A Novel Mechanism Is Involved in Cationic Lipid-Mediated Functional siRNA Delivery

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Abstract: A key challenge for therapeutic application of RNA interference is to efficiently deliver synthetic small interfering RNAs (siRNAs) into target cells that will lead to the knockdown of the target transcript (functional siRNA delivery). To facilitate rational development of nonviral carriers, we have investigated by imaging, pharmacological and genetic approaches the mechanisms by which a cationic lipid carrier mediates siRNA delivery into mammalian cells. We show that ~95% of siRNA lipoplexes enter the cells through endocytosis and persist in endolysosomes for a prolonged period of time. However, inhibition of clathrin-, caveolin-, or lipid-raft-mediated endocytosis or macropinocytosis fails to inhibit the knockdown of the target transcript. In contrast, depletion of cholesterol from the plasma membrane has little effect on the cellular uptake of siRNA lipoplexes, but it abolishes the target transcript knockdown. Furthermore, functional siRNA delivery occurs within a few hours and is gradually inhibited by lowering temperatures. These results demonstrate that although endocytosis is responsible for the majority of cellular uptake of siRNA lipoplexes, a minor pathway, probably mediated by fusion between siRNA lipoplexes and the plasma membrane, is responsible for the functional siRNA delivery. Our findings suggest possible directions for improving functional siRNA delivery by cationic lipids.

Keywords: siRNA; delivery; cationic lipids; pathways/mechanisms

Introduction

RNA interference (RNAi) is a conserved cellular mechanism by which a small double stranded RNA (dsRNA) directs the degradation of complementary mRNA and therefore inhibits the expression of a specific gene.[1] Since its discovery, RNAi has become a powerful tool to study gene functions in biological processes.[2-4] The ability to induce RNAi in mammalian cells using synthetic small interfering RNA (siRNA) has stimulated great interest in

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therapeutic applications of RNAi.[5−7] In numerous studies, siRNAs have shown promise for treating a variety of diseases, including influenza and HIV infection, cancer and genetic defects.[8−10]

A key challenge of RNAi-based therapeutic application is the efficient delivery of siRNA into target cells. siRNA is usually 21 nucleotides in length and highly charged and therefore cannot cross the cytoplasmic membrane by free diffusion. In the circulation and interstitial space, siRNA is vulnerable to degradation by RNase.[11] Although siRNA can be delivered directly and locally to the target sites in limited applications,[12,13] a carrier system is required in most applications to protect siRNA from degradation and to facilitate its uptake by target cells.[14,15] The existing carrier systems usually contain a key cationic component, such as a cationic lipid, a cationic polymer or a cationic peptide, in order to bind siRNA effectively. Other supplementary components help to improve the stability, solubility or pharmacological profiles of siRNA−carrier complexes.[16]

The use of carriers can dramatically increase the uptake of siRNA. However, the efficacy of the carriers in mediating functional siRNA delivery, i.e., leading to the knockdown

of the target transcript, remains low.[17] Many of the existing carriers were originally designed for DNA delivery, and hence have not been optimized to account for the differences between siRNA and DNA. The size and electrostatic charge of siRNA are much smaller than those of DNA. siRNA mediates its effect in the cytosol,[18] while DNA requires entry into the nucleus in order to gain access to the transcriptional machinery. Such differences between siRNA and DNA could have great influences on the functionality and efficacy of a carrier.

To rationally develop efficient siRNA carriers, it is necessary to elucidate the mechanisms by which the carriers mediate functional siRNA delivery. However, the current knowledge on the mechanisms of the carriers comes predominantly from studies with DNA delivery. Although different carriers have vastly different chemical structures and physical properties, it is believed that a majority of the DNA−carrier complexes is taken up into the cells by endocytosis.[19,20] A primary focus in the research and development of carriers for DNA delivery has been to facilitate the endosomal escape of the DNA−carrier complexes prior to their degradation in the lysosomes.[21,22] However, even with the most efficient DNA carriers developed so far, it remains controversial to what extent the escape from endosome contributes to transfection efficiency.[19−24] Considering the physical differences between siRNA and DNA and their sites of action, whether endosomal escape plays any significant role in functional siRNA delivery by the various carrier systems has yet to be determined.

In this report, we have determined the mechanisms by which a widely used cationic lipid carrier mediates functional delivery of siRNA by a combination of imaging, pharmacological and genetic approaches. We show that although

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most of the cellular uptake of siRNA lipoplexes is via endocytic pathways, these pathways do not appear to play a significant role in the delivery of siRNAs that mediate RNAi. Instead, a novel pathway, probably mediated by direct fusion between siRNA lipoplexes and the plasma membrane, is responsible for the functional delivery of siRNA into the cell.

Experimental Section

Cells, siRNAs and Plasmids. African green monkey kidney epithelial cells BSC-40, human embryonic kidney cells 293FT, and human cervical carcinoma cells HeLa were purchased from the American Type Culture Collection (ATCC). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 100 IU/mL of penicillin, 100 IU/mL of streptomycin, 2 mM L-glutamine, and 10% heat-inactivated fetal bovine serum (FBS) (Biowest, Miami, FL). The cells were cultured in 6-well FALCON tissue culture plates in a 5% CO_2 humidified incubator at 37 °C.

siRNAs specific for cyclophilin B (CyB) and green fluorescent protein (GFP) and Cy5 conjugated GFP siRNA were purchased from Dharmacon (Lafayette, CO). The siRNA sequences are as follows: GFP siRNA: (sense) 5′-GGCUACGUCCAGGAGCGCAdTdT-3′ and (antisense) 5′-UGCGCUCCUGGACGUAGCCdTdT-3′. CyB siRNA: (sense) 5′-GGAAAGACUGUUCCAAAAAdTdT-3′ and (antisense) 5′-UUUUUGGAACAGUCUUUCCdTdT-3′.

The wild-type and mutant dynamin expression plasmids were generously provided by Dr. Mark McNiven of the Mayo Cancer Center[25] and were cloned into pEGFP-N1 expression vector from Clontech. The vectors expressing the wild-type and mutant CAV-FED and CAV-DN caveolins were generously provided by Dr. Jan Eggermont[26] and were cloned into the pCINeo/IRES-GFP expression vector. Mutant cell lines were established by electroporation of BCS cells followed by selection in 2.5 mg/mL Geneticin (Invitrogen, Carlsbad, CA). The cells were maintained in 1 mg/mL Geneticin. The day prior to experiments, the cells were sorted with a FACSAria (Becton-Dickinson and Co.) to enrich for the GFP positive cells. Uptake of fluorescence-labeled transferrin (Sigma-Aldrich) was used to test if the K44A mutant cells lacked the clathrin-mediated endocytosis. Uptake of fluorescence-labeled cholera toxin (Invitrogen) was used to test if the caveolin mutant cells lacked the caveolin-mediated endocytosis.

Transfection Reagent, Lipoplex Preparation and Transfection. DharmaFECT1, a cationic lipid based siRNA transfection reagent, was purchased from Dharmacon. siRNA lipoplexes were prepared following the manufactur-

er's protocol. In brief, 4 μL of DharmaFECT1 was diluted into 200 μL of Opti-MEM I (Invitrogen) and then mixed with an equal volume of Opti-MEM I with appropriate amount of siRNA. The siRNA lipoplex solution was then diluted 5-fold in DMEM, 10% FBS for transfection. Twelve hours prior to transfection, BSC cells were seeded in 6-well plates at a density of 2×10^5 cells/well so that, by the time of transfection, the cells were in the early log phase and reached ∼70% confluence. During transfection, cell culture media were aspirated and cells were washed with PBS. Unless otherwise specified, 2 mL of 100 nM siRNA lipoplexes was incubated with cells for 4 h at 37 °C. The cells were subsequently washed using CellScrub buffer (Genlantis, San Diego, CA) and PBS. After washing, fresh media were added and cells were cultured at 37 °C for the indicated length of time.

Inhibitors and Temperature Treatment. Pharmacological inhibitors were purchased from Sigma-Aldrich (St. Louis, MO) and were used at the following concentrations: cytochalasin D, 100 μM; chlorpromazine, 10 μg/mL; filipin, 10 μg/mL; nystatin, 100 μg/mL; amiloride, 3 mM; D,L-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), 10 μM; sodium azide, 40 mM; and 2-deoxy glucose, 40 mM. The cells were pretreated with the inhibitors for 1 h at 37 °C and then transfected with siRNA lipoplexes for 4 h at 37 °C in the presence of the inhibitors. The cells were washed and cultured in fresh media in the absence of the inhibitors for another 20 h prior to RNA isolation. In experiments where siRNA transfection was carried out at 4, 10, 20, and 37 °C, the cells were incubated at specified temperatures for 1 h and then transfected with siRNA lipoplex at the same temperatures for 4 h. The cells were washed, replenished with fresh media, and incubated for 20 h at 37 °C prior to RNA isolation.

RNA Extraction and Real-Time PCR Assays. At indicated hours post-transfection, single cell suspension was prepared and total RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA concentration was determined by UV absorbance at 260 nm, and all samples had an A_{260}/A_{280} ratio greater than 1.95. The level of CyB transcript was measured using a Real-Time PCR assay. Total RNA was first converted into cDNA using random hexamers and TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). The levels of CyB and Actin mRNAs were then measured using TaqMan Gene Expression Assays (Applied Biosystems). The real time RT-PCR reactions were performed in Applied Biosystems' MicroAmp optical 96-well reaction plate, on a PRISM 7000 Real-Time PCR system.

Flow Cytometry. Cellular uptake of lipoplexes containing DharmaFECT1 and Cy5-labeled siRNA was quantified with flow cytometry. Unless specifically indicated, 2 mL of 100 nM siRNA lipoplexes were incubated with cells for 4 h at 37 °C. The cells were washed with CellScrub buffer, trypsinized and suspended in PBS plus 2% bovine serum albumin and 0.05% NaN_3. The cells were analyzed with a FACSCaliber and CellQuest software (Becton-Dickinson).

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Dead cells were excluded from analysis by propidium iodide staining. Cellular uptake of Cy5 labeled siRNA was quantified by measuring the relative fluorescence signal in Cy5 channel. Cells incubated with nonlabeled siRNA lipoplexes were used as a control, and their mean fluorescence values were subtracted from the uptake measurements of Cy5-siRNA lipoplexes.

Fluorescence Microscopy. Cells were grown in 35 mm diameter MatTek (MatTek, Ashland, MA) glass bottom culture dishes, transfected with Cy5-siRNA lipoplexes, and viewed on an inverted epifluorescence microscope (Olympus IX71, Olympus, Center Valley, PA), integrated in a Deltavision deconvolution system (Applied Precision, Issaquah, WA). Cells were housed in an environmental chamber (Solent Scientific, Segensworth, U.K.) to maintain constant temperature, humidity and CO_2 levels during the observation. Unless specified, all experiments were conducted at 37 °C, using an atmosphere of 95% air and 5% CO_2. For fluorescence imaging, cell nuclei and endolysosomes were stained with Hoechst 33342 dye and LysoSensor Blue DND-167 dye, respectively. Cells were viewed in Opti-MEM I medium with an Olympus $63\times$ PlanApo oil immersion objective with a numerical aperture of 1.40, fitted with a CoolSNAP HQ Monochrome 12-bit cooled CCD camera (Photometrics, Tucson, AZ). Images of 512×512 pixels were captured by Applied Precision's softWoRx software and analyzed using Imaris 4.2 (Bitplane, Saint Paul, MN).

Statistical Method. ANOVA was used for statistical analysis.

Results

To elucidate the mechanisms underlying the functional siRNA delivery, we focused on DharmaFECT1, a widely used siRNA transfection reagent based on cationic lipids. For easy imaging analysis, we used African green monkey kidney epithelial cells BSC-40. Human embryonic kidney cells 293FT and human cervical carcinoma cells HeLa were also used in certain experiments. To measure the functional siRNA delivery, we assayed the knockdown of an endogenous gene cyclophilin B (CyB) because it is ubiquitously expressed and its knockdown has no apparent effect on cell survival and growth. CyB is also highly conserved between human and African green monkey; therefore, the same siRNAs are effective in cells derived from both species. We used quantitative PCR to measure the level of CyB transcript so as to detect the effect of functional siRNA delivery as directly and quickly as possible. In addition, we established that the uptake of siRNA lipoplexes is proportional to siRNA concentration in a range of 10 pM to 200 nM, and the target transcript knockdown is linear to siRNA concentration in a range of 100 pM to 100 nM (Supplementary Figure S1 in the Supporting Information). Because 100 nM siRNA produces the most dramatic knockdown of the target transcript but is still within the linear range, we used 100 nM siRNA in most experiments.

Majority of the siRNA Lipoplexes Persist in Endolysosomes. To determine the dynamics of siRNA lipoplex transfection, we analyzed the localization of siRNA lipoplexes inside the cells at different times post-transfection. BSC cells were incubated with Cy5 labeled siRNA lipoplexes for 1, 2, or 4 h, washed and then fixed for imaging analysis. Some of the 4 h transfected cells were washed and cultured for an additional 4, 12, and 46 h before fixation and imaging analysis. siRNA lipoplexes were detected in cells following 1 h transfection; the number of siRNA lipoplexes increased significantly following 4 h transfection (Figure 1A). A significant number of siRNA lipoplexes still persisted inside the cells 46 h post-transfection.

The punctate pattern of intracellular siRNA lipoplexes suggests that they localize in endolysosomes. Thus, cells were stained with LysoSensor specific for endolysosomes and then transfected with siRNA lipoplexes for 1, 2, 3, and 4 h. Quantification of colocalization of LysoSensor and Cy5-labeled siRNA lipoplexes revealed that >90% siRNA lipoplexes were localized in the endolysosomes (Figure 1B).

To determine the relationship between the observed uptake of siRNA lipoplexes and the target transcript knockdown, BSC cells were transfected for either 2 or 4 h and assayed for CyB transcript level immediately, or transfected for 4 h, washed and cultured for additional 2 or 20 h before measuring the level of CyB transcript. A decrease in CyB mRNA level was detected following 2 h transfection, but a significant decrease was clearly evident following 4 h transfection (Figure 1C). Additional culture of 4 h transfected cells resulted in further decrease of CyB transcript level. By comparing Figures 1A, 1B and 1C, it is clear that significant target mRNA knockdown occurred in the absence of significant change in the level of siRNA lipoplexes in the endolysomes. Because a 4 h siRNA lipoplex transfection followed by a 20 h culture results in a consistent CyB mRNA knockdown by 80−90%, we used this assay format in the rest of the study.

Functional siRNA Delivery Is Independent of the Clathrin-Mediated Endocytosis. One of the best characterized and most important endocytosis pathways is the clathrin-mediated endocytosis, which is responsible for the uptake of a wide variety of macromolecules from extracellular environment.[27] To quantify the uptake of siRNA lipoplexes through this pathway, BSC cells were treated with chlorpromazine or cytochalasin D, which inhibit clathrin-mediated endocytosis,[28] followed by siRNA lipoplex transfection. After washing, uptake of siRNA lipoplexes was measured by microscopy and flow cytometry. CyB transcript level was measured following 20 h culture. Both inhibitors reduced the uptake of Cy5 labeled siRNA lipoplexes by ∼50%

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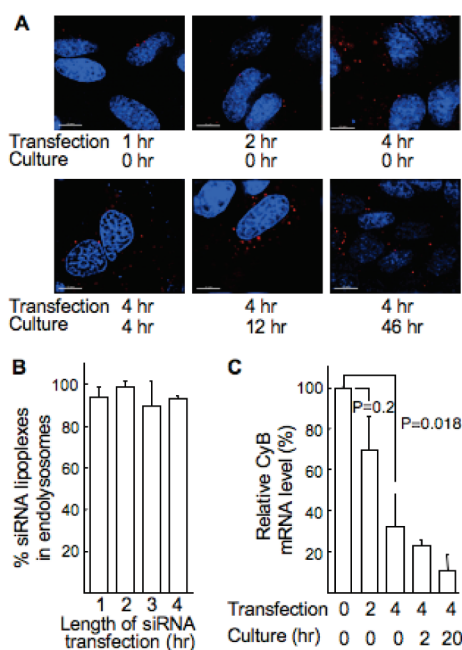
Figure 1. Uptake of the bulk of siRNA lipoplexes and functional siRNA delivery are not correlated. (A) Imaging analysis of siRNA lipoplexes inside the cells. Cy5-siRNA lipoplexes were incubated with BSC cells for the indicated length of time, washed, and cultured for the indicated length of time before fixation and imaging analysis. Cy5-siRNA lipoplexes were red and nuclei were stained blue. Representative images at the indicated times are shown. Scale bar, 10 μm. (B) siRNA lipoplexes colocalize with endolysosomes. BSC cells were transfected with Cy5-siRNA lipoplexes for the indicated length of time in the presence of LysoSensor. Live cell imaging was carried out at different time points after transfection. The number of intracellular siRNA complexes and the number of siRNA complexes that overlapped with LysoSensor staining were enumerated. Percentages of intracellular siRNA complexes that colocalize with LysoSensor are shown at the indicated time points. (C) Kinetics of target mRNA knockdown. BSC cells were transfected with CyB-specific siRNA lipoplexes for 2 or 4 h, washed, were used for RNA isolation immediately. Some 4 h transfected cells were cultured for another 2 or 20 h before RNA isolation. The levels of CyB transcript were quantified by real-time PCR and normalized to that in nontransfected cells. Percentages of target mRNA level are shown as mean ± SD. *P* values between selected samples are indicated. Representative data from one of three to five experiments are shown.

(Figure 2A and Supplementary Figure S2A in the Supporting Information). However, siRNA mediated knockdown of CyB transcript was not diminished in the presence of these inhibitors (Figure 2B).

Dynamin is required for the clathrin-mediated endocytosis.[29,30] To confirm the results with pharmacological inhibitors, we constructed BSC cells that constitutively expressed the wild-type or a dominant-negative form of
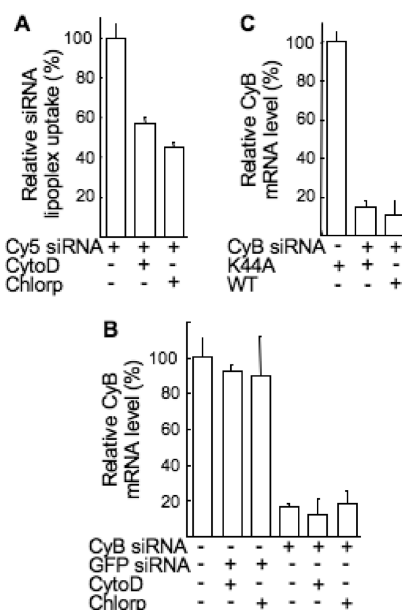

Figure 2. Inhibition of clathrin-mediated endocytosis significantly blocks uptake of siRNA lipoplexes but not functional siRNA delivery. BSC cells were treated with chlorpromazine (Chlorp, 10 μg/mL) or cytochalasin D (CytoD, 100 μM) for 1 h followed by siRNA lipoplex transfection for 4 h in the presence of the inhibitors. After washing, siRNA lipoplex uptake was measured by flow cytometry (see Experimental Section for details). Transfected cells were cultured for another 20 h before CyB transcript level was measured by real-time PCR. (A) Uptake of Cy5-siRNA lipoplexes in the absence or presence of inhibitors. (B) Relative levels of CyB transcript in the presence or absence of inhibitors as normalized to the level of untreated cells. (C) Relative levels of CyB transcript in BSC cells that expressed the wild-type (WT) or K44A dynamin. BSC cells that stably express either the WT or K44A dynamin were transfected with siRNA lipoplexes for 4 h, washed, cultured for 20 h, and then assayed for CyB transcript level. Data shown are mean ± SD (n = 4). Representative data from one of three to five experiments are shown.

dynamin with mutation of lysine at the position 44 to alanine (termed K44A).[29] Expression of the mutant but not the wild-type dynamin inhibited the uptake of transferrin (Supplementary Figure S2B in the Supporting Information), a marker for the clathrin-mediated endocytosis. However, siRNA-mediated knockdown of the target transcript was not affected by expression of either the wild-type or K44A dynamin (Figure 2C). Similarly, transient expression of K44A dynamin in human embry-

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onic kidney cells 293FT did not inhibit the knockdown of target transcript following siRNA lipoplex transfection (Supplementary Figure S2C in the Supporting Information). Inducible expression of K44A dynamin in human cervical carcinoma cells HeLa also did not affect target transcript knockdown (Supplementary Figure S2D in the Supporting Information). Together, these results show that although the clathrin-mediated endocytosis is a major pathway for cellular uptake of siRNA lipoplexes, it does not appear to contribute significantly to the functional siRNA delivery.

Functional Delivery of siRNA Lipoplexes Is Sensitive to Cholesterol Depletion. Next, we determined if functional siRNA delivery is dependent on cholesterol because cholesterol is a critical component of the cytoplasmic membrane and plays a critical role in many membrane-associated events, including membrane fusion, macropinocytosis, and caveolin- and lipid-raft-mediated endocytosis.[31,32] BSC cells were treated with nystatin or filipin for 1 h to deplete cholesterol from the plasma membrane[33,34] and then transfected with siRNA lipoplexes for 4 h in the presence of the inhibitors. After washing, cells were assayed for siRNA lipoplex uptake by flow cytometery or cultured for 20 h and then assayed for CyB transcript level. Under these conditions, cytotoxicity was minimal because >90% of the cells did not take up propidium iodide. As shown in Figure 3, although treating cells with nystatin or filipin had minimal effect on the uptake of siRNA lipoplexes, target mRNA knockdown was completely abolished in the presence of filipin and significantly reduced in the presence of nystatin. To exclude the possibility that the inhibitors may have interfered with the functional siRNA delivery by destabilizing the siRNA lipoplexes, we measured the particle size of siRNA lipoplexes in the presence of the inhibitors by dynamic light scattering. When the inhibitors were added to siRNA lipoplexes, the size of the particles remained the same, with >95% of the particles were 100−200 nm in diameter (data not shown). In addition, after the inhibitors were removed from the siRNA lipoplexes suspension by dialysis, the siRNA lipoplexes remained active and mediated efficient RNAi (Supplementary Figure S3 in
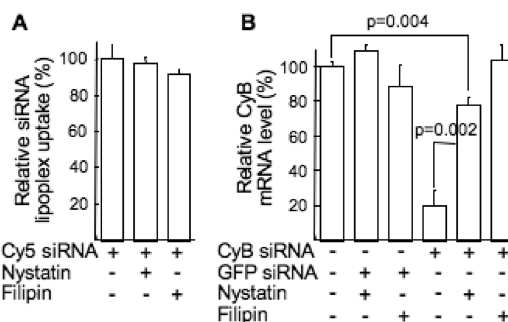

Figure 3. Cholesterol depletion inhibits functional siRNA delivery. (A) Uptake of siRNA lipoplexes in the absence or presence of either nystatin (100 μg/mL) or filipin (10 μg/mL) as measured by flow cytometry. (B) Relative CyB transcript level in the presence or absence of the inhibitors. Representative data are shown as mean $\pm$ SD ($n = 4$). P values between selected samples are shown. Representative data from one of three experiments are shown.

the Supporting Information). Hence, depletion of cholesterol from the plasma membrane abolishes functional delivery of siRNA mediated by cationic lipids.

Functional Delivery of siRNA Lipoplexes Does Not Depend on Macropinocytosis or Caveolin- or Lipid-Raft-Mediated Endocytosis. Among the various endocytic pathways that are sensitive to cholesterol depletion, the caveolin-mediated endocytosis is perhaps the best characterized.[35,36] To determine whether this pathway contributes to the functional delivery of siRNA by cationic lipids, we constructed BSC cells that stably expressed one of the two dominant-negative caveolin constructs: One mutant, termed CAV-DN, harbored a deletion of amino acid residues 1−81 at the N-terminal; and the other mutant, termed CAV-FED, had amino acid residues 68−73 replaced with AAAAAG.[26] Expression of the mutant but not the wild-type caveolin in BSC cells resulted in the inhibition of the uptake of cholera toxin (Supplementary Figure S4A in the Supporting Information), suggesting that caveolin-mediated endocytosis is inhibited significantly. However, transfection of siRNA lipoplexes into BSC cells expressing either the wild-type or the mutant caveolin resulted in CyB knockdown to the same extent (Figure 4A). Similarly, transient expression of the dominant negative caveolins in 293FT cells did not diminish CyB transcript knockdown (Supplementary Figure S4B in the Supporting Information).

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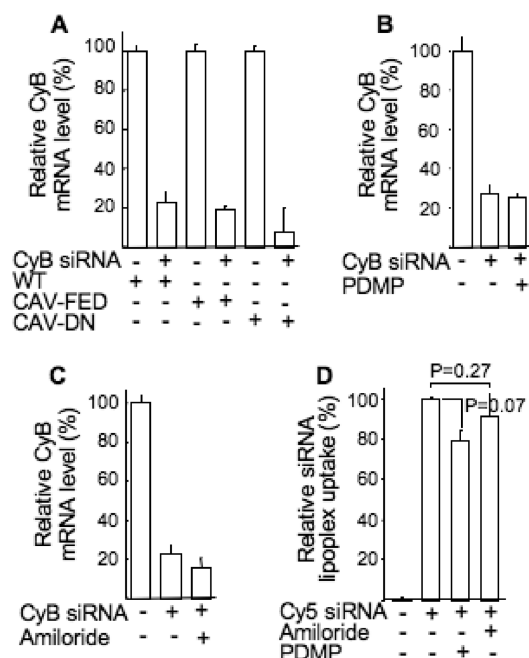
Figure 4. Functional delivery of siRNA lipoplexes is not dependent on caveolin- or lipid-raft-mediated endocytosis or macropinocytosis. (A) Relative levels of CyB transcript in BSC cells that express the wild-type (WT) or the mutant caveolins. BSC cells that stably expressed either the wild-type or one of the mutant caveolins were transfected with siRNA lipoplexes for 4 h, washed and cultured for 20 h. The level of CyB transcript was quantified. (B, C) Effect of PDMP (B) or amiloride (C) on functional siRNA delivery. BSC cells were treated with PDMP (10 μM) or amiloride (3 mM) for 1 h, followed by siRNA lipoplex transfection for 4 h, washed, and cultured for 20 h. The level of CyB transcript was quantified. (D) Uptake of Cy5 labeled siRNA lipoplexes in the absence or presence of inhibitors as quantified by flow cytometry. Representative data are shown as mean $\pm$ SD ($n = 4$). P values between selected samples are shown. Representative data from one of three experiments are shown.

ponent enriched in domains of lipid raft.[38] Treatment of BSC cells with PDMP had no effect on knockdown of target transcript following transfection with siRNA lipoplexes (Figure 4B) although it inhibited the uptake of siRNA lipoplexes by approximately 20% (Figure 4D). Similarly, amiloride, a strong inhibitor of macropinocytosis,[39] showed no significant effect on either the knockdown of CyB transcript or siRNA lipoplex uptake (Figure 4C,D). Together, these results suggest that functional delivery of siRNA lipoplexes does not appear to depend on caveolin- or lipid-raft-mediated endocytosis or macropinocytosis.

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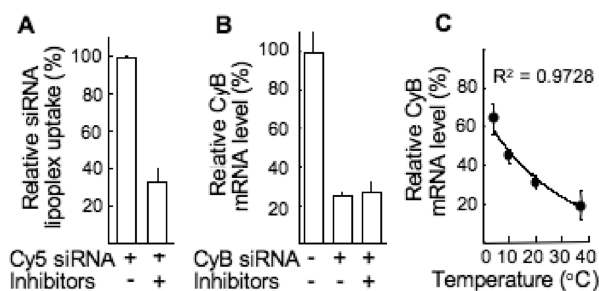

Figure 5. Effect of lowing temperature and inhibiting ATP biosynthesis on functional delivery of siRNA lipoplexes. (A) Uptake of siRNA lipoplexes in the absence or presence of deoxy-glucose (40 mM) and sodium azide (40 mM). (B) Relative level of CyB transcript in the presence or absence of ATP biosynthesis inhibitors. (C) Effect of temperature on knockdown of target transcript. Representative data are shown as mean $\pm$ SD ($n = 3$ or 4). R^2 value of exponential fit is shown. Representative data from one of three experiments are shown.

Functional Delivery of siRNA Lipoplexes Is Temperature Sensitive. To determine if the functional delivery of siRNA lipoplexes is an energy-dependent process, we treated the cells with deoxy-glucose and sodium azide to inhibit ATP biosynthesis. BSC cells were treated with both inhibitors for 1 h and then transfected with siRNA lipoplexes for 4 h in the presence of the inhibitors. After washing, cells were assayed immediately for siRNA uptake or cultured for 20 h in the absence of the inhibitors (to avoid inhibition of RNAi reaction) and then assayed for CyB transcript level. The treatment inhibited the uptake of siRNA lipoplexes by ~70% (Figure 5A). However, inhibition of ATP biosynthesis during transfection did not diminish the target transcript knockdown (Figure 5B), suggesting that the functional delivery of siRNA lipoplexes does not appear to depend on new ATP biosynthesis.

We examined the effect of temperature on functional delivery of siRNA lipoplexes. BSC cells were precooled to 4, 10, or 20 °C for 1 h and then transfected with siRNA lipoplexes for 4 h at the same temperatures. After washing, the cells were incubated at 37 °C for 20 h and assayed for CyB transcript level. Knockdown of the target transcript was significantly reduced at low temperatures (Figure 5C). Curve fitting suggests that the reduction of target transcript level was exponential (no sharp transition) as temperature decreases, suggesting that the effect of temperature on functional delivery of siRNA lipoplexes is gradual.

Discussion

In this study, we examined pathways by which siRNA lipoplexes are taken up by cells to determine whether specific uptake pathways contribute to functional siRNA delivery. Our results show that ~95% of siRNA lipoplexes that enter cells end up in the endolysosomes, most of which are contributed by clathrin-mediated endocytosis (~50%) and lipid-raft-mediated endocytosis (~20%). These findings are consistent with observations that endocytosis is the major

pathway for the uptake of DNA lipoplexes.[20,22−24,40,41] Despite the contribution to the uptake of the majority of siRNA lipoplexes, the endocytotic pathways do not appear to contribute significantly to functional siRNA delivery as measured by target transcript knockdown. Consistent with this observation, while endocytosis is an energy-dependent process,[27,32] functional siRNA delivery mediated by cationic lipids is independent of new ATP biosynthesis during the transfection period. Furthermore, inhibition of clathrin-mediated endocytosis by either pharmacological inhibitors or expression of a dominant negative dynamin did not inhibit the knockdown of the target transcript (Figure 2). Similarly, inhibition of caveolin-mediated endocytosis, lipid-raft-mediated endocytosis or macropinocytosis did not diminish the knockdown of the target transcript (Figure 4). While these results do not exclude the possibility that a small amount of siRNA lipoplexes "leaked" out of endolysosomes following endocytosis and contributed to the target gene silencing, taking together, they suggest alternative mechanisms of cationic lipid-mediated functional siRNA delivery.

The functional delivery of siRNA mediated by cationic lipids is shown here to be a rapid process as inferred from the rapid knockdown of target transcript (Figure 1C). Furthermore, the process is temperature sensitive but does not require new ATP biosynthesis during the transfection period (Figure 5). Based on these characteristics, the mechanism underlying the cationic lipid-mediated functional siRNA delivery is probably through direct fusion between siRNA lipoplexes and the plasma membrane. Membrane fusion requires cholesterol,[31] and it is notable that depletion of cholesterol from the plasma membrane by treating cells with nystain or filipin abolished or significantly inhibited target transcript knockdown (Figure 3). Because treating cells with nystain or filipin did not significantly inhibit the bulk uptake of siRNA lipoplexes, the functional siRNA delivery likely occurs through a minor uptake pathway, consistent with the mechanism of a direct fusion.

The mechanisms by which cationic lipids mediate DNA delivery have been investigated extensively.[19,21,23,24,40−44] During cationic lipid-mediated DNA delivery, the lipids function by compacting plasmid DNA into DNA-containing liposomes or lipid−DNA complexes (lipoplexes). The li-

poplexes facilitate cellular uptake of DNA primarily via endocytosis following binding of positively charged lipid−DNA lipoplexes to the negatively charged cellular surfaces.[19,23,24,43] Although fusion of DNA lipoplexes with the plasma membrane was initially suggested as a way to deliver DNA directly into the cytoplasm,[19] lipid mixing assays did not reveal a correlation between fusion of DNA lipoplexes with plasma membranes and their transfection efficiency.[41,45−47] Rather, it seems that when DNA lipoplexes fuse with the cellular membrane, DNA tends to be excluded at the cell surface and does not enter the cell.[48] Because the majority of DNA lipoplexes end up in the endolysosomes, most of the effort to enhance DNA transfection has focused on stimulating the escape of DNA lipoplexes from the endolysosomes.

What could account for the differences in the mechanisms of cationic lipid mediated siRNA and DNA delivery? Both DNA and siRNA are negatively charged. This similarity could account for the uptake of siRNA or DNA lipoplexes by the same endocytosis pathways. However, siRNA is much smaller than DNA and functions in the cytosol whereas DNA has to be transported into the nucleus to be transcribed. Upon fusion of siRNA lipoplexes with the plasma membrane, some siRNA is likely delivered directly into the cytosol, where siRNA can be readily recruited into RNAi mechinary to exert its effect. In contrast, DNA delivered into the cytosol via fusion has to be transported into nucleus before it can be transcribed. Even if some DNA is delivered directly into the cytosol, it may not contribute significantly to the functional DNA delivery. This difference could potentially account for the different mechanisms by which cationic lipids mediate functional siRNA versus DNA delivery. Thus, one practical significance of our observations is that optimizing conditions for fusion between siRNA lipoplexes and the plasma membrane should improve functional siRNA delivery by cationic lipids.

In summary, our studies show that although most siRNA lipoplexes enter cells via endocytosis, this mode of entry does not appear to contribute significantly to functional siRNA delivery. Instead, a minor but rapid pathway, probably mediated by fusion of siRNA lipoplexes with the plasma membrane, accounts for the finding that only a small fraction of the siRNA lipoplexes that enter cells are responsible for most of the observed siRNA-mediated target gene knockdown.

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Abbreviations Used

RNAi, RNA interference; siRNA, small interfering RNA; CytoD, cytochalasin D; Chlorp, chlorpromazine; CyB, cyclophilin B; GFP, green fluorescent protein; PDMP, D,L-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol.

Supporting Information Available: Four supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

MP900023V