Immunological applications of single-domain llama recombinant antibodies isolated from a naïve library

Ana Monegali1, Diletta Ami1, Chiara Martinelli1,3, He Huang2, Marisa Aliprandi1, Paola Capasso1, Chiara Francavilla2, Giuseppe Ossolengo1 and Ario de Marco1,4

1Consortium for Genomic Technologies, Biochemistry Unit, IFOM-IEO Campus, Via Adamello 16, 20139 Milano, Italy, 2Department of Biological Engineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, P.R. China and 3SEMM, IFOM-IEO Campus, Via Adamello 16, 20139 Milano, Italy
4To whom correspondence should be addressed.
E-mail: ario.demarco@ifom-ieo-campus.it

We describe the rapid isolation of single-domain recombinant antibodies in VHH format from a pre-immune llama library created in our laboratory. Such naïve library has demonstrated to be a versatile tool and enabled the isolation of several different antibodies for any of the six proteins panned in parallel. The binders specific for human fibroblast growth factor receptor 1 (FGFR1) were successively analyzed in more detail and resulted suitable for both western blot and immunofluorescence analyses. Several milligrams per liter of antibodies were purified by affinity chromatography and used for kinetic and thermodynamic characterization. Their $K_D$ was in the nanomolar range and they apparently bound a FGF receptor 1 domain not overlapping the region recognized by its physiological ligand FGF. Altogether, the collected data indicate that the new library can enable the recovery of binders of high affinity, specificity and functionality in the conventional immunological tests, avoiding the necessity of further maturation steps. Such results confirmed recent reports of high affinity pre-immune IgNARs and supported the choice of using large single-domain recombinant antibody naïve libraries as an alternative to the preparation of immune libraries for selecting monoclonal antibodies, at convenient cost and time conditions.

Keywords: FGFR1/immunofluorescence/llama/phage display/single-domain recombinant antibodies

Introduction

The increasing need of antibodies in both basic research and applied biotechnology has been addressed by evolving large combinatorial libraries for obtaining fast in vitro selection of binders (Marks et al., 1991; Griffiths et al., 1994; Silacci et al., 2005). The single-chain format (scFv), in which the light-chain and heavy-chain variable regions of a conventional antibody are physically connected by a short linker, still remains the most used material. However, scFvs often have structural instability that reduces their solubility and applications. An alternative became available after the identification of antibodies exclusively formed by the heavy chain (HCAbs) in Camelidae and sharks (Hamers-Casterman et al., 1993; Greenberg et al., 1995). HCAbs are biotechnologically interesting because they are more stable and can recognize alternative epitopes in comparison to conventional antibodies, including the active sites of enzymes (Lauwereys et al., 1998). The peculiarity of recognizing clefts rather than planar epitopes is due to the VHH (single domain from HCAb) paratope shape that is predominantly formed by the convex H3 loop (De Genst et al., 2006).

Most of the VHVs recovered so far have been isolated from immunized camels or llamas. Chromatographic methods have been used for separating different classes of Camelidae antibodies starting from serum and obtaining monospecific antibodies (Hamers-Casterman et al., 1993; Lange et al., 2001). However, the preparation of recombinant VHH phage display libraries from immunized animals and their successive panning has been the preferred method for the isolation of high-affinity specific binders (Arbabi Ghahroudi et al., 1997; Lauwereys et al., 1998; van der Linden et al., 2000). The procedure is time-consuming and, recently, several synthetic and naïve VHH libraries from llamas and sharks have been proposed (Goldman et al., 2006; Verheesen et al., 2006; Liu et al., 2007; Shao et al., 2007). The rationale is that the large diversity of the libraries would allow for the identification of binders for any potential antigen. Such diversity can be reached in (semi)synthetic libraries by hypermutation of the variable regions inside a single VHH framework (Goldman et al., 2006), similarly to what has been successfully performed in the case of scFv (Silacci et al., 2005). The random mutations would compensate for the missing antigen-induced somatic hypermutation. However, in the past it has already been possible to retrieve useful binders even using a relatively small naïve shark library ($10^7$) (Liu et al., 2007). Therefore, we speculated that the elevated germline complexity of the Camelidae HCAbs (Harmsen et al., 2000; Nguyen et al., 2000) could be exploited to prepare a naïve library from which isolating binders directly applicable in standard immunological techniques.

In this paper, we describe the preparation of a naïve library starting from llama (Llama glama) blood and show that it allows the recovery of recombinant antibodies suitable for immunological applications in only few weeks. We estimated that the cost for their isolation is significantly lower than the cost necessary to obtain binders by using the hybridoma technology and their production in bacteria significantly less expensive than culturing mammalian cells. At such conditions, extensive isolation and production of single-domain antibodies become affordable.

Materials and methods

RNA isolation and PCR amplification

$1 \times 10^9$ lymphocytes were isolated by Ficoll gradient centrifugation from 1 l blood of non-immunized llamas (Llama glama) and total RNA extracted using the Nucleospin RNA
Selection of phage displayed VHHs

An aliquot of $5 \times 10^{10}$ bacterial cells was grown in 2xTY containing 0.1 mg/ml ampicillin and 1% glucose at 37°C up to an OD$_{600}$ of 0.4 and infected with 20-fold excess of KM13 helper phage for 30 min at 37°C. Infected cells were harvested by centrifugation, resuspended in 2xTY with 0.1 mg/ml ampicillin, 0.05 mg/ml kanamycin and 0.1% glucose and incubated overnight at 30°C at 150 rpm.

Phage particles were precipitated from culture supernatant with 4% PEG 6000, 0.5 M NaCl, resuspended in sterile PBS, titrated and used for panning against purified soluble protein constructs [GST, lysozyme, fibroblast growth factor receptor 1 (FGFR1-Fc), nucleophosmin (NPM-GST), epsin1-GST, myosin and a mixture of GST and lysozyme]. Phage library aliquots used for panning fusion proteins were previously depleted from binders recognizing the fusion tag by panning them against the corresponding tags (GST or Fc fragment) coated on immobilubotes. Unbound phages after pre-panning incubation step (30 min rocking and 90 min standing upright at room temperature) were recovered.

Antigens were coated overnight at 4°C in 4 ml Nunc-Immuno$^{TM}$ Maxisorp$^{TM}$ tubes at a concentration range of 10–100 µg/ml (Table I) using 50 mM sodium carbonate buffer, pH 9.6. All antigens were directly coated on the tube surface, apart from FGFR1-Fc that in the first and third panning was bound to a goat anti-human Fc antibody (Sigma I 2136) previously coated on the tube.

Tubes were blocked with 3% BSA in PBST at room temperature for 2 h, washed three times with PBS before the addition of $3 \times 10^{15}$ phages for the first round of panning. After 30 min rocking and 90 min standing upright at room temperature, tubes were washed 10 times with PBS 0.1% Tween and 10 times with PBS, bound phages were eluted with 0.1 M triethylamine, pH 11.0. Eluted phages were titrated, used to infect TG1 cells and plated on 2xTY ampicillin, glucose large square plates.

Colonies were scraped, infected with $10^{10}$ KM13 helper phage, grown overnight and phage particles precipitated from culture supernatant with 4% PEG 6000, 0.5 M NaCl. The new sublibrary of phages was resuspended in sterile PBS, titrated, depleted against fusion tags and used in the second round of panning. The same complete procedure was repeated.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Coating concentration µg/ml (µM)</th>
<th>ELISA positives out of 96 clones</th>
<th>Positive binders (%)</th>
<th>Binder sequence diversity</th>
<th>Sequence diversity/ELISA positives (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGFR1</td>
<td>19.8 (0.18)</td>
<td>60</td>
<td>63</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>NPM</td>
<td>100 (1.7)</td>
<td>33</td>
<td>34</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Epsin1</td>
<td>100 (2.3)</td>
<td>15</td>
<td>16</td>
<td>7</td>
<td>47</td>
</tr>
<tr>
<td>Myosin</td>
<td>100 (3.3)</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>100 (6.9)</td>
<td>69</td>
<td>72</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>GST</td>
<td>100 (3.7)</td>
<td>85</td>
<td>89</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>GST+</td>
<td>100 (6.9)</td>
<td>84 GST</td>
<td>88</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>100 (3.7)</td>
<td>5 lysozyme</td>
<td>5</td>
<td>2</td>
<td>40</td>
</tr>
</tbody>
</table>

Binders selected as positive by ELISA test using periplasmic extract of cultured bacteria infected with VHH-fused phages after three rounds of panning. Clone diversity was identified by DNA sequencing. Soluble antigens were used for panning at concentrations ranging from 10 to 100 µg/ml.
repeated for the third round of panning alternating 3% BSA in PBST and 2% skimmed milk in PBS as blocking agents.

In each round of panning, the enrichment of the phage sublibrary obtained was calculated as the ratio of output/ input phages. Ninety-six clones were analyzed by ELISA after the third round of panning.

**Screening of VHHs by ELISA**

Ninety-six single colonies from the third panning of each antigen were grown at 37°C in 2xTY supplemented with 0.1 ng/ml ampicillin, 0.1% glucose for 3–4 h, induced with 1 mM IPTG and incubated overnight at 30°C. Cultures were harvested and periplasmic lysates containing soluble HA-tagged VHHs were diluted 1:3 and incubated with mouse supernatant anti-HA 12CA5 (10 μg/ml) for being used in ELISA.

Maxisorp 96-well plates (Nunc) were coated with either the fusion tag proteins alone or the fusion constructs overnight in 50 mM sodium carbonate buffer (4°C). Antigens were used at the concentration as for panning, except for NPM and myosin that were coated at 1 μg/ml. Plates were blocked with 2% BSA 2 h, washed three times with PBS and incubated 1 h with periplasmic lysates. Plates were washed three times with PBS plus 0.1% Tween and treated with anti-mouse HRP conjugate (Bio-Rad) for 1 h at room temperature. Plates were washed three times with PBST, the reaction was developed by adding ABTS and the absorbance at 405 nm was measured after 30 min incubation.

Clones with an absorbance value higher than 0.25 and that recognized exclusively the recombinant proteins and not the fusion tags were considered positives and their sequences analyzed to identify unique binders.

**Recombinant antibody production**

Clones with unique sequence were subcloned into pHEN6 vector (Conrath et al., 2001) to obtain His-tagged recombinant VHH binders after transformation into XL1Blue competent cells. Bacteria were grown in Terrific Broth supplemented with 0.1% glucose, 0.1 mg/ml ampicillin and 0.01 mg/ml tetracycline till an optical density at 600 nm of 0.6, and induced with 1 mM IPTG for 16 h at 28°C. Cultures were harvested and periplasmic proteins extracted by osmotic shock (Skerra and Plückthun, 1988). The periplasmic extract was purified by affinity chromatography on a Hitrap HP chelating column using an AKTA FPLC Explorer (GE Healthcare).

Protein purity was evaluated by colloidal blue (Instant Blue) stained SDS gels and the correspondence of the identified bands to VHH proteins was verified by western blot analysis using rabbit anti-His (Rockland 600-401-382) as primary antibody. The monodispersity of the recombinant VHHs was estimated by gel filtration using a Superose 12 10/300 GL column applied to AKTA FPLC (GE Healthcare) and the presence of aggregates was also evaluated by measuring the aggregation index (Nomine et al., 2001) using a spectropolarimeter Jacso 810. Yields were calculated by measuring the absorbance at 280 nm and applying the protein extinction coefficient.

**Biochemical characterization: western blot**

Purified recombinant FGFR1-Fc (500 ng) was run in an 8% SDS–PAGE gel and transferred to a nitrocellulose membrane. Blots were blocked with 5% skimmed milk in PBS, incubated with VHH periplasmic extract diluted 1:3, and mouse supernatant anti-HA 12CA5 (10 μg/ml). Next, the membranes were washed in PBS plus 0.1% Tween and incubated with an HRP-conjugated anti-mouse. An unrelated VHH was used as a negative control.

**Biochemical characterization: immunofluorescence**

10⁶ HeLa cells were seeded in 10 cm² plates and cultured in standard culture medium (MEM plus 10% FBS, 1% L-glutamine, 1% P/S, 1% Na pyruvate, 1% non-essential amino acids), before being transfected with 10 μg of plasmid DNA coding for the human FGFR1-GFP construct (Bryant et al., 2005) in the presence of Lipofectamine™, according to manufacturer’s instructions. The day after, transfected Hela cells were seeded on coverslips coated with 0.5 ml of gelatin solution and serum starved overnight.

Slides were rinsed three times with washing buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂). Cells were fixed with 3% formaldehyde-2% sucrose-PBS for 10 min, permeabilized with 20 mM HEPES pH 7.4, 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose, 0.5% Triton X-100 for 3 min at 4°C, washed three times and blocked with 2% BSA, 0.05% Tween 20, 10% donkey serum in PBS for 1 h. Successively, cells were incubated with periplasmic VHH extract and mouse anti-HA (Covance MMS-101P) for 1 h at 37°C, washed three times and incubated with secondary donkey-anti mouse-cy3. Finally, slides were incubated with DAPI for 3 min, mounted and assessed by fluorescence microscopy at the DAPI, GFP and cy3 channels.

**Surface plasmon resonance spectroscopy**

Kinetic measurements were performed at 26°C by using a BIACORE 2000 instrument (Biacore, Uppsala, Sweden). FGFR1-Fc in sodium acetate 10 mM, pH 5.0, was immobilized on a sensor chip CM5 research grade (Biacore, Uppsala, Sweden) by amine coupling according to the manufacturer’s protocol. A total of 1500–3000 resonance units (RU) of FGFR1 were immobilized on the sensor chips. Fc was immobilized in a control cell to subtract unspecific binding signal.

Llama VHH antibodies were injected at concentrations ranging from 1 to 200 nM on both FGFR1- and Fc-immobilized flow cells. The protein dilution and running buffer was 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20.

For each concentration, the VHH antibody was injected for 45–60 s at both 20 and 30 μl/min. The antibody was let dissociate for at least 3 min and, when necessary, binding surfaces were regenerated with 10 mM glycine, pH 2.0–3.0.

The binding curves were obtained by subtracting the reference response and were fitted using the BIA evaluation 4.1 software (Biacore, Uppsala, Sweden) according to 1:1 Langmuir binding model. Similar conditions were used for analysing GST binders (about 1000 RU immobilized). For the analysis of the anti-episp 1 binders, about 700 RU of the protein was captured using a mouse monoclonal anti-GST.

Competition experiments were performed by amine coupling immobilization of the VHH C8 in 5 mM sodium maleate, pH 6.0, on a CM5 sensor chip (Biacore, Uppsala, Sweden), according to the manufacturer’s protocol. An immobilization level of about 2000 RU was obtained.
blank control surface was also prepared to be subtracted to VHH C8 response. FGFR1-Fc (3.6 μM) was mixed with an equimolar concentration of each VHH binder or of the natural ligand FGF and equilibrated 30 min at room temperature before Biacore analysis. Injections of 1 min were performed at 10 μL/min followed by a dissociation period of about 5 min using the same running buffer as for kinetics experiments. Surface regeneration was obtained by 10 mM glycine, pH 2.0. As a control, the response of FGFR1-Fc alone was recorded under the same experimental conditions before any single competition injection.

**Sandwich ELISA**

One microgram of purified His-tagged C8 was coated overnight at 4°C in Nunc Maxisorp plate using 50 mM sodium carbonate buffer, pH 9.6. Plate was blocked with 2% skimmed milk in PBS, 200 ng of FGFR1-Fc was added to each well and incubated 1 h at room temperature. After three washes with PBS, a 1:3 dilution of the periplasmic lysate containing a HA-tagged VHH was added and incubated 90 min at room temperature with mouse supernatant anti-HA 12CA5. Wells were washed three times with PBS 0.1% Tween, incubated with anti-mouse HRP conjugate 1 h at room temperature, washed again and binding reactions were visualized in the presence of ABTS.

An unrelated purified VHH and no VHH were used as negative controls. The positive control for the efficiency of the FGFR1-Fc capture by coated C8 was the direct color development in the presence of ProtA-HRP. Periplasmic lysate functionality was tested by interaction with directly coated FGFR1-Fc.

**Results**

**VHH library construction**

In order to generate a large and highly diverse VHH naïve library, total RNA was isolated from 10⁹ lymphocytes collected from non-immunized llamas and used to synthesize cDNA employing both random and oligodT primers. Heavy chains of all immunoglobulins (from the leader sequence to the CH2 exon) were amplified by PCR at different conditions to maximally preserve the initial diversity. VHVs were separated from conventional VH on agarose gel, purified and re-amplified by nested PCR using degenerated primers that introduced the Ncol-PalI/NotI restriction sites as well. Final PCR fragments were cloned into the phagemid vector pHEN4 introduced the NcoI-PstI/NotI restriction sites as well. Final PCR fragments were cloned into the phagemid vector pHEN4.

**Library functional diversity**

The library functional dimension was evaluated by colony PCR and the assay performed with 200 random clones confirmed the presence of an insert having the expected size (around 400 bp) in more than 95% of the analyzed clones (data not shown).

Considering that we started from 10⁹ lymphocytes and that the proportion of B-cell producing antibodies is less than 20% of the total lymphocytes, and that the VHHs represent 25% of the total llama antibodies, the maximal library diversity would be 5 × 10⁷. Random errors introduced by PCR could slightly increase this number. In order to estimate whether the actual library diversity and functionality were close to the theoretical values, or if higher redundancy and low expression levels reduced its complexity, we analyzed some randomly chosen clones.

The diversity was estimated by comparing the HinfI digestion pattern of 200 PCR inserts followed by sequencing the clones sharing similar patterns (Fig. 1A). Furthermore, 60 other clones were arbitrarily picked from the naïve library and directly sequenced. In both cases, all the analyzed sequences were unique (Fig. 1B).

Accumulation of antibody fragments in the periplasm of TG1 cultured bacteria grown starting from 50 single colonies randomly picked was evaluated by western blot and showed that 100% of them expressed recombinant VHHs. Sequence analysis showed that few clones contained amber stop codons. However, TG1 is a permissive strain and, therefore, these stop codons did not result in preventing VHH expression.

Altogether, these data indicate that the library (named Cogentech 1) is composed by functional clones and that its diversity could approach the theoretical number of 5 × 10⁷, since no redundancy was identified.

**Recovery of VHHs against soluble proteins**

The possibility to recover functional binders from the library was investigated by panning in parallel six soluble recombinant proteins (Table I).

A pre-clearing step was performed to eliminate binders specific for the tags fused to target proteins before every round of panning, for all the three rounds of panning. After the selection, the periplasmic extract recovered from induced bacteria corresponding to 96 single clones was analyzed by ELISA.

At least one positive clone was identified against any of the antigens used for panning (Table I). This was true also in the case in which GST and lysozyme were coated together in a unique panning, and the presence of VHHs against each separate protein was confirmed by ELISA on the corresponding protein. The number of diverse binders selected for each antigen was successively determined by sequencing the positive clones (Table I). We further investigated whether the large diversity of VHHs obtained against FGFR1 represented an advantage in terms of application opportunity.

**Immunological application of the VHH antibodies selected against FGFR1**

A first group of seven binders against FGFR1 (EU927375, EU927376, EU927377, EU927378, EU927379, EU927380, EU927386, corresponding to the VHHs identified with the codes A11, A7, B2, C8, D6, H1, E1) was selected because it was representative of the large sequence difference, while A11 and E1 that differ only for two amino acids in their CDR3 were chosen to evaluate the functional meaning of minimal differences (Fig. 2A). Diversity would correspond to different binding features and, therefore, the VHHs were characterized for their applicability in immunological methodologies and for their affinity constant. Periplasmic extracts from bacteria expressing the HA-tagged VHHs were used and four out of seven anti-FGFR1s detected the antigen in western blot (Supplementary data available at PEDS online, Fig. S1), while five recognized the FGFR1-GFP construct in immunofluorescence.
The specific co-localization of the VHH antibodies with FGFR1 in transfected cells was demonstrated by the complete overlap with the GFP signal (Fig. 2B). The best performances were obtained in all the immunological techniques using the VHHs C8 and H1. Such observation induced us to investigate the possible correlation between application efficacy and affinity for the antigen.

Affinity constant analyses
Anti-FGFR1 VHHs were sub-cloned into pHEN6 (Conrath et al., 2001) to obtain their His-tagged versions and proceed...
to purification by metal affinity chromatography. The yields were in the range of mg protein/L culture and the VHHs were stable and showed negligible tendency to aggregation, as indicated by both gel filtration and aggregation index analysis (data not shown).

VHHs purified to homogeneity were used for surface plasmon resonance spectroscopy analyses (Fig. 3A). All the VHHs showed an affinity for FGFR1 in the nM range (Table II). Notably, two amino acid substitutions in CDR3 between A11 and E1 were sufficient to vary three times the $K_D$. C8 had the lowest $K_D$ (9.3 nM) among all clones, mainly due to its low $k_{\text{off}}$ value (Fig. 3B). This feature was used to set a competition experiment among VHHs and the natural ligand FGF.

The affinity constant measurement of binders specific for GST and epsin1 confirmed that recombinant VHH antibodies isolated from the naïve Cogentech1 library have in average $K_D$ in the nM range (Table II).

**Table II.** Kinetic and thermodynamic constants of the VHH binders selected against FGFR1, epsin 1 and GST

<table>
<thead>
<tr>
<th>Antigen</th>
<th>VHH clone</th>
<th>$k_{\text{on}}$ (1/Ms)</th>
<th>$k_{\text{off}}$ (1/s)</th>
<th>$K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGFR1</td>
<td>A7</td>
<td>$5.81 \times 10^5$</td>
<td>$8.5 \times 10^{-2}$</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>A11</td>
<td>$9.95 \times 10^5$</td>
<td>$4.0 \times 10^{-2}$</td>
<td>41.7</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>$5.02 \times 10^5$</td>
<td>$2.0 \times 10^{-1}$</td>
<td>43.4</td>
</tr>
<tr>
<td></td>
<td>C8</td>
<td>$8.38 \times 10^5$</td>
<td>$7.5 \times 10^{-1}$</td>
<td>9.35</td>
</tr>
<tr>
<td></td>
<td>D6</td>
<td>$2.84 \times 10^5$</td>
<td>$9.5 \times 10^{-2}$</td>
<td>336</td>
</tr>
<tr>
<td></td>
<td>E1</td>
<td>$8.23 \times 10^5$</td>
<td>$1.0 \times 10^{-1}$</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>H1</td>
<td>$1.56 \times 10^5$</td>
<td>$7.9 \times 10^{-2}$</td>
<td>53.2</td>
</tr>
<tr>
<td>Epsin1</td>
<td>B8</td>
<td>$3.54 \times 10^5$</td>
<td>$2.0 \times 10^{-2}$</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>C10</td>
<td>$2.76 \times 10^4$</td>
<td>$1.1 \times 10^{-2}$</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>E1</td>
<td>$4.59 \times 10^4$</td>
<td>$9.0 \times 10^{-2}$</td>
<td>1970</td>
</tr>
<tr>
<td>GST</td>
<td>IB</td>
<td>$2.69 \times 10^5$</td>
<td>$4.5 \times 10^{-3}$</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>$3.04 \times 10^5$</td>
<td>$1.1 \times 10^{-3}$</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Data shown in the Table are the mean value of at least three independent experiments. Standard deviation for $K_D$ values is less than 25%, with the exception of FGFR1-A7 (40%) and GST-1B (46%).

**Fig. 3.** Determination of the VHH affinity for FGFR1. 6xHis tagged VHH antibodies (1 μg) were purified (A) and analyzed for structural integrity and aggregation before being used in surface plasmon resonance spectroscopy measurements against immobilized FGFR1. The sensorgrams of C8 at different antibody concentrations are reported as an example (B).

**Fig. 4.** Competition among anti-FGFR1 VHH binders analyzed by surface plasmon resonance spectroscopy. C8 was immobilized on the chip surface and a pre-incubated mixture of a second VHH with FGFR1 was fluxed. Whether the fluxing VHH binds a FGFR1 epitope completely independent from the epitope recognized by C8, its complex with FGFR1 will be bound by the C8 immobilized on the chip. The corresponding sensorgram will show a signal higher than that corresponding to the unique contribution of FGFR1 fluxed alone (A). When the fluxing VHH competes with C8 for the same or partially overlapping FGFR1 epitopes, the sensorgram signal will decrease since the antigen competition between the two antibodies will impair part of the FGFR1 to bind C8 on the chip (B). Experimental results obtained using FGFR1-Fc (3.6 μM) alone and mixed together with both different VHHs (3.6 μM) and its natural ligand FGF (3.6 μM) are reported in (C).
higher or lower than the reference given by FGFR1 alone (Fig. 4A and B).

The results indicate that all the VHHs seem to compete for the same or partially overlapping epitopes, while FGF would recognize an independent region on FGFR1 (Fig. 4C). This result is supported by a sandwich ELISA where purified His-tagged C8 was coated on the plate and incubated with FGFR1 prior to the addition of a HA-tagged VHH. None of the VHHs allowed the detection of the C8–FGFR1–VHH complex by HA-dependent immunostaining, indicating that all VHHs acted as competitors of C8 (data not shown).

Discussion

Libraries of recombinant antibodies have been proposed as a cost effective and rapid alternative to conventional preparation of monoclonal antibodies. The initial approach of using Fab fragments and scFvs reproducing the paratope of complete immunoglobulins (Skerra and Plückthun, 1988; Marks et al., 1991) was soon integrated by the introduction of molecules with different structural conformation and, consequently, new binding modalities. VHHs from Camelidae (Hamers-Casterman et al., 1993; Arbabi Ghahroudi et al., 1997) and IgNAR from sharks (Greenberg et al., 1995; Nuttal et al., 2001) are single-domain antibodies from HCAbs that can recognize epitopes non accessible for conventional Abs (De Genst et al., 2006; Stanfield et al., 2007; Simmons et al., 2008).

The statistic possibility to bind a molecule is related to the diversity of the clones represented in a library and, therefore, synthetic libraries were created by hypermutation of the CDRs of both conventional and VHH recombinant antibodies (Jobling et al., 2003; Silacci et al., 2005; Goldman et al., 2006). Random hypermutation would compensate for somatic hypermutation already acquired into libraries recovered from immunized animals. In vivo somatic hypermutation has been estimated improving 10–300-fold the affinity for the antigen, depending on the animal model (Li et al., 2003; De Genst et al., 2004; Stanfield et al., 2007). However, the preparation of single libraries for each different antigen is time-consuming and expensive, while primary repertoire may already contain binders of surprising high affinity (Stanfield et al., 2007). The only previously described llama naïve VHH library already succeeded in providing useful binders, although built starting from only 10⁷ lymphocytes (Muruganandam et al., 2002). In this paper, we show that the llama naïve library Cogentech 1, built up from 10⁹ lymphocytes, can be used to recover antibodies with nM affinity and wide range of application (Table II and Supplementary data available at PEDS online, Fig. S1).

Three weeks were sufficient to isolate the VHHs from our library, panning in parallel against several proteins. VHH antibodies were recovered for all the six different soluble proteins used in this experiment and panning succeeded also when cell markers were used (data not shown). The VHHs were produced at high yields and their biophysical characterization indicated their stability. In particular, the VHHs against FGFR1 performed at least as good as the commercially available antibodies in immunofluorescence and western blot (data not shown) and their affinity is comparable to monoclonal antibodies obtained from hybridoma, scFv or small immunized VHH libraries (van Koningsbruggen et al., 2003). The low koff of C8 makes this VHH a good candidate for in vivo applications.

The binders selected against FGFR1 had affinity values that ranged between 10 and 450 nM. Their significant heterogeneity also in terms of amino acids present in the CDRs (Fig. 2) would indicate that they use different interaction surfaces for binding the antigen. However, the competition experiments we performed seem to indicate that all the VHHs against FGFR1 recognize epitopes close enough to reciprocally hinder the antibody binding to the antigen. This result reminds the binding analysis of different VHHs to lysozyme that showed how six out of eight antibodies with large sequence diversity recognized partially overlapping epitopes (De Genst et al., 2006). In contrast, the natural ligand for FGFR1, FGF, apparently uses different contact surfaces of the receptor and binds to another independent site.

The preferential recognition of specific conformational epitopes that are compatible with the protruding VHH paratope could explain the selection of binders sharing particular substrate regions. An alternative possibility would be that the first binding event modifies the conformation of the epitope recognized by the second VHH. Further experiments have been started to identify the minimal FGFR1 domains bound by each different VHH. However, anti-epsin1 VHHs isolated from the same library recognized different epitopes (data not shown), suggesting that the interaction binders/antigen is probably peculiar for each protein. It is interesting to note that even polyclonal antibodies often recognize only very few epitopes on their targets (Rockberg et al., 2008).

Recently, heavy-chain fragments have been selected that are active against virus and toxins (Goldman et al., 2006; Liu et al., 2007), anti-tumor necrosis factor (Coppieters et al., 2006), food-and-mouth disease (Harmsen et al., 2008), Streptococcus and E.coli infections (Harmsen et al., 2006; Kruger et al., 2006), endotoxins (El Khattabi et al., 2006) and haptens (Alvarez-Rueda et al., 2007). The described llama naïve library may represent a valid tool to select useful VHHs without the need of animal immunization.

Acknowledgements

The authors wish to thank Serge Muyldermans for having provided material and protocols, Aurelien Olichon and Ugo Cavallaro for critical discussion.

Funding

This work was supported by an individual grant of Associazione Italiana per la Ricerca sul Cancro to Ardo de Marco.

References


Received August 4, 2008; revised December 19, 2008; accepted January 5, 2009

Edited by Laurent Jespers