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Selection of TNF-α binding affibody molecules using a β-lactamase protein fragment complementation assay

Research Paper

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Protein fragment complementation assays (PCAs) based on different reporter proteins have been described as powerful tools for monitoring dynamic protein–protein interactions in living cells. The present study describes the construction of a PCA system based on genetic splitting of TEM-1 β-lactamase for the selection of proteins specifically interacting in the periplasm of *Escherichia coli* bacterial cells, and its application for the selection of affibody molecules binding human tumour necrosis factor-α (TNF-α) from a combinatorial library. Vectors encoding individual members of a naïve 10^9 affibody protein library fused to a C-terminal fragment of the β-lactamase reporter were distributed via phage infection to a culture of cells harbouring a common construct encoding a fusion protein between a non-membrane anchored version of a human TNF-α target and the N-terminal segment of the reporter. An initial binding analysis of 29 library variants derived from surviving colonies using selection plates containing ampicillin and in some cases also the β-lactamase inhibitor tazobactam, indicated a stringent selection for target binding variants. Subsequent analyses showed that the binding affinities (K_D) for three selected variants studied in more detail were in the range 14–27 nM. The selectivity in binding to TNF-α for these variants was further demonstrated in both a cross-target PCA-based challenge and the specific detection of a low nM concentration of TNF-α spiked into a complex cell lysate sample. Further, in a biosensor-based competition assay, the binding to TNF-α of three investigated affibody variants could be completely blocked by premixing the target with the therapeutic monoclonal antibody adalimumab (Humira®), indicating overlapping epitopes between the two classes of reagents. The data indicate that β-lactamase PCA is a promising methodology for stringent selection of binders from complex naïve libraries to yield high affinity reagents with selective target binding characteristics.

Introduction

Technology developed for functional *in vitro* selection and identification of novel proteins with desired target binding properties, initially present as rare members in large repertoires of candidate variants, has contributed enormously to the field of protein engineering, including the development of a large number of important reagents for biotechnology, diagnostics and therapy. The common theme of these methods is a physical link between genotype and phenotype during the typically multi-round selection process. Examples include phage display [1], ribosome display [2] and cell display, used in conjunction with libraries of peptides [3], antibody fragments [4] or novel binders based on non-Ig frameworks, such as anticalins [5], DARPins [6] and affibody molecules [7].

Originally introduced as a method enabling studies of protein–protein interactions in living mammalian cells [8,9], the protein fragment complementation assay (PCA) principle has emerged as one of the most promising for large scale studies of protein–protein

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interactions, and their perturbations, at a proteomic scale [10]. More recently, PCA technology has also been explored for selections from complex libraries in protein engineering and cDNA analysis [11–14]. The common principle behind all PCAs is a molecular recognition-driven reconstitution of the activity of a genetically split reporter protein, to monitor or select for particular protein–protein interactions in living cells. The two subfragments of the reporter protein, which are unable to associate spontaneously, are co-expressed within the same cell, each fused to one or the other member of an interrogated interaction pair. Depending on the reporter protein, host cell and experimental conditions used, interactions and their perturbation can be monitored by different read-outs, including fluorescence, luminescence and cell survival [15,16]. In an *Escherichia coli* host cell, the co-expressed reporter fusion protein halves can be directed to either the cytoplasmic or periplasmic compartments. PCA systems combining a particular reporter protein with a specific class of library protein and a certain target protein may only work in one or the other compartment, based on the differences between system components in terms of, for example, secretion competence, folding requirements and proteolytic stability. For example, only the periplasmic space is suitable if the formation of disulphide bridges is required for structure and/or solubility. In this case all components have to be compatible with secretion through the bacterial inner membrane. To date, PCA in *E. coli* has been used to select binders from libraries of leucine zippers, peptides, antibody fragments (scFv) or ankyrin repeats, based on cytoplasmic or periplasmic co-expression of library members and target proteins fused to subfragments of either mouse dihydrofolate reductase (mDHFR) or TEM-1 β-lactamase, respectively. This has allowed for selection strategies based on clonal survival in selective media-containing trimethoprim or ampicillin (Amp), respectively [11,12,16–18]. However, owing to high levels of false-positive background clones, the PCA selection step has typically been used only after first reducing the complexity of a naïve library by one or more rounds of pre-selection using ribosome or phage display [11,12,17]. Although the combined and sequential use of different selection systems may offer certain advantages [19], a direct selection of novel binders from a naïve complex library by a single methodology is still the preferred route in most settings aiming for high-throughput binder generation.

Here we describe the first use of a naïve, 10^9 member, TEM-1 β-lactamase PCA library of the small, three-helix bundle affibody binding protein class for direct selection of high affinity binding proteins. Using a soluble variant of human tumour necrosis factor (TNF-α) as the target, we describe a selection procedure involving novel means to reduce false-positives, as well as the characterization of obtained binders with respect to affinity and selectivity in different assays. This study indicates that β-lactamase PCA may be used for reliable high-throughput selection from naïve combinatorial libraries.

Materials and methods

Bacterial strains, chemicals and enzymes

E. coli strains RR1ΔM15 [20], DH5α and BL21 (DE3) were used for all work, as indicated. All chemicals were from Sigma–Aldrich and all enzymes from New England Biolabs (NEB) except were indicated.

Vector construction and affibody library

The target vector pPCA-TARGET corresponds to a modified pOmpA-α197-TARGET vector [21]. Briefly, to facilitate target gene cloning between the NotI and AscI restriction sites downstream of the β-lactamase fragment gene, a NotI restriction site upstream of the expression cassette was destroyed using a PCR-based strategy. In addition, the chloramphenicol (Cml) resistance cassette (chloramphenicol acetyl transferase) in the vector was exchanged for the tetracycline (Tc) resistance gene from pBR322, amplified by PCR to introduce flanking restriction sites BsaI and AatII sites used in the cloning. The library vector pPCA-LIB has been described elsewhere (also known as pOmpA-LIB-ω198) [21]. The pPCA-TARGET-TNF-α vector contains a gene fragment encoding aa 1–157 of soluble human TNF-α inserted utilizing the AscI and NotI restriction sites. The naïve library of affibody molecules (PCA-lib) was of a complexity of 1.3×10^9 and had been constructed in the pPCA-LIB vector by NN(G/T) codon variation of 13 positions as described earlier and used to transform RR1ΔM15 cells [22]. Phage particles (non-displaying) for the infection of target cells were prepared according to standard procedures using M13K07/VCSM13 helper phage [23].

Western blotting

A 100 ml culture of *E. coli* carrying plasmid pPCA-TARGET-TNF-α was grown in Tryptic Soy Broth (30 g/l, Merck) media supplemented with yeast extract (5 g/l, Merck) (TSB + Y) and 10 μg/ml Tc at 37°C to mid-log phase when isopropylthiogalactoside (IPTG) was added to a final concentration of 1 mM and the temperature was changed to 30°C. After four hours of growth, the periplasmic protein content was recovered using an osmotic shock protocol [24]. A sample of approximately 10 μl of the 'shockate' was run on a NuPage® 4–12% Bis–Tris (Invitrogen) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a PVDF membrane. A polyclonal rabbit anti-β-lactamase antibody (Millipore) and a goat anti-rabbit antibody conjugated to HRP (Sigma) were used as primary and secondary antibodies, respectively, for detection. For the chemiluminescent detection, SuperSignal® West Dura Extended Duration Substrate (Pierce) was used and all incubations were carried out according to the supplier's protocol. The result was monitored and recorded using a CCD camera equipped ChemiDoc™ (Bio-Rad) instrument.

PCA-selection of binders to TNF-α

Pre-selection

The plasmid pPCA-TARGET-TNF-α was used to transform chemically competent *E. coli* cells and an aliquot was transferred to semi-solid media supplemented with 10 μg/ml Tc and incubated overnight at 37°C. A single colony was picked and inoculated to 20 ml of TSB + Y supplemented with 10 μg/ml of Tc. After one night of growth (37°C, 150 rpm) 10 ml of the culture was used as inoculum to 1000 ml TSB + Y supplemented with 2% glucose and 10 μg/ml Tc in a 5 l flask. After three hours of cultivation (37°C, 75 rpm), the cell density reached an $OD_{600\ nm}$ of about 0.6 and 2×10^{11} cfu of pPCA-LIB phage particles were added. The culture was kept still for one hour at RT, divided into four 500 ml centrifuge bottles and centrifuged ($2500 \times g$) for 10 min. Supernatants were discarded and cell pellets dissolved and pooled in 1000 ml TSB + Y supplemented with 10 μg/ml Tc and 20 μg/ml Cml in a 5 l flask. Cultivation was thereafter continued at 37°C

and 150 rpm for 3.5 hours, after what concentrated culture aliquots were frozen at −80°C.

Selection, step I

Approximately 2×10^{10} cfu, was thawed on ice and the contents added to 150 ml TSB + Y supplemented with 10 μg/ml Tc, 20 μg/ml Cml and 10 μM IPTG in a 1 l flask. After seven hours cultivation (37°C, 150 rpm), when the $OD_{600\ nm}$ had reached 8, the culture was transferred to a centrifuge bottle and centrifuged at $2000 \times g$ for 10 min. The supernatant was discarded and the cell pellet dissolved in medium as above to a cell density of about 2×10^{10} cfu/ml. Four 500 μl portions of this concentrated cell material was transferred to large (140 mm diameter) semi-solid media selection plates supplemented with 40 μg/ml Amp, 10 μg/ml Tc and 1 mM IPTG. As control experiments, diluted aliquots of the cells were transferred to semi-solid media plates with and without Tc (data not shown). All plates were incubated for 20 hours at 37°C, after which resulting colonies on each selection plate were harvested using a scraper and 3 ml of TSB media, supplemented with 20 μg/ml Cml. From the collected cells (4 × 3 ml), target and library plasmid vectors were subsequently co-recovered using a commercial mini-prep kit (Qiagen).

Selection, step II

Coding sequences of affibody molecules initially selected by PCA were rescued via five 50 μl PCR reactions in which a 150 ng vector mixture (about 3×10^{10} plasmid molecules) was used as template in combination with a forward primer including an XhoI restriction site and a reverse primer which was 5′-biotinylated. For cloning purposes 16 μg of PCR fragments (170 bp long) were restricted in two steps, starting with XhoI digestion reaction in 150 μl. The cleavage reaction was incubated with streptavidin coated magnetic M-280 Dynabeads® (Invitrogen) for capturing the 5′-biotinylated cleavage products, followed by washing and restriction enzyme buffer exchange. Through addition of NheI restriction enzyme, fragments digested at both ends were released from the beads and recovered. Approximately 1.6 μg of the digested PCR product was ligated (T4 DNA ligase) in four 250 μl reactions to 8 μg of NheI and XhoI digested and dephosphorylated pPCA-LIB vector. After an overnight incubation at 16°C, the reactions were pooled and purified on a PCR-cleanup spin column (QIAquick®). The ligation reaction was electroporated into *E. coli* strain DH5α cells in ten transformations, each involving 2.5 μl ligation mixture and 100 μl of competent cells. All transformations were pooled, transferred to a 250 ml flask and 25 ml of SOC media [23] added. After one hour cultivation at 37°C and 100 rpm shaking, aliquots were transferred to semi-solid media supplemented with 20 μg/ml Cml to determine the total number of transformants ($>10^6$). Remaining culture was transferred to a 5 l flask and 1 l TSB + Y supplemented with 20 μg/ml Cml was added and after an overnight incubation plasmids were prepared from 10 ml of the culture. To initiate the second selection step, ten 180 ng aliquots of the plasmid mixture were separately electroporated into 50 μl portions of RR1ΔM15 *E. coli* cells, harbouring the pPCA-TARGET-TNF-α plasmid. Reactions were pooled and SOC media added to a final volume of 15 ml. To determine cfu/ml count, aliquots of cells after one hour cultivation at 37°C and 100 rpm were transferred to semi-solid media supplemented with 20 μg/ml Cml. Remaining culture was transferred to a 1 l flask and 250 ml TSB + Y supplemented with 20 μg/ml Cml and 10 μg/ml Tc was added and cultivation continued until $OD_{600\ nm}$ had reached 3 when dilutions were transferred to four large (140 mm diam.) selection plates (10^7 cfu/plate) of different compositions all supplemented with 10 μg/ml Tc and 1 mM IPTG. In addition to this, either 40 or 80 μg/ml Amp together with either 0 or 50 ng/ml of tazobactam was added to the media. Plates were incubated for 16 hours at 37°C and from each plate colonies were picked and transferred to storage plates supplemented with 20 μg/ml Cml.

Protein production and purification

Affibody molecule sequences from 46 clones picked from the four different selection conditions were individually propagated by colony PCR and products used as templates for DNA sequencing where 34 unique clones were found. Coding sequences of all unique clones were PCR primer-elongated with forward primer adding a NotI and reverse primer adding an AscI restriction site, individually amplified and resulting fragments digested and ligated into AscI and NotI cleaved and gel-purified pAff8c [25] expression vector. Using this strategy a combined affinity gene fusion tag consisting of both a hexahistidyl (His_6) tag and an ABP (albumin binding protein) tag were fused to the N-terminus of the affibody molecules. Ligation products were transformed into DH5α *E. coli* cells after which 29 sequence verified vectors were transformed into *E. coli* strain BL21 DE3. Cultivation and parallel purifications under denaturating conditions of the binder candidates were performed, essentially as described earlier [26], utilizing the N-terminal His_6 tag. For biosensor analysis purposes, the buffer of an aliquot from each of the purified samples was exchanged with HBS-EP (0.01 M HEPES [pH 7.4], 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20) using Nanosep® (Pall) microcentrifuge columns with a MWCO of 10 kDa.

Biosensor analyses

A BIAcore® 2000 (Biacore AB) instrument was used for biosensor analysis and all proteins were covalently immobilized to a CM-5 sensor chip surface via amine coupling chemistry. All 29 affibody molecule TNF-α binder candidates were diluted to about 1 μM and sequentially injected over an HSA (human serum albumin) surface at a flow rate of 10 μl/min, directly followed by an injection of TNF-α (250 nM trimer) (Prospec). The response from a control surface (activated and ethanolamine deactivated CM-5 surface) was subtracted from all measurements. Biacore software was used to analyze binding profiles, and nine clones were selected for further studies. They were all individually immobilized onto CM-5 chip surfaces surfaces and 150 μl each of ten different concentrations of TNF-α in the range 50 pM–500 nM were sequentially injected (15 μl/min) in duplicate and random order. Equilibrium RU values were determined and for the seven clones showing highest affinity the software GraphPad Prism (GraphPad Software) was used to simultaneously fit the four parameters equation $Y = (R_{max}{*}X)/(K_D{*}X) + NS{*}X + C$, where C and NS are recognized as constant and concentration-dependent unspecific binding respectively to the received RU values. From each measured response, given values of C and NS were subtracted and the difference normalized by dividing it with the fitted value of R_{max}.

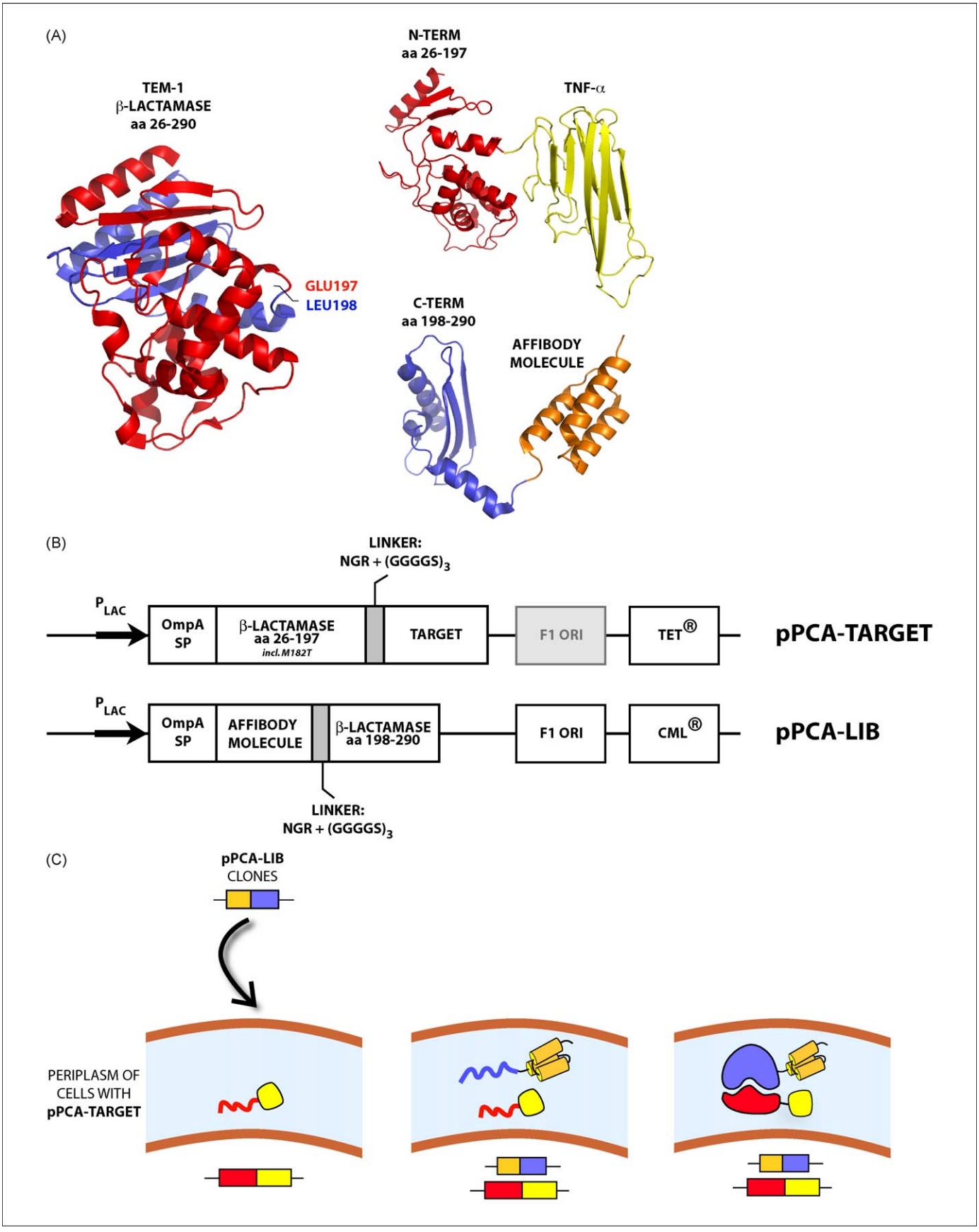

FIGURE 1

Overview of the β-lactamase-based protein fragment complementation assay system. **(A)** Molecular graphics representation of TEM-1 β-lactamase (1NYY.pdb), with subfragments aa 26–197 (red) and aa 198–290 (blue) highlighted (left). Also shown are the two co-expressed constructs whose complementation is

Binder selectivity evaluations

RR1ΔM15 *E. coli* cells harbouring either pPCA-TARGET-TNF-α or pPCA-TARGET-Z_{SPA-1} plasmids were in all possible combinations transformed with pPCA-LIB vectors with inserts corresponding to affibody molecules Z_{WT}, TA3, TE3 or TG7. The eight different double vector containing cells were separately cultured overnight in TSB + Y supplemented with 10 μg/ml Tc and 20 μg/ml Cml after what dilutions calculated to hold a few hundred cells was plated in duplicate on semi-solid media supplemented with 1 mM IPTG and 15 or 50 μg/ml of Amp. Plates were incubated at 37°C overnight and the next morning resulting colonies were counted and plates photographed using a Canon EOS 40D digital camera.

Recombinant TNF-α protein was added to a total *E. coli* cell periplasmic protein extract to a low nM concentration. Both TNF-α spiked and native 'shockate' were injected over CM-5 sensor chip surfaces containing affibody binder candidates. These samples, as well as a pure TNF-α standard sample were additionally run on a NuPAGE® 4–12% Bis–Tris SDS-PAGE gel (Invitrogen). Micromolar concentrations of human transferrin and polyclonal IgG were also injected over the affibody binder surfaces as above, and no binding was detected (data not shown).

Binding competition analysis

A mixture containing 100 nM (trimer) of TNF-α and either 1 μM of the monoclonal anti-TNF-α antibody adalimumab (Humira®) (Abbott) or 1 μM of a monoclonal anti-human-PSMA (prostate specific membrane antigen) antibody (Acris) in HBS-EP buffer were incubated for one hour on ice and then injected over CM-5 chip surfaces containing individually immobilized affibody binder candidates. As control experiments, a 100 nM (trimer) solution of TNF-α alone of the same concentration was injected in a following run.

Results

System design

The PCA system used in this work for binding protein selection was based on the bacterial enzyme TEM-1 β-lactamase, commonly used in biotechnology as resistance marker in combination with selective media containing Amp or similar β-lactam antibiotics. In accordance with the principle behind PCAs, individual binding protein candidates and the target protein were co-expressed in the same cell during the selection process, genetically fused to two separate subfragments of the reporter enzyme (Fig. 1). Members of a complex library of affibody binding protein variants (approx. 1.3×10^9 variants) were produced from one vector (pPCA-LIB, Fig. 1B) fused via a 15-amino acid (aa) residue linker to the N-terminus of a C-terminal segment of β-lactamase (aa 198–290). The presence of a filamentous phage F1 origin of replication in the vector allowed the use of helper phage technology to pack library vectors into phage particles for efficient delivery of the library to recipient cells by infection (see below). The TNF-α target protein (aa 1–157 of soluble TNF-α) was produced from a second vector (pPCA-TARGET, Fig. 1B) as fused to the C-terminal end of the larger and N-terminal part of β-lactamase (aa 26–197), via a linker consisting of an NGR tripeptide described to promote the enzyme reconstitution [27] and a flexible stretch of 15 residues. Both fusion protein constructs were directed to the *E. coli* periplasm using an Omp A signal peptide, and their production was under the control of an inducable *lac* promoter.

Selection towards human TNF-α

To investigate first if the β-lactamase$_{26–197}$–TNF-α target fusion protein was produced and secreted into the periplasm as intended, western blotting was performed on soluble proteins in a periplasmic extract from cells harbouring the pPCA-TARGET-TNF-α plasmid. Detection of the fusion protein using a polyclonal anti-β-lactamase antibody recognizing the enzyme portion of the fusion protein showed the presence of a distinct band with an apparent molecular weight in concordance with the expected 39 kDa (data not shown). This showed that the fusion between the genetically truncated β-lactamase and the TNF-α target protein could be produced as a soluble protein in *E. coli* periplasm, the absence of bands of lower molecular weights indicated that both moieties of the fusion protein were stable against proteolysis by host proteases under these conditions.

To initiate selection, a culture of pili-bearing *E. coli* cells harbouring the plasmid pPCA-TARGET-TNF-α, were subjected to infection by library phage particles prepared from cells harbouring the vectors encoding the affibody library (pPCA-LIB). Results from previous experiments had shown that the delivery of library vectors to 'target cells' via phage infection was efficient and time saving compared to standard electroporation (data not shown). It should be noted that these procedures do not involve any display of affibody library members on the phage surface as in phage display technology.

After overnight growth of the infected culture in liquid media containing Cml and Tc to select for clones containing both vectors, aliquots of cells were prepared for use in the selection of clones showing a reconstituted Amp resistance. An initial selection round was performed by spreading approx. 10^{10} cells on antibiotic supplemented semi-solid media and resulted in a relatively large number of surviving clones. However, the presence of clones in the library showing inherent Amp resistance independent of co-expression with the pPCA-TARGET-TNF-α target vector had earlier been observed. This background fraction of survivors, corresponding to approximately 1 in 10^5 clones, could in theory significantly exceed the number of positive affibody library clones binding to TNF-α.

monitored in the PCA system: the subfragment aa 26–197 as fused to the TNF-α target (yellow) and an affibody protein library member (orange) fused to the aa 198–290 subfragment (right). Note, the folding state of the enzyme-derived moieties of the fusions in the cell when not engaged in target::binder recognition-driven complementation most probably differs from the native state appearance indicated in this figure. **(B)** Schematic representation of the expression cassettes of the two vectors used. From the pPCA-TARGET vector, the target protein is expressed as fused to the C-terminus of the aa 26–197 fragment of β-lactamase, connected via an NGR tripeptide [34] and the 15 aa linker $(GGGGS)_3$. From the pPCA-LIB vector, affibody proteins are expressed as fused to the N-terminus of the aa 198–290 fragment of β-lactamase. Both vectors harbour a filamentous phage F1 ORI but only the one present in the pPCA-LIB vector is used. **(C)** Overview of the selection principle applied in the study. Cells harbouring the pPCA-TARGET-TNF-α vector are subjected to infection by library phagemid particles containing packed pPCA-LIB vectors (I). This results in cells co-expressing TNF-α target protein and an affibody protein library member, as fused to their respective β-lactamase fragments (II), allowing for a survival-based selection for rare clones with an ampicillin-resistance phenotype obtained via productive complementation of the two enzyme subfragments (III). Colour code as above.

TABLE 1

Listing of deduced amino acid sequences of some of the selected clones discussed in the text.

Clone name	Freq.[a]	Sequence[b]	Plate type[c]	Affinity K_D (nm)
		1 10 20 30 40 50		
		• • • • • •		
Z_{WT}	-	VDNKFNKEQQNAFYEILHLPNLNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDAQAPK		
TA3	1	--------SHA-LR--FK-----ET-VS---R--V----------	80+T	16
TE3	1	--------YVS-LF--GK-----GN-RY---G--R----------	40+T	27
TG7	1	--------VGW-TG--GG-----AG-FA---L--A----------	80	14
TA7	1	--------VAE-AH--VK-----EK-KV---G--P----------	80	ND
TA9	1	--------LTG-PR--LA-----FD-AM---Q--R----------	80+T	ND
TB12	2	--------WWT-GW--RD-----SQ-RR---G--G----------	40-80	51
TC7	1	--------WLP-TK--TE-----VV-MN---W--T----------	80	42
TF1	1	--------RSM-AC--EG-----AV-CG---A--R----------	80+T	42
TF5	1	--------TGK-LG--AA-----TH-VR---L--Y----------	40+T	37

(a) Frequency with which the clone appeared among 35 sequenced clones showing correct affibody molecule sequences.
(b) Amino acid sequences in one-letter-code of affibody molecule. Horizontal lines indicate identity to the Z_{WT} clone.
(c) Plate type on which the clone was identified. Numbers indicate ampicillin conc. (μg/ml), T means that the plate in addition contained 50 ng/ml tazobactam.

Therefore, it was considered worthwhile to perform a second round of selection before initiating further analyses. To this end, surviving clones were recovered, affibody coding sequences amplified by PCR and subsequently ligated into a zero background-controlled batch of pPCA-LIB vector, followed by transformation into cells, yielding a secondary library. Electroporation of this library into pPCA-TARGET-TNF-α target vector containing cells, followed by overnight growth yielded a rich source of dual-plasmid-containing cells. Aliquots of the latter cells were subjected to second round survival selections on four types of plates conferring different stringencies involving, in addition to IPTG and Tc, Amp with or without the addition of the β-lactamase inhibitor tazobactam, the latter was included to suppress the survival of clones originating from general stickiness effects and to decrease an otherwise observed growth of satellite colonies. It was shown that an increased stringency resulted in a lower number of surviving colonies, ranging from 500 colonies (Amp80/tazobactam) to $>10^4$ colonies (Amp40/no tazobactam) depending on plate composition. In addition, a slower growth rate was observed for higher stringency plates (tazobactam containing), as colonies on these plates tended to be of smaller size. In total 46 clones were subjected to DNA sequencing of their affibody gene inserts, of which 35 contained a single pPCA-LIB vector with a correct affibody encoding sequence. The results summarized in Table 1, showed that only one clone (TB12) was represented more than once, resulting in 34 unique clones.

Analysis of binding specificity and affinity

The affibody gene inserts of the majority of unique clones were subsequently transferred to an expression vector for intracellular *E. coli* production of His_6–ABP-affibody fusion proteins. In an initial biosensor-based ranking of the binding properties, a single concentration of TNF-α was injected over sensor chip surfaces containing the different immobilized metal ion affinity chromatography (IMAC)-purified His_6–ABP-affibody fusion proteins, as well as a control construct, all non-covalently attached to HSA-containing surfaces via their ABP tags. This analysis indicated that all the clones showed some binding to the TNF-α target, albeit with different kinetic profiles (data not shown). On the basis of their binding profiles, nine clones with interesting characteristics were chosen for a more detailed analysis of binding affinities. Here, the corresponding His_6–ABP-affibody fusion proteins were covalently attached to the sensor chip surfaces and the equilibrium responses obtained from injections of TNF-α were recorded. The results showed that the apparent binding affinities (K_D) towards TNF-α for the three highest affinity clones (TE3, TA3 and TG7) as seen in this analysis format were in the range between 14 and 27 nM, (Fig. 2) which is similar to affinities seen for affibody molecules selected against other targets from naïve libraries using phage display [7].

To investigate selectivity of binding, several tests were performed. In a cell-based assay, bacteria containing different pPCA-TARGET/pPCA-LIB vector combinations were transferred to selection plates to investigate the TNF-α target dependency on survival. Cells containing either a non-TNF-αPCA pair showing affinity in the μM range (Z_{WT}/Z_{SPA-1}) [28] or pPCA-TARGET-TNF-α in combination with one of the pPCA-LIB vectors encoding the three TNF-α binding affibody proteins TA3, TE3 and TG7 or the parental Z domain (Z_{WT}) were plated onto Amp-containing plates and the resulting growth after an overnight incubation at 37°C was investigated. The test showed that all three investigated TNF-α binding clones were dependent on the presence of TNF-α as target for growth, indicating a selective

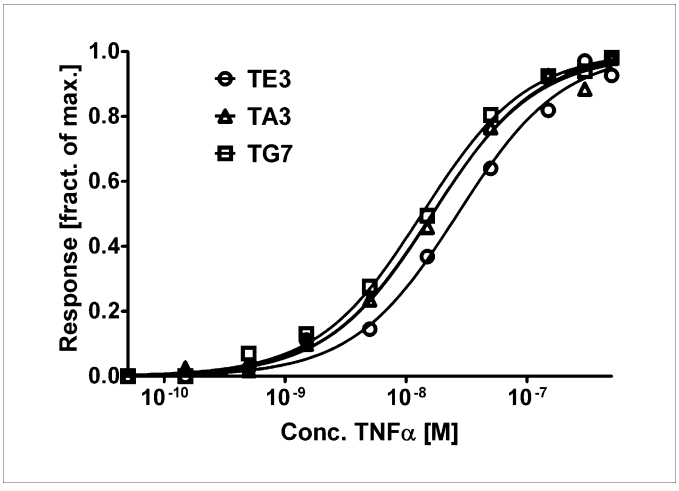

FIGURE 2

Results from affinity analyses of the TG7, TE3 and TA3 clones. TNF-α proteins in the range of 50 pM–500 nM (trimer), were injected over Biacore surfaces containing the selected anti-TNF-α affibody molecules TG7, TE3 and TA3 and equilibrium responses were recorded. Fitted data showed that dissociation constants (K_D) were approximately 14, 16 and 27 nM respectively.

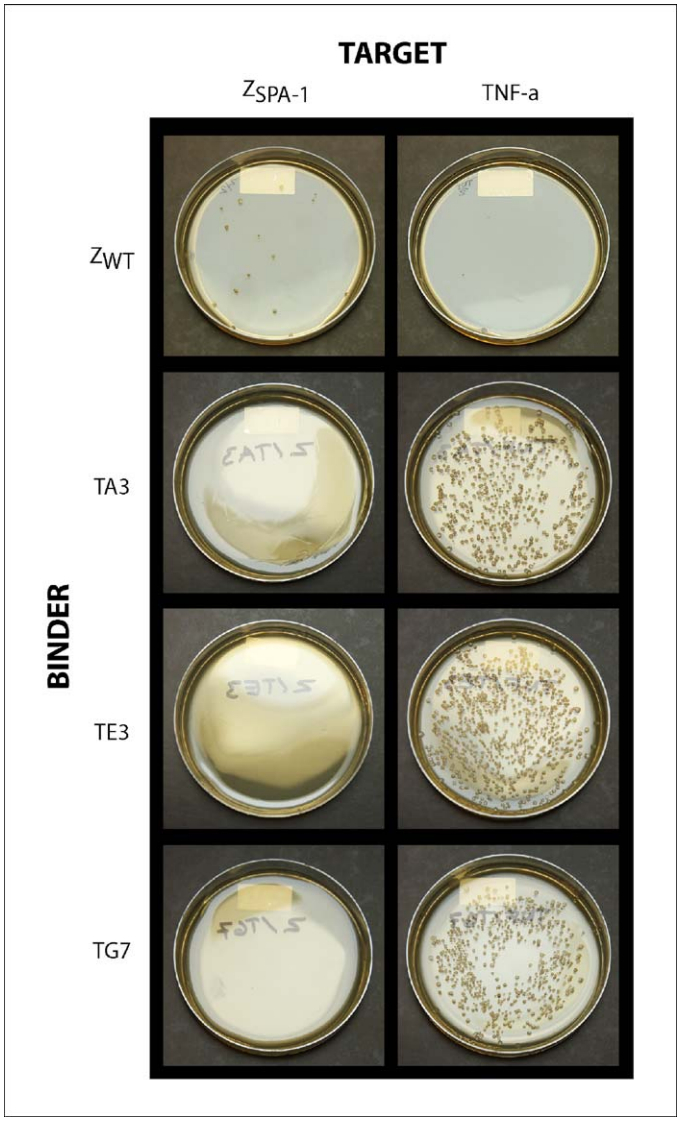

FIGURE 3

Results from a cell-based selectivity analysis. Cells containing pPCA-TARGET-Z_{SPA-1} (encoding a Z_{WT} binding anti-idiotypic control affibody protein; [28]) or pPCA-TARGET-TNF-α vectors in combination with one of the pPCA-LIB vectors encoding the three TNF-α binding affibody proteins TA3, TE3 and TG7 or the parental Z domain (Z_{WT}) was plated onto ampicillin-containing plates and the resulting growth was investigated (see text for details).

binding (Fig. 3). In addition, to obtain surviving colonies for the control affinity pair (Z_{WT}/Z_{SPA-1}), a lower Amp concentration had to be used (15 μg/ml compared to 50 μg/ml) (see Discussion). Further, additional biosensor experiments showed specific detection of a low nanomolar concentration of TNF-α spiked into a complex *E. coli* extract (Fig. 4), and no detectable binding towards injected control proteins transferrin or human polyclonal IgG (data not shown). To further characterize the selected affibody variants, a binding competition analysis was performed utilizing either the humanized anti-TNF-α antibody adalimumab (Humira®) or a control antibody (anti-PSMA). In a biosensor-based format, premixing TNF-α protein with adalimumab, but not with the control antibody, completely inhibited binding between injected TNF-α protein and sensor chip-immobilized anti-TNF-α affibody variants (Fig. 4). This indicated that the adalimumab and the affibody variants share overlapping epitopes on the TNF-α target.

Discussion

The results in this study demonstrate the use of a β-lactamase-based PCA system for direct selection of binders from a complex naïve library, without the need for a preceding cycle of pre-selection using an alternative selection technology. Often only a fraction of the clones obtained by multiple-round selections using phage display are true target binders. Therefore, the observation that all of the 29 randomly sampled affibody molecule clones from the different PCA selection plates showed binding to TNF-α is highly encouraging and indicates that the stringency in the selection indeed was high. The introduction of the β-lactamase inhibitor tazobactam into the selection medium may at first appear counter-productive because it inhibits the very activity used for selection. However, it is well known that β-lactamase, as well as other periplasmic proteins, may leak out from the cell resulting in degradation of the Amp in the medium. This should be the situation also for β-lactamase subfragments, which however need to remain associated outside the cell to be enzymatically active. Few, if any, 'satellite opportunists' were seen around colonies after the addition of tazobactam, showing that false-positive clones, corresponding to either non-binding or sticky library variants conferring lower β-lactamase activities can be reduced by this strategy. Notably, of the variants investigated in this study, the highest affinity towards TNF-α was seen for a clone isolated from a plate without tazobactam (TG7, Table 1). However, this should not be interpreted as showing that the addition of tazobactam in plates was not adding any increased stringency in the selection. Taking into account that only a limited fraction of the colonies emerging on each selection plate was further investigated, it cannot be ruled out that the TG7 clone, or clones of even higher TNF-α affinities, may have existed on a plate with tazobactam added but were missed in the analysis. In addition, the cross-target analysis performed in cells showed that no colonies arose for binder/target combinations other than between cognate pairs, again showing the potential of the system for stringent selections. Interestingly, it was necessary to use a lower Amp concentration to obtain survivors of colonies harbouring the Z_{WT}/Z_{SPA-1} pair, associated with an affinity characterized by a K_D in the low micromolar range, that is considerably weaker than that between TNF-α and the affibody binder clones analyzed in parallel [28]. This indicates a potential to modulate the selection stringency to allow for discrimination between variants of different binding affinities.

Clearly, the performance of the PCA method will be influenced by the general status of both the target protein and the library members in the cell. Owing to the absence of disulphide bridges, affibody molecules should be compatible with both cytoplasmic and periplasmic PCA-systems, as indicated by soluble production of various bioactive affibody fusion proteins in *E. coli* cytoplasm or periplasm [29,30]. By contrast, when an mDHFR PCA system was used for selection of disulphide-containing scFv fragments in the cytoplasmic space, the level of false-positives could only be reduced after mutating the antibody framework for higher stability in the reducing environment [11]. Another requirement of the β-lactamase PCA technology is for a target protein or a fragment thereof to be both soluble and proteolytically stable in the *E. coli* periplasm. However, if such problems are encountered, truncated versions or other mutants of the target with increased solubility and

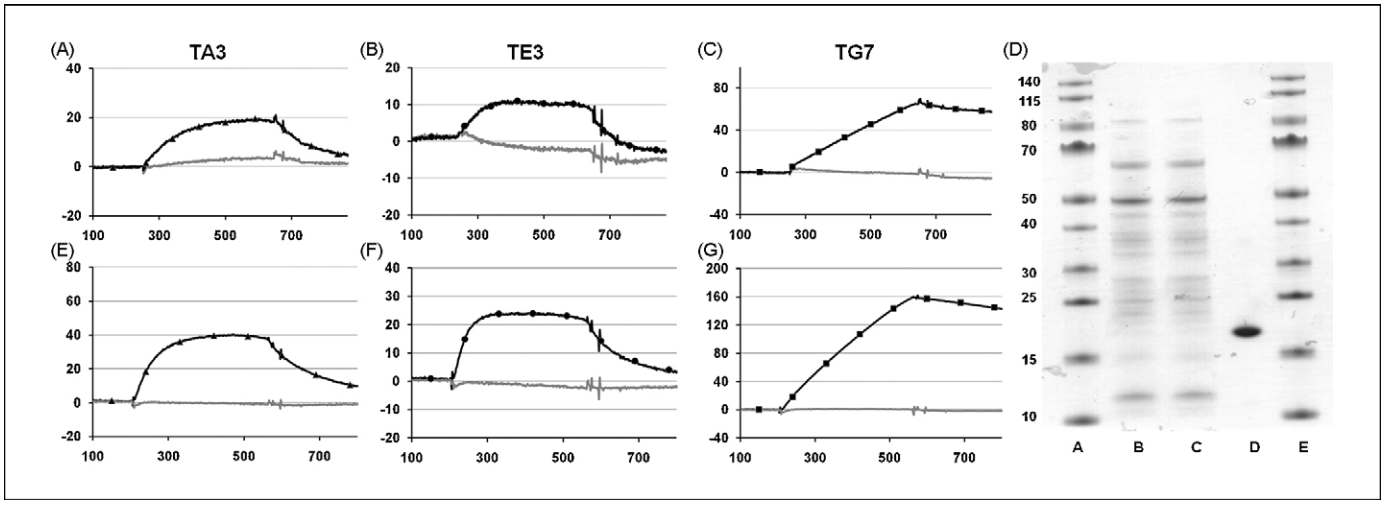

FIGURE 4

Results from binding selectivity and epitope analyses using a target protein-spiked *E. coli* cell extract or the anti-TNF-α monoclonal antibody adalimumab (Humira®). **(A)–(C)** The selectivity of the three PCA selected TNF-α binding affibody clones TA3 (A), TE3 (B) and TG7 (C) was challenged in a biosensor experiment using a complex sample consisting of a periplasmic extract from *E. coli* spiked (black) or not spiked (grey) with a low nM concentration of TNF-α (black traces). **(D)** SDS-PAGE analysis of 20 μl samples showing the non-spiked periplasmic extract (lane B), the spiked extract (no visible band of TNF-α) (lane C) and a TNF-α standard (500 ng) (lane D), illustrating the low concentration of the target used in the analysis. **(E)–(G)** Overlay plots from a biosensor-based binding epitope analysis where 100 nM (trimer) of TNF-α, either alone (black sensorgrams) or pre-mixed with the monoclonal anti-TNF-α antibody adalimumab (Humira®) (1 μM) (grey sensorgrams) were injected over chip surfaces containing TNF-α binding affibody variants TA3 (E), TE3 (F) and TG7 (G). The complete cancelling of binding of TNF-α to the affibody variants indicates that the affibody variants and the antibody recognize overlapping epitopes.

stability, or the use of alternative secretion routes or host strains may provide the solution [31,32].

In the present study the target was the non-membrane bound form of TNF-α (sTNF-α), a single cysteine-bridge-containing protein capable of forming non-covalent trimers at nM concentrations or higher [33]. Western blotting on the periplasmic fraction indicated that soluble, full-length TNF-α fusion protein was indeed present. However, the oligomeric state of TNF-α during selections in the bacterial periplasm, when expressed as fused to the C-terminal β-lactamase subfragment, is not known. Such concentration-dependent equilibria between different oligomeric states could have an effect on the accessibility of different faces of the TNF-α monomer for interaction with co-expressed library members. In addition, the relation between a target protein size and the linker lengths used for connecting the library members and the target to their respective enzyme halves may also influence the possibilities for interaction between different surfaces. Interestingly, all three affibody molecules tested in this study were found to bind to an epitope of TNF-α overlapping with that recognized by the monoclonal anti-TNF-α antibody adalimumab (Humira®), used in the treatment of rheumatoid and psoriatic arthritis. From the present study, it cannot be elucidated whether or not this finding implies that this region of TNF-α is particularly prone to be involved in biomolecular interactions or if other factors, such as steric accessibility, may have been dominating during selection.

One advantageous feature of PCA-based selection is that the production of the target protein is integrated into the procedure, which obviates the need for preparation of purified target protein to be used during selection. In combination with direct selection from naïve libraries, this would in principle allow for scenarios of high-throughput selection to many targets for which only the genes are available. However, in practice a source of purified target protein is convenient for post-selection characterization of obtained binders. Nevertheless, the selectivity and binding strength of binders obtained by PCA may in principle be addressed using immobilized binders and a natural source for the target protein followed by identification by mass spectrometry or sandwich immuno technology. Taken together, the present study shows that β-lactamase PCA is an interesting selection technology applicable to selection of binders from complex naïve libraries. The speed of the procedure opens up for high-throughput identification of binding proteins to targets for which only the gene is available during selection.

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