J. Mol. Biol. (1997) **268**, 607–618

JMB

Selectively-infective Phage (SIP): A Mechanistic Dissection of a Novel *in vivo* Selection for Protein-ligand Interactions

Claus Krebber[1], Stefania Spada[1], Dominique Desplancq[1] Anke Krebber[1], Liming Ge[2] and Andreas Plückthun[1]*

[1]*Biochemisches Institut Universität Zürich Winterthurerstr. 190 CH-8057, Zürich, Switzerland*

[2]*MorphoSys GmbH Frankfurter Ring 192a D-80807, München, Germany*

**Corresponding author*

Selectively-infective phage (SIP) is a novel methodology for the *in vivo* selection of interacting protein-ligand pairs. It consists of two components, (1) a phage particle made non-infective by replacing its N-terminal domains of geneIII protein (gIIIp) with a ligand-binding protein, and (2) an ''adapter'' molecule in which the ligand is linked to those N-terminal domains of gIIIp which are missing from the phage particle. Infectivity is restored when the displayed protein binds to the ligand and thereby attaches the missing N-terminal domains of gIIIp to the phage particle. Phage propagation is thus strictly dependent on the protein-ligand interaction. We have shown that the insertion of β-lactamase into different positions of gIIIp, mimicking the insertion of a protein-ligand pair, led to highly infective phage particles. Any phages lacking the first N-terminal domain were not infective at all. In contrast, those lacking only the second N-terminal domain showed low infectivity irrespective of the presence or absence of the F-pilus on the recipient cell, which could be enhanced by addition of calcium. An anti-fluorescein scFv antibody and its antigen fluorescein were examined as a protein-ligand model system for SIP experiments. Adapter molecules, synthesized by chemical coupling of fluorescein to the purified N-terminal domains, were mixed with non-infective anti-fluorescein scFv-displaying phages. Infection events were strictly dependent on fluorescein being coupled to the N-terminal domains and showed a strong dependence on the adapter concentration. Up to 10^6 antigen-specific events could be obtained from 10^{10} input phages, compared to only one antigen-independent event. Since no separation of binders and non-binders is necessary, SIP is promising as a rapid procedure to select for high affinity interactions.

Keywords: geneIII; filamentous phage; phage display; infection

Introduction

The identification of protein-ligand interactions is one of the most important challenges in biological sciences, both for identifying new interaction partners in regulatory networks (Phizicky & Fields, 1995) and for finding and improving pharmaceutical lead structures (O'Neil & Hoess, 1995). Numerous methods for screening large peptidic libraries for protein-ligand interactions by physically coupling the protein or peptide to its gene have been described (reviewed by Phizicky & Fields, 1995). In phage display (Smith, 1985), one of the binding partners is fused to a phage coat protein of a filamentous phage (Figure 1 a and b), and the selection procedure involves the enrichment of phages which bind to a given immobilized target molecule

Present addresses: D. Desplancq, Département Récepteurs et Protéines Membranaires, UPR 9050 du CNRS ESBS, Pôle Universitaire d'Illkirch, Rue Sébastien Brandt, F-67400 Illkirch, France. C. Krebber, Maxygen, 3410 Central Expressway, Santa Clara, CA 95051, USA.

Abbreviations used: cfu, colony forming units; Bla, β-lactamase; gIIIp, gene III protein; N1, first N-terminal domain of gIIIp; N2, second N-terminal domain of gIIIp; CT, C-terminal domain of gIIIp; scFv, single-chain Fv fragment of an antibody; scFlu, single-chain Fv fragment of an antibody directed against fluorescein; SIP, selectively-infective phage; cam, chloramphenicol; str, streptomycin; tet, tetracycline; amp, ampicillin; R, resistance; FITC, fluorescein isothiocyanate; Ag, antigen; wt, wild-type; ON, over night; Flu, fluorescein antigen; FluCad, 5-((5-aminopentyl)thioureidyl)fluorescein; PEG, polyethylene glycol.

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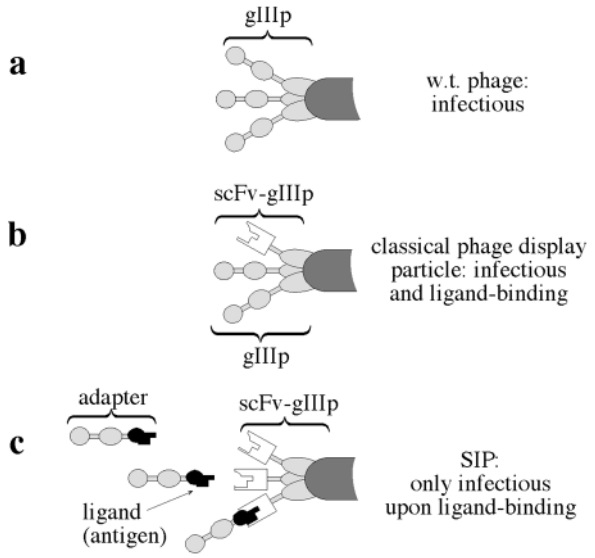

Figure 1. Concept of selectively-infective phages (SIP) in comparison to wt phages and phages used for phage display. a, wt phage. The gIIIp is needed for infectivity, i.e. entry of the phage into bacteria (see the text and Figure 2). b, Classical phage display particle. By using phagemid/helper phage systems, both wt gIIIp and the fusion of the ligand binding protein to the C-terminal domain of gIIIp are present in the same phage particle. The phage particle can both bind a ligand and remain infective. c, SIP: the wt gene in the phage genome is replaced by the ligand binding protein, fused to the C-terminal portion of gIIIp. Therefore, the fusion protein is present in all copies, which renders the phage non-infective by the complete absence of the N-terminal domains of gIIIp. The missing N-terminal domains of gIIIp are supplied to the phage particle by the ''adapter'', which contains the covalently linked ligand. Only by the ligand binding to the displayed protein, the phage becomes infective.

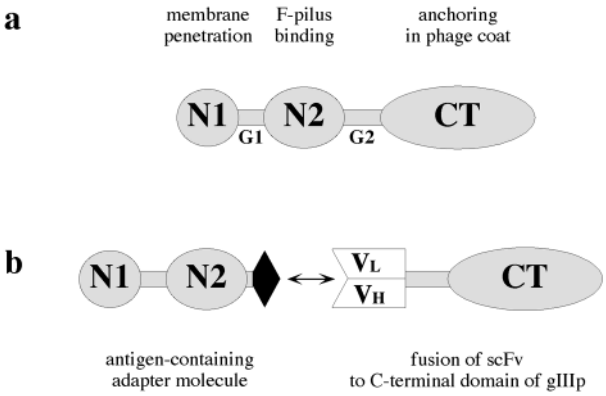

Figure 2. a, The geneIII adsorption protein of F-specific filamentous phage, with the three-domain structure indicated. The nomenclature is as used throughout the text, with the first N-terminal domain labelled N1, the second N-terminal domain N2 and the C-terminal domain CT, interspersed by two glycine-rich linkers G1 and G2. b, The adapter molecule contains the N-terminal domains (in this case N1 and N2) and links them to a ligand (in this case antigen), while the C-terminal domain is anchored in the phage coat and thereby attaches the displayed scFv to the phage particle.

(Clackson & Wells, 1994; Winter *et al*., 1994). In phage display the phage particle remains infective because of the presence of wt geneIII protein (gIIIp) (Figure 1b). Consequently, the DNA of both non-specifically and specifically bound phages enters the bacterial cell by infection, after they have been eluted from the ligand surface. The main challenge of phage display is thus to minimize non-specific adsorption of the phages in the binding procedure (Adey *et al*., 1995) and to enrich phage which display high-affinity molecules over highly abundant, low-affinity binders (Hawkins *et al*., 1992).

Here, we investigate the mechanism of selectively-infective phage (SIP) (Krebber *et al*., 1993, 1995; Duenas & Borrebaeck, 1994; Gramatikoff *et al*., 1994). In SIP, in contrast to phage display, the desired protein-ligand interaction, in this study exemplified by an antibody-antigen interaction, is itself directly responsible for restoring infectivity in an otherwise non-infective display phage (Figure 1c).

This is achieved by exploiting the modular structure of gIIIp of wt filamentous phage (Figure 2a) (Armstrong *et al*., 1981; Stengele *et al*., 1990). The gIIIp 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, the second N-terminal domain, N2, in binding of the bacterial F-pilus (Stengele *et al*., 1990; Jakes *et al*., 1988). The C-terminal domain (CT) plays a role in phage morphogenesis and caps one end of the phage particle (Nelson *et al*., 1981; Crissman & Smith, 1984).

After synthesis in the cytoplasm the preprotein of gIIIp is transported through the inner membrane, and the signal sequence is then cleaved off (Ito *et al*. 1980), while processed gIIIp is inserted in the inner membrane of *Escherichia coli via* a short C-terminal peptide (Davis *et al*., 1985; Endemann & Model, 1995). In the inner membrane it oligomerizes and associates with gVIp, to be taken up by the budding phage (Gailus *et al*., 1994a; Gailus & Rasched, 1994). This complex of three to five copies of gIIIp and gVIp forms the end of the phage particle (Davis *et al*., 1985) which leaves the host cell last (Lopez & Webster, 1983). In the phage particles, the C-terminal domain (CT) is part of the coat structure and thereby anchors the N-terminal domains of gIIIp 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 3D-structure.

The basis of SIP involves the separation of the N-terminal domains required for infection (N1 or N1-N2) from the anchoring C-terminal domain (Figure 2b). In SIP the gene for a peptide or a protein (e.g. an anti-fluorescein scFv) is used to replace the N1 or N1-N2 encoding parts by genetic fusion to the C-terminal part of geneIII, leading to a phage particle which is non-infective (Nelson *et al*., 1981; Crissman & 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 consist of the ligand covalently bound to these N-terminal domains. The ligand can be a protein, a peptide or a small organic compound (e.g. fluorescein). 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 to the ligand. The N-terminal domains of gIIIp become again connected to the phage particle and thereby the phage becomes infective. Thus, a rapid one-step method to select protein-ligand interactions results. Since SIP works without the involvement of solid phase interactions, the selection for high affinity binders seems to be feasible (Duenas *et al*., 1996).

In this study we investigated the influence of different parameters on the process of selectively-infective phage. The efficiency of SIP is potentially influenced by a number of parameters: the site where the protein-ligand will be inserted within gIIIp, the strength of the protein-ligand interaction, the folding yield of each binding partner and their resistance to proteolytic digestion. Furthermore, we wanted to test whether the N-terminal fragments of gIIIp themselves might have stimulatory or inhibitory effects on the infectivity of non-infective protein-displaying phage, independent of the presence of antigen in the adapter molecule.

In order to discriminate between these many factors, we first constructed phage vectors which led to phage particles with β-lactamase inserted within gIIIp (Soumillion *et al*., 1994) at various locations. By spatially separating the N-terminal and C-terminal parts of gIIIp while retaining the covalent linkage between them, an infinite binding constant between the protein-ligand pair is mimicked, and the theoretical limit of infectivity of the corresponding SIP complex can be deduced. In addition, the infectivity of phage particles displaying β-lactamase but lacking various domains of gIIIp was investigated, using both F-pilus expressing and F-pilus lacking recipient cells.

We then constructed two different non-infective anti-fluorescein scFv-displaying phage particles and prepared adapter molecules from different purified N-terminal fragments of gIIIp and coupled them to a fluorescein derivative. Both components were then combined in quantitative selectively-infective phage experiments and the infectivity of the different phage-adapter complexes and their dependence on the adapter concentration was measured.

Results

Infectivity of β-lactamase gIII-fusions and the influence of Ca^{2+}

In a first set of experiments, we created a number of phages in which β-lactamase was inserted between the domains of gIIIp, or in which domains of gIIIp have been replaced by β-lactamase. The enzyme β-lactamase (Bla) was chosen since it acts in the periplasm. Since gIIIp is anchored to the periplasmic face of the inner membrane before it is inserted into the phage coat, its N-terminal domains are in effect in the periplasmic compartment, as is β-lactamase when fused to gIIIp. Indeed, this enzyme can be directly selected for (Siemers *et al*., 1996), even when fused to gIIIp, by addition of ampicillin to the growth media. Thereby mutants which would recreate the wild-type gIII by deleting the Bla gene cannot skew the results. From the infectivity of those Bla-gIIIp fusions we can deduce the function of the various domains of gIIIp, thereby confirming and extending previous deletion experiments (Stengele *et al*., 1990). Most importantly, by using those Bla-gIIIp fusions, we also expected to gain new information about the tolerance of gIIIp to domain spacing and thus about the synergistic action of the three gIIIp domains during the infection process.

The different phage vectors (Figure 3c), were constructed starting from fCKC, a cam^R derivative of the fd phage (Krebber *et al*., 1995). The fCKC vector contains unique restriction sites in gIII (Figure 3a) which allowed the shuffling of domains and β-lactamase (Figure 3b). In all constructs (Figure 3c) the Bla-gIIIp fusion proteins were transported to the periplasm by signal sequences of either PelB, OmpA or gIIIp. The same fd cam^R phage vectors were also used to construct the non-infective scFv displaying phages described below (Figure 3e).

Every insertion of Bla into gIIIp retained high phage infectivity for F^+ recipient cells (Table 1). Insertions behind the second N-terminal domain (N1-N2-Bla-CT, N1-N2Bla-CT in Figure 3c) still allowed the production of plaques, but they were smaller than those resulting from fCKC (N1-N2-CT; Figure 3a and c), which served as ''wt'' control. This was reflected in a 100-fold lower infectivity of those Bla-insertions (Table 1). The insertion of Bla between the two N-terminal domains (N1-Bla-N2-CT, Figure 3c) led to a 10^4-fold lower infectivity (Table 1), which was insufficient for producing plaques. This lower infectivity suggests either a direct binding interaction of N1 and N2 being disturbed by the insertion, or a functional but structurally independent cooperation of the two domains requiring a given distance.

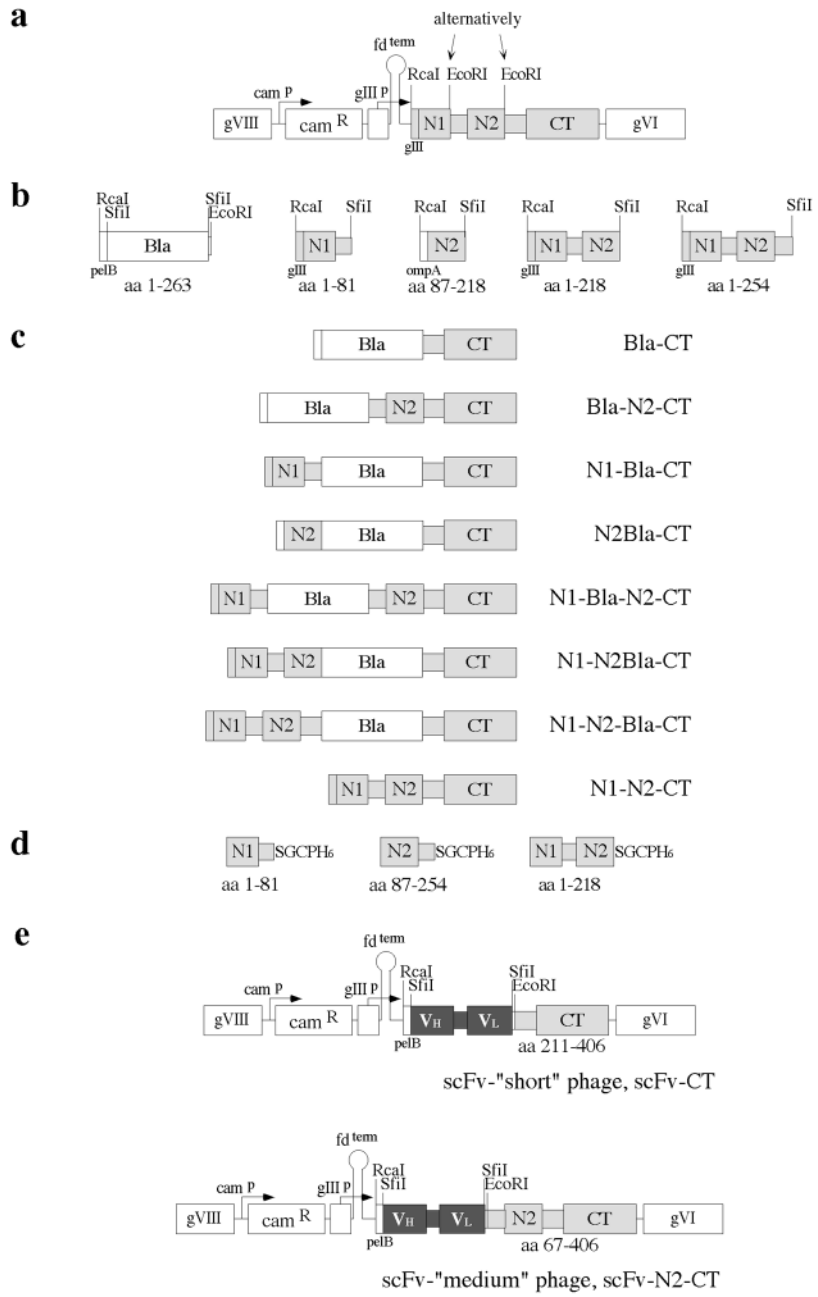

Figure 3. a, The chloramphenicol resistance mediating derivative of the fd-phage fCKC which served as starting construct for all others. Only the region flanked by gene VIII and gene VI, which is different from fd phage, is depicted. wt fd phage has been modified to contain a chloramphenicol resistance (cam^R) cassette in front of the central terminator, but keeping the original gene III promoter by a partial duplication, and introducing the indicated unique restriction sites within gene III (Krebber *et al.*, 1995). All parts of gene III are shaded in gray, and domains are shown as thick boxes and the glycine-rich linkers as thin boxes. b, Cassettes for constructing the Bla-fusion phages. The signal sequences used are indicated. Cassettes with the restriction sites indicated were constructed by PCR. The amino acid numbering below the boxes corresponds to the numbering of the proteins or domains without the respective signal peptide. c, Fusion proteins between Bla and various parts of gIII as present in fCKC derived vectors. The names of the corresponding phage are shown on the right; note that a hyphen indicates the presence of a glycine-rich linker. d, The gIIIp domains N1, N2 and N1-N2 were independently expressed without signal sequence and purified. The amino acids from processed gIIIp are indicated. A hexahistidine purification tail is engineered to the C terminus as indicated, which also contains a single cysteine, to which ligands can be chemically attached. e, Antibody scFv display phages. In the ''short'' phage (scFv-CT), the scFv is fused directly in front of the C-terminal domain CT, in the ''medium'' phage (scFv-N2-CT) it is fused in front of N2.

Bla-gIIIp fusion constructs lacking the first N-terminal domain (Bla-CT, Bla-N2-CT, N2Bla-CT) showed a complete loss of infectivity in all cases (Table 1). In contrast, for the construct only lacking the second N-terminal domain (N1-Bla-CT in Figure 3c), some low infectivity could still be detected, and it could be increased by three to four orders of magnitude on addition of Ca^{2+} (Table 1). For none of the other constructs could the infection of F^+-cells be enhanced by 50 mM Ca^{2+} (Table 1).

When F^- cells, cells which do not possess an F-pilus on their surface, were tested in the absence of Ca^{2+}, all Bla-gIIIp fusions showed very low or no infectivity at all (Table 1), but upon addition of 50 mM Ca^{2+}, enhancements of two to four orders of magnitude were observed once the N1 domain was present (Table 1). In the presence of 50 mM Ca^{2+}, the N1-Bla-CT construct (Figure 3c) showed even the same infectivity of 10^5 cfu/ml in F^+ and F^- recipient cells. This indicates that no infection at all occurs in the absence of N1 under any conditions, and that in the presence of only N1 and Ca^{2+} a F-pilus-independent infection occurs, since the results using F^+ or F^- cells as recipients are the same. Whenever N2 is present in addition to N1, the F-pilus-dependent infection is at least four orders of magnitude more efficient than F-pilus-independent, Ca^{2+}-mediated infection.

Table 1. Infectivity of Bla-fusion phages and dependence on recipient strain and Ca^{2+}

Bla-gIIIp fusion	F^+ (cfu/ml) No Ca^{2+}	F^+ (cfu/ml) Ca^{2+}	F^- (cfu/ml) No Ca^{2+}	F^- (cfu/ml) Ca^{2+}
Bla-CT	<2	<2	<2	<2
Bla-N2-CT	<2	<2	<2	<2
N1-Bla-CT	20	1.2×10^5	50	1.6×10^5
N2Bla-CT	<2	<2	<2	<2
N1-Bla-N2-CT	1×10^7	1×10^7	2	1×10^3
N1-N2Bla-CT	4×10^9	4×10^9	20	1.6×10^4
N1-N2-Bla-CT	2×10^9	2×10^9	20	8×10^3
N1-N2-CT (wt)	2×10^{11}	2×10^{11}	2×10^3	7×10^5

The phages containing the different Bla-fusions, with the dash (-) representing a Gly-rich linker, were used to infect the isogenic pair of JM83 (F^-) and JM83-F^+. 2YTG with and without addition of 50 mM Ca^{2+} was used for growth of the recipient bacteria. The number of phage particles produced by the host cells (*E. coli* XL1-Blue) was within less than one order of magnitude for all constructs. This was estimated by coating phage dilutions on an ELISA plate with subsequent detection of the phages with an anti-M13 horseradish peroxidase conjugate (Krebber *et al.*, 1995). The constructs used are shown in Figure 3.

Inhibitory effects of gIIIp domains

For the design of SIP experiments it was important to know which concentrations of adapter molecules will show inhibitory effects on the infectivity of SIP complexes (Armstrong *et al.*, 1981). Furthermore, it had to be demonstrated that the N-terminal domains from which the adapters are made, were functional. Therefore, the N-terminal domains N1, N2, and N1-N2 (Figure 3d) were separately expressed and purified.

To characterize the N1, N2, and N1-N2 preparations, each was used to inhibit the infection of F^+-recipient cells by N1-N2Bla-CT phage (Figure 3c). N1 showed a half inhibitory concentration at 5×10^{-7} M, N2 at 2×10^{-8} M and N1-N2 even at 7×10^{-9} M (Figure 4). These results for N1-N2 and N1 did not change when fluorescein was coupled to them (N1-N2-Flu, N1-Flu; data not shown). Interestingly, all N-terminal gIIIp fragments enhanced the infectivity of phage slightly (by a factor of 2) at a concentration just before the onset of inhibition (Figure 4). We have currently no explanation of this phenomenon. The comparison of inhibitory properties (Figure 4) shows that N1 and N2 functionally interact, as the combination of both (N1-N2) inhibits at the lowest concentration. The difference in inhibition by N1 and N2 would be consistent with them acting at different sites, as suggested from the Bla-gIIIp fusion phage (Table 1).

Since the adapter molecules N1-Flu and N1-N2-Flu, which were derived from the same N1 and N1-N2 preparations, were able to enhance infectivity of non-infective scFv-display phage (see below), the functionality of the N1 and N1-N2 preparations used in Figure 4 was demonstrated.

Specificity and quantification of infection in SIP experiments

Since the Bla-gIIIp fusion phages indicated the possibility of ''interrupting'' the gIIIp structure either between N1 and N2 or between N2 and CT, and since the inhibitory effect of the N-terminal fragments of gIIIp, N1 and N1-N2 was found to be very different (Figure 4), we decided to investigate all possibilities of combining adapter molecules and non-infective protein-displaying phage particles to create a selectively-infective phage complex (Figure 5).

Two different scFv-display phages were produced: in the scFlu-''short'' phage the anti-fluorescein scFv FITC-E2 (Vaughan *et al.*, 1996) was

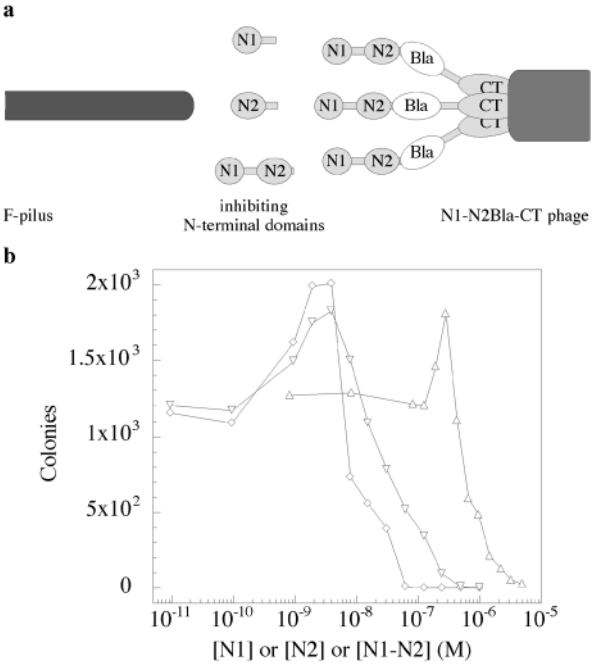

Figure 4. Inhibition of infectivity of N1-N2Bla-CT Bla-fusion phage towards *E. coli* XL1-Blue by N1 (△), N2 (▽) and N1-N2 (◇) domains. a, Schematic depiction of experiment. b, Colonies obtained as a function of soluble gIIIp N-terminal domains, using a constant number of 1.2×10^3 cfu N1-N2Bla-CT fusion phage.

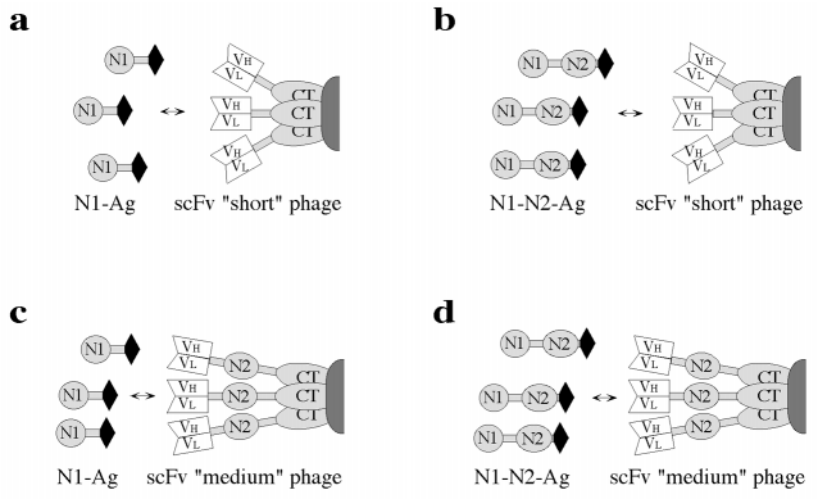

Figure 5. Four types of SIP experiments studied. a, In the absence of N2; b, recreating the wt arrangement of domains by interrupting the protein between N2 and CT; c, recreating the wt arrangement of domains by interrupting the protein between N1 and N2; and d, in the presence of two copies of N2, one on the soluble portion, the other on the scFv-display phage.

fused in front of the second glycine-rich linker, so both N-terminal domains of gIIIp are missing in the phage particles (scFlu-CT, Figure 3e). The scFlu-''medium'' phage contains N2 and thus places the scFv in front of the first glycine-rich linker (scFlu-N2-CT; Figure 3e). Both phage particles depicted in Figure 4 have in common that they do not contain the N1 domain (cf Bla-CT, Bla-N2-CT; Table 1) and indeed, the background infectivity of those scFlu-phage particles was very low (one to ten cfu per 10^{10} input phages; data not shown).

Two different antigen fluorescein containing adapter molecules were synthesized by chemical coupling of a fluorescein derivative to N1-N2 and N1 (Figure 9) using a free thiol function genetically engineered in front of the C-terminal His-tag (Figure 3d).

Both scFlu-short and scFlu-medium phages were tested for their regain of infectivity by addition of N1-Flu or N1-N2-Flu adapter protein (Figure 5). N1-N2-Flu with scFlu-short phage (Figure 5b) and N1-Flu with scFlu-medium phage (Figure 5c) recreate the wt domain arrangement, albeit interrupted at a different point. In the combination of N1-Flu with scFlu-short phage the domain N2 is completely absent (Figure 5a). In the combination N1-N2-Flu with scFlu-medium phage the N2 domain occurs twice, once in the adapter and once in the phage (Figure 5d).

In the absence of chemically coupled fluorescein, neither N1 nor N1-N2 was able to confer any significant infectivity upon the anti-Flu scFv-short or medium phage (Table 2, Figures 6 and 7). This shows that the infection is completely dependent on the antibody-antigen interaction. The isolated, purified N1, N2 and N1-N2 domains do not have the property of allowing ''non-specific entry'' of a phage. Thus, we can conclude that the N1 or the N1-N2 domains need to be physically attached to the phage particle, in this case by the non-covalent binding interaction.

In a more quantitative comparison, differences between the effects of N1-Flu and N1-N2-Flu became apparent. Infectivity of scFlu-medium phage (scFv-N2-CT) increased as a function of N1-Flu concentration up to the highest concentration tested (10^{-5} M), at which point it began to level off, yielding 1.2×10^6 colonies from 10^{10} input phages (Figure 6). In contrast, N1-N2-Flu showed an optimum concentration for enhancement of infectivity at 3×10^{-8} M irrespective of using scFlu-short (scFv-CT) (Figure 7) or medium phage (scFv-N2-CT; data not shown), in both cases yielding 5×10^4 colonies from 10^{10} input phages.

The infection of bacteria by the SIP complex formed in the presence of 2×10^{-8} M N1-N2-Flu and 10^9 scFlu-short phages (scFv-CT) could be inhibited by addition of free fluorescein (Figure 8), demonstrating that the infectivity is indeed due to the antibody-antigen interaction.

Table 2. Specific infectivity of SIP complexes, dependence on Ca^{2+} and Mg^{2+}

	N1 (2 μM) cfu/ml			N1-Flu (2 μM) cfu/ml			N1-N2 (20 nM) cfu/ml			N1-N2-Flu (20 nM) cfu/ml		
scFv-phage	0	Ca^{2+}	Mg^{2+}	0	Ca^{2+}	Mg^{2+}	0	Ca^{2+}	Mg^{2+}	0	Ca^{2+}	Mg^{2+}
Short	4	2	⩽1	48	1376	154	⩽1	⩽1	⩽1	1760	960	6300
Medium	⩽1	⩽1	36	8080	3360	49,000	⩽1	⩽1	⩽1	1530	200	9700

The two different non-infective scFlu phages were incubated ON with the different N-terminal domains of gIIIp crosslinked or not crosslinked to the antigen. The cells were grown in 2YTG without addition of Mg^{2+}. The cells were used directly or they had 50 mM Ca^{2+} or 50 mM Mg^{2+} added immediately before use. The different types of interactions investigated here are shown schematically in Figure 5. However, this Table also includes the controls without fused antigen (N1 and N1-N2).

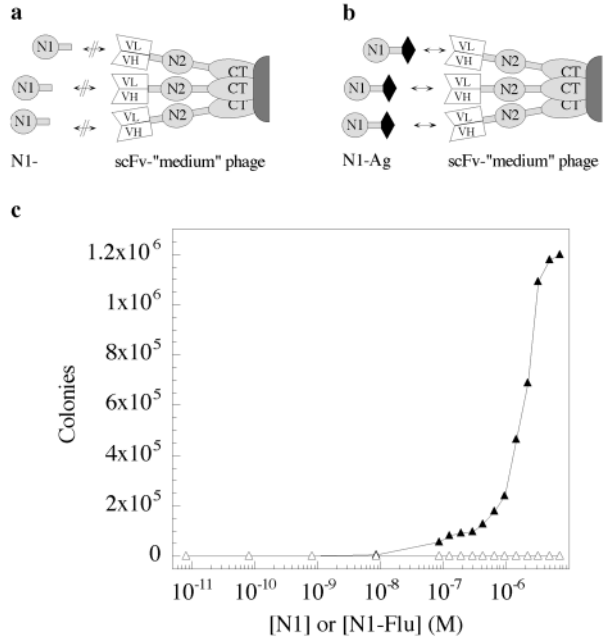

Figure 6. Titration of infectivity and specificity of scFlu-medium phage using N1 (△) and N1-Flu (▲). Schematic depiction of the experiment: a, in the absence of coupled antigen (△); b, in the presence of coupled antigen (▲); c, colonies obtained as a function of N1 or N1-Flu using 10^{10} scFv-display phage.

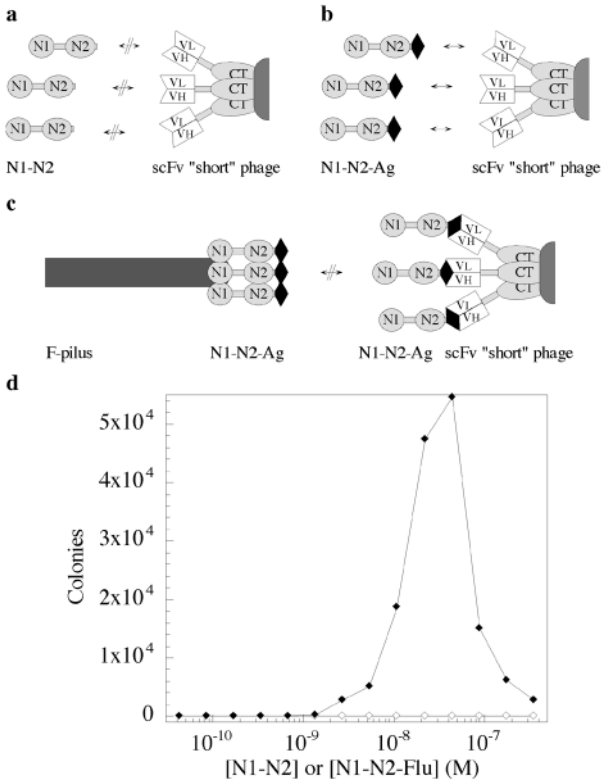

Figure 7. Titration of infectivity and specificity of scFlu-short phage using N1-N2 (◇) and N1-N2-Flu (◆). Schematic depiction of the experiment: a, in the absence of coupled antigen (◇); b, in the presence of coupled antigen (◆); c, in the presence of high, inhibitory concentrations of N1-N2-Flu; d, colonies obtained as a function of N1-N2 or N1-N2-Flu using 10^{10} scFv-display phage.

Domains required for SIP and influence of metal ions

The low infectivity of the combination scFlu-short phage and N1-Flu with F^+ cells (Table 2) could be enhanced 30-fold in the presence of 50 mM Ca^{2+}, similar to the effects observed with the N1-Bla-CT fusion (Table 1). Thus, SIP complexes are infective in the absence of N2, but then require Ca^{2+}. In contrast, whenever N2 was present in the SIP experiment (N1-Flu with scFlu-medium phage, or N1-N2-Flu with scFlu-short or scFlu-medium phage), Ca^{2+} had no stimulatory effect, and was even seen to inhibit infectivity slightly (Table 2). The addition of Mg^{2+} to the cells shortly before adding N2-containing SIP-complexes enhanced the specific infectivity by four- to sixfold (Table 2), and the effect of adding Mg^{2+} during growth of the bacteria was slightly stronger still (data not shown). These results suggest the existence of two pathways of infection, one using N2 for interaction with the pili and N1 for entry into the cell, and the other using only N1 in the presence of Ca^{2+}. As a consequence, the different combinations of adapters and scFv-displaying phage have different dependencies on the concentration of the soluble domains (Figures 6 and 7, Table 2), and this knowledge allows us now to choose between them and to ''tailor'' selection experiments.

Discussion

The principle of selectively-infective phage makes use of the modular structure of the infection mediating protein gIIIp of filamentous phage. In SIP infectivity of an otherwise non-infective phage particle is restored upon occurrence of a protein-ligand interaction, which brings together the three domains of gIIIp (Figures 2 and 5). For the understanding of the function of the various components of the system, which is the prerequisite for the further development of SIP as a highly efficient selection tool, we had to identify the consequence of interrupting gIIIp at various points. Furthermore, the roles of the individual gIIIp domains in the infection process had to be clarified.

From β-lactamase insertion experiments, we found that increasing the spacing between gIIIp domains and the presence of a bulky protein did not destroy the infection process. However, as different positions of insertion led to different reductions of the infection efficiency, we must conclude that the three domains are not completely independent in their function. The ability of gIIIp

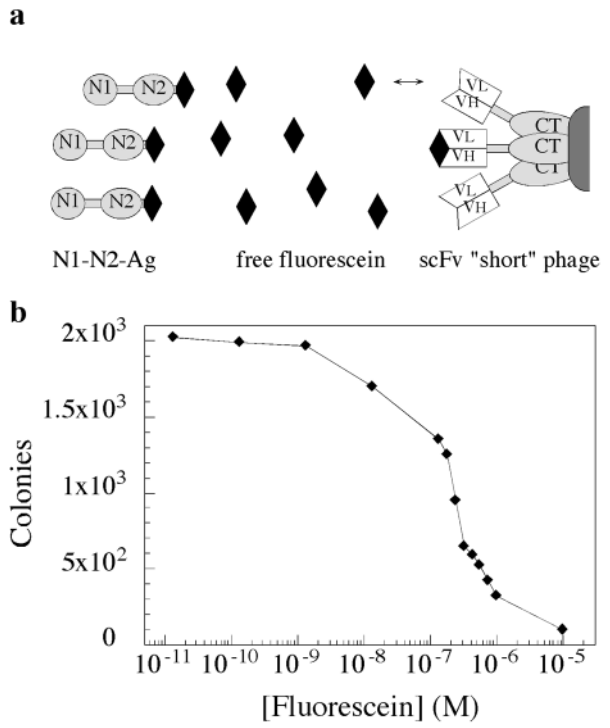

Figure 8. Competitive inhibition by free fluorescein of the infectivity of a SIP complex formed by scFlu-short phage and N1-N2-Flu. a, Schematic depiction of experiment. b, Colonies obtained as a function of the fluorescein concentration using 10^9 scFv-display phage and 3×10^{-8} M N1-N2-Flu adapter.

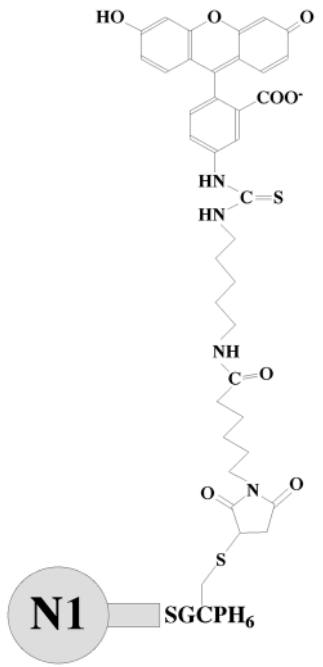

Figure 9. Structure of the chemical conjugate used in the experiments, coupled to N1 (as shown) or to N1-N2 (analogously). The preparation of the conjugate is explained in Materials and Methods.

to tolerate such big inserts in its structure without losing its function will have implications for standard phage display. For example, protein libraries may be inserted within gIIIp at the indicated sites, instead of at the N terminus. This will be advantageous for identifying robust and proteolysis resistant proteins, since only those phages would remain highly infective, from which N1 is not cleaved off.

The N1 domain of gIIIp was found, both from Bla-gIIIp fusion phages and SIP experiments, to be essential for the entry of the phage DNA into the cell under all conditions, consistent with previous reports (Armstrong *et al.*, 1981; Stengele *et al.*, 1990). However, exogenously added N1 domain protein itself does not promote entry of unlinked non-infective phage into the cell; instead, N1 must be physically linked to the phage particle, either directly *via* genetic fusion, or *via* the non-covalent antibody-antigen interaction of SIP.

In contrast, if only the second N-terminal domain N2 is missing in the phage particle, a residual infectivity can be observed, but the ability to infect F^+ recipient cells was much lower than in the presence of N2. This low N2-independent infectivity could be increased by one to four orders of magnitude by the addition of Ca^{2+} (Table 1), consistent with previous results for wt phage (Russel *et al.*, 1988). In the absence of N2 in Bla-gIIIp phage, Ca^{2+} increases infectivity, independent of the presence of an F-pilus (Table 1). 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). Interestingly, the enhancement of infectivity towards F^- cells by Ca^{2+} is lower by about one to two orders of magnitude if N2 is present (compare N1-Bla-CT with N1-Bla-N2-CT in Table 1). In SIP experiments Ca^{2+} even acts as an inhibitor (by a factor of 6 to 50) in the presence of N2 (Table 2).

This suggests that there are two pathways of infection (F-pilus independent, strongly stimulated by Ca^{2+} and F-pilus dependent, requiring N2) which partially interfere with each other. 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 (Table 2) (Duenas & Borrebaeck, 1994; Duenas *et al.*, 1996), but it appears to be necessary to use Ca^{2+} treated cells to overcome the loss of N2 (data not shown). We cannot distinguish whether Ca^{2+} has merely a direct effect on the cell envelope or may indirectly influence uptake *via* the TolQRA system (Sun & Webster, 1987; Webster, 1991; Braun & Herrmann, 1993). Consistent with these ideas of

N2 function, the N-pilus specific phage IKe has no homologue of N2 (Peeters *et al*., 1985), but does have domains homologous to N1 and CT of fd. Instead of N2, a different receptor domain is inserted in front of N1 of IKe (Endemann *et al*., 1992). Further interactions between the domains of gIIIp are suggested by the failure of functional domain interchange between fd and IKe (Endemann *et al*., 1993). One possible explanation for the results described is that a common precursor, possessing only N1 and using the above-mentioned pilus-independent pathway, diverged into fd and IKe, each using a different pilus type for enhanced infectivity.

The inhibition experiments with different purified N-terminal gIIIp domains showed that N1 was less efficient at inhibiting infection than N2 or N1-N2 (Figure 4). This suggests that N1 interacts with its partner with a lower binding constant than N2 and probably at different sites: N1 may directly interact with the *E. coli* TolQRA-system (Sun & Webster, 1986; Russel *et al*., 1988; Webster, 1991) at micromolar concentrations. The Tol-system is known to be used both by several colicins and phages as an uptake mechanism, probably by transducing energy-dependent conformational changes (Braun & Herrmann, 1993; Lazdunski *et al*., 1995). In contrast, the much stronger inhibitory effect of N2 suggests that it binds at the tip of the F-pilus with higher functional affinity than N1 does at the *E. coli* envelope (Gray *et al*., 1981) (Figure 4).

In SIP experiments, the stronger inhibitory effects of N2 and N1-N2 than of N1 are also reflected in the different adapter concentration optima when using N1-Flu (Figure 6) or N1-N2-Flu (Figure 7). To select for protein-ligand interactions with moderate binding constants, only N1-adapter and medium phage can be used, because of the lower inhibitory effect of N1 at high concentrations. However, even with a very tight antibody-antigen binding system, as used here, high concentrations of the adapter are necessary, possibly reflecting a lower affinity of the N1-Flu / medium-phage SIP-complexes (Figure 5c) to *E. coli* than of the N1-N2-Flu/ short-phage SIP-complex (Figure 5b). This inhibitory effect thus seems to limit the achievable infection efficiency (cf N1-Bla-N2-CT over N1-N2Bla-CT in Table 1). The optimum curve using N1-N2-Flu (Figure 7) must be interpreted as being composed of the antigen-antibody binding curve (upwards slope) and an inhibition curve (downwards slope) (cf Figure 4). The upward (binding) slope has a mid-point at approximately 10^{-8} M, despite the fact that the anti-Flu scFv binds the antigen with a K_D of 3×10^{-10} M (Vaughan *et al*., 1996). This suggests, if equilibrium conditions apply, that single antigen-antibody binding events on the phage may not be sufficient for high infectivity, but that several antibody-antigen pairs have to form on one phage. This multivalent docking of the phage to the pilus may also explain why the inhibition of infection by free fluorescein requires a concentration as high as 10^{-7} M (Figure 8). Thus, SIP will strongly favour antibody-displaying phages with high binding constants, when low concentrations of the adapter N1-N2-Ag are used. The examination of this concentration dependence was made possible by the purification of the gIIIp domains and the adapter molecules and allowed us now to adjust the selection pressure in a rational way by changing adapter concentration. A biophysical understanding, however, will also require a more detailed knowledge of the mechanism and kinetics of the infection process.

In previously reported experiments, the cognate partners were genetically fused (Duenas & Borrebaeck, 1994; Gramatikoff *et al*., 1994; Krebber *et al*., 1995). We have now shown that SIP can also be used with chemically coupled partners, thus expanding the range of possible interaction systems to organic molecules, sugars, eukaryotic proteins containing post-translational modifications or even proteins composed of D-amino acids (Schumacher *et al*., 1996).

SIP experiments conducted without fluorescein present in the adapter molecule, using scFv-fusion phage and only N1 and N1-N2, showed only one to ten infection events from an input of 10^{10} phage particles, whereas for cognate antibody-antigen pairs under the most favourable conditions 10^4 to 10^5 (using anti-Flu short phage and N1-N2-Flu) and even 10^6 infection events (using anti-Flu medium phage and N1-Flu) could be detected. This low background infectivity rate shows that SIP is an extremely effective and highly specific method to select for cognate interaction events. When compared to phage display, a much lower background is observed, and in this one-step procedure the time consuming and generally inefficient physical separation of cognate and non-specifically binding phages is obviated. SIP thus appears to be a very powerful method to select for ligand-binding proteins. In addition, the SIP strategy can possibly be extended to eukaryotic systems, where it might be used to design a vector for gene therapy, in which a virus is only infective in the presence of an exogenously added adapter protein.

Materials and Methods

Bacterial strains

JM83 *(ara, Δ(lac-proAB), rpsL (strA), thi, ϕ80, lacZΔM15)* and XL1-Blue *(recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac [F'proAB lacI^Q ZΔM15* Tn*10 (Tet^r)])* were conjugated to construct JM83-F$^+$. This was achieved by mixing exponentially growing cultures of the two strains for one hour at 37°C and subsequently plating these on 2xYT agar containing 30 μg/ml str and 15 μg/ml tet (Miller, 1972). JM83-F$^+$ bacteria and the F$^-$ strain JM83 constitute an isogenic pair that could be used as recipients to test the influence of the F-pilus on infectivity of the Bla-gIIIp fusion phages. Both bacterial strains were grown in 2YTG (2xYT, 0.5% (w/v) glucose, 1 % (v/v) glycerol, 50 mM $MgCl_2$) containing 30 μg/ml str and 15 μg/ml tet, or 30 μg/ml str, respectively. XL1-Blue, which was used as host for the phage production

and as the recipient for the infection by SIP complexes, was grown in 2YTG supplemented with 15 μg/ml tet. All bacterial cells were grown to an A_{550} of 0.75 to 0.9 when used as recipient for infection experiments.

Construction of phage vectors

The scFv-displaying phage was constructed from the cam^R derivative of the fd phage fCKC as described previously (Krebber *et al.*, 1995). Replacing either the first N-terminal domain of gIIIp in the medium or both N-terminal domains in the short phage by a scFv fragment cloned between the *Rca*I and *Eco*RI restriction sites led to the production of non-infective scFv-displaying phage (Figure 3e). The anti-Flu scFv FITC-E2 was amplified by PCR from pCANTAB-FITC-E2 (Vaughan *et al.*, 1996) and cloned into the SfiI sites of a scFv-gIII fusion (Figure 3e).

The β-lactamase gIIIp-insertion and deletion fusions were constructed by replacing the SfiI scFv-cassette in the medium and short phage (Figure 3e) with the β-lactamase gene, PCR-amplified from pUC19 (Figure 3b). The *Rca*I-*Sfi*I *pelB* signal sequence cassette was replaced by a fragment encoding the N-terminal domains of gIIIp, including their gIII signal sequence, PCR-amplified from M13mp19 (Figure 3b). In the case of N2Bla-CT, N2 was initially fused behind a *ompA* signal sequence and then inserted as *Rca*I-*Sfi*I cassette (Figure 3b). The expression of all phage gIIIp-fusions was under the control of the natural gIII promoter.

Growth and testing of the phages

Phages conferring cam resistance were produced from single colonies of XL1-Blue grown in 2YTG containing 15 μg/ml tet and 25 μg/ml cam ON at 37°C. All cultures of Bla-gIIIp fusion phages also contained 50 μg/ml amp. To test for the ability of the phages to produce plaques, culture supernatants were passed through an 0.22 μm filter and streaked on 2YTG agar containing 15 μg/ml tet. The plates were dried, and 5 ml top-agar containing 500 μl XL1-Blue cells were poured on the streaked plates.

The scFv-displaying phages were tested for binding to their antigen by standard ELISA (Krebber *et al.*, 1995), using FITC chemically coupled to lysine of human transferrin (Fluka). In order to check specificity of binding, inhibition ELISAs were performed using soluble fluorescein as competitor (data not shown). The number of phage particles was estimated by spectrophotometry (Smith & Scott, 1993).

Production of gIIIp domains and coupling to antigen

For the production of N1, N2 and N1-N2 proteins (Figure 3d), corresponding fragments were PCR-amplified from M13mp19 using Vent-polymerase (New England Biolabs) with primers which delete the signal sequence and introduce an N-terminal methionine and a C-terminal Ser-Gly-Cys-Pro-His_6 tag. Fragments were cloned into the vector pTFT74 (Freund *et al.*, 1993) under the control of the T7 promoter. The N1, N2, and N1-N2 genes (without the signal sequence) were expressed in BL21(DE3), where N1 required the presence of pLysS (Studier & Moffat, 1986), and obtained as cytoplasmic inclusion bodies. Purification was carried out using the coupled IMAC-AIEX protocol, in the presence of 8 M urea (Plückthun *et al.*, 1996). The protein was refolded by dialysis against 0.2 M Tris-HCl (pH 8.5), 0.4 M arginine, 0.2 M guanidinium-hydrochloride, 0.1 M $(NH_4)_2SO_4$, 2 mM EDTA, using a redox shuffle containing 1 mM oxidized glutathione and 0.2 mM reduced glutathione (Freund *et al.*, 1993). Micro sequencing and mass spectroscopy revealed that the methionine of the engineered start codon was cleaved, releasing the expected sequence NH_2-AETVE for N1 and N1-N2 and giving the appropriate molecular mass. To remove the covalently linked glutathione, introduced by the refolding procedure, the N1 and N1-N2 proteins were mildly reduced using 5 mM dithiothreitol in 25 mM Tris-HCl (pH 8.5) at 4°C ON. The dithiothreitol was removed by gel filtration on a Sephadex G-25 (Pharmacia) column in 25 mM Na-phosphate (pH 6.8). Protein concentrations were calculated from A_{280} (Gill & von Hippel, 1989). Since the scFlu antibody was selected with FITC coupled to lysine (Vaughan *et al.*, 1996), but the directional coupling had to be to cysteine, we coupled 5-((5-aminopentyl)thioureidyl)fluorescein (FluCad) (Molecular Probes Inc.) to the heterobifunctional crosslinker *N*-succinimidyl-6-maleimidocaproate (Fluka) *via* its primary amine (Figure 9). The reaction was carried out in DMF for one hour at 30°C in the dark, at a ratio of FluCad to crosslinker of 3:2. Completion of the reaction was controlled by thin layer chromatography. This reaction mixture was reacted, with FluCad in a threefold molar excess, with the free sulphydryl group of N1 or N1-N2 proteins for one hour at 25°C and then at 4°C ON. The resulting adapter proteins (Figure 9) were gel filtrated on a Sephadex G25 column in 50 mM Tris (pH 7.5). The N-terminal domains of gIIIp, which were labelled with fluorescein, were separated from the unlabelled in the same buffer on a perfusion chromatography HQ column with a NaCl gradient (0 to 600 mM), using a BioCAD 60 system (Perseptive Biosystems). The success of the process was monitored by micro sequencing, SDS-PAGE, mass spectroscopy, and spectrophotometry, all of which together indicated that the adapter proteins, N1-Flu and N1-N2-Flu, were 99% pure.

Infection experiments

For quantification of the infectivity of β-lactamase-gIIIp fusions, phage culture supernatants were PEG precipitated (0.25 vol. 17% PEG6000, 3.3 M NaCl, 1 mM EDTA; 30 minutes, 4°C; 30 minutes, 4°C, 4500 ***g***) and redissolved in TBS (25 mM Tris-HCl, 140 mM NaCl, 2.7 mM KCl, pH 7.4). Bla-gIIIp fusion phage were added to JM83 and JM83-F^+ cells and these cultures were shaken for one hour at 37°C prior to plating on 2YTG agar containing 30 μg/ml str and 50 μg/ml amp, and 15 μg/ml tet in the case of F^+ cells. In order to titre the phage fCKC, amp was omitted from the medium. The influence of Ca^{2+} on the different Bla-gIIIp fusion constructs was tested by mixing 1 ml of JM83 and JM83-F^+ with 50 μl of 1 M $CaCl_2$ immediately before addition to the phage.

The inhibitory effect of N1, N2 and N1-N2 was measured by mixing 10 μl of 10^4-fold diluted N1-N2-Bla-CT fusion phage with 10 μl of different concentrations of N1, N1-Flu, N2, N1-N2 or N1-N2-Flu. These mixtures were added to 80 μl of XL1-Blue cells, shaken for one hour at 37°C and subsequently grown on 2YTG agar containing 25 μg/ml cam, 50 μg/ml amp, 15 μg/ml tet ON at 37°C.

For SIP experiments, 5 μl of scFv-displaying phage supernatants, concentrated 200 times by PEG precipitation, was incubated with 10 μl of different concentrations of the N1, N1-Flu, N1-N2 or N1-N2-Flu proteins. After ON incubation at 4°C, 85 μl of XL1-Blue cells was added and the mixture was incubated for one hour at

37°C with shaking. Aliquots and dilutions were subsequently plated on 2YTG agar containing 25 μg/ml cam, 15 μg/ml tet. The inhibitory effect of fluorescein on SIP experiments was measured by preincubating the phage with N1-N2-Flu and free fluorescein ON. The dependence of SIP on Mg^{2+} and Ca^{2+} was investigated by growing XL1-Blue without any addition of Mg^{2+}, and subsequently adding 50 μl of 1 M $MgCl_2$ or $CaCl_2$ to 1 ml of cells immediately before use. The indicated concentrations of the proteins N1, N1-Flu, N2, N1-N2, N1-N2-Flu and fluorescein always refer to their final concentrations in the presence of bacteria.

Acknowledgements

We thank P. Hunziker for mass spectroscopy, N. Birchler for protein sequencing, G. Wall and L. Nieba for critically reading the manuscript, B. Lindner for technical assistance (University Zürich), V. Ilag (MorphoSys GmbH) for discussions on SIP experiments, A. Williams (Cambridge Antibody Technology Ltd., Melbourn, UK) for pCANTAB-FITC-E2 and the Schweizerische Nationalfonds (grant 3100-037717.93/1) and MorphoSys GmbH, Munich for financial support.

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Edited by I. B. Holland

(Received 4 November 1996; received in revised form 17 February 1997; accepted 19 February 1997)