Intracellular Traffic of Oligodeoxynucleotides In and Out of the Nucleus: Effect of Exportins and DNA Structure

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The delivery of oligodeoxynucleotides (ODNs) into cells is widely utilized for antisense, antigene, aptamer, and similar approaches to regulate gene and protein activities based upon the ODNs' sequence-specific recognition. Short pieces of DNA can also be generated in biological processes, for example, after degradation of viral or bacterial DNA. However, the mechanisms that regulate intracellular trafficking and localization of ODNs are not fully understood. Here we study the effects of major transporters of microRNA, exportin-1 (Exp1) and exportin-5 (Exp5), on the transport of single-stranded ODNs in and out of the nucleus. For this, we employed a fluorescent microscopy-based assay to quantitatively measure the redistribution of ODNs between the nucleus and cytoplasm of live cells. By measuring the fluorescent signal of the nuclei we observed that after delivery into cells via cationic liposomes ODNs rapidly accumulated inside nuclei. However, after removal of the ODN/liposome containing media, we found re-localization of ODNs from the nuclei to cytoplasm of the cells over the time course of several hours. Downregulation of the *Exp5* gene by siRNA resulted in a slight increase of ODN uptake into the nucleus, but the kinetics of ODN efflux to the cytoplasm was not affected. Inhibition of Exp1 with leptomycin B somewhat slowed down the clearance of ODNs from the nucleus; however, within 6 hours most of the ODN were still being cleared from the nucleus. ODNs that could form intramolecular G-quadruplex structures behaved differently. They also accumulated in nuclei, although at a lesser extent than unstructured ODN, but they remained there for up to 20 hours after transfection, causing significant cell death. We conclude that Exp1 and Exp5 are not the major transporters of our ODNs out of the nucleus, and that the transport of ODNs is strongly affected by their secondary structure.

Introduction

VARIOUS APPROACHES BASED on sequence- and structure-specific recognition of nucleic acids, that is, antisense (Stein and Cohen, 1988; Stein and Cheng, 1993), antigene (Helene and Toulme, 1990; Giovannangeli and Helene, 1997), aptamer (Thiel and Giangrande, 2009), and alike, rely on delivery of oligodeoxynucleotides (ODNs) to their targets inside cells. On the other hand, ODN can be generated in biological processes ongoing inside cells, for example, by degradation of bacterial or viral DNA in infected cells. Such endogenous short DNAs recently have been described as potent signals in innate immune defense (Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Ishikawa et al., 2009) and even as signals for cellular responses to genomic DNA damage (Peng et al., 2007; Jazayeri et al., 2008; Quanz et al., 2009). Therefore, knowledge of the intracellular trafficking of ODN could be a

key to understanding of their functions. Due to their small size, ODNs <40 nt long should be able to migrate freely through the nuclear pore complexes. Thus, traffic of ODN in and out of the nucleus could have proceeded by simple diffusion through the nuclear pore complexes. However, it was found that ODN microinjected or otherwise delivered into the cytoplasm quickly re-localized into the nucleus of the cell (Leonetti et al., 1991; Fisher et al., 1993; Alam et al., 2008; Chen et al., 2009). This finding indicated the existence of mechanisms of active transport of ODN inside cells.

In the past decade, mechanisms for transport of microRNA (miRNA) have been extensively studied (Gorlich and Kutay, 1999; Winter et al., 2009). The key player in miRNA transport from the nucleus to cytoplasm is exportin-5 (Exp5) (Lund et al., 2004; Ohrt et al., 2006). Recently, the role for another exportin, exportin-1 (Exp1), in the shuttling of miRNA from the nucleus to cytoplasm was established (Castanotto et al.,

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2009). However, the involvement of these karyopherins in intracellular trafficking of ODN has not been determined.

Here we studied effect of Exp5 and Exp1 on the intracellular traffic of ODNs. We also studied how the formation of a secondary structure (G-quadruplex) by an ODN affects intracellular re-localization.

Materials and Methods

Cell culture

HT1080 human fibrosarcoma cells (ATCC # CCL-121) were cultured in 10% fetal bovine serum in Eagle's Minimum Essential Medium (EMEM) (ATCC) in T-75 flasks. Cells were collected by treatment with trypsin/ethylenediaminetetraacetic acid (EDTA) and centrifugation in a 15 mL tube. The cells were counted using a hemocytometer and $\sim 5 \times 10^4$ cells were seeded into 60 mm MatTek glass bottom dish (Becton Dickinson). Leptomycin B (LMB) (Sigma) was added to 5×10^4 cells seeded in the glass bottom culture dish 12 hours before transfection with ODN-1 or ODN-G4 in final concentration 10 nM following in general the protocol described in (Wolff et al., 1997).

Oligonucleotides

3'-Glyceryl CPG and 5'-Cy3 Phosphoramidite were purchased from Glen Research. ODN were synthesized on an ABI 394 DNA synthesizer (Applied Biosystems), and purified by denaturing polyacrylamide gel electrophoresis as described in Gaynutdinov et al. (2008). The concentration of single-stranded ODN was measured at 260 nm on an Agilent 8453 diode array spectrophotometer, and was calculated with extinction coefficient calculator software (www.basic.northwestern.edu/biotools/oligocalc.html).

In this study we used the following ODNs synthesized in house as described above: ODN-1, 5'-Cy3-CCA-TTA-CCT-GAC-AGT-GCT-AGA-TTG-CAG-GAC-glyceryl-3'; ODN-G4, 5'-Cy3-GTG-CAG-TAG-GGG-TTA-GGG-TTA-GGG-TCA-GGG-CT-glyceryl-3'; Exp5 forward primer, 5'-CCA-TCC-TCG-GAC-CTC-TTT-TCA-C-3'; Exp5 reverse primer, 5'-CTT-CAT-CAG-ACA-TTT-GCC-CAG-G-3'. The sequence of ODN-1 was selected for the lack of potential to form secondary structures and homology to a nucleotide sequence in human genome. Beta-actin polymerase chain reaction (PCR) primers were purchased from Qiagen.

siRNA transfection

Exp5 siRNA (sense, 5'-GCC-CUC-AAG-UUU-UGU-GAG-G-dTdT; antisense, 5'-CCU-CAC-AAA-ACU-UGA-GGG-C-dTdT) (Lund et al., 2004) and U6 siRNA (sense, 5'-AAU-UG-G-AAC-GAU-ACA-GAG-A-dTdT; antisense, 5'-UCU-CUG-UAU-CGU-UCC-AAU-U-dTdT) that target nucleotides 29–48 of U6 snRNA (Robb et al., 2005) were purchased from Invitrogen. HT1080 cells (50,000) seeded on a 10 cm² plastic dish were transfected with 100 pmol of siRNA premixed with 5 μ L of Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 4 hours the transfection medium was removed and a fresh complete medium was added. Cells were kept in the complete medium for 24 or 48 hours, and then collected with trypsin/EDTA and washed with the medium; 50,000 cells were seeded to a glass bottom plate for the following treatment with fluorescent ODNs.

ODN transfection

ODNs (500 pmol in 10 μ L) were mixed in a sterile 1.5 mL microcentrifuge tube with 240 μ L of warm reduced serum medium (OPTI-MEM) (Invitrogen) and left in dark for 5 minutes. ODN-G4 was preincubated in 100 mM KCl at 37°C for 1 hour to allow quadruplex formation. In a separate 1.5 mL microcentrifuge tube 5 μ L of Lipofectamine 2000 and 245 μ L of OPTI-MEM was gently mixed using pipette and incubated in dark for 5 minutes. The Lipofectamine 2000 solution and ODNs were combined, mixed gently, and incubated in dark for additional 20 minutes. Just before the end of the 20-minute incubation period, the complete medium (10% fetal bovine serum in EMEM; ATCC) was aspirated from glass bottom plates containing 50,000 cells. Five hundred microliters of DNA:Lipofectamine solution was added to the glass bottom plate, plus 1.5 mL of OPTI-MEM. OPTI-MEM was replaced with the complete medium 2 hours post-transfection.

Microscopy

Images of the nuclear uptake of fluorescent ODN were collected with Zeiss Axiovert 200M (Karl Zeiss) microscope equipped with a heated stage and a chamber. The glass bottom dishes were placed into the chamber that maintained at 37°C with 5% CO₂ and 95% air mix pumping through it. The images were collected using AxioVision software at fixed exposure (brightfield 363 ms, rhodamine 1648 ms). The nuclei were observed on the bright-field images and outlined manually, and the intensity of the rhodamine fluorescence within the outlined regions was determined with AxioVision software. These measurements were repeated for at least 30 cells for each experiment and time point. Statistical values were calculated with StatPlus:mac software. The measured intensity is presented on the graphs in arbitrary units.

Reverse transcriptase-PCR

RNA from the harvested siRNA-transfected cells was purified and cDNA was synthesized using SuperScript[®] III Cells Direct cDNA Synthesis Kit (Invitrogen). The cDNA samples were subjected to PCRs using Exp5 PCR primers or β -actin PCR primers (Qiagen): forward, 5'-CCA-TCC-TCG-GAC-CTC-TTT-TCA-C; reverse, 5'-CTT-CAT-CAG-ACA-TTT-GCC-CAG-G; SYBR GreenER[™] qPCR SuperMix for iCycler[®] (Invitrogen). The PCR conditions were as follows: 50°C for 2 minutes, 95°C for 5 minutes, then 50 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, then 72°C for 5 minutes, and then hold at 4°C. Ct (cycle threshold) values were obtained for each sample, averaged over triplicates, and normalized on Ct value of β -actin, according to the formula, $E = 100 \times 2^{(Ct[\beta\text{-actin}] - Ct[\text{studied gene}])}$, where E is the percent of normalized expression.

Recovery of ODN after transfection

HT1080 cells (100,000) were seeded in 60-mm culture dishes 24 hours before transfection. Cells were transfected with ODN-1/Lipofectamine 2000 liposomes as described above. After 2 hours the ODN/liposome-containing medium was substituted with complete EMEM. At the selected time points the medium was aspirated, and cells were washed with 5 mL of cold phosphate-buffered saline, aspirated, and covered with 1 mL of cold phosphate-buffered saline. Cells were gently scrapped on

FIG. 1. Uptake and efflux of fluorescently labeled oligodeoxynucleotide (ODN) from the nuclei of live HT1080 cells. **(A)** Merged fluorescent and bright-field images were taken after 30, 120, 240, and 420 minutes. One-hundred-twenty-minute image was taken before media replacement; free liposomes are visible outside cells on 120- and 30-minute images. **(B)** Merged fluorescent and bright-field images of a single cell taken after 265, 275, 285, and 295 minutes. Images were taken with Zeiss Axiovert 200M microscope equipped with rhodamine filter under 20 $\times$ magnification as described in Materials and Methods. **(C)** Nuclear fluorescence measured as described in Material and Methods as a function of time. The error bars at the bottom of the graph correspond to the standard deviation (STDEV) of the average fluorescence of the nuclei. The error bars at the top of the graph show standard error (SEM = STDEV/square root of number of measured cells). Nuclear fluorescence before addition of ODN-1 (time point 0 minute) was considered 0.

ice, collected into a 15 mL tube, and precipitated by centrifugation at 4°C at 1600 RPM for 7 minutes. Cells were lysed with 600 μ L of lysis solution (20 mM EDTA, 2% sodium dodecyl sulfate, and 50 mM Tris-HCl; pH 7.5) and 400 μ L of 5M NaCl; cell debris was precipitated by centrifugation for 30 minutes at 4°C at 3000 RMP. The supernatant was extracted twice with equal volumes of phenol-chloroform-isoamyl alcohol, precipitated with ethanol, washed twice with 70% cold ethanol, and resuspended with 100 μ L of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA (TE) pH 8.0. Ten microliters of the samples was mixed with 10 μ L formamide loading buffer and analyzed in 10% TBE-Urea gel (Invitrogen). Reference markers contained 495, 55, 49.5, and 4.95 fmol of pure ODN-1. Bands in the gel were observed with FluorImager 595 (Molecular Dynamics) and their intensity was measured with ImageQuant software (GE).

Results

Delivery of ODNs in and ejection out of the nuclei

To deliver fluorescently labeled ODNs into cultured HT1080 cells we used commercial cationic liposome formulation, Lipofectamine 2000, and 3'-glyceryl end-capped ODN-1 (see Materials and Methods section). As can be seen in Fig. 1A (top panels) fluorescently labeled ODN-1 quickly accumulated in the nuclei of the liposome-treated cells. However, after removing liposomes from the media after 120 minutes by media replacement, we discovered quick translocation of ODN-1 from the nuclei to the cytoplasm of the cells (Fig. 1A, bottom panels). Figure 1B shows an example of ODN-1 clearance from a single cell that occurred within 30 minutes 265 minutes after media replacement. Ejected ODN-1 localized outside the nucleus in the endoplasmic reticulum-like structure, and in discrete vesicles (see also Supplementary Movies S1 and S2, available online at www.liebertonline.com). To quantify kinetics of this out-of-nucleus ejection of ODN-1 we measured the intensity of fluorescence in the nuclei of multiple cells as described in detail in Materials and Methods. The results of such measurement are shown in Fig. 1C. The error bars at the bottom of the graph correspond to the standard deviation (STDEV) of the average fluorescence of the nuclei. They reflect a significant variation in the nuclear fluorescence signal between individual cells. The error

TABLE 1. INHIBITION OF EXPORTIN-5 EXPRESSION BY siRNA: REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION DATA

	siRNA			
Primer	None	U6	E5-50-24	E5-50-48
Ct β -actin	23.96	23.47	22.93	21.4
Ct Exp5	27.67	27.73	27.97	28.5
E (%) Exp5/ β -actin	7.6	5.2	3.0	0.7

$$E (\%) = 2^{(Ct[\beta\text{-actin}] - Ct[\text{Exp5}])} \times 100.$$

Ct, cycle threshold; Exp5, exportin-5.

bars at the top of the graph show standard error (SEM = STDEV/square root of number of measured cells).

Effect of Exp5 downregulation on ODNs nuclear ejection

To explore whether Exp5 plays a role in the ODNs ejection from the nuclei, we used Exp5 siRNA (Lund et al., 2004) to suppress expression of this gene. To confirm that treatment of the cells with Exp5 siRNA resulted in significant downregulation of Exp5 we used qPCR. Table 1 shows results of qPCR experiments that measured expression of Exp5 mRNA relative to β -actin (bottom row). Relative expression of Exp5 mRNA decreased only slightly after treatment with control, unrelated siRNA (U6, compare column 1 and 2). Treatment of the cells with Exp5 siRNA caused >2 times decrease in Exp5 mRNA relative expression after 24 hours and 10 times decrease after 48 hours of treatment (columns 3 and 4). These results are in agreement with previously published data (Lund et al., 2004). It was also proved previously that the decrease in Exp5 mRNA expression translated into the corresponding reduction in the Exp5 protein level 48 hours after treatment (Lund et al., 2004).

Figure 2 shows kinetics of ODN-1 ejection from the nuclei of the cells pretreated with Exp5 siRNA for 48 hours. The results of 2 independent experiments [E5(1) and E5(2)] were compared with control that was not treated with Exp5 siRNA. Results presented in Fig. 2 show that cells pretreated with Exp5 siRNA accumulate more ODN-1 at the earlier time points. However, they also reveal that downregulation of

FIG. 2. Kinetics of efflux of ODN-1 from the nuclei of live HT1080 cells. *Diamonds*—cells were not treated with siRNA; *Squares and Circles*—results of 2 independent experiments when cells were pretreated with exportin-5 (Exp5) siRNA to downregulate the *Exp5* gene. The error bars show standard error (SEM = STDEV/square root of number of measured cells). Time points with *P* level between a set of data and control <0.001 are marked with asterisks.

FIG. 3. Kinetics of efflux of ODN-1 from the nuclei of live HT1080 cells pretreated with leptomycin B (circles) and not (diamonds). The error bars at the top of the graphs correspond to the standard deviation (STDEV) of the average fluorescence of the nuclei. The error bars at the bottom of the graph show standard error (SEM = STDEV/square root of number of measured cells). Time points with P level between 2 sets of data <0.01 are marked with asterisks.

Exp5 expression has no effect on the kinetics of ejection of ODNs from the nucleus after ODN-1/liposomes were removed from the media at the 120 minutes time point.

Effect of inhibition of Exp1 on ODNs nuclear ejection

To explore the role of Exp1 in the ejection of ODNs from the nucleus we made use of LMB, a potent inhibitor of Exp1 that covalently modifies a cysteine residue in its central conserved region (Kudo et al., 1999). Cells were pretreated with LMB for 12 hours before transfection with ODN-1-liposomes. Figure 3 shows comparison of the nuclear ejection kinetics of such

LMB-treated cells with untreated control. After 180, 240, and 300 minutes there were about 30% more ODNs in the nuclei of the treated cells vs. untreated. Two-tailed t -test probabilities (P -level) for the differences of means at these time points are <0.01 (marked with asterisks in Fig. 3). However, at the later time points most of ODN were still expelled from the nuclei of the treated cells. Therefore, we could confidently conclude that the treatment with LMB transiently slowed down the kinetics of ejection of the ODNs from nuclei.

Effect of ODN secondary structure on the ODNs nuclear uptake/ejection

We then tested the effect of ODNs secondary structure on its uptake to and ejection from the nuclei. As an example we used fluorescently labeled ODN-G4 containing 4 human telomeric repeats that were shown to form intramolecular quadruplex structures stabilized by 3 guanine tetrads under physiological conditions. An example of such quadruplex conformation that can be formed by ODN-G4 is shown on Fig. 4A (Gaynutdinov et al., 2008). Figure 4B shows that unlike ODN-1 6 hours after Lipofectamine delivery most of the ODN-G4 remained inside nuclei. The results of quantitative analysis of the kinetics of nuclear accumulation and expulsion of the quadruplex-forming ODN, and effects of Exp5 and Exp1 on these processes are shown in Fig. 5. As compared with unstructured single-stranded ODN-1 (control), quadruplex-forming ODN-G4 accumulated in the nuclei to a lower extent but remained there for a considerably longer time. In fact, quadruplex-forming ODN-G4 were localized almost exclusively in the nuclei even after 20 hours post-transfection. However, quantitative analysis at the longer time points was complicated by the fact that most of the treated cells became detached, most likely, due to toxicity of the telomeric ODNs (Longe et al., 2009).

Pretreatment of the cells with Exp1 inhibitor LMB had insignificant effect on the uptake/ejection kinetics of the

FIG. 4. (A) Schematic illustration of a possible G-quadruplex conformation of ODN-G4. Guanines in G_3 runs are numbered from 2 to 22 and are shown to form 3 stacked G-tetrads. (B) Accumulation of fluorescently labeled ODN-G4 in the nuclei of live HT1080 cells 6 hours after delivery.

FIG. 5. Uptake and efflux of fluorescently labeled ODN-G4 from the nuclei of live HT1080 cells. *Open circles*—no pretreatment; *Black triangles*—cells were pretreated with Exp5 siRNA; *Black squares*—cells were pretreated with leptomycin B; *Open diamonds*—efflux of ODN-1 as a control.

quadruplex-forming ODN-G4. So did the treatment with Exp5 siRNA to downregulate the *Exp5* gene. However, it could be noted that Exp5 knockdown leads to a lower overall ODN uptake. Nevertheless, the main conclusion from these experiments is that quadruplex-forming ODN-G4 was not effectively ejected from the nuclei.

ODN integrity inside cells

The above observation could have a trivial explanation if the ODNs became degraded in the course of our studies. Even though chemical protection of both the 3' and the 5' ends of ODNs was proven to be sufficient to preserve their integrity inside cells (Fisher et al., 1993), given the importance of this point for the interpretation of our data we carried out independent verification of the lack of ODN-1 degradation. Samples of the cells were collected immediately (0 hour) and 2, 4, and 8 hours after transfection, lysed, and subjected to polyacrylamide gel electrophoresis (Fig. 6A). The amount of the intact ODN-1 was determined by measuring the intensity of the corresponding bands and comparing them with that of the intensity of the bands of the control standards. As can be seen in Fig. 6B, a small amount of ODN-1 stuck to the cells almost immediately (0 hours), then the amount of intact ODN increased at 2 hours, and remained steady up to 8 hours. This proves that ODN-1 was not degraded upon ejection from the nuclei and that fluorescent label ejected from the nuclei at the later time point was still attached to intact ODN-1.

Discussion

Quick nuclear accumulation of ODN delivered into cells by various methods was reported previously (Leonetti et al., 1991; Fisher et al., 1993; Alam et al., 2008; Chen et al., 2009). Here, by monitoring the intracellular localization of fluorescently labeled ODNs within live cells at different times after delivery, we found that after an initial nuclear accumulation ODN-1 translocated into the cytoplasm within 6 hours. Such redistribution clearly indicates the presence of an active transport of ODNs inside cells; however, the mechanism(s) of ODN trafficking remains unclear.

We explore a possible involvement in ODNs transport of Exp1 and Exp5, two factors that are known to play role in miRNA, siRNA, and snRNA trafficking. We assessed the effect of downregulation of these factors on the kinetics of the ODN-1 nuclear efflux to the cytoplasm. In the case of miRNA, downregulation of these factors results in the significant ac-

cumulation of miRNA in the nucleus (Lund et al., 2004; Ohrt et al., 2006; Castanotto et al., 2009). Our results show that downregulation of Exp5 with siRNA resulted in only a modest increase in nuclear accumulation of ODN-1 but did not affect the kinetics of their translocation from the nucleus to the cytoplasm. Therefore, we conclude that Exp5 does not play a major role in the export of ODNs from the nucleus.

Inhibition of Exp1 with LMB resulted in somewhat slower kinetics of the efflux of ODNs from the nucleus. However, after 6 hours most of ODN were still transported from the

FIG. 6. Analysis of integrity of ODN-1 extracted from HT1080 cells after transfection. **(A)** Fluorescent image of 10% TBE-Urea denaturing gel; 4 lanes on the left have known amount of ODN-1 loaded shown on the top in fmol (Loading Standards); 8 lanes on the right contain ODN-1 extracted from the cells after number of hours indicated on the top (in duplicates). **(B)** Measurements of the amount of the ODN-1 purified from the cells based on the Loading Standards; error bars show SEM of duplicate measurements.

nucleus. Therefore, we conclude that Exp1 is not the major factor transporting ODN from the nucleus. Because Exp1 carries a wide variety of cargoes it is possible that its inhibition indirectly affected the export of ODN resulting in the observed modest slow down in the efflux.

The factors that export ODN recognize unstructured ssDNA. Formation of a secondary structure such as G-quadruplex completely abolishes nuclear export. This may be explained by the existence of specific targets inside the nucleus, such as telomeric overhang and/or G-quadruplex binding proteins (Lipps and Rhodes, 2009) that could retain ODN-G4. On the other hand, the lack of active efflux of G-quadruplex-forming ODNs may be by itself a reason for their high toxicity to cells (Longe et al., 2009). It is also possible that the secondary structure could prevent binding by export factors.

An important question is why there is transport of ODNs first to and then out of the nucleus. Most likely this mechanism is important for presentation of signaling ODNs after viral/bacterial degradation, DNA damage, etc., to the ODN-receptor proteins that initiate various cellular responses, such as, innate immunity, DNA damage responses, etc. Identification of these receptors/transporters is important for understanding of the mechanisms of such cellular responses.

From a practical standpoint, elucidation of the mechanisms of ODNs trafficking could help to improve delivery of ODNs to targets in nucleus for antigene approach or to the cytoplasm for antisense approach. A better understanding of the mechanisms of ODNs trafficking would help to improve both targeted delivery for therapies and understand cellular response to foreign DNA.

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Author Disclosure Statement

No competing financial interests exist.

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