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Importance of Hydrophobic Region in Amphiphilic Structures of α-Helical Peptides for Their Gene Transfer-Ability into Cells

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It has been showned that cationic α-helical peptides can be useful as nucleic acid-carrier molecules for gene transfer into cells. In order to investigate the significancemake sure of importance of the hydrophobic region in amphiphilic peptides in relation to their transfection ability, we have employed five kinds of peptides with a systematically varied hydrophobic-hydrophilic balance in the amphiphilic structures, and have evaluated the relationship between the structure and the gene transfer ability of the peptides into COS-7 cells. The peptides with a large hydrophobic region took α-helical structures, formed large aggregates and showed high transfection efficiency. Their high efficiency can be explained on the basis of their ability to form stable aggregates which can be internalized by endocytosis and remain resistant to digestion in lysosomal vesicles. Furthermore, it was suggested that the hydrophobic region of peptides plays an important role in the disruption of the endosomal membrane, which ca prevent the degradation of DNA in lysosomal vesicles. When peptides do not have so strong membrane-disruptive activity, but form aggregates which can be incorporated by endocytosis, the transfection efficiency can be recovered by the addition of an endosome-disruptive peptide.

Novel gene transfer techniques which contribute to advancement in the treatments of both inherited and acquired diseases have recently attracted much attention. Most ongoing therapy protocols rely on several recombinant viruses. On the other hand, the development of nonviral gene transfer techniques has also been encouraged, particularly the use of cationic lipids (1-9), polycations such as polylysine (10), and dendritic and linear/branched polymers (11, 12). Furthermore, gene carrier molecules that incorporate some ligands have also been used in targeted gene transfer. In these systems, the ligands on the polymeric cations trigger receptor-mediated endocytosis into cells (9, 10, 13-16). In the studies of these systems, the genes transferred into cells have been found to be extensively degraded in the acidic lysosomal compartment. Most approaches to cytoplasmic delivery have used endosome-disruptive peptides derived from the amino-terminal sequence of influenza virus hemagglutinin HA-2 or anionic α-helical designed model peptides, and have introduced DNA into the cytoplasm prior to the fusion of the endosome with the lysosome (17-19). It is known that an acidic environment can trigger these peptides to disrupt liposomes (20-22).

Recently, novel nonviral gene transfer techniques, in which a cationic α-helical peptide is used as a DNA carrier into cells, have been reported (23). We have also reported the transfection technique which is mediated by some amphiphilic α-helical peptides consisting of 24 amino acids, including 6 arginines (e.g. Ac-LARL-LARL-LARL-LRAL-LRAL-LRAL-$NHCH_3$). In that study, we tested five peptides of which the chain length and the width of the hydrophobic region were changed. The peptides with long chain length and/or a large hydrophobic region could strongly bind to DNA, and the formation of large aggregates was observed in the case of the peptides with long chain length. Furthermore, the peptides with long chain length showed a high gene transfer ability into COS-7 cells. The results showedAs the results, it was found that the hydrophobic region in the amphiphilic structure of the peptides is important for the aggregation of the peptides with DNA, and the transfection ability of these peptides parallels

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their ability to form aggregates with DNA (24). Furthermore, the transfection efficiency was remarkably enhanced by the addition of an anionic α-helical peptide (LAEL-LAEL-LAEL; 4_3E) which acted as an endosome-disruptive peptide (25). In the present study, in order to clarify in detail the importance of the hydrophobic region in the amphiphilic peptide in relation to its transfection efficiency, we have employed the Hel series peptides, which consist of 18 residues, and have systematically varied the hydrophobic-hydrophilic balance in the amphiphilic structures. The structural features of these peptides and their interactions with lipid membranes have been previously studied by Kiyota et al. (26). We examined the binding abilities of the peptides to DNA, their ability to form aggregates with DNA, and their transfection ability into COS-7 cells in combination without or without 4_3E.

MATERIALS AND METHODS

Materials. Reagents used for the synthesis and analysis were of reagent grade. Amino acid derivatives were purchased from Watanabe Chemical (Hiroshima, Japan). Poly-L-lysine (15-30 kDa) was purchased from SIGMA (St. Louis, MO), and plasmid DNA which contains a luciferase gene and SV40 promoter (PicaGene control vector, PGV-C) from Toyo Ink (Tokyo, Japan). Closed circular plasmid DNA was purified by ultracentrifugation in CsCl gradients. The plasmid preparations showed a major band of closed circular DNA and minor amount (< 20%) of nicked plasmid. Lipofectin was purchased from GIBCO BRL (Gaithersburg, MD).

Peptides synthesis. Hel peptides were synthesized by Fmoc (9-fluorenylmethylcarbonyl) chemistry starting from Fmoc-(Leu or Lys)-PEG-PS (polyethylene glycol-polystyrene)-resin and purified by HPLC with reversed-phase column (20 × 250 mm, YMC C8) as described by Kiyota et al. (26). Peptide concentrations in solution were determined from UV-absorbance of Trp at 280 nm in buffer (ϵ = 5500) containing 6M Gu·HCl.

DNase I protection assay. The DNA-binding abilities of the peptides were evaluated by the nuclease-inhibitory activities (24). The tests were performed by mixing 0.5 μg of the plasmid DNA (PGV-C) with the peptides, in which the positive (peptide)/negative (DNA) charge ratios were 0, 0.10, 0.25, 0.50, 1.0, 2.0 and 4.0, respectively, in 45 μl of HBS (21 mM Hepes-KOH buffer containing 135 mM NaCl, 5.0 mM KCl, and 0.76 mM Na_2HPO_4, pH 7.4). After 30 min at room temperature, 5 μl of a solution of 10 mM $MgCl_2$ and 10 mM $CaCl_2$ was added, followed by 5 μl of 0.5 μg/ml DNase I (Worthington, DPPF grade) in water. After 30 min at 42°C, 50 μl of a stop solution consisting of 4M ammonium acetate, 20 mM EDTA, and 2 mg/ml glycogen was added, and the reaction mixture was placed on ice. To dissociate the plasmid DNA from the peptide, 15 μl of 1% SDS was added prior to extraction with TE-saturated phenol/chloroform, followed by ethanol precipitation. The final pellet was resuspended in 25 μl of dye mixture (TBE, 0.02% bromophenolblue, and 5% glycerol). TBE consists of 90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.0. The aliquot of 5.0 μl was applied to 1% agarose gel electrophoresis.

Circular dichroism. Circular dichroism (CD) spectra were recorded on a JASCO J-720W spectropolarimeter using a quartz cell of 1.0 mm path length. The peptides were dissolved at the concentration of 10 μM in HBS (pH 7.4). Measurements were performed in the presence of the plasmid DNA at peptide/DNA charge ratio of 1.0 and in the absence of the plasmid DNA at 25 °C.

Electron microscopy. Samples were prepared by mixing 63 μM of peptide (per cationic charge concentration) and 10 μg/ml of the plasmid DNA in HBS at final concentrations. These were then left standing for 30 min at room temperature. Peptide-DNA complexes were processed for transmission electron microscopy (TEM) using a negative stain technique. Fifteen-ml drops of freshly prepared samples were placed on glow-discharged carbon-coated 200-mesh copper grids for 3 min. Solution was wicked off with filter paper and replaced with 1% aqueous uranyl acetate for 30 s. After removal of the solution, grids were rinsed in distilled water and allowed to dry. Grids were imaged in a JEOL JEM-100S TEM.

Peptide-mediated transfection. COS-7 cells, a simian kidney cell line transformed with simian virus 40 (SV40), were grown to just before confluence in 16 mm dishes in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and 60 μg/ml kanamycin in an atmosphere of 5% CO_2 at 37°C, and washed twice with 1 ml of HBS. Plasmid DNA in 125 μl of HBS and peptide in 125 ml of HBS were mixed and allowed to stand for 15 min at room temperature. The mixture was poured gently onto the cells. After incubation for 3 h at 37 °C, 1 ml of DMEM with 10% FCS was added. The medium was replaced with 1 ml of a fresh medium after 24 h, and the cells were incubated for 48 h from the first addition of the medium. Treatment of the cells with chloroquine was performed at 100 μM during transfection procedure. Harvesting of cells and luciferase assays were performed 48 h after transfection as described in the protocol of PicaGene luminescence kit (Toyo Ink; Tokyo, Japan). The light units were analyzed by luminometer (Maltibiolumat LB9505, Berthold, Germany). The light unit values shown in the figures represent the specific luciferase activity (RLU/mg protein) which is standardized for total protein content of the cell lysate. The measurement of gene transfer efficiency was performed in triplicate.

Cytotoxicity of peptides. Cytotoxicity of Hel peptides were evaluated by MTT assay (27). COS-7 cells were plated at 1 × 104 cells per well in a 96-well tray and incubated for 12 h at 37C°. The cells were incubated with the solution of the peptides or the peptide-DNA complex in 50 μl HBS without or with 100 μM chloroquine. After incubation for 3h at 37C°, 80 μl of DMEM containing 10% FCS was added. The medium was replaced with 100 ml of a flesh medium after 24 h, and the cells were incubated for 24 h. The medium was removed and replaced by 15 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in PBS. After incubation for 4 h, 100 ml of lysis solution containing 20% (w/v) SDS, 2% (v/v) AcOH, 0.08 N HCl and 50% (v/v) DMF were added to each well and allowed to stand for 24 h in the dark. Absorbaencies at 595 nm of each well were measured using a Bio-Rad Model 3550 microplate reader. Results are expressed as the percentage of dye reduction in cells treated according to the transfection protocol to dye reduction in cells treated with HBS. The measurement was performed in triplicate.

RESULTS

Peptide structures. In this study, we used five kinds of cationic α-helical peptides consisting of 18 residues (Hel series peptides) as nucleic acid-carrier molecules. The primary structures of the peptides and their helical wheel representation are shown in Fig. 1. Hel series peptides are composed of hydrophobic Leu and hydrophilic Lys residues in ratios of 13:5, 11:7, 9:9, 7:11, and 5:13 (abbreviated as Hels 13-5, 11-7, 9-9, 7-11, and 5-13, respectively). The amphiphilic α-helix structures of these peptides have a systematically varied hydrophobic-hydrophilic balance (26).

Agarose gel shift assay. The peptide-DNA complex formation was examined by the electrophoretic mobility of the complexes on an agarose gel (1% w/v) stained with ethidium bromide at the various ratios of peptides

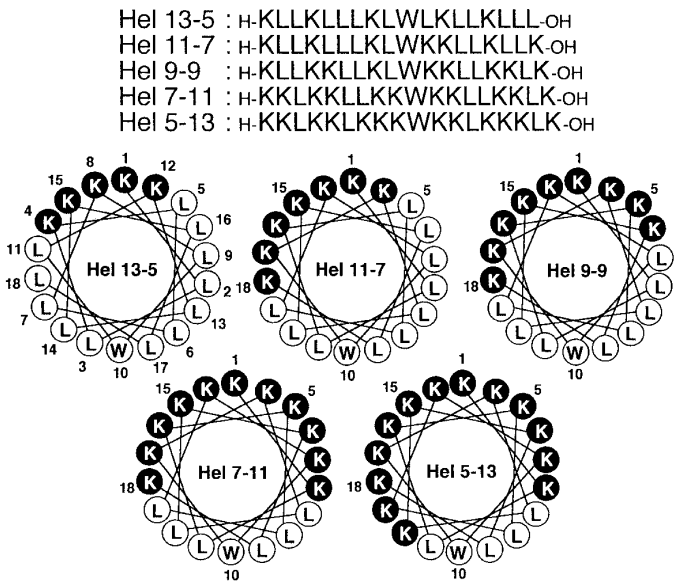

FIG. 1. Structures of the cationic α-helical peptides. Amino acid sequences of the designed peptides and illustration of α-helical wheels of the peptides.

to a double-stranded DNA. The tests were performed by mixing 0.1 mg of the plasmid DNA (PGV-C) with the cationic peptides, in which the positive (peptide)/ negative (DNA) charge ratios were 0, 0.10, 0.25, 0.50, 1.0, 2.0, and 4.0, respectively (Fig. 2A). In the case of Hels 13-5 and 11-7, no migration of the band of plasmid DNA occurred at a charge ratio of 0.5. This was probably due to neutralization of the nucleic acids by cationic peptides or the formation of a large complex between the peptides and the DNA. Hel 9-9 suppressed the migration of the DNA at a charge ratio of 1.0. In the case of Hels 7-11 and 5-13, migration of the DNA was suppressed at charge ratios of 1.0 and 0.5, respectively, and the mobilities of the DNA were gradually reduced by increasing amount of Hels 7-11 or 5-13 at charge ratios of 0.1 to 0.5.

DNase I protection assay. When the peptide-DNA complex is formed, it is expected that the digestion of DNA by DNase I is inhibited. We also evaluated the DNA binding ability of the cationic peptides by the nuclease-inhibitory activity. After adding the peptides to the plasmid DNA at various charge ratios (0, 0.1, 0.25, 0.5, 1.0, 2.0, and 4.0), the mixtures were incubated with DNase I. After the undigested DNA was extracted, the DNA was analyzed by 1% agarose gel electrophoresis (Fig. 2B). In the case of Hel 13-5, the band of the plasmid DNA was remarkably detected at a charge ratio of 0.5. Other peptides similarly protected the DNA from the DNase I at a charge ratio of 1.0.

Secondary structure of peptides. In order to analyze the structural features of these peptides which bind to the plasmid DNA, the circular dichroism (CD) spectra of the peptides was measured. In these measurements, it was hard to analyze spectra under 205 nm due to a large absorbance. As shown in Fig. 3A and B, Hels 13-5 and 11-7 showed a typical α-helix CD pattern with double minima at 208 and 222 nm in the HBS with a peptide concentration of 10 μM, while the peptides with a small hydrophobic face (Hels 9-9, 7-11, and 5-13) mainly took a random structure (Fig. 3C and D). The helical content of the peptides increased with the increasing hydrophobicity.

In the presence of plasmid DNA at a charge ratio of 1.0, remarkable changes in the CD spectra of Hels 13-5 and 11-7 were observed (Fig. 3A and B). The change in Hel 11-7, for which the valley at 208 nm became shallower, was similar to that of peptide 4_6, which is a cationic α-helical peptide that has been reported by Niidome et al. (24), suggesting an aggregation of the peptide with the DNA as described by Yoshimura et al. (28). The spectra of Hel 9-9 was moderately changed

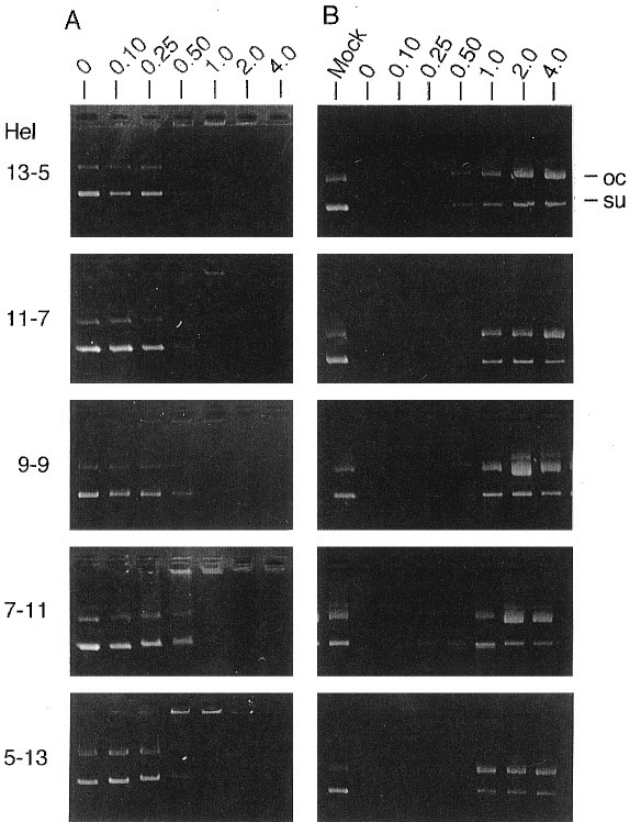

FIG. 2. Agarose gel electrophoresis. A, agarose gel shift assay. The plasmid DNA (100 ng) and several amounts of the peptides were mixed, followed by electrophoresis on an agarose gel (1% w/v) stained with ethidium bromide. The charge ratio (peptide : DNA) is indicated *above* each lane. *B,* DNase I protection assay. The plasmid DNA was preincubated with the peptides at the charge ratio indicated *above* each lane, followed by treatment with DNase I described in "Materials and methods". Mock-digested sample was applied in the *left lane.* The positions of the open coiled (*oc*) and supercoiled (*su*) forms are indicated on the *right.*

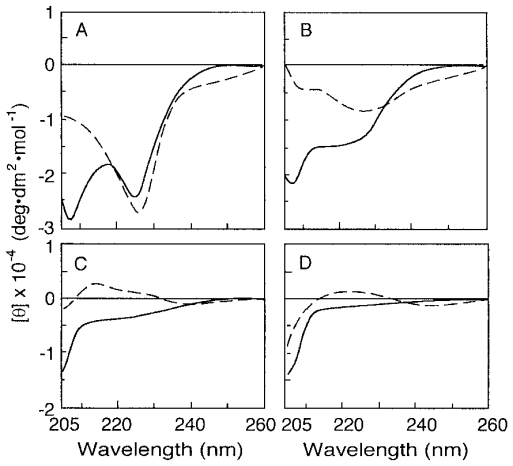

FIG. 3. Circular dichroism spectra of the peptides with or without the plasmid DNA. The peptide was dissolved at 10 μM in HBS. Measurement was performed in the absence (*solid line*) or in the presence (*broken line*) of the plasmid DNA at 25 °C. *Panel A, B, C* and *D* show the spectra of Hels 13-5, 11-7, 9-9 and 7-11, respectively. Spectrum of Hel 5-13 was similar to that of Hel 7-11.

by the addition of the DNA (Fig. 3C). In the case of Hels 7-11 and 5-13, no remarkable changes in the CD spectra were observed (Fig. 3D). The CD pattern of the B-form-like DNA, a peak and valley at 220 and 245 nm, respectively, was rather conspicuous. With the addition of Hels 7-11 and 5-13 to the DNA, little conformational change of DNA would have occured, because a peak and valley at the same wavelengths as described above were also observed in the absence of the peptides (data not shown).

Electron microscopy. To assess the structures of the peptide - DNA complexes, we used transmission electron microscopy with negative staining. Fig. 4A - E shows complexes of the peptides and the plasmid DNA at a charge ratio of 2.0. Hel 13-5 formed large aggregates with high density (Fig. 4A). Hels 11-7 and 9-9 formed aggregates in large and small sizes, respectively, but the density was lower than that of Hel 13-5 and the twisted fiber-like structure was similar to that of peptide 4_6 (24) (Fig. 4B, C). In the case of Hels 7-11 and 5-13, no large aggregates were observed. The size of the aggregates that were found was smaller than that of Hels 13-5 and 11-7, and the shapes were also different from those of Hels 13-5, 11-7, and 9-9 (Fig. 4D - E).

Transfection efficiency. COS-7 cells were transfected with 2.5 μg of plasmid DNA, which contains a reporter gene encoding firefly luciferase, complexed with the peptides at a charge ratio (peptide/DNA charge ratio) of 2.0. The efficiencies of the peptides in the expression of luciferase were determined at 48 h after transfection by measurements of the total enzyme activity in the cell extracts of the cultured cells using a luminometer. In Fig. 5A, the cross-hatched bar shows the transfection efficiency of the peptides without chloroquine treatment. Hels 13-5, 11-7 and 9-9 showed significant transfection activity among the five peptides, whereas Hels 7-11 and 5-13 had no transfection activity. The order of the transfection activity was Hel 13-5 > Hel 11-7 > Hel 9-9 and was dependent on the width of the hydrophobic region in the amphiphilic structure. Furthermore, we compared the transfection efficiency of the Hel series peptides with that of other materials, i.e., peptide 4_6, calcium phosphate, lipofectin, and polylysine as DNA-carrier reagent. Although the transfection efficiency of Hel 13-5 exceeded that of peptide 4_6 and polylysine by 8 and 80-fold, respectively, the efficiency of Hel 13-5 was about 4 and 3 times lower than that of the calcium phosphate and lipofectin methods, respectively. As shown in Fig. 5A (*open bar*), concurrent treatment of the cells with chloroquine, which may inhibit the degradation of the DNA by lysosomal hydrolases, increased the efficiencies of Hels 11-7 and 9-9 by 70 and 120-fold, respectively.

The cytotoxic activity of the peptides and the complex of the peptides and the plasmid DNA was evaluated by the MTT assay using theas same conditions as those in the transfection procedure. In the absence of DNA, Hel 11-7 had the strongest cytotoxic activity among all pep-

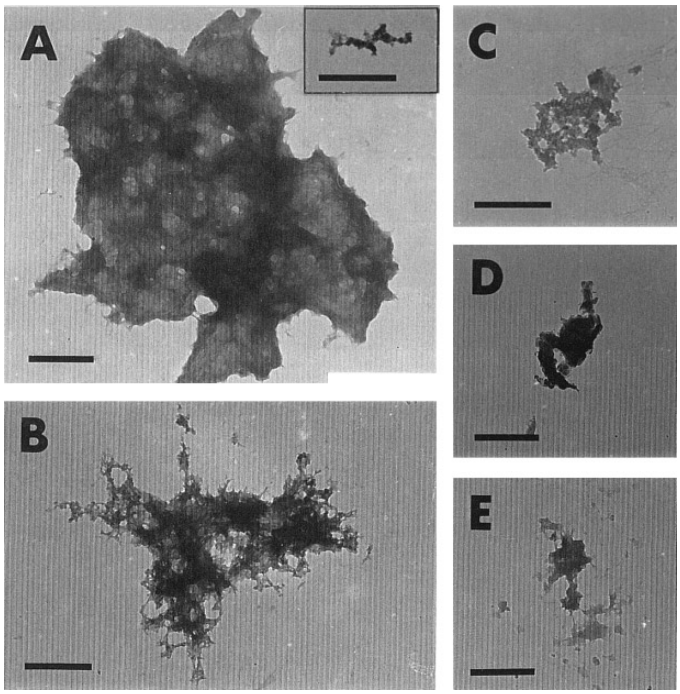

FIG. 4. Electron photomicrographs of peptide-DNA complexes. Peptide-DNA complexes were prepared at a charge ratio of 2.0 (peptide : DNA), and the methods used for electron microscopy are described in "Materials and methods". *Panels A-E* show examples of aggregations composed of Hels 13-5, 11-7, 9-9, 7-11 and 5-13, respectively, and the plasmid DNA. Bar indicates 1.0 μm. Inlet photomicrograph in *panel A* shows appearance of the plasmid DNA without peptide. *Bar* indicates 0.2 μm.

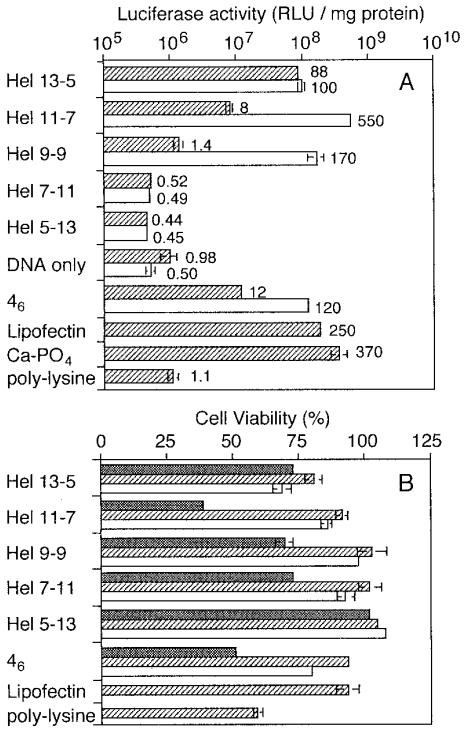

FIG. 5. Transfection efficiencies of the peptides, and the cytotoxic activities of the peptides and their complexes with the plasmid DNA. A, cells were incubated with peptide - DNA complexes containing 16 nmol (cationic charge) of the peptides and 7.9 nmol (negative charge) of the plasmid DNA (2.5 μg) (peptide : DNA charge ratio of 2.0) in the 250 ml of HBS/16 mm dish. After incubation for 3 h at 37°C, the cells were harvested in DMEM with 10% FCS, and subjected to luciferase assay as described in "Materials and methods". *Cross-hatched bars* indicate the efficiency without chloroquine treatment. *Open bars* indicate the efficiency with chloroquine treatment. The *numbers* to the *right* side of the *bars* indicate the luciferase activities (RLU/mg protein $\times 10^{-6}$). *B*, cytotoxities under individual transfection conditions were evaluated by MTT assay as described in "Materials and methods". *Shaded bars*, *cross-hatched bars* and *open bars* indicate the cell viabilities in the presence of the peptide, the peptide-DNA complex, and the peptide-DNA complex and chloroquine, respectively.

tides (40% of cell viability), and the other peptides except for Hel 5-13 had moderately cytotoxic activity (Fig. 5B, *shaded bar*). However, in the case of complexes of the peptides except for Hel 5-13 and the DNA, the cytotoxic activity decreased remarkably (about 80 % of cell viability and up) (Fig. 5B, *cross-hatched bar*). As a result of the aggregate formationforming aggregates with DNA, the hydrophobic face in the amphiphilic structure of the peptides would be buried in the complex. It is consequently considered that the reduction in the cytotoxic activity due to the formation of a complex with DNA is caused by decreasing the perturbation activity of the peptides to the cell membrane. In the case of the chloroquine treatment, the cell viabilities of the peptides except for Hel 5-13 decreased slightly (Fig. 5B, *open bar*). The increase in the cytotoxity would be due to the cytotoxic activity of the chloroquine.

Enhancement of transfection efficiency using an acidic peptide. We previously reported an enhancer peptide (LAEL-LAEL-LAEL; 4_3E), which acts as an endosome-disruptive reagent in the cells for the gene transfer mediated by an endocytosis pathway (25). The tests were performed by creating a mixture of Hel 11-7 and the plasmid DNA at a charge ratio of 2 with increasing amounts of 4_3E, so that the ratio of the negative charges of 4_3E to the positive charges of the complex of Hel 11-7 and DNA were from 0 to 16 (Fig. 6A). It was expected that the transfection efficiency of Hel 11-7 would be remarkably increased by the addition of 4_3E because Hel 11-7 showed the highest transfection efficiency in the presence of chloroquine. The enhancement effects on the transfection efficiency of Hel 11-7 clearly increased with increasing amounts of 4_3E. The enhanced efficiency by 4_3E at a charge ratio of 16 was 90-fold higher than that in the absence of 4_3E and reached the efficiency level of Hel 11-7 with chloroquine

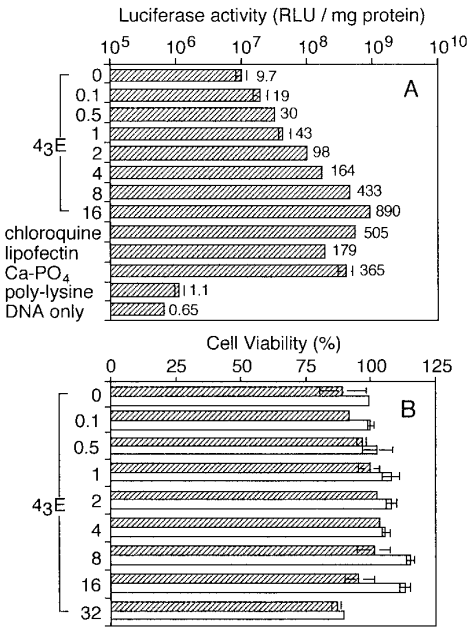

FIG. 6. Effect of 4_3E on transfection efficiency of Hel 11-7 and the cytotoxic activities of the complexes. *A*, the plasmid DNA (2.5 μg, 7.9 nmol of negative charge) and Hel 11-7 (5.0 μg, 16 nmol of positive charge) were mixed, followed by addition of several amounts of 4_3E in 250 μl of HBS. After 10 min, the mixture was added to the cells, and 3 h later, DMEM containing 10% FCS (250 μl) was added. The cells were incubated for 48 h from the first addition of the medium, and subjected to luciferase assay as described in "Materials and methods". The *numbers* on the *left* side of the *bars* indicate the charge ratio of 4_3E : complex of Hel 11-7 and DNA. Treatment of the cells with chloroquine was performed at 100 μM during the transfection procedure in the absence of 4_3E. The *numbers* on the *right* side of the bars indicate the luciferase activities (RLU/mg protein $\times 10^{-6}$). *B*, cytotoxicities under individual transfection conditions were evaluated by MTT assay as described in "Materials and methods". *Cross-hatched bars* and *open bars* indicate the cell viabilities in the presence of Hel 11-7-DNA-4_3E complex and 4_3E alone, respectively.

treatment. The efficiency exceeded that of lipofectin and calcium phosphate by 5 and 2.5-fold, respectively. Above a charge ratio of 16, it was difficult to accurately estimate the transfection efficiency due to the increase in the cytotoxity of the complex of Hel 11-7, DNA, and 4_3E. The cytotoxic activity of the complex of Hel 11-7, DNA, and 4_3E, as well as 4_3E alone, was also evaluated by the MTT assay (Fig. 6B). The cytotoxities at all charge ratios were weak.

DISCUSSION

In order to clarify in detail the importance of the hydrophobic region in the amphiphilic peptide in relation to its transfection efficiency, we have employed five kinds of peptides with a systematically varied hydrophobic-hydrophilic balance in the amphiphilic structures, and have evaluated the relationship between the structure, the DNA-binding ability, and the gene transfer ability of the peptides into COS-7 cells.

At first, in order to analyze the binding abilities of the peptides, we performed a gel mobility shift and DNase I protection assay. All peptides formed a stable complex with the plasmid DNA at charge ratios between 0.5 and 1.0. Based on the molar ratio between the peptide and the DNA, it can be said that peptides with a large number of lysine residues have strong DNA-binding ability. That is, the DNA-binding ability of a peptide depends on the number of lysine residues, and the hydrophobic region in the amphiphilic peptide scarcely affects its DNA-binding ability. However, in the results of the gel mobility shift assay, it was found that the mobilities of DNA were gradually reduced by increasing the amount of Hels 7-11 or 5-13 at charge ratios of 0.1 to 0.5. This mobility shift has been typically observed with non-α-helical cationic peptides such as Ac-$(SPKK)_6$-NH_2 and Ac-$(RPPF)_6$-NH_2 (unpublished data). It can be said that Hels 7-11 and 5-13 behave rather like a non-α-helical peptide in DNA-binding, although the peptides moderately bind to the DNA.

The CD study gave clear conformational data for the peptides. The peptides with a larger apolar face (Hels 13-5 and 11-7) took an α-helical structure in HBS. It is expected that small oligomers of the peptides were formed with a well-defined number of monomers. Therefore, the above observation suggests that Hels 13-5 and 11-7, with their larger apolar faces self-associate with their hydrophobic faces, which are formed by adopting the amphiphilic α-helical structure. Other peptides, however, with smaller apolar faces are present in a monomeric state with random structure in HBS. In the presence of the plasmid DNA, dynamic spectra changes were observed in the case of Hels 13-5 and 11-7. Hel 13-5 especially showed a large valley at 225 nm. Although we cannot fully explain the newly formed conformation, the change in the CD spectra would indicate an aggregation which is of different type from that of Hel 11-7. The observation of a peptide-DNA complex by electron microscopy also supports this explanation. On the other hand, remarkable spectra changes for the peptides with smallar hydrophobic regions (Hels 7-11 and 5-13) were not observed with the addition of the DNA. However, these peptides bound to the DNA at a charge ratio of 1.0 in the agarose gel shift and DNase I protection assays, indicating that Hels 7-11 and 5-13 mainly interact with the DNA just in random coil structures.

Differences in the binding modes of the peptides to DNA could be clearly distinguished by aggregate formation with DNA. In electron microscopy observations, the peptides with large hydrophobic regions such as Hels 13-5 and 11-7 formed large aggregates, while aggregates of the peptides with small hydrophobic regions such as Hels 7-11 and 5-13 were rarely found. These results indicate that the width of the hydrophobic region in amphiphilic α-helical structures of the peptides play an important role in the formation of large aggregates. We assume that the oligomer which self-associates with the hydrophobic face of the peptides behaves like a cationic polymer, and the large aggregates are the result of the noncovalent cross-linking of these cationic oligomers to the plasmid DNA.

The transfection ability of the peptides also depended on the width of hydrophobic region in the amphiphilic structure of the peptide. Indeed, Hel 13-5, which can form larger aggregates compared with other peptides, has the highest transfection efficiency in the absence of the chloroquine. It is likely that the formation of stable aggregates would be advantageous to the internalization of aggregates into cells by endocytosis or would make the aggregates more resistant to digestion by nucleases in the lysosomal vesicles.

The transfection efficiencies of Hels 11-7 and 9-9 were remarkably increased and exceeded that of Hel 13-5 in response concurrent treatment with chloroquine, suggesting that the internalization of the peptide-DNA aggregates are mediated by an endocytosis pathway, and a large number of the aggregates are digested by hydrolases in the lysosomal vesicles in the absence of chloroquine. Hel 13-5 forms the most stable complex with DNA, but the luciferase activity is smaller compared to Hels 11-7 and 9-9 in the presence of chloroquine. One explanation for this difference in transfection efficiencies would be that the complex of Hel 13-5 and DNA is too stable to dissociate in the cytoplasm.

Furthermore, the efficiency of Hel 13-5 showed little increase in response to the chloroquine treatment, suggesting the possibility of an additional import pathway other than endocytosis, i.e. direct penetratation into the cytosol through the cell membrane because Hel 13-5 has strong membrane-perturbation activity (26). However, since the aggregates made with this peptide were too large to penetrate through the cell membrane,

it can be assumed that the incorporation was mediated by an endocytosis pathway. The strong perturbation activity of this peptide would rather lead to escape from endosomal vesicles in the cells. As a consequence, a large amount of the peptide-DNA complex could be transported from the endosomal vesicles to the cytosol even without chloroquine treatment. Based on the perturbation activity in the absence of chloroquine, the lower transfection efficiencies of Hels 11-7 and 9-9 compared with Hel 13-5 would accounted for not only their weak aggregate formation, but also their lower membrane-disruptive activity. In the case of Hels 7-11 and 5-13, of which have a low abilityabilities to form aggregates are low, the transfection efficiencies were not influenced by the chloroquine treatment. We suppose that the complexes of these peptides with DNA were hardly incorporated into the cells.

In summary, it appears that although the hydrophobic regions of the peptides do not seriously affect the initial interactions with the DNA, they are important in the followingfor several steps of thein transfection pathway: as follows. 1) stabilization of the α-helical structure of the peptides, 2) formation of stable aggregates which can be internalized by endocytosis and remain resistant to digestion in lysosomal vesicles, and 3) disruption of the endosomal membrane, which can prevent the degradation of DNA in lysosomal vesicles. Furthermore, when peptides do not have so strong membrane-disruptive activity but instead form aggregates which can be incorporated by endocytosis, the transfection efficiency can be recovered by the addition of an endosome-disruptive peptide such as 4_3E. Based on the results of this study regarding the relationship between peptide structure and transfection ability, we have identified several important factors for efficient gene transfer into cells. The obtained information will guide us in developing a molecular design for a more efficient gene carrier molecule.

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