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Translocation of an Aib-containing peptide through cell membranes

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ABSTRACT

The biophysical characteristics and channel-forming activity of peptaibols inserted into artificial membranes have been studied over the last 30 years. However, to our knowledge, no studies have addressed directly their behavior in living cells. In this work, a novel strategy has been employed to precisely assess the living cell membrane-penetrating activity of a fluorescein-labeled Aib (α-aminoisobutyric acid)-containing peptide derived from a peptaibol, trichorovin-XIIa (TV-XIIa). We have demonstrated for the first time that the peptide containing an unusual amino acid residue, Aib, is taken up by cells via a non endocytic pathway. The replacement of Aib in the TV-XIIa sequence with Ala inhibits the cellular uptake.

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α-Aminoisobutyric acid (Aib, U) (Fig. 1A) is a hydrophobic and non-coded protein amino acid which has been found in microbial origin.[1] A variety of experiments conducted on Aib-containing peptides have indicated that these peptides exclusively form helical backbone structure,[2,3] resist to protease,[4,5] and strongly bind to membranes.[6,7] The Aib residue is present in the peptaibol family of channel-forming peptide, and the biophysical characteristics and channel-forming activity of peptaibol inserted into artificial membranes have been studied over the last 30 years.[8]

Trichorovin-XIIa (TV-XIIa), which belongs to a class of peptaibols, was isolated from the fungus *Trichoderma viride*.[9] TV-XIIa consists of 11 residues, three of which are Aib residues, and its amino acid sequence is Ac-U-N-I-I-U-P-L-L-U-P-Iol (Ac: acetyl; Iol: isoleucinol). TV-XIIa can also form voltage-dependent ion channels in artificial planar lipid bilayer membranes.[10]

The research on the activity of these peptaibols in living cells has been limited. In the search for the other bioactive function of the Aib-containing peptide to living cells, we have found that a TV-XIIa derivative, which is conjugated between TV-XIIa and a 10-mer of lysine (Lys), could deliver 20-mer oligonucleotides into cells, and that the 10-mer of Lys alone was not capable of delivering them.[11] Then, TV-XIIa itself is considered to play an important role in the delivery system. Although the ionic permeability of membranes by peptaibols and the interaction of peptaibols with lipid membranes have been studied in detail,[8,12,13] to our knowledge, there are no direct evidences for the translocation of TV-XIIa and the other peptaibols through membranes into living cells. To obtain the direct evidences for the cellular uptake of TV-XIIa itself and investigate the delivery principles of the 20-mer oligonucleotides, a fluorescein-labeled peptide with the TV-XIIa amino acid sequence was synthesized and applied to living cells to directly observe the behavior of TV-XIIa itself in living cells. Furthermore, to understand the importance of the three unusual Aib residues in the sequence, they were replaced with Ala, and the behavior of the Aib → Ala substitutional analog in living cells was also examined.

Peptides were prepared by Fmoc-based solid-phase synthesis on a Rink amide resin. The amino acid sequence of peptide **1** contained that of TV-XIIa with cysteine amide linked to the C-terminus for fluorescein labeling to monitor the internalization of the peptides by fluorescence microscopy (Fig. 1A). In peptide **2**, the three Aib residues of **1** were replaced with Ala. Peptide **1** contains three bulky Aib residues. Because the coupling efficiency is generally very low in the reaction of Aib-based activated esters owing to steric hindrance, more reactive acid fluorides were employed in the coupling reactions at positions 1, 4, 5, 8, and 9.[14] For all other couplings, in situ activated esters were prepared by exposing the peptides to 1-hydroxy-1*H*-benzotriazole (HOBt) and diisopropylcarbodiimide (DOPCDI) as coupling reagents. Furthermore, synthesized peptides **1** and **2** were treated with 5-maleimidofluorescein diacetate to obtain fluorescein-labeled peptides **1** (**1**-Fl) and **2** (**2**-Fl), respectively.

The circular dichroism (CD) spectra of **1** and **2** analogs having deletion at the C-terminal Cys residue used as a fluorescein linker (**1a** and **2a**, respectively) are shown in Figure 1B. Note that both peptides show CD spectra typical of a helical peptide, with two minima near 205 and 222 nm.[15,16] As determined from the ratio R [$\theta_{222}/\theta_{neg.max}$],[15,16] both Aib-containing peptide **1a** ($R = 0.2$) and its Aib → Ala substitutional analog **2a** ($R = 0.4$) might form 3_{10}-helices. However, the helicity of the Aib-containing peptide **1a** was higher than that of the Ala-containing peptide **2a**.

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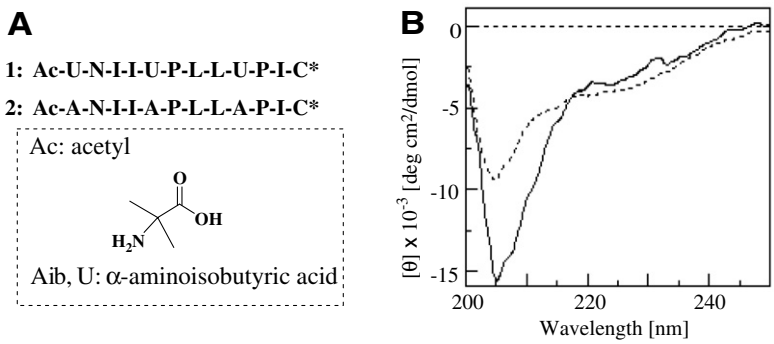

Figure 1. (A) Primary structures of an Aib-containing peptide **1** and its Aib → Ala substitutional analog **2**. One-letter codes are used (U, α-aminoisobutyric acid; Ac: acetyl). C-terminal cysteine amide (C^*) was labeled with fluorescein to monitor internalization of the peptides by fluorescence microscopy. (B) CD spectra of an Aib-containing peptide and its Aib → Ala substitutional analog in methanol. Solid line, 1 μM Aib-containing peptide having deletion at the C-terminal Cys of **1** (**1a**); Dashed line, 1 μM Ala-containing peptide having deletion at the C-terminal Cys of **2** (**2a**).

The uptake of fluorescein-labeled peptides **1**-Fl and **2**-Fl by two different eukaryotic cell lines, mouse embryonic fibroblasts (NIH3T3) and human lung carcinoma (A549) cells, was studied by confocal laser-scanning microscopy (CLSM) and fluorescence-activated cell sorting (FACS). In a typical experiment, cell cultures were treated with the peptides at 0.1–10 μM at 37 °C for 2 h, washed twice with buffer, and directly observed by CLSM and FACS without fixation. Figure 2 shows CLSM images of cells treated with **1**-Fl and **2**-Fl at the same concentration (1.0 μM). As can be noted, **1**-Fl efficiently traversed the membranes of NIH3T3 and A549 cells and diffused to the cytoplasms, and slightly to the nuclei (Fig. 2A–D), whereas **2**-Fl was poorly taken up by cells (Fig. 2E–H). To estimate the amount of peptides taken up in terms of fluorescence intensity, FACS was conducted (Fig. 3). Cells treated with **1**-Fl at concentrations of 0.1, 1.0, and 10 μM showed fluorescence intensity increase by approximately 7-, 47-, and 86-fold in NIH3T3 cells (Fig. 3A; Fig. S-1 (A) in Supplementary Data), and 6-, 19-, and 31-fold in A549 cells (Fig. 3B; Fig. S-1 (C) in Supplementary Data), respectively, relative to the autofluorescence intensity of cells. In contrast, cells treated with **2**-Fl showed very low fluorescence intensities (slightly higher than autofluorescence intensity) at concentrations of 0.1 and 1 μM. At the high concentration of 10 μM, **2**-Fl was taken up by cells, particularly, NIH3T3 cells, but the amounts taken up were lower than that of **1**-Fl (Fig. 3A and B; Fig. S-1 (B) and (D) in Supplementary Data). Furthermore, the effects of low-temperature treatment and reducing the amount of cellular ATP to deplete the energy-dependent cellular pathways on **1**-Fl uptake were examined by FACS (Fig. 3C and D; Figs. S-2 and S-3 in Supplementary Data). Incubation temperature at 4 °C resulted in 92% (NIH3T3) and 83% (A549) reduction in fluorescence intensity as compared with that at 37 °C. However, treatment with ATP-depleting reagents (6 mM 2-deoxy-D-glucose and 10 mM sodium azide) had no effect on **1**-Fl transport efficiency in NIH3T3 cells, but induced a twofold increase in A549 cells (Fig. 3C and D; Fig. S-3 in Supplementary Data). If the cellular transport is caused by endocytosis, both low-temperature treatment and ATP depletion should inhibit cellular uptake because endocytosis is a temperature- and energy-dependent process. These results suggest that endocytosis is not the major pathway for cellular entry of **1**-Fl and that cell membrane fluidity and the mobility of membrane protein and other membrane constituents may affect **1**-Fl transport. These facts were supported by CLSM experiments using an endocytic marker FM4-64.[17] (Fig. S-4 in Supplementary Data).

The activity of lactate dehydrogenase (LDH), a cytoplasmic enzyme, in the incubation medium was measured to determine whether **1**-Fl disrupts the membranes of cells, resulting in the leakage of LDH. The exposure of cells to **1**-Fl (1.0–30 μM) did not result in the release of LDH compared with that of the control batch. Thus, **1**-Fl was not cytotoxic under our experimental conditions, and **1**-Fl was translocated into the cytoplasms without causing cell damage (Fig. S-5 in Supplementary Data).

In conclusion, an Aib-containing peptide can translocate into cells, the replacement of Aib in the amino acid sequence with Ala (**2**-Fl) inhibits the cellular uptake, and there were no distinct differences in conformation between **1a** and **2a**. These observa-

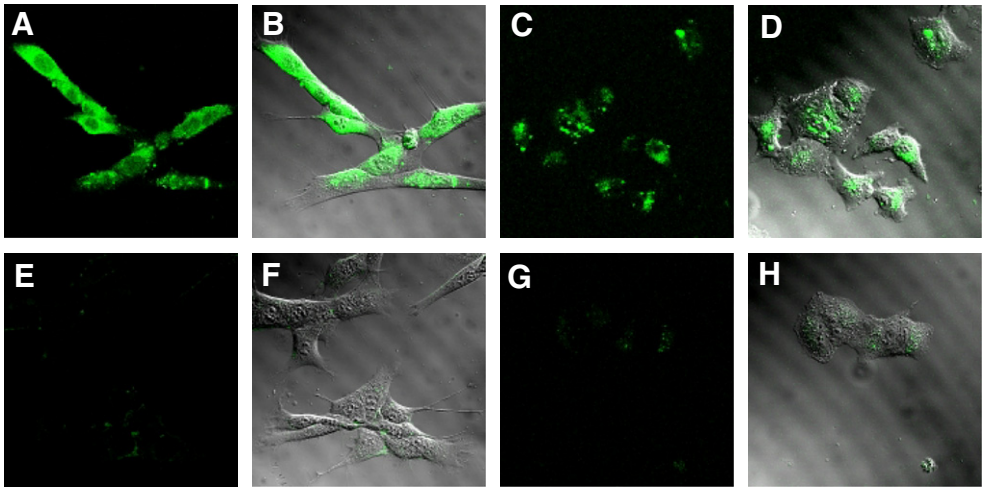

Figure 2. Confocal microscopy images of NIH3T3 (A, B, E, and F) and A549 (C, D, G, and H) cells. Fluorescence images (A, C, E, and G), and fluorescence and differential interference contrast images were merged (B, D, F, and H). The cells were treated with 1 μM **1**-Fl (A–D) and 1 μM **2**-Fl (E–H) at 37 °C for 2 h.

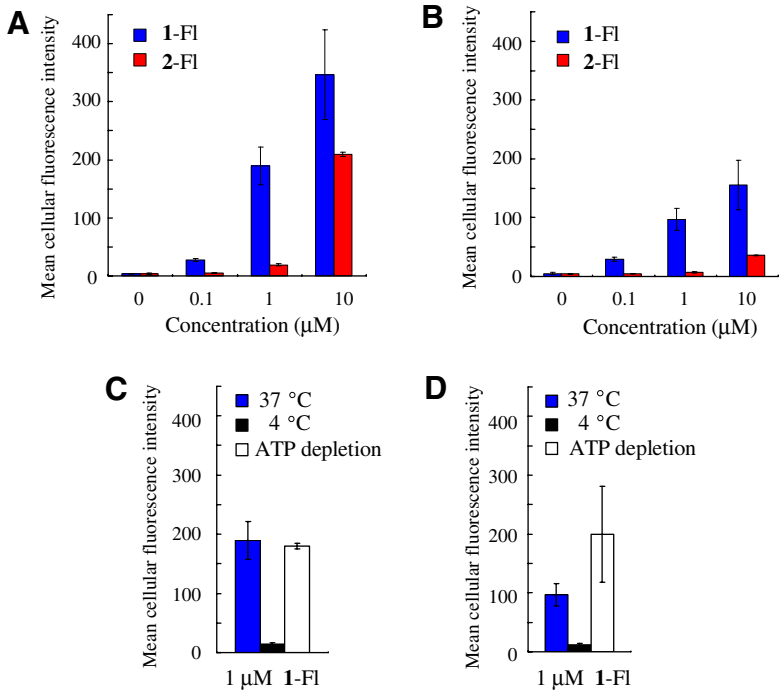

Figure 3. Mean cellular fluorescence intensities of NIH3T3 (A and C) and A549 (B and D) cells treated with fluorescein-labeled **1**-Fl and **2**-Fl as determined by FACS. (A) and (B): Average intensities of 5000 cells treated with 0-10 μM **1**-Fl (blue) and **2**-Fl (red) at 37 °C for 2 h; (C) and (D): treatment with 1 μM **1**-Fl at 37 °C for 2 h (blue); 1 μM **1**-Fl at 4 °C for 2 h (black); co-incubation of 1 μM **1**-Fl with ATP-depleting reagents, 6 mM sodium azide and 10 mM 2-D-deoxyglucose at 37 °C for 2 h (white).

tions indicate that Aib, which is more hydrophobic than Ala, may affect the interaction of the peptide with cell membrane lipids and the ability of the peptide to penetrate the membrane. Furthermore, the enhancement of **2**-Fl cellular uptake at a high concentration (10 μM) indicates that the amino acid sequence of **2** and its conformation may be essential for penetrating the cell membrane. The translocation of the Aib-containing peptide seems to involve an energy-independent process, and the detailed translocation mechanism is unclear. Further mechanism investigations are now in progress. The Aib-containing peptide having the TV-XIIa amino acid sequence may be a good delivery tool because it does not induce endosome/lysosome degradation. In the delivery of 20-mer oligonucleotides, the TV-XIIa amino acid sequences might also play an important role. To our knowledge, we have directly demonstrated using a fluorescence-labeling technique for the first time that the peptide containing an unusual amino acid residue, Aib, is taken up by living cells via a non endocytic pathway.

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Supplementary data

The details of the experiments and the FACS, CLSM, and LDH data can be found, in the online version, at doi:10.1016/j.bmcl.2008.06.005.

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