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Translocation of a β-Peptide Across Cell Membranes

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Biological membranes are critical for life because the hydrophobic barrier between cytoplasm and external medium allows cells to regulate entry of water-soluble molecules from the environment. This barrier effect, however, drastically limits the delivery of polar molecules (peptides, nucleic acids, drugs) to intracellular targets. Over the past decade, several short peptides have been found to cross biological membranes rapidly.[1] For example, HIV Tat and related DNA-binding proteins were shown in the late 1980s to move spontaneously into nuclei from the extracellular milieu,[2] and more recent work has demonstrated that this translocation activity depends on relatively short segments within such proteins.[3] The Tat 47−57 translocation sequence is among the shortest examples.[4] Hydrophobic segments from signal peptides[5] and arginine oligomers[6] also move across cell membranes, as do peptoid (i.e., *N*-alkyl-glycine) oligomers bearing multiple guanidinium-tipped side chains.[7] Translocating peptides can deliver diverse cargos, including proteins,[8] peptide nucleic acids,[9] and drugs,[10] to intracellular targets. Here we show that a β-amino acid analogue of Tat 47−57 (**1**) translocates across human cell membranes with efficiency comparable to Tat 47−57 itself (**2**).

β-Amino acid oligomers ("β-peptides") have been studied extensively in recent years.[11] They can adopt well-defined protein-like secondary structures, and several β-peptides with interesting biological activities have been reported.[12] We were motivated to undertake the present study because β-peptides are resistant to

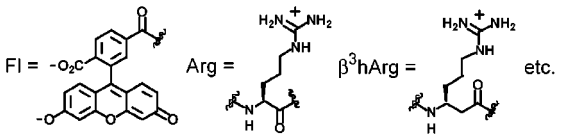
1 = Fl-β^{3}hTyr-β^{3}hGly-β^{3}hArg-β^{3}hLys-β^{3}hLys-β^{3}hArg-β^{3}hArg-β^{3}hGln-β^{3}hArg-β^{3}hArg-β^{3}hArg-NH_2

2 = Fl-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-NH_2

3 = Fl-β^{3}hTyr-β^{3}hGly-β^{3}hArg-β^{3}hLys-β^{3}hLys-β^{3}hArg-β^{3}hArg-β^{3}hGln-NH_2

4 = Fl-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-NH_2

5 = β^{3}hTyr-β^{3}hGly-β^{3}hArg-β^{3}hLys-β^{3}hLys-β^{3}hArg-β^{3}hArg-β^{3}hGln-β^{3}hArg-β^{3}hArg-β^{3}hArg



degradation by proteases[13] and because our ability to control β-peptide secondary structure might ultimately be useful for elucidating the mechanism of translocation.

β-Peptide **1** is constructed from β-substituted β-amino acid residues ("β^3-residues"), which are available enantiospecifically from the corresponding α-amino acid residues via methodology developed by Seebach et al.,[14] as modified by Müller et al.[15,16] Both the Tat fragment **2** and β-peptide analogue **1** bear N-terminal fluorescein labels, allowing detection of cell membrane transit via confocal fluorescence microscopy.

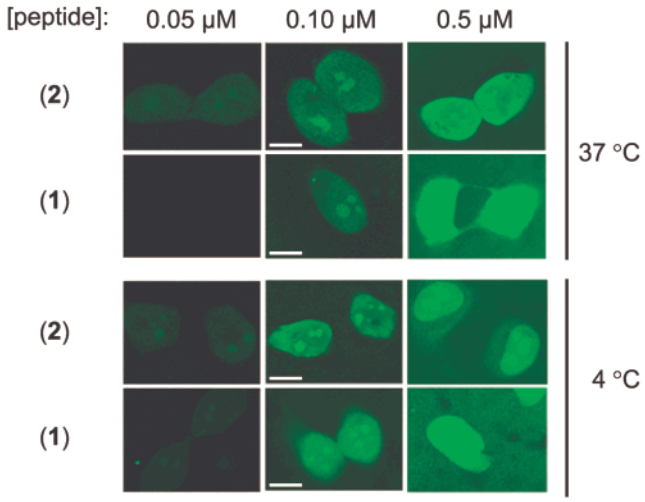


Figure 1. Confocal microscopy images of HeLa cells incubated for 10 min at 37 or 4 °C with different concentrations of a fluorescein conjugate of HIV Tat 47−57 (**2**) or its β-peptide analogue (**1**). The microscope settings were identical for each peptide and dose. Bar: 10 μm.

Figure 1 shows representative results with HeLa cells for **1** and **2**.[17] Neither molecule is observed more than faintly within the cells when incubated with them at a concentration of 0.05 μM, but at 0.10 μM both molecules can be unambiguously detected inside the cells. The intracellular fluorescence intensity rises when the concentration of **1** or **2** is increased to 0.5 μM. (The background fluorescence in these images was unaffected by additional washing of the cells prior to fixation.) Similar results were obtained when fixation was omitted.

We conducted control experiments to determine the effect of length on **1**. In truncation studies of Tat 48−60, Vivès et al. showed that Arg-55, Arg-56, and Arg-57 were critical for translocation; in contrast, the presence or absence of Pro-58, Pro-59, and Gln-60 had little effect on translocation.[3a] Therefore, we examined truncated β-peptide **3**, which lacks the three C-terminal β^3-homoarginine residues of **1**, and the α-peptide analogue **4**. No cell penetration could be detected after treatment with up to 1 μM **3** or **4** (data in Supporting Information), a 10-fold higher concentration than is required for detectable cell penetration by full-length β-peptide **1** and its α-analogue **2**. In addition, free fluorescein did not enter the cells under these conditions. These results show that a minimum β-peptide length and charge is required for movement across cell membranes; comparable conclusions have been drawn from studies of arginine oligomers[6] and peptoid analogues.[7]

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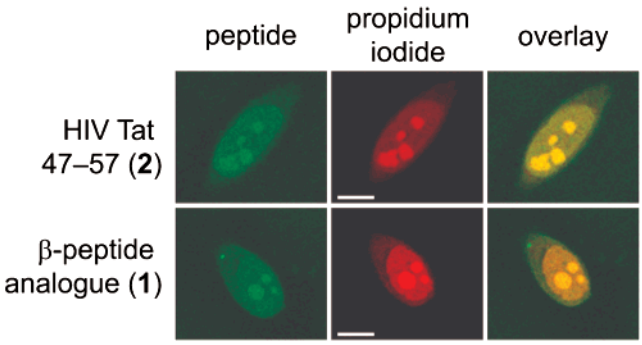

Figure 2. Confocal microscopy images of HeLa cells incubated for 10 min at 37 °C with a solution containing 0.10 μM fluorescein-labeled peptides **2** or **1**, washed, permeabilized, and stained with propidium iodide.[17] The overlay images show that peptides **1** and **2** co-localize with propidium iodide to the nucleus, a result confirmed by bright-field microscopy (not shown); the bright spots within each nucleus represent nucleoli. The microscope settings were identical for each peptide and dose. Bar: 10 μm.

To identify the major intracellular destination of these molecules, cells were co-stained with propidium iodide, which binds specifically to nucleic acids. The co-staining results with Tat fragment **2** (Figure 2) match previous reports that related peptides localize in the nucleus;[3a] the results for β-peptide **1** show that this unnatural analogue behaves similarly. Nuclear localization has been explained by the presence of a GRKKR nuclear localization signal (NLS) in the sequence of **2**.[3a] In further support of this hypothesis, nuclear localization of a sparsely cationic *Antennapedia* peptide is impaired by proline incorporation adjacent to or within the main cationic stretch.[3b] β-Peptide **1** contains multiple oligocationic motifs for possible recognition by the nuclear import machinery.

We used circular dichroism (CD) to examine the solution conformation of the β-peptide analogue of Tat 47−57 lacking terminal modifications (**5**). Mutation studies provide evidence against a conformational requirement for translocation activity of cationic α-peptides,[6,9] and the α-peptide Tat 48−60 has been shown by CD to lack regular secondary structure in methanol.[6b] CD data indicate that **5** is largely disordered in aqueous solution but highly helical in methanol (data included in the Supporting Information).

The mechanism by which short Tat-derived peptides and related oligomers traverse cell membranes remains unclear. Several groups have concluded that endocytosis does not play a role in this process because the transit rate is not temperature-dependent.[3,6a] We found no difference in translocation activity for either **1** or **2** between 37 and 4 °C (Figure 1; the apparent difference in fluorescence intensity at 0.10 μM is within measurement error). These findings are consistent with prior reports with regard to α-peptide **2** and suggest that cell penetration by β-peptide **1** does not depend on endocytosis.

Both **1** and **2** show increasing levels of internal fluorescence at increasing doses. Vivès et al. demonstrated a linear relationship of fluorescence intensity to initial concentration of labeled Tat 48−60;[3a] this relationship, which appears qualitatively similar for β-peptide **1**, suggests either unsaturated first-order kinetics or an equilibration process. Data obtained with a radiolabeled peptide related to **2** (ref 18) and with arginine oligomers[6a] suggest that equilibrium is reached within the 10-min incubation period for $\leq$1 μM peptide (saturation occurs at higher concentrations).

Our work demonstrates that membrane translocation activity is preserved despite a drastic alteration in the oligomer backbone (amide group spacing). Our findings are complementary to those of Wender et al.[7] in showing that protease-resistant oligomers can be used to shepherd attached moieties across cell membranes. Because β-peptides are more easily programmed to adopt specific conformations than are either α-peptides or peptoids,[11] unstructured β-peptide **1** represents a platform that can be used to explore the effects of conformational constraints on translocation activity.

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Supporting Information Available: Discussion of the synthesis and purification of β-peptide **1**, confocal microscopy images of cells incubated with truncated peptides **3** and **4**, and circular dichroism spectra of compound **5** in water and methanol (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) A discussion of the synthesis and purification of **1** is included in the Supporting Information.
(17) HeLa cells were obtained from the American Type Culture Collection (Manassas, VA). HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified incubator containing 5% CO_2(g). All studies were performed using asynchronous log-phase cultures. Exponentially growing HeLa cells were dissociated with trypsin, plated at 40% confluence on six-well Lab-Tek coverslips (Nunc Inc.) and cultured overnight. The culture medium was discarded, and the cells were washed once with DMEM. The peptides and fluorescein stock solutions were diluted in DMEM, and the cell monolayers were incubated at 37 °C with a known concentration of peptide **1** or **2** for 10 min. Subsequently, cells were rinsed three times with DMEM at room temperature and fixed in paraformaldehyde (4% w/v in PBS) for 5 min at room temperature. For experiments at 4 °C, the protocol was the same except that all incubations were performed at 4 °C until the end of the fixation procedure. Fixed cell monolayers were permeabilized with Triton X-100 (0.1% w/v) for 10 min, and washed twice with PBS (pH 7.3). Cells were stained with propidium iodide (1 μg/mL in PBS) for 5 min at room temperature. Cells were then washed three times with PBS and mounted onto glass microscope slides using mounting medium for fluorescence. The distribution of the fluorescence was analyzed on a Zeiss Axiovert 100TV confocal microscope equipped with a Kr/Ar laser and 63× oil immersion objective lens (Leica). Images were captured with BioRad Laser Sharp MRC1024 and Adobe Photoshop v4.0.1 software.
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