to amplify rat V_H and V_L domain coding DNA representing the expressed repertoire of the animal. These primer sets were successfully tested and used to produce a scFv phage display library from rats that had been infected by the blood fluke, *Schistosoma mansoni*. The library was selected for scFvs that bind to extracellular loop epitopes on either of two known immunogenic *S. mansoni* membrane proteins, Sm23 and SmTsp2, both of the tetraspanin protein family. Two unique scFvs were identified that bind to each tetraspanin and shown to have excellent specificity for the target, including recognition of the native tetraspanins produced by the fluke. The primers and methods developed for rat scFvs should be of use to others seeking to characterize the antibody repertoire and/or prepare recombinant antibodies from this model.

1. Introduction

Antibodies are widely used for medical and biotechnological purposes. Since the development of monoclonal antibodies (Kohler and Milstein, 1975) and the subsequent generation of functional fragments of immunoglobulin through recombinant DNA technologies (Better et al., 1988; Bird et al., 1988; Huston et al., 1988; Skerra and Pluckthun, 1988) clonal antibodies have demonstrated a wide range of biological activities and specificities that have proved useful for the treatment of diseases, diagnostics and research. Immunoglobulin (Ig) proteins and genes have been extensively characterized from human and murine B cells (IMGT database, Montpellier, France (Giudicelli et al., 2004) and these models have become the primary source of monoclonal antibodies and recombinant antibodies for research and therapeutic applications. More recently, other immunoglobulin sources have been utilized, such as camel (Davies and Riechmann, 1996), rabbit (Ridder et al., 1995), sheep (Li et al., 2000), chicken (Foord et al., 2007), shark (Schluter et al., 2005), etc., since these sources have been found advantageous for particular applications. Technology exists to re-engineer these antibodies to be indistinguishable from human antibodies by grafting the CDR regions into a human antibody framework (Jones et al., 1986), thus making it easier to develop antibodies using different animal model for human purposes.

Phage display technology has been used since the early 1990s as an alternative to hybridomas for the development of clonal antibodies that recognize specific targets (McCafferty et al., 1990). The main advantages of this methodology compared with conventional monoclonal antibodies are that it is less time consuming, less expensive and permits direct and rapid selection of antibody-based binding agents having the desired target specificity. Specifically, antibody V-domain coding regions are amplified by PCR, usually from a B cell cDNA source, and the V_H and V_L domains are joined together separated by a flexible spacer region. The resulting recombinant single-chain Fv fragments (scFvs) (Bird et al., 1988; Huston et al., 1988) or Fabs (Better et al., 1988) are then fused to a bacteriophage protein such that they become displayed on the surface of the resulting phage. Typically, the antibody repertoire of an immune animal can be represented as antibody fragments of rearranged V_H and V_L domains displayed on a library of phage. Researchers then select the phage-displayed binding agents that have the desired specificity and affinity, usually by “panning” for phage able to bind to the target. The resulting phage contain the DNA encoding the selected scFvs or Fabs. If desired, the CDRs of the single chain antibodies can be recloned into a vector expressing a full size recombinant monoclonal antibody. In this way, the antibody species and isotype of the final product can be selected to maximize the therapeutic or functional properties that are desired.

Animal models are used extensively for studying different diseases and, in some cases, it is useful to obtain clonal antibodies from these models to characterize the immune response or to create useful research reagents. The rat model is widely used for studying hypertension, diabetes, some autoimmune diseases, and also some tumors (see http://rgd.mcw.edu/tools/diseases/disease_search.cgi). Rat is also an unusual “semi-permissive” model of schistosomiasis mansoni, a helminth parasitic disease that affects hundreds of millions of people worldwide. Evidence exists that, unlike the permissive mouse model, rats develop antibodies that contribute to the rejection of adult schistosomes about four to five weeks post-infection (Maddison et al., 1970; Barker et al., 1985). Obtaining clonal antibodies representing these putative “protective” antibodies could lead to identification of schistosomiasis vaccine targets or new therapeutic avenues.

Surprisingly, despite their widespread use as a disease model, rats have not previously been used as a source of coding DNA for the generation of recombinant antibody libraries. Indeed, little information is currently available on rat immunoglobulin sequences (Bruggemann et al., 1986; Dammers et al., 2000). As expected, mouse and rat immunoglobulin gene organization seems to be highly conserved (Bruggemann et al., 1986; Zhao and Hammarstrom, 2003). Based on limited sequence information, the immunoglobulin coding DNA is also well conserved between mice and rats suggesting that PCR primers based on mouse immunoglobulin sequences could successfully amplify many rat V_H and V_L domains. However, using primers based on information from closely related animal models will likely lead to uneven amplification of the V-domains (Maass et al., 2007) and thus create antibody display libraries that don't well represent the source material. We wished to produce a phage display scFv library that represented the antibody V_H and V_L repertoire of rats that were in the act of rejecting an *Schistosoma mansoni* infection. To do this, we used mouse immunoglobulin sequences for homology searches for V-domains in rat genomic and EST databases. Based on this information, we created a set of degenerate primers and used these to produce a library of phage displayed rat scFvs. The success of the library was validated by the identification of multiple unique scFvs against two known schistosome tegumental membrane proteins, Sm23 (Wright et al., 1990) and SmTsp2 (Tran et al., 2006).

2. Materials and Methods

2.1 Vector construction

The phagemid vector JSC was used for preparation of pIII phage display libraries. This vector derives from HQ2-2 which drives pIII expression from a Lac promoter and was previously described (Maass et al., 2007). To create JSC, the signal peptide coding region in HQ2-2 was first replaced by the pelB leader sequence (Better et al., 1988) by insertion of an appropriate PCR product engineered to ligate in frame between the ScaI and AscI sites to create vector JSB. The V_L and V_H cloning sites of JSB were re-engineered using two large oligonucleotides, homologous to each other to leave single-stranded ends appropriate for ligation into SfiI and SgrAI. Following digestion with SfiI and SgrAI, the annealed oligonucleotide was ligated into JSB. Sequence was obtained throughout the regions changed from HQ2-2 to ensure no mutations were present. The complete sequence of JSC was submitted to GenBank (submitted).

2.2. Primer design

Little information has yet been compiled on the amino acid sequences of rat Igs (Gilliland et al., 1996; Dammers et al., 2000). To design a set of oligonucleotide primers for PCR that amplify a high percentage of rat V_L and V_H coding domains, the rat genome and EST sequence databases were investigated for close homologues to mouse V_L or V_H coding sequences. Using the Blast software (<http://www.ncbi.nlm.nih.gov/BLAST/>) we identified more than 100 distinct rat V_H sequences and more than 200 rat V_L sequences that permitted identification of numerous potentially functional V domains: 65 V_Hs with 17 J_Hs, and 77 V_Ls with 26 J_Ls. PCR primers were then designed that should be able to amplify each of these rat V_L or V_H coding domains in at least one primer combination. The primers contained sequence degeneracy at some positions to maximize amplification efficiency while minimizing the creation of stop codons. The presence of some pseudogenes or V-like sequences in our sampling cannot be excluded.

2.3. Rat-scFv library construction

Eight male Fisher rats (~60 g) were infected with about 1000 *S. mansoni* cercariae. Two rats were sacrificed four weeks post-infection and a third rat was sacrificed eight weeks post-infection, and each rat was subjected to liver perfusion (Smithers and Terry, 1965) to recover adult worms. After four weeks, hundreds of worms were recovered while after 7 weeks only a single, immature pair of adult worms was found. These results confirmed earlier reports that the Fischer rats had rejected the vast majority of the infection between four and eight weeks after receiving a large dose of cercariae. Eight weeks post-infection, the remaining five rats received a second challenge infection of 1000 cercariae and were sacrificed 4 weeks later. Serum was also obtained from the rats at this time. Spleens were removed from the immune rats and immediately used or frozen at -80°C. Approximately equal amount of each of the 5 spleens (total of 300mg) were mixed, glass-homogenized and passed through QIAshredder columns (Qiagen). Total RNA was isolated using the RNeasy kit according to manufacturer instruction (QIAGEN). In four separate reactions, a total of 8 ug of total spleen RNA (as estimated by spectrophotometry and electrophoresis) was used for cDNA preparation, using random hexamer and oligo(dT) as primers. First-strand cDNA synthesis was performed using SuperScriptTM II RNase H⁻ reverse transcriptase (InVitrogen) according to the manufacture's protocol. The resulting cDNA was split into 50 tubes (for 24 V_H and 26 V_L PCR amplification reactions) in equal amount, and subjected to amplification: 1 step of denaturation (95°C, 15 min), 30 cycles (95°C, 30s; 60°C, 30s; 72°C, 30s) and a final finishing step (72°C, 10 min). PCR reactions were performed by Hotstartaq DNA polymerase (QIAGEN) using the designed degenerated primers at a concentration of 1 uM each. All V primers were used separately with a mix of the corresponding J primers. The final V_H and V_L PCR products were separately

pooled as a function of primer degeneracy in an effort to form approximately equimolar mixes of the different amplified DNAs.

Amplified V domain DNAs were digested with appropriate restriction enzymes and ligated into similarly digested JSC vector, introducing first the V_L s and then the V_H s to generate the final library. The restriction enzymes for cloning the V_L were NgoMIV and NotI, and for the V_H were AscI and SpeI. The ligated DNA was transfected by electroporation into high efficiency electroporation-competent TG1 cells (Stratagene) following the recommendation of the supplier, and plated on LB-amp plates (100 ug/ml ampicillin) containing 2% glucose. The total number of independent clones obtained following V_L cloning was ~106 and characterization of random clones demonstrated that > 90% contained V_L inserts. DNA was prepared from the V_L library and used as the vector for the V_H cloning. The resulting library contained about 10^7 clones and a sampling of random clones indicated that >90% of the clones contained full length scFvs. Digestion of the amplified scFv DNA from 80 random clones with BstNI indicated that all contained different scFvs (Tomlinson et al., 1992).

Phage were prepared for panning by standard methods (Marks et al., 1992) with some minor modifications. Briefly, 100 mL of LB-amp, 2% glucose were inoculated at 37° C with 10^9 CFU of the amplified rat scFv library and brought to log phase, and then helper phage VCSM13 was added at a multiplicity of infection of ~5. After 30 min incubation, bacteria was recovered by centrifugation and resuspended in LB-amp (100ug/mL)-kan (25ug/mL) for an overnight incubation at 30° C. The next day, supernatant was separated from bacteria and phage were purified by PEG precipitation twice and resuspended in PBS-10% glycerol.

2.4. Expression and purification of Sm23 and SmTsp2 epitopes

DNA encoding the complete, major predicted extracellular loop of the *S. mansoni* immunogenic tetraspanin proteins Sm23 and of SmTsp2 was amplified from adult worm cDNA, cloned into an expression vector and the two encoded proteins were expressed as fusions with maltose binding protein (MBP) using the pMAL protein fusion and purification system (Biolabs). PCR amplification of the Sm23 coding DNA utilized the following oligos sets: Sm23-EC2-1 (5'-CGC GGA TCC GTT GTT TAC AAA GAT AGG ATC-3') and Sm23-EC2-2 (5'-GGG AAG CTT CAC ACC AGG TTG CGT TTT AAG-3'). For SmTsp2, the primers used were: SmTSP2-EC2-1 (5'-CGC GGA TCC GCT ATA GTA GAA AAG CCC-3') and SmTSP2-EC2-2 (5'-GGG AAG CTT CAG ATA GCA TTG AGG TGC G-3'). Amplification was performed as previously described using the Phusion DNA Polymerase (Finnzymes) but using an annealing temperature of 50° C. The amplified products (each about 240 bp) were ligated into pMALp2x vector following digestion with BamHI and HindIII such that the coding DNA was fused in frame to MBP coding DNA. Expression and purification of the pMAL fusion proteins was performed as described by the manufacturer.

2.5. Screening and selection of scFvs that bind to Sm23 or SmTsp2

Selection was carried out by "panning" of scFv-displayed phage libraries for phage that bind to immunotubes (Nunc) coated overnight at 4° C with one of the two MBP fusion proteins, Sm23/MBP or SmTsp2/MBP (10ug/mL each). The tubes were then washed three times with PBS (HyQ phosphate buffered saline (1x) 67mM (PO₄), HyClone), and blocked with 4% non-fat dried milk in PBS (MPBS). A 1 ml suspension of phage in MPBS was prepared containing $\sim 10^{12}$ CFU and incubated in the coated immunotube at room temperature for 1h. The tubes were washed 5 times with PBS containing 0.1% Tween 20 (PBST) followed by 5 times with PBS. For succeeding selections, 20 washes with each buffer were performed. Bound phage were eluted with 1 ml of 100 mM HCl for 10 min, then recovered and neutralized with 0.5 ml of 1 M Tris-HCl, pH 8.0. An aliquot of 1 mL of the eluted phage was used to infect a 10 ml culture of log-phase *E. coli* ER2738 cells (Biolabs). A small aliquot of the infected bacteria

was used in serial dilutions to titrate the number of phage eluted while the remainder was processed as described above to amplify the phagemid DNA for further selection or analysis. Positive clones were identified by ELISA and western blotting. Individual colonies were picked by toothpick and vortexed in 100 μ L dH₂O. The scFvs were amplified by PCR using HotStartTaq DNA polymerase (Qiagen) and analyzed on a 2.5% agarose gel (Mercury). Selected clones were further analyzed by fingerprinting (Tomlinson et al., 1992) using the BstN1 enzyme (Biolabs) and the scFv coding regions of unique clones were sequenced.

2.6. Production of soluble single domain antibodies

Coding DNA for selected scFvs were engineered for expression as soluble proteins fused to six histidines to facilitate purification. The scFv coding DNA was subcloned into the expression vector JSC-His using the same restriction sites as in the library cloning. The JSC-His was constructed essentially by replacing the M13 gene III coding DNA in the JSC vector with hexahistidine coding DNA followed by a stop codon. The following oligonucleotides (oligos): 6His-3 (5'-GAT CCG CTG GAA CCG CGT GCC GCA GCT CAC CAT CAC CAT CAC CAT TAA TGA CA-3') and 6His-4 (5'-TAT GTC ATT AAT GGT GAT GGT GAT GGT GAG CTG CGG CAC GCG GTT CCA GCG-3') were annealed and ligated into JSC digested with BamHI-NdeI. The modified region of DNA was sequenced to confirm correct insertion of the adapter DNA.

E. coli Rosetta-gami (Novagen) and/or SORL (Stratagene) containing the various scFv expression plasmids were grown at 37° C to an optical density of 0.7 at 600 nm. At this point, cells were pelleted and resuspended in LB-amp lacking glucose and containing 0.5 mM IPTG. The cells, induced for scFv expression, were then incubated overnight at 30° C. The cells were pelleted and supernatant scFv was quantified by western blot comparisons to a scFv standard.

2.7. Immunological detection

ELISAs (Nunc Maxisorb plates) and western blotting (BioTrace PVDF membranes (PALL Life Sciences)) were performed using standard procedures. Detection of bound scFvs utilized HRP-conjugated anti-E-tag (GE Healthcare). Detection of mouse or rat immunoglobulins utilized anti-rat-IgG/HRP-conjugated antibody (Zymed) or anti-mouse-IgG/HRP-conjugated antibody (Zymed). ELISA detection was with 3,3',5,5'-tetramethyl benzidine (Sigma) and westerns were developed using the TMB membrane peroxidase substrate system (3-C) (KPL).

3. Results

3.1. Rat scFv primer design and V-domain amplification

As part of an effort to identify antibodies contributing to the unusual ability of rats to reject adult *Schistosoma mansoni*, we sought to obtain a scFv phage display library from schistosome immune rats. Unfortunately, rat scFv display libraries have not been previously reported and information on the coding sequences of rat immunoglobulin has not been compiled in a practical form for use in the design of appropriate PCR primers for amplification of the V_L or V_H coding domains. We thus used the numerous available, closely related, murine V-domain sequences as templates to perform BLAST searches of the rat genome and EST databases for V_L or V_H domain homologues. After analyzing more than 100 complete rat immunoglobulin V-domain homologue sequences it was clear that standard murine V_H and V_L primers

(
http://www.mrc-cpe.cam.ac.uk/index.php?module=pagemaster&PAGE_user_op=view_page&PAGE_id=26
) would likely fail to amplify most of the rat immunoglobulin coding DNAs and/or introduce mutations not present in the selected rat Ig sequences. Thus we designed two sets of degenerate primers for rat immunoglobulins, one for V_L and another for V_H (Table 1).

Each of the V_L or V_H forward oligonucleotide primers (5'-3') was tested in combination with a pool of the four or five corresponding reverse primers by performing PCR amplification on cDNA prepared from spleen RNA obtained from *S. mansoni* immune rats. Because of the strong homology between the different J sequences, using pools of the 3'-5' primers in the PCR reactions should not significantly reduce the repertoire diversity or create scFv expression problems. As shown in the Figure 1A, almost all of the primer combinations yielded a PCR product of the expected size (arrows). In reactions yielding additional products of aberrant size, the proper sized DNA fragment was purified by excision and elution from agarose gels. The PCR products were pooled, attempting to include approximately equal amounts of DNA from each reaction, and then the rat V_L and V_H DNAs were sequentially cloned into the JSC phage display vector to produce a library with a complexity of ~10⁷. More than 90% of the clones had full-length scFv inserts and a random sampling of clones was fingerprinted by digestion with BstN1 to test for evidence of clonal diversity (Fig 1B). As observed, all the tested clones differ from each other. Additional fingerprinting was performed on individual V_L and V_H regions which, together, indicated that the library contained a broad representation of the rat V-domain repertoire (not shown).

The ability of the rat scFvs to be displayed on phage was also examined. Several single clones were induced to produce phage which were then purified and analyzed for the presence of scFv/pIII fusion proteins. Western blotting was performed on extracts of individual phage preparations using an antibody against an epitopic tag (E-tag) that is fused to all pIII proteins expressed from the JSC phagemid vector. The results (not shown) demonstrated that at least 60% percent, and probably more, of the randomly picked clones were efficiently displayed using the phage production methods described in the Materials and Methods. This value is similar or better to those reported for human scFv libraries (Vaughan et al., 1996; O'Connell et al., 2002). The failure to observe scFv/pIII fusion proteins in some clones is likely caused by the poor expression levels of these scFv fusions, or by aberrant or introduced stop codons.

3.2. Validation of the library

Serum from schistosome immune rats has been shown to be highly enriched in antibodies that recognize antigens within the apical tegumental membrane of this parasite (manuscript in preparation). Two schistosome tetraspanin proteins, Sm23 and SmTsp2, were selected to validate the utility of the rat scFv library. These proteins were chosen because they are known to be immunogenic in schistosome infected animals (Wright et al., 1990; Tran et al., 2006) and because they are among the very few integral membrane antigens shown to be present within the host-interactive worm surface tegumental membrane (Braschi et al., 2006; Tran et al., 2006). Four weeks after *S. mansoni* infection, rats have a moderate titer of serum antibodies against both Sm23 and SmTsp2 (Fig. 2A). This was demonstrated by testing the serum for recognition of the major extracellular loops of each tetraspanin protein expressed in recombinant *E. coli* as a fusion protein to maltose binding protein (Sm23/MBP or SmTsp2/MBP). No recognition of these proteins was detected using serum from uninfected rats (not shown).

To identify phage displayed rat scFvs that recognize epitopes on the major extracellular loops of *S. mansoni* tetraspanin Sm23 or SmTsp2, the immune rat scFv library was panned for phage that bind to an equimolar pool of Sm23/MBP and SmTsp2/MBP. After an initial low stringency panning, the selected phage pool was subjected to two high stringency pannings (see Materials and Methods). Employing the same stringency and titer of input phage, we obtained about 100 fold more target binding phage in the third cycle compared to the second cycle, indicating strong enrichment of phage recognizing the target. Random picks from the third cycle of panning were tested by ELISA for their ability to bind to either Sm23/MBP or SmTsp2/MBP. As shown in Fig. 2B, more than 80% of the clones from the third panning cycle produced a

positive signal. Most of the clones clearly and exclusively recognized one or the other of the two MBP fusion proteins indicating that recognition was directed at schistosome epitopes rather than MBP. Clones recognizing Sm23 or SmTsp2 were about equally represented.

Individual positive clones were characterized by BstN1 fingerprinting and those with unique fingerprints were sequenced. The results revealed two unique clusters of rat scFv clones recognizing Sm23/MBP and another two recognizing SmTsp2/MBP. Some sequence variation was found within each of the four clusters but these were almost certainly mutations introduced by PCR after library construction since the V_H and V_L CDR (complementary determining region) sequences were the same. No rat scFvs were found that recognize MBP epitopes. The few clones in the initial screen that showed some recognition of both Sm23/MBP and SmTsp2/MBP proved to be mixtures or artifacts (Fig 2B).

A representative of each of the two Sm23-binding scFv clones (named S1 and S3) and the two SmTsp2-binding scFv clones (T1 and T2) were selected for further study. The amino acid sequences of these four scFvs are shown in Fig. 3. Each has typical V_H and V_L domains separated by the linker region which serves as a flexible spacer to permit the two domains to separately fold into a conformationally native form. BLAST searching finds that the most homologous amino acid sequences within GenBank are other known rat V_H and V_L domains, despite the preponderance of mouse Ig sequences in the database. These four scFvs were each expressed as recombinant proteins lacking the fusion to M13 pIII that exists for the scFvs in phage display vectors (see Materials and Methods). The four soluble scFvs were first tested by western blot for their recognition of either Sm23/MBP and/or SmTsp2/MBP (Fig. 4A). As expected, scFvs S1 and S3 recognize Sm23/MBP but not SmTsp2/MBP while scFvs T1 and T2 have the opposite specificity.

The four scFvs were next tested by ELISA for recognition of Sm23/MBP, SmTsp2/MBP and *S. mansoni* apical membrane extracts (Fig. 4B). As for the western blot, scFvs S1 and S3 scFvs recognize Sm23/MBP while T1 and T2 scFvs recognize SmTsp2/MBP. Importantly, all four of the scFvs also recognize antigens within the apical membrane fraction of *S. mansoni*. When a pool of rat scFvs S1 and S3 was tested for recognition of apical membrane proteins by western blot, a single band of about 25 kD was stained (Fig. 4C). This is precisely the SDS-PAGE mobility expected for Sm23 (Reynolds et al., 1992). Western blotting with a pool of T1 and T2 specifically recognizes an apical membrane protein of similar but not identical molecular weight to Sm23 that is expected to be native SmTsp2.

4.0. Discussion

This study reports the first preparation of a scFv library representing the antibody V_H and V_L repertoire of rats, and the validation of the library through the isolation and characterization of scFvs that bind to antigens recognized by the serum of these rats. Although the rat model is highly valued for research in many areas, it is less studied than the mouse model and, as a result, there is far less rat immunoglobulin sequence data available and what is available is less well compiled. Thus, to design primers that would amplify the vast majority of rat V_L and V_H coding regions, it was first necessary to perform an extensive search of existing rat genomic and EST sequence data to understand sequence variations that exist within the immunoglobulin coding DNA at the V_L and V_H primer design sites. Searching was done primarily for rat DNA with homology to the closely related murine V_L and V_H sequences. With this information, a portfolio of primers was designed to amplify precisely the same regions of rat V_L and V_H that have proven effective in the preparation of mouse scFvs. The primers were tested using rat spleen cDNA deriving from five Fisher rats immunized by multiple infections with the blood fluke, *S. mansoni*. The fact that virtually all primer combinations produced products of the expected size indicates that most of the V_L and V_H sequences identified by our searches are

actually represented in the rat immunoglobulin repertoire. Thus we believe our primer design will have utility for researchers wishing to produce recombinant antibodies from rat.

To demonstrate that the rat scFv primer design was robust, a scFv phage display library was constructed from an approximately equimolar pool of rat V_L and V_H DNA amplified by the primer sets. The V_L region was cloned first and the resulting library used as the vector for the subsequent cloning of the V_H coding region. The final library had a complexity of about 10⁷ and most of the clones displayed a full-sized scFv. This library size should be sufficient to represent a large portion of the antibody repertoire of the schistosome immune rat. We then selected two different and challenging antigen targets to test the library. For several reasons, we decided to seek scFvs that bind to the Sm23 and SmTsp2 antigens present in the tegumental membrane syncytium that surrounds adult *S. mansoni* parasites. Both tetraspanin proteins were previously shown to be immunogenic during schistosome infection in at least one animal model. Each of these antigens has a large predicted extracellular loop (EC2) that has been used previously to elicit antibodies against the native tetraspanins and to identify antibodies elicited to these proteins following infection (Reynolds et al., 1992; Tran et al., 2006). Maltose binding protein fusions to EC2 of Sm23 and SmTsp2 were used as the target in our panning selections. Prior to panning, we demonstrated that serum from schistosome-infected rats contained good antibody titers against both of the MBP/EC2 fusion proteins while naïve rats did not.

The robustness of our rat scFv library construction method seems to be well demonstrated by our ability, in one series of three panning cycles, to identify multiple unique scFvs recognizing each of the two selected targets. First, the targets are only one of probably hundreds of different antigens that are immunogenic during schistosome infection. Secondly, they derive from proteins that are not particularly abundant in the parasites based on proteomic surveys (van Balkom et al., 2005; Braschi et al., 2006). Thirdly, the epitopes derive from a relatively small extracellular loop domain (~10kD) contained within these apical membrane proteins, which are often more difficult than cytosolic or secreted proteins to study as antigens. Fourthly, it is predicted that the selected tetraspanin EC2 domains are heavily glycosylated and conformationally-constrained which should limit the number of available epitopes that are represented by the linear fusion proteins that were used as the panning targets. Finally, the isolated scFvs show excellent specificity for the native tetraspanin targets.

In summary, we have designed a set of primers for the PCR amplification of rat V_L and V_H coding DNA and demonstrated that the products of these primers can be used effectively to produce a robust library of recombinant single-chain antibody fragments that represent the antibody repertoire of the rats that were used as the source of cDNA template.

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5.0. References

- Barker RH Jr, Srivastava BS, Suri P, Goldberg M, Knopf PM. Immunoprecipitation analysis of radiolabelled protein antigens biosynthesized in vitro by *S. mansoni*. I. Identification of antigens uniquely recognized by protective antibodies. *J Immunol* 1985;134:1192–201. [PubMed: 3965570]
- Better M, Chang CP, Robinson RR, Horwitz AH. *Escherichia coli* secretion of an active chimeric antibody fragment. *Science* 1988;240:1041–3. [PubMed: 3285471]
- Bird RE, Hardman KD, Jacobson JW, Johnson S, Kaufman BM, Lee SM, Lee T, Pope SH, Riordan GS, Whitlow M. Single-chain antigen-binding proteins. *Science* 1988;242:423–6. [PubMed: 3140379]

- Braschi S, Curwen RS, Ashton PD, Verjovski-Almeida S, Wilson A. The tegument surface membranes of the human blood parasite *Schistosoma mansoni*: a proteomic analysis after differential extraction. *Proteomics* 2006;6:1471–82. [PubMed: 16447162]
- Bruggemann M, Free J, Diamond A, Howard J, Cobbold S, Waldmann H. Immunoglobulin heavy chain locus of the rat: striking homology to mouse antibody genes. *Proc Natl Acad Sci U S A* 1986;83:6075–9. [PubMed: 3016742]
- Dammers PM, Visser A, Popa ER, Nieuwenhuis P, Bos NA, Kroese FG. Immunoglobulin VH gene analysis in rat: most marginal zone B cells express germline encoded VH genes and are ligand selected. *Curr Top Microbiol Immunol* 2000;252:107–17. [PubMed: 11125468]
- Davies J, Riechmann L. Single antibody domains as small recognition units: design and in vitro antigen selection of camelized, human VH domains with improved protein stability. *Protein Eng* 1996;9:531–7. [PubMed: 8862554]
- Foord AJ, Muller JD, Yu M, Wang LF, Heine HG. Production and application of recombinant antibodies to foot-and-mouth disease virus non-structural protein 3ABC. *J Immunol Methods* 2007;321:142–51. [PubMed: 17328909]
- Gilliland LK, Norris NA, Marquardt H, Tsu TT, Hayden MS, Neubauer MG, Yelton DE, Mittler RS, Ledbetter JA. Rapid and reliable cloning of antibody variable regions and generation of recombinant single chain antibody fragments. *Tissue Antigens* 1996;47:1–20. [PubMed: 8929708]
- Giudicelli V, Chaume D, Lefranc MP. IMGT/V-QUEST, an integrated software program for immunoglobulin and T cell receptor V-J and V-D-J rearrangement analysis. *Nucleic Acids Res* 2004;32:W435–40. [PubMed: 15215425]
- Huston JS, Levinson D, Mudgett-Hunter M, Tai MS, Novotny J, Margolies MN, Ridge RJ, Brucoleri RE, Haber E, Crea R, et al. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc Natl Acad Sci U S A* 1988;85:5879–83. [PubMed: 3045807]
- Jones PT, Dear PH, Foote J, Neuberger MS, Winter G. Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* 1986;321:522–5. [PubMed: 3713831]
- Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 1975;256:495–7. [PubMed: 1172191]
- Li Y, Kilpatrick J, Whitlam GC. Sheep monoclonal antibody fragments generated using a phage display system. *J Immunol Methods* 2000;236:133–46. [PubMed: 10699586]
- Maass DR, Sepulveda J, Pernthaler A, Shoemaker CB. Alpaca (*Lama pacos*) as a convenient source of recombinant camelid heavy chain antibodies (VHHs). *J Immunol Methods* 2007;324:13–25. [PubMed: 17568607]
- Maddison SE, Norman L, Geiger SJ, Kagan IG. *Schistosoma mansoni* infection in the rat. I. Worm burden and serological response in infected, reexposed, and antigen-sensitized animals. *J Parasitol* 1970;56:1058–65. [PubMed: 5504537]
- Marks JD, Griffiths AD, Malmqvist M, Clackson TP, Bye JM, Winter G. By-passing immunization: building high affinity human antibodies by chain shuffling. *Biotechnology (N Y)* 1992;10:779–83. [PubMed: 1368267]
- McCafferty J, Griffiths AD, Winter G, Chiswell DJ. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 1990;348:552–4. [PubMed: 2247164]
- O'Connell D, Becerril B, Roy-Burman A, Daws M, Marks JD. Phage versus phagemid libraries for generation of human monoclonal antibodies. *J Mol Biol* 2002;321:49–56. [PubMed: 12139932]
- Reynolds SR, Shoemaker CB, Harn DA. T and B cell epitope mapping of SM23, an integral membrane protein of *Schistosoma mansoni*. *J Immunol* 1992;149:3995–4001. [PubMed: 1281197]
- Ridder R, Schmitz R, Legay F, Gram H. Generation of rabbit monoclonal antibody fragments from a combinatorial phage display library and their production in the yeast *Pichia pastoris*. *Biotechnology (N Y)* 1995;13:255–60. [PubMed: 9634767]
- Schluter SF, Jensen I, Ramsland PA, Marchalonis JJ. Recombinant shark natural antibodies to thyroglobulin. *J Mol Recognit* 2005;18:404–12. [PubMed: 15954089]
- Skerra A, Pluckthun A. Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. *Science* 1988;240:1038–41. [PubMed: 3285470]

- Smithers SR, Terry RJ. The infection of laboratory hosts with cercariae of *Schistosoma mansoni* and the recovery of the adult worms. *Parasitology* 1965;55:695–700. [PubMed: 4957633]
- Tomlinson IM, Walter G, Marks JD, Llewelyn MB, Winter G. The repertoire of human germline VH sequences reveals about fifty groups of VH segments with different hypervariable loops. *J Mol Biol* 1992;227:776–98. [PubMed: 1404388]
- Tran MH, Pearson MS, Bethony JM, Smyth DJ, Jones MK, Duke M, Don TA, McManus DP, Correa-Oliveira R, Loukas A. Tetraspanins on the surface of *Schistosoma mansoni* are protective antigens against schistosomiasis. *Nat Med* 2006;12:835–40. [PubMed: 16783371]
- van Balkom BW, van Gestel RA, Brouwers JF, Krijgsveld J, Tielens AG, Heck AJ, van Hellemond JJ. Mass spectrometric analysis of the *Schistosoma mansoni* tegumental sub-proteome. *J Proteome Res* 2005;4:958–66. [PubMed: 15952743]
- Vaughan TJ, Williams AJ, Pritchard K, Osbourn JK, Pope AR, Earnshaw JC, McCafferty J, Hodits RA, Wilton J, Johnson KS. Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nat Biotechnol* 1996;14:309–14. [PubMed: 9630891]
- Wright MD, Henkle KJ, Mitchell GF. An immunogenic Mr 23,000 integral membrane protein of *Schistosoma mansoni* worms that closely resembles a human tumor-associated antigen. *J Immunol* 1990;144:3195–200. [PubMed: 2324498]
- Zhao Y, Hammarstrom L. Cloning of the complete rat immunoglobulin delta gene: evolutionary implications. *Immunology* 2003;108:288–95. [PubMed: 12603594]

Figure 1.

Testing PCR primer pairs for amplification of rat V_L and V_H coding DNA. A) PCR amplification of rat spleen cDNA with each of the different forward primers from Table 1 in combination with a pool of the corresponding reverse primers. For V_L domain amplification, 26 forward primers were tested with the pool of 5 reverse primers. For V_H domain amplification, 23 forward primers were tested with the pool of 4 reverse primers. Reaction products were analyzed by 1% agarose gel electrophoresis. Arrows show the expected PCR product (~400 bp). B) Fingerprint analysis of randomly selected single clones. The ~1200 bp V region coding DNA from random clones was amplified by PCR, digested with BstN1 enzyme and analyzed by 2% agarose gel electrophoresis.

Figure 2.

Rat serum and scFv recognition of MBP fusion proteins. A) Schistosome infected rat sera recognition of Sm23/MBP (open squares) and SmTsp2/MBP (filled triangles). B) Recognition of Sm23/MBP (light grey) and SmTsp2/MBP (dark grey) by random scFv clones selected after 3 rounds of panning on a mixture of both MBP fusion proteins, detected by E-tag ELISA.

Figure 3.
Amino acid sequences of the unique rat scFvs shown to recognize Sm23 (S1, S3) or SmTsp2 (T1, T2). The rat V_L and V_H domains contained within the scFvs are shown by overlining. These domains are separated by a linker domain which is encoded by the vector.

Figure 4. Specificity of the rat scFv for the target schistosome antigens. A) Western blot analysis of the rat scFv recognition of Sm23/MBP and SmTsp2/MBP. MBP-fusion protein were separated by a 12% PAGE-SDS, incubated overnight with scFv and detected with anti-E-tag mAb. Lane 1 contains purified Sm23/MBP, Lane 2 contains purified SmTsp2/MBP. The positions of molecular weight markers are shown to the right. B) ELISA analysis of scFv (30nM) recognition of Sm23/MBP (grey bars), SmTsp2/MBP (striped bars) and *S. mansoni* tegumental extract (black bars). C) Western blot analysis of rat scFv recognition of *S. mansoni* tegumental antigens. Lane 1 used a control rat scFv. Lane 2 used a pool of scFvs S1 and S3. Lane 3 used a pool of scFvs T1 and T2. The tegumental extract was resolved by 12% PAGE-SDS. Bound scFv was detected with anti-E-tag mAb.

Table 1

Primer sequence pools designed for amplifying rat IgG V-domains.

Rat VL domain forward primers		Rat VL domain forward primers	
RATVLF01	GATCGCCGGCRACATTGTSHTGACYCAGTCTC	RATVHF01	GATCGGCGCGCCAGGTRCARCTRAMRGAGTCAGG
RATVLF02	GATCGCCGGCGAAACTGTGATGACCCAGTC	RATVHF02	GATCGGCGCGCCAGGTGSAKMTGAAGGAGWC
RATVLF03	GATCGCCGGCCAGGCTGTTGTGACTCAGG	RATVHF03	GATCGGCGCGCCAGRTGCAGYKGAUWGAGTC
RATVLF04	GATCGCCGGCGAMACTRYDSTGACCCAGTC	RATVHF04B	GATCGGCGCGCCAGGTHCAGCTGCASCARTCT
RATVLF05	GATCGCCGGCRACRTCCAGWTRACCCAGWCT	RATVHF04C	GATCGGCGCGCCAGGTHCAGCTGTACCARTCT
RATVLF06	GATCGCCGGCGACATCCAYATGACWCAGWM	RATVHF05	GATCGGCGCGCCAGATCCAGTTGGYACAGTC
RATVLF07	GATCGCCGGCGAYATCCRGRTGACWCAGTC	RATVHF06	GATCGGCGCGCCAGGCCAGCTGCAGTCTGG
RATVLF08B	GATCGCCGGCGACATYSRGATGACYMAGTCTC	RATVHF07	GATCGGCGCGCCAGGTCCAGYTGCAGCARTS
RATVLF08C	GATCGCCGGCGACATYTKGATGACYMAGTCTC	RATVHF08	GATCGGCGCGCCAGATTCAGCTGCARCAGTG
RATVLF09	GATCGCCGGCGACATTKYGATRACCCARTMT	RATVHF09	GATCGGCGCGCCAAACAGTCCAGCTACAGCAGTC
RATVLF10	GATCGCCGGCGATATTGTGATGACHCARRS	RATVHF10	GATCGGCGCGCCAAAGARGTCCWGTGCAKACAGTM
RATVLF11	GATCGCCGGCGATGTTGTGWTGACACAACTC	RATVHF11	GATCGGCGCGCCAGGTTMMTCTGMAASAGTC
RATVLF12	GATCGCCGGCGATGTTGTTTGGTGACACA	RATVHF12	GATCGGCGCGCCARGTYAASCTRCWGCAGTC
RATVLF13B	GATCGCCGGCGATGTTGTGATGACCCAG	RATVHF13	GATCGGCGCGCCAAAGAGGTAAAGCTGCARCAGTC
RATVLF14	GATCGCCGGCGATGTTTGTGTMGRCCAGAC	RATVHF14	GATCGGCGCGCCAAAGARGTTCARCTGCAGCAGTC
RATVLF15	GATCGCCGGCGAMATTGTGMTSAYYCAGTCTC	RATVHF15B	GATCGGCGCGCCAAAGAGGTGCARMTTTCWGGAGWC
RATVLF16	GATCGCCGGCGAMATTRTRCTVACCCAGTCT	RATVHF15C	GATCGGCGCGCCAAAGAGGTGCARMTTTTGGAGWC
RATVLF17	GATCGCCGGCGAWAWTGTCTMMCTCAGTC	RATVHF16	GATCGGCGCGCCAAAGAGGTGAAACTGTGCGAGTC
RATVLF18	GATCGCCGGCGACGTTGTGCTGACTCAGTC	RATVHF17	GATCGGCGCGCCAAAGVGGTGCAGCTWGTGAGWC
RATVLF19	GATCGCCGGCGAYATCRTKHTRACTAGTCTC	RATVHF18	GATCGGCGCGCCAAAGARGTGCARYTGGTGGARTC
RATVLF20	GATCGCCGGCCAGCCCGTGTGCTGCATCAG	RATVHF19B	GATCGGCGCGCCAAAGAAGTGAARCTGGWRGARTCTGG
RATVLF21	GATCGCCGGCCAGWTCACGTSACYCARCM	RATVHF19C	GATCGGCGCGCCAAAGAAGTGAARCTGTTRGARTCTGG
RATVLF22	GATCGCCGGCCAGKYTGTSCTTACTCAGYC	RATVHF20	GATCGGCGCGCCAAAGMRGTACAGCTRGTKGAGTC
RATVLF23	GATCGCCGGCACTGGAGAAACAACACAGTC	RATVHF21	GATCGGCGCGCCAAAGAGGTGCAGCTGAAGGAATC
RATVLF24	GATCGCCGGCACTGTGGCCTTAGAGCAGG		
RATVLF25	GATCGCCGGCAGCTATGAGCTGATCCAACC		
Rat VL domain forward primers		Rat VL domain forward primers	
RATVLB01	GATCGCGGCCGACSTTTCARYTCCWGCYTGG	RATVHB01	GATCACTAGTKGAGGASACGGTGACCRKGG
RATVLB02	GATCGCGGCCGACGTTTYATTTCCARCTKSGTC	RATVHB02	GATCACTAGTTGAGGAGACTGTGAGAGTGG
RATVLB03B	GATCGCGGCCGABSTTTGATCTCCAGYTTGGTC	RATVHB03	GATCACTAGTTGARGAGACTRTGRYCRGTGAC
RATVLB04	GATCGCGGCCGCTAGGACAGTSAGYKTKGTTCCWC	RATVHB04	GATCACTAGTTGARGAGACAGWGACYRRAG
RATVLB05	GATCGCGGCCGCCCGGCTTTCAGGCAG		