

# De Novo-designed Peptide Transforms Golgi-specific Lipids into Golgi-like Nanotubules\*

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Published, JBC Papers in Press, June 13, 2001, DOI 10.1074/jbc.M104705200Sannamu Lee<sup>‡§</sup>, Tomomi Furuya<sup>‡</sup>, Taira Kiyota<sup>‡</sup>, Noboru Takami<sup>¶</sup>, Kunihiko Murata<sup>||</sup>,  
Yasuro Niidome<sup>\*\*</sup>, Dale E. Bredeesen<sup>‡‡</sup>, H. Michael Ellerby<sup>§‡‡</sup>, and Gohsuke Sugihara<sup>‡</sup>From the <sup>‡</sup>Department of Chemistry, Faculty of Science, <sup>¶</sup>Radioisotope Center, and <sup>||</sup>Central Laboratory for Infectious Diseases, School of Medicine, Fukuoka University, Fukuoka 814-0180, Japan, the <sup>\*\*</sup>Department of Materials Physics and Chemistry, Graduate School of Engineering, Kyushu University, Fukuoka 812-8581, Japan, and the <sup>‡‡</sup>Buck Institute for Age Research, Novato, California, 94945

Cellular organelles, such as the Golgi apparatus and the endoplasmic reticulum, adopt characteristic structures depending on their function. While the tubular shapes of these structures result from complex protein-lipid interactions that are not fully understood, some fundamental machinery must be required. We show here that a *de novo*-designed 18-mer amphipathic  $\alpha$ -helical peptide, Hel 13-5, transforms spherical liposomes made from a Golgi-specific phospholipid mixture into nanotubules on the scale of and resembling the shape of the nanotubules that form the Golgi apparatus. Furthermore, we show that the size and the shape of such nanotubules depend on lipid composition and peptide properties such as length and the ratio of hydrophobic to hydrophilic amino acids. Although the question of precisely how nature engineers organellar membranes remains unknown, our simple novel system provides a basic set of tools to begin addressing this question.

Cellular organelles like the Golgi apparatus and the endoplasmic reticulum adopt characteristic structures depending on their function (1, 2). For example, extensive membrane nanotubules, typically 50–70 nm in diameter and up to several  $\mu$ m in length, have been observed to form the Golgi complex, the *trans* Golgi network, and the connections between the Golgi stacks. The morphological engineering of these membranes involve complex interactions between proteins and lipids that are not yet understood (3, 4). However, there must be some fundamental machinery required to form such structures.

We recently described the properties of a *de novo*-designed 18-mer peptide, Hel 13-5 (5, 6). This peptide can adopt an ideal  $\alpha$ -helix having a 240° hydrophobic sector region (Fig. 1A). It forms a self-association state in buffer solution by adopting this amphipathic structure (70%  $\alpha$ -helical structure by CD), and it binds to model- and biomembranes with high affinity. In the present study, we show that Hel 13-5 induces nanotubular structures, not only for PC liposomes, but also for various

naturally occurring phospholipids. Most importantly, we demonstrate that Hel 13-5 transforms spherical liposomes made from a Golgi-specific phospholipid mixture into nanotubules on the scale of, and resembling the shape of, the nanotubules of the Golgi apparatus.

## MATERIALS AND METHODS

**Reagents**—Peptide was synthesized by the Fmoc<sup>1</sup> strategy based on the solid phase technique starting from Fmoc-PAL-PEG resin using a PerSeptive 9050 automatic peptide synthesizer described previously (5). The stock solutions of Hel peptides were prepared as follows: the powders were dampened with a small amount of 30% acetic acid and then diluted in buffer (5 mM Tris/100 mM NaCl, pH 7.4). The peptide concentrations in the buffer solution were determined from the UV absorbance of Trp at 280 nm ( $\epsilon = 5500$ ).

**Turbidity Measurement**—A lipid solution in chloroform was filmed in a round bottom flask by drying in a stream of N<sub>2</sub> gas. The lipid film was hydrated with the Tris buffer by vortexing. The turbid liposome solution obtained was then diluted to a concentration of about 100  $\mu$ M with the same buffer. The peptide solutions were then added to the solution to attain a given molar ratio of the peptide to lipid and then incubated at 25 °C. The absorbance of the sample solution was recorded at 400 nm using a JASCO spectrometer (Tokyo, Japan) after vigorous vortexing.

**Viscosity Measurement**—The viscosities of Hel 13-5 and egg PC liposome solutions were obtained by a rotary type viscosity meter DV-II with a spindle type s40 (Brookfield EnG Labs Inc.). The sample solution was placed between the sample cup and the spindle after incubation at 25 °C for 24 h. The rotation velocity of the spindle was 50 rpm. The relative viscosity ( $\eta$ ) was calculated using the following equation  $\eta = \eta_1 / \eta_0$  where  $\eta_1$  and  $\eta_0$  are the viscosities of liposome solutions in the presence and absence of peptide, respectively.

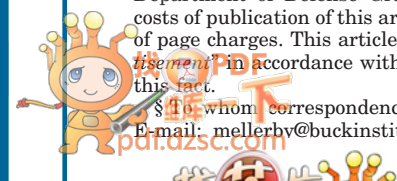
**Liposome Size Determination**—Unilamellar liposomes with a controlled size distribution of about 80–90 nm in diameter were prepared by the extrusion method (7). The liposomes formed by ultrasonic irradiation in the cuphorn of a Branson model 185 sonifier at room temperature were extruded through two stacked polycarbonate filters with a 100- and 200-nm pore size by applying nitrogen pressure and collected and re-extruded into an Extruder (Lipex Biomembranes Inc., Vancouver, Canada). The liposome solution (0.2 mM, 1 ml) was then added to the peptide solution (1 ml) at the appropriate concentrations at 25 °C. A NICOMP Submicron Particle Sizer, Model 370, with an argon ion laser ( $\lambda = 488$  nm) of 75 milliwatt maximum power was used to measure liposome size (6).

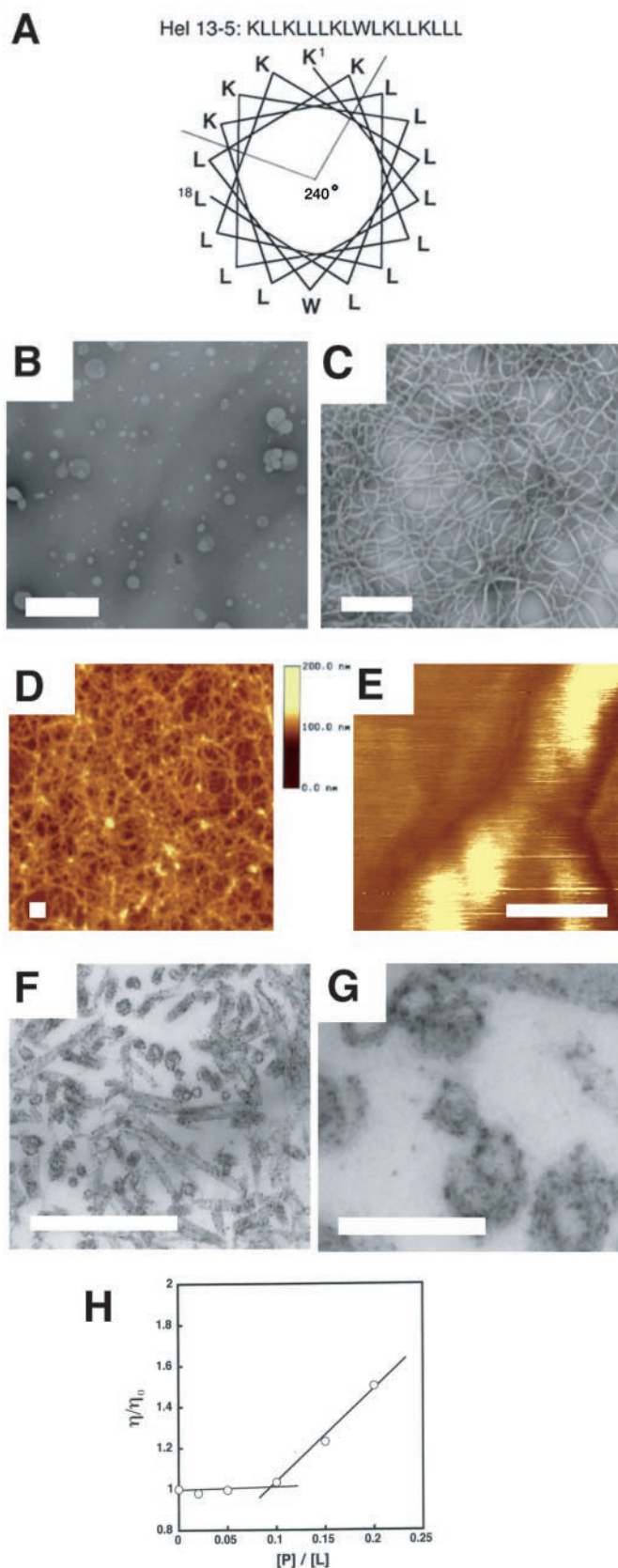
**CD Spectroscopy**—CD spectra were obtained using a JASCO J-600 spectrometer using a quartz cell with 1-mm path length. The peptide solutions were prepared at concentrations of 40–50  $\mu$ M in the presence and absence of egg PC liposomes. Lipids were stored at 1 mM concentration.

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§ To whom correspondence may be addressed. Fax: 415-209-2230; E-mail: mellerby@buckinstitute.org (to H. M. E.) or Fax: 81-92-865-xa-u.ac.jp (to S. L.).

<sup>1</sup> The abbreviations used are: Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; PC, egg yolk phosphatidylcholine; AFM, atomic force microscopy; Ch, cholesterol; DPPC, dipalmitoyl-D,L-phosphatidylcholine; Nle, nor-leucine; PE, egg yolk phosphatidylethanolamine; PG, egg yolk phosphatidylglycerol; PI brain phosphatidylinositol; PS, brain phosphatidylserine; SM, sphingomyelin; TEM, transmission electron microscopy; Tris, *N*-Tris [hydroxymethyl]methyl-2-aminoethanesulfonic acid.





**FIG. 1. Hel 13-5 forms fibrils with PC liposomes.** **A**, primary structure and helical wheel representation of Hel 13-5. **B**, transmission electron micrographs of PC liposomes prepared from the extruder method, and **C**, incubated with Hel 13-5. Hel 13-5 was incubated with PC liposomes in EDTA-containing buffer for 24 h, and then the preparation analyzed. **Bar** is 500 nm. **D**, the low magnification AFM image of the liposome surface after incubation with Hel 13-5. **E**, the high magnification AFM image of the liposome surface after incubation with Hel 13-5. **F**, low magnification TEM image of the Hel 13-5/PC mixture. **G**, high magnification TEM image of the Hel 13-5/PC mixture. **H**, viscosity change in Hel 13-5/PC mixture as a function of molar ratio.

**Electron Microscopy**—For whole-amount observation, liposomes prepared in 5 mM Tes buffer (pH 7.4) containing 100 mM NaCl and 1 mM EDTA. The peptide concentration was 100  $\mu$ M (lipid/peptide = 5). All the peptides were incubated for about 24 h with liposomes before the negative staining. The sample solutions were placed on carbon-coated grids and stained with 0.5% phosphotungstic acid adjusted to pH 7.4.

For thin sectioning, the incubation mixture was fixed in a suspension of 2% glutaraldehyde in Tes buffer, pelleted in an Eppendorf centrifuge, and postfixed in  $\text{OsO}_4$ . The precipitate was mixed with 3% agar solution, cut in 1  $\text{cm}^3$  cubes, dehydrated by ethanol and propylene oxide, embedded in Epon 815, and sectioned.

## RESULTS

**Golgi-like Nanotubules Are Induced by Hel 13-5**—When Hel 13-5 was added to PC liposomes, the liposomes were transformed from spherical structures into twisted ribbon-like fibrils  $\sim 25$ – $50$  nm in diameter and more than a few  $\mu$ m in length as determined by TEM (Fig. 1, **B** and **C**). We confirmed that Hel 13-5 induces twisted helical-shaped fibrils in egg PC liposomes using atomic force microscopy (AFM) (Fig. 1, **D** and **E**). Under low magnification, TEM revealed a great many circular- and sheath-like nanotubular structures (Fig. 1**F**). Under high magnification, there were many unilamellar shapes including double-lamellar structures. The tubules were about 40–50 nm in diameter and 5 nm in thickness for unilamellar, and 10 nm in thickness for double-lamellar layers (Fig. 1**G**). Relative viscosity ( $\eta$ ) measurements of the peptide-lipid solution demonstrated that the fibrils begin to form at a ratio of peptide to lipid (P/L) of about 1:10 as measured by the onset of a change in viscosity (Fig. 1**H**).

Because Hel 13-5 reshaped membranes in a simple system composed only of egg PC liposomes, it is logical to ask whether or not Hel 13-5 could also demonstrate this ability in a more complex and realistic biomembrane system, such as the liposomes formed from an organelle-specific mixture of phospholipids. We considered the effect of Hel 13-5 on the spherical liposomes formed from a Golgi-specific phospholipid mixture (PC/PE/PS/PI/SM/Ch = 50:16:5:6:7:17). We found that Hel 13-5 did indeed alter the morphology of the spherical liposomes made from a Golgi-specific phospholipid mixture. However, it was a surprise to find that Hel 13-5 induced the formation of nanotubules on the scale of, and with the shapes characteristic of, the nanotubules forming the Golgi apparatus (Fig. 2**A**). These nanotubules (*large arrow*) had diameters of  $\sim 40$ – $50$  nm and lengths of  $\sim 400$  nm. They were formed from larger tubules with lengths of  $\sim 600$  nm that had both ends pinched off into twisted tubules with extremely small diameters of  $\sim 2$ – $3$  nm (*small arrows*). These 400 nm nanotubules bore a remarkable morphological resemblance to the nanotubules of the Golgi apparatus (8) and were absolutely distinct from the fibrils formed by the interaction of Hel 13-5 and the egg PC phospholipid liposomes (Fig. 1, **B**–**E**).

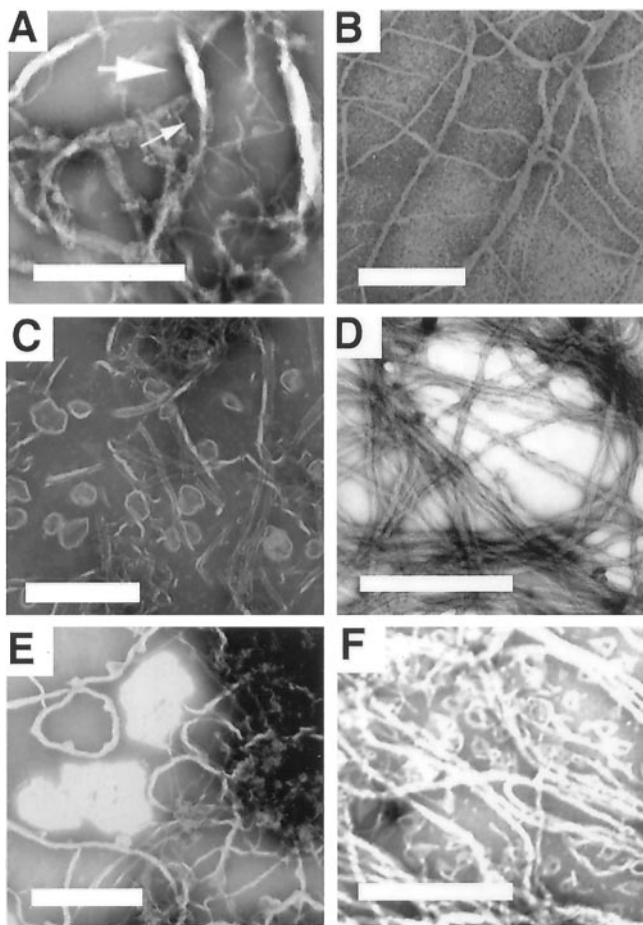
**Nanotubule Formation Depends on Phospholipid Composition**—Although Hel 13-5 formed fibrils with neutral (PC) bilayers, we previously found that it formed fused vesicles in acidic liposomes (PC/PG = 3:1) (6). Thus, fibril formation depends on the lipid species. To investigate in greater detail the dependence of nanotubule formation on lipid composition, we studied the interaction between Hel 13-5 and various neutral and acidic lipid species by means of electron microscopy, turbidity,

the mixture was washed two times with water after casting on mica. **Bar** is 100 nm. Low (**F**), and high (**G**) magnification, electron micrographs of Hel 13-5/PC mixture. After incubating 24 h, the peptide-lipid mixture was centrifuged and fixed with 3% glutaraldehyde, acetone-treated, embedded in resin, sectioned, and analyzed by TEM. **Bars** are 500 nm in **F**, and 100 nm in **G**. **H**, viscosity change in Hel 13-5/PC liposome solution (500  $\mu$ M) after a 24-h incubation as a function of molar ratio.

and dynamic light scattering (Table I). Electron micrographs taken 24 h after the addition of Hel 13-5 to several liposome systems are shown in Fig. 2, B–F. We found that the resulting morphology was indeed dependent on the lipid mixture. Hel 13-5 incubated with the PC/PE/SM (5:3:2) mixture induced

fibril formation containing resinoid, branched tree structures (Fig. 2B). Hel 13-5 incubated with pure SM liposomes induced a mixture of ring-like structures and fibrils with diameters of ~25 nm and lengths of ~500 nm (Fig. 2C). These fibrils were much shorter than those created by Hel 13-5 with pure neutral (PC) liposomes. Hel 13-5 incubated with a neutral/acidic liposome mixture PC/PG (3/1) at a high peptide/lipid ratio (3:1), also induced the formation of twisted tubular fibers with lengths greater than a few  $\mu\text{m}$  and with diameters of ~25 nm (Fig. 2D). With the mixture PC/PE/Ch (5:3:2), Hel 13-5 created curved fibril structures that exhibited surface bumps, mixed with large ring structures (Fig. 2E). The interaction of Hel 13-5 with a lipid mixture based on lung surfactant lipids, DPPC/PC/PG/PE (4:4:1:1) lead to the creation of a mixture of tubular and doughnut-type structures (Fig. 2F). Taken as a whole, the above data imply that Hel 13-5 induces a diverse array of lipid-specific nanotubular structures. Interestingly, a system composed of Hel 13-5 and a lipid mixture based on myelin lipids, Ch/PC/PE/PS/SM/cerebroside (43:9:12:5:10:21), did not form any fibril structures, thus demonstrating that tubule formation was not a universal phenomenon for Hel 13-5 (data not shown).

**Nanotubule Formation Occurs in Solution**—Electron microscopy requires the use of an adsorbed or fixed sample. Thus, structures found this way cannot completely reflect the actual structures in solution, and these structures are the ones of interest. We previously reported that the formation of fibril structures by lipid-peptide complexes in solution can often be detected by the combination of a decrease in turbidity of neutral lipid (egg-PC) suspensions and an increase in mean diameter of the peptide-lipid complex, as measured by dynamic light scattering (6). Based on this concept, mixed systems of Hel 13-5 with other lipids such as PE, SM, PS, PG, and Ch were exam-

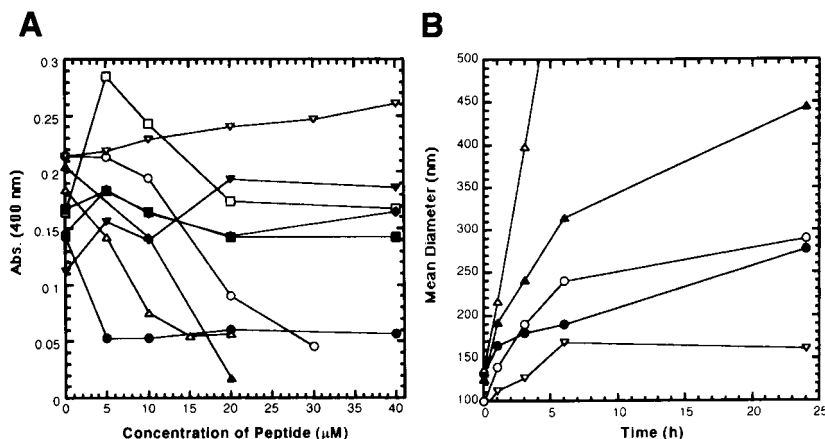


**FIG. 2. Electron micrographs of Hel 13-5 with various lipid mixtures.** A, electron micrographs taken at 24 h after the addition of Hel 13-5 to liposomes prepared from the composition of Golgi membrane lipids; B, PC/PE/SM liposomes; C, SM liposomes; D, PC/PG (3:1); E, PC/PE/Ch (5:3:2) liposomes; and F, liposomes prepared from the composition of the lung surfactant lipids, DPPC/PC/PG/PE (4:4:1:1). Bars are 500 nm.

**TABLE I**  
List of the lipid systems studied

Lipid type	Lipid components
Neutral lipids	Egg PC, SM, Egg PC/egg PE (1:1) <sup>a</sup> , PC/PE/SM (5:3:2) PC/PE/Ch (5:3:2)
Acidic lipids	PC/PG (3:1), PC/PS (3:1)
Lung surfactant lipids	DPPC/PC/PG/PE (4/4/1/1)
Golgi lipids	PC/PE/PS/PI/SM/Ch (50/10/5/6/7/17)
Myelin lipids	Ch/PE/SM/PS/SM/cerebroside (43/9/12/5/10/21)

<sup>a</sup> The numbers in parentheses are molar ratio of lipids.



**FIG. 3. Turbidity and liposome size changes of Hel 13-5 with various lipid (or lipid mixture) complexes.** A, turbid liposome-clearing ability of Hel 13-5 as a function of peptide concentration after the incubation in various lipids. The absorbance of the sample solution was recorded at 400 nm after vigorous vortexing. PC (○), SM (△), PC/PE (●), PC/PE/SM (▲), PC/PE/Ch (▽), PC/PG (▼), PC/PS (□), lung surfactant lipids (■), B, change in mean hydrodynamic diameter of various liposomes as a function of times after the incubation of Hel 13-5 with a NICOMP submicron particle sizer as described previously (6). Peptide and lipid concentrations are 20  $\mu\text{M}$  and

TABLE II  
List of the Hel 3-5-related peptides studied

Peptides were synthesized by the Fmoc strategy based on the solid phase technique starting from Fmoc-PAL-PEG resin using the PerSeptive 9050 automatic peptide synthesizer.

Peptide	Sequence
Hel 13-5	H-KLLKLLKLLKWLKLLKLLL-OH
Hel 8-4	H-KLLKL WLKLLK-OH
Hel 17-7	H-KLLKLLKLLKWLKLLKLLKLLKLLL-OH
L9GHel 13-5	H-KLLKLLKLLKWLKLLKLLL-OH
Hel 13-5Nle	H-KnnKnnnKnWnKnnKnnn-OH <sup>a</sup>
Hel 13-5R	H-RLLRLLLRLLRLLRLLL-OH
Hel 13-5V	H-KVVKVVVVKVVVVKVVV-OH
E3Hel 13-5	H-ELLKLLLELWLKLELLL-OH
KL4W	H-KLLKLLKLLLWLLKLLKLLL-OH

<sup>a</sup> n, norleucine.

ined in solution for the formation of nanotubular/fibril structures (Fig. 3).

The addition of Hel 13-5 to the PC/PE-mixed liposomes at ratio of 1:1 led to a decrease in turbidity of lipid suspension rapidly within 1 h at the low peptide concentrations (Fig. 3A). The solution-clearing ability of Hel 13-5 on this phospholipid mixture was much stronger than that of the Hel 13-5/PC pure liposome mixture. Addition of other neutral lipids such as SM or Ch to PC/PE liposomes (PC/PE/SM or PC/PE/Ch = 5:3:2) exhibited no significant difference in clearing ability as compared with those of PC/PE liposomes. In the case of SM alone, the turbidity of lipid suspension was slightly decreased with increasing concentration of Hel 13-5 (Fig. 3A). A mixture PC/PE/Ch (5:3:2) and a mixture based on the lung surfactant lipids, DPPC/PC/PG/PE (4:4:1:1), did not show much of a decrease in liposome-turbidity with increasing concentration of Hel 13-5 (Fig. 3A). In general, solution turbidities either decreased or remained constant.

The time-dependent changes in liposome size after the addition of the Hel 13-5 (20  $\mu\text{M}$ ) to several liposome solutions (100  $\mu\text{M}$ ) were then considered (Fig. 3B). The sizes of PC liposomes and PC/PE-, PC/PE/SM-, and PC/PE/Ch-mixed liposomes increased with time in similar ways. On the other hand, the size of pure SM liposomes treated with Hel 13-5 increased more gradually than the other systems. These results are in agreement with the electron microscopy that showed that SM formed smaller size fibers. Overall, the turbidity and liposome size data were consistent with the electron microscopy data in the determination of fibril/nanotubule formation.

**Nanotubule Formation Depends on Peptide Chain Length and Hydrophobicity**—We investigated the relationship between the peptide sequence and the fibril structure formation. In this regard, we designed and synthesized a set of Hel 13-5-related peptides as shown in Table II. All the peptides we designed adopted an amphipathic  $\alpha$ -helix structure with a 240° hydrophobic sector. The peptides Hel 17-7 and Hel 8-4 were made to study the effect of chain length. Hel 13-5V, Hel 13-5Nle, and L9GHel 13-5 were created to look at the role of hydrophobicity. Hel 13-5R and E3Hel 13-5 were synthesized to consider the role of hydrophilicity. In addition, we considered KL<sub>4</sub>W, a transmembrane helical peptide evaluated as a therapeutic surfactant for respiratory distress syndrome (9). For simplicity, we began our evaluation of the peptides in an egg PC system. CD studies showed that Hel 13-5, 17-7, L9GHel 13-5, 13-5R and KL<sub>4</sub>W adopted  $\alpha$ -helical structure, whereas Hel 8-4 and 13-5V took on  $\beta$ -strand structures in PC liposomes. Hel 13-5Nle and E3Hel 13-5, which have broad negative bands at 205–225 adopted a mixture of  $\alpha$ -helix and  $\beta$ -structure

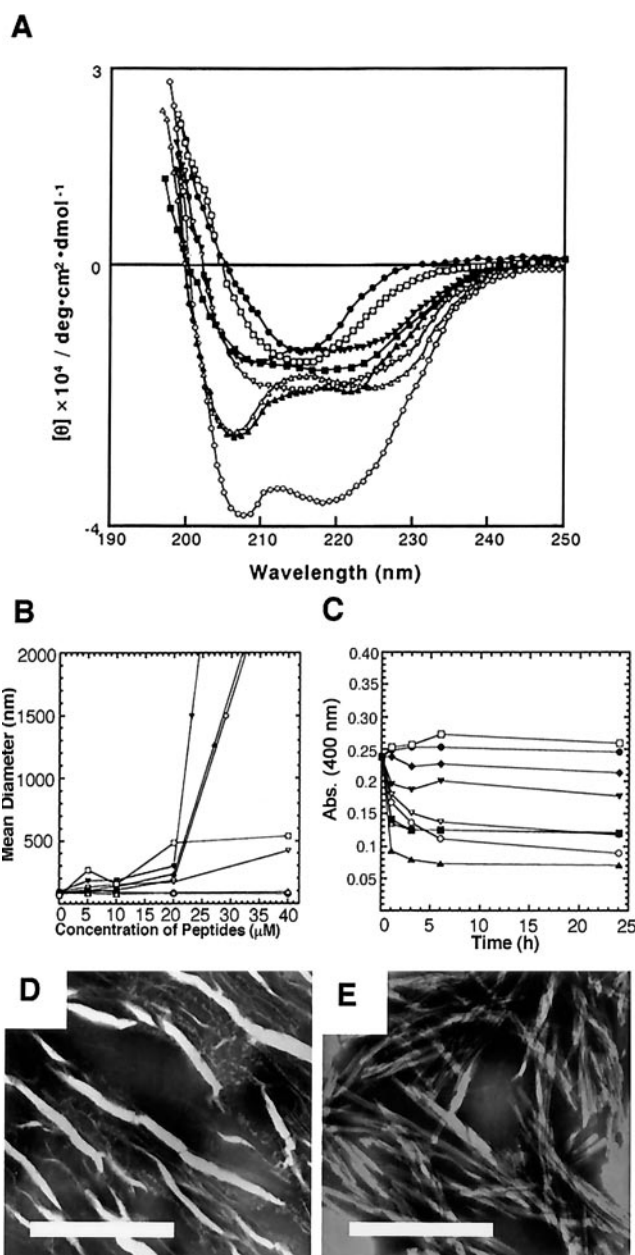


FIG. 4. CD spectra, turbidity, and liposome size changes of Hel 13-5-related peptides in the presence of egg PC liposomes. A, the CD spectra of peptides Hel 13-5 (○), 8-4 (●), 17-7 (△), L9GHel 13-5 (△), 13-5Nle (▽), 13-5R (▼), E3Hel 13-5 (■), and 13-5V (□), measured by a JASCO J-600 spectrometer using a quartz cell with 1-mm path length. The sample solutions of peptides were prepared at concentrations of 40–50  $\mu\text{M}$  in the presence and absence of neutral (PC) liposomes. Lipid concentrations were kept at 1 mM. B, change in mean hydrodynamic diameter of various liposomes as a function of peptide concentrations after the incubation of Hel 13-5. Lipid concentration is 100  $\mu\text{M}$ . All symbols are the same as those of Fig. 3. C, turbidity change of hydrated PC by the peptides as a function of time. Peptide and lipid concentrations are 20  $\mu\text{M}$  and 100  $\mu\text{M}$ , respectively. No addition of peptide (◆). Other symbols are the same as those of Fig. 3. Electron micrograph of PC liposomes incubated with Hel 13-5Nle. E, electron micrograph of PC liposomes incubated with E3Hel 13-5 for 24 h. Bars are 500 nm.

somes after the incubation of peptides with the liposomes at 25 °C were measured. The mean diameter increased drastically for Hel 13-5R and L9GHel 13-5, and gradually for Hel 13-5Nle as shown in Fig. 4B. These results were consistent with electron micrographs that showed the former peptides formed long fibers and the latter one formed short ones (data not shown). As

Hel 13-5Nle, and E3Hel 13-5 decreased drastically within 1 h and then reached a plateau at the peptide concentration of 20  $\mu\text{M}$  (lipid/peptide = 5:1). This suggests that the size of vesicles became smaller than that of liposomes in the absence of peptides. Little change or a slight increase in turbidity, on the other hand, was observed for other peptides, suggesting a weaker or no interaction of the peptides with neutral liposomes. For Hel 13-5Nle, "immature" fibril-like structures formed (Fig. 4D), which were about 0.5–1  $\mu\text{m}$  in length and 40 nm in diameter, much shorter than the structure formed by Hel 13-5. Interestingly, E3Hel 13-5 also created short fibril structures of about 300 nm in length and 50 nm in diameter, similar to Hel 13-5Nle (Fig. 4E). Both peptides created a mixture of  $\alpha$ -helix and  $\beta$ -structures, indicating that the shape of the fibril may also depend on their secondary structure. No fibril structure was observed for the other peptides. Taken together, our results indicate that the chain length and the hydrophobic Leu residues of Hel 13-5 are critical to nanotubule formation, but the charged Lys residues in the hydrophilic region are replaceable by Arg or Glu residues. Experiments differing from the above only in the replacement of the egg PC liposomes with Golgi-specific phospholipid liposomes demonstrate similar trends (data not shown). We previously showed that Hel 13-5 adopts an  $\alpha$ -helix structure when forming fibrils with egg-PC liposomes after 24 h incubation (6). The structure of Hel 13-5 remained  $\alpha$ -helical as it formed fibrils with mixed liposome systems such as PC/PE (1:1), PC/PE/SM (5:3:2), PC/PE/Ch (5:3:2), and PC/PG, indicating that the fiber structure does not result from a conformational change of the peptide itself to  $\beta$ -structure.

#### DISCUSSION

In our systems, the formation of nanotubules depends on peptide properties, the lipid composition, and lipid/peptide ratio. This is also seen in nature, with generation of lipid nanodomains in intracellular organelles, and with the structural changes in membranes triggered by relevant protein/lipid components. For example, the characteristic features obtained by using SM liposomes (Fig. 2C) and Ch-contained liposomes (Fig. 2E) might reflect the dynamic movement of specialized nanodomains, lipid rafts, which are enriched in sphingolipids and Ch, and have been implicated in several cellular functions (10). It has been shown that the pulmonary surfactant system consisting of several lipid and protein components is packed together in dense lamellar bodies that, in the alveoli, are converted to a lattice of tubular myelin bilayers (11). We found that when the liposomes were prepared from the composition of the lung surfactant lipids, Hel 13-5 converted them into a mixture of fiber and doughnut-type structures (Fig. 2F). This result suggests that peptides like Hel 13-5 could be used to assist in the transformation of lung surfactant between the lung surface film and the tubular myelin.

The conversion of spherical liposomes into tubules has been

demonstrated in a complex cell-free system composed of brain lipid extract and the proteins dynamin and amphiphysin, which have been proposed to participate in the endocytosis of synaptic vesicles. The addition of these proteins to a brain lipid extract transformed spherical protein-free liposomes into narrow tubules (12, 13). Although brain extract is not a simple phospholipid model system, the narrow tubules obtained from that system are very similar to ours.

The most striking result of our studies is that the short de novo-designed peptide Hel 13-5 interacts with a Golgi-specific lipid mixture to create Golgi apparatus-like nanotubules. Both the nanotubule length (600 nm) and diameter ( $\sim 40$ –50 nm) were on the order of Golgi apparatus tubules. Furthermore, the ends of the nanotubules were pinched off to narrow diameters of  $\sim 2$ –3 nm, giving the tubules a remarkable morphological resemblance to the nanotubules of the Golgi apparatus. It suggests that the membrane-bound proteins of such an organelle must at some level have properties embodied in Hel 13-5, e.g. the formation of hydrophobic helices. In various morphological studies, it has been shown that such membrane tubules are generated from the Golgi complex and *trans* Golgi network, and serve as bridging between cisternal stacks (3, 4). In addition to the precise role of these tubules in membrane trafficking events, the molecular mechanisms forming membrane tubules are not known.

Our work provides a novel model system for the study of tubule formation in various intracellular events. Using this system, we have shown that lipid composition and peptide structure control nanotubule morphology. A thorough understanding of tubulation mechanisms will be critical to the creation of nanovesicular structures designed for example, to entrap and deliver drugs. Finally, although the question of precisely how nature engineers organellar membranes remains unknown, our approach provides a basic set of tools to begin addressing such questions.

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