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Note

A cytoplasm-sensitive peptide vector cross-linked with dynein light chain association sequence (DLCAS) enhances gene expression

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ABSTRACT

We previously engineered a novel, non-viral, multifunctional gene vector (STR-$CH_2R_4H_2C$) containing stearoyl (STR) and a block peptide consisting of Cys (C), His (H), and Arg (R). STR-$CH_2R_4H_2C$ forms a nano-complex with pDNA and is stabilized by electronic interactions and disulfide cross linkages. In blood, pDNA, a cytosol-sensitive gene vector, is released from the complex into the cytosol. The current study aimed to make STR-$CH_2R_4H_2C$ capable of active nuclear localization. The dynein light chain association sequence (DLCAS) was disulfide cross-linked to STR-$CH_2R_4H_2C$/pDNA through disulfide linkages, and the gene expression ability of this DLCAS cross-linked gene vector was evaluated. We examined the gene transfection efficiency of S-180 cells transfected with the STR-$CH_2R_4H_2C$/DLCAS/pDNA complex. STR-$CH_2R_4H_2C$/DLCAS/pDNA showed significantly higher and faster gene expression compared with STR-$CH_2R_4H_2C$/pDNA. We also evaluated the cellular uptake ability of STR-$CH_2R_4H_2C$/DLCAS/Cy5-labeled pDNA complex. STR-$CH_2R_4H_2C$/DLCAS/pDNA showed significantly lower cellular uptake compared with STR-$CH_2R_4H_2C$/pDNA. This result indicates that high gene expression of STR-$CH_2R_4H_2C$/DLCAS/pDNA does not facilitate its cellular uptake. In addition, the gene expression of DLCAS/STR-$CH_2R_4H_2C$/pDNA in S-180 cells pretreated with the tubulin polymerization inhibitor, nocodazole (NCZ), was significantly lower than that in the absence of NCZ. These results indicate that the high transfection efficiency of DLCAS/STR-$CH_2R_4H_2C$/pDNA is dependent on intra-cellular transport utilizing the microtubule motor protein, dynein. Taken together, our results suggest that DLCAS-modified STR-$CH_2R_4H_2C$ may be a promising gene delivery system.

1. Introduction

Gene expression of plasmid deoxyribonucleic acid (pDNA) requires efficient gene delivery into the cells and the nucleus. Gene delivery requires several essential steps, including cellular uptake, endosomal escape and nuclear localization (Yang et al., 2008). Some viruses and certain viral proteins can interact with the microtubule (MT) cytoskeleton and the MT motor protein, dynein, which mediates cargo transport from the cell membrane to the nucleus (Pouton et al., 2007; Dohner et al., 2005). Proteins such as retinoblastoma, parathyroid hormone-related protein (PTHrP) and p53 undergo nuclear import that is facilitated by the MTs/dynein retrograde motor mechanism (Roth et al., 2007; Giannakakou et al., 2000; Rathinasamy and Panda, 2008). Moseley et al. (2007) reported that dynein light chain (DLC) association sequences (DLCASs), which mediate binding of the dynein motor, can facilitate NLS-mediated nuclear import and the accumulation of endogenously expressed cellular proteins that are dependent on MTs. Furthermore, they reported that delivery of exogenous cargo to the nucleus entering the cell by protein transduction can be enhanced by attachment to a dynein-association sequence. The effectiveness of binding to this sequence is dependent on the MT network (Moseley et al., 2010).

Recently, we synthesized a disulfide-linked carrier, STR-$CH_2R_4H_2C$, which can form stable complexes with nucleic acids through ionic interactions and disulfide cross-linkages. The disulfide linkages are cleaved in reducing environments such as the cytoplasm, leading to the release of pDNA. In a previous study, we evaluated the efficacy of STR-$CH_2R_4H_2C$ as a pDNA carrier. STR-$CH_2R_4H_2C$/pDNA complexes showed significantly higher gene expression than Lipofectamine (Tanaka et al., 2010). However, this carrier cannot rapidly transport nucleic acids into the nucleus.

To overcome this limitation, DLCAS was cross-linked to STR-$CH_2R_4H_2C$ and its ability to enhance gene expression was evaluated. In addition, we confirmed the dynein-dependency of the gene expression of DLCAS on cross-linked STR-$CH_2R_4H_2C$ complexes using nocodazole (NCZ).

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Table 1
Peptide sequences used in this study.

Peptide	Sequence
STR-$CH_2R_4H_2C$	$CH_3(CH_2)_{16}$-CONH-Cys-$(His)_2$-$(Arg)_4$-$(His)_2$-Cys-COOH
C-DLCAS-C	H_2N-Cys-Lys-Ser-Ser-Glu-Asp-Lys-Ser-Thr-Gln-Thr-Thr-Gly-Arg-Cys-COOH

2. Materials and methods

2.1. Materials

Plasmid DNA (pLuc), containing a subcloned luciferase cDNA fragment at the Hind III and BamHI sites of pcDNA3.1, was amplified in *Escherichia coli* (DH5a), purified using an Endfree Plasmid Maxi kit (QIAGEN, USA), then precipitated with ethanol and diluted with Tris/EDTA buffer. Plasmid DNA concentration was determined from its UV absorption at 260 nm. C-DLCAS-C was purchased from PH Japan (Japan). The sequence of DLCAS was described previously (Moseley et al., 2007, 2010).

2.2. Synthesis of the peptide vector

The sequences of the two peptides utilized in this study are shown in Table 1. $CH_2R_4H_2C$ was synthesized using Fmoc chemistry according to our previous study (Tanaka et al., 2010) on an ABI 433A solid phase peptide synthesizer (Applied Biosystems, Japan) and purified by reverse-phase HPLC. The molecular weight of $CH_2R_4H_2C$ was 1398.9 as determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS). Stearic acid was conjugated to the N-terminus of $CH_2R_4H_2C$ using a solid-phase peptide synthesis method (Futaki et al., 2001) to generate STR-$CH_2R_4H_2C$.

2.3. Preparation of pLuc complexes

STR-$CH_2R_4H_2C$ and C-DLCAS-C were prepared by mixing pLuc and STR-$CH_2R_4H_2C$ (to generate pLuc/STR-$CH_2R_4H_2C$) at an N/P ratio of 20. After 30 min, C-DLCAS-C (1.7 μg) was added to pLuc/STR-$CH_2R_4H_2C$ and the mixture was incubated for 24 h at room temperature.

2.4. In vitro transfection

S-180 cells were cultured to 70–80% confluence in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen Co., USA) containing 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin/streptomycin (stock 10,000 U/mL, 10,000 mg/μL, Invitrogen).

S-180 cells (2×10^5 cells) were seeded onto 6-well culture plates. After 24 h incubation in DMEM containing 10% FBS, the cells were rinsed with phosphate buffered saline (PBS), then 1.9 mL of DMEM without FBS was added to each well. The pLuc complex (100 μL containing 1 μg of pLuc) was applied to each well. After 4 h, the medium was removed and replaced with 10% FBS containing DMEM for further incubation. After 4 and 20 h, the cells were washed with PBS, lysed by the addition of 100 μL of lysis buffer per well, and left for 15 min at room temperature. Cell lysates were then collected and centrifuged at 15,000 rpm for 3 min. After addition of 20 μL of luciferase substrate solution to 40 μL of cell lysate, the luciferase activity was measured using MicroLumat Plus LB96V, a chemiluminescence instrument (MicroLumat Plus LB96V; Berthold, Germany). The protein concentration of each cell lysate was determined using a standard BioRad protein assay kit (Bio-Rad, Hercules, CA, USA). The results are shown as relative light units (RLU) per mg of protein.

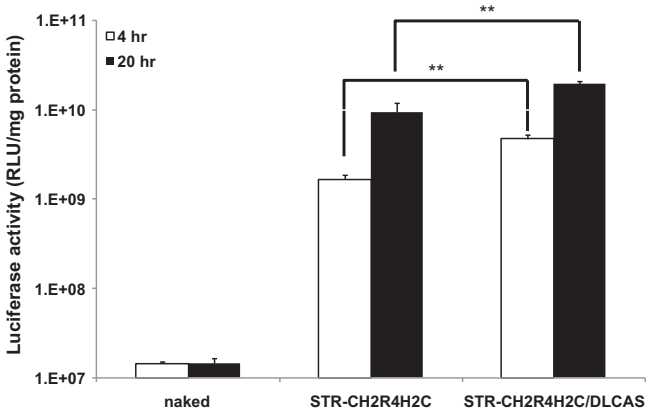

Fig. 1. pLuc (1 μg) complexes with STR-$CH_2R_4H_2C$ (N/P ratio: 20) and C-DLCAS-C (1.7 μg) were transfected into S-180 cells. After transfection for 4 h or 20 h, the luciferase activity in the S-180 cells was determined. Each bar represents the mean ± S.D. ($n = 3$). $^{**}P < 0.01$ (t-test).

Nocodazole (NCZ), which inhibits tubulin polymerization, was used to evaluate the effect of dynein-based transport on transfection efficiency. S-180 cells (2×10^5 cells) were seeded onto 6-well culture plates. After 24 h incubation in DMEM containing 10% FBS, the cells were rinsed with PBS and then 1.9 mL of DMEM without FBS was added to each well. Before transfection, some cells were pre-treated with NCZ (10 μM) for 30 min, then pLuc complex (100 μL containing 1 μg of pLuc) was applied to each well and the cells were incubated in the presence or absence of NCZ during transfection. After transfection for 4 h, the medium was replaced with DMEM containing 10% FBS and the cells were incubated for 4 h. Results are expressed as a percentage of light units (RLU) per mg of protein relative to the control cells that were incubated under normal *in vitro* transfection conditions.

2.5. Cellular uptake assay

S-180 cells (2×10^5 cells) were seeded onto 6-well culture plates. After a 24-h incubation in DMEM containing 10% FBS, the cells were washed with PBS, and 1.9 mL of DMEM without FBS was added to each well. For transfection of the pLuc complexes, 100 μL containing 1 μg of pLuc was applied to each well. Plates were incubated for 4 h, then the culture medium was aspirated and the cells were washed with PBS. After detachment by pipetting and resuspension in PBS, the cells were analyzed using flow cytometry (BD FACSCanto, Japan).

2.6. Statistical analysis

All data are expressed as the mean ± S.D. Statistical analysis of the data was performed using an unpaired Student's t-test. Statistical significance was defined as $^{*}P < 0.05$ and $^{**}P < 0.01$.

3. Results and discussion

3.1. In vitro transfection

The transfection experiments were performed using a luciferase reporter gene assay in S-180 cells. To evaluate the time-dependence of gene expression, we measured the luciferase activity at 4 and 20 h after transfection. At each time point, DLCAS cross-linked to the STR-$CH_2R_4H_2C$/pDNA (STR-$CH_2R_4H_2C$/DLCAS/pDNA) complex showed higher luciferase activity than the STR-$CH_2R_4H_2C$/pDNA complex (Fig. 1). We also evaluated the physical properties of each complex. The diameters of the STR-$CH_2R_4H_2C$/DLCAS/pDNA com-

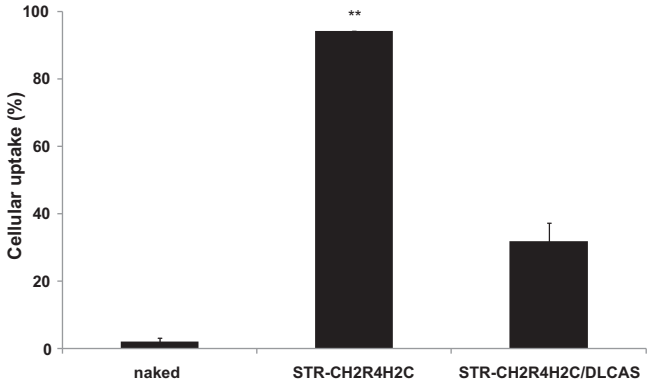

Fig. 2. Cy5-pDNA (1 μg) complexes with S STR-$CH_2R_4H_2C$ (N/P ratio: 20) and C-DLCAS-C (1.7 μg) were transfected into S-180 cells. After transfection for 4 h, cellular uptake ability was determined by flow cytometry. Each bar represents the mean ± S.D. ($n = 3$). $^{**}P < 0.01$ (t-test vs. cellular uptake of STR-$CH_2R_4H_2C$/DLCAS).

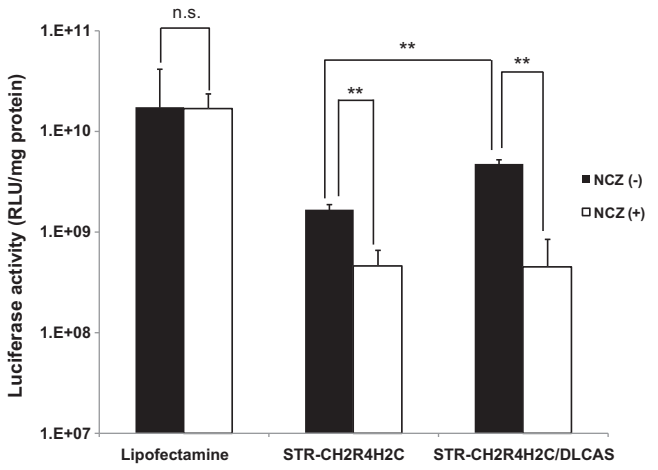

Fig. 3. pLuc (1 μg) complexes with STR-$CH_2R_4H_2C$ (N/P ratio: 20) and C-DLCAS-C (1.7 μg) were transfected into S-180 cells with or without added NCZ. After transfection for 4 h, luciferase activity in the S-180 cells was determined. Each bar represents the mean ± S.D. ($n = 3$). $^{**}P < 0.01$ (t-test).

plex and the STR-$CH_2R_4H_2C$/pDNA complex were 113.0 ± 60.6 nm and 160.1 ± 59.8 nm, respectively, and the zeta-potentials of the STR-$CH_2R_4H_2C$/DLCAS/pDNA complex and the STR-$CH_2R_4H_2C$/pDNA complex were 28.5 mV and 27.9 mV, respectively. Furthermore, we determined the cellular uptake ability of the STR-$CH_2R_4H_2C$/DLCAS/Cy5-labeled pDNA complex (Fig. 2). STR-$CH_2R_4H_2C$/DLCAS/pDNA showed significantly lower cellular uptake compared with STR-$CH_2R_4H_2C$/pDNA, indicating that high gene expression of STR-$CH_2R_4H_2C$/DLCAS/pDNA does not facilitate its cellular uptake, but may improve intracellular traffic by facilitating cellular transport and localization to the nucleus using the MT motor protein, dynein. In addition, the luciferase activity of the STR-$CH_2R_4H_2C$/DLCAS/pDNA complex 4 h after transfection was significantly higher than that of the STR-$CH_2R_4H_2C$/pDNA complex, indicating that DLCAS modified complexes can reach the nucleus faster than unmodified complexes.

In a previous study, Barbar (2008) reported that sequences including DLCAS can bind to LC8, which is an essential component of the MT-based molecular motor, dynein. Dynein has diverse roles in the cell and varying subcellular localization, including the cell nucleus, and much of its activity is regulated through LC8 binding. Modification of DLCAS in the complexes may change not only the efficacy of nuclear transportation, but also intracellular trafficking to other organelles and other subcellular functions.

We also evaluated transfection efficiency in other cell lines such as COS7 and JAWS2 cells. In these cases, STR-$CH_2R_4H_2C$/DLCAS/pDNA showed higher transfection efficiency than STR-$CH_2R_4H_2C$/pDNA (data not shown). Furthermore, we evaluated the *in vitro* cytotoxicity, and the results showed that STR-$CH_2R_4H_2C$/pDNA (N/P ratio: 20) and STR-$CH_2R_4H_2C$/DLCAS/pDNA (N/P ratio: 20) did not have any cytotoxicity (data not shown). Therefore, we think the carriers used in this study are safe for cells.

3.2. Transfection mechanism

In order to examine the transfection mechanism of STR-$CH_2R_4H_2C$/DLCAS/pDNA complexes, cells were preincubated with NCZ for 30 min before transfection and 4 h during transfection. NCZ is a well-known tubulin polymerization inhibitor that can inhibit a cellular transport process involving the MT motor protein, dynein, for the length of the MT. Fig. 3 shows the luciferase activity of each complex transfected into S-180 cells with or without NCZ. The luciferase activity of Lipofectamine was the same under both conditions, whereas the luciferase activity of the STR-$CH_2R_4H_2C$/DLCAS/pDNA complex without NCZ was about ten times higher than that in the presence of NCZ. This result indicates that the gene expression of the STR-$CH_2R_4H_2C$/DLCAS/pDNA complex depends on tubulin polymerization and cellular transport via dynein-driven MTs. In addition, the luciferase activity of STR-$CH_2R_4H_2C$/pDNA complex in the absence of NCZ was about four times higher than that in the presence of NCZ (Fig. 3), suggesting that at least some of the STR-$CH_2R_4H_2C$/pDNA complexes are delivered to the nucleus via MTs. Human immunodeficiency virus (HIV) is reliant on dynein function and MTs during infection (Dohner et al., 2005). The transactivator of transcription (Tat) peptide derived from HIV is also reliant on MT dynamics (Huo et al., 2011). Similar to the Tat peptide, STR-$CH_2R_4H_2C$ comprises eight basic amino acids and may be able to interact with MTs or dynein. We speculate that STR-$CH_2R_4H_2C$ has low affinity to dynein and that some STR-$CH_2R_4H_2C$/pDNA complexes localize close to the nucleus due to transport via MT. Therefore, NCZ affected transfection efficiency of STR-$CH_2R_4H_2C$/pDNA in this study.

In conclusion, our results indicate that DLCAS cross-linkage can promote the rate and amount of gene expression of pDNA by facilitating nuclear localization involving the MT motor protein, dynein. These results suggest that DLC-AS facilitates transport to MT organizing centers (MTOCs) located near the nucleus via the MT cytoskeleton. The findings reported in this study suggest that this novel DLCAS-modified peptide vector may be useful for clinical applications such as the delivery of therapeutic genes.

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