生物機能中分子の細胞導入の分子基盤：細胞内送達ペプチドHAadの設計と評価

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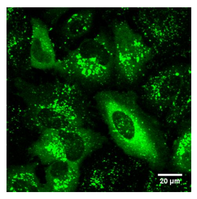
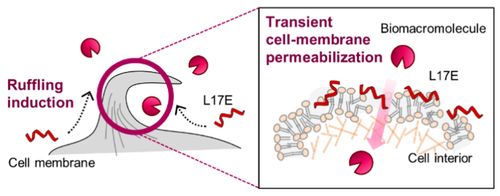
Introduction

Development of systems that effectively bring macromolecules into cytosol should be beneficial for the understanding of biological significance of cytosolic components by modulating their functions using macromolecules, and for developing therapeutic systems based on these understandings. Our laboratory developed a peptide “L17E” that delivers macromolecules into cytosol, by modifying the sequence of a hemolytic spider venom peptide M-lycotoxin (Akishiba, M., *et al.*, *Nat. Chem.* (2017)). L17E is capable of efficiently delivering various bioactive proteins, including IgG, into cytosol. Specific recognition of cytosolic proteins and suppression of glucocorticoid receptor-mediated transcription were thus achieved using the targeting IgGs in the presence of L17E.



[Cells; HeLa, L17E; 40 μM, Alexa488-hIgG; 1mg/mL, treatment; 1hr in α-MEM(-)]

In detailed analysis, we have found that L17E has an unique mechanism of action: L17E stimulates polymerization of actin followed by induction of membrane ruffling. At that site L17E transiently permeabilize the ruffled plasma membrane and macromolecules enters into cytosol within 5 min. This mode of action is thought to be the crucial mechanism for cytosolic delivery of macromolecules rather than endosomal destabilization.

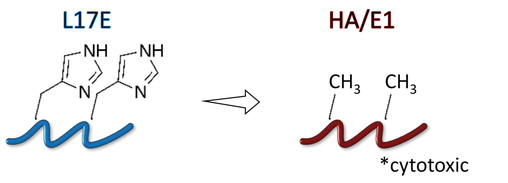


Akishiba, M. and Futaki, S., *Mol., Pharmaceutics* (2019).

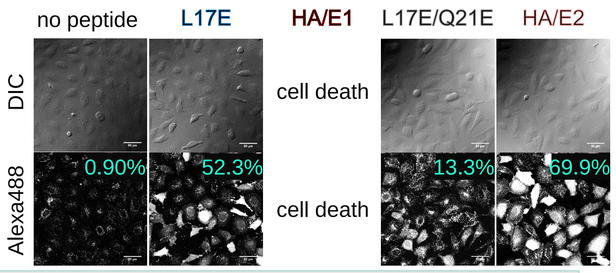
To improve the cytosolic delivery activity of L17E, we focused on the helical structure formation of the peptides dependent on endosomal pH decrease to more interact with endosomal membranes to improve the endosomolytic activity. We have succeeded in obtaining a peptide named “HAad”, by stepwise remodeling of L17E. HAad attained a significantly improved cytosolic delivery of various macromolecules (polydextran and Cre recombinase).

Results & Discussion

A. Substitution of histidine to alanine residues



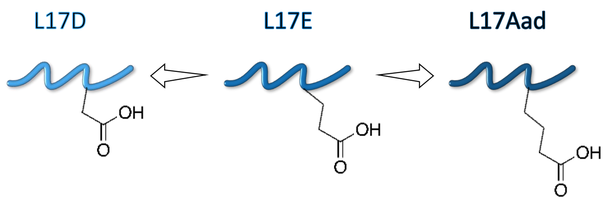
	peptide name	sequence
■	L17E	IWLTALKFLGK**H**AAK**H**E AKQQLSKL-amide
■	HA/E1	IWLTALKFLGK**A**AAK**A**E AKQQLSKL-amide
■	L17E/Q21E	IWLTALKFLGK**H**AAK**H**E AKQ**E**LSKL-amide
■	HA/E2	IWLTALKFLGK**A**AAK**A**E AKQ**E**LSKL-amide

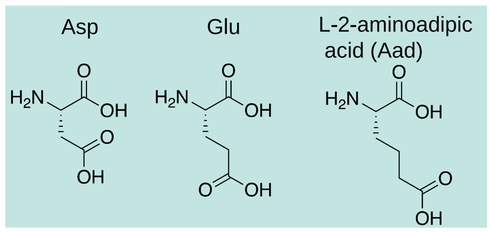


[Cells; HeLa, peptides; 40 μM, Alexa488-dextran (10 kDa); 200 μg/mL, treatment; 1 hr in serum-free α-MEM]

✓ His-to-Ala substitution yielded an improved intracellular macromolecule-delivering activity.

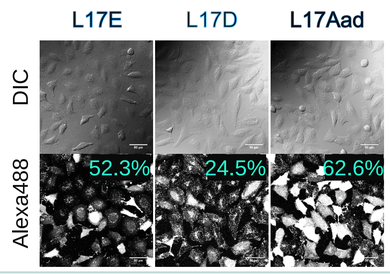
B. Introduction of acidic amino acid with longer side chain





	peptide name	sequence
■	L17E	IWLTALKFLGKHAAKH**E**AKQQLSKL-amide
■	L17D	IWLTALKFLGKHAAKH**D**AKQQLSKL-amide
■	L17Aad	IWLTALKFLGKHAAKH**X**AKQQLSKL-amide

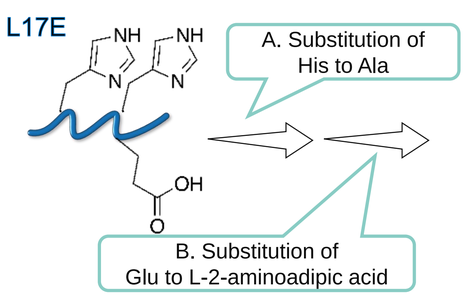
X; L-2-aminoadipic acid (Aad)

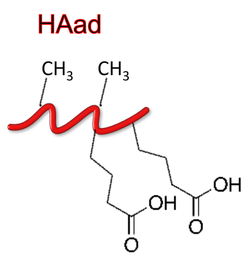


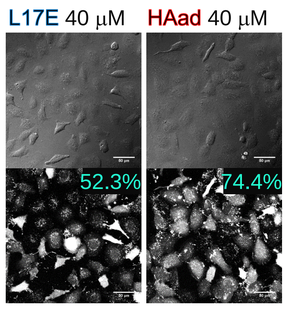
[Cells; HeLa, peptides; 40 μM, Alexa488-dextran (10 kDa); 200 μg/mL, treatment; 1 hr in serum-free α-MEM]

✓ Substitution of Glu to Aad also yielded an improved intracellular macromolecule-delivering activity.

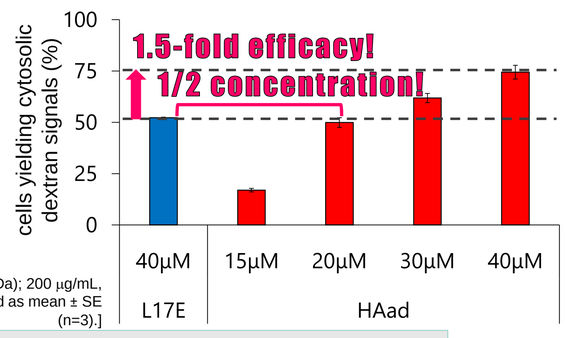
C. Combination of A and B







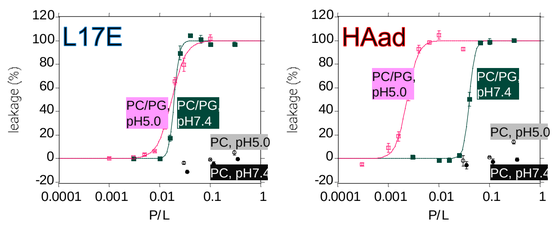
	peptide name	sequence
■	L17E	IWLTALKFLGK**H**AAK**H**E AKQQLSKL-amide
■	HAad	IWLTALKFLGK**A**AAK**A**X AKQ**X**LSKL-amide



[Cells; HeLa, peptides; 40 μM, Alexa488-dextran (10 kDa); 200 μg/mL, treatment; 1 hr in serum-free α-MEM. Results are presented as mean ± SE (n=3).]

✓ By combination of both substitutions HAad was obtained to display the highest efficacy.

D. Leakage assay

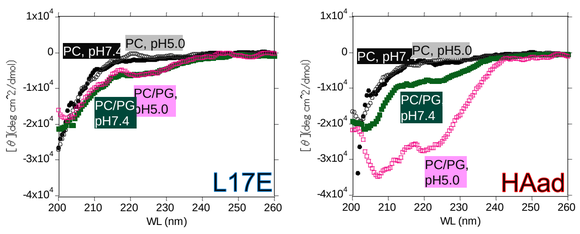


✓ L17E has no pH selectivity.

✓ HAad has negatively-charged lipid-selective and low pH-selective membrane destabilizing activity.

[lipid concentration; 200μM, lipid composition; 100% POPC or 75% POPC and 25% POPG (mol:mol) encapsulating 12.5mM ANTS/45.0mM DPX, pH; 7.4 or 5.0, incubation; 1hr at 37℃, leakage (%)=(F_{obs}-F_{blank})/(F_{max}-F_{blank})*100]

E. CD spectroscopy

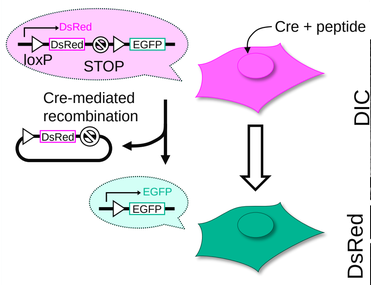


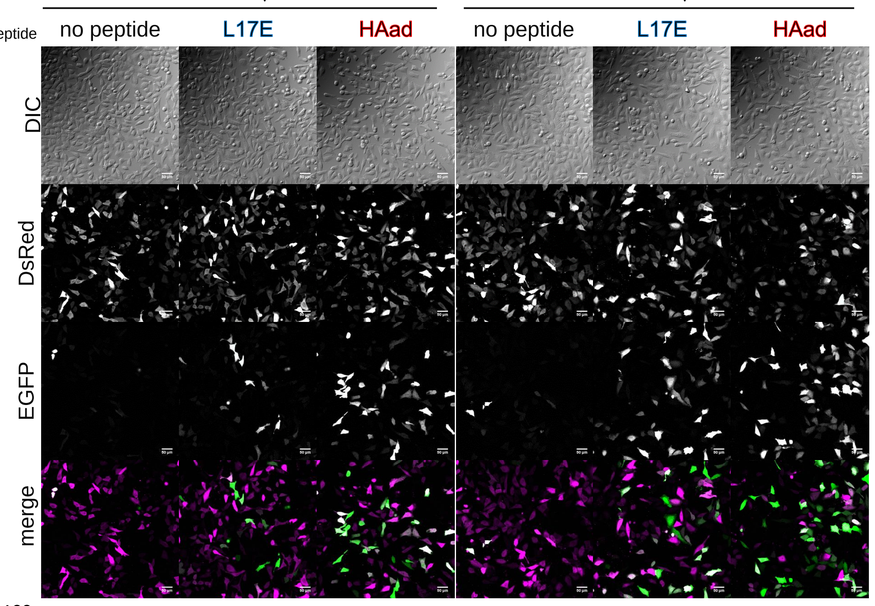
✓ L17E does not have a marked helical structure even in the presence of LUVs.

✓ HAad showed almost complete helical structure in the presence of POPG at pH 5.0.

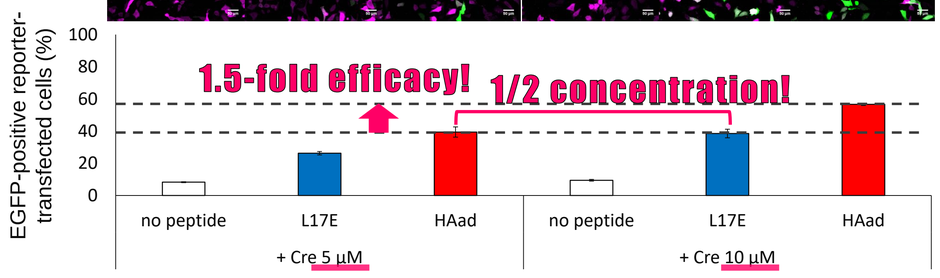
[peptide concentration; 20μM, lipid concentration; 2mM, lipid composition; 100% POPC or 75% POPC and 25% POPG (mol:mol), pH; 7.4 or 5.0, temperature; 25°C,]

F. HAad-mediated intracellular delivery of functional protein

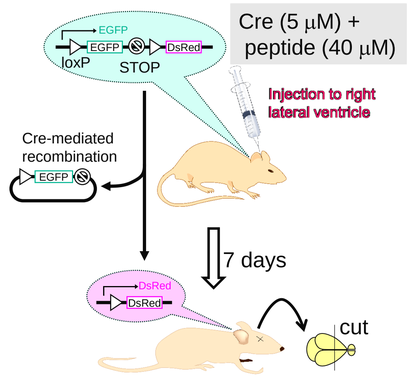




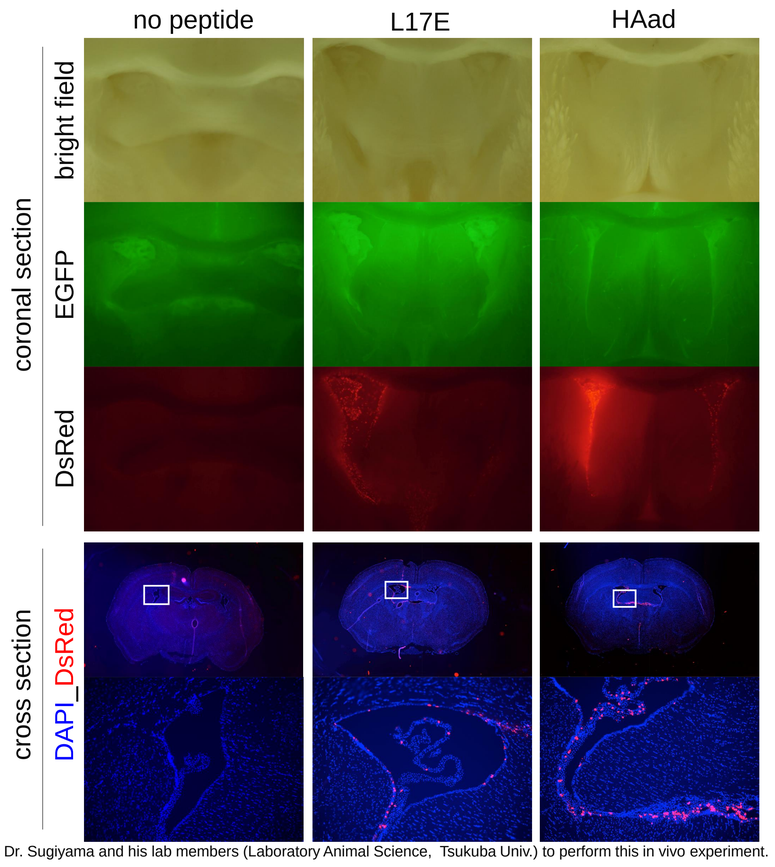
[Cells; HeLa expressing loxP-DsRed-STOP-losP-EGFP reporter, peptides; 40 μM, Cre recombinase; 5 or 10 μM, treatment; 1 hr in serum-free a-MEM. Results are presented as mean ± SE (n=3).]



G. Intracerebroventrical administration of Cre and HAad



[mouse; adult female R26GGR mouse expressing loxP-EGFP-STOP-loxP-DsRed reporter, peptide; 40 μM, Cre recombinase; 5 μM, treatment; i.c.v. administration for 7 days]

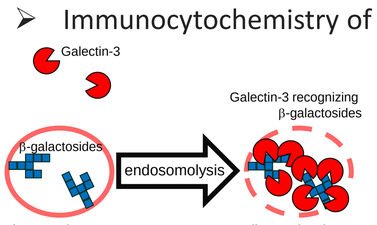


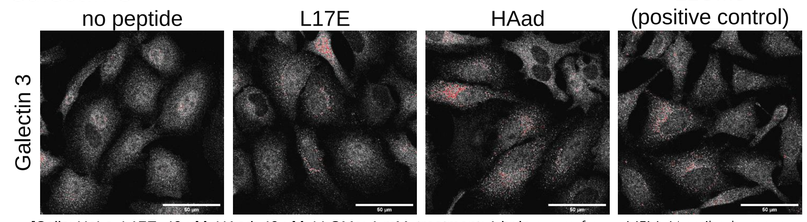
✓ HAad delivered Cre even *in vivo*.

◆ We are deeply grateful to Dr. Sugiyama and his lab members (Laboratory Animal Science, Tsukuba Univ.) to perform this in vivo experiment.

H. Mode of action of HAad

➢ Immunocytochemistry of Galectin-3

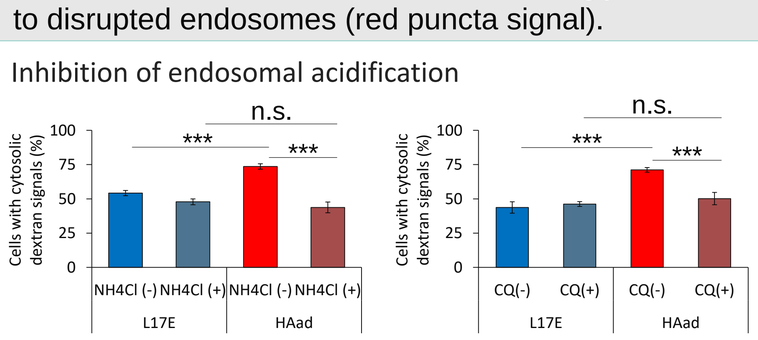




[Cells; HeLa, L17E; 40 μM, HAad; 40 μM, LLOMe; 1 mM, treatment; 1 hr in serum-free α-MEM. 1st antibody; mouse anti-Galectin 3 antibody [A3A12], 2nd antibody; Alexa Fluor 488 goat anti-mouse IgG (H+L).]

✓ In HAad-treated cells Galectin-3 was strongly localized to disrupted endosomes (red puncta signal).

➢ Inhibition of endosomal acidification



[Cells; HeLa, L17E or HAad; 40 μM, Alexa488-dextran; 200 mg/mL, NH_4Cl: 25 mM, chloroquine (CQ); 100 μM, pretreatment; 30 min w/ or w/ o NH_4Cl or CQ, treatment; 1 hr in serum-free a-MEM w/ or w/o BafA1 or CQ. Results are presented as mean ± SE (n = 3).]

✓ In the presence of endosomal acidification inhibitors, the cytosolic macromolecule-delivering activity of HAad was attenuated to the same level of L17E.

✓ The increment of the activity from L17E to HAad would be due to the obtained/enhanced endosomolytic activity.