Isolation of receptor–ligand pairs by capture of long-lived multivalent interaction complexes

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We have combined phage display and array screening for the rapid isolation of pairs of interacting polypeptides. Our strategy, named SAC (selection by avidity capture), is based on the avidity effect, the formation of highly stable complexes formed by multivalent interactions; in our case, between a receptor (multivalently displayed on phage) and a ligand (coexpressed as a multimeric fusion protein). Capture of the long-lived interaction complex allows the isolation of phage bearing cognate interaction pairs, as we demonstrate for a range of interactions, including Ab–antigen pairs and the rapamycin-dependent interaction of FKBP-12 and FRAP. Cognate phage are enriched by SAC up to 1000-fold and interacting pairs can be identified by array screening. Application of SAC to Ab–antigen interactions as a model system yielded over 140 specific Abs to a single antigen and 92 Abs to three different fetal human brain antigens in a single round of SAC each. Our results suggest that SAC should prove useful for the identification and study of receptor–ligand interactions in particular among extracellular proteins, as well as for the rapid generation of specific Abs to multiple antigens.

Interactions among extracellular proteins, such as receptor–ligand interactions, play a crucial role in the development, cell-to-cell communication, and immune defense of multicellular organisms. Yet, standard technologies for the analysis of protein–protein interactions such as the two-hybrid system (1) but also more recent strategies such as the protein-complementation assay approaches (2–5) and RNA polymerase recruitment (6) operate inside the cell. Although there are examples of successful applications of these systems to extracellular proteins, frequently such proteins (especially those with disulfide bridges), do not attain full functionality when expressed in the reducing environment of the cytoplasm.

Because of the importance of extracellular protein–protein interactions for understanding biological function as well as for drug discovery, many useful methods have been developed for their analysis and selection. The most widely used are the various protein-display technologies (reviewed in ref. 7). Phage display in particular has allowed the isolation of many novel peptide and protein ligands as well as the isolation of Abs directly from diverse libraries of V genes (7). Current display technologies are mostly used for the isolation of ligands to a single target, available in at least partially purified form and linked to a solid support. To increase throughput and to increase the utility of display methods to proteomics applications, it would be desirable to be able to select directly for interacting receptor–ligand pairs starting from their encoding genes or repertoires thereof. Within phage display several approaches (called SAP, SIP, and CLAP; reviewed in ref. 8) have been described, which rely on reconstitution of phage infectivity by cognate interactions and allow selection of protein–protein interaction pairs. However, reconstitution of phage infectivity is inefficient (and depends on a narrow window of interaction affinities), constraining repertoire size and the types of interactions that can be analyzed.

Here we describe a novel strategy for the direct selection of interacting polypeptides, particularly extracellular proteins, on phage without compromising phage infectivity. Our strategy, called SAC (selection by avidity capture), is superficially similar to immunoprecipitation and exploits the much-increased apparent affinity and half-life of multivalent compared with monovalent interactions, i.e., the avidity (or chelate) effect (9). It involves the coexpression of receptors (displayed on phage) and ligands in multimeric form. As a result, cognate receptor–ligand pairs form multivalent high-avidity interaction complexes on the surface of phage. Once assembled, such complexes are very stable and thereby permit the isolation of phage bearing such complexes (Fig. 1). Cognate interaction pairs are verified by array screening with capture and detection with generic ligands.

To test the utility of SAC for the detection and selection of cognate interactions we coexpressed a diverse range of receptor–ligand pairs on phage and examined the specificity and stability of the resulting interaction complexes. With Ab–antigen interactions as a model case, we applied SAC to the selection of cognate Ab–antigen pairs directly from a naive human library of single-chain Fv (scFv) Ab fragments and antigens derived from a fetal human brain cDNA library.

Materials and Methods

DNA Manipulation and Protein Expression. Glutathione *S*-transferase (GST) (*Schistosoma japonicum*) was expressed from pGEX4T-2 (Amersham Pharmacia Biotech) and purified on glutathione Sepharose (GS; Amersham Pharmacia Biotech). Recombinant cDNA clones (D, human chloride ion current inducer protein, RZPD clone MPMGp800E04369Q3; M, unknown function, RZPD clone MPMGp800B12492Q3; and T (ubiquitin), RZPD clone MPMGp800D17184Q3) derived from the human fetal brain cDNA library hEX1 (10) were expressed and purified as described (11). Receptor proteins were cloned in and displayed with phagemid vectors pHEN-1 (12), pH (pHEN-1 without amber codon and c-myc tag), and pIT-2 (13). pHENS, in which the ampicillin (Amp)-resistance gene (*bla*) of pHEN-1 is replaced with the spectinomycin-resistance gene (*Sp*), was constructed as follows: the *Sp* gene from pSC101 was amplified with primers 1 (5′-TCA GCG CAC GCT GAC GTC GTG GAA ACG GAT GAA GGC ACG AAC-3′) and 2 (5′-GCC GCC CGG GCA GTC GAC TTA TTA TTT GCC GAC TAC CTT GGT GAT CTC GCC-3′), cut with *Aat*II, and ligated with pHEN-1 that was cut with *Aat*II and *Dra*I. Anti-human tumor necrosis factor-α (huTNFα) scFv Mab32 (14), anti-M scFv M12 (13), anti-T scFv T15 (13), anti-D scFv D4 (13), and anti-BSA scFv13CG2 (13) have been described. Other receptor proteins were amplified with specific primers and cloned into the above phagemid vectors (detailed procedures and primer sequences are published as Supporting Methods on the PNAS web site, www.pnas.org). The ligand expression vector was constructed as follows: The *lac* promotor, *pel*B leader, and polylinker of pUC119mycHis6 (15) were amplified with primers 3 (5′-CGG TGG CTG CCA TCG ATG GCA ACG CAA TTA ATG TGA

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Abbreviations: SAC, selection by avidity capture; scFv, single-chain Fv; GST, glutathione *S*-transferase; Amp, ampicillin; Chl, chloramphenicol; PEG, polyethylene glycol; GS, glutathione Sepharose; huTNF, human tumor necrosis factor.

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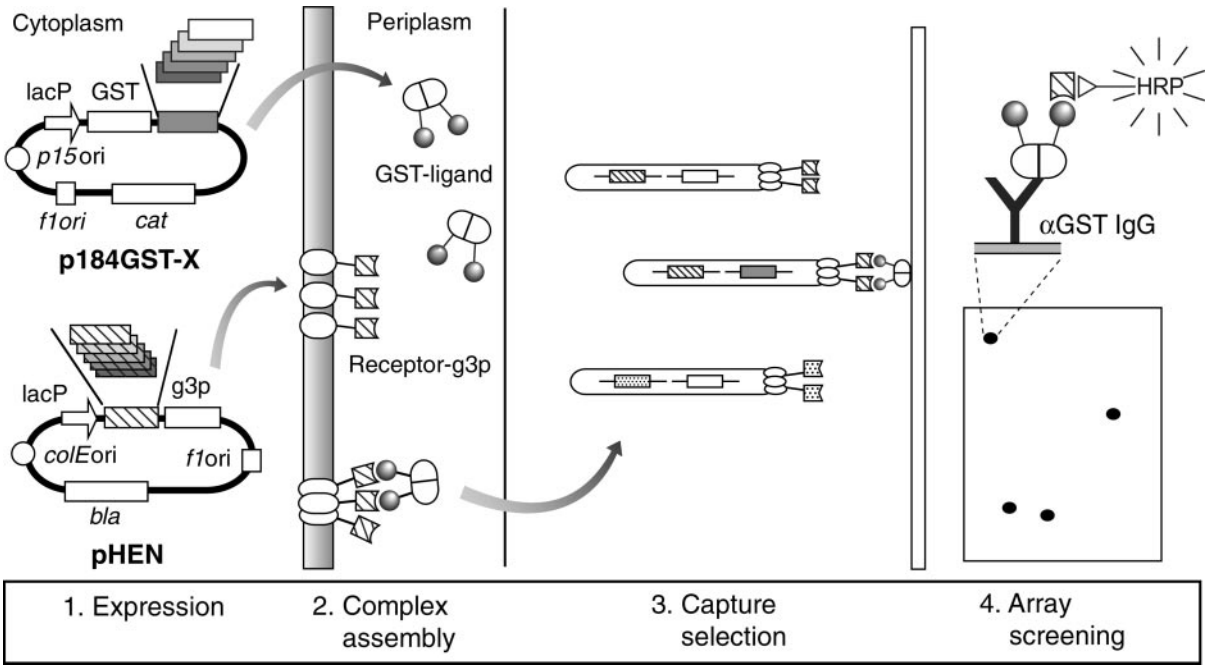

Fig. 1. General scheme of SAC. (1) A receptor polypeptide fused to g3p and a ligand polypeptide (fused to a multimerizing domain, e.g., GST) are coexpressed in the same cell and exported to the periplasm, where (2) they associate to form a multivalent high-avidity complex that is incorporated into nascent phage particles (3). Phages bearing a cognate interaction complex are captured on a solid support by way of the GST domain. Because both plasmids contain an f1 packaging origin, both are packaged into phage particles (4). Selected phage are plated for double antibiotic resistance and arrayed. Receptor and ligand proteins are coexpressed and cognate pairs detected (e.g., by capture of the GST-ligand fusion protein with an anti-GST IgG and detection of cognate receptor binding with an antireceptor-horseradish peroxidase conjugate).

GTT AGC TC-3′) and 4 (no. 1211, New England Biolabs) cut with *Cla*I and ligated into pACYC184 (New England Biolabs) cut with *Cla*I/*Bsa*BI to give p184lacP. The f1 origin of pUC119 was amplified with primers 5 (5′-GCT GCC GAC TCG GCT AGC GAA TGG CGA ATG GCG CCT GAT GCG G-3′) and 6 (5′-GCC GGG TCG CTT TAA AGT GTT GGC GGG TGT CGG GGC TGG C-3′) cut with *Nhe*I/*Dra*I and cloned into p184lacP cut with *Nhe*I/*Xmn*I to give p184f1lacP. GST was amplified from pGEX4T-2 with primers 7 (5′-CGC CGG GAC TCG CGG CCC AGC CGG CCA TGG CCC AGT CCC CTA TAC TAG GTT ATT GG-3′) and 8 (5′-CTC CGG CTG CGG CCG CAG CCT CGA GCG GGA ATC CAC GCG GAA CCA GAA CTT CCA GAT CCG ATT TTG GAG GAT G-3′) cut with *Sfi*I/*Not*I and ligated into p184f1lacP cut with *Sfi*I and *Not*I to give p184GST. For the human brain cDNA antigens, we used a variant of GST with three surface Cys mutated to Ser (GST_{3S}) (16) as fusion tag (kindly provided by A. Skerra, Univ. of Munich). GST_{3S} was amplified with primers 7 and 9 (5′-CGG CTC CCC AGT CGA CCC GGG AAT TCC TGG GGA TCC ACG CGG AAC CAG ATC CGA TTT TGG AGG ATG GTC GCC ACC-3′) and cut with *Sfi*I and *Sal*I; antigen genes M, T, and E were excised from the hEX1 cDNA library with *Sal*I/*Not*I and both fragments were coligated into p184f1lacP1 cut with *Sfi*I and *Not*I to give vectors p184GST_{3S}-M, -T, and -E. Other ligand proteins were amplified with specific primers and cloned into p184f1lacP (see Supporting Methods).

SAC Phage Rescue. Plasmids were cotransformed (or coinfected) into *Escherichia coli* TG1 and plated on TYE/Amp (0.1 mg/ml)/chloramphenicol (Chl) (10 μg/ml)/5% glucose plates (TYAC5 plates). Single colonies were picked and inoculated into 2× TY/Amp (0.1 mg/ml)/Chl (10 μg/ml)/5% glucose (TYAC5 medium) and grown overnight (ON). ON cultures were diluted 1/100 into fresh TYAC5 medium, grown for 1 h at 37°C, and R408Δg3p phage (17) (kindly provided by J. Rakonjac, Rockefeller University, New York) was added to a final concentration of 10^9 plaque-forming units per ml. The culture was incubated standing at 37°C for 1.5 h, spun down, and resuspended in an equal volume of terrific broth/carbenicillin (Sigma) (0.1 mg/ml)/Chl (10 μg/ml)/0.4% glucose [including 1 nM rapamycin (Calbiochem) for FKBP-12 × FRAP] and grown ON at 25°C. Phage were isolated by polyethylene glycol (PEG) precipitation, titered, and assayed by phage ELISA with standard protocols (18).

SAC Selections and Array Screening. The single framework (V3-23/DP-47, J_H4b × O12/O2/DPK9, $J_\kappa1$) libraries I and J have been described (13). Phages were produced from libraries I and J with standard protocols (18) and used to infect TG1 cells harboring ligand expression vector p184GST-M or an equal mix of p184GST-D, -M, and -T. Infected cells were plated on TYAC5 plates and grown overnight at 30°C. Plates were scraped as described (18) and bacteria added to prewarmed TYAC5 medium to a final OD_{600} of 0.4. Phage particles were produced with R408Δg3p helper phage as described above. Phages were selected on GS. GS was incubated for 2 h in PBS/2% Tween 20 at room temperature and washed 2 times with PBS/0.1% Tween20 (PBST). Phage were incubated for 15 min in PBST before addition to GS, mixed for 1 h with GS on a rotating platform, then washed (4 × 25 ml of PBST). Phages were eluted by incubation of the resin with 1 mg/ml of trypsin in PBS for 10 min. Eluted phages were used to infect TG1 and plated onto TYAC5 plates. Colonies were robotically picked and gridded (see Supporting Methods for detailed procedure). Array screening and ELISA were carried out as described (13).

Ab Purification and Affinity Determination. Selected scFv fragments were expressed and purified on protein-A Sepharose (Amersham Pharmacia Biotech) as described (19). Binding affinities of

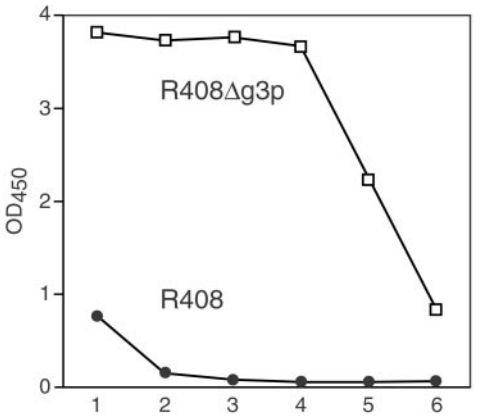

Fig. 2. Effect of multivalency. The cognate receptor–ligand pair (scFv anti-BSA 13CG2 × GST-protein L B1 domain) is rescued with two different helper phages. ELISA signal is plotted against phage titer. Phage are diluted stepwise by a factor of 2, starting from 10^{12} colony-forming units (cfu) per ml (R408, filled circles) and 10^{11} cfu/ml (R408Δg3p, open squares). Phages rescued with R408 are predominantly monovalent, leading to inefficient avidity complex formation. Phages rescued with R408Δg3p are multivalent and avidity complexes are readily formed.

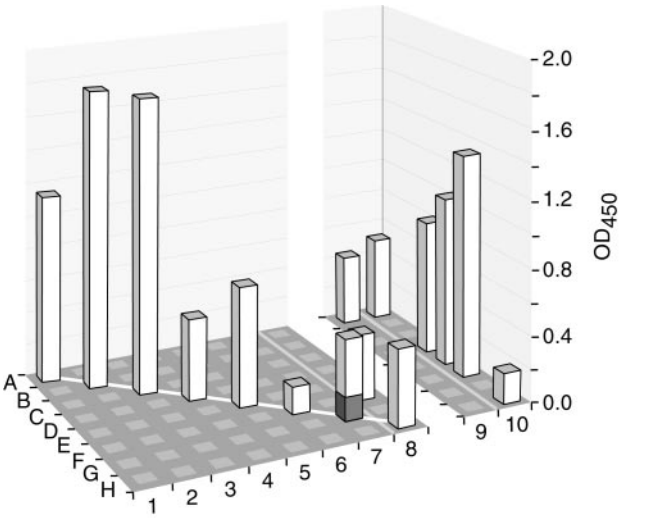

Fig. 3. Interaction matrix ELISA of avidity complexes on phage. Receptors and K_D of cognate interactions row A, Fab 9E10 [anti-c-myc, K_D = 80 nM (21)]; row B, FKBT-12 [$K_{D(FKBP\text{-}rapamycin,\ FRAP)}$ = 2 nM (24)]; row C, FRAP (see B); row D, scFv M12 [anti-M, K_D = 21 nM (13)]; row E, scFv T14 [anti-T, K_D = 5 μM (this work)]; row F, scFv D4 [anti-D (not determined)]; row G, c-Abl SH3, [we tested binding to two proline-rich target peptides, p41, $K_{D(p41)}$ = 1.5 μM, and 3BP1, $K_{D(3BP1)}$ = 35 μM (39)]; row H, scFv Mab32 [anti-huTNFα, K_D = 26 nM (14)] are combined with ligands column 1, GST-c-myc; column 2, GST-FRAP; column 3, GST-FKBP-12; column 4, GST-M; column 5, GST-T; column 6, GST-D; column 7, GST-41p and GST-3BP1 peptide (shaded); column 8, anti-hen egg lysozyme (HEL) FvD1.3-huTNFα fusion protein (22); column 9, anti-HEL diabody Hy-HEL10/5-c-myc (40); column 10: GST-protein L B1 domain $K_{D(huVk1)}$ = 130 nM (27)]. SAC phage were rescued and all combinations assayed by ELISA [with either anti-GST IgG (columns 1–7 and 10) or HEL (columns 8 and 9) for capture of phage bearing avidity complexes].

scFv fragments were determined with plasmon surface resonance with BIAcore as described (19).

Results

Overview of SAC. The general principle of SAC is outlined in Fig. 1. Receptor–ligand pairs were coexpressed in the bacterial periplasm with two phagemid expression vectors with compatible origins of replication and different antibiotic resistance. For phage display of receptor proteins we used standard ColE1 origin phagemid vectors like pHEN-1 (12) or derivatives thereof. Ligand proteins were expressed from a newly constructed phagemid vector, derived from the p15 origin plasmid pACYC184 (p184GST). Because of the compatibility of the ColE1 and p15 origins of replication, the two plasmids can coexist within the same *E. coli* cell without toxic effects. Both receptor and ligand protein expression are under control of the *lac* promotor. Optimal levels of expression for avidity complex formation are provided by leaky expression of the *lac* promotor in rich medium with a low level of glucose (not shown).

Control of Receptor and Ligand Valency. For coexpressed polypeptides to be able to form multivalent, high-avidity interaction complexes on the phage tip, both ligand and receptor must be expressed in multimeric form. Polypeptides are most frequently displayed on phage by fusion to the minor coat protein g3p (3–5 copies per virion), the major infectivity determinant of filamentous phages (20).

Phage display with phage vectors is always multivalent. Indeed, we found that stable avidity complexes were formed efficiently between Ab fragments [Fab 9E10 (anti-c-myc) (21) and scFv Mab32 (anti-human TNFα) (14)] displayed on phage fd and their cognate antigens [the c-myc tag, fused to a diabody (19) or huTNFα, fused to an Ab Fv fragment (22)] (not shown).

When using a phagemid as display vector, a helper phage is needed to package the g3p-fusion protein and phagemid into phage particles. Standard helper phages like M13 K07 or R408 express g3p in excess of the g3p-fusion expressed from the phagemid and thus yield a majority of monovalent phage particles. As a result, assembly of avidity complexes is inefficient (Fig. 2). The recently described R408Δg3p helper phage, however, lacks the g3p gene (17). Phage particles produced with this helper phage show polyvalent display, which promotes efficient assembly of high-avidity interaction complexes on the phage tip (Fig. 2). Valency of receptor display on phage is critical for SAC and can thus be readily controlled by choice of helper phage.

For multimeric ligand proteins (such as trimeric TNFα), further multimerization is unnecessary and a monomeric capture tag (such as the Ab Fv fragment) is sufficient (Fig. 3). For monomeric ligand proteins, however, valency must be increased by the appendage of a multimeric fusion tag. Our preferred fusion tag is the dimeric *S. japonicum* GST, although other multimeric avidity tags, like diabodies or triabodies (19), also work (Fig. 3). GST is among the most widely used fusion tags for both protein expression and proteomics (23), because many GST-fusion proteins are both functional and well expressed and often more soluble than the parent protein. Furthermore, GST fusion proteins are easily captured and eluted under mild conditions on GS beads.

Replicon Copackaging. To maintain genotype–phenotype linkage, receptor and ligand replicons must remain linked during phage selection and screening. It has long been known that replicons carrying a phage f1 origin will be copackaged into the same phage particle to a significant degree. Indeed, we found that up to 10% of phage particles carry both ligand and receptor phagemid replicons (not shown). Two-replicon phages are easily isolated by selection for double antibiotic resistance. The resulting colonies can be directly picked and gridded for array screening. Alternatively, single-replicon phage can be isolated and re-recombined with ligand replicons either by coinfection or transformation. The easy reassortment of receptor and ligand replicons also allows convenient identification of cross-reactivities by interaction matrix screening (Fig. 3).

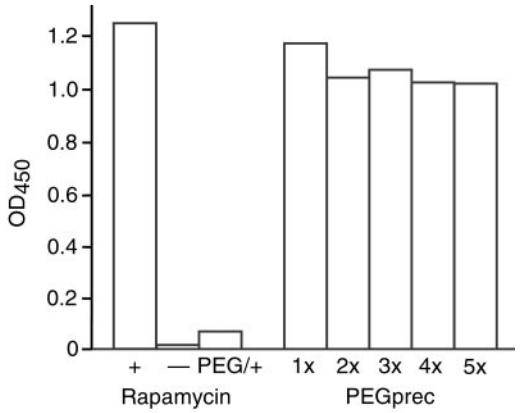

Fig. 4. Stability of avidity complexes on phage. Dependence of avidity complex formation between FKBP-12 on phage and GST-FRAP on the extraneous mediator rapamycin and avidity complex stability is assayed by ELISA. Phage are rescued in the presence (+) or absence (−) of rapamycin, or are precipitated before the addition of rapamycin (PEG/+) and captured with anti-GST IgG. Avidity complex formation strictly depends on rapamycin. Once formed, FKBP-12 × GST-FRAP avidity complexes on phage are stable to repeated precipitation with PEG.

Detecting Cognate Protein–Protein Interactions. We investigated the ability of SAC to detect a diverse range of receptor–ligand interactions including Ab–antigen interactions with a matrix screen comprising all possible permutations of 10 ligand and 8 receptor proteins (Fig. 3). SAC detects interactions of a wide range of affinities (down to 35 μM). Only the cognate interactions gave rise to an ELISA signal, with exception of the anti-D scFv D4 (13), which shows cross-reactivity with huTNFα. The identification of unexpected cross-reactivities is important, in particular for application of Abs in therapy. The above example shows the potential of SAC matrix screening for detecting such cross-reactivities.

The high-avidity interaction complexes assembled on the phage tip seem to be exceptionally stable. They survive multiple, sequential precipitations of the phage particles with PEG (Fig. 4) and are stable to storage at 4°C for several weeks. Phage-bearing avidity complexes can also be filtered through 0.45 μM filters without loss of either phage titer or ELISA signal (not shown), suggesting that avidity complexes do neither significantly increase phage size nor involve multiple phages. Furthermore, unlike in the SIP/SAP approach, phage infectivity and hence library size are not affected by the assembly of multimeric interaction complexes on the phage tip. PEG precipitation is also an efficient way to remove soluble ligand protein present in the culture supernatant from the phage preparation, as we demonstrate for the rapamycin-dependent interaction between FKBP-12 and FRAP (24). If rapamycin is added during phage rescue or before phage precipitation with PEG, it drives association of FKBP-12 and FRAP. In the absence of rapamycin or if it is added after PEG precipitation, no (or much reduced) complex formation is observed (Fig. 4).

Selection of Cognate Interactions by SAC. We tested the utility of SAC for the selection of cognate interactions with an Ab–antigen interaction pair [human fetal brain antigen M fused to GST (GST-M) and its cognate anti-M Ab M12 (13)] as a model. To aid selections a protease cleavage site was introduced between GST and the ligand domain in the ligand expression vector p184GST to allow specific elution by protease cleavage. To distinguish between cognate and noncognate phage, we constructed the Spectinomycin resistance (Sp^R)-conferring phagemid pHENS for display of scFv M12. As noncognate phage we used the anti-BSA scFv 13CG2 cloned into the Amp resistance (Amp^R)-conferring phagemid pHEN-1. Both phagemids were used to infect cells harboring p184GST-M [which confers Chl resistance (Chl^R)]. From these, phage were rescued with R408Δg3p (17). To test the ability of SAC to select for cognate interactions we spiked cognate phage (pHENS:scFv M12 × p184GST-M) (Sp^R/Chl^R) into a 10^3-10^4-fold excess of noncognate phage (pHEN-1:scFv 13CG2 × p184GST-M) (Amp^R/Chl^R). With either capture by an anti-GST Ab (coated on plastic) or GS beads and specific elution of captured phage by protease cleavage, we found that up to a 1,000-fold enrichment could be achieved in a single round of SAC [as judged by comparing the ratio of Sp^R/Chl^R to Amp^R/Chl^R phage before and after selection (not shown)]. Enrichment factors for SAC are thus comparable to those observed for standard phage display (13).

Selection of Ab–Antigen Pairs with SAC. We then applied SAC to the isolation of Ab–antigen interaction pairs directly from a naive Ab scFv library. Because of its two-replicon format, SAC can be applied to any preexisting display library constructed in standard phagemid vectors without the need for recloning. We chose two synthetic, single human framework scFv libraries, I and J (13). We first combined library I with GST-M and performed a single round of SAC capturing cognate phage on GS. Input and eluted phage were plated on Amp/Chl plates and 3,072 colonies each were robotically picked and gridded for array screening. Cognate interactions were identified by capture of the SAC complex with an anti-GST Ab and detection of the Ab portion by protein L. We also investigated specific binding of the Abs directly to recombinant antigen M or nonspecific binding to GST or an irrelevant antigen (BSA). We detected about 60 positive clones (Fig. 5*B*) on the screen for the cognate interactions. A larger number (about 110; Fig. 5*C*) appeared to bind specifically to antigen M but were not (or only weakly) detectable on the cognate screen, suggesting that the cognate screen is more stringent. Coexpression of GST-M, followed by capture on anti-GST IgG, may result in a lower local antigen density than direct coating. Nevertheless, antigen binding appeared highly specific with no binders detected on the GST and BSA screens (not shown). No positive clones were detected from screening the unselected library (Fig. 5*A*), indicating an enrichment of specific binders of at least 100-fold. Comparable enrichment factors are obtained by conventional phage display.

We chose 30 clones (with a range of positive signals) from the cognate screen (Fig. 5*B*) and tested their specificity by ELISA against antigen M and control antigens (BSA, GST). A majority (21) was specific for M, whereas for the other 9 clones, no binding could be detected on any of the antigens tested. Because of the higher density of antigen coating and less stringent washing, array screening detects lower-affinity interactions than ELISA, as observed (13). Sequence analysis of the 21 specific clones showed that they were all different but contained serine-rich VH CDR3 sequences very similar to anti-M scFvs isolated by conventional phage display (13). Indeed, scFv M1/14 (Table 2, which is published as supporting information on the PNAS web site) displayed an identical VH CDR3 sequence (SSYS) (but different VH CDR1 and VL sequences) as scFv M4 isolated by conventional phage display (13).

Parallel Selection of Abs to Multiple Antigens with SAC. We applied SAC to the simultaneous selection of Abs to multiple antigens. To this end we coselected two Ab libraries (I and J) with three different antigens, D, M, and T, from the same fetal human brain cDNA library (10) and analyzed the outcome by array screening. Selected (6,144) as well as unselected (3,072) clones were screened for the presence of cognate interacting pairs, for direct binding to D, M, and T and for nonspecific binding. After one round of SAC, we detected 92 Abs against the three antigens. Of

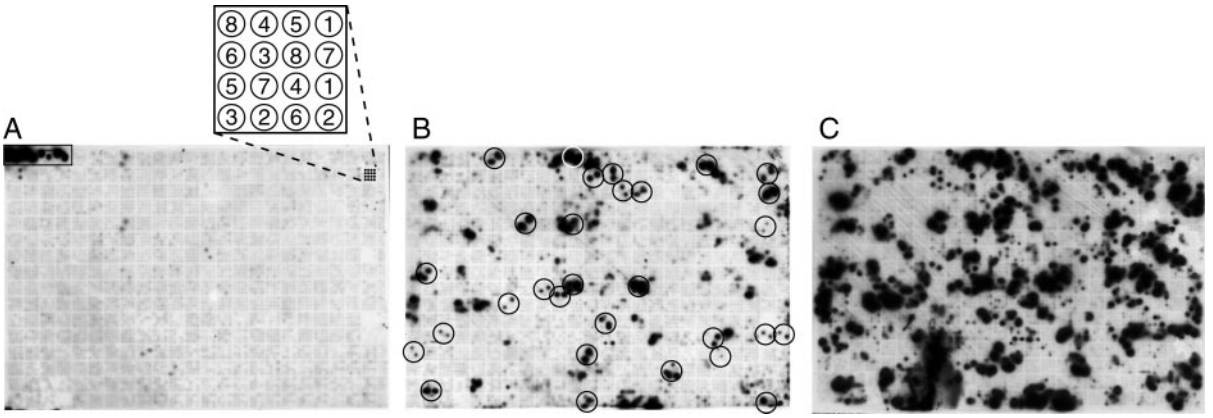

Fig. 5. Results of SAC selection. Array screen of 3,072 double-spotted clones before and after one round of SAC selection. Clones were arrayed in a 4 × 4 pattern (see expanded image). (*A* and *B*) Screening for cognate binding pairs before (*A*) and after (*B*) selection. Cognate interaction pairs are captured with anti-GST IgG and detected with protein L-horseradish peroxidase. Selected clones are circled. Positive controls GST-M × anti-M scFv M12 and GST-T × anti-T scFv T4 are boxed. (*C*) Direct screen for antigen M-specific binders after selection.

these 8 (D), 19 (M) and 4 (T) were confirmed in ELISA. As before, direct screening for antigen binding allowed the detection of specific Abs not detected by the screen for cognate interaction pairs. Presumably, these arise not only from its greater sensitivity, but also because the direct screen detects potential "false negatives." These may derive from noncognate phage, which get captured after picking up cognate antigen from the supernatant.

In agreement with results from conventional phage selection (13), binders to antigen D were exclusively isolated from library J, whereas binders to M or T derived preferentially from library I. Selected binders were specific and did not bind GST, BSA, or noncognate antigens. For selections with library I, we detected few false positives, i.e., polyreactive Abs (1/3,072) or Abs specific for the anti-GST IgG (1/3,072) or the GST fusion tag (0/3,072). The specific protease cleavage elution, together with the use of just a single round of selection, may contribute to the low prevalence of polyreactive binders. Conversely, library J showed a higher number of polyreactive binders. However, the number of polyreactive clones was very similar before and after SAC selection.

Sequence analysis of the anti-D, -M, and -T clones showed that again, SAC had yielded a highly diverse collection of specific Abs with all 31 clones being different. Anti-M clones showed again serine-rich VH CDR3 sequences, similar (but not identical) to isolated anti-M Abs (13), whereas the anti-T or anti-D Abs displayed no such similarities (Table 2). We purified 8 of the ELISA-confirmed scFv fragments and measured affinities on BIAcore for both the purified antigens and the corresponding GST-antigen fusion proteins. Affinities were modest for all Abs, with dissociation constants (K_D) ranging from 0.3 to 1.5 μM (Table 1). Affinities for recombinant antigen closely matched affinities for the corresponding GST-fusion proteins.

Discussion

SAC is based on the avidity or chelate effect, i.e., the greatly increased stability of multivalent compared with monovalent interactions. Ubiquitous in nature, it has previously been exploited in diverse areas ranging from coordination chemistry to immunoassays. The precise increase in affinity gained through avidity depends on many factors, including steric effects, local epitope concentration, etc., but theoretically the resulting binding constant can approach the square of the original binding constant (9). Thus, our observation that multivalent interaction complexes on the phage tip are long-lived and persist even through repeated precipitation steps (Fig. 4) is not unprecedented.

Our results show that SAC is well suited for the screening and selection of protein–protein interactions, as exemplified by the rapid generation of specific Abs against single or multiple antigens. A single round of SAC selection was sufficient for the identification of a highly diverse set of Abs, presumably because the avidity effect allows isolation of low-affinity interactions and thus makes maximum use of the diversity encoded in the starting libraries. Indeed, the affinities of eight different scFv Ab fragments isolated by SAC were modest, between 0.3–1.5 μM (Table 1). Although limiting the direct utility of the selected scFv fragments, selection of low-affinity interactions are a prerequisite for the application of SAC as a generic interaction screen. Detection of the avidity complex of the Abl SH3 domain with the 3BP-1 peptide (35 μM) (Fig. 3) suggests that interactions with affinities as low as 100 μM should be selectable by SAC. Even lower affinity interactions may become accessible by the use of higher valency fusion tags, higher valency display, e.g., g8p (2,800 copies per phage virion; ref. 20), or through the covalent stabilization of fleeting interactions, e.g., the formation of disulphide bridges or the addition of chemical crosslinking or bridging agents. Conversely, capture of receptor–ligand complexes on phage may be used to select for high-affinity interactions with monovalent display and monomeric ligands. Precedents for this approach exist. Ab Fab fragments can be displayed and selected on phage (25). Fabs are formed by the noncovalent association of Ab heavy and light chains (K_D = <1 pM) (26). Thus, affinities in this range should allow monovalent capture to work, whereas the affinity of the protein L B1-Vκ1 interaction (K_D = 130 nM) (27) appears too low (Fig. 2).

Table 1. Binding affinities of human scFv fragments isolated with SAC

scFv fragment	Target antigen	K_D (μM) for antigen	K_D (μM) for GST-antigen
M2/2	M	0.34	0.39
M2/6	M	1.40	1.54
M2/10	M	0.94	2.10
M3/14	M	1.30	1.61
T2/14	T	1.50	3.50
T2/15	T	0.94	0.69
D2/1	D	0.79	0.46
D2/2	D	0.41	0.28

Because of its two-replicon format, preexisting phage display libraries can be applied directly to SAC with no need for recloning or alterations to existing protocols. Many phage display libraries are constructed in colE1 phagemid vectors and thus are compatible and can be readily combined with our GST-ligand expression vector allowing the application of SAC to the parallel screening and selection of Ab–antigen interactions (as described here), to the generation of very large combinatorial Ab libraries (O. Ignatovich, I.M.T., and G. Winter, unpublished results) or as a generic screen for protein–protein interactions. The example of the rapamycin-mediated interaction of FKBP-12 and FRAP (Fig. 2) shows that not all of the interaction partners need to be proteinaceous. SAC may be useful for the screening of compounds (like rapamycin) that mediate receptor–ligand interactions or complement mutations in a ligand-receptor interface (28). Drug-discovery applications may be facilitated by the periplasmic location of SAC, as some compounds equilibrate poorly across the cell membrane into the cytoplasm, whereas molecules of up to 10 kDa (29) can enter the periplasmic space. SAC may also offer the possibility of "capture display," e.g., the capture of solubly, expressed multimeric Ab fragments or other proteins by a generic ligand displayed on phage. Indeed, a stable avidity complex could be assembled between a triabody and the protein L B1 domain displayed on phage (not shown).

The utility of SAC (and any other bacterial interaction screen) critically depends on the ability to express a wide range of eukaryotic proteins in functional form in bacteria. Although large multidomain proteins are often poorly expressed in bacteria, a broad range of single-domain proteins or isolated domains can be secreted to the periplasm and displayed on phage. Furthermore, considerable progress has been made recently to expand the range of proteins that can be expressed in bacteria or displayed on phage (reviewed in ref. 30). SAC also does require only very low-level expression. The precise amounts produced by an incompletely repressed *lac* promotor have been measured (31) and found to be as low as 100–1,000 molecules per cell. Such modest levels of expression should be attainable for a broad range of proteins.

Should expression in bacteria prove intractable, other expression hosts may be used. The SAC strategy of assembling high-avidity interaction complexes that are sufficiently stable and long-lived to allow capture and selection should be generally applicable to other expression hosts and display technologies including eukaryotic display methods like baculovirus display (32) and yeast display (33). Indeed, SAC should be applicable to *in vitro* methods like ribosome display (34) and RNA display (35), provided coexpression of individual receptor–ligand pairs is appropriately segregated, for example, by using artificial methods of compartmentalization such as vesicles (36), prefabricated nanowells (37), or emulsion droplets (38) to ensure genotype–phenotype linkage.

In conclusion, we have described a novel, potentially general strategy for the screening and selection of protein–protein interactions based on the assembly and capture of long-lived, noncovalent interaction complexes. As shown, SAC in conjunction with array screening has great potential for the fast generation of a diverse spectrum of Abs against multiple antigens. We foresee many more applications of the technology, including the screening and selection of generic protein–protein interactions in particular among extracellular or endoplasmic reticulum proteins, the screening of small molecular compound libraries, and the coevolution of protein–protein interaction surfaces.

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