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Selectively infective phage (SIP) technology: scope and limitations

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Abstract

We review here the selectively infective phage (SIP) technology, a powerful tool for the rapid selection of protein–ligand and peptide–ligand pairs with very high affinities. SIP is highly suitable for discriminating between molecules with subtle stability and folding differences. We discuss the preferred types of applications for this technology and some pitfalls inherent in the in vivo SIP method that have become apparent in its application with highly randomized libraries, as well as some precautions that should be taken in successfully applying this technology.

Keywords: Phage display; Affinity maturation; Disulfide bonds; ScFv fragments

1. Introduction

1.1. The principle of SIP

The selectively infective phage (SIP) technology was developed for selecting interacting protein–ligand pairs (Dueñas and Borrebaeck, 1994; Gramatikoff et al., 1994; Krebber et al., 1995). It has also been called selection and amplification of phage (SAP) (Dueñas and Borrebaeck, 1994) or direct interaction rescue (DIRE) (Gramatikoff et al., 1994). While SIP is related to phage display, it has the advantage of directly coupling the productive protein–ligand interaction with phage infectivity and amplification, without the need of an elution step from a solid matrix (Fig. 1).

SIP exploits the modular structure of the gene-3-protein (g3p), which consists of three domains, N1, N2 and CT, which are connected by glycine-rich linkers and possess different functions for the phage life cycle (Fig. 1) (Armstrong et al., 1981; Stengele et al., 1990). The g3p is present most likely in five copies on the phage, reflecting the five-fold symmetry of the phage coat and the pilus (Marvin, 1998). The N-terminal N1 domain of g3p consists of 68 amino acids and is absolutely essential for *Escherichia coli* infection (Armstrong et al., 1981; Jakes et al., 1988; Stengele et al., 1990; Holliger and Riechmann, 1997; Krebber et al., 1997). The 132 amino acid sized N2 domain, which forms a complex with N1 on the phage (Lubkowski et al., 1998), specifically interacts with the *E. coli* F-pilus (Jakes et al., 1988; Stengele et al., 1990). This pilus interac-

Abbreviations: SIP, selectively infective phage; SAP, selection and amplification of phage; DIRE, direct interaction rescue; g3p, gene-3-protein of filamentous phage; N1, first N-terminal domain of g3p; N2, second N-terminal domain of g3p; CT, C-terminal domain of g3p; hag, hemagglutinin peptide epitope of antibody 17/9

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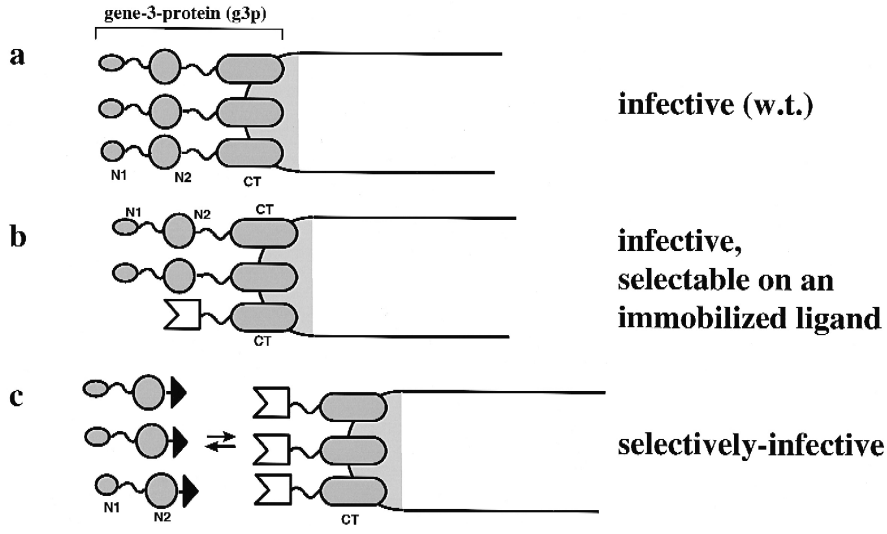


Fig. 1. The modular structure of the minor M13 phage coat protein gene-3-protein (g3p) and its recombinant variations used in phage display and in the SIP technology. For clarity, only three of the probably five copies of g3p are shown. (a) w.t. M13 phage, (b) display phage used in traditional phage display, (c) a variant of a SIP phage, which itself is non-infective and gains infectivity solely in the presence of the adaptor. For details and variations to the particular constructs shown, see text.

tion, however, is not absolutely required for infection, as an alternative, albeit less effective, direct infection pathway exists (Russel et al., 1988; Krebber et al., 1997), which will be described later. The CT domain consists of 149 amino acids (including the C-terminal transmembrane anchor), forms part of the phage coat and is absolutely essential for phage morphogenesis (Nelson et al., 1981; Crissman and Smith, 1984).

In SIP, the basic infectivity of the M13 filamentous phage is destroyed by deleting from the phage genome either the N1 domain or the N1 and N2 domains of the g3p. A peptide or protein library is fused N-terminally to some or all copies of the CT domain or the N2–CT domains of g3p, and no w.t. g3p must be present on the phage. The infectivity of the phage can now only be restored by adding the N1 or the N1–N2 complex, as the N1 domain is absolutely required for infection. These domains are themselves fused or chemically coupled to a ligand which binds to the peptide or protein displayed on the phage. These infectivity restoring molecules will be referred to as the ''adaptors'', and the consequences of choosing different adaptors, consisting of either N1 or N1–N2, will be discussed later.

There are two routes to selectively restoring the infectivity of the phage: in vivo and in vitro SIP (Fig. 2). For in vitro SIP, both components — the phage displaying the protein and the N1 adaptor or N1–N2 adaptor with the ligand coupled to it — are separately purified and combined in defined amounts in vitro to yield infective phages, provided the ligand binds to the protein. Consequently, the adaptor is encoded on an expression plasmid and the ligand can be either genetically fused to it or, in case of a small organic molecule such as a hapten, chemically coupled to the purified N1–N2 (Gao et al., 1997; Krebber et al., 1997).

In contrast, in the in vivo SIP approach the ligand has to be a protein or peptide genetically fused to N1 or N1–N2, and this fusion protein is encoded on the phage genome. During in vivo phage production, the N1–ligand or N1–N2–ligand adaptor is exported to the bacterial periplasm, while the CT–peptide or CT–protein fusion is also transported to the periplasmic space but remains anchored to the inner membrane through the C-terminal transmembrane helix of CT, before it is incorporated into the budding phage. In case of a tight interaction in the periplasmic space between the polypeptides fused to the adaptor or to

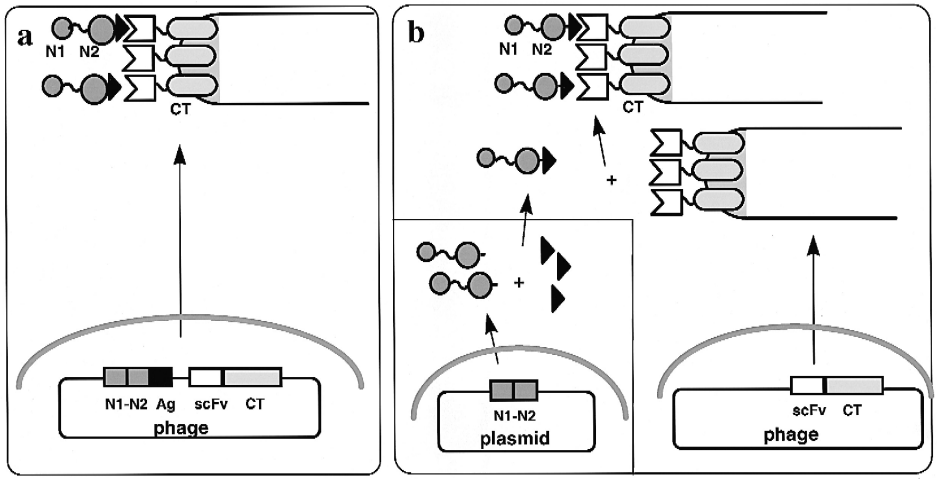


Fig. 2. The principles of in vivo- and in vitro-SIP. The contour of the *E. coli* cells expressing the phage or the adaptor are symbolized by thick grey lines. (a) In the in vivo SIP variant, the phage and the adaptor are produced from the same cell. While the system is drawn with a single replicon, polyphage with copackaged replicons can also be used. In principle, interacting pairs can be coevolved in a library-vs.-library setting, as the genetic information for both libraries is linked in the same phage and propagated in the same cell. (b) For in vitro SIP, the two components are produced separately. Thus, the system can be better controlled. Recombination events between the phage and the adaptor leading to w.t. phages are impossible, and, furthermore, the concentrations of adaptor and phage relative to each other can be controlled in order to drive selection stringently towards higher affinities (see also Fig. 3). However, no coevolution of interacting peptides is possible, as the genetic information of the polypeptide linked to the adaptor is not coupled to the selection process.

the CT domain, respectively, the infectivity of the phage is restored.

The major advantage of SIP in comparison to phage display is the strict coupling of the selection and the infection process, which occur simultaneously. Two further important advantages are apparent for the in vivo SIP approach. First, in identifying an interacting peptide or protein partner to a specific protein, this protein does not have to be first expressed and purified as in phage display. Instead, its DNA is all that is needed, and only very small quantities have to be functionally expressed in the selection system. Nevertheless, it obviously does have to be compatible with transport to and folding in the periplasmic compartment. Second, the in vivo SIP strategy would in principle also be suitable for ''library-vs.-library'' selections, which are not possible in a direct manner in traditional phage display. However, current limitations in the efficiency of selection, leading to only a limited effective library size, and some unresolved issues in adaptor exchange between phages (see below) have so far not lead to a practical realization of this strategy. On the other hand, progress has been made in developing methods how such ''two-dimensional'' libraries can in principle be constructed conveniently, as under some circumstances filamentous phages can pack two single-stranded vectors, which may each encode one of the potentially interacting proteins (Rudert et al., 1998).

Since its first proof-of-principle experiments with antibody Fab and scFv fragments as well as with coiled-coil peptides (Dueñas and Borrebaeck, 1994; Gramatikoff et al., 1994; Krebber et al., 1995), progress in understanding the underlying mechanisms has been made, and this knowledge has lead to the construction of improved in vitro and in vivo SIP phage vectors, which have been successfully applied to the selection from various synthetic scFv libraries.

1.2. Structural insight relevant to SIP

New insight has been gained into the structural requirements of fusions to N1 and N2 through the solution of the N1–N2 structure by X-ray crystallography (Lubkowski et al., 1998). Both domains consist mainly of β-sheet and show a striking similarity in their core folds, which suggests an evolutionary

origin by domain duplication. Between the N1 and N2 domains exists a large contact interface formed by two β-strands of N2 that participate in the N1 β-sheet. Nevertheless, there is some flexibility in the relative orientation of N1–N2 (Holliger et al., 1999), and N1 alone has the same structure as in the complex, as determined by NMR (Holliger and Riechmann, 1997). In the infection process, the N2 domain binds to the *E. coli* F-pilus, and while the pilus is ''withdrawn'', the N1 domain is brought into contact with the C-terminal domain of TolA (Click and Webster, 1997; Riechmann and Holliger, 1997; Click and Webster, 1998; Deng et al., 1999). This interaction appears to be absolutely critical, as no infection is possible at all without either the N1 domain or in the absence of TolA, while the pilus and the N2 domain both merely improve infectivity, but are not indispensable. The crystal structure of the complex of N1 and TolA was solved recently (Lubkowski et al., 1999), and it clearly shows that TolA displaces the N2 domain, which had been proposed from biochemical experiments (Riechmann and Holliger, 1997), even though both bind with very different geometry. Thus, the flexible linkers connecting N1, N2 and CT are an integral part of the rearrangements necessary in the infection process. It is at present not clear what the further fate of the domains is in the infection process nor which further *E. coli* proteins may interact with them. It follows that there may be geometric restrictions in the protein–ligand pairs compatible with SIP, and the affinity threshold (see below) may also be related to the infection mechanism.

2. Recent advances in SIP technology

2.1. Model systems

A thorough study of infection properties of different g3p fusion modules has brought some further understanding of the infection process, especially of the in vitro SIP method (Krebber et al., 1997). In this study, β-lactamase was inserted at different positions within g3p, and also different fusions of a scFv fragment to the phage have been investigated in conjunction with different adaptor constructs. It could be shown that N1 is absolutely required for infection

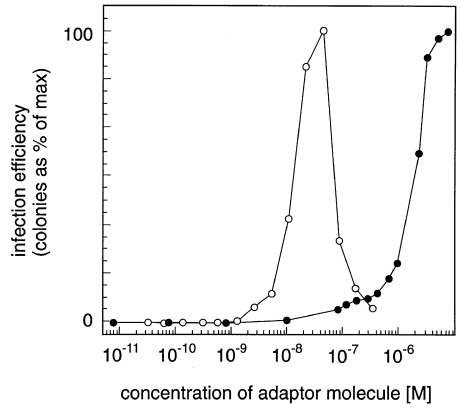


Fig. 3. Different arrangements of the g3p domains for in vitro SIP lead to different infectivity profiles dependent on the adaptor concentration. The N1–N2 adaptor (○) leads to inhibition of infection at higher concentrations and is therefore only suitable for interacting pairs with higher binding constants, and thus ideal for exerting selection pressure towards higher affinities by lowering the adaptor concentration in the infection experiment. The N1 adaptor (●) has to be employed at lower concentrations and might thus be more suitable for interacting pairs with lower affinities. (Figure adapted from Figs. 6 and 7 of Krebber et al. (1997).)

under all circumstances, whereas infection in the absence of N2 is possible, but is dependent on Ca^{2+}. In this case, a pilus-independent infection is made possible by Ca^{2+} disturbing the membrane (Fig. 3).

A library displayed on a SIP phage can be constructed by fusing it N-terminally either to CT or to N2–CT. In general, the N2–CT fusions give higher infectivities (Krebber et al., 1997). Both types can be combined with either an N1–ligand or an N1–N2–ligand, thereby having either zero, one or two copies of N2 in each reassembled g3p. With the N1–N2–ligand adaptor only very low concentrations of adaptor (10^{-8} M) had been necessary for infection in the investigated scFv–hapten system ($K_D = 10^{-10}$ M) (Vaughan et al., 1996), while the same adaptor *inhibits* infection at higher concentrations (10^{-7} M) (Krebber et al., 1997) (Fig. 3, ○). One possible explanation may be that the N1–N2 adaptor binds simultaneously to the pilus and the phage at high concentrations, which is something the N1 adaptor cannot do. Consequently, the N1-N2 adaptor may be very suitable for improving binding constants by SIP by constantly lowering the adaptor concentrations in consecutive rounds. Conversely, the adaptor N1-

ligand gives no inhibition up to adaptor concentrations of about 10^{-6} M, although it may inhibit at even higher concentrations. Higher concentrations are not only possible, but also necessary for infection with the N1 adaptor: In the scFv–hapten system with a K_D of 10^{-10} M an N1 adaptor concentration of 10^{-6} M was required for optimal infection (Krebber et al., 1997) (Fig. 3, ●). Therefore, this adaptor type should be valuable in systems with lower binding constants. It should be pointed out, however, that neither any unequivocal low-affinity system has yet been successfully selected — and, in some cases, the K_D values are simply unknown — nor has this question of the K_D threshold yet been systematically investigated in the various adaptor combinations.

In vitro SIP was also shown to be useful for the selection of catalytic antibodies in a model experiment with a defined antibody (Gao et al., 1997). SIP phages displaying a catalytic antibody scFv fragment fused to CT can be rendered highly infectious, when the catalytic scFv is covalently trapped by a suicide substrate coupled to N1–N2. The coupling chemistry of the suicide inhibitor to N1–N2 was varied, testing the coupling of an engineered cysteine to a substrate containing a maleimide moiety, or interactions between N1–N2 with a His-tag to a substrate coupled to Ni-NTA, or between a N1–N2–streptavidin fusion with a biotinylated substrate. All three coupling procedures lead to selective infectivity, however, for the streptavidin–N1–N2 fusion the infectivities were generally low, which may be due to the tetrameric structure of streptavidin interfering with the infection process.

Finally, in vivo SIP was used in a defined model system for testing a two-vector system for packaging the genetic information for in vivo SIP (Rudert et al., 1998). This would be more convenient for making libraries in both partners at once or for using the same library with many targets without recloning, with a view of ''library-vs.-library'' screening. This system was tested with the intracellular domain of p75 neurotrophin receptor coupled to the N1–N2 adaptor and an interacting peptide displayed on phage in a CT fusion. Both vectors were packaged in a polyphage after cotransformation, yielding phage particles that were infectious in a cognate pair, but not in a negative control. Infection events could be scored as colonies, when the donor cell streaks were grown on a filter, the phage passed through the filter and infected the recipient on the agar underneath. Polyphage production, which is required in this approach, is generally related to low incorporation of g3p fusion proteins into the phage (note that there is no g3p w.t. in this system), but the exact requirements are not yet clear. The coexistence of a phage and a phagemid genome in the same host require a genetic alteration in the phage genome, termed the ''interference resistance'' phenotype (Enea and Zinder, 1982).

A different, SIP-related approach of exploiting the selective infectivity of filamentous phages was taken by Sieber et al. (1998). Here, a ribonuclease T1 library was inserted between N2 and CT in the M13 phage. Infectivity was selectively destroyed by protease cleavage, thus selecting for stability and protease resistance, and not, as it is usually done in phage display, for ligand interactions. A similar system was developed by Kristensen and Winter (1998) based on a phagemid/helper phage system using a helper phage with a protease site between N2 and CT. This helper phage will also be useful for normal phage display, as it can help to reduce the background.

2.2. Examples for SIP library selections

In an example of library applications of the in vivo SIP system Gramatikoff et al. (1995) selected ligands to a jun-peptide from a human cDNA library. In contrast to all other examples cited here, they fused the library to the adaptor, while the interacting (constant) jun-peptide was displayed on the phage. No comments on false-positives were given, but in other in vivo SIP projects the adaptor exchange during phage production had led to an uncoupling of phenotype and genotype (S. Spada and D. Christ, unpublished results).

With the in vivo SIP methodology, a larger synthetic library was selected for the most stable scFv structure (Spada et al., 1998). Three successive amino acids in V_L around position 8, which usually is a *cis*-Pro in κ-chains, were randomized by Kunkel mutagenesis in the hemagglutinin (hag) peptide binding scFv 17/9. Only Pro-containing sequences were selected after three rounds of SIP, and it was shown that stability had been the selection criterion rather

than folding yield. An interesting corollary of this result was that the naturally most abundant sequences around position L8 had been selected.

Dueñas et al. (1996) used in vitro SIP in a library setting with a small model Fab library. The selection was shown to require high affinity, and the authors suggested that selection for low and high on- or off-rates could be guided by fine-tuning the selection conditions. Another mini-library of defined point mutants of a fluorescein-binding scFv could be selected by in vitro SIP for a threshold affinity within one round, and for the combined optimum between affinity and the amount of folded and active protein within three rounds (Pedrazzi et al., 1997). In vitro SIP was also employed to select for a useful non-repetitive scFv linker. A linker library was obtained by cloning of a semi-randomized linker cassette into the fluorescein binding scFv FITC-E2 (Vaughan et al., 1996), and SIP-selection yielded all functional scFvs after only a single round (Hennecke et al., 1998).

3. Troubleshooting SIP:pitfalls and countermeasures

While SIP has been shown to be able to select tight binders from libraries in a single round, as well as to be a very powerful technique for the enrichment of the best binder and folder from a library of similar molecules, we have discovered a few pitfalls, which the user needs to be aware of in order to take the appropriate countermeasures for making optimal use of the technology. The selection for tight binding is so powerful that covalent bonds between the adaptor and the phage are strongly selected. This has two consequences, which will both be an issue only in the in vivo SIP method, but not in the in vitro SIP approach. First, there is the danger of picking up mutations in which disulfide linkages are introduced. Second, at some low frequency, w.t.-like phages may appear through a variety of genetic rearrangements, in which the genetic fusion of N1–N2 to CT is restored.

3.1. Selection of spurious disulfide bonds

We have observed the occurrence of unwanted disulfide bonds in two scenarios. In the first, DNA shuffling of a scFv fragment was carried out (S.J. and A.P., unpublished results), which possessed the CDRs of the anti-hag antibody 17/9 (Rini et al., 1992; Schulze-Gahmen et al., 1993; Spada et al., 1998), grafted onto the framework of the scFv B72.3 (Brady et al., 1992; Desplancq et al., 1994). Using in

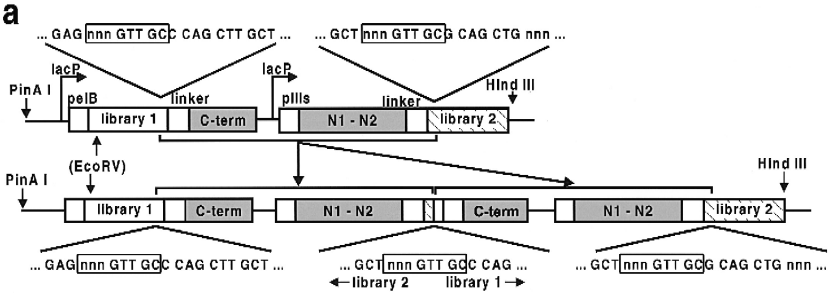


Fig. 4. Detection of recombinations. (a) Proposed molecular mechanism of recombination. The g3 cassette is doubled and recombined at homologous sequence stretches in libraries 1 and 2. (b) Purified phage DNA of jun–fos libraries was digested with the enzymes *Pin*AI and *Hin*dIII, which should result in a vector fragment of 6152 bp and an insert of 1913 bp. For the proposed recombination (a) the insert would be 3329 bp long. Samples are shown with their respective infectivities in the initial library (round 0) and after each SIP round. As molecular weight standard lambda-DNA was digested with *Pst*I. (c) Mini-prep phage DNA of scFv library pools A (phages produced at room temperature) and B (phages produced at 37°C) digested with *Eco*RV/*Hin*dIII after each round of SIP. The expected band at 2174 bp for the insert also occurs in the control phage fB72.3HAG, the library parent. The recombination band at ca. 3600 bp, expected according to a recombination similar to that shown in (a), neither occurs in the parent phage nor in the initial and recloned libraries before SIP selection, but only after the 1st SIP round in both libraries A and B. After recloning, the recombination increases in strength only in library B where the selection pressure is higher than in library A. Nevertheless, the infectivity rises also in the latter library due to disulfide bond formation. Molecular weight standard: λ-DNA cut with *Bst*EII.

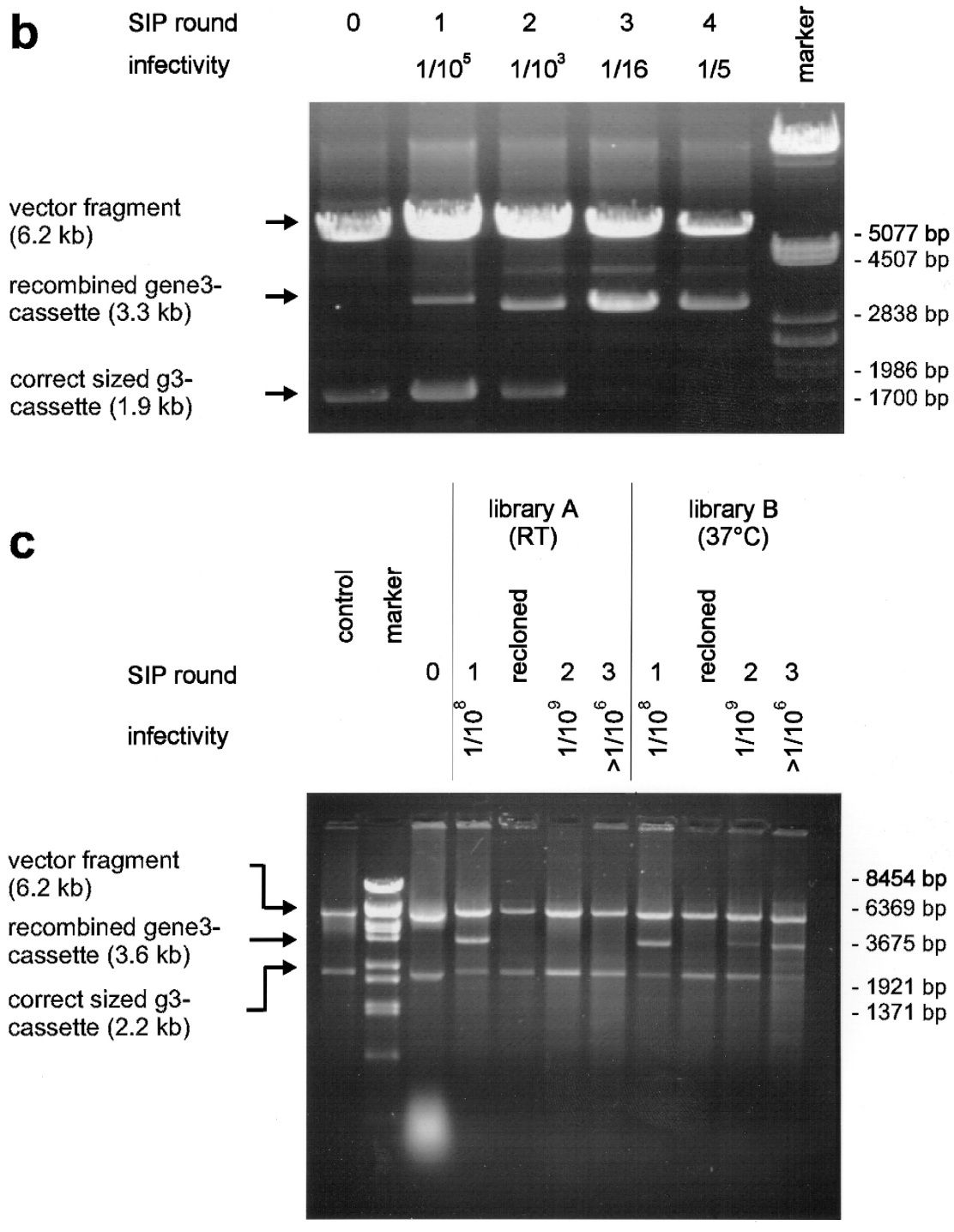


Fig. 4 (continued).

vivo SIP with vectors as described before with the scFv fragment fused to CT (Spada et al., 1998), scFv fragments were enriched carrying unpaired cysteines. In the clones, for which the corresponding N1–N2–hag fragment was sequenced, a frameshift was detected behind N2, leading to a small peptide fusion containing a cysteine. Thus, it appears that a disulfide link was selected which covalently links the adaptor at its C-terminus to the phage-displayed scFv–CT fusion. Indeed, an overnight incubation of these phages with 5 mM DTT at 10°C decreased the infectivity by one order of magnitude, while this treatment decreased the infectivity of w.t. phages and phages displaying a scFv without free cysteines by only twofold.

Similarly, a semisynthetic library of Jun-related peptides was displayed on the phage (K.M.A. and A.P., unpublished results), while a library of Fos-related peptides was fused at the C-terminus of N1–N2. In this library-vs.-library selection, after two rounds, Jun and Fos sequences were enriched, which each contained single cysteines. Interestingly, in the peptide–CT fusions the origin of most of the cysteines were point mutations, most likely introduced in the original PCR-based cassette generation of the library from the long synthetic oligonucleotide. Apparently, the CT domain, which is necessary for the formation of functional phages (see below), largely prevents frameshifts. On the other hand, the peptide fused C-terminally to N1–N2 generated cysteines by frameshifting to other reading frames (see below). For a long synthetic oligonucleotide, 1 bp deletions at a low level are essentially unavoidable and remain present even after purification by polyacrylamide gel electrophoresis.

In the case of the unspecifically interacting disulfide-linked peptides, treatment of the phages with 5 mM DTT at 37°C reduced the cysteine formation by four to eight orders of magnitude, compared to one order of magnitude for wild-type phages, so that further selection rounds were carried out without reappearance of cysteine pairs. Thus, while the use of DTT in experiments where spurious cysteines may occur did reduce the problem, it could not be completely eliminated. It is worth noting that currently all successful in vivo experiments have used defined, high-quality libraries, which were devoid of cysteines (Spada et al., 1998) or have not gone through more than one round (Gramatikoff et al., 1995). It should be stressed that the occurrence of unspecifically paired cysteines is not a problem at all during in vitro SIP, as the adaptor is chemically defined and does not carry a spurious cysteine. Thus, no such problem has been observed during in vitro SIP, even when using multiple rounds (Dueñas et al., 1996; Pedrazzi et al., 1997; Hennecke et al., 1998).

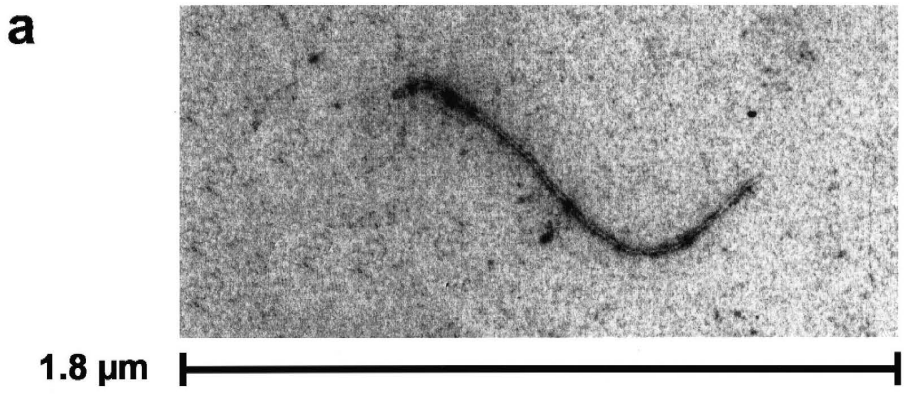
3.2. Genetic recombinations

A second potential pitfall during in vivo SIP is the selection for a genetic recombination which restores some form of N1–N2–CT connection. This genetic rearrangement background was greatly reduced by the use of appropriate vectors (Krebber et al., 1995), but it could not be totally eliminated in some circumstances. Apparently, very short stretches of sequence identity (as short as 8 bp) are sufficient (K.M.A. and A.P., unpublished results), and this cannot always be prevented in library studies, as we have found in two independent library projects using fully randomized synthetic libraries (Fig. 4). However, it is easy to check for and minimize this recombination reaction. Since the size of the restriction fragments encoding the protein–CT and the N1–N2–ligand dramatically changes upon recombination (Fig. 4a), the desired DNA fragment of the original size can be cut from preparative agarose gel electrophoresis gels every few rounds and be recloned into fresh vector, which largely eliminates the problem (Fig. 4b,c). Reducing the phage production temperature also helped to reduce the extent of recombination events (Fig. 4c).

3.3. Frameshifts in the displayed polypeptide lead to functional polyphages

Occasionally, the occurrence of frameshifts, which still allowed the functional production of proliferative phages, have been observed in traditional phage display (Carcamo et al., 1998; Jacobsson and Fryk-

Fig. 5. Electron microscopy of fd phages. The scale is given below each panel. (a) In vivo SIP phage with the g3p divided into two parts as indicated in the scheme of Fig. 1c (b, c) In vivo SIP polyphages in which the CT-domain is out frame, two different scales.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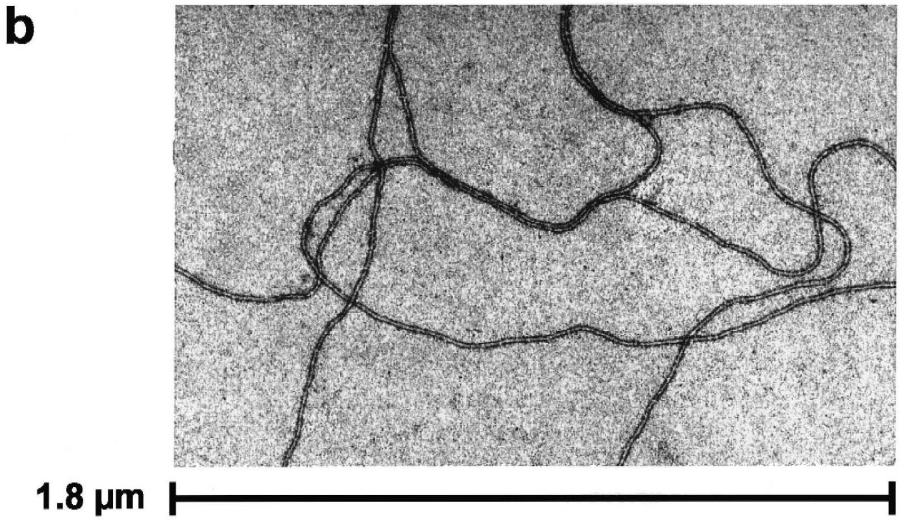
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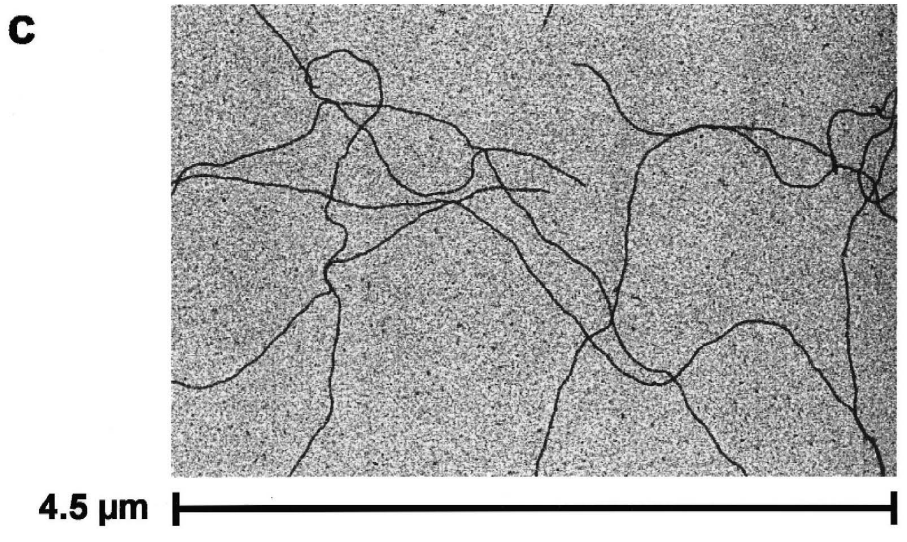
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berg, 1998). These frameshifts occurred in the polypeptide N-terminally fused to the CT domain so that the CT domain was out of frame. Also, in SIP experiments, we have observed the occurrence of frameshift variants, which were selected due to unspecific disulfide bond formation as described above. This was seen especially in synthetic libraries generated with long synthetic oligonucleotides, where 1-bp deletion products are common and difficult to separate from full-length oligonucleotides. Although the clones with frameshifts in front of CT should not be functional with the CT domain being out of frame, they were evidently selectable by SIP. As we wished to elucidate the reason for this behavior, we tested whether phages can be formed without the CT domain at all or whether unspecifically ''sticky'' frameshift polypeptides could replace the CT domain within the phage and be directly incorporated into the phage coat. In such a case, an N1–N2–peptide fusion would have to be able to bind directly to the phage coat.

However, neither the total genetic deletion of the CT domain from the SIP phage nor the replacement of the CT domain with the short frameshifted polypeptides in w.t. phages lead to any detectable infectivity using 10^{11} phages for infection. In contrast, the infectivities of these selected clones, possessing unpaired cysteines and frameshifts in the genetic presence of a CT domain, lead to a relatively high SIP infectivity of about $1/10^5$ phages. Therefore, the CT domain must be present on the protein level in the genetically frameshifted SIP phages, possibly through a second, *translational* frameshift, bringing the CT domain back into the right frame.

In fact, two frameshifted clones investigated more closely possessed two subsequent rare arginine codons, AGG, which are known to promote translational frameshifts (Spanjaard and van Duin, 1988). These clones were shown to produce polyphages, as detected by electron microscopy (Fig. 5). In conclusion, the frameshift variants occasionally observed to be selectable by traditional phage display (Carcamo et al., 1998; Jacobsson and Frykberg, 1998) or in SIP do possess a functional CT domain on the protein level, but the phage-producing cell makes so little of it, due to the rare events of translational frameshifts, that polyphages — which are infective — are produced. As long as the adaptor is covalently linked (via the spurious disulfide bonds), these phage can be selected. Importantly, there is apparently no danger of ''direct'' binding of the adaptor to the phage via non-specific interactions, as the CT domain is absolutely required for functionality.

4. Conclusions

While the in vivo SIP technology is especially convenient, as no protein at all needs to be expressed and purified for the selection of binding partners, it is important to understand the potential side reactions which can result in false positives: spurious cysteines, leading to covalently disulfide-linked adaptor–phage complexes, and rare genetic recombinations which regenerate N1–N2–CT rearrangements. Recombination events can be efficiently eliminated by recloning of the correct-sized g3p cassette. While DTT incubations can reduce much of the disulfide coupling, it does not reduce the background to zero. Furthermore, *genetic* frameshifts leading to nonsense-polypeptides and spurious cysteines are not necessarily strictly selected against, probably due to a low frequency of *translational* frameshifts promoted by certain sequences, which bring the CT domain back into frame so that functional phages can be produced in spite of frameshifts. Therefore, caution must be exercised in applying in vivo SIP to libraries obtained from error-prone PCR, DNA shuffling or very long oligonucleotides, and especially from cDNA.

In contrast, very encouraging results have been obtained with defined in vivo SIP libraries, such as the randomization of a short stretch of a V_L domain (Spada et al., 1998). In this case, the library was well defined and free of spurious cysteine codons at the level required. It should be stressed again that none of the problems occur during in vitro SIP, even after several rounds, and a number of libraries have been successfully screened (see below).

The potential advantage of in vitro SIP has been the very low background, at least under all conditions tested, which allowed functional molecules to be selected after only one single round of SIP selection (Krebber et al., 1997; Hennecke et al., 1998). While in traditional phage display enrichment factors of $10–10^4$ per round are normal (Winter et al., 1994), enrichment factors of $10^5–10^6$ per round can

be easily achieved using SIP (Dueñas and Borrebaeck, 1994; Dueñas et al., 1996). Additionally, a well-defined library permits a convenient enrichment of molecules with even small advantages in molecular properties (Pedrazzi et al., 1997; Spada et al., 1998). Thus, we see in vivo SIP, at the current level of understanding, mostly as a technology for molecular improvement, and less one of initial screening of large libraries, except in such cases where very high affinities will be present.

In vitro SIP is far more resistant to spurious genetic alterations, as the N1–N2–ligand or the N1–ligand adaptor is constant, since its genetic information is not amplified together and coevolved with the partner displayed on the phage. Several library experiments have been successfully carried out, including both defined mutant libraries (Dueñas et al., 1996; Pedrazzi et al., 1997) and partially randomized libraries (Hennecke et al., 1998). For in vitro SIP, the high required (affinities of at least 10^{-9} M) have been well documented (Dueñas et al., 1996; Krebber et al., 1997; Pedrazzi et al., 1997), even though the exact number may depend on the molecular system in question, and for some of the model systems reported, the K_D is not known.

SIP is a powerful strategy to select for protein–ligand interactions as well as for other desired features as protein folding and stability. Moreover, the threshold for the phage to infect seems to be so high that the selection pressure for the very best restored g3p is enormous, meaning that excellent binding or even covalent linkage of the N-terminal domains and the C-terminal domain is strongly favored within the selection process. Provided that artifacts can be controlled, by using high-quality libraries and/or in vitro SIP, this is a big advantage of SIP compared to traditional phage display, as SIP can within very short time and with minimal effort select for the best binders and even discriminate subtle differences (Pedrazzi et al., 1997; Spada et al., 1998). In cases where selection for a covalent binder is actually *desired*, like in the trapping of catalytic antibodies by suicide inhibitors (Gao et al., 1997), for the selection of interacting pairs with extremely low dissociation constants or by separating proteolytically cleaved proteins from intact ones (Kristensen and Winter, 1998; Sieber et al., 1998), this technology is very attractive because of its speed and selection power and because no dissociation of the tightly or covalently interacting pair is required in SIP.

In summary, SIP is an extremely rapid and powerful selection alternative to conventional phage display. The applicability of in vivo SIP can be extenuated to arbitrarily randomized libraries and libraries with low initial infectivity only when special precautions are taken to guide selection towards non-covalent interactions and to prevent the selection of genetically or chemically restored w.t. phages. As these precautions do not completely suppress undesired covalent variants of g3p, the in vivo SIP methodology is more suitable for libraries made by controlled mutagenesis and with sufficiently interacting pairs initially present. However, if only one library is to be screened against a constant partner, the in vitro SIP variant of the SIP methodology should be the method of choice, as it is more robust against spontaneous genetic changes within the phage. Recombination of w.t. g3p and spontaneous mutations towards cysteines on the N1–N2 adaptor are not possible in in vitro SIP, as the N-terminal domains are not genetically linked in the selection. The future development and extension of SIP, however, will clearly require a more detailed mechanistic understanding of the phage infection process.

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