Phage Display of Selenopeptides

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We report a new method, selenopeptide phage display, that unites the power of in vivo biomolecular amplification with the unlimited diversity of small-molecule chemistry. The fusion of peptides to phage coat proteins is a widely used method for screening combinatorial libraries of peptides.[1,2] One common approach is to express random sequences at the N-terminus of the M13 coat protein pIII, resulting in library complexities of about 10^9 different clones. Selection is achieved by performing multiple rounds of target binding (panning), elution, and amplification. Because each phage particle contains both the displayed peptide and the DNA encoding it, the selected peptides can be readily identified by DNA sequencing. Despite its utility and convenience, in vivo biological expression limits library diversity to combinations of 20 of the naturally occurring amino acids, linked by peptide bonds.

To increase diversity, peptide libraries can be specifically chemically modified prior to each round of panning. Phage libraries with enzymatically phosphorylated tyrosine residues have been constructed;[3] there is, however, no method to specifically modify displayed tyrosine with other chemical moieties while protecting endogenous tyrosine residues elsewhere on the phage coat. The side chains of lysine and cysteine (Cys) are reactive, but small-molecule reagents are likely to target residues within the native coat protein in addition to the displayed peptide. A new type of phage library, with a unique site available for a broad range of chemical modifications, is therefore needed.

To maintain the essential amplification and selection techniques of phage display, bacterial genetic machinery should be employed to incorporate the unique reactive site into the displayed peptide. The naturally occurring amino acid selenocysteine (Sec), which is not present in the native M13 proteins,[4,5] would be a good candidate. The potential modifications of Sec derive from its unique chemical properties. The pK_a of Sec is 5.2, compared to 8.1 for Cys, so that at pH 6−7, nucleophilic substitution reactions can specifically alkylate Sec but not Cys residues.[6] The formation of stable sulfide−selenide cross-links[7] also permits covalent Sec modification by thiol reagents.

Prokaryotic Sec incorporation has been well characterized and requires the constitutively expressed *selA*, *selB*, *selC*, and *selD* gene products.[8,9] Sec is encoded by the TGA opal stop codon,[10] which is suppressed in the presence of a specific downstream hairpin structure termed the selenocysteine insertion sequence (SECIS, Figure 1). We have designed an assay for Sec incorporation based on the dependence of M13 phage infectivity on expression of the coat protein pIII.[11] The fusion of putative selenopeptides to the N-terminus of pIII couples phage plaque formation to opal suppression. Because of the relatively high level of protein synthesis required for plaque formation, we also anticipated that selenium-supplemented media would be required for selenopeptide phage display. We thus expected to identify selenopeptide−pIII fusions based on the selenium dependence of plaque formation.

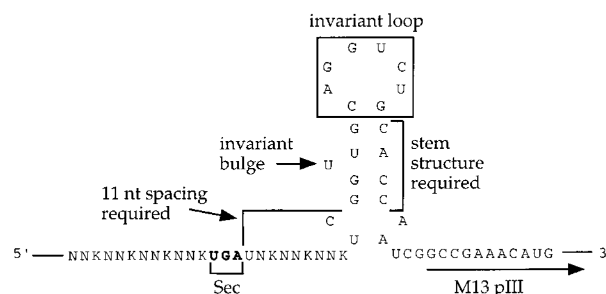

Figure 1. Randomized SECIS library insert expressed as a pIII fusion in this report. N = A, G, C, or U. K = G or U. Permissible mutations are based on the *E. coli* formate dehydrogenase (fdh) SECIS as reported in ref 12.

Based on the reported minimal SECIS requirements,[12] a library consisting of the SECIS element with four upstream and three downstream randomized codons, and a minimal mRNA SECIS (Figure 1), was prepared. The library insert was ligated into M13KE, an M13mp19 derivative designed with Acc65I and EagI sites for pentavalent N-terminal pIII expression.[13] Because the *Escherichia coli* endogenous tryptophan-inserting opal suppression pathway is enhanced by downstream purines and CTG codons,[14] the nucleotide immediately downstream of the opal codon was fixed as T. As a control, the native *E. coli* formate dehydrogenase SECIS, with no randomized codons and a full-length stem, was also cloned into the M13KE vector (Sec-1).

The electroporation and plating of the ligation products resulted in small plaques, with about 10 times more plaques forming in the presence of 2 μM supplemental sodium selenite than in unsupplemented medium. Individual plaques were amplified for further analysis, with representative sequences shown in Table 1. The growth of all of the TGA-containing clones was strictly selenium-dependent, with plaques appearing only in the presence of 1−2 μM supplemental sodium selenite. M13KE phage growth, by contrast, was selenium-independent over a range of 0−4.5 μM supplemental sodium selenite.

To quantitate the strict selenium dependence of phage growth, clone 6 (ASPTSecFKP) was plated with varying concentrations of supplemental sodium selenite in the medium. The number and diameter of visible clone 6 plaques increased in a selenium-dependent fashion from 0 to 3.2 μM sodium selenite, with half-maximal plaque diameter at ~0.4 μM selenite.

The pMal−pIII shuttle vector[13] was employed to overexpress and purify the Sec-1 peptide sequence as a fusion to the N-terminus of maltose binding protein (MBP), with a pIII leader sequence to direct the fusion to the periplasm. Indirect evidence that the MBP fusion possessed a Sec residue in the expected position was provided by protein sequencing via successive cycles of N-terminal Edman degradation. The results showed the expected Sec-1 N-terminus, SARVXHGPSV, with X assumed to be Sec. The acid breakdown product of a Sec residue, generated

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Table 1. Selected Library Clones[a]

Clone	Displayed Peptide Sequence						
Sec-1	S	A	R	V	Sec	H	G	P
(fdh)	AGC	GCT	CGT	GTC	TGA	CAC	GGC	CCA
Cys-1	S	A	R	V	L	C	N	H
	AGC	GCT	CGT	GTC	TTA	TGT	AAT	CAT
2	L	T	G	T	Sec	C	Q	N
	TTG	ACT	GGT	ACG	TGA	TGT	CAG	AAT
3	E	A	S	R	Sec	C	S	T
	GAG	GCG	TCG	CGT	TGA	TGT	TCG	ACT
4	K	L	A	R	Sec	S	A	S
	AAG	TTG	GCT	CGT	TGA	TCG	GCG	TCG
5	N	G	A	Q	Sec	S	R	H
	AAT	GGG	GCG	CAG	TGA	TCG	AGG	CAT
6	A	S	P	T	Sec	F	K	P
	GCG	AGT	CCT	ACT	TGA	TTT	AAG	CCG
7	C	A	H	P	Sec	S	T	R
	TGT	GCT	CAT	CCG	TGA	TCT	ACT	CGT
8	Q	S	T	R	Sec	W	N	D
	CAG	TCG	ACG	CGG	TGA	TGG	AAT	GAT
9	I	V	E	S	Sec	L	N	P
	ATT	GTG	GAG	TCG	TGA	TTG	AAT	CCG
10	F	G	A	N	Sec	L	A	R
	TTT	GGG	GCT	AAC	TGA	TTG	GCG	CGG
11	V	Q	Y	T	Sec	L	P	K
	GTG	CAG	TAT	ACG	TGA	TTG	CCG	AAG
12	A	G	Q	S	Sec	S	T	D
	GCT	GGG	CAG	TCG	TGA	TCG	ACT	GAT

[a] All of the clones formed plaques only when plated with supplemental sodium selenite, except for Cys-1, which possessed a G → T point mutation within the opal codon.

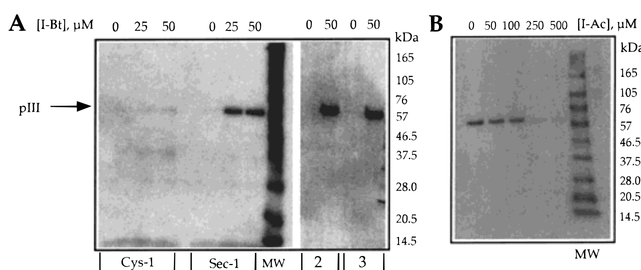

Figure 2. Immunoblots of biotinylated phage, probed with HRP-conjugated anti-biotin antibody (NEB) and visualized by chemiluminescence. Phage (10^{11} pfu) was diluted in 150 mM NaCl, 50 mM glycine-HCl (pH 2.5). I-Bt in DMF was added at 5% v/v to the indicated final concentration, and the reactions were incubated in the dark at room temperature. MW, biotinylated molecular weight markers. (A) Phage samples (peptide sequences in Table 1), treated with I-Bt for 30 min. (B) Sec-1 samples, treated with the indicated concentration of iodoacetamide for 1 h at room temperature, followed by 50 μM I-Bt for 20 min.

by acid-catalyzed β-elimination, should be the same as that of Cys or Ser residues in that all produce dehydroalanine. The DTT adduct of the dehydroalanine PTH was observed at the position corresponding to the TGA codon (cycle 5). Ser also produces this adduct, but cycles 1 and 9, and not cycle 5, also showed a parent Ser peak. No significant amount of Trp-PTH (<200 fmol) was observed in this cycle, eliminating the possibility of endogenous Trp-inserting opal suppression. Similar results were obtained for the clone 12 MBP fusion. The protein sequencing data are consistent with the presence of a Sec residue at cycle 5.

To rule out the possibility of Cys incorporation at the TGA codon, and to demonstrate specific chemical modification of the Sec residue in a displayed peptide, the chemical reactivity of phage clone Sec-1 was compared to that of clone Cys-1 (Table 1), which displayed a single unpaired Cys residue. Phage samples were treated with iodoacetyl-LC-biotin (I-Bt, Pierce), an electrophilic reagent which should specifically target thiol or selenol groups. Figure 2A shows the results of immunoblotting of the products. At both pH 2.5 and 8, the biotinylation was highly specific for the Sec residue. Biotinylation of Cys-1 was enhanced at pH 8, although the reaction remained highly selective (>10:1) for Sec. The biotinylation experiments confirm that, at acidic pH, the reactivity of a Sec residue in a pIII fusion greatly exceeds that of the eight paired Cys residues[11] in M13 pIII or of an unpaired Cys residue in the displayed peptide.

Presumably because of the stability of sulfide−selenide cross-links, the selenopeptide library contained clones with a single Cys residue at a much higher incidence than is normally seen in pIII libraries constructed in this system (Noren, C. J.; Noren, K. A., unpublished results). To determine whether the putative sulfide−selenide bridging inhibited Sec reactivity, phage clones 2 and 3 were modified with I-Bt; immunoblotting revealed that both samples were biotinylated to a similar extent as Sec-1 (Figure 2A). This result suggests that the sulfide−selenide cross-link is sufficiently reversible to allow trapping of the free selenide with an excess of electrophile.

To estimate the efficiency of chemical modification, Sec-1 phage was modified with iodoacetamide (I-Ac), and the remaining unmodified phage was then reacted with I-Bt and detected by immunoblotting (Figure 2B). Treatment for 1 h with 250 μM I-Ac at pH 2.5 was sufficient to block the biotinylation reaction. Because the electrophilicities of I-Ac and I-Bt are essentially identical, this result suggests that modification with I-Bt under these conditions would go to completion. To assess the infectivity of the modified phage, the Sec-1 clone was treated for 1 h at room temperature at pH 5.2 with I-Ac or I-Bt. After quenching with 2 equiv of β-mercaptoethanol to scavenge any unreacted I-X electrophile, the samples were diluted and plated, with no significant effect on the resulting plaque counts.

We have demonstrated that selenopeptide libraries can be generated using an adaptation of standard phage display methods. All of the clones assumed to display selenopeptides formed plaques only in the presence of supplemental selenium. N-terminal sequencing of MBP fusions revealed dehydroalanine, and not Trp, in several putative Sec-inserting clones. The chemical modification of the phage samples believed to contain Sec was consistent with selenium reactivity, with nucleophilic substitution readily occurring at acidic pH, where Cys is expected to be unreactive. Finally, the occurrence of clones encoding Cys proximal to the TGA codon also implicates Sec incorporation, since sulfide−selenide bridging would stabilize the otherwise unpaired Cys.

The molecular diversity of displayed peptides can now include 21 amino acids instead of the traditional 20, but since the 21st amino acid can be specifically chemically modified, any desired functionality can be appended prior to each round of panning. Small libraries of appended functionalities could be screened by modifying the peptide libraries in separate and spatially addressable reaction vessels. Enzyme inhibitors could be identified by modifying Sec with substrate or transition-state analogues and panning the resulting modified peptide libraries against enzymes. The linkage of cytotoxic agents to the Sec residue could permit the discovery of peptide−drug complexes that are taken up into specific cell types. Phage simultaneously displaying enzyme libraries and substrate-derivatized selenopeptides could be screened for enzymes with enhanced activity or altered specificity. The covalent linkage of substrates to phage-displayed peptides would permit rigorous reaction and selection conditions that might disrupt the noncovalent interactions utilized in recent examples of phage-mediated enzyme evolution.[15,16]

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Supporting Information Available: HPLC PTH analysis of the N-terminal sequencing of the Sec-1-MBP fusion, experimental details of phage library construction, and graph showing Se dependence of plaque formation (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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