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Synthesis of a peptide that can translocate to the endoplasmic reticulum

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ABSTRACT

The accumulation of unfolded proteins in the endoplasmic reticulum (ER) leads to ER stress, which has been implicated in the development of diseases. In the present study, we synthesized a peptide that entered cells and translocated to the ER. This peptide possessed fluorescein isothiocyanate (FITC), HIV-TAT, mini-αA-crystallin, and KDEL sequences. We demonstrated that this peptide entered cells and translocated to the ER. Time course experiments revealed that this peptide existed in the ER of cos-7 cells for 16 h. Furthermore, we detected the full-length peptide in cells by fluorescent immunostaining followed by SDS-PAGE. The peptide also entered glial and neuronal cells. These results suggest that this peptide has the ability to enter cells and exert chaperone activity at the ER, and provide an insight into the development of new drugs.

1. Introduction

The endoplasmic reticulum (ER) is mainly involved in the folding of proteins. Endoplasmic reticulum stress (ER stress) has been implicated in the development of obesity, diabetes, and neurodegenerative disorders [1–6]. For example, pancreatic β cells and neuronal cell death were previously shown to be induced by ER stress, and subsequently developed into diabetes and neurodegenerative disorders, respectively [7,8]. ER stress has been attributed to the accumulation of unfolded proteins, which is mediated by stress stimuli such as viral infections, inflammation, and toxic substances. Eukaryotic cells activate the unfolded protein response (UPR) to avoid these stresses. When unfolded proteins accumulate in the ER, cells activate UPR to remove or repair unfolded proteins, thereby avoiding stress [9]. For example, cells activate a molecular chaperone such as glucose-regulated protein 78 (GRP78) to reduce the accumulation of unfolded proteins. However, when ER stress exceeds the capacity of cells to prevent stress, apoptosis is activated.

Several chemical compounds have been shown to assist in protein folding, and are referred to as “chemical chaperones”. For example, 4-phenylbutyrate (4-PBA), tauroursodeoxycholic acid (TUDCA), and trimethylamine N-oxide (TMAO) have been identified as chaperones [10–12]. We recently demonstrated that flurbiprofen exhibited chaperone activity [13]. Therefore, compounds with chaperone activity may be able to inhibit ER stress by ameliorating the accumulation of aggregated proteins. However, it currently remains unknown whether these compounds specifically translocate to the ER and function as chaperones.

In the present study, we synthesized a new peptide that had the ability to translocate to the ER. This peptide was designed to enter cells and translocate to the ER selectively. This peptide possessed fluorescein isothiocyanate (FITC), HIV-TAT, mini-αA-crystallin, and KDEL sequences. HIV-TAT is a cell-penetrating peptide (CPPs) that is abundant in basic amino acids [14]. Previous studies suggested that HIV-TAT entered cells through macropinocytosis [15,16]. Therefore, the insertion of this sequence permitted the peptide to enter cells. We also incorporated β-alanine into this peptide to increase its stability and transitivity into cells. When β-alanine was attached to octa arginine (R8), which is one of the CPPs, its stability and transitivity into cells were found to increase [17]. Therefore, we attached β-alanine to the HIV-TAT sequence. Mini-αA-crystallin is a part of αA-crystallin, a heat shock protein (HSP) with chaperone activity [18]. Since chaperone activity is only exerted by a partial region of αA-

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crystallin, called mini-αA-crystallin [19], we incorporated these minimal sequences into this peptide. The KDEL sequence has been identified as an ER retention sequence [20]. Therefore, we incorporated these sequences into the peptide. In the present study, we investigated whether this new peptide entered cells and localized to the ER efficiently.

2. Materials and methods

2.1. Synthesis of the peptide

We designed the peptide sequence of N-terminal-FITC-Ahx-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-βAla-Asp-Phe-Val-Ile-Phe-Leu-Asp-Val-Lys-His-Phe-Ser-Pro-Glu-Asp-Leu-Thr-Val-Lys-Asp-Glu-Leu −C-terminal. Ahx (6-aminohexanoic acid) was the linker sequence for FITC. The HIV-TAT sequence was Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg. The mini-αA-crystallin sequence was Lys-Phe-Val-Ile-Phe-Leu-Asp-Val-Lys-His-Phe-Ser-Pro-Glu-Asp-Leu-Thr-Val-Lys. The lysine residue of the N-terminal of mini-αA-crystallin was changed to aspartic acid because it was previously shown to increase hydrophilicity without decreasing chaperone activity [18]. The KDEL sequence was Lys-Asp-Glu-Leu-C terminal. The peptide was synthesized at JBios [Saitama, Japan] and dissolved in acetic acid. The final concentration of acetic acid was 10.82%.

2.2. Cell culture

Cos-7 and HT-22 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum, penicillin 100 U/mL, and streptomycin 100 μg/mL. U251 cell lines were cultured in DMEM containing 10% fetal calf serum. They were maintained at 37 °C in humidified 5% CO_2/95% air. The medium were changed to DMEM containing 0 or 2% of fetal calf serum for fluorescent immunostaining and SDS PAGE, respectively.

2.3. Fluorescent immunostaining

We used a protein disulfide isomerase (PDI) antibody (Thermo Fisher scientific; diluted to 1: 1000) to stain the ER. Hoechst 33342 (Dojindo; diluted to 1: 1000) was used to stain the nucleus. After cells were treated with this peptide, they were washed with PBS (0.8% NaCl, 0.02% KCl, 0.11% Na_2HPO_4, and 0.02% KH_2PO_4). They were then fixed with methanol and fluorescent staining was observed under a microscope (BZ-9000, KEYENCE, Osaka, Japan). We also used the ER-ID Red assay kit (Enzo Life Science, New York) to stain the ER.

2.4. SDS-PAGE and detection of FITC

We prepared lysis buffer containing 20 mM HEPES-NaOH (pH 7.5), 300 mM NaCl, 2 mM EDTA, 2 mM Na_3VO_4, 20 mM NaF, 2% NP-40, 20 ng/mL aprotinin, 20 ng/mL leupeptin, and 2 mM PMSF. After treating cells with the peptide, they were washed with PBS and lysis buffer was added. After a 20 min incubation on ice, the lysates were collected and centrifuged at 15,000 rpm for 20 min at 4 °C. The supernatants were boiled with Laemmli buffer for 3 min. The samples were fractionated by SDS-PAGE and FITC was detected by laser scanner Typhoon FLA 7000 (GE Healthcare Life Science, Tokyo, Japan).

3. Results

3.1. The peptide entered cos-7 cells and translocated to the ER

Cos-7 cells were treated with the peptide for 1 h (5 μM) and the ER was stained with a PDI antibody or the ER-ID Red assay kit (Fig. 1A and B). PDI predominantly exists in the ER. ER-ID specifically stains the ER in living and fixed cells. Hoechst 33342 binds to double strand DNA, thereby staining the nucleus. FITC was shown by green fluorescence. ER staining was shown with red fluorescence. Nucleus staining was shown with blue fluorescence. We confirmed that green fluorescence was not detected without this peptide (Fig 1A). We observed a merged photo of FITC and the PDI signal, indicating that this peptide successively entered cells and translocated to the ER (Fig. 1A and B). In addition, FITC merged with ER-ID staining, another marker of the ER (Fig 1B). Therefore, these results suggested that the peptide translocated to the ER. However, we also observed slight staining in the nucleus with this peptide, suggesting that this peptide also translocated to the nucleolus.

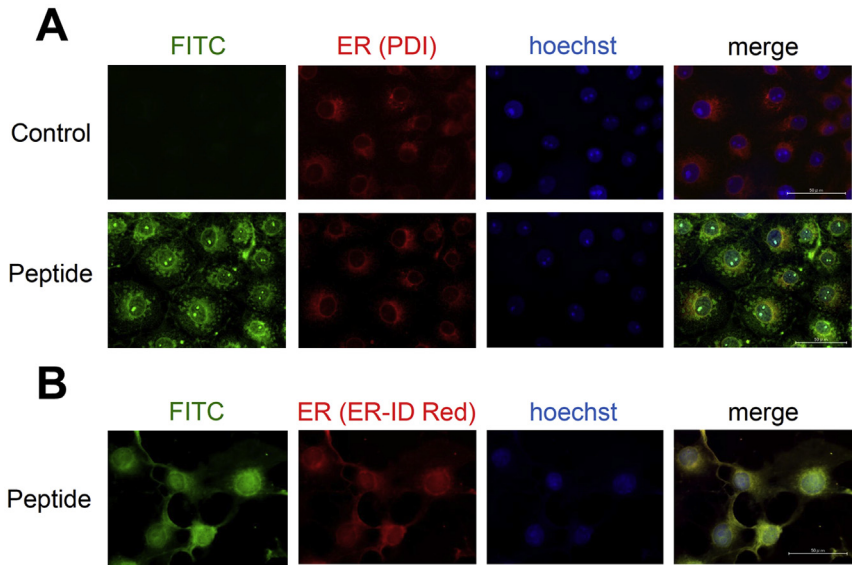

Fig. 1. The peptide entered cells and translocated to the ER. Cos-7 cells were treated with the peptide (5 μM) for 1 h and the ER was stained with (A) a PDI antibody (red) or (B) ER-ID (red). The FITC-labeled peptide (green) was detected in cos-7 cells. Scale bar, 50 μm.

3.2. Time course experiment of peptide translocation

We performed time course experiments to analyze the stability of the peptide. Cos-7 cells were treated with the peptide for 1, 4, and 16 h (5 μM), and stained with ER markers (Fig. 2A and B). FITC and the ER signal were merged at all the time points investigated (Fig. 2A and B). A decrease was not observed in the intensity of the FITC, even at the longer time point at 16 h. Therefore, we concluded that this peptide existed in the ER of cells for 16 h.

3.3. The peptide entered glial and neuronal cells

We demonstrated that the peptide entered cos-7 cells. To elucidate whether it also entered other cell lines, we treated U251 and HT-22 cell lines, which are human glioblastoma and immortalized mouse hippocampal neuronal cell lines, respectively, with the peptide. U251 and HT-22 cells were treated with the peptide for 1 h (5 μM), and then stained with ER-ID (Fig. 3A and B). Cells were stained with the FITC signal, suggesting that the peptide efficiently entered these cells. Furthermore, we observed a merged signal with FITC and ER-ID, indicating that the peptide translocated to the ER in U251 and HT-22 cells (Fig. 3A and B). Therefore, we concluded that this peptide also entered U251 and HT-22 cells and translocated to the ER. These results suggested that the peptide successfully entered glial and neuronal cells.

3.4. The full-length peptide may enter and exist in cells

We determined whether the full-length peptide existed in cells after the treatment. Cos-7 cells were treated with the peptide for 0 and 4 h (5 μM), lysed, and fractionated by SDS-PAGE, and the FITC signal was detected by a laser scanner. We loaded a recombinant peptide as a positive control. As shown in Fig. 4, we observed the 4.7 kDa band of the peptide, which was detected at a similar molecular weight to that of the positive control. Therefore, we concluded that the full-length peptide existed in the cell. On the

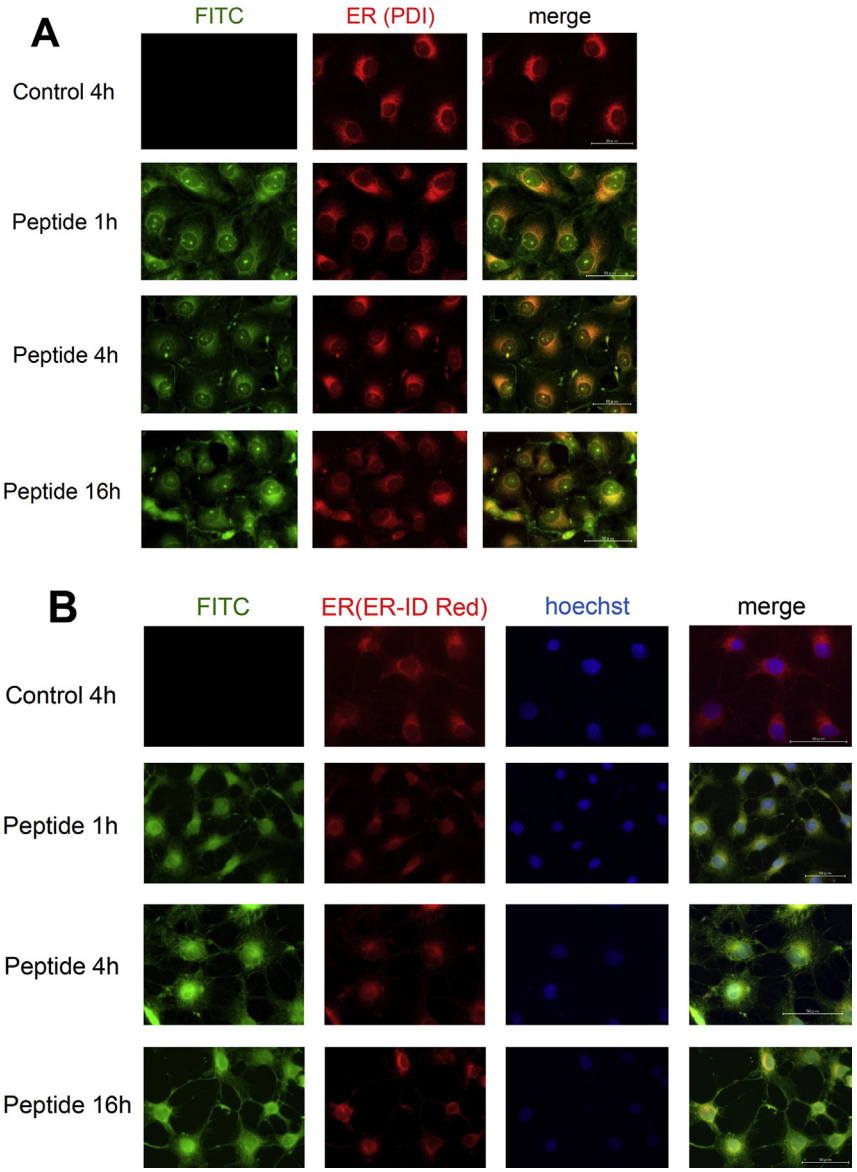

Fig. 2. Time course experiment of peptide translocation. Cos-7 cells were treated with the peptide (5 μM) for 1, 4, and 16 h. The ER was stained with (A) a PDI antibody (red) or (B) ER-ID (red). The intensity of the FITC-labeled peptide did not decrease in cos-7 cells at any of the time points investigated. Scale bar, 50 μm.

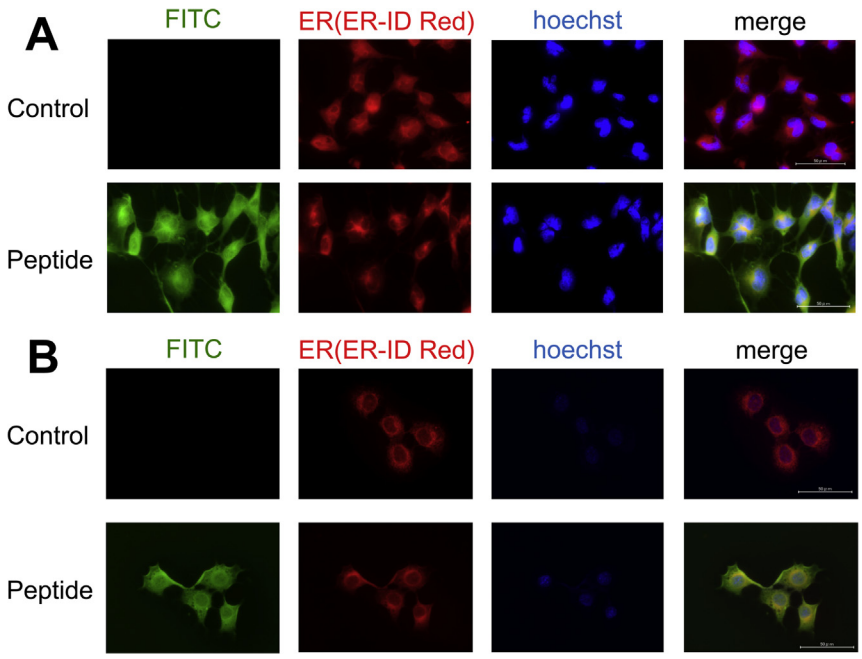

Fig. 3. The peptide entered U251 and HT-22 cells. (A) U251 and (B) HT-22 cells were treated with the peptide (5 μM) for 1 h. The ER was stained with ER-ID (red). The FITC-labeled peptide was detected in U251 and HT-22 cells. The peptide entered glial and neuronal cells and translocated to the ER. Scale bar, 50 μm.

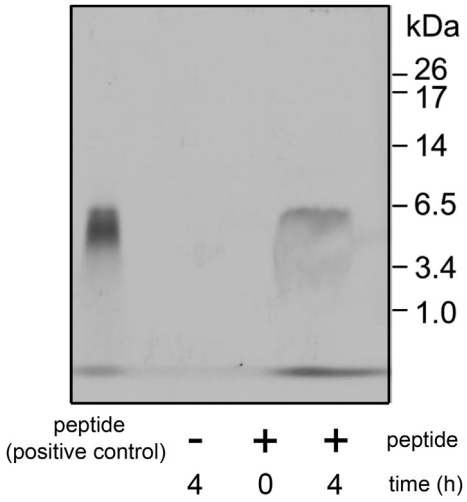

Fig. 4. Detection of the full-length peptide in cells. Cos-7 cells were treated with the peptide (5 μM) for 0 and 4 h. The FITC-labeled peptide was detected by a laser scanner, following by SDS-PAGE. The full-length peptide band (4.7 kDa) was detected after the treatment. The fluorescence of FITC was not detected in control cells briefly treated with the peptide (0 h). Therefore, the FITC signal detected under the present conditions may not have been due to the signal detected from the peptide attached to the cell surface.

other hand, FITC was also detected at a lower molecular weight, suggesting partial degradation of the peptide.

4. Discussion

In the present study, we demonstrated that a peptide with HIV-TAT, mini-αA-crystallin, and KDEL sequences entered cells and efficiently translocated to the ER. This result suggested that peptide can be delivered to the ER. Furthermore, we succeeded in delivering the peptide into various cell lines, such as cos-7, U251, and HT-22 cells. Furthermore, the peptide entered neuronal cells. Since neurons are vulnerable to ER stress, resulting in neurodegenerative diseases, our results provide an insight into the development of new drugs. The delivery of a peptide sequence with chaperone activity into the ER may be useful for the treatment of ER stress-related diseases. Although disorders associated with ER stress are known to be caused by the accumulation of unfolded proteins, drugs that can efficiently ameliorate ER stress do not currently exist. If this peptide can reduce the severity of ER stress, it may lead to the development of novel drugs for the treatment of obesity, diabetes, and neurodegenerative disorders.

Conflict of interest

The authors declare no conflict of interest.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.080.

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