We have developed a novel transfection method using cationic applications as nonviral gene transfer techniques.

vitro

and show promise as vectors for ongoing gene therapy protocols rely on recombinant retrovirus, in the treatment of both inherited and acquired diseases. Most avoid such problems, the development of nonviral gene transfer
effects by random insertion into the host genome (5, 6). To generation of several types of immune response, and oncogenic production of replication-competent viruses by recombination, the possible insertion of large size DNA fragments into the gene, the possible problems were determined by the expression of luciferase from its cDNA in COS-7 cells. The long peptides showed high transfection abilities. As a result, it could be said that the transfection ability of these peptides was parallel to their ability to form aggregates with DNA. Furthermore, the transfection ability was increased by the addition of chloroquine in the transfection procedure. This result indicated that the internalization of the peptide-DNA aggregates would be mediated by the endocytosis pathway.

Polycationic reagents such as cationic lipids and poly-L-lysine are widely used for gene transfer into cells in vitro and show promise as vectors for in vivo gene therapy applications as nonviral gene transfer techniques. We have developed a novel transfection method using cationic amphiphilic α-helical oligopeptides with repeated sequences. Oligopeptide has the advantages of being easily designed and modified because of its simple structure. In this study, we synthesized five kinds of peptides of which the total chain length and the width of the hydrophobic region were changed. The binding of the peptides to plasmid DNA was evaluated by agarose gel electrophoresis. It was found that the long and/or hydrophobic peptides can strongly bind to the DNA. The formation of large aggregates with a 0.5–5-μm diameter, which consisted of the long peptides and the DNA, was observed by electron microscopy. The transfection abilities of the peptides were determined by the expression of luciferase from its cDNA in COS-7 cells. The long peptides showed high transfection abilities. As a result, it could be said that the transfection ability of these peptides was parallel to their ability to form aggregates with DNA. Furthermore, the transfection ability was increased by the addition of chloroquine in the transfection procedure. This result indicated that the internalization of the peptide-DNA aggregates would be mediated by the endocytosis pathway.

Gene transfer techniques have caused an important advance in the treatment of both inherited and acquired diseases. Most ongoing gene therapy protocols rely on recombinant retrovirus, adenovirus, adeno-associated virus, etc., which have a number of advantages for gene transfer because of their general efficiency and wide range of cell targets (1–4). However, there were some problems with these protocols, i.e. difficulty in insertion of large size DNA fragments into the gene, the possible production of replication-competent viruses by recombination, generation of several types of immune response, and oncogenic effects by random insertion into the host genome (5, 6). To avoid such problems, the development of nonviral gene transfer techniques has been also encouraged, particularly the use of cationic lipid and polycation such as polylysine. Many gene transfer techniques using cationic lipids bearing essentially a single tertiary or quaternary ammonium head group have been tested in vitro (7–14). Furthermore, Remy et al. (15) succeeded in targeted gene transfer into hepatoma cells using lipopolyamime-condensed DNA particles presenting galactose ligands. Polymeric DNA-binding cations such as polylysine, which were linked to cell-targeting ligands such as asialoorosomucoid, transferrin, insulin, galactose, or lactose were also used in the targeted gene transfer. In these systems, the ligands on the polymeric cations trigger receptor-mediated endocytosis into cells (16–20). In these works, the gene transferred into cells was found to be extensively degraded in the acidic lysosomal compartment. Recently, effective gene transfer has been achieved using a hemisynthetic virus that was prepared by coupling polylysine-asialoorosomucoid with adenovirus (21) and using a complex of DNA, polylysine-transferrin, and fusogenic peptide (22).

As synthetic vectors for nonviral gene transfer techniques, various complexes have been used. These were usually made by a combination of several functional groups or compounds that were able to transfer genes into the targeted cell and to perturb the lysosomal membrane to avoid degradation of the gene in the lysosome. However, the design of the synthetic vectors is generally complicated due to the introduction of several functional compounds such as glycoprotein and the fusogenic peptide. For the purpose of further study of the gene transfer system based on polycationic reagent, here we investigated gene transfer into cells using simple molecules, i.e. cationic oligopeptides that have the potential to take an α-helical structure. Previously, we studied perturbation and fusion of phospholipid membrane by cationic amphiphilic α-helical model peptides and elucidated the relationship between their structure and function (23–25). In the present study, we have examined the correlation between DNA binding ability of the peptides or morphology in the peptide-DNA aggregation and their transfection ability. The results indicated that the cationic amphiphilic α-helical peptides were highly efficient in transferring genes into cultured cells and that their transfection ability increased with their ability to cause aggregation.

EXPERIMENTAL PROCEDURES

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, and plasmid DNA that contained a luciferase gene and SV40 promoter (PicaGene control vector, PGV-C)1 was from Toyo Ink (Tokyo, Japan). Closed circular plasmid DNA was purified by ultracen-

1 The abbreviations used are: PGV-C, PicaGene control vector; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.
trifugation in CsCl gradients. Lipofectin was purchased from Life Technologies, Inc.

Peptides Synthesis—The peptides were prepared by solid phase synthesis using oxime resin (26–28). The synthesized peptides were purified by high pressure liquid chromatography with reversed-phase columns (YMC-Pack ODS A-323 or YMC-Pack C8, A-823, 10 × 250 mm). Elution was carried out with a linear gradient established between 50 and 100% acetonitrile in 0.05% trifluoroacetic acid for 30 min monitored at 220 nm. The final products were identified by elemental analysis, amino acid analysis, and matrix-assisted laser desorption ionization mass spectrometry using a Shimadzu Kratos Kompact MALDI III apparatus.

DNase I Protection Assay—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-NaOH buffer containing 135 mM NaCl, 5.0 mM KCl, and 0.76 mM Na2HPO4, pH 7.4). After 30 min at room temperature, 5 μl of a solution of 10 mM MgCl2 and 10 mM CaCl2 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 4 M ammonium acetate, 20 mM EDTA, and 2 mg/ml glycerin 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% bromphenol blue, and 0.5% glycerin), 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—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 a concentration of 10 μM in HBS. Measurements were performed in the presence of the plasmid DNA at a peptide:DNA charge ratio of 1.0 and in the absence of the plasmid DNA.

Electron Microscopy—Samples were prepared by mixing 63 μM of peptide (per cationic charge concentration) and 10 μg/ml 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 using a negative stain technique. Fifteen-μl 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-100CX transmission electron microscope.

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 with 10% fetal calf serum and 100 μg/ml streptomycin in an atmosphere of 5% CO2 at 37 °C, and washed twice with 1 ml of HBS. Plasmid DNA in 125 μl of HBS and peptide in 125 μl 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 Dulbecco’s modified Eagle’s medium with 10% fetal calf serum was added. The medium was replaced with 1 ml of a fresh medium after 12 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 (Malthiolumat LB9950, Berthold, Germany). The light unit values shown in the figures represent the specific luciferase activity (cpm/mg of protein) which is standardized for total protein content of the cell lysate. The measurement of gene transfer efficiency was performed in triplicate.

RESULTS AND DISCUSSION

Peptide Design—As shown in Fig. 1, peptides 4S and 46S, which were previously synthesized, take an amphiphilic α-helical structure. These consist of 12 and 24 residues, respectively, with a repeat of tetrapeptide units (23–25). Peptides 4S and 46S were designed on the basis of 4S and 46 sequences so as to reduce the hydrophobicity of 4S and 46 by introducing 3 and 6 hydrophilic Ser residues, respectively, instead of hydrophobic Leu and Ala residues. As a consequence, the hydrophobic region of the helix was increased from 1/4 in 4S and 46 to 1/2 in 4S and 46S. Peptide 4S contains the Pro-Pro sequence, which can disrupt α-helical structure, instead for Leu-Leu of 4S in the middle of the peptide chain. The 4S and 46 series peptides have 3 amid residues of cationic amino acid (Arg), respectively, in the molecules.

Formation of the Peptide-DNA Complex—The peptide-DNA complex formation was examined by the electrophoretic mobility of the complex on an agarose gel (1%, w/v) stained with ethidium bromide at the various ratio of peptides to a double-stranded DNA. The tests were performed by mixing 0.1 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, 4.0, and 8.0, respectively (Fig. 2A). In the case of 43, no migration of the plasmid DNA band occurred at charge ratio of 2.0. This lack of migration was due to neutralization of nucleic acid by cationic peptide and/or formation of a large complex between the peptide and the DNA. Peptide 43S gave no effect on migration of the DNA. The long peptides, 4 6, 46S, and 4 6P, suppressed the migration of the DNA at charge ratios of 0.5, 1.0, and 1.0, respectively. These results indicated that peptides with long length and large hydrophobic region strongly bound to the plasmid DNA. Introduction of proline residues, which breaks the α-helical structure, at the center of 46 slightly reduced the binding ability.

DNase I Protection Assay—When peptides bind to the plasmid DNA, it is expected that digestion of the DNA by DNase I will be inhibited. The DNA binding abilities of the peptides were evaluated by the nuclease-inhibitory activities. After adding the peptides to the plasmid DNA at various charge ratios, the mixtures were incubated with DNase I. After undigested DNA was extracted, the DNA was analyzed by 1% agarose gel electrophoresis (Fig. 2B). In the cases of 46, 46S, and 46P, the bands of the DNA were remarkably detected at a charge ratio of 2.0, 0.25, 1.0, and 0.25, respectively. In each case, the band upper to the position of supercoiled plasmid DNA appeared. It was considered that the upper bands corresponded to the position of the nicked or linearized DNA digested by the nuclease at one site. However, 43S offered no resistance to digestion of the DNA by the nuclease. In the case of 46, the upper band was scarcely detected at a charge ratio of 4.0. These findings indicated that 46 had the strongest binding ability to the plasmid DNA and were consistent with the results from the agarose gel shift assay described above.

CD Spectra—To analyze the structural features of these peptides that bound to the plasmid DNA, CD spectra of the peptides were measured. In these measurements, it was hard to analyze spectra under 205 nm due to large absorbance. As shown in Fig. 3A, 4 showed a typical α-helix CD pattern with double minima at 208 and 222 nm in the HBS.
222 nm indicated that the peptide took an almost complete α-helical structure (>95%). The helical content of 4S and 4P was lower than that of 4C. An increase in the hydrophilic region or the introduction of proline residues into peptides, which disrupts α-helical structure, actually reduced the α-helicity of the peptides. Peptides 4C and 4S took mainly a random coil structure in the HBS.

In the presence of the plasmid DNA at charge ratio of 1.0, 4S took a slightly α-helical structure (<20%) (Fig. 3B), while the helical content of 4C was remarkably increased by the addition of the DNA. This result means that 4C binds to the plasmid DNA with a stable α-helical structure. Peptides 4C, 4S, and 4P showed a large valley at 222 nm, and another valley at 208 nm became shallower. This spectrum would mean aggregation of peptide with DNA as described by Yoshimura et al. (29). Peptide 4P also showed a large valley at 222 nm, but its depth was shallower than that of 4C and 4S. It is possible that two proline residues in the center of 4P effectively broke the α-helical structure in binding to the DNA.

Electron Microscopy—To assess the structure of the peptide-DNA complexes, we used transmission electron microscopy with negative staining. Fig. 4A shows the free plasmid DNA without peptide, and Fig. 4B and C, shows complexes of 4C and the plasmid DNA at a charge ratio of 2.0. Interestingly, aggregates with a diameter of 0.5–5 μm were found. Although there were several shapes in aggregates, twisted fiber-like structures were common. The peptides 4S, 4P, and 4C except for 4S caused aggregation analogous with that of 4C (Fig. 4D–F). Although 4C generated similar aggregates as 4C, 4S, and 4P, the population of the aggregates was lower. The results indicated that most of the complex of 4C and the plasmid DNA was dissolved in HBS without forming large aggregates, because 4C indicated the typical α-helical CD pattern in the presence of DNA despite no strong aggregation with DNA. Furthermore, in the case of 4C, the aggregates were found at a peptide-DNA charge ratio of 0.50, while no aggregate was found at a charge ratio of 0.10. These results were consistent with those from the agarose gel shift assay and DNase I protection assay.

We furthermore found that the formation of aggregates was inhibited by reduction of sodium chloride concentration in buffer solution (data not shown). This result indicates that not only electrostatic interaction but also hydrophobic interaction between peptide and DNA is necessary for the formation of complex and aggregate. The mechanism of formation of large aggregates by peptide and DNA would be explained as follows. 1) Positive charges of the arginine residues in the peptides stand in line by taking an α-helical structure and electrostatically interact with the negative charge of phosphate in DNA. 2) Formation of a hydrophobic face on the opposite side of the DNA-binding face in an α-helix peptide causes hydrophobic interaction between peptide-DNA complexes, and the interaction results in aggregation.

Transfection Efficiency—The efficiency of these peptides in the transfection of COS-7 cells was tested with the plasmid DNA that contains a reporter gene encoding firefly luciferase. 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. 5, the cross-hatched bar shows the transfection efficiency of the peptides when the peptides were mixed with 2.5 μg of the plasmid DNA at a charge ratio of 2.0 without chloroquine treatment. In these cases, 125 μl of 126 μM (cationic charge concentration) peptides and 125 μl of 20 μg/ml plasmid DNA were mixed, and the mixture was added to the COS-7 cells in a 16-mm dish. Peptides 4C and 4S were found to exhibit no transfection ability, while the long peptides 4S and 4P had remarkable abilities; in particular, 4P had 11- and 6-fold higher abilities compared with those of 4S and 4P, respectively. To examine the efficiency of other DNA carrier molecule, we used poly-L-lysine as a cationic polypeptide. The poly-L-lysine was found to exhibit the same transfection ability as that of 4C. However, the efficiencies of methods using calcium phosphate and cationic liposome (Lipofectin) were 3- and 5-fold higher, respectively, than that of 4C.
It was clear that the binding of the plasmid DNA to the peptides reduced the cytotoxicity of the peptides. As shown in Fig. 5 (open bar), concurrent treatment of the cells with chloroquine, which may inhibit the degradation of the DNA by lysosomal hydrolases, made the efficiencies of all peptides increase remarkably. The gene transfer efficiencies of the long peptides (46, 43S) were 30–200-fold higher than those of the short peptides (43, 43S). Surprisingly, the treatment with chloroquine increased the efficiency of 46P by about 400-fold. In this condition, cell viabilities were reduced by 5–25% compared with in the absence of chloroquine treatment (Fig. 6). Since the increase in transfection efficiencies by the addition of the chloroquine was considerably higher than that in the cytotoxicity, it could be said that the increase in the transfection efficiency, which is standardized for total protein content of the cell lysate, is not due to a decrease in living cells. These findings indicated that internalization of the peptide-DNA aggregates was mediated by endocytosis pathway.

That 46 has the highest transfection efficiency in the absence of chloroquine treatment can be explained by the fact that 46 can bind more strongly to the plasmid DNA and more efficiently inhibit degradation of the DNA by DNase I compared with 43S and 43P. As a consequence, a large amount of the DNA could survive in the lysosomal vesicles. On the other hand, 43 had little transfection ability, although this peptide could bind to DNA. It could be considered that most of aggregates were degraded in the lysosomal vesicles because 43 could not protect the DNA from DNase I digestion adequately, although the aggregates of 46 and DNA might be incorporated into cells. It is clear that 43S lacked transfection ability because it could not bind to DNA. In the presence of chloroquine, degradation of incorporated DNA was restrained. Therefore, transfection efficiency of any peptide was increased. The efficiencies of 46 and 43S were similar, suggesting that both peptides caused similar aggregations and that the aggregates were incorporated with the same efficiency. On the other hand, 43P, which had a similar aggregation pattern to that of 46 and 43S, showed remarkably stronger transfection efficiency. Since 43P had stronger membrane perturbation activity compared with 46 and 43S, it is likely that a large amount of DNA could be transferred from endosomal vesicles to cytosol without remarkable degradation of the plasmid DNA. These results indicated that transfer of the peptide-DNA complex from the endosomal compartment to the cytosol is an important step for efficient expression of the gene in the presence of chloroquine. Therefore, it is possible that transfection efficiency depends on endosomal or lysosomal membrane perturbation activity of the peptide. Further investigation will be necessary for pursuing the localization of incorporated DNA in the cell.

The effect of the amount of 46 and the plasmid DNA on the transfection efficiency is shown in Fig. 7. The most effective transfection occurred when 2.5 μg of the plasmid DNA and 2.6 nmol of 46 were mixed. Furthermore, in any amount of the DNA, the peptide:DNA mixing ratio was optimum at 2.0. When the peptide:DNA charge ratio was smaller than 2.0, the efficiencies were low. It is probable that transfection-competent aggregates of peptide and DNA were hardly formed. Reduction of the efficiency at the higher amounts of both peptide and DNA than those at optimum conditions suggests that aggregates too large for endocytosis were formed because of the high concentration of both peptide and DNA. When the peptide:DNA charge ratio was higher than 2.0, the transfection efficiencies were also reduced. This was due to the cytotoxic activities of the excess free peptides that remained in the solution.

Conclusions—In this study, we examined some abilities of amphiphilic α-helical oligopeptides containing cationic amino acid as a gene carrier molecule. These peptides transferred plasmid DNA into cells and demonstrated high expression of protein encoded in the plasmid DNA. In particular, the peptides having a continuous large hydrophobic region, i.e., 46, could form stable aggregation with the plasmid DNA and also efficiently transfer the DNA into cells. Furthermore, this study indicated that membrane perturbation activity of the peptide also was important for the efficient transfection.

To increase the transfection efficiency of the peptide, it will be important to make the aggregates transfer from endosomal vesicle to cytosol efficiently and to achieve cell-specific gene delivery by the peptide with some ligands. Recent methods of peptide synthesis make it possible to design and exactly synthesize complicated peptides, e.g., peptide with ligands recognizable by specific cells, bundled peptide with multifunctions, peptide with a nonpeptidyl component, and peptide-nucleic acid hybrid. Accordingly, peptide has a high potential as a functional gene carrier because of its diversity on construction.

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