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Functional improvement of antibody fragments using a novel phage coat protein III fusion system

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Abstract

Functional expressions of proteins often depend on the presence of host specific factors. Frequently recombinant expression strategies of proteins in foreign hosts, such as bacteria, have been associated with poor yields or significant loss of functionality. Improvements in the performance of heterologous expression systems will benefit present-day quests in structural and functional genomics where high amounts of active protein are required. One example, which has been the subject of considerable interest, is recombinant antibodies or fragments thereof as expressions of these in bacteria constitute an easy and inexpensive method compared to hybridoma cultures. Such approaches have, however, often suffered from low yields and poor functionality. A general method is described here which enables expressions of functional antibody fragments when fused to the amino-terminal domain(s) of the filamentous phage coat protein III. Furthermore, it will be shown that the observed effect is neither due to improved stability nor increased avidity.

Keywords: Antibodies; scFv; Expression; Fusion; Phage display; Antibody; Stability; Protein III; Multimerisation

Expressions of heterologous genes in bacteria have been the subject of considerable interest as this is by far the simplest and most inexpensive system for obtaining vast amounts of a desired polypeptide. Although expression is straightforward, problems are often encountered when recombinant proteins are to be expressed in bacteria. The most prominent of these seems to be loss of function. This is probably caused by poor folding efficiency in the bacteria or by insufficient amounts of folding factors upon overexpression, leading to degradation of unfolded protein and/or aggregation and concomitant formation of inclusion bodies [1]. Following the development of phage display antibody repertoires and the use thereof, much attention has been devoted to the heterologous expressions of antibody fragments in various hosts and especially to the conditions under which functional protein can be obtained from *E. coli* [2–4].

Antibodies are traditionally considered to be molecules that consist of two heavy and two light chains, which comprise both constant and variable domains. The antigen-binding site resides in the variable domains and can be expressed separately without loss of the binding properties [5]. Furthermore, a single chain fragment variable (scFv) can be formed with a flexible linker to join the variable heavy and light chains [6,7]. Due to its smaller size (25 kDa) and reduced complexity compared to whole antibodies, scFvs are among the

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most widely expressed antibody formats in *E.coli* [8]. These properties are exploited in the generation of recombinant antibodies by, e.g., phage display, where repertoires of scFvs, functionally displayed on the surface of the filamentous phage by fusion to coat protein III, are affinity selected for required specificities (reviewed in [9,10]). Loss of functionality has been a general observation when phage-display derived scFvs are expressed as soluble scFv. This loss of functionality has been attributed to two phenomena, namely the loss of avidity and stability. With regard to the avidity component, the filamentous bacteriophage contains up to five copies of protein III to which the scFv can be fused [11]. However, the display format often used favours monovalent display on the phage particle. Therefore it seems likely that the stability component deserves more attention.

Many approaches have been used to deal with the stability issue reviewed by Wörn and Plückthun [8]. The overall stability of scFvs can be increased either by insertion of disulphide bridges between the two chains [12], or by the introduction of mutations in the framework using rational or evolutionary strategies [13,14]. The peptide linker that connects the heavy and light chains is another target for optimisation as it influences the stability and both linker length [15] and sequence [16] have been investigated. Likewise, the succession of the variable domains in the scFv has been examined (V_HV_L and V_LV_H constructions), but no general rule has been found and the optimal order has to be determined for each individual scFv [15]. Additionally, complementarity determining regions (CDRs) from non-stable frameworks have been grafted to stable ones [17]. This approach has been implemented in the construction of phage displayed antibody repertoires using stable frameworks as building blocks [18–20].

A general method to increase expression yield, solubility, and stability is to fuse the protein in question to peptides or proteins with beneficial properties [21]. Such approaches have been very attractive with respect to antibody fragments as generic improvements can be obtained avoiding laborious analysis of each scFv separately. A panel of fusion partners has been reported, e.g., maltose-binding protein [22,23], alkaline phosphatase [24], green fluorescent protein [25], lambda head protein D [26], and human interleukin 2 fragment [27]. All of these have improved either the expression yield, increased the stability or added functions to the scFv. In addition to this, co-expression of recombinant antibody fragments with chaperones seems to increase the yield of functional scFv [4,28].

In the present study we have investigated the functional improvement of scFvs through fusion to filamentous phage protein III (Protein Accession Code: CAA23862.1 and http://www.mrc-cpe.cam.ac.uk/~/g1p.html). Protein III is responsible for the interaction with the bacterial receptors, F-pilus and TolA, and filamentous bacteriophage infection. It is structurally divided in three domains: domain I and domain II interact with the bacterial receptors, whereas domain III integrates protein III in the phage capsid [29]. We observed that some scFvs, which bound their cognate antigen when expressed on the surface of the filamentous bacteriophage, lost their binding properties when expressed as scFv alone. Thus suggesting that protein III is partly or totally responsible for inducing the active fold of the scFv, and as such, it is a reasonable fusion partner. In the following we will report that an inactive antibody fragment can be functionally rescued by fusion to the N-terminal domains of the original phage display fusion partner—protein III—in a novel expression system designated FuncFAb—Functionally Fused Antibody.

Materials and methods

Construction of pKBJ vectors. A scFv was initially subcloned from pHEN2 (http://www.mrc-cpe.cam.ac.uk/~/g1p.html) into the HindIII and *Not*I sites of the pUC119 His6MycXbaI [30] plasmid. This modified vector was used as founder vector for the pKBJ vectors. Gene III (Nucleotide Accession Code: V00604) of pHEN2 (http://www.mrc-cpe.cam.ac.uk/~/g1p.html) was PCR amplified using primers appropriate for each vector. pKBJ1 was constructed by PCR amplification with primers gIII N-term *Not*I and DII-III *Eag*I and subsequent cloning of the *Eag*I digested PCR product into the *Not*I site of the founder vector. For pKBJ2, gene III was PCR amplified with primers VL-link and DII-III Opal-*Eco*RI, digested with *Not*I and *Eco*RI and cloned into these sites of the founder vector. Quick-Change (Stratagene) with primers gIII N-term QC Amber-back and gIII N-term QC Amber-fw was subsequently used to remove the amber stop codon between the scFv and gene III in pHEN2. The last construct, pKBJ3, was constructed essentially as pKBJ1, using primers gIII N-term *Not*I and DI-DII *Eag*I. All constructs were subsequently sequenced with primers M13-Rev, VL-link, and M13-20 using an Applied Biosystems 373A sequencer and the ABI Prism Dye terminator cycle sequencing kit (Perkin–Elmer). All enzymes were purchased from New England Biolabs unless otherwise stated and used according to manufacturer's instructions.

Expression of antibodies. Clones were picked from TYE-plates, grown overnight at 37 °C in 2xTY [31] supplemented with 100 μg/mL ampicillin and 1% glucose, before 1:100 dilution in 2xTY with 100 μg/mL ampicillin and 0.1% glucose, and incubated for four hours at 37 °C shaking. The cultures were induced by addition of 1 mM IPTG and grown overnight at room temperature. Cells were pelleted at 6000*g* and resuspended in 50 mM $Na_xH_yPO_4$, pH 8.0, before lysis in French Press (American Instruments, Silver Spring, MD, USA). The suspension was subsequently cleared by centrifugation (26,000*g*) and the supernatant was being supplemented with 30 mM imidazole and 300 mM NaCl prior to immobilised metal affinity chromatography (IMAC). Ni-NTA was incubated with the supernatant for 2 h at 4 °C and subsequently washed with a minimum of 100 mL wash buffer (50 mM $Na_xH_yPO_4$ pH 8.0, 300 mM NaCl, and 30 mM imidazole) followed by 50 mL high saline wash buffer (50 mM $Na_xH_yPO_4$, pH 8.0, 750 mM NaCl, and 30 mM imidazole). Protein was eluted with wash buffer supplemented with Imidazole to 300 mM. The concentration was determined according to Bradford [32] and the purity was analysed by SDS–PAGE [33].

Activity measurements using ELISA. ELISA using the pKBJ derivatives of the scFv antibodies L36 [34] and D4 (K.B. Jensen et al.,

submitted for publication) was performed by coating of either Laminin-1 (Sigma) or Fibronectin (Sigma), respectively, overnight at 4 °C in 50 mM $NaHCO_3$, pH 9.6, at a concentration of 0.5 μg/well in ELISA-plates (MAXI-sorp, NUNC, Roskilde, Denmark). Residual binding to the plastic surface was blocked using 2% MPBS (PBS supplemented with 2% weight/volume low-fat milk powder) for 2 h and antibody derivatives were added at varying concentrations for 1 h. Plates were subsequently washed six times in PBS and bound antibody was detected with a murine antibody 9E10, which recognises the c-myc tag of the expressed scFv derivatives (provided by the European collection of animal cell cultures, ECACCs) at a concentration of 0.5 μg/mL. After washing six times in PBS the plates were incubated with a 1:1000 dilution of a HRP-conjugated rabbit anti mouse antibody (DAKO, Denmark) in 2% MPBS. The reaction was developed with *o*-phenylenediamine (OPD)-tablets (DAKO, Denmark) according to manufacturer's instructions after six additional washes in PBS.

To test activity of the scFv antibody R5 and its derivatives, Anti-Thomsen-Friedenreich/MUC-1 antibody (A76 A/C7) [35] was coated at a concentration of 0,1 μg/well overnight at 4 °C in PBS. Residual binding was blocked with 2% BSA (Sigma) in PBS for 2 h and the plates were subsequently incubated with antibody derivatives for 2 hours. The R5 antibody was detected with a polyclonal rabbit anti-c-myc (A-14) antibody (Santa Cruz Biotechnology) and subsequently a HRP-conjugated swine anti-rabbit antibody (DAKO, Denmark). Between each incubation step the plates were washed five times in PBS and developed as described above.

Stability measurements. Purified L36 fusion scFv was diluted to a final concentration of 1 μM in different concentrations of Guanidinium Chloride (GdmCl) in 10 mM Tris–HCl, pH 8, and transferred to a 3 mm path length quartz cuvette. The cuvette was placed in a RTC2000 fluorimeter with a 75 W Xenon arc lamp (Photon Technology International, Lawrenceville, NJ) and the emission scan from 300 to 400 nm was recorded (λ_{ex} 285 nm, excitation slit width 2 nm and emission slit width 6 nm). Folded scFv had emission maximum at 330 nm while maximum for unfolded was at 355 nm, thus the ratio between fluorescence at these wavelengths was used as signal to determine $[GdmCl]_{50\%}$ [36].

Gel filtration analysis. Gel filtration analysis was performed to determine whether antibody fusions multimerised. Gel filtration was performed on a TSK-gel G3000 SW column with a precolumn (ToSoHaas) using HPLC (Biotek Instruments). For correlation of retention times with globular molecular mass, bovine plasma fibronectin, murine IgG, BSA, and GST were applied onto the column and protein detected with a Diode Array Detector 540+ (Biotek Instruments). At least 1 mg scFv-fusion protein was applied to the column of each of the constructs and fractions were collected for subsequent analysis in ELISA.

Results

Construction of pKBJ family of vectors

Plasmids enabling fusion of scFv to various fragments of gene III were constructed by amplification of gene III domain I (pKBJ3) and gene III domain I-II (pKBJ1 and pKBJ2) (Fig. 1). A construct for domain I fusions, which encodes the first 80 amino acids of protein III plasmid, was first constructed (pKBJ3) enabling amino-terminal fusions to domain I of selected scFvs. Furthermore domain I is followed by a mycHis6-tag for immunodetection and purification. A pelB leader precedes the fusion protein directing expression of fusion protein to the periplasmic space of *E. coli* (Fig. 1).

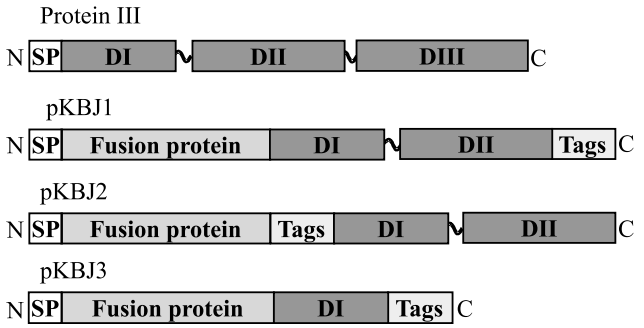

Fig. 1. Illustration of filamentous bacteriophage protein III and linear structure of the novel fusion proteins. A schematic representation of the domain structure of protein III and the constructed fusion proteins using the following abbreviations: DI, domain I, DII, domain II, DIII domain III, SP, signal peptide—pelB leader. The pKBJ2 has the native tag structure from the phage display format, whereas pKBJ1 and pKBJ3 have carboxy-terminal positioning of the tags.

pKBJ1 was constructed analogously (see Materials and methods) allowing scFvs to be fused to domains I and II of gene III. In the last construct, pKBJ2, the tags were positioned as in the phagemid vector and separate the scFv from domains I and II of gene III (see Table 1).

Expression and activity analysis of recombinant scFv

Three different phage display selected scFv antibodies, L36, D4, and R5, were sub-cloned into the three pKBJ vectors and the pUC119 vector in order to analyse the level of expression and the activity of the antibodies. L36 recognises Laminin-1 and is expressed in reasonable amounts as soluble active antibody [34]. The antigen of D4 has recently been identified as Fibronectin (K.B. Jensen et al., submitted for publication) and it is inactive when expressed as soluble scFv, though still expressed in fair amounts. R5 mimics a combined conformational epitope of MUC-1 and Thomsen-Friedenreich carbohydrate structure recognised by a murine antibody (unpublished results). This antibody is likewise expressed as active soluble scFv in decent amounts. Expression of these antibodies from the three novel vectors gave expression yields of the recombinant protein comparable to that of a traditional scFv expression system and of similarly high purity after IMAC purification (Table 2 and Fig. 2). In general, a slight increase in yield was observed by fusion, except for D4. It is well established that the amino-terminal part of a protein is most important for expression yields, thus similar expression yields were expected for the scFv and their respective derivatives [23]. In addition, the solubility of the scFvs was found unaltered upon fusion as determined by Western blotting of the supernatant and pellet after cell lysis (data not shown). The difference in yields for D4 scFv and derivatives could be caused by accumulation and aggregation of poorly folded inactive protein in inclusion bodies, a phenomenon often associated with

Table 1
Oligonucleotide sequences

Name	Sequence
GIII N-term *Not*I	5′AAGGAAAAAAGCGGCCGCCGGGGCCGCAACTGTTGAAAGTTGTTTAGC 3′
DII-III *Eag*I	5′AAGCCGGCCGAGCCGCCAGCATTGACAGG 3′
DI-DII *Eag*I	5′AAGCCGGCCGAACCGCCACCCTCAGAACC 3′
VL-link	5′ACCGCCAGAGCCACCTCCGCC 3′
DII-IIIOpal-*Eco*RI	5′CGGAATTCTCAGCCGCCAGCATTGACAGG 3′
GIII N-term QC Amber-back	5′CTTTCAACAGTCTGTGCGGCCCC 3′
GIII N-term QC Amber-fw	5′GGGGCCGCACAGACTGTTGAAAG 3′
M13-Rev	5′AAACAGCTATGACCATG 3′
M13–20	5′GTAAAACGACGGCCAGT 3′

Sequences of the primers applied for construction of vectors and subsequent verification of vectors sequences.

Table 2
Expression yields for scFv and their derivatives

Antibody	scFv	pKBJ1	pKBJ2	pKBJ3
L36	0.8	3.4	1.5	2.6
D4	13.7	3.3	6.2	4.0
R5	5	7	11	1.5

Expression yield in mg purified protein pr. L culture.

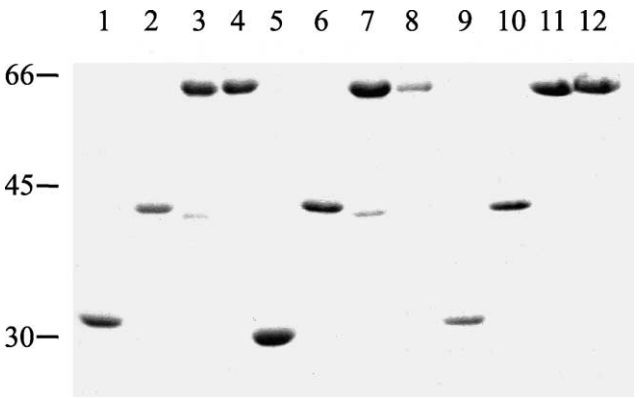

Fig. 2. IMAC purified antibodies and antibody derivatives. SDS–PAGE showing IMAC purified protein of the four different constructs for each of the three antibodies—D4 (lanes 1–4), L36 (lanes 5–8), and R5 (lanes 9–12) in pUC119 (lanes 1, 5, and 9), pKBJ3 (lanes 2, 6, and 10), pKBJ1 (lanes 3, 7, and 11), and pKBJ2 (lanes 4, 8, and 12).

significant increase in expression of recombinant proteins [1]. This is not very likely since the amount of D4 accumulating in the insoluble fraction was unaltered by fusion to protein III.

ELISA was performed to test the effect of fusing fragments of protein III to the scFvs. All preparations of protein expressed in the FuncFAb-system appeared to be active, and a higher reactivity was observed for some when compared to antibodies expressed in the traditional expression system (Fig. 3). D4, which has only been active when displayed on the surface of the filamentous bacteriophage, was found to be active as fusion to either domain I or domain I-II (Fig. 3A), demonstrating that protein III exhibits a positive effect on the functionality of the antibody. In addition, the activities of the domain I fusions were higher for all antibodies than those of both the domain I-II fusion and scFv alone. Likewise the position of the c-myc and his-tags either internally or terminally did not have any influence on the antibody activity. Subsequently, a panel of 14 antibodies has been cloned into the vectors and restoration of the activity was seen in 9 out of 10 antibodies, whereas 4 were active both before and after fusion (Unpublished results). Thus, it has been found that inactive scFv selected from phage displayed antibody repertoires can be activated when expressed in the molecular context, in which it was selected.

Activity measurements were performed after incubation for varying times and at different temperatures to test the effect of fusion on the stability of antibody derivatives. For all derivatives of L36 and R5 no apparent effect was observed after incubation at room temperature, 30 °C and 37 °C for up to four days (data not shown). The activity of D4 derivatives was unaffected by incubation at room temperature. However, after exposure to thirty degrees the activity decreased slightly and after one day at thirty-seven degrees inactivation was observed (data not shown). To further examine the stabilities of the different fusion proteins with that of the scFv, L36 and derivatives thereof were subjected to GdmCl induced unfolding measured by steady state fluorescence spectroscopy. The concentrations needed to half denature scFv-L36, pKBJ1-L36, pKBJ2-L36, and pKBJ3-L36 were 2.73 ± 0.11, 2.70 ± 0.07, 2.67 ± 0.03, and 2.41 ± 0.09 M ($[\mathrm{GdmCl}]_{50\%}$) respectively. Consequently, the increase in activity was not a consequence of increased stability. Furthermore, given the fact that the pKBJ1 and pKBJ2 expression systems behaved similarly in the initial activity assays, pKBJ1 was chosen for further characterisation, since the tags are located terminally.

Gel filtration of scFv fusions

The crystal structure of protein III domain I-II shows intramolecular domain interactions [37] and therefore it

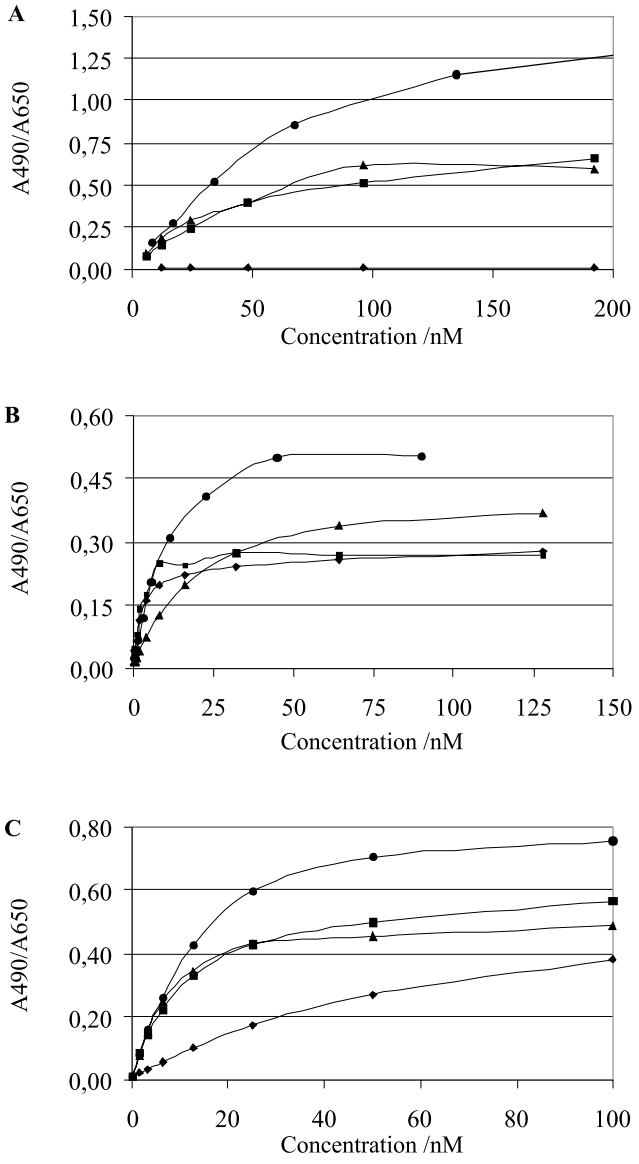

Fig. 3. Activity assays of the antibodies and derivatives in ELISA. Activity assays of the three scFvs, which show binding of D4 to fibronectin (A), L36 to Laminin-1 (B), and R5 to A/C7 monoclonal mouse antibody (C). The four different constructs from each antibody were tested in dilution series in duplicate and the absorbance was read at 490 nm with the absorbance at 650 nm as reference (A490/A650). Generally the domain I fusion, pKBJ3 (●), is more active than the domain I-II fusions, pKBJ1 (■) and pKBJ2 (▲), which are again more active than the non-fused scFv, pUC119 (◆).

can be speculated that the increased activity of the antibody fusions is an avidity effect caused by multimerisation. Consequently, gel filtration analysis was performed. The three antibodies did indeed multimerise into varying amounts of monomer, dimer, and higher order multimer (Table 3). The multimer eluted in void volume, which corresponds to a globular size above approximately 500 kDa as this is the exclusion limit for the column applied (data not shown). Aggregation was not observed in any of the preparations, which indicates that the proteins formed discrete multimer. In addition, when multimer was resampled monomer and dimer appeared, indicating reversibility in the formation of the high molecular weight complex. It was therefore concluded that the formation of multimer is an ordered process and not a result of aggregation. Multimerisation has been reported previously for other filamentous phage proteins such as protein IV, which forms discrete 14 mers in the outer membrane of *E. coli* [38].

It was contemplated which of the complexes corresponded to active fusion protein. Fractions were consequently collected from the gel filtration column and tested for activity (Fig. 4). The concentration of protein was normalised according to an arbitrary absorbance at 220 nm to ensure that approximately the same amount of protein was used in each activity experiment and that the observed activities were comparable. The domain I fusions are the only ones active as multimer; however, no general conclusions could be made, since the three tested antibodies behaved differently under the conditions chosen. This variation could be an effect of the different antigens applied as well as distinct behaviour of these upon coating, thus making comparisons difficult. D4 fused to domain I-II is active as monomer and inactive as multimer, but when fused to domain I no monomer is found, and in this case, the multimer is active. L36 is most active as dimer when fused to both domain I and domain I-II. In the case of R5 domain I-II fusion, multimer and monomer are equally active, whereas fusion to domain I makes the multimer most active. Based on these results, protein III domain I seems to be capable of inducing multimerisation, and when domain II is present the antigen-binding sites of some antibodies are shielded in the multimers, because these are inactive.

Discussion

Previous studies on expression of scFv isolated from phage displayed antibody repertoires and optimisation thereof have either focused on the antibody framework [17], co-expression of bacterial chaperones increasing the yield of functional scFv [4,28], or on fusion to proteins increasing the solubility of the fusion product [22].

Here an analogous system is described, where scFvs isolated from different phage displayed antibody repertoires, the Tomlinson I (D4), Griffin (L36), and Tomlinson J (R5) (http://www.mrc-cpe.cam.ac.uk/~/g1p.html) [20], are expressed together with the entity with which they were selected, that is protein III. The three antibodies used for proof of concept had very homologous primary sequences, R5 and D4 were both encoded by VH3 gene family member DP-47, and VK1 family member DPK9, and differ on one residue only (residue 5 in the variable heavy chain) besides the variability in the

Table 3
Antibody and derivatives analysed by gel filtration

		Monomer (%)	Dimer (%)	Multimer (%)
R5	pUC119	100	0	0
	pKBJ1	51	47	3
	pKBJ3	49	16	35
L36	pUC119	93	7	0
	pKBJ1	60	14	25
	pKBJ3	30	2	68
D4	pUC119	n.d	n.d.	n.d.
	pKBJ1	3	0	97
	pKBJ3	0	0	100

The amount of each species is calculated as the area beneath the gel filtration curve, and the distribution between monomer, dimer, and multimer varies according to the constructs. Three different constructs for each scFv are compared—pUC119, pKBJ1, and pKBJ3. n.d.: not determined.

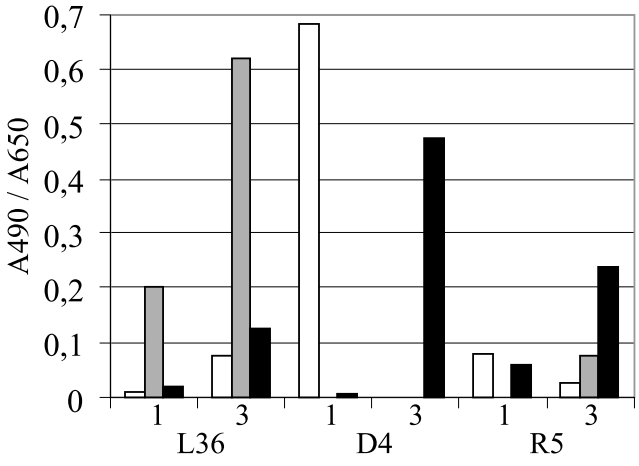

Fig. 4. Activity of monomer, dimer, and multimer collected from gel filtration. The activity of the different multimerisation states of pKBJ1 and pKBJ3 constructs is compared using equal amounts of monomer (empty), dimer (grey), and multimer (filled) for each antibody construct. Arbitrary absorbance units at 220 nm obtained from gel filtration analysis were applied in order to use equivalent amounts of monomer, dimer, and multimer. The different constructs were assayed in duplicate and the absorbance was read at 490 nm with the absorbance at 650 nm as reference (A490/A650).

CDRs. L36 is encoded by a different VH3 family member (DP46) and differs on two residues in the heavy chain compared to R5 and D4. Greater difference was found in the light chain, which belongs to the VL1 variable gene family (V-base—http://www.mrc-cpe. cam.ac.uk/imt-doc/). Consequently small sequence differences are important for successful expression of recombinant antibody fragments in vitro, as reported by others [8,13].

The rationale for our study originated from the observation that some antibodies isolated from antibody repertoires are active when displayed on the filamentous phage; nevertheless, all attempts to produce soluble antibody have failed. Thus, it could be speculated that protein III has some sort of beneficial effect, like those observed with proteins such as the periplasmic substrate binding family of proteins [39]. Filamentous phage coat protein III is a globular protein consisting of three domains separated by flexible linker regions [29]. The structures of the two amino-terminal domains have been solved by X-ray crystallography for the two homologous filamentous phages, M13 and Fd, and show interactions between the two domains [37,40]. In order to test the effect of the filamentous phage coat protein, three different constructs were created which enable antibody fusion to truncated versions of protein III. Tags were either located as in the selected molecule or carboxy-terminally (Fig. 1). It is not likely that any gain of function would be due to the classical increase in solubility, since the probability for expression as soluble protein did not increase significantly: 11%, 17%, 18%, and 19% for L36 in pUC119, pKBJ1, pKBJ2, and pKBJ3, respectively, according to the Wilkinson-Harrison solubility model [41].

After fusion to the coat protein, antibodies could be expressed in amounts similar to that of the unfused antibody (Table 2). The solubility was likewise unaltered as determined by Western blotting experiments (data not shown) supporting the calculated probabilities for soluble expression using the solubility model. However, the activity of the antibodies, when fused to domain I and to domain I-II, was increased, in particular that of the domain I fusion.

The selection pressure applied in antibody selections favours an increase in antibody affinity. This can be obtained by optimising the surface interactions between antibody and antigen, but also indirectly by increasing the stability and thereby the functional fraction of antibody fragments [14]. Phage displayed antibodies can be considered as either antibodies fused to an irrelevant protein or an entity of both antibody and phage protein III. Therefore, the selection pressure applied can either stabilise the antibody or the fusion entity. As a result, antibodies, where selection has stabilised the scFv, will not necessarily gain activity by fusion neither will they lose activity, since this is the context in which they were actively selected as for L36 (Fig. 3B). The most extreme

effect was observed for antibody D4, which was not active as scFv (Fig. 3A).

Two different phenomena could cause increased activity. First, the fraction of folded antibody is increased, which ensures a higher reactivity in the sample. Second, the increased activity is due to the formation of multimer, which increases the avidity of the antibody. A clear formation of multimer was observed when the domain I-II fusion proteins were analysed using gel filtration; nonetheless, active fractions of antibodies were found in the monomer or dimer fraction (Fig. 4). This indicates that the increased activity compared to scFv observed for D4 is due to a higher percentage of correctly folded protein (Fig. 3A). In the case of L36 and partly R5 the activity of scFv and domain I-II fusion is almost identical (Figs. 3B and C), which points to the fact that they are already stable as scFvs. The further increase in activity observed for these antibodies when fused to domain I could be caused by a combined effect of stability and avidity as both monomer and multimer are found active (Fig. 4). Thus, it could be argued that fusion increases the activity by improving antibody folding and that the phage protein therefore could function as a chaperone. Alternatively, fusion to protein III could optimise the surface interactions between antigen and antibody.

This is to our knowledge the first report which shows the potential of fusion to the phage coat protein III. It has not yet been established whether the coat protein has intrinsic chaperone activity, contains elements responsible for recruiting folding components in the bacterial periplasm or improves the antigen-antibody interface. Chaperone properties have been reported for other fusion partners such as maltose-binding protein [23] and lambda head protein D [26]. However, the effects have never been as dramatic as those observed with protein III, where the reactivity of an antibody was restored upon fusion. Whether the function of protein III holds true for all proteins or only those selected by phage display has so far not been determined.

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