Communication

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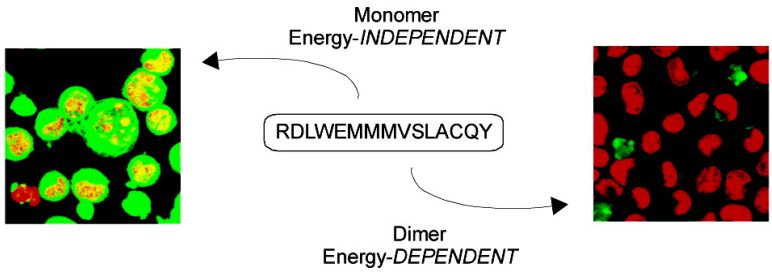


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# A Dimerization "Switch" in the Internalization Mechanism of a Cell-Penetrating Peptide

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Peptide sequences that effect membrane translocation from the extracellular space to the cytoplasm of eukaryotic or prokaryotic cells have been the subject of great interest in recent years. One of the prime reasons for this has been their early-recognized potential as delivery vectors to improve the pharmacodynamics of otherwise poorly bioavailable drugs.[1] The majority of these cell-penetrating peptides (CPPs) have been designed on the basis of known, naturally occurring peptides and proteins that mediate physiological membrane processes such as fusion, disruption, and pore formation. For example, the HIV-1 transcription activator Tat has long been known to cross the cell membrane and localize directly to the cell nucleus.[2] More focused investigations have shown that truncated forms of Tat, such as the 49−57 residue sequence, are necessary and sufficient for membrane translocation;[3] this finding has spurred the development of polycationic peptides and "peptidomimetic" polymers comprising natural[4,5] and unnatural[6,7] amino acid residues for the delivery of various payloads into cells both in vitro and in vivo.

Phage display technology has found widespread application in the selection of peptides and proteins with specific recognition properties.[8] To broaden the scope of biomolecules accessible by phage display, we have developed a combinatorial phage display format for the construction of diverse homo- and heterodimeric peptide arrays.[9] This approach employs the neighboring pVII and pIX coat proteins for the display of juxtaposed fusion peptides that serve as model dimeric motifs. Using this library, we identified the sequence SDLWEMMMVSLACQY (N→C), **1**, by whole-cell panning of a phage library displaying random 15-residue homo- and heterodimers against WI-L2-729HF2 B-lymphocyte cells, and we found the entire homodimeric **1**-phage to be endocytosed into the target cells.[10] In this Communication, we detail our investigations into the internalization mechanism of this peptide by a combination of biological and chemical methods, focusing on the structural determinants and biophysical characterization of its membrane translocation activity.

Our current investigations have shown that the internalization of **1**-phage is a temperature-dependent process, being nullified by administration to target WI-L2 cells preincubated at 4 °C (data not shown). The signficance of this finding stems from its suggestion of an energy-dependent internalization mechanism, which contrasts starkly with the passive translocation mechanism employed by the majority of CPPs reported to date.[4,6,11] We examined the binding of this sequence to the target WI-L2-729HF2 cell surface via whole-cell ELISA using a construct comprising the sequence of **1** fused to a Jun dimerization domain bearing a C-terminal Flag affinity tag (see Supporting Information). Surprisingly, we observed saturation kinetics, which suggest a mechanism for cell penetration that involves a saturable cell surface receptor cohort. Quantitative whole-cell ELISA revealed that there are approximately $10^6$ receptors for the **1**-Jun construct per cell.

***Table 1.*** CPP Sequences Obtained by Mutational Analysis of **1**-Phage at Positions 1, 3, and 7

| Peptide # | Sequence (N→C) | Library Frequency |
|---|---|---|
| 1 (wt) | SDLWEMMMVSLACQY | 22% |
| 2 | RDIWEMMMVSLACQY | 22% |
| 3 | RDLWEMMMVSLACQY | 9% |
| 4 | MDVWEMMMVSLACQY | 13% |
| 5 | LDIWEMMMVSLACQY | 9% |
| 6 | TDLWEMVMVSLACQY | 5% |
| 7 | GDLWEMMMVSLACQY | 5% |
| 8 | KDWWEMLMVSLACQY | 5% |
| 9 | MDPWEMMMVSLACQY | 5% |
| 10 | VDVWEMIMVSLACQY | 5% |

The primary structural determinants of binding and internalization by **1**-phage were explored through the construction of a new phage library wherein each position in this sequence was incorporated at a mutation rate of 50%. This new phage library was screened for internalization into WI-L2-729HF2 cells, after which 20 clones were randomly picked and sequenced. The amino acid residues at all positions were identical to the wild-type sequence of **1** except for variations at positions 1, 3, and 7. From this result, a second affinity maturation experiment was performed using a more focused phage library wherein these three positions were substituted with all of the possible 20 amino acids, and the resulting library selected against WI-L2-729HF2 cells. A variety of enriched sequences were found, of which nine are shown; these results indicate a significant difference in the mutational tolerance at these positions (Table 1).

With SAR data from our initial and more focused phage libraries in hand, peptide 3 (Table 1) was prepared by chemical synthesis in monomer (**3a**) and symmetrical disulfide-linked dimer (**3b**) formats using a combination of solid- and solution-phase methods. Whereas the original lead, peptide 1, was our main target for chemical synthesis, its exceedingly poor solubility and difficult purification motivated us to replace the N-terminal Ser residue with the more hydrophilic Arg residue, as found in peptide 3 (Table 1). Synthetic constructs **3a** and **3b** were prepared with an additional lysine residue at the C-terminus, which was functionalized at the side chain with biotin via an $\epsilon$-aminohexanoic acid spacer. Competitive whole-cell ELISA showed that **3b** is capable of competing against **1**-Jun in binding to WI-L2-729HF2 cells with an $IC_{50}$ corresponding to 25% of the saturating concentration of the recombinant **1**-Jun dimer (see Supporting Information). Confocal fluorescence microscopy revealed that both **3a** and **3b** were efficiently internalized into the target WI-L2-729HF2 cells (Figure 1).

Having verified that **3a** and **3b** are in fact true CPPs, we sought additional support for an energy-dependent internalization mechanism. We assayed the competency of internalization by **3a** and **3b** after depletion of the intracellular ATP pool in the target cells by inhibition of cytochrome oxidase via pretreatment with $NaN_3$.[12]



10.1021/ja0443171 CCC: $30.25 

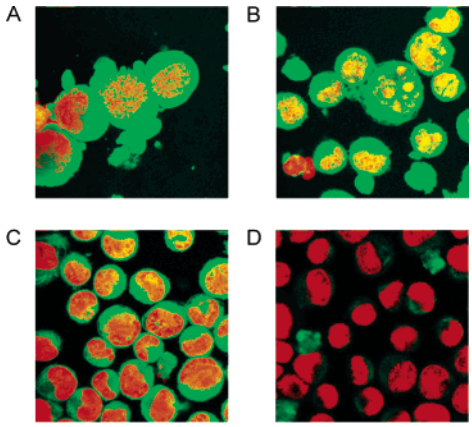


***Figure 1.*** Overlaid confocal fluorescence microscopy images of WI-L2-729HF2 cells obtained at 488 and 568 nm after treatment with monomer **3a** in the absence (A) and presence (B) of $NaN_3$, and with dimer **3b** in the absence (C) and presence (D) of $NaN_3$. Cells were stained with FITC-streptavidin (green) to visualize the internalized peptide and with propidium iodide (red) to delineate the nucleus. The average fluorescence intensity values per cell in each image are (A) 178.9 ± 50.3, (B) 217.1 ± 39.6, (C) 141.5 ± 47.2, and (D) 22.1 ± 10.8.

This experiment yielded surprising results; while internalization of **3a** was found to be independent of ATP depletion, suggesting passive diffusion of this peptide across the cell membrane (Figure 1, panels A and B), the internalization of **3b** was virtually abolished in the $NaN_3$-treated cells (Figure 1, panels C and D). Furthermore, **3a** seems to exhibit some degree of cytotoxicity, as the target cells appear hyperosmotic and intensely stained, possibly due to intracellular accumulation of the monomeric peptide.[13]

To determine whether the solution-phase conformation of **3a** and **3b** might shed some light on their differential properties, we examined the structure of these two peptides by far-UV circular dichroism under nondenaturing aqueous conditions. Both peptides show the hallmarks of a high degree of α-helicity, as judged by global and local minima in mean-residue ellipticity at 208 and 220 nm, respectively (see Supporting Information). Notably, the monomer appears to exhibit approximately 2-fold more intense α-helicity, which may be related to its passive diffusion across the cell membrane of the target WI-L2-729HF2 cells. Such a mechanism is not entirely unreasonable, as a variety of naturally occurring and designed CPPs adopt stable α-helical conformations, although the extent to which this is a requisite for membrane translocation remains the subject of active debate.[14]

While phage display has been used to identify peptide sequences that induce binding and internalization of cell surface-bound phage,[15] the symmetrical dimer **3b** is, to our knowledge, the first CPP whose specific membrane translocation mechanism is controlled by a dimerization-dependent "switch". Whether the energy-dependent, receptor-mediated importation of **3b** is a function of dimerization per se, or simply superseded by a helicity-induced passive diffusion mechanism as suggested by the monomer **3a**, is under investigation. Toward this end, we are currently exploring the biological effects of alternative, non-native dimerization chemistries on the activity of **3b**. These remaining questions notwithstanding, we have shown that the pVII/pIX phage display format is a valuable tool for the de novo identification of novel CPPs with unique importation mechanisms. Further studies from our laboratory will involve use of the pVII/pIX phage display format for the selection of peptides against additional cell lines to identify new lead sequences for CPPs to be used as targeted drug delivery vectors.

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**Supporting Information Available:** Details of phage library selection, quantitative whole cell ELISA, peptide synthesis protocols, confocal fluorescence microscopy sample preparation, and far-UV circular dichroism data. This material is available free of charge via the Internet at http://pubs.acs.org.

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