RESEARCH COMMUNICATION

Biotin binders selected from a random peptide library expressed on phage

Isabella SAGGIO and Ralph LAUFER*

Istituto di Ricerche di Biologia Molecolare P. Angeletti, Via Pontina Km 30.600, 00040 Pomezia (Roma), Italy

Recombinant biotin-binding phages were affinity-selected from a random peptide library expressed on the surface of filamentous phage. Phage binding to biotinylated proteins was half-maximally inhibited by micromolar concentrations of a monobiotinylated molecule. Sequencing of the peptide inserts of selected phages led to the identification of a previously unknown biotin-binding motif, CXWXPPF(K or R)XXC. A synthetic peptide containing this sequence motif inhibited streptavidin binding to biotinylated BSA with an IC_{50} of 50 μM. This compound represents the shortest non-avidin biotin-binding peptide identified to date. Our results illustrate that phage display technology can be used to identify novel ligands for a small non-proteinaceous molecule.

INTRODUCTION

The recently developed technology of phage peptide libraries [1–4] is gaining increasing interest as a novel approach for the identification of ligands for antibodies and other biomolecules [5]. The target ligate is used to screen for potential binders among a vast mixture of short random peptide sequences, each displayed on the surface of a filamentous bacteriophage clone. With the aid of such fusion phage libraries, it has been possible to identify short peptide mimics of antigenic epitopes [1,3,4,6,7], as well as ligands for proteins, such as streptavidin [2] and concanavalin A [8,9], whose natural substrates are not peptides.

In the course of a study on mAb 35, a rat monoclonal antibody against nicotinic acetylcholine receptor [10], we used a biotin conjugate of this antibody to screen a random peptide library expressed on the surface of filamentous phage. This paper describes the isolation of recombinant phage clones that bind to the biotin moieties carried by the target ligate, and to other biotinylated molecules. Sequencing of the peptide inserts of these phages led to the identification of a previously unknown biotin-binding motif.

MATERIALS AND METHODS

Biotinylation of proteins

Biotinylation was performed as described by Parmley and Smith [11], by using 6 nmol of mAb 35 [isolated by $(NH_4)_2SO_4$ precipitation of serum-free culture medium of a mAb 35-secreting hybridoma line obtained from the American Type Culture Collection], affinity-purified human IgG (kindly given by Dr. F. Felici, IRBM), BSA or bovine thyroglobulin, and 360 nmol (3.6 μmol for thyroglobulin) of sulphosuccinimidyl-6-(biotinamido)hexanoate (NHS-LC-biotin; Pierce). A conjugate of mAb 35 with sulphosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-SS-biotin; Pierce) was prepared by the same protocol. The average number of biotin molecules incorporated into proteins was determined after digestion with proteinase K [12], by the method of Mock [13], except that ^{125}I-streptavidin (Amersham) was used instead of ^{125}I-avidin. Molar ratios of biotin per protein molecule were found to be 320 for biotinylated thyroglobulin and between 20 and 60 for the other proteins used in this study.

Selection of B-mAb 35-binding phage clones

The phage display library used (pVIII-9aa.Cys) was kindly given by Dr. A. Luzzago and Dr. F. Felici (IRBM). Recombinant phages contain peptide inserts of 9 random amino acid residues, flanked by two invariant cysteine residues, within the major M13 phage coat protein pVIII [6]. The insert sequences are preceded by AEGEF and followed by GDPAK.... [7]. The library (10^{11} ampicillin-transducing units) was screened for binders to B-mAb 35 by three rounds of biopanning [11], by using B-mAb 35 concentrations of 1000, 10 and 1 nM respectively. Phage amplification, isolation of random ampicillin-resistant clones, and purification were performed as described by Felici and co-workers [4,6,7]. DNA sequences were determined by the Sequenase 2.0 kit protocol (USB).

Protein binding to phages: e.l.i.s.a.

Polystyrene 96-well microtitre plates (ProBind, Falcon) were coated overnight at 4 °C with 4×10^{10} CsCl-purified phage particles diluted in 50 mM Tris/HCl/150 mM NaCl, pH 7.4 (TBS). Wells were then washed with blocking solution [5% (v/v) non-fat dry milk in TBS], incubated with blocking solution for 90 min at room temperature, and washed three times with TBS containing 0.5% (v/v) Tween 20 (TBS-Tween). Test antibodies or biotinylated proteins, diluted in 50 μl of 0.5% (v/v) Triton X-100, 1 mg/ml BSA in PBS, were added, and left to incubate overnight at 4 °C. After five washes with TBS-Tween, wells were incubated for 4 h at 4 °C with 50 μl of TBS containing 1 mg/ml BSA and either alkaline phosphatase-conjugated avidin (Jackson Immunoresearch Laboratories; 1:1000, v/v), to detect biotinylated proteins, or alkaline phosphatase-conjugated antibody against rat IgG (Calbiochem; 1:1000, v/v), to detect mAb 35. Wells were then washed five times with TBS-Tween, and developed at 37 °C with 1 mg/ml *p*-nitrophenyl phosphate in 1 M diethanolamine hydrochloride, pH 9.8. The A_{405} was determined in a microplate reader.

Abbreviations used: mAb, monoclonal antibody; B-mAb 35, biotinamidohexanoate conjugate of mAb 35; B-SS-mAb 35, biotinamidoethyldithiopropionate conjugate of mAb 35.

* To whom correspondence should be addressed.

Phage binding to biotinylated proteins: e.l.i.s.a.

Polystyrene 96-well microtitre plates were coated overnight at 4 °C with 0.01 μg of B-mAb 35 or 0.1 μg of biotinylated goat anti-rat IgG antibody in 50 mM $NaHCO_3$, pH 9.5. After washing and blocking, as described in the preceding section, wells were incubated overnight at 4 °C with 50 μl of 0.5% Triton X-100 and 1 mg/ml of BSA in PBS containing different amounts of phage, as specified in the Results section. They were then washed five times with TBS-Tween, and incubated for 2 h at 4 °C with 100 μl of polyclonal rabbit antiserum against M13 phage (kindly provided by Dr. F. Felici, IRBM), diluted 1:20000 (v/v) in blocking solution containing 0.05% Tween 20. After washing as above, wells were incubated for 2 h at 4 °C with alkaline phosphatase-conjugated goat antibody against rabbit IgG (Calbiochem), diluted 1:5000 (v/v) in blocking solution containing 0.05% Tween 20. Final washing and development were as described in the preceding section.

Solid-phase assay of interaction between a synthetic peptide and biotinylated proteins

Polystyrene 96-well microtitre plates were coated overnight at 4 °C with 0.02 μg of biotinylated BSA in 50 mM $NaHCO_3$, pH 9.5. Wells were washed five times with PBS, blocked for 1 h at room temperature with 3 mg/ml BSA in PBS, and washed again. Wells were then preincubated for 1 h at room temperature with 40 μl of 0.2 M Hepes/2 M NaCl/1 mg/ml BSA, pH 7.0, in the absence or presence of different test compounds, before addition of 10 μl of the same buffer containing 8 fmol (40000 c.p.m.) of ^{125}I-streptavidin. Incubations were continued at 4 °C overnight. Wells were then washed five times with PBS, after which bound ^{125}I-streptavidin was solubilized by addition of 0.2 M NaOH/1% SDS and counted in a γ-radiation counter.

Binding of other biotin-binding proteins was determined after overnight preincubation at 4 °C with or without different test compounds, by addition of alkaline phosphatase-conjugated avidin (final dilution 1:1000, v/v) or alkaline phosphatase-conjugated antibody against biotin (Pierce; final dilution 1:1000, v/v). After incubation for 2 h at 4 °C, wells were washed and developed as described in the preceding sections.

Solution assay of interaction between a synthetic peptide and B-mAb 35

B-mAb 35 (0.2 nM) was preincubated overnight at 4 °C in 0.2 M Hepes/2 M NaCl/1 mg/ml BSA, pH 7.0, with or without different test compounds, before addition of 0.5 nM ^{125}I-streptavidin. After 3 h at 4 °C, the antibody was immunoprecipitated by addition of 2 μl of normal rat serum and 10 μl of goat antibody against rat immunoglobulins (Calbiochem). The precipitate was washed three times with 0.5% Triton X-100 in PBS, and counted in a γ-counter.

Synthetic peptides

Peptides were prepared by solid-phase synthesis in the Peptide Synthesis Unit at IRBM (Dr. A. Pessi).

RESULTS

The pVIII-9aa.Cys phage library [6] was screened for binders to B-mAb 35, a biotinamidohexanoate conjugate of the anti-(acetylcholine receptor) antibody mAb 35, by three rounds of biopanning [11]. After the third round of affinity selection, 72 random phage clones (numbered 1–72) were isolated. Nine of these phages (numbers 21, 25, 30, 36, 42, 47, 55, 71, 72) were found to bind B-mAb 35, as determined by e.l.i.s.a. (see below). Sequencing of the pVIII insert region of the nine B-mAb 35-binding phages revealed that they displayed three different peptide sequences, each of which was present in 2–4 clones (Figure 1). Peptide inserts share the common motif CXWXPPF(K or R)XXC. It should be noted that the two flanking cysteine residues are common to all recombinant phages from the pVIII-9aa.Cys library [6].

Table 1 summarizes the results of an e.l.i.s.a. demonstrating dose-dependent binding of B-mAb 35 to immobilized phage no. 36. Similar results were obtained with the other B-mAb 35-binding phages (see below, and results not shown). In contrast, phage no. 36 did not bind mAb 35 at concentrations up to 1 μM (see Table 1). Identical results were obtained with phages nos. 30, 42 and 55 (results not shown). The inability of B-mAb 35-binding phages to recognize the non-biotinylated antibody was confirmed by competition experiments. Indeed, preincubation of immobilized phage no. 30 with 1 μM mAb 35 did not prevent binding of 0.1 nM B-mAb 35 (105±15% of control binding; $n = 3$). Neither B-mAb 35 nor mAb 35 bound specifically to phage no. 2 (see Table 1), whose peptide insert sequence (CFHSAHGNYAC) bears no similitude to those of the nine B-mAb 35-binding phages.

These results suggested that B-mAb 35-binding phages interact with the biotin moieties carried by the antibody. If so, this binding should be competed for by biotin-containing compounds. Indeed, binding of B-mAb 35 to immobilized phage no. 30 was inhibited by 0.1 mM of both free d-biotin (48±3% of control, $n = 2$) and the biotinylated peptide [α-Val^1,ϵ-Lys^9-(biotinamidohexanoate)]VQGEESNDK (11±2% of control,

Phage no.	
25, 47, 71, 72	C-S-**W**-R-**P**-**P**-**F**-**R**-A-V-C
30, 36, 42	C-S-**W**-A-**P**-**P**-**F**-**K**-A-S-C
21, 55	C-N-**W**-T-**P**-**P**-**F**-**K**-T-R-C

Figure 1 Peptide insert sequences of B-mAb 35-binding phages

Identical or similar (K, R) residues are in **bold** type.

Table 1 Biotin mediates the interaction of selected phage with B-mAb 35

Antibodies at the indicated concentrations were incubated with immobilized phages no. 36 or no. 2. Binding was determined by e.l.i.s.a.

	Binding (A_{405}) to phage no.:	
Antibody	2	36
B-mAb 35		
(0.1 nM)	0.12	0.90
(1 nM)	0.10	1.17
(10 nM)	0.08	1.54
(100 nM)	0.08	1.90
mAb 35		
(1 nM)	0.13	0.12
(10 nM)	0.10	0.09
(100 nM)	0.17	0.14
(1000 nM)	0.37	0.36

Table 2 Binding of biotinylated proteins to selected phages

Binding of biotinylated proteins to immobilized phages was determined by e.l.i.s.a. Data are means ± S.E.M. from 2–4 experiments, and are expressed as the ratio of absorbance readings, obtained in the presence of test protein to that measured in its absence. Abbreviations: B-hIgG, B-TG, biotinamidohexanoate conjugates of human IgG and bovine thyroglobulin respectively; B-AR, biotinamido conjugate of goat anti-rat IgG antibody; n.d., not determined.

	Signal ratio (-fold over background) with phage no.:			
Protein	2	30	55	72
B-mAb 35 (1 nM)	1.0±0.2	16.1±6.8	3.6±0.5	4.2±0.7
B-SS-mAb 35 (1 nM)	1.0±0.1	42.4±0.8	n.d.	n.d.
B-hIgG (1 nM)	1.3±0.1	27.9±14.0	16.0±0.3	12.1±1.4
B-hIgG (10 nM)	1.0±0.2	51.1±13.9	n.d.	n.d.
B-TG (1 nM)	1.2±0.1	22.5±9.6	6.6±0.1	13.9±3.6
B-TG (10 nM)	1.2±0.2	40.0±14.3	n.d.	n.d.
B-AR (10 nM)	1.2±0.2	13.9±3.9	n.d.	3.8±0.7

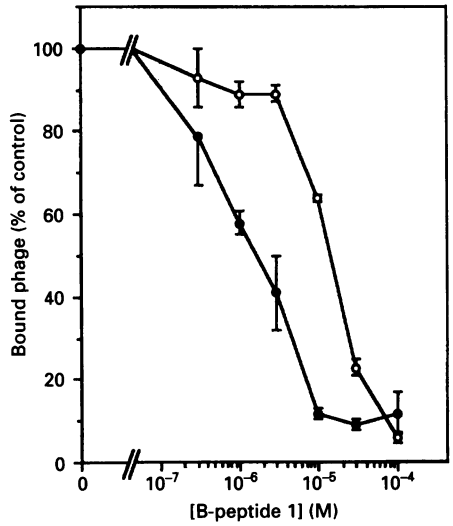

Figure 2 A biotinylated peptide inhibits phage binding to biotinylated proteins

Immobilized B-mAb 35 (○) or biotinylated goat anti-rat IgG antibody (●) was incubated with 10^{11} or 5×10^{11} particles/ml, respectively, of phage no. 30, in the presence of the indicated concentrations of [ε-Lys⁹(biotinamidohexanoate)]VQGEESNDK (B-peptide 1). Phage binding was determined by e.l.i.s.a. Data are mean values from duplicate determinations, and are expressed as percentages of the absorbance readings obtained in the absence of competitor (control A_{405} after 60 min of development, 1.46±0.02 and 0.22±0.02; background, 0.02 and 0.03; for B-mAb 35 and biotinylated goat antibody respectively).

$n = 2$). The effect of the peptide was due to the biotinamidohexanoate groups, since 1 mM of its non-biotinylated analogue did not significantly affect phage–antibody interaction (89±2% of control, $n = 2$).

To test whether selected phages could bind other biotinylated proteins unrelated to mAb 35, we prepared biotinamidohexanoate conjugates of human IgG and bovine thyroglobulin. As summarized in Table 2, the biotinylated proteins bound at nanomolar concentrations to phages nos. 30, 55 and 72, but not to phage no. 2, which does not interact with B-mAb 35. Likewise, none of these proteins bound to five phages that were selected

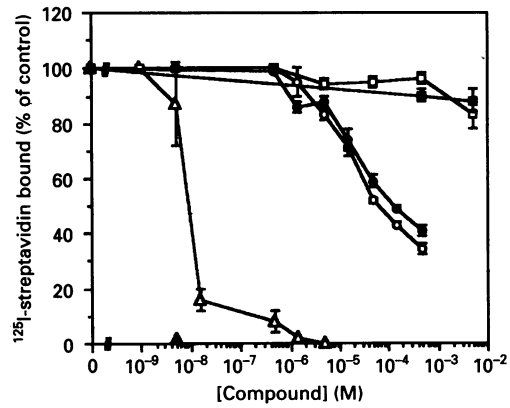

Figure 3 Inhibition of ^{125}I-streptavidin binding to biotinylated proteins by the peptide insert of phage no. 30

The effects of streptavidin (△, ▲), AEGEFCSWAPPKASCGDPAK (○, ●) or VQGEESNDK (□, ■) on the binding of ^{125}I-streptavidin to immobilized biotinylated BSA (△, ○, □) or B-mAb 35 in solution (▲, ●, ■) were determined as described in the Materials and methods section. Results are mean values from two separate experiments, and are expressed as percentages of radioligand binding in the absence of competitor.

from the pVIII-9aa.Cys library by using different ligates (I. Saggio and R. Laufer, unpublished work). To exclude the possibility that the observed effects were mediated by the spacer arm of the biotinylation reagent sulphosuccinimidyl-6-(biotinamido)-hexanoate, we also tested two antibodies that were biotinylated with different reagents. Both B-SS-mAb 35 and biotinylated goat anti-rat IgG antibody (Sigma B-7139), which were prepared by using sulphosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate and *N*-hydroxysuccinimidobiotin respectively, were found to bind specifically to phage no. 30 (see Table 2).

To determine the apparent affinity of interaction between phage no. 30 and a monobiotinylated compound, we assayed phage binding to plastic plates coated with either B-mAb 35 or biotinylated goat anti-rat antibody, in the absence or presence of [ε-Lys⁹(biotinamidohexanoate)]VQGEESNDK. As shown in Figure 2, the biotinylated peptide inhibited binding of phage no. 30 to B-mAb 35 or biotinylated goat anti-rat antibody with IC_{50} values of 20 μM and 2 μM respectively. No significant inhibition of phage binding was obtained with 1 mM of the non-biotinylated peptide analogue (results not shown). Finally, binding was completely abolished in the presence of 10 nM streptavidin (results not shown), confirming again that biotin mediates the interaction of biotinylated proteins with phage no. 30.

To determine whether the peptide insert of phage no. 30 would retain its biotin-binding properties independently of the phage context, the peptide AEGEFCSWAPPKASCGDPAK, which contains the insert of phage no. 30 and five flanking pVIII residues, was synthesized. As shown in Figure 3, the peptide inhibited ^{125}I-streptavidin binding to immobilized biotinylated BSA in a dose-dependent manner, with an IC_{50} value of 50 μM. The unrelated peptide VQGEESNDK, used as a control, did not modify ^{125}I-streptavidin binding at concentrations up to 5 mM (Figure 3). As expected, unlabelled streptavidin at a concentration of 15 nM abolished binding of the radiolabelled analogue to biotinylated BSA. Peptide AEGEFCSWAPPKASCGDPAK also inhibited binding of alkaline phosphatase-conjugated avidin and anti-biotin antibody to biotinylated BSA (percentage of control binding in presence of 150 μM peptide: 44±2 and 39±2 respectively; $n = 2$).

To rule out possible artefacts that could arise in solid-phase competition assays, we examined whether peptide AEGEFCSWAPPKASCGDPAK would inhibit the binding of ^{125}I-streptavidin to a different biotinylated protein (B-mAb 35) in homogeneous solution. In this latter assay, the peptide was found to compete for binding to B-mAb 35 with an IC_{50} of 100 μM, which is close to the value obtained in the solid-phase system (Figure 3). Also, in solution, the streptavidin–biotin interaction was unaffected by the control peptide VQGEESNDK (Figure 3). Taken together, these results demonstrate that a synthetic peptide containing the insert of phage no. 30 is able to interact with the biotin groups carried by two different biotinylated proteins.

DISCUSSION

The use of a biotinylated antibody to screen a random peptide library expressed on phage led to affinity selection of display phages whose interaction with the target ligate is mediated by biotin. This conclusion is borne out by the following findings: (i) selected phages react with the anti-(acetylcholine receptor) antibody only when it is conjugated to biotin; (ii) biotin, biotinylated compounds and streptavidin inhibit phage binding to B-mAb 35; (iii) selected phages specifically bind biotinylated molecules unrelated to mAb 35. Our results demonstrate for the first time that phage display technology can be used to identify binders for a small non-proteinaceous molecule. It will be interesting to determine whether this could provide a general means to generate non-antibody ligands for different haptens.

Peptide inserts of biotin-binding phages share a sequence motif that has not previously been implicated in biotin binding. Indeed, this motif bears no obvious similarity to sequences of known biotin-binding proteins, such as streptavidin [14], avidin [14], biotin operon repressor and biotin holoenzyme synthetase [15], or biotin carboxylase [16]. However, the inserts of all biotin-binding phages contain a tryptophan residue, which is known to be required for the activity of some biotin-binding proteins [17]. To our knowledge, the peptides displayed by biotin-binding phages represent the shortest non-avidin biotin-binding sequences identified to date. A synthetic 21-residue peptide containing the insert sequence of phage no. 30 inhibited ^{125}I-streptavidin binding to biotinylated BSA with an IC_{50} value of 50 μM, i.e. at approx. 10^4-fold higher concentration than unlabelled streptavidin (see Figure 3). In comparison, the shortest avidin fragment shown to recognize biotin is a tridecapeptide containing residues 26–38 [18]. This peptide competes with streptavidin for binding to biotinylated BSA with an IC_{50} of $\sim$ 100 μM [18].

Competition experiments showed that micromolar concentrations of a peptide carrying one biotin group were needed for half-maximal inhibition of binding of phage no. 30 to biotinylated proteins. This might be an underestimate of the true affinity, considering that multivalent display of insert peptides on the phage surface [4] could give rise to nearly irreversible interactions with proteins carrying multiple biotin groups. The same argument applies for the inhibition, by the pVIII insert sequence derived from phage no. 30, of the high-avidity binding of streptavidin to biotinylated BSA. Further experiments are under way to determine the affinities of monovalent binding of biotinylated compounds to the peptide inserts of selected phages.

New biotin binders could be useful complements to avidin in certain technological applications ([19], and references therein). The possibility to control the number of peptides displayed on a recombinant phage [20], and hence the avidity of ligand binding, should permit versatile uses of biotin-binding phages. For instance, high-avidity phages could serve to detect biotinylated molecules in e.l.i.s.a. (see Figure 2) or blotting assays, whereas low-avidity phages could be used for affinity purification. More efficient binders might be obtained by screening a display library of variant peptides [21], constructed by mutagenesis of the sequences identified in the present work. Potentially, these compounds could provide leads for the development of novel reagents for biotin technology.

We thank Dr. A. Luzzago and Dr. F. Felici for generous supply of the phage library and advice on its use, and Dr. A. Pessi for kindly providing synthetic peptides. We also thank Dr. A. Folgori, Dr. P. Monaci and Dr. A. Nicosia for helpful advice and discussions, Dr. J. Lindstrom for helpful suggestions, and Dr. N. La Monica and Dr. R. Cortese for critical reading of the manuscript.

REFERENCES

1 Scott, J. K. and Smith, G. P. (1990) Science **249**, 386–390
2 Devlin, J. J., Panganiban, L. C. and Devlin, P. E. (1990) Science **249**, 404–406
3 Cwirla, S. E., Peters, E. A., Barrett, R. W. and Dower, W. J. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 6378–6382
4 Felici, F., Castagnoli, L., Musacchio, A., Jappelli, R. and Cesareni, G. (1991) J. Mol. Biol. **222**, 301–310
5 Smith, G. P. (1991) Curr. Opin. Biotechnol. **2**, 668–673
6 Luzzago, A., Felici, F., Tramontano, A., Pessi, A. and Cortese, R. (1993) Gene, in the press
7 Felici, F., Luzzago, A., Folgori, A. and Cortese, R. (1993) Gene, in the press
8 Oldenburg, K. R., Loganathan, D., Goldstein, I. J., Schultz, P. G. and Gallop, M. A. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 5393–5397
9 Scott, J. K., Loganathan, D., Easley, R. B., Gong, X. and Goldstein, I. J. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 5398–5402
10 Tzartos, S. J., Rand, D. E., Einarson, B. L. and Lindstrom, J. M. (1981) J. Biol. Chem. **256**, 8635–8645
11 Parmley, S. F. and Smith, G. P. (1988) Gene **73**, 305–318
12 Bayer, E. A. and Wilchek, M. (1990) Methods Enzymol. **184**, 138–160
13 Mock, D. M. (1990) Methods Enzymol. **184**, 224–233
14 Green, N. M. (1990) Methods Enzymol. **184**, 51–67
15 Howard, P. K., Shaw, J. and Otsuka, A. J. (1985) Gene **35**, 321–331
16 Li, S. J. and Cronan, J. E. (1992) J. Biol. Chem. **267**, 855–863
17 Dakshinamurti, K. and Chauhan, J. (1990) Methods Enzymol. **184**, 93–102
18 Hiller, Y., Bayer, E. A. and Wilchek, M. (1991) Biochem. J. **278**, 573–585
19 Bayer, E. A. and Wilchek, M. (1990) Methods Enzymol. **184**, 49–51
20 Greenwood, J., Willis, A. E. and Perham, R. N. (1991) J. Mol. Biol. **220**, 821–827
21 Wells, J. A. and Lowman, H. B. (1992) Curr. Opin. Biotechnol. **5**, 355–362

Received 29 April 1993/25 May 1993; accepted 4 June 1993