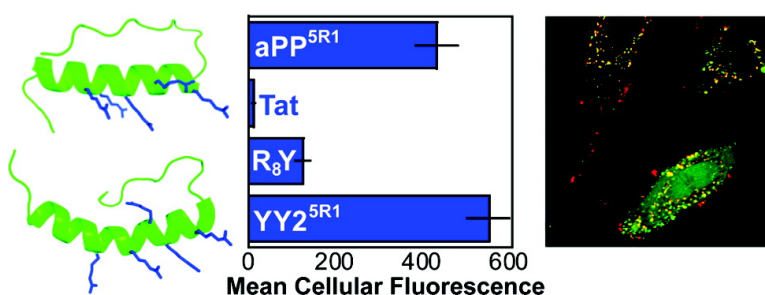


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## Minimally Cationic Cell-Permeable Miniature Proteins via $\alpha$ -Helical Arginine Display

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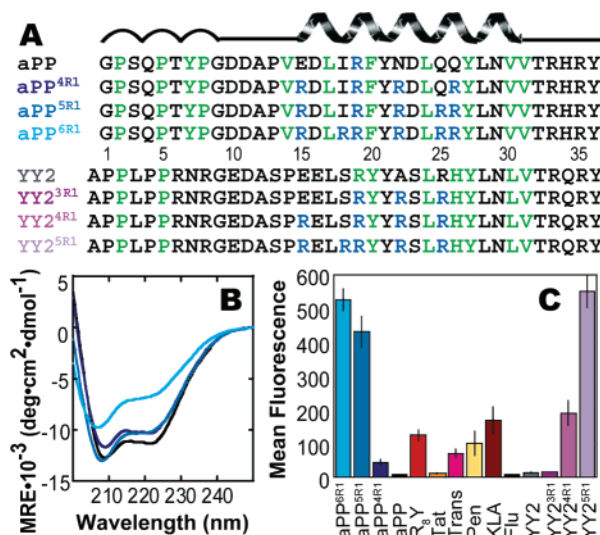
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Protein therapeutics are a blossoming industry, with revenues exceeding \$51 billion in 2005 and a growth rate nearly three times that of the overall pharmaceutical industry.<sup>1</sup> Although it has been known for 40 years that cationic amino acid polymers can transport molecular cargos across the plasma membrane,<sup>2–4</sup> inefficient cellular delivery severely impedes the development of peptide and protein drugs. Oligoarginine sequences are effective at permeating cells<sup>5</sup> and have gained popularity for the delivery of protein cargos.<sup>6–8</sup> Unfortunately, oligoarginine tags can increase toxicity<sup>8</sup> and diminish the stability of appended proteins.<sup>9</sup> 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.<sup>10</sup> Here we demonstrate that an even smaller cationic motif can be embedded within the  $\alpha$ -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  $\alpha$ -helix.

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

We first characterized the secondary structure of each variant using circular dichroism (CD) spectroscopy (Figure 1B). The CD spectra of aPP<sup>4R1</sup> and aPP<sup>5R1</sup> were virtually identical to that of aPP, with characteristic negative ellipticity at 208 and 222 nm. By contrast, the spectrum of aPP<sup>6R1</sup> indicated significantly less  $\alpha$ -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<sup>4R1</sup> and aPP<sup>5R1</sup> characterized by  $T_m$ 's of 54 and 47 °C, near that of aPP; the  $T_m$  of aPP<sup>6R1</sup> is significantly depressed (35 °C) (Figure SI-1).

We next used flow cytometry to determine whether aPP<sup>4R1</sup>, aPP<sup>5R1</sup>, and aPP<sup>6R1</sup> penetrated eukaryotic cells. HeLa cells were incubated with 1  $\mu$ M fluorescein-labeled peptide for times between



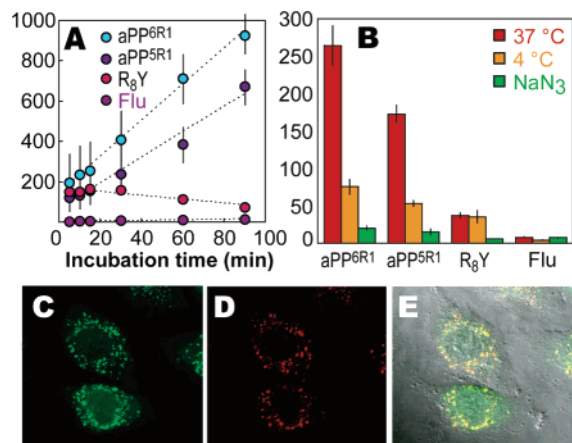
**Figure 1.** (A) Miniature protein sequences. Residues that contribute to aPP/YY2 folding are in green; arginines located on the  $\alpha$ -helices are in blue. (B) Circular dichroism spectra of aPP, aPP<sup>4R1</sup>, aPP<sup>5R1</sup>, and aPP<sup>6R1</sup> (10  $\mu$ M) in PBS (pH 7.4) at 37 °C.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

5 and 90 min and treated with trypsin to remove peptide from the cell surface.<sup>23</sup> Cellular penetration of <sup>Flu</sup>aPP<sup>5R1</sup>, <sup>Flu</sup>aPP<sup>6R1</sup>, and the well-studied oligoarginine <sup>Flu</sup>R<sub>8</sub>Y were similar at early times ( $t < 20$  min) (Figure 2A). Surprisingly, uptake of <sup>Flu</sup>R<sub>8</sub>Y remained constant at longer times whereas uptake of <sup>Flu</sup>aPP<sup>5R1</sup> and <sup>Flu</sup>aPP<sup>6R1</sup> increased significantly. At an incubation time of 30 min, <sup>Flu</sup>aPP<sup>4R1</sup> entered cells more efficiently than <sup>Flu</sup>Tat<sup>24</sup> but less efficiently than <sup>Flu</sup>R<sub>8</sub>Y,<sup>5</sup> <sup>Flu</sup>pAntp,<sup>25</sup> <sup>Flu</sup>Transportan,<sup>26</sup> and <sup>Flu</sup>KLA<sup>27</sup> (also known as MAP) (Figure 1C). By contrast, <sup>Flu</sup>aPP<sup>5R1</sup> and <sup>Flu</sup>aPP<sup>6R1</sup> penetrated cells significantly more effectively than any cell penetrating peptide tested, generating cellular fluorescence 3–4 times that of <sup>Flu</sup>R<sub>8</sub>Y and 35–45 times that of <sup>Flu</sup>Tat. Confocal microscopy confirmed that <sup>Flu</sup>aPP<sup>6R1</sup> was internalized to the cytosol and endosomes and was not limited to the cellular membrane (Figure 2C).

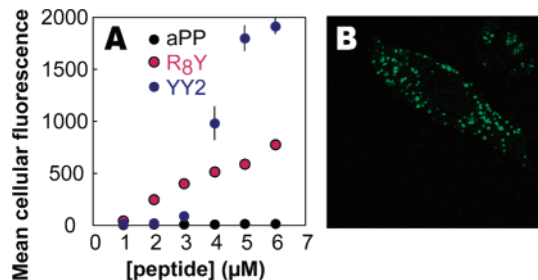
The mechanism by which cationic peptides enter cells is not well understood and may vary depending on sequence, conditions, and cargo.<sup>28,29</sup> Recent results obtained with live cells imply that endocytosis is often a major pathway.<sup>23</sup> Indeed, cellular uptake of <sup>Flu</sup>aPP<sup>5R1</sup> and <sup>Flu</sup>aPP<sup>6R1</sup> is both temperature- and ATP-dependent (Figure 2B). Moreover, confocal microscopy of cells treated with <sup>Flu</sup>aPP<sup>6R1</sup> 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).<sup>30</sup> Together these data suggest a significant contribution from an energy-dependent mechanism such as endocytosis. However, <sup>Flu</sup>aPP<sup>6R1</sup> also shows diffuse cytosolic

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**Figure 2.** (A) Time-dependent uptake of 1  $\mu\text{M}$  fluorescein-labeled peptides by HeLa cells as quantified by flow cytometry. Plot illustrates the mean cellular fluorescence  $\pm$  the standard error of three experiments. (B) HeLa cells were incubated with 1  $\mu\text{M}$  peptide for 30 min at 37  $^{\circ}\text{C}$  (red), 4  $^{\circ}\text{C}$  (orange), and at 37  $^{\circ}\text{C}$  in glucose-free media supplemented with  $\text{NaN}_3$  (green), and intracellular localization was quantified by flow cytometry. (C–E) Confocal microscopy of HeLa cells co-incubated with 5  $\mu\text{M}$   $\text{Flu}$ -aPP<sup>6R1</sup> (green) and 10 kDa dextran labeled with Alexa Fluor 647 (red) for 30 min at 37  $^{\circ}\text{C}$ . (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  $^{\circ}\text{C}$  as quantified by flow cytometry. Plot illustrates the mean cellular fluorescence  $\pm$  the standard error of three experiments. (B) Confocal microscopy of HeLa cells incubated with 5  $\mu\text{M}$   $\text{Flu}$ YY2 for 30 min at 37  $^{\circ}\text{C}$ .

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

Oligoarginine tags can increase cytotoxicity,<sup>8</sup> 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  $\mu\text{M}$  (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.<sup>17</sup> To evaluate whether a minimal cationic motif could be transferred from aPP to an alternative protein context, we synthesized YY2<sup>3R1</sup>, YY2<sup>4R1</sup>, and YY2<sup>5R1</sup> (Figure 1A). Although  $\text{Flu}$ YY2<sup>3R1</sup> did not penetrate cells appreciably at 1  $\mu\text{M}$ ,  $\text{Flu}$ YY2<sup>4R1</sup> and  $\text{Flu}$ YY2<sup>5R1</sup> did so at levels similar to  $\text{Flu}$ aPP<sup>6R1</sup>, the most cell-permeant aPP derivative studied (Figure 1C). Unexpectedly, even  $\text{Flu}$ YY2, whose sequence contains only two arginine residues within the  $\alpha$ -helix, penetrates cells, albeit at higher concentration. At 6  $\mu\text{M}$ ,  $\text{Flu}$ YY2 shows intracellular fluorescence twice that of  $\text{Flu}$ R<sub>8</sub>Y (Figure 3A). This observation highlights the expanding utility of miniature

proteins, which, like certain peptoids,<sup>31</sup> 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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