Early Endosomal Escape of a Cyclic Cell-Penetrating Peptide Enables Effective Cytosolic Cargo Delivery

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Supplementary Information

Supplementary Materials. Rink resin LS (100-200 mesh, 0.2 mmol/g) was purchased from Advanced ChemTech. LC-SMCC (succinimidy-4-[N-maleimidomethyl] cyclohexane-1-carboxy-[6-amidocaproate]) was purchased from Thermo Scientific (Rockford, IL), while 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho(1′-rac-glycerol) (sodium salt) (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), sphingomyelin (Brain, Porcine), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Heparan sulfate (HO-03103, Lot#HO-10697) was obtained from Celcus Laboratories (Cincinnati, OH).

Peptide Synthesis. Peptides were synthesized on Rink Resin LS (0.2 mmol/g) using standard Fmoc chemistry. The typical coupling reaction contained 5 equiv of Fmoc-amino acid, 5 equiv of 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and 10 equiv of diisopropylethylamine (DIPEA) and was allowed to proceed with mixing for 75 min. After the addition of the last (N-terminal) residue, the allyl group on the C-terminal Glu residue was removed by treatment with Pd(PPh₃)₄, phenylsilane (0.1 and 10 equiv, respectively) in anhydrous DCM (3 x 15 min). The N-terminal Fmoc group was removed by treatment with 20% piperidine in DMF and the peptide was cyclized by treatment with benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP)/HOBt/DIPEA (5, 5, and 10 equiv) in DMF for 3 h. The peptides were deprotected and released from the resin by treatment with 82.5:5:5:2.5 (v/v) TFA/thioanisole/water/phenol/ethanedithiol for 2 h. The peptides were triturated with cold ethyl ether (3x) and purified by reversed-phase HPLC on a C₁₈ column. The authenticity of each peptide was confirmed by MALDI-TOF mass spectrometry.

Preparation of cFΦR₄-Conjugated PTP1B. The gene coding for the catalytic domain of PTP1B (amino acids 1-321) was amplified by the polymerase chain reaction using PTP1B cDNA as template and oligonucleotides 5′-gggaattccatatggagatggaaaaggagttcgagcag-3′ and 5′-gggatccgctcgacattgtgtggctccaggattcgtttgg-3′ as primers. The resulting DNA fragment was digested with endonucleases Nde I and Sal I and inserted into prokaryotic vector pET-22b(+)-ybbR. This cloning procedure resulted in the addition of a ybbR tag (VLDSLEFIASKL) to the N-terminus of PTP1B. Expression and purification of the ybbR tagged PTP1B were carried out as previously described.2

Peptide containing a C-terminal lysine (cFΦR₄-Lys, ~10 μmol; Figure S3) was synthesized on the solid phase, deprotected and released from the support, dissolved in degassed DPBS (pH 7.4, 1 mL), and mixed with bifunctional linker LC-SMCC (5 equiv) dissolved in DMSO (0.2 mL). After incubation at room temperature for 2 h, the reaction product cFΦR₄-SMCC (Figure S3) was purified by reversed-phase HPLC equipped with a C₁₈ column. The product was then mixed with coenzyme A (2 equiv) in DPBS and incubated for 2 h. The resulting cFΦR₄-SMCC-CoA adduct was purified again by reversed-phase HPLC. Next, ybbR-tagged PTP1B (30 μM),
cFΦR₄-SMCC-CoA (30 μM), and phosphopantetheinyl transferase Sfp (0.5 μM) were mixed in 50 mM HEPES (pH 7.4), 10 mM MgCl₂ (total volume of 1.5 mL) and incubated at 37 °C for 15 min. The labeled protein (cFΦR₄-PTP1B; Figure S3) was separated from unreacted cFΦR₄-SMCC-CoA by passing the reaction mixture through a PD-10 desalting column eluted with DPBS.

**Image Analysis.** Raw images were uniformly modified using imageJ. Pearson’s correlation coefficient (R) was obtained from endosomal regions using Just Another Colocalization Plugin (JACoP).³ For GR-GFP translocation assay, individual GFP and Hoescht images were loaded into a customized CellProfiler pipeline and colored to grey.⁴ Nuclei were distinguished from the Hoescht image via Otsu automatic three-class thresholding, with pixels of the middle intensity class assigned to background. Clumped objects were identified using Laplacian of Gaussian modeling and separated by shape. The nuclear region was defined as the diameter of the Hoescht objects shrunken by 1 μm, while the cytosolic ring region was defined as the region between the nuclear diameter and the nuclear diameter expanded 2 μm. The translocation ratio was defined as the mean GFP signal inside the nuclear region divided by the mean GFP signal within the cytosolic region measured per cell, and 30-70 cells from 15-30 images were captured for each condition tested.

**Preparation of Small Unilamellar Vesicles (SUVs).** SUVs were prepared by modifying a previously reported procedure.⁵ A proper lipid mixture was dissolved in chloroform in a test tube. The lipid mixture was dried gently by blowing argon over the solution, and kept in a desiccator overnight. The dried lipids were rehydrated in DPBS to final total lipid concentration of 10 mM. The suspension was rigorously mixed by vortexing and sonication on ice until it became clear. A typical preparation yields a homogeneous solution containing vesicles with average diameter of ~80 nm and polydispersity (PdI) index of <0.15 as determined by dynamic light scattering measurements using Zeta Sizer Nano Series (Malvern, Brookhaven, CT). The SUV solution was stored at 4 °C and used for FP experiments on the same day.

**Fluorescence Polarization.** A typical experiment was performed by incubating 100 nM FITC-labeled peptide with varying concentrations of heparan sulfate (0-5,000 nM) in DPBS for 2 h at room temperature. The FP values were measured on a Molecular Devices Spectramax M5 spectrofluorimeter, with excitation and emission wavelengths at 485 and 525 nm, respectively. EC₅₀ were determined by plotting the FP values as a function of heparan sulfate concentrations and fitted to a four-parameter logistic curve with GraphPad PRISM ver.6 software.

To obtain the EC₅₀ value of CPP with lipid membranes, the FP experiment was similarly conducted using 100 nM FITC-labeled peptide with increasing concentrations of SUV solutions (0-10 mM) in DPBS. The FP values were similarly measured, plotted, and analyzed.

**Flow Cytometry.** To estimate the effect of cFΦR₄ on endocytosis, HeLa cells were seeded in six-well plates (5 x 10⁵ cells per well) and allowed to adhere overnight. Following adherence, cells were treated with clear DMEM containing no supplement, 1 μM cFΦR₄ peptide, 100 μM dextran³⁸⁸ (Life Technologies, D-22910), or both 1 μM cyclic peptide and 100 μM dextran³⁸⁸ for 30 min under standard cell culture conditions. The cells were washed with DPBS twice, removed from the plate with 0.25% trypsin (xx min), diluted into clear DMEM containing 10% FBS, pelleted at 300 g for 5 min, washed once with DPBS and resuspended in
200 µL of DPBS. Whole-cell dextran uptake was analyzed on a BD Accuri C6 flow cytometer using the manufacturer FL1 laser and filter set.

**Serum Stability Test.** The stability tests were carried by modifying a previously reported procedure.⁶ Diluted human serum (25%) was centrifuged at 15,000 rpm for 10 min, and the supernatant was collected. A peptide stock solution was diluted into the supernatant to a final concentration of 5 µM for cΦR₄ and Antp and 50 µM for peptides R₉ and Tat and incubated at 37 °C. At various time points (0-6 h), 200-µL aliquots were withdrawn and mixed with 50 µL of 15% trichloroacetic acid and incubated at 4 °C overnight. The final mixture was centrifuged at 15,000 rpm for 10 min in a microcentrifuge, and the supernatant was analyzed by reversed-phase HPLC equipped with a C₁₈ column (Waters). The amount of remaining peptide (%) was determined by integrating the area underneath the peptide peak (monitored at 214 nm) and compared with that of the control reaction (no serum).

**Cytotoxicity Assay.** MTT assays were performed to evaluate cyclic peptide’s cytotoxicity against several mammalian cell lines.⁷ One hundred µL of MCF-7, HEK293, H1299, H1650, A549 (1×10⁵ cells/mL) cells were placed in each well of a 96-well culture plate and allowed to grow overnight. Varying concentrations of the peptide (5 or 50 µM) were added to the each well and the cells were incubated at 37 °C with 5% CO₂ for 24 to 72 h. Ten µL of MTT stock solution was added into each well. Addition of 10 µL of the solution to the growth medium (no cell) was used as a negative control. The plate was incubated at 37 °C for 4 h. Then 100 µL of SDS-HCl solubilizing buffer was added into each well, and the resulting solution was mixed thoroughly. The plate was incubated at 37 °C for another 4 h. The absorbance of the formazan product was measured at 570 nm using a Molecular Devices Spectramax M5 plate reader. Each experiment was performed in triplicates and the cells without any peptide added were treated as control.

| PTP    | cΦR₄-PCP | Tat-PCP | R₉-PCP | Antp-PCP |
|--------|----------|---------|--------|----------|
| PTP1B  | 37100    | 13800   | 14700  | 17400    |
| TCPTP  | 2780     | 560     | 457    | 970      |
| SHP2   | 7400     | 2290    | 248    | 2210     |
| CD45   | 35100    | 21800   | 2940   | 22300    |
| VHR    | 2460     | 1460    | 6240   | 2030     |

⁴₇$^{a}$ $k_{cat}/K_M$ was measured as previously described.²

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Table S2. Kinetic Activities ($k_{cat}/K_M$, $M^{-1} s^{-1}$) of PTP1B and cFΦR₄-PTP1B against pNPP$^a$

| enzyme               | $k_{cat}/K_M$ (M$^{-1} s^{-1}$) |
|----------------------|---------------------------------|
| PTP1B                | 1340                            |
| cFΦR₄-PTP1B          | 1600                            |

$^a$ pNPP = p-nitrophenyl phosphate; $k_{cat}/K_M$ was measured as previously described.$^2$

Figure S1. Structures of some of the peptides used in this study.
Figure S1 cont’d
Figure S2. Scheme showing the synthesis of cFΦR₄-S-S-GFP.

Figure S3. Scheme showing the synthesis of cFΦR₄-PTP1B.
Figure S4. Fluorescence microscopic images of GR-GFP transfected HeLa cells treated with 1 μM cFΦR₄Dex alone or in the presence of different inhibitors. GFP fluorescence was present in both cytoplasm and nucleus of untreated cells (TR = 1.17 ± 0.23). Treatment of cells with 1 μM Dex or cFΦR₄Dex resulted in translocation of GR-GFP into the nucleus. Further treatment with various endocytic inhibitors decreased nuclear translocation of GR-GFP.

Figure S5. Fluorescence microscopic images of HeLa cells co-transfected with GR-GFP and DsRed-Rab5 WT or GR-GFP and DsRed-Rab5Q79L in the presence of 1 μM Dex, cFΦR₄Dex, or TatDex.
**Figure S6.** Effect of cFΦR₄ on the endocytosis of dextran<sup>Alexa488</sup> by HeLa cells. HeLa cells were treated with clear DMEM containing no supplement, 1 μM cFΦR₄ only, 100 μM dextran<sup>Alexa488</sup> only, or both 1 μM cFΦR₄ and 100 μM dextran<sup>Alexa488</sup>. MFI, mean fluorescence intensity.

**Figure S7.** Effect of pH on CAP fluorescence. cFΦR₄-PCP was dephosphorylated by alkaline phosphatase and purified by HPLC and its fluorescence at indicated pH’s was measured.

**Figure S8.** MTT assay of various mammalian cells after treatment with cFΦR₄ (5 or 50 μM) for 48 or 72 h.
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