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SHORT COMMUNICATION

Repertoires of aggregation-resistant human antibody domains

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We recently described a method for the generation of a large human domain antibody repertoire involving combinatorial assembly of CDR building blocks from a smaller repertoire comprising a high frequency of aggregation-resistant antibody domains. Here we show that the frequency of aggregation-resistant domains in the combinatorial repertoire remained high. Furthermore, one of the antigen-binding domains selected from the combinatorial repertoire retained its binding properties through 25 cycles of thermal denaturation, suggesting that antibody domains can be created that rival the heat-resistance of thermophilic proteins such as *Taq* polymerase.
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The complementarity determining regions (CDRs) of antibodies largely determine their antigen-binding activities (Kabat *et al.*, 1983), but also other properties such as the propensity to aggregate. In particular, their role in determining the aggregation properties of single antibody domains has been noted. For example, typical mammalian heavy chain variable domains readily aggregate in the absence of their light chain partners, in contrast to the camelid single domain antibodies that lack natural light chains (Ewert *et al.*, 2002). In camelid single domains, the residues from CDR3 pack against the region corresponding to the light chain interface, helping to prevent aggregation and improving solubility of the domain (Desmyter *et al.*, 1996; Spinelli *et al.*, 1996). Other packing interactions mediated by CDRs can also lead to improved hydrophilicity of the interface and help prevent aggregation (Jespers *et al.*, 2004a). More generally, the nature of the CDR sequences can affect thermodynamic stability as well as expression and purification yields (Bond *et al.*, 2003; Jespers *et al.*, 2004b).

Recently, a phage-based method was reported that allowed the selection of human antibody variable heavy chains that are resistant to heat-induced aggregation (Jespers *et al.*, 2004b). The domains selected by this method, did not aggregate when heated above their unfolding temperature, suggesting that their resistance to aggregation was a property of their denatured rather than native states.

We have recently described a DNA-amplification method for the generation of large recombinant repertoires (Christ *et al.*, 2006). We have demonstrated that an extensive domain antibody library can be assembled by combinatorial ligation of CDR-encoding regions and that antigen binders can be selected from this repertoire by phage display. As the CDR building blocks used in the assembly had been derived from antibody domains pre-selected by thermal denaturation on phage (Jespers *et al.*, 2004b, Christ *et al.*, 2006), we wondered whether the combinatorial repertoire had retained aggregation-resistant properties. Here, we have investigated the aggregation properties of the combinatorial repertoire and further characterized antibody domains selected from the repertoire against a target antigen.

Material and Methods

Generation of repertoire S and C

Purified phages from repertoire U [library 1, 2×10^9 clones (Jespers *et al.*, 2004b)] were heated in Phosphate Buffer Saline (PBS) at 80°C for 10 min and captured on a protein A coated immuno-tube. MaxiSorp Immuno-tubes (Nunc) had been coated over night with protein A at 10 μg/ml in PBS at room temperature and blocked using 2% (v/v) Tween-20 in PBS for 1 h at 37°C. The heat-selected phages were diluted in 2% (v/v) Tween-20 in PBS and added to the blocked Immuno-tubes for a 2 h incubation at room temperature. After 10 washes with 0.1% (v/v) Tween-20 in PBS, protein A-bound phages were eluted in 1 ml of 1 mg/ml trypsin in PBS during 10 min at room temperature. These phage comprised repertoire S. They were used to infect exponentially growing *Escherichia coli* TG1 bacteria (Gibson, 1984) (OD_{600}=0.5) for 30 min at 37°C, and plated on agar plates containing 15 μg/ml tetracycline. The next day, the bacterial lawn was scraped off the plates, and DNA was prepared using a Qiagen miniprep kit. Gene segments encoding CDRs1/2 and CDR3 were then amplified by PCR and re-combined by combinatorial ligation in a pR2 phagemid format to give repertoire C as previously described (Christ *et al.*, 2006).

Phage ELISA for aggregation resistance

The aggregation resistance of the repertoires was determined by measuring the retention of signal after heat incubation in a phage Enzyme-linked Immunosorbent Assay (ELISA) format (McCafferty *et al.*, 1990). As repertoire C is in a phagemid format, inserts were first amplified by PCR from a DNA preparation and re-cloned into phage vector with a tetracycline resistance gene that contains a c-myc tag between the leader sequence and gene III (Jespers *et al.*, 2004b), to give a corresponding phage repertoire. ELISA wells were coated over night with protein A at 10 μg/ml in PBS at room temperature and blocked using 2% (v/v) Tween-20 in PBS. Phage infected colonies were grown in a 96-well microtitre plate at 37°C over night and phages were biotinylated in the culture supernatant using

biotin-N-hydroxysuccinimide (50 μM final concentration; Perbio). The supernatant was incubated at either 80°C or room temperature for 10 min, mixed 1:1 with 4% Tween-20 in PBS and added to the blocked ELISA wells. After washes with PBS, bound phage particles were detected using a streptavidin-HRP conjugate (1 μg/ml; Sigma) and 3,3′,5,5′-tetramethylbenzidine as a substrate. Forty-four clones were analyzed from each repertoire; clones displaying low ELISA signals (<0.5 in the absence of heat treatment) were excluded from the analysis. Clones retaining $\geq 40\%$ of the ELISA signal after heating were scored as aggregation-resistant.

Characterization of beta-galactosidase specific domain antibodies

Repertoire C was selected for binding to beta-galactosidase as described in (Christ *et al.*, 2006). Domain antibodies were expressed from the pR2 phagemid vector in shaking flask culture using baffled flasks. Cultures were grown in 2xTY medium (100 μg/ml ampicillin, 0.1% glucose) at 37°C to mid-logarithmic phase; protein expression was induced by the addition of 1 mM IPTG and cultures grown for 24 h at 30°C. After centrifugation, supernatants were filtered (0.22 μM) and incubated with Streamline protein A Sepharose (Amersham) overnight. The resin was washed on a column with PBS and eluted with 0.1 M glycine pH 3.0. After neutralization with 1 M Tris–HCl pH 7.4, the protein preparation (1–5 mg/l of culture) was concentrated by diafiltration and purified on a HR75 gel-filtration column (Amersham). Affinity measurements were performed on a Biacore 2000 instrument. Approximately 1000–2000 resonance units (RU) of biotinylated beta-galactosidase (Sigma) were immobilized on a streptavidin SA chip (Biacore). All measurements were performed using HBS-EP running buffer (Biacore). Thermal resistance was tested by heating in PBS at a concentration of 5 μM protein. Binding activity was analysed after up to 25 cycles of PCR using the following conditions: 20 s 94°C, 20 s 50°C, 90 s 72°C at a concentration of 1 μM in PBS. Half-life of domain antibodies at 97.5°C was measured at 1 μM protein concentration in PBS. The fraction of active protein was determined by monitoring peak height of the binding curve by surface plasmon resonance and calculating active protein concentration by Scatchard-plot analysis. Thermal unfolding was monitored at 235 nm at a protein concentration of 5 μM in PBS. Measurements were performed on a J-720 spectropolarimeter (Jasco). The unfolding curves were fitted according to a two-state model using a ΔCP value of 12 cal/K/mol per amino acid.

Results and discussion

In order to enrich for aggregation-resistant building blocks, we first selected a **repertoire U** (Fig. 1) of human antibody variable heavy chains displayed on phage (2×10^9 clones) (Jespers *et al.*, 2004b) by thermal unfolding. The phages were heated to 80°C, followed by capture of those bearing aggregation-resistant domains, using immobilized superantigen protein A, thereby generating **repertoire S** (Fig. 1). We observed a huge loss in yield; thus, less than 1 in 10^3 phage particles could be successfully captured after the heat denaturation step (about 10^8 output phages from 4×10^{11} input phages in a typical experiment).

We then used the CDRs of repertoire S to generate a large combinatorial repertoire, as previously described. Our strategy involved amplifying regions corresponding to CDR1/2 and CDR3 of repertoire S by PCR, re-combining them by ligation, followed by *in vitro* amplification of the product and transformation into bacteria. This yielded a combinatorial **repertoire C** (Fig. 1) of 3×10^9 clones, which was generated in a phagemid format in order to favour the selection of higher affinity domain antibodies (Christ *et al.*, 2006). The amplification step was essential in the assembly of the repertoire, as transformation of ligated PCR products alone resulted in a 10^5-fold reduction in the number of colonies (Christ *et al.*, 2006).

We analyzed the aggregation resistance of the repertoires by phage ELISA. In this assay, phage particles were heated to 80°C and aggregation-resistant clones were detected by protein A capture (repertoire C had been re-cloned into a

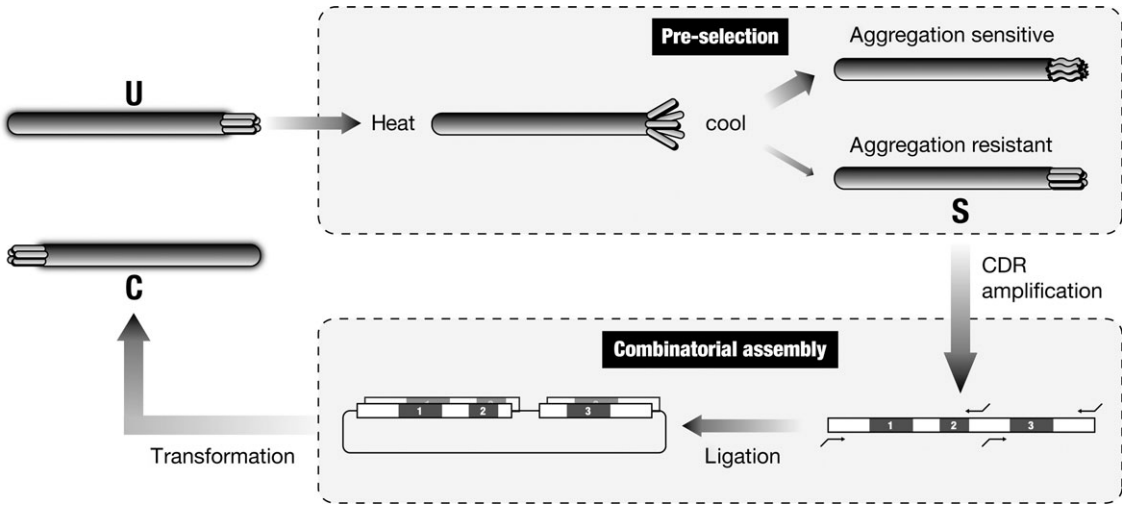

Fig. 1. Combinatorial assembly of aggregation-resistant antibody repertoire. Unselected repertoire on phage (**repertoire U**) was subjected to heat-cooling and an aggregation-resistant sub-repertoire (**repertoire S**) was captured. A large aggregation-resistant repertoire (**repertoire C**) was generated by combinatorial ligation of CDR-encoding regions amplified by PCR from repertoire S.

phage format to allow a direct comparison, see Material and Methods for details). The frequencies of aggregation-resistant clones in repertoires U, S and C were 8%, 80% and 71%. Thus, repertoire C retained a similar frequency of aggregation-resistant clones to repertoire S despite reshuffling of the CDR1/2 and CDR3 building blocks.

While the combinatorial repertoire clearly displayed an aggregation-resistant phenotype, we wondered whether this property would be maintained when selecting for antigen binding. For this purpose, we sequenced 75 clones after two rounds of binding selections using beta-galactosidase as a target antigen (Christ *et al.*, 2006). This revealed that two sequences dominated the population (clone 2: 42-times observed; clone 1: 17-times observed). We expressed the two proteins, purified them by affinity chromatography and gel-filtration and analyzed their binding activity by surface plasmon resonance (Fig. 2A; clone 2 shown).

Both proteins bound to beta-galactosidase immobilized on the sensor chip surface with affinities in the micro- to nanomolar range (clone 1: $k_a = 3.5 \times 10^4\ M^{-1}\ s^{-1}$, $k_d = 4 \times 10^{-2}\ s^{-1}$, $K_D = 1.16\ \mu M$) (clone 2: $k_a = 1.8 \times 10^4\ M^{-1}\ s^{-1}$, $k_d = 6.6 \times 10^{-3}\ s^{-1}$, $K_D = 360$ nM). The proteins also retained significant binding activity after heating. Thus, clone 1 maintained ~50% of binding activity after heating at concentrations of up to 1 μM (data not shown), while clone 2 maintained 100% of activity after heating at 80°C at concentrations of up to 5 μM (Fig. 2B). When monitoring unfolding by circular dichroism, clone 2 unfolded reversibly and cooperatively upon repeated heating cycles (Fig. 2C), with a midpoint of transition of 64.6°C and a ΔG of unfolding of

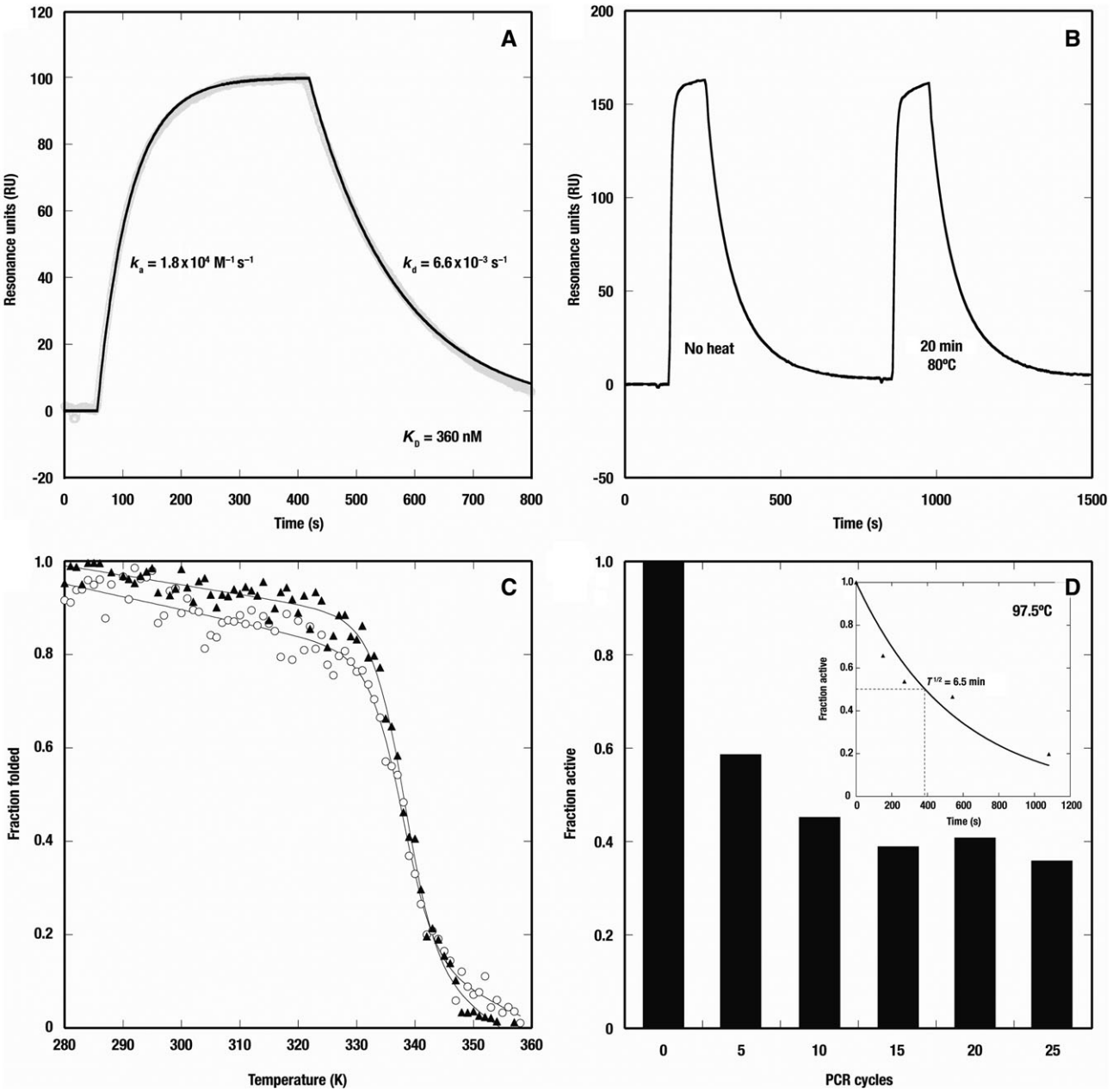

Fig. 2. Biophysical characterization of beta-galactosidase-specific domain antibody (clone 2). (**A**) Analysis of antigen binding by surface plasmon resonance; (**B**) antigen binding before and after heat incubation; (**C**) thermal denaturation as monitored by circular dichroism (triangles: first denaturation; circles: second denaturation); (**D**) binding activity after up to 25 cycles of PCR as analyzed by surface plasmon resonance. Inset graph: half-life of domain antibody at 97.5°C.

25.1 kJ/mol. The observed values for clone 2 are higher than for a set of previously reported aggregation-resistant domain antibodies (melting temperature, T_m=54.1–61.2°C; ΔG_u = 14–23 kJ/mol) (Jespers *et al.*, 2004b), but lower than that of aggregation-prone domain antibodies based on the human DP47/3-23 framework (40–53 kJ/mol)(Ewert *et al.*, 2002).

We wondered whether clone 2 would be able to withstand even more extreme conditions, and subjected the antibody to multiple heat cycles and conditions typical for PCR reactions. This revealed that clone 2 withstands multiple PCR cycles and maintains as much as 40% of its original binding activity after 25 cycles (Fig. 2D). The half-life of the antibody at 97.5°C is ~6.5 min (Fig. 2D, inset). This rivals the heat-resistance of *Taq* polymerase, with a half-life of about 9 min (Lawyer *et al.*, 1993). Thus, the use of building blocks from aggregation-resistant antibody domains to generate a large combinatorial repertoire has allowed the selection of domain antibodies that combine good antigen-binding activities with remarkable aggregation-resistant properties. Such properties should generally facilitate the expression and manufacture of recombinant antibody products, as well as allow novel applications. For example, the ability to control antibody function in a temperature-dependant manner may allow the generation of synthetic switches for the regulation of protein or nucleic acid function, or the development of re-usable affinity columns where (thermostable) antigen can be eluted by a high temperature wash.

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