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Synthetic copoly(Lys/Phe) and poly(Lys) translocate through lipid bilayer membranes

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Abstract

Several membrane-transporting peptides (MTP) containing basic amino acid residues such as Lys and Arg that carry macromolecules such as DNA and proteins across cell plasma membranes by an unknown mechanism have been actively studied. On the basis of these results, we have been investigating the translocation ability of synthetic polypeptides, copoly(Lys/Phe) and poly(Lys), through negatively charged phospholipid (soybean phospholipid (SBPL)) bilayer membranes by zeta potential analysis, circular dichroism (CD) spectroscopy, fluorescence spectroscopy, an electrophysiology technique, and confocal laser scanning microscopy (CLSM). The binding of these polypeptides to the membrane, which is the first step for translocation across the membrane, resulted in the conformational transition of the polypeptide from a random coil form or helix-poor form to a helix-rich form. The fluorescence studies demonstrated that the time-dependent decrease in the fluorescence intensities of the FITC-labeled polypeptides bound to the SBPL liposome reflected translocation of the polypeptide across the lipid bilayer with the low dielectric constant. Both the rate constant and the efficiency of the polypeptide translocation across the lipid bilayer were greater for copoly(Lys/Phe) than for poly(Lys). These results suggest that the random incorporation of the hydrophobic Phe residue into the positively charged Lys chain results in a lowering of the potential barrier for passage of the polypeptide in the hydrophobic core portion of the lipid bilayer. We presented the first direct observation that the positively charged polypeptides, copoly(Lys/Phe) (MW: 41,500) and poly(Lys) (MW: 23,400), could translocate across the lipid bilayer membrane.

Keywords: Positively charged polypeptide; Lipid membrane; Translocation; Secondary structure; Micropolarity

1. Introduction

Several membrane-transporting peptides (MTP) have been reported to have the ability to translocate macromolecules across cell plasma membranes by an unknown mechanism [1–3]. *Drosophila* antennapedia (Antp) (16-mer peptide) [2] and human immunodeficiency virus (HIV)-1 Tat (13-mer peptide) are the well-known MTPs to have transporting ability [3]. Antp is a highly basic peptide having three Arg and four Lys in 16 amino acid residues and Tat is also a basic peptide containing six Arg and two Lys. The basic amino acid residues play an important role in the membrane translocation [4,5]. Futaki et al. [4] showed that arginine-substituted Tat could translocate through the cell membrane. Not only could these MTPs translocate through cellular and nuclear membranes, but they could also carry enzymes [6], oligonucleotides [7] and even larger structures such as liposomes [8] or nanoparticles [9] into the cells. This discovery can be expected to open new perspectives for the efficient delivery of therapeutic genes to target cells.

The application of positively charged MTP as a DNA vector results in the electrostatic neutralization of DNA, following DNA condensation [5]. The condensation enhances the delivery of the DNA vectors to cells. On the basis of this line, the positively charged poly(L-lysine) (poly(Lys)) can also condense DNA for a more efficient uptake [10–14]. A complex system consisting of poly(Lys), a low-

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density lipoprotein, and DNA produced a five-fold increase in the reporter gene expression in vitro [15]. The combination of the polycationic peptide protamine sulfate with cationic liposomes also enhances DNA delivery in vitro [16]. The complexed vector consisting of cationic polypeptide which contains an integrin-targeting motif, liposome, and plasmid DNA demonstrated receptor-specific enhancement of transfection in porcine arterial endothelial cells but not porcine vascular smooth muscle cells [17]. Histidine-containing cationic peptides exhibited an efficient transfection for gene delivery and the transfection efficiency depended on the number and positioning of histidine residues in the peptide [18]. Potential application of the positively charged polypeptides as a DNA vector should also be advantageous for DNA condensation and complexation and for electrostatic interaction with the lipid membrane due to abundant positive charges in the polypeptide chain [[19]. In spite of the large amount of evidence for the translocation of MTP [1–3,18] or the poly(Lys)-DNA complex [10–16] across cellular and nuclear membranes, the exact mechanism of cell entry has not yet been identified. Certain MTPs enter cells through non-endocytotic and receptor- and transporter-independent pathways [20,21]. Thorén et al. [22] presented the first study that the Antp translocates across the lipid bilayer. The electrostatic interaction of the basic amino acid residues with a lipid bilayer plays a crucial role in the process of membrane translocation. The cell-translocating mechanism is most likely a non-specific interaction with the lipid membrane [23]. A notable common feature is the importance of the positively charged residues such as Lys and Arg in their amino acid sequences of MTP. The role of the hydrophobic residues is not well-known. It is not known whether any particular secondary structure has to be induced in order to allow a transition over a potential barrier in the lipid bilayer. Magzoub et al. [24] demonstrated that the nature of the secondary structure induced by the lipid membrane systems was not directly correlated with the common transport property of cell-translocating peptides.

In this study, we have investigated the translocation ability of positively charged polypeptides, copoly(Lys/Phe) and poly(Lys), through a mechanism involving only peptide–lipid interactions in a model lipid membrane system (soybean phospholipid (SBPL) liposome and SBPL planar bilayer). It is almost certain that there exist three distinct steps for the translocation of the charged polypeptide through the lipid bilayer membranes: (1) positively charged polypeptide binding to the lipid membranes, (2) change in the secondary structure of the polypeptide in the lipidic environment of low dielectric constant, and (3) translocation of polypeptide over a potential barrier in the hydrophobic core portion of the lipid bilayer. The binding ability of the polypeptide to the membrane and its secondary structure in the lipidic environment were analyzed by zeta potential and circular dichroism (CD) measurements, respectively. The translocation of the polypeptide across the membrane was examined by three types of independent methods, i.e., fluorescence spectroscopy, an electrophysiology technique, and confocal laser scanning microscopy (CLSM).

2. Materials and methods

2.1. Chemicals

SBPL (Sigma, St. Louis, MO) used here contains phosphatidylcholine (40%), phosphatidylethanolamine (30%), phosphatidic acid (15%), cardiolipin (5%), phosphatidylinositol (4%), phosphatidylserine (3%), and others. Poly(L-lysine) (poly(Lys) (MW, 23,400; DP, 112) and copoly(lysine/phenylalanine = 1:1) (copoly(Lys/Phe) (MW, 41,500; DP, 233) were obtained from Sigma. FITC-labeled polypeptides were prepared: the degrees of substitution with FITC were 1.1 for poly(Lys) and 0.88 for poly(Lys/Phe), respectively. All the reagents used were of analytical grade.

2.2. Liposome preparation for zeta potential, CD and fluorescence measurements

The liposomes were prepared by the reverse phase evaporation method [25]. A lipid (20 mg) solution in 3 ml chloroform was gently added to 1 ml of buffer solution (10 mM Tris–HCl/10 mM KCl, pH 7.4) and then the mixed solution was sonicated using a bath-type sonicator for 5 min at ice bath temperature. The obtained liposomes were extruded seven times through two stacked 0.4-μm pore size filters and nine times through two stacked 0.1-μm pore size filters (Lipofast, Canada). Negative staining electron microscopy indicated that the obtained liposomes were unilamellar.

2.3. Giant liposome preparation for CLSM

The lipid (5 mg) was dissolved in 1 ml hexane in a 100 ml round-bottom flask and the solvent was evaporated under reduced pressure for 10 h to form a thin lipid film on the surface of the lower portion of the flask. The dried lipid film was hydrated with 10 ml of the buffer solution (10 mM Tris–HCl/10 mM KCl, pH 7.4) and the flask was sealed under argon and incubated at 20 °C for 24 h. The lipid film was gradually stripped off the glass surface during the incubation and formed a giant liposome suspension [26].

2.4. Zeta potential and CD measurements

The zeta potential (a Nicomp 380 ZLS, US) of the SBPL liposome suspension (10 mM Tris–HCl/10 mM KCl, pH 7.4) was calculated from the electrophoretic mobility based on the Helmholtz–Smoluchowski formula.

The CD measurement was made using a Jasco J-600 CD spectropolarimeter with a 2 mm quartz cuvette. The spectra were collected by subtracting the spectrum of the liposome suspension from that of the liposome suspension containing

the polypeptide and averaged over nine scans. Measurements were started after 10 min of polypeptide addition to the liposome suspensions.

2.5. Fluorescence spectroscopy

The fluorescence measurement was performed using a fluorescence spectrometer F-4500 (Hitachi, Tokyo). FITC fluorescein was excited at a wavelength of 488 nm and emission spectrum was recorded with a peak at 520 nm. The excitation and emission slit widths were both 5 mm. A 10-mm path length quartz cell was used. When the FITC-labeled polypeptide was added to the liposome suspension, the time dependence of the fluorescence intensity at 520 nm was immediately recorded. The translocation of the polypeptide across the lipid bilayer was examined using the large unilamellar liposomes with the high stability of bilayer membrane (about 0.1 μm diameter), but not small unilamellar liposomes. The quartz cell was placed in a thermostatically controlled cell holder at a temperature of 20 °C.

2.6. Electrophysiology technique

The experiments used the technique of the planar bilayer membrane formation by Montal and Mueller [27]. The planar bilayer was monitored for 10 min to check for stability and possible leak currents. When the membrane remained stable, an aliquot of polypeptide solution was added to the buffer solutions (10 mM Hepes–Tris/100 mM KCl, pH 7.4). The trans chamber was connected to the voltage-holding electrode and the cis chamber was held at ground. The polypeptide was then added to the cis chamber. The voltage clamp data were recorded by the Ag–AgCl electrodes immersed in a 3 M KCl solution with 0.2 M KCl agar bridges attached to a high-resolution voltage clamp amplifier. The signals were amplified and displayed on a digital oscilloscope. The data were subsequently digitized and analyzed using a pClamp 4.5 software (Axon Instruments, Foster City, CA). All experiments were performed with symmetrical buffer solutions at room temperature (20 °C).

2.7. Confocal laser scanning microscopy

The CLSM used was a laser scanning confocal imaging system (Zeiss, LSM-410) equipped with an argon ion laser (488 nm) and a He–Ne laser (543 nm). The FITC-labeled polypeptide fluorescence was excited by an argon ion laser (488 nm) and the emission was observed by a band filter (515–565 nm). To prevent photobleaching, the confocal microscope was operated under conservative laser intensity and time exposure conditions. The time exposure condition was 8.96 μs per pixel (frame size, 2048 × 2048) and scan speed was 40 s. The temperature of the observation chamber was maintained at 20 °C during the experiments.

3. Results

The translocation of the positively charged polypeptides, copoly(Lys/Phe) and poly(Lys), across the negatively charged lipid membrane was examined using three types of independent methods.

3.1. Adsorption of polypeptide to lipid bilayer

The first step for the translocation of polypeptide through the lipid bilayer membranes is the interaction with the membrane. Fig. 1 shows the zeta potential of the negatively charged SBPL liposome in the presence of positively charged poly(Lys) or copoly(Lys/Phe) in buffer solution (10 mM Tris–HCl/10 mM KCl, pH 7.4). The zeta potential of the SBPL liposome was −30.2 mV. The addition of poly(Lys) or copoly(Lys/Phe) to SBPL liposome suspension gradually led to an increase in the zeta potential, indicative of the electrostatic adsorption of the polypeptide. The change in the zeta potential was larger for pol(Lys) than that for copoly(Lys/Phe).

3.2. Conformation of polypeptide adsorbed to the lipid bilayer

The electrostatic attraction between the negatively charged SBPL membrane and the positively charged polypeptide significantly enhanced the effective binding to the membranes with a low dielectric constant (Fig. 1). Fig. 2 shows the CD spectra of poly(Lys) (A) and copoly(Lys/Phe) (B) bound to the SBPL liposome membranes, together with their CD spectra in the buffer solution. The concentrations of lipid and polypeptide were 0.14 mM and 1.2 μm, respectively. A high lipid-to-polypeptide ratio (about 100:1 molar ratio) was chosen in order to guarantee the complete binding of the polypeptide to the lipid membrane. In the aqueous phase, the CD spectrum of poly(Lys) is characterized by a negative minimum ellipticity at 195 nm, which is assigned to

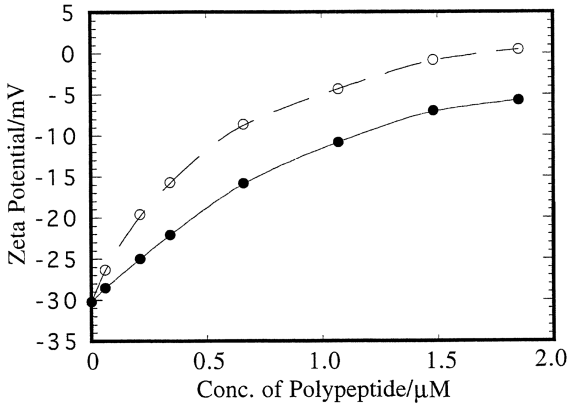

Fig. 1. Zeta potential of negatively charged SBPL liposomes as a function of the concentrations of positively charged copoly(Lys/Phe) or poly(Lys) in buffer solution (10 mM Tris–HCl/10 mM KCl, pH 7.4). (●) Copoly(Lys/Phe), (○) poly(Lys).

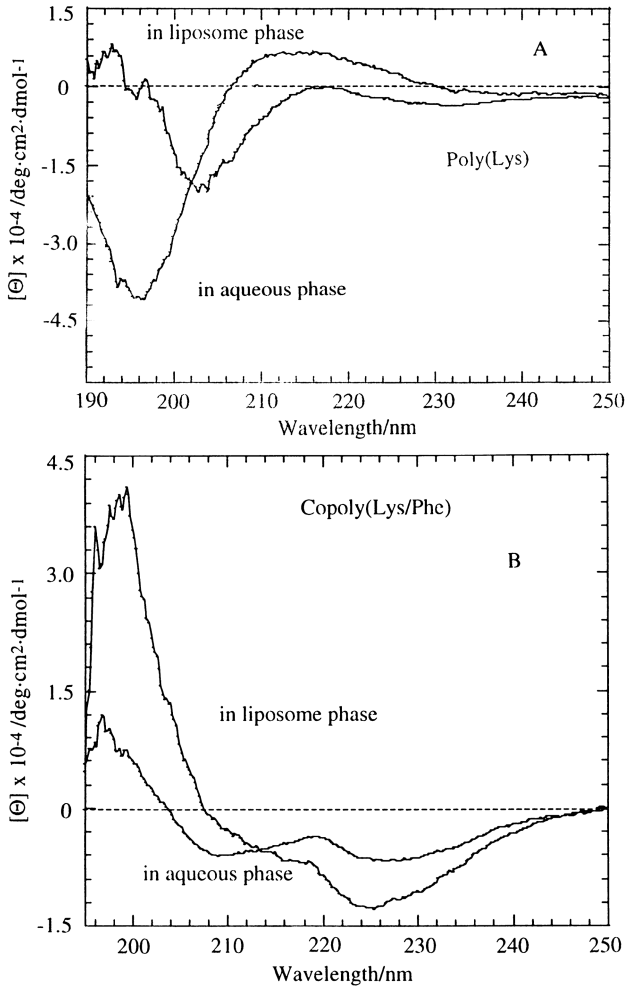

Fig. 2. CD spectra of poly(Lys) (A) and copoly(Lys/Phe) (B) bound to SBPL liposome and in aqueous phase (10 mM Tris–HCl buffer solution, pH 7.4). The concentrations of lipid and polypeptide were 0.14 mM and 1.2 μm, respectively.

the random coil structure (Fig. 2A) [28]. Upon binding of poly(Lys) to the membrane, the random coil was transformed to the new type of spectrum which has a large ellipticity at 204 nm and a small one at 230 nm, in which this double minimum ellipticity is assigned to the α-helix-like secondary structure[28]. The secondary structure of poly(Lys) bound to the liposomal membrane evidently differed from that in the aqueous phase. For the CD spectra of copoly(Lys/Phe) in the aqueous phase, the double minimum ellipticities at 209 and 225 nm, which are almost equal in amplitude, were observed (Fig. 2B). For the membrane-bound copoly(Lys/Phe), the ellipticity at 209 nm increased from -6000 to -2200 deg cm^2 dmol^{-1} and the latter one decreased from -6200 to -12000 deg cm^2 dmol^{-1}. Both the poly(Lys) and copoly(Lys/Phe) bound to the lipid membrane increased their helix contents. The membrane-bound secondary structure of these polypeptides may depend on the polarity of the lipid membrane interacting with the polypeptide. Fig. 3 shows the CD spectra of the copoly(Lys/Phe) in various water/dioxane

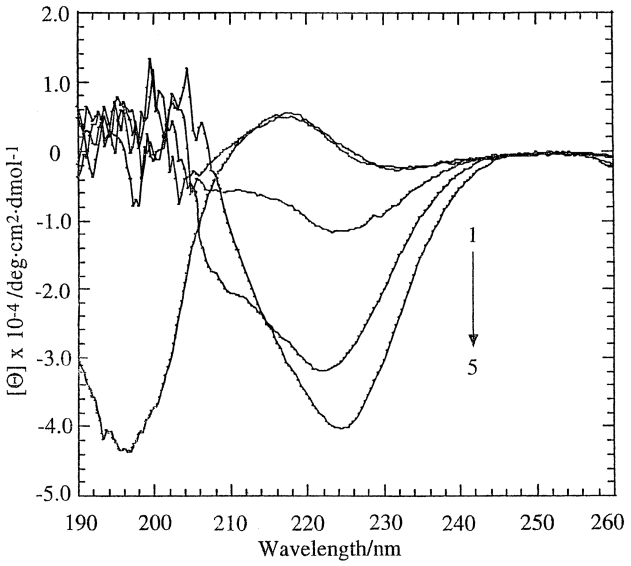

Fig. 3. CD spectra of copoly(Lys/Phe) in various water/dioxane mixtures. Dioxane content: (1), 0%; (2), 20%; (3), 50%; (4), 80%; (5), 100% (v). The concentration of polypeptide was 1.2 μm.

mixtures with different polarities. Because of the absorption of light energy by the solvent (dioxane), in the region below about 210 nm the CD spectra of polypeptide could not be detected. The negative ellipticity at 224 nm increased with an increase in dioxane content, indicating that the copoly(Lys/Phe) is transformed to the helix-rich form. This suggests that these polypeptides are able to adapt a more favorable conformation during the process of passage across the lipid membrane because of a micropolarity gradient between the

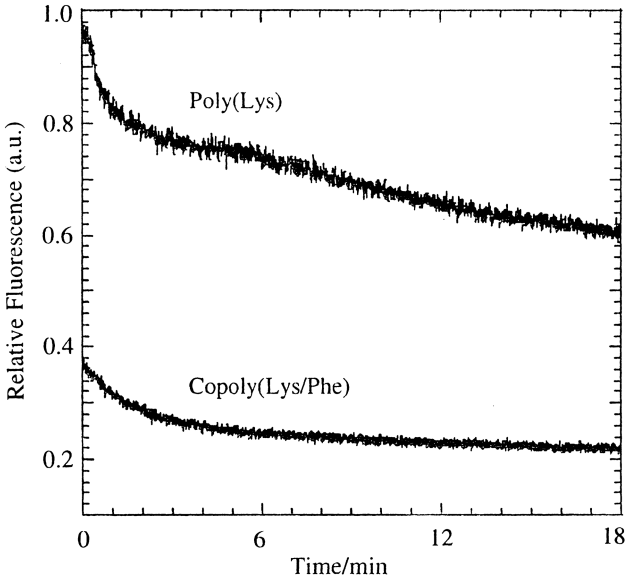

Fig. 4. The time dependence of fluorescence intensity of FITC-labeled poly(Lys) and copoly(Lys/Phe) bound to SBPL liposomal membrane. The fluorescence at 520 nm was normalized by the initial fluorescence in the absence of the polypeptide. The concentrations of lipid and polypeptide were 0.24 mM and 1.1 μM, respectively.

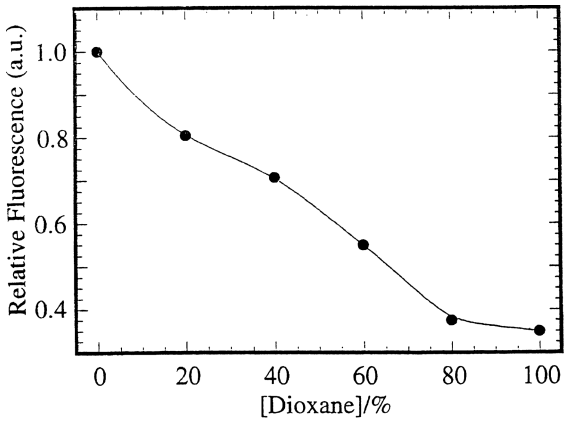

Fig. 5. Relative fluorescence intensity of FITC-labeled copoly(Lys/Phe) as a function of dioxane content (%(v)). The concentration of copoly(Lys/Phe) was 1.1 μM.

polar head group and the hydrophobic core of the lipid bilayer [29].

3.3. Translocation of polypeptide across liposomal membrane by fluorescence spectroscopy

Next, we examined the translocation of the polypeptide molecule across the SBPL liposomal membrane with fluorescence measurement. Fig. 4 shows the time scans of the change in the fluorescence intensity of the polypeptide after addition of the FITC-labeled poly(Lys) or copoly(Lys/Phe) to the SBPL liposome suspensions. The concentrations of the lipid and polypeptide were 0.24 mM and 1.1 μM, respectively. The fluorescence intensity at the peak FITC emission wavelength of 520 nm steeply decreased at the first stage and then showed a gradual decrease. We presumed that the time-dependent decrease in the fluorescence intensity of the polypeptide may be associated with the process during passage of the polypeptide through the lipid bilayer after adsorption of the polypeptide from the aqueous phase to the liposomal membrane. The rate constant of transport of the polypeptide across the SBPL bilayer was determined by the Guggenheim plotting method [30]: 1.9×10^{-2} min^{-1} for poly(Lys) and 1.3×10^{-1} min^{-1} for copoly(Lys/Phe). The rate constant for copoly(Lys/Phe) was greater than that for poly(Lys) by a factor of about 10 times. The rate-limiting step of the polypeptide translocation across the lipid membrane is the passage over a potential barrier in the hydrophobic core portion of the lipid membrane [31]. There exists a steep gradient for micropolarity (dielectric constant, ε) in the lipidic environment between the lipid membrane surface and the hydrocarbon core of the lipid bilayer [29]. Thus in order to examine whether the time-dependent decrease in the fluorescence intensity of the polypeptide reflects the process during passage of the polypeptide across the lipid bilayer, the

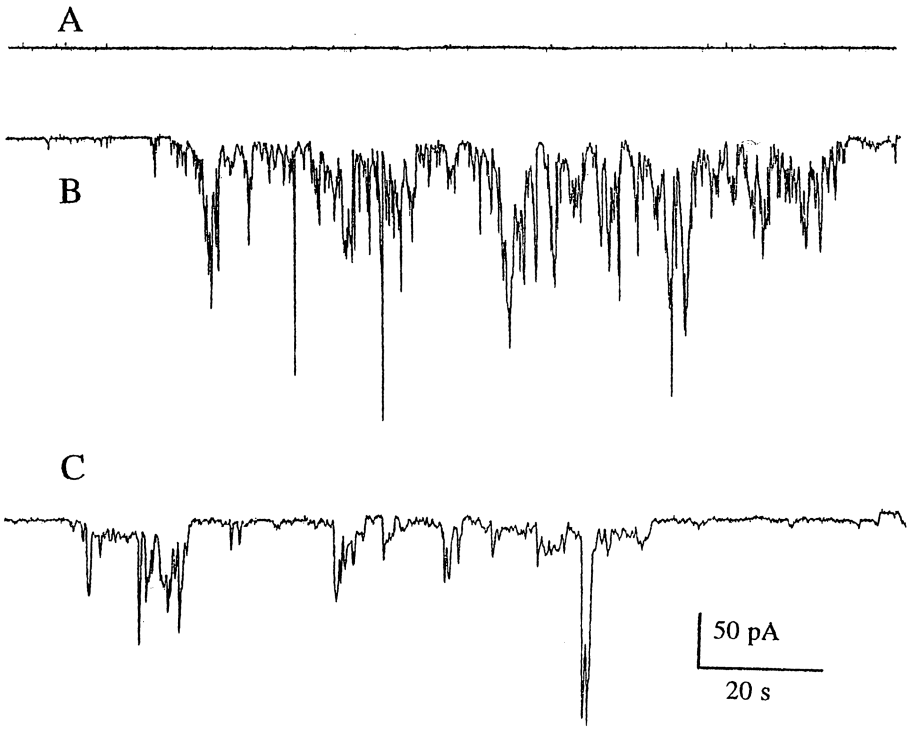

Fig. 6. Current traces at constant voltage of − 100 mV applied through SBPL planar bilayer in the absence (A) or presence of copoly(Lys/Phe) (B) and poly(Lys) (C). These positively charged polypeptides (0.28 μM) were added to the cis chamber (10 mM Hepes/Tris/100 mM KCl, pH 7.4) and then the negative voltage $V_m = -100$ mV was applied on the membrane side without the polypeptide. Measurement was performed with symmetrically buffer solution (10 mM Hepes–Tris/100 mM KCl, pH 7.4) at room temperature (20 °C).

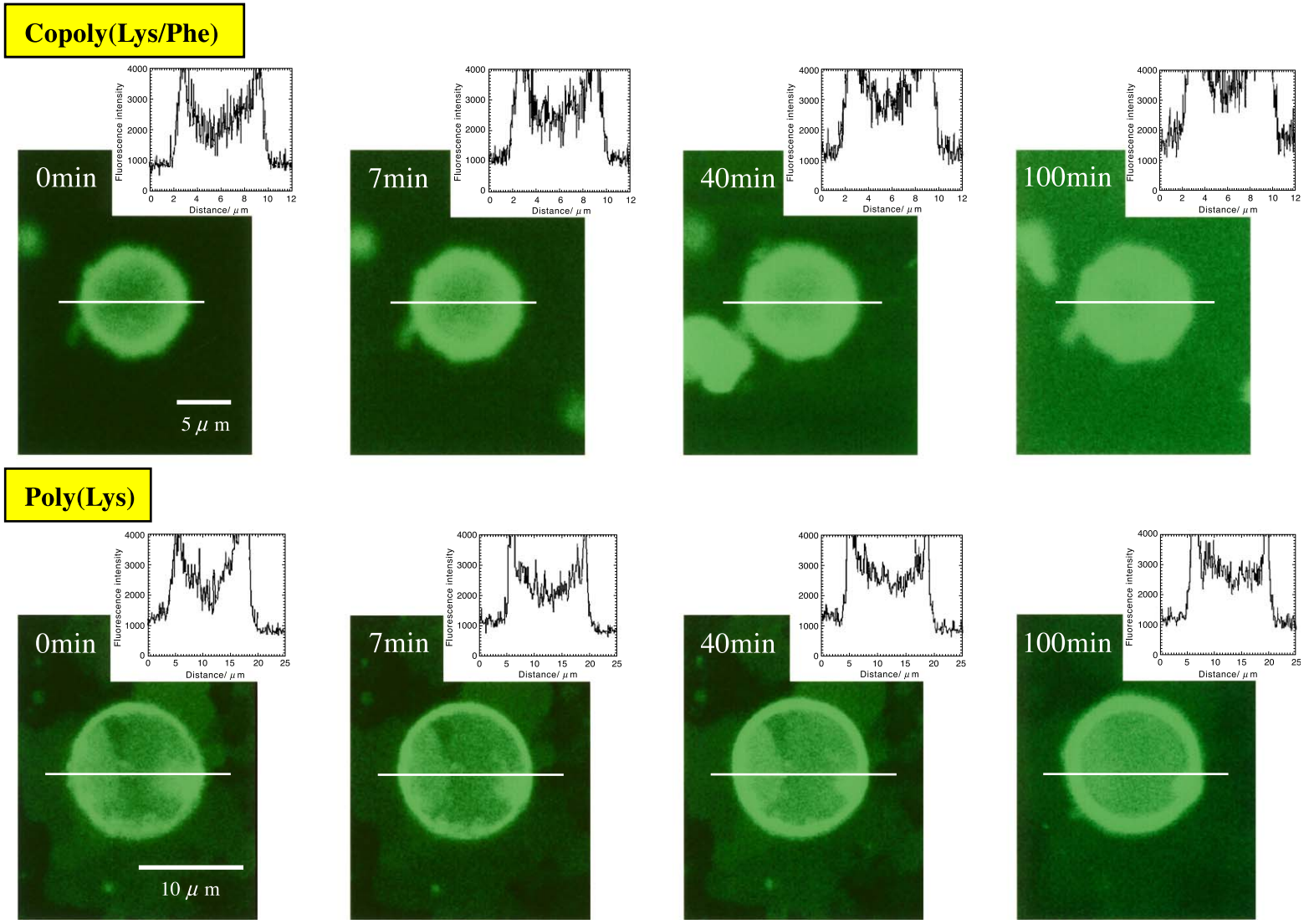

Fig. 7. Confocal laser scanning images, taken at different times, of FITC-labeled copoly(Lys/Phe) and poly(Lys) added to SBPL liposome. Lipid concentration was 0.24 mM. Lipid–polypeptide concentration ratio = 100:1 (mol/mol). The first micrograph ($t = 0$) has already progressed only 2 min after the polypeptide is added to the liposome suspension. For the first micrograph, fluorescent spots were predominantly distributed around the periliposomal membrane region. Afterwards, the spots then gradually increased in the liposome inner phase, with a maximum signal intensity up to 100 min. Histograms showed that the relative fluorescence intensity between the periliposomal membrane region and the liposome inner phase varied with time.

fluorescence intensities of copoly(Lys/Phe) in various water/dioxane mixtures with different dielectric constants were measured (Fig. 5). The dielectric constants of mixed water/dioxane systems are $\varepsilon = 78.3$ (25 °C) for aqueous phase, $\varepsilon = 64.4$ for 20% dioxane, $\varepsilon = 40.2$ for 50% dioxane, $\varepsilon = 16.1$ for 80% dioxane and $\varepsilon = 2.2$ for 100% dioxane, respectively [32]. The fluorescence intensity of copoly(Lys/Phe) decreased with an increase in the dioxane content, indicating that its fluorescence intensity is closely correlated to the polarity of the medium. Therefore, we concluded that the time-dependent decrease in the fluorescence intensity of the polypeptide bound to the liposomal membranes is a result of the process during translocation of the polypeptide through the lipidic environment with different micropolarities.

3.4. Translocation of polypeptide across lipid planar bilayer by electrophysiology technique

The transport of a polypeptide across the lipid membrane was investigated using the SBPL planar bilayer membrane by the electrophysiology technique. Fig. 6 shows the representative current traces at constant voltages of − 100 mV applied through the SBPL planar bilayer in the absence (A) or presence of copoly(Lys/Phe) (B) and poly(Lys) (C). These cationic polypeptides (0.28 μM) was added to the cis chamber (10 mM Hepes/Tris/100 mM KCl, pH 7.4) and then the negative voltage − 100 mV was applied on the membrane side without the polypeptide. The applied negative voltage provides an electric potential drop for transport of the positively charged molecules or ions. There was zero current in the absence of polypeptide at the applied voltage of $V_m = -100$ mV (Fig. 6A), indicating that the SBPL bilayer retains a high ohmic seal. When the membrane potential of − 100 mV was applied in the presence of copoly(Lys/Phe) in the chamber (Fig. 6B), the current pulses with a large amplitude and long duration of fluctuation appeared and then returned to zero current. On the other hand, the presence of poly(Lys) at − 100 mV also caused fluctuating current pulses (Fig. 6C). At the positive voltage of + 100 mV applied after the negative voltage of − 100 mV, only small transient current pulses less than 10 pA were observed (data not shown). The amplitude of the current pulse and the duration of the fluctuation were much greater for copoly(Lys/Phe) than those for poly(Lys). The amplitude and the duration time of the electrical current across the SBPL planar bilayer depended upon the applied voltage and the voltage polarity. It appears that the interaction of copoly(Lys/Phe) or poly(Lys) with the SBPL membrane at a constant applied voltage results in the electro-diffusive transport of the charged substances involving the actual transport of copoly(Lys/Phe) or poly(Lys) across the SBPL bilayer.

In order to learn whether the membrane currents observed were correlated to the actual transport of the polypeptide across the lipid membrane, we determined the amounts of polypeptide molecules which were translocated to the opposite solution side across the SBPL membrane after 20 min of polypeptide addition (0.5 μM) to the cis side chamber by fluorescence spectrometry. The actual transport of polypeptides across the lipid membrane was 4.1% for copoly(Lys/Phe) and 0.43% for poly(Lys). The translocation efficiency of copoly(Lys/Phe) was about 10 times that of poly(Lys).

3.5. Observation of polypeptide translocation across liposomal membrane by CLSM

Fig. 7 shows confocal laser scanning images, taken at different times, of FITC-labeled copoly(Lys/Phe) and poly(Lys) added to the SBPL liposome. The lipid concentration was 0.24 mM. The lipid/polypeptide concentration ratio was 100:1 (mol/mol). Micrographs were taken 2 min after the addition of the FITC-labeled polypeptide to the liposome suspension. Therefore, the first micrograph ($t = 0$) has already progressed only for 2 min after the polypeptide is added to the liposome suspension. For the first micrograph, fluorescent spots were predominantly distributed around the periliposomal membrane region. Afterward the spots then gradually increased in the liposome inner phase, with a maximum signal intensity up to 100 min. Histograms showed that the relative fluorescence intensity between the periliposomal membrane region and the liposome inner phase varied with time. The translocation of copoly(Lys/Phe) was much more effective than that of poly(Lys). We obtained the first direct observation on the translocation of (Lys/Phe) and poly(Lys) across the SBPL bilayer membranes.

4. Discussion

The primary aim of gene therapy is the efficient delivery of DNA to target cells. Potential application of the positively charged MTP- [1–3,7] or positively charged polypeptide-based delivery vectors [11–14,18] can be expected to improve the efficiency and safety of gene delivery. Concerning the construction of a polypeptide-based vector, we have been studying the translocation of positively charged polypeptides across the negatively charged lipid bilayers as a first step. Zeta potential data showed that the electrostatic attraction between the positively charged polypeptide and the negatively charged SBPL membrane significantly enhanced the effective binding to the membranes with a low dielectric constant (Fig. 1). The binding was more effective for poly(Lys) than that for copoly(Lys/Phe). The binding of these polypeptides from the aqueous phase to the lipid membrane surface resulted in the conformational transition to the helix-rich form (Fig. 2). The transport of the polypeptide from the aqueous phase into the lipidic environment with a low polarity leads to a drastic reorganization of the hydrogen bonds since hydrogen bonds between the water and amino acid side chains must be replaced by intrachain hydrogen bonds leading to a highly ordered

protein structure. Poly(Lys) is a typical case in point, which upon binding to lipid membranes, undergoes conformational transition from a random coil structure in the aqueous phase to a partially α-helix-like secondary structure in the SBPL liposomal membrane phase (Fig. 2A). The lipidic environment with a low dielectric constant seems to cause the conformational transition of the polypeptide. The CD spectra of copoly(Lys/Phe) in various dioxane/water mixtures with different polarities showed that the formation of the α-helix-like secondary structure facilitated with a decrease in the dielectric constant in the polypeptide-dissolved environment (Fig. 3). The conformational transition from the charged coil form to the helix-rich form may be advantageous for polypeptide translocation through the hydrophobic core portion in the lipid membrane due to a lowering of the electrostatic barrier in the hydrophobic core portion in the membrane. Recent structure–activity studies demonstrated that both membrane-transporting peptides Antp and histidine-containing cationic peptide interacted with lipid membranes and had a high propensity for α-helical structure in a lipidic environment [18,22]. We consider that the conformational transition from random coil structure in aqueous phase to hydrophobic structure such as α-helix or β-sheet in lipid membrane phase may be a requirement for polypeptide transport across lipid bilayer membranes.

The translocations of copoly(Lys/Phe) and poly(Lys) across the SBPL bilayer were examined using three types of independent methods, i.e., fluorescence spectroscopy, an electrophysiology technique, and CLSM. The addition of the FITC-labeled copoly(Lys/Phe) or poly(Lys) to the SBPL liposome suspensions decreased the time-dependent fluorescence intensity for these polypeptides (Fig. 4). This is considered to reflect the translocation process of the polypeptide across the lipid membranes with a steep gradient for the micropolarity between the polar head ($\varepsilon = 30-40$) and the hydrophobic core portions ($\varepsilon = 2-3$) of the lipid molecule in the lipid bilayer. The rate constant of the transport of copoly(Lys/Phe) across SBPL bilayer was greater (about 10 times) than that for poly(Lys). In order to improve this hypothetical elucidation, the fluorescence intensity of the FITC-labeled copoly(Lys/Phe) in various water/dioxane mixtures with different dielectric constants was measured (Fig. 5). The fluorescence intensity was found to depend upon the polarity of the medium. Therefore, the time-dependent decrease in the fluorescence intensity of the polypeptide reflects the process during passage of the polypeptide across the lipid bilayer.

The physiological technique also demonstrated the translocations of copoly(Lys/Phe) and poly(Lys) across the SBPL planar bilayer. When the polypeptide was added to the chamber with the planar bilayer, the electrical currents across the SBPL planar bilayer were generated in the presence of only the polypeptide, but not without the polypeptide (Fig. 6), in an electric field applied through the SBPL planar bilayer. The current amplitude depended upon the applied voltage and the voltage polarity. The actual transport of the polypeptides across the SBPL planar bilayer after 10 min of polypeptide addition (0.5 μM) to the chamber was 4.1% for copoly(Lys/Phe) and 0.43% for poly(Lys), respectively. The reason why both the translocation efficiency (Fig. 6) and the rate constant (Fig. 4) of copoly(Lys/Phe) are greater than those of poly(Lys) will be explained as follows. Taking into account the data that the free energies of transfer of the amino acids from the lipid membrane to the aqueous phase are 1.13 kcal mol^{-1} for Phe and -0.99 kcal mol^{-1} for Lys [33], the random incorporation of the hydrophobic Phe residues into the positively charged poly(Lys) chain may result in a lowering of the potential barrier in the hydrophobic core portion of the lipid bilayer for the polypeptide translocation. So far, the role of the hydrophobic residues has not been well-known. The obtained results may show the importance of the hydrophobic residues in the amino acid sequence for the polypeptide translocation. A particular secondary structure seems to be induced in order to allow a translocation over a potential barrier in the lipid bilayer.

The confocal laser scanning images taken at different times gave first direct observation that the copoly(Lys/Phe) and poly(Lys) translocate across the SBPL bilayer membranes (Fig. 7). The translocation of copoly(Lys/Phe) was much more effective than that of poly(Lys). We presented that there exist three distinct steps for the translocation of charged polypeptide across the lipid bilayer membranes: (1) the positively charged polypeptide binding to the SBPL membranes and the accumulation of polypeptides around the periliposomal membrane region, basically by an electrostatic interaction with the lipid; (2) after binding to the membrane, the change in the polypeptide secondary structure in the lipidic environment of a low dielectric constant; and (3) then the translocation across the lipid bilayer through the potential barrier of the polypeptide translocation in the hydrophobic core portion of the lipid bilayer.

5. Conclusion

We present the first direct observation that copoly(Lys/Phe) and poly(Lys) bound to the liposomal membrane translocate across the lipid membrane accompanying its conformational change in the lipidic environment. The translocation efficiency of copoly(Lys/Phe) was about 10 times that of poly(Lys). The random incorporation of the hydrophobic residue, Phe, in the positively charged poly(Lys) chain may be advantageous for the membrane translocation due to a lowering of the electrostatic barrier in the hydrophobic core region of the lipid bilayer. The obtained results strongly suggest that the translocation of the positively charged polypeptide across the SBPL bilayer membranes is a three-step event that requires (1) polypeptide binding to the lipid membranes, (2) the conformational transition of the polypeptide from a charged random coil (hydrophilic) form in the aqueous phase to a α-helix-like

secondary structure in the lipid membrane of a low dielectric constant, and (3) the translocation across the lipid bilayer through destabilization of the membrane interacting with the polypeptide.

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