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Sequence Dependency of the Internalization and Distribution of Phosphorothioate Oligonucleotides in Vascular Smooth Muscle Cells

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ABSTRACT. Antisense studies imply the utilization of oligonucleotides (ODN) for sequence-specific down-regulation of genes. This usually consists in assessing antisense sequences versus control sequences (mismatched, inverted, scrambled, randomized or any sequence unrelated to the relevant target). Even though the investigated biological effect (knockdown of an unwanted protein) is observed only with the antisense sequence and weakly, if at all, with any of the control sequences, this is a necessary but not a sufficient condition to demonstrate an antisense effect. Indeed, biochemical parameters such as stability, uptake and subcellular compartmentalization of ODN in a given cellular system are most often sequence-dependent processes. In this work, a series of phosphorothioate ODN of different lengths and sequences were evaluated as to their binding, internalization and subcellular distribution properties in vascular smooth muscle cells. In addition to membrane binding and nuclear accumulation, the partition of ODN in the cytosol of cells was measured by a method based upon controled permeabilization of the plasma membrane, permitting the recovery of the cytosolic content with minimal damage to the membranes of the endocytic vesicles and lysosomes. We found that the tested ODN showed striking differences in their uptake and distribution in smooth muscle cells. Our results gave rise to the problem of validating the observed biological effects when different sequences of ODN were compared. Cellular studies such as the one presented in this work could help in choosing the proper control sequences among ODN exhibiting similar cell interactions as compared to the antisense sequences. Moreover, this method could be useful for the selection of antisense sequences that can be efficiently internalized and preferentially distributed in the appropriate compartments in cells for *in vitro* antisense studies. BIOCHEM PHARMACOL **55**;9:1465–1473, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. oligodeoxynucleotides; uptake; permeabilization; digitonin; compartmentalization; smooth muscle cells

ODN‖ have been widely studied for specific gene repression and therapeutics [1–5]. Depending on cell types, poor cellular uptake of ODN and their localization within inappropriate intracellular compartments limit the experimental and therapeutic usefulness of these compounds. Specific ODN receptors or ODN binding proteins have been described [6–9], whereas several authors have shown that other uptake pathways of ODN such as adsorptive endocytosis and/or fluid-phase pinocytosis could also be operative [9, 10]. Based upon endocytosis and fluorescence microscopy studies [6, 11–13], it has been stated that most of the internalized ODN remain entrapped within endosomes and lysosomes (observation of punctuated fluorescence patterns). To explain the observed antisense effect, it was postulated that a small part of the vesicular ODN could escape the endocytic pathway by a yet undefined mechanism and enter the cytosol to exert its action. It was also shown that ODN, when administered by microinjection into the cytoplasm of cells [11, 14] or by cell permeabilization [15–17], preferentially accumulate in the nucleus presumably by free diffusion through the nuclear pores. In contrast, when an ODN was allowed to follow the normal endocytosis process, the nucleus/cytoplasm partition could be highly variable depending on the type of recipient cell [18, 19]. Other parameters such as the sequence or the chemical nature of the antisense molecule could also be determinant with respect to cellular uptake [19, 20] or breakdown by nucleases [21].

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‖ *Abbreviations:* DEX, dextran-rhodamine; DMEM, Dulbecco's modified Eagle's medium; FCS: fetal calf serum; HAM, hexosaminidase; LDH, lactate dehydrogenase; ODN, oligodeoxynucleotide; SMC, smooth muscle cells; PS, phosphorothioate.

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From these data, it appears that key parameters such as stability, internalization and compartmentalization of ODN in a given cellular system should be established prior to any antisense study in which ODN consisting of different sequences (the antisense sequence vs its control sequences) are usually compared. The absence of a biological effect with the control molecules in contrast to the antisense molecule could be validated only when the tested ODN showed similar biochemical behavior such as stability inside and outside the cell, cellular uptake and subcellular distribution towards a specific cell system. These issues were poorly addressed in the past, due probably to a lack of reliable methods for determining subcellular distribution.

In this paper, we present the results on the cellular fate of a series of phosphorothioate oligomers in rat aortic vascular SMC. The choice of the ODN sequences and the cellular system used in this work was dictated by our interest in using antisense strategies to prevent restenosis, a coronary obstruction process due to excessive proliferation of SMC after balloon angioplasty [22–26]. The method we used for the determination of the ODN concentration in the cytosol compartment was based on controlled membrane permeabilization by digitonin. This approach has been used to study cytosolic proteins [27–29] and to facilitate ODN penetration into cells [15–17]. Our results showed that PS ODN exhibited contrasting cellular behavior, and the differences observed in stability, cell binding, uptake and cytosolic partition were essentially related to the sequences of the tested oligomers.

MATERIALS AND METHODS

Oligonucleotides

2′-Deoxy-PS ODN were obtained from Eurogentec (Seraing). Oligomers were purified by butanol extraction followed by reverse phase HPLC. The oligomers were 5′-labeled with ^{32}P-γ-ATP followed by HPLC separation to remove free radioactive ATP. The purity of ODN was assessed by PAGE and found to be higher than 90%. Nuclease degradation of ODN in culture media and in cell extracts was monitored by PAGE or by ionic HPLC equipped with a Berthold flow-through 506 C-1 radioactivity monitor. The sensitivity of the detector corresponds to a minimum detection of 100 counts per min (cpm), which is equivalent to roughly 0.1 pmol of ^{32}P-ODN; the specific radioactivity in standard experiments was 1 million cpm/nmol.

The sequences (5′ > 3′) and lengths of the PS oligonucleotides are:

N°	Sequences	Lengths
1	GGACGTGACTGT	12mer
2	GGTCGTGGATAC	12mer
3	TCCGGAGCCAGACTTCATTC	20mer
4	AGCGGTCCCACTCTTGTTTG	20mer
5	GAGTCCAGAGCCTCCTTACT	20mer
6	CTCTCGCACCCATCTCTCTCCTTCT	25mer

Cells

Rat aortic SMC were grown in DMEM (Sigma Cell Culture) in which 50 IU/mL of penicillin, 50 μg/mL of streptomycin (ICN Pharmaceuticals), 2 mM of L-glutamine (Life Technologies), and 5 or 10% fetal calf serum (FCS, Boehringer Mannheim cell culture) were added. Figures are given as final concentrations in the culture medium. Cells were grown in an 80-mL flask (Nunclon). At confluence, cells were washed with 5 mL of Ca^{2+} and Mg^{2+} free PBS (Sigma Cell Culture) and treated with 4 mL of EDTA-trypsin (Bio Whittaker) at 37° in a 5% CO_2 humidified incubator. Cells were harvested in DMEM containing 10% FCS, stained with trypan blue and counted. Subcultures (passages 4 to 10) were carried out by seeding 2×10^6 SMC in Petri dishes (35 mm diameter). To minimize ODN degradation by nucleases and phosphatases, heated FCS (65°, 30 min), EDTA and potassium phosphate (both at 1 mM) were added to the cell culture medium.

Uptake Experiments

ODN at 1-μM final concentration peaked with 5′-^{32}P-labeled ODN (1–2 million cpm) and DEX (MW 10 kD, from Sigma) at 1 mg/mL was added to 2–3 million adherent SMC per dish in 1 mL of DMEM containing 5% heated FCS. Cells were incubated at 37° in a 5% CO_2 humidified oven. At determined times, the dish was removed and kept on crushed ice. All washings were carried out with precooled solutions (4°). The supernatant (S_0) was removed and cells were washed 6 times (S_1 to S_6) with 1 mL of PBS containing 5 mg/mL of dextran (molecular mass 40 kDa, DT40, Sigma). The polysaccharide was added with the aim of displacing the bound DEX from the cell surface. The fraction of ODN (or DEX) that remained bound to the membrane proteins was desorbed with 2×1 mL PBS containing 10% heated FCS for 30 and 20 min respectively (S_7 and $S_{7'}$). Cells were then washed with 2×1 mL (S_8 and S_9) of a protective buffer PB (0.25 M of sucrose, 20 mM of HEPES pH 7.2, 2 mM of potassium phosphate, 0.24 mM of EGTA, 10 mM of $MgCl_2$) to remove the serum proteins. In order to evaluate the fraction of ODN that remained strongly bound to the membrane proteins, a final treatment consisted in treating the cell layer with diluted (250 mg/mL) trypsine in the protective buffer for 70 sec (S_T) followed by 2×1 mL PB washes (S_{10} and S_{11}). The total duration of the washing procedure was approximately 1 hr. Exocytosis of internalized ODN for this time and at 4° was found to correspond to a loss of *ca.* 10% of the internalized species. Cell viability during washings was assessed by measuring the lactate dehydrogenase (see below), a cytosolic enzyme which is supposedly released only by dead cells. Cells were recovered from the dish by treatment with 1 mL of 0.1% Triton X-100 (Fluka). The amount of internalized ODN was measured from the radioactivity associated with the cells after washing. Counts per min

were converted to the amount of cell-associated ODN (pmol) on the basis of known ODN dilution (see above).

Measurement of the Cytosolic Content by Controled Permeabilization of the Plasma Membrane with Digitonin

SMC permeabilization (after wash S_9) was carried out by adding 1 mL of 0.007% (w/v) digitonin (Merck) to the protective buffer over the cell layer and incubating for 30 min at 4°. This treatment results in the release into the supernatant (digitonin fraction) of free diffusible cytosolic macromolecules up to 200 kDa, with minimal damage to the membranes of intracellular vesicles [29]. Cytosolic LDH was used as a cytosolic marker [29, 30], endocytosed DEX as a endosomal marker [31], and HAM as a lysosomal marker [32]. After collection of the digitonin supernatant, SMC were treated with 1 mL of 0.1% Triton X-100 in the protective buffer (Triton fraction) to recover the remaining intracellular ODN and markers. The sum of the digitonin and triton fractions was taken as 100% of the internalized compounds.

LDH was measured using the oxidation reaction of β-NADH in the presence of pyruvate, monitored by the linear absorbance decrease with the reaction time of β-NADH at $\lambda = 340$ nm using a Sigma kit. HAM was determined by using the chromogenic substrate *p*-nitrophenyl-β-D-hexosaminide (Sigma). The reaction was carried out at pH 5.2 and the amount of the generated *p*-nitrophenol was monitored by its absorbance increase at $\lambda = 400$ nm after alcalinization of the reaction mixture. The content of DEX in fractions was compared by measuring their relative fluorescence intensities with a Jobin-Yvon spectrofluorimeter ($\lambda_{exc} = 540$ nm, $\lambda_{emi} = 580$ nm).

Nuclear Fraction

Following the digitonin treatment, cells were washed twice with 1 mL of the protective buffer PB. The nuclear fraction was obtained by two methods: a) Cells were treated by 0.5% Nonidet P40 (Pharmacia) and spun down at 2,000 *g*. The 2,000 *g* pellet was then submitted to centrifugation at 30,000 *g* for 10 min over a 2-M sucrose bed and the nuclei were recovered in the 30,000 *g* pellet. b) Cells were disrupted with a Dounce homogenizer (50 strokes) in an isotonic buffer (0.25 M of sucrose). Intact cells were discarded by centrifugation at 200 *g* for 3 min. Nuclei were recovered by spinning down at 700 *g* for 10 min and further washed with the isotonic buffer containing 0.5% Nonidet P40, followed by a second centrifugation at 700 *g*.

RESULTS

Stability of Oligomers under Cell Culture Conditions

Oligomers 1 and 2 are the antisense sequences complementary to the AUG region of the Farnesyl-transferase gene (rat) and the corresponding 5 base-mismatched sequence, respectively. Oligomers 3 and 4 represent the antisense and mismatched sequences, respectively of the initiation codon of the Insuline-like Growth Factor Receptor (IGFR) as described by Delafontaine *et al.* [22], while the scrambled sequence 5 was constructed by us from the sequence of 3. Oligomer 6 was included, as this sequence is directed against the *gag* gene of HIV-1 [33], a target sequence that is completely irrelevant to the investigated cell system. We observed an inhibitory activity of oligomer 1 and the four sequences 3 to 6, based on both the measurements of tritiated thymidine incorporation and cell growth (data not shown). Oligomer 2 was found to be almost inactive, probably due to its rapid degradation in the presence of SMC (see below). However, our results conflict with the observed specific inhibitory effect of oligomer 3 vs 4 as previously reported [22]. The recently discussed [34] non-sequence-specific effect of PS ODN at inhibiting cell proliferation points to the need for more careful work regarding this class of oligomers, especially the search for other possible targets such as proteins [35] instead of nucleic acid targets and this in relation to their localization inside the cell.

The binding of PS ODN to proteins has been thoroughly investigated [6–9, 11, 14, 34, 35]. In this study, we wanted to focus on issues such as external membrane binding, penetration and distribution in cells of PS oligonucleotides. Before performing such studies, it was necessary to assess the stability of the tested ODN under the cell culture conditions. Oldenburgh *et al.* [36] have shown that EDTA and potassium phosphate can be usefully added to the culture medium to retard nuclease degradation and dephosphorylation of the 5′-^{32}P-labeled oligomers. Based on these data, we worked out the following optimal conditions under which the culture medium contained heat-inactivated serum (65° 30 min), and 1 mM each of EDTA and potassium phosphate. Under these conditions, the 5′-^{32}P label and integrity of oligomers were preserved for a period of time (5–6 hr) sufficiently long for endocytosis study. In the absence of cells, most of the tested compounds were found to be fairly intact in the above medium for more than 8 hr. However, in the presence of SMC, degradation processes took place gradually, the extent of degradation reaching 20% after 5 hr for some compounds. Dephosphorylation was also observed but was limited (10–20% at 2–3 hr). Only two oligomers, the 12mers 1 and 2, were found to be more sensitive to phosphatases and nucleases (70 and 50% of oligomer 1 were found intact after 2.5 and 5 hr incubation periods respectively), while its mismatched congener 2 was almost completely degraded after 5 hr in the presence of SMC. A strong dephosphorylation process also occurred with the latter oligomer (>80% at 5 hr), while this reaction was only limited for oligomer 1 (<20%). These observations emphasize the fact that enzymatic reactions such as dephosphorylation and chain hydrolysis may be very different from one sequence to another. Apart from the latter two cases, the above results indicate that working within 5–6 hr allowed us to carry out the endo-

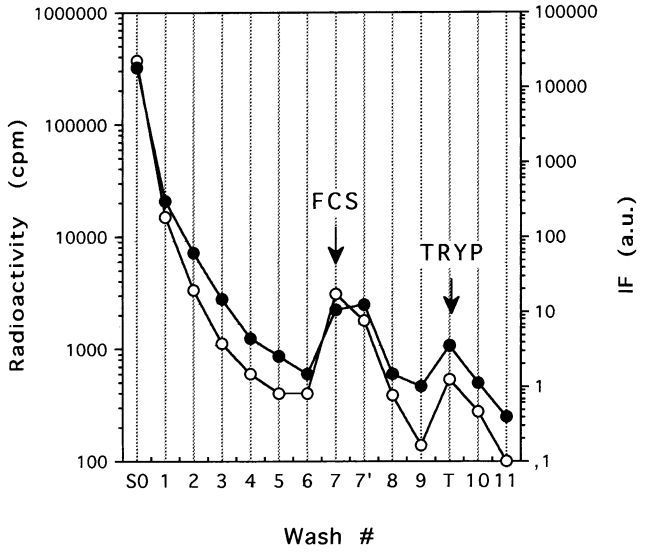

FIG. 1. SMC washing profiles. Cell washes were carried out at 4° as indicated in Materials and Methods with PBS-DT40 from S_0 to S_6, 10% FCS in PBS for S_7 and $S_{7'}$, and an isotonic protective buffer for S_8 and S_9. Further treatment with a diluted trypsin solution in the protective buffer was carried out (S_T) followed by 2 washings with 1 mL each of the protective buffer (S_{10} and S_{11}). The desorbed ODN was followed by radioactivity counting (○) and that of the DEX by measurement of the rhodamine fluorescence IF (●) of the washed fraction.

cytosis experiments during which oligomers (compounds 3 to 6) remained virtually intact (>80%) before internalization.

Cell Binding and Internalization of Phosphorothioate Oligonucleotides in SMC

In order to define a correct and reliable value of the internalization yield, we performed extensive cell washings to eliminate membrane-bound species. It is important to keep in mind that molecular interactions of PS ODN and DEX could take place with proteins and/or sugar moieties of the plasma membrane as already noted [9]. Many researchers carried out cell washings with 3 × 1 mL of PBS, while others such as Gao *et al.* [18] performed 6 washes to remove the horseradish peroxidase added as an endosomal marker. The removal efficiency of membrane-bound species (oligomers and DEX) by our cell washing procedure is displayed in Fig. 1. The results clearly show that 3 and even 6 PBS washes were not sufficient to desorb membrane-bound species. Indeed, we found that more radioactive and fluorescent species can be recovered by treating the cell surface with 2 × 1 mL washings with 10% heated FCS in PBS for 30 and 20 min, respectively (Fig. 1, washes S_7 and $S_{7'}$) followed by two more washes with the 0.25 M of sucrose-containing protective buffer (see Materials and Methods) to remove FCS proteins from the medium (S_8 and S_9). Further treatment with diluted trypsine (250 mg/L) for 70 sec (ST) followed by two washes (S_{10} and S_{11}) made it possible to reach radioactivity (for ODN) and fluorescence

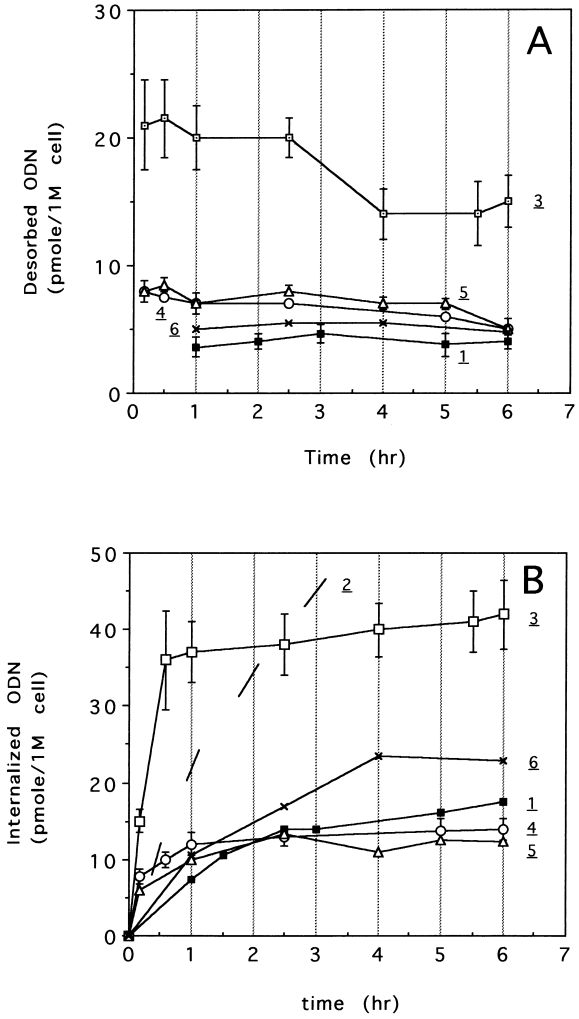

FIG. 2. Cell binding (A) and internalization (B) of PS oligomers as a function of incubation time at 37°: oligomer 1, (■); 2, (---); 3, (□); 4, (○); 5, (△); and 6, (x). The dashed line represents the profile observed with the highly degraded compound 2. Each Petri dish contained approximately 2.10^6 SMC cells. Error bars are added to some curves (N = 2–4) and omitted on others for the sake of clarity.

intensities (for DEX) close to the background values. On average, we found that the fraction of oligomers that can be recovered by trypsine treatment lay between 15–20% of the total amount of oligomers associated with the cell surface (total number of fractions recovered by FCS and trypsin treatments). Exocytosis could take place during the washing procedure. In a separate experiment performed after wash S_{11}, we found that this process was slow at 4°, the loss of radioactivity being *ca.* 10% after 1 hr in contact with the medium.

The fraction of ODN that could be exchanged with FCS proteins (S_7 + $S_{7'}$) was used as an indicator of the oligomer affinity for membrane proteins. The results are presented in Fig. 2A. The presented radioactivity values were not corrected for the minor contribution of exocytosis (less than 10%). It can be seen that the affinity of the 20mer 3 for the cell surface was particularly high, while that of the

12mer 1 exhibited the lowest affinity, with the other oligomers lying closer to compound 1 than to compound 3. These results indicate that the ODN affinity for the cell membrane (proteins) depended on the sequence, and more likely on the build-up of a tertiary structure from that sequence, rather than on the size of the oligomer.

We noticed that the more the ODN bound to the cell, the more it internalized. The radioactivity that remained associated with cells after the above washing procedure was considered representative of internalized species and was expressed as picomoles of internalized oligomers per million of cells. For comparative purposes, the kinetics profiles of the internalization of oligomers 1 to 6 by SMC at 37° are represented in the same figure (Fig. 2, part B). The external ODN concentration in our experiments was 1 μM (1 nmol in 1 mL of medium) and did not vary dramatically, since less than 5% of the initial extracellular ODN was internalized at the plateau value for the most efficient compound (Fig. 2B). The uptake curves reached a steady state after 1–2 hr incubation, and an average plateau value of internalized ODN corresponding to 13 ± 2 pmol/10^6 cells (except for oligomer 3 whose value was 37 ± 4 pmol/10^6 cells) could be derived. Taking a cell volume of 1.2 pL [18], this yielded a mean intracellular concentration of roughly 10 μM (or 30 μM for oligomer 3). The accumulation factor of PS ODN by SMC cells could thus be as high as 10- to 30-fold within the cells as compared to the extracellular concentration. This kind of ODN concentration effect has already been observed by some authors in other cell types [12, 18]. The uptake profile of oligomer 2 was also included (dashed curve) to illustrate the type of kinetics deviation that could take place when the oligomer was rapidly degraded during endocytosis. The uptake curve of oligomer 1, at least for endocytosis times longer than 3 hr, may not be comparable to the other curves due to the partial degradation of this compound (see above).

The efficiency of internalization again does not seem to depend on the length but on the sequence of the oligomer. On the other hand, the chemical nature of the oligomer backbone could be another parameter that determines the interactions of ODN with cells. Our data show that the phosphodiester sequence of 3 (a PO oligomer derivatized with —$(CH_2)_6$—NH_2 at the 3′ end for exonuclease protection) bound and internalized 3-fold fewer SMC than did its PS congener. Finally, it is important to note that 20mer 3 (antisense sequence) was internalized about 3 times more in SMC cells as compared to the two corresponding control sequences of the same length (4 and 5). This result illustrates the utility of cell uptake and intracellular distribution studies of ODN that will be later assessed in antisense studies.

Intracellular Distribution of ODN

Digitonin forms pore-like complexes when it binds to membrane cholesterol. This causes the cytosolic content of these digitonin-permeabilized cells to leak out while leaving most of the endosomal membrane intact. The plasma membrane contains a relatively high concentration of cholesterol, whereas its presence in the endosomal membrane depends on the cell type. In rabbit alveolar macrophages for example, the plasma membrane could be selectively permeabilized by a low concentration of digitonin without affecting the integrity of intracellular membranes [29]. For each cell type, a distribution curve of cytoplasmic, endosomal and lysosomal markers versus digitonin concentration must be established. The aim of this methodology is to find a digitonin concentration leading to a maximal recovery of a cytosolic marker (LDH) and minimal amounts of endosomal (DEX) and lysosomal (HAM) markers in the digitonin-extracted fraction. The optimal conditions worked out for SMC corresponded to a digitonin concentration of 0.007% (w/v) for a treatment time of 30 min at 4°. A typical profile of plasma and vesicular membrane permeabilization by digitonin under these conditions is represented in Fig. 3A. It should be noted that with this particular cell system, the susceptibility of the internal membranes (endosomes) could be very dependent on the "age" of the cell (number of passages), which is reflected by the greater height of the SE bar that affects the measured value of the liberated DEX. Averaging data collected from more than a dozen permeabilization experiments showed the recovery yield of the cytosolic LDH to be higher than 80%, while permeabilization of the vesicle membranes remained quite limited (less than 15% and 10% for the endosome and lysosome membranes, respectively). These limit values make it possible to correct the measured value for the cytosolic content in ODN. Details of this quantitative approach in the permeabilization process are given in the Appendix. Based on the results of ODN stability in the culture medium and the kinetics data (see Fig. 2B), an endocytosis duration of 2.5 hr was chosen for the determination of the cytosolic distribution of the internalized oligomers. In Fig. 3 (part B) are represented the "corrected" percentages of cytosolic ODN (as derived from the value of X_{odn}^{cyt} explained in the Appendix). The results clearly show that the intracellular fraction of the oligomers (40–60% of the internalized oligomer was found in the digitonin-extracted fraction) far exceeded that of the intracellular DEX. The cytosolic content of compound 2 (75%) was much higher than that of the other oligomers, and this behavior could very well be linked to the particular case of a highly degraded oligomer.

The partition of the ODN within the cell was followed by tracking oligomer radioactivity. The state of the oligomers in the cytosolic fraction is of particular interest, as it needs to be checked for intact or fragmented oligonucleotides in the extracted cytosol fraction. Ionic HPLC analysis of these fractions (cytosolic [D] and non-cytosolic [T fractions]) as exemplified by oligomer 1 (Fig. 4) showed that a significant amount of this oligomer remained intact in both fractions for short endocytosis times of 2.5 hr. It should be noted that a peak appeared at 2.5 min in the triton fraction (Fig. 4, T), indicating the existence of a

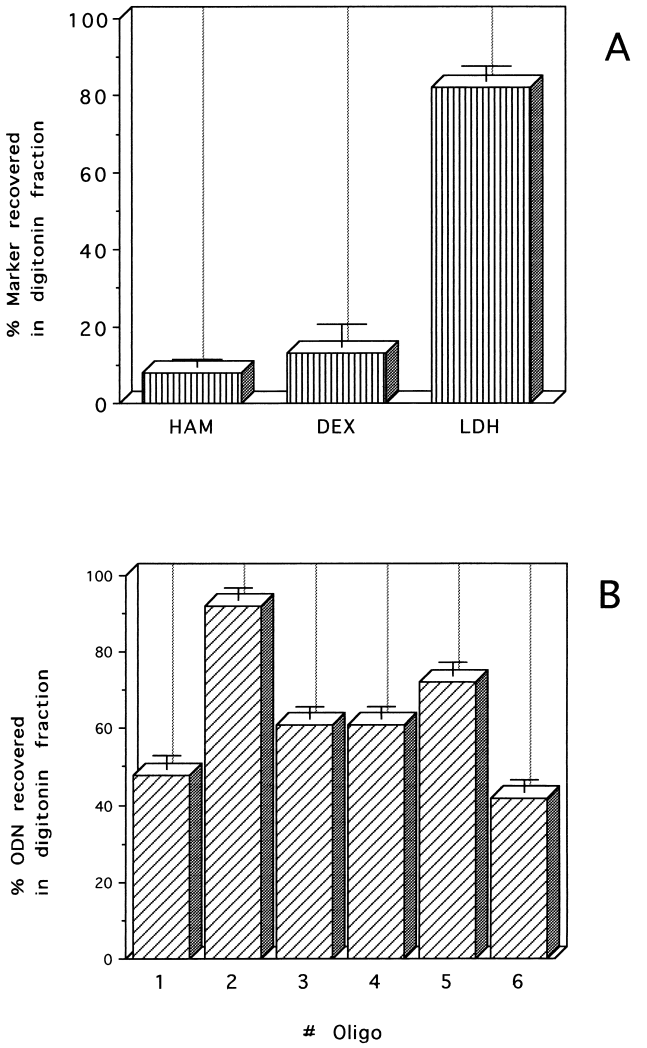

FIG. 3. Analysis of intracellular markers (A) and ODN (B) following permeabilization by digitonin (0.007% w/v) for 30 min at 4°. The amount of species recovered upon permeabilization of the cell membrane is expressed as the percent of the internalized species after 2.5 hr of endocytosis.

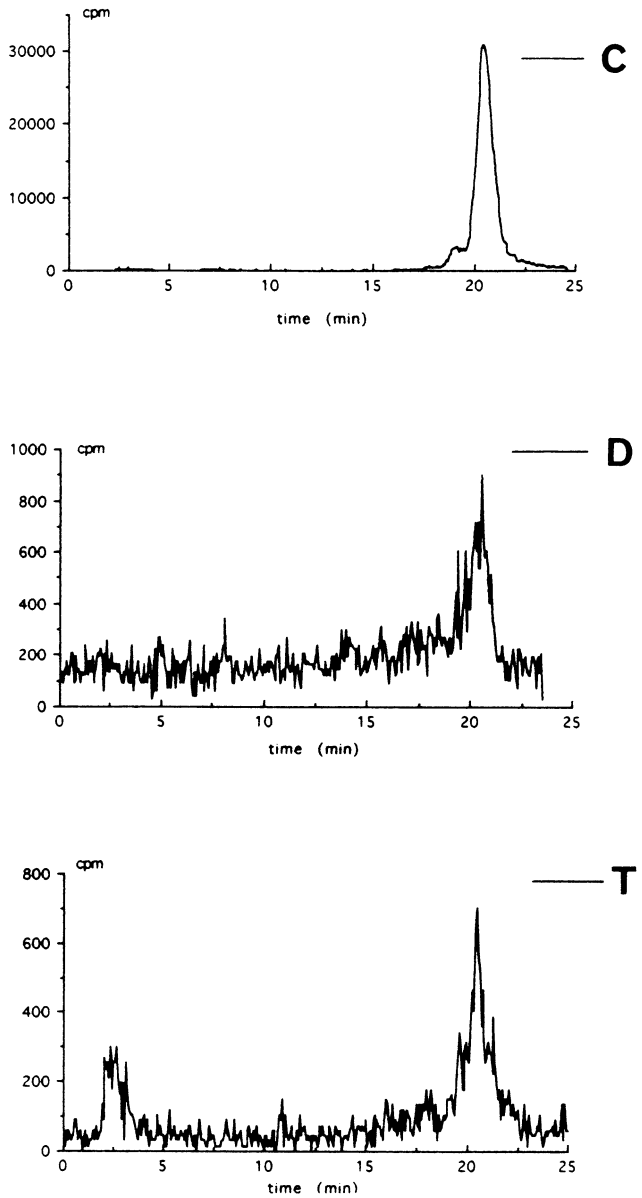

FIG. 4. Analysis of oligomer 1 in the digitonin and triton fractions by HPLC equipped with a radioactivity detector. Compound 1 at 1 μM was incubated for 2.5 hr at 37° with 3.10^6 SMC; 20 μL of the digitonin (D) or triton (T) fractions were analyzed along with the control sample (C) by anion exchange HPLC on a TSK DEAE-NPR (Perkin–Elmer) column (4.6 × 25 mm). Elution was performed using a KCl concentration gradient increasing linearly from 0 to 1 M for 30 min. Buffer A: potassium phosphate 10 mM, pH 7, acetonitrile 20%. Buffer B: Buffer A containing 1 M of KCl. The counting efficiency of the detector was monitored to ensure that it did not vary when the KCl concentration increased from 0 to 1 M.

membrane-associated phosphatase activity. These results suggest that dephosphorylation could be the main degradative process, at least for short incubation periods. Intracellular degradation appears to be minimal, as short fragments are barely visible from the chromatogram background. These results were further confirmed by PAGE analysis (results not shown), corroborating the fact that the reported values of cytosolic ODN concentrations largely represent the intact ODN and not its degraded fragments.

Taken together, these results suggest that the cytosolic fraction of the internalized oligomers does not seem to depend on the sequence (compare for example the three 20mers 3, 4 and 5) in contrast to their uptake efficiencies.

DISCUSSION

Many studies on the cell localization of ODN have been published [9–14, 18–20], but only a few have described a quantitative measurement of the cytosol content in oligomers while taking into account the contributions of membrane-bound species and/or the species leaked out from the vesicles. In this study, we used the digitonin permeabilization method to recover a digitonin-extracted fraction in which the amount of species that escapes from the vesicles could be monitored by measuring the appropri-

ate markers, i.e. the endocytosed dextran-rhodamine for the endocytotic compartment and the enzyme HAM for the lysosomal compartment. Concerning the membrane adsorption of the PS ODN, we have shown that the amount of protein-bound species could be as high as 50% of the total amount internalized in cells (Fig. 2). Wu-Pong *et al.* observed the same phenomenon with a G-quartet containing ODN in an erythroleukemic cell line [12]. The high affinity for binding to surface proteins of another G4-oligo, an anti-c-myb PS oligomer, has also been reported [9]. These results point to the necessity of performing efficient removal of membrane-bound ODN in cell uptake studies.

We found high percentages (40–60%) of internalized ODN in the digitonin-extracted fraction. Other authors also found high percentages of partition in the cytoplasmic fraction of PS ODN, depending on the cell line [18, 19]. As far as the anti-*gag* oligomer 6 is concerned, we found that the cytosolic fraction of this oligomer accounted for 42% of the internalized species (Fig. 3B). This value could be compared to the 60% value as derived by Temsamani *et al.* [37] for the same ODN in diverse cell types. It should be noted that the method of cell fractionation performed by these authors resulted in a "cytoplasmic" fraction comprising membranes, cytosol and other organelles except the nuclei. By contrast, the digitonin method led to a maximal recovery of soluble molecules in the cytosol with minimal damage to vesicles.

The digitonin method was compared to a conventional fractionation method consisting of trypsination to harvest the adherent cells followed by membrane breaking using a Dounce homogenizer and ultracentrifugation (100,000 *g* for 30 min). Comparable percentage values of markers (LDH: 83%; DEX: 14%; and HAM: 7%) were found in the supernatant, a fraction that would contain all soluble species liberated in the cytosol. These values are quite close to those obtained in the digitonin-extracted fraction (Fig. 3B). The good agreement between the two methods, at least for this specific cell type, validates the digitonin-controlled permeabilization method as a reliable means of determining the cytosolic content of internalized compounds in cells.

Despite the substantial concentration of ODN found in the cytosol, the nuclear accumulation of oligomers was found to be limited, from 7% to 13% of the internalized oligomer depending on the sequence and the method used for the isolation of nuclei. Although microinjected fluorescent ODN are said to mainly accumulate within the nucleus [11, 14], ODN incubated in the presence of cells exhibit contrasted cellular distribution after endocytosis. For example, the homo-oligomer S-dC28 displays a nucleus/cytoplasm ratio ranging from 0.2 to 3.8 depending on the cell line used [18]. In LTK cell lines, Cerruzzi *et al.* [19] also showed that oligomers in either PO or in PS structures were found to be predominantly distributed in the cytoplasm or membrane fractions, whereas very few oligomeric species were detected in the nuclei. In our nuclei isolation procedure, we cannot rule out the possibility of redistribution of the ODN from the nucleus to the cytosol during the cell fractionation procedure. Redistribution is a real concern for everyone engaged in cell fractionation studies. For our part, we attempted to maintain a pressure in the ODN concentration by adding tRNA at a concentration of 3×10^{-4} M (in nucleotides) to the permeabilization solution. This concentration corresponds to the average concentration of ODN found in the digitonin-extracted fraction. Preliminary results showed that no significant differences were observed in the percentages of ODN recovered from the cytosol in the presence or absence of tRNA, suggesting that redistribution of ODN between the nucleus and the cytosol may be negligible under our experimental conditions. However, this point needs further confirmation by additional experiments using, for example, photoactivatable ODN to covalently link oligomers to their binding sites before permeabilization experiments are carried out.

In conclusion, we have shown in this study that antisense sequences were not always equivalent to their corresponding control sequences in terms of stability towards nucleases or efficiency of internalization and distribution in a given cellular system. Using the permeabilization method described in this work, it was possible to evaluate these differences and to take them into account when comparing the biological effects of the antisense sequences versus the control sequences. When monitoring the endocytosis of the ODN and the DEX, both of which have comparable molecular weight (5–10 kD) and water solubility properties, it was remarkable to find by the digitonin-induced permeabilization method that the partition of the two compounds in the cytosol compartment was completely different. The cytosolic fraction of oligomers far exceeded that of the fluorescent polysaccharide. The origin of this "cytosolic" ODN species remains to be elucidated. It could be speculated that these soluble and diffusible ODN may originate from some endosome leakage during vesicle-vesicle fusion processes along the endocytotic pathway as postulated by Akhtar and Juliano [10] and/or an entry route of ODN in cells that is completely different from the conventional endocytosis routes described so far. The binding of ODN (particularly PS oligomers) to membrane proteins may trigger some unexpected transconformation of these proteins that could result in direct delivery of the oligomer to the cytosol compartment. These points deserve more investigation in the future.

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APPENDIX

Analysis of Permeabilization Experiments

Notations used:

i:	species i
dig:	digitonin
n_i:	number of mol of species i
tot:	total in cell
p:	fraction of permeabilized cells

q: fraction of nonpermeabilized cells (p + q = 1)

f_{vi}: fraction of vesicles containing species i and permeabilized by digitonin. It is assumed that $f_{vi} = f_{ves}$, regardless of i

cyt: cytosolic

ves: vesicular

n_i^{cyt}, (n_i^{ves}): number of mol of species i located within the cytosol (or in vesicles) before digitonin treatment.

Basic equations: The number of internalized species i (n_i) is divided into cytosolic species n_i^{cyt} and vesicular species n_i^{ves} (Eqn 1). The number of moles that can be extracted upon the digitonin-induced permeabilization of cell membranes n_i^{dig} is composed of n_i^{cyt} and those coming from the permeabilized vesicles n_i^{ves} (Eqn 2).

$$n_i^{cyt} + n_i^{ves} = n_i^{tot} \quad (1)$$

$$n_i^{dig} = p.[n_i^{cyt} + f_{vi}.n_i^{ves}] \quad (2)$$

a) LDH (i = ldh)

Hypothesis $n_{ldh}^{ves} = 0$ and $n_{ldh}^{cyt} = n_{ldh}^{tot}$

According to Eqn 2, it follows that:

$$n_{ldh}^{dig} = p.n_{ldh}^{cyt} + 0 = p.n_{ldh}^{tot}$$

Then $$p = \frac{n_{ldh}^{dig}}{n_{ldh}^{tot}} = X_{ldh}^{dig}$$

p or X_{ldh}^{dig} = LDH fraction recovered following digitonin treatment. This parameter could be considered as a measure of the yield of recovery of the cytosol content.

b) Dextran-rhodamine (i = dex)

Hypothesis $n_{dex}^{cyt} = 0$ and $n_{dex}^{ves} = n_{dex}^{tot}$

According to Eqn 2: $$n_{dex}^{dig} = p.[n_{dex}^{cyt} + f_{ves}.n_{dex}^{ves}] = 0 + p.f_{ves}.n_{dex}^{tot}$$

Then $$p.f_{ves}. = \frac{n_{dex}^{dig}}{n_{dex}^{tot}} = X_{dex}^{dig}$$

$p.f_{ves}$ or X_{dex}^{dig} is the dextran-rhodamin fraction recovered following digitonin treatment. This parameter represents the rate of vesicle permeabilization by the digitonin treatment.

c) ODN (i = odn)

Hypothesis $n_{odn}^{cyt} \neq 0$ and $n_{odn}^{ves} \neq 0$

$n_{odn}^{cyt} + n_{odn}^{ves} = n_{odn}^{tot}$ according to Eqn 1

$n_{odn}^{dig} = p.n_{odn}^{cyt} + p.f_{ves}.n_{odn}^{ves}$ according to Eqn 2

or

$$n_{odn}^{dig} = p.n_{odn}^{cyt} + p.f_{ves}[n_{odn}^{tot} - n_{odn}^{cyt}] = n_{odn}^{cyt}[p - p.f_{ves}] + p.f_{ves}.n_{odn}^{tot}$$

then $$n_{odn}^{cyt} = \frac{n_{odn}^{dig} - p.f_{ves}.n_{odn}^{tot}}{p - p.f_{ves}}$$

or $$n_{odn}^{cyt} = \frac{n_{odn}^{dig}/n_{odn}^{tot} - p.f_{ves}.}{p - p.f_{ves}} \cdot n_{odn}^{tot}$$

Defining the oligonucleotide fraction recovered in the digitonin extract by X_{odn}^{dig}, it follows that:

$$X_{odn}^{dig} = \frac{n_{odn}^{dig}}{n_{odn}^{tot}}$$

and since $p = X_{ldh}^{dig}$ and $p.f_{ves} = X_{dex}^{dig}$, then:

$$n_{odn}^{cyt} = \frac{X_{odn}^{dig} - X_{dex}^{dig}}{X_{ldh}^{dig} - X_{dex}^{dig}} \cdot n_{odn}^{tot}$$

The sought-after value of the cytosolic ODN fraction $X_{odn}^{cyt} (= n_{odn}^{cyt}/n_{odn}^{tot})$ can then be derived:

$$X_{odn}^{cyt} = \frac{X_{odn}^{dig} - X_{dex}^{dig}}{X_{ldh}^{dig} - X_{dex}^{dig}} \quad (3)$$

The same correction applies for species leakage from lysosomes (i = hexosaminidase, see text, or any lysosomal marker). The procedure could be generalized to any other fractionation method, regardless of the means used to break or open cells and separate the soluble-diffusible vs particulate material. The basic equation is then as follows:

$$n_i^{sol} = p.n_i^{cyt} + p.f_v.n_i^v$$

n_i^{sol} represents the fraction of soluble and diffusible species that could be separated from other sedimentable organelles.