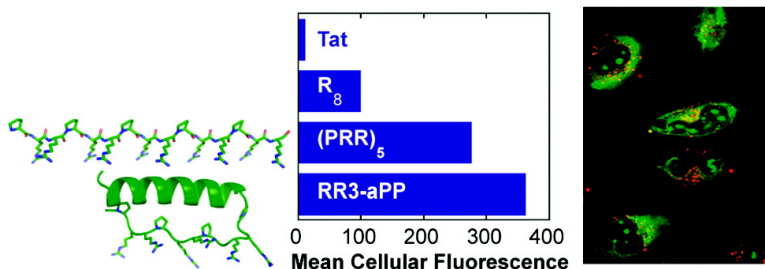


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Intrinsically Cell-Permeable Miniature Proteins Based on a Minimal Cationic PPII Motif

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The characterization or alteration of intracellular processes requires the delivery of exogenous agents to the interior of cells. Cell-penetrating peptides (CPPs) have emerged as promising tools for the cellular delivery of molecular cargos ranging in size from small molecules and peptides to proteins and quantum dots.^{1,2} While Ryser and Hancock discovered over 40 years ago that histones and polycationic peptides permeate eukaryotic cells,³ the demonstration that the HIV-1 protein Tat could cross the cell membrane to activate transcription^{4,5} revitalized interest in CPPs as transduction vectors. It is now well-established that proteins presenting cationic surface residues, either by nature^{3–8} or engineering,^{9,10} can permeate eukaryotic cells. Arginine-rich sequences are particularly effective, with short oligoarginine peptides currently providing the most efficient encodable CPPs.¹¹ Although several α -helical CPPs have been reported,¹² oligoarginines and the Tat peptide are unstructured, and stable helicity is considered to impede cellular uptake.¹³ Here we expand the repertoire of cell-penetrating motifs by designing encodable CPPs possessing type-II polyproline (PPII) helical structure that surpass the uptake efficiency of previous CPPs and can be embedded within the primary sequence of a small structured protein.

The marriage of arginine- and proline-rich sequences for the generation of structured CPPs is appealing for several reasons. PPII helices are stable in short, isolated sequence motifs.^{14–16} Moreover, due to its large 3.1 Å rise per residue, the PPII helix readily tolerates charged side chains on successive turns.¹⁵ In fact, arginine has high PPII propensity in host–guest studies,^{14,16} facilitating the design of PPII helices possessing one or more cationic faces. In addition, natural proline-rich sequences such as Bactenecin-7¹⁷ (Bac) and γ -zein¹⁸ (SAP) permeate cells, albeit with low efficiency, providing some precedent for the feasibility of PPII-based CPPs. Although non-natural amino acid modifications can substantially increase the uptake of PPII-based CPPs,^{19–21} these derivatives are not readily DNA-encodable.

To establish design rules for successfully wedding the uptake capacity of oligoarginines to the structural propensity of oligoprolines, we synthesized two series of cationic PPII helices. Exploiting the periodicity (3.0 residues per turn) of the left-handed PPII helix, these minimalist repeats of (PPR)_n and (PRR)_n create aligned faces of proline or arginine residues. All peptides were labeled at the N-terminus with fluorescein, and their uptake into live HeLa cells was evaluated by flow cytometry (Figure 1A). Neither the previously reported proline-rich sequences, SAP and Bac, nor members of the proline-rich (PPR)_n series showed significant uptake after 1 h at 1 μ M. (PPR)₃ and (PPR)₄ are slightly more cell-permeable under these conditions, performing as well as Tat. In contrast, (PPR)₅ and (PPR)₆ are extraordinarily cell-permeable, matching or surpassing the levels observed for simple oligoarginines R₈, R₁₀,

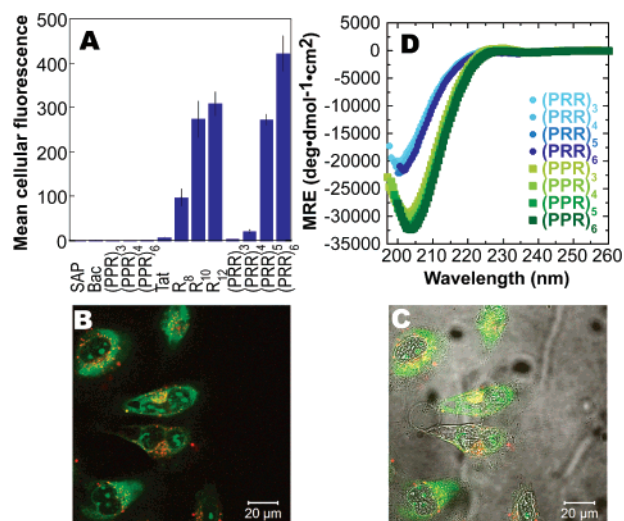


Figure 1. Encodable, proline-rich cell-penetrating peptides. (A) HeLa cell uptake of 1 μ M fluorescein-labeled peptides after 1 h quantified by flow cytometry. Plot illustrates the mean cellular fluorescence \pm the standard error of three experiments. (B) HeLa cells were incubated with 1 μ M (PPR)₅ (green) for 20 min, and intracellular localization was monitored by confocal microscopy. Endosomes are visualized using 10 μ M 10 kDa dextran labeled with AlexaFluor-647 (red). (C) Two-color fluorescence with bright-field superposition. (D) Circular dichroism spectra of 100 μ M (PPR)_n and (PRR)_n peptides at 5 °C in PBS, pH 7.4.

and R₁₂, the most potent encodable CPPs previously reported.¹¹ The relative efficiencies of R₈, R₁₀, and R₁₂ are length-dependent, consistent with earlier reports that oligoarginine uptake increases with increasing charge but peaks between R₈ and R₁₅.^{22,23} The (PPR)_n series displays an even stronger length dependence, such that (PPR)₄ significantly underperforms its “iso-ionic” counterpart R₈, whereas (PPR)₅ matches R₁₀ and (PPR)₆ outperforms R₁₂. Thus, at longer lengths, the uptake of simple (PPR)_n peptides exceeds the maximal uptake of oligoarginine vectors, outperforming the commonly used sequences R₈ and Tat by 3–4 and 29–44 times, respectively.

While our flow cytometry protocol includes trypsin treatment to remove any cell-surface bound peptide,²⁴ we performed live-cell confocal microscopy to verify internalization and assess intracellular distribution (Figure 1B). The peptides generate diffuse cytoplasmic and nuclear staining, as well as strong nucleoli staining. The lack of co-localization with dextran, an endocytotic marker, reveals that little peptide is present in endosomes. Another requisite feature of import tags is that they are not intrinsically cytotoxic at concentrations required for uptake. We found no significant reduction in cell viability after a 6 h incubation with 1–100 μ M (PPR)_n and (PRR)_n peptides.²⁵

Consistent with their design, both the (PPR)_n and (PRR)_n series display characteristic PPII signatures, with weak MRE maxima at

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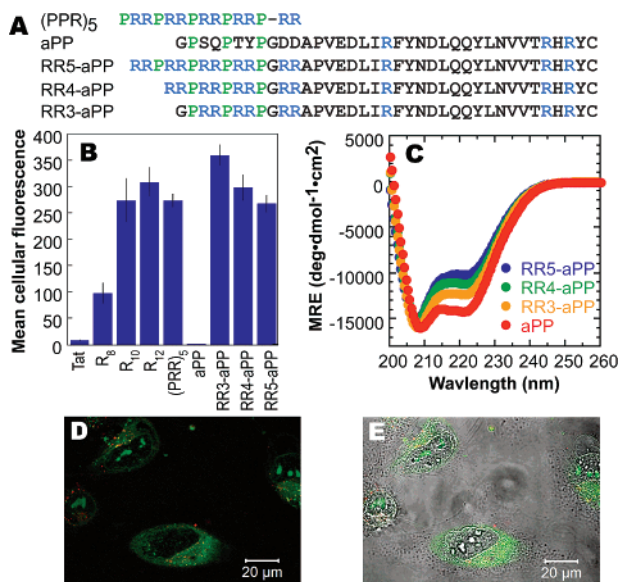


Figure 2. (A) Installation of (PRR)₅ in a miniature protein by sequence alignment. (B) HeLa cell uptake of 1 μM fluorescein-labeled miniature proteins after 1 h quantified by flow cytometry. Plot illustrates the mean cellular fluorescence ± the standard error of three experiments. (C) CD spectra of 50 μM miniature proteins at 5 °C in PBS, pH 7.4. (D) HeLa cells were incubated with 1 μM RR3-aPP (green) for 20 min, and intracellular localization was monitored by confocal microscopy. Endosomes are visualized using 10 μM 10 kDa dextran labeled with AlexaFluor-647 (red). (E) Two-color fluorescence with bright-field superposition.

~228 nm and strong minima at ~204 nm (Figure 1C). The bands observed for arginine-rich (PRR)_n peptides are blue-shifted relative to proline-rich (PPR)_n peptides, as expected from the lower tertiary amide content in the former. As the positions of the maxima for both series correspond well to that of oligoproline peptides, the PPII content can be approximated using Creamer's method.^{14,16} Both (PPR)_n and (PRR)_n peptides show extensive, temperature-dependent structure that is nearly 50% PPII helical at 5 °C and retain approximately 20% PPII helicity at 90 °C.²⁵ In comparison, a heptaproline peptide (P₇GY) is 67% PPII helical, with single amino acid substitutions (P₃XP₃GY) reducing helicity to 49–66%.¹⁶

To test the ability of the (PRR)_n motif to deliver cargos both capable of supporting functionality and dependent on PPII structure, we incorporated them into the miniature protein avian pancreatic polypeptide (aPP) (Figure 2A). Previous work has shown that aPP is a robust platform for the design of miniature proteins that bind DNA²⁶ and proteins,^{27–30} inhibiting their interactions with high affinity and specificity in vitro and in cells. We replaced the PPII helix of aPP with (PRR)₅ to create RR5-aPP (Figure 2A). As measured by flow cytometry (Figure 2B), RR5-aPP penetrates cells as efficiently as an isolated (PRR)₅ motif. RR4-aPP and RR3-aPP, with successively truncated N-terminal extensions, demonstrate slightly greater cellular uptake than RR5-aPP. Notably, RR3-aPP does not add additional residues to the native aPP sequence and is significantly more cell-permeable than arginine homopolymers of comparable arginine content.

Confocal microscopy confirms that, like our uptake motifs in isolation, these cell-permeable miniature proteins are intracellular and not sequestered in endosomes (Figure 2D). RR3-aPP and RR4-aPP did not show discernible toxicity at 10 μM, while cells incubated with RR5-aPP remained 91% viable.²⁵ None of the three was cytotoxic at 1 μM.²⁵ Circular dichroism demonstrates that, although installation of our PPII-based CPP results in some loss of α-helicity, the miniature proteins retain significant structure and

thermostability (Figure 2C). RR3-, RR4-, and RR5-aPP exhibit melting transitions at 44, 45, and 38 °C, respectively, comparable to those of monomeric aPP molecules.^{31,32}

In summary, we have designed encodable, cell-permeable peptides possessing polyproline type-II structure. These motifs penetrate eukaryotic cells with efficiencies vastly higher than Tat and previous natural PPII peptides, and significantly higher than oligoarginine peptides. These PPII-based motifs are not cytotoxic at concentrations 10–100 times greater than that necessary for uptake. Moreover, encoding the motifs within miniature proteins endows cell permeability without destroying structure or adding significant mass. We anticipate these motifs will facilitate both the delivery of peptides and proteins for intracellular study, as well as expand the utility of functionalized miniature proteins.

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Supporting Information Available: Experimental methods, circular dichroism, and cell viability data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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