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Detailed Experimental Protocol

Library Synthesis. The library was constructed, transcribed, and ligated to the puromycin-containing linker as described (1). A 10-ml translation was performed with the Ambion (Austin, TX) *in vitro* translation kit, by using standard conditions for capped mRNA, supplemented with 2 mCi [^{35}S]methionine and a total methionine concentration of 10 μM. The reaction was programmed with 300 nM library mRNA linker. After 1 h at 30°C, $MgCl_2$ and KCl were added to 20 and 710 mM, respectively, and the reaction was further incubated at room temperature for 5 min (increasing the yield of displayed peptides; see ref. 2). The reaction was then diluted 10-fold into oligo(dT)-binding buffer (1M NaCl/50 mM Hepes/10 mM EDTA/0.25% Triton X-100/5 mM 2-mercaptoethanol, pH 7.9) and 80 mg of oligo(dT) cellulose (type 7, Amersham Pharmacia) and incubated with agitation at 4°C for 30 min. The mixture was applied to a column (Poly-Prep chromatography column, Bio-Rad), drained, and washed with 10 ml of oligo(dT)-binding buffer, 10 ml of oligo-dT-wash buffer (300 mM NaCl/20 mM Hepes/1 mM EDTA/0.25% Triton X-100/5 mM 2-mercaptoethanol, pH 7.9), and then 1 ml of 0.5× oligo(dT) wash buffer. The mRNA-displayed peptides were then eluted with 4.5 ml of water plus 5 mM 2-mercaptoethanol into tubes containing Triton X-100 and BSA (New England Biolabs) for final concentrations of 0.15% and 15 μg/ml, respectively. This sample was then exposed to 0.5 ml of Ni-NTA-agarose (Qiagen, Chatsworth, CA) in Ni-binding buffer [6 M guanidinium chloride/0.5 M NaCl/100 mM sodium phosphate/10 mM Tris(hydroxymethyl)aminomethane/0.1% Triton X-100/5 mM 2-mercaptoethanol/4 μg/ml tRNA (Roche Molecular Biochemicals)/5 μg/ml BSA, pH 8.0] and incubated for 30 min at room temperature. The matrix was then drained and washed with 12 column volumes (CV) of Ni-binding buffer and then eluted with the same buffer plus 100 mM imidazole. Eluted fractions were then combined and desalted by using two successive NAP columns (Amersham Pharmacia) equilibrated in 1 mM Tris(hydroxymethyl)aminomethane/0.01% Triton X-100/50 μM EDTA/0.5 mM 2-mercaptoethanol/0.5 μg/ml tRNA (Roche Molecular Biochemicals)/50 μg/ml BSA, pH 7.6. The mRNA display constructs were then reverse transcribed by using Superscript II (GIBCO/BRL) according to the manufacturer's instructions, except that the mRNA concentration was about 5 nM, and the enzyme concentration was 1 unit/μl. To ensure a high yield in the reaction, a mixture of two primers was used, the "splint" from the splinted ligation (1) and the 3' PCR primer, each at 1 μM. After 30 min at 42°C, the reaction was raised to 50°C for 2 min and then cooled over 5 min to room temperature (to allow gradual peptide folding). Finally, the contents were desalted by using a NAP column and then subjected to scintillation counting. By comparing the ^{35}S counts of the purified reverse-transcribed mRNA-peptide fusions to the [^{35}S]methionine stock and taking into consideration the total methionine concentration in the translation reaction (10 μM), it was possible to determine the number of displayed peptides in this sample.

Selection for Streptavidin (SA)-Binding Peptides. The displayed peptides were incubated with immobilized SA (Ultralink Immobilized Streptavidin Plus, about 4

mg/ml; Pierce) in SA-binding buffer [40 mM Tris(hydroxymethyl)aminomethane/300 mM KCl/2 mM EDTA/0.1% Triton X-100/5 mM 2-mercaptoethanol/100 μg/ml BSA/1 μg/ml tRNA, pH 7.4]. The amount of gel used was 0.5 ml in a total volume of 5.5 ml. After incubation for 20 min at room temperature, the contents were loaded onto a disposable chromatography column, drained, washed with 14 column volumes (CV) of SA-binding buffer, and then eluted with five successive aliquots (at 10-min intervals) of SA-binding buffer plus 2 mM D-biotin (Sigma). Elution fractions were combined, desalted on a NAP column, and then PCR-amplified by using the described (1) conditions and primers (8-ml reaction). This procedure concluded the first round of selection, and the remaining rounds followed the same protocol except that the translation was scaled down 10-fold, and the number of CV for washing the SA column was increased (32 volumes for round 2; 40 for rounds 3, 4, and 6; and 25 for rounds 5 and 7). The SA-binding selection for rounds 5 and 7 was performed directly on the SA-column eluate from the preceding selection rounds, without intervening amplification (the biotin was removed by three successive NAP column treatments). PCR products amplified after the seventh selection round were cloned by using the TOPO TA cloning kit (Invitrogen).

Analysis of Individual Peptide Sequences. To characterize selected peptides rapidly, a method for generating mRNA-peptide covalent fusions was used (3). Plasmids containing single inserts were used as templates for PCR amplification by using the same 5' PCR primer as described for the library construction (2) and a new 3' primer (5'-ATAGCCGGTGCCAAGCTTGCAGCCGCCAGACCAGT-3'), which altered the 3' RNA sequence to ACUGGUCUGGCGGCUGCAAGCUUGGCACCGGCUAU. This sequence was designed to anneal to the photo-crosslinking linker, which has the sequence 5'-psoralen-TAGCCGGTG-A17-CC-puromycin-3', in which the underlined positions are 3'-methoxy nucleotides and the remaining are deoxy (oligonucleotide synthesized by using reagents from Glen Research, Sterling, VA). This new primer changed the constant C-terminal peptide sequence from WSGGCHHHHHHSSA to WSGGCKLGTGY, of which the last three amino acids may not be translated, because the encoding RNA is annealed to the linker. Each DNA template was transcribed and gel purified as described (1) and then incubated with the psoralen linker under the following conditions: 2 μM mRNA/4 μM linker/50 mM Tris(hydroxymethyl)aminomethane/200 mM KCl/10 mM spermidine, pH 7.4, at 70°C for 2 min, and then cooled to 4°C over 5 min. Samples were then placed in the cold room in a 96-well plate (50 μl per well), 1 inch above which a UV lamp was suspended (366 nm, Ultraviolet Products, San Gabriel, CA, model no. UVL-21) for 15 min. Reactions were then desalted by using a G-50 Sephadex spin column (Roche Molecular Biochemicals). The translation/display reactions were then carried out as above, as was the oligo(dT) purification. Finally, RNase A (200 ng/ml, 10 min, room temperature) was added to degrade the mRNA (complete degradation was confirmed by SDS/PAGE analysis).

The resulting purified DNA-tagged proteins (DTPs) were analyzed in the SA column-binding assay, in which ≈500 pM ^{35}S-labeled DTPs were mixed with 50 μl of the SA matrix in SA-binding buffer, in a total volume of 300 μl, for 10 min at room temperature (with agitation), after which the contents were loaded onto a chromatography column. The column was drained and then washed with 80 CV of SA-binding buffer and then

eluted with three consecutive aliquots (3 CV each) of SA-binding buffer plus 2 mM biotin (over a 15-min period). All fractions (flowthrough, washes, elutions, and irreversibly bound counts) were analyzed by scintillation counting to determine the fraction of DTP that bound SA and eluted with biotin. The *Strep*-tag II (4) template encoded the peptide sequence MSNWSHPQFEKNWSGGCGTGY. The nonselected clone, in which two HPQ motifs (separated by 19 amino acids) were introduced, encoded the sequence DEA**HPQ**AGPVDQADARLVQQGALQH**HPQ**GDRMMSGGCKLGTGY (the underlined portions are identical to the HPQ regions of clone SB2).

For quantitative analysis of binding, an electrophoretic mobility-shift assay (EMSA) was used. DTPs were incubated with varying amounts of pure SA (Pierce Immunopure Streptavidin) in SA-binding buffer plus 5% glycerol (to increase the density of the solution so it could collect at the bottom of the gel well). After incubation at room temperature for 20 min, the reactions were moved into the cold room, where they remained for 10 min before being carefully loaded onto a 10% polyacrylamide/bisacrylamide (37.5:1, National Diagnostics) gel (thickness 0.7 mm, height 16 cm, width 18 cm) containing 2× 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA (TBE), 0.1% Triton X-100, and 5% glycerol. The gel (prerun for 30 min at 13 W) and running buffer were precooled to 4°C and run in the cold room at 13 W (increasing the temperature of the gel to about 20°C) for 45–120 min, depending on the mobility of the particular DTP. The gel was then fixed in 10% acetic acid/10% methanol for 15 min, transferred to electrophoresis paper (Ahlstrom, Mt. Holly Springs, PA), dried, and analyzed by using a PhosphorImager (Molecular Dynamics). The concentration of DTPs was <1 nM in each titration, and thus the K_D can be approximated by the concentration of SA that results in half of the DTP being mobility-shifted. To determine the K_D, several different measurements were taken in the range of 25–75% of DTP bound (values outside of this range were unreliable because of the background and close proximity of the bound and unbound band in the gel). The K_D was determined by using the equation K_D = [SA]*R, where R is the ratio of unbound to bound DTP (ratio of unshifted to shifted band). Independent measurements on gels prepared at different times were used for each clone (the number of different measurements, *n*, is shown in Table 2). SA concentrations were measured by UV_{282}, by using the molar extinction coefficient of 57,000 per monomer (5).

C-terminal deletion analysis of clone SB19 was performed by amplifying the clone with the original 5' primer and a series of 3' primers that truncated the sequence at various positions and also replaced two codons (encoding Asp-Trp) in the C-terminal constant region with methionine codons (to increase the ^{35}S incorporation). Analogous primers were used for the N-terminal deletion analysis, except that no change was made in the N-terminal constant sequence.

For the SA-coated plate-based binding assay, the following [^{35}S]methionine-labeled protein was generated by *in vitro* translation in reticulocyte lysate (Novagen) by using the manufacturer's instructions plus an additional 100 mM KCl and 1 mM $MgCl_2$: MDYKDDDDKMDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREPGSHH HHHHMGM. After incubation at 30°C for 1 hour, this was diluted 10-fold into FLAG-

binding buffer (150 mM NaCl/50 mM Hepes/0.25% w/v Triton X-100, pH 8.3), and the solution was then incubated with FLAG M2 agarose (Sigma) for 1 hour at 4°C with rotation. After elution in the same buffer with 10 equivalents of FLAG elution peptide, the eluted fraction was then mixed with an equal volume of 2× Ni-NTA-binding buffer [1× is 6 M guanidinium chloride/0.5 M NaCl/100 mM sodium phosphate/10 mM Tris(hydroxymethyl)amino methane/10 mM 2-mercaptoethanol/0.25% (wt/vol) Triton X-100, pH 8.0] and then incubated with Ni-NTA agarose (Qiagen) for 1 hour at 4°C with rotation. The Ni-NTA agarose was then washed with Ni-NTA first wash buffer [8 M urea/0.5 M NaCl/100 mM sodium phosphate/10 mM Tris(hydroxymethyl)amino methane/10 mM 2-mercaptoethanol/0.25% (wt/vol) Triton X-100, pH 6.3]; the protein was then eluted with Ni-NTA-binding buffer with an additional 250 mM imidazole. This sample was then dialyzed into SA-binding buffer.

This sample was then diluted into SA binding buffer to give 100 pM with a range of concentrations of SA spanning 10 pM to 10 μM with an additional no-SA control. After incubation at 4°C for 1 hour, these samples were transferred to a Reacti-Bind Streptavidin High Binding Capacity Coated 96-well Plate (Pierce) and incubated at 4°C for 5 minutes. The supernatant was then counted in a scintillation counter. This experiment was repeated in duplicate and gave a K_D of 2.4 nM each time, with 22 and 24% of the counts being bindable in each instance. All binding was inhibited in the presence of either 10 μM SA or 1 mM biotin.

References

1. Cho, G., Keefe, A. D., Wilson, D. S., Liu, R. & Szostak, J. W. (2000) *J. Mol. Biol.* **297,** 309-319.

2. Liu, R., Barrick, J. E., Szostak, J. W. & Roberts, R. W. (2000) *Methods Enzymol.* **318,** 268-293.

3. Schmidt, T. G. M., Koepke, J., Frank, R. & Skerra, A. (1996) *J. Mol. Biol.* **255,** 753-766.

4. Kurz, M., Gu, K. & Lohse, P. A. (2000) *Nucleic Acids Res*. **28,** E83.

5. Green, N. M. (1970) *Methods Enzymol.* **18**, 418-424.