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EF-Tu Binding Peptides Identified, Dissected, and Affinity Optimized by Phage Display

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Summary

The highly abundant GTP binding protein elongation factor Tu (EF-Tu) fulfills multiple roles in bacterial protein biosynthesis. Phage-displayed peptides with high affinity for EF-Tu were selected from a library of $\sim 4.7 \times 10^{11}$ different peptides. The lack of sequence homology among the identified EF-Tu ligands demonstrates promiscuous peptide binding by EF-Tu. Homolog shotgun scanning of an EF-Tu ligand was used to dissect peptide molecular recognition by EF-Tu. All homolog shotgun scanning selectants bound to EF-Tu with higher affinity than the starting ligand. Thus, homolog shotgun scanning can simultaneously optimize binding affinity and rapidly provide detailed structure activity relationships for multiple side chains of a polypeptide ligand. The reported peptide ligands do not compete for binding to EF-Tu with various antibiotic EF-Tu inhibitors, and could identify an EF-Tu peptide binding site distinct from the antibiotic inhibitory sites.

Introduction

Elongation factor Tu (EF-Tu) performs key roles in bacterial protein biosynthesis [1]. EF-Tu recognizes and transports aminoacyl-transfer RNAs to the A site of the ribosome. Subsequently, EF-Tu is released from the ribosome upon hydrolysis of EF-Tu bound guanine triphosphate (GTP) to guanine diphosphate (GDP). Hydrolysis of GTP is triggered by codon-anticodon pairing at the ribosome and accompanies a gross conformational rearrangement of EF-Tu, forcing dissociation of the protein from the ribosome [2]. Thus, EF-Tu functions as a codon-anticodon pairing machine powered by GTP. The GDP binding form of EF-Tu is then converted back to the GTP binding conformation by the guanine nucleotide exchange factor, EF-Ts, in preparation for the next round of protein biosynthesis.

EF-Tu also binds other ligands, including four structurally distinct families of antibiotics—kirromycin, enacyloxin, GE2270A, and pulvomycin. Kirromycin [3] and enacyloxin [4] lock EF-Tu into a conformation similar to the GTP bound state and hinder EF-Tu dissociation from the ribosome. GE2270A [5] and pulvomycin [6] prevent binding of the incoming aminoacyl-tRNA by favoring the GDP conformation of EF-Tu. Because EF-Tu is abundant in the cell, other functions have been suggested, particularly during periods of cellular stress when protein synthesis may be slowed. One established alternate function is the role of EF-Tu in the initiation step of Qβ phage replication [7]. Other functions suggested by in vitro data include the regulation of transcription via an interaction with the C-terminal domain of RNA polymerase [8], protein disulfide isomerase activity [9], and chaperone-like activity [10, 11]. EF-Tu also binds to hydrophobic patches of unfolded proteins [12]. EF-1A, the eukaryotic homolog of EF-Tu, can interact with newly synthesized and unfolded polypeptides of different lengths and sequences [13].

With the exception of the ribosomal binding site, the residues of EF-Tu that interact with other ligands, including GDP [14], GTP [15], aminoacyl-tRNA [16], EF-Ts [17], GE2270A [5], and kirromycin [3], have all been identified at atomic resolution. A major question arises as to whether the EF-Tu binding sites utilized during protein synthesis involve the same or different sets of amino acids used in alternate functions of EF-Tu. Particularly intriguing is the interaction with GE2270A, an antibiotic that mimics a natural oligopeptide, up to 13 residues in length, if the sulfur atoms are replaced by carbon atoms. Is GE2270A binding to a site intended for a natural polypeptide involved in one of the many EF-Tu functions? GE2270A binds to a deep hydrophobic groove that includes the same pocket used by the aminoacyl group of tRNA [5]. Thus, one possibility is that EF-Tu facilitates the alignment of the aminoacyl group on the A-site tRNA with the peptidyl group on the P-site tRNA, prior to peptide bond formation during protein synthesis. Another possibility is that the GE2270A pocket is the same site that binds to hydrophobic patches of unfolded peptides for the chaperone-like activity of EF-Tu. In order to probe these questions further, EF-Tu-specific polypeptides are needed. Although EF-Tu binding to specific polypeptides has been previously examined [12], peptide binding trends and sequence preferences have not been assessed. Thus, we envisioned casting a broad net for EF-Tu-peptide ligands with a vast naive peptide library ($>10^{11}$ different peptides), followed by dissection of peptide sequence preferences with focused shotgun scanning libraries.

Selection and engineering of polypeptides through phage display harnesses diverse libraries of polypeptides, each displayed by a filamentous bacteriophage [18–21]. Peptides with high affinity and specificity for a target molecule can be isolated through multiple rounds of phage-based selection for binding to a target molecule. Phage display has also been used to rapidly assess the contribution of specific side chains to protein-protein interactions through combinatorial libraries of alanine and homolog substitutions (termed "shotgun scanning") [22–24]. Statistical analysis of selectants from shotgun scanning libraries can evaluate binding energetics of specific side chains.

Our strategy for identifying EF-Tu ligands applies

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Table 1. EF-Tu Ligands Selected from the Naive Phage-Displayed Library

Naïve Library Selectants	Sequence	Net Charge	Scaffold[a]
N-1	SSVICSEGPGGWSCVRWE	−1	$X_4CX_2GPX_4CX_4$
N-2	GVWRCILGPDGWLCLAVQ	0	
N-3	GYEWRCWPTRGQWLCILTGL	1	$X_5CX_8CX_5$
N-4	GAKLVCATDWVVILCHKVVT	2	
N-5	WDTLLCKHYDVRWICSLITR	2	
N-6	ATESQCYWSESGVLCWVTGP	−2	
N-7	SSMYWCATLETMWWCWRVVE	−1	
N-8	SKGWLCIWRVPGYVCIKFWT	3	
N-9	MEELHCARDGSQLWCWWGVL	−1	
N-10	EWYQLCATGPRGSRCWWVQL	1	
N-11	HREFLCWSLGEEGARCFVIW	0	$X_5CX_9CX_4$
N-12	EVMRTCYRAWEWGWICLLQA	0	
N-13	IEWECIALRDHVWRCWVPLP	0	$X_4CX_9CX_5$

[a] The naive peptide library was composed of 15 different scaffolds that feature different arrangements of conformation biasing disulfides and randomized residues. Net charges were calculated based upon the number of carboxyl side chains (expected to offer negative charge at neutral pH), amine bearing (positive), imidazole (positive), and guanidine side chains (positive) for each peptide.

highly diverse ($>10^{11}$ different peptide variants) libraries of peptides screened for binding to the GDP bound conformation of EF-Tu. In addition, we use homolog shotgun scanning to dissect and optimize side chain requirements for peptide binding to EF-Tu. Homolog shotgun scanning augments the original library with focused additional diversity and elucidates receptor-peptide molecular recognition from exhaustive analysis of every side chain in a peptide.

Results

Naive Library Selection

The naive peptide libraries were constructed using a previously reported design, which included both linear (X_8) and disulfide-constrained peptides expected to provide preorganized potential binding motifs [25]. Libraries were pooled to provide a diversity of $\sim 4.7 \times 10^{11}$ different peptides. Phage were enriched for binding to EF-Tu fixed to a solid support. After three or four rounds of selection for binding to EF-Tu, individual selectants were used for an ELISA-based screen to assess binding affinity. Thirteen unique phage-displayed peptides with affinity for EF-Tu were identified (Table 1). All were based upon the $X_nCX_8CX_n$ or $X_nCX_9CX_n$ scaffold.

Homolog Shotgun Scanning of N-8

Homolog shotgun scanning of **N-8** featured combinatorial libraries of wild-type and homologous side chains in every position of the **N-8** peptide. Statistical analysis following rounds of selection has been used to identify side chain and functional group preferences from similar shotgun scanning libraries [22–24]. Homolog shotgun scanning mutations were introduced by oligonucleotide-directed mutagenesis of a derivative of pS1607 [26] with four sequential stop codons replacing the phage-displayed protein open reading frame (plasmid designated pM1165a). The experimental diversity of the homolog shotgun scanning library, measured by phage titers, was $\sim 6 \times 10^7$, which exceeded the theoretical diversity for the library (1.05×10^6); thus, all possible combinations of homolog and wild-type substitutions were included in the homolog shotgun scanning library. Library enrichment was observed following rounds of selection for binding to EF-Tu complexed with GDP.

After four rounds of biopanning selection, a total of 14 additional EF-Tu binding peptides based on the sequence of **N-8** were identified from the homolog scanning library (Table 2). Unlike the naive library selection, multiple copies of the same clone ("siblings") were not found among selectants from the homolog scanning library. Specific amino acids preferences were observed for each position. For example, Cys6 and Cys15 were not replaceable by serine. Of the three tryptophans in the original peptide, only Trp4 was required for binding to EF-Tu (ratio of Trp:Leu of 13:2). Trp8 had relatively low preference and could be replaced with leucine for approximately half of the EF-Tu ligands (Trp:Leu of 7:6). Leucine was preferred over tryptophan at position 19 of **N-8** (Trp:Leu of 2:13). Glycines were strongly preferred over alanine at both position 3 (Gly:Ala of 13:2) and

Table 2. EF-Tu Ligands Selected from the Homolog Shotgun Scanning Library

Selectants	Amino Acid Sequence[a]																			
Position	1				5				10				15				20			
H-1	A	R	G	L	L	C	V	W	R	I	S	G	F	V	C	I	R	F	L	S
H-2	S	R	G	L	L	C	V	W	K	V	P	G	F	V	C	V	R	F	L	T
H-3	S	R	G	W	I	C	I	W	K	V	P	G	Y	V	C	V	R	Y	L	T
H-4	A	K	G	W	L	C	V	W	R	V	P	A	F	V	C	V	K	F	L	S
H-5	S	R	G	W	I	C	V	W	R	V	P	A	Y	I	C	I	K	F	W	S
H-6	A	R	G	W	L	C	V	L	R	V	P	G	F	V	C	V	R	F	L	S
H-7	A	R	G	W	I	C	I	L	K	V	P	G	Y	V	C	V	R	Y	L	T
H-8	A	R	G	W	L	C	I	L	R	V	P	G	F	V	C	I	R	F	L	S
H-9	S	R	G	W	I	C	V	S	R	V	S	G	F	V	C	I	R	F	L	S
H-10	A	T	G	W	L	C	V	S	R	V	T	G	Y	V	C	V	R	Y	L	S
H-11	S	T	G	W	L	C	V	L	R	I	S	G	Y	V	C	I	R	Y	L	T
H-12	S	R	G	W	I	C	I	L	R	I	S	D	F	I	C	V	R	Y	L	S
H-13	S	R	A	W	I	C	V	L	R	V	P	G	Y	V	C	V	K	Y	L	T
H-14	A	R	A	W	I	C	V	W	R	V	P	G	Y	V	C	V	R	F	L	S
N-8	S	K	G	W	L	C	I	W	R	V	P	G	Y	V	C	I	K	F	W	T

[a] Shaded boxes highlight positions with 89% homology; unshaded and dotted boxes represent 80% and 73% homology, respectively. Underlined substitutions resulted from spontaneous, unprogrammed mutations.

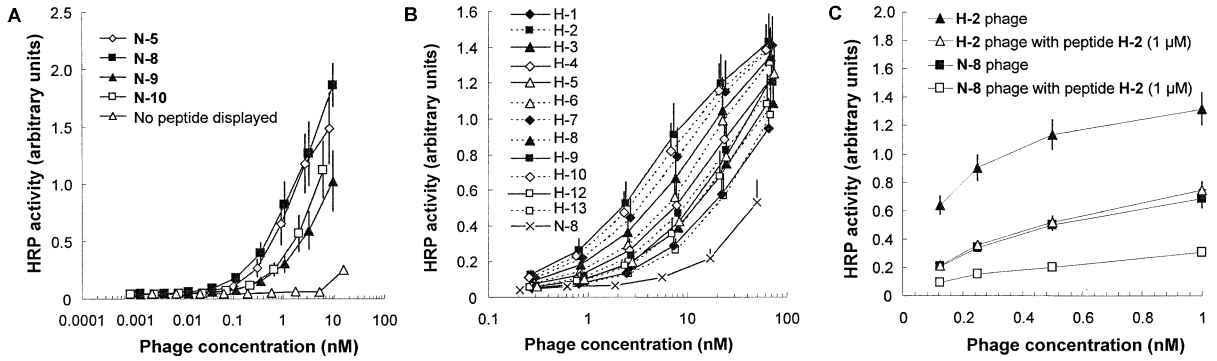

Figure 1. Phage-Based ELISA

(A) Phage-based ELISA of representative EF-Tu peptide ligands selected from naive libraries. Serial dilutions of phage-displayed EF-Tu ligands and VCS M13 phage not displaying a peptide, as a negative control, were incubated in EF-Tu-coated microtiter wells. The selectants assayed here maximized positive charge for increased solubility during future assays. Each data point represents the average of four experiments, and error bars represent standard error.

(B) Phage-based ELISA of EF-Tu peptide ligands selected from homolog shotgun scanning library. Serial dilutions of phage-displayed peptides selected from homolog shotgun scanning library were incubated in EF-Tu-coated microtiter wells. Each data point represents the average of four experiments. Control phage lacking a displayed peptide do not bind EF-Tu (shown in A). Error bars represent standard error and, to simplify the diagram, error bars in only one direction are shown.

(C) To directly compare **N-8** and **H-2**, serial dilutions of phage-displayed peptides were incubated with N-terminal acetylated **H-2** (1 μM) in EF-Tu-coated microtiter wells. The binding of the phage-displayed **H-2** (1 nM) was decreased to 55% of original binding, whereas the binding of the phage-displayed **N-8** (1 nM) was decreased to 40% of original binding. Error bars represent the standard error for the average value measured four times.

12 of **N-8** (Gly:Ala of 12:2). A low rate of spontaneous mutations, expected from propagation of phage in *E. coli*, was also observed. Phage-displayed peptides selected from homolog shotgun scanning library all bound EF-Tu stronger than the **N-8** peptide selected from naive library (Figure 1B). The higher EF-Tu affinity for homolog selectants was further confirmed by a direct competition assay between the **H-2** ligand from homolog shotgun scanning, resynthesized as an N-terminal acetylated peptide, and the **N-8** ligand (Figure 1C).

Additional Peptide-EF-Tu Binding Assays

To obtain more precise binding measurements for EF-Tu peptide interactions, the H-2 ligand was chemically resynthesized with a biotin tag at the N terminus to allow quantification by streptavidin linked to HRP (horseradish peroxidase) and as the nonbiotinylated **H-2** peptide with an acetylated N terminus. The binding affinity of the N-terminal biotinylated and acetylated peptide **H-2** was examined by competition ELISA (Figure 2). N-terminal biotinylated **H-2** bound EF-Tu with a 0.9 μM apparent K_D, as shown by ELISA EC_{50}. EF-Tu binding to the biotinylated peptide **H-2** (0.5 μM) was measured in competition with N-terminal acetylated **H-2**, and an IC_{50} value of 1.5 μM was observed. Using the same assay conditions, various antibiotics, including GE2270A (7.5 μM), kirromycin (7.5 μM), tetracycline (1.5 mM), 26A-VRC3064 (4 μM), and 30A-VRC3348 (4 μM), did not compete with *N*-terminal biotinylated **H-2** for binding to EF-Tu (Figure 3). The competition and noncompetition assays were repeated without tween in the wash buffer, and the results were qualitatively similar with higher background binding by the peptide (data not shown). To demonstrate binding to EF-Tu in solution, N-terminal biotinylated **H-2** and EF-Tu were mixed and, after an appropriate amount of time, free peptide was separated by filtration for quantification (Table 3). Results from the solution phase and ELISA assays were consistent.

Discussion

EF-Tu Ligand Discovery

Phage-display techniques can access exceptionally diverse libraries of different peptides; for example, the

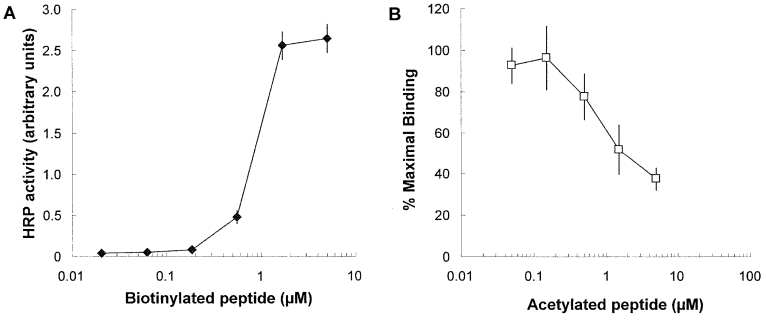

Figure 2. Assay of Chemically Synthesized EF-Tu Ligands

(A) ELISA binding profile of N-terminal biotinylated **H-2** to immobilized EF-Tu (diamonds).

(B) Inhibition of N-terminal biotinylated **H-2** (0.5 μM) binding to immobilized EF-Tu by N-terminal acetylated **H-2**. Each data point represents the average of four experiments. Error bars represent standard error.

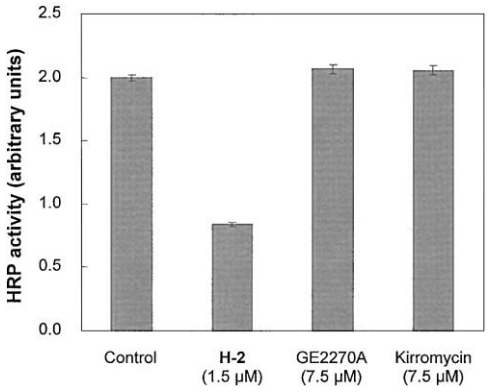

Figure 3. Assay of Competitive Binding for EF-Tu Peptide and Non-peptide Ligands

Biotinylated peptide **H-2** (0.5 μM) was incubated in EF-Tu-coated microtiter wells (control). N-terminal acetylated peptide **H-2** (1.5 μM) (positive control), GE2270A (7.5 μM), or kirromycin (7.5 μM) were added for competition binding. Error bars represent the standard error for the average value measured four times.

library applied here includes approximately 5×10^{11} different peptides. Although a previous study has focused upon EF-Tu binding to a few peptides synthesized on cellulose support [12], our approach has applied highly diverse libraries of peptides incorporating cysteine disulfides as conformationally restrictive elements. This approach tailors the initial diversity to a potentially productive area of structural diversity space—peptides rigidified by a disulfide crosslink [19, 23–28]. All of the EF-Tu ligands identified here have been selected from disulfide crosslinked libraries, in preference to other nondisulfide crosslinked peptides included in the naive library. Phage-displayed cysteine containing peptides usually form intramolecular disulfide bonds, which result in cyclic peptides, but can also form intermolecular disulfides to result in ligand dimerization [25–29].

Thirteen essentially nonhomologous peptides were selected as EF-Tu ligands from this library (Table 1). A uniform distribution of hydrophobic amino acids at positions bracketing the cysteine residues was observed in the selected peptides. This could stem from a general preference of EF-Tu for hydrophobic residues demonstrated by Richarme and coworkers [12]. This pattern could also result from selection of more structured peptides, as β-turn formation in peptides has been shown to benefit from hydrophobic residues adjacent to cysteine disulfides [27]. Additional screening and sequencing of selectants has identified only additional sibling sequences. The lack of sequence homology among the identified EF-Tu ligands demonstrates that many solutions exist for peptide binding by EF-Tu. This diversity could reflect a deliberately promiscuous binding activity necessary for effective chaperone activity by EF-Tu. Without an obvious consensus sequence for peptide ligands to EF-Tu, additional study has been required to identify key attributes responsible for molecular recognition between EF-Tu and peptides.

Dissecting an EF-Tu Peptide Ligand

Rather than adopting a mass production approach to synthesize many peptide variants to glean structure activity relationship (SAR) data, we constructed a single homolog shotgun scanning library to dissect one peptide ligand. For these experiments, the **N-8** peptide was chosen, as the +3 charge of **N-8** at neutral pH could potentially improve the solubility of synthetic ligands for future experiments. An **N-8** homolog shotgun scanning library tests the tolerance for a homologous side chain in every position of the peptide. This homolog shotgun scanning approach to expedited SAR has been demonstrated previously for an anti-Her2 antibody [23] but has not been previously applied to short peptides. This approach is similar to the medicinal chemistry practice of synthesizing large numbers of analog compounds for SAR studies. However, in our experiment, all possible combinations of homologous peptides are synthesized in a single phage-displayed library.

Like other shotgun scanning studies [23, 24], trends for preferred amino acids have emerged at specific positions of the homolog shotgun scanning library (Table 2). First, the two cysteines have proven entirely resistant to substitution with serine (Cys:Ser ratio of 14:0), which could indicate a requirement for the conformational rigidity provided by formation of the cysteine disulfide-formed macrocycle. Further demonstrating the importance of the disulfide bond to EF-Tu peptide ligands, treatment of **H-2** with a reducing agent (Tris (2-carboxyethyl) phosphine hydrochloride, TCEP·HCl) significantly reduces peptide binding to EF-Tu (data not shown).

Three positively charged amino acids at peptide positions 2, 9, and 17 display a marked preference for arginine instead of lysine. This preference for the guanidine group of arginine in place of the primary amine of lysine suggests that the interaction between Arg in these positions and EF-Tu is not mere charge-charge pairing. For example, the bidentate hydrogen binding possibilities of the guanidine group could prove important for interactions between EF-Tu and **N-8**. Further structural analysis is required to more precisely assign a functional role to these highly conserved arginines.

Gly12 and Trp4 are the next most preferred amino acids (Gly:Ala of 13:2 and Trp:Leu of 13:2). Tryptophan likely contributes significant binding energy to the EF-

Table 3. Solution Phase Assay of **H-2** Ligand Binding to EF-Tu (n = 3)

Condition/Measurement	Experiment 1	Experiment 2	Experiment 3
Peptide **H-2** (5 μM)	+	+	+
EF-Tu (0.2 mg/ml)	−	−	+
Filtration	−	+	+
HRP activity (mean ± SD) (**H-2** amount in filtrate)	1.691 ± 0.048	1.960 ± 0.066	0.209 ± 0.089

Tu peptide interaction, and glycine could be important for reducing a steric clash or imparting a key structural motif to the ligand. For example, Gly12 could introduce flexibility for turn formation in concert with the next residue, either proline or serine, which are both tolerated at position 13. In some peptide positions (e.g., 1, 5, 8, 13, 18, and 20), the original side chain functionality has readily been replaced with a homolog, which indicates minimal contribution to EF-Tu binding by specific functional groups in these positions.

Assay of EF-Tu Binding to Peptides

Several different assay formats were used to measure binding affinity between EF-Tu and peptide ligands. First, all peptides selected from the naive and homolog shotgun scanning libraries were subjected to a phage-based ELISA to assess relative binding affinities for EF-Tu (for representative examples, see Figure 1A). All peptide ligands reported here bound EF-Tu specifically in this ELISA and failed to bind to a BSA control, which also tests for peptides that could bind to polystyrene ELISA plates (data not shown).

Unexpectedly, all selectants from the homolog shotgun scanning library bound more strongly to EF-Tu than the **N-8** peptide used as a template for the library of homologous peptide variants (Figure 1B). This conclusion assumes that avidity effects and peptide expression levels on the phage surface are all roughly similar among the phage-displayed clones; this is a reasonable assumption for these highly homologous peptides grown under identical conditions. In addition, a relatively homologous set of peptide variants with an evenly spaced range of binding affinities to EF-Tu was observed. Such data could be used to analyze contributions to binding energy by mutations in specific positions of the peptide. Thus, SAR information can be derived from the range of affinities resulting from the different homologous EF-Tu ligands. This analysis is distinct from standard shotgun scanning epitope mapping, which has focused exclusively upon the distribution of wild-type and mutant in specific positions to derive $\Delta\Delta G$ values. The unexpected improvement in ligand affinity could result from fine adjustment of side chain functionality and competition during selection for the highest affinity ligand. The affinity maturation observed here (up to approximately two orders of magnitude) did not result from an inability to recover more weakly binding EF-Tu ligands, as less strongly binding ligands were selected from the naive library (shown in Figure 1A).

Affinity maturation is perhaps the most common application of protein engineering and phage display in particular. The method applied here, homolog shotgun scanning, features binary substitution to similar amino acids. This approach is distinct and, in some ways, simpler than previously described methods for affinity maturation. For example, methods have been described for affinity maturation based upon switching the displayed peptide from a high valency to monovalent format, minimizing avidity effects during the maturation process [25].

The peptide chosen for further assays, **H-2**, minimizes β-branched side chains associated with aggregation during synthesis and maximizes positive charge, which could improve ligand solubility. Here again, homolog shotgun scanning assisted the process of ligand discovery, as we could pick the best candidate for chemical synthesis from a large set of potential ligands. Perhaps as a result of this prioritization for minimal β-branching and maximal positive charge, synthesis of **H-2** resulted in a single product by HPLC and a water-soluble peptide (solubility $>$0.2 mg/ml). To more closely mimic the peptide displayed on the N terminus of the M13 phage coat protein P8, we synthesized **H-2** with a carboxamide C terminus. For direct assay of EF-Tu-peptide binding, **H-2** was synthesized with either an acetylated or biotinylated N terminus.

Measurement of EF-Tu binding to **H-2** applied several assay formats. First, a direct ELISA was used to measure binding of N-terminal biotinylated **H-2** to ELISA plate bound EF-Tu (Figure 2A). N-terminal biotinylated **H-2** bound EF-Tu with an EC_{50} value of approximately 0.9 μM. This EC_{50} value for direct assay of the chemically synthesized peptide was considerably higher than the approximately 10 nM EC_{50} observed for the ELISA of phage-displayed **H-2**. The difference in measurements between phage-based and direct ELISAs suggest that avidity or velcro effects boost the apparent K_D for phage-displayed **H-2**, which is fused to the multicopy phage coat protein P8. Similarly, we expect other phage-displayed peptides reported here to also experience avidity effects.

Non-phage-displayed, N-terminal biotinylated **H-2** was used for competition ELISAs with a variety of different EF-Tu ligands. First, N-terminal acetylated **H-2** competed successfully with N-terminal biotinylated **H-2** for binding to EF-Tu (Figure 2B). Numerous known antibiotic ligands to EF-Tu, including GE-2270A, kirromycin, tetracycline, 26A-VRC3064, and 30A-VRC3348, failed to compete with N-terminal biotinylated **H-2** for binding to EF-Tu (for representative data, see Figure 3). Five phage-displayed peptides (**N-3**, **N-4**, **N-10**, **N-11**, and **N-13**) were also examined for competition between the above listed antibiotics for binding to EF-Tu. Moreover, different formats attempted to demonstrate antibiotic-peptide competition included first adding the antibiotic and premixing the peptide and antibiotic. However, no competitive binding between peptides and antibiotics under any format was observed. Therefore, we conclude that the peptide **H-2** and most likely other peptide ligands reported here bind to a site on EF-Tu that is distinct from the binding site necessary to inhibit EF-Tu function for antibiotic activity.

Biological Implications for EF-Tu-Peptide Binding

Given the high concentration of EF-Tu found in cells (approximately 0.56 mM, see Experimental Procedures for calculation), the EC_{50} values reported here are likely to be important biologically. In other words, if EF-Tu ligands with binding similar to the peptide ligands reported here are found in the cell, the complex would be strongly favored by thermodynamics. Furthermore, we expect peptide binding by EF-Tu not to interfere with EF-Tu function, as competition assays demonstrate binding to a site distinct from the antibiotic sites. Thus,

the peptide binding activity identified here most likely represents a biological function of EF-Tu, such as interaction with other proteins to form a larger protein complex or, possibly, chaperone-like activity by EF-Tu.

EF-Tu has been proposed to act as a chaperone [10] and as a protein disulfide isomerase [9]. EF-Tu has also been shown to bind the hydrophobic clusters composed of both consecutive and nonconsecutive hydrophobic residues in the same manner as the chaperone DnaK [12]. The EF-Tu ligands identified here are slightly hydrophobic and lack a discernible consensus sequence. Thus, the peptide binding reported here could be consistent with the function of EF-Tu as a chaperone. To rule out the possibility of EF-Tu binding through the disulfide isomerase activity, we have repeated the phage-displayed peptide assays with the naive selectants, after first treating with plate bound EF-Tu with iodoacetamide; no loss of binding was observed (data not shown).

Attempts had been made to use sequences of peptide ligands from phage display libraries to identify cellular binding partners [30, 31]. Thus, we performed BLAST searches [32] with the EF-Tu ligands reported here to identify homology with proteins included in the *E. coli* genome. No exact homologs were found. However, the peptide **H-4** displayed the highest degree of homology with an *E. coli* protein; **H-4** had 45% identity and 55% similarity to the GTP binding domain of selenocysteinyl-tRNA-specific translation factor (SELB) (residues 51 to 66). Both EF-Tu and SELB are GTP binding proteins and perform similar roles ferrying amino acids to the ribosome. Though such similarities could be coincidental, further studies could focus upon binding between EF-Tu and SELB through the binding site identified by **H-4**.

Significance

EF-Tu is a versatile biosynthetic machine, interacting with amino-acyl tRNAs, the ribosome, and other molecules during protein synthesis. Evidence presented here could suggest another role for EF-Tu—binding to diverse peptide sequences. A few features common to the different ligands identified here define subtle requirements for peptide binding in vitro. The two-step method for identifying and optimizing peptide ligands, biased peptide libraries followed by homolog shotgun scanning, extracts SAR information while improving binding by the initial lead peptide. This approach offers a general strategy to the challenging problem of leveraging protein libraries with vast, though not exhaustive, diversity for ligand identification. The ligands described here most likely bind a site distinct from known antibiotic binding sites.

Experimental Procedures

Materials

Reagents for dideoxynucleotide sequencing were from ABI/PE Biosciences. Enzymes were from New England Biolabs, excluding Taq polymerase, which was from Fisher. Maxisorp immunoplates were from NUNC. Anti-M13/HRP conjugate was obtained from Amersham Life Science. Amino acid derivatives, HOBt and HBTU, were from Novabiochem AG. Succinimidyl-6-(biotinamido) hexanoate (NHS-LC-biotin) was purchased from Pierce. The water-soluble derivatives of GE2270A, 26A-VRC3064, and 30A-VRC3348 were a gift of Versicor, Inc. (Fremont, CA).

Oligonucleotides

DNA degeneracies are represented in the IUB code (K = G/T, M = A/C, N = A/C/G/T, R = A/G, S = G/C, W = A/T, Y = C/T). The oligonucleotides used are listed below with mutation highlighted in bold.

STOP-4, 5′-TT GCT ACA AAT GCC TAT GCA **TAA TAA TGA TGA** GGT GGA GGA TCC GGA GG-3′; M13-F1 primer, 5′-TGTAAAAC GACGGCCAGT-3′; SAV-F1 primer, 5′-TGTAAAACGACGGCCAGT CGAGCACTTCACCAACAA-3′; SAV-R2 primer, 5′-CAGGAAACAGC TATGACGACAACAACCATCGCCC-3′; and homolog scanning, 5′-TT GCT ACA AAT GCC TAT GCA **KCC ARG GST TKG MTC TSC RTT TKG ARG RTT YCG GST TWC RTT TSC RTT ARG TWC TKG ASC** GGT GGA GGA TCC GGA GG-3′.

Construction of Phage-Displayed Peptide Libraries

$X_nCX_nCX_n$ and X_n type libraries fused to the P8 major coat protein of M13 phage were constructed using a previously described procedure [33]. The libraries were combined and stored frozen at −78°C in 10% glycerol for later use.

The homolog scanning libraries were constructed using a previously described method [33] with phagemid pM1165a as library template. Phagemid pM1165a was identical to a previously described phagemid designed for phage display of proteins (pS1607) [26] with the following exception. The codons encoding the displayed protein were changed to a series of four stop codons, TAA TAA TGA TGA, through mutagenesis with the oligonucleotide STOP-4.

Phage-Displayed Peptide Selection for EF-Tu Binding (Biopanning)

Phage libraries cycled through serial rounds of selection for binding to EF-Tu. Nunc 96-well Maxisorb plates were coated at room temperature with EF-Tu-GDP [34] diluted to 5 μg/ml in PBS (pH 7.6). After 2 hr, the coating solution was removed, and the plates were blocked for 15 min with a 0.2% solution of bovine serum albumin (BSA) or a 0.2% solution of casein in PBS used for alternating rounds of selection. After the plates were washed three times with wash buffer (0.02% Tween 20 in PBS), 100 μl of the phage library in PBS was transferred to 24 of the coated wells. After 1 hr, the plate was washed five times with wash buffer. Phage were eluted by adding 100 μl of 100 mM HCl and shaken vigorously for 5 min. The eluted phages were immediately neutralized with 33 μl of 1.0 M Tris-HCl (pH 8.0). Half of the eluted phage solution was used to infect 15 ml of XL1-Blue cells (OD_{550} 0.5–1.0). After 20 min of shaking at 37°C, the infected cells were transferred to 400 ml of 2×YT supplemented with 50 μg/ml ampicillin and 10^{10} phage/ml M13-K07 helper phage. After 16 hr of incubation at 37°C, cells were removed by centrifugation for 10 min at 12,000 × g, the supernatant was transferred to a new tube containing 60 ml of PEG/NaCl (20% polyethylene glycol 8000, 2.5 M NaCl), and it was incubated at room temperature for 10 min. Phage were harvested from the culture supernatant by centrifugation (10 min, 12,000 × g) and resuspended in 2 ml of PBS. After the first round of selection, biopanning was conducted at $1/12^{th}$ of the scale described above, and repeated five times.

Phage ELISA

To assess EF-Tu binding activity of individual selectants from the phage-displayed libraries, a phage-based ELISA was used. Nunc 96-well maxisorb plates were coated with 100 μl EF-Tu-GDP diluted to 5 μg/ml in PBS (pH 7.6). After 2 hr incubation at room temperature, the coating solution was removed, and the plates were blocked for 15 min with a 0.2% solution of BSA in PBS. Control wells, which were not coated with EF-Tu, were coated with the BSA solution. After the plates were rinsed with wash buffer (0.02% Tween-20 in PBS) three times, a solution of phage-displayed peptides was added to both EF-Tu-coated plates and BSA control plates. Then the plates were incubated at room temperature with gentle shaking for 1 hr. For initial assessment of individual selectants, culture supernatant obtained from growth of 0.5 ml 2YT culture in 96-well format and centrifugation at 1500 × g for 30 min were assayed as described. For a more careful follow-up assay of EF-Tu ligands, isolated phage

at approximately 2×10^{-8} M (4.7×10^{-8} M of helper phage) were diluted by eight 1:3 dilution in PBS for assay. After the plates were washed five times with wash buffer, the plates were incubated 30 min with HRP-conjugated anti-M13 antibody (1:3000) (Amersham Pharmacia Biotech) dissolved in PBS containing 0.02% Tween-20, 0.2% BSA. The plate was washed five times with wash buffer and twice with PBS, before incubation with 2 mg/ml o-phenylenediamine dihydrochloride/0.02% H_2O_2 solution in citric acid buffer (50 mM citric acid, 50 mM Na_2HPO_4 [pH 5.0]). After 10 min, the absorbance of the unquenched solution at 492 nm was measured by a 96-well microtiter plate reader (μQuant, Bio-Tek). All solutions of phage-displayed peptides were prepared from the overnight culture at the same time, and the relative binding affinities were compared on the same ELISA plate.

PCR of Positive Clones

The phage supernatant (0.2 μl) was added to 23 μl of premixed PCR cocktail A (19.7 μl water, 2.5 μl 10 × PCR buffer, 0.25 μl 25 mM dNTPs, 0.25 μl 330 ng/μl SAV-F1 primer, 0.25 μl 330 ng/μl SAV-R2 primer, and 0.125 μl [5 units/μ]) Taq polymerase), thermocycled (94°C for 3 min, 25 × [94°C for 0.5 min, 50°C for 0.5 min, 72°C for 1 min], 72°C for 7 min), and stored at 4°C. PCR product (5 μl) was added to 2 μl of premixed PCR cocktail B (0.25 μl exonuclease-1, 0.25 μl shrimp alkaline phosphatase, 0.20 μl 10 × PCR buffer, 2.00 μl water) and heated to 37°C for 30 min and 80°C for 15 min.

The following cocktail was prepared for each sample: 4 μl 0.8 pmol/μl M13-F1 primer, 4 μl sequencing dilution buffer (200 mM Tris [pH 9.0], 5 mM $MgCl_2$), 5.5 μl water, 4 μl Terminator Ready Reaction Mix (ABI). Then, samples were thermocycled (25 times [96°C for 10 s, 50°C for 5 s, 60°C for 4 min]) and stored at 4°C. DNA was isolated by precipitation with 80 μl 75% 2-propanol. Sequencing was performed by the UCI DNA Core facility using an ABI Prism 3700 Capillary Sequencer.

Peptide Synthesis

N-terminal acetylated and biotinylated peptide based upon **H-2** were synthesized by automated Fmoc-based solid phase synthesis on a 0.3 mmol scale. Fmoc-PAL-PEG-PS resin (Applied Biosystems, Foster City, CA) was used to install carboxyamides at the C terminus of the synthesized peptides to mimic the C-terminal fusion of phage-displayed peptides to the anchoring M13 coat protein P8. Upon completion of automated peptide synthesis, the peptide N terminus was modified by treatment with acetic anhydride (10 eq) and diisopropylethylamine (DIPEA) (10 eq) or NHS-LC-biotin (2 eq) in N, N′-dimethylformamide (DMF)/dichloromethane (DCM) (1:1, v/v). Side chain protection groups were removed and the peptide was simultaneously cleaved from the resin with 94% trifluoroacetic acid (TFA), 2.5% water, 2.5% ethanedithiol (EDT), and 1% triisopropylsilane (TIS) applied for 3 hr. Purification of linear peptides was performed by automated preparative LC/MS (Waters) with a water/acetonitrile gradient containing 0.1% formic acid. Linear peptide solution (0.1 mg/ml) in 0.1 M ammonium bicarbonate was rapidly stirred and exposed to air to oxidatively form the disulfide bond. During HPLC-monitored peptide oxidation, the peak corresponding to the linear peptide decreased in size, and emergence of a peak with a shorter retention time corresponding to the disulfide-cyclized macrocycle was observed. The disulfide cyclized peptide was isolated by lyophilization, before additional HPLC purification. The purity and identity of each peptide was determined by analytical LC/MS. The observed mass (m/z) for synthetic peptides matched expected values within 0.05% as follows: linear N-terminal acetylated peptide, 1161.8 (MH^{2+}) and 775.2 (MH^{3+}) (calculated mass: 2321.9); cyclic N-terminal acetylated peptide, 1161.1 (MH^{2+}) and 774.6 (MH^{3+}) (calculated mass: 2319.9); linear N-terminal biotinylated peptide, 1310.5 (MH^{2+}) and 874.3 (MH^{3+}) (calculated mass: 2619.3); and cyclic N-terminal biotinylated peptide, 1309.4 (MH^{2+}) and 873.6 (MH^{3+}) (calculated mass: 2617.3).

ELISA of Chemically Synthesized Peptide Ligands to EF-Tu

Coating of EF-Tu-GDP and BSA blocking of 96-well maxisorb plates was conducted as described above. After the plates were rinsed with wash buffer three times, serial dilutions (1:3) of N-terminal biotinylated peptide (10 μM) were added to both EF-Tu coated plates and BSA control plates, and the plates were incubated at room temperature for 1 hr. After the plates were rinsed three times with wash buffer, the plates were incubated 1 hr with HRP-conjugated streptavidin (Pierce) dissolved in PBS containing 0.02% Tween-20, 0.2% BSA. The plate was rinsed three times with wash buffer and twice with PBS, before incubation for 10 min with 2 mg/ml o-phenylenediamine dihydrochloride/0.02% H_2O_2 solution in citric acid buffer (50 mM citric acid, 50 mM Na_2HPO_4 [pH 5.0]). After 10 min, the absorbance of the unquenched solution at 492 nm was measured by a 96-well microtiter plate reader (μQuant, Bio-Tek).

Competitive displacement assay was conducted with a N-terminal biotinylated peptide (5×10^{-7} M) and serial dilutions (1:3, from 5×10^{-6} M) of N-terminal acetylated peptide or antibiotic in the ELISA format described above.

Solution Phase Binding Assay

N-terminal biotinylated peptide **H-2** (5 μM) was incubated with and without EF-Tu (0.2 mg/ml) in an EF-Tu assay buffer (50 mM Tris-HCl [pH 7.6], 10 mM $MgCl_2$, and 10 μM GDP) on ice for 1 hr. To separate EF-Tu bound from free peptide, the incubated mixture was then subjected to centrifugal filtrations by Centricon YM-30 (cut-off 30 kDa, Millipore) after prerinsing Centricon tubes with 0.1% Tween-20. Free peptide in the filtrate was detected by ELISA, which was conducted as described above.

Calculation of EF-Tu Concentration in *E. coli*

The concentration of EF-Tu was calculated based upon expectations for the cellular composition of *E. coli* [35], and an approximate concentration of EF-Tu roughly equal to the cellular concentration of tRNAs [36]. Thus, we calculated an EF-Tu concentration of 0.56 mM in *E. coli*.

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