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Cloning *Aspergillus fumigatus* Allergens by the pJuFo Filamentous Phage Display System

Key Words

cDNA cloning
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Abstract

A cDNA library from *Aspergillus fumigatus* has been displayed on the surface of filamentous phage M13 and screened for gene products binding to human serum IgE. The physical linkage of cDNA gene products to the genetic information required for their production, achieved by exploiting the high-affinity interaction of the Jun and Fos leucine zippers, allows rapid and easier screening of large libraries in semifluid systems. The pJuFo cloning vector is designed to display proteins on the surface of phage and allows selective isolation of genes by gene product-ligand interaction. Thus the system is applicable to clone cDNA that encodes proteins for which a ligand is available. Herein we show that phage expressing IgE binding proteins from *A. fumigatus* can be enriched and separated from nonspecific phage by using serum IgE from *A. fumigatus*-allergic individuals coated to plastic dishes. Subsequently, the proteins can be produced in high amounts in *Escherichia coli* and purified for usage in allergy testing.

Introduction

During the last few years cDNA libraries have been extensively used to isolate allergenic proteins from different sources, including house dust mite [1, 2], pollen [3, 4], insects [5, 6] and some other sources [7–9]. Alternatively, partial sequence knowledge from the primary structure of allergenic proteins [7, 10–12] combined with the polymerase chain reaction [13], has led to the determination of the primary structure of a number of important allergens [14–16]. Although these techniques have proven to be extremely successful for the determination of the primary structure of allergens, cloning of several components from complex allergenic systems is labor-intensive, e.g. cloning of *Aspergillus fumigatus* allergens, a mold which is known to express a great number of highly allergenic components [17]. Whereas more than 40 IgE binding proteins from *A. fumigatus* were demonstrated, ragweed pollen is reported to contain at

least 52 antigens [18] of which 22 have been shown to bind to human serum IgE [19]. A rational cloning approach for heterogeneous protein mixtures sharing common binding properties like allergens implicates selective enrichment of specific clones expressing proteins which are able to bind to IgE antibodies. Filamentous phage appears attractive to clone allergens for different reasons [20] and has been successfully used to display the surface of phage peptide epitopes [21], DNA binding proteins with altered binding properties [22, 23] and antibody fragments [24], and a number of other proteins. Phage expressing the proteins of interest can be selectively isolated from a mixture of non-specific phage by using antibodies [20, 21], DNA [23], antigens [24] or other ligands [25]. However, all approaches applied in these cases use fusions that are N-terminal to the gene III coat protein located at the tip of the phage and responsible for the attachment of the phage to the bacterial F pilus in infection. Therefore, the vectors do not allow the

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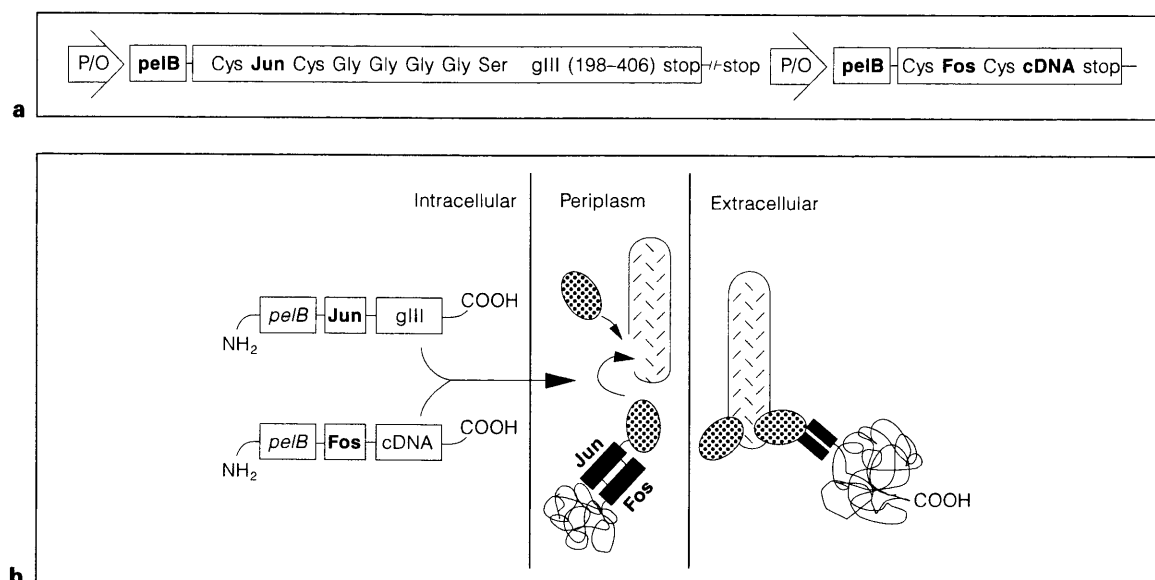


Fig. 1. a Essential elements of the pJuFo phagemid. The Jun leucine zipper flanked by Cys residues is expressed and secreted as N-terminal fusion to a truncated gIII gene product of the phagemid. The Fos leucine zipper flanked by Cys residues is used to produce Fos-cDNA fusions secreted via the *pelB* leader and captured by the Jun leucine zipper. cDNA expression libraries can be constructed using the unique *Bgl*III, *Xba*I and *Kpn*I restriction sites of the phagemid. The backbone of pJuFo is recruited from the previously reported pComb3 construct [24]. **b** Generation of phage surface display libraries. Jun/gIII and Fos/cDNA heterodimers were incorporated into the phage surface together with wild-type gene III product of the helper phage.

construction of cDNA expression libraries because translational stop sites present at the 3'-end of nontranslated regions of eukaryotic mRNA prevent the construction of fusion proteins N-terminal to the gIII gene product. We present now a novel cloning system which circumvents this problem by using the Jun leucine zipper N-terminally cloned to the gIII gene, in order to capture cDNA gene products decorated with the Fos leucine zipper. The strong interaction of the Jun and Fos leucine zippers [26] is exploited to covalently link cDNA gene products to the surface of the phage. It allows a rapid and simplified screening of cDNA libraries by gene product-ligand interaction (fig. 1). With regard to cloning allergens from organisms producing complex patterns of IgE-binding proteins, the screening of libraries constructed in pJuFo reduces the time required for the selection of candidate clones generally to a few days.

Construction and Screening of Phage-Displayed cDNA Libraries

Unidirectional cDNA libraries can be constructed into pJuFo as *Bgl*III/*Xba*I, *Bgl*III/*Kpn*I or *Xba*I/*Kpn*I inserts generated from reverse transcribed mRNA by standard procedures [27, 28]. Furthermore, the restriction sites *Bgl*III/*Xba*I/*Kpn*I can be used to transfer the cDNA in-

serts of pre-made λ -Zap libraries [29] into pJuFo. We have chosen this approach to construct a cDNA phage surface display library from the *A. fumigatus* strain ATCC 42202. Therefore, the *A. fumigatus* λ -Zap library generated from polyA-reverse transcribed mRNA [14] was excised *in vivo* [29]. Two micrograms of cDNA inserts, obtained by *Xba*I/*Kpn*I digestion of the excised phagemid, were gel purified, electroeluted and ligated to 4 μ g of *Xba*I/*Kpn*I restricted pJuFo. After electroporation of the ligation mixture into *Escherichia coli* strain XL-1Blue (Stratagene, La Jolla, Calif., USA), 3 ml SOC medium were added and the culture shaken at 220 rpm for 1 h at 37°C. At this time, 10 ml SB medium [24] containing ampicillin and tetracycline were added and aliquots were plated on ampicillin containing agar to determine the size of the library. Depending on the efficiency of ligation and electroporation, the library size varied from 2×10^7 to 2×10^9 independent clones, recovered as ampicillin-resistant colonies. The culture was shaken for an additional hour at 300 rpm, added to 100 ml SB medium containing antibiotics as above and shaken for another hour. After this time, helper phage VCMS13 (10^{12} plaque forming units, Stratagene) was added and incubated for 2 h at 37°C, 300 rpm. Afterwards 70 μ g ml⁻¹ kanamycin were added and incubated overnight. The supernatant was cleared by centrifugation, phage precipitated and stored as described [20, 24].

To obtain an allergen-specific IgE-coated surface, anti-human IgE mAb TN-142 (Dr. C. Heusser, Ciba-Geigy, Basel, Switzerland) was coated to poly-styrene microtiter wells (Maxisorb, Nunc). Free sites were blocked [14] and 100 μ l serum, pooled from 5 individuals expressing high titers of IgE antibodies and allergic reactions to *A. fumigatus*, were added to each well followed by incubation at 37°C for 2 h. After washing, 10^{11} phage from the library were applied to a single

well and incubated for 2 h at 37°C. Phage washing, elution, phage purification and reinfection procedures for further rounds of selection were performed as described [8, 20]. Selective enrichment can be monitored by following the absolute number of phage eluted when input phage number and ligand surface were equivalent in each round of panning. If no enrichment is observed after five rounds of panning, the desired specificity is unlikely to be present in the library. Enrichment factor and specific binding of the enriched phage population to human IgE are presented in table 1.

Characterization of Specific Clones

The phage population enriched by panning against human IgE from *A. fumigatus*-allergic individuals is likely to contain phage encoding different allergenic proteins. Various proteins of *A. fumigatus* are recognized by the coated IgE antibodies to this mold [17]. Single clones are expected to be multiply represented due to amplification of binding phage during several rounds of phage growth and selection [8]. A first discrimination of clones can be made by the determination of the length of the inserts by restriction analysis and agarose gel electrophoresis [27] among phage scoring positively in IgE ELISA (tables 1, 2). Alternatively, Western blots of phage with IgE-antibody-containing serum, followed by anti-human IgE (mAb TN-142) second antibody and detection with enzyme conjugated anti-mouse immunoglobulin can be performed [8]. This procedure allows a direct estimation of the apparent molecular mass of the displayed proteins. The physical linkage between gene product and its encoding DNA allows straightforward determination of the primary structure of the protein from the cDNA sequence. Inserts encoding allergen-specific cDNAs can be isolated as *Bgl*II/*Pvu*II fragment from the corresponding phagemid and subcloned into *Bam*HI/*Pvu*II-cleaved high-level expression vectors [8, 20]. This simple procedure allows the production of allergens in large amounts (table 2). The final proof that the recombinant proteins produced are allergenic comes from their application in vivo, especially by the dose-dependent reactivity in skin tests [30]. The importance of an allergen is given by its frequency to yield specific reactions in patients with the respective allergy.

Discussion

Screening of plasmid, cosmid or λ -phage libraries is performed after transfer of phage plaques or bacterial colonies to nitrocellulose membranes [27]. If sequence information is available, positive clones can be identified by hybridiza-

Table 1. Enrichment of phage binding to human serum IgE antibodies from individuals sensitized to *A. fumigatus*

Round of panning	Phage applied	Phage eluted	Enrichment factor ^a	IgE binding phage ^b
1	2.1×10^{11}	7.0×10^3	3.3×10^{-6}	0/10
2	9.5×10^{10}	2.0×10^4	2.1×10^{-5}	0/10
3	8.7×10^{10}	2.8×10^4	3.2×10^{-5}	0/10
4	7.4×10^{10}	1.7×10^5	2.1×10^{-4}	1/10
5	1.6×10^{11}	4.8×10^6	3.0×10^{-3}	9/10

About 10^{11} phage from a cDNA phage surface display library were applied to a single well of a microtiter plate coated with human serum IgE and adsorbed for 2 h at 37°C. After washing, binding phage were eluted and used to infect *E. coli* cells for a further round of phage growth and selection. Ten phage from each round of panning were tested for IgE binding affinity by an IgE-specific ELISA [14].

^a Percent yield of phage from each round of panning [percent yield = (n of phage eluted $\times$ 100/n of phage applied)].

^b Single colonies from plates used to titrate phagemids were grown in liquid culture, phage induced and purified phages were coated directly into microtiter plates and tested for IgE binding affinity by an IgE-specific ELISA [14].

tion with oligonucleotides. Alternatively, the design of PCR primers may allow direct isolation of the desired gene [14–16]. Although these systems have been proven as extremely successful, they are limited by the need of sequence information. cDNA expression libraries can be screened with antibodies to detect gene products immobilized to nitrocellulose. This procedure is affected by denaturation effects due to adsorption of proteins to solid phase [27] and from the limited number of plaques or colonies that can be screened with reasonable efforts. In both cases, immobilization of libraries to solid phase supports hampers the selective enrichment of clones expressing the desired gene product. The utility of direct linkage of cDNA gene products via the phage surface to the DNA encoding the product itself, achieved by Jun-Fos heterodimerization, solves these problems. As much as 10^{11} single clones can be screened on a single well of a microtiter plate, and the semifluid screening keeping the library on fluid phase avoids denaturation of proteins. Immobilization of the ligand instead of the library allows the specific enrichment of clones interacting with the solid-phase-immobilized ligand by repeated rounds of clonal selection. Moreover, the isolation of specific clones recognized by human IgE of a patient may allow the identification of patient-specific allergens.

Allergy testing in vivo and in vitro is still based on the application of allergen preparations mostly consisting of heterogenous mixtures of allergenic compounds in variable

Table 2. Main characteristics of phage displaying IgE binding proteins which were isolated from an *A. fumigatus* phage surface display library by selective enrichment

Phage n	Insert length ^a , bp	MW of the protein ^b , kD	Expression ^c mg/l culture	IgE binding properties ^d	Preliminary designation ^e	Skin test reactivity ^g
2	1,080	40	36	++++	rAsp f 4	+
7	860	10	65	++	rAsp f 7	NT
19	760	28	220	++	rAsp f 2	+
28	630	21	45	++++	rAsp f 3	+
32	870	38	140	++++	rAsp f 8	NT
38	920	43	120	++++	rAsp f 9	NT
46	690	18	140	++++	rAsp f 1/a ^f	+
48	1,270	42	45	++++	rAsp f 5	+
51	1,000	34	75	++	rAsp f 10	NT

After 5 rounds of selection in a microtiter plate coated with IgE from serum of *A. fumigatus*-allergic individuals [8], virtually all selected clones yielded phage recognized by human IgE. MW = Molecular weight.

^a Estimated from agarose gels.

^b Estimated from polyacrylamide gels.

^c Produced as inclusion body protein after subcloning of the fragments into pDS76/RBSII, 6×His [14]. The yields represent mg of Ni²⁺ chelate affinity purified proteins per liter culture [16].

^d Sera from 30 patients with allergic bronchopulmonary aspergillosis were tested for the ability to bind to recombinant proteins in an IgE-specific ELISA [30]. Proteins recognized by >50% of the sera (++++), proteins recognized by <50% of the sera (++).

^e Nomenclature not officially approved.

^f This allergen has been previously described [14, 30].

^g Skin test reactivity determined as described [30]; NT = Not tested.

compositions. This is particularly problematic if complex organisms like molds, which can express different proteins depending on the stage of development, are used to produce allergen extracts. Accordingly, the allergic reactivity of a patient can apparently change and may not reflect the full truth, depending on the allergen preparation used for testing. Moreover, allergenicity of a compound does not correlate with its concentration, and trace amounts can elicit strong allergic reactions. The composition of allergen preparations, especially of molds, depends on its stage of development and time of earning the organism. A further drawback related to such extracts is that impurities also exert a number of unknown biological effects if used for in vivo allergy testing. In principle, an allergen preparation can be defined and standardized according to its antibody binding pattern. However, the usage of a defined panel of characterized, pure allergens, reflecting the major reactivity of an extract preparation, has clear advantages. Such defined reagents can only be prepared, fully characterized and provided in sufficient high amounts with the aid of gene cloning and biotechnological procedures. Starting from the elucidated gene sequence, molecular biology techniques can further be applied to generate mutated proteins devoid of unwanted biological functions like enzymatic activity. Moreover, using defined recombinant reagents, it is possible to

determine individual allergen reactivity patterns and phenotypes reflecting a patient's type and stage of disease and site of sensitization. Indeed, using only two selected recombinant *A. fumigatus* allergens (Aspf1, Aspf2), we have been able to demonstrate different allergen reactivity patterns in allergic bronchopulmonary aspergillosis, *A. fumigatus*-sensitive asthma and atopic dermatitis, compared to mold infection [31, unpubl. observation].

The demonstration that cDNA libraries can be displayed on phage surface and screened by product-ligand interactions has implications far beyond the isolation of allergenic proteins and opens new perspectives in gene cloning. The pJuFo cloning system can be used for selective isolation of any protein for which a ligand is available.

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