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Improved cytosolic translocation and tumor-killing activity of Tat-shepherdin conjugates mediated by co-treatment with Tat-fused endosome-disruptive HA2 peptide

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Abstract

Tat peptides are useful carriers for delivering biologic molecules into the cell for both functional analysis of intracellular disease-related proteins and treatment of refractory diseases. Most internalized Tat-fused cargos (Tat-cargos) are trapped within the endosome, however, which limits the biologic function of the cargo. In this study, we demonstrated that Tat-fused HA2 peptide ($HA2^{Tat}$), an endosome disrupted peptide, enhanced the endosome-escape efficiency of Tat-cargos. In cells treated with a mixture of fluorescein isothiocyanate-labeled Tat and $HA2^{Tat}$, widespread fluorescence was observed throughout the cytosol. In addition, this $HA2^{Tat}$-mediated cytosolic delivery technique led to enhanced cytotoxicity of Tat-fused anti-cancer peptides, specifically shepherdin. Thus, we improved the function of the delivered molecules by co-treating with $HA2^{Tat}$ and propose that this is a useful method for the delivery of therapeutic macromolecules into the cytosol.

Keywords: Tat; HA2; Protein transduction domain; PTD; Shepherdin; Peptidomimetic antagonist; Peptide blocker

Progress in genomics and proteomics research has led to an increased need for functional annotation of proteomes to allow for the rational choice of particular therapeutic targets from a growing set of candidates. At the same time, the emergence of the interactome of intracellular proteins, such as signal transduction- and protein transport-related proteins, will continue to generate tremendous candidate interactions whose functions need to be clarified and validated in relation with disease [1]. Although small interference RNA- or antisense oligonucleotide-mediated gene knockdown technology are invaluable as primary tools for validation analysis [2,3], these techniques are not always useful for true functional proteomics, because they are not suitable for analysis of post-transcriptional modification, such as phosphorylation, transport to organelles, and protein degradation, and the findings do not always correlate with transcript levels. Therefore, alternative technologies are needed to clarify the function of intracellular candidates, not at the transcript level but at the protein level. Progress in this field will lead to the development of various therapeutic agents.

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Specific inhibition of protein–protein interactions by peptide-blockers, i.e., protein fragments, is a powerful methodology for investigating target validation at the protein level [4–6]. Furthermore, these peptide-blockers possess the potential to be used directly as therapeutic agents. Because many validation targets exist in the cell, it is important that peptide-blockers be delivered directly into cells. There are several recent reports of potent delivery vehicles, known as protein transduction domains (PTD), that can deliver bulky molecular cargos, such as peptides, proteins, oligonucleotides, and nano-particles, into a wide variety of cell types [7–9]. The best-known example of a PTD is the 11 amino acid sequence (Tat; YGRKKRRQRRR) derived from the human immunodeficiency virus type 1 (HIV-1)-Tat protein, and Tat-mediated delivery of peptide-blockers is thought to be useful for evaluating intracellular candidate proteins and developing peptide-based novel therapeutic drugs [10,11]. Recent studies suggested that Tat-fused cargo import is mediated by endocytotic pathways, such as lipid raft-dependent macropinocytosis [12,13]. After internalization via the macropinocytotic pathway, cargos are carried to macropinosomes. For molecules delivered by Tat to function in the cell as both validation probes and therapeutic drugs, they must reach the cytosol. Therefore, macropinosomal escape techniques are needed for the Tat-mediated intracellular delivery of peptide-blockers.

In this context, in the present study we investigated whether the effect of co-treatment with Tat-fused endosome-disruptive peptide ($HA2^{Tat}$) and Tat-fused anti-cancer peptide-blocker shepherdin ($shepherdin^{Tat}$) induced delivery into the cytosol of tumor cells and enhanced the anti-cancer effect of shepherdin. Although cancer cell treatment with shepherdin peptides, which interfere with the binding between heat shock protein 90 (Hsp90) and survivin, promote the degradation of survivin and increase the sensitivity to apoptosis [14], it is still possible that most of the treated peptides are entrapped in the macropinosomes, thereby limiting the function of shepherdin. With this in mind, we aimed to enhance the cytosolic delivery of peptide-blockers using the N-terminal 20 amino acid peptide of the influenza virus hemagglutinin protein (HA2). HA2 is well-characterized as a pH-sensitive membrane-disruptive peptide that destabilizes lipid membranes at low pH [15,16], and a recent study showed that Tat-fused with HA2 ($HA2^{Tat}$) markedly enhances the disruption of macropinosomes [12]. Therefore, we hypothesized that co-treatment with $HA2^{Tat}$ and Tat-fused peptide-blocker $shepherdin^{Tat}$ will be a promising approach for validating intracellular targets from proteomics analysis as well as the development of effective peptide-based anti-cancer drugs. Here, we evaluated the utility of this strategy using shepherdin peptides.

Materials and methods

Cell lines. HeLa cells, human cervical carcinoma cells, and A549 cells, human lung non-small-cell carcinoma cells, were obtained from the American Type Culture Collection (Manassas, VA). HeLa cells were cultured in α-minimal essential medium (MEMα; Wako Pure Chemical, Osaka, Japan) medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. A549 cells were maintained in Dulbecco's modified Eagle's medium (Wako Pure Chemical) supplemented with 10% FBS, 1% L-glutamine, and antibiotics. These cells were cultured at 37 °C, 5% CO_2.

Synthetic peptides. All peptides used in the present study were purchased from GL Biochem Ltd. (Hiroshima, Japan) and their purities of 90% or more were confirmed by HPLC analysis and mass spectroscopy. The sequences of these peptides were YGRKKRRQRRRK-FITC for Tat-fused fluorescein isothiocyanate ($FITC^{Tat}$), GLFEAIEGFIENGWEG MIDGWYGYGRKKRRQRRR for HA2-fused Tat ($HA2^{Tat}$), KHSSG CAFL for shepherdin, and KHSSGCAFLYGRKKRRQRRR for shepherdin-fused Tat ($shepherdin^{Tat}$). The Tat sequence is underlined.

Intracellular localization analysis. HeLa cells were cultured on chamber coverglass (Nunc International, Naperville, IL) at 3.0×10^4 cells/well in MEMα supplemented with 10% FBS and incubated for 24 h at 37 °C. Peptide internalization was performed as follows. HeLa cells were co-treated with $FITC^{Tat}$ (10 μM) with or without $HA2^{Tat}$ (2 μM) in Opti-MEM I (Invitrogen, CA) containing 100 ng/ml Hoechst 33342 (Invitrogen). After incubation at 37 °C for 6 h, the medium was replaced with fresh medium and the fluorescence was observed by confocal laser scanning microscopy (Leica Microsystems GmbH, Germany) without cell fixation.

Cytotoxicity assay. HeLa or A549 cells were seeded on 96-well tissue culture plates (Nunc) at 1.0×10^4 cells/well. After incubation for 24 h at 37 °C, the cells were co-treated with various concentrations of shepherdin or $shepherdin^{Tat}$ in the presence or absence of 2 μM (for HeLa cells) or 5 μM (for A549 cells) $HA2^{Tat}$. After 6 h incubation, cell viability was measured using a WST-8 assay kit (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions.

Flow cytometry analysis. HeLa or A549 cells were seeded on 24-well tissue culture plates (Nunc) at 1.0×10^5 cells/well for 24 h at 37 °C. The cells were co-treated with 10 μM $FITC^{Tat}$ in the presence of 2 μM (for HeLa cells) or 5 μM (for A549 cells) $HA2^{Tat}$ diluted in Opti-MEM I for 6 h. Cells were washed three times with 1 mM EDTA in PBS and treated with 0.25% trypsin to remove the $FITC^{Tat}$ adsorbed on the cell surface and to harvest the cells. Fluorescence was analyzed on a FACSCalibur flow cytometer, and data were analyzed using CellQuest software (Becton–Dickinson, San Jose, CA).

Results and discussion

We first analyzed the subcellular localization of $FITC^{Tat}$ by confocal laser scanning microscopy (Fig. 1). In HeLa cells treated with $FITC^{Tat}$ alone, only punctuate fluorescence was observed intracellularly. We previously confirmed that Tat peptides co-localized in live cells to vesicles with FM4-64, which is a general endosome marker (data not shown). Together, this result and the previous observation indicate that Tat-cargo enters the cell by endocytosis, but most of it is entrapped within the endosomal vesicles. As these results demonstrated that Tat-cargo accumulated in the endosomal vesicles, which severely limited its function, a method that enhances the escape of the Tat-cargo from the endosomes into the cytosol is indispensable for the cargo to exert its function.

Recently, a method for the intracellular delivery of Tat-fused biologically active protein using membrane-disruptive HA2 was studied by several researchers. Wadia et al. reported that $HA2^{Tat}$ markedly enhanced the recombination activity of Tat-fused Cre protein [12]. Additionally, Michiue et al. succeeded in enhancing both nuclear transportation and transcription activity of PTD-fused p53,