536–537 Nucleic Acids Research, 1994, Vol. 22, No. 3

Amphiphilic peptides enhance the efficiency of liposome-mediated DNA transfection

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Received November 25, 1993; Accepted December 21, 1993

Recently, synthetic amphiphilic peptides composed of 20 amino acids, E5 (GLFEAIAEFIEGGWEGLIEG) and K5 (GLFKAIAKFIKGGWKGLIKG), derived from an influenza virus hemagglutinin have been revealed to induce the membrane fusion (1). The peptide E5 contains 5 Glu residues to be anionic and the peptide K5 contains 5 Lys residues to be cationic, and both have the same sequence except for these charged amino acids. Furthermore, both peptides can form alfa-helical structure, in which the charged amino acids (E or K) aligned on one side of the helix while the other side of the helix is very hydrophobic, which is favorable for interacting with lipid bilayers. At neutral pH's, E5 or K5 alone failed to induce the liposome fusion, however, an equimolar mixture of E5 and K5 rapidly and efficiently induced the liposome fusion. This might be due to an electrostatic interaction between the reversely charged amino acid residues (glutamic acids and lysines) on these peptides resulting in the fusion among two liposome populations (1).

Introduction of exogenous genes into mammalian cells is one of the crucial step required for molecular biology and gene engineering. Several methods for the expression of an exogenous gene in culture cells have been developed. These methods utilize polycations, calcium phosphate, retroviruses, microinjection, electroporation, protoplast, and liposomes. Among them, the liposome-mediated transfection (2, 3) has some advantages when compared to others: firstly, the method is simple and highly reproducible. Secondly, exogenous DNA's can be introduced in large number of cells, and thirdly, the liposomes are not toxic for the cells. Although the detailed mechanisms are unknown, it is suggested that the cationic charges of the lipids interact with the anionic charges of DNA to form the liposome–DNA complex followed by adsorption of the complex onto the cellular surface, and then the DNA would be incorporated into cells when the fusion between cellular membranes and liposomes occurs. Thus the substances such as E5 and/or K5 which facilitate the adsorption- and/or the fusion-processes would enhance the efficiency of DNA transfection. This prompted us to examine the effect of these peptides on the liposome-mediated DNA transfection.

For a transient transfection, the 50% confluent COS-7 cells grown on 24-well plastic tissue culture plates were washed, then incubated for 12 hr in the serum-free medium (500 μl) with 2 μg of the plasmid pmiwZ (4) and the peptides, E5 and/or K5, in the presence of liposomes made of various phospholipids (phosphatidylcholine (PC), phosphatidylserine (PS), cationic lipids mixture 'Lipofectin' (the mixture of dioleoyl phosphatidylethanolamine (DOPE) and N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA)) purchased from GIBCO BRL. The pmiwZ encodes the β-galactosidase gene under the RSV enhancer and the chicken β-actin promoter to express it constitutively. After the transfection, the cells were further incubated in a fresh medium supplemented with a serum for 48 hr. Then the β-galactosidase activity of the transfected cells was measured (Fig. 1). Only the cells transfected in the presence of Lipofectin (DOPE/DOTMA) expressed the high β-galactosidase activity, indicating that the DNA was efficiently introduced into the cells. The addition of the peptides, E5 and/or K5, at the concentration of 1 μM, resulted in 3–5 fold enhancement of the Lipofectin-mediated transfection. The omission of the plasmids from the transfection mixture yielded no increase in the endogenous β-galactosidase activity, indicating that the transfection procedure itself has no effect on the

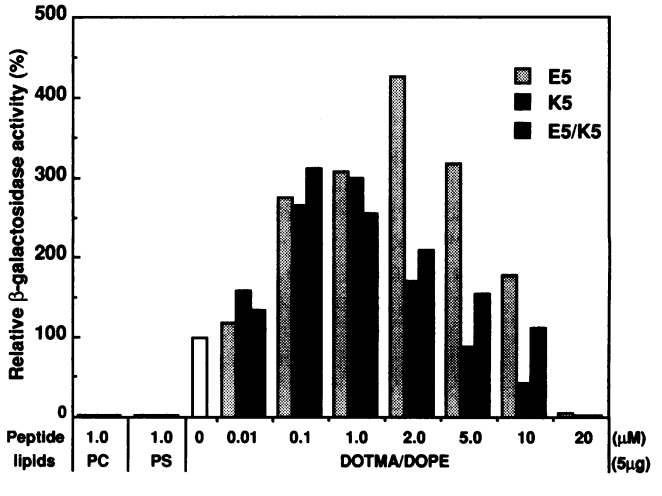

Figure 1. E5 and/or K5 enhance the liposome-mediated transient transfection. COS-7 cells were transfected with the plasmids pmiwZ using various lipids and the peptides for 12 hr, then further incubated for 48 hr. The cells were washed with 1 ml of phosphate-buffer saline twice then suspended in 600 μl of the reaction buffer (0.1 M sodium phosphate (pH 7.4), 10 mM KCl, 1 mM $MgCl_2$, 0.1% Triton X-100, and 5 mM 2-mercaptoethanol). The suspensions were frozen at −20°C and thawed at room temperature, and then centrifuged at 10,000 x *g* for 5 min to obtain the supernatant (cell extract). 100 μl of 5 mg/ml o-nitrophenyl β-D-galactopyranoside solution was added to the cell extract (500 μl), and incubated at 37°C for 30 min, then the absorbance at 415 nm was measured. The relative activities of β-galactosidase in the cell extracts were determined and calculated on the basis of those using Lipofectin without the peptides as 100%. The peptides, E5 and K5, were synthesized as described (5).

Table 1. Transient transfection of β-galactosidase gene to various cell lines

		Relative β-galactosidase activity (%)*			
Transfection Condition	no peptide	E5 (1 μM)	K5 (1 μM)	E5+K5 (1 μM each)	
COS-7	(exp. 1)	100	308	299	255
	(exp. 2)	100	176	201	160
HeLa	(exp. 1)	100	271	276	682
	(exp. 2)	100	683	611	635
LTK^-	(exp. 1)	100	419	476	388
	(exp. 2)	100	233	312	263

*The values were expressed as% of the activity of the β-galactosidase gene transfected cells without the peptides in each experiment. Cell lines of COS-7, HeLa and LTK^- were grown in the Eagle's MEM medium supplemented with 10% fetal bovine serum. All cultures were incubated in a 5% CO_2 atmosphere at 37°C.

Table II. Stable transfection of neomycin resistance gene

		Transfection Frequencies (per 10^5 cells)			
Transfection Condition	no peptide	E5 (1 μM)	K5 (1 μM)	E5+K5 (1 μM each)	
HeLa	(exp. 1)	40	100	80	80
	(exp. 2)	230	430	960	390
LTK^-		102	154	124	135
PC12		90	310	n.d.	n.d.

Cells were transfected with pSV2neo with Lipofectin and were selected in the medium containing 400 μg/ml G418 for 2 weeks. The colonies on the plates were counted after the Giemsa staining. A rat pheochromocytoma cell line PC12 were grown in the Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10% horse serum.

endogenous β-galactosidase activity in these cells. It was thus prominent that the peptides which facilitate the membrane fusion increased the transfection efficiency significantly. However, the DNA was not incorporated into the cells by the simple liposomes composed of either PC or PS even in the presence of the peptides E5 and/or K5.

The peptide E5 increased the efficiency, reaching maximal at 2 μM as the peptide concentration increased. The peptide K5 showed its maximal effect at 1 μM and also decreased thereafter. The equimolar mixture of the peptides E5 and K5 showed the maximal effect at the concentration of 0.1 μM each, however, the enhancing effect by these peptides was not additive but reduced as the K5 concentration increased. This may be due to the cytotoxic effect of K5 at its higher concentrations. In fact, the cells were detaching from the plastic plates and died in the medium containing K5 above 10 μM (data not shown). In contrast to K5, E5 yielded no cytotoxity even at 100 μM. However, higher concentrations of E5 reduced the efficiency of transfection which may be due to that the anionic charges of E5 would primarily interact with the cationic charges of the lipids resulting in the inhibition of the formation of DNA−liposome complex. In addition these peptides did not stimulate the cell growth during the incubation period of the transfection procedure. The ability of the peptides E5 and K5 to facilitate the Lipofectin-mediated DNA transfection was determined with various cell lines (Table I). These peptides enhanced the transfection efficiency about 6−7 fold in HeLa cells, and 3−5 fold in LTK^- cells, respectively. Thus the peptides yield a significant positive effect on the liposome-mediated transfection of DNA with wide variety of cell lines.

We further estimated the effect of the peptides E5 and K5 on the stable transfection. The cells grown on 6 cm plastic plate were transfected with the plasmids pSV2neo (6). The mixture (50 μl) containing the plasmids (2 μg) and Lipofectin (10 μg) was added to the cells in 3 ml of serum-free medium, and incubated for 12 hr. Then the medium was replaced with the fresh serum-containing medium and incubated for 2 days. The stable transformant cells were selected by the incubation in the medium containing G418 (400 μg/ml) for 2 weeks. The number of the G418-resistant colonies were counted after Giemsa staining. The number of neomycin-resistant colony increased when the cells were transfected with the neomycin-resistant gene in the presence of E5 and/or K5 (Table II). A significant increases in numbers of the neomycin-resistant colony was observed with various cell lines. Thus the peptides E5 and K5 remarkably enhance not only the transient transfection but also the stable transfection of DNA's mediated by cationic liposomes. This will provides another useful and reliable tool in molecular and cellular biological studies.

REFERENCE

1. Murata,M., Kagiwada,S., Takahashi,S. and Ohnishi,S. (1991) *J. Biol. Chem.* **266**, 14353−14358.
2. Felgner,P.L., Gadek,T.R., Holm,M., Roman,R., Chan,H.W., Wenz,M., Northrop,J.P., Ringold,G.M. and Danielsen,M. (1987) *Proc. Natl. Acad Sci. USA* **84**, 7413−7417.
3. Behr,J.P., Demeneix,B., Loeffler,J.R. and Perez-Mutul,J. (1989) *Proc. Natl. Acad Sci. USA* **86**, 6982−6986.
4. Suemori,H., Kadodawa,Y., Goto,K., Araki,I., Kondoh,H. and Nakatsuji,N. (1990) *Cell Differ. Dev.* **29**, 181−186.
5. Takahashi,S. (1990) *Biochemistry* **29**, 6527−6264.
6. Southern,P.J. and Berg,R. (1982) *J. Mol. Appl. Genet.* **1**, 327−341.