Selectively infective phage (SIP) technology: A novel method for *in vivo* selection of interacting protein–ligand pairs

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We describe here a new technology, selectively infective phages (SIP)[1–8], which can be used in screening large libraries and in driving molecular evolution within these libraries. SIP is related to phage display technology using filamentous phages[9,10], where a protein or peptide of interest is fused to a phage coat protein and, thus, displayed on the outer surface of the phage, while its genetic information is contained in the phage DNA. In contrast to phage display, the phage particles are rendered non-infective in SIP by disconnecting the N-terminal domains (N1 and N2) of the phage g3p coat protein (Fig. 1) — those involved in docking and bacterial cell penetration — from the C-terminal domain (CT), which caps the end of the phage. The N- and C-terminal domains are then each fused to one of the interacting partners being studied. One partner is thus displayed on the phage surface associated with CT, while the other is genetically fused or chemically coupled to the N-domain(s), thus constituting a separate "adapter" molecule. Only when the specific protein–ligand interaction occurs between the partners is the g3p reconstituted and does the phage particle regain its infectivity, and the genetic information of a successful binder is propagated. In contrast, in phage display the whole phage binds and can be eluted from an immobilized target molecule, whereupon, since it remains infective, it can inject its DNA into bacterial cells and be amplified. The main limitation of phage display at present is the occurrence of non-specific adsorption of phages during the binding stage[11], necessitating an enrichment over several rounds and individually tailored washing and elution conditions.

Variations of SIP

The g3p N1 domain is absolutely essential for the infection process[6,12], whereas a residual infectivity is observed in the absence of the N2 domain[6]. Two pathways of infection are possible: One requires only N1, is stimulated by high Ca^{2+} concentrations, does not require F-pili and must therefore involve direct binding of N1 to the bacterial surface[6]. The second requires N2 and F-pili, but no Ca^{2+}, suggesting a docking of N2 to the pili[6]. Both pathways can be used in SIP; the second one is much more efficient, however[6].

The influence of different g3p domain rearrangements in SIP has been studied. In a fluorescein/anti-fluorescein scFv model system, purified N1 or N1-N2 domains, chemically coupled to the antigen fluorescein (N1-Ag and N1-N2-Ag), were used in combination with scFv-"short" (Fig. 2*a*) or scFv-"medium" phages (Fig. 2*b*) to recreate a wt g3p interrupted at various positions[6]. As shown in Fig. 2*c*, infectivity as a function of adapter concentration was different in the two cases; furthermore, N1-N2 inhibits the entry of wt phage into cells with a half-inhibitory concentration two orders of magnitude lower than N1 alone[6]. At low N1-N2-Ag concentrations the phage is not sufficiently saturated with ligand-adapter to mediate infectivity in SIP selection, while at high concentrations the bacteria themselves become saturated, blocking infection[6]. Therefore, very low concentrations of N1-N2-Ag are needed and the system will select for high affinity binders — provided, of course, such molecules exist in the library under study. In the case of the N1-Ag adapter molecule, higher concentrations can and must be used in order to overcome the lower infection efficiency of the system[6], while possibly allowing selection for lower binding affinity pairs. This requirement for a high concentration of N1 may reflect a low-affinity binding to *Escherichia coli* in the absence of pili and/or a necessity for multivalent binding to the bacteria — requiring simultane-

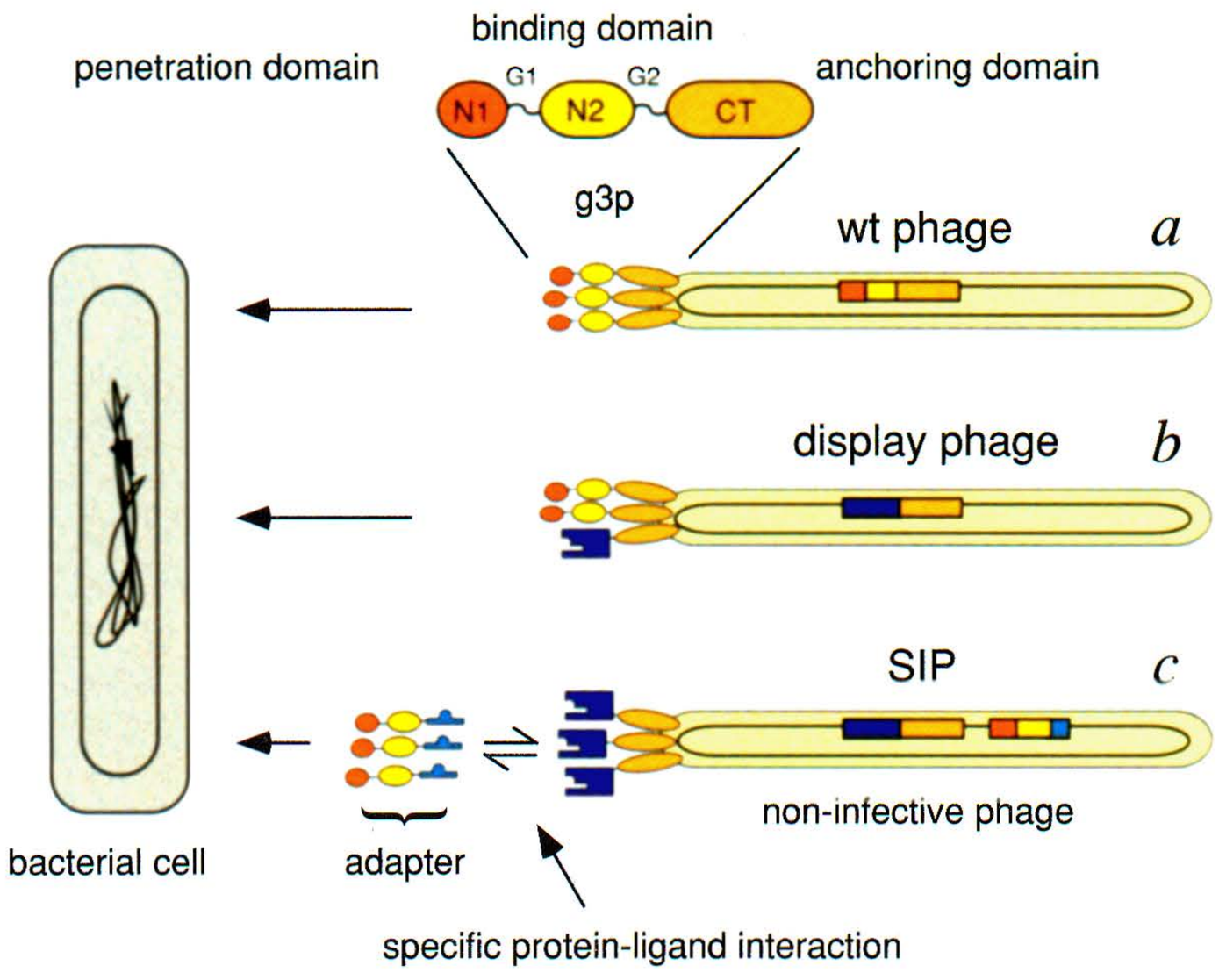

Fig. 1 Comparison of wt phage, display phage and SIP. *a*, wt phage: Infectivity is mediated by the wt g3p, which is enlarged. It consists of two N-terminal domains, N1 (1–68 aa) and N2 (87–217 aa), and a C-terminal domain, CT (257–406 aa), linked by two glycine-rich linkers, G1 and G2. *b*, Display phage: CT fused to the displayed protein (encoded on a phagemid that gets packaged) and wt g3p (encoded by a helper phage) are both present on the phage, which remains infective. *c*, SIP: The specific protein–ligand interaction is essential to restore infectivity of an otherwise non-infective phage.

ous occupation of all cognate ligand-binding sites on the phage. In the absence of coupled antigen, no infectivity was observed[6], demonstrating that the N-terminal domains must be physically coupled to the phage for infection.

For SIP to work, the phage must be strictly non-infective. Using a phagemid/helper phage system, an unpackageable g3p expression plasmid must be used in combination with a g3p-deleted helper phage, but all tested plasmids appeared to contain cryptic packaging signals[4] and thus gave rise to background infectivity. These problems are overcome by introducing the g3p fusion protein directly into a single genetic package, the phage genome[4].

SIP experiments can be carried out *in vivo* or *in vitro* (Fig. 3): In the former approach the phage or phagemid encodes both interacting molecules (for example, antibody–antigen) in the same vector[3,4,7] (Fig. 3*a*), and the protein–ligand interaction occurs in the bacterial periplasm; the infective phage particle is extruded from the cell and can be harvested and used to infect different bacteria. The simplicity of design of the experiment is counteracted to some extent by the lack of experimental control over parameters such as concentrations, incubation times and the infection process itself.

These factors can be controlled in the *in vitro* SIP experiment (Fig. 3*b*). In this case, the adapter protein is prepared from a separate *E. coli* culture, either by secretion[2,5,8] or by *in vitro* refolding[6]. This approach broadly extends the range of applications of SIP, as, in addition to genetically fused peptidic antigens, non-peptidic antigens can now be chemically coupled to the N-terminal domains[6]. The adapter is then incubated with the non-infective phage library, and added to the bacteria.

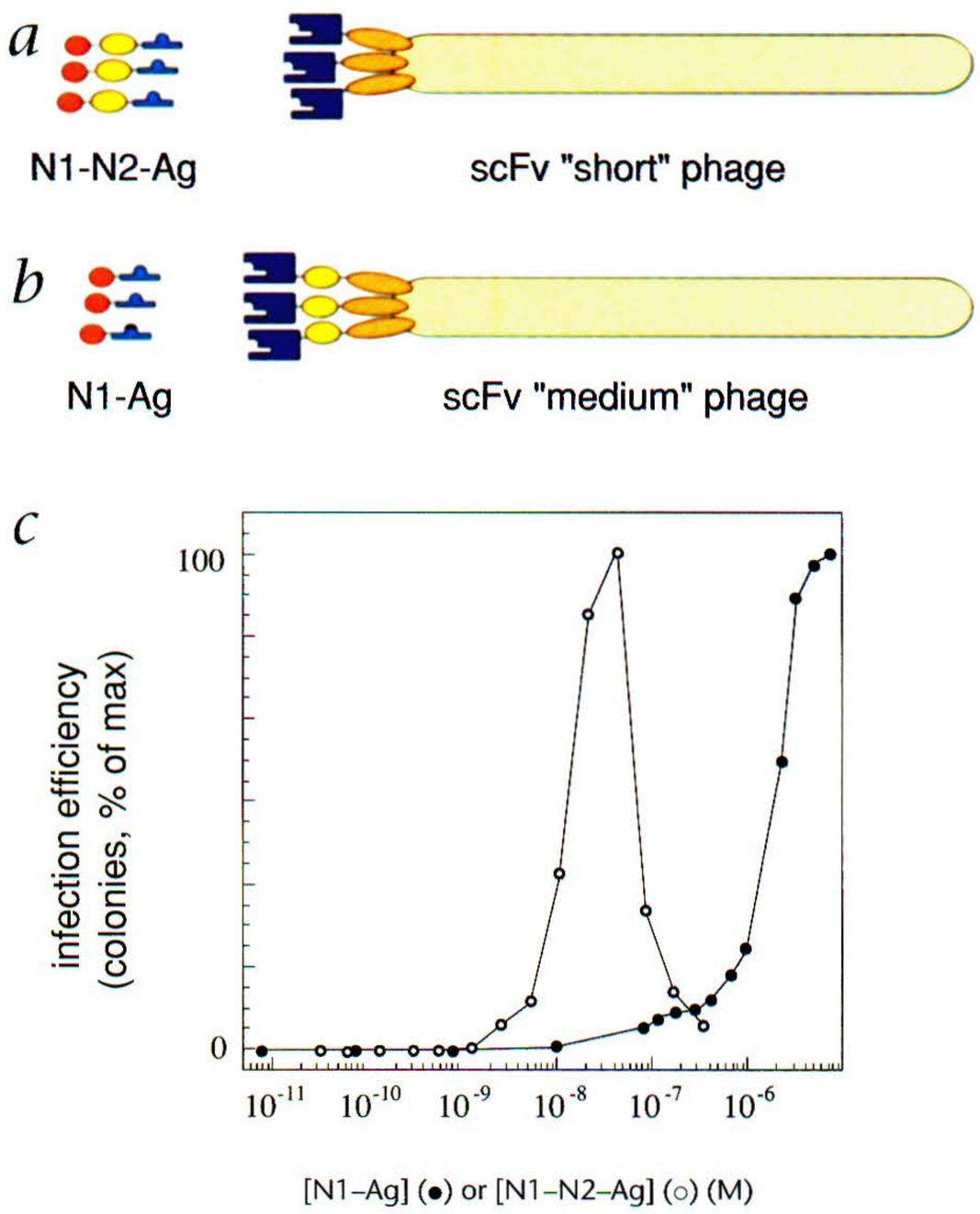

Fig. 2 Two different g3p arrangements in SIP and their respective infectivity as a function of adapter concentration are shown. *a*, N1-N2-Ag in combination with scFv-"short" phage, with g3p interrupted between N2 and CT. The same infectivity was observed when scFv-"medium" phage was used instead. *b*, N1-Ag with scFv-"medium" phage, where g3p is interrupted between N1 and N2. Fewer colonies were found with N1-Ag and scFv-"short" phage. *c*, Infectivity (number of colonies) of combinations (*a*) and (*b*) as a function of adapter concentration. (Adapted from ref. 6.)

Examples of SIP studied

The SIP system has been tested with both proteins and peptides. The leucine-zipper motifs of the jun and fos proteins, with both helices flanked by cysteine residues[13], were used as a model system[3] in which the heteromeric interaction between the two peptides restored the infectivity of the phage. An enrichment of up to 200-fold over non-interacting pairs was observed after one round. Furthermore, zipper-like domains that pair with the jun-zipper domain have been selected from a human cDNA library[7].

With an anti-lysozyme Fab fragment model system, in association with an N1-lysozyme adapter molecule, a specific enrichment factor of approximately 10^{10} was observed after only two rounds of selection[2]. The antibody 17/9, specific for a hemagglutinin peptide, was used as a model where both peptide and antibody were encoded in the same phage genome[4], resulting in a very clean selection of the desired Ab–Ag pair. An anti-fluorescein antibody/antigen system was used as the first example of a non-peptidic antigen being coupled to purified g3p N-terminal domains[6]. In the latter case, up to 10^6 antigen-specific infection events were observed from 10^{10} input phages, compared to only one antigen-independent event. No background infection was observed in the absence of antigen or in the presence of an antibody of different specificity in the latter two examples, presumably due to the use of phage vectors as the sole replicons and thus in strict absence of any wt g3p.

In a Fab library made from B cells of gp120 seronegative donors, with only the N1 domain fused to the V3 loop peptide, three rounds of traditional phage panning yielded no antibodies against the antigen, but half of the phages gave positive ELISA signals after three rounds of SIP[2]. The ability to enrich for affinity or even kinetic constants has been tested using six different Fab fragments against the V3 loop peptide as a model library, with high-affinity antibodies being preferentially selected[5]. High-affinity antibodies were also enriched from a library that had been made from B cells after *in vitro* immunization[8].

Further prospects

A requirement of SIP appears to be a high affinity between protein and ligand, and all of the systems studied thus far have had dissociation constants of at least 10^{-8} M. It is not yet clear, however, what the minimum affinity necessary for significant infectivity will be. Further investigations will also have to be carried out to test the, initially encouraging, apparent ability to select for kinetic parameters of ligand binding, and to investigate the dependency of infectivity on properties of the proteins, such as thermodynamic stability and expression rate, as well as the size limits of protein–ligand complexes usable in SIP.

Since SIP is definitely compatible with disulfide formation, it complements the two-hybrid system[14] well for library screening, as the latter is designed for use with nuclear and cytoplasmic proteins. The toxicity of certain proteins, when displayed on phage, is a limitation SIP shares with phage display. Furthermore, in SIP care must be taken, especially during *in vivo* SIP experiments, in phage handling and in controlling rare recombination events in the phage that could restore infective wt-like g3p (ref. 4).

These challenges are to be contrasted with the numerous ad-

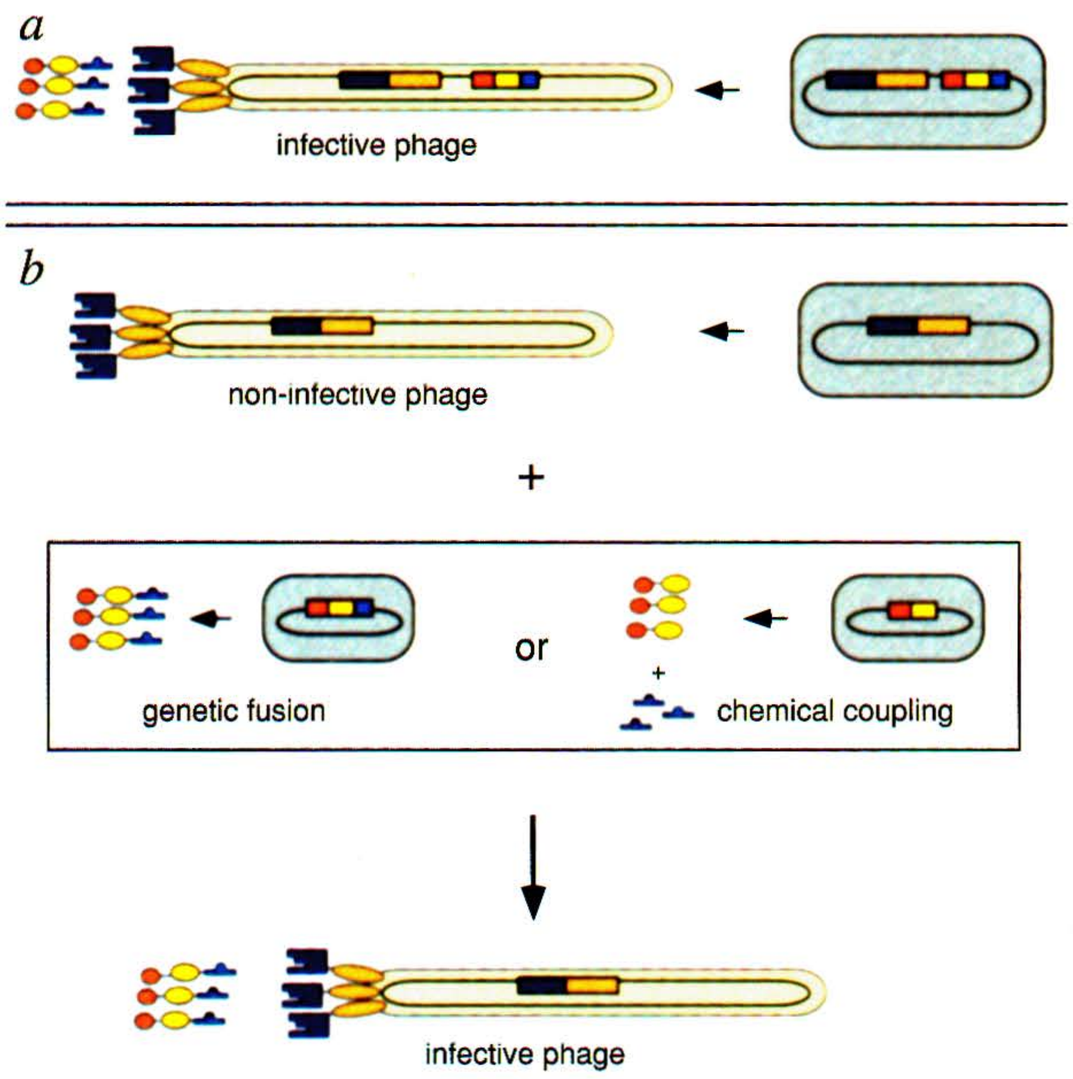

Fig. 3 *In vivo* versus *in vitro* SIP. *a*, In the *in vivo* approach both interacting partners are encoded in the same genetic vector and co-expressed in the same bacterium where they can interact. *b*, In the *in vitro* approach the N-terminal domains of g3p and the displaying phage are produced in separate cells. The antigen, fused or chemically coupled to the N-terminal domains, needs to be incubated with the non-infective phage before they are used to infect bacteria.

vantages that SIP has over phage display. No solid-phase interaction with any support is necessary in SIP, limiting the potential of the occurrence of non-specific interactions and eliminating the need for elution. In addition, the low background infectivity observed with some of the SIP systems[4,6] demonstrates that SIP can be an extremely effective and highly specific method of selecting for cognate interaction events. Furthermore, the exciting possibility of simultaneous screening of two interacting libraries by *in vivo* SIP might broaden the ability to identify interacting receptor–ligand pairs.

Acknowledgments

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1. Krebber, C., Moroney, S., Plückthun, A. & Schneider, C. European Patent Application EP 93102484 (1993).
2. Duenas, M. & Borrebaeck, C.A.K. Clonal selection and amplification of phage displayed antibodies by linking antigen recognition and phage replication. *Bio/Technology* **12,** 999–1002 (1994).
3. Gramatikoff, K., Georgiev, O. & Schaffner, W. Direct interaction rescue, a novel filamentous phage technique to study protein–protein interactions. *Nucleic Acids Res.* **22,** 5761–5762 (1994).
4. Krebber, C., Spada, S., Desplancq, D. & Plückthun, A. Co-selection of cognate antibody–antigen pairs by selectively-infective phages. *FEBS Let.* **377,** 227–231 (1995).
5. Duenas, M., Malmborg, A.-C., Casalvilla, R., Ohlin, M. & Borrebaeck, C.A.K. Selection of phage displayed antibodies based on kinetic constants. *Mol. Immunol.* **33,** 279–285 (1996).
6. Krebber, C. *et al.* Selectively-infective phage (SIP): A mechanistic dissection of a novel *in vivo* selection for protein–ligand interactions. *J. Mol. Biol.* **268,** 619–630 (1997).
7. Gramatikoff, K., Schaffner, W. & Georgiev, O. The leucine-zipper of c-Jun binds to ribosomal protein L18a: A role in Jun protein regulation? *Biol. Chem.* **376,** 321–325 (1995).
8. Duenas, M. *et al. In vitro* immunization of naive human B cells yields high affinity immunoglobulin G antibodies as illustrated by phage display. *Immunology* **89,** 1–7 (1996).
9. Smith, G.P. Filamentous fusion phage: Novel expression vectors that display cloned antigens on the virion surface. *Science* **228,** 1315–1317 (1985).
10. Dunn, I.S. Phage display of proteins. *Current Opin. Biotechnol.* **7,** 547–553 (1996).
11. Adey, N.B., Mataragnon, A.H., Rider, J.E., Carter, J.M. & Kay, B.K. Characterization of phage that bind plastic from phage-displayed random peptide libraries. *Gene* **156,** 27–31 (1995).
12. Stengele, I., Bross, P., Garces, X., Giray, J. & Rasched, I. Dissection of functional domains in phage fd adsorption protein. *J. Mol. Biol.* **212,** 143–149 (1990).
13. Crameri, R. & Suter, M. Display of biologically active proteins on the surface of filamentous phages: cDNA cloning system for selection of functional gene products linked to the genetic information responsible for their production. *Gene* **137,** 69–75 (1993).
14. Bai, C. & Elledge, S.J. Gene identification using the yeast two-hybrid system. *Methods Enzymol.* **273,** 331–347 (1996).