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Minimally Cationic Cell-Permeable Miniature Proteins via α-Helical Arginine Display

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Protein therapeutics are a blossoming industry, with revenues exceeding $51 billion in 2005 and a growth rate nearly three times that of the overall pharmaceutical industry.[1] Although it has been known for 40 years that cationic amino acid polymers can transport molecular cargos across the plasma membrane,[2−4] inefficient cellular delivery severely impedes the development of peptide and protein drugs. Oligoarginine sequences are effective at permeating cells[5] and have gained popularity for the delivery of protein cargos.[6−8] Unfortunately, oligoarginine tags can increase toxicity[8] and diminish the stability of appended proteins.[9] We recently reported that small, folded proteins containing a minimal cationic motif embedded within a type II polyproline (PPII) helix efficiently cross the plasma membrane of eukaryotic cells.[10] Here we demonstrate that an even smaller cationic motif can be embedded within the α-helix of a small, folded protein to generate molecules that penetrate cells significantly more efficiently than oligoarginine-rich sequences or Tat. Our results suggest that the function of cell permeability can be encoded by judicious placement of as few as 2−3 additional arginine residues on a protein α-helix.

The avian pancreatic polypeptide (aPP) is a 36-residue polypeptide composed of an N-terminal PPII helix and a C-terminal α-helix. This well-packed, thermostable structure provides a starting point for the presentation of well-folded PPII and α-helical epitopes that bind protein targets with high affinity and specificity *in vitro* and *in vivo*.[11−20] We reasoned that substitution of arginines for residues located on the aPP α-helix would impart cell permeability, but perhaps only at the expense of structural stability due to charge−charge repulsion.[21] To determine the minimum number of arginine residues that would facilitate cell permeability and retain a stable fold, we synthesized aPP variants aPP^{4R1}, aPP^{5R1}, and aPP^{6R1} (Figure 1). These molecules contain four, five, or six arginines, respectively, on the solvent exposed face of the aPP α-helix in place of side chains that contribute minimally if at all to protein stability.[22]

We first characterized the secondary structure of each variant using circular dichroism (CD) spectroscopy (Figure 1B). The CD spectra of aPP^{4R1} and aPP^{5R1} were virtually identical to that of aPP, with characteristic negative ellipticity at 208 and 222 nm. By contrast, the spectrum of aPP^{6R1} indicated significantly less α-helix structure. Temperature-dependent CD analysis demonstrated that each peptide, like aPP, underwent a cooperative melting transition. Each arginine substitution decreases thermostability slightly, with aPP^{4R1} and aPP^{5R1} characterized by T_m's of 54 and 47 °C, near that of aPP; the T_m of aPP^{6R1} is significantly depressed (35 °C) (Figure SI-1).

We next used flow cytometry to determine whether aPP^{4R1}, aPP^{5R1}, and aPP^{6R1} penetrated eukaryotic cells. HeLa cells were incubated with 1 μM fluorescein-labeled peptide for times between 5 and 90 min and treated with trypsin to remove peptide from the cell surface.[23] Cellular penetration of ^{Flu}aPP^{5R1}, ^{Flu}aPP^{6R1}, and the well-studied oligoarginine ^{Flu}R$_8$Y were similar at early times (t < 20 min) (Figure 2A). Surprisingly, uptake of ^{Flu}R$_8$Y remained constant at longer times whereas uptake of ^{Flu}aPP^{5R1}and ^{Flu}aPP^{6R1} increased significantly. At an incubation time of 30 min, ^{Flu}aPP^{4R1} entered cells more efficiently than ^{Flu}Tat[24] but less efficiently than ^{Flu}R$_8$Y,[5] ^{Flu}pAntp,[25] ^{Flu}Transportan,[26] and ^{Flu}KLA[27] (also known as MAP) (Figure 1C). By contrast, ^{Flu}aPP^{5R1} and ^{Flu}aPP^{6R1} penetrated cells significantly more effectively than any cell penetrating peptide tested, generating cellular fluorescence 3−4 times that of ^{Flu}R$_8$Y and 35−45 times that of ^{Flu}Tat. Confocal microscopy confirmed that ^{Flu}aPP^{6R1} was internalized to the cytosol and endosomes and was not limited to the cellular membrane (Figure 2C).

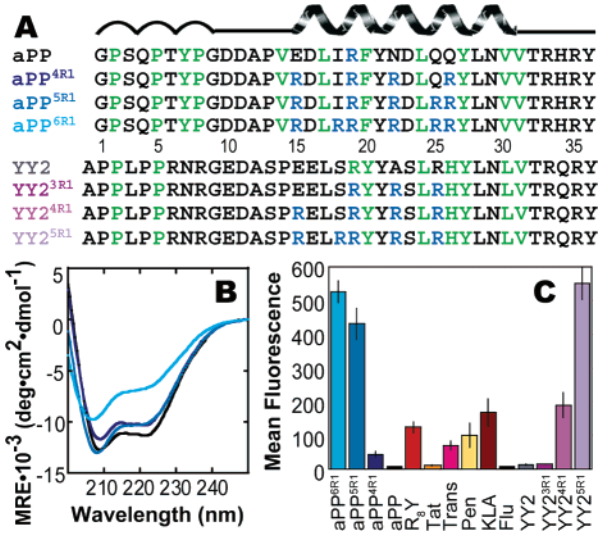

Figure 1. (A) Miniature protein sequences. Residues that contribute to aPP/YY2 folding are in green; arginines located on the α-helices are in blue. (B) Circular dichroism spectra of aPP, aPP^{4R1}, aPP^{5R1}, and aPP^{6R1} (10 μM) in PBS (pH 7.4) at 37 °C. See panel A for legend. (C) Mean cellular fluorescence of HeLa cells incubated for 30 min with 1 μM fluorescently labeled peptide in DMEM containing 10% FBS.

The mechanism by which cationic peptides enter cells is not well understood and may vary depending on sequence, conditions, and cargo.[28,29] Recent results obtained with live cells imply that endocytosis is often a major pathway.[23] Indeed, cellular uptake of ^{Flu}aPP^{5R1} and ^{Flu}aPP^{6R1} is both temperature- and ATP-dependent (Figure 2B). Moreover, confocal microscopy of cells treated with ^{Flu}aPP^{6R1} reveals a punctate pattern of fluorescence in the cytosol (Figure 2C) that colocalizes in part with 10 kDa dextran, a marker for endocytosis (Figure 2D, 2E).[30] Together these data suggest a significant contribution from an energy-dependent mechanism such as endocytosis. However, ^{Flu}aPP^{6R1} also shows diffuse cytosolic

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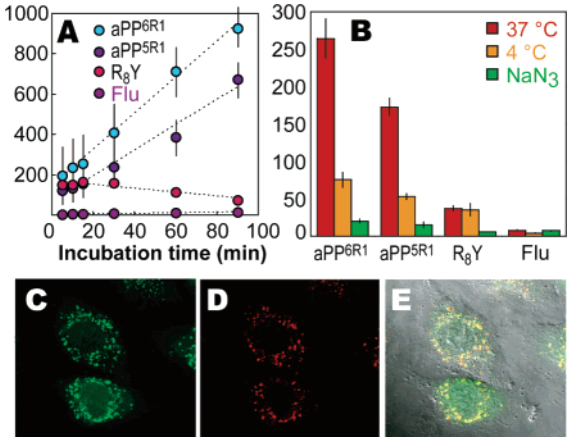

Figure 2. (A) Time-dependent uptake of 1 μM fluorescein-labeled peptides by HeLa cells as quantified by flow cytometry. Plot illustrates the mean cellular fluorescence ± the standard error of three experiments. (B) HeLa cells were incubated with 1 μM peptide for 30 min at 37 °C (red), 4 °C (orange), and at 37 °C in glucose-free media supplemented with NaN_3 (green), and intracellular localization was quantified by flow cytometry. (C−E) Confocal microscopy of HeLa cells co-incubated with 5 μM ^{Flu}aPP^{6R1} (green) and 10 kDa dextran labeled with Alexa Fluor 647 (red) for 30 min at 37 °C. (C) Green channel. (D) Red channel. (E) Superposition of (C), (D), and transmitted light.

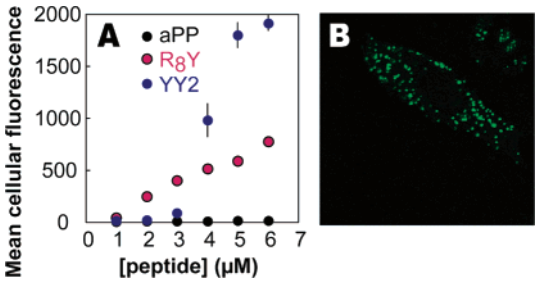

Figure 3. (A) HeLa cell uptake of fluorescein-labeled peptides at the indicated concentration after 30 min at 37 °C as quantified by flow cytometry. Plot illustrates the mean cellular fluorescence ± the standard error of three experiments. (B) Confocal microscopy of HeLa cells incubated with 5 μM ^{Flu}YY2 for 30 min at 37 °C.

staining that is not colocalized with dextran, suggesting either endosomal escape or an alternative entry pathway.

Oligoarginine tags can increase cytotoxicity,[8] and thus we examined the toxicity of all peptides using the CellTiter-Blue cell viability assay (Promega). Incubation of HeLa cells with any of the aPP- or YY2-based peptides at concentrations as high as 50 μM (50-fold higher than required for penetration) for 6 h (70-fold longer than necessary for uptake) led to less than 10% loss in viability (Figure SI-3).

Previously we described a series of miniature proteins that made use of their PPII helix to bind SH3 domains selectively *in vitro*; one such molecule, YY2, activated Hck kinase in cell extracts.[17] To evaluate whether a minimal cationic motif could be transferred from aPP to an alternative protein context, we synthesized YY2^{3R1}, YY2^{4R1}, and YY2^{5R1} (Figure 1A). Although ^{Flu}YY2^{3R1} did not penetrate cells appreciably at 1 μM, ^{Flu}YY2^{4R1} and ^{Flu}YY2^{5R1} did so at levels similar to ^{Flu}aPP^{6R1}, the most cell-permeant aPP derivative studied (Figure 1C). Unexpectedly, even ^{Flu}YY2, whose sequence contains only two arginine residues within the α-helix, penetrates cells, albeit at higher concentration. At 6 μM, ^{Flu}YY2 shows intracellular fluorescence twice that of ^{Flu}R$_8$Y (Figure 3A). This observation highlights the expanding utility of miniature proteins, which, like certain peptoids,[31] may not require additional engineering to offer cell permeability.

In conclusion, we have created a second family of minimally cationic miniature proteins that effectively cross the plasma membrane of eukaryotic cells. Although introduction of multiple arginine residues decreases the value of T_M, judicious placement and empirical charge minimization afford miniature proteins that are both well-folded and cell permeable. As these scaffolds are capable of supporting high affinity and specificity interactions *in vitro* and *in vivo*, we expect the additional encoding of cellular permeability to significantly expand their utility.

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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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