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Efficient cationic lipid-mediated delivery of antisense oligonucleotides into eukaryotic cells: down-regulation of the corticotropin-releasing factor receptor

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ABSTRACT

Oligonucleotides (ODNs) can be employed as effective gene-specific regulators. However, before ODNs can reach their targets, several physical barriers have to be overcome, as although ODNs may pass cell membranes, most become sequestered in endocytic compartments. Accordingly, sophisticated strategies are required for efficient delivery. Here we have employed a pyridinium-based synthetic amphiphile, called SAINT-2, which carries ODNs into cells in a highly efficient, essentially non-toxic and serum-insensitive manner. Intracellular delivery was examined by monitoring the trafficking of fluorescent ODNs and lipid, and by measuring the effect of specific antisense ODNs on target mRNA and protein levels of the receptor for the neuropeptide corticotropin-releasing factor (CRF-R), expressed in Chinese hamster ovary cells. ODN delivery is independent of lipoplex size, and fluorescently tagged ODNs readily acquire access to the nucleus, whereas the carrier itself remains sequestered in the endosomal–lysosomal pathway. While the release is independent of the presence of serum, it is not observed when serum proteins gain access within the lipoplex, and which likely stabilizes the lipoplex membrane. We propose that the amphiphile-dependent aggregate structure governs complex dissociation, and hence, the biological efficiency of ODNs. We demonstrate an essentially non-toxic and effective antisense-specific down-regulation of the CRF-R, both at the mRNA and protein level.

INTRODUCTION

Cell biological analyses or therapeutic treatment may often benefit from an ability to down-regulate or modify the expression of proteins. The application of antisense technology, as has been described for a variety of receptors (1,2), provides such a possibility. This technology relies on the use of antisense oligonucleotides (ODNs), which are short DNA or modified DNA fragments (7–30 nt in length) that contain a complementary base sequence to their target RNAs. Upon selective hybridization, a specific interference with gene transcription, RNA transport, splicing or translation of the desired target protein can, thus, be accomplished, thereby allowing a selective regulation of gene expression. For example, the corticotropin-releasing factor (CRF) is a neuropeptide present in the central nervous system that is intimately involved in the expression of stress in mammals (3). Its effects, including behavioral, immunological and endocrine responses, are propagated via the CRF-receptor (CRF-R), which belongs to the G-protein coupled receptor family. Thus far, the biochemical and physiological characteristics of this receptor have been poorly characterized. Therefore, from both a biochemical (2) and a therapeutic point of view, down-regulation of receptor expression and, thereby, its function would be of interest.

The specificity of the antisense technology has been under debate, as often high ODN concentrations and long term incubations are required (2,4), conditions that may readily lead to cell cytotoxic effects of the ODN *per se*. Also, non-specific hybridization with intracellular proteins rather than mRNAs has been reported (5), thus challenging the occurrence of a genuine antisense effect. Other limiting factors include ODN stability, and selective cellular uptake and processing. To improve the latter features, antisense phosphorothioate ODNs are preferred over phosphodiester derivatives as the latter are readily degraded by nucleases (6). In contrast, thioate ODNs display a capacity to induce degradation of the target sequence by RNase H (7,8). Delivery of ODNs *in vitro* has been accomplished by simple exogenous addition, microinjection and electroporation (9,10). Also, liposomes have been employed for this purpose (11). However, such vehicles may display their own inherent drawback of a relatively poor encapsulation and delivery. Some cationic lipids may in part overcome these disadvantages, although such systems often suffer from high cytotoxic side effects and a high sensitivity towards serum (12), whereas selective uptake by only a restricted number of cells within a population may also occur. Moreover, the efficiency of ODN delivery may strongly depend on the chemical nature of the cationic surfactant, but the underlying cause for such differences remains entirely obscure (1).

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In the present work, we have employed a synthetic amphiphile, 1-methyl-4(dioleoyl)methyl-pyrimidinium chloride (SAINT-2), and examined the mechanism of uptake and cellular processing of CRF-R-specific, unmodified phosphodiester (D-)ODNs and phosphorothioate (S-)ODNs. We demonstrate that the intracellular delivery of antisense ODNs improved by more than two orders of magnitude when complexed with SAINT-2–dioleoylphosphatidyl ethanolamine (DOPE) vesicles, in a serum-insensitive and virtually non-cytotoxic manner. As a result, an effective and specific down-regulation of the CRF-R, cloned in Chinese hamster ovary (CHO) cells, was accomplished, using only nanomolar amounts of S-ODNs.

MATERIALS AND METHODS

Synthesis and formulation of amphiphile

SAINT-2 was synthesized as described in detail elsewhere (13,14). For vesicle preparation, equimolar amounts of SAINT-2 and DOPE (Avanti Polar Lipids Inc., USA) were mixed and the solvent was removed by evaporation under a stream of nitrogen, followed by placing the vial under vacuum overnight. The lipids were then resuspended in water (1 ml) and sonicated to clarity in a bath sonicator in a closed vial. Where indicated, 1 mol % *N*-(lissamine Rhodamine sulfonyl)-PE (*N*-Rh-PE; Avanti Polar Lipids, Inc.) was included in the lipid mixture to monitor the fate of the lipid complex by confocal fluorescence microscopy.

Cell culture

CHO cells, stably expressing the CRF receptor under the control of CMV promoter were kindly provided by Solvey Pharmaceuticals (Weesp, The Netherlands). The cells were grown in CHO-S-SFM medium (Gibco, Breda, The Netherlands) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine and penicillin (50 U/ml)/streptomycin (50 µg/ml) under the selection of 0.5 mg/ml geneticin in 5% CO_2/95% air at 37°C.

ODNs

Two antisense S-ODNs, complementary to CRF-R mRNA (sequence see Gene Bank, accession L25438), targeted to bp 473–490 (antisense 1) and bp 788–801 (antisense 2) with the sequence: 5′-GGA TGA AAG CCG AGA TG-3′ and 5′-GTA TAC CCC AGG AC-3′, respectively, as well as the GC-mismatched randomized-sequence ODNs with the sequence: 5′-ACT ACG ACC TAC GTG AC-3′ and 5′-GAA CCA AGA GCA CC-3′, and a fluorescein-labeled randomized-sequence ODN with the sequence: 5′-ACT ACG ACC TAC GTG AC-3′ were designed and manufactured by Biognostik (Göttingen, Germany). All ODNs were thioated and purified by high-performance liquid chromatography, cross-flow dialysis and ultrafiltration. A 25mer Cy5-labeled phosphodiester ODN was custom-synthesized by Pharmacia Biotech (NJ, USA).

Preparation of SAINT-2–DOPE–antisense complexes

The complexes were prepared as follows. Twenty nanomoles of vesicles, prepared as described above, were diluted in 100 µl serum-free CHO medium. Then, 0.1 nmol ODN in TE buffer (pH 7.4) was added, mixed gently, and the complex was allowed to assemble by incubating the mixture at room temperature. After 20 min, 900 µl pre-warmed (37°C) 10% serum-containing or serum-free CHO medium was added. Alternatively, complexes were diluted in 100 µl 0.9% NaCl/10 mM HEPES buffer (instead of serum-free CHO medium). Then, 0.1 nmol ODN in TE (pH 7.4) was added, mixed gently, and the complex was allowed to assemble by incubating the mixture at room temperature. After 20 min, 900 µl pre-warmed (37°C) serum-free CHO medium was added. Finally, complexes were prepared by diluting vesicles in 100 µl 10% serum-containing medium. After 20 min, 900 µl pre-warmed (37°C) 10% serum-containing medium was added. The experiments were carried out in 6-well plates by adding the complexes, as prepared, to the washed cells and, unless indicated otherwise, the experiments were carried with ODN lipoplexes that had been assembled in serum-free CHO medium. The sizes of the lipoplexes were determined by particle size analysis, using a Nicomp 370 submicron particle sizer (CA, USA). The efficiency of ODN association with the complex was determined by using the Oligreen® ssDNA Quantitation kit (Molecular Probes, OR, USA).

Cellular binding and uptake studies

500 000 cells/well were seeded in 6-well plates. After 24 h, when the cells had reached 70–80% confluency, the complexes were prepared as described above, added to cells and incubated at 37°C during a time interval as indicated. Cells were then rinsed with PBS, trypsinized and resuspended in medium prior to quantifying fluorescence by fluorescence-activated cell sorting (FACS) measurements.

For characterization of intracellular uptake and distribution, FITC-labeled ODNs and *N*-Rh-PE-containing SAINT-2–DOPE complexes were visualized by TCS Leica confocal laser scanning microscopy (Heidelberg, Germany). To this end, CHO cells were grown on coverslips in 6-well plates and treated with SAINT-2–DOPE–ODNs complex as described above.

To verify the endosomal–lysosomal localization of the SAINT-2–DOPE complex, the cells were incubated with 2 mg/ml FITC-dextran (mol. wt 71 600 Da; Sigma) for 12 h. After removal by washing, the cells were incubated for another 4 h with the lipoplexes. Finally, the cells were rinsed three times with PBS, and analyzed directly or fixed for 10 min in 2.5% paraformaldehyde in PBS, washed and mounted on microscope slides for examination.

Cytotoxicity studies

The cytotoxicity of SAINT-2–DOPE, S-ODNs and their complex was determined in CHO cells, using the MTT assay. In brief, following the incubation with lipid, antisense ODN or the complex, the cells were incubated for 24 h. The surviving fraction was determined by the MTT dye assay, measuring the absorbance at 520 nm with an automated microplate reader, as described (15).

Antisense assay

The ability of SAINT-2–DOPE to deliver antisense ODNs in pharmacologically active form was evaluated by examining the level of CRF-R mRNA in transfected CHO cells, treated with SAINT-2–DOPE–antisense ODN complex. CHO cells, stably expressing CRF-R under the control of CMV promoter, were plated in 10 cm dishes and grown for 24 h. Cells were

then treated with the complex of S-ODNs and SAINT-2–DOPE, prepared as described above. After a 24 h incubation at 37°C, total RNA was extracted from 6×10^6 cells using the Qiagen RNeasy kit (Hilden, Germany). The purity and quantity of the RNA preparation was determined by recording the absorbance at 260 and 280 nm. Equal amounts of RNA were resolved on a 1.2% agarose gel, containing 6.7% formaldehyde, and transferred to a nylon membrane by vacuum transfer. Total RNAs were fixed onto the membrane by heating for 1 h at 80°C. The RNA was intact without any degradation as indicated by 18S and 28S bands on the membrane, as visualized by methanol blue staining. CRF-R and GAPDH probes were prepared by amplifying CRF-R and GAPDH cDNA by RT–PCR from total RNA in CHO cells. The CRF-R and GAPDH primers were ATTATGGGACGGCGCCCG/TCACACTGCTGTGGACTG and CCACCCATGCAAATTCCATGGCA/TCTAGACGGCAGGTCAGGTCCACC, respectively. PCR was performed using oligo(dT)-primed cDNAs (synthesized with reverse transcriptase; Boehringer Mannheim, Germany) as a template under the following conditions: 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 45 s, and a final 72°C extension of 5 min). The probes from the PCR products were gel purified (QiaexII Gel Extraction kit, Germany) and labeled with [^{32}P]ATP using a random priming kit (Gibco BRL, Breda, The Netherlands). The level of CRF-R mRNA was probed by the cDNA probe for the CRF-R gene, visualized by autoradiography and quantified by densitometry, by scanning an area that contained the band of interest, and which was of the same size for each band examined. To normalize the level of RNA loading, the CRF-R probe was removed by stripping the membrane in boiling SDS solution (0.5%) for 10 min. The membrane was then probed with the control GAPDH probe.

Levels of CRF receptors were evaluated by western immunoblot. An aliquot of 10^6 CRF-R-expressing and control CHO cells were seeded in 10 cm dishes and grown for 24 h. The cells were then treated with the S-ODN–SAINT-2–DOPE complex, and after a 24 h incubation at 37°C, the complex was removed and fresh medium was added. The cells were harvested after 48 h and lyzed, and cell membranes were isolated as described (16). A 100 μg sample of proteins was run on 12.5% SDS–PAGE (Bio-Rad, Hercules, CA), electrotransferred to Immobilon™-P transfer membranes (Millipore Corp., MA), and probed with goat anti-rat CRF-R (1:100, Santa Cruz), followed by alkaline phosphatase-conjugated rabbit anti-goat antibody (1:3000, Sigma). The blot was color processed by nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl (Sigma), and analyzed by the software of image tool IT2.00.

RESULTS

SAINT-2–DOPE (1:1) efficiently mediates antisense S-ODN uptake in an almost non-toxic manner

In previous work, we have demonstrated that SAINT-2–DOPE (molar ratio 1:1), when complexed with plasmid DNA, effectively transfects eukaryotic cells (13). Indeed, transfection efficiencies up to 90% are obtained at a charge ratio (+/–) of approximately 2.5 (10–20 μM lipid/1 μg DNA). The next goal was, therefore, to examine whether SAINT-2–DOPE could similarly tranfer antisense ODNs into cells to regulate gene expression for therapeutic or cell biological purposes. Using fluorescently tagged ODNs, we established that >95% of the added ODNs, at all conditions described in the present study, associated with the SAINT-2–DOPE vesicles prepared as described in the Materials and Methods, thus giving rise to efficient lipoplex assembly (not shown). In order to determine the optimal ratio between SAINT-2–DOPE and S-ODNs in terms of ODN delivery efficiency, CHO cells were treated either with 20 μM SAINT-2–DOPE, complexed with various concentrations of FITC-labeled S-ODNs, or with a complex consisting of 100 nM S-ODNs and various concentrations of SAINT-2–DOPE. Treated cells were harvested after a 4 h incubation period, and the S-ODN delivery in terms of the efficiency and number of cells that contained internalized complex was then measured by FACS. As shown in Figure 1A at a fixed lipid concentration of 20 μM, both the absolute delivery and number of cells that had taken up the complex increased with increasing S-ODN concentration. Thus, at an ODN concentration of 80–100 nM, essentially all cells displayed the presence of the complex. Conversely, when adding complexes to the cells at identical conditions, assembled from various amounts of SAINT-2–DOPE and a fixed concentration of S-ODN of 100 nM, no further increase in absolute delivery was obtained relative to that obtained for 100 nM ODN/20 μM SAINT-2–DOPE (Fig. 1B). Therefore, the latter composition was used in this study to further examine the mechanism and to define the efficiency of antisense delivery. To appreciate the efficiency of the delivery capacity of the present system, we first investigated the cellular uptake of non-complexed S-ODN and the effect of serum. To this end, the cells were incubated with S-ODNs alone or the S-ODN–SAINT-2–DOPE complex for 24 h at various conditions, and cellular uptake was determined by measuring total cellular-associated fluorescence, using fluorescently tagged S-ODN. As shown in Figure 1C, SAINT-2–DOPE enhanced S-ODN cellular uptake by 100–250-fold compared with the uptake of S-ODN alone. Interestingly, the absolute amount of uptake, as reflected by the total cell-associated fluorescence, increased by ~1.5-fold in the presence of 10% FCS. Note, however, that in either case, essentially all cells displayed the presence of cell-associated fluorescence.

The application of both synthetic amphiphiles and treatment with antisense ODNs has often suffered from serious cytotoxic side effects. Although our previous work (13,17,18) clearly implied the relative non-toxic nature of the SAINT amphiphile compared with other cationic lipids, we next verified its potential toxicity in conjunction with the application of ODNs. CHO cells were incubated with 20 μM SAINT-2–DOPE alone, 100 nM antisense S-ODNs alone, or the ODN–amphiphile complex in the presence or absence of 10% serum. After 24 h, the cells were harvested and the cytotoxicity was determined by the MTT assay. As shown in Figure 2, the cells treated with antisense S-ODNs did not show any cytotoxicity. Cells treated with the SAINT-2–DOPE alone, and the complex of SAINT-2–DOPE and S-ODNs showed only modest cytotoxicity, which was <10% in the presence of serum. We conclude, therefore, that at an appropriate molar ratio SAINT-2–DOPE (20 μM) effectively delivers ODNs (100 nM) to cells, leading to complex uptake by essentially all cells in the culture system in an almost non-toxic manner, which is promoted rather than inhibited by the presence of (10%) serum. To further define the parameters of uptake, the mechanism of internalization and

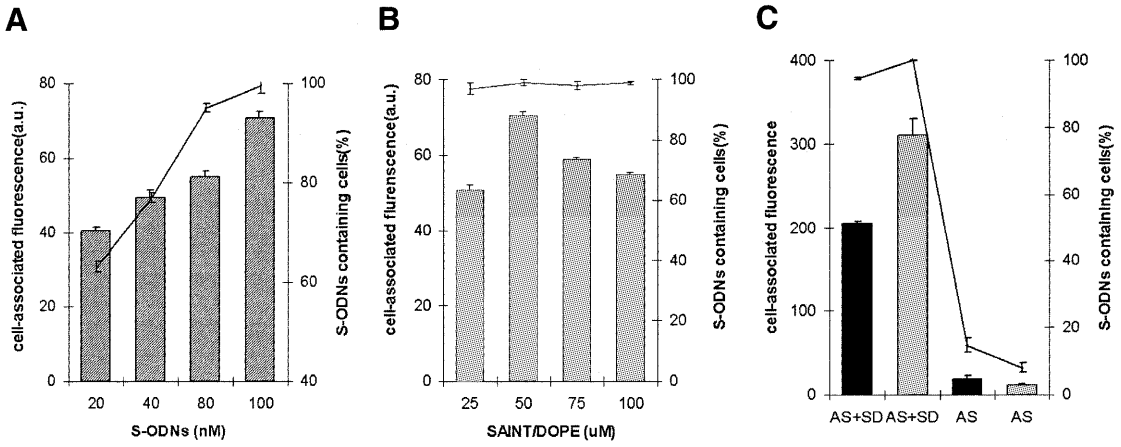

Figure 1. Optimization of parameters for SAINT-2–DOPE-mediated delivery of antisense S-ODNs into cells. Lipoplexes, containing 5′ fluorescein-labeled S-ODN, or non-complexed ODN were incubated with the cells as described in Materials and Methods, and the amount of cell-associated antisense was determined by FACS measurements. (**A**) The cell-associated fluorescence intensity (bars, left *y*-axis) and the percentage of fluorescently labeled cells (line, right *y*-axis), as a function of the concentration of antisense ODN complexed with 20 µM SAINT-2–DOPE (1:1). (**B**) The association of the ODNs (100 nM) as a function of SAINT-2–DOPE concentration. The number of cells that showed cell-associated lipoplexes is indicated by the line (right *y*-axis). (**C**) CHO cells were treated with S-ODNs alone (AS), or when complexed with SAINT-2–DOPE (AS + SD), in the presence (shaded bars) or absence of serum (filled bars). The line indicates the number of cells that were labeled in each population at the various conditions indicated. Note that when complexed with SAINT-2–DOPE (100 nM/20 µM of lipid) S-ODN delivery is enhanced by 100–250-fold, while complex uptake in the presence of serum is enhanced. Data are the mean values (±SD) of three determinations.

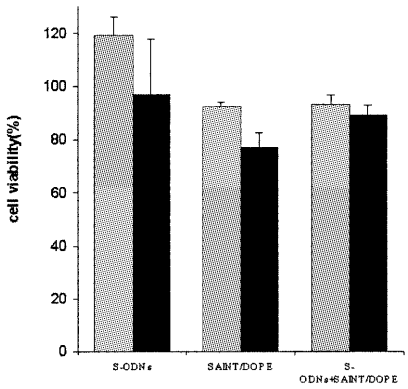

Figure 2. Antisense treatment and cell toxicity. CHO cells were incubated for 24 h in either the presence (shaded bars) or absence of 10% serum (filled bars). The toxicity was determined using the MTT assay, and the data are expressed as the percentage of surviving cells, relative to untreated CHO cells (100%). Mean values ± SD were obtained from two to three experiments, carried out in duplicate.

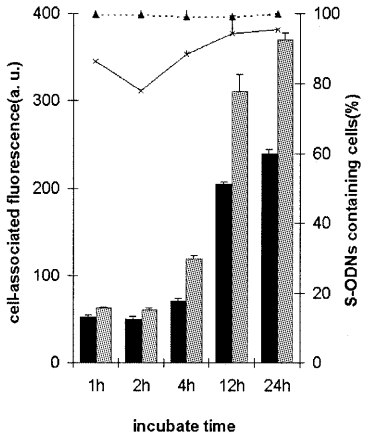

Figure 3. Kinetics of S-ODN uptake and the effect of serum. The cells were incubated with FITC-labeled S-ODN–SAINT-2–DOPE complexes for the indicated time intervals, either in the presence or absence of serum. After extensive washing, the cells were harvested and the cell-associated fluorescence (filled bars, without serum; shaded bars, in the presence of serum) and the number of fluorescently labeled cells in the population (triangles and crosses, presence and absence of serum, respectively) were measured by FACS. Results are the mean values ±SD of three different experiments, carried out in duplicate.

ensuing biological effects, the next experiments were undertaken.

SAINT-2-mediated uptake of S-ODN is time-dependent and promoted by serum

To determine the kinetics of cellular uptake of the amphiphile–ODN complex, the cells were incubated with FITC-labeled antisense S-ODN complex, prepared in CHO medium (see Materials and Methods), in the presence or absence of serum. After various time intervals, the cells were harvested and the cell-associated fluorescence was determined by FACS measurements. As shown in Figure 3, already after 1 h of incubation, a significant association of fluorescently tagged S-ODNs with the cells was apparent, the amount increasing over a time interval of 12 h. Following this time interval, the subsequent increase over the next 12 h was only minor, amounting to an additional ~10–15% of the total uptake. Interestingly, as already noted above, the efficiency of uptake could be enhanced when the cells were incubated with the complex in the presence of 10% serum, the difference between both conditions becoming most pronounced after 4 h of incubation. It is further relevant to note that over the entire incubation period,

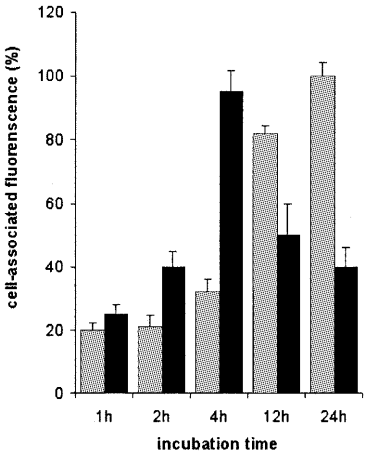

Figure 4. Comparison of the kinetics of processing of D-ODNs versus S-ODNs in CHO cells. CHO cells were incubated with either FITC-labeled S-ODNs or Cy5-labeled D-ODNs, which were complexed with SAINT-2–DOPE. The cells were incubated with these complexes at 37°C for various times, and the cell-associated fluorescence was measured by FACS. The amount of cell-associated S-ODNs continuously increased over the 24 h incubation (shaded bars). However, the cell-associated D-ODNs only transiently increased, the cell-associated fraction decreasing again after 4 h of incubation. Results are the mean values ±SD of three determinations.

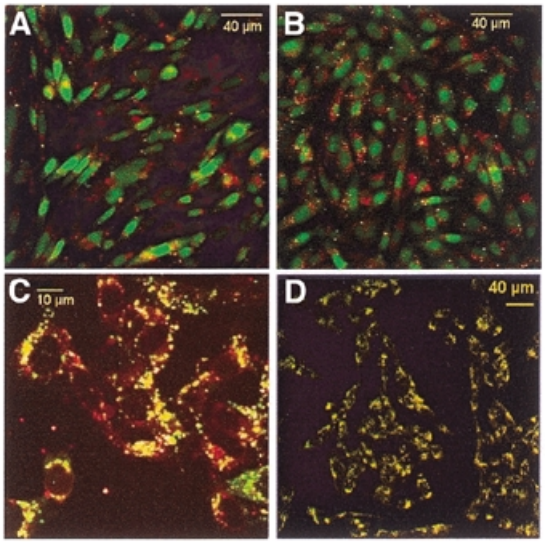

Figure 5. Confocal microscope images of intracellular uptake and distribution patterns of S-ODNs, mediated by SAINT-2–DOPE. CHO cells were incubated with complexes, prepared from FITC-labeled S-ODN, and SAINT-2–DOPE, labeled with 1 mol% *N*-Rh-DOPE, at 37°C for 24 h in the presence or absence of 10% serum. Images show fields of live cells in which the S-ODNs are visualized in green, while the SAINT-2–DOPE vehicles are visualized in red. (**A**) Distribution of ODNs and carrier after incubation in the absence of serum. Note the presence of S-ODNs in the cell nuclei (green), whereas SAINT-2–DOPE was present as large, heterogenous dots (red) in the cytoplasm and at the cell surface. (**B**) Cells were incubated with complexes, prepared as in (A), in the presence of serum. The S-ODNs were localized in the cell nuclei (green), while in this case, the SAINT-2–DOPE carrier was seen as relatively small and homogenous dots (red) in the cytoplasm, giving rise to a fine-punctate distribution. Note that in both (A) and (B), only occasionally yellow dots are seen, reflecting the colocalization of the fluorescent ODN and lipid. The perinuclear localization of the red-labeled carrier lipid (in A and B) most likely originated from the presence of the carrier in the endosomal–lysosomal pathway. When cells were incubated with the lysosomal marker FITC-dextran (green) for 12 h, followed by a 4 h incubation with complexes of S-ODNs (non-labeled) and *N*-Rh-PE–SAINT-2–DOPE (red; in the absence of serum), a prominent colocalization of lysosomal marker and SAINT-2–DOPE was apparent, as reflected by the yellow fluorescence (**C**). (**D**) When the complexes had been prepared in the presence of 10% serum, only non-dissociated complexes were seen within the cells, as reflected by the almost exclusive appearance of yellow fluorescence.

all cells display a capacity in taking up the complex, even after 1 h, all cells of the population show cell-associated fluorescence and, moreover, that this uptake is not affected by the presence of serum.

Thus far, we have carried out our studies using S-ODN in anticipation of potential nuclease-mediated degradation of the ODN. To rationalize its use, it was of interest to examine whether D-ODN delivery was processed differently by the cells, using the current carrier system. As shown in Figure 4, this indeed appeared to be the case. Whereas the cell-associated fraction of thiolated ODN continuously increased as a function of the incubation time, the cell-associated D-ODN fraction, although increasing with fairly similar kinetics over the first 4 h of incubation as those observed for S-ODN, rapidly decreased over the next 8 h of incubation. These data thus imply that a major fraction of the ODNs becomes exposed to a mechanism that must involve degradation prior to cellular expulsion within 4 and 12 h after the onset of the incubation. To further examine the intracellular processing, we subsequently determined the (intra-)cellular localization of cell-associated complexes by confocal laser scanning microscopy, employing fluorescently labeled S-ODN, while the carrier itself was labeled by the non-exchangeable lipid probe *N*-Rh-PE (19).

The presence of serum does not affect effective nuclear localization of S-ODNs

CHO cells were incubated with the fluorescently labeled (S-ODN and lipid) complex, prepared in CHO-medium, for 3 h at 37°C in the presence or absence of serum. After this time interval, serum-containing medium was added, and the cells were further incubated at 37°C. After 21 h, the cells were extensively washed, and either examined directly or after fixation with 2.5% paraformaldehyde by confocal laser scanning microscopy (Fig. 5). The data reveal that irrespective of the presence of serum in the incubation medium (initial 3 h), essentially all cells contain fluorescence, which is largely localized in the nuclei. In the presence of serum very little if any fluorescence is accumulating at the cell periphery (which would have been apparent as patchy fluorescence), implying that most of the cell-associated fluorescence has been internalized by the cells. The internalized red-labeled *N*-Rh-PE, marking the lipid carrier, can be discerned as a fine-punctate fluorescence, which primarily localizes at perinuclear regions. Some colocalization (as reflected by the yellow colour) of lipid and ODN is occasionally observed. Note that laterally diffused membrane staining of *N*-Rh-PE is not detectable. The perinuclear localization reflects the presence of the carrier in the endosomal–lysosomal pathway, as demonstrated by a prominent colocalization (yellow) of the lysosomal marker FITC-dextran (green), internalized over a 12 h period, and *N*-Rh-labeled (red) complexes, internalized for a subsequent time interval of 4 h (Fig. 5C). Relative to its distribution in the presence

of serum, a more irregular appearance of *N*-Rh-PE fluorescence is observed in the absence of serum (Fig. 5A). Thus, in contrast to a fine-punctate appearance in its presence, in the absence of serum, large clusters are apparent, which are often localized to the cell periphery, presumably reflecting the incapacity of the cells to internalize such complexes. Consistent with this notion, the absolute uptake of complexes in the absence of serum is less than in its presence (Fig. 3). Clearly, this decrease does not affect the ultimate fate of the internalized S-ODNs as its nuclear localization is, at least in a qualitative sense, as prominent as observed in the presence of serum (Fig. 5A versus B). Finally, when non-complexed fluorescently labeled S-ODN was incubated at similar conditions, an occasional fluorescent dot in the cytoplasm in <5% of the cells was observed. At none of these conditions was any fluorescence localized in the nucleus (not shown).

Only lipoplex-penetrating serum proteins affect ODN localization

The data presented above indicated that the presence of serum in the extracellular medium may have a profound influence on the size of the complex, and accordingly, on the efficiency of complex internalization. In addition, it is possible that serum proteins might penetrate to different degrees into the complex, thereby affecting complex stability and, hence, the release of the ODN. Both complex internalization and release of ODN from the complex are likely important parameters in governing the eventual antisense effect. During these studies, we noted that different sizes of lipoplexes could be obtained, depending on the methodology of preparation, as indicated in the Materials and Methods (see section entitled Preparation of SAINT-2–DOPE–antisense complexes). Thus, when prepared in serum-free CHO medium, complexes with a diameter of up to 1000 nm were obtained, as determined by particle size analysis. When such complexes were mixed in serum-containing medium within 20 min after preparation, the size of the complexes was ~750 nm. Interestingly, when ODN–lipid complex assembly was carried out in buffer (0.9 % NaCl/10 mM HEPES), particles were obtained with a diameter of only 80 nm. In contrast, when for complex assembly the buffer was replaced by serum-containing CHO medium, thereby simulating the potential penetration of serum proteins into the lipid core, the size of the complexes was ~200–300 nm. Importantly, at all conditions, lipoplexes effectively assembled, involving >95% of the added ODN (not shown). When added to cells and examining the cellular localization of the complexes, i.e. as prepared in either CHO-medium, NaCl–HEPES buffer, or in serum-containing CHO medium, it was observed that in all but one case, nuclear fluorescence was apparent, and essentially indistinguishable in appearance from the data shown in Figure 5A and B. The exception concerned complexes that had been assembled in serum-containing CHO medium. In this case, the level of cell-associated fluorescence was similar to that seen for complexes prepared in the absence of serum, but intriguingly, no fluorescence was apparent in the nucleus. Rather, only punctate fluorescence, distributed throughout the cytoplasm was present, indicating that neither vesicle clustering nor growth occurred during the incubation, consistent with incubations of complexes assembled in serum-free media, but incubated with the cells in the presence of serum. The typical cytoplasmic distribution and perinuclear localization, in conjunction with the colocalization of nucleotide and lipid as reflected by the yellow colour, likely represents entrapment of the complex in the endosomal–lysosomal pathway (Fig. 5C versus D), implying that dissociation of ODN from the complex did not occur. Accordingly, as an incubation with the 80 nm particles, as obtained by preparing the complex in NaCl–HEPES, result in efficient delivery, the data indicate that dissociation rather than particle size represents a rate limiting step in ODN delivery.

Following dissociation and arrival at the nucleus, we finally examined whether the ODN thus delivered proved to be effective in conveying a biological response.

SAINT-2-mediated delivery of antisense S-ODNs efficiently down-regulates CRF-R mRNA and protein levels

To determine the capacity of SAINT-2–DOPE to deliver ODNs in pharmacologically active quantities, the ability of antisense ODNs targeted to rat CRF-R to down-regulate the levels of mRNA was examined by nothern blot analysis. CHO cells were incubated for 3 h with the complex, consisting of 100 nM antisense S-ODNs and 20 μM SAINT-2–DOPE in the absence of serum, followed by the addition of 10% serum-containing medium. After an incubation of 24 h, the cells were harvested and total RNA was extracted and transferred onto nylon membranes. The blots were first probed by ^{32}P-labeled CRF-R probes, and then stripped and reprobed by ^{32}P-labeled GAPDH probes. GAPDH was used as an internal control for the RNA loading. The relative amounts of mRNA were determined by densitometric measurement of the autoradiographs by scanning a defined spot-encompassing area, which was the same for all spots, and the amount of CRF-R mRNA was normalized to that of GAPDH mRNA. The use of the antisense S-ODN as applied led to a reduction in CRF-R mRNA by ~50%. Either antisense sequence (1 and 2, see Materials and Methods) displayed approximately the same efficiency. No effect was seen when the cells had been treated with antisense S-ODNs only, or a complex consisting of mismatched ODNs and SAINT-2–DOPE, emphasizing the specificity of the observed effect. The latter control also indicates that the cationic lipid mixture itself does not significantly affect CRF-R expression in a non-specific manner, such as by affecting cell cytotoxicity. This is entirely consistent with the data presented in Figure 2, in which toxicity was monitored directly. The seemingly slight decrease in activity seen in lane 9 (Fig. 6), in which free vesicles rather than complexes were added, may therefore have been caused by fluctuations seen in GAPDH expression, rather than by a toxic effect of the amphiphile. At any rate, the data in conjunction with those shown in Figure 2 support the notion that the ODN–lipid complexes display little if any toxicity towards the cells. Thus, SAINT-2–DOPE was able to effectively deliver antisense ODNs into the CRF-R expressing CHO cells and consequently elicit a potent and selective inhibition of gene expression.

To verify the consequences of mRNA down-regulation in terms of down-regulation in receptor expression *per se*, we subsequently determined the level of CRF-R expression by western immunoblot, following antisense ODN treatment. The cells were incubated with complexes as described above, i.e. 100 nM ODN and 20 μM SAINT-2–DOPE in the absence of serum for 3 h, after which serum-containing medium was

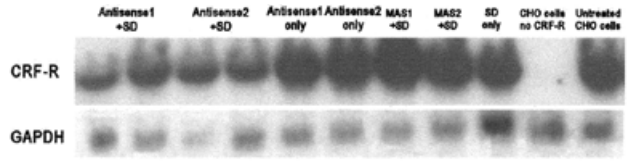

Figure 6. Specific down-regulation of CRF-R messenger RNA by antisense S-ODNs. CRF-R expressing CHO cells were treated with lipoplexes prepared from 100 nM S-ODNs and 20 μM SAINT-2–DOPE for 3 h (in the absence of serum), and were subsequently incubated for 24 h in 10% serum-containing medium. Total RNA was then isolated, fractionated on agarose formaldehyde gels, and blotted on nylon membrane as described in the Materials and Methods. This membrane was probed with ^{32}P-radiolabeled CRF cDNA (CRF-R) and then stripped and reprobed with the ^{32}P-radiolabeled GAPDH cDNA (GAPDH). GAPDH is used as the internal control for RNA loading. Note that a decrease of CRF-R mRNA was only seen when antisense ODNs had been delivered by SAINT-2–DOPE(SD). Each of the above experiments was repeated in duplicate, and similar results were obtained. Two antisense sequences were employed, antisense 1 and antisense 2 (for sequences see the Materials and Methods). MAS1(2) + SD indicates results obtained with lipoplexes containing mismatched antisense (AS) 1 or 2. As a negative control, CHO cells without CRF-R expression were treated and analyzed similarly (CHO-cells, no CRF-R).

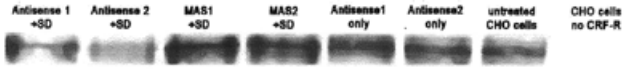

Figure 7. Effect of antisense S-ODNs on CRF-R expression. CRF-R was analyzed by western immunoblot. CHO cells expressing CRF-R under the control of CMV promoter were treated with 100 nM S-ODNs and 20 μM SAINT-2–DOPE for 3 h in the absence of serum, followed by an incubation of 24 h in 10% serum-containing medium. The lipoplexes were removed by washing and fresh medium with serum was added. After another 48 h, the cell membranes were isolated, the proteins were separated on PAGE, probed with goat anti-rat CRF-R and then with alkaline phosphatase-conjugated rabbit anti-goat antibody. The reduction of CRF-R was only observed when antisense ODNs had been delivered by SAINT-2–DOPE (antisense 1 and 2). MAS represents treatment with mismatched antisense 1 and 2. Each of the above experiments was repeated in duplicate, and similar results were obtained.

added, and the cells were left for 24 h. The ODN complexes were then removed by washing the cells, and fresh medium was added. After another 48 h, the cell membranes were isolated, and the presence of CRF-R was determined as described in the Materials and Methods. As shown in Figure 7, a prominent reduction of >50% in CRF-R was only seen when the cells had been treated with either antisense 1 or antisense 2 ODN, complexed with SAINT-2–DOPE. Accordingly, the data demonstrate that SAINT-2–DOPE-mediated delivery of CRF-R antisense effectively down-regulated both mRNA and protein levels.

DISCUSSION

To design a successful antisense-ODN-based approach for either biochemical or therapeutic purposes, it is crucial to develop a convenient carrier system that transfers ODNs efficiently into cells and across membranes, followed by an equally efficient delivery into the nucleus, high enough to promote effective RNA binding. The present work reveals that the cationic lipid SAINT-2, which has been previously demonstrated to act as an efficient, virtually non-toxic carrier for gene delivery (13), also effectively translocates small ODNs into cells, in a non-toxic and serum-insensitive manner. The relative insensitivity of the present system towards serum is highly relevant as many previous studies revealed a more or less perturbing interference with ODN or gene delivery (1,20,21). Recently, some novel umbrella amphiphile systems have been developed that showed only a moderate capacity in overcoming serum-inhibited delivery of antisense ODN (22). Relative to free ODNs, the delivery capacity of the SAINT-2–DOPE carrier system is enhanced by at least two orders of magnitude. In passing, the efficient uptake of ODN–SAINT-2–DOPE complexes is not restricted to their uptake by CHO cells. Cellular internalization of such complexes with a similar efficiency as in CHO cells has also been obtained in COS7 cells, mouse pituitary AtT20 cells and in a mouse L cell line, implying a potential for a wide application of the present ODN carrier system (F.Shi and D.Hoekstra, unpublished observations). Here evidence has been presented that CRF-R antisense ODNs introduced in this manner effectively and specifically down-regulate receptor expression at both the mRNA and protein level. Within 24 h, the mRNA level was down-regulated by ~50%. The level of protein expression diminished even to a higher extent after the same period of antisense treatment, but because of the half time of the receptor (~60 h) quantified after another 48 h, thereby indicating the stability of the introduced ODNs. Moreover, the significance of the efficiency of this down-regulation is emphasized when taking into account that exposure of a pituitary gland primary cell culture to 10 μM free CRF-R antisense ODN for 40–67 h resulted in decrease in CRF binding to the receptor of 17–36% (2). Similarly, others (1,2) reported *in vitro* studies in which antisense effects and down-regulation (of among others the CRF-R) could only be accomplished in the micromolar range, whereas SAINT-2–DOPE elicits such effects in the nanomolar range. It should also be noted that the drastic reduction of CRF-R expression was obtained while being under control of the CMV promoter, which is much stronger than the promoter (23), effective at physiological conditions, i.e. during stress.

Our studies also provide insight into the mechanism of ODN delivery. Thus, the data suggest that particle size is not a crucial factor in ODN delivery, as even particles of a size of only 80 nm, as obtained by complex assembly in NaCl–HEPES, effectively carry ODNs into cells. Rather, stabilization of the carrier system by serum proteins, as accomplished when complex assembly is carried out in the presence of serum-containing medium, and which presumably leads to a partial insertion of serum proteins into the carrier's membrane, leads to endocytic internalization of the complex, without the release of ODNs. In this manner, the presence of protein may preclude the complex from destabilizing the endosomal or plasma membrane, necessary for ODN translocation. Note that these conditions differ from those in which complexes, assembled in serum-free medium, are added to cells, cultured in the presence of serum. Thus, when serum proteins present in the medium are recruited onto the surface of SAINT-2–DOPE–ODN complexes, prepared in serum-free medium, such an inhibitory effect is not observed. In contrast, in the presence of serum, the delivery appears to be enhanced. Therefore, the inhibitory activity of serum proteins seen after complex preparation in the presence of serum must be related to a requirement for the cationic lipid of being able to (locally) perturb the

structural integrity of its target membrane in order to accomplish efficient delivery. Clearly, such a perturbation does not involve a fusion step. At none of the incubation conditions at which ODN translocation had taken place was the presence of a mobile fraction of *N*-Rh-PE in cellular membranes apparent. It can be argued whether the resolution suffices for discerning such a distinction at the level of the endocytic membrane. However, as there is a continuous recycling from such membranes to the cell surface, recycling should have caused the reappearance of *N*-Rh-PE at the plasma membrane, resulting in laterally diffused membrane-associated fluorescence, as previously demonstrated for recycling of fluorescently tagged sphingolipids between endosomes and plasma membrane (24,25). Accordingly, we exclude that SAINT-2–DOPE-mediated ODN translocation includes a fusion-mediated step, but rather may facilitate (transient) pore formation as argued previously (13,26), presumably involving the polymorphic features of SAINT-2 which, in conjunction with the presence of DOPE, may involve the ability of the cationic lipid to convert into non-lamellar, hexagonal phases (27,28 and our unpublished observations).

Our data also indicate that the structure of the ODN is not the rate-determining step in actual delivery. Both D-ODNs and S-ODNs are efficiently translocated during the early phase of complex–cell interaction, i.e. between 0 and 4 h. After 4 h, however, the D-ODN is rapidly expelled from the cells, presumably because of its degradation by nucleases, consistent with observations of others (29). However, S-ODNs are resistant to such degradation for at least 24 h (30,31), i.e. a time that suffices for the ODNs to reach their mRNA targets, consistent with the efficient down-regulation observed in the present work.

Although some colocalization of fluorescently tagged ODNs and lipid was occasionally seen in the endosomal pathway, suggesting that dissociation had not occurred, free ODNs (green fluorescence) were almost exclusively present in the nucleus. In contrast, when antisense ODNs were added to cells directly, little uptake was seen, but once internalized, they were sequestered in compartments that presumably represented endocytic compartments (not shown), consistent with results presented by others (32–34). These data thus imply that for reaching the cytosol, ODN translocation and passage of the endosomal membrane barrier requires the presence of the cationic lipid. For subsequent arrival at the nucleus, no cationic lipid appears to be needed as, based upon the localization of the lipid marker *N*-Rh-PE, the carrier system itself is largely retained in the endosomal–lysosomal track, as revealed by its colocalization with the lysosomally processed marker dextran (Fig. 5). Indeed, it has long been documented that when microinjected into the cytosol, ODNs readily diffuse into the nucleus (35,36), a localization that appears to be closely related to the capacity of antisense ODN to down-regulate target mRNA (37). In this context it is finally interesting to note that polyplex-mediated delivery of ODN may be accomplished by co-entry of the polymer (PEI) into the nucleus (38). The fact that the cationic lipid does not reach/enter the nucleus may imply a different mechanism, but it excludes potential interference of the cationic lipid with antisense effectiveness.

In this regard, an ideal lipoplex-based ODN delivery system requires low cytotoxicity, little or no serum sensitivity, high efficiency of delivery into the nucleus, and a simple, economically advantageous large-scale production of ODN–amphiphile assemblies. SAINT-2–DOPE appears to comply with such requirements. Considering also its ability to efficiently deliver genes into cells for biochemical and therapeutic purposes, the present carrier can, thus, be regarded as a versatile and generally applicable delivery system.

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