

Covalent antibody display—an *in vitro* antibody-DNA library selection system

Herald Reiersen*, Inger Løbersli, Geir Å. Løset, Else Hvattum, Bjørg Simonsen, John E. Stacy, Duncan McGregor¹, Kevin FitzGerald¹, Martin Welschhof, Ole H. Brekke and Ole J. Marvik

Affitech AS, Oslo Research Park, Gaustadalleen 21, N-0349 OSLO, Norway and ¹Isogenica Ltd, Barbraham CB2 4AT, UK

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ABSTRACT

The endonuclease P2A initiates the DNA replication of the bacteriophage P2 by making a covalent bond with its own phosphate backbone. This enzyme has now been exploited as a new *in vitro* display tool for antibody fragments. We have constructed genetic fusions of P2A with single-chain antibodies (scFvs). Linear DNA of these fusion proteins were processed in an *in vitro* coupled transcription-translation mixture of *Escherichia coli* S30 lysate. Complexes of scFv-P2A fusion proteins covalently bound to their own DNA were isolated after panning on immobilized antigen, and the enriched DNAs were recovered by PCR and prepared for the subsequent cycles of panning. We have demonstrated the enrichment of scFvs from spiked libraries and the specific selection of different anti-tetanus toxoid scFvs from a V-gene library with 50 million different members prepared from human lymphocytes. This covalent antibody display technology offers a complete *in vitro* selection system based exclusively on DNA-protein complexes.

INTRODUCTION

Antibodies are protein ligands with a wide range of biomedical applications. They have been displayed and evolved successfully by different *in vivo*- or *in vitro*-based selection methods using, for example, phage particles, yeast or bacteria cells (1–3). One limitation of the *in vivo* selection systems is the library size that could be generated. A large library is considered to be important to obtain high-affinity ligands. However, the efficiency of transfer of DNA into cells often limits

the library size to 10^9 – 10^{10} members (3–6). *In vitro*-based antibody selection methods such as ribosome display or mRNA display have proven to be successful in the construction and selection of libraries with a high diversity and complexities (potentially up to 10^{14} different members) (5–9). Both technologies are based on an initial selection of stabilized mRNA-protein complexes (4–10).

All the present antibody selection systems only indirectly link phenotype and genotype via cell membranes, phage-protein coat, mRNA-ribosomes, protein-puromycin-mRNA complexes or water/oil emulsions (1–3,7,8,11,12). In nature, however, there is a protein from the bacteriophage P2 that offers a direct DNA-antibody selection system. This phage replicates by attaching its early gene product P2A to its own DNA. The first genetic evidence of the *cis*-activity *in vivo* was obtained when the wild-type P2 phage did not complement mutations in P2A (13). P2A initiates the rolling circle replication of the P2 phage *in vivo*. Its catalytic tyrosine residue (Y454) makes a single-stranded-specific nick at the viral origin of replication, located at base pair 1860 inside the 2.3 kb P2A gene, and forms a covalent bond with the 5'-phosphate group of the coding strand (14–16) (Figure 1a).

A single chain antibody (scFv) can be genetically fused to the P2A protein creating the smallest imaginable antibody selection particle: a protein and its gene (Figure 1b). Covalent antibody display (CAD) exploits the demonstrated *in vivo cis*-activity of P2A in an *in vitro* selection system: a fusion protein of P2A and an scFv antibody binds to the same molecule of DNA from which it has been expressed. Following *in vitro* coupled transcription and translation, the P2A protein makes a covalent link between scFv genotype and scFv phenotype, by producing a stable protein-DNA complex (14–17). P2A may thus be exploited to select scFvs from a library by using only *in vitro* methods. These antibody-DNA complexes can be isolated with standard affinity selection strategies. Specific

*To whom correspondence should be addressed. Tel: +47 22958877; Fax: +47 22958358; Email: herald@affitech.com
Correspondence may also be addressed to Martin Welschhof. Email: m.welschhof@affitech.com

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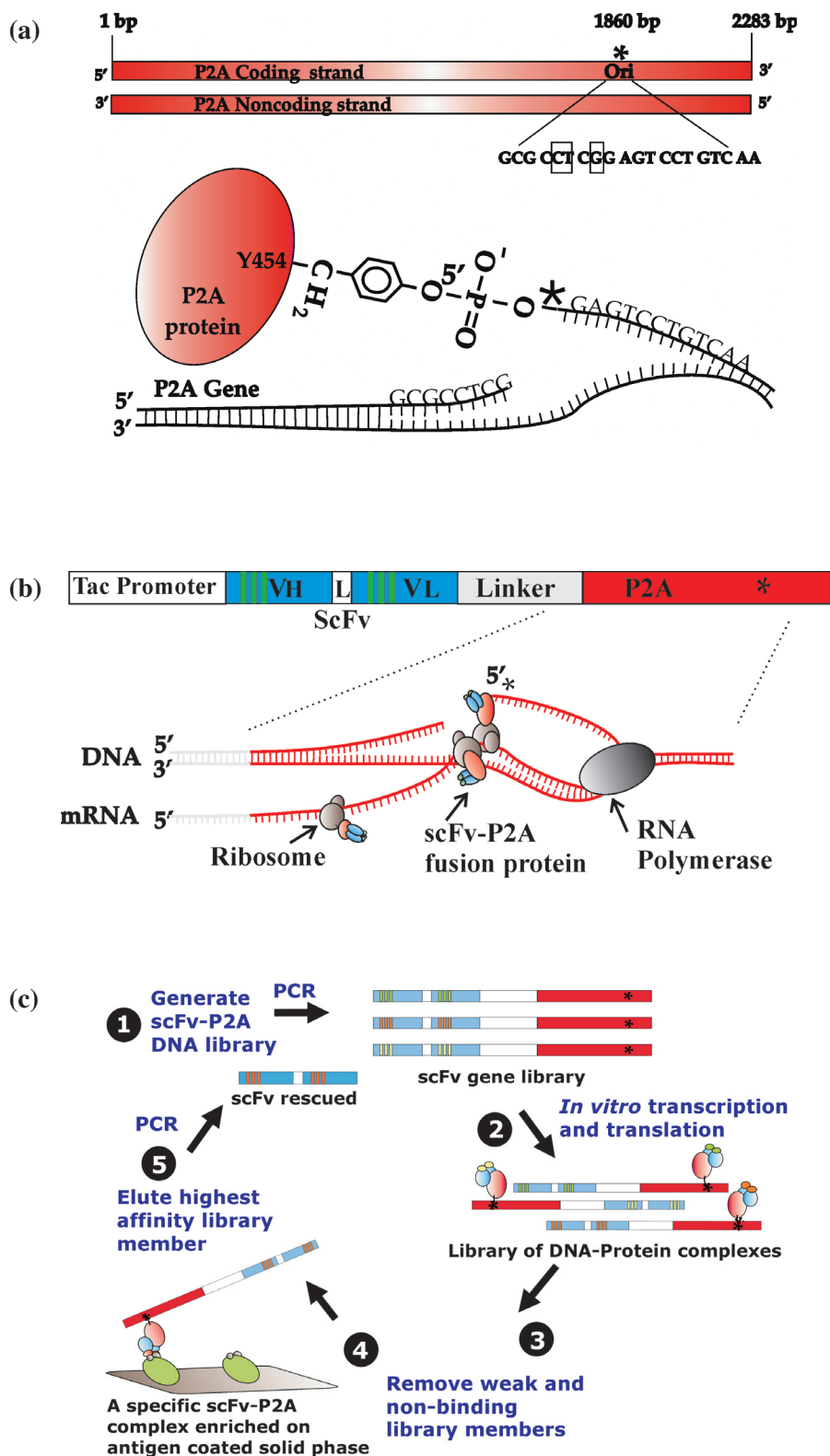


Figure 1. (a) The endonuclease P2A (761 residues, 86.3 kDa) makes a single-stranded site-specific nick at Ori of replication (... CCT CCG, *), located inside its own gene at position 1860, and becomes covalently attached (via Y454) to the 5' phosphate of its own DNA (14–16). (b) CAD is the exploitation of P2A to select antibodies or antibody fragments genetically fused to it. In prokaryotes, the transcription and translation are coupled, and the start of translation normally takes place before the transcription is finished (17). The figure is a model of the complex formation among DNA, RNA polymerase, ribosomes, mRNA and nascent P2A–scFv fusion proteins (colours match the gene products). The P2A protein part of the P2A–scFv fusion protein indirectly attaches the genotype of the scFv covalently to its phenotype. (L = linker). (c) Selection cycle for CAD. An scFv repertoire is assembled with the P2A gene using PCR methods and fresh P2A and tac-promoter (1). Protein–DNA complexes are being produced in an *in vitro* *E. coli* S30 coupled transcription–translation mixture (2) and selected on the immobilized target (3 and 4). Retained members are eluted and DNA for the next cycle is prepared using PCR (5). Figures are not drawn to scale.

complexes are enriched, eluted and rescued by PCR amplification (Figure 1c).

In the present study, we have demonstrated the suitability of P2A for specific selection of scFvs. Fusion proteins of scFv-P2A were expressed and DNA-antibody complexes were specifically recovered on antigen-coated solid phase. In addition, we have applied this technology to select antibodies from spiked and medium complex libraries. We propose that CAD can be exploited as a complete and independent *in vitro* antibody display tool for affinity selections.

MATERIALS AND METHODS

PCR cloning and assembly

The scFvs anti-phOx [phOx, 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one (18); anti-phOx (19)] and anti-DOB [DOB, 2,5-dimethoxy-4-bromo-amphetamine (20); in house made anti-DOB (unpublished data), specific against DOB] were fused to either the N-terminal or C-terminal position of P2A with standard PCR cloning techniques, attaching a GSGSGS linker containing suitable flanking restriction sites (EcoRI, NotI, XhoI or NcoI) and two stop codons at the 3' end (Figure 2). A vector tacP2aHa (5926 bp) containing the P2A-HA gene under the control of a tac promoter was supplied by Isogenica Ltd. *Pfu* Turbo DNA polymerase (Stratagene) was applied for generating GS-linker-scFv products for cloning. The PCR mixture was composed of 200 μ M dNTP mixture, 30 ng vector DNA-template, 0.6 μ M primers GsDOB3F

(aaattaaaactcggggttctggctccgggtccatggcgggaagtgcagctggtgc; XhoI and NcoI sites are underlined and GSGSGS sequence is in italics)/EcoRIPhog (ttttttgaattctatcagtgatggtgatggtgatg-agttagg; EcoRI site is underlined and HHHHHH sequence is in italics) or GsPhOxF (aaattaaaactcggggttctggctccgggtccatggcggcaggtgcagctggtgc; XhoI and NcoI sites are underlined and GSGSGS sequence is in italics)/EcoRIPhog in the *Pfu* reaction buffer. Initial denaturation was at 94°C for 5 min followed by 30 cycles at 94°C (1 min), 63°C (1 min) and 72°C (2 min 30 s) in an Eppendorf Mastercycler Gradient PCR machine. The final elongation step was at 72°C for 5 min. The GS-linker-P2A for cloning scFvs at the 5' of P2A was produced by *Pwo* Polymerase (Roche, Norway) at an annealing temperature of 65°C [primers: GsP2af (aaattaaaactcggggttctggctccgggtccatggcgggtaagcctccggg; NotI and NcoI sites are underlined and GSGSGS sequence is in italics)/Lanrb (gtggataaccgtattaccgc)]. The *Pwo* PCR programme was as follows: 94°C for 2 min, with initial 10 cycles at 94°C (30 s), 65°C (1 min) and 72°C (2 min), then 20 cycles at 94°C (30 s), 65°C (1 min) and 72°C (2 min) increasing the extension time with 5 s per cycle, and with a final elongation step at 72°C for 7 min. To clone small scFv libraries (scFv)*n* into the scFv-GSGSGS-P2A vector via NcoI/NotI sites, it was necessary to delete the second NcoI site at the beginning of P2A (Figure 2). This site was removed with the primers GSP2Anew (aaattaaaactcggggttctggctccgggtctatggcgttaagcctccggg; NotI site is underlined, GSGSGS sequence is in italics) and Lanrb and *Pwo* polymerase as described previously. Standard methods were applied for heat transformation of

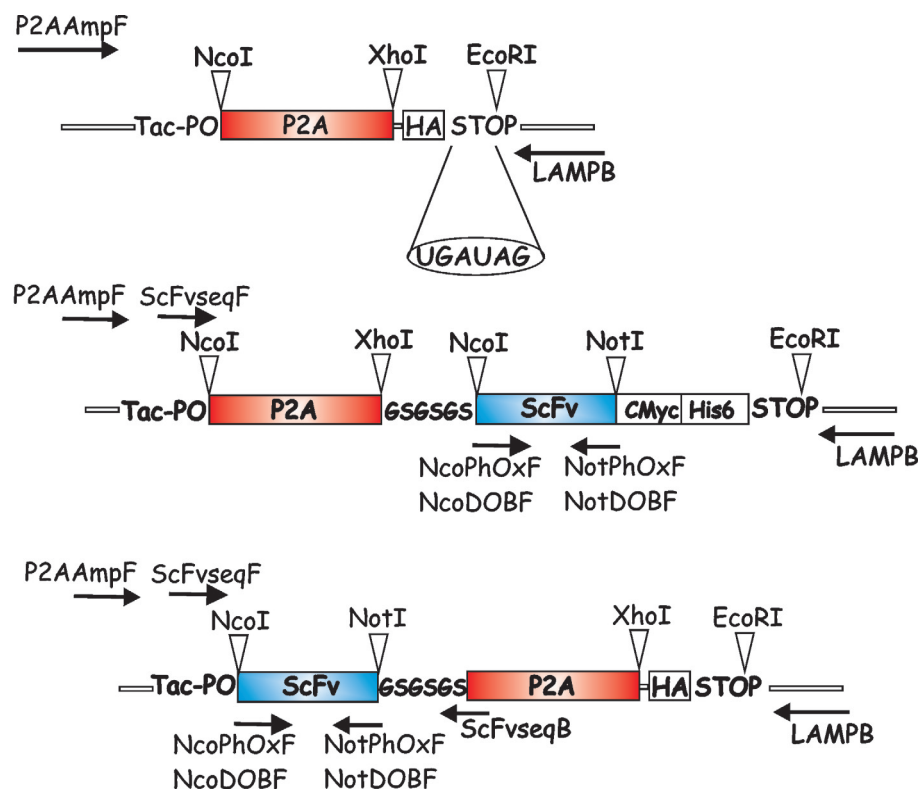


Figure 2. Primer and restriction sites for rescue PCR and assembly of DNA for next rounds of CAD. Top: P2A-HA construct; middle: P2A-scFv; and bottom: scFv-P2A. HA, haemagglutinin peptide epitope of human influenza virus (YPYDVPDYA); c-Myc, myc oncogene epitope (EQKLISEEDL); His₆, hexahistidine sequence (HHHHHH).

plasmid DNA into chemical competent *Escherichia coli* DH5- α or Origami cells (Novagen). The transformed cells were grown in SOC medium at 37°C for 1 h shaking at 260 r.p.m. and plated on SOC/ampicillin (100 μ g/ml) agar dishes for 15–20 h at 37°C.

Input linear DNA of *P2A-scFv* and *scFv-P2A* clones for coupled transcription and translation were made by either *Pwo* or *Tgo* DNA polymerase (Roche, Norway) with a hot-start technique. Preparations of plasmids (ligation mixtures or minipreps; 0.5 μ g) were used as template for PCR, and the primers P2AampF (gcttcagtaagccagatgctac, 30 pmol) and LAMPB (tacaccgaactgagata cctac, 30 pmol) were chosen for producing the \sim 4 kb DNA fragment comprising tac-promoter, *scFv* and *P2A* (Figure 2). The PCR programme for *Tgo* DNA polymerase was as follows: 94°C (2 min), cycling 30 times at 94°C (30 s), 65°C (1 min) and 72°C (3 min) followed by a 7 min incubation at 72°C. The linear DNA fragments were separated on agarose gel and purified with Qiaquick gel extraction kit (Qiagen). Extra washing steps were necessary in order to remove all the salt components inhibiting transcription and translation. The products were adjusted to a high-concentration sample (1–3 μ g/ μ l) by alcohol precipitation and applied in coupled transcription and translation.

In vitro transcription-translation

Reaction buffer (2.5 \times) and *E.coli* [strain SL-119, (21)] S30 extract (22) with 1 mM DTT (Sigma) were applied. For a 50 μ l reaction in an Eppendorf tube, the following components were assembled on ice: S30 2.5 \times reaction buffer (20 μ l), *E.coli* S30 extract (15 μ l), \sim 1 mM L-methionine (Sigma), 1 μ M protein disulfide isomerase (PDI; Sigma), 40 U RNasin (Promega), nuclease-free water, 0.1% BSA and DNA (1–2.5 μ g). Generally, 5–10 μ g of DNA was added for each round of selection. Transcription and translation reaction was started by adding DNA (1–5 μ g per 50 μ l reaction) and processed for 60 min at 30°C. After 40 min of incubation, 2 mM of oxidized and reduced glutathione were added. The reaction was stopped by diluting the mixture in ice-cold panning buffer and incubated with an immobilized target. The transcription and translation extract from Promega (*E.coli* S30 extract for linear templates) was also applied, but it contains an excess of DTT, which inhibits the folding of antibodies.

Affinity selections

Dynabeads M-280 Tosylactivated (DynaL Biotech, Oslo, Norway) were coated with anti-haemagglutinin (anti-HA), or with the antigens phOx-BSA (18) or DOB-BSA (20) (6 μ g antibody or 20 μ g hapten-BSA conjugate per mg beads; 20 mg beads/ml) in 0.1 M sodium carbonate, pH 9.6 at 37°C for 20 h on an Eppendorf thermomixer at 1200 r.p.m. Then, they were washed three times in phosphate-buffered saline (PBS), pH 7.4 with 0.1% BSA at 20°C and blocked for another 20 h at 20°C (1200 r.p.m.) in 0.3 M Tris-HCl, pH 8.8 with 0.1% BSA. Finally, they were washed in PBS, pH 7.4 and stored at a concentration of 20 mg beads per ml. For the E7 scFv, an scFv specific for an outer membrane epitope of *Neisseria meningitidis* (E7), the outer membrane vesicles (OMV) made from the bacteria, were applied as antigen (23). Immuno tubes (Maxisorp, Nunc, Denmark) were coated with antigens in PBS (pH 7.4) overnight at 20°C on a blood mixer. The affinity

matrices were pre-incubated with panning buffer SuperBlock (Pierce) added \sim 0.2 mg/ml herring sperm DNA (Sigma), 0.25% (w/v) heparin and 1% Tween-20 (6,24,25) for 1–2 h at 20°C on a blood mixer, and then rinsed three times in PBS before panning mixture was added.

DNA-protein complexes were captured by first diluting the transcription and translation mixture into panning buffer, then incubating on an affinity coated matrix for 2–4 h rotation at 4°C or at 20°C on a blood mixer. The enriched complexes were thoroughly washed in PBS with 0.1% Tween-20, panning buffer (including an incubation step at 20°C for 30 min) and in PBS.

The DNA was eluted using proteinase K (50 μ g/ml; Sigma) in TE buffer, pH 8.0 for 45 min at 50°C (26) and ethanol precipitated in seeDNA (Amersham Pharmacia Biotech).

PCR-rescue, detection of P2A-scFv fusions and processing scFv clones

If the *P2A-scFv* fusions have been expressed and purified, the enriched DNA of the *P2A-scFv* construct can be identified and amplified by PCR. The seeDNA precipitated DNA was dissolved in 10 μ l of nuclease-free sterile MQ-water and 1–5 μ l was used for each PCR-screen. If the input DNA in the transcription and translation mixture was made by PCR, it was not possible to use the same primers P2AampF and LAMPB in the PCR mixture as was done for producing it (Figure 2). This was probably due to the partial degradation of DNA after incubation. However, by using primer combinations NcoDOBF-(atggcgaagtgcagctggtg)/NotDOBB(gccgcgaagacagatggtgc), NcoPhOxF(atggcccaggtgcagctggtgc)/NotPhOxB (gccgcactagacggtgacc) or scFvseqF (cctgttgacaattaatcatggtctg)/scFvseqB (cctgcggcaaatgctgacgg) (Figure 2), they generated products comprising just the *scFv* gene and short flanking regions (\sim 0.8–1 kb). For primer combinations NcoDOBF/NotDOBB and NcoPhOxF/NotPhOxB, an annealing temperature at 68°C was chosen, and 55°C for scFvseqF/scFvseqB. For detection purposes, DynaZyme II DNA polymerase (Finnzymes, Finland) was used, and for amplification of new products for next round of selection, *Pwo* polymerase (Roche) was chosen.

The recovered and amplified *scFv* DNA from each round of CAD was either cloned into an assay expression vector pHOG21 (having a lac promoter and *c-myc/His₆*-tags) (27) or assembled with tac promoter and *P2A* gene using restriction/ligation methods into vector tacP2aHa (NcoI/NotI sites; Figure 2). DNA for the next round of transcription and translation was generated by PCR using ligation mixture as mentioned above. For the expression of soluble scFvs in 96 deep-well plates, the pHOG21 expression vector constructs were transformed into XL-1 Blue cells and induced in Luria-Bertani (LB) medium containing 100 μ g/ml of ampicillin (A) with 100 μ M isopropyl- β -D-thiogalactopyranoside (IPTG) at 30°C and shaken for 16 h. The expressed scFvs were detected using enzyme-linked immunosorbent assay (ELISA), or were assayed as described below.

SDS-PAGE, western blotting and enhanced chemiluminescence (ECL) detection

The samples, added SDS-sample buffer with 2-mercaptoethanol, were separated on a 12.5% SDS-PAGE criterion

precasted gel (Bio-Rad), electro blotted onto nitrocellulose membranes, washed (PBS), blocked with Blotto (1% dry milk in PBS), and incubated in anti-*c*-Myc (diluted 1:5000 in Blotto; Invitrogen) or anti-HA (1:2000 in Blotto; Roche) solutions. The membrane was washed (PBS with 0.1% Tween-20) and incubated with detection antibody [rabbit anti-mouse-horseradish peroxidase (HRP); DAKO, Denmark]. The HRP activity on membrane was visualized in ECL-western blotting reagents RPN 2106, Hyperfilm ECL (both Amersham Pharmacia Biotech) and a film developer.

ELISA and filter screening

Maxisorp 96-well plates (Nunc, Denmark) were coated with antigen, and expressed scFvs were detected via their *c*-Myc tags using antibodies/enzymes as described above. 3,3',5,5'-Tetramethylbenzidine (TMB) was used as a substrate. Filter screening of 3040 bacterial colonies was performed as described previously (28). To prepare target filters (0.45 μ m pore size cellulose nitrate; Schleicher & Schuell Protan BA 85), these were cut to 22 \times 7 cm² and coated with 10 ml of pHox BSA or DOB BSA at 0.1 mg/ml. Generally, clones were high-density gridded with a Qpix robot (Genetix, New Milton, UK) on a BSA-coated filter surfacing a 22 \times 22 cm² LB agar bioassay plate containing 30 μ g/ml of tetracycline (T), 100 μ g/ml of ampicillin (A) and 0.1 M glucose (G), and grown for 16 h at 37°C. Then, the BSA filter with clones was lifted on top of a sandwich with an antigen-coated filter in the middle and fresh LB agar plate with 100 μ M IPTG and A at the bottom, and induced for 16 h at 30°C. The active scFvs will leak from the periplasm of the colonies to the antigen-coated filter underneath. This filter was developed like a western membrane as described above.

RESULTS AND DISCUSSION

Constructing a basic scFv–P2A model system

The A protein of the P2 phage can be used as a potential antibody display tool (Figure 1b and c). To test its suitability, we chose two well-characterized scFvs against the haptens: pHox (18) and DOB (20). The scFvs were genetically fused to P2A at two different positions connected by a (GS)₃-linker and expressed from a tac-promoter. The scFv N-terminal to P2A with an HA-tag: tacPO-scFv-(GS)₃-P2A-HA and scFv C-terminal to P2A with *c*-Myc tag: tacPO-P2A-(GS)₃-scFv-cmyc-His₆ were constructed (Figure 2). The fusion proteins and P2A-HA alone were translated in a standard *E. coli* S30 extract for linear templates (Promega) and sharp bands corresponding to the ~90 kDa P2A-HA and the ~110 kDa P2A-scFv proteins were clearly visible, showing efficient *in vitro* expression (Figure 3a). Estimating the yield of protein, the sensitivity of ECL (10⁻¹¹ M) indicated a protein band (~1 ng) where the input of DNA was 1 μ g (Figure 3a). This suggests that ~3% of input DNA produces proteins, which would require around 30–40 copies of each scFv to be present in the library to ensure isolation of any unique scFv members.

Antibodies are normally functionally folded in oxidizing or slightly reducing environments (6,24). To allow RNA polymerase activity, which requires a reducing environment, a specially made transcription and translation mixture was prepared (see Materials and Methods). Immediately after

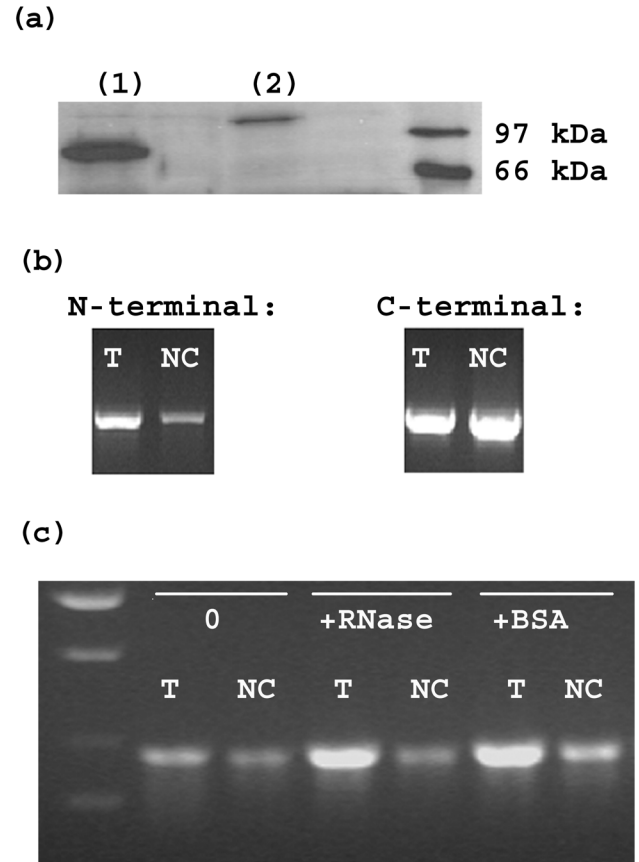


Figure 3. (a) Western/ECL identification of P2A-HA (lane 1) and P2A-anti-DOB3-*c*-Myc (lane 2) proteins expressed in a coupled transcription-translation mixture (Promega *E. coli* S30 extract for linear templates). The proteins were separated on 12.5% criterion polyacrylamide gel (Bio-Rad), blotted onto nitrocellulose membrane and detected by anti-*c*-Myc/anti-HA and rabbit anti-mouse IgG-HRP/ECL. (b) Agarose gel of rescue PCR of DNA from *anti*-pHox-P2A (left panel) or P2A-*anti*-pHox (right panel) protein-DNA complexes selected onto target T (pHox-BSA-coated Dynabeads) or negative control NC (BSA-coated Dynabeads). The protein-DNA complexes were generated in *E. coli* S30 lysate added 1 μ M PDI and 1 mM DTT. They were eluted with proteinase K digestion, alcohol precipitated and detected by PCR. (c) Agarose gel of rescue PCR of DNA from scFv(E7)-P2A protein-DNA complexes selected on *N. meningitidis* outer membrane vesicles (T) or superlock (Pierce, NC). The effect of RNase (Promega) treating samples immediately after transcription-translation (at 30°C, 10 U) or adding 0.1% BSA during transcription and translation. The DNA bands from no additions/post-treatments are also shown (0).

transcription and translation, the protein-DNA complexes were enriched onto target antigen-coated beads, eluted and detected by PCR.

The display of functional scFv was evaluated at both the N- and C-terminus of P2A. Figure 3b shows the recovery of rescued DNA for N- and C-terminal position of *anti*-pHox scFv relative to P2A. For the N-terminal position (scFv-P2A), there was more DNA associated with pHox-BSA than BSA beads (target PCR signal > background PCR signal). This illustrates that the DNA enrichment was well correlated with antibody activity. Similar results were also obtained for the anti-DOB fusion proteins (data not shown). In contrast, the C-terminal fusions (P2A-scFv) showed no specific enrichment. It is possible that the linker length was too short to accommodate proper folding and functionality of these fusions, or they were structurally destabilized. Premature

termination of the translation may also occur if P2A nicks DNA before scFv is synthesized. This may occur more frequently if the scFv is at the C-terminal end of P2A rather than at the N-terminal end.

Different additives improve the functionality of covalent antibody display

Adjusting the redox potential of the transcription and translation mixture generated active fusion proteins associated with DNA, and adding BSA increased the yield of DNA recovered by PCR and improved the signal-to-noise ratio relative to no addition (Figure 3c). BSA may stabilize enzymes and components in the transcription and translation mixture during *in vitro* synthesis, even though BSA also contributes to a stronger unspecific adsorption of DNA (Figure 3c). Furthermore, the samples were treated with RNase after the protein-DNA complexes were formed (Figure 3c). A similar pre-treatment has been applied for ribosome display, using DNase to remove DNA (29). RNase increased the signal-to-noise ratio confirming that mRNA is not involved during selection (as for ribosome display). The enriched active species therefore consist of pure DNA-protein complexes. In addition, the coupled transcription and translation mixture of an *anti-phOx-P2A-HA* DNA construct (Figure 2, bottom) was incubated in 6.3 M urea before it was diluted in panning buffer and enriched on anti-HA coated tubes. Still, this harsh treatment made it possible to specifically enrich its DNA on anti-HA-coated tubes (data not shown), and thus did not release the anti-phOx-P2A-HA protein from its mother DNA. This again confirms that the anti-phOx-P2A-HA protein is covalently bound to DNA as shown previously for P2A-HA proteins (13–16).

Enrichment experiments

To demonstrate the recovery of specific antibody DNA-protein complexes from a complex mixture, specific enrichment experiments were performed. In a population where there is only one copy of a specific *P2A-scFv* gene among, e.g. 10^7 other different non-active *P2A-scFv* genes, the *cis*-activity is related to the probability of recovering the specific *P2A* gene product binding to its own mother DNA. If the P2A protein only finds its own mother DNA molecule, a direct enrichment of P2A protein-DNA complexes is productive. However, all selection systems contain a certain amount of noise (non-relevant gene products) raising the threshold required to detect rare binders from a large diverse library early in the selection process (round 1–2). If there were no *cis*-activity, the amount of randomness involved would possibly block the enrichment of the specific gene and gene product, even after 10–20 cycles. On the other hand, an early enrichment of active library members from a very diverse mixture should demonstrate that there is a *cis*-directed selection.

An scFv antibody library from human lymphocytes (PBL) was constructed by RT-PCR using *IGHV-D-J*, *IGKV-J* and *IGLV-J* genes. The library having a diversity of $n = 5.0 \times 10^7$ different clones was cloned as an N-terminal fusion to P2A, (*scFv*)*n*-P2A. DNA of *anti-phOx-P2A* or *anti-DOB-P2A* fusions were spiked into the library at a ratio of 1:25 000 (anti-phOx) and 1:1000 and 1:100 000 (anti-DOB) and subjected to a complete covalent display selection cycle (Figure 1c). Two spiking strategies were employed. For the

selection of anti-DOB scFvs spiked into the (*scFv*)*n*-P2A library at a ratio of 1:10³, 4.8 ng of *anti-DOB-P2A* DNA (1.1×10^9 molecules) and 5 μ g (1.1×10^{12} molecules) of library DNA were used. Lowering the same spiking ratio to 1:10⁵, 48 pg of *anti-DOB-P2A* (1.1×10^7 DNA molecules) was added into 5 μ g (1.1×10^{12} DNA molecules) of library DNA. For the selection of anti-phOx, 400 pg of *anti-phOx-P2A* DNA was spiked into 10 μ g of (*scFv*)*n*-P2A library (1:25 000). The recovered DNA from each round was analysed using filter screen as shown for anti-phOx in Figure 4a. As expected, there were no positive candidates before the selection of 3040 clones in the unselected library. However, after round 1–3 (R1–R3) of CAD, the number of enriched clones increased from 6 (R1), 49 (R2) to 82 clones (R3). The enrichment after each round is also illustrated in Figure 4b. The clones were MvaI digested (30) and their restriction fragment length polymorphism (RFLP) patterns confirmed their *anti-phOx-P2A* identity. Enrichment was also seen after analysing 1:1000 and 1:100 000 spiking of *anti-DOB-P2A* into the same library, respectively. After R1 of CAD 28 positive *anti-DOB-P2A* clones (1:10³ spiking)

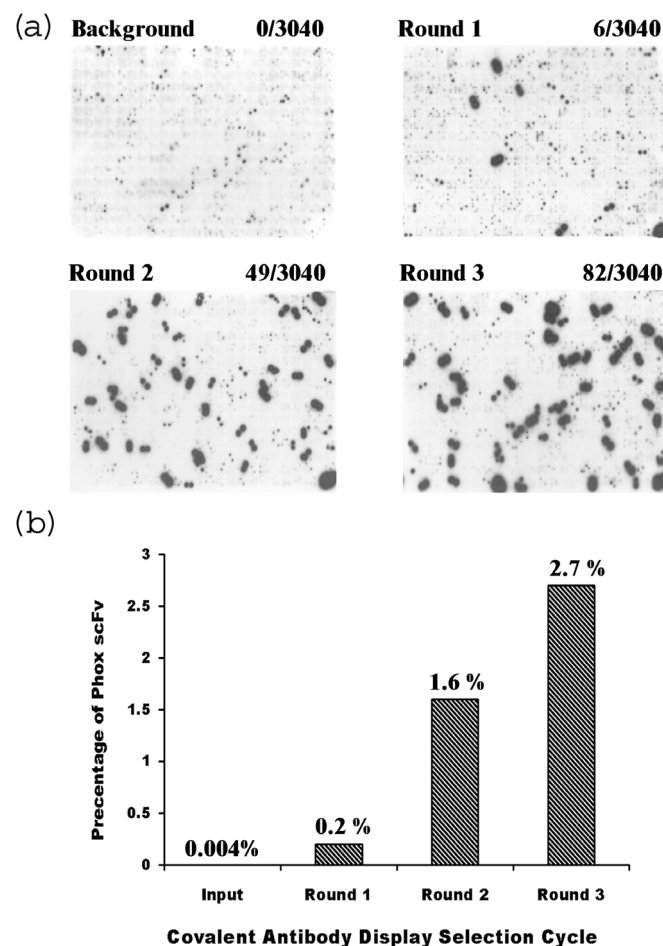


Figure 4. (a) Filter screen of expressed scFvs derived from rescued DNA cloned from each round of CAD selection. Spiking 1:25 000 of *anti-phOx-P2A* DNA into (*scFv*)*n*-P2A library with diversity of $n = 50$ million unique scFvs, the fraction of positives from a random selection of 3040 clones is shown. (b) Enrichment obtained from each round of selection (total of 800-fold). R0–R1: 50-fold; R1–R2: 8-fold; and R2–R3: 2-fold.

were identified from a random sample of 3040 clones (14-fold enrichment), and similarly 8 clones (~300-fold enrichment) for the 1:10⁵ spiking (data not shown). On studying the results above, where the ratio of scFv-PLA spiked with other irrelevant scFv-PLA DNA molecules is 1:25 000, the likelihood that the correct scFv is randomly chosen, is very low. The probability of selecting 82 positive clones from a random sample of 3040 candidates with a population of 5×10^7 and having 1 positive among 25 000 clone is as low as 10^{-13} [$P, N(0,1)$]. This argues strongly that the one given molecule of P2A should bind to its own molecule of DNA that directed its synthesis.

The translation complex formed among DNA, mRNA, ribosomes and proteins is common in prokaryotes. This implies the production of extra mRNAs and an overexpression of the P2A protein. Liu and Haggård-Ljungquist (15) describe the inability to complement an amber A mutant by the overexpression of normal A protein on a second plasmid. This indicates high-fidelity *cis*-activity. The authors suggest either compartmentalization of 'A' protein or rapid inactivation of A as the mechanism for controlling *cis*-activity. It may be that the strong *cis*-effect is due to the coupling of P2A with single-stranded DNA that is available only during the melting of double-stranded DNA with RNA polymerase, and that other free P2A proteins may be inactive for nicking. P2A cannot bind to double-stranded DNA (15), and thus, free scFv-P2A molecules should not be able to bind to untranscribed double-stranded DNA. It is also possible to purify P2A without covalently bound DNA, suggesting that P2A can be released from the ribosome without binding to DNA. The experiments, however, demonstrate a specific recovery of protein-DNA complexes. The downward trend during selections (Figure 4) may be explained wherein, for each round of selection, more active scFv-P2A fusion proteins are formed. It is most likely that the majority of these protein complexes were not bound to DNA. However, this does not influence the *cis*-activity since only single-stranded DNA is nicked by P2A. On the other hand, it affected the panning. The free, uncomplexed scFv-P2A fusion proteins compete with the corresponding DNA-protein complexes. Thus, the more the concentration of scFv-P2A protein fusions is increased, the less the DNA-protein complexes are isolated. Panning a more complex library (e.g. $n = 10^{14}$) should not however involve this type of problem since the initial concentration of expressed active proteins is very low and should not compete with DNA-protein complexes. This was also seen from the spiking experiments. Starting with a more diverse library (1:10⁵), the initial enrichment from R0 to R1 was larger (300-fold) compared to less diverse spikings 1:2.5 $\times 10^3$ and 1:10³ that only had 50- and 14-fold enrichments, respectively. The CAD method has to be developed further to deal with these technical problems, which probably only arise at the final enrichment cycles. It may be possible to first purify all DNA before selection on protein complexes. The initial isolation of DNA could be performed with biotinylated DNA and SA-beads before subjecting it to an ordinary panning against active protein.

Panning tetanus toxoid scFvs from a 5×10^7 library

The human antibody library, (scFv)*n*-P2A, was also selected against tetanus toxoid (TTx). The serum antibody titre of the

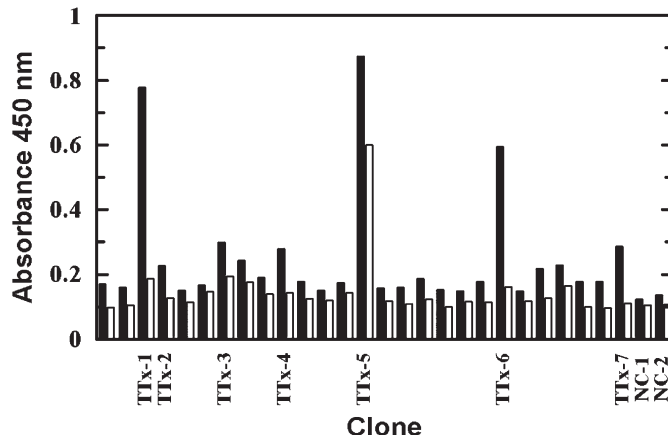


Figure 5. Panning an scFv library. (scFv)*n*-P2A ($n = 50$ million unique clones) against tetanus toxoid. ELISA of R1 of CAD. A total of 92 clones were randomly picked and assayed (ELISA). Only clones with $A_{450} > 0.15$ are shown (anti-TTx scFv, closed bar; BSA, open bar).

donor against TTx was found to be about 1:25 000, implying that it should be possible to identify anti-TTx scFv antibodies derived from donor lymphocytes. For each round of selection against TTx, 10 μ g of DNA of the (scFv)*n*-P2A library was applied as an input for coupled transcription and translation. Indeed, as shown in Figure 5, after one round (R1) of CAD several anti-TTx scFvs were identified by ELISA. The total yield of available *anti-TTx*-P2A DNA after each round of selection was estimated from *anti-phOx*-P2A DNA standards at known concentrations (pg range). The input DNA was correlated with the corresponding PCR yields of *anti-TTx* scFv DNA versus *anti-phOx* DNA standards on an agarose gel. After the first round of selection (R1), the amount of input DNA for PCR (recovered DNA) was ~0.2 pg, which equals a DNA recovered/input ratio of 1/50 000 000. After two and three rounds of selection (R2 and R3), the estimated ratio increased to 1/2 500 000 and 1/420 000, respectively. The CAD-enriched library was also analysed by filter screen with no scFvs isolated before selection (background, 3040 colonies screened). However, after R1 of CAD, the number of positives increased to 26 (26/3040). Most of these clones bound BSA (TTx-5, Figure 5). Nevertheless, some anti-TTx scFvs not cross-reacting with BSA were confirmed by ELISA. After R2 and R3, other scFvs were rescued and TTx-12 isolated after two rounds of CAD was specific for TTx. Clones were also analysed by RFLP. The R1 clones were unique. However, TTx-8 and TTx-12 (both R2 clones) had identical RFLP patterns and DNA sequence (Table 1). The same HCDR3 sequence also appeared for TTx-17 (R3 clone), although there was a difference in their LCDR3 region (Table 1), suggesting a sequence enrichment during R1-R3 of CAD. Furthermore, the HCDR3 sequences in this study were aligned with known human anti-TTx antibody sequences [Table 1 and (31,32)]. The residues at the C-terminal region of HCDR3 of TTx-12/TTx-17 (R2/R3 clones, respectively) were very similar to 6-ATT (33), suggesting a selection on the same epitope of TTx. Similarly, most of the HCDR3 sequences compared in Table 1 have a positively charged N-terminal (at position 105 or 106) and an Asp residue at position 116. For the

Table 1. Comparison of human anti-TTx CDR3 regions

Clone	R	Name (IMGT-score)	HCDR3 Sequence	Name (IMGT-score)	KCDR3/LCDR3 Sequence	Reference
TTx-1	R1	IGHV3-74*03 (1227)	ARYCSGGSCLYAFDI	IGLV1-44*01 (1153)	AVWDDS - - LNGPV	-
TTx-6	R1	IGHV1-2*02 (1236)	ARGHSGSQPRRGLPKFDL	IGKV4-1*01 (1269)	QQYYN - - - APLT	-
TTx-22	R1	IGHV3-74*01 (1263)	ARVLGV - - EHPLG	IGLV1-44*01 (1237)	AAWDDS - - LSNPV	-
TTx-38	R1	IGHV2-5*10 (1278)	VHRRYFDRSPHFAS	IGLV1-51*01 (1252)	GTWDDS - - LSARV	-
TTx-12	R2	IGHV1-18*01 (1290)	VRLVVKRTATLHYIDV	IGLV1-51*01 (1202)	GAWDGS - - LREAV	-
TTx-17	R3	IGHV1-18*01 (1290)	VRLVVKRTATLHYIDV	IGLV1-51*01 (1193)	AAWDDS - - LNGPV	-
CH8	n.a.	IGHV	ETSVRRDYRDYPMTDY	IGLV	QQSYS - - -TPPRT	(34)
CH5	n.a.	IGHV	RSVWGE - - HYFDY	IGLV	QQYNS - - -WPLT	(34)
6-ATT	n.a.	IGHV4-B*01 (1138)	AKVALMKWFGYYFDS	IGKV1-9*01 (1179)	QQYSS - - -SPPVT	(33)
B7-15A2	n.a.	IGHV1	ARVLFQQLVLYAPFDI	IGLV	QSYDS - - -SLSAR	(35)

TTx-sequences (*IGHV-D-J*, *IGKV-J* and *IGLV-J* genes) were classified and aligned using IMGT/V-QUEST (<http://imgt.cines.fr>), and the positions 105–117 for CDR3 sequences are shown (31,32). The gene names are the human germline equivalent. IMGT-scores > 1179 reflects at least 95% nucleotide sequence identity (33). The 6-ATT clone is from *Macaca fascicularis* Fab (33). R = Round of CAD. na, not available. The basic residues are in blue, the acidic residues are in red, and serines and threonines are in green.

Table 2. Comparison of different *in vitro* display technologies

Property	Phage (3) ^a	Ribosomal (7) ^a	mRNA (7,8) ^a	IVC (11,12) ^a	CAD (this study)	CIS (36) ^a
Covalent link	No	No	Yes (synthetic)	No	Yes (inherent)	No
Nucleic acid selected	DNA	mRNA	mRNA/cDNA	DNA	DNA	DNA
Antibody display	scFv	scFv	scFv ^b	No	scFv	scFv
Typical e.f.	10 ² –10 ⁴	10 ¹ –10 ³	10 ¹ –10 ³	10 ¹ –10 ³	10 ¹ –10 ²	10 ³
Library size	10 ⁹ –10 ¹¹	10 ¹² –10 ¹³	10 ¹² –10 ¹³	10 ⁹ –10 ¹⁰ /ml	10 ⁷	10 ¹²
Potential size	10 ¹¹	>10 ¹³	>10 ¹³	>10 ¹⁰	>10 ¹³	>10 ¹³
Making libraries	Time consuming	Fast	Fast	Fast	Fast	Fast
Stability	Stable	Stable in Mg ²⁺	Covalent	Stable	Covalent	>2 days

Abbreviations: e.f., enrichment factor per round of selection; and IVC, *in vitro* compartmentalization.

^aRepresent the reference numbers.

^bVia *IGHV-D-J*, *IGKV-J* and *IGLV-J* genes.

light chains, they all contain a Ser in the middle of the loop (position 109 or 110).

CAD as a new *in vitro* antibody display tool

We have demonstrated the successful selections from two independent spiking experiments (phOx and DOB fusions) and from an immunized scFv library with a complexity of 10⁷. The method should thus be well suited for *in vitro* scFv selections from libraries. However, in order to fully demonstrate its potential, a more diverse library should be screened. P2A can be applied as a general panning tool for antibody library selections at the N-terminal end of the endonuclease. The advantages for such a selection system are numerous, compared to the existing phage display and *in vitro* mRNA display methods. CAD is the only antibody selection method providing a direct covalent link between an scFv gene and its protein (14–16), increasing the chemical stability of the panning complex. Table 2 compares the more common methods of *in vitro* selection, and all methods have their advantages and disadvantages. The new DNA-based *cis*-display methods of panning are however very promising. Generally, CAD may facilitate a very rapid library generation using PCR methods with increased library complexity size (potentially up to 10¹³ scFv members) and a fast cycle time. In a few hours, unique scFvs can be enriched, isolated and directly amplified for the next rounds of selection (Figure 1c). The system should be very well suited for scaling up and automation, and may be a future standard for *in vitro* antibody selections. However, at

present, the CAD methods must be made more robust to overcome the product inhibition of uncomplexed fusion proteins without DNA. CAD offers a covalent antibody selection tool as an alternative to the latest non-covalent *in vitro* display selection method of peptides and proteins with the RepA protein (36).

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