Protein Science (1996), 5:1833–1843. Cambridge University Press. Printed in the USA.

Phage display of intact domains at high copy number: A system based on SOC, the small outer capsid protein of bacteriophage T4

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(RECEIVED November 20, 1995; ACCEPTED June 5, 1996)

Abstract

Peptides fused to the coat proteins of filamentous phages have found widespread applications in antigen display, the construction of antibody libraries, and biopanning. However, such systems are limited in terms of the size and number of the peptides that may be incorporated without compromising the fusion proteins' capacity to self-assemble. We describe here a system in which the molecules to be displayed are bound to pre-assembled polymers. The polymers are T4 capsids and polyheads (tubular capsid variants) and the display molecules are derivatives of the dispensable capsid protein SOC. In one implementation, SOC and its fusion derivatives are expressed at high levels in *Escherichia coli*, purified in high yield, and then bound in vitro to separately isolated polyheads. In the other, a positive selection vector forces integration of the modified *soc* gene into a *soc*-deleted T4 genome, leading to in vivo binding of the display protein to progeny virions. The system is demonstrated as applied to C-terminal fusions to SOC of (1) a tetrapeptide; (2) the 43-residue V3 loop domain of gp120, the human immunodeficiency virus type-1 (HIV-1) envelope glycoprotein; and (3) poliovirus VP1 capsid protein (312 residues). SOC-V3 displaying phage were highly antigenic in mice and produced antibodies reactive with native gp120. That the fusion protein binds correctly to the surface lattice was attested in averaged electron micrographs of polyheads. The SOC display system is capable of presenting up to $\sim 10^3$ copies per capsid and $>10^4$ copies per polyhead of V3-sized domains. Phage displaying SOC-VP1 were isolated from a $1{:}10^6$ mixture by two cycles of a simple biopanning procedure, indicating that proteins of at least 35 kDa may be accommodated.

Keywords: bacteriophage T4; capsid assembly; human immunodeficiency virus antigens; phage display; protein engineering

Filamentous phage-based display systems (Smith, 1985) have found widespread use in molecular biology, including many immunologic applications such as antigen presentation and the immunoisolation of desired recombinants (biopanning) (Marks et al., 1992; Smith et al., 1993; Williamson et al., 1993). However, with filamentous phages, peptides that may be displayed from the major coat protein are limited in size to 6–10 amino acid residues (Kishchenko et al., 1994; Iannolo et al., 1995), although somewhat longer peptides may be displayed by co-assembly with the wild-type coat protein (Perham et al., 1995). Full-length polypeptides may displayed on their minor proteins, but only at very low copy number (Parmley & Smith, 1988). Moreover, the requirement that the fusion protein should pass through the secretion system of *Escherichia coli* may pose problems of toxicity for the host or for correct folding of the displayed protein (Skerra & Plückthun, 1991).

The T4 SOC protein has a number of features that recommend it as an alternative vehicle for protein display. SOC is a small protein (9 kDa) that is nonessential for T4 capsid morphogenesis (Ishii & Yanagida, 1975) but, if available, binds with high affinity to sites on the outer surface of the mature capsid (Aebi et al., 1977; Ishii et al., 1978) or polyheads (Steven et al., 1976), which are tubular capsid-like polymorphs. Thus, assembly of its platform is decoupled from expression and folding of the SOC-derived display

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Abbreviations: SOC, small outer capsid protein. Genes are designated in lower case, italics, e.g., *soc*, and the corresponding proteins in upper case, e.g., SOC.

protein. SOC binds in equimolar stoichiometry to the major capsid protein gp23*, which is present in ~960 copies per capsid, or $>10^4$ copies per polyhead. Triplets of SOC binding sites surround the local threefold symmetry axes on the hexagonal gp23* surface lattice (Aebi et al., 1976). Thus, in principle, one may envisage SOC display particles on which up to 10^3–10^4 densely packed copies of a domain or protein of interest cover the outer surface of a single particle (Fig. 1).

SOC has several additional desirable features. The complication of negotiating a secretory pathway does not arise in lytic phage T4 assembly. Moreover, SOC is restored readily to full capsid-binding activity after denaturation in guanidine hydrochloride (Ishii & Yanagida, 1977) or by heating (E. Locke & A.C. Steven, unpubl. results), indicating that it should be possible to retrieve SOC fusions from inclusion bodies in a state competent to bind to capsids.

In the present study, we establish the feasibility of the SOC display system, using C-terminal fusions of three different sizes. (1) SOC-CYS has a four-residue extension containing a cysteine residue, included to allow the subsequent conjugation of protein ligands (wild-type SOC contains no cysteines). (2) For a polypeptide comparable in size to individual domains, we chose a 43-residue sequence containing the V3 loop of human immunodeficiency virus type-1 (HIV-1), strain IIIB (Laman et al., 1992), and used CYS to link V3 to SOC. We note that a 12-residue V3 peptide from HIV-1 (strain MN) displayed on filamentous phage has been used successfully to raise neutralizing antibodies (di Marzo Veronese et al., 1994). (3) The poliovirus VP1 capsid protein (312 residues) provided an example of an entire protein. SOC-VP1 was constructed in the same way as SOC-V3.

Results

SOC fusions: Expression and phage genome integration vectors

We first tried the approach of using *soc*-deficient phage to infect *E. coli* cells harboring plasmids designed to overexpress SOC-fusion proteins, intending that the progeny phage should bind directly the display proteins. Driven by the T7 promoter (Studier et al., 1990), the *soc* gene on plasmid pE-SOC (Fig. 2A, a) produced high levels (>10% of total cell protein) of SOC protein (Fig. 3, lanes 6 and 7). However, high level *soc* expression resulted in inclusion body formation and the demise of the plasmid-containing bacteria, which rapidly lost the ability to support phage multiplication. Moreover, the SOC which remained soluble was degraded rapidly by proteolytic activity, even in protease-deficient strains of *E. coli* (data not shown).

Accordingly, two alternative strategies were employed. In the first, the overexpressed SOC or SOC-fusion protein was extracted from inclusion bodies, purified, renatured, and then bound in vitro to polyheads or capsids (see below). In the second, the modified *soc* gene was reintegrated into the T4 genome to achieve regulated *soc* gene expression and SOC binding to the viral capsid in vivo in the infected bacteria. To reintegrate the *soc* gene and its derivatives into the phage genome, a modified positive selection plasmid was constructed (pRH, Fig. 2B, a; see Hong & Black, 1993). In this plasmid, the *soc* gene is flanked on its 5′ side by a 3′ portion of the T4 lysozyme gene (*e′*), and on its 3′ side by a 5′ part of another T4 gene (*denV′*), which allows homologous recombination be-

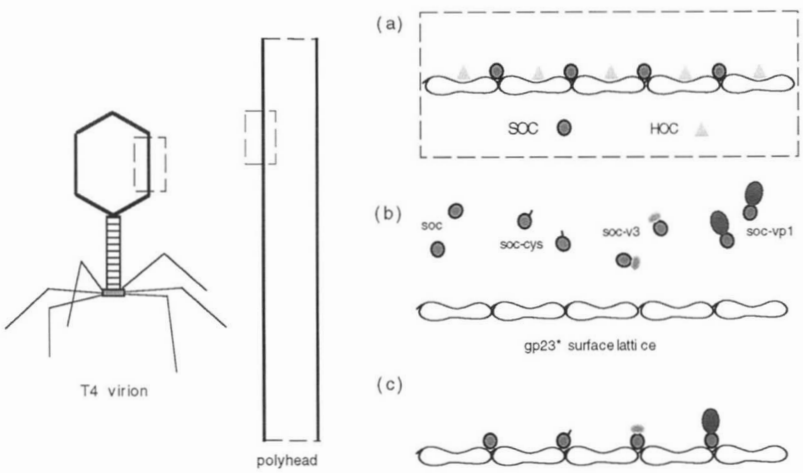

Fig. 1. Schematic diagram illustrating the principle of the SOC display system. Surface lattice of the mature T4 capsid contains two dispensable proteins, SOC and HOC (see Black et al., 1994). The same surface lattice may be reproduced by in vitro maturation of polyheads, which are open-ended tubular polymorphic variants of the wild-type capsid. A segment of the surface lattice (dashed boxes) is shown in greater detail (a). Its basic structure is a hexagonal array of hexamers of the major capsid protein gp23*, with a periodicity of 14 nm. (The capsid, but not polyheads, has pentamers of another protein, gp24*, at the vertex sites.) If available, HOC and SOC bind to the outer surface of the gp23* lattice: a HOC monomer binds at the center of each hexamer, and trimers of SOC bind around the trigonal sites, giving equimolarity with gp23*. Unlike the precursor (prohead) state of the surface lattice, which dissociates readily, the mature surface lattice does not dissociate over a wide range of concentrations and environmental conditions (see Fig. 1 of Black et al., 1994, for the T4 capsid assembly pathway). Moreover, SOC and HOC bind only to the mature state of the surface lattice, and not to the precursor states. The basic concept of this display system is illustrated in (b) and (c). Display platforms are SOC-less capsids or polyheads. Peptides or polypeptides to be displayed are expressed as C-terminal fusions of SOC and bind to the display platform (c). In this study, the viability of this system is demonstrated for a 4-residue peptide (CYS = Cys-Leu-Asn-Ser), a 47-residue domain (CYS-V3), and a 316-residue polypeptide (CYS-VP1).

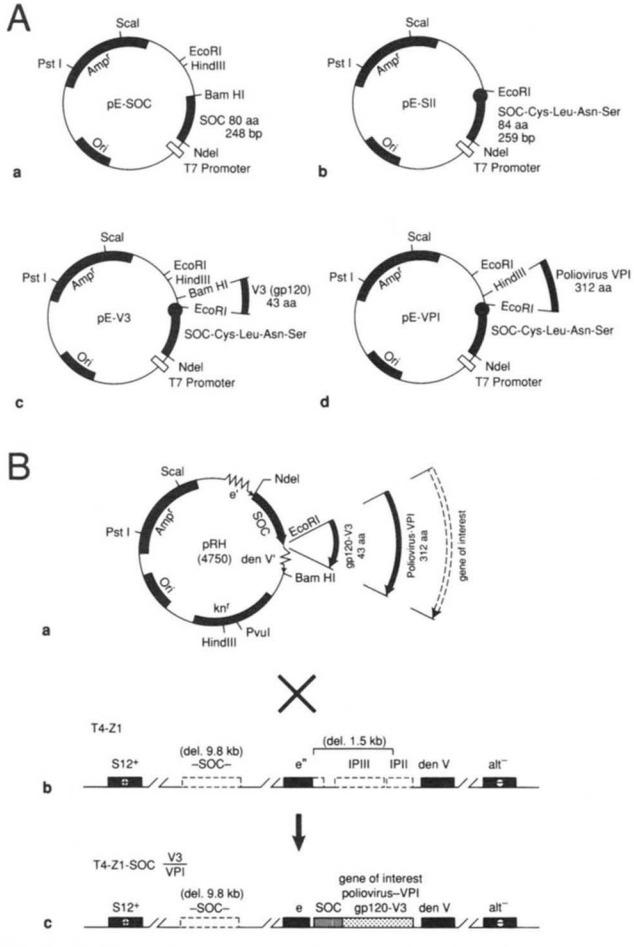

Fig. 2. A: Expression vectors employing an inducible T7 promoter are shown for (a) SOC (pE-SOC), (b) SOC-CYS peptide (pE-S11), (c) SOC-V3 (gp120 of HIV-1) (pE-V3), and (d) SOC-VP1 (capsid protein VP1 of poliovirus) (pE-VP1). **B:** (a) A phage integration plasmid is shown for *soc* recombination plasmid (pRH). The plasmid contains a portion of the phage T4 lysozyme gene *e'* at the 5′ end of the modified *soc* gene, and a portion of the 3′ *den V'* gene of T4. (b) pRH allows homologous recombination at both ends of *soc* with the T4-Z1 phage, which is deleted for genes *soc*, *IPIII*, *IPII*, and a part of *e"*. (c) Following recombination, which forms an intact gene *e* and transfers the modified *soc* gene into the phage genome, an egg white lysozyme-independent phage plaque selects for the *soc* gene.

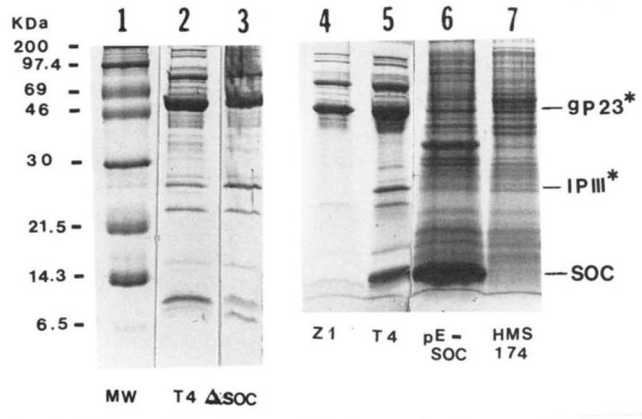

Fig. 3. SOC-containing phages and SOC expression from vectors are shown by SDS-PAGE, with staining for protein. Lane 1, molecular weight standards; lanes 2, 3, comparison of wild-type T4 phage with a phage deleted for the gene which codes for SOC (9 kDa); lanes 4, 5, comparison of wild-type T4 phage with the integration phage Z1, which lacks viral proteins SOC, IPIII, IPII, and ALT; lanes 6, 7, SOC expressed from bacteria containing the expression vector (Fig. 2A) compared with lysates of the same HMS174 bacteria but which lack the plasmid.

tween the phage and plasmid on either side of the plasmid *soc* gene.

The recombination-integration phage T4-Z1 (Fig. 2B, b) is deleted for *soc*, for a partial 3′ overlapping portion (to pRH) of the lysozyme gene *e"*, and for genes *IPIII* and *IPII*, which code for dispensable components of the prohead scaffold (Black et al., 1994). Absence of both SOC and IPII, which overlap in SDS-PAGE, accounts for the absence from the Z1 phage of a band at the 9-kDa position (Fig. 3, lane 4). Z1 lacks a functional lysozyme gene and requires egg white lysozyme for growth. Homologous recombination can integrate genes from the recombination plasmid at the *IPIII* position to reconstitute an intact lysozyme (*e*) gene and allow lysozyme-independent growth. Reintegration of the *soc* or *soc-SII* genes at the *IPIII* position occurred with high frequency in phage-plasmid crosses (see Materials and methods), as demonstrated by the growth of phage plaques in the absence of egg white lysozyme. Essentially all of these yielded virus particles that contained SOC or SOC-CYS proteins (data not shown).

Expression and binding to T4 capsids of a SOC-V3 (HIV-gp120) fusion

The *soc-cys* peptide extension gene *pE-S11* was fused to a portion of the *env* gene of HIV-1 containing the sequence coding for the V3 loop of gp120. This 43-residue polypeptide contains five amino acids on the N-terminal side of the first cysteine of the loop and two amino acids on the C-terminal side of the second cysteine (Laman et al., 1992). This construction allowed high level SOC-V3 expression from the pE-V3 vector (Fig. 2A, c), as determined by SDS-PAGE or Western blotting (Fig. 4).

The desired recombinant phage could be selected from a cross between the recombination selection phage T4-Z1 and the pRH-V3 plasmid, which contained the *soc-v3* gene, as determined by PCR. The resulting phage were found to contain SOC-V3 (Fig. 4). According to SDS-PAGE, the apparent molecular weights of the proteins produced from the expression and recombination vectors and those bound to the phage were the same (i.e., ~14,000). These Z1-*soc-v3* phage contained about one third of the normal complement of SOC-V3 (i.e., ~300 copies per virion), according to quantitation of the Coomassie blue-stained gel (Fig. 4A). Purification of the recombinant phage by CsCl gradient centrifugation did not significantly reduce the amount of SOC-V3 relative to gp23* (cf. lanes 4 and 5), supporting the inference that SOC-V3 is indeed bound to the phage particles. Western blotting with a monoclonal anti-gp120 antibody confirmed the identity of the designated gel band as SOC-V3 (Fig. 4B, lanes 1 and 2). In fact, two closely migrating bands of SOC-V3 were observed, especially with CsCl-purified phage, which may reflect partial proteolysis.

That SOC-V3 is specifically bound to the virion was shown by a mixing experiment (Fig. 5). Purified SOC-containing virions were mixed with SOC-V3 extract (lane 3) and subjected to CsCl density gradient centrifugation. SOC-V3 was found in the protein band (lane 2), cleanly separated from the much denser phage par-

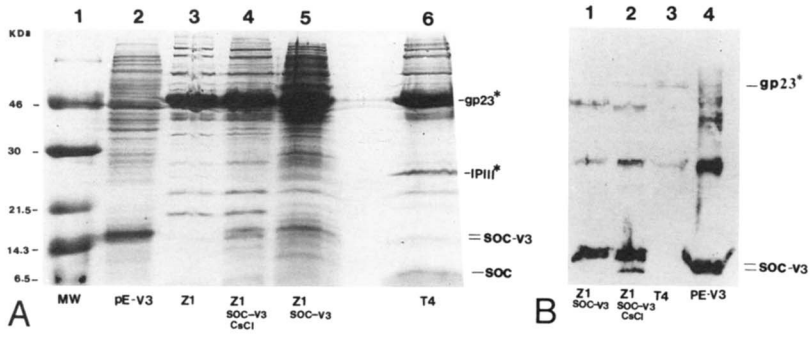

Fig. 4. A: Phage and bacteria containing the SOC-V3 protein analyzed by SDS-PAGE and stained for protein. Lane 1, molecular weight standards; lane 2, overexpression of SOC-V3 (~14 kDa) in *E. coli*; lane 3, recombination phage lacking SOC and IPIII. Following integration of the *soc-v3* gene, SOC-V3 is present on phage purified by high-speed centrifugation followed by CsCl density gradient centrifugation (lane 4) or by the first step only (lane 5). Comparable amounts of phage analyzed, as judged by the quantity of the major capsid protein (gp23*) in the Z1, Z1-*soc-v3*, and wild-type phages (lane 6), allow comparison of SOC content. **B:** The same samples are compared following western blotting using antiserum against gp120 of HIV-1.

ticles (lane 1). We infer that SOC-V3 did not associate with these virions because their specific SOC binding sites were already occupied, and that SOC-V3 does not bind nonspecifically to the capsid surface lattice.

Immunogenicity of SOC-V3 phage

After purified SOC-V3 phage were used to immunize mice, high levels of anti-gp120 antibodies were observed (Fig. 6). Whereas none of the mice had serum IgG antibodies specific for gp120 prior to immunization, each mouse developed such antibodies after the second inoculation (Fig. 6). The mouse-to-mouse variation seen in these ELISA reactions is typical of serum IgG responses in immunized populations. Importantly, these data show that immunization with the display phage induced serum IgG antibodies that recognize native glycosylated gp120. Furthermore, these responses were maintained for at least 120 days after the first immunization (Fig. 6). Together, these data demonstrate that protein domains displayed on SOC can be highly immunogenic, inducing antibodies that recognize the native parent protein.

In vitro binding of SOC-V3 to polyheads

To examine the binding of SOC-V3 to the gp23* capsid surface lattice, polyheads were used. As with capsids, only polyheads that have undergone the maturational expansion transformation bind SOC (see review by Black et al., 1994). Expanded gp23* polyheads were prepared and incubated in the presence of a twofold molar excess of freshly purified SOC-V3 or SOC, then centrifuged to separate them from unbound protein. The resulting pellets were resuspended and examined by SDS-PAGE (Fig. 7). Quantitation of the SOC and gp23* bands (Fig. 7, lane b) yielded an estimate of 1.2 for the molar ratio of SOC:gp23*. Because the intensity of the gp23* band was close to saturation, this figure is not significantly discordant with the known equimolarity of SOC and gp23*. The corresponding figure determined for SOC-V3:gp23* (Fig. 7, lane d) was 1.8. However, SOC-V3 is less soluble than SOC and a control incubation of SOC-V3 in the absence of polyheads also produced a pellet (Fig. 7, lane e), unlike the SOC control (not shown).

To ascertain whether SOC-V3 was associating specifically with the polyheads, negatively stained electron micrographs were recorded (e.g., Fig. 8a,b) and examined by optical diffraction and image analysis. The resulting diffraction patterns were found to have a different intensity distribution from those of unlabeled polyheads, implying that SOC-V3 was bound at specific sites and not randomly deposited on the polyhead surface. Filtered images revealed hexamers of stain-excluding units, surrounded by triplets of similarly sized units (Fig. 8c). For comparison, gp23* surface lattices with and without SOC are shown in Figure 8e and d, respectively. These results show that the SOC-V3 fusion protein associates specifically with the SOC binding sites. In Figure 8e, the hexameric units, which represent protruding portions of gp23* molecules, are somewhat larger than the triplet units, which represent SOC monomers. In contrast, the two kinds of stain-excluding units (hexamer and triplet) are similar in size in the SOC-V3-decorated lattice (Fig. 8c), consistent with SOC-V3 being ~50% larger than SOC. We infer that, in the incubation experiment

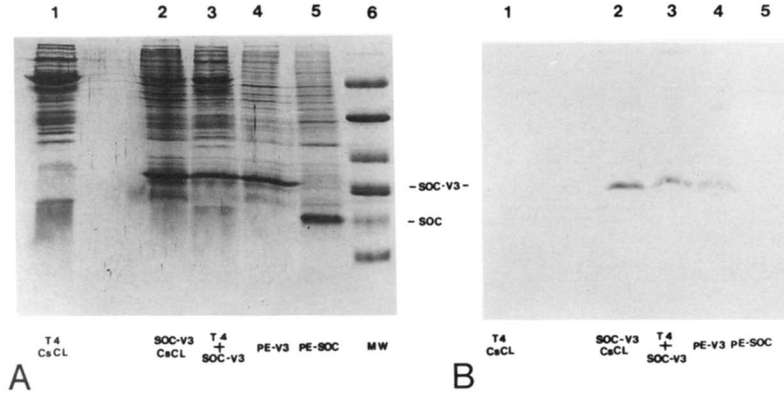

Fig. 5. Separation by CsCl gradient centrifugation of SOC-V3 protein from T4-SOC$^+$ phage as shown by (**A**) SDS-PAGE stained for protein and (**B**) western blotting using gp120 antiserum. Protein extracts of bacteria induced for SOC-V3 (lane 4) or SOC (lane 5) were prepared as described previously (Figs. 2, 3). A mixture of SOC-V3 extract and T4-SOC$^+$ phage (lane 3) was centrifuged at 35,000 rpm for 20 h. Visible and widely separated bands containing phage ($\rho \sim 1.5$) (lane 1) and protein ($\rho \sim 1.35$) (lane 2) were collected, dialyzed, and analyzed by SDS-PAGE. Lane 6, MW standards of 3, 6.5, 14.3, 21.5, 30, and 46 kDa.

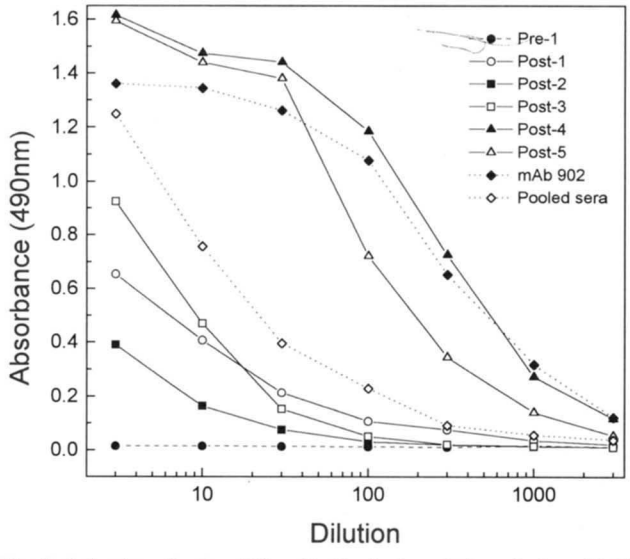

Fig. 6. Induction of anti-gp120 antibodies by inoculation of mice with T4 SOC-V3-displaying phage. Individual responses of five animals are shown compared with the baseline of the pre-bleed titer of one mouse, which was typical of the whole group. The mice were pre-bled on day −3, immunized on day 0, boosted on day 60, and harvested on day 95 (see Materials and methods), and assayed by ELISA against glycosylated gp120. Pooled sera data represent results obtained after pooling the sera collected from all five mice on day 120, following a second boost on day 104.

(Fig. 8d), the SOC-V3 protein bound rapidly to the available SOC binding sites, and the remainder precipitated.

Construction and display on phage T4 of a SOC-VP1 (poliovirus) fusion

To test the suitability of this system for display of larger polypeptides, we tried the 312-residue VP1 protein of the Mahoney strain of poliovirus (Kitamura et al., 1981). VP1 is the major capsid immunogen (van der Werf et al., 1983; Li et al., 1994). Our SOC-VP1 fusion was constructed by the same procedures as described above, i.e., an expression plasmid producing SOC-VP1 (pE-VP1) and an integration plasmid form of the gene fusion (pRH-VP1) were produced (Fig. 2A, d; Fig. 2B, a). A PCR assay confirmed that the *soc-vp1* gene fusion had been inserted correctly into both vectors.

The expression vector was found to overproduce SOC-VP1 (Fig. 9A,B). Following integration of plasmid pRH-VP1 into T4-Z1, recombinant SOC-VP1 (43 kDa) was found to be associated with the Z1-*soc-vp1* virions and was retained through purification on CsCl gradients (Fig. 9A,B). As with SOC-V3, a doublet of SOC-VP1 was observed by SDS-PAGE. The amount of display protein bound, cumulatively 25–100 molecules per virion, was considerably lower than with SOC-V3. Whether this reflects impaired binding, poor expression of the *soc-vp1* gene, or instability of SOC-VP1 in *E. coli* remains to be determined. The latter explanation is favored by the observation that the integration vector produced predominantly a protein of a size corresponding to the original VP1, and only a smaller amount of the fusion protein (Fig. 9B, lanes 3 and 6), whereas the expression vector produced large amounts of the SOC-VP1 fusion of the expected 43-kDa molecular weight, and low amounts of VP1 (Fig. 9B, lanes 6 and 7). In fact, phage T4 infection protects some foreign proteins from proteolysis in *E. coli* (Hong et al., 1995), and the recombinant phage clearly displayed significant levels of SOC-VP1, as confirmed below.

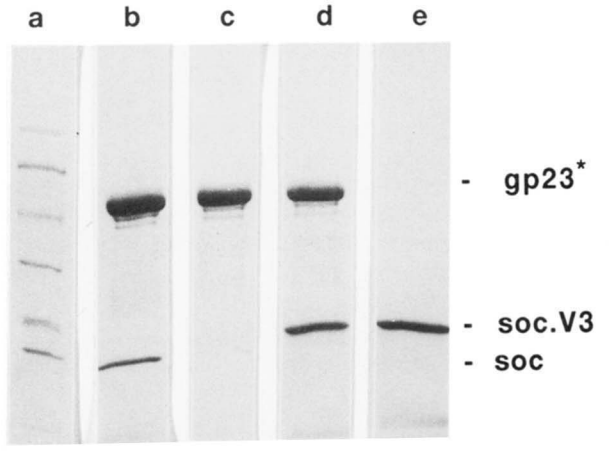

Fig. 7. SDS-PAGE of (a) molecular weight standards (97, 66, 43, 31, 22, and 14 kDa) and T4 polyheads complemented with purified SOC (b), control (c), and SOC-V3 (d). Purified SOC-V3 is shown in lane (e). Polyheads are of the cleaved/expanded type, in which the surface lattice of the major capsid protein gp23 has undergone proteolytic cleavage to the gp23* form and the subsequent expansion transformation, and thus can bind SOC.

Selection of the VP1 fusion clone by a modified biopanning procedure

To validate SOC and its gene for immunoselection, it is important to demonstrate that a recombinant phage displaying an epitope of interest can be isolated and propagated (Parmley & Smith, 1988). Results obtained with a modified and simplified biopanning procedure employing the Z1-*soc-vp1* phage (Fig. 10) show that the displayed VP1 protein is present and immunologically recognizable. These data are further supported by an immunoprecipitation assay in which ~1% of the Z1-*soc-vp1* phage remained when the VP1 antiserum was used, whereas the control Z1 phage (before recombination) was not depleted significantly (data not shown).

The biopanning procedure began with an initial ratio of one Z1-*soc-vp1* phage to 10^6 *del soc* phage. Nevertheless, the desired Z1-*soc-vp1* phage could be recognized in the second cycle of selection both by PCR and by Western blotting (Fig. 10). After each of three cycles, the DNA was probed by PCR, using phage DNA as template. A *soc* band was detected in the second and third cycles (Fig. 10A). In agreement with this measurement, in the second cycle, 1/10 plaques, and in the third cycle, 9/10 plaques contained the *soc-vp1* gene, by PCR assay of randomly selected single plaques. We conclude that phage of the desired genotype can be selected specifically; presumably, an even smaller fraction than 10^{-6} could be isolated by using more selection cycles. As well as being effective, this modified biopanning procedure is both simple and economical of antiserum (less than 60 μL was used).

Discussion

Several *soc-fusion* expression and integration vectors, and the corresponding reintegrant phage were constructed. All three fusion proteins tested, which corresponded to C-terminal extensions of

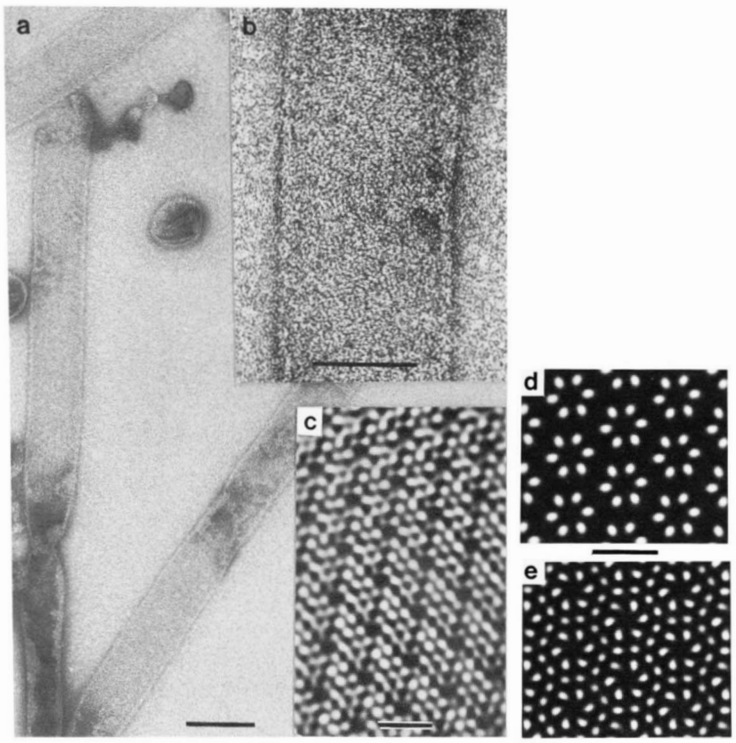

Fig. 8. (a) Electron micrograph of T4 polyheads (cleaved/expanded type) after complementation with SOC-V3. The specimen was negatively stained with uranyl acetate. Bar = 100 nm. (b) An individual polyhead at higher magnification. Bar = 50 nm. (c) Computer-filtered image of the surface lattice at ~3 nm resolution. Bar = 15 nm. For comparison, the undecorated gp23* surface lattice (d) and the lattice decorated with wild-type SOC (e) are also shown (reproduced from Ross et al., 1985). Bar = 15 nm. Triplets of SOC binding sites surround the points of local three-fold symmetry in the hexagonal surface lattice (e). In the SOC-V3 binding experiment (c), these sites are occupied by stain-excluding units that are somewhat larger than those observed with wild-type SOC, consistent with the greater size of the SOC-V3 fusion protein. We infer that occupancy of the SOC sites by SOC-V3 molecules is complete or close to it, because the peak density above background of the SOC-V3-related units in (c) is, on average, 10–15% higher than for the gp23* related units.

4 amino acids (SOC-CYS), 47 amino acids (SOC-V3), and 316 amino acids (SOC-VP1), bound to phage particles in vivo. In vitro binding of SOC-V3 to polyheads was also demonstrated. SOC-V3 binding was abundant in vivo (~30% of saturation) and stoichiometric in vitro (~100%), and averaged electron micrographs confirmed that SOC-V3 occupied the normal SOC sites on the surface lattice. As displayed, both SOC-V3 and SOC-VP1 were competent immunologically. Together these results suggest that the SOC system may be widely applicable for the display of individual domains and intact proteins in high copy numbers.

Use of soc in cloning

Our decision to first try 3′ gene fusions was based on the relative ease of analysis of recombinant products and lack of interference with SOC expression by termination codons introduced in cloning random DNA sequences, or by depression of translation of the modified *soc* gene through the possible occurrence of rare codons in 5′ sequences. Because substantial extensions may be added to SOC's C terminus without affecting its ability to bind to capsids, we infer that its C terminus must reside on the other side of the protein from that which binds to the gp23* surface lattice (see Fig. 1). The properties that SOC stabilizes phage at high pH (Ishii et al., 1978) or elevated temperatures (Ross et al., 1985) may allow for direct selection of phage displaying SOC derivatives.

Our isolation of Z1-*soc-v3* and Z1-*soc-vp1* phage integrants demonstrates that the SOC system may be used to clone DNAs whose protein products can be targeted by antibodies subsequently. Although display at high copy numbers is desirable for many immunologic purposes and for binding low affinity ligands (Green-

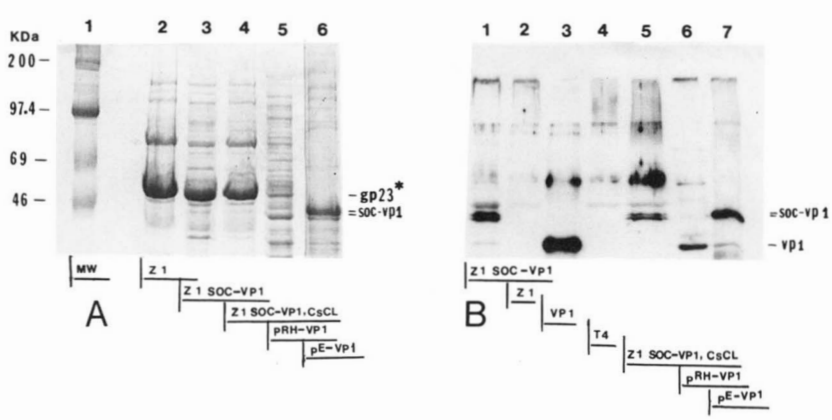

Fig. 9. A: Phage and bacteria containing the SOC-VP1 fusion protein were analyzed by SDS-PAGE and stained for protein. Molecular weight standards are shown in lane 1. Comparison of CsCl-purified virions of the recombination phage Z1 (lane 2) with the *soc-vp1* Z1 integrant, as purified by high-speed centrifugation (lane 3) and additionally by CsCl gradient centrifugation (lane 4), reveals the SOC-VP1 protein below gp23*, the major capsid protein. Products of the expression vector pE-VP1 (Fig. 2A, d) (lane 6) and of the recombination vector pRH-VP1 (Fig. 2B, a) (lane 5) have the expected apparent molecular weight of 43 kDa. **B:** Western blotting of the same samples using anti-VP1 antiserum. This gel also shows the position of unmodified VP1 from purified poliovirus (lane 3) and the same amount of T4 (lane 4) as Z1 and Z1-*soc-vp1* phages (lanes 1, 2, 5) as a negative control.

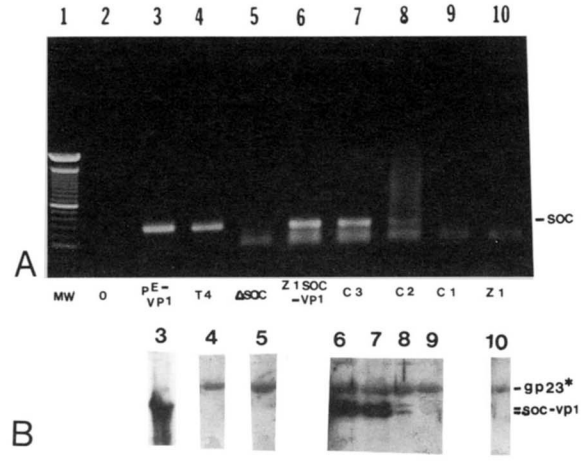

Fig. 10. A: PCR assay of a biopanning procedure using an antibody against poliovirus VP1 and an initial mixture of 5×10^9 *delsoc* and 5×10^3 Z1-*soc-vp1*. Compared with molecular weight standards (lane 1), DNA derived from the *soc* gene (259 bp) can be detected in expression vector PE-VP1 (lane 3), T4 (lane 4), and Z1-*soc-vp1* integrant (lane 6), but not in *delsoc* (lane 5), Z1 (before recombination) (lane 10), or buffer used for single plaque test (lane 2). C1, C2, and C3 are successive cycles of biopanning, showing that the *soc* gene can be detected in a mixture of phages arising from the second cycle of precipitation followed by growth. **B:** The same phage samples assayed by western blotting with the anti-VP1 antibody.

wood et al., 1991), display at low copy number may be preferable for selection of clones by biopanning (Wells & Lowman, 1992). Preliminary experiments suggest that SOC-related toxicity and expression level can be controlled by introducing suppressible termination codons adjacent to the *soc* initiation codon (Z.J. Ren, unpubl.).

Binding and other properties of SOC-displayed proteins

The SOC moieties of the three fusions studied retained the ability to bind specifically to the gp23* surface lattice. SOC-VP1 displayed on virions was recognized by anti-VP1 antibodies and was used successfully in a biopanning experiment. The displayed V3 polypeptide was also immunologically cross-reactive with its parent protein, gp120. An indication that SOC-V3 displayed on polyheads did not assume a random unfolded structure was given by its long-term resistance to proteolytic degradation when stored at 4 °C, contrasting with the high susceptibility to proteolysis of the soluble protein. Wild-type SOC that is not bound to capsids or polyheads is subject to C-terminal processing. During the isolation procedure (see Materials and methods), we often observed a protein ~2 kDa smaller than, but with the same N-terminal sequence as, wild-type SOC. Spectroscopic data (not shown) suggest that cleavage may take place before residue Trp-67.

We conclude that the SOC display system affords an effective platform for presentation of large antigens, with $\sim 10^3$ copies per capsid for polypeptides in the same size range as V3. Triplets of SOC binding sites are spaced ~7 nm apart, and within a triplet, the three sites are ~4 nm apart (Fig. 8d), so that steric blocking should not occur until large fusion proteins are involved. In such cases, one might still anticipate successful display at reduced occupancy, e.g., one copy of the display protein per triplet of SOC binding sites. In fact, interference between adjacent SOC sites on the capsid was inferred from kinetic studies of the binding of SOC monomers (Ishii et al., 1978).

Virions and polyheads have complementary advantages as display platforms. Phage bind the fusions directly in vivo, obviating the need for separate purification, and might be propagated in situ in an immunized host. Polyheads have an even higher carrying capacity and lack the highly antigenic (competing) components found on mature phage, i.e., the HOC capsid protein (Ishii & Yanagida, 1975) and the tail fibers.

Anti-gp120 (HIV) activity of antibodies raised against SOC-V3

ELISA assays showed that all five mice immunized with $\sim 10^{11}$ phage produced antibodies that recognized gp120. In contrast, T4 phage containing ~400 copies of a fusion in which V3 was linked to the internal protein (IPIII-V3, Hong et al., 1995) was not immunogenic by the same immunization schedule (G. Lewis, unpubl. results). Nevertheless, it remains possible that IPIII-V3 may be able to induce T-cell responses to helper and cytotoxic T-cell epitopes of V3, in which case it would provide a way to prime for T-cell responses in the absence of antibody production. This could help overcome the phenomenon of feedback suppression by anti-carrier antibodies, which can severely curtail primed immune responses (Edinger et al., 1990). This possibility is being tested currently.

Properties and potential applications of the SOC system

Filamentous phage display systems, under active development for more than a decade, allow quantitative display of short peptides (6–10 residues) and display of one or a few copies of intact proteins from minor phage proteins (see Introduction). Although these systems have proved to be powerful for cloning and immunologic applications, they have some limitations that have motivated the development of alternative display systems with different features.

Antigens have been displayed on the C terminus of the hepatitis B virus core antigen (Stahl & Murray, 1989). The lambda tail protein (Maruyama et al., 1994; Dunn, 1995) and the T4 *wac* protein (Efimov et al., 1995) afford systems in which a few copies of the displayed protein are incorporated into the virions of double-stranded DNA phages. As this work was being completed, we learned of a display system employing the lambda protein gpD, this phage's analogue of SOC (Sternberg & Hoess, 1995). N-terminal fusions of 8 or 73 amino acids to gpD were displayed on the capsid and the corresponding integrants were biopanned. However, the properties of these phages as immunogens were not reported, nor were the fusion proteins isolated and characterized with respect to their surface binding properties.

As noted above, systems based on capsid proteins have the potential to display complete domains or proteins in larger copy numbers than the minor proteins of filamentous phage allow. Large moieties may be fused to accessory capsid proteins like SOC and gpD without impeding assembly of the display platform (capsid or polyhead), which proceeds separately. As adaptations of lytic phages, these systems are independent of the *E. coli* secretion machinery, and consequently are unaffected by related problems. For instance, secretion-related toxicity is avoided, although, as we have seen with SOC-VP1 (above), stability problems may arise. Although at an earlier stage of development than filamentous phage display, these second generation systems appear to have considerable potential for further development.

In this article, several attractive features of the SOC system have been demonstrated. A 43-amino acid domain of the gp120 envelope protein of HIV-1 has been displayed at full occupancy of the SOC binding sites (~10,000 copies per polyhead). As demonstrated for SOC-V3 (Fig. 8), this system potentially allows display of proteins on regular arrays, making them suitable for structural analysis (Unwin, 1993) as well as functional studies. The poliovirus VP1 protein, which is toxic and unstable in bacterial expression (M. Chow, pers. comm., G. Lewis, unpubl.), has also been displayed—so far, in lower copy numbers—and its gene has been biopanned. Work is in progress to further assess the prospects of the SOC display system.

Materials and methods

Construction of T4 soc integration vector, pRH

In this construction, the *soc* gene is flanked on its 5′ side by a 3′ portion of the *e* (lysozyme) gene of phage T4 and by the strong *IPIII* promoter to drive *soc* expression. [IPIII is expressed throughout T4 infection, leading to incorporation of about 400 copies of IPIII per head (Black et al., 1994).] At the 3′ end of *soc*, a portion of a downstream T4 gene, *den V′*, allows homologous recombination between the phage and plasmid on either side of the *soc* gene (Fig. 2B, a).

The integration vector pRH, which allowed homologous recombination with the T4 derivative Z1, resulted from combining the *e′-IPIII-denv′* insert, which in turn resulted from digesting plasmid *Ep-denv*-151 with *Bam*H I and *Sca* I together with plasmid pACYC177. The *IPIII* gene was removed by digesting at the unique *Nde* I and *Eco*R I sites with *soc* derivatives inserted at the *IPIII* position. We constructed integration vector pRH-V3 and pRH-VP1 using pRHB digested with *Nde* I and *Eco*R I and filling in the *Eco*R I end as receptor portion. For the pRH-V3 insert, *soc-v3* was digested from pE-V3 with *Bam*H I (which was filled in) and *Nde* I; for the pRH-VPI insert, pE-VP1 was digested from pE-VP1 with *Hin*d III (then filled in) and *Nde* I, and ligated to pRH (Fig. 2B, a).

Construction of soc-fusion gene recombination bacteriophage T4-Z1

T4 phage$(39\text{-}56)_{12}$, deleted of ~9.8 kb between genes 39 and 56 (Homyk & Weil, 1974), was a gift from Dr. G. Mosig, Vanderbilt University. This phage (named in this paper *delsoc*) was crossed with phage eG326 in *E. coli* CR63 and tested for egg white lysozyme dependent and independent plating as described (Hong & Black, 1993). After two generations of lysozyme-dependent growth, 20 single plaque recombinants were picked at random and the *delsoc* confirmed by PCR with primers 77 and 95, and *delIPIII* confirmed using primers 67 and 81. The absence of functional gene *alt* as well as of *IPIII* and *soc* from the recombinant phage Z1 (Fig. 2B, b) was determined by SDS-PAGE of purified phage particles (cf. Fig. 3). In addition to the existing *soc* deletion of 9.8 kb in T4-Z1, the *alt* gene deficiency allows 6 kb more DNA to be packed into the T4 head (Wu et al., 1991).

PCR techniques

PCR was conducted on several kinds of templates. When T4 plaques were used directly as templates, a single plaque was picked from a plate and suspended in 200 μL of HPLC grade water. A 40-μL aliquot was then boiled for 3 min, and 25 pmol of each primer added, together with 15 nmol of each dNTP, 5 μL of 10× Mg^{2+}-containing Taq buffer, and 2.5 U Ampli Taq DNA polymerase (Perkin Elmer Co). Forty-five cycles of 94 °C for 1 min; 50 °C for 1 min, and 72 °C for 4 min were performed with a reaction volume of 50 μL, using a modification of the procedure of Eddy and Gold (1991). If a nonpurified phage stock (>10^{10} pfu/mL) was used as template, 1–2 μL was used in place of the single plaque.

PCR primers

T4 *soc*:

5′ end of *soc*:

Pr. 78: 5′-GAATCATATGGCTAGTACTCGCGGT-3′ (*Nde* I tailed)

3′ end of *soc*:

Pr. 77: 5′-CTTGGGATCCTAACCAGTTACTTTCCAC-3′ (TAG and *Bam*H I tailed)

For assay of T4 genomic soc: (421bp 5′ to *soc* ATG (MacDonald et al., 1984):

Pr. 95: 5′-CCTGGTGGTCAGGGTYGGAGAAGGAAGA-3′

T4 *soc*-S11:

3′ end of *soc*-S11:

Pr. 84: 5′-AGCAGAATTCAAGCAACCAGTTACTTTCCAC AAATC-3′ (tailed with Cys-Leu-Asn-Ser and *Eco*R I)

HIV I gp120-V3 loop:

Pr. 85: 5′-TGAAGAATTCTGTAGAAATTAATTGT-3′ (tailed with *Eco*RI)

Pr. 82: 5′-ATTTGGATCCCTAAATGTTACAATGTGCTTG-3′ (tailed with TAG and *Bam*H I)

Poliovirus VP1 (Mahoney):

Pr. 107: 5′-AGCAGAATTCGCTAGCACAGGGGTTA-3′ (tailed with *Eco*R I site)

Pr. 108: 5′-TACAAAGCTTTCTATTGGTGTCCGAATCCA TATGT-3′ (tailed with TAG and *Hin*d III)

Pr 107-108 cover 312 amino acids, from the RNA sequence of poliovirus type I (Mahoney) (Kitamura et al., 1981).

T4-IPIII:

Pr. 67: 5′-AGGAAACATATGAAAACATATCAA-3′

Pr. 81: 5′-AGAATTACCACGGGCTGCATTAGCAAC-3′

Pr. 67-81 cover T4 IPIII (193 amino acids).

Construction of soc gene expression vectors

A pET expression vector with phage T7 inducible promoter and T7 translational start signals was modified to allow overproduction of SOC and SOC-fusions in *E. coli*.

pE-Soc

The *soc* coding sequence was removed from its normal phage T4 controls (MacDonald et al., 1984). The fragment inserted started with the ATG of *soc* from a PCR product of T4 phage DNA obtained using primers 78 and 77. The PCR product was digested with *Nde* I and *Bam*H I and then was ligated into plasmid pET 3a derivative ppL-1 (Hong & Black, 1993) digested with the same two enzymes (Fig. 2A, a).

pE-S11

Same as pE-Soc, except the inserted fragment was obtained using primers 78 and 84, which were trimmed with *Nde* I and *Eco*R I (Fig. 2A, b).

pE-V3

The start plasmid was pE-Soc digested with *Nde* I and *Bam*H1. The V3 was obtained by PCR using template plasmid pBL21PiNIII containing the portion of HIV-1 provirus DNA for gp120, using primers 85 and 82. The product was digested with *Eco*R I and *Bam*H I. The *soc-Cys* insert was excised from pE-S11 with *Nde* I and *Eco*R I, and the three DNA pieces were then ligated together (Fig. 2A, c).

pE-VP1

The *soc-cys-leu-asn-ser* fragment of pE-S11 was removed with *Nde* I and *Eco*R I. A poliovirus *vp1* insert was produced by PCR, using template plasmid pSV2O (a gift from the Institute of Medical Biology, Chinese Academy of Medical Sciences), which contained whole poliovirus cDNA and primers 107 and 109. The PCR fragment was trimmed with *Eco*R I and *Hin*d III, and was ligated to the large fragment derived from pE-SOC by *Nde* I and *Hin*d III (Fig. 2A, d).

All expression vectors were transformed into *E. coli* HMS174 (DE3), which was induced with 0.5 mM IPTG for 3 h at 37 °C to test for the desired protein fusions by the procedures described.

Recombination of phage T4 Z1 with integration vector pRH and its derivatives

E. coli HB101(pRH-V3) and HB101 (pRH-VP1) were grown in CM medium (Hong & Black, 1993) to OD_{600nm} = 0.4. After addition of L-trp to 20 μg/mL, bacteria were infected with T4-Z1 at a multiplicity of infection of 4. After 5 h at 37 °C, the infected bacteria were concentrated by centrifugation and suspended in CM containing egg white lysozyme and chloroform. The supernatant, after centrifugation for 5 min at 4,000 × *g*, was spread on a CM plate containing *E. coli* B^E in CM top agar without lysozyme. Single plaques were isolated and checked for integration of the desired genes using the appropriate PCR primers listed above.

Expression and purification of SOC and SOC-V3

E. coli HMS174 (DE3) cells (Studier et al., 1990) containing the plasmid pE-V3 were grown in a fermentor at 37 °C. When the OD_{600nm} reached 0.6, the cells were induced with 0.4 mM IPTG for 3–4 h. The cells were resuspended in 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 4% glycerol, 3 mM 2-mercaptoethanol, and 2 mM EDTA (lysis buffer) that contained 100 μg/mL lysozyme. Cells from 6 L of culture were lysed by three cycles of freezing and thawing. DNase was added (10 μg/mL) to reduce the viscosity and the suspension was centrifuged at 10,000 × *g* for 45 min. The pellet fraction, which contained the insoluble SOC protein, was washed twice by suspensions into 100 mM Tris-HCl, followed by centrifugations at 10,000 × *g* for 30 min. The washed pellet was extracted with 40 mL of 50 mM Tris-HCl, pH 8.0, containing 6 M guanidine-HCl and 10 mM dithiothreitol. The solution was filtered through a 0.45-μm filter unit and applied to a Superdex 200 preparative grade 60/600 column (6-cm diameter × 60-cm long), equilibrated in 20 mM Tris-HCl, pH 8.0, containing 4 M guanidine-HCl and 5 mM DTT (column buffer A). Fractions were assayed by SDS-PAGE (18% acrylamide gels from Novex) after removing the guanidine-HCl (Pepinsky, 1990). Pooled fractions (~90 mL) were concentrated to 35 mL by ultrafiltration using an Amicon stirred cell with a PM10 diaflo membrane and applied to a Superdex 75 prep grade 60/600 column (6 × 60 cm) equilibrated in column buffer A. Fractions were pooled (~90 mL at 0.5 mg/mL) and stored in aliquots at −80 °C. Protein was folded by slowly diluting 25 mL lots into 100 mL of 100 mM sodium phosphate, pH 7.5, containing 5 M urea and 1 mM $MgSO_4$. The diluted protein (~0.12 mg/mL) was dialyzed against two changes of 2 L of buffer minus the urea. For folding the SOC-V3 fusion protein, 1–2 mM dithiothreitol was included in the dilution buffer. The clear solution was concentrated to 1–2 mg/mL using an Amicon centriprep-3 concentrator and then sterile filtered with a Millipore GV 0.22-μm filter unit. Protein concentrations were determined by UV-spectroscopy (Wetlaufer, 1962). The 79-residue SOC protein (M_r 8,989) and the 126-residue SOC-V3 fusion protein (M_r 14,208) both have calculated extinction coefficients of 18 $mM^{-1} \cdot cm^{-1}$. N-terminal sequencing (1–20 cycles) of the wild-type and fusion SOC proteins gave the same and expected sequences predicted from the translations of the respective coding DNA. The initiating N-terminal methionines in both proteins had been removed by the *E. coli* processing enzymes.

Binding of SOC and SOC-V3 to polyheads

Cleaved/expanded polyheads were prepared and purified as described (Steven et al., 1990). For the complementation experiments, 100-μg aliquots of polyheads initially at ~3.5 mg/mL protein in 0.1 M potassium phosphate, 1 mM $MgSO_4$, pH 7.0, buffer were added to a twofold molar excess of SOC or SOC-V3 in a total volume of 100 μL, incubated at 37 °C for 30 min, then spun at 20,000 rpm for 30 min in the SS34 rotor of a Sorvall RC5B centrifuge. The pellet was resuspended in 0.5 mL of the same buffer, then pelleted again. The resuspended pellet was analyzed by SDS-PAGE with 10–20 μg protein per lane, and the protein detection by staining with Coomassie brilliant blue. Negative staining electron microscopy with 1% uranyl acetate was performed on a Philips EM400T electron microscope, with micrographs recorded at a nominal magnification of 46,000×. Optical diffraction and micrograph digitization were conducted as described by Ross et al. (1985). Image averaging by computational lattice filtering was performed using the PIC program (Trus et al., 1996), running on an Alpha workstation (Digital Equipment Corp., Maynard, Massachusetts).

Gel electrophoresis and western blotting

Phage T4-*soc*, T4-*soc-v3*, and T4-*soc-vp1* were run on 17% (or 13% for T4-*soc-vp1*) SDS-PAGE gels, transferred to nitrocellulose membranes, and reacted with antibody, using an ECL chemiluminescence kit (Amersham Life Sciences) for detection. Typically, 20–50 μg of protein were loaded per lane. The antibodies used were: monoclonal Mab902, which is specific for the V3 loop of gp120 from the LAI isolate of HIV-1 (Cheesebro & Wehrly, 1988) and was obtained from the NIH AIDS Research Reagent Depository (Gaithersburg, Maryland); and polyclonal antibody prepared against SDS-PAGE-purified VP1 of poliovirus, generously provided by Dr. Marie Chow (University of Arkansas Medical School). To quantitate gels, photographic negatives were digitized using a COHU CCD camera and normalized relative to a Kodak stepwedge. These operations were performed under control of the NIH Image program. Two-dimensional integration of bands and background subtractions were performed using PIC software (Trus et al., 1996).

Mice and immunizations

Female BALB/cJ mice 6–8 weeks of age were purchased from Jackson Laboratories (Bar Harbor, Maine). Each mouse was bled via the tail vein three days prior to immunization to provide negative controls (denoted pre-bleeds in Fig. 6 legend). Groups of five mice were each immunized with 1×10^{11} PFU of CsCl-purified phage emulsified with an equal volume of Freund's Complete Adjuvant containing 1 mg/mL *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, Michigan). A total of 100 μL of this emulsion was inoculated subcutaneously on the dorsal hind quarters of each mouse. Secondary immunizations were via the intraperitoneal route using the same number of phage particles in Incomplete Freund's Adjuvant (Difco, Detroit, Michigan) and for tertiary immunizations, in phosphate-buffered saline (140 mM NaCl, 10 mM PO_4, pH 7.4). The mice were bled via the tail vein at the times stated in the text and the sera stored at −20 °C until they could be evaluated for anti-gp120 antibodies.

Measurement of serum antibody responses to gp120

Anti-gp120 antibody responses were measured according to a modified (Abacioglu et al., 1994) form of the antigen-capture ELISA assay (Moore & Jarrett, 1995). Briefly, native gp120 (Intracel Corp., Cambridge, Massachusetts) was captured onto polystyrene ELISA trays using an affinity-purified sheep antibody specific for the C terminus of gp120. Mouse sera were titrated in 1/2-log dilutions, beginning at 0.5 log. Binding was detected using alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (Southern Biotechnology, Birmingham, Alabama) and an enhance phosphatase substrate system (Life Sciences Inc., Gaithersburg, Maryland). This assay is configured for selective detection of immune responses to epitopes expressed on the surface of properly folded, glycosylated gp120. The epitope studied is lost when gp120 is denatured (Cheesebro & Wehrly, 1988).

Biopanning

A mixture of 5×10^3 T4-*soc-vp1* and 5×10^9 *delsoc* (1 mL) in phage dilution buffer was mixed with 30 μL of VP1 antiserum at 4 °C overnight. Then 30 μL of 100 mg/mL Protein A–Sepharose CL-4B (Pharmacia) were added, mixed gently on a rocking platform at room temperature for 30 min, and then centrifuged at 2,000 × *g* for 2 min in an Eppendorf microfuge. The pellet was washed five times by resuspending in 1 mL of SM buffer for 1 min and centrifuging as before at 2,000 × *g*. Finally, the pellet was resuspended in 1 mL of *E. coli* B^E at 2×10^8 bacteria/mL in M9S with gentle shaking for 20 min at 30 °C, and then 100 μL was plated. Subsequent cycles of isolation repeated the same procedure using ~5×10^9 phages obtained from the single plate stock. The same procedure was followed using normal rabbit serum and whole phage T4 antisera as controls. Phage were purified by CsCl density gradient centrifugation. Other phage techniques were as described previously (Hong & Black, 1993).

Acknowledgments

We thank Dr. Eva Kocsis for help with image processing, Dr. Marie Chow for generously supplying anti-VP1 antibodies and helpful advice, Dr. Changbai Dai for providing a cDNA plasmid carrying the whole poliovirus provirus, and Ms. Yu-ping Ning and Mr. Robert Tiskin for excellent technical assistance.

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