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Review

Phage Display as a Tool for Rapid Cloning of Allergenic Proteins

ULRICH APPENZELLER, KURT BLASER and RETO CRAMERI*

Swiss Institute of Allergy and Asthma Research (SIAF), Obere Strasse 22, CH-7270 Davos, Switzerland

Abstract. Allergic diseases represent an immune disorder associated with the production of immunoglobulin E (IgE) against normally innocuous antigens (allergens). Almost 20% of the population in industrialized countries suffer from type I allergic symptoms such as allergic rhinitis, conjunctivitis, urticaria or asthma. Although the mechanisms responsible for these allergic reactions are quite well understood, knowledge about the repertoire of molecules able to elicit type I symptoms is still limited. To clone and characterize entire allergen repertoires from complex allergenic sources in a fast and efficient way, new technologies are required. The phage surface display of cDNA libraries described here has proven to be a versatile cloning system to selectively isolate allergens physically linked to their genetic information. The screening of cDNA libraries displayed on phage surfaces with immobilised serum IgE from allergic patients reduces the time required for the selection of candidate clones to a few weeks. Robot-assisted high-throughput screening of the enriched library provides a fast and cost-effective way to isolate complete allergen repertoires. The biotechnological production of recombinant allergens derived from these sequences bears a high potential for the improvement of the diagnosis of allergic diseases.

Key words: cloning; cDNA; phage surface display; recombinant allergens; allergy.

Introduction

Allergic and asthmatic diseases show increasing prevalence[17, 35] and represent a relevant social and economic factor in industrialised countries[6, 33]. These genetically determined immune disorders are associated with the formation of immunoglobulin E (IgE) against normally innocuous environmental antigens[6]. IgE-mediated reactions can cause pain, disability and loss of life quality. An important step in the cascade eliciting allergic symptoms is the cross-linking of allergen-specific IgE bound on the surface of effector cells through the high-affinity IgE receptor FcεRI[31]. Once sensitised by allergen exposure, individuals produce allergen-specific IgE antibodies that bind to the FcεRI receptors on mast cells and basophils. Upon re-exposure to allergen, membrane-bound IgE molecules are cross-linked and initiate a complicated signal transduction pathway ending in degranulation of effector cells. The release of preformed mediators, such as histamine and leukotrienes, promotes allergic inflammation and causes allergic symptoms[21]. These considerations show the pivotal role played by allergens in eliciting IgE-mediated clinical symptoms. The severity of the symptoms ranges from mild forms, such as urticaria, hay fever or mucosal swelling, to life-threatening anaphy-

* Correspondence to: Prof. Dr. Reto Crameri, Ph.D., Head Molecular Allergology, Swiss Institute of Allergy and Asthma Research (SIAF), Obere Strasse 22, CH-7270 Davos, Switzerland, tel.: +41 81 410 08 48, fax: +41 81 410 08 40, e-mail: crameri@siaf. unizh.ch

laxis. Proteins derived from almost any source can be allergens, as demonstrated by the rapidly increasing list of cloned allergens[23]. In spite of this rapid development, which has allowed discovery of whole families of cross-reactive allergens[5, 27, 32] and disease-specific allergens[12, 18], we assume that the repertoire of IgE-binding molecules involved in allergic diseases is far from being complete[15]. To clone and characterize entire allergen repertoires from complex allergenic sources such as molds, yeasts and foods in a fast and cost-effective way, new technologies are required[15]. Here we discuss the construction of cDNA libraries displayed on the surface of filamentous phages as a cloning tool allowing selective isolation of phages displaying allergens using the discriminative power of affinity selection.

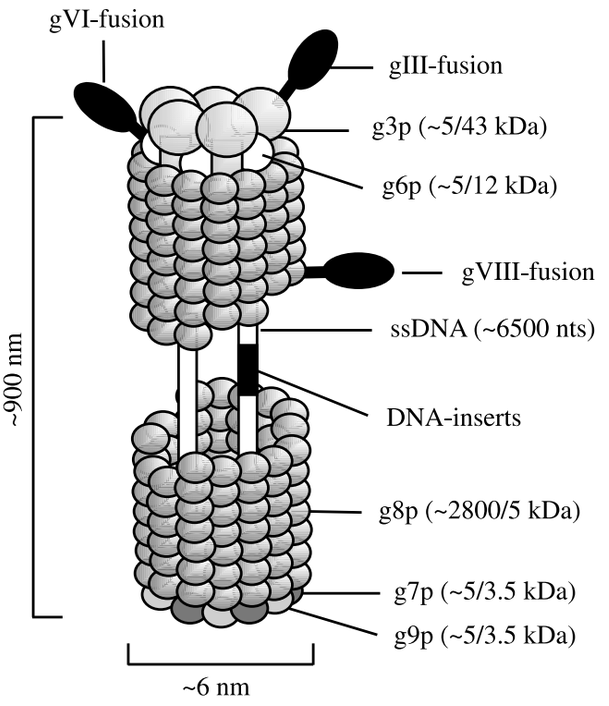

Fig. 1. Schematic representation of the physical linkage between expression products displayed on the phage surface and genetic information required for their production. Fusions can be generated between products and the minor coat proteins gIII and gVI or the major coat protein gVIII

The pJuFo Phagemid Vector

Phage surface display technology, first described by SMITH[30], represents, together with the polymerase chain reaction, one of the most frequently used methods in modern molecular biology. The basic concept of linking the recognition function, displayed as a peptide or protein on the phage surface, to its replication function, as self-replicating DNA integrated into the phage genome, allows the construction and survey of complex molecular libraries[3]. In contrast, the genetic information, integrated into the phage genome in bacteriophage λ-based libraries, and the gene product, expressed exploiting the machinery of the host cell during the lytic process, are not physically linked[36]. The physical linkage between gene products and genetic information required for their production is technically achieved by display of foreign molecules as chimeric proteins fused to a major or minor coat protein of the phage (Fig. 1). This linkage allows selection of phages displaying a desired gene product out of a large library using target molecules linked to a solid phase as ligand (Fig. 2). The general applicability of linking genotype and phenotype is demonstrated by the successful screening of a broad spectrum of libraries[15, 22, 34]. To date, antibodies, enzymes, enzyme inhibitors, receptors, hormones, lymphokines and DNA-binding proteins have been displayed on filamentous phages. The major advantage of screening molecular libraries displayed on the surfaces of filamentous phages is the possibility of rapid handling of a large number of individual phages (clones). Thereby, phages displaying functional proteins are enriched by the interaction with the immobilised ligand during consecutive rounds of phage growth and selection[13, 14]. However, translational stop codons present at the 3'-end of non-translated regions of eukariotic mRNA prevent the construction of fusion protein N-terminal to the gIII gene product, the most common approach used to link phenotype and genotype[22, 34], while fusions to the membrane-bound C-terminus would hamper the incorporation of the fusion protein into the phage coat[14] (Fig. 3). To overcome these problems, we developed a phagemid cloning system, which uses an indirect method to anchor cDNA-encoded proteins to the phage coat[7, 13, 14]. Co-expression of Fos--cDNA and Jun-gIII fusions under control of separate *Lac*-promoter/operators and forced secretion of both fusion proteins into the periplasmic space of *Escherichia coli* by *pel*B-leader sequences are the essential steps required for the construction of phage surface display libraries. The ability of the Jun and Fos leucine zippers to form stable heterodimers[28] (Fig. 4) allows efficient display of cDNA expression products on the surfaces of M13 phagemids[8, 16, 29] and application of powerful affinity selection procedures [7, 14].

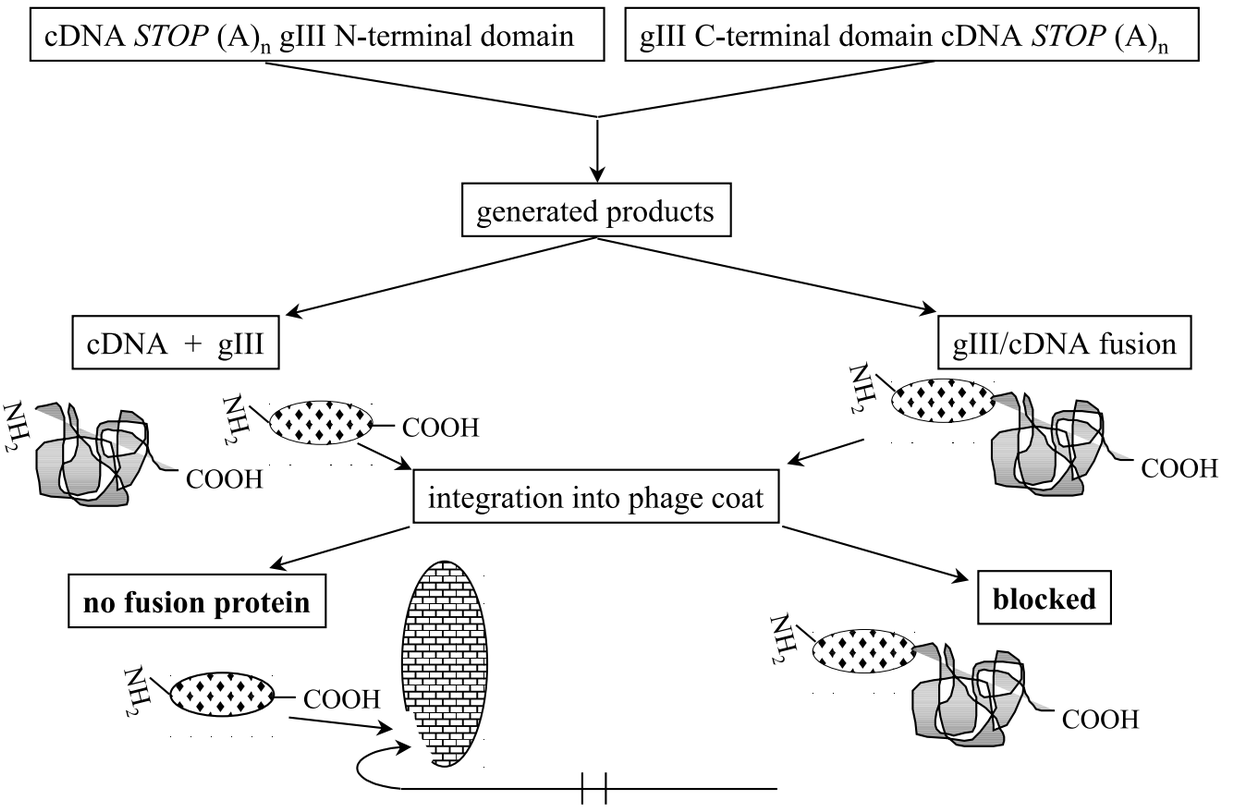

Fig. 2. Structure of the gIII gene of filamentous phage M13. Translational stop codons at the 3'-end on non-translated regions of mRNA prevent construction of N-terminal fusion, while fusions to the membrane-bound C-terminus of gIII would hamper incorporation of the fusion into the phage coat (modified from ref. 15)

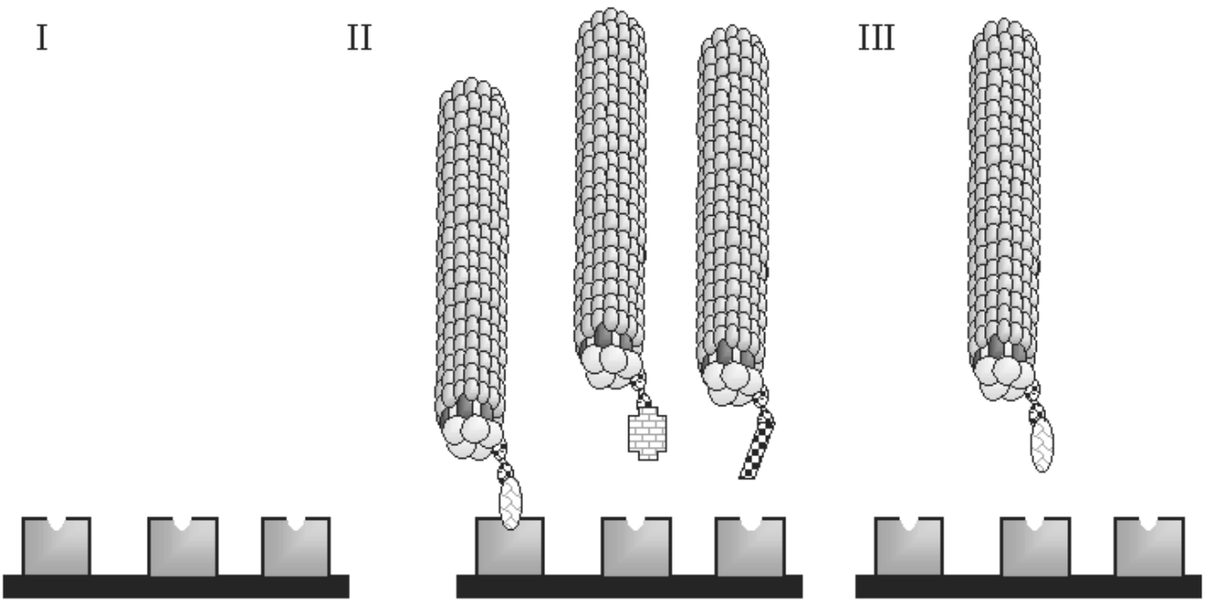

Fig. 3. Affinity selection of specific phages. I – the target molecule (ligand) is immobilised to solid phase. II – the library is applied in liquid phase, allowing specific phages to interact with the ligand. III – after washing to eliminate unspecific binders, phages carrying cDNA expression products with affinity for the ligand are eluted and used for further rounds of affinity selection

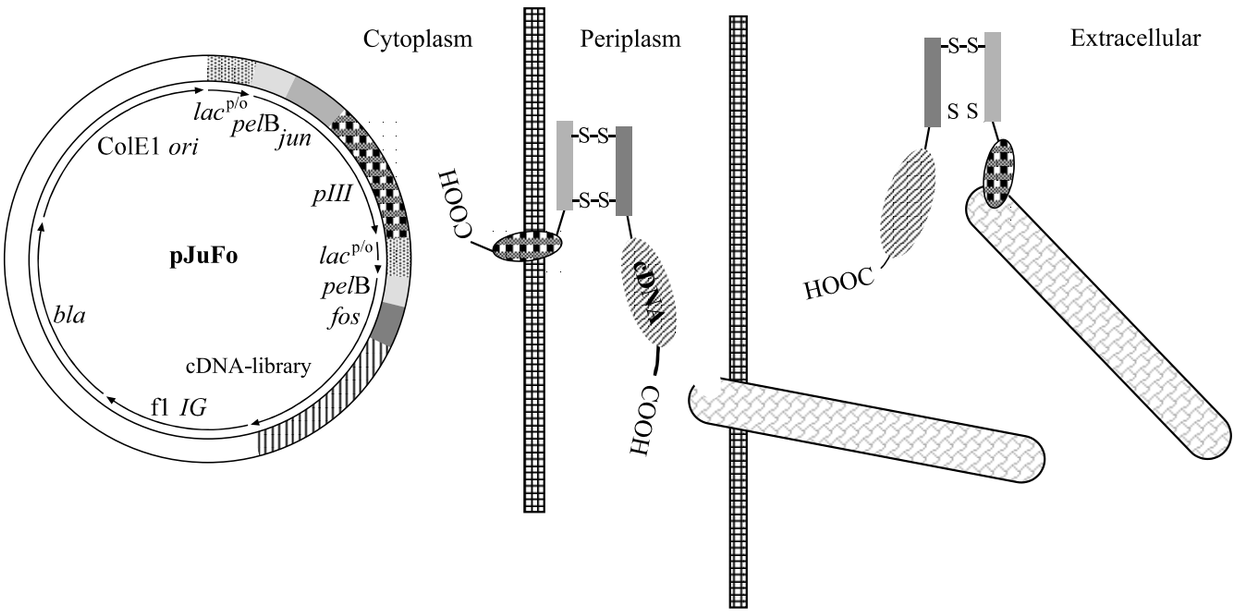

Fig. 4. Phagemid pJuFo. The Jun leucine zipper is expressed and secreted as N-terminal fusion to a truncated gIII gene product of the phagemid. The Fos leucine zipper is used to produce and secrete Fos-decorated cDNA fusions. In the periplasmic space of *E. coli*, heterodimerisation of Jun/gIII-Fos/cDNA products occurs and allows, through incorporation into the phage surface, generation of cDNA expression libraries displayed on the phage surface

Construction and Screening of cDNA Libraries Displayed on Phage Surfaces

The pJuFo phagemid vector (Fig. 4) was specially conceived to display cDNA gene products on the surface of filamentous phages[14]. The original vector was designed for direct subcloning of commercial λ-Zap libraries[7, 13]. Therefore, the cloning sites available for the generation of Fos-decorated fusion proteins were limited to Bgl II, Xba I and Kpn I. These enzymes represent rare cutters for eukaryotic cDNA sequences, and turned out to be sufficient for most purposes. The shuffling of cDNAs from a pre-made library into pJuFo is an easy procedure for fast generation of new phage surface-displayed cDNA libraries[7, 13]. However, direct construction of phage surface-displayed cDNA libraries from reverse transcribed mRNA involves the same steps as used for the construction of λ-based libraries and is strongly facilitated by the small size of the cloning vector[25]. Screening a library for isolation of clones with a pre-determined affinity for a given ligand is an easy procedure based on multiple rounds of phage growth and selection. As much as 10^{11} single phages can be applied to a single well of a microtiter plate coated with the ligand[14]. After extensive washing to eliminate non-specific phagemids, bound particles can be eluted from the ligand surface. Filamentous phages are robust biologic systems and support strong denaturing conditions, facilitating recovery of infectious particles[2, 14]. Since filamentous phages are auto-reproductive in a suitable host, phagemids recovered from a round of affinity selection can be amplified by infection and growth of competent *E. coli* cells and used for a further round of affinity selection. Monitoring of enrichment simply occurs by determination of the absolute number of infectious phage particles recovered after each round of affinity selection. If phages able to interact with the ligand are present in the library, the number of phages eluted will increase after each round of panning[2]. The sensitivity of the system is high[14], allowing selective enrichment of cognate phages from a mixture of non-cognate phages at a ratio as low as 1 in 10^7. Therefore, the time required for the isolation of a functionally expressed cDNA encoding a gene product with a reasonable affinity for a ligand is reduced to a few days[10, 15].

Cloning of Allergen Repertoires

The most attractive feature of the pJuFo cloning system is the fact that the expressed products of cDNA libraries and the encoding cDNAs become packaged as one entity into a filamentous phage particle and, thus, physically linked[7, 10, 14]. As a consequence of the physi-

Table 1. Allergens with known biological function cloned from phage surface-displayed cDNA libraries with serum IgE as ligand

Protein	Function	Source	MW (kDa)	References
CSP	cold shock protein	*Cladosporium* cDNA	8.1	15
NTF2	nuclear transport factor 2	*Cladosporium* cDNA	14.2	15
HSP	heat shock protein	*Cladosporium* cDNA		15
NTF2	nuclear transport factor 2	*Alternaria* cDNA	14.2	15
Thioredoxin	enzyme	*Plasmodium ovale* cDNA	11.5	15
MnSOD	enzyme	human cDNA	21.6	1
P_2 protein	ribosomal protein	human cDNA	11.2	1
Cyclophilin B	enzyme	human cDNA	21.5	1
Asp f 1	ribotoxin	*Aspergillus fumigatus* cDNA	16.8	12, 17
Asp f 3	peroxisomal protein	*Aspergillus fumigatus* cDNA	18.5	12, 17
Asp f 5	metalloprotease	*Aspergillus fumigatus* cDNA	42.1	8, 9
Asp f 6	MnSOD	*Aspergillus fumigatus* cDNA	23.0	11, 27
Asp f 8	P2 ribosomal protein	*Aspergillus fumigatus* cDNA	11.0	26
Asp f 10	aspartyl protease	*Aspergillus fumigatus* cDNA	34.4	8, 9
Asp f 11	cyclophilin	*Aspergillus fumigatus* cDNA	20.0	9
Asp f 13	serine protease	*Aspergillus fumigatus* cDNA	19.8	9
Profilin	structural protein	peanut cDNA	14.0	24
Villicin	seed storage protein	peanut cDNA	60.3	24
Conglutin	seed storage	peanut cDNA	14.5–17.4	24
Glycinin	seed storage	peanut cDNA	35.9	24
Mal f 5	peroxisomal protein	*Plasmodium ovale* cDNA	18.2	25
Mal f 6	cyclophilin	*Plasmodium ovale* cDNA	17.2	25
Thioredoxin	enzyme	*Coprinus comatus* cDNA	12.1	4, 15

cal linkage between genotype and phenotype, selection of phages displaying a given gene product automatically yields the encoding cDNA. Therefore, sequencing the DNA of the integrated section of the phage genome can readily elucidate the amino acid sequence of the displayed gene product[14, 15]. The cloning system has been used for selective isolation of many genes[15, 16, 20, 29]. However, historically, pJuFo had been mainly applied to clone IgE-binding molecules (Table 1). Identification of allergens is facilitated by the common property of the molecules to interact with serum IgE of sensitised individuals. Although IgE from individuals sensitized to complex allergenic sources such as yeasts or molds is raised against many components, the majority of allergen-expressing clones present in a cDNA library can be selectively isolated in a few days[4, 10, 24, 25]. The consequent selection of IgE-binding molecules from an *Aspergillus fumigatus* cDNA library displayed on phage surfaces using pools of sera from patients suffering from different *A. fumigatus*-related pulmonary complications yields a large panel of fungal allergens[8, 9, 15]. Serologic and clinical evaluation of the derived recombinant proteins revealed the existence of allergens which are highly specific for allergic bronchopulmonary aspergillosis, a clinical syndrome difficult to diagnose[12, 18]. Therefore, cloning and characterization of *A. fumigatus* allergens from a cDNA library displayed on phage surfaces allowed the development of a highly specific and sensitive diagnostic procedure for a distinct pulmonary complication which still poses severe difficulties in clinical practice[12, 17, 18]. Moreover, examination of the amino acid sequences of the cloned allergens revealed the presence of phylogenetically highly conserved proteins with strong sequence homology and identity also to human proteins[1]. Clinical evaluation of the recombinant human proteins revealed their ability to act as autoallergens *in vivo*, clearly showing the existence of autoimmune reactions in chronic allergic diseases [11, 26]. Extension of the technology to robot-based high-throughput screening of the phage surface-displayed cDNA library enriched with sera from patients suffering from different diseases related to *A. fumigatus* yielded more than 70 cDNAs encoding IgE-binding proteins (KODZIUS et al., in preparation), likely to cover the whole allergen repertoire of the mold[15]. The availability of a complete allergen panel from such a complex allergenic source will allow the further evaluation of the role played by each single allergen in aspergillosis, still considered a complex clinical syndrome[8, 9].

Conclusions

Rapid handling of large numbers of individual clones is required in order to identify gene products of interest present in molecular libraries. Selection based on specific interaction by functional screening of

cDNA libraries displayed on phage surfaces, as shown for the isolation of IgE-binding proteins from complex allergenic sources, enables, in weeks, the characterization of all the desired gene products present in a library. Extension of phage surface display technology to robot-based high-throughput screening of enriched phage libraries provides the technology for a fast and cost-effective way to identify unknown genes. The combination of the two powerful technologies is ideally suited to study protein-ligand interactions and, thus, to facilitate rapid progress in the field of life science.

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