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Intracellular Delivery of Peptidyl Ligands by Reversible Cyclization: Discovery of a PDZ Domain Inhibitor that Rescues CFTR Activity**

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Abstract

We report a general strategy for intracellular delivery of linear peptidyl ligands by fusing them with a cell-penetrating peptide and cyclizing the fusion peptides through a disulfide bond. The resulting cyclic peptides are cell permeable and have improved proteolytic stability. Once inside the cell, the disulfide bond is reduced to produce linear, biologically active peptides. This strategy was applied to generate a cell-permeable peptide substrate for real-time detection of intracellular caspase activities during apoptosis and a CAL-PDZ domain inhibitor for potential treatment of cystic fibrosis.

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Keywords

Cell-penetrating peptide; cyclic peptide; cystic fibrosis; PDZ domain; protein-protein interaction

The application of peptides as drugs is limited by their proteolytic instability and poor membrane permeability. Cyclization^[1] or stapling^[2] of peptides greatly improves their proteolytic stability. Moreover, it was recently discovered that cyclic peptides containing certain amphipathic sequences (e.g., FΦRRRR, where Φ is L-2-naphthylalanine) efficiently enter mammalian cells through endocytosis.^[3-5] Biologically active cyclic peptides can be delivered into the cytosol and nucleus of mammalian cells by incorporating into them these short sequence motifs.^[3,4,6] However, in many circumstances, binding to a molecular target (e.g., PDZ^[7,8] and BIR domains^[9]) requires that the peptidyl ligand exist in an extended conformation (e.g., β-strand) and cyclization (or stapling) interferes with target binding. Here we report a potentially general strategy for delivering linear peptides into mammalian cells through reversible, disulfide-mediated cyclization. When present in the oxidizing extracellular or endosomal environment, the peptides exist as macrocycles whose rigidified structures enhance their proteolytic stability and cell permeability. Upon entering the cytosol of a cell, the disulfide bond is reduced by the intracellular thiols to produce linear, biologically active peptides^[10,11] (Figure 1).

We recently reported a cyclic peptide, cyclo(FΦRRRRQ) (cFΦR₄), as a novel cell-penetrating peptide (CPP), which binds directly to plasma membrane phospholipids, enters cells via multiple endocytic mechanisms, and efficiently escapes from the early endosome.^[3,4] With a cytosolic/nuclear delivery efficiency 4-12-fold higher than that of nonaarginine (R₉), Tat, and penetratin,^[4,12] cFΦR₄ is one of the most active CPPs known to date. To test the validity of the reversible cyclization strategy, we synthesized peptide HSCH₂CH₂CO-FΦRRRRCK-NH₂ and cyclized it via an intramolecular disulfide bond (Figure S1; Table 1, peptide **1**). A linear control peptide of the same sequence (Figure S1; Table 1, peptide **2**) was also synthesized by replacing the N-terminal 3-mercaptopropionyl group with a butyryl group and the C-terminal cysteine with 2-aminobutyric acid (Abu or U). Both peptides were labeled at the side chain of a C-terminal lysine with fluorescein isothiocyanate (FITC), and their cellular uptake was assessed by live-cell confocal microscopy and flow cytometry. HeLa cells treated with cyclic peptide **1** showed strong, diffuse green fluorescence throughout the entire cell, while rhodamine-labeled dextran (dextran^{Rho}, an endocytosis marker) exhibited only punctate fluorescence in the cytoplasmic region (Figure 2a). The nearly uniform distribution of FITC fluorescence in both cytoplasmic and nuclear regions suggests that the cyclic peptide was efficiently internalized by HeLa cells and like the parent peptide, cFΦR₄, was able to efficiently escape from the endosome. In contrast, cells treated with peptide **2** showed much weaker fluorescence under the same conditions. Quantitation by fluorescence-activated cell sorting (FACS) gave mean fluorescence intensity (MFI) of 27,100, 5530, and 1200 arbitrary units (AU) for cells treated with peptides **1**, **2**, and FITC alone, respectively. We also used a hydrophilic peptide, Asp-Glu-pCAP-Leu-Ile (PCP, where pCAP is phosphocoumaryl aminopropionic acid)^[4], as cargo and attached it to peptides **1** and **2** through a polyethyleneglycol linker (Figure S1). pCAP is non-fluorescent but, upon entry into the cytosol, undergoes rapid

dephosphorylation to generate a fluorescent product, coumaryl aminopropionic acid (CAP).^[13] The pCAP assay therefore provides a quantitative assessment of the cytosolic/nuclear CPP concentration.^[3,4] FACS analysis of HeLa cells treated with 5 μ M peptide **1**-PCP and peptide **2**-PCP gave MFI of 3020 and 700 AU, respectively (Figure S2). Thus, cyclization of F Φ RRRR through a disulfide bond has a similar effect to N-to-C cyclization and increases its cellular internalization efficiency by $\sim$ 5-fold. Disulfide cyclization also greatly enhances the proteolytic stability of the peptide. Incubation of peptide **1** in human serum for 6 h at 37 °C resulted in 48% degradation ($t_{1/2} \sim$ 6 h), whereas under the same conditions peptide **2** was completely degraded within 3 h ($t_{1/2} \sim$ 15 min) (Figure 2b).

To illustrate the utility of the reversible cyclization strategy, we applied it to deliver a caspase substrate into mammalian cells and monitor the intracellular caspase activity in real time.^[14] Although peptidyl coumarins have been used as caspase substrates *in vitro*,^[15] they are impermeable to the cell membrane and not suitable for *in vivo* applications. To generate a cell-permeable caspase substrate, we fused a caspase 3/7 substrate, Ac-Asp-Nle-Abu-Asp-AMC^[16] (Table 1, peptide **3**, where AMC is 7-amino-4-methylcoumarin and Nle is norleucine; Figure S3), with the CPP motif RRRR Φ F (Figure 3a). The fusion peptide was cyclized by appending a 3-mercaptopropionyl group to its N-terminus, replacing the Abu residue with a cysteine, and forming an intramolecular disulfide bond between the thiol groups, to give peptide **4** (Table 1; Figure S3). For comparison, we synthesized an irreversibly cyclized peptide (Table 1, peptide **5**; Figure S3) through the formation of a thioether bond between an N-terminal 4-bromobutyl moiety and the C-terminal cysteine. A linear control peptide of the same sequence was also prepared as described above (Table 1, peptide **6**; Figure S3). Finally, the caspase 3/7 substrate was conjugated to R₉ to produce a positive control peptide (Table 1, peptide **7**; Figure S3). *In vitro* kinetic analysis revealed that conjugation of the caspase 3/7 substrate to RRRR Φ F and R₉ decreased its enzymatic activity by 53% and 72%, respectively (relative to peptide **3**), whereas irreversible cyclization rendered the substrate completely inactive toward caspase 3 (Table S1). The enzymatic activity of caspase 3 toward peptide **4** could not be determined because the caspase assay requires a reducing condition, which would cleave the disulfide bond. Given the structural similarity between peptides **4** and **5**, we expect peptide **4** to be inactive toward caspases but have similar activity to peptide **6** upon reduction of the disulfide bond (Table S1).

Jurkat cells were pretreated with kinase inhibitor staurosporin to induce caspase activities. The cells were then incubated with peptides **3-7** and the amount of AMC released was monitored over time (0-10 h). As expected, the impermeable caspase substrate **3** produced little fluorescence increase over the 10-h period (Figure 3b). Peptide **4** produced the fastest fluorescence increase, reaching 459 fluorescence units (FU) in 10 h, followed by the R₉-conjugated peptide **7** and the linear peptide **6**. The irreversibly cyclized peptide **5**, which is inactive toward caspase 3 *in vitro*, also produced AMC in a time-dependent manner, albeit at a much lower rate (99 FU in 10 h). We attribute this slow AMC release to hydrolysis by other intracellular enzymes. Consistent with this hypothesis, Jurkat cells pretreated with a pan-caspase inhibitor Z-VAD(OMe)-CH₂F (FMK)^[17] followed by peptide **4** released AMC at a rate similar to that of peptide **5** alone. The simplest interpretation of the above data is

that both peptides **4** and **5** entered the cell interior efficiently but only peptide **4** was converted into the linear, active caspase substrate inside the cell.

Many protein-protein interactions (PPIs) are mediated by protein domains binding to short peptides in their extended conformations (e.g., α -helix and β -strand).^[18] For example, the PDZ domain is a globular interaction module containing 80-90 amino acids and found in numerous proteins from bacteria to man.^[7,8,19] PDZ domains recognize specific sequences at the C-termini of their binding partners, and the bound peptide ligands adopt an extended β -strand conformation.^[7,20] It was recently reported that the activity of cystic fibrosis transmembrane conductance regulator (CFTR), a chloride ion channel protein mutated in cystic fibrosis (CF) patients, is negatively regulated by CFTR-associated ligand (CAL) through its PDZ domain (CAL-PDZ).^[21] Inhibition of the CFTR/CAL-PDZ interaction was shown to improve the activity of F508-CFTR, the most common CF-causing mutation,^[22,23] by reducing its lysosomal degradation.^[21,24] Previous library screening and rational design have identified several moderately potent peptidyl inhibitors against the CAL-PDZ domain (K_D values in the high nM to low μ M range).^[24-27] However, these peptidyl inhibitors are membrane impermeable and thus have limited therapeutic potential.

Starting from a previously reported CAL-PDZ domain ligand, WQVTRV,^[25] we designed a disulfide-cyclized peptide by adding the sequence CRRRRF to its N-terminus and replacing the Val residue at the -3 position (relative to the C-terminal Val, which is designated as position 0) with a cysteine (Figure 4a and S4, Table 1 peptide **8**). The tryptophan residue at the -5 position was designed to serve dual functions of PDZ binding and membrane translocation (as a replacement of L-naphthylalanine). A FITC group was added to the N-terminus to facilitate affinity and cellular uptake measurements. Two structurally similar control peptides were also prepared. The linear peptide **9** contains two Abu instead of the Cys residues, whereas peptide **10** has an amidated C-terminus, which should abolish PDZ domain binding (Figure S4).^[8] Fluorescence anisotropy (FA) analysis showed that in the absence of reducing agent, peptide **8** had no detectable binding to the CAL-PDZ domain (Figure 4b). In the presence of 2 mM tris(carboxylethyl)phosphine, however, peptide **8** (i.e., its reduced form) bound to the CAL-PDZ domain with a K_D value of 490 ± 130 nM. As expected, peptide **10** did not bind the PDZ domain under any condition (Figure S5). Peptide **8** was readily cell permeable; incubation of HeLa cells with peptide **8** (5 μ M for 2 h) resulted in intense and diffuse fluorescence throughout the entire cell (Figure 4c). FACS analysis showed that peptide **10** entered cells with similar efficiency as peptide **8**, whereas peptide **9** had approximately 4-fold lower cell uptake efficiency (Figure S5). Peptide **8** had superior serum stability over a control peptide, in which the PDZ ligand was attached to the Gln side chain of cF Φ R₄ (Figure S6, peptide **11**).

Peptide **8** was tested for its ability to rescue the chloride ion transport activity of F508-CFTR was assessed by a 6-methoxy-*N*-(3-sulfopropyl)-quinolinium (SPQ) assay on human bronchial epithelial CFBE41o- (CFBE) cells, which are homozygous for the F508-CFTR mutation.^[28] Briefly, CFBE cells were first treated with 20 μ M small-molecule CFTR corrector VX809^[29] for 24 h, which enhances the folding of mutant CFTR protein, and then incubated for 2 h with 50 μ M peptide **8**. The cells were next loaded with the halide-sensitive SPQ fluorophore using hypotonic shock, washed with sodium iodide containing

fluorescence quenching buffer, and incubated with a dequenching isotonic sodium nitrate buffer. Pharmacological activation of CFTR with forskolin and genistein resulted in iodide efflux which correlated with increased SPQ fluorescence. A negative control experiment in the presence of the CFTR inhibitor GlyH101 was performed to determine the amount of non-CFTR-mediated iodide efflux. A combination of VX809 and peptide **8** increased the activity of the mutant CFTR by 77% (Figure 4d). In contrast, VX809 alone, VX809 in combination with linear peptide **9**, or VX809 in combination with the C-terminally amidated peptide **10** resulted in no significant (27%) increase in the CFTR activity (Figure 4d and S7). Treatment of CFBE cells with another small-molecule corrector, Corr-4a^[30] (20 μ M), and 50 μ M peptide **8** also improved the ion transport activity of F508-CFTR, albeit less effectively (Figure S8). Immunofluorescence staining with an anti-CFTR antibody showed that while wild-type CFTR is distributed throughout the cytoplasm of bronchial epithelial (16HBE) cells, F508-CFTR is localized predominantly in the perinuclear region in ~47% of the untreated CFBE cells or CFBE cells treated with Corr-4a only (Figure 4e and S8). Co-treatment of the CFBE cells with Corr-4a and peptide **8** (but not peptide **10**) significantly increased the population of CFBE cells with wild-type cell-like CFTR distribution (from 53% to 67%). Taken together, our data suggest that peptide **8** inhibited the CFTR/CAL-PDZ interaction, reduced lysosome-mediated degradation of the F508-CFTR protein, and further increased the ion transport activity of the mutant CFTR in the presence of small-molecule correctors.

In summary, we have demonstrated that incorporation of short amphipathic CPP motifs coupled with disulfide-mediated cyclization provides a novel approach for intracellular delivery of linear peptidyl cargos into mammalian cells. Cyclization of the bifunctional peptides confers both improved delivery efficiency and proteolytic stability, while the reversibility of the disulfide bond permits the release of functional linear peptides only inside the cell.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic representation of the reversible cyclization strategy for delivering peptidyl cargos into mammalian cells. GSH, glutathione.

Figure 2.

Comparison of the cellular uptake efficiency and proteolytic stability of disulfide-cyclized (1) and linear CPPs (2). a) Live-cell confocal microscopic images of HeLa cells after treatment for 1 h with 5 μM FITC-labeled peptide 1 or 2, Dextran^{Rho} (0.5 mg mL^{-1}), and nuclear staining dye DRAQ5. All images were recorded in the same Z-section. b) Plot of the percentage of remaining peptide 1 or 2 (relative to time 0) as a function of incubation time in human serum (37 °C). Data reported represent the mean $\pm$ SD from three independent experiments.

Figure 3.

a) Structure of disulfide-cyclized caspase substrate **4**. b) Time-dependent release of AMC by Jurkat cells treated with peptides **3-7** (5 μM) in the absence and presence of caspase inhibitor FMK (100 μM). Data reported represent the mean ± SD from three independent experiments.

Figure 4.

a) Structure of FITC-labelled peptide **8**. b) Binding of FITC-labelled peptide **8** to CAL-PDZ domain in the presence or absence of reducing reagent as analysed by fluorescence anisotropy. Data reported represent the mean $\pm$ SD from three independent experiments. c) Live-cell confocal microscopic images of HeLa cells treated with FITC-labelled peptide **8** (5 μ M) and nuclear stain DRAQ5 in the same Z-section. d) Rescue of F508-CFTR activity in CFBE cells by treatment with VX809 (20 μ M) in combination with peptide **8**, **9**, or **10** (50 μ M) as monitored by the SPQ assay. Fluorescence increase in the y axis reflects the ion channel activity of the F508-CFTR protein. Asterisk (**) indicates $p < 0.01$ relative to control (no VX809 or peptide), two-tailed t-test ($n = 4$). e) Immunofluorescence staining of CFTR protein in wild-type human bronchial epithelial (16HBE) cells (I) or CFBE cells before (II) and after treatment with 20 μ M Corr-4a and 50 μ M peptide **8** (III).

Table 1Sequences of peptides in this work.^[a]

Peptide ID	Sequence
1	$\begin{array}{c} \text{S} \text{---} \text{S} \\ \quad \\ \text{CH}_2\text{CH}_2\text{CO-F}\Phi\text{RRRRCK(FITC)-NH}_2 \end{array}$
2	CH ₃ CH ₂ CH ₂ CO-FΦRRRRUK(FITC)-NH ₂
3	CH ₃ CO-DMUD-AMC
4	$\begin{array}{c} \text{S} \text{---} \text{S} \\ \quad \\ \text{CH}_2\text{CH}_2\text{CO-RRRR}\Phi\text{FDMCD-AMC} \end{array}$
5	$\begin{array}{c} \text{S} \\ \\ \text{CH}_2\text{CH}_2\text{CO-RRRR}\Phi\text{FDMCD-AMC} \end{array}$
6	CH ₃ CH ₂ CH ₂ CO-RRRRΦFDMUD-AMC
7	CH ₃ CO-RRRRRRRRRDMUD-AMC
8	$\begin{array}{c} \text{S} \text{---} \text{S} \\ \quad \\ \text{FITC-CRRRRFWQCTRV-OH} \end{array}$
9	FITC-URRRRFWQUTRV-OH
10	$\begin{array}{c} \text{S} \text{---} \text{S} \\ \quad \\ \text{FITC-CRRRRFWQCTRV-NH}_2 \end{array}$

^[a] AMC, 7-amino-4-methylcoumarin; FITC, fluorescein isothiocyanate; Φ, L-2-naphthylalanine; M, norleucine; U, 2-aminobutyric acid