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Display of biologically active proteins on the surface of filamentous phages: a cDNA cloning system for selection of functional gene products linked to the genetic information responsible for their production

(Gene enrichment; clonal selection; dimeric proteins; prokaryotic expression; Fos; Jun)

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SUMMARY

A cloning and expression system allowing display of functional cDNAs or other gene products on the surface of filamentous phage has been developed, exploiting the high-affinity interaction of the Jun and Fos leucine zippers. Gene *jun* was expressed from a *lacZ* promoter as a fusion protein with the viral coat protein, pIII, thereby being structurally incorporated into phage particles during infection with a helper phage. Using a second *lacZ* promoter of the phagemid, gene *fos* was co-expressed as an N-terminal fusion peptide to cDNA library gene products, so that the resulting Fos-fusion proteins could become associated with the Jun-decorated phage particles. To avoid interphage exchange of *fos*-cDNA fusion products, cysteines were engineered at the N- and C-termini of each of the leucine zippers, providing a covalent link of the cDNA gene product to the genetic instructions required for its production. Dissociation between phage and cDNA gene products was readily achieved using reducing agents. Phages displaying gene products covalently anchored on their surface via the modified leucine zippers can be selectively enriched 10^4 – 10^6 -fold over nonspecific phages using antibodies. Thus, this cloning system allows rapid isolation of rare mRNA products from complex cDNA libraries by enrichment with appropriate ligands. This approach should allow the expression and cloning of dimeric proteins by cDNA shuffling.

INTRODUCTION

Several difficulties are inherent in the screening of proteins expressed from cDNA libraries in prokaryotic hosts using appropriate ligands such as antibodies (Ab) (Miererdorf et al., 1987; Snyder et al., 1987). The translated products may not assume the native three-dimensional structure or correct disulfide bond formation, or they may

be denatured when transferred to a solid phase such as nitrocellulose prior to screening by ligands (Thorpe et al., 1984; Suter and Butler, 1986; Butler, 1992). In addition, the lambda (λ) phage based cDNA expression systems (Helfman et al., 1983; Snyder et al., 1987; Short et al., 1988), as well as plasmid systems (Sambrook et al., 1989) available to date do not allow for enrichment by ligands, and hence large numbers of recombinant clones have to

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Abbreviations: A, absorbance (1 cm); aa, amino acid(s); Ab, antibody(ies); Ap, ampicillin; *Asp f* I/a, *Aspergillus fumigatus* allergen I; bp, base pair(s); ELISA, enzyme-linked immunosorbent assay; Ig,

immunoglobulin; kb, kilobase(s) or 1000 bp; Leu zipper, leucine zipper, mAb, monoclonal Ab; nt, nucleotide(s); pIII, gene III product of M13; PBS, phosphate-buffered saline (50 mM Na-phosphate/150 mM NaCl pH 7.4); PCR, polymerase chain reaction; PEG, poly(ethylene glycol); pfu, plaque-forming unit(s); *phoA*, alkaline phosphatase-encoding gene; PLA₂, phospholipase A₂; re*Asp f* I/a, recombinant *Asp f* I/a; ::, novel junction (fusion or insertion).

be screened on solid-phase systems to find products encoded by rare mRNAs. Moreover, the λ phage systems available may not be useful for the simultaneous expression of recombinant gene products which assemble to functional unit such as heterodimers.

Some of these problems have been overcome by the production of monoclonal antibodies (mAb) which specifically recognize denatured antigens (Hollander and Katchalski-Katzir, 1986; Regenmortel, 1992), or by λ phage systems which allow secretion and thus folding of the gene product (Huse et al., 1989; Burton et al., 1991). However, problems associated with screening of the solid-phase-bound proteins and inability for ligand-based enrichment of genes coding specific products still remain.

Recently, filamentous phage systems have been developed and used mainly for the expression of peptides (Smith, 1985; Parmley and Smith, 1988; Scott and Smith, 1990; Devlin et al., 1990; Cwirla et al., 1990) as well as single-chain Ab (McCafferty et al., 1990) and human growth hormone (Bass et al., 1990). The peptides or proteins are expressed as part of the virus coat and hence are believed to assume the correct structure for production of infectious phage particles and for enrichment using Abs or other ligands (Parmley and Smith, 1988).

The filamentous phage systems developed for expression of functional immunoglobulin (Ig) fragments in *Escherichia coli* make use of two independent promoters allowing the simultaneous expression of the Ig heavy chain as a coat fusion protein and the Ig light chain as a secreted molecule (Barbas et al., 1991; Burton et al., 1991; Hoogenboom et al., 1991). Hence, filamentous phages allow expression of functional heterodimers which can be selected by powerful enrichment procedures (Barbas and Lerner, 1991; Kang et al., 1991). Therefore, filamentous phage systems appear attractive for cDNA expression because of the possibility for the expression of folded molecules and the ability to select appropriate clones from very large libraries. However, cDNA gene products cannot be directly expressed as fusion proteins to the N-terminus of the viral coat proteins due to transcriptional stop sites present at the 3'-end of non-translated regions in eukaryotic cDNA obtained by poly(A)⁺ selection of mRNA followed by poly(A) priming (Abelson and Butz, 1980; Sambrook et al., 1989). This fact prevents the use of filamentous phage developed so far for cDNA library screening. To covalently link the cDNA gene product to the phage surface, a strategy based on the filamentous phage pComb3 (Barbas et al., 1991) and on the ability of the Leu zippers Jun and Fos to associate with each other (Kouzarides and Ziff, 1988; Abate et al., 1990; Kerpolla and Curran, 1991) was developed. The cloning system, named pJuFo, allows the expression and enrichment of functionally folded cDNA products covalently linked to

the filamentous phage, and thus to the genetic information required for their production.

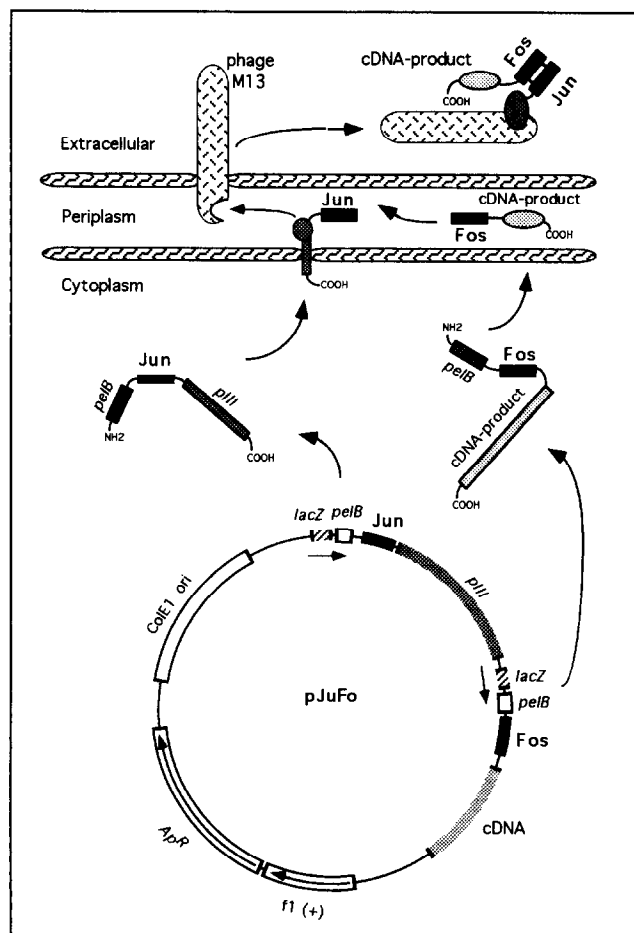
RESULTS AND DISCUSSION

(a) Vector construction

The phagemid vector, pJuFo, was constructed to fuse the modified Jun Leu zipper with the C-terminal domain of the pIII protein of the filamentous phage (Fig. 1). At the N- and C-termini of the original Jun Leu zipper, Cys residues were added via GlyGly spacers (O'Shea et al., 1989). The Jun::pIII fusion was placed under the control of a *lac* promoter/operator element and directed to the periplasmic space by the *pelB* signal sequence. Gene products to be captured on the outer phage surface were linked to the C-terminus of the modified Fos Leu zipper, which was flanked by Cys residues added via GlyGly spacers at the N- and C-termini (Fig. 1). The genes to be coexpressed with Fos were cloned as *Bgl*II-*Xba*I, *Bgl*II-*Kpn*I or *Xba*I-*Kpn*I fragments and placed under the control of a separate *lac* promoter/operator element and directed to the periplasmic space of the *E. coli* host by the *pelB* signal sequence. The periplasmic space allows functional assembly of Jun/Fos heterodimers (Turner and Tjian, 1989; O'Shea et al., 1989) as parallel coiled-coil structures (Gentz et al., 1989) followed by proper disulfide bond formation mediated by the engineered Cys residues flanking the Leu zippers (Fig. 1). During helper phage superinfection the Fos-fusion protein captured by Jun::pIII is incorporated into the virion (Barbas et al., 1991). Purified phage displaying gene products on the surface were used for functional investigations.

(b) Enzymes expressed on the filamentous phage pJuFo are active

To test the function of gene products expressed by pJuFo, the *phoA* gene of *E. coli* was fused to the *fos*-encoding region (Fig. 2). pJuFo::*phoA* phages produced by helper phage infection (Barbas and Lerner, 1991) were precipitated twice with PEG/NaCl (Cwirla et al., 1990) and tested for the presence of the *phoA* gene product by a standard enzymatic assay. Fig. 2 shows the time-dependent substrate conversion as a function of the number of pJuFo::*phoA* phage used. In contrast, precipitated pJuFo phages did not show any enzymatic activity. This experiment indicated that enzymatically active molecules can be produced by pJuFo and captured on the phage surface. Originally we planned to express bee venom phospholipase A₂ (PLA₂) and to measure the enzymatic activity of the captured protein on the phage surface with an enzymatic assay for PLA₂ (Dudler et al., 1992). Due to the high toxicity of expressed enzymatically active PLA₂



(A) *lacZ* P/O\pelB leader A Q V K L \ X h o I \ H i n d I I I \ C G G \ R I A R L E E K V K T L K A Q N S E L A S T A N M L
R E Q V A Q L K Q K V M N H \ G G C \ B a m H I \ S p e I \ G G G S \ (p I I I P 198 - S 406)

(B) *lacZ* P/O\pelB leader A E \ S a c I \ C G G \ L T D T L Q A E T D Q L E D E K S A L Q T E I A N L
L K E K E K L E F I L A A H \ G G C \ B g I I I \ X b a I \ c D N A \ K p n I

Fig. 1. Essential elements of the pJuFo phagemid are shown in the lower panel. (A) The nt sequence of the *pelB* leaders, the GGS flexible linker (Huston et al., 1988) and the C-terminal domain of pIII were recruited from the phagemid pComb3 (Barbas et al., 1991). The gene encoding the Jun Leu zipper was inserted as a *HindIII/BamHI* fragment. (B) P/O elements as in (A). The gene encoding the Fos Leu zipper was inserted as a *SacI/XbaI* fragment. The *BglIII* (created by PCR, see below), *XbaI* and *KpnI* sites allow construction of cDNA expression libraries whose gene products are displayed on the phage surface. The Jun and Fos Leu zippers are flanked by *CGG* and *GGC* aa at the N- and C-terminus, respectively, to covalently link the Jun and Fos Leu zippers. The aa leucine (L) involved in the interaction of the zippers are highlighted. The diagram illustrates the composition of the pJuFo vector and the proposed pathway for the capture of cDNA products on the phage surface. **Methods:** DNA encoding the Leu zippers from the Jun and Fos genes (Abate et al., 1990) was isolated by PCR using the original constructs generously supplied by Dr. T. Curran (Roche Research Center, NJ, USA) as a template and the following oligodeoxynucleotides: 5'-primer Jun, 5'-CCC AAG CTT TGC GGT GGT CGG ATC GCC CGG CTC GAG GA, 3'-primer Jun, 5'-CGG GAT CCG CAA CCA CCG TGG TTC ATG ACT TTC TGT TT, 5'-primer Fos, 5'-GCG AGC TCT GCG GTG GTC TGA CCG ACA CCC TGG AG, 3'-primer Fos, 5'-GCT CTA GAA GAT CTG CAA CCA CCG TGT GCC GCC AGG ATG AAC. PCR amplifications were performed in a thermocycler (Technique PHC-2, Brouwer, Luzern, Switzerland) using standard protocols (Sambrook et al., 1989; Innis et al., 1989) and amplification reagents obtained from Anawa (Wangen, Switzerland). In a first step the Jun-

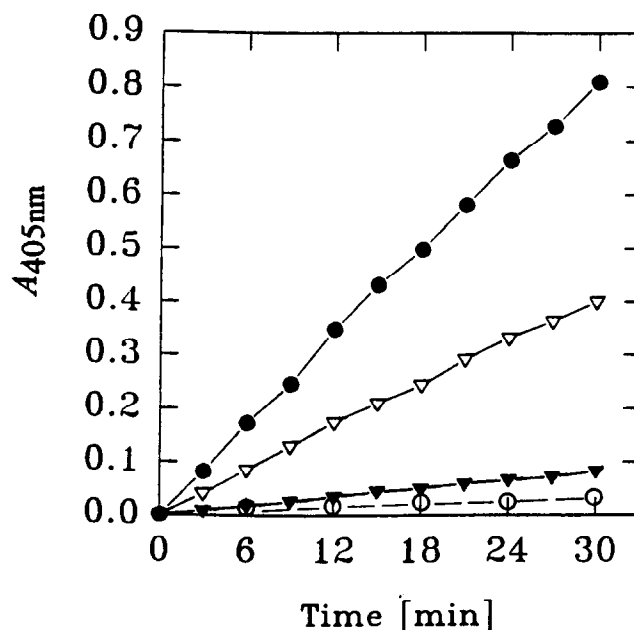


Fig. 2. Enzymatic activity of phage expressing the *phoA* gene of *E. coli* as covalently bound molecule on the surface as function of phage number and incubation time. 10^{10} pfu (●), 5×10^9 pfu (▽), 10^9 pfu (▼). pJuFo phage (10^{12} pfu) was used as control (○). Alkaline phosphatase activity was detected in microtiter plates as follows: 100 μ l substrate (1.5 mg/ml of 4-nitrophenyl phosphate in 1 M diethanolamine buffer pH 9.8 containing 0.5 mM $MgCl_2$) was added to microtiter wells. Serially diluted phage was added to the wells and the increase of the optical density due to substrate conversion was monitored at 405 nm in a kinetic Microtiter plate reader. Production of filamentous phage and titration of pfu were as described (Barbas et al., 1991). Phage was recovered from liquid cultures by PEG/NaCl precipitation (Cwirla et al., 1990) and stored at $-20^\circ C$ until use. Construction of pJuFo::phoA was as follows: the *phoA* gene was amplified by PCR from genomic DNA of *E. coli* strain M15 (a generous gift of Dr. D. Stüber, Hoffmann-La Roche, Basel, Switzerland; Villarejo and Zabin, 1974) using the following primers derived from the published nt sequence (Chang et al., 1986): 5'-primer, 5'-GCT CTA GAC GGA CAC CAG AAA TGC CTG TTC TG; 3'-primer, 5'-CCG GTA CCC GGG CGC GGT TTT TAT TTC AG. The amplified *phoA* gene was subcloned into pJuFo as a *BglIII-KpnI* fragment, expressed, and the Fos-PhoA fusion captured on the surface of phages. The construct was verified by restriction analysis.

for the host *E. coli*, several attempts to produce phage failed. However, an enzymatically inactive variant of the PLA₂ molecule obtained by site directed mutagenesis

encoding gene fragment was inserted in-frame 5' to the pIII coding sequence of pComb3 (kindly supplied by Dr. R. Lerner, the Scripps Research Institute, La Jolla, CA, USA; Barbas et al., 1991) as a *HindIII-BamHI* fragment. For cloning of DNA encoding the modified Fos Leu zipper region, phagemid DNA containing the modified Jun zipper was digested with *SacI*+*XbaI*, gel-purified, extracted with glass milk (Bio 101, La Jolla, CA, USA) and ligated with Fos PCR product digested with *SacI*+*XbaI*. The ligation mixture was used to transform 50 μ l of *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA) electrocompetent cells (Dower et al., 1988) which were then plated on LB/Ap plates as described (Barbas and Lerner, 1991). Single colonies were picked, grown in liquid culture, DNA was prepared (Holmes and Quigly, 1981) and the final construct was verified by restriction analysis.

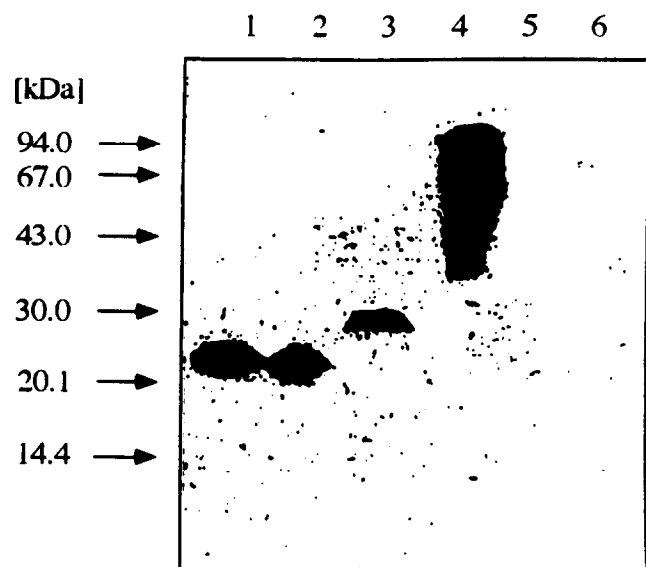


Fig. 3. Western blot analysis of phage. *reAsp f I/a* protein (1 μ g, lanes 1 and 2) or phage samples expressing *asp f I/a* gene on the surface (10^{12} – 10^{13} pfu, lanes 3 and 4) were separated by 0.1% SDS-12.5% PAGE under reducing or non-reducing conditions, respectively, and transferred to a nitrocellulose membrane. Lane 3 shows the Fos-*reAsp f I/a* fusion released from the phage surface under reducing conditions. In lanes 5 and 6 pJuFo phagemid (10^{14} pfu) was run under reducing and non-reducing conditions as a control. **Methods:** For Western blot analysis, phage expressing recombinant products and appropriate controls were separated by 0.1% SDS-12.5% PAGE (Schägger and von Jagow, 1987) under reducing and non-reducing conditions using a Midget Electrophoresis Unit (Pharmacia LKB, Uppsala, Sweden) and then transferred to Hybond-ECL nitrocellulose membranes (Amersham, Amersham, UK) using a Multiphor II Electrophoretic Transfer Unit (Pharmacia) at 50 mA for 2.5 h (Burnette, 1981). Nitrocellulose filters were blocked with PBS, pH 7.4, containing 5% (w/v) non-fat dry milk powder (blocking buffer) at 22°C for 3 h, then mAb 4A6 diluted in blocking buffer containing 0.5% Tween 20 (dilution buffer) was added followed by overnight incubation at 22°C on a rotary oven (Bachofar, Reutlingen, Germany). After washing three times for 1 h at room temperature in excess of PBS, horseradish-peroxidase-labelled secondary Ab in dilution buffer was added and filters were incubated at 37°C for 3 h followed by washing as described above. Western blots were developed using the ECL system (Amersham) according to the manufacturer's instructions. Construction of pJuFo::*asp f I/a*: the gene encoding for *reAsp f I/a* (Moser et al., 1992) was cloned into pJuFo as *Bam*HI-*Xba*I fragment into *Bgl*II + *Xba*I-cleaved pJuFo vector DNA to generate a Fos-fusion protein. The *asp f I/a* DNA fragment contains the full-length gene with a C-terminal stop codon followed by the T_0 terminator and an approx. 2-kb fragment of vector DNA derived from the original construct. After verification of the construct by restriction analysis, phage was produced in a 250-ml liquid culture by helper phage induction and concentrated by PEG/NaCl precipitation.

(T. Dudler and M.S., unpublished) could be expressed and captured on the surface of phage as demonstrated by PLA₂-specific ELISA (data not shown).

(c) Demonstration of covalent bond formation between Jun and Fos-fusion proteins

The aim of the pJuFo expression system is to screen for a desired gene product and simultaneously isolate the

gene encoding the product. It is, therefore, mandatory that the gene product be covalently linked to the phage coat to prevent dissociation of Jun/Fos, and thus losing the appropriate product/gene pair. To test this, a well-characterized gene encoding a fungal ribotoxin (Moser et al., 1992) was cloned into pJuFo to generate a Fos-fusion protein. The phages resulting from infection with this construct were subjected to Western blot analysis (Fig. 3), using the mAb 4A6 (kindly provided by Dr. K.L. Arruda) raised against the 18-kDa fungal ribotoxin (Arruda et al., 1990; 1992) as the primary antibody to detect the *reAsp f I/a* protein. This experiment showed that the protein was released from the phage surface only under reducing conditions and demonstrated that Fos-fusion proteins can be captured on the phage surface by disulfide bonding to the Jun Leu zipper. Notably, protein degradation was not observed under these conditions.

(d) Phage ELISA

The binding of anti-*Asp f I*-specific mAb 4A6 to phagemids encoding the Fos::*asp f I/a* fusion was further analyzed by solid phase ELISA. A phage preparation produced from cells harboring the pJuFo-vector was used as a negative control. As shown in Fig. 4, anti-*Asp f I* mAb 4A6 reacted with solid-phase-coated phage depending on the quantity of phage particles applied. Comparable results were obtained with other gene products (data not shown).

(e) The potential of the pJuFo cloning system for selection of genes from cDNA libraries

The utility of the pJuFo system for enrichment of genes from cDNA libraries via the expressed gene product can be demonstrated by Ab-specific sorting of phage coding for a known protein present in a mixture of pJuFo phage. Phage was prepared by overnight infection of pJuFo- and pJuFo::*asp f I/a*-phagemid containing cells with helper, yielding typical titers of 10^{11} – 10^{12} pfu/ml. Phages expressing *reAsp f I/a* captured on the surface via Jun/Fos Leu zippers were mixed with pJuFo phage at different ratios (Table I) and submitted to multiple rounds of panning on wells coated with 1 μ g/ml of the *Asp f I*-specific mAb 4A6. Phages expressing *reAsp f I/a* were enriched to virtually 100% over helper phage and pJuFo phage after four rounds of panning, even if the frequency of *reAsp f I/a* phage was as low as 1 per 10^7 in the starting mixture (Table I). Similar results were obtained using the streptavidin-biotin system as panning procedure (Gretch et al., 1987; data not shown).

The applicability of the pJuFo system for cDNA library screening was analyzed by selective isolation of an *reAsp f I/a*-specific phage from a previously described *Aspergillus fumigatus* cDNA library in λ ZapII (Moser

et al., 1992) subcloned into the pJuFo (Table I). Phage particles containing the *asp f I/a* gene were isolated from the library after four rounds of panning. The presence of the gene was verified by *SacI* + *SmaI* restriction analysis and the product analyzed by phage ELISA. (The *SmaI* restriction site not present in the pJuFo cloning vector is located internally at nt 345 of the *asp f I/a* gene; Moser et al., 1992).

pJuFo contains two identical promoter/operator elements and allows simultaneous expression of two proteins at similar ratios. As shown previously with other filamentous phage (Barbas et al., 1991; Kang et al., 1991a; Hoogenboom et al., 1991) functional dimeric molecules can be expressed in such systems. pJuFo could be used as a shuffling vector (Kang et al., 1991b; Marks et al., 1992) to screen a Fos-cDNA library to find a dimeric partner if one monomer is already known and expressed as C-terminal Jun-fusion. In the absence of a characterized monomer a different strategy may be envisaged. It is possible to construct a cDNA library using random priming to reduce transcriptional stop sites present at the 3'-end of eukaryotic mRNA. Random primed mRNA can

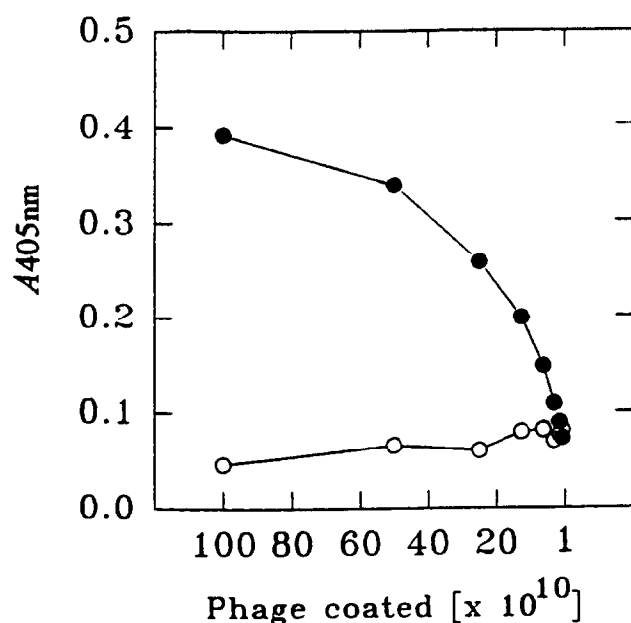


Fig. 4. Phage-specific ELISA. Binding of mAb 4A6 to solid phase-coated phage expressing reAsp f I/a (●). pJuFo phages were used as control (○). **Methods:** Phage samples (10^{12} cfu) serially diluted in PBS pH 8.0 were coated overnight at 4°C on Maxisorp polystyrene microtiter plates (Nunc, Roskilde, Denmark). After washing, the remaining free sites were blocked at 37°C for 1 h with 5% non-fat dry milk powder in PBS pH 7.4 (blocking buffer), followed by a further washing. Primary antibody in blocking buffer containing 5% Tween 20 was added to each well and incubation continued for 2 h. After washing with PBS, phosphatase-labelled secondary antibody in dilution buffer was added and plates were incubated at 37°C for another 2 h. Plates were developed with 100 μ l alkaline phosphatase substrate solution (1.5 mg/ml of 4-nitrophenyl phosphate in 1 M diethanolamine buffer (pH 9.8) containing 0.5 mM $MgCl_2$) as described (Held et al., 1989).

TABLE I

Ab-specific enrichment of phage displaying the *asp f I/a* gene product over non-cognate phage or phage carrying the *asp f I/a* gene from an *A. fumigatus* cDNA expression library^a

Clonal mixture ^b		Rounds of panning ^c				^f
		1	2	3	4	
1:10 ⁴	^c	1.5 $\times 10^{-4}$	2.5 $\times 10^{-2}$	4.4 $\times 10^{-2}$	—	10/10
	^d	2.5 $\times 10^5$	3.2 $\times 10^7$	3.1 $\times 10^7$	—	
1:10 ⁵	^c	3.1 $\times 10^{-4}$	1.2 $\times 10^{-2}$	2.8 $\times 10^{-2}$	—	10/10
	^d	2.8 $\times 10^5$	1.8 $\times 10^7$	5.3 $\times 10^7$	—	
1:10 ⁶	^c	1.7 $\times 10^{-5}$	6.2 $\times 10^{-5}$	8.4 $\times 10^{-3}$	3.8 $\times 10^{-2}$	9/10
	^d	9.3 $\times 10^3$	5.7 $\times 10^4$	4.3 $\times 10^6$	2.9 $\times 10^7$	
1:10 ⁷	^c	1.2 $\times 10^{-6}$	1.1 $\times 10^{-5}$	8.3 $\times 10^{-5}$	4.1 $\times 10^{-3}$	8/10
	^d	1.6 $\times 10^3$	9.8 $\times 10^3$	3.1 $\times 10^4$	8.2 $\times 10^6$	
cDNA library	^c	3.5 $\times 10^{-6}$	1.2 $\times 10^{-5}$	2.6 $\times 10^{-3}$	3.3 $\times 10^{-2}$	9/10
	^d	1.3 $\times 10^3$	5.3 $\times 10^4$	4.2 $\times 10^5$	2.0 $\times 10^7$	

^aConstruction of an *A. fumigatus* cDNA library into pJuFo: λ ZapII recombinant phage particles (10^9) were used to infect 20 ml of *E. coli* XL1-Blue cells ($A_{600}=1$) together with 10^{11} pfu of R408 helper phage. After incubation for 15 min at 37°C, 200 ml 2 $\times$ YT medium (Barbas et al., 1991) containing 100 μ g Ap/ml were added and incubation continued for 6 h. The culture was then heated at 70°C for 20 min, then centrifuged for 10 min at 8000 $\times$ g. The decanted supernatant containing the in vivo excised cDNA library into the pBluescript phagemid (Short et al., 1988) was stored at 4°C. To prepare DNA excised phagemid (10^{10} pfu) was used to infect *E. coli* XL1-Blue (10 ml, $A_{600}=1$). After addition of 250 ml LB-medium containing 100 μ gAp/ml and further incubation at 37°C overnight, plasmid DNA was prepared using a commercial kit (Diagen, Düsseldorf, Germany). For the construction of a phage cDNA library, 2 μ g of this DNA was cleaved with *XbaI* + *KpnI*, the inserts gel purified and ligated to 4 μ g of pJuFo vector digested with *XbaI* + *KpnI*. The ligation mixture was used to transform *E. coli* XL1-Blue cells by electroporation (Dower et al., 1988) and a phage expression library was prepared by helper phage infection as described (Barbas and Lerner, 1991). The primary cDNA library consists of 3×10^9 independent clones recovered as Ap-resistant colonies: this corresponds to an efficiency of transformation of about 5×10^8 per μ g of ligation mixture.

^bNumber of phages carrying the *asp f I/a* gene product : non cognate phage in the starting mixture.

^cPercentage yield of phage from each round of panning. % Yield = (No. of phages eluted $\times 100$) / (No. of phages applied).

^dEnrichment as monitored by the absolute number of phage eluted from the plate after panning.

^ePhage panning: The previously described panning procedure (Parmley and Smith, 1988; Barbas et al., 1991) was modified to enrich for phage expressing proteins of interest on their surface. Two wells of a microtiter plate (Nunc) were coated overnight at 4°C or at 37°C for 2 h with 200 μ l of mAb 4A6 (10 μ g/ml in PBS pH 8.0). The wells were washed three times with PBS and blocked by filling with 5% (w/v) dry milk powder in PBS for 2 h at 37°C. Blocking solution was removed and 100 μ l of phage mixture or phage library (typically 10^{11} – 10^{12} pfu) were added to each well followed by incubation at 37°C for 3 h. Washing, phage elution and reinfection procedures were as described (Barbas et al., 1991). Phage preparation and further rounds of panning were repeated as described above. For some experiments the streptavidin-biotin system was used for phage enrichment essentially as described for affinity isolation of proteins (Gretch et al., 1987).

^fNumber of colonies carrying the *asp f I/a* gene as verified by restriction analysis and phage ELISA/number of colonies tested after the last round of panning.

thus be cloned as part of the filamentous coat protein in frame to the C-terminus of Jun, and a poly(A)⁺-selected and post-primed cDNA library cloned at the C-terminus of Fos. This approach seems to be feasible as dimers such as Jun/Fos Leu zippers (Kouzarides and Ziff, 1988; O'Neil et al., 1990; Kerpolla and Curran, 1991) or Ig domains (Hood et al., 1985) have interactive structures which are generally independent of the flanking regions.

(f) Further potential applications of the pJuFo system

The ease of directly analyzing properly folded gene product on the phage will avoid time consuming purification and refolding procedures for proteins produced in prokaryotes as inclusion bodies in order to analyze gene products for biological activity or antigenic reactivity (Creighton, 1990; Buchner and Rudolph, 1991; Dudler et al., 1992). Although our work so far has focused on application of pJuFo for enzyme and antigens, we can foresee the application of the pJuFo cloning strategy in various fields. Large-scale screening of potential new drugs binding to biological receptors has proven to be difficult due to the unavailability of robust systems applicable for various screening tasks (Hodgson, 1993). pJuFo displaying receptors can be used to screen for ligands and to select ligands with higher affinity. pJuFo could be used to find biologically important proteins in tests where coat proteins from a virus expressed on the surface of pJuFo compete with wild-type virus for adsorption to a targeted cells. The advantage of phage over other systems has been discussed in this paper and elsewhere (Smith, 1993).

(g) Conclusions

(1) pJuFo is a phagemid cloning system allowing selective isolation of genes from cDNA via the linkage of the gene product expressed on the filamentous phage surface with the genetic information for its production.

(2) Enzymes or antigens covalently attached to the phage coat by the modified Jun/Fos molecules are biologically active as shown by enzymatic tests or binding to Ab.

(3) We expect that the pJuFo cloning and expression system will allow: (i) efficient cloning of cDNA from rare mRNA species by enriching for the correctly folded product using appropriate ligands such as antibodies; (ii) convenient analysis of genetically engineered variants of molecules to determine the size of the gene product, their biological activity or antigenicity; (iii) screening and analysis of homo- or heterodimeric molecules; (iv) screening of ligands or analogs thereof to mono- or dimeric receptors displayed on the surface of pJuFo.

Therefore, the application of the three major principles of pJuFo: (a) cDNA or gene translation and production

of functionally folded molecules accessible for direct analysis; (b) possibility for the enrichment of the desired molecule physically linked to the genetic information required for its production; and (c) the potential for the expression of dimers is mainly limited by the imagination of the investigator.

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