Biol. Chem., Vol. 378, pp. 445 – 456, June 1997 · Copyright © by Walter de Gruyter & Co · Berlin · New York

Review

Selectively Infective Phages (SIP)

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and Hoess, 1995) through cycles of random mutagenesis and selection.

Both applications of combinatorial biology, screening and optimization, require a precise understanding of the physicochemical parameters for which the molecules are selected, be it binding strength, stability, functional expression or, most likely, a combination of these properties. Ultimately it should be possible to identify and generate molecules with the desired properties by designing the selection pressure in the process properly. This is even more important for the development of evolutionary strategies, that is to carry mutagenesis and selection over many generations. Several selection tools which are able to handle big libraries have been described over the last few years, including phage display on filamentous phages (Smith, 1985; Dunn, 1996), display on phage lambda (Maruyama et al., 1994; Sternberg and Hoess, 1995), the yeast two-hybrid system (Bai and Elledge, 1996; McNabb and Guarente, 1996), the selection of peptides connected to a plasmid (Cull et al., 1992), and the use of ribosome display of peptides (Mattheakis et al., 1994) and proteins (Hanes and Plückthun, 1997). All of these techniques couple the peptide or protein of interest to its gene in a different way, with the aim of 'panning' large libraries. The construction of ever bigger libraries may have come to its practical limits, however (Vaughan et al., 1996), so that the solution for molecular optimization may be to imitate nature by not having all diversity present from the beginning, but to build it up successively – by the process of evolution. A number of techniques have also been developed over the last few years for genetic diversification. They allow the mutation and recombination of genes with high efficiency, or the limitation of mutations to particular codon types (Stemmer, 1994; Cadwell and Joyce, 1994; Virnekäs et al., 1994). Cycling between both steps, genetic diversification of the selected pool and selection for a desired property make up the evolutionary approach. To run those evolutionary strategies efficiently, both the diversification and the selection procedure have to be simple and robust to allow improvement over many generations.

We review here advances in the selectively infective phage (SIP) technology, a novel method for the in vivo selection of interacting protein-ligand pairs. A 'selectively infective phage' consists of two components, a filamentous phage particle made non-infective by replacing its N-terminal domains of gene3 protein (g3p) with a ligand-binding protein, and an 'adapter' molecule in which the ligand is linked to those N-terminal domains of g3p which are missing from the phage particle. Infectivity is restored when the displayed protein binds the ligand and thereby attaches the missing Nterminal domains of g3p to the phage particle. Phage propagation becomes strictly dependent on the protein-ligand interaction. This method shows promise both in the area of library screening and in the optimization of peptides or proteins.

Key words: Gene3protein / Libraries / Molecular evolution / Phage infectivity / Phage libraries / Screening.

Introduction

Combinatorial techniques have found a firm place in biology in two different areas: in the identification of interacting biomolecules and in their evolutionary improvement. Protein-ligand interactions are the ultimate basis of specificity in biology, and thus their identification is vital for delineating interacting partners in regulatory networks (Phizicky and Fields, 1995) and for generating and refining biological lead structures to improve pharmaceutical functions (O'Neil and Hoess, 1995).

Much effort has been directed at developing methodologies for both types of applications. In both cases, a screening or biological selection from a preexisting diversity is required. In the first case, either a natural or synthetic repertoire is tapped (such as, for example, a cDNA library or an antibody library) (Phizicky and Fields, 1995). In the second case, a series of candidate molecules are iteratively improved for optimizing lead structures (O'Neil

This article will summarize the status of a new technology, selectively infective phages (SIP), which may be of use both in library screening and in molecular evolution. Since it is related to phage display on filamentous phages (Smith, 1985), we will first summarize this technique and its underlying biology. In normal phage display, one of the binding partners is fused to a phage coat protein of a filamentous phage (Figure 1B, C, D), and the selection procedure involves the enrichment of phages which bind a given

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Fig. 1 The Principle of SIP.

A comparison of wt, classical display phages and SIP phages is shown. (A) In wt phages, 3-5 copies of g3p are at the tip of the phage, mediating infection (see text). (B) In display phages with multivalent display, infectivity is secured by the presence of all domains of g3p in all copies of the fusion protein. The fusion is therefore to the N-terminus of g3p. It may be pointed out that it is not clear whether proteolysis during phage morphogenesis may regenerate the wt g3p in one or more copies of the g3p fusion protein. (C,D) In phagemid/helper phage systems, infectivity is maintained by the presence of wt g3p, encoded by the helper phage. Thus, the fusion protein can (C) contain all domains of g3p ('long fusion') or (D) only the C-terminal domain of g3p ('short fusion'). (E) In the selectively infective phage, all copies of g3p are lacking the N-terminal domains, and they are replaced in all copies by the protein or peptide to be displayed. Such a phage is totally non-infectious, but infectivity can be restored when the missing domains are supplied in an adapter protein. The adapter protein connects the missing domains of g3p to the phage by a non-covalent cognate interaction (such as a specific receptor-ligand or antibody-antigen interaction).

immobilized target molecule (Clackson and Wells, 1994; Winter et al., 1994). In phage display the phage particle has to remain infective because it must enter the cell for amplification after elution from the immobilized ligand, and consequently it requires the presence of wt gene3 protein (g3p) (Figure 1A – D). This can be achieved either by using phage particles and fusing the protein or peptide to be displayed to the N-terminus of g3p (Figure 1B), or using a helper phage/phagemid combination (Figure 1C, D). By using phage genomes, the fusion will be present in all copies of g3p, resulting in multivalent binding of the displayed polypeptide to the immobilized ligand (McCafferty et al., 1990). Multivalent display can also be achieved in a phagemid/helper phage system, if a g3-deleted helper phage is used (Griffiths et al., 1994). With normal helper phage/phagemids systems (Figure 1C, D), less than one copy is usually displayed per phage on average - depending on the expression ratio of wt protein and fusion protein (Lowman et al., 1991). When the fusion protein is directly encoded in the phage genome, the displayed protein or peptide must be fused (Figure 1B) such that the whole g3p is present, for the phage to remain infective. In the presence of wt g3p from a helper phage, the displayed protein can be fused to either the first, second or third domain of g3p (Figure 1C, D) (see below), since the required domains of g3p are delivered by the helper phage. In phage display, the DNA of both non-specifically bound and specifically bound phages enters the bacterial cell by infection, and the main challenge of this technology is thus to minimize non-specific adsorption of the phages in the binding procedure (Adey et al., 1995) and to enrich phages which display high-affinity molecules over highly abundant, low-affinity binders (Hawkins et al., 1992).

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-SGCPHHHHHH

A wt protein3 amino acids (aa) 1-406 B "Adapter " molecules for chemical coupling

1-218

1-81

SGCPHHHHHH





Fig.2 Structure of the Gene3protein.

(A) wt g3p. The three domains N1, N2 and CT are interspersed by two glycine-rich linkers, G1 and G2. An intramembrane domain is present in CT (378–406 aa) to anchor the g3p to the inner membrane of *E. coli* cells prior to assembly and extrusion from the cell. (B) Adapter proteins for chemical coupling. Both the first N-terminal domain (N1) as well as the first and the second domains together (N1-N2) have been engineered to add a single unpaired cysteine and a histidine tag for purification. (For details, see Krebber *et al.*, 1997) (C): Ribbon diagram (stereo) of the N1 domain, recently solved by NMR. Reproduced from Holliger and Riechmann (1997) with permission.

The Principle of SIP

In this review, the method of selectively infective phages (SIP) (Krebber *et al.*, 1993; Duenas and Borrebaeck, 1994; Gramatikoff *et al.*, 1994; Krebber *et al.*, 1995; Gramatikoff *et al.*, 1995; Duenas *et al.*, 1996a, b; Krebber *et al.*, 1997) will be discussed. In SIP, in contrast to phage display, the desired specific protein-ligand interaction is itself directly responsible for restoring infectivity in an otherwise non-infective display phage (Figure 1E). SIP has also been called SAP (**s**election and **a**mplification of **p**hage) or DIRE (**d**irect interaction **r**escue).

SIP works by exploiting the modular structure of g3p of wt filamentous phage (Figure 2A) (Armstrong *et al.*, 1981; Stengele *et al.*, 1990). The g3p consists of three domains of 68 (N1), 131 (N2) and 150 (CT) amino acids, connected by glycine-rich linkers of 18 (G1) and 39 (G2) amino acids, respectively (Figure 2A). The first N-terminal domain, N1, is thought to be involved in penetration of the bacterial membrane, while the second N-terminal domain, N2, may be responsible for binding of the bacterial F-pilus (Jakes *et al.*, 1988; Stengele *et al.*, 1990). Recently, the structure of the N1 domain has been determined by NMR spectroscopy (Holliger and Riechmann, 1997) (Figure 2C). It consists of an N-terminal α -helix, while the remainder of the protein is composed of β -structures interspersed by turns

that form a single β -sheet with an extensive hydrogen bond network. At the end of the β -barrel, which is close to both the N-terminal helix and the C-terminus, a hydrophobic core extends into an exposed hydrophobic patch, with residues conserved between fd, IKe and I2-2 phages. It has been speculated that a hypothetical peptide of *Escherichia coli* TolQRA might dock to this region and that this is the potential binding site to the cell. The C-terminal domain (CT) of g3p plays a role in phage morphogenesis and caps one end of the phage particle (Nelson *et al.*, 1981; Crissman and Smith, 1984).

After synthesis in the cytoplasm the preprotein of g3p is transported through the inner membrane, and the signal sequence is then cleaved off (Ito *et al.*, 1980), while processed g3p is inserted in the inner membrane of *E. coli* via a short hydrophobic C-terminal peptide (Davis *et al.*, 1985; Endemann and Model, 1995). In the inner membrane it oligomerizes and associates with g6p, to be taken up by the budding phage (Gailus *et al.*, 1994; Gailus and Rasched, 1994). This complex of 3–5 copies of g3p and g6p forms the end of the phage particle (Davis *et al.*, 1985) to last leave the host cell (Lopez and Webster, 1983). In the phage particles, the C-terminal domain (CT) is part of the coat structure and thereby anchors the N-terminal domains to the phage particle (Davis *et al.*, 1985). The glycine-rich linkers G1 and G2 have been shown to enhance the infectivity of the phage (Rampf, 1993; Endemann *et al.*, 1993), probably by conferring flexibility in connecting the three domains and adjusting the required distance in the 3Dstructure.

The principle of SIP involves the separation of the N-terminal domains required for infection (N1 or N1-N2) from the anchoring C-terminal domain (CT) (Figure 2A). In SIP the gene for a peptide or a protein of interest replaces N1 or N1-N2 by genetic fusion to the C-terminal domain of gene3, leading to a phage particle which is non-infective (Nelson et al., 1981; Crissman and Smith, 1984) and displays the encoded protein or peptide at one end of the phage particle (Smith, 1985). The missing N-terminal domains are supplied within 'adapter' molecules, which contain the ligand covalently bound to the N-terminal domains of g3p (Figure 1E). The ligand can be a protein or a peptide genetically fused to the adapter (Gramatikoff et al., 1994; Duenas and Borrebaeck, 1994; Krebber et al., 1995) or a small organic compound chemically coupled (Krebber et al., 1997), and the size limits of the ligand have not been tested. By adding the adapter molecule to the non-infective protein-displaying phage particles, protein-ligand complexes can form on the phage if the displayed protein is able to bind the ligand. The N-terminal domains of g3p become re-connected to the phage particle and thereby the phage becomes infective (Figure 1E). Thus, a rapid onestep method to select protein-ligand interactions results.

cently extended by inserting β -lactamase at various positions of g3p, in the presence or absence of individual domains (Krebber *et al.*, 1997). This strategy mimics an infinite binding constant between the protein-ligand pair, and the theoretical limit of infectivity of the corresponding SIP complex can be deduced.

Increasing the spacing between g3p domains and the presence of a bulky protein apparently does not destroy the infection process (Smith, 1985; Krebber et al., 1997). It may be pointed out, however, that the N-terminus and the C-terminus of β -lactamase are close together in space, and therefore the observed reduction in infectivity may be more pronounced for other proteins. Nevertheless, the insertion of β -lactamase between N1 and N2 leads to a more significant reduction in infectivity than the insertion between N2 and CT, suggesting that N1 and N2 functionally cooperate. The ability of g3p to at least partially tolerate such big inserts in its structure without losing its function will have implications even for standard phage display. For example, protein libraries may be inserted within g3p at the indicated sites, which will be advantageous for identifying robust and proteolysis-resistant proteins, since only those phages from which N1 is not cleaved would remain highly infective (see below). The N1 domain of g3p was found, both from deleted phages and SIP experiments (Figure 3), to be essential for the entry of the phage DNA into the cell under all conditions (Armstrong et al., 1981; Stengele et al., 1990; Krebber et al., 1997). A very low background of infectivity, 1-10 cfu out of 10¹⁰ phages used, has been detected in SIP experiments in the complete absence of N1 domain. Exogenously added N1 domain protein itself does not promote entry of unlinked non-infective phage into the cell, however. Instead, N1 must be physically linked to the phage particle, either directly via genetic fusion, or indirectly via the non-covalent protein-ligand interaction of SIP (Krebber et al., 1997).

Properties of Phages with Altered Domain Arrangements

In order to identify the effect of changes in the normal arrangement of the g3p domains, a number of deletion mutants had been previously constructed (Stengele *et al.*, 1990; Crissman and Smith, 1984). This groundbreaking work has led to the hypotheses of the function of the g3p domains detailed in the Introduction. This work was re-





N1-N2-Ag scFv -''short'' phage N1-N2-Ag scFv -''medium'' phage

Fig.3 Four Types of SIP Experiments Studied.

(A) In the absence of N2, (B) recreating the wt arrangement of domains by interrupting the protein between N2 and CT, (C) recreating the wt arrangement of domains by interrupting the protein between N1 and N2 and (D) in the presence of two copies of N2, one on the soluble portion and the other on the scFv-display phage.

In contrast, if only the second N-terminal domain, N2, is missing in the phage particle, a residual infectivity can be

- A Pilus-independent pathway Ca²⁺-dependent requires only N1
- B Pilus-dependent pathway Ca²⁺-independent requires N1 and N2



observed. This could be increased by 1 to 4 orders of magnitude by the addition of Ca^{2+} (Krebber *et al.*, 1997), consistent with previous results for wt phage (Russel *et al.*, 1988). Under these conditions, the infectivity is the same in the presence (F⁺-cells) and absence of pili (F⁻-cells). These results demonstrate that the N2 domain is not necessary for the infection process itself. Instead, N2 confers specificity towards F⁺-cells with a concomitant increase in infectivity, which is most simply explained by the N2 domain directly docking to the F-pilus (Armstrong *et al.*, 1981).

This suggests that there are two pathways of infection (F-pilus independent, strongly stimulated by Ca²⁺ and Fpilus dependent, requiring N2) which partially interfere with each other (Figure 4). Both pathways have an absolute requirement for N1, but the pathway mediated by N2 and the F-pilus is much more efficient. Thus, it is indeed possible to carry out SIP experiments in the absence of N2 (Figure 3A) (Duenas and Borrebaeck, 1994; Duenas et al., 1996a, b; Krebber et al., 1997), but it appears to be very advantageous to use Ca²⁺ treated cells to overcome the loss of N2 (Krebber et al., 1997). It is not clear whether Ca²⁺ merely has a direct effect on the cell envelope or indirectly influences uptake via the TolQRA system (Sun and Webster, 1987; Webster, 1991; Braun and Herrmann, 1993). No improvement has been observed when the N2 domain occurred twice (Figure 3D), once in the adapter and once in the phage (Krebber et al., 1997).

Fig.4 Two Pathways of Infection.

Domain deletion and insertion experiments, as well as the dependence on Ca^{2+} and pill suggest that there are two different pathways of phage infection. Both are strictly dependent on the presence of the N1 domain, which must be physically linked to the phage. (A) In the presence of only N1, infection is independent of the presence of F-pili. Infection is strongly stimulated in the precence of Ca^{2+} , which might have a direct effect on the membrane structure and/or an indirect effect on the TolQRA system with which the phage is presumed to interact on its pathway into the cell (Webster, 1991). (B) In the presence of both N1 and N2, the phage can take advantage of pill, leading to a much higher infectivity than in pathway (A).

The ability of N1 to lead to transfer of DNA into the cell in the absence of N2 and thus to confer moderate infectivity



N

penetration domain



Fig. 5 Hypothesis of the Existence of a Primordial Phage with Only N1. The filamentous phages of the Ff group (containing f1, fd and M13) and IKe share a homologous N1 domain, but no homology in the N2 domain, which is also at a different location relative to N1. While the Ff group is specific for F-pili, the IKe phage is specific for N-pili.

upon a phage particle displaying N1 invites speculation about a primordial filamentous bacteriophage using only N1. This speculation is supported by evolutionary evidence. IKe, a filamentous phage which specifically infects cells bearing N-pili, is 55% homologous to the F-pilus specific bacteriophage across its whole genome. In its g3p, regions with homology to N1 and CT can be aligned. For the fd phage domain N2, located between N1 and CT, which gives F-pilus specificity, no sequence homology can be found (Peeters et al., 1985). Instead a different receptor binding domain is inserted in front of N1 of IKe (Endemann et al., 1992). In the NMR structure of N1 of fd (Holliger and Riechmann, 1997) the conserved residues of N1 were suggested to be structurally important, and might take part in the binding and membrane penetration of E. coli, suggesting a conserved infection pathway of fd, IKe and I2-2. Perhaps a common precursor, possessing only N1 and using the pilus-independent pathway men-tioned above, diverged into the different filamentous phage, e.g. fd and IKe, each using a different pilus type for enhanced infectivity (Figure 5). Recently, hybrid phages have been constructed with various pieces of the IKe g3p fused to the N-terminus of the complete g3p of fd phage (Marzari et al., 1997). These phages infect both bacteria with N-pili and those with Fpili. The background infection in the absence of IKe g3p, presumably pilus-independent, is enhanced by the presence of the full pilus-binding domain of IKe. Further improvement is obtained when the IKe penetration domain (N1) and its glycine-rich linker are also fused, suggesting that in the N-pilus-dependent infection, the pilus-binding and membrane penetration domains cooperate, and must have a certain distance and flexibility between them. This exactly mirrors the results with fd phages (see above) (Krebber et al., 1997), where N1 and N2 also seem to functionally cooperate. Earlier experiments had also shown (Endemann et al., 1993) that only certain deletion mutants of g3p of IKe and g3p of Ff can coexist on the same phage particle. This is a strong hint that there are lateral interactions between the copies of g3p, and thus, that more than one molecule may be required for the infection event (see below). This is in agreement with the dependencies of infection on the concentration of soluble N1-N2-Ag and especially N1-Ag in SIP experiments (see next section) (Krebber et al., 1997), which require far higher concentrations of adapter protein than should be expected from the protein-ligand interaction.

cally linked to the phage. Both scFv-'short' and scFv-'medium' phages were tested for a regain of infectivity by addition of either N1-Ag or N1-N2-Ag adapter protein (Figure 3). N1-N2-Ag with scFv-'short' phage (Figure 3B) and N1-Ag with scFv-'medium' phage (Figure 3C) recreate the wt domain arrangement, albeit interrupted at a different point. In the combination of N1-Ag with scFv-'short' phage the N2 domain is completely absent (Figure 3A). In the combination N1-N2-Ag with scFv-'medium' phage the N2 domain occurs twice, once in the adapter and once in the phage (Figure 3D).

The infectivity of scFv-'short' or scFv-'medium' phages increased as a function of N1-Ag concentration up to the highest concentration tested (10^{-5} M) and then leveled off. The absolute yield with scFv-'medium' phage is higher than with scFv-'short' phages, however, resulting in up to 1.2×10^6 colonies from 10^{10} input phages (Krebber *et al.*, 1997). In contrast, N1-N2-Ag showed an optimal concentration for enhancement of infectivity at low concentrations (10⁻⁸м), irrespective of the use of scFv-'short' or 'medium' phage. In both cases significantly lower colony counts were observed than with N1, and at high adapter concentrations the infectivity vanished. This suggests that inhibition occurs at high N1-N2 concentrations, but not at high N1 concentrations. Indeed, when using these domains in inhibiting the entry of wt phages, N1 inhibits at a half-inhibitory concentration of $5 \times$ 10⁻⁷м, while N1-N2 inhibits at a concentration as low as 7×10^{-9} M. A crucial site on the bacteria apparently becomes saturated at high N1-N2 concentrations, and it is attractive to think that this is the F-pilus. The practical consequence of these studies for SIP selections is that experiments with N1-N2-Ag will work only for very high affinity systems. At low concentrations of N1-N2-Ag, the phage is not saturated with ligand-adapter, while at high concentrations, the bacteria become saturated and infection is blocked. On the other hand, only very low amounts of adapter are needed, and the system must select for high affinity binders (if there are any). With N1-Ag, higher concentrations can and must be used to overcome the lower rate of infection events. It is not entirely clear why this is the case. There are two explanations, which are not mutually exclusive. In the first, the binding to E. coli cells in the absence of pili is of low affinity, and the observed concentration dependence with N1-adapters would be attributable to the binding curve of phages to bacteria. In the second explanation, binding of phage to bacteria must be multivalent, and thus all binding sites on the phage for cognate ligand must be simultaneously fil-

Variations of SIP: The Use of Different Domains

of g3p

Using purified adapter molecules, coupled to fluorescein in a fluorescein/anti-fluorescein scFv model system, the consequences of using different adapter molecules were studied (Krebber et al., 1997). When no antigen was fused to either N1 or N1-N2, no infection was observed, demonstrating that the N-terminal domains must become physiled, requiring adapter concentrations far higher than the dissociation constant. It will require further experimentation to distinguish between these possibilities. We also wish to stress that it is not clear that equilibrium considerations are adequate; it is possible that these concentration dependencies reflect steady state phenomena which are impossible to interpret in the absence of a more detailed knowledge of the infection mechanism.

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Fig.6 In Vitro vs. In Vivo SIP.

(A) *In vivo* SIP. Both interacting partners (e.g. antibody and antigen or receptor and ligand) are encoded on the phage genome. Thus, the cognate interaction occurs in the periplasm of the phage-producing host. This system requires both partners to be of peptidic nature. On the other hand, both partners can potentially be randomized at the same time ('library versus library screening', see below). (B) *In vitro* SIP. The adapter protein is produced in an expression host (left), either from inclusion bodies with refolding *in vitro*, or by secretion of the native protein. It is purified and then chemically coupled with the antigen. Thus, the antigen can be of non-peptidic nature. The phage only encodes the protein or peptide to be displayed (right), and the adapter and phage are mixed *in vitro*. The infection process can be more tightly controlled and monitored than in the case of *in vivo* SIP.

Variations of SIP: In Vitro vs. In Vivo SIP

One may carry out the SIP experiment in two different ways. In the '*in vivo* SIP' approach the phage (Krebber *et*

antigens can be used, the former genetically fused to the N-terminal domains, the latter chemically coupled. This chemical coupling broadly extends the range of applications of SIP, as now glycoproteins, small organic mole-

al., 1995) or phagemid/helper phage (Gramatikoff et al., 1994) encodes both interacting molecules (receptor-ligand or antibody-antigen) (Figure 6A). Thus, in the 'in vivo SIP' approach adapter and non-infective protein or peptide-displaying phage are produced in one bacterium, whereupon adapter and phage bind each other, mediated by the protein-ligand interaction in the periplasm of the respective bacterial cell. The experiment only requires the phages (of which some will be infective since they carry the N-terminal domains) to be harvested and used to infect bacteria. This simplicity of the design of the 'in vivo SIP' experiment led to the idea of library-library selection schemes (see below). However, the simplicity is somewhat counterbalanced by a lack of experimental control of concentrations, incubation times and the infection process itself. These factors can be adjusted in the 'in vitro SIP' experiment (Figure 6B), in which the adapter protein is prepared from a separate E. coli culture, either by secretion (Duenas and Borrebaeck, 1994) or by in vitro refolding (Krebber et al., 1997). The phage library is prepared like a normal phage display library, but instead of affinity enrichment of the phage particles on immobilized ligand, the non-infective phage library is incubated with the adapter protein, and then the *E. coli* cells to be infected are added. In this approach, not only peptidic (Duenas and Borrebaeck, 1994) but also non-peptidic (Krebber et al., 1997)

cules or even proteins consisting of D-amino acids (Schumacher *et al.*, 1996) can be coupled and used in SIP.

Making SIP Phage Libraries: Phagemid vs. Phage Systems

Phage particles can be produced by a phagemid/helper phage system. In the case of SIP, where the infection is strictly dependent on a cognate interaction between receptor and ligand, it is necessary that the helper phage does not deliver any wt g3 to the phage particle (Δ g3 helper phage). This $\Delta g3$ helper phage must be able to infect a phagemid library, however, for which it needs g3 protein. This can, in principle, be achieved by producing a helper phage in a cell which harbors an unpackageable g3p expression plasmid (Figure 7) (Duenas and Borrebaeck, 1994, 1995). With $\Delta g3$ helper phages, from which the important central terminator (Beck and Zink, 1981; see below) between the strongly and weakly expressed genes and the promoter which drives g3 and the downstream genes were deleted (Duenas and Borrebaeck, 1994), phage production was poor. It improved in the precise deletion of the coding region in M13MD Δ 3.2 (Duenas and Borrebaeck, 1995). However, using an identical precise deletion of the coding



Fig.7 The Preparation of SIP Libraries.

(A) In a phagemid/helper phage system, a helper phage which does not contain the genetic information for wt g3p but contains the g3p in the phage coat is necessary in order to infect the cells containing the phagemid library. This phage is prepared from cells doubly transformed with a g3 deleted helper phage (Nelson *et al.*, 1981) and a plasmid containing the wt g3, but no packaging signal. Thus, only the Δ g3p helper phage should be packaged. It appears that the plasmid is packaged, however, at low frequency (see text) leading to some infective helper phages (bracketed) which form background during SIP selection. The Δ g3p helper phage is then used to infect a phagemid library, and phages are produced for SIP experiments. (B) Filamentous phages can be directly engineered to receive the library. The phages must contain a selectable marker, usually an antibiotic resistance. The information for N1 and N2 is replaced by the peptide or protein to be displayed. For details, see text.

region g3 from M13KO7 still some packaging of the g3p expression vectors was observed (Krebber *at al.*, 1995) in the authors' hands. Packaging was found with different g3p expression plasmids, derived from either pUC,

pACYC or pBR vectors, albeit with a low efficiency, presumably due to the presence of unidentified cryptic packaging signals. The presence of wt g3p, and of course especially its genetic information g3, in the pool of display

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phages affects the 'non-infectivity', a fundamental prerequisite for the selectivity of the infection process, raising the background level of the system (Duenas and Borrebaeck, 1994, 1995; Duenas *et al.*, 1996a). An alternative is to transform the phagemid library into cells which already contain $\Delta g3$ helper phage (Gramatikoff *et al.*, 1994). However, helper phage $\Delta g3$ -M13KO7, with a precise deletion of g3, still showed some genetic instability and lowered the viability of bacteria (Krebber *et al.*, 1995). On the other hand, fCA55 and fKN16 phage (Nelson *et al.*, 1981) encode a short fragment of the C-terminal domain of g3p which competes with the display of the fusion protein on the phage (Nelson *et al.*, 1981).

All these problems are simultaneously avoided by using a phage genome, since the fusion proteins are thus direc-

pomyosin, a component of the cytoskeleton, have been selected from a human cDNA library to pair with the junzipper domain (Gramatikoff *et al.*, 1995).

SIP has also been used for antibody/antigen systems. An anti-lysozyme Fab fragment was used as a model system, with N1 fused to the enzyme as the adapter (Duenas and Borrebaeck, 1994), giving rise to a specific enrichment factor of approximately 10¹⁰ after only two rounds of selection. It is an open question whether the spectacular enrichment of the anti-lysozyme antibody is aided by some affinity of the lysozyme molecule to the *E. coli* cells, but the same system was elegantly used to enrich Fab fragments specific for a peptide antigen (Duenas and Borrebaeck, 1994; Duenas *et al.*, 1996a, b). An anti-fluorescein antibody/antigen system (Krebber

tly incorporated into a single genetic package (Krebber et al., 1995, 1997). The circular genome of the filamentous phage consists of 2 main transcriptional units separated by a central terminator on one side and the origin of replication on the other (Beck and Zink, 1981). In early constructs, the resistance gene has been inserted into the 'intergenic region' (Zacher et al., 1980), but this is the region of the replication origin. In order not to disturb the delicate system of replication and transcription of the phage genes, a resistance gene has more recently been inserted immediately downstream of g8, without disturbing the central terminator or the transcription of g3 and g6, and indeed, without significantly affecting the titer of the phage (Krebber et al., 1995). Fusion proteins of interest are then inserted either under a regulatable promoter downstream of the resistance gene, directly behind a further terminator to keep the system tightly controlled (Krebber et al., 1995), or under the control of the natural g3 promoter directly do*et al.*, 1997) was the first example where a non-peptidic antigen had been coupled to the g3 N-terminal domains and where purified components were used. Under the optimal conditions, up to 10^6 antigen-specific infection events occurred from 10^{10} input phages, compared to only 1 antigen-independent event. The antibody 17/9, specific for a hemagglutinin peptide, was used in an example where both peptide and antibody were encoded in the same phage genome (Krebber *et al.*, 1995). In the latter two examples, presumably due to the use of phage vectors as the sole replicon rather than phagemid/helper phage systems, no background was observed in the absence of the peptide or in the presence of an antibody with different specificity.

Duenas and Borrebaeck (1994) tested a Fab library made from B-cells of gp120 seronegative donors, with only the N1 domain fused to the V3 loop peptide. From this library, no antibodies against the antigen could be obtain-

wnstream of the central terminator, by replacing the N-terminal domains of g3 in the natural site (Krebber *et al.*, 1997).

Examples of SIP Studied

SIP has been tested with both proteins and peptides. Gramatikoff et al. (1994) used the leucine-zipper motifs of jun and fos proteins as a model for an interacting pair, with the jun-zipper domain fused to the C-terminal domain of g3p and the fos domain fused to N1-N2. The infectivity is restored by the heteromeric interaction between the two leucine-zipper domains. In their model system (even though not stated explicitly), the jun and fos helices were flanked by cysteine residues, separated by Gly-Gly spacers (Crameri and Suter, 1993), which then covalently link the N-terminal domain of g3p to the phage by periplasmic disulfide formation, presumably catalyzed by DsbA. An enrichment of up to 200-fold was observed over non-interacting pairs in one round (Gramatikoff et al., 1994). The whole fos protein, not containing the engineered cysteines, also caused infection, however. Furthermore, zipper-like domains from the ribosome protein L18a, component of the large ribosomal unit, and troed after three rounds of phage panning, but half of the phages gave positive ELISA signals after three rounds of SIP. Thus, SIP is faster than standard phage display using the same library. Using six different Fab fragments against the V3 loop peptide as a model library, it was investigated whether an enrichment for affinity or even kinetic constants is possible (Duenas et al., 1996a). In individual experiments, antibodies with a $K_{\rm D}$ around 10^{-9} M gave higher infectivity than those with one of 10^{-8} M, and in a competition experiment, the former were selected. In an experiment in which the incubation time was varied, the antibody with the fastest on-rate appeared to be slightly preferred early on, while the one with the slowest off-rate was slightly favored when a competing free antigen was added, but the inherent experimental variations in these kinds of experiment makes this analysis notoriously difficult.

SIP has then been used for the analysis of an *in vitro* response to a synthetic immunogen, using lymphocytes

from seronegative donors (Duenas *et al.*, 1996b). Different libraries were selected for anti-V3-peptide Fab fragments using SIP. Only few clones were selected after two rounds of SIP from the naive library. In contrast, a number of positive clones were found after only one round of selection when libraries were constructed after the first and/or second *in vitro* immunization. An increase in affinity constants





-(14)







Fig.8 Schematic Depiction of 'Library vs. Library' Screening.

The illustration uses an antibody and an antigen library as an example. By fusing the antigen to the adapter, and the antibody to the phage, only those bacterial cells in which partners recognize each other will give rise to infective phages. In principle, an 'interaction landscape' (schematically shown as a two-dimensional grid on the right) may be obtainable. Interactions are symbolically shown by the darkness of the field in the 2D map. It needs to be stressed that it is still unclear whether two libraries of useful size can be comprehensively mapped in this way.

was noted, as a consequence of the second *in vitro* immunization, mainly due to a decrease in dissociation rate constants. In this experiment, only high affinity antibodies seemed to have been enriched, in line with the properties of the SIP method.

ent molecules, which will be a concern if the interaction has a fast off-rate.

Library vs. Library Screening

One of the exciting possibilities of SIP is that two interacting libraries may be screened simultaneously (Figure 8). Such an approach might clearly have broad utility in the identification of interacting receptor-ligand pairs. From a more theoretical point of view, this information might be vital in understanding the tolerance of interacting systems against mutation and the frequency of finding interacting surfaces. Furthermore, the massive data on protein-ligand pairs which would emerge from such interaction libraries could lead to a more complete understanding of the prerequisites for sequences and structures required for binding. In the end, such databases may be useful in designing interacting partners of good stability. It must be said, however, that the feasibility of truly comprehensive screening of two large libraries against each other is currently unclear. One important issue is the possible exchange of adapter between different phages in a population of differ-

Further Challenges in the Development of SIP

SIP is a very young technology, and has obviously been studied far less than, e.g., the two-hybrid system (Bai and Elledge, 1996; McNabb and Guarente, 1996) or phage display (Smith, 1985; Dunn, 1996). Therefore, the limits of the system are only now starting to be explored. For instance, it is not yet clear what the minimum affinity is for the interacting protein-ligand pair to show significant infectivity. All the systems studied to date had dissociation constants of 10^{-8} M or better, and even though this number is not precisely known for some of the coiled-coil helices studied, they may also be expected to be in this range (Pernelle et al., 1993; Patel et al., 1994) and it is possible that the SIP system requires this range of binding strength. Whether this is seen as an advantage or disadvantage of SIP probably primarily depends on whether one's favorite library contains such high affinity molecules or not. While there may be some encouraging results on kinetic selection (Duenas et al., 1996a), the generality of selection for kinetic parameters of ligand binding will have to be more widely tested and checked against issues such as different

thermodynamic stabilities of the proteins, or expression rates.

Some of the possible limitations of SIP are shared with phage display. Certain proteins will be toxic when displayed on phage, or at least reduce the phage production below useful levels, while most peptides are probably amenable to display on g3p. For library screening, SIP complements the two-hybrid system nicely (Bai and Elledge, 1996; McNabb and Guarente, 1996), since the latter is designed for nuclear and cytoplasmic proteins (devoid of disulfides) while SIP is definitely compatible with disulfide formation but may cause problems with cytoplasmic proteins containing unpaired cysteines or with very hydrophobic proteins. No information is yet available about the size limits of the protein-ligand complex to be used in SIP. Another point to consider is that the infection event (which is ultimately selected for) can conceivably be caused by wt phages or 'wt-like' phages, so that care has to be taken in phage handling and control of rare recombination events in the phage which may restore infective constellations of the g3p domains, especially during 'in vivo SIP' experiments (Figure 6). Future work will also be directed to developing ever more robust phage vectors, and it is tempting to use molecular evolution for this purpose as well. These challenges are to be contrasted with numerous advantages that SIP may have over phage display. No solid phase interaction with any support is necessary, while in phage display, the interacting phages have always to be separated from the non-interacting ones by binding to affinity columns, polystyrene wells or coated magnetic beads. On all of these surfaces non-specific interactions can occur and have been described (Adey et al., 1995). Furthermore, because of the lack of a surface binding step, no elution is necessary in SIP. Much work in phage display has been invested in optimizing washing and elution steps, and they have to be optimized somewhat for almost every protein-ligand pair.

Acknowledgements

The authors wish to thank G. Wall, F. Hennecke, K. Arndt and G. Pedrazzi for helpful suggestions and critical reading of the manuscript.

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Conclusions and Perspectives

The low background infectivity rate observed with some of the SIP systems, as explained above, shows that SIP can be an extremely effective and highly specific method to select for cognate interaction events. When compared to phage display, this one-step procedure obviates the time consuming and generally inefficient physical separation of cognate and non-specifically binding phages. SIP thus appears to be a very powerful method to select for ligandbinding proteins or peptides. Nevertheless, our understanding of the infection event itself and the range of permissible interacting molecules and the requirements regarding their physical properties is very inadequate at best and in need of detailed studies, some of which are ongoing.

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