Selection of Linkers for a Catalytic Single-chain Antibody Using Phage Display Technology

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Phage display has been evaluated as a means of rapidly selecting tailored linkers for single-chain antibodies (scFvs) from protein linker libraries. Preliminary experiments with a conventional linker failed to yield a functional single-chain version of a catalytic antibody with chorismate mutase activity. A random linker library was therefore constructed in which the genes for the heavy and light chain variable domains were linked by a segment encoding an 18-amino acid polypeptide of variable composition. The scFv repertoire (~5 × 10^6 different members) was displayed on filamentous phage and subjected to affinity selection with hapten. The population of selected variants exhibited significant increases in binding activity but retained considerable sequence diversity. Screening 1054 individual variants subsequently yielded a catalytically active scFv that was produced efficiently in soluble form. Sequence analysis revealed a conserved proline in the linker two residues after the VH C terminus and an abundance of arginines and prolines at other positions as the only common features of the selected tethers. There are apparently many viable solutions to the problem of linking individual VH and VL domains, but subtle differences in sequence dramatically influence the production, stability, and recognition properties of the scFv. The success of these experiments suggests that phage display will be generally useful for identifying peptide sequences for covalently linking any two protein domains.

Functional single-chain antibodies (scFvs) have been engineered in many laboratories by linking together immunoglobulin heavy and light chain variable domains (VH and VL) via polypeptide tethers (1, 2). Compared to intact IgGs or Fab fragments, scFvs have the practical advantages of smaller size and structural simplicity with comparable affinity for antigen. Covalent linkage of the VH and VL chains also favors domain assembly and enhances protein stability relative to analogous two-chain Fv fragments. Moreover, organizing Fv genes as a single continuous DNA sequence can facilitate the generation of mutant libraries.

Successful construction of an scFv depends on the choice of a linker that neither interferes with the folding and association of the VH and VL domains nor reduces the stability and recognition properties of the Fv molecule. A certain degree of flexibility in the linker may also be needed for the functional cooperation of the two subunits. To satisfy these requirements, several design strategies have been developed (1, 2). In one approach, flexible glycine-rich sequences such as (GGGGS)_3 have been used as tethers. Alternatively, useful linkers have been derived from multidomain proteins or designed by molecular modeling. There is no reason to believe, however, that a linker suitable for one antibody will be optimal for others. Indeed, expression levels, solubility, stability and binding affinity of scFvs can vary significantly depending on linker length and sequence.

Selection of a functional linker from a large population of candidate sequences represents a potentially more general solution to the problem of scFv design. Stemmer and co-workers have developed a procedure to identify scFv vs synthesized from libraries of scFv genes with randomized linker DNA sequences (3). This method uses filter lifts to detect binding activity to hapten, but requires hapten labeling and is limited by the number of molecules that can be practically screened. Phage display technologies have the potential to extend this approach greatly. Large repertoires of >10^7 scFv clones can be produced on the surface of filamentous phage particles (4–6), making possible direct selection of linkers on the basis of their ability to yield functional (i.e. hapten binding) receptor molecules. Here we report the successful application of this strategy to the construction of a single-chain version of the catalytic antibody 1F7 (7) which was raised against transition state analog 1 and possesses modest chorismate mutase activity (Fig. 1).

MATERIALS AND METHODS

Strains, Bacteriophage, and Vectors—Escherichia coli XL1-Blue (Stratagene) was used for cloning of libraries, phage panning, screening for soluble scFv protein production, and for routine plasmid preparations. BL21(DE3) (Novagen) was the host strain for large scale production of functional scFv. Helper phage VCS-M13 (Stratagene) was chosen for phage rescue. Phagemid pComb-M3 (Fig. 2a) was constructed by deleting a 272-base pair fragment from Nhe I (859) to Xba I (1131) in pComb3 (8) to avoid problems with in vivo recombination and rearrangements associated with a second homologous cloning site. A sequence encoding a decapeptide tag (YPYDVPDYAS) was placed upstream of the gII sequence for easy detection of protein products by the monoclonal antibody 12a5 (provided by Dr. I.A. Wilson). The restriction sites Xba I and Nhe I flanking the gII fragment are compatible and facilitate excision of the gII sequence for production of soluble scFv protein. Phagemid pET-22b (+) (Novagen) was used for the overproduction of selected scFv proteins.

Oligonucleotide Synthesis—The randomized linker oligonucleotide and PCR primers were synthesized on a Pharmacia Biotech Inc. Gene Assembler Plus. The antisense oligonucleotide 1F7vMLU 5'- TTCAACCGGTTSNNSNNGACAGTTGACAGGTAC-3', where S is dA, dC, dG, or dT.

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§ The abbreviations used are: scFv, single-chain antibody; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography.

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The scFv gene, containing both dG or dC and N is dA, dG, dC, or dT) contains a sequence complementary to the 3' end of the VL gene and encodes an 18 amino acid linker within the VL sequence. Second, the random linker library was fused to the Vx gene obtained by PCR from the corresponding Fab gene (9) using the primers 1F7Vx-Mlu and 1F7Vx-Nco. The PCR products were ligated into Nco/Mlu-digested pComb3-mVL3. The final ligation mixture was transformed into XL1-Blue cells by electroporation. An aliquot of transformed cells was plated on an ampicillin-containing agar plate to allow characterization of the starting library by sequencing of individual clones. The remaining sample was incubated at 37 °C for 9 h in 200 ml of 2xYT medium (10) containing 1% glucose and 100 μg ampicillin/ml, and stored in 15% glycerol at −70 °C.

Phage Rescue and Panning—Phage rescue from the phagemid library by helper phage VCS-M13 was carried out as described by Marks et al. (11). Phage panning was performed in 96-well microtiter plates following standard protocols (11). Phage particles that bound to the immobilized bovine serum albumin-conjugate of transition state analog 1 (7) were eluted with 1.5 ml of free hapten and introduced back into exponentially growing XL1-Blue cells by infection. Five rounds of panning were performed. Cells containing the final pool of selected phage-mids were stored at −70 °C in 15% glycerol.

Screening for Soluble Functional scFv—Double-stranded phagemid DNA was isolated from the final pool. In order to produce scFvs in soluble form, the gII1 fragment was excised by digestion of the phagemid pool with XbaI and NheI, and the large fragment was recircularized after purification by agarose gel electrophoresis. To screen for soluble hapten binding scFvs, this phagemid pool was transformed into XL1-Blue. Individual colonies were picked with a toothpick and grown in 150 μl of CS medium (4.8% yeast extract, 0.3% NaPO4, 0.3% NaH2PO4, pH 7.1) containing 100 μg of ampicillin/ml in a 96-well microtiter plate at 37 °C for 6 h. Expression of the scFv genes was induced by addition of 50 μl of fresh CS medium containing 1 mM isopropyl-1-thio-D-galactopyranoside to each well, and the cultures were incubated at room temperature for an additional 24 h. Hapten binding activity was assayed by ELISA with 50 μl of the culture supernatant. Active clones were further characterized by semiquantitative ELISA, Western blotting, and DNA sequencing (10).

Large Scale Production of scFv—Novagen’s pET system (12) was used for the production of preparative amounts of the most active scFv. The scFv gene, containing both pelB signal and decapetide tag sequences, was cloned into the expression vector pET-22b after appropriately altering the restriction sites at both ends of the fragment to be inserted by PCR. Strain BL21(DE3) was transformed with the resulting pET-sc1F7/L2 construct (Fig. 2c) and grown in 3 liters of 2xYT medium containing 1% glucose and 100 μg of ampicillin/ml at 37 °C with frequent additions of NaOH to maintain pH 7.0 until the cell density reached A600nm = 0.7. The culture was then cooled to room temperature and its pH was adjusted to 5.8. Expression of the scFv gene was induced by addition of isopropyl-1-thio-β-D-galactopyranoside to 0.3 mM, and the culture was incubated at room temperature for 8 h at a constant pH of 5.8. Cells were harvested by centrifugation and stored at −70 °C prior to protein isolation.

Protein Purification—Soluble scFv protein was purified from the cells in two steps. First, the frozen cells were suspended in 80 ml of buffer A (50 mM sodium phosphate, 300 mM NaCl, pH 8, containing 0.2 mM phenylmethylsulfonyl fluoride and 2 μg/ml leupeptin, pepstatin A, and aprotinin) and disrupted by passage through a French press. After centrifugation for 60 min at 25,000 × g, the supernatant containing all soluble proteins was loaded onto a column packed with 4 ml of ProBond C15-琼脂糖 resin (Pierce) that had been equilibrated with buffer A. The column was thoroughly washed with buffer A containing 10 mM imidazole, and bound protein was eluted with 250 mM imidazole, pH 7.0. Second, proteins eluted from the nickel column were loaded onto a monoS cation exchange column (Pharmacia) in 50 mM sodium phosphate, 0.1 M NaCl, pH 7.0. After washing the column with the loading buffer, bound proteins were eluted by a 100–600 mM NaCl gradient. The scFv protein eluted at ~400 mM NaCl. Protein purity was checked by SDS-polyacrylamide gel electrophoresis using Pharmacia’s PhastGel system. Protein concentration was determined by measuring the absorbance at 280 nm (ε280 =

![Fig. 1. Transition state analog 1 was used to generate the monoclonal antibody 1F7, which catalyzes the rearrangement of chorismate to 2-phenylpyruvate.](https://example.com/fig1.png)

![Fig. 2. Vectors used for the construction and phage display of scFv libraries and the expression of individual clones.](https://example.com/fig2.png)
efficiency of the individual phosphoramidite solutions used for oligonucleotide synthesis may be at fault. Two truncated linkers were also found. One apparently resulted from internal digestion by MluI in the second cloning step; the other may have arisen from a truncated 1F7 VH-MluI primer.

Phage panning was initiated by incubating \( 2 \times 10^{11} \) phage particles in a microtiter plate coated with a bovine serum albumin conjugate of the transition state analog 1. Bound phage were eluted with free hapten (1.5 mM) and used to infect XL1-Blue cells. Phage output was determined by titration of a sample of the phage on the same cells. Approximately \( 1 \times 10^{12} \) phage particles were obtained following amplification and phage rescue, and \( 2 \times 10^{11} \) phages were used for the next cycle of panning. After five rounds of selection, the phage output/input ratio increased roughly 30-fold, from \( 4.8 \times 10^{-7} \) to \( 1.5 \times 10^{-5} \). Soluble single-chain protein was produced from the phage library pool after excision of the gII fragment from the fusion genes. The selected and pooled scFv proteins exhibited significant hapten binding activity by ELISA, whereas protein isolated from the starting library gave no detectable signal. Nevertheless, no bias for specific codons was observed at any position in the selected linkers when the scFv gene pool was sequenced.

Screening for Efficiently Expressed scFvS—a panel of 1054 individual clones was assayed for the production of soluble scFv capable of binding hapten 1. A total of 93 clones gave an ELISA signal at least 2-fold higher than background; the best 22 were confirmed to have elevated levels of hapten binding activity by semiquantitative ELISA (using \( \sim 50 \mu g \) of total cellular protein) and were sequenced. The deduced amino acid sequences of the linkers are shown in Table I. The clone which gave the highest hapten binding activity (scF17/L2) was found 7 times; the other clones are unique. Alignment of the linker sequences (Table I) reveals a strong bias for a proline residue in the second position after the VH C terminus (present in 12 of the 16 unique clones); Arg and Asn, preceded or followed by a proline, are the only alternatives observed at this position. Comparison of these sequences with those obtained prior to panning also shows that negatively charged residues (Asp and Glu) are disfavored in the selected linkers, whereas Pro and positively charged Arg are more abundant (Fig. 3). Thus, only two linkers have a negatively charged amino acid (one Asp each), but many clones, including scF17/L2, have multiple prolines and arginines. Aside from these minimal shared features, no other consensus properties are evident in the sequences. Apparently, a diverse set of polypeptides can link the variable domains of 1F7

### Table I

| Clone    | Linker peptide sequence          |
|----------|----------------------------------|
| scF17/L1 | NPSLRYPPFPGFPPSVPFR              |
| scF17/L2 | NPSLYIRHPHSPPSPITT               |
| scF17/L3 | TPGLSHLIPSLGPIHTN                |
| scF17/L4 | RSVPFFTPPRSLNSWLPA               |
| scF17/L5 | SPAAHPFRISPGRPGPIRT              |
| scF17/L6 | GPSPAPRSILIPSRAFG                |
| scF17/L7 | PRNSHFLHLPVLAPLGA                |
| scF17/L8 | NLPSLGVQVRVLYLLPOLL              |
| scF17/L9 | SPOYQPLLTLIPHHFLS                |
| scF17/L10| NPSLNPSSYLRAPRIS                 |
| scF17/L11| LPRTSPLLPSLPLRRPP                |
| scF17/L12| PLFAKGPVGLSSRSPFP               |
| scF17/L13| VPAVPVSVLSNHRARPPPY             |
| scF17/L14| LRPFPFPRVSGYCCCTPP              |
| scF17/L15| PNVAHVLLTPLVYPDNLR               |
| scF17/L16| CNLPILPCARSPAVRTPF              |

*Conserved prolines are shown in boldface.

*Six other linkers had the same sequence.
to yield hapten binding single-chain molecules.

The effect of individual linker sequences on protein production and hapten affinity was investigated by Western blotting and quantitative ELISA for nine of the selected scFvs (Fig. 4). Similar levels of total scFv gene expression were observed for each of the clones (Fig. 4c), but the production of soluble scFv varies dramatically (Fig. 4b). Furthermore, efficient production of soluble protein does not correlate with antigen binding activity. Thus, clones sc1F7/L2 and sc1F7/L6 have considerably more hapten binding activity than sc1F7/L8 (Fig. 4c), despite comparable yields of soluble scFv in all three cases (Fig. 4b, lanes 2, 6, and 8). Similarly, sc1F7/L7, which is expressed at higher levels of soluble protein than any of the other clones (Fig. 4b, lane 7), gives one of the weakest ELISA signals (Fig. 4a, lane 7). Although many linkers can be used to produce hapten binding scFvs, specific sequences are apparently needed to achieve an optimal balance of efficient expression as a soluble protein and high affinity binding.

Characterization of sc1F7/L2—Clone sc1F7/L2 was selected for more extensive investigation on the basis of its high hapten binding activity in preliminary assays. Preparative quantities of the single-chain protein were obtained by expression of the corresponding gene, modified to incorporate a C-terminal His tag, with the Novagen T7 system (12) (Fig. 2a). Efficient production of the soluble scFv required optimization of the growth conditions. Very rich medium, such as 2xYT or CS, proved superior to LB or YT medium. In addition, induction of expression at high cell density (A_{600} nm of 6 or higher), low pH (5.5–5.8), and low temperature (23 °C) gave the best results. Under these conditions, ~1 mg of soluble scFv per liter of culture could be obtained after purification. Higher overall expression levels were achieved at higher pH and temperature (i.e. ~50 mg/l at 37 °C and pH 7), but most of the product was found in inclusion bodies with the signal peptide still attached (data not shown).

The soluble sc1F7/L2 protein was purified to homogeneity in two steps (affinity chromatography on a nickel column followed by FPLC cation exchange chromatography on MonoS) and shown to catalyze the rearrangement of chorismate to prephenate (Fig. 5). Although E. coli possesses two bifunctional chorismate mutases, contamination by host enzymes was excluded by the absence of detectable prephenate dehydrogenase (15) or prephenate dehydrogenase (14) activity in the purified scFv samples. Moreover, the values of the steady-state kinetic parameters for the scFv compare favorably with those determined for the parent monoclonal antibody under comparable conditions (Table II). The k_{cat} value is reduced only by a factor of 2.7 and corresponds to a rate acceleration of approximately 100-fold over the uncatalyzed reaction. Affinity for chorismate and the free transition state analog 1 are affected to a greater extent as judged by K_{m} and (K_{d} \cdot k_{cat}) values that are, respectively, 5.3- and 8.5-fold larger for the scFv as compared with the corresponding Fab fragment, leading to a 15-fold reduction in k_{cat}/K_{m}. These differences presumably reflect minor adjustments in the relative orientation of the V_{H} and V_{L} domains imposed by the linker peptide.

**DISCUSSION**

Phage display has emerged in recent years as a powerful tool for protein engineering (19). Nowhere is this more evident than in the successful isolation of immunoglobulin-based receptors from large combinatorial libraries displayed on filamentous phage and in the modification of the affinity and selectivity of these molecules through multiple rounds of mutagenesis and selection (5, 6, 20). As shown in the current study, phage display can also greatly facilitate the identification of tailored linkers for single-chain antibodies.
single-chain Fv from Random Linker Libraries

Even the largest scFv phage libraries can contain but a tiny fraction of the 2.6 × 10^{23} possible 18-amino-acid linkers. Nevertheless, our experiments show that rather small libraries (~5 × 10^6 clones) are sufficient for the identification of single-chain proteins that are functional with respect to both hapten recognition and catalysis. These results are consistent with those of Stemmer and co-workers (3) who found that approximately 0.2% of the members of an scFv library derived from a metal chelate binding antibody and containing a 15-amino-acid randomized linker segment were active. There are apparently many viable solutions to the problem of linking individual V\textsubscript{H} and V\textsubscript{L} domains.

The principal advantage of the phage display approach is the rapid identification and amplification of functional gene variants that phenotypic selection makes possible. In our experiments this is illustrated by the increased hapten binding activity associated with the scFv pool obtained after five rounds of selection as compared with the starting library. Nevertheless, the diversity of the final pool is still large. The failure of a single clone to dominate the population of selected scFvs may reflect an incomplete course of selection, but a more likely explanation, as mentioned above, is that successful linkers have relatively few sequence requirements. The selection protocol used in these experiments is probably insufficiently stringent to differentiate between receptors with roughly comparable hapten affinities.

Although divergent linker sequences yield scFvs that can be selected on the basis of hapten affinity, the resulting molecules are not equally suited for large scale applications. Genes expressed at low levels may provide sufficient fusion protein for display on the surface of phage, allowing passage to subsequent rounds of selection, but they may not be the best candidates for high level expression or may not encode proteins possessing optimal combinations of hapten affinity, catalytic activity, and stability. Screening for scFvs possessing high binding activity and which can be produced in high yield is therefore an important additional step in the approach we describe. We found that only ~9% of the clones obtained after the final round of selection secreted sufficient soluble scFv with enough hapten binding activity to afford an ELISA signal 2-fold over background. In detailed studies of representative clones producing the highest ELISA signals, large variations in soluble protein production and hapten binding were observed (Fig. 4). Perhaps fortuitously, the most active variant (sc1F7/L2) was produced in good yield; multiple copies of this clone were also found in the library, indicating that it was a particularly successful competitor in the initial selection step.

Sequence analysis of 22 of the best binders (Table I) reveals a highly conserved proline in the second (or an adjacent) position of the linkers. This proline is likely to have functional significance, making possible a tight turn that orients the linker segment into the groove separating V\textsubscript{H} and V\textsubscript{L}. Aside from a modest enrichment in prolines and arginines at other positions, the selected polypeptides share no other common features. The abundance of positively charged arginines suggests that hydrophilicity of the linker may be important, while proline residues will disfavor formation of regular secondary structure and lower the susceptibility of the scFv to proteolysis. Given this, the linker segments are likely to be rather flexible and at least partially disordered, consistent with structural studies of other single-chain antibodies (21–23), although specific interactions between individual linkers and the Fv domain presumably dictate the observed variation in hapten affinity (and catalytic activity).

In summary, phage display, coupled with a high throughput screen for efficient expression, has yielded a single-chain variant of the catalytic antibody 1F7 which is suitable for structural and mechanistic studies. Conceivably, even better linkers can be found by searching larger starting libraries or by improving the first-generation scFv obtained here through additional mutagenesis and selection. This strategy obviates the need for the heuristics and structural information that usually guide site-directed mutagenesis experiments. In principle and more generally, therefore, the same approach that yielded sc1F7/L2 should be easily extensible to other proteins, affording a general methodology for identifying suitable peptide sequences for covalently tethering any two protein domains together in the absence of structural data.

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