Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 3176–3181, April 1996
Biochemistry

A serum-resistant cytofectin for cellular delivery of antisense oligodeoxynucleotides and plasmid DNA

(cyclin B/luciferase/liposome/C-5 propyne/gene therapy)

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Communicated by Marvin H. Caruthers, University of Colorado, Boulder, CO, December 7, 1995 (received for review September 25, 1995)

ABSTRACT **Development of antisense technology has focused in part on creating improved methods for delivering oligodeoxynucleotides (ODNs) to cells. In this report, we describe a cationic lipid that, when formulated with the fusogenic lipid dioleoylphosphatidylethanolamine, greatly improves the cellular uptake properties of antisense ODNs, as well as plasmid DNA. This lipid formulation, termed GS 2888 cytofectin, (*i*) efficiently transfects ODNs and plasmids into many cell types in the presence or absence of 10% serum in the medium, (*ii*) uses a 4- to 10-fold lower concentration of the agent as compared to the commercially available Lipofectin liposome, and (*iii*) is ≥20-fold more effective at eliciting antisense effects in the presence of serum when compared to Lipofectin. Here we show antisense effects using GS 2888 cytofectin together with C-5 propynyl pyrimidine phosphorothioate ODNs in which we achieve inhibition of gene expression using low nanomolar concentrations of ODN. This agent expands the utility of antisense ODNs for their use in understanding gene function and offers the potential for its use in DNA delivery applications *in vivo*.**

A major barrier for the development of antisense oligodeoxynucleotides (ODNs) is the permeability of ODNs to cellular membranes. ODNs must be present at sufficiently high concentrations in the nucleus of the cell for RNA binding to occur (1). Recent studies have shown that when ODNs are added to cell culture media, they are not efficiently transported to the cytoplasm or nucleus of most cells, resulting in poor or nonexistent antisense effects (2–6). Cytofectin agents, also known as cationic amphiphiles or cationic liposomes, have been developed that are capable of delivering DNA, including both plasmids and ODNs, past the cellular membrane, and the use of these agents strongly correlates with the ability to achieve high activity of the DNA (2–9). The continued development of cytofectins should make the generalized use of antisense ODNs and gene delivery more practical as research tools and for therapeutic applications.

Little progress has been reported for designing ODNs that are more efficient at passive cell membrane diffusion, although promising lead backbone derivatives have been identified (see ref. 10). However, substantial advances have been made in the use of cationic lipids that can efficiently deliver polyanionic ODNs and plasmid DNA from cell media into the cytoplasm and nucleus of cells (11). Lipofectin, a 1:1 (wt/wt) liposome formulation of the cationic lipid *N*-[1-(2,3,-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) and dioleoylphosphatidylethanolamine (DOPE), has been shown in several studies to deliver ODNs into cells, resulting in antisense gene inhibition (2–6).

Despite the success using Lipofectin, we have found several limitations of its use for transfection of ODNs and plasmids into cells. This agent is serum sensitive; therefore, optimal transfection must be performed using serum-free conditions, which has obvious shortcomings for potential *in vivo* applications. In addition, the agent does not work efficiently with many cell types. In this report, we describe a markedly improved cytofectin, termed GS 2888, which overcomes many of the shortcomings of Lipofectin for delivering ODNs and plasmids to cells. The GS 2888 cytofectin efficiently transfects ODNs and plasmids into many cell types in the presence or absence of serum in the medium, uses a 4- to 10-fold lower concentration of the agent as compared to Lipofectin, and is ≥20-fold more effective at eliciting antisense effects in the presence of serum when compared to Lipofectin. Thus, we show antisense effects using GS 2888 cytofectin together with C-5 propynyl pyrimidine phosphorothioate ODNs (C-5 propyne S-ODNs) in which we achieve inhibition of gene expression using low nanomolar concentrations of ODN. In addition, we demonstrate efficient gene delivery in the presence of serum. This agent offers the potential for its use in *in vivo* applications for both gene and antisense delivery and expands the utility of antisense ODNs for their use in understanding gene function.

MATERIALS AND METHODS

ODN Synthesis and Fluorescent Labeling. ODNs were synthesized using the H-phosphonate approach with standard methods (6). A 15-nt C-5 propyne S-ODN (5′-CUU CAU UUU UUC UUC-3′) was labeled on the 5′ end using fluorescein coupled via a 6-amino-1-hexanol linker (12). Fluorescent derivatization and purification were performed as described (12). This ODN was used for each of the fluorescence localization studies.

Cell Culture. All cell lines were purchased from the American Type Culture Collection and were cultured according to the recommended conditions. Normal human dermal fibroblasts, smooth muscle cells, and endothelial cells were purchased from Clonetics (San Diego).

Synthesis of GS 2888. Compound GS 2888 was synthesized as outlined in Fig. 1. Carbobenzyloxyglycine (1.05 g, 5 mmol) in tetrahydrofuran (30 ml), *N*-hydroxysuccinimide (0.58 g, 5 mmol), and 1,3-dicyclohexylcarbodiimide (1.1 g, 5.3 mmol) were stirred at room temperature (rt) overnight. The precipitate was filtered off and washed with methylene chloride. The

Abbreviations: ODN, oligodeoxynucleotide; DOPE, dioleoylphosphatidylethanolamine; rt, room temperature; FBS, fetal bovine serum; PI-3-kinase, phosphatidylinositol-3-kinase; C-5 propyne S-ODN, C-5 propynyl pyrimidine phosphorothioate ODN; Boc, *t*-butoxycarbonyl; DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride.

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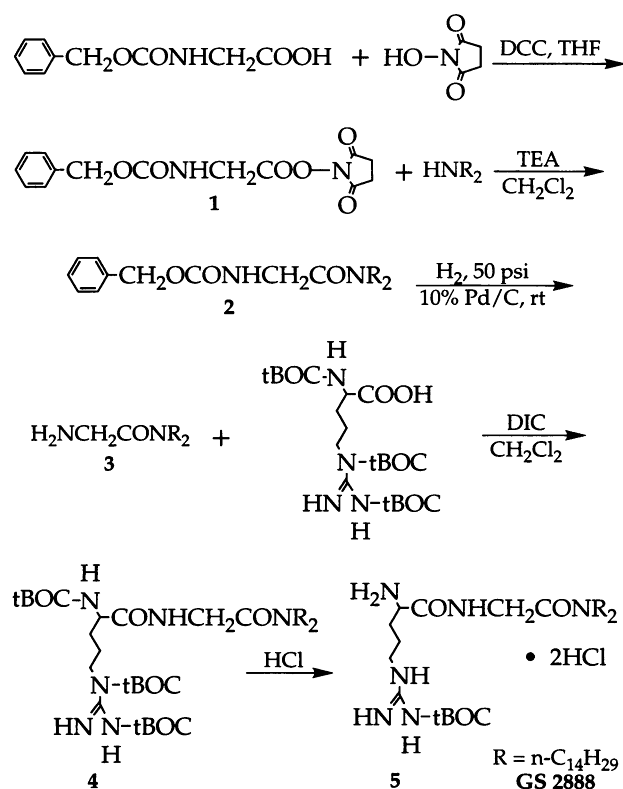

FIG. 1. Scheme for synthesis of GS 2888. DCC, 1,3-dicyclohexylcarbodiimide; THF, tetrahydrofuran; TEA, triethylamine; DIC, 1,3-diisopropylcarbodiimide.

combined organic solution was concentrated to dryness, yielding compound **1.** Next, compound **1** (5 mmol) was dissolved in methylene chloride (30 ml) containing triethylamine (0.74 g, 7.3 mmol), followed by addition of dimyristylamine (2.1 g, 5 mmol). After 5 hr at rt, the reaction mixture was washed with water, dried over Na_2SO_4, and concentrated. The residue was purified by flash column chromatography on silica gel and eluted with methylene chloride to yield the desired product, **2**, as a colorless liquid [0.54 g; yield 18% (2 steps)]. Compound **2** (0.45 g) in methylene chloride (5 ml) and ethanol (5 ml) was hydrogenated at 50 psi (1 psi = 6.89 kPa) in the presence of 10% Pd/C for 8 hr. The catalyst was filtered off through a celite pad. The filtrate was concentrated, producing a yellow, wax-like solid (compound **3**, 0.33 g; 94%).

L-Boc-Arg $(Boc)_2$-OH [L-arginine protected with the *t*-butoxycarbonyl (Boc) group; Bachem, no. A2935; 172 mg, 0.36 mmol], compound **3** (161 mg, 0.34 mmol), and 1,3-diisopropylcarbodiimide (48 mg, 0.38 mmol) were dissolved in CH_2Cl_2 (10 ml). The reaction mixture was stirred at rt overnight, washed with H_2O, dried over Na_2SO_4, and concentrated and purified by flash column chromatography on silica gel, producing compound **4** (120 mg; 37.5% yield). Compound **4** (120 mg) was dissolved in dioxane (3 ml) and treated with 4 M HCl in dioxane (4 ml) at rt for 2.5 hr. The reaction mixture was concentrated and azeotroped with CH_3CN twice to yield compound **5.** The reaction was monitored using reverse-phase HPLC [Hewlett–Packard 1090 equipped with a C_{18} column (Vydac, 218TP5415, 4.6 × 150 mm; Hesperia, CA) using the following conditions: mobile phase, 90% MeOH/10% 50 mM ammonium acetate (pH 6.8); flow, 1 ml/min (isocratic); temperature, 60°C; diode array detector at 205–210 nm; retention times, 21, 12, 8.2, and 6.6 min for the tri-, di-, mono-, and no-Boc derivatives of **5**, respectively]. Cellular uptake studies showed that the mono-Boc cationic lipid (**5**) was more efficient at delivering ODNs to cells than the fully deprotected lipid. NMR (DMSO-d_6 + 1 M HCl): δ 11.0 (s, NH), 8.80 (6, NH), 8.70 (t, NH), 8.30 (br, NH_2), 3.80–4.15 (m), 3.35 (q, 2H), 3.20 (m, 4H), 1.88 (m), 1.60 (m), 1.18–1.60 (m), 0.82 (t, 6H).

Formulation of GS 2888. In preliminary studies with the GS 2888 lipid, we assessed its delivery properties in the absence of a co-lipid (such as DOPE) and found that it had poor DNA delivery properties as a single lipid agent. Additionally, the GS 2888 lipid was formulated with DOPE by first combining the two lipids in various molar ratios in $CHCl_3$, followed by drying them to a thin film under argon. Sterile-filtered water was then added to the dried lipids to obtain a lipid suspension at 1 mg of lipid per ml, followed by a 5-min vortex. The suspension was in some cases used directly as a multilamellar vesicle preparation. Alternatively, the vortexed mixture was either (*i*) sonicated five times for 15 sec per sonication at 0–4°C with 30 sec between pulses and centrifuged at 2000 rpm using an Eppendorf microcentrifuge or (*ii*) freeze–thawed on dry ice six times. Either of these two methods created small vesicles as assessed by microscopy. The liposomes were used directly or sized by filtration through a polycarbonate membrane (50- to 200-nm pore sizes; Liposofast; Avestin, Ottawa, QC Canada).

After the initial screen of cytofectin agents (Table 1), GS 2888 was optimized for its delivery properties. Fluorescent ODN uptake analysis in CV-1 African monkey cells showed that an unsized formulation, prepared by the freeze–thaw method, at a GS 2888 to DOPE molar ratio of 2:1 was optimal for delivery. This formulation was used for subsequent studies and is referred to as the GS 2888 cytofectin.

Cationic Lipid Delivery of ODNs to Cells. Lipofectin, LipofectAce [a 1:2.5 (wt/wt) formulation of dimethyldioctadecylammonium bromide and DOPE], and LipofectAmine {a 3:1 (wt/wt) formulation of 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propanaminiumtrifluoroacetate and DOPE} were purchased from Life Technologies (Gaithersburg, MD); Transfectam reagent (5-carboxyspermylglycinedioctadecylamide) was purchased from Promega.

To prepare the cytofectin–ODN complexes for transfection in one well of a six-well microtiter plate, the ODN was first diluted into 100 μl of prewarmed Opti-MEM (GIBCO/BRL) in polystyrene plastic. The cytofectin was diluted similarly. The prediluted ODN and cytofectin mixtures were combined (again in polystyrene plastic), and within 15 min 800 μl of the appropriate prewarmed (37°C) medium (with or without serum) was added. Medium was then removed from the cells and replaced with the medium containing the ODN–cytofectin complex. Washing the cells with phosphate-buffered saline at any point during the transfection appeared to interfere with cell uptake and affected the viability of the cells.

Antisense Assays. The HeLa X1/5 cell line stably expresses luciferase and has been described in detail (13). The cells were seeded onto six-well tissue culture plates at a density of 5×10^5 cells per well in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics. The following day the cells were transfected for 4 hr using either antisense ODNs targeted to the coding region (two sequences were used: GS 2427, 5′-CGU GAU GUU CAC CUC-3′ and GS 2429, 5′-UCC UUG UCG UAU CCC-3′) or mismatch sequence ODNs [underlines indicate mismatch positions: GS 2631, 5′-CGC UUU CUA UAG CGC-3′ (8-base mismatch to GS 2427) and GS 3432, UCC CUA UGC UGU UCC-3′ (6-base mismatch to GS 2429)] together with either GS 2888 cytofectin (GS 2888 to DOPE at a 2:1 molar ration, unsized) or Lipofectin. At defined time points, cell extracts were made using reporter lysis buffer (Promega), and luciferase levels were quantified using a single photon liquid scintillation counter (LS6500; Beckman) as described (14). Alternatively, cells were treated with cyclin B antisense ODNs (two sequences were used: GS 3207, 5′-UUC CAG UGA CUU CCC-3′ and GS 3208, UCU UCA UUU CCA UCU-3′) or their mismatched sequences [GS 3319, UCU GAU GAC CCU CUC-3′ (9-base mismatch to GS 3207) and GS 3320, UUU

Table 1. Qualitative assessment of the nuclear uptake of a 5′-fluorescein-labeled 15-mer C-5 propyne S-ODN (250 nM) into CV-1 cells in DMEM with or without 10% FBS using various lipid formulations

			− serum		+ serum	
Lipid	Conc., μg/ml	Charge ratio*	% uptake†	Intensity‡	% uptake†	Intensity‡
Lipofectin	1.0	0.2	80	+/−	0–5	−
	2.5	0.6	80	+	25	+/−
	5.0	1.1	95	+	55	+/−
	10.0	2.2	95–100	++	55	+/−
	20.0	4.4	90§	++	45	+/−
LipofectAmine	1.0	1.2	0–5	−	0–5	−
	2.5	3.0	0–5	−	0–5	−
	5.0	6.0	50	+	0–5	−
	10.0	12	90	++	0–5	−
	20.0	24	90§	+++	0–5	−
LipofectAce	1.0	0.16	0–5	−	0–5	−
	2.5	0.33	0–5	−	0–5	−
	5.0	0.65	75	+	0–5	−
	10.0	1.3	75	++	5	+/−
	20.0	2.6	85	++	15	+/−
Transfectam	1.0	1.4	0–5	−	0–5	−
	2.5	3.5	0–5	−	0–5	−
	5.0	7.0	50	+/−	0–5	−
	10.0	14	95	+	0–5	−
	20.0	28	95	++	5	+/−
GS 2888	1.0	1.2	95	+	95	+
	2.5	3.0	100	+++	100	+++
	5.0	6.0	100	+++	100	+++
	10.0	12	100	+++	100	+++
	20.0	24	100§	+++	100	+++

*The molar ratio of cationic amphiphile to anionic DNA phosphate. For GS-2888, a charge of +1 was assumed for the molecule at physiological pH.
†Measured as the number of cells that contained fluorescent ODN in the nucleus by visual inspection.
‡Intensity refers to the relative fluorescence intensity observed in the microscope.
§At 20 μg/ml, Lipofectin and GS 2888 showed a 20–30% decrease in cell viability and LipofectAmine showed a 90% decrease, each in the absence of serum.

CCA UUU ACC UUC (6-base mismatch to GS 3208)]. All of the ODNs described above consisted of C-5 propyne and phosphorothioate modifications.

Levels of luciferase and cyclin B were evaluated by Western immunoblot. Hela X1/5 cells were seeded in six-well plates at 5.0×10^5 cells per well, and the following day they were treated for 4 hr using ODN–cytofectin complexes in DMEM containing 10% FBS, washed, given fresh media, and lysed after 18 hr. Thirty micrograms of protein was separated on 7.5% acrylamide Tris·Cl ready gels (Bio-Rad) and electrotransferred to Westran poly(vinylidene difluoride) membrane (Schleicher & Schuell) and probed with either mouse anti-human cyclin B1 (1:500; PharMingen), rabbit anti-luciferase (1:500; Promega), or rabbit anti-rat phosphatidylinositol-3-kinase (PI-3-kinase; 1:5000; Upstate Biotechnology, Lake Placid, NY). Blots were developed using horseradish peroxidase-conjugated goat anti-rabbit and/or anti-mouse antibodies (1:1000; Zymed) and the ECL Western blotting detection reagents (as per instructions, Amersham). Membranes were exposed to X-Omat AR film (Eastman Kodak) for 1–20 sec.

RESULTS

Fluorescence ODN Uptake Study. We designed and synthesized a cationic lipid, GS 2888, and evaluated its ability (as a formulation with the fusogenic lipid DOPE) to transfect a 5′-fluorescein-labeled C-5 propyne S-ODN into African green monkey epithelial cells (CV-1), rat embryo fibroblast cells (Rat-2), and human epitheloid carcinoma cells (HeLa). Additionally, this lipid formulation was compared with several previously described cytofectins including LipofectAmine, Lipofectin, LipofectAce, and Transfectam. Each of the agents was used at four concentrations (20, 10, 5, and 2.5 μg/ml) together with 250 nM ODN such that an excess cation to anion charge ratio could be evaluated, and cells were incubated with the agents for 6 hr in the presence or absence of serum. The cells were then washed free of the complex and viewed immediately as well as 18 hr later. The results, taken in a blinded fashion, showed that each of the agents was able to transfect ODN into the nuclei of CV-1 cells in the absence of serum (Table 1), although the agent that qualitatively delivered the most ODN to cells was the GS 2888 lipid formulated with DOPE. Most of the agents were nontoxic to the cells in the absence of serum as evidenced by visual inspection and trypan blue dye exclusion. Exceptions to this were observed at the highest concentration (20 μg/ml) in which the LipofectAmine agent was toxic to >90% of the cells and the Lipofectin and GS 2888 agents were toxic to 20–30% of the cells. In the presence of 10% FBS, most of the lipids and lipid mixtures failed to efficiently deliver ODN to the nuclei of cells at the concentrations tested, with the striking exception of the GS 2888 lipid (Figs. 2 and 3). The GS 2888 agent was also able to deliver ODN to CV-1 cells in 50% FBS (Fig. 3). Cellular toxicity appeared to be substantially mitigated for each of the agents in the presence of 10% and 50% serum. For each of the above experiments, similar results were observed for the three cell lines tested.

Optimization of the GS 2888 lipid with DOPE using the fluorescence assay described above showed that an unsized 2:1 molar ratio of GS 2888 to DOPE (prepared by the freeze–thaw method) was optimal (results not shown). This formulation is referred to as GS 2888 cytofectin.

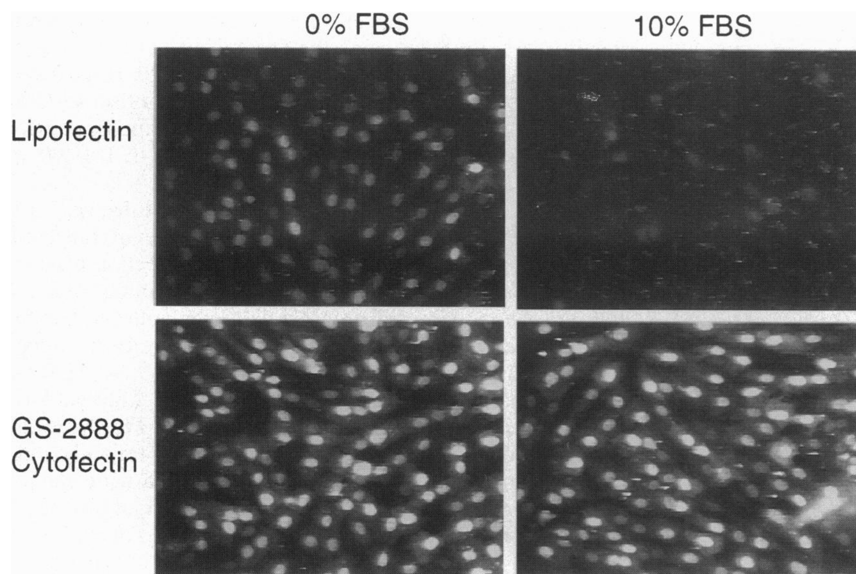

FIG. 2. Uptake of a 5′-fluorescein-labeled 15-mer C-5 propyne S-ODN into Rat-2 cells using Lipofectin or the GS 2888 cytofectin. Rat-2 cells were incubated with either Lipofectin (10 μg/ml) or GS 2888 cytofectin (2.5 μg/ml) and fluorescent ODN (250 nM) for 24 hr in DMEM with or without 10% FBS. Confocal images (Noran Instruments, Middleton, WI) were captured [×16 Planneofluar lens; Zeiss Axiovert 10 microscope (Zeiss)] at identical settings as described (12). Images show fields of live cells in which the fluorescent ODN was delivered to their nuclei.

Transfection of ODNs into Various Cell Lines Using the GS 2888 Cytofectin. The GS 2888 cytofectin was found to transfect the fluorescent ODN into the nuclei of a broad array of cell types. Most of the cell types were efficiently transfected with the fluorescent ODN using between 1 and 5 μg of the GS 2888 cytofectin per ml in the presence of 10% FBS, including the following secondary human cell cultures—dermal fibroblasts (Fig. 3), umbilical vein endothelial cells, keratinocytes, and smooth muscle cells—and the following cell lines—promyelocytic leukemia (HL60), simian virus 40-transformed African green monkey kidney (Cos-7), rat skeletal muscle (L6), human melanoma (Lox), mouse macrophage (C21), lymphoid neoplasm (P388D1), human breast carcinoma (MCF-7, SK-BR-3), human cervical carcinoma (SK-OV-3), human diploid lung fibroblasts (WI-38, IMR-90), rat smooth muscle (A10), differentiated human colon adenocarcinoma (HT-29), and acute human T-cell leukemia (Jurkat). Exceptions included human colon adenocarcinoma (CaCo2) cells, mouse helper T cells (D10), and human acute lymphoblastic leukemia (Molt-4) in which only 5–10% nuclear uptake was observed. Under these conditions, minimal (<5%) cellular toxicity was observed for each of the cell types.

Antisense Effects Using the GS 2888 Cytofectin. Next, the GS 2888 cytofectin was shown to effectively deliver antisense ODNs to cells and consequently effect potent and selective antisense gene inhibition. This experiment used a previously characterized HeLa X1/5 cell line, which stably expressed luciferase under the tight control of a tetracycline-responsive promoter (13). We targeted luciferase using two 15-nt antisense C-5 propyne S-ODNs targeted to the coding region of the gene. Luciferase expression was potently inhibited using the HeLa X1/5 cells, GS 2888 cytofectin (2.5 μg/ml), and the antisense C-5 propyne S-ODNs GS 2427 and 2429 (IC_{50} = 0.5 and 3 nM, respectively), while mismatch sequence control ODNs (GS 2631 and GS 3432) were significantly less active (extrapolated IC_{50} values for both mismatches were ≈180 nM). Comparison of the activity of ODN GS 2427 using GS 2888 cytofectin to that using Lipofectin showed that the GS 2888 cytofectin delivered >20-fold more ODN to cells in the presence of serum even though 4-fold less agent was used (2.5 μg/ml versus 10 μg/ml, respectively; Fig. 4*A*). Western blot analysis using ODN GS 2427 (at 3, 9, and 27 nM) with GS 2888 (2.5 μg/ml) showed that the inhibition of luciferase protein was gene specific as compared to two other control proteins, PI-3-kinase and cyclin B (Fig. 4*B*). As a further demonstration of specificity, two antisense ODNs were targeted to cyclin B and analyzed by Western blot analysis. The results using the antisense sequence GS 3207 showed selective inhibition of cyclin B (IC_{50} ≈ 3 nM) and did not affect luciferase or PI-3-kinase levels; the mismatched cyclin B ODN (GS 3319)

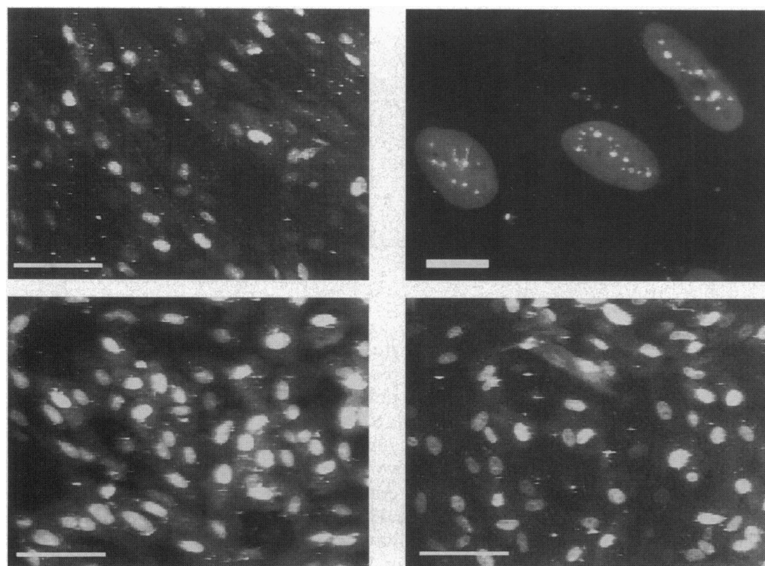

FIG. 3. Uptake of a 5′-fluorescein-labeled 15-mer C-5 propyne S-ODN into live cells. (*Upper*) Normal human dermal fibroblast cells were incubated for 24 hr with ODN (250 nM) and GS 2888 cytofectin (2.5 μg/ml) in DMEM containing 10% FBS. (*Upper Left*) Field of cells captured using a ×16 lens as described in Fig. 2 (bar = 100 μm). (*Upper Right*) Field of cells captured using a ×100 Planneofluar lens (Zeiss) and shows the nuclear fluorescence staining of the ODN in three cells (bar = 10 μm). (*Lower*) Monkey kidney epithelial (CV-1) cells. (*Lower Left*) Cells incubated for 24 hr with ODN (250 nM) and GS 2888 cytofectin (2.5 μg/ml) in DMEM containing 10% FBS. (*Lower Right*) Cells incubated for 24 hr with ODN (250 nm) and GS 2888 cytofectin (2.5 μg/ml) in DMEM + 50% FBS. Images were captured as described in Fig. 2 (bars = 100 μm).

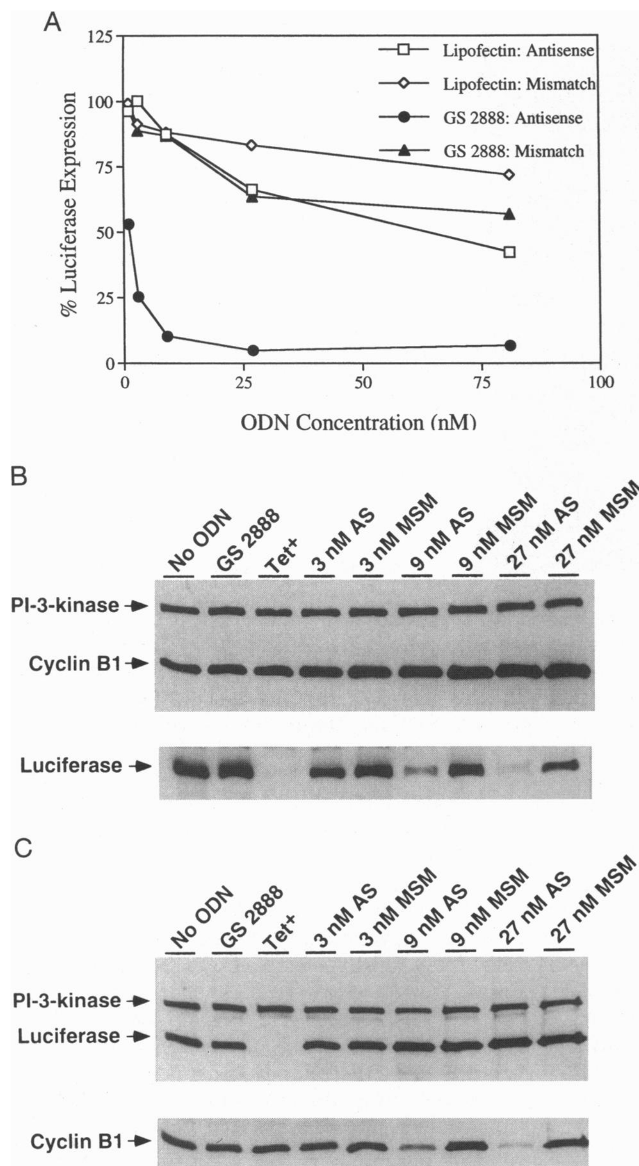

FIG. 4. (*A*) Dose–response for inhibition of luciferase in HeLa X1/5 cells using an antisense (GS 2427) versus a mismatch sequence control (GS 2631) together with either Lipofectin (10 μg/ml) or GS 2888 cytofectin (2.5 μg/ml) in the presence of 10% FBS. % Luciferase Expression refers to the relative number of light units detected through the luciferase enzyme assay relative to control cells treated with lipid alone. Luciferase expression was quantitated as described in *Materials and Methods*. The experiment was repeated in triplicate, and values for each of the points varied by $<5\%$. (*B*) Antisense inhibition of luciferase using C-5 propyne S-ODNs. HeLa X1/5 cells were incubated with either antisense (AS) or mismatch (MSM) ODNs targeted to luciferase (GS 2427 and GS 2631, respectively; 3, 9, and 27 nM; 24 hr) together with the GS 2888 cytofectin (2.5 μg/ml) in DMEM containing 10% FBS. Cell extracts were prepared and analyzed by immunoblot. Since luciferase and cyclin B run at approximately the same molecular weight on an SDS/7.5% PAGE gel, side-by-side gels were electrophoresed using identical amounts of protein per lane and immunoblotted using PI-3-kinase, luciferase, and cyclin B antibodies. Since luciferase is negatively regulated by tetracycline in these cells (13), tetracycline (1 μg/ml; Tet^+) was added to the medium of HeLa X1/5 cells at the time of ODN transfection as a positive control. (*C*) Antisense inhibition of cyclin B using C-5 propyne S-ODNs. HeLa X1/5 cells were incubated with either antisense (AS) or mismatch (MSM) ODNs targeted to cyclin B (GS 3207 and GS 3319, respectively) in an otherwise identical experiment to that in *B*. Each of the above experiments was repeated in duplicate, and similar results were obtained.

was inactive at the concentrations tested (Fig. 4*C*). Similar results were obtained using the second pair of antisense and mismatched sequences (GS 3308 and GS 3320, respectively). Additional experiments using the cyclin B antisense ODNs together with the GS 2888 cytofectin showed antisense effects with the same potency and specificity in CV-1, IMR90, and secondary human dermal fibroblast cells.

Plasmid DNA Delivery Using GS 2888 Cytofectin. To determine the general utility of GS 2888 cytofectin in transfecting DNA into cells, we compared plasmid transfection efficiency of GS 2888 [at a charge ratio range (cation to anion) of 2.5–10] to four other widely used commercially available cytofectins in the presence of 10% FBS: Lipofectin (charge ratio range of 1.2–9.6), LipofectAce (charge ratio range of 0.7–5.6), Transfectam (charge ratio range of 7.7–62), and LipofectAmine (charge ratio range of 6.6–52.5). As shown in Fig. 5, GS 2888 effectively delivered the simian virus 40 promoter and enhancer-driven luciferase reporter construct over a wide range of lipid concentrations in Cos-7 cells. Under optimal transfection conditions for GS 2888 (10 μg/ml; charge ratio of 5), GS 2888 resulted in 6.6- and 3.6-fold more luciferase enzyme activity than Lipofectin (charge ratio of 4.8) or Transfectam (charge ratio of 31), respectively. When comparing maximum luciferase enzyme activity for both GS 2888 and the next best cytofectin, Lipofectin [GS 2888 at 10 μg/ml (charge ratio of 5) and Lipofectin at 20 μg/ml (charge ratio of 9.6)], GS 2888 resulted in 2.4-fold more luciferase enzyme activity at one-half the lipid concentration. GS 2888 cytofectin demonstrates a marked improvement over several of the currently available lipid formulations in delivering plasmids in the presence of serum.

DISCUSSION

We conclude from these studies that GS 2888 cytofectin is a greatly improved agent for delivering antisense ODNs to cells. This cytofectin offers several advantages over commercially available agents because it efficiently delivers high concentrations of ODN with excellent efficiency to a large number of cell lines in the presence of serum. This agent expands the utility of the antisense approach for cell culture studies. Currently, we have been able to inhibit a wide variety of gene targets using

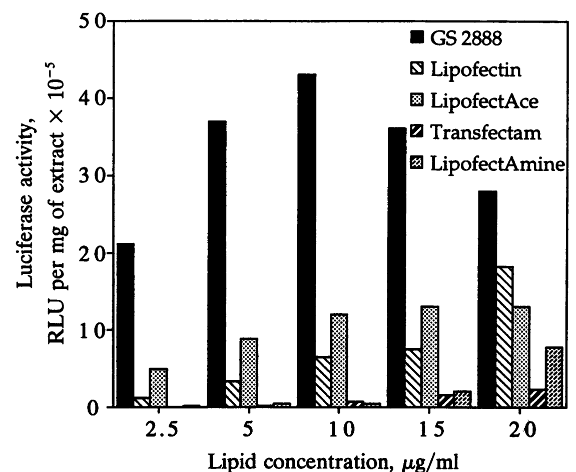

FIG. 5. GS 2888 cytofectin effectively delivers plasmid DNA to Cos-7 cells. Comparison of GS 2888 cytofectin with four commercially available cytofectins in delivering plasmid DNA in the presence of 10% serum. One microgram of pGL2-control vector (Promega) was complexed with various cytofectins at 2.5–20 μg/ml under conditions identical to ODN delivery. The lipid–DNA complex (in 1 ml) was added directly to 90% confluent Cos-7 cells (American Type Culture Collection) in triplicate. Extracts were made 24 hr later and assayed for luciferase enzyme activity, which is expressed as relative light units (RLU) per mg of extract. Values for the triplicate wells varied by $<5\%$.

the GS 2888 cytofectin together with the C-5 propyne S-ODNs. Examples were described above in which potent, sequence- and gene-specific antisense inhibition of luciferase and cyclin B was achieved.

The GS 2888 cytofectin agent was derived from a screen of cationic lipids that analyzed the ability of the agents to transfect ODNs into cells. The GS 2888 lipid is structurally related to the Tranfectam reagent, 5-carboxyspermylglycinedioctadecylamide, in which the 5-carboxyspermyl group was replaced with Boc-arginine and the dioctadecylamide group was replaced with dimyristylamide. Structure–activity relationship studies evaluated dialkyl chain length using the Boc-arginine head group, and we found the following relative order of effectiveness: $C_{14} > C_{18} >> C_{12}$ (J.G.L., K.-Y.L., and R.W.W., unpublished results). A similar relationship was previously found for derivatives of DOTMA (9). Decreased bilayer stiffness and phase-transition temperatures may result for the C_{14} chain length compared to C_{18} and may lead to more favorable fusion with endosomal membranes (see ref. 9 for discussion).

The mechanism by which cytofectin agents can deliver DNA to cells is still poorly understood. Transfection appears to be efficient when there is a net positive charge on the cytofectin–DNA complex (15), and this is true for the GS 2888 cytofectin. DOPE appears to be a very important component of cationic liposomes and provides a membrane fusion functionality (16–20). For the GS 2888 cytofectin, optimal activity occurred at a GS 2888 to DOPE molar ratio of 2:1. We predict that a heterodimer may form between the negative charge on DOPE and the primary amine on GS 2888. Thus, the primary amino group on DOPE and the additional GS 2888 molecule in the 2:1 complex are free to bind the nucleic acid and give the overall cytofectin–DNA complex a net positive charge (depending on the pH of the environment).

The GS 2888 cytofectin should prove to be a very effective tool for performing gene inhibition studies in cell culture and possibly in certain local applications *in vivo*. With this agent and the C-5 propyne phosphorothioate ODNs, active antisense ODN inhibitors can easily be identified by targeting any region of the target RNA (6). As shown in the luciferase and cyclin B example above, substantially reduced protein levels can be achieved by using low nanomolar concentrations of ODN. The ability to use the GS 2888 cytofectin in the presence of serum appears to mitigate much of the toxicity encountered using cationic lipids and may promote its *in vivo* activity.

Antisense inhibition using ODNs has, at times, proved problematic and frustrating to a large number of researchers. With the development of improved antisense agents and cell delivery techniques, many of the barriers toward developing research tools and therapeutic agents have been overcome. These advances should help to reestablish the antisense ODN technique as a powerful new technology for achieving specific gene inhibition.

We thank M. Hogsett and L. Magnani for preparation of the manuscript and figures; L. Su, D. Grant, and T. Huang for technical assistance; K. Krishnamurthy, S. Swaminathan, K. Facchine, G. Lewis, G. Martin, and R. Crouch for lipid structure determination; H. Bujard for supplying the HeLa X1/5 cell line; and T. Terhorst for synthesis of the ODNs. This work was supported in part by a grant from the Advanced Research Projects Agency.

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