Supplemental information for:

Gp10 based-thioetherification (10BASE$_d$-T) on a displaying library peptide of bacteriophage T7

Keisuke Fukunaga,[a] Takaaki Hatanaka,[b] Yuji Ito[b] and Masumi Taki*[a]

[a] Department of Engineering Science, Bioscience and Technology Program, The Graduate School of Informatics and Engineering, The University of Electro-Communications (UEC), 1-5-1 Chofugaoka, Chofu, Tokyo 182-8585, Japan.

[b] Department of Chemistry and Bioscience, Graduate School of Science and Engineering, Kagoshima University, 1-21-35 Korimoto, Kagoshima, Kagoshima 890-0065, Japan.

Tel: +81-42-442-5980; E-mail: taki@pc.uec.ac.jp (M.T.)

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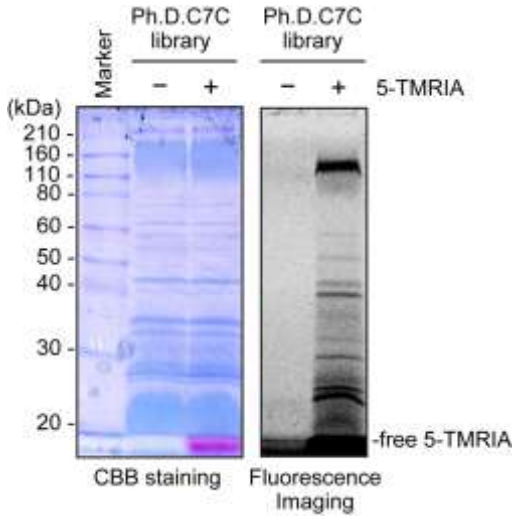

Fig. S1 Chemical modification against M13-displayed library peptide. 5-TMRIA was used for the modification. Experimental standard condition is the same as the 10BASE$_d$-T (see "Chemical modification of T7-displayed peptide via the 10BASE$_d$-T" in the materials and methods). Ph.D. -C7C phage display peptide library was purchased from New England Biolabs (Cat. No. E8120S).

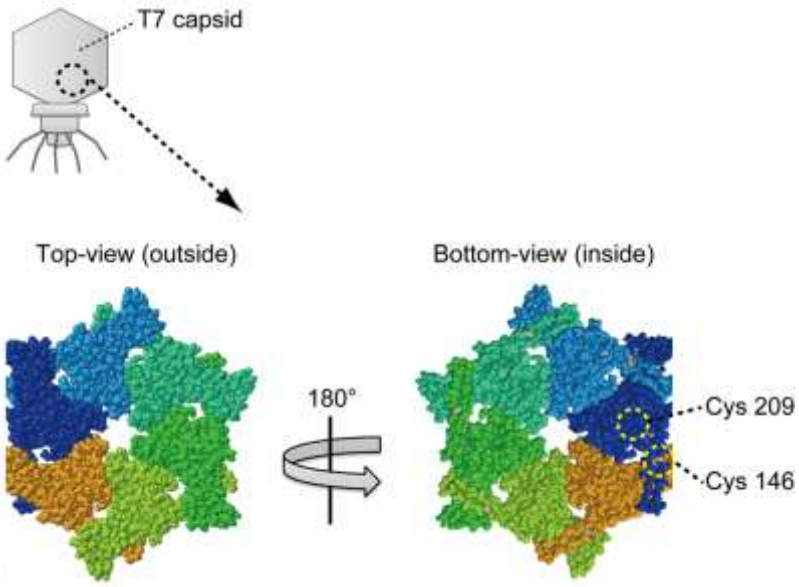

Fig. S2 A structural model of hexametric gp10 (RCSB Protein Data Bank ID: 2XVR) revealed that the intrinsic thiol groups of the gp10 (Cys146 and Cys209) are buried inside of the mature capsid.[1] Each color represents the gp10 monomer. The structures were generated by RasMol software (http://rasmol.org/).

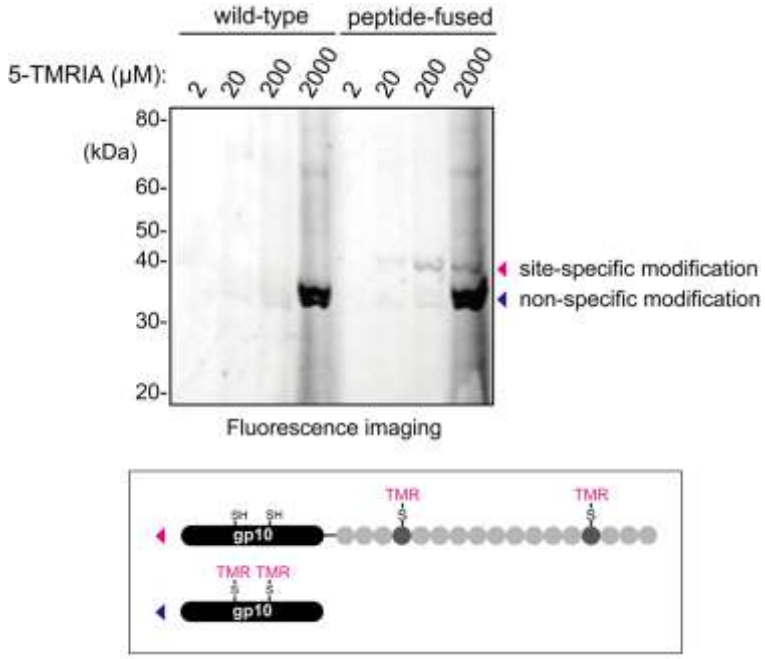

Fig. S3 Optimization of 5-TMRIA concentration. The T7 phage clones (i.e., without and with displaying peptide on gp10) were modified via the 10BASE$_d$-T with various molar concentrations of 5-TMRIA under the standard condition (described in Materials and Methods). Equal amounts of phage proteins were subjected to SDS-PAGE followed by fluorescence imaging. TMR represents tetramethylrhodamine.

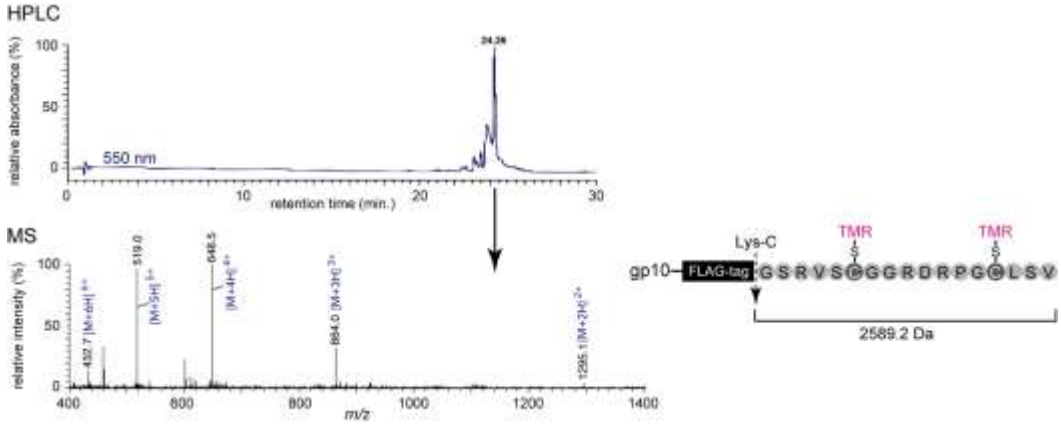

Fig. S4 LC-MS analysis of lysyl endopeptidase-digested gp10 fusion. Upper panel: HPLC chromatogram of the digested gp10 fusion. The 550 nm absorption corresponding to TMR was monitored. Lower panel: MS spectrum of the corresponding peak at 24.26 min. A series of multiple charged ions were detected (experimental *m/z* = 1295.1, 864.0, 648.5, 519.0, and 432.7), and consistent with theoretical *m/z* values of the TMR-conjugated peptide (calculated *m/z* = 1295.6, 864.1, 648.3, 518.8, and 432.5). Lys-C represents lysyl endopeptidase.

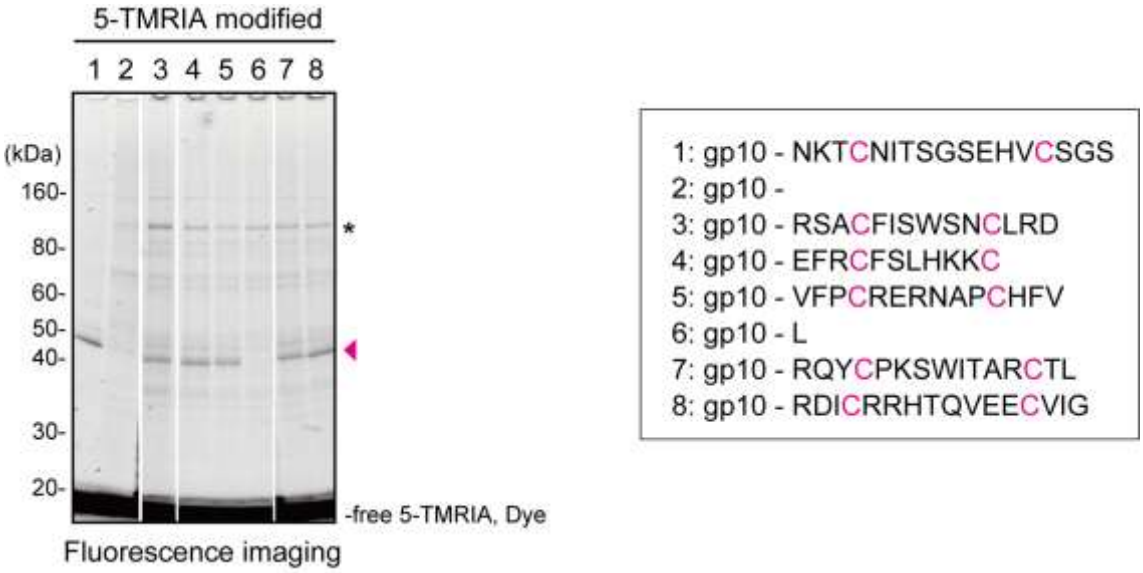

Fig. S5 Introduction of tetramethylrhodamine (TMR) into different peptide-fused gp10. Amino acid sequences of the peptides are shown in right panel. A triangle and an asterisk (*) represent the TMR-conjugated gp10 and contaminated protein derived from *E. coli*, respectively. Two clones lacking Cys in the T7-displayed peptide were not modified (clones 2 and 6). In case of these clones, the T7-displayed peptides were truncated by the appearance of a stop codon before the designated Cys that was supposed to be translated.

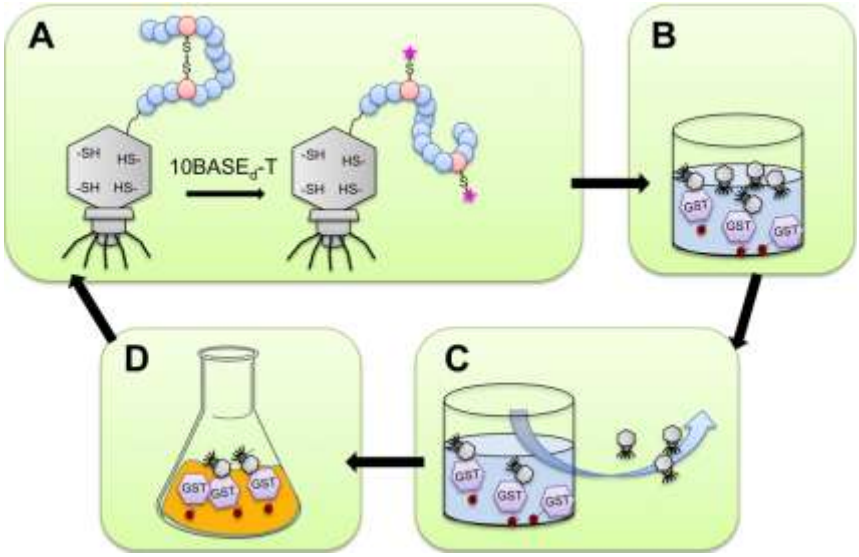

Fig. S6 Schematic diagram of biopanning used in this study. (A) Construction of TMR-conjugated peptide library on T7 phage through the 10BASE$_d$-T. (B) Incubation of the phage display peptide library with immobilized glutathione *S*-transferase (GST). (C) Washing of unbound phage. (D) Amplification of the GST-bound phage for subsequent rounds of biopanning.

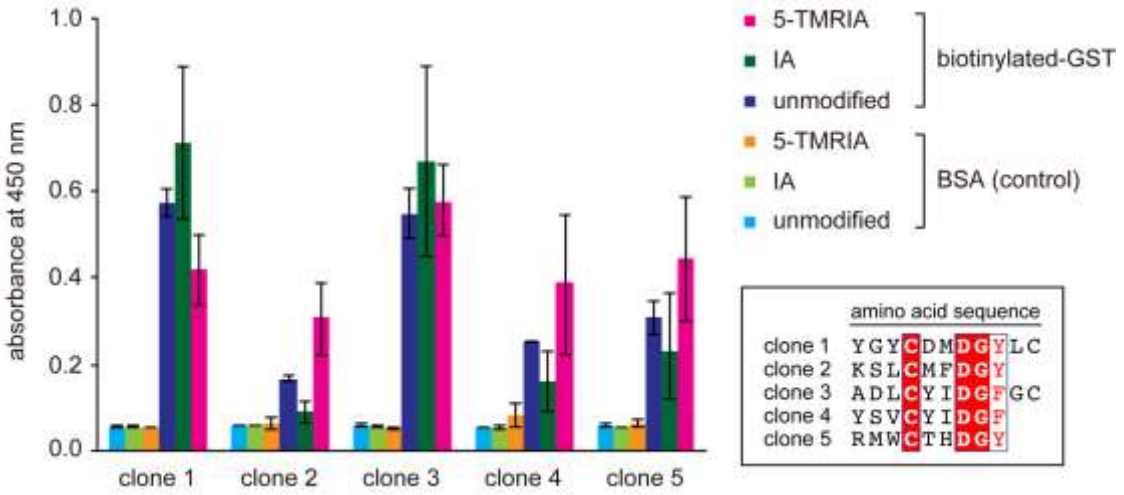

Fig. S7 ELISA of T7 phage clones randomly chosen from phage pool after 5 rounds of biopanning. Peptide sequences displayed on the phage are shown in right panel (clones 1 to 5). The graph summarizes the results of three independent experiments. Error bars represent standard deviations. IA and BSA represent iodoacetamide and bovine serum albumin, respectively. The discrepancy between ambiguous ELISA results in here (clone 5; unmodified vs. 5-TMRIA) and clear-cut FP results in Fig. 3C and S9 can be explained as follows. In phage-based ELISA assay, multivalent peptides were attached on a single phage particle. Thus, avidity effects should not be ignored. A positive signal in ELISA could be obtained even if the monomeric peptide was a weak binder to GST. In this case, ELISA results could not reflect true dissociation constant of the peptide.

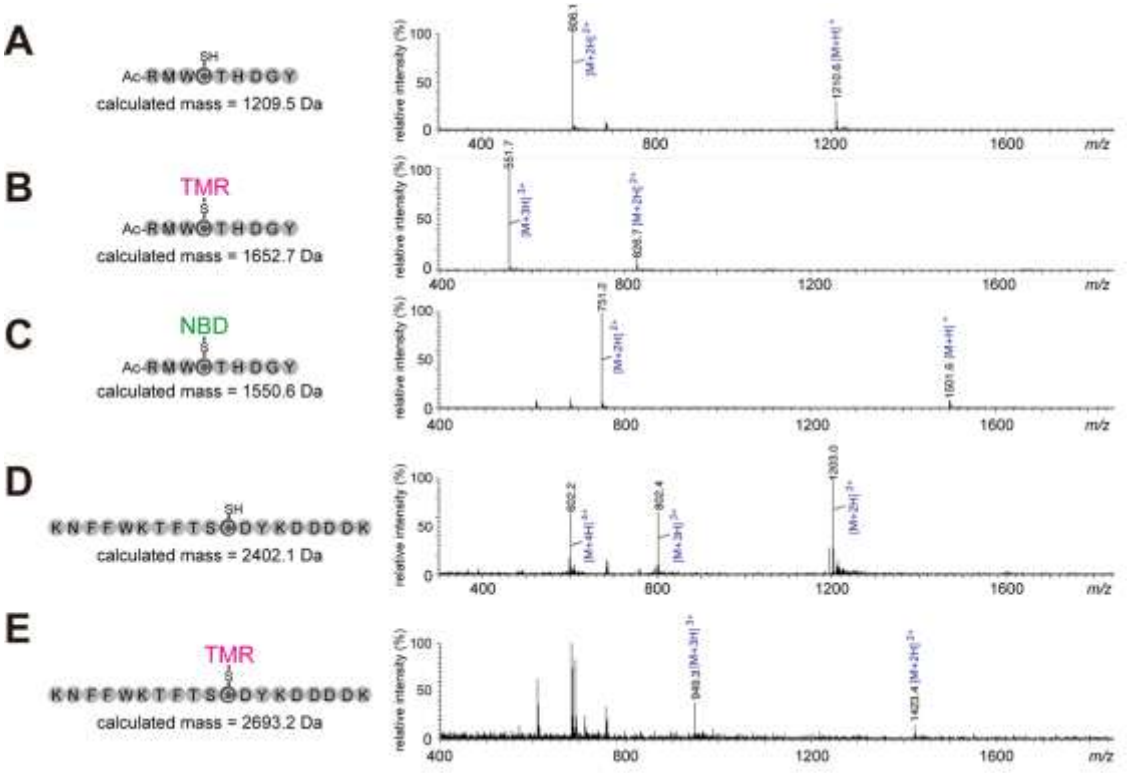

Fig. S8 LC-MS analysis of chemically synthesized peptides. (A) unmodified Ac-RMWCTHDGY peptide (equivalent to the T7 phage clone 5 in Fig. 3B and S7). (B) TMR-conjugated peptide. (C) 7-nitrobenzofurazan (NBD)-conjugated peptide. (D) unmodified unrelated peptide.[7] (E) TMR-conjugated unrelated peptide.

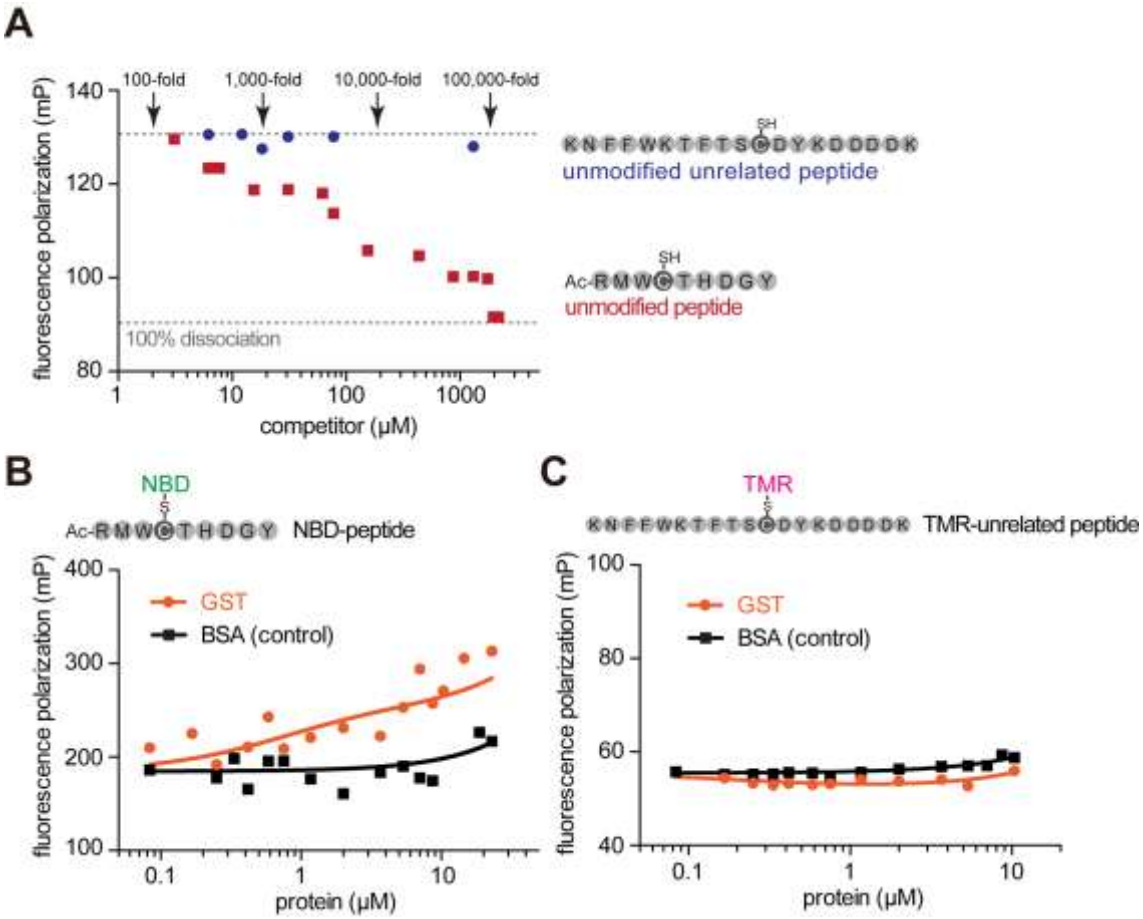

Fig. S9 Fluorescence polarization assay by using negative control peptides. (A) Competitive binding assay. Unmodified peptides were mixed with TMR-conjugated peptide-GST complex in the presence of dithiothreitol (2 mM) in phosphate-buffered saline. The molar ratio of the competitor to TMR-conjugated peptide is represented in the upper part. (B) Binding of NBD-conjugated peptide with GST or BSA. When the TMR moiety was substituted to NBD moiety, the GST-specific binding of the peptide was drastically prevented. (C) Binding of TMR-conjugated unrelated peptide. The GST specific binding of the TMR-conjugate never occurred because the peptide sequence was totally different from that of the GST-binding peptide.

Materials and Methods

1. General

All experiments were performed with commercially available reagents and kits. Note that no special materials and skills are needed. Contrary to popular belief and T7Select system manual (Merck Millipore), CsCl step gradient and ultracentrifugation are not necessary to perform all of the experiments to obtain target-specific binder.[2] For purification of T7 phage at every step by polyethyleneglycol / NaCl precipitation, we only used conventional centrifugation system which can rotate at 13,000 rpm.

2. Construction of T7 phage display library

A. For the identification (used in Fig. 1 and 2):

FLAG-tagged T7 phage peptide library (-S-G-G-G-G-FLAG-G-S-X_3-C-X_7-C-X_3; where X represents any randomized amino acid) was constructed with T7Select10-3b (used in Figures 1A and 1D) or T7Select415-1b (used in Figure 1B) system according to the kit instructions (Merck Millipore). In brief, an oligonucleotide was synthesized in the following format: 5'-GGTGGAGGTGGCGACTACAAGGATGACGATGACAAGGGATCA $(NNK)_3TGC(NNK)_7TGT(NNK)_3$TGAAAGCTTGGA-3'. The oligonucleotide was amplified by polymerase chain reaction (PCR) using an appropriate set of primers harboring restriction sites for *Eco*RI and *Hind*III, respectively. The PCR products were purified, and then digested with *Eco*RI and *Hind*III. The DNA fragments were further purified and ligated to the vectors. The ligation products were subjected to *in vitro* packaging with T7 packaging extracts followed by infection into *Escherichia coli* BLT5403 cells. Noted that an amplification of T7 phage carrying a T7Select415-1b high-copy vector by using BLT5403 cells causes medium-high-copied peptide display (200 peptides per virion).[4]

B. For the selection (used in Fig. 3):

One of the T7 phage display peptide library (-S-G-G-G-X_3-C-X_7-C-X_3) was constructed in a previous study.[3] Other T7 phage display peptide libraries

(-S-G-G-G-X_3-C-$X_{5/6}$-C-X_3) were independently constructed in the same manner of section 2-A. They were mixed altogether and used for the 10BASE$_d$-T followed by selection against GST.

Note: In both cases of **A** and **B** (above), quality and complexity of the phage display peptide libraries were checked by DNA sequence analysis of PCR-amplified DNA fragments from randomly chosen phage clones. For the DNA amplification, following oligonucleotides were used: 5'-CGCTAAGTACGCAATGGGCC-3' (forward primer), 5'-GTCTCAACGTTCATATCGTATGAGCG-3' (reverse primer).

3. Chemical modification of T7-displayed peptide via the 10BASE$_d$-T

Procedures of the standard reaction condition of the 10BASE$_d$-T are the following. The reaction was carried out in 1.5 mL microcentrifuge tubes. T7 phage particles (approximately 1.0×10^{11} plaque-forming units) were suspended in 700 μL of phosphate-buffered saline (PBS, Nacalai, #14249-95) supplemented with 400 mM NaCl, and well dissolved by sonication (vortex is also available). At this moment, the solution contains 1.0×10^{12} (T7 phage made by T7Select10-3b system) or 2.0×10^{13} (by T7Select415-1b system) molecules of the T7-displyed peptides. After centrifugation at 12,000 rpm for 5 minutes at room temperature, the supernatant was mixed with neutralized tris(2-carboxyethyl)phosphine (TCEP-NaOH; final concentration of 500 μM) at 4 °C. At this time, the TCEP stock aqueous solution of pH 7 was prepared by neutralization with NaOHaq in advance. Tetramethylrhodamine-5-iodoacetamide stock solution (5-TMRIA; Molecular Probes, cat. No. T-6006) was added at a final concentration of 200 μM, and the mixture was incubated at 4 °C for 3 hours in the dark with shaking. 5-TMRIA was dissolved in dimethyl sulfoxide/water (1:1 by volume) in advance, to prepare the stock solution. To inactivate the unreacted 5-TMRIA, 2-mercaptoethanol was added to the mixture at a final concentration of 5 mM, and further incubated at 4 °C for several minutes. The T7 phage particles were precipitated with a mixture of polyethylene glycol 6000 and sodium chloride to final concentrations of 5% (w/v) and 0.5 M, respectively. After centrifugation, the precipitate was dissolved

in an appreciate buffer.

4. In-gel fluorescence imaging and Western blot analysis

T7 phage particles were dissolved in 1 × sample buffer (62.5 mM tris(hydroxymethyl)aminomethane-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.002% bromophenol blue). The solution was incubated at 95 °C for 5 min, and then subjected to SDS-PAGE. Proteins were resolved by a 10% or a 10-20% gradient SDS–polyacrylamide gel. After the electrophoresis, the TMR-conjugated proteins were visualized by in-gel fluorescence imaging using an imager (FMBIO III-SC01, Hitachi, Japan) as reported previously.[5] A band-pass filter (555 BP20) was used for the detection. A conventional gel imager excited by UV light, such as Gel Doc XRS+ (Bio-Rad), can also take the image.

Western blot analysis was performed as described previously.[6] In brief, proteins in the gel were transferred onto a polyvinylidene difuoride (PVDF) membrane (Bio-Rad), and then the membrane was subjected to blocking with bovine serum albumin. The blots were incubated with anti-FLAG M2 mouse monoclonal antibody (1/1,000 dilution; Sigma Aldrich), followed by incubation with anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (1/5,000 dilution Cell Signaling Technology). After several washes, the blots were incubated with ECL plus reagent (GE Healthcare Life Sciences), and detected using a Gel Doc XRS+. Image contrast and brightness were adjusted in Photoshop CS4 (Adobe).

5. Site-specific cleavage of peptide-fused gp10 by enterokinase

Site-specific cleavage of peptide-fused gp10 was performed as previously described.[7] In brief, non-denatured T7 phage particles were dissolved in a reaction buffer (20 mM tris(hydroxymethyl)aminomethane-HCl, pH 7.4, 50 mM NaCl, 2 mM $CaCl_2$) in the presence or absence of 0.1 units of recombinant enterokinase (Merck Millipore), and incubated at 37 °C for 16 hours. The reaction was stopped by an addition of 4 × sample buffer.

6. Mass spectrometric analysis

For mass spectrometric analysis, a PAGE gel was stained with Rapid Stain CBB kit (Nacalai, Japan). The stained protein bands were excised from the gel. Proteins in the gel were reduced with 25 mM dithiothreitol at 65 °C for 10 min, and then alkylated with 55 mM iodoacetamide at room temperature for 60 min in the dark. Digestion was carried out with modified trypsin (Promega, Madison, WI) or lysyl endopeptidase (Wako, Japan) at 37 °C overnight. The resulting peptides were analyzed using Agilent 1100 HPLC system (Agilent Technologies) equipped with a C_{18} reverse-phase column (Hypersil GOLD, 2.1 × 100 mm, Thermo Fisher Scientific) connected to LCQ-Fleet mass spectrometer. The peptides were separated using a 0-50% gradient of acetonitrile containing 0.1% formic acid during 40 min at a flow rate of 300 μL per a minute, and then eluted peptides were directly sprayed into the mass spectrometer. The mass spectrometer was operated in the data-dependent mode and externally calibrated. Survey MS scans were acquired in the 400-2000 *m/z* range. Multiply charged ions of high intensity per scan were fragmented with CID in the ion trap. A dynamic exclusion window was applied within 30 seconds. All tandem mass spectra were collected using normalized collision energy of 35%. Data were acquired and analyzed with Xcalibur software (Thermo Fisher).

7. GST expression and biotinylation followed by biopanning

For preparation of GST protein, pGEX-4T-3 vector (GE Healthcare) was introduced into *E. coli* BL21 (DE3) strain. Transformants were precultured overnight at 37°C in 10 mL of LB medium supplemented with 100 μg/mL ampicillin, and then transferred to 200 mL of the fresh medium. After incubation for 2 hours at 37 °C, isopropyl β-D-1-thiogalactopyranoside was added at a final concentration of 0.2 mM, and the cells were further cultured for 2 hours. The cells were harvested, and suspended in B-PER protein extraction reagents (Thermo Scientific) supplemented with lysozyme. After incubation for 1 hour at 4 °C, the sample was cleared by centrifugation at 20,000 × *g* (13,000 rpm) for 10 min at 4 °C. The supernatant was incubated with glutathione sepharose 4B (GE Healthcare). After several washing with tris-buffered saline

supplemented with 0.5 % Triton X-100, GST protein was eluted with glutathione elution buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-KOH, pH 7.4, 100 mM NaCl, 1 mM dithiothreitol, and 50 mM reduced glutathione). The eluted protein was desalted by ultrafiltration, and then subjected to biotinylation with Biotin Labeling Kit-NH_2 (DOJINDO, Japan) followed by purification. The biotinylation of the GST was confirmed by Western blotting. Purity of the biotinlylated-GST was estimated to be above 95%. 20 pmol of the biotinlylated-GST was immobilized on streptavidin-coupled Dynabeads (Invitrogen).

For biopanning, approximately 8.4×10^{10} pfu of *mixed* T7Select10 libraries (-S-G-G-G-X_3-C-$X_{5\text{-}7}$-C-X_3; X represents any randomized amino acid) were modified via the 10BASE$_d$-T. To eliminate T7 phage bound to Dynabeads itself, the modified T7 libraries were dissolved in a selection buffer (PBS supplemented with 1% Triton X-100 and 1% bovine serum albumin), and incubated with Dynabeads for 2 hours at 4 °C. Then, the supernatant was further incubated with the biotinylated-GST immobilized Dynabeads for 2 hours at 4 °C. The beads were washed three times with 200 μL of the selection buffer, and the GST-bound phage was directly infected and amplified with *E. coli* BLT5403 cells. Stringent conditions were stepwisely applied to each round by shortening the binding time and by increasing the washing frequency. After 5 rounds of biopanning, randomly chosen T7 phage clones were subjected to DNA sequencing. Overview of the biopanning in this study is shown in figure S6.

8. Enzyme-linked immunosorbent assay (ELISA)

830 pmol of streptavidin (Promega) was dissolved in PBS, and immobilized on each well of a 96 well immunoplate (Nunc MaxiSorp, Thermo Scientific). After washing with PBS, surface of the wells were coated with 1% (w/v) BSA in PBS supplemented with 0.05% Tween-20 at 4°C overnight. 5 pmol of the biotinylated-GST was immobilized on it, and unbound protein was washed by the same buffer. Approximately 2.0×10^{10} pfu of the T7 phage was dissolved in tris-buffered saline supplemented with 0.5% Triton X-100, and applied to the well plate. The plate was incubated for 1 hour at 25 °C with shaking by using a maximizer (MBR-022UP, Taitec,

Japan), and then washed three times with tris-buffered saline supplemented with 0.5% Triton X-100. The bound phage was incubated with T7 tail fiber monoclonal antibody (1:5,000 dilution, Merck Millipore) and anti-mouse IgG HRP-linked antibody (1:5,000 dilution, Cell Signaling) for 1 hour at 25 °C with shaking. After washing, *o*-phenylenediamine dihydrochloride substrate (SigmaFast OPD, Sigma Aldrich) was added, and the absorbance was quantified using a microplate reader equipped with a 450 nm band-pass filter (Bio-Rad).

9. GST-binding peptide synthesis and fluorescence polarization assay

A peptide (acetyl-RMWCTHDGY-OH) was synthesized and characterized by HPLC and ESI-TOF-MS by GenScript Corp (USA). A mock peptide (H_2N-KNFFWKTFTSCDYKDDDDK-OH) was synthesized and used in a previous study.[7] Purity of the peptide was estimated to be above 95%. For the conjugation of tetramethylrhodamine (TMR) or nitrobenzofurazan (NBD), the alkylating reagent and neutralized TCEP (pH 7) were mixed with each peptide (100 μM) in a phosphate buffer (20 mM phosphate-KOH, pH 7.4) at final concentrations of 150 μM and 500 μM, respectively. NBD-iodoacetamide (cat. No. D-2004) was purchased from Molecular Probes. The mixture was incubated overnight at 37 °C in the dark with shaking. After addition of formic acid at a final concentration of 1%, each peptide was purified with reverse-phase HPLC (Shimadzu, Japan) equipped with XTerra Prep MS C_{18} column (10 × 50 mm, Waters). The peptide was separated using a 0-100% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid during 12 min at a flow rate of 4 ml per a minute. Fractions containing the fluorophore-conjugated peptide were lyophilized and then dissolved in dimethyl sulfoxide. Characterization of the peptides was performed by LC-MS (see Figure S8). Purity was estimated to be above 90%.

Fluorescence polarization was measured with a HYBRID-3000ES (Photoscience, Japan) equipped with appropriate filters (TMR: Ex. 535/540 nm and Em. 570 nm, NBD: Ex. 480 nm and Em. 530 nm). The fluorophore-conjugated peptide (4 pmol, 20 nM) was incubated with various concentrations of GST or BSA in phosphate-buffered saline at 30°C. Klotz plot was generated by GraphPad Prism

software 6.0 (GraphPad Software, San Diego, CA), and the sigmoid curve was fitted with non-linear least squares analysis to obtain dissociation constant.

Supplemental References

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