Transferrin-Modified Liposomes Equipped with a pH-Sensitive Fusogenic Peptide: An Artificial Viral-like Delivery System†

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ABSTRACT: Liposomes are one of the most promising systems for selective cellular targeting via introduction of specific ligands for cell-surface receptors. After being taken up by the cells, these liposomes usually follow intracellular pathways of receptor-mediated endocytosis. Control of intracelluar trafficking is required for optimized drug delivery. In this study, we elucidated the intracellular fate of transferrin-modified liposomes and succeeded in altering it by introducing the pH-sensitive fusogenic peptide, GALA (WEAALAEALAEALAEHLAEALAEALEALAA). Transferrins that are chemically attached to a liposomal surface (Tf-L) were internalized via receptor-mediated endocytosis more slowly than unmodified transferrins. In contrast to the recyclable nature of transferrin, liposome-attached transferrins together with encapsulated rhodamines were retained in vesicular compartments. When GALA was introduced into liposomal membranes using a cholesteryl moiety for anchoring (Chol-GALA), rhodamines were efficiently released and diffused into the cytosol. The addition of GALA to the Tf-L-containing medium or the encapsulation of GALA in Tf-L did not induce similar effects. These results clearly indicate that GALA must be present on the surface of liposomes to exert its function. *In vitro* energy transfer and dynamic light scattering experiments suggested that the endosomal escape of the encapsulates in Tf-L equipped with Chol-GALA can be attributed to pH-dependent membrane fusion. With GALA present on the surface, intracellular trafficking of liposomes after receptor-mediated endocytosis could be successfully controlled.

Viruses can be considered to be sophisticated gene delivery systems. The human immunodeficiency virus binds to the plasma membrane via CD4 and other surface receptors, and the core is then internalized into the cytoplasm. These interactions trigger the pH-independent fusion of the viral envelope with the plasma membrane (*1*). The viral RNA is then converted to a complementary DNA form by reverse transcriptase and is imported into the nucleus (*2*). The influenza virus binds to the plasma membrane and is internalized into endosomes by receptor-mediated endocytosis (*3*). The viral envelope fuses with the endosomal membrane in the low-pH environment of the late endosome so that the matrix protein and the eight RNA segments are released into the cytoplasm (*4*). The individual segments are then imported into the nucleus (*5*). Most viruses utilize intracellular pathways of the target cells and control their own direction by using their sorting devices.

The utilization of receptor-mediated endocytosis would be expected to be a pathway for the intracellular delivery of anticancer drugs in cancer chemotherapy as well as nucleic acids in gene therapy (*6*). However, once the drug is endocytosed, the control of intracellular trafficking is difficult, since it is under the regulation of the cell. Therefore, to optimize intracellular trafficking after receptor-mediated endocytosis to a target organelle such as the cytosol and the nucleus, artificial sorting devices are required (*7*).

The focus of this study was on transferrin receptor-mediated endocytosis as a model entering pathway for the rational development of sorting devices for intracellular targeting. Transferrin receptors (Tf-R)[1] appear to be expressed in all nucleated cells in the body. In malignant cells, the level of Tf-R expression is elevated, due to the strict requirement for iron for their growth (*8*). Tf-R are internalized into clathrin-coated pits and delivered to early endosomes or recycling endosomes, and are then recycled to the plasma membrane via two independent pathways (*9*). Some Tf-R are also known to be delivered to the Golgi complex (*10*).

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Liposomes are promising systems for intracellular targeting because it is feasible to equip them with various sorting devices. Since the intracellular trafficking of Tf-R is diverse, as introduced above, elucidation of the routes of the intracellular trafficking of Tf, when attached to liposomes (Tf-L), would be important for the design of delivery systems using liposomes. Once the liposomes are effectively internalized to the target cells due to modification by transferrin, another sorting device would be required to permit the liposomes, or their contents, to escape from endosomes to the cytosol. Inspired by fusion peptides as has been reported for viruses, we examined the applicability of the peptide, GALA, as the second sorting device. GALA is an artificially designed and pH-dependent fusogenic peptide composed of 30 amino acids (*11*, *12*).

The findings in this study show that a significant proportion of the Tf-modified liposomes become trapped in endosomes and lysosomes without recycling with Tf-R. We also show that the addition of the GALA peptide to the culture medium or to the aqueous compartment of liposomes did not promote the cytosolic release of the liposomal contents. On the other hand, a highly efficient cytosolic release was achieved when the GALA peptide was present on the liposomal surfaces, which was attained by anchoring the GALA peptide to the liposomal membranes using a cholesterol derivative (Chol-GALA). The results of confocal laser microscopic observation of cells treated with these Tf-modified liposomes and studies of the molecular mechanism for the endosomal escape of the Chol-GALA-containing liposomes are reported.

MATERIALS AND METHODS

Chemicals. EPC, Chol, PDP-PE, NBD-PE, and Rh-PE were purchased from Avanti Polar Lipids (Alabaster, AL). Tf, SPDP, and DTT were purchased from Sigma (St. Louis, MO). S-Rh, 6-CF, FITC-Tf, Rh-Tf, and Lysosensor were purchased from Molecular Probes (Eugene, OR).

Cell Culture. K562 cells, human chronic myelogenous leukemia cells, were cultured in RPMI 1640 medium supplemented with 10% FBS in a humidifier incubator (5% CO_2) at 37 °C.

Peptide Synthesis. The peptide chains of the GALA and Chol-GALA peptides were constructed by Fmoc solid phase peptide synthesis (*13*). The C-terminus of the respective peptide was designed to have an amide structure for the ease of preparation. In the case of Chol-GALA, the cholesteryloxycarbonyl moiety was introduced on the peptide resin using cholesteryl chloroformate and diisopropylethylamine. Subsequent treatment of the peptide resins with a trifluoroacetic acid/ethanedithiol mixture (95:5) gave the desired peptides. The fidelity of the products was ascertained by time-of-flight mass spectrometry (TOFMS).

Preparation of Tf-Modified Liposomes. Liposomes were composed of EPC and Chol (2:1 molar ratio), and for cross-linking with transferrin, PDP-PE was incorporated at 1.0 mol % of the total lipid. Liposomes were prepared by reverse phase evaporation (*14*) followed by extrusion with a Mini-Extruder (Avanti Polar Lipids), through polycarbonate membrane filters (Nuclepore) of 400, 200, and 100 nm, 20 times for each pore size. Sulforhodamine B (S-Rh) was used as an aqueous phase marker. Unencapsulated S-Rh was separated on a Bio-Gel A-1.5m column (100−200 mesh) equilibrated with PBS(−) buffer. GALA was encapsulated as the aqueous phase. Cholesteryloxycarbonyl-GALA (Chol-GALA) was introduced into the liposomal membrane at 2 mol % of the total lipids. The particle sizes of liposomes were measured by means of a dynamic light scattering spectrophotometer (ELS-8000, Photal Otsuka Electronics).

For the attachment of Tf to liposomes, Tf with an FITC or S-Rh probe (final concentration of 62.5 μM) were treated with SPDP (final concentration of 66 μM) for 30 min at room temperature. The resulting 3-(2-pyridinedithio)propioyl (PDP)-Tf was separated from unreacted SPDP by gel filtration on a Sephadex G-25 Fine column equilibrated with PBS(−). PDP-Tf was reduced with 50 mM DTT in H_2O for 30 min at room temperature to yield 3-mercaptopropyl-Tf, which was purified on a Sephadex G-25 Fine column. Disulfide cross-link formation between Tf and liposomes was then conducted by treating 3-mercaptopropyl-Tf with the PDP-PE-containing liposomes at a 4:1 weight ratio at 4 °C overnight. The resulting Tf-L were separated from unreacted Tf on a Bio-Gel A-1.5m column (200−400 mesh). Tf-L were equilibrated with 50 mM $Fe_2(SO_4)_3$-EDTA (pH 7.4, final Fe^{3+} concentration of 100 μM) because of Tf resaturation with Fe^{3+}. Tf-L labeled with FITC and Rh, which were represented as Tf (FITC)-L and Tf (Rh)-L, respectively, were used further experiments.

To analyze the intracellular trafficking of liposomal lipids, *N*-(lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (Rh-PE) was introduced into the liposomal lipids (0.3 mol %) instead of encapsulating S-Rh in their aqueous phase. For a detailed study of intracellular fate of Tf after internalization, liposomes containing rhodamine-labeled Tf (Rh-Tf) were prepared in basically the same way as in the case of those containing FITC-Tf except that S-Rh was not encapsulated in the liposomes.

Number of Bound Tf per Liposome. The number of bound Tf per liposome (molecules per vesicle) was calculated on the basis of the equation $(A/B)C$, where $A-C$ represent the total number of Tf molecules in a liposome suspension, the total number of lipid molecules in a liposome suspension, and the number of lipid molecules per liposome, respectively. *A* was calculated by measuring the protein concentration of Tf in a Tf-L suspension multiplied by Avogadro's number. *B* was calculated by multiplying the total lipid concentration of the Tf-L suspension by Avogadro's number. *C* was estimated from the mean diameter of Tf-L according to the method of Enoch et al. (*15*).

Intracellular Trafficking of Tf. K562 cells (2.0×10^4 cells/mL) were treated with FITC-Tf (final concentration of 0.5 μM) in serum free medium at 4 °C for 10 min and at 37 °C

[1] Abbreviations: EPC, egg phosphatidylcholine; Chol, cholesterol; Chol-GALA, cholesteryloxycarboxyl-GALA; PDP-PE, *N*-[3-(2-pyridyldithio)propionate]-1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; PBS, phosphate-buffered saline; Tf, transferrin; SPDP, 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester; DTT, dithiothreitol; FITC, fluorescein isothiocyanate; S-Rh, sulforhodamine B; 6-CF, 6-carboxyfluorescein; PI, propidium iodide; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tf (FITC)-L, liposomes modified by Tf labeled with FITC; Tf (Rh)-L, liposomes modified by Tf labeled with Rh.

for 60 min in the presence of $Fe_2(SO_4)_3$-EDTA (final Fe^{3+} concentration of 100 μM). Cells were then washed twice with cold PBS(−) and analyzed by confocal laser microscopy (LSM510, Carl Zeiss). To examine the recyclability of Tf, cells were first incubated with Tf as stated above, and washed with PBS(−) to remove Tf in the medium. The cells were further incubated with RPMI medium supplemented with 10% FBS in the absence of Tf and analyzed as described above.

Intracellular Trafficking of Tf-L. K562 cells (10×10^4 cells/mL) in serum free medium were incubated with Tf-L encapsulating S-Rh as an aqueous phase marker (final lipid concentration of 630 μM) for up to 18 h in the presence of $Fe_2(SO_4)_3$-EDTA (final Fe^{3+} concentration of 100 μM). After incubation for the indicated time, cells were washed twice with PBS(−) at 4 °C and analyzed by confocal laser microscopy. After an 18 h incubation, the cells were washed twice with RPMI medium supplemented with 10% FBS and further incubated for 6 h in the absence of Tf-L. When Lysosensor was used, 1 mM Lysosensor (final concentration of 1 μM) was added 30 min before the indicated incubation time. The cells were then analyzed as described above.

Inhibition of Tf-L Endocytosis by Free Tf. K562 cells (10×10^4 cells/mL) in serum free medium were incubated with free FITC-Tf and Tf (Rh)-L as described above. The free: liposomal Tf ratios were 0, 1, 3, 10, and 20 in this experiment. Cells were analyzed by confocal laser scan microscopy (CLSM) as described above.

Flow Cytometry. We used 6-CF as a liposomal marker for flow cytometry. K562 cells (5×10^5 cells) in serum free medium were incubated with unmodified liposomes or Tf-L for 18 h, as described above. After being washed, the cells were suspended in 100 μL of PBS(−), and 1 mg/mL PI was then added to the suspension (final concentration of 50 μg/mL). After a 30 min incubation on ice, an additional 400 μL of PBS(−) was added, and the suspension was then filtered through a nylon mesh. Cells were analyzed with a flow cytomerer (FACScan, Becton Dickinson).

Fluorescence Resonance Energy Transfer (FRET). Membrane fusion was assessed by fluorescence resonance energy transfer (FRET) (*16*). The probe liposomes were composed of EPC and Chol (2:1 molar ratio), which contained both NBD-PE and Rh-PE at 1 and 0.5 mol %, respectively. Chol-GALA (2 mol % lipid) was incorporated into nonprobe liposomes composed of EPC and Chol (2:1 molar ratio). The probe liposomes and nonprobe liposomes in PBS(−) were mixed at a molar ratio of 1:9 (final lipid concentration of 2 mM) and incubated at 37 °C for 1 h with the appropriate pH adjustment. After incubation, the pH was returned to 7.4 and energy transfer was assessed by measuring the fluorescence intensity (excitation at 470 nm and emission at 530 nm).

Membrane fusion between probe and nonprobe liposomes will lead to the diffusion of NBD and rhodamine into the lipid membranes, which causes an increase in the fluorescence intensity at 530 nm by cancellation of energy transfer. As a control, probe liposomes containing NBD-PE and Rh-PE were incubated with nonprobe liposomes without Chol-GALA. The maximum fluorescence was defined as the fluorescence when the liposomes were dissolved in Triton X-100 (final concentration of 0.5%). Fusion (%) was estimated by the decrease in the level of energy transfer in accordance with membrane fusion, and was calculated as follows:

$$\text{fusion (\%)} = (F - F_0)/(F_{max} - F_0) \times 100$$

where F, F_0, and F_{max} represent the fluorescence intensity of the sample, the fluorescence intensity of the blank [liposomes composed of EPC and Chol (2:1 molar ratio)], and the maximum fluorescence intensity, respectively.

Circular Dichroism. CD spectra were recorded on a Jasco J-720 spectrometer using a 2 mm cuvette at 20 °C. Solutions of peptides or liposomes [EPC/Chol (2:1 molar ratio)] in PBS(−) at pH 7.4 were acidified to pH 5.0, and 15 min later, spectra were recorded.

RESULTS

Characterization of Tf-L. The sizes of liposomes were estimated with a dynamic light scattering spectrophotometer and found to range from 130 to 145 nm in diameter. The number of Tf molecules per liposome ranged from 100 to 183. The final concentration of GALA in GALA-encapsulating Tf-L and Chol-GALA-containing Tf-L in the incubation medium was 4.5 and 9.5 μM, respectively. The final concentration of S-Rh in GALA-encapsulating Tf-L and Chol-GALA-containing Tf-L in the incubation medium was 61 and 54 μM, respectively.

Intracellular Trafficking of Tf. The intracellular trafficking of Tf, which was labeled with FITC (FITC-Tf), was examined by confocal laser microscopy using K562 cells, a human chronic myelogenous leukemia cell, which is known to overexpress Tf-R (*17*, *18*). FITC-Tf was added to the cultured medium at 4 °C (final concentration of 0.5 μM). Significant surface binding of the Tf was observed (Figure 1A,B). A rapid internalization of FITC-Tf was observed when the temperature was increased to 37 °C (Figure 1C−F). Sixty minutes later, the cells were washed and then incubated for an additional 60 min in the absence of FITC-Tf. Intracellular Tf disappeared, presumably accompanied by the receptor recycling of Tf-R as reported previously (*9*) (Figure 1G,H).

Intracellular Trafficking of Tf-L. To examine whether the liposomal attachment of Tf has any influence on its intracellular fate, the internalization of Tf-L by K562 cells was examined. Tf was labeled with FITC, and Rh was employed as an aqueous phase marker of the liposomes. Tf (FITC)-L were internalized by K562 cells, but more slowly than Tf that was not attached to liposomes. A few hours were required for the internalization of Tf (FITC)-L, whereas only several minutes were necessary for that of free Tf, as seen in Figure 1. Colocalization of the liposomal surface FITC-Tf and the encapsulated S-Rh in vesicular compartments was observed even after incubation for 18 h, which suggests an inefficient cytosolic release of S-Rh from Tf (FITC)-L. The vesicular compartments were considered to be fused endosomes, the size of which was in the range of micrometers. On the other hand, little internalization was observed for liposomes not modified by Tf (data not shown).

After an 18 h incubation, the cells were washed and incubated for an additional 6 h in medium without Tf-L. Although liposomally encapsulated S-Rh remained in endosome-like compartments, FITC-Tf-derived signals disappeared in the cells. Similar results were obtained when

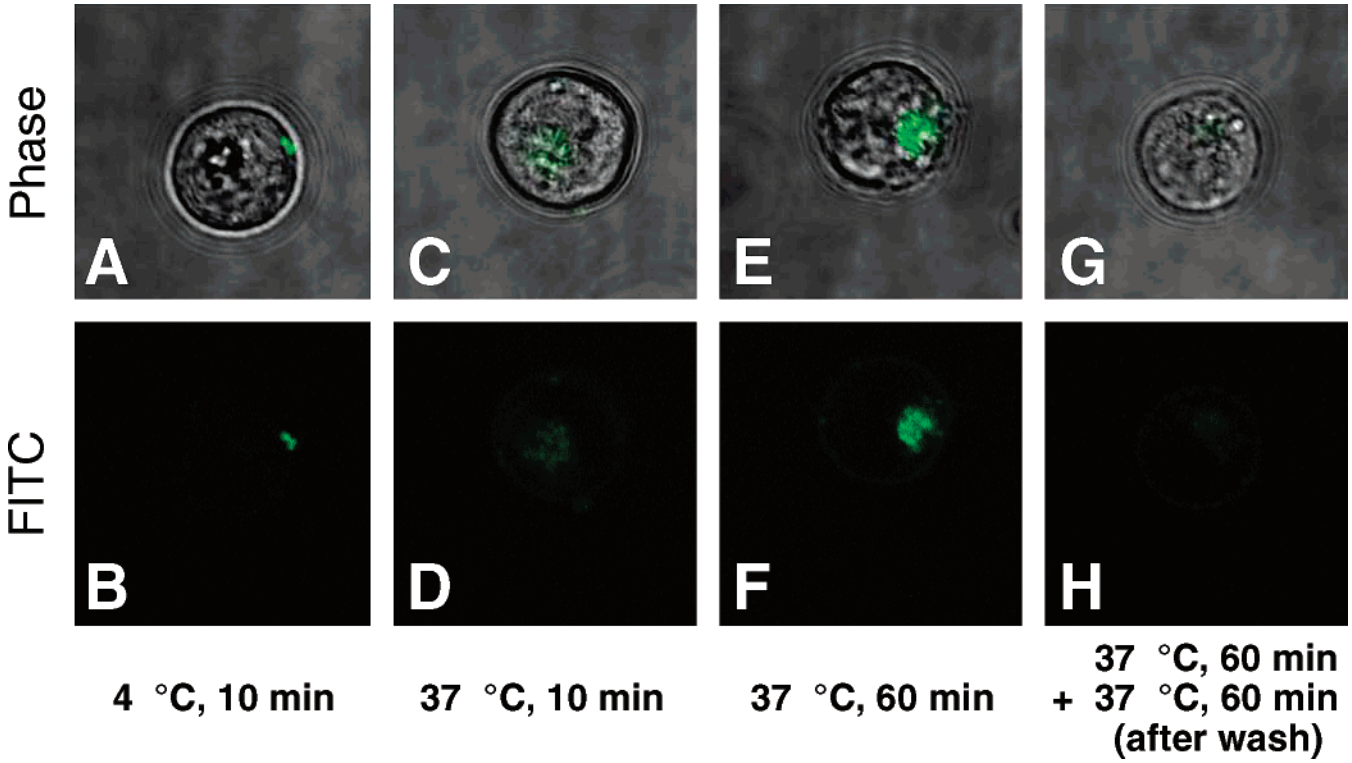

FIGURE 1: Intracellular trafficking of FITC-Tf. K562 cells were incubated with FITC-Tf (final concentration of 0.5 μM) at 4 °C for 10 min (A and B). After the medium had been replaced, the cells were observed after incubation with FITC-Tf at 37 °C for 10 min (C and D) and 60 min (E and F). Then, cells were washed and incubated for an additional 60 min in the absence of FITC-Tf at 37 °C for 60 min (G and H). The cells were observed by confocal laser microscopy using a 40× lens: (top row) phase contrast images and (bottom row) fluorescent images of FITC-Tf.

liposomal membranes were labeled with Rh-PE, and the intracellular trafficking of Rh-PE as well as FITC-Tf was observed by confocal laser microscopy. Colocalization of Rh-PE in endosome-like compartments with FITC-Tf was observed 18 h after incubation of the cells with Tf (FITC)-L. After the washout of Tf (FITC)-L, the signals from FITC-Tf were not detected in the cells, while Rh-PE remained in endosome-like compartments, like in the case of the aqueous phase marker S-Rh (data not shown).

One plausible explanation for the result described above is that the FITC-Tf was recycled back to the plasma membrane together with Tf-R and released to the medium, even that attached to liposomes. However, there is a possibility that FITC-Tf remained in acidic compartments such as endosomes or lysosomes where the fluorescent intensity of FITC decreased. Therefore, we examined rhodamine, a relatively pH-independent fluorescent agent, as a probe for Tf. The cells were treated in a manner similar to that for Tf (Rh)-L for 18 h (Figure 2A−C) and in the absence of Tf (Rh)-L for 6 h (Figure 2D−F). Significant colocalization of Rh and Lysosensor was observed even after the washout of Tf (Rh)-L. This observation suggests that a certain population of Tf was captured by the endosomes or lysosomes when internalized in the form of Tf (Rh)-L. Therefore, the liposomal attachment of Tf had a measurable effect on its intracellular fate in trafficking.

The time course for the internalization of Tf-L is shown in Figure 3A. The percentage of cells which internalized Tf-L increased in a time-dependent manner and essentially reached a plateau after 6 h (~50%). These results indicate that Tf-L are internalized more slowly than free Tf. Moreover, the maximum percent of cells in which Tf-L was internalized was lower than of those internalized free Tf (>95%).

Inhibition of Tf-L Endocytosis by Free Tf. To examine the contribution of Tf-R-mediated endocytosis in the uptake of Tf-L, the inhibition of the uptake of Tf-L by free Tf was examined. Tf (Rh)-L and FITC-Tf were co-incubated with K562 cells. The rate of internalization of Tf (Rh)-L decreased as the result of the addition of free FITC-Tf, and the decrease was dependent on the free FITC-TF concentration (Figure 3B). This result strongly indicates that Tf-R-dependent endocytosis constitutes the principal uptake pathway of Tf-L.

Flow Cytometry. CLSM was mainly used to analyze the intracellular trafficking of Tf-L. Flow cytometry analysis was also used to obtain more quantitative data. Figure 3C shows the 6-CF fluorescence intensity of PI-negative cells. This suggests that Tf-L had a high affinity for K562 cells compared with unmodified liposomes.

Intracellular Trafficking of Liposomal S-Rh in Tf-L Encapsulating GALA. The results described above suggested that Tf-L had the ability to deliver the aqueous phase marker into endosome-like compartments. However, a significant release of the marker to the cytosol was not observed. The findings also indicate that a certain amount of Tf was captured in the endosomes and/or lisosomes by the conjugation of liposomes without recycling with Tf-R. GALA is reported to be a pH-dependent fusogenic peptide. We were interested in the possibility of enhancing the cytosolic release of the aqueous phase marker by the addition of GALA to the cultured medium together with Tf-L. The addition of GALA (final concentration of 13 μM) to the incubation medium together with Tf (FITC)-L, however, did not result

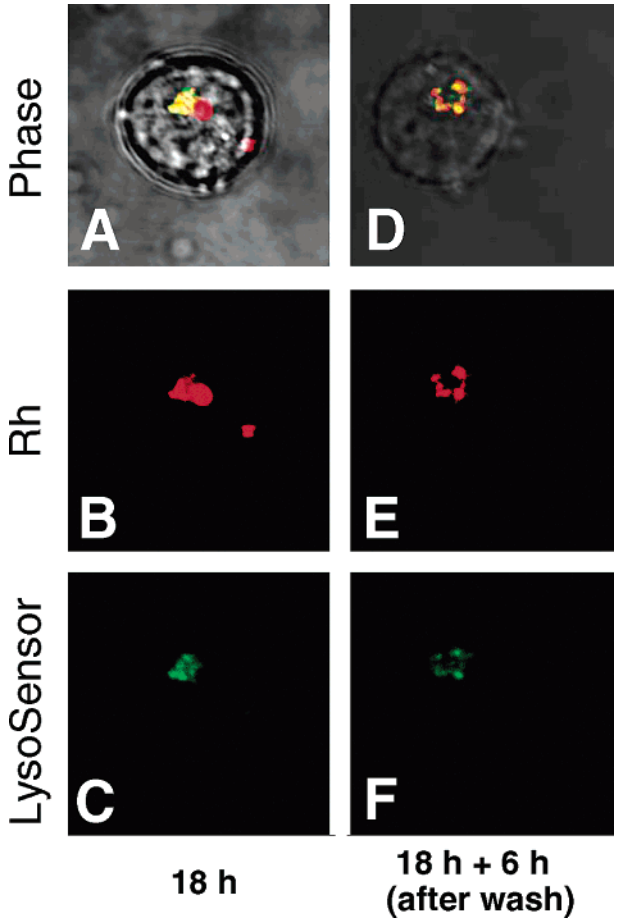

FIGURE 2: Failure of recycling of Tf-L. K562 cells were incubated with Tf (Rh)-L at 37 °C for 18 h (A−C). After the medium had been replaced, the cells were further incubated for 6 h in the absence of Tf (Rh)-L (D−F). Lysosomes were stained with Lysosensor (final concentration of 1 μM) for 30 min prior to analysis by confocal microscopy: (top row) phase contrast images, (middle row) fluorescent images of Rh-Tf, and (bottom row) images of Lysosensor.

in a significant release of S-Rh into the cytosol (data not shown).

We next examined whether the encapsulation of GALA in Tf-L improved the cytosolic release of the aqueous phase marker, since encapsulation would increase the local concentration of GALA at the attachment sites of Tf-L in the cells. Tf (FITC)-L, encapsulating GALA, were found to be internalized (Figure 4). Colocalization of FITC-Tf and S-Rh was observed in endosome-like compartments (Figure 4G−I). This result was very similar to that for the case of Tf (FITC)-L without GALA, in which no remarkable enhancement in the cytosolic release of S-Rh was found. The failure of Tf-L encapsulating GALA in enhancing the cytosolic release might be due to an inefficient decrease in the intraliposomal pH or inefficient interactions between GALA and the endosomal membrane. Therefore, we synthesized the cholesterol derivative of GALA (Chol-GALA, Figure 5A) with the expectation that the cholesteryl moiety would anchor the GALA segment such that it would be efficiently displayed on the surface of the liposomes (Figure 5B).

Cytosolic Release of Liposomal S-Rh in Tf-L Equipped with Chol-GALA. Tf (FITC)-L containing Chol-GALA were applied to K562 cells, and intracellular trafficking was monitored as described above. Tf (FITC)-L containing Chol-

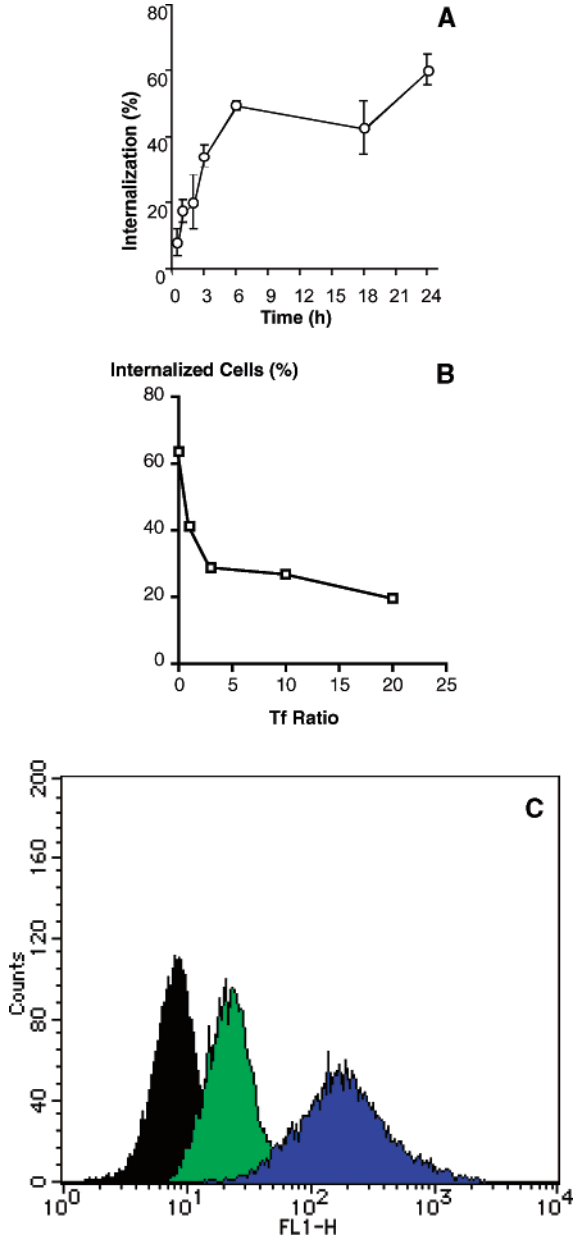

FIGURE 3: (A) Time course of internalization of Tf-L. K562 cells were incubated with Tf-L under the same conditions described in the legend of Figure 2. The percent of cells that were internalized Tf-L is shown. (B) Inhibition of Tf-L endocytosis by free Tf. K562 cells were incubated with Tf (Rh)-L and free FITC-Tf by changing the free FITC-Tf from 0 to 20 equiv of free Tf. The percent of cells that were internalized Tf (Rh)-L is shown. (C) Flow cytometry analysis. K562 cells were incubated with Tf-L or unmodified liposomes for 18 h. Both liposomes contained 1 mM 6-CF. The histograms show the 6-CF fluorescence intensity of the PI-negative cells. The histograms for control cells, unmodified liposomes, and Tf-L are shown in black, green, and blue, respectively.

GALA were internalized at a similar rate with other Tf-L. As shown in Figure 6A−C, a remarkable cytosolic release of encapsulating S-Rh was observed after incubation for 3 h. Although some S-Rh was colocalized with FITC-Tf in endosome-like compartments, the majority of the S-Rh was released into the cytosol. These facts suggest that the endosomal release of S-Rh proceeded quite efficiently with the aid of GALA that was present on the liposome surface. The amount of internalized S-Rh continued to increase until the washout of Tf (FITC)-L (Figure 6G−I). It is noteworthy

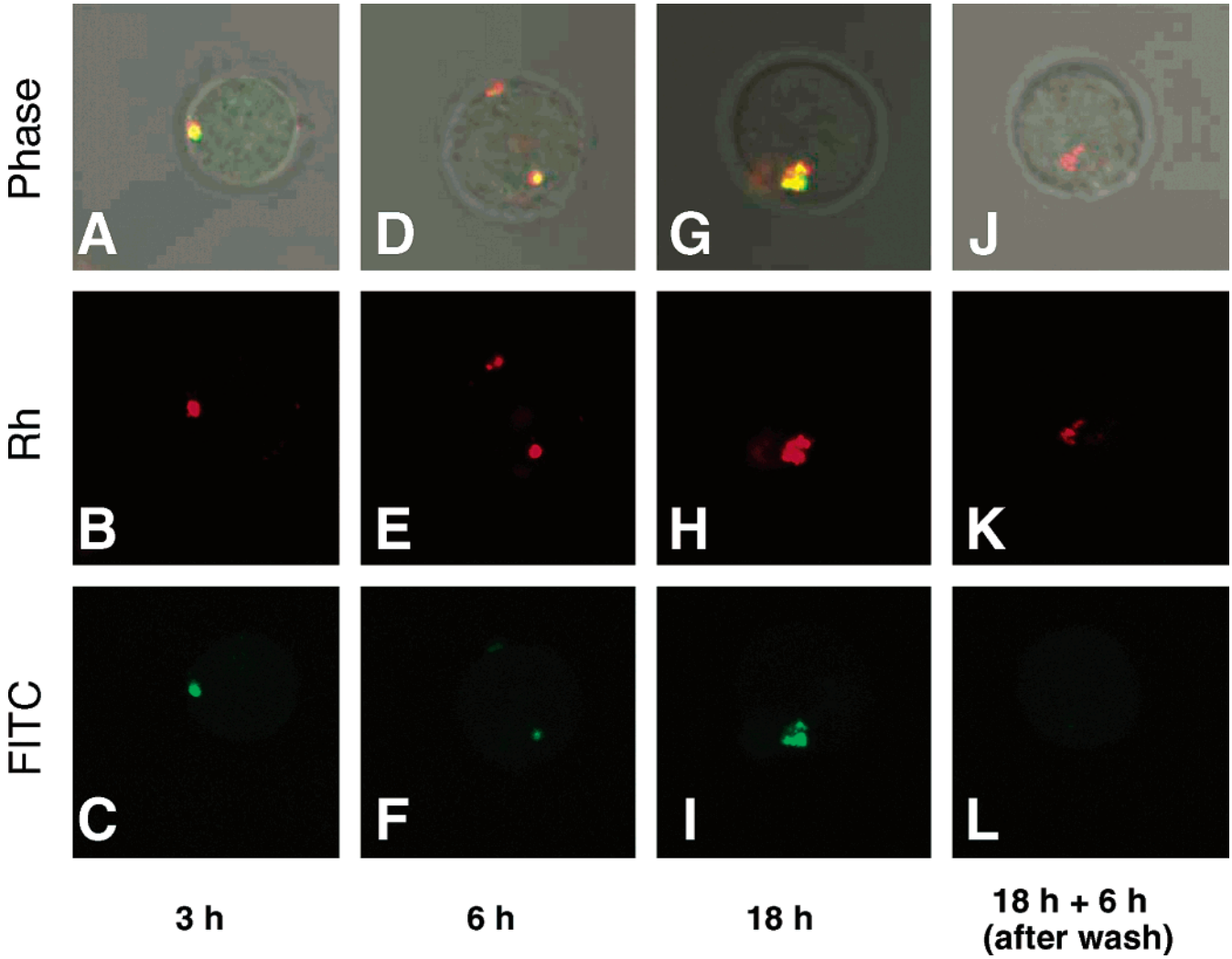

FIGURE 4: Little improvement in the cytosolic release of S-Rh by liposomal encapsulation of GALA. Tf (FITC)-L containing S-Rh as an aqueous phase marker were used. GALA was also encapsulated in the aqueous phase (final concentration of 4.5 μM). Cells were incubated with liposomes and analyzed by confocal laser microscopy. Cells were observed after incubation with Tf (FITC)-L encapsulating GALA at 37 °C for 3 h (A−C), 6 h (D−F), and 18 h (G−I). The cells were then washed and further incubated for 6 h in the absence of Tf (FITC)-L (J−L): (top row) phase contrast images, (middle row) fluorescent images of liposomally encapsulated S-Rh, and (bottom row) fluorescent images of FITC-Tf.

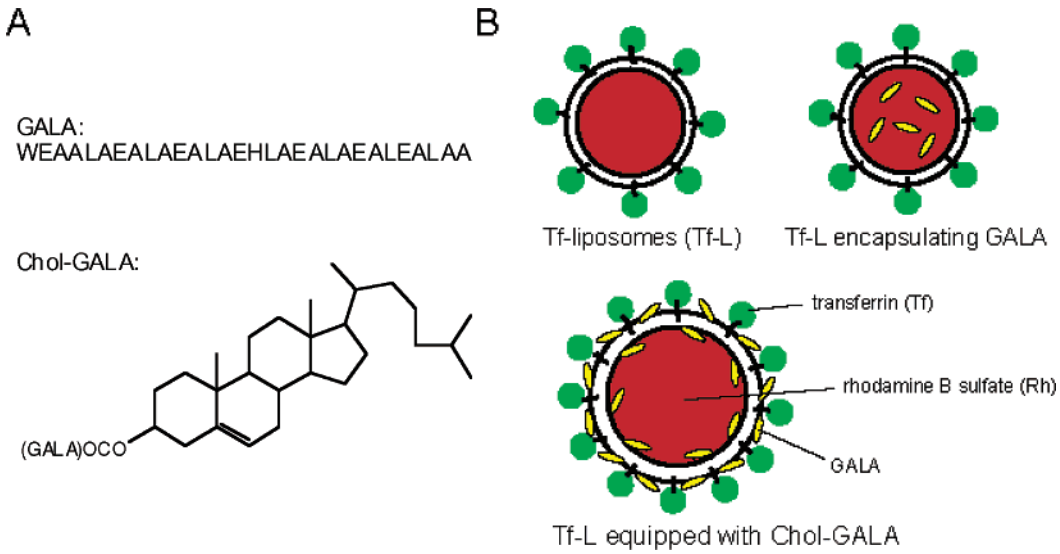

FIGURE 5: Structure of GALA and its cholesterol derivative (Chol-GALA) (A) and schematic representation of Tf-L, Tf-L encapsulating GALA, and those equipped with Chol-GALA (B). Tf-L encapusulating GALA contain GALA in an aqueous phase, while Tf-L equipped with Chol-GALA display the GALA peptide segments on both sides of liposomal surfaces with the aid of the Chol moiety.

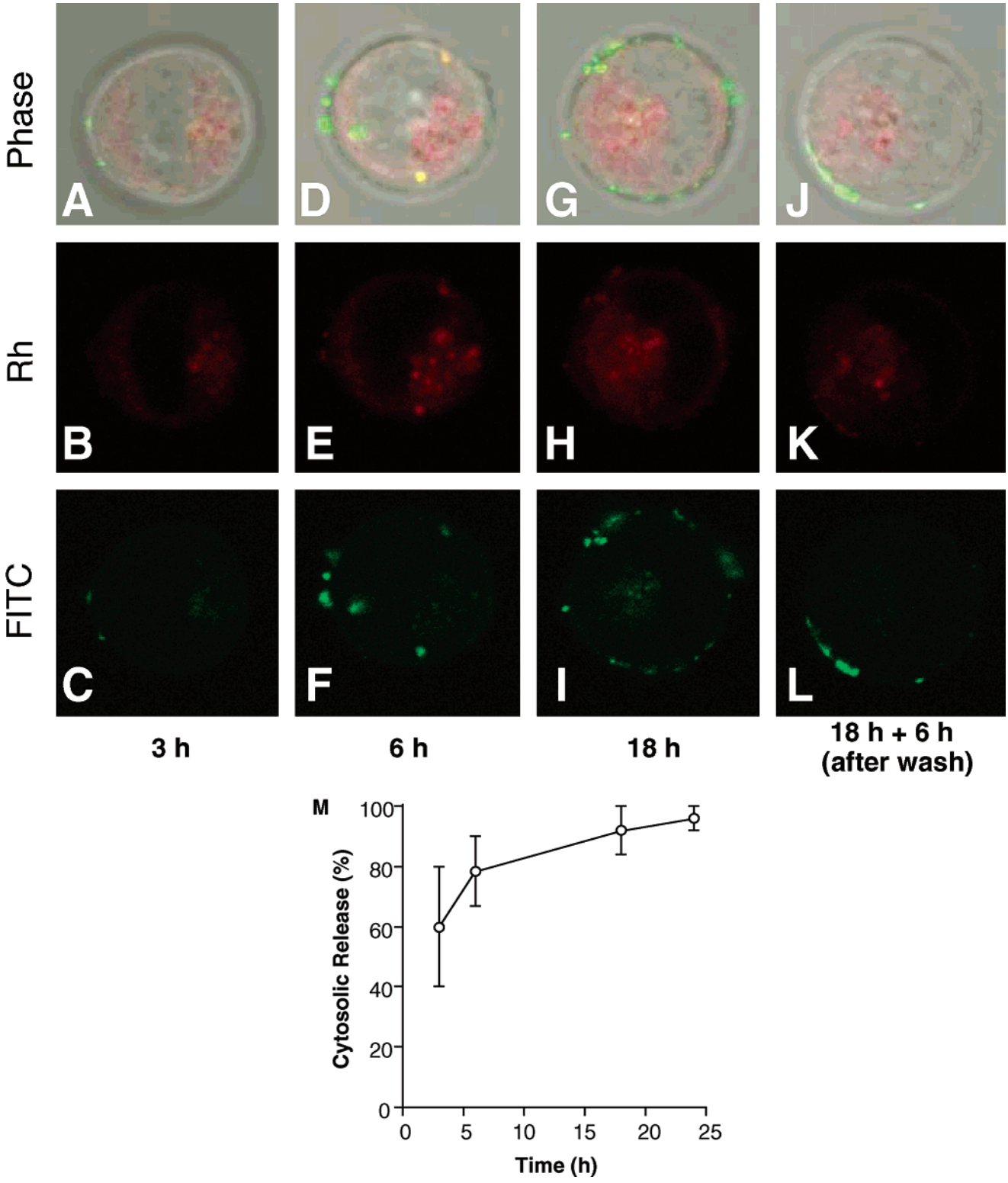

FIGURE 6: Efficient cytosolic release of liposomally encapsulated Rh from Tf-L equipped with Chol-GALA. Tf (FITC)-L containing S-Rh as an aqueous phase marker were used. Chol-GALA (final concentration of 9.5 μM) was introduced into the liposomal membrane as illustrated in Figure 5B. Cells were incubated with the liposomes and analyzed by confocal laser microscopy. Cells were observed after incubation with Tf (FITC)-L containing GALA at 37 °C for 3 h (A−C), 6 h (D−F), and 18 h (G−I). The cells were washed and further incubated for 6 h in the absence of Tf (FITC)-L (J−L): (top row) phase contrast images, (middle row) fluorescent images of encapsulated S-Rh, and (bottom row) fluorescent images of FITC-Tf. (M) Time course of the cytosolic release of liposomal S-Rh from Tf-L eqipped with Chol-GALA. Tf-L equipped with Chol-GALA were added to K562 cells under the same conditions described for panel A, and the percentage of cells which released liposomal S-Rh into the cytosol is shown.

that, with the marked differences between Tf (FITC)-L and GALA-encapsulating Tf (FITC)-L, FITC-Tf was recycled and remained on the plasma membranes when observed 6 h after the washout of Tf (FITC)-L containing Chol-GALA (Figure 6J−L). Therefore, Chol-GALA significantly affected the intracellular fate of Tf-L. These results suggest that the topology of GALA, namely, its surface disposition on the liposomal membrane, was a critical factor in these effects.

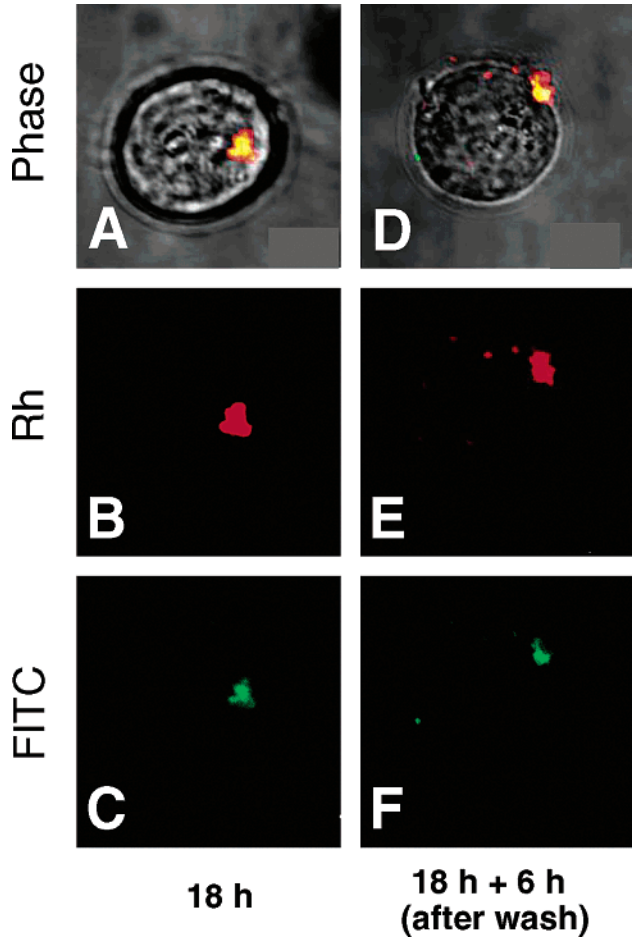

FIGURE 7: Intracellular trafficking of liposomal Rh-PE in Tf-L equipped with Chol-GALA. Tf (FITC)-L containing Rh-PE as a lipid phase marker were used. Chol-GALA was also introduced into the liposomal membrane. Cells were treated with liposomes and analyzed by confocal laser microscopy. Cells were observed after incubation with Tf (FITC)-L equipped with GALA at 37 °C for 18 h (A−C), and then cells were washed and further incubated for 6 h in the absence of Tf (FITC)-L (D−F): (top row) phase contrast images, (middle row) fluorescent images of Rh-PE, and (bottom row) fluorescent images of FITC-Tf.

The time course of the percent cells showed that the diffusion of S-Rh to the cytosol was highly efficient (Figure 6M). When Tf-L equipped with Chol-GALA were incubated, the cytosolic release of S-Rh was observed in almost all cells at 24 h.

Intracellular Fate of Liposomal Membrane Rh-PE in Tf-L Equipped with Chol-GALA. The data presented above suggest that membrane fusion was successfully induced between Tf-L equipped with Chol-GALA and endosomal membranes, as expected. To confirm this assumption, membranes of Tf (FITC)-L equipped with Chol-GALA were labeled with Rh-PE, and the intracellular trafficking of Rh-PE and that of FITC-Tf were analyzed by confocal laser microscopy (Figure 7). Rh-PE was observed in endosome-like compartments together with FITC-Tf after the cells had been incubated with Tf (FITC)-L equipped with Chol-GALA for 18 h (Figure 7A−C). However, after the washout of Tf (FITC)-L equipped with Chol-GALA from the medium, the Rh-PE recycled back onto the plasma membrane together with FITC-Tf (Figure 7D−F). These results strongly suggest that the liposomal Rh-PE together with FITC-Tf, which was located on the liposomal surface conjugated with PE, fused with the endosomal membranes. The results described above are in stark contrast to those observed for Tf-L without Chol-GALA, where the Rh-PE was retained in endosome-like compartments, even after the washout of Tf (FITC)-L (Figure 2).

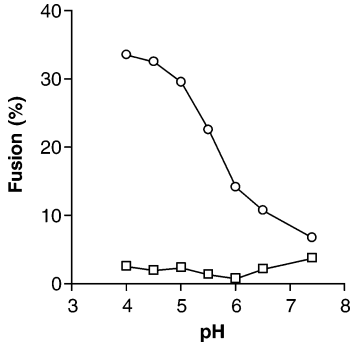

FIGURE 8: pH-dependent membrane fusion evoked by liposomes equipped with Chol-GALA. Membrane fusion activity was assessed by fluorescence resonance energy transfer (FRET). Probe liposomes containing both NBD-PE and Rh-PE and nonprobe liposomes containing Chol-GALA were incubated at a molar ratio of 1:9 at 37 °C and pH 5.0 for 1 h. After incubation, the pH was adjusted to 7.4 and the extent of energy transfer was estimated by measuring the fluorescence intensity (excitation at 470 nm and emission at 530 nm). As a control, the above probe liposomes were incubated with nonprobe liposomes without Chol-GALA: (○) liposomes equipped with Chol-GALA and (□) liposomes without Chol-GALA.

Underlying Mechanism of Endosomal Release by Chol-GALA. The mechanism of the cytosolic release of S-Rh encapsulated in Tf-L containing Chol-GALA was assumed to be membrane fusion. To obtain further support for this assumption, an *in vitro* study using fluorescence resonance energy transfer (FRET) was conducted. Probe liposomes containing both NBD-PE and Rh-PE were incubated with nonprobe liposomes containing Chol-GALA at a molar ratio of 1:9 at 37 °C for 1 h in the pH range of 4−7.4 (Figure 8). The level of energy transfer was estimated by measuring the fluorescence intensity at 530 nm (excitation at 470 nm). As a control, probe liposomes containing NBD-PE and Rh-PE were incubated with nonprobe liposomes without Chol-GALA.

Little fusion was observed between the probe liposomes and nonprobe liposomes containing Chol-GALA at pH 7.4. However, a remarkable increase in the extent of membrane fusion was observed when the pH was decreased. Almost 30% of the liposomes fused with each other at pH 5.0. On the other hand, when probe liposomes containing NBD-PE and Rh-PE were incubated with nonprobe liposomes without Chol-GALA as a control, no promotion of fusion was observed, even at low pH.

Membrane fusion was further confirmed by determining the particle size of the liposomes using dynamic light scattering. Liposomes [EPC/Chol (2:1)] with and without Chol-GALA were incubated at a molar ratio of 9:1 at 37 °C for 1 h at pH 5.0. The size of the liposomes increased from 210 to 2310 nm. No significant increase in particle size was observed when liposomes were incubated in the absence of Chol-GALA. This suggests that Chol-GALA-containing liposomes have the ability to fuse with other liposomes or lipid membranes even when the pH of the solution is decreased to 5.0.

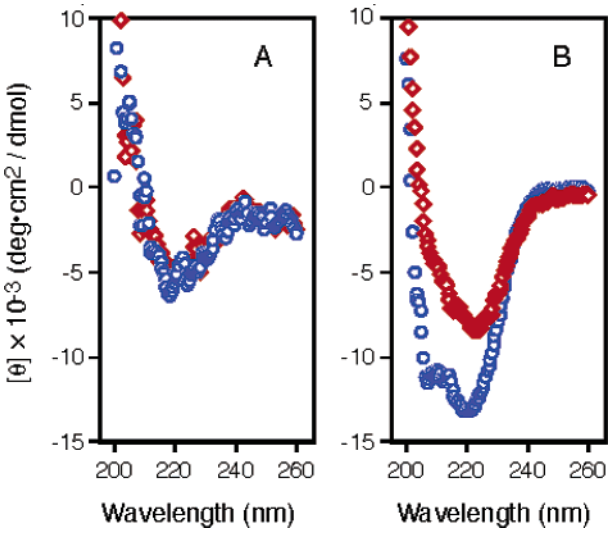

FIGURE 9: CD spectra of GALA and Chol-GALA peptides in the presence of liposomes. (A) CD spectra of GALA (20 μM) encapsulated in liposomes in PBS(−)-containing liposomes at pH 7.4 (○) and 5.0 (◇). (B) CD spectra of liposomes equipped with Chol-GALA (123 μM) in PBS(−) at pH 7.4 (○) and 5.0 (◇). The liposomes were composed of EPC and Chol (2:1) (lipid concentration of 7 mM).

CD spectra of the liposomes containing Chol-GALA were next examined to obtain structural information about the GALA moiety in liposomes (Figure 9). The GALA peptide was reported to have a rather disordered conformation in water at pH ~7, because of charge repulsion between negatively charged Glu residues; when the pH of the solution was decreased, the peptide formed a helical structure due to the decrease in the level of repulsion by the nonionized Glu residues (*11*, *12*). The peptide was reported to interact negligibly with membranes at pH 7.5, but considerable structural change in the peptide and eventual disruption of lipid membranes were observed at pH 5 by the interaction of the peptide with the lipid membranes (*11*, *12*). As reported, an increase in the helicity of the GALA peptide was observed when the pH of the solution was decreased from 7.4 to 5.0, as judged by the molar ellipticity ([θ]) at 222 nm. In the presence of liposomes, significant structural change was observed by the interaction of the peptide with the liposomes at pH 5.0 (data not shown). When the GALA peptide was encapsulated in liposomes, however, no pH-dependent transition in structure was observed (Figure 9A). The GALA peptide remained in a rather disordered conformation in liposomes even when the pH of the solution was adjusted from 7.4 to 5.0.

In the case where Chol-GALA was incorporated into the liposomes, the peptide exhibited a helical structure even at pH 7.4 ($[\theta]_{222} = -12500$ deg cm^2 dmol^{-1}) (Figure 9B). Alternatively, the GALA peptide without being attached to the Chol moiety was reported to interact only slightly with lipid membranes and to exhibit a rather random structure at pH 7.4 (*11*, *12*). Anchored by the cholesteryl moiety, the peptide was probably forced to lie on the membrane, forming a helical structure. When the pH of the solution was reduced to 5.0, a dramatic structural change was observed (Figure 9B). The possible loss of negative charges on Glu residues changed the mode of interaction of the peptide with the membranes, eventually facilitating membrane fusion.

DISCUSSION

Human Tf-R is a transmembrane glycoprotein composed of two disulfide-bonded subunits, each of which has an apparent molecular mass of 90 kDa. The Tf-R complexes are routed into the endosomal compartment. Upon maturation and loss of the clathrin coat, endosomes are rapidly acidified to a pH of 5.5. Iron is then released from Tf. The free Fe^{3+} released to the endosomes is reduced to Fe^{2+} probably by an oxidoreductase (*19*). The reduced iron is pumped out of the endosomes to the cytosol by DMT1, a new proton couple metal ion transport protein (*20*). The resulting apo-Tf develops a complex structure with Tf-R, and is recycled through exocytic vesicles back to the cell surface. Apo-Tf is then released to the extracellular fluid due to its low affinity for Tf-R at pH 7.4. This recycling occurs rapidly, requiring approximately 4−8 min (*21*).

Although the intracellular trafficking of Tf and the Tf-R complex has been extensively studied, little information about the intracellular fate of Tf attached to the surface of liposomes is available. Because of this, we examined the intracellular trafficking of Tf-modified liposomes in comparison with that of free Tf. The internalization of FITC-labeled free Tf (FITC-Tf) was efficient and reached a steady state within 10 min (Figure 1), while hours were required for Tf-L. These results suggest that the internalization of Tf-L is less efficient than that of free Tf. After a washout of Tf-L, the aqueous phase marker (S-Rh) as well as the lipid marker (Rh-PE) remained in endosome-like compartments (data not shown). Since FITC-Tf was not observed in the cells, the possibility of its removal by exocytosis, accompanied by the receptor recycling of Tf, was first suspected. However, when Rh was employed as a marker of Tf, Rh-Tf was found to remain in the endosomes and lysosomes (Figure 2D−F). These facts suggest the possibility of the fluorescence intensity of FITC being decreased in acidic compartments and the lysosomal entrapment of Tf when internalized in the form of Tf-L. The attachment of Tf on the liposomal surface had a great influence on the intracellular trafficking of Tf.

From flow cytometry analysis, the interaction of Tf-L with K562 cells was ~10-fold stronger than with unmodified liposomes (Figure 3C). This result suggests that Tf modification of liposomes greatly enhances delivery to the cells.

We used a CLSM to analyze the proportion of Tf-L-endocytosed cells. From the time course experiment, the maximum percent of Tf-L-endocytosed cells was ~60% (Figure 3A). In the case of free Tf, more than 95% of the cells were endocytosed within a few minutes (*21*). The reason for this may be a decreased steric hindrance of the liposomes, which may inhibit the initiation of endocytosis, such as invagination.

The inhibition experiment indicated that Tf-R-dependent endocytosis is the principal pathway of Tf-L (Figure 3B). The uninhibited uptake of Tf-L suggests a nonspecific uptake by K-562 cells, which was also observed for unmodified liposomes.

GALA was designed to preferentially interact with neutral bilayers at low pH, considering factors such as the hydrophobicity of the residues, the conformational preference of the amino acids, the length of the peptides, and the topology of the residues on the peptide (*11*, *12*). The peptide contains

30 amino acids with a repeated Glu-Ala-Leu-Ala (GALA) sequence. Glu is positioned so that it aligns on the same face of the helix. At a neutral pH, the possible repulsion of the negative charges among these Glu residues prevents the peptide from forming a helical structure. When the pH of the solution is decreased, the protonation of these Glu residues weakens the repulsive forces, thus inducing a helical structure. In the presence of lipid membranes, the peptide readily interacted with lipid membranes to fuse with the membranes (*22*−*26*).

As shown in Figure 4, the encapsulation of GALA in the aqueous phase of Tf (FITC)-L failed to improve the efficiency of the cytosolic release of S-Rh as compared in the case of Tf (FITC)-L without GALA. Under these conditions, the GALA encapsulated in the aqueous phase of the liposomes was not able to lyse the lipsomal membranes. Even when GALA was added to the incubation medium, little enhancement of S-Rh into the cytosol was observed. The GALA peptide has been reported to interact little with lipid membranes at neutral pH (*11*, *12*). Because of the cognate characteristics of GALA, the efficiency of adsorption of GALA to the cell surface and its eventual incorporation into endosomes would not be sufficient to facilitate the cytosolic release of S-Rh from Tf-L.

Many viruses have both recognition segments for host cells and fusion segments to facilitate entry of the virus into the cytosol. The results described above suggest that GALA should be on the liposomal membrane to interact with the endosomal membrane when the endosomal pH is decreased. Therefore, we prepared Chol-GALA for the effective presentation of the GALA segment on the liposomal surface.

First, we examined whether Chol-GALA is able to interact with the plasma membrane directly. On the basis of a CLSM analysis, 32% of the Chol-GALA liposomes were taken, while this value increased to 56% in the presence of Tf, after a 6 h incubation. If the nonspecific uptake (20%) is corrected, the uptake due to Chol-GALA (12%) is one-third of that of Tf-L (36%). In addition, the contribution of Chol-GALA in the presence of Tf may be small, since the steric effect of Tf will have an effect on the Chol-GALA. Therefore, we conclude that the ability of Chol-GALA to fuse with the plasma membrane directly would be weak. Next, we incubated K562 cells with Chol-GALA-containing Tf-L and then carried out a CLSM analysis. In these analyses, a remarkable cytosolic release of S-Rh was observed as the result of introducing Chol-GALA into Tf (FITC)-L (Figure 6A), which suggests that GALA on the liposome surfaces developed an altered conformation when the endosomal pH decreased from 7.4 to 5.0 and interacted with liposomal and endosomal membranes. The surface binding of FITC-Tf was also observed after washing with medium, as shown in Figure 6J−L. FITC-Tf is cross-linked with liposomal PE. This fact strongly suggests that the Tf-attached PE was fused to endosomal membranes and exocytosed to the cell surface, remaining on the plasma membrane along with recycled Tf-R. This scenario was further supported by the fact that Rh-labeled PE in Tf (FITC)-L equipped with Chol-GALA was also observed on the cell surface, and not in acidic compartments, after the cells had been washed with cultured medium (Figure 7). By the introduction of Chol-GALA into Tf (FITC)-L, membrane fusion proceeded efficiently in the cells.

To obtain further support for the above hypothesis for the enhanced cytosolic release of S-Rh by Chol-GALA, *in vitro* experiments using egg phosphatidylcholine (EPC) small unilamellar vesicles were conducted. As shown in Figure 8, Chol-GALA brought about fusion between liposomes made from EPC and Chol when the pH was reduced from neutral to acidic. The diameter of the liposomes increased from 210 to 2300 nm when the pH was reduced from 7.4 to 5.0. These results suggest that liposomes fused with each other via Chol-GALA at pH 5.0 to form aggregates.

Structural changes in the GALA peptide in the liposomes caused by membrane fusion were further supported by its CD spectra. It is noteworthy that the GALA peptide anchored to liposomes by the Chol moiety had a helical structure, even at pH 7.4 (Figure 9B). Without attaching to the Chol moiety, the GALA peptide has a rather disordered conformation in the presence and absence of liposomes at pH 7.4 (*11*), suggesting that interactions between the membrane and GALA peptide itself were not strong at neutral pH. The result implies that certain care should be paid in analyzing the conformation of membrane-associated peptide segments such as those corresponding to the loops in membrane proteins. When simply added to the liposomal or micellar solutions, these peptides could adopt conformations that are different from their cognate structures. This could be the case especially when these peptides are tightly fixed to cell surfaces by a transmembrane domain or other lipophilic peptides, as was seen in the GALA peptide anchored to liposomes by a Chol moiety. The inefficiency of Tf-L encapsulating GALA coincided with the CD spectral results shown in Figure 9A. No substantial difference was observed in the spectra at pH 7.4 and 5.0.

As shown in this study, Tf-L efficiently fuses with endosomal membranes when Chol-GALA is embedded in the liposomal membranes. This system has great potential for applications to gene delivery via delivery of encapsulated compacted plasmid DNAs. By membrane fusion, even large molecules such as plasmid DNA could be effectively released into the cytosol. Transferrin on liposomes would be expected to contribute to increase the rate of internalization of the liposomes by receptor-mediated endocytosis. A recent trial involving combining cationic liposomes with transferrin or with GALA, leading to a significant improvement in luciferase gene expression (*27*), also supports the rationality of the approach described above.

In conclusion, we have demonstrated the highly efficient delivery of liposomal encapsulates to the cytosol by using Tf as a recognition element for cellular entry and the GALA peptide as a fusion-inducing molecular device. The disposition of GALA on the liposomal surfaces was indispensable for achieving this. Derivatization of GALA by a Chol moiety greatly contributed to this enhancement. The Tf-L equipped with Chol-GALA represent a potential prototype for a delivery system, where multiple functional moieties act in concert to achieve a highly efficient and specific delivery. Thus, we obtained a novel artificial virus-like delivery system. During the course of this study, we determined the intracellular fate of the transferrin-modified liposomes. The findings obtained here therefore provide valuable implications, not only for the design of intracellular targeting systems but also for our understanding of cellular uptake systems.

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