Intracellular Delivery of Proteins with a New Lipid-mediated Delivery System*

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There are many very effective methods to introduce transcriptionally active DNA into viable cells but approaches to deliver functional proteins are limited. We have developed a lipid-mediated delivery system that can deliver functional proteins or other bioactive molecules into living cells. This delivery system is composed of a new trifluoroacetylated lipopolyamine (TFA-DODAPL) and dioleoyl phosphatidylethanolamine (DOPE). This cationic formulation successfully delivered antibodies, dextran sulfates, phycobiliproteins, albumin, and enzymes (β-galactosidase and proteases) into the cytoplasm of numerous adherent and suspension cells. Two systems were used to demonstrate that the proteins were delivered in a functionally active form. First, intracellular β-galactosidase activity was clearly demonstrated within X-gal-stained cells after TFA-DODAPL:DOPE-mediated delivery of the enzyme. Second, the delivery system mediated delivery of several caspases (caspase 3, caspase 8, and granzyme B) into cultured cell lines and primary cells triggering apoptosis. Mechanistic studies showed that up to 100% of the protein mixed with the lipid formulation was captured into a lipid-protein complex, and up to 50% of the input protein associated with cells. This lipid-mediated transport system makes protein delivery into cultured cells as convenient, effective, and reliable as DNA transfection.

Considerable progress has been made toward the development of effective transfection reagents for the delivery of transcriptionally active DNA into cultured cells (1–4) and today, plasmid transfection is a routine laboratory procedure used in most modern biomedical laboratories. Often, the primary reason for performing DNA transfection is to express a desired protein in the transfected cell to investigate its function. In this respect DNA transfection technology can be considered an indirect protein delivery system.

New methodologies to deliver functional proteins into cells...
(human T cell leukemia) were obtained from ATCC. Ki-Ras 267β1 cells were a kind gift of Dr. John S. Rim (Laboratory of Cellular and Molecular Biology, NCI, National Institutes of Health). Chronic myelocytic leukemia (CML) and acute myelocytic leukemia (AML) were isolated from patients diagnosed with CML/CD19⁻B-CML or CD34⁺ myeloblasts, respectively, and isolated by Ficoll density gradient centrifugation. All cell lines were grown according to ATCC recommendation. Ki-Ras 267β1 were grown in RPMI 1640, 10% fetal calf serum, CML and AML were grown in α-minimal essential medium, 10% fetal calf serum.

Preparation of TFA-DODAPL:DOPE/Protein Formulation—The cationic lipoplexes formulation is composed of a 2:1 mixture of a cationic lipid, TFA-DODAPL, and a neutral lipid, DOPE (US and international patents pending). TFA-DODAPL consists of 2 saturated C-18 alkyl chains linked to a core lysine residue through a 1,3-dipropylamine. ε-Linked dilsyline residues were added to this core lysine to produce DODAPL (2,6-diamino-hexanoic acid [5-amino-5-[5-[2-amino-6-(2,6-diamino-hexanoylamino)-hexanoylamino]-1-(3-dioctadecylamino-propylcarbamoyl)-pentylcarbamoyl]-pentyl]-amide). TFA-DODAPL:DOPE (called BioPORTER) has been used according to the manufacturers instructions (Gene Therapy Systems, San Diego, CA). Briefly, the BioPORTER dry film is re-suspended with 250 μl of methanol or chloroform and vortexed for 10–20 s. Then, the desired amount of BioPORTER (depending on the type of experiment) is transferred into an Eppendorf tube and the solvent is evaporated under a hood for at least 2 h at room temperature. The molecule to be delivered is diluted in HBS (10 mM Hepes, 150 mM NaCl, pH 7.0) or PBS (150 mM NaCl, 20 mM Na phosphate, pH 7.4). The antibody, dextran sulfate 10 and 70 kDa, phycoerythrin, β-galactosidase, cytochrome c, and BSA solutions were diluted at 80–160 μg/ml in HBS or PBS (β-galactosidase). Caspase 3 and caspase 8 solutions were diluted at 165 to 1000 pg/ml and granzyme B solution was diluted at 7.5 to 60 ng/ml. The diluted protein solutions (10 to 25 μl) were then used to hydrate the dried BioPORTER formulation. The solution was pipetted up and down, incubated at room temperature for 5 min, and vortexed gently and briefly. Finally serum-free medium was added to the complexes according to the final transfection volume (250 μl for the 24-well plate).

Delivery of Fluorescent Molecules or β-Galactosidase with TFA-DODAPL:DOPE—The molecules were seeded 0.5–1 × 10⁵ cells/well in a 24-well plate (or on coverslips). The next day, the medium was aspirated from the cells for coverslip, blot it dry and place it in a 35-mm dish and then transferred the TFA-DODAPL:DOPE/protein mixture directly onto the cells. 2 μg of FITC-labeled antibody, 1 μg of Oregon Green 488-dextran, and 0.5 μg of β-galactosidase were delivered by TFA-DODAPL:DOPE (2.5 μl) to the cells in the serum free condition. The cells were incubated at 37 °C for 4 h (for longer incubation time 10% serum was added to the cells after 4 h). The cells were washed twice with PBS and coverslips were mounted directly onto a hanging drop slide. Living cells were directly examined with a fluorescent microscope (NIKON E-600) equipped with a ×60 objective and a 3-CCD camera or a confocal microscope (NIKON E-600). For Western blot, the culture medium was aspirated from the cells and the mixture was directly added onto the cells. After 4 h of incubation at 37 °C, the medium was removed, cells were washed twice with PBS, and medium + washes were pooled for fluorescence measurement. Cells were lysed with 200 μl of lysis buffer (1% Triton X-100 in PBS, protease inhibitor mixture, and 50 mM dithiothreitol) for 10 min on ice. The cytosol and membrane fractions were separated by centrifugation at 14,000 rpm for 10 min. An aliquot of the samples were used to determine the protein concentration with the Bio-Rad DC protein assay Kit. Fluorescent samples were analyzed with a fluorometer (FluroMax-2™; Instruments S.A., Edison, NJ). For Western blot, samples were run in a 12% SDS-polyacrylamide electrophoresis gel (Bio-Rad) and transferred onto a nitrocellulose Hybrid ECL membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Immunode tection was done with a mouse alkaline phosphatase-labeled goat antibody IgG F(ab')₂ (Pierce, Rockford, IL) and an alkaline phosphatase-conjugated substrate kit (Bio-Rad).

RESULTS

Intracellular Delivery of Macromolecules with TFA-DODAPL:DOPE—We investigated whether cationic lipid formulations effective for DNA transfection would also be useful for the delivery of functional proteins into cells. Twenty-five new cationic lipids were synthesized, formulated, and tested for their ability to deliver fluorescently labeled antibody (FITC-Ab) and β-galactosidase into NIH-3T3 cells. Among the lipids tested, a new trifluoroacetylated lipopolyamine (TFA-DODAPL) mixed with DOPE was the only formulation able to deliver both proteins into 70–75% NIH-3T3 cells (Fig. 1). Seven commercial transfection reagents (DOTAP, DMRIE, Trans-IT, FuGene 6, Transfast, LipofectAMINE, and Lipofectin) were

![Fig. 1. TFA-DODAPL:DOPE-mediated intracellular delivery of a fluorescent antibody, dextran sulfate, and β-galactosidase in NIH-3T3 cells. FITC-labeled antibody (A and B), β-galactosidase (C and D), or Oregon Green 488-labeled high (70 kDa) and low (10 kDa) molecular mass dextran sulfate (E and F) complexed to TFA-DODAPL:DOPE (2.5 μl) were delivered as described under "Experimental Procedures." After washes with PBS, live (antibody, dextran) or fixed (β-galactosidase) cells were examined under a microscope. A, 2 μg of FITC-antibody only; B, 2 μg of FITC antibody with TFA-DODAPL:DOPE; C, 2 μg of β-galactosidase only; D, 0.5 μg of β-galactosidase with TFA-DODAPL:DOPE; E, 1 μg of dextran 10 kDa with TFA-DODAPL:DOPE; and F, 2 μg of dextran 70 kDa with TFA-DODAPL:DOPE.](Image)
also tested and all were inefficient (<5% delivery efficiency) for FITC-Ab and β-galactosidase delivery (not shown). Other lipid formulations, negatively charged (DOPG ± DOPE, DOPC, or cholesterol) and neutral (DOPC ± DOPE or cholesterol), were also analyzed and no proteins were successfully delivered (data not shown).

The formulation of TFA-DODAPL was optimized by testing multiple helper lipids (DOPE, DOPE, and cholesterol) at different cationic lipid/neutral lipid ratios. In this way the optimized TFA-DODAPL:DOPE formulation (BioPORTER) composed of a 2:1 mixture of TFA-DODAPL and DOPE was selected. When the dried cationic TFA-DODAPL:DOPE lipid formulation is hydrated with a solution of the protein to be delivered, a complex between the lipid and protein spontaneously forms. These complexes when applied to cultured cells to deliver the protein into the cells (Fig. 1). TFA-DODAPL:DOPE-mediated delivery of FITC-Ab, fluorescently labeled dextrans, and β-galactosidase into NIH-3T3 cells is shown in Fig. 1. Nearly all NIH-3T3 cells contained FITC-Ab (Fig. 1B) or β-galactosidase activity (Fig. 1D) after treatment with these TFA-DODAPL:DOPE-protein complexes. In contrast, without the delivery agent, neither antibody (Fig. 1A) nor β-galactosidase (Fig. 1C) was delivered into the cells.

This cationic lipid formulation also delivered dextran sulfate into cells. Low molecular mass dextran (10 kDa) was able to enter into the nucleus of the transduced cells (Fig. 1E), whereas, high molecular mass dextran (70 kDa) did not (Fig. 1F). This is consistent with published results showing that diffusion of high molecular weight molecules lacking a nuclear localization signal through the nuclear pore is size restricted (17). Cellular uptake of dextran sulfate also required the lipid-mediated delivery system (not shown). These results showed that the delivery system did not alter the biodistribution of dextran molecules.

We monitored by flow cytometry the ability of TFA-DODAPL:DOPE to deliver phycocerythrin, a high molecular weight protein (670,000). Primary AML, Jurkat, Ki-Ras 267/11, and primary CML cells registered 99, 70, 55, and 35% uptake, respectively (not shown). The different percentages of effective transduction suggest that the lipid-mediated protein delivery is cell type dependent.

**Determination of Intracellular Delivery Conditions for Multiple Cell Types**—To demonstrate the versatility of this system, we have investigated TFA-DODAPL:DOPE-mediated delivery of FITC-Ab into different cell lines. Fig. 2A shows intracellular delivery of a FITC-Ab in various cells. In all cases, the cells treated with antibody in the absence of the lipid formulation displayed no intracellular fluorescence. Other cells were also successfully transduced including 293, MDCK, and P19 cells (not shown). Furthermore, various antibodies obtained from different sources gave similar results. The optimal delivery of a fluorescent antibody into NIH-3T3 cells was achieved after 4 h of incubation (Fig. 2B). Longer incubation periods (24–96 h) led to decreases in both the number of positive cells and the intracellular fluorescence intensity. Serum-free conditions were required during the first 4 h of incubation (not shown).

The delivery efficiency also depended on the amount of lipid and on the quantity of protein delivered (Fig. 2, C and D). For NIH-3T3 cells optimal delivery in 24-well plates was seen with 1–2 μg of fluorescent antibody and 2.5 μl of TFA-DODAPL:DOPE formulation (Fig. 2, C and D). Optimal delivery is also dependent on the protein concentration used to form the TFA-DODAPL:DOPE-protein complexes. The optimal concentration for antibody or β-galactosidase was between 80 and 200 μg/ml. At the highest antibody concentration, the intracellular fluorescence intensity in the positive cells was high (Fig. 2E, bars). In contrast, at lower protein concentrations, the percentage of fluorescent cells was high, but the intracellular intensity was low (Fig. 2E, lines).

**Induction of Apoptosis in Primary Cells with Apoptotic Proteases Delivered by TFA-DODAPL:DOPE**—To demonstrate the functionality of lipid-mediated protein delivery, we chose to examine protease mediators of apoptosis induction. Several reasons supported this choice. First, the function of apoptosis inducing proteases is extensively described in the literature and they have potent activity (18, 19). Second, assays are well established for measuring their activities. Third, these enzymes represent a major interest in research and therapeutic applications (18–20). Thus, the demonstration that apoptotic proteases and other types of apoptosis modulators can be delivered intracellularly in their biologically active form would provide a versatile tool for investigating the apoptotic cascade in cellular and disease models.

We tested the ability of TFA-DODAPL:DOPE to deliver functional granzyme B into primary human AML cells (Fig. 3). Granzyme B is a serine protease that is produced and stored in granules of cytotoxic lymphocytes. This protease cleaves and activates multiple members of the caspase family (21). The bioactivity of granzyme B therefore was assessed by measuring activation of endogenous caspases using a fluorogenic cell permeable substrate, CaspaTag, which can be monitored by flow cytometry.
Fig. 3. TFA-DODAPL:DOPE-mediated proteases delivery into primary and cultured suspension cells. A and B, primary human AML cells treated with PE/TFA-DODAPL:DOPE (A) or with a mixture of PE/granzyme B (1 unit)/TFA-DODAPL:DOPE (B). C-F, Jurkat cells were treated with BSA-phycoerythrin conjugate (BSA-PE) (C), or a mixture of BSA-PE and 1 unit of caspase 3 (D), granzyme B (E), or caspase 8 (F). Proteins were delivered with TFA-DODAPL:DOPE. The y axis quantifies the amount of the fluorescent PE that enters the cells, and the x axis quantifies the caspase activity using CaspaTag Kit. Untreated cells are confined to the lower left hand quadrant (data not shown).

Fig. 4. TFA-DODAPL:DOPE-mediated delivery of apoptotic proteases into suspension and adherent cultured cells. Cells were treated and analyzed as described under "Experimental Procedures." The percentage of apoptotic cells represents cells that are annexin V-FITC positive and propidium iodine positive or negative, after 24 h of incubation. A-F, Jurkat cells. A, cells only; B, 1 μM staurosporine; C, TFA-DODAPL:DOPE only; D, granzyme B (450 ng) only; E, TFA-DODAPL:DOPE/granzyme B (450 ng); and F, TFA-DODAPL:DOPE/galactosidase (1 μg). The y axis quantifies the amount of the propidium iodine taken up by the cells and the x axis quantifies the amount of annexin V-FITC bound to the cells.

Induction of Apoptosis in Cultured Suspension Cell Lines with Apoptotic Proteases Delivered by TFA-DODAPL:DOPE—
The same experimental approach was used to deliver caspase 3, granzyme B, and caspase 8 into Jurkat cells (Fig. 3). Caspase-3 was the most potent apoptosis inducer, leading to ~40% of the cells to score positive for caspases activity. Granzyme B and caspase 8 generated ~20% caspase positive cells, with the background of caspase activity in the culture being 7%. We also compared electroporation and TFA-DODAPL:DOPE efficacy using a wide variety of conditions for voltage and capacitance (not shown). Electroporation was shown to be toxic to Jurkat cells since 90% were killed under electroporation conditions that resulted in successful BSA-PE protein delivery. In contrast, TFA-DODAPL:DOPE treatment was deleterious to only 10% of the cell population (not shown).

The delivery of a functional granzyme B was further assayed by monitoring the early and late phases of the apoptotic process. The early stage is characterized by the cell membrane exposure of phosphatidylserine normally restricted to the inner cell membrane (22), which is recognized by annexin V-FITC. The later phase of the apoptosis can be assessed by measuring the DNA labeling with the propidium iodine indicator of the cell membrane permeabilization (19). We confirmed that granzyme B delivered by TFA-DODAPL:DOPE induced apoptosis of Jurkat cells. Granzyme B alone and the lipid formulation alone...
or complexed to a control protein (β-galactosidase) were ineffective to induce apoptosis (Fig. 4, C, D, and F, and Table I). As a positive control, we used staurosporine a well known chemical inducer of apoptosis (23) (Fig. 4B and Table I). These results demonstrate the efficacy of the delivery system, however, not all apoptotic modulators were effective. Indeed, cytochrome c, another powerful effector of apoptosis failed to induce apoptosis when delivered with the transport system (Table I). This observation illustrates the importance of the intrinsic properties such as charge and structure of the molecules to be delivered in order to support TFA-DODAPL-mediated transduction.

**Induction of Apoptosis in Cultured Adherent Cell Lines with Apoptotic Proteases Delivered by TFA-DODAPL:DOPE**—The cationic lipid carrier system was also used to deliver apoptotic proteases into adherent Ki-Ras 267β1 cells. The effects of granzyme B and caspase delivery were tested by measuring the percentage of cells that underwent apoptosis as determined by the annexin V-FITC staining kit (Table I). β-Galactosidase and staurosporine were used as negative and positive controls, respectively. TFA-DODAPL:DOPE-mediated delivery of granzyme B and caspase 3 induced apoptosis of Ki-Ras 267β1 cells to a similar extent (60 and 58%). These two proteins delivered by TFA-DODAPL:DOPE were as potent as staurosporine. No apoptosis was observed without the addition of the lipid formulation and with the lipid formulation alone or complexed to a control protein (β-galactosidase). To confirm that the induced apoptosis was only mediated by the delivery of the apoptotic proteases, we conducted another experiment with an inactive mutant of caspase 3 and compared its effect with caspase 3 and granzyme B (Fig. 4J). No significant apoptosis was observed with the mutant caspase 3 confirming that the induction of apoptosis was effectively mediated by the intracellular release of the active caspase 3. The results in Fig. 4J confirm that at the optimal doses tested, the lipid formulation complexed with control proteins such as β-galactosidase, phycocerythrin-BSA, and inactive caspase 3 does not trigger apoptosis. The percentage of cells undergoing apoptosis was influenced by the amount of granzyme B or caspase 3 delivered. With Ki-Ras 267β1 cells, the plateau was reached with 150 ng of granzyme B and 3.3 ng of caspase 3 (Fig. 4, G and H). The TFA-DODAPL:DOPE dose-response was also investigated (Fig. 4I). Fixed amounts of granzyme B, caspase 3, and caspase 8 were delivered with increasing amounts of the lipid formulation. The level of caspase activation was TFA-DODAPL:DOPE dependent. However, spontaneous caspase activation was induced by the higher concentrations of the cationic lipid formulation. This phenomenon depends on the cell type and density.

**TFA-DODAPL:DOPE Protein Interaction Analysis**—The mechanism of TFA-DODAPL:DOPE-mediated protein delivery may be analogous to that of cationic lipid-mediated DNA delivery (24–27) which involves 3 steps. (i) Formation of a complex between the cationic lipids and the protein of interest. (ii) Interaction of the complex with cells. (iii) Intracellular uptake and release of the protein into the cytoplasm. According to this mechanism, proteins that fail to interact with the cationic lipids will not get into the cells. The physical properties, such as charge, lipophilicity, and hydrophobicity, of the protein would be expected to influence its ability to interact with the cationic lipids.

A native protein gel shift assay was developed to compare the ability of TFA-DODAPL:DOPE to capture different proteins. Increasing amounts of lipids were mixed with a protein of interest and the mixture was applied to a native polyacrylamide gel. The results in Fig. 5 illustrate the parameters of this assay with 3 different proteins, β-galactosidase, goat IgG, and BSA. The majority of β-galactosidase is shifted by the lowest amount of TFA-DODAPL:DOPE indicating its capture by the lipid preparation (Fig. 5A, lane 2). TFA-DODAPL:DOPE shifts the IgG somewhat less efficiently, but at the two highest lipid concentrations the IgG is completely shifted (Fig. 5A, lanes 9 and 10). Thus, TFA-DODAPL:DOPE can capture nearly 100% of these two proteins. In contrast, BSA did not interact very well with the lipid formulation (Fig. 5B). Qualitatively, these results correlated with the delivery results for these three proteins showing that β-galactosidase and IgG were efficiently delivered to cells (Fig. 1), but BSA was not (not shown). This native protein gel shift assay can also be used for positively charged proteins like cytochrome c. A weak interaction between cytochrome c and the delivery system was observed (not shown) supporting its lack of apoptosis induction (Table I).

**Assays to Quantify TFA-DODAPL:DOPE-mediated Protein Uptake**—Fluorescence microscopy and flow cytometry results showed that TFA-DODAPL:DOPE could mediate uptake of
cent samples were analyzed with a fluorometer. Cells were fractionated (cytosol and membrane). Fluorescence microscopy and gel-shift assays that showed a strong interaction of the FITC-IgG with the lipids and effective intracellular delivery, whereas BSA interacted poorly with the lipid formulation and was not taken up efficiently by cells. Clearly, the efficacy of TFA-DODAPL:DOPE-mediated protein delivery depends on the physicochemical properties of the protein, working well for some but not for all.

To monitor intracellular uptake of non-fluorescent proteins, we have used a Western blot assay to determine intracellular protein levels. The data in Fig. 5C show monoclonal antibody uptake into NIH-3T3 cells with or without the delivery system. Without TFA-DODAPL:DOPE there was very little antibody recovered from the cells (lane 2). In the presence of the lipid formulation (lane 3) a significant amount of antibody (~100 ng) was recovered from the cells. Additionally, the Western blot ensures that the observed fluorescent signal was derived from intact not degraded proteins.

Monitoring Cell Surface Adsorption of Protein Versus Intracellular Uptake—The whole cell uptake studies shown in Table II and Fig. 5C revealed how much of the input protein is taken up by cells following TFA-DODAPL:DOPE-mediated delivery, however, these results did not address the intracellular localization of the protein. Microscopy can be used to assess whether fluorescent protein is adsorbed on the cell surface or inside the cells. The results in Fig. 5D, E and F, show how β-galactosidase can be used to assess differences in the cellular protein distribution following TFA-DODAPL:DOPE-mediated protein delivery. We compared TFA-DODAPL:DOPE-β-galactosidase complexes prepared in high (Fig. 5D) or low (Fig. 5E) phosphate buffer. Although all buffer conditions led to substantial β-galactosidase uptake by the cells, the staining pattern differed. The low phosphate buffer and other buffers (Tris and Hepes), showed cell surface-associated aggregates whereas the

| % of Fluorescent-protein recovered |
|-----------------------------------|
| Medium                           |
| Cytosol                          |
| Membrane                         |
| Fluorescein-IgG                   |
| - TFA-DODAPL:DOPE                 | 97%  2%  1% |
| + TFA-DODAPL:DOPE                | 50%  46% 5% |
| Fluorescein-BSA                   |
| - TFA-DODAPL:DOPE                 | 84%  14% 2% |
| + TFA-DODAPL:DOPE                | 71%  28% 1% |

high phosphate buffer resulted in a more even distribution pattern and considerable intracellular uptake. This result illustrates the critical role for some proteins of the buffer composition used to rehydrate the dried lipid formulation. For antibody delivery no significant differences were seen among the tested buffers and pH 6–8 conditions. These assays can be used to optimize the protein transduction conditions for each specific molecule of interest.

Taken together, the fluorescent microscopy, cell fractionation, and functional assays demonstrated clearly that the protein was effectively delivered by TFA-DODAPL:DOPE into the cytoplasm of cells treated. The different intracellular biodistributions of low and large molecular weight dextran emphasized the release of the molecules transported inside the cells since only the low molecular weight dextran was taken up by the nuclei. However, to avoid uncertainty over whether molecules delivered by lipids were on the inside or outside of the cell membrane, we used confocal microscopy to examine the intracellular distribution of fluorescently labeled antibody and dextran sulfate. The results in Fig. 6 confirm that low molecular weight dextran sulfate delivered with the cationic lipid formulation is distributed inside cells and is localized in both the cytoplasm and the nucleus (Fig. 6, A and B). FITC-Ab is also delivered intracellularly by the lipid-mediated delivery system and is excluded from the nucleus, presumably because it is too large to get through the nuclear pore (Fig. 6, C and D).

**DISCUSSION**

Although there are many effective reagents available to introduce transcriptionally active DNA into viable cells, approaches to deliver functional peptides and proteins into living cells are not generally available. For this reason, we investigated a new approach to deliver protein directly into cells using a unique lipid-based carrier system. The cationic lipid formulation interacts rapidly and non-covalently with the protein
creating a protective vehicle for immediate delivery into cells. The efficiency of delivery varied, primarily due to the properties of the molecule to be delivered and the cell type used. The delivery efficiency in living cells ranged from 35 to 99% and up to 50% of the input protein was introduced into the cells. This cationic lipid formulation can deliver antibodies, dextran sulfate, phycobiliproteins, and enzymes (β-galactosidase, caspases, and granzyme B) into a broad range of cell types including primary cells. It surpasses both microinjection and electroporation in delivering biologically active proteins into living cells. Furthermore, delivered proteins were recovered intact from the cytosol as detected by Western blot and apoptotic proteases delivered into cells with TFA-DODAPL:DOPE remained functional, since they triggered apoptosis. The protein delivery system is fast and under optimal conditions displays no significant toxicity.

TFA-DODAPL:DOPE, called BioPORTER, has advantages over the other protein delivery approaches, which use membrane transport peptides such as PTD since it does not require any modification of the protein to be delivered. Indeed, conjugation or generation of fusion proteins containing membrane transport sequences can sometimes adversely affect biological activity of the protein. In addition, unlike PTD, we have not been hampered by size limitation of the protein for TFA-DODAPL:DOPE-mediated delivery since we can show that a 240,000 molecular weight phycoerythrin protein can be effectively delivered into cells (12, 13).

Several formulation and experimental parameters modulate the delivery efficiency of the carrier system including the biological properties of the molecules to be delivered, the composition of the hydration buffer, the cell type, and time of incubation. Since the biological properties of the molecule to be delivered can vary greatly, we set up several assays to assist in the optimization of the cationic lipid formulation performance and to better understand its mechanism. The lipid-mediated delivery system also has some limitations; it works well for some but not all proteins as illustrated with cytochrome c, a basic protein. One of the fundamental differences between developing nucleic acid and protein delivery systems is related to their differences in physical properties. Cationic lipids that interact with one type of nucleic acid molecule also usually interact with every other type of negatively charged nucleic acid molecule. In contrast, proteins vary greatly in their net charge, hydrophobicity, lipophilicity, and conformation. As shown in Table III, proteins used in this study present multiple biophysical properties. They have different size, are negatively charged (β-galactosidase, phycocyanin, caspase 3, and caspase 8) or cationic (granzyme B and cytochrome c). In the same way, caspase 3 and 8 have hydrophobic domains whereas BSA contains few hydrophobic regions encrypted in its globular conformation. Thus, different proteins would be expected to interact very differently with the positively charged lipid formulation. Indeed, positively charged cytochrome c (Table III) did not interact with TFA-DODAPL:DOPE and consequently was not transported into cells, whereas, very negatively charged proteins such as β-galactosidase, phycocyanin, caspase 3, and caspase 8 were transported. However, the net charge of the protein to be delivered is not the only factor involved since granzyme B, a positively charged protein (Table III), is effectively delivered whereas BSA, negative at neutral pH, is not. Overall hydrophobicity conformation and the presence or absence of accessible hydrophobic or amphiphatic regions of the molecules to be delivered might also be implicated. Both granzyme B and cytochrome c are positively charged, but granzyme B has a very hydrophobic region, whereas cytochrome c does not. Presumably it is this hydrophobic region that allows granzyme B to interact with the cationic lipid even though they cannot interact electrostatically. In fact, cytochrome c and to a lesser extend BSA did not get efficiently delivered into cells possibly due to their globular nature (BSA), charge (cytochrome c), lack of hydrophobicity (BSA and cytochrome c, see Table III), or combinations of them. Further studies are underway to characterize a larger number of proteins in more detail. Nevertheless it is reasonable to predict that highly negatively charged proteins would be successfully delivered by the lipid formulation whereas highly positively charged molecules with few or no hydrophobic domains would not. In this context, we are also attempting to alleviate these limitations by adding a negatively charged tail to some positively charged proteins to enhance their interaction and incorporation in the delivery system. Varying the pH and/or the salt concentration of the hydration buffer used to dilute the protein could also potentially solve this problem.

Several attempts to use cationic lipids for delivering proteins into cells have been reported (28–31). Cationic liposomes have been shown to co-deliver transcriptional factor with plasmid DNA containing the transcription factor response element (28, 30). The plasmid DNA-protein complexes were transfected into cells using cationic lipid-mediated transfection protocols. However, the delivery efficiency was very low, required chloroquine treatment (28) and the benefit of using the cationic lipid was unclear since the protein alone can spontaneously enter the cell without the aid of any delivery system (30, 32). Cationic lipids have also been used to deliver protein into the intracellular processing pathway leading to antigen presentation, but the discrimination between inside and outside antigen presentation was difficult and the efficiency of functional intracellular protein delivery remains unclear (29). In vivo lung delivery of β-galactosidase by cationic lipids has also been reported, however, the protein delivery was not increased by the cationic lipids since the number of cells positive for β-galactosidase was

### Table III

| Protein                | M<sub>r</sub> | pI   | Charge at pH 7.0<sub>a</sub> | Number of residues – | Number of residues + | Hydrophobic region<sup>b</sup> |
|------------------------|-------------|------|----------------------------|---------------------|---------------------|-----------------------------|
| Phycoerythrin          | 240         |      | −3.7                       | 21                  | 17                  | +                          |
| − α chain              |             |      | −12.2                      |                     |                     | +                          |
| − β chain              |             |      | −1.2                       |                     |                     | +                          |
| BSA                    | 69          | 5.82 | −9.9                       | 99                  | 86                  | +/–                        |
| β-Galactosidase        | 116         | 5.28 | −32.1                      | 126                 | 86                  | +/–                        |
| Caspase 3              | 31          | 6.09 | −2.3                       | 40                  | 36                  | +                          |
| Caspase 8              | 55          | 5.90 | −18.7                      | 80                  | 60                  | +                          |
| Granzyme B             | 27.7        | 9.56 | 15.9                       | 20                  | 34                  | +                          |
| Cytochrome c           | 11.7        | 7.95 | 9.6                        | 12                  | 21                  | −                          |

<sup>a</sup> The charge of the protein at neutral pH was determined with the protein calculator software [www.scripps.edu/cgi-bin/cdputnam/protcalc](http://www.scripps.edu/cgi-bin/cdputnam/protcalc).

<sup>b</sup> The Kyte and Doolittle method was used to calculate hydrophobicity of the protein. +, 3 or more hydrophobic regions (>0.5); +/-, 1–2 hydrophobic regions (>0.5); −, no hydrophobic regions (>0.5).
identical with or without the delivery system and no other functional protein was used (31). In most cases, procedures to assess the protein delivery efficiency required fixation, permeabilization, or scrape loading and the observed intracellular localization may have been a consequence of these membrane disrupting procedures (28, 31, 33). Consequently, the use of cationic lipids for protein delivery remains extremely isolated and limited. In contrast, the TFA-DODAPL:DOPE formulation efficiently delivered functional proteins specifically in live cells by the lipid formulation and predictive guidelines for which proteins are likely to work are proposed.

There are many reasons for wanting to have access to convenient and reliable methods for delivering proteins, peptides, and antibodies into cells. The apoptosis results reported here show that proteins delivered with this new lipid-mediated delivery system (TFA-DODAPL:DOPE) enter cells, remain active, and can exert their functional effect on the cells, so that their physiological effects can be investigated even in different cell types. The ability to directly inhibit or initiate other targeted intracellular functions specifically in live cells by the delivery of antibodies, recombinant proteins, or peptides will be of tremendous benefit in all aspects of cellular biology and functional genomics. The intracellular function of other proteins controlling intracellular signaling, cell cycle regulation, apoptosis, oncogenesis, and transcription regulation may be similarly investigated.

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