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The nuclear export signal-dependent localization of oligonucleopeptides enhances the inhibition of the protein expression from a gene transcribed in cytosol

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ABSTRACT

Upon endocytosis, most oligodeoxynucleotides (ODNs) accumulate in vesicular compartments; a tiny number of them cross the vesicle membrane, reach the cytosol and by passive diffusion enter the nucleus where they are entrapped. So far, the compartment in which an antisense ODN interacts with its mRNA target has not been precisely characterized. In an attempt to answer this question, ODN-peptides were designed with the aim of maintaining them in the cytosol. This has been achieved by a short peptide sequence called the nuclear export signal (NES). Upon microinjection, ODN–NES peptide conjugates were efficiently and rapidly exported from the nucleus to the cytosol whereas ODN-peptides containing an inactive NES were found to be located in the nucleus. The inhibitory activity of antisense ODN was tested in a system allowing the specific transcription of a luciferase reporter gene in the cytosol. Antisense propynylated ODN–NES peptide conjugates, directed against the luciferase gene, efficiently inhibited (75%) the cytosolic expression of luciferase whereas at the same concentration the peptide-free propynylated ODN or the propynylated ODN-peptides containing an inactive NES were nearly inactive.

INTRODUCTION

Antisense oligodeoxynucleotides (ODNs) used as putative therapeutic agents, as stated in 1978 by Zamecnik and Stephenson (1), are supposed to interact selectively and in a predictable manner with a target RNA, and are routinely used to selectively prevent the expression of a given gene (reviewed in 2–4). The detailed mechanism of ODN uptake by cells has not yet been established. In most cells, ODNs, upon endocytosis, are entrapped in endosomal compartments and must then escape from them to reach the cytosol or the nucleus in order to encounter their target mRNA. ODNs injected into the cytosol by microinjection (5,6) or by using streptolysin-O (7) readily enter the nucleus and concentrate there. To explain the inhibition of expression induced by antisense ODNs, two main mechanisms have been suggested. The formation of a RNA–DNA duplex may exert a steric effect impeding either the binding of factors required for the initiation of translation or the translocation of ribosomes along the mRNA, resulting in chain termination. Alternatively, for a large proportion of active ODNs, the mRNA may be cleaved by endogenous RNase H at the site of the RNA–DNA duplex. Because RNase H is present in the nucleus and in the cytoplasm (8–11), the heteroduplex cleavage could occur both in the nucleus and in the cytosol. Nevertheless, the intracellular compartment where an ODN elicits its activity still remains to be determined.

To address this question, we developed a new approach to transport the ODNs in the cytosol by using a peptidic nuclear export signal (NES; reviewed in 12,13). Signals for rapid nuclear export have been found in the human immunodeficiency virus (HIV) Rev protein, transcription factor III A (TFIIIA), inhibitor of protein kinase A (PKI) (reviewed in 14) and more recently in cyclin B1 (15) and in actin (16).

It has been shown (17) that the leucine-rich core of the Rev activation domain acts as an NES. The relevant nine amino acid-long peptide was coupled to bovine serum albumin. This conjugate, injected into the nucleus of *Xenopus* oocytes as well as in that of various mammalian cultured cells, was rapidly exported from the nucleus into the cytosol. A similar conjugate containing an inactive NES did not leave the nucleus. However, because an ODN spontaneously leaves the cytosol to accumulate in the nucleus, it was not known and even not obvious that the NES in an ODN-peptide conjugate could be strong enough to efficiently counteract the spontaneous accumulation of such a small conjugate in the nucleus, resulting in an efficient sorting of the conjugate from the nucleus to the cytosol.

In this paper we report that, when an ODN-peptide containing an active NES domain (the Rev activation domain ALPP**LE**RLTL) was microinjected in the nucleus, it rapidly migrated to the cytosol, whereas a peptide-free ODN or an ODN-peptide containing an inactive analog (ALPP**DL**RLTL) did not leave the nucleus.

In order to determine the ability of an antisense ODN maintained in the cytosol to efficiently and specifically inhibit a gene expression, we made use of the T7 RNA polymerase which is devoid of a nuclear localization signal and does not enter the nucleus. The activity of this enzyme is therefore limited to the transcription of a gene when it is in the cytosol. We used a plasmid encoding luciferase as target and a C5-propyne pyrimidine modified phosphodiester ODN which

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binds its target mRNA with a high affinity, higher than its unmodified (propyne-free) ODN counterpart (18). Propynylated ODN directed against the coding region of the luciferase gene (19) substituted by the active NES inhibited the expression of cytosolic transcript luciferase, whereas the same ODN bound to the inactive NES or the peptide-free ODN were both nearly inactive. Such conjugates will be interesting tools to shed light on the mechanism of antisense ODN activity and putative improved drugs to inhibit cell infection by virus which are developing in the cytosol.

MATERIALS AND METHODS

Reagents

Paraformaldehyde was purchased from Merck (Darmstadt, Germany), 1,4-diazabicyclo[2-2-2]octane (DABCO) and polyethylenimine (PEI; MM 800 000) from Aldrich (Saint Quentin Fallavier, France), polyacrylamide from Bio-Rad (Oxfordshire, UK), Tris(2-carboxyethyl)phosphine (TCEP) and fluorescein-5-isothiocyanate (FITC; Isomer I) were from Molecular Probes (Leiden, The Netherlands). Leptomycin B was kindly provided by Dr Y. Wang (Novartis, Basel, Switzerland). The plasmids pAR3126 and pEMCLucβgAn were kindly provided by Dr J. A. Wolff (Waisman Center, Madison, WI).

The ODN used for the intracellular localization studies is a phosphodiester-type pentacosamer: 5'-CTC TCG CAC CCA TCT CTC TCC TTC T-3', specific for a sequence encompassing the AUG of the gag_{HIV}, kindly provided by Dr U. Asseline (CBM-CNRS, Orléans, France). Its 5'-end was substituted with an alkyl dithiopyridine moiety, and its 3'-end with an alkylamine leading to: pyridyl-S-S-R_1-$^{5'}$(ODN)$^{3'}$-R_2-NH_2, where R_1 is $(CH_2)_6$-O-PO_2^- and R_2 is PO_2-O-$(CH_2)_6$. These modifications protect the phosphodiester ODN from exonuclease degradation and allow further substitution with a fluorescent tag and/or a peptide.

The ODN used for biological activity studies, specific for a sequence in the coding region of the luciferase gene, is a C-5 propyne modified-pyrimidine phosphodiester-type octadecamer (propyne-ODN) 5'-GUA CGU GAU GUU CAC CUC-3' and the control is a non-specific propynylated ODN 5'-CGC CUG CUG UCC UGC GUC UCC-3' (Eurogentec, Seraing, Belgium). The pyrimidine bases of the ODN were modified and the methyl group of thymine and of 5-methyl cytosine were replaced with a propynyl group. As described above, their 5'- and 3'-ends were substituted with an alkyl dithiopyrydine moiety and with an alkylamine, respectively.

Peptide synthesis

The active NES peptide ALPP**LE**RLTL and the inactive NES analog ALPP**DL**RLTL (17) (alanine was designed as a spacer between the ODN and the peptide) were prepared by using a solid phase peptide synthesizer Applied 433 A (Applied Biosystems, Foster City, CA) using the Fmoc strategy starting from a 4-(hydroxymethyl)-phenoxymethyl resin (0.96 mmol/g, Applied Biosystems) and monitoring the coupling efficiency by conductimetry. At the last step, bromoacetic anhydride reacted with the α-amino group leading to N_α-(bromoacetyl)-protected peptide-resin (20,21). The peptides were further deprotected and released from the resin by incubation in a trifluoroacetic acid/water/phenol mixture (90:5:5 per volume) for 3 h. After precipitation and washing with *tertio*-butyl methyl ether, the peptides were dried under reduced pressure. The peptides were characterized by matrix assisted laser desorption ionization/time-of-flight mass spectrometry using a Finnigan MAT Lasermat 2000 instrument (San Jose, CA) and α-cyano-4-hydroxycinnamic acid as a matrix (Aldrich). Their purity was checked by capillary electrophoresis on a P/ACE 5500 instrument (Beckman, Fullerton, CA) equipped with a P/ACE diode array detector using a fused-silica capillary equilibrated in sodium borate buffer pH 8.6 (Beckman Kit). Capillary electrophoresis showed that the peptides were quite pure (data not shown), so further high performance liquid chromatography (HPLC) purification was not requested. The mass spectrometry data, 1244.6 and 1230.5 Da, respectively determined for the generic active NES peptide ALPP**LE**RLTL and for the inactive analog ALPP**DL**RLTL, were in agreement with the calculated mass: 1244.4 and 1230.4 Da, respectively.

ODN-peptide synthesis

The ODNs, propynylated or not, (2 mg, 250 nmol) were dissolved in 2 ml of 0.2 M sodium carbonate buffer pH 9.3 containing 2 M sodium acetate. FITC (4 mg, 10 μmol, in 2 ml acetone) was added, and the reaction performed for 12 h at 37°C under stirring. The fluorescent ODN was precipitated, upon adding 2.5 vol of ethanol, at –20°C for 30 min. The precipitate was dissolved in 1 ml of distilled water and loaded onto a Biogel P2 (25 × 2 cm) column (Bio-Rad, Oxfordshire, UK), eluted with water containing 5% *n*-butanol, to remove the remaining fluorescein. The first fluorescent peak eluted, corresponding to the fluorescein-labeled ODN (F-ODN), was collected and freeze-dried.

The F-ODN was further dissolved in 1 ml of 50 mM sodium phosphate buffer pH 7.0 containing 2 M sodium acetate, degassed upon freezing under low pressure, and reduced with 1.1 equivalent of TCEP (79 μg in 7.9 μl sodium phosphate buffer, pH 7.0) under argon for 30 min at 25°C. Then the bromoacetyl-peptide (5 equivalents, 1.5 mg in 0.5 ml of the same buffer) was slowly added. At 3 h intervals during the first 9 h, TCEP (0.5 equivalent, 36 μg) and NES-peptide (5 equivalents, 1.5 mg) were added. After 20 h, TCEP (0.5 equivalent) and NES peptide (5 equivalents) were further added, and the solution was stirred for 4 h. A total of 25 equivalents of NES peptide were added to the F-ODN or to the fluorescein-free propynylated ODN in order to prepare ODN–peptide conjugate used for the biological activity study; the coupling reaction was monitored by HPLC.

HPLC analyses were carried out using a Waters system (Saint Quentin en Yvelines, France) with Millennium software on a Nova-Pack C-18 (Waters) column (3.9 × 150 mm; particle size: 4 μm), by using a linear gradient of 5–30% solvent B in solvent A over 30 min [flow rate: 1 ml/min; solvent A: 95% 0.1 M triethylammonium acetate (TEAA), pH 7.0 + 5% acetonitrile; solvent B: 5% 0.1 M TEAA, pH 7.0 + 95% acetonitrile]. The elution was monitored by UV absorbance, using a Waters photodiode array detector. The F-ODN-peptide was then precipitated by adding 2.5 vol of ethanol. The precipitate was dissolved in 500 μl distilled water before purification by HPLC on a LiChrospher 100 RP-18 E (Merck) column (10 × 250 mm; particle size: 10 μm). The F-ODN-peptide obtained was characterized by mass spectrometry and by a polyacrylamide (15%) gel electrophoresis stained with methylene blue.

Confocal microscopy analysis of microinjected somatic cells

HeLa cells or Vero cells (10^5 cells/well) were plated onto CELLocate microgrid coverslips (Eppendorf, Hamburg, Germany) and cultured for 24 h in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (complete medium). F-ODN or F-ODN-peptide (40 μM) were injected (injection time 0.5–1 s) either into the nucleus or into the cytosol by using a microinjector 5242 and a micromanipulator 5170 (Eppendorf, Hamburg, Germany). Cells were incubated for 30 min at 37°C (or at 4°C in the low temperature experiments), in complete medium, with 5% CO_2 in a humid atmosphere and were then washed and fixed at 37°C (or at 4°C) with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. In inhibition experiments with leptomycin B, cells were incubated at 37°C, in the absence or in the presence of leptomycin B (100 ng/ml) for 2 h before microinjection. Cells were maintained further under leptomycin B for 30 min and then fixed as described above. Cells were mounted in PBS/glycerol (1:1 per volume) containing 1% DABCO as an antifading agent (22) and observed with an MRC 1024 confocal microscope (Bio-Rad) equipped with a Nikon Optiphot epifluorescence microscope (Nikon, Tokyo, Japan) and 60× Planapo objective (numerical aperture 1.4). The analyses were achieved by using photocounting mode and ~20 accumulations were performed.

Biological activity

HeLa cells were co-transfected with pAR3126 (23) which contains the T7 RNA polymerase gene under the control of the SV40 large T antigen promoter and pEMCLucβgAn (24), which contains the luciferase gene controlled by the T7 promoter but using PEI (25): 47 μl of 10 mM PEI were mixed to 15.6 μg of pAR3126 and to 15.6 μg of pEMCLucβgAn in 1.25 ml of serum-free DMEM for 10 min; then 8.75 ml serum-free DMEM were added. HeLa cells, cultured in 75 cm^2 flasks, were washed with PBS and the mixture was added. After 2 h, the supernatant was removed; cells were washed with PBS and treated with PBS/trypsine/EDTA (PBS with 200 μg/ml EDTA and 2.5 μg/ml trypsin) at 37°C for 5 min and harvested.

Cells were washed twice with PBS containing 10 mM HEPES pH 7.4 (PBS/HEPES); 5×10^6 HeLa cells were suspended in 250 μl PBS/HEPES at 4°C. The ODNs were added to give a final concentration of 2 μM. The cell suspension was transferred to an ice-cold 0.4 mm gap electroporation chamber (Bio-Rad), and cells were permeabilized by a single pulse from a Gene Pulser attached to an optional capacitance extender (Bio-Rad) set to 960 μF, 270 V. These conditions resulted in an electroporation time constant of ~40 ms. Cells were further incubated for 15 min at 4°C in the electroporation chamber and then 1.5×10^5 cells/well were transferred in a 24-well tissue culture plate. After 20 h incubation, non-adherent cells, corresponding to dead cells, were discarded and the luciferase gene expression was measured by using the method of De Wet *et al*. (26). The luminescence was recorded for 4 s with a luminometer (Lumat LB 9501, Berthold, Wildbach, Germany), 1 s after automatic injection of luciferine and reported as relative light units (RLU) per 10^6 cells of adherent living cells.

RESULTS AND DISCUSSION

While ODNs which prevent splicing act as gene expression inhibitors in the nucleus, it is not yet known whether ODNs elicit their activity in the nucleus or in the cytosol. ODNs, internalized into cells by endocytosis, have to leave the vesicular endocytic compartments to reach the cytosol, and from there, they rapidly enter into the nucleus by diffusion (reviewed in 4,27); how and where ODNs are released from endosomal compartments is unknown (28). Most of the biological effects of antisense ODNs were obtained by using cationic lipids to help the ODNs delivery into cells (29,30). Although cationic lipids did not increase the intracellular concentrations more than permeabilization by streptolysin-O or electroporation (31), the concentration of required ODNs to inhibit gene expression was lower with cationic lipids (1 μM) than that required (20 μM) with streptolysin-O or electroporation (31,32). One explanation of this paradoxical result is that ODNs microinjected into the cytosol in order to bypass endocytosis rapidly localized within the nucleus (5,6), whereas a large number of ODNs remain associated with cationic lipids in the cytoplasm, leading to a delay in their release and accumulation in the nucleus (30).

Although ODNs rapidly migrate to the nucleus, it is unlikely that they exert their action solely in the nucleus. Indeed, antisense activity has been observed with ODNs targeted to vesicular stomatitis virus, whose replication cycle entirely takes place in the cytoplasm (33), and the ODN retention in the cytosol has been suggested to be positively correlated with its activity (34). It is possible that the transfer from endocytic vesicles to the cytosol may be the limiting step but the relationship between the subcellular distribution and the ODN activity remains unknown. To address this question we synthesized ODN-peptide conjugates containing an NES and determined initially the capacity of such conjugates to counteract the spontaneous entrapement of the ODNs in the nucleus, and secondly the capacity of such conjugates to inhibit the translation of mRNA specifically produced in the cytosol.

RNAs exit the nucleus through the nuclear pore. In both the nucleus and the cytoplasm, RNAs are complexed with proteins, some of them providing the signal for nuclear export. One of the compelling indications that some proteins mediate RNA export came from HIV-1. The protein Rev, which recognizes a specific HIV RNA sequence, the Rev Response Element, mediates export of the unspliced HIV RNAs. The NES, identified within the Rev protein, is a leucine-rich peptide, LPP**L**E**L**RLTL (17,35). This leucine-rich NES-mediated export is saturable (17). This property of NES-mediated protein export indicates that specific receptors recognize NES and mediate the rapid export of a NES-bearing protein to the cytoplasm. Recently, Chromosome Region Maintenance 1 (CRM1) was found to be a nuclear export receptor and has been renamed exportin-1 (36,37). In addition, it was shown that leptomycin B, which blocks the nucleocytoplasmic translocation of the HIV Rev protein and Rev-dependent mRNA (38), interacts directly with CRM1. The present work is based on this nuclear export mechanism making use of the Rev NES motif.

Synthesis of F-ODN-peptide

N_α-bromoacetyl-peptides and ODN 5'-end-substituted with an alkyl dithiopyridine moiety and 3'-end-substitued with an

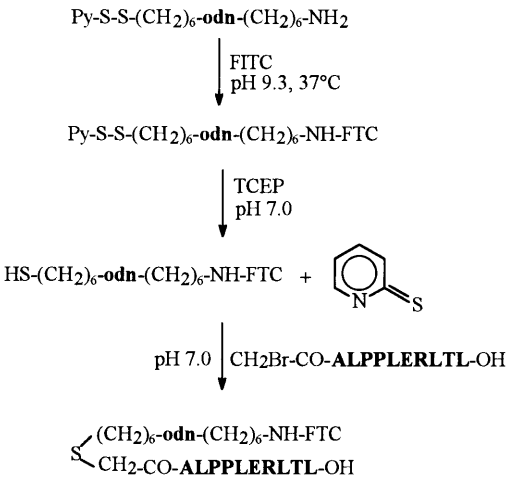

Figure 1. Scheme of the synthesis of an F-ODN-peptide conjugate. FITC reacted on 3'NH2 ODN in 0.2 M sodium carbonate buffer at pH 9.3 containing 2 M sodium acetate at 37°C; the disulfide bridge was reduced with TCEP in a 50 mM sodium phosphate buffer at pH 7.0 containing 2 M sodium acetate; the free thiol group released reacted with an Nα-bromoacetyl-NES peptide leading to the expected ODN-peptide conjugate through a thioether bond. The NES peptide sequence used was ALPPLERLTL according to the one letter abbreviation code.

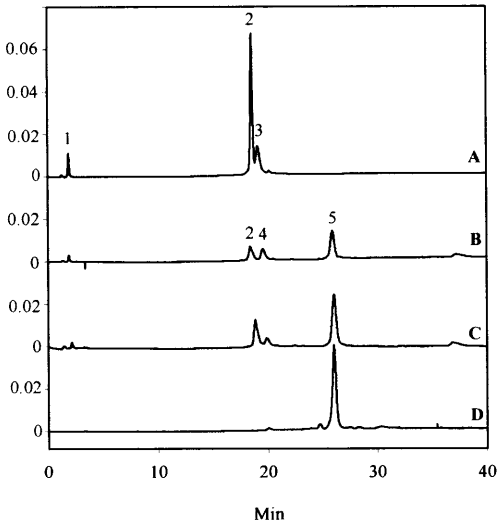

Figure 2. HPLC analysis of the formation of a F-ODN-peptide (active NES) using the conditions described in the Materials and Methods. (A) F-ODN after reduction with TCEP; (B) after 12 h reaction; (C) after 20 h reaction; (D) purified F-ODN-peptide. **1** is the released pyridine 2-thione, **2** is the reduced F-ODN, **3** is the non-reduced F-ODN, **4** is a reducible product and **5** is the expected F-ODN-peptide.

alkylamine were used for the synthesis of the ODN-peptide conjugate. The synthesis of the F-ODN-peptide conjugate was conducted in three steps (Fig. 1).

The first step was a fluorescein tagging of the 3'NH_2 ODN; this reaction was quantitative. This step was omitted for the

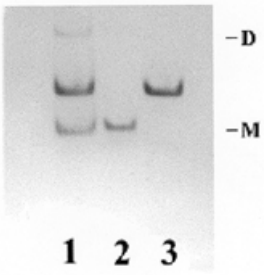

Figure 3. Polyacrylamide gel electrophoresis of ODN and derivatives. F-ODN migrated for 3 h on a 10 cm long 15% polyacrylamide gel, under non-denaturing conditions. Lane 1 is the mixture of F-ODN, peptide and F-ODN-peptide after 24 h reaction. Lane 2 is the HPLC purified F-ODN and lane 3 is the HPLC purified F-ODN-peptide. ODNs were stained with methylene blue, peptides being not stained under these conditions. M and D indicate the monomeric and the dimeric ODN, respectively.

preparation of conjugates used in biological assays. The second step involved a deprotection of the thiol group by using a slight excess of TCEP (1.1 equivalent) which releases pyridine thione (Fig. 2A, peak 1). The third step was the coupling between the F-ODN and the N_α-bromoacetyl-peptide leading to a conjugate with a thioether bond, stable under reducing conditions; this reaction was monitored by HPLC (Fig. 2). The retention time of the conjugate was 8 min after the reduced F-ODN. The reaction progressed very slowly; further additions of TCEP and of NES peptide led, after 12 h, to the formation of ~50% of conjugate (Fig. 2B); after 20 h ~70% of conjugate was formed (Fig. 2C). Despite another addition of TCEP and NES peptide the reaction did not progress. The successive additions of TCEP were required because such an S–S bridge requires an excess of TCEP to be cleaved, in contrast with the stoichiometric reaction when TCEP acts on a dithiopyridine derivative, and because the reduced ODN spontaneously dimerizes, as shown after electrophoresis. The F-ODN-peptide conjugate was purified by HPLC leading to a pure compound, as shown by HPLC (Fig. 2D) and by polyacrylamide gel electrophoresis (Fig. 3, lane 3). The electrophoretic mobility of the ODN-peptide conjugate was, as expected, lower than that of the ODN monomeric form but faster than that of the dimeric form.

Fate of the F-ODN-peptide upon injection into cells

The purpose of the present investigation was to determine whether a small molecule such as an ODN, which spontaneously leaves cytosol to enter into the nucleus, could be enforced to stay in or to concentrate in the cytosol, thanks to an NES device. Two techniques were selected for direct introduction of ODNs into the cells: microinjection for microscopy analyses and electroporation to assess the biological effects. The fluorescein-labeled phosphodiester ODN, upon injection either into the nucleus (Fig. 4A) or into the cytosol (Fig. 4B), rapidly localized in the nucleus, in agreement with previous findings (5,6). The ODN–NES peptide conjugate, injected into the cytosol of HeLa cells, stayed in the cytosol (Fig. 4D); injected into the nucleus, it was rapidly (within 30 min) and efficiently exported from the nucleus to the cytosol (Fig. 4C). Conversely,

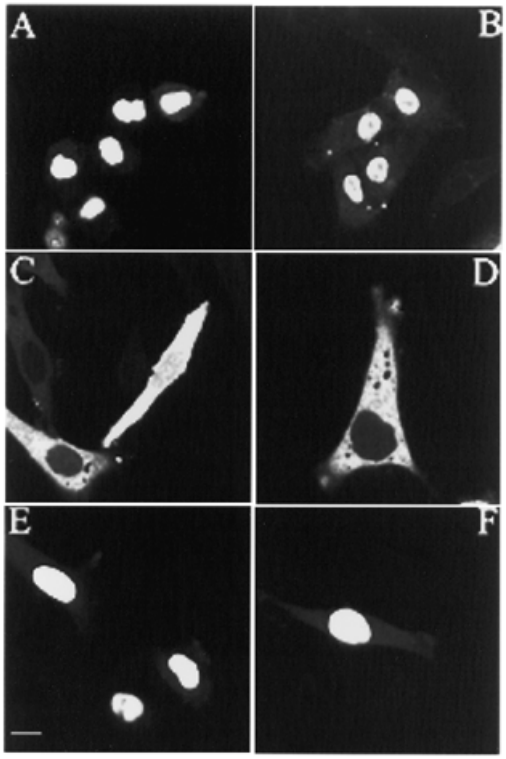

Figure 4. Intracellular localization of F-ODN and of F-ODN-peptide conjugate. HeLa cells were microinjected with the F-ODN either in the nucleus (**A**) or in the cytosol (**B**), and with F-ODN-peptide either in the nucleus (**C**) and (**E**) or in the cytosol (**D**) and (**F**); upon injection, cells were incubated for 30 min at 37°C in DMEM containing 10% FBS. In (C) and (D), the linked peptide was the active NES peptide and in (E) and (F), the linked peptide was the inactive NES analog. Cells were fixed with 2% paraformaldehyde and observed with a confocal microscope. Scale bar, 10 μm.

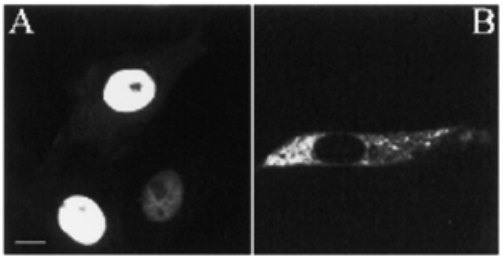

Figure 5. Intracellular localization of F-ODN–NES peptide conjugate. Vero cells were microinjected with F-ODN–NES peptide conjugate in the nucleus and then incubated for 30 min at 4°C (**A**) or at 37°C (**B**) in DMEM containing 10% FBS. Cells were fixed and then observed by using a confocal microscope. Scale bar, 10 μm.

when the ODN-peptide bearing an inactive NES analog was injected into the nucleus it remained in the nucleus (Fig. 4E), while upon injection into the cytosol, it was rapidly accumulated in the nucleus (Fig. 4F). These results suggest that the export of an ODN-peptide from the nucleus to the cytosol mediated by an NES peptide is more efficient than its spontaneous diffusion and entrapping into the nucleus. Similar results were obtained with other cell types including Vero cells, monkey kidney epithelial cells (Fig. 5) or A549 cells, human non-small cell lung carcinoma cells. Propynylated phosphodiester ODN-peptides behave in a similar manner (data not shown).

The total amount of ODN-peptide injected in a cell was controlled by the duration of the injection time. Using a constant ODN-peptide concentration, a short injection time (0.5–1 s) in the cell allowed the ODN–NES peptide conjugate to be excluded from the nucleus within 30 min. When the amount of injected ODN was much higher, the ODN–NES peptide conjugate appeared also in the nucleus. The total amount of injected ODN or ODN-peptide in cells under identical conditions did fluctuate from one cell to another. As an example, in Figure 4C, two injected cells did not receive the same amount: clearly, the cell containing more ODN-peptide had a labeled nucleus whereas the cell containing less ODN-peptide had a quite dark nucleus. Conversely, the nuclear localization of the ODN-peptides with an inactive NES was independent of the injected amount (see for instance cells in Fig. 4E). Therefore, an ODN-peptide with an inactive NES behaves as a peptide-free ODN, spontaneously migrating from the cytosol to the nucleus by a non-saturable mechanism. In contrast, an ODN-peptide with an active NES counterbalances the spontaneous diffusion from the cytosol to the nucleus by a saturable exportation from the nucleus to the cytosol.

This interpretation is further supported by the differential temperature-dependent behaviour of ODN–NES peptide conjugate. While the exportation from the nucleus to the cytosol was quite efficient at 37°C (Fig. 5B), it was inefficient at 4°C (Fig. 5A), suggesting that the nuclear export process of ODN-peptide is energy dependent. At this point, it is clear that the exit of the ODN–NES peptide conjugate from the nucleus must depend on an active exportation process. However, the retention of such ODN-peptides in the cytosol could also be related to an entrapping of the NES peptide on cytosolic materials.

The active exportation process is clearly dependent on the known nuclear export mechanism because of the inhibitory action of leptomycin B, a specific inhibitor of the NES dependent nuclear export (38). Cells incubated in the presence of leptomycin B were unable to export the ODN–NES peptide conjugates from the nucleus to the cytosol. This stands for all the tested cells, including HeLa cells, A549 cells and Vero cells. This point is illustrated with Vero cells which have been pre-incubated in the absence (Fig. 6B) or in the presence (Fig. 6A) of leptomycin B. As expected, the localization of the ODN-peptide bearing an inactive NES analog was not dependent on the presence of leptomycin B. The ODN-peptide remained in the nucleus with (Fig. 6C) or without (Fig. 6D) leptomycin B.

Biological activity of ODN-peptides

In order to assess the capacity of such an ODN-peptide conjugate to inhibit the expression of a gene in the cytosol, we used a system allowing the cytosolic transcription of a gene. The T7 RNA polymerase, which lacks a nuclear localization signal and does not enter the nucleus, allows a strictly restricted cytosolic transcription (39,40). Two plasmids were used, one containing the T7 RNA polymerase gene under the control of SV40 large T antigen promoter and the other containing the luciferase gene

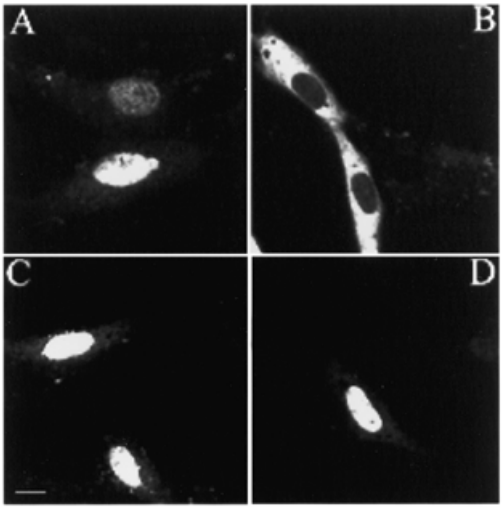

Figure 6. Effect of leptomycin B on the intracellular localization of F-ODN-peptide conjugate. Vero cells were microinjected in the nucleus with F-ODN-peptide and incubated for 30 min at 37°C in DMEM containing 100 ng/ml of leptomycin B (**A**) and (**C**) or without leptomycin B (**B**) and (**D**). In (A) and (B), the peptide moiety was the active NES peptide; in (C) and (D), the peptide moiety was the inactive NES analog. Cells were fixed with 2% paraformaldehyde and observed with a confocal microscope. Scale bar, 10 μm.

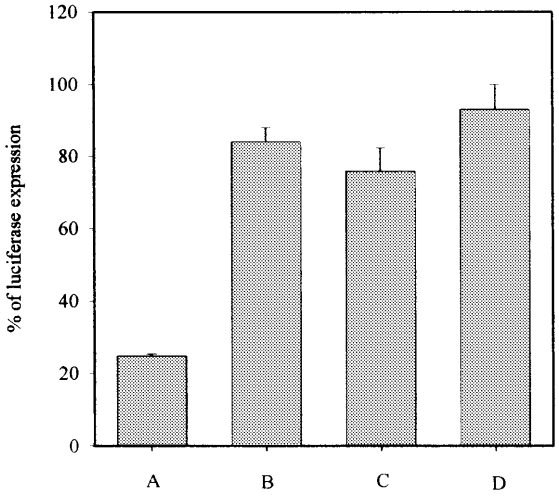

Figure 7. Biological activity of propynylated ODN–NES peptide conjugate. HeLa cells were co-transfected with pAR3126 and pEMCLucβgAn using PEI. After 2 h cells were then electroporated in the presence of 2 μM ODN or ODN-peptide and plated for 16 h before measuring the gene expression by assaying the luciferase activity of cell lysate. The percentage of luciferase expression is related to the number of RLU in living cells electroporated in the absence of ODN. HeLa cells were electroporated in the presence of the specific ODN–NES peptide conjugate (A), the peptide-free ODN specific for the luciferase mRNA (B), the specific ODN-peptide conjugate bearing the inactive NES analog (C) or the non-specific ODN–NES peptide conjugate (D).

controlled by the T7 promoter (23,24). Transfection of cells with a plasmid encoding the luciferase in the absence of a plasmid encoding the T7 RNA polymerase led to a negligible expression of luciferase (data not shown), supporting the fact that the expression occurred in the cytosol and not in the nucleus.

Propynylated phosphodiester ODNs directed against a coding region of the luciferase gene (19) were chosen because phosphodiesters were rapidly degraded inside the cells and were found to be inactive. Phosphorothioates, as well as propynylated phosphorothioates, were strongly associated with both cytosolic and/or nuclear proteins and led to a non-specific inhibition (reviewed in 41,42). Propynylated phosphodiester ODNs, which have a higher affinity for their target mRNA than their unpropynylated counterparts (18), appeared in preliminary experiments to be more stable than their corresponding phosphodiester ODNs substituted at both ends to avoid exonuclease degradation.

After co-transfection of the two plasmids with the help of polyethylenimine (25), the ODN or the ODN-peptide (2 μM) were introduced in the cells by electroporation. The ODN–NES peptide conjugate inhibited (75%) the expression of luciferase, whereas the peptide-free ODN as well as the ODN-peptide containing an inactive NES analog were nearly inactive (Fig. 7). As a control, a non-specific propynylated ODN, which did not hybridize with the luciferase mRNA, substituted with the same active NES peptide did not inhibit the expression of luciferase.

In conclusion, these data demonstrate that a NES peptide, which was first shown to be efficient in protein export (17,35), is also efficient in the export of small molecules. The ODN–peptide–NES conjugates concentrate in the cytosol and are efficient to inhibit the expression of a gene transcribed and translated in the cytosol. Such conjugates are interesting tools to shed light on the mechanism of antisense ODN activity. Based on the ability of an active NES peptide to maintain an ODN in the cytosol, it is now possible to determine whether a given ODN elicits its activity on a cellular gene by encountering its target in the cytosol or in the nucleus. Experiments along this line are currently in progress in our laboratory. Moreover, such conjugates could be used to efficiently inhibit viruses which have their replicative cycle in cytosol or which have to be retrotranscribed in the cytosol immediately after their penetration in cells, as in the case of retroviruses and especially AIDS viruses.

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