Biologicals 39 (2011) 110–116

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Improving the characteristics of a mycobacterial 16 kDa-specific chicken scFv

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ARTICLE INFO

Article history:
Received 15 December 2010
Received in revised form
27 January 2011
Accepted 27 January 2011

Keywords:
Chicken scFv
Phage display
Mutagenesis
Short linker
Mycobacterial 16 kDa protein

ABSTRACT

Recombinant antibodies can be engineered to improve their binding or other characteristics. A chicken single chain variable fragment (scFv) phage display library was panned against the mycobacterial 16 kDa antigen. Three fusion phages which bound specifically to the antigen were selected, each of which produced low signals in ELISA when secreted as a soluble scFv. One scFv was therefore chosen to be modified in an attempt to improve its binding. Firstly, a mutant sublibrary was created by random mutagenesis. High stringency panning of this sublibrary yielded binders which produced ELISA signals up to eleven times higher than the parent scFv. An increase in the intrinsic affinity was confirmed by surface plasmon resonance. Secondly, the flexible linker between the heavy and light chains of the parent scFv was either shortened to one glycine residue or deleted entirely. No ELISA signal was obtained when the linker was absent, but the glycine-linked scFv showed enhanced binding. Size exclusion chromatography revealed that the enhanced binder had aggregated to form tetramers. This study confirms that the strategies used to improve the binding of human and mouse scFvs can also enhance chicken scFvs.

1. Introduction

Mycobacterium tuberculosis and *Mycobacterium bovis*, both of which are in the *M. tuberculosis* (TB) complex, each possess an identical 16 kDa protein (accession numbers P30223 and P0A5B8) [1] which is synonymous with heat shock protein hspX, hsp16.3 and the 14 kDa protein [2–4]. This antigen is considered to be a potentially useful diagnostic target for serodiagnosis of TB in humans [5] and can also be used to stimulate lymphocytes in the gamma interferon test for bovine TB [6]. Antibodies directed against this protein are therefore likely to be useful in research and diagnosis.

Unlike polyclonal antibodies in immune serum, recombinant antibodies are a renewable resource which can be characterised by their encoding DNA sequence. This in turn makes it possible to standardise assays with reproducible reagents and the antibodies can even be recovered by constructing a synthetic gene if necessary. A further advantage of recombinant antibodies is that their physical and chemical properties can be changed using standard recombinant DNA methods such as mutagenesis [7–13], multimerisation [14,15], chain and DNA fragment shuffling [16–18]. Affinity maturation by random mutagenesis, followed by increased selection pressure mimics somatic mutation *in vitro*, a process which often allows antibodies with higher affinity and specificity to be derived [7]. Moreover, more stable scFvs [19] and an increase in bacterial expression of an scFv have been obtained in this way [20].

ScFvs consist of the variable heavy (V_H) and variable light (V_L) domains of immunoglobulins, most commonly joined by a flexible polypeptide linker [21,22]. The sequence and the length of this linker can influence the properties of the scFv [15,23]. For example, mouse scFvs with linkers between 12 and 15 amino acids occur mostly as monomers while those joined with between 5 and 11 residues occur as dimers [14,24,25]. Variable domains joined either directly to each other or with linkers of up to four residues occur as trimers and tetramers [15]. This multimerisation results in an increase in cooperative binding and hence the avidity of the scFv. While the antibody engineering methods described above have been shown to be effective in improving mouse and human scFvs, they have so far not been widely used with scFvs from other species. Using an scFv specific for the 16 kDa *M. tuberculosis* protein this study to explores whether random mutation and manipulating linker length can also be used to improve chicken scFvs.

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1045-1056/$36.00
doi:10.1016/j.biologicals.2011.01.007

2. Materials and methods

2.1. Antibody selection by panning

The *Nkuku*® library [26] was panned against 20 μg/ml of the *M. tuberculosis* 16 kDa antigen. The purified 16 kDa antigen of *M. tuberculosis* was obtained from Europa Bioproducts (Cambridge, England). Four rounds of panning were performed followed by screening by monoclonal phage and scFv ELISA as described previously [26].

2.2. Mutant sublibrary construction

The first strategy was to attempt to improve the characteristics of the scFv B4 by error-prone PCR. Random mutations were introduced into the gene coding for the scFv using the Diversify ™ PCR Random Mutagenesis kit as described by the manufacturer (BD Biosciences, Palo Alto, CA, USA). Plasmid DNA (1 ng) containing the scFv gene and 0.4 pmol/μl Sfi1L and LCNOT1 primers [26] were added to the PCR reaction. Conditions were used to obtain seven mutations per 1000 base pairs. The PCR products were digested overnight (ON) with 40 U of *SfiI* (Roche Diagnostics, Mannheim, Germany) at 50 °C followed by another ON digestion with 40 U *NotI* (Roche) at 37 °C. The digested products were purified with the QIAquick PCR kit (QIAGEN, Hilden, Germany). Primers Sfi1L and LCNOT1 (0.4 pmol/μl) were used to allow cloning into the *SfiI* and *NotI* sites of the phage display vector pHEN1 (1 μg) which was digested with 40 U of the same restriction enzymes and purified with the QIAquick PCR kit. To remove the stuffer fragment the vector was further purified with a crystal violet gel [27]. The genes were ligated into the vector with the Rapid DNA Ligation Kit (Roche). The ligations were electroporated into *Escherichia coli* TG1 and plated as described before [26]. Serial dilutions of 10^{-1}–10^{-4} were plated onto TYE agar with 100 μg/ml ampicillin (amp) to determine the size of the library. The plates were incubated ON at 30 °C. The next day the colonies were scraped off the plates in 2x TY and the bacteria were stored in 15% glycerol at −70 °C.

2.3. Sequencing

To check for the presence of mutations, DNA inserts of individual clones were sequenced. Single *E. coli* TG1 transformant colonies were grown in 5 ml 2x TY supplemented with 100 μg/ml amp and 2% (w/v) glucose (2x TY-AG) at 30 °C, 240 rpm. Phagemid DNA was isolated using the QIAprep Spin Miniprep plasmid purification kit (QIAGEN). Sequencing primers OP52 and M13rev were used [26]. Automated sequencing was done by the Molecular Biology Division, Onderstepoort Veterinary Institute. Sequences were analysed using the Staden and BioEdit software packages [28,29].

2.4. Screening mutant library

The mutant library was panned using stringent conditions to select for scFvs with higher affinities than the parent scFv. Panning was done as described previously with some minor modifications [26]. For the first two rounds of panning 20 μg/ml of 16 kDa *M. tuberculosis* antigen in PBS was used to coat the wells of a microtitre plate (Nunc, Maxisorp) and for the third round 2 μg/ml was used. For the first round the wells were washed 19 times with PBS containing 0.1% Tween-20 (Tween-PBS) (Merck, Schardt, Germany) followed by a long 20 min wash on a rocker. Thereafter the wells were washed 20 times with PBS. For the second and third rounds the wells were washed 20 times with 0.5% Tween-PBS followed by 20 washes with PBS. The phages were eluted with 0.1 N HCl for all rounds. In order to determine input phage titres dilutions of 10^{-9}–10^{-11} phages were made in PBS. From each phage dilution 10 μl was added to 40 μl *E. coli* TG1 cells (OD_{600} 0.4–0.6) and 50 μl 2x TY. This was incubated at 37 °C for 30 min, plated on TYE plates containing 100 μg/ml amp and incubated at 30 °C ON. The eluted phages (output) were titred by plating ten-fold dilutions (10^{-1}–10^{-4}) and the rest plated on a 16 cm petri dish.

2.5. Production and purification of scFvs

To produce scFvs, individual TG1 transformant colonies were inoculated into 5 ml 2x TY-AG and incubated ON at 30 °C at 240 rpm. A $^{1}/_{100}$ dilution on the ON culture was made in the same medium and grown until the OD_{600} was 0.9. The culture was centrifuged at 2000× *g* for 10 min and the pellet resuspended in a fifth volume 2x TY with 100 μg/ml amp and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce scFv production. The final induction volumes of 10 ml or 2 ml were shaken at 240 rpm ON at 30 °C. Bacteria were removed by centrifuging the culture at 2000× *g* for 15 min. The supernatant fluids containing the secreted antibodies were used directly in ELISAs. Alternatively, scFvs were affinity purified from the supernatant fluid using an anti-c-myc tag monoclonal antibody 9E10 as described previously [26]. The scFv samples were stored at 4 °C or in 2% sucrose solution at −20 °C.

2.6. ELISA

The binding of the mutant scFvs to the antigen was characterised by ELISA. An immunoplate (Nunc, Maxisorp) was coated ON at 4 °C with 10 μg/ml of 16 kDa *M. tuberculosis* antigen diluted in PBS. Apart from blocking and washing steps, all the reactions volumes were 50 μl per well. The plate was blocked with 300 μl per well of 2% (w/v) bovine serum albumin (BSA) in PBS at 37 °C for 1 h followed by three washes with 0.05% Tween-PBS. The scFvs in the supernatant fluid were diluted 1:1 with 4% BSA–PBS and incubated at 37 °C for 1 h followed by three washes. An anti-c-myc tag monoclonal antibody 9E10 [30] in Dulbecco's Modified Eagles Medium (GIBCO, Grand Island, USA) diluted 1:1 in 4% BSA–PBS was added, incubated at 37 °C for 1 h and washed three times with 0.05% Tween-PBS. For detection a $^{1}/_{1000}$ dilution of polyclonal rabbit anti-mouse immunoglobulins conjugated to HRP (DakoCytomation, Ely, UK) in 2% BSA–PBS was added and incubated at 37 °C for 1 h and washed as in the previous step. After the final wash 50 μl of substrate made up of 1 mg/ml o-phenylene diamine and 0.5 μl/ml of 30% ($^{v}/_{v}$) H_2O_2 in 0.1 M citrate buffer (pH 4.5) was added and left at room temperature (RT) for 40 min. The enzyme reaction was stopped with 50 μl 2N H_2SO_4 and the absorbance measured at 492 nm. All scFv samples in the study were tested in duplicate. An additional ELISA with a short 10 min incubation time was used to determine whether a shorter reaction time would reduce the ELISA signal. The scFvs were diluted 1:1 with 4% BSA–PBS and incubated at 37 °C for 10 min for the short ELISA and washed as above. An ELISA with harsh washing conditions was also used to determine whether the scFvs could still bind to the antigen. Here the scFvs diluted 1:1 with 4% BSA–PBS were incubated at 40 °C for 1 h and washed 10 times with 0.05% Tween-PBS at 40 °C, followed by 30 min incubation with 0.05% Tween-PBS at 40 °C.

2.7. Surface plasmon resonance (SPR)

SPR was used to compare the binding kinetics of the mutant scFvs on a Biacore X (Biacore, Uppsala, Sweden). Experiments were performed at 25 °C using HBS-EP running buffer. The 16 kDa protein of *M. tuberculosis* was covalently bound to the dextran surface of a CM5 chip via its primary amine groups (BIApplications handbook, Biacore). A volume of 35 μl of 16 kDa (50 μg/ml in 10 mM acetate buffer, pH 4) was injected and un-reacted ester groups were blocked with 1 M ethanolamine-HCL, pH 8.5. These conditions resulted in 3, 300 RU being immobilized. The control flow cell was left empty. For