Size of the ligand complex between the N-terminal domain of the gene III coat protein and the non-infectious phage strongly influences the usefulness of *in vitro* selective infective phage technology

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The selective infective phage (SIP) technology allows a rapid positive selection of interacting pairs of biological molecules that restore to non-infectious phages their ability to infect the bacterial host. After a successful infection, the phage is amplified and the DNA encoding the interacting ligand is isolated from the phage genome and sequenced. In our studies we have evaluated the usefulness of SIP for the identification and cloning of proteins interacting with a biotinylated target binding to a newly designed adapter molecule consisting of streptavidin fused to the C-terminus of the extracellular domain of the phage minor coat protein III. The new adapter was expressed in *Escherichia coli* and refolded from inclusion bodies. The two different domains joined within the chimaera were found to be biologically functional. We also demonstrated that non-covalent interactions

between a non-infectious phage displaying a short peptide, which specifically binds the streptavidin, and the adapter molecule restore phage infectivity. To evaluate the potential of SIP as a general and generic tool for the screening of cDNA libraries that encode the ligands displayed at the surface of the phage and binding to biotinylated targets, we have increased both the size of the displayed ligand on the phage and the size of the biotinylated target bound to the streptavidin domain of the adapter molecule. In our model systems we show that the size of either the ligand or the target is a limiting factor for the technology.

Key words: biotinylated targets, cDNA cloning, fusion proteins, protein–protein interactions.

INTRODUCTION

Protein–protein interactions have a crucial role in the cellular mechanisms responsible for the control of pathways involved in the normal state or the disease state. The ability to modulate these interactions within cells with suitable inhibitors would be a powerful method of elucidating the functional role of the proteins involved in different regulatory pathways.

Classical expression cloning [1] as well as yeast dual hybrid cloning systems [2–4] have permitted the cloning of many different pairs of proteins. More recently, cDNA-encoded proteins were expressed and displayed at the surface of filamentous phage [5,6]. The method has permitted the identification and cloning of novel proteins that interact with known targets [6–10]. A significant advantage of this novel technique is that the selection procedure is done completely *in vitro*, thus giving almost unlimited freedom in modifying and modulating the selection conditions. Later, positive selection procedures were developed in which only phages displaying the correct protein partners at their surface could be propagated. These methods are called selective infective phage (SIP) [11,12], direct interaction rescue ('DIRE') [13] or selection and amplification of phage ('SAP') [14]. In essence, in all of these methods, a fusion protein consisting of the N-terminal domain of the gene III minor coat protein of the phage and a target molecule able to interact directly with a ligand displayed on the N-terminal extremity of the truncated gene III protein C-terminal domain is used. The phage is non-infectious, because it lacks an intact gene III protein. Non-covalent inter-

action between the target and the ligand protein allows the formation of a modified but functional minor coat protein III at the surface of the phage that restores the ability of the phage to infect host cells. The principle of the method is shown in Figure 1.

The aim of our studies was to evaluate SIP as a generic method for the identification of cDNA-encoded proteins interacting with a target molecule. Any biotinylated target molecule will interact with the streptavidin domain of a generic adapter. This adapter is a fusion protein made with the N-terminal domain of the phage gene III coat protein and streptavidin. It will allow the reconstitution of a functional gene III protein by the association of the complex with the ligand displayed by the phage. If this method works, then a single cDNA library cloned and expressed by the phage should make it possible to identify binding partners immediately by incubating the library, the adapter and any available biotinylated proteins. We expected that the size of the ligand encoded by the cDNA and presented by the non-infectious phage, as well as the size of the complex formed by the adapter with the N-terminal domain of the gene III protein and the biotinylated target, would influence the probability of recovering infectious phage. Thus, as shown in Figure 1, we gradually increased both the size of the phage displayed ligands and the size of the biotinylated targets in our experiments. This was achieved with a Fab antibody as ligand interacting with the biotinylated anti-idiotypic antibody (Figure 1A), or with a known biotinylated peptide epitope (Figure 1B). At the extreme, we took a streptavidin-binding peptide as ligand displayed on the

Abbreviations used: IPTG, isopropyl β -D-thiogalactoside; LB medium, Luria Broth medium; mAb, monoclonal antibody; SIP, selective infective phage.

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Figure 1 Manipulation of the size of the protein complex present between the C-terminal domain of the gene III protein (CT) and the streptavidin domain of the adapter

(A) The largest complex formed with the adapter and used in these studies was made with a biotinylated anti-idiotypic antibody recognized by the idiotype antibody displayed at the surface of the phage. (B) The size of the complex was decreased by substituting the anti-idiotypic antibody with the peptide epitope recognized by the displayed antibody. (C) The smallest complex used was to display a streptavidin-binding peptide. Strep-tag II has also been further separated from the phage by inserting 123 residues of the Grb2-SH2 domain. N1 and N2 are the two subdomains of the N-terminal domain of the gene III coat protein.

phage without any other intermediate between it and the adapter molecule (Figure 1C). All combinations were assessed for their influence on SIP.

EXPERIMENTAL

Bacterial strains, constructions of the plasmid and the phagemid vectors

Escherichia coli TG1 or XL1-Blue cells (Stratagene, La Jolla, CA, U.S.A.) were used in our studies. For the rescuing and the formation of normally infectious phage from phagemid vectors, the helper phages VCSM13 or M13K07 (Stratagene) were used and the rescuing was performed as described earlier [15]. The pKN16 phage was a gift from Dr G. Smith (University of Missouri, St Louis, MO, U.S.A.).

The replicative form of the M13K07 DNA was used as a PCR template for the construction of the helper phage lacking the complete gene III. After amplification with the oligonucleotides MG235 and MG236 (Table 1), the whole helper DNA lacking exactly the gene III sequence was purified from a 1% (w/v) agarose gel, digested with *Bam*HI and ligated [16]. After elimination of the *Bam*HI site by deletion mutagenesis, the ribosome-binding site of gene III was optimally placed in front of the ATG of gene VI, the next gene in the gene III operon. This helper phage was called M13 Δ3.

The base plasmid used for the construction of all the vectors was pXI196. It was derived from pTK2552/B described previously [15], in which the *Not*I restriction site present behind the gene encoding the viral coat protein III was removed.

The plasmid pXI264 expresses the gene III minor coat protein in *E. coli* cells under the regulation of the *lacZ* promoter and can complement *in trans* a gene-III-deficient helper phage. For the construction of this plasmid, the DNA encoding the mature

Table 1 DNA sequences of the oligonucleotides

The restriction nucleases indicated in parentheses cleave the DNA at the sequences indicated in bold. Underlined are the sequences not derived from the sequence to be amplified, but added with the oligonucleotides for the cloning strategy.

Oligonucleotide	Sequence
MG220	ATATG CGGCCGC ATAGAAGCAGCAGACGG (<i>Not</i> I)
MG221	ATAT GAATTC ATATGGCTGAAGCT (<i>Eco</i> RI)
MG222	ATAT GAATTC CGCCACCCTCAGAACCGCCACCCTC (<i>Eco</i> RI)
MG223	ATAT CATATG GCTGAACTGTTGAAAG (<i>Nde</i> I)
MG235	TATAG GATCC GTTGAAATCTCCAAAAAAGGCTC (<i>Bam</i> HI)
MG236	ATAT CCATGG CTGAACTGTTGAAAGTTG (<i>Nco</i> I)
MG237	TATAG GATCC ATGCCAGTCTTTTGGGTATTC (<i>Bam</i> HI)
MG238	GAATATT GGGCCCTGGCATGATTAAGACTCC (<i>Ssp</i> I)

version of the gene III protein was amplified by PCR from M13K017 with oligonucleotides MG236 and MG238 (Table 1). After complete digestion with *Nco*I and partial digestion with *Ssp*I, to keep the internal *Ssp*I site within the gene III, the fragment was cloned into pXI196 digested with the same enzymes. A correct clone was called pXI264.

The two genes encoding a variable light chain VI fused to a C κ domain and a variable heavy chain Vh fused to the CH₁ domain of an immunoglobulin were cloned into pXI196. The heavy chain was fused to gene III encoding the minor coat protein, and the gene encoding the immunoglobulin VI-C κ chain was cloned into a newly created *Asc*I restriction site. Both genes are under the control of a *lacZ* promoter and express an antibody Fab fragment that is displayed at the surface of a rescued phage as a fusion protein to the C-terminal domain of the gene III minor coat protein. The phagemid was constructed by using standard cloning techniques [1]. Finally the genes encoding the Vh and VI domains of the anti-tumour monoclonal antibody (mAb) 763.74 [17] were amplified by reverse-transcriptase-mediated PCR from the hybridoma cell line producing the antibody and cloned between *Xho*I/*Nhe*I and *Rsr*II/*Avr*II restriction sites in the new vector. The latter plasmid now encoded an antibody Fab fragment specific for the human melanoma-associated chondroitin sulphate proteoglycan antigen and was called pXI261.

A synthetic DNA fragment encoding a peptide defining strep-tag II [18] was cloned between the *Nco*I and *Spe*I sites in pXI196 and we thus obtained the plasmid called pXI265.

In pXI260 we constructed a fusion gene encoding both the N1N2 domain of minor coat protein III corresponding to residues 1–233 of the mature protein [19] and the streptavidin core protein. The DNA fragment encoding the N1N2 domains was amplified by PCR from M13mp18 (New England Biolabs, Beverly, MA, U.S.A.) with MG223 and MG222 (Table 1) and was cloned into the pET23b vector (Novagen, Madison, WI, U.S.A.) between the *Nde*I and *Eco*RI restriction sites. In a second step, the gene encoding streptavidin was amplified by PCR with the oligonucleotides MG221 and MG220 (Table 1) and cloned into the last plasmid. The streptavidin gene was generously supplied by DuPont Merck [20].

The restriction maps of the different plasmids are shown in Figure 2.

Phagemid rescuing with a helper phage lacking the gene III minor coat protein

Non-infectious phages were obtained by performing a double transformation with a phagemid DNA and the purified DNA of the mutant helper phage M13 Δ3 into *E. coli* cells. A single

Figure 2 Map of the different phagemid vectors used in our studies

The framework for the different vectors is plasmid pXI196. The heavy-chain Vh-CH1-Jun dimerization domain–gene III coat protein fusion and the light-chain VI-C κ encoding the anti-melanoma mAb 763.74 Fab fragment were expressed from pXI261, whereas the short strep-tag II–gene III coat protein fusion was expressed from pXI265. The mature gene III minor coat protein was expressed from pXI264. All these recombinant genes were under the control of the *lacZ* promoter, and the proteins were directed to the periplasmic space of *E. coli* with the pectate lyase B leader sequence (pelB). In pXI260, the adapter gene encoding the N-domain of the gene III coat protein fused to the streptavidin core protein was cloned between the *NdeI* and *XhoI* sites of the vector pET23b. ColE1 ori, plasmid replication origin in *E. coli*; f1 ori, phage origin or replication.

colony of doubly transformed cells was grown in Luria Broth (LB) medium [1] containing ampicillin (50 μ g/ml), kanamycin (30 μ g/ml) and 1% (w/v) glucose to a D_{600} of 0.8 at 37 °C. After centrifugation at 2500 g for 10 min, the cells were resuspended in the same medium depleted of glucose but containing 1 μ M isopropyl β -D-thiogalactoside (IPTG) and incubated further overnight at 30 °C with shaking at 150 rev./min. After centrifugation under the same conditions the phages present in the growth medium were recovered by PEG8000/NaCl precipitation as described earlier [15] or harvested by ultrafiltration on a 400 ml stirred cell from Millipore and a VM100 Amicon filter [21].

Expression and purification of the fusion protein made with the N-terminal domain of minor coat protein III and streptavidin

The fusion protein made between the N1N2 domain of minor coat protein III and streptavidin was recovered as intracellular inclusion bodies. A single colony of the *E. coli* strain BL21(DE3)(pLysE) (Novagen) transformed with the plasmid pXI260 was grown in five 2-litre Erlenmeyer shaking flasks, each containing 500 ml of LB medium, in the presence of 10 μ M biotin under chloramphenicol (34 μ g/ml) and ampicillin (50 μ g/ml) selection up to a D_{600} of 0.5 at 37 °C. At that stage, a non-induced aliquot was taken from the growing culture, and cultures were continued for a further 2 h after the addition of 0.4 mM IPTG. After centrifugation, the cells were resuspended

in 8 vol. (wet cell basis) of lysis buffer [50 mM Tris/HCl (pH 8.0)/5 mM dithiothreitol/5 mM benzamidine HCl/5 mM EDTA] and lysed at 175 MPa in a continuous-flow French press. The inclusion bodies were recovered by centrifugation at 17000 g and resuspended in the same volume of lysis buffer with the help of a Heidolph DIAX600 homogenizer (Merck, Dietikon, Switzerland). After a further centrifugation under the same conditions, the pelleted inclusion bodies were recovered in 4 vol. of lysis buffer and resuspended. This last step was repeated once, and finally the inclusion bodies were resuspended in water containing 5 mM dithiothreitol. The denaturation and renaturation of the inclusion bodies were performed as described [19]. The adapter protein was stored at a concentration of approx. 100 μ g/ml protein at –80 °C until use. From an HPLC analysis we estimate that we purified 2.6 mg of adapter protein/litre of original culture.

Biological testing of the two functions of the fusion adapter protein domains

The integrity of the streptavidin-binding domain of the fusion protein was tested by ELISA as described below. Instead of the labelled anti-M13 antibody, horseradish peroxidase conjugated to biotin was used at a concentration of 1 μ g/ml.

The functionality of minor coat protein III N1N2 domain was tested by its activity in competitively inhibiting VCSM13 helper phage from infecting bacteria. The serial dilution of the adapter

and a constant amount of infectious phage (10^4 plaque-forming units) were added to 100 μ l of *E. coli* XL1-Blue cells grown to a D_{600} of 0.5 in LB medium and incubated for 5 min at 37 °C before being spread on selective plates. The infectious phages were counted as colony-forming units after incubation at 37 °C. As a negative control the adapter fusion protein was replaced by streptavidin.

Chemiluminescent phage ELISA

The wells of a Microlite 2 immunoassay plate (Dynatech Laboratories, Chantilly, VA, U.S.A.) were coated with of the suitable proteins (or BSA as negative control) at 2 μ g per well in 100 μ l of 50 mM Tris/HCl, pH 8.1. After blocking with Blotto [5% (w/v) non-fat dried milk and 0.05% (v/v) Tween 20 in 10 mM Tris/HCl (pH 7.5)/150 mM NaCl (TBS)], the phages in 100 μ l were added for 2 h at 37 °C. The wells were washed 10 times with TBS/0.05% Tween 20; anti-M13 antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Dübendorf, Switzerland) was added at a 1:4000 dilution in Blotto. After incubation at room temperature for 1 h, followed by the same washing steps as described above, luminol, a chemiluminescent substrate (BM Chemiluminescence ELISA Reagent; Roche Diagnostics, Rotkreuz, Switzerland) was added. Readings of emitted light were recorded as relative units (Microlumat LB96P; Berthold, Regensdorf, Switzerland).

Infection of bacterial cells with selectively infective phages

Phages were obtained from *E. coli* cells co-transformed with both the mutant helper phage M13 Δ 3 and the phagemid encoding the ligand molecule to be displayed. Roughly 10^{12} non-infectious phages were added to 20 μ l (approx. 2 μ g) of the adapter protein and to the biotinylated target molecule in a final volume of 30–50 μ l. After incubation overnight at 4 °C, a vast excess (20 ml) of *E. coli* cells grown in LB medium to a D_{600} of 0.8 was added and incubated at 37 °C for 1 h with shaking at 80 rev./min. The cells were recovered by centrifugation and resuspended in 2 ml of LB medium. Aliquots (200 μ l) were plated on LB agar containing 150 μ g/ml carbenicillin. Phages recovering infectivity were counted as colony-forming units after incubation overnight at 37 °C.

Biotinylation of target proteins

The anti-idiotypic antibodies MK2.2-CH γ 1 and mAb 763.74 have been described [22]. The biotinylation reaction was performed with the EZ-link sulpho-NHS-LC-biotinylation kit from Pierce (Rockford, IL, U.S.A.).

RESULTS

Construction of a generic adapter combining the functions of streptavidin and the N1N2 domain of gene III coat protein

For the expression cloning of proteins interacting with either a proteinaceous or a non-protein target we devised a generic cloning system *in vitro* based on SIP. For this purpose an adapter molecule linking streptavidin to the domain of minor coat protein III was constructed. On induction with IPTG the fusion protein encoded by pXI260 was expressed in *E. coli* BL21(DE3)pLysE cells and analysed by SDS/PAGE. A major band corresponding to the expected molecular mass of a protein comprising 375 amino acid residues was observed (results not shown). The protein was expressed as non-soluble inclusion bodies and purified. A successfully refolded adapter molecule requires that both the biotin-binding site of the streptavidin

domain is restored and that the N-terminal domain of the minor coat protein recognizes and binds to the bacterial pilus. The experiments shown in Figures 3(A) and 3(B) demonstrate that we did indeed recover an adapter molecule in which both domains were biologically functional. However, as shown by the experiment described in Figure 3(C), 0.125 mol of adapter coated in the wells significantly bound less biotin peroxidase than 0.083 mol of streptavidin. In further control experiments, we demonstrated by ELISA that the binding of a biotinylated target to the adapter was competitively blocked by the addition of biotin (results not shown; see also Figure 4A). The important observation is that both tasks of the adapter were biologically functional.

Construction of the non-infectious M13 Δ 3 helper phage

The SIP technology requires that the phage displaying the ligand molecule be non-infectious in itself. Therefore this phage must lack functional minor coat protein III [11,14,23–25]. In preliminary experiments we used the non-infectious pKN16 phage for rescuing the bacterial cells transformed with the phagemid encoding the ligand molecule. This helper phage is non-functional, because of an in-frame deletion of the extracellular domain of the gene III protein. As a further alternative we also constructed the M13 Δ 3 helper phage, which lacked the complete gene III coding sequence. Phage virions were recovered from the medium of the *E. coli* cells grown after successful transformation with the respective plasmid DNA species. We were unable to detect the formation of infectious phage (results not shown). However, the phage's ability to infect bacteria was restored if an active gene III product was complemented *in trans* from the plasmid pXI264. During its construction this plasmid has lost the oriF1 present in the parental phagemid and cannot be packaged into phage. Under these conditions we recovered 7×10^{10} plaque-forming units of M13 Δ 3/ml.

Display of target molecules on non-infectious M13 Δ 3 phage and study of their interactions with the adapter in ELISA

For the evaluation of SIP, we used the phagemid pXI261 encoding a Fab antibody fragment derived from the anti-tumour mAb 763.74. Both the anti-idiotypic antibody MK2.2-CH γ 1 and the Val-His-Leu-Asn-Tyr-Glu-His heptapeptide epitope specifically recognized by mAb 763.74 were available [22]. In Figure 4(A) we show that the biotinylated anti-idiotypic mAb MK2.2-CH γ 1 bound to the adapter coated in the wells of a microtitre plate. The binding was specifically inhibited by an increase in biotin concentration. Figure 4(B) shows that the phage displaying the Fab antibody fragment bound to the anti-idiotypic antibody, whereas a control phage did not. In Figure 4(C) we show with a competition ELISA that the phage displaying the anti-melanoma Fab antibody fragment specifically bound to the biotinylated anti-idiotypic mAb MK2.2-CH γ 1 coated to the well through the adapter molecule. In this case the interactions between the non-infectious phage and the target were specifically inhibited by an increasing concentration of the competitor mAb 763.74. Similar results were obtained if the antibody MK2.2-CH γ 1 was replaced in the ELISA by the biotinylated Val-His-Leu-Asn-Tyr-Glu-His peptide epitope (results not shown). Taken together, these results show that the phages displaying the Fab antibody fragment bind specifically to the adapter through the intermediation of biotinylated targets.

Phages displaying the anti-melanoma Fab antibody fragment were prepared either with the non-infectious helper phage M13 Δ 3 or with pKN16. *E. coli* cells were doubly transformed with pXI261 and either pKN16 or M13 Δ 3 DNA. Phage were

Figure 3 Both protein domains of the adapter are biologically functional

(A) Horseradish-peroxidase-conjugated biotin bound specifically to the microtitre plate coated with the adapter (2 μg per well) (●) and not to the plate coated with BSA (2 μg per well) (○). The binding of biotin is shown in relative units (RU). (B) The addition of soluble adapter competitively inhibited the infection of host *E. coli* cells by VCSM13 phage in a 160 μl final reaction volume (○). Streptavidin as competitor did not affect the infection of the bacteria by the phage (●). In the absence of the adapter as competitor the number of recovered infectious phage was 10000 colony-forming units (cfu). (C) Horseradish-peroxidase-conjugated biotin bound in a concentration-dependent manner to the microtitre plate coated either with the adapter (2 μg per well) (○) or with streptavidin (2 μg per well) (●) as in (A); the binding of biotin is shown in RU.

Figure 4 Analysis of the specificity of the different ligand–target interactions required for successful SIP with the example of the mAb 763.74 and its anti-idiotypic antibody

(A) The binding of biotinylated anti-idiotypic mouse/human chimaeric mAb MK2.2-CHγ1 (1 μg per well) against microtitre plate wells coated with 2 μg per well N1N2-streptavidin adapter was inhibited in the presence of an increasing concentration of biotin as competitor. The binding antibody was detected with horseradish-peroxidase-conjugated anti-(human Fc) antibody. (B) Phage displaying the mAb 763.74 Fab fragment bound in a dose-dependent manner to 1 μg of biotinylated anti-idiotypic mAb bound to the microtitre wells through 2 μg per well N1N2-streptavidin adapter (●), whereas an irrelevant phage displaying a random peptide library did not bind under the same conditions (○). (C) The same Fab-phage (10¹² virions per well) bound to wells coated with 0.5 μg per well of the anti-idiotypic mAb only in the absence of free mAb 763.74 competitor. The binding phages were detected with a horseradish-peroxidase-conjugated anti-phage antibody.

Figure 5 Binding of the mAb 763.74 Fab fragment displayed by non-infectious phage prepared with the M13 $\Delta 3$ or pKN16 helper phage in the presence of free mAb 764.74

The phagemid pXI261 was rescued with the helper M13 $\Delta 3$ (○) or with pKN16 (●). The resulting non-infectious phages displaying the mAb 763.74 Fab fragment (10^{10} virions per well) were added to the wells of a microtitre plate precoated with $0.5 \mu\text{g}$ per well anti-idiotypic antibody MK2.2-CH γ 1 and incubated in the presence of the competitor mAb 763.74. The binding phages were detected with a horseradish-peroxidase-conjugated anti-phage antibody.

Figure 6 SIP-dependent infection of *E. coli* cells with a phage displaying strep-tag II

Bacterial infection was dependent on the number of target phage interacting with the adapter molecule in either the presence (○) or the absence (●) of a constant size of foreign irrelevant non-infectious phage population unable to bind to the adapter. The number of phages was measured as colony-forming units (cfu).

obtained and concentrated from the growth medium. We checked spectrophotometrically that the numbers of each virion used in the ELISAs described in Figure 5 were similar. We analysed the binding of both phage populations displaying the Fab antibody fragment on microtitre plates coated with the MK2.2-CH γ 1 antibody in a competition experiment with increasing amounts of free mAb 763.74. As shown in Figure 5, the ELISA signal obtained with the phage prepared with M13 $\Delta 3$ was significantly

higher than that obtained with pKN16-derived virions. In consequence of these results, all further experiments were performed exclusively with phage prepared with M13 $\Delta 3$.

The pXI265-phage displayed the ligand strep-tag II, a decapeptide known to bind to streptavidin. Indeed, phage displaying the strep-tag II binds specifically to streptavidin in ELISA experiments (results not shown).

All of the available ELISA results indicate clearly that both the large and small ligands displayed at the surface of the non-infectious phages are able to bind to their known biotinylated target molecules, and through these interactions the phages bind to the N1N2-streptavidin adapter. These interactions can all be specifically inhibited with the relevant competitor molecules. Thus the prerequisites for successful SIP are fulfilled. With these tools we were able to evaluate experimentally the role of the size of the ligand–target complex and define the limits of tolerance between the coat protein N1N2 domain on the adapter and at the tip of the non-infectious phage.

Size of the complex between the adapter molecule and the phage strongly influences the effectiveness of SIP

The strategy behind our studies aiming at analysing the influence of the size of the complex between the adapter molecule and the non-infectious phage is presented in Figure 1. We wondered whether the size of the complex formed by the displayed ligand, the biotinylated target and the adapter had any influence on the restoration of the infectivity of phage M13 $\Delta 3$. Plasmid pXI261, encoding the mAb 763.74 Fab fragment, and pXI265, encoding strep-tag II, are shown in Figure 2. On rescue with the helper phage M13 $\Delta 3$, the two plasmids generate non-infectious phage displaying either the Fab (Figures 1A and 1B) or strep-tag II (Figure 1C). We started SIP experiments with non-infectious phages displaying the Fab (the phage population was the same as for the experiments shown in Figure 4C) and the biotinylated anti-idiotypic antibody MK2.2-CH γ 1. After incubation of the N1N2-streptavidin adapter with the biotinylated antibody and the pXI261 phage population, *E. coli* XL1-Blue cells were added in large excess. Only an extremely small number of phages had recovered their infectivity in the presence of the adapter (15 colony-forming units). In the absence of the latter molecule we recovered only two colony-forming units. We therefore decreased the size of the ligand–target complex formed between the phage and the adapter by replacing the biotinylated anti-idiotypic antibody with the Val-His-Leu-Asn-Tyr-Glu-His epitope specifically recognized by mAb 763.74. The number of observed colony-forming units reflecting the recovery of infectious phage was again similarly low. Finally, we repeated a SIP infection with the phage displaying the short peptide encoding strep-tag II binding directly to the streptavidin domain of the adapter. This last combination of target and ligand permitted a significantly higher recovery of infectious phages. In the presence of the adapter we counted 700 colonies on the plates representing infectious phages, whereas in the absence of the adapter only 10 colonies were found as background. After a successful SIP we noted that the absolute number of recovered phages was reproducibly low in comparison with the number of phages used during the incubation in presence of the adapter molecule. The successful SIP infection was not observed without the addition of the excess bacteria (results not shown). As shown in Figure 3(B), in the presence of free adapter molecules a bacterial infection by phage is very efficiently inhibited. To give a maximal chance for each individual reconstituted phage to target the bacterial pili and initialize the infection we therefore had to add an excess of bacteria to neutralize the free adapter molecules maximally. In

another attempt to modulate and to slightly increase the size of the complex between the adapter and the phage we constructed a phage displaying strep-tag II with the addition of the 123 residues of the SH2 domain of the Grb-2 protein inserted between the phage and the tag. In all ELISA reactions we were unable to distinguish between both phage populations. In SIP, however, we did not recover infected bacteria at a higher level than was obtained in the absence of the added adapter molecule (results not shown).

In all the SIP experiments we used a homogeneous population of phages displaying the whole strep-tag II molecule. To mimic more closely a real phage library we mixed an increasing amount of this model phage with an excess of phage expressing a random peptide library and repeated a SIP experiment. The results are shown in Figure 6, from which it can be seen that the non-related phages did not affect the recovery of the phages interacting with the adapter. It was therefore possible to isolate phages that interacted specifically with the bait molecules from a larger library of phages.

DISCUSSION

The display of peptides and proteins on filamentous bacteriophages has evolved over the past decade into an extremely versatile and powerful technology [26–32]. In selection protocols for specific binders, a library of ligands displayed at the surface of the phage is incubated with the target molecules either in solution or with the targets bound to a solid support. Through iterative cycles of selection and enrichment steps the phage carrying the specific ligand can eventually be isolated and characterized. As a further technological development, positive selection schemes for the identification of interacting partners have been worked out [11,13,14]. With these selection procedures only productive interactions between the target and the ligand allow the biological survival of the phage displaying the ligand and thus permitting its enrichment.

On the basis of this principle we tried to develop a selection procedure *in vitro* for non-infectious phages displaying a ligand able to interact with a specific biotinylated target molecule. The streptavidin domain of a newly engineered adapter serves as link between a biotinylated target molecule and the core of non-infectious phage lacking a functional gene III coat protein but displaying an interacting ligand instead (see Figure 1). The construction of fusion proteins with streptavidin and their expression in *E. coli* have been described [33–35]. In our constructions we fused streptavidin to the C-terminus of the extracellular part of minor coat protein III. This domain is responsible for the infectivity of the filamentous phage M13. The gene encoding this new adapter molecule was cloned into an *E. coli* expression vector, and the protein fusion was produced as inclusion bodies. This approach is required because active and soluble streptavidin would bind the biotin molecules necessary for the survival of the bacteria. In addition, these biotin molecules would also completely and irreversibly block their binding sites on streptavidin. These sites are needed for interactions with the biotinylated target molecules. As a consequence, the inclusion bodies must be denatured and extensively dialysed to eliminate trapped biotin molecules. A successful refolding procedure is required to obtain a biologically active adapter. A functional adapter obviously requires that both its domains possess biological activity on the same molecule. The streptavidin domain must specifically bind a biotinylated target, whereas simultaneously the N1N2 domain must be able to inhibit the binding of wild-type phage to the pili of *E. coli* cells. Figure 3(C) indicates that the refolding of the streptavidin moiety of the adapter

produces a molecule that does not bind biotin as efficiently as wild-type streptavidin. The high level of correct folding of the N1N2 domain is demonstrated in Figure 3(B). As little as sub-nanomolar concentrations of the adapter abrogate a successful infection by phage. Clearly an optimal refolding for either the streptavidin or the N1N2 domains taken individually is no guarantee that the refolded adapter is suitable for SIP. The only stringent indicator to check the quality of the refolding effectively is to test it in a SIP experiment for its capacity to restore infectivity to an otherwise non-infectious phage. This test has been successfully performed with phages displaying strep-tag II, demonstrating the efficiency of refolding. The exact refolding yield was not determined. This is not of primary concern: even if only a small proportion of the adapter is active in both functions these molecules are still useful in SIP experiments because the amount of active adapter can easily be adjusted accordingly. In our experiments and to avoid both denaturation and a decrease in the quality of the adapter we took care not to use thawed and refrozen molecules.

The focus of our interest was to develop a generic selection system by which any biotinylated molecule that represented an interesting target could be used for the identification of ligands displayed at the surface of filamentous phage. Thus the analysis of the maximal size of the complex tolerated between the N-domain of the gene III protein and the body of the non-infectious phage was an important milestone in our evaluation of *in vitro* SIP.

With pKN16 as helper phage, only 1 in 10–100 phages will display the ligand of interest at its surface [36,37]. To enlarge the number of phages carrying and displaying a recombinant protein we constructed the new helper bacteriophage M13 $\Delta 3$ lacking the complete DNA sequence encoding the gene III coat protein. After rescue of a phagemid encoding the ligand–gene-III protein fusion from bacterial cells with the new M13 $\Delta 3$ helper phage, each individually recovered phage would display three to five copies of the ligands at its surface. This number might represent a maximal estimate of the redundancy of the recombinant fusion protein at the surface of the phage, because the display of a large recombinant protein will probably interfere sterically with the display of several other copies of the same protein at the very tip of the same phage. The C-terminal domain of the gene III protein is required for the correct termination of phage morphogenesis [21,38] and because this domain is part of the recombinant fusion protein, each viable virion must display at least one copy of the ligand. With the construction of the new helper phage M13 $\Delta 3$, our phage ELISA reactions were more sensitive (see Figure 5). Therefore the total number of each individually displaying phage also increases, thus influencing positively the probability of success in SIP selection experiments.

For our first SIP experiments we first chose to biotinylate an anti-idiotypic antibody, to incubate it with the adapter molecule and phage displaying the appropriate Fab fragment so as to avoid possible problems that might simply be explained by affinity considerations between different partners involved in SIP. Despite the high-affinity interactions (binding affinity larger than $6 \times 10^9 \text{ M}^{-1}$) between the anti-idiotypic antibody and the antibody Fab fragment on one site and between the biotinylated anti-idiotypic antibody and streptavidin, we were not able to restore infectivity to phages displaying the ligand molecule. The most likely explanation for this failure is that the complex streptavidin/antibody/Fab fragment was too large to allow the simultaneous binding of the phage to the F-pilus while still tolerating membrane penetration of the phage DNA required for successful bacterial infection. We therefore decreased the size of the complex by replacing the anti-idiotypic antibody with the

biotinylated heptapeptide epitope recognized by the Fab fragment [22]. Similarly, under these new experimental conditions we were again unable to restore infectivity to the phage. The smallest molecule that we selected to display on the phage was the strep-tag II peptide. We chose this peptide because it binds streptavidin as the N-terminus of a fusion protein [18]. Without any further intermediate the strep-tag II phages bind directly to the adapter molecule. This direct interaction permitted us to recover infectious phages. Our results have demonstrated that the phage displaying the Fab of mAb 763.74 does not recover its infectivity after incubation in the presence of the adapter molecule and of a biotinylated target molecule such as the anti-idiotypic antibody or the peptidic epitope. ELISA experiments have shown unambiguously that the Fab can be correctly folded and displayed. The level and quality of the refolding of displayed molecules rely exclusively on the bacteria and are hardly controllable. A stringent control to demonstrate that the refolding level of the displayed molecules was not primarily the explanation for our difficulties in recovering phage infection was conducted with the strep-tag II phage pXI265. Our results showed that this phage recovers its infectivity in the presence of the adapter. However, a derivative of the pXI265 phage in which 123 amino acid residues encoding the SH2 domain of the Grb2 protein were inserted between the phage and the strep-tag II did not recover any more infectivity. Nevertheless, ELISA cannot distinguish between the phages, so the affinity of strep-tag II in both of them for the adapter is identical. Clearly the refolding and display of strep-tag II cannot explain the ELISA and the SIP results and a reasonable explanation is that the distance between the N1N2 domain and the phage body carrying the DNA to be transferred to the pilus of the bacterial cell has become too large in the second phage to allow a successful infection.

In published papers [9,11,39–41] and to the best of our knowledge, only fusions of peptides or small haptens to the N1N2 domain of the gene III protein are able to generate suitable interactions with single chain Fv antibodies or small ligands displayed at the surface of the phage that give back infectivity to the phage. Our results confirm these results and permit for the first time the definition of further inherent limitations in the SIP technology. These considerations will influence the conditions under which selection schemes such as ligand library against target library can be initiated with a reasonable chance of success [42]. In this last study, the target p75CD protein domain for the displayed peptide library was approx. 140 residues long.

It is worth noting that with our experimental model (the displayed strep tag II ligand) we isolated only approx. 700 colony-forming units out of a total of 10^{12} identical phages. We wish to stress that the low level of recovery might be the result of the relatively low affinity of the strep-tag II for streptavidin [18,41]. However, on the basis of these results, we calculate that a phage displaying a ligand must be present 10^8 – 10^9 times within a cDNA library to have a chance of being isolated by SIP. With a maximal number of approx. 10^{13} phages that can be expected from 200 ml of infected *E. coli* culture, only 10^4 independent cDNA clones can be present in the library if a specific clone is to be successfully selected.

In conclusion, and on the basis of our experimental results and on our studies on selection procedures for non-matured phage antibodies [15], we consider that SIP will be useful only for the identification of high-affinity ligand–target pairs. Both the required high-affinity interactions between target and ligand on the one hand, and the limited size flexibility of the complex tolerated between the phage and the adapter molecule on the other, make SIP quite unattractive for the cloning and identification of cDNA encoding proteins.

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