Gene delivery: A single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus

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Communicated by Jean-Marie P. Lehn, Université Louis Pasteur, Strasbourg, France, November 3, 1998 (received for review September 9, 1998)

ABSTRACT Translocation of exogenous DNA through the nuclear membrane is a major concern of gene delivery technologies. To take advantage of the cellular import machinery, we have synthesized a capped 3.3-kbp CMVLuciferase-NLS gene containing a single nuclear localization signal peptide (PKKKRKVEDPYC). Transfection of cells with the tagged gene remained effective down to nanogram amounts of DNA. Transfection enhancement (10- to 1,000-fold) as a result of the signal peptide was observed irrespective of the cationic vector or the cell type used. A lysine to threonine mutation of the third NLS amino acid completely abolished these remarkable features, suggesting importin-mediated translocation. Our hypothesis is that the 3-nm-wide DNA present in the cytoplasm is initially docked to and translocated through a nuclear pore by the nuclear import machinery. As DNA enters the nucleus, it is quickly condensed into a chromatin-like structure, which provides a mechanism for threading the remaining worm-like molecule through the pore. A single NLS signal is thus sufficient, whereas many signals on a gene would actually inhibit entry, the same DNA molecule being threaded through adjacent pores.

Several attempts to improve entry of plasmid DNA into the nucleus have been published. These include use of electrostatic binding of DNA to cationic NLS-containing proteins (11, 12) or peptides (13) or lipids (14), as well as sequence-specific binding of DNA to karyophilic proteins (15–17). Yet such “piggyback” nuclear transport relies on the unpredictable stability of the complexes within the cytoplasm. Recent work by the group of Jon Wolff (18) with digitonin-permeabilized cells demonstrated nuclear accumulation of fluorescently labeled DNA that was randomly tagged with hundreds of NLS peptides; nuclei of intact cells did not take up the modified DNA. Our approach took advantage of a chemically controlled pathway to irreversibly link a single NLS-peptide to one end of a gene. Moreover, following Heisenberg’s principle, we avoided taking a too close look at nuclear import using potentially disturbing techniques. The goal being gene delivery, the level of transgene expression after transfection was chosen as the most pertinent test of success.

EXPERIMENTAL PROCEDURES

Chemicals, Enzymes, and Oligonucleotides. 4-(N-Maleimidomethyl)cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester (SMCC) was purchased from Sigma. The SAL 34-mer 5′-d(TCCATGTCCCGTTGCTTGCAACCGGA-CA) oligodeoxynucleotide was synthesized by Appligene by using an amino-modified deoxythymidine (X; amino-modified dT, Glen Research, Sterling, VA). The XMA 34-mer 5′-d(CCGCTACCTTGGAGCTTTTGCTGCAAGGTAG) oligodeoxynucleotide, the NLS peptide NH2-PKKKRKVEDPYC, and the mutated-NLS peptide NH2-PKTKRVDPYC with C-terminal amidation were synthesized by Genosys (The Woodlands, TX). Hairpin structures composed of a loop of four thymines, a stem of 13 bp, and a sticky 5′ end were formed by boiling and subsequently cooling the XMA or the SAL oligonucleotides in ice. XmaI, XmnI, Sall, and BspHI restriction endonucleases, T4 polynucleotide kinase, T4 DNA ligase, and exonuclease III were purchased from New England Biolabs. Linear 22-kDa (ExGen500) and branched 25-kDa polyethyleneimines (PEI) were purchased from Euromedex (Souffelwyersheim, France) and Fluka (Saint-Quentin Falfivier, France), respectively. Transfектant (dioctadecylamido glycylylpermine) was synthesized as described (19).

Preparation of the Oligonucleotide-PEptide Conjugate. Two A260 units (6.46 nmol, assuming ε260 = 309,800 M−1 cm−1) of the amino-modified SAL oligonucleotide in 20 μl of phosphate buffer [10 mM sodium phosphate (pH 7.5)] was mixed with a 40:1 molar excess of the bifunctional crosslinker SMCC in dimethylformamide (as a 30 mM stock solution) and incubated

Abbreviations: NLS, nuclear localization signal; SMCC, 4-(N-maleimidomethyl)cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester; PEI, polyethyleneimine; N/P, nitrogen/phosphate.

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† Communicated by Jean-Marie P. Lehn, Université Louis Pasteur, Strasbourg, France, November 3, 1998 (received for review September 9, 1998)

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at room temperature for 2 h. Excess SMCC was removed with a Nick-Spin column (Amersham-Pharmacia) that had been equilibrated with PBS. The recovered oligonucleotide solution (100 μl) was immediately reacted with a 10-fold molar excess of NLS-peptide (or mutated-NLS-peptide) overnight at room temperature and then stored at −20°C. The oligonucleotide-peptide conjugate was purified by preparative PAGE. The coupling yield was 30%, based on quantification of the radiolabeled oligonucleotides after migration in a 20% denaturing polyacrylamide gel. To assess the peptide content, 1 pmol of labeled oligonucleotides was purified by preparative PAGE. The purified oligonucleotides were added to cell culture plates (Costar) to reach 60–70% confluence during transfection. Before transfection, cells were rinsed and 0.2 ml of fresh culture medium supplemented (transfection in the presence of serum) or not (transfection in the absence of serum) with 10% fetal calf serum was added to each well. The desired amount of DNA was diluted into 46 μl (final volume) of 0.15 M NaCl or 5% glucose. The desired quantity of ExGen500, 25-kDa PEI, or Transfектам (from a 1 mM aqueous amine nitrogen stock solution of PEI or a 2 mM ethanolic stock solution of Transfектам) was then added to the DNA-containing solutions, vortex-mixed gently, and centrifuged. After 10 min, volumes corresponding to 10, 20, or 200 ng of CMVLuc fragment (10 ng/μl of DNA) or to the gene-number-corrected amount of plasmid DNA were added to the cells. After 2–3 h, 20 μl of fetal calf serum was added to the serum-free wells. Cells were cultured for 24 h and tested for reporter gene expression. All experiments were done in duplicate.

**Luciferase Assay.** Luciferase gene expression was measured by a luminescence assay. The culture medium was discarded and cell lysate was harvested after incubation of cells for 30 min at room temperature in 100 μl of Lysis Reagent 1× (Promega). The lysate was vortex-mixed gently and centrifuged for 5 min at 14,000 rpm at 4°C (Sigma 3K10). Twenty microliters of supernatant was diluted into 100 μl of luciferase reaction buffer (Promega) and the luminescence was measured by a luminescence assay. The culture medium was discarded and cell lysate was harvested after incubation of cells for 30 min at room temperature in 100 μl of Lysis Reagent 1× (Promega). The lysate was vortex-mixed gently and centrifuged for 5 min at 14,000 rpm at 4°C (Sigma 3K10). Twenty microliters of supernatant was diluted into 100 μl of luciferase reaction buffer (Promega) and the luminescence was measured by a luminescence assay.
RESULTS

Synthesis of a Capped Gene-Peptide Conjugate, Purification, and Proof of Structure. The construction was based on ligation of a pair of hairpin oligonucleotides to unique cohesive termini generated on the reporter gene (Fig. 1). Incorporation of $^{32}$P into the modified oligonucleotide allowed us to follow the reaction kinetics, to purify the fragment of interest, and to verify its structure. The firefly luciferase reporter gene (Luc) flanked by the cytomegalovirus (CMV) enhancer/promoter sequence and the simian virus 40 polyadenylation signal was excised from the pCMVLuc plasmid (Fig. 1). Quadruple endonuclease digestion ensured straightforward large-scale separation of the 3,380-bp CMVLuc fragment from the remaining <1-kbp fragments by ultracentrifugation through a 15–30% sucrose gradient. T4 loops are well suited for hairpins and the C-terminal thiol group of the PKKKRKVEDPYC peptide was conjugated to a thymine with a C(5) amino group through an activated ester/maleimide bifunctional linker (SMCC) as detailed in Fig. 2. Preliminary experiments showed that the global reaction yield was highest after activation of oligonucleotide-NH$_2$ with SMCC for 2 h (significant parasitic maleimide hydrolysis may occur with time). The 5' $^{32}$P radiolabeling and polyacrylamide gel electrophoresis of oligonucleotides (Fig. 3A) showed the peptide conjugation reaction to be completed after 3 h, giving 30% oligonucleotide-NLS, based on the starting oligonucleotide. Proteinase K digestion converted oligonucleotide-NLS to a faster migrating compound, presumably the oligonucleotide conjugated to the C-terminal amino acid (oligonucleotide-Mal-Cys), thus establishing the chimeric nature of the conjugate (Fig. 3B). The oligonucleotide-NLS was purified by electrophoresis. The uncapped CMVLuc fragment was then simultaneously reacted with a 15-fold excess of oligonucleotide-cap and oligonucleotide-NLS by using T4 DNA ligase in previously optimized conditions. Ligation reaction yield (80–90%) was assessed by quantitative radioactivity counting and full capping of the CMVLuc fragment was checked by 3' exonuclease digestion. CMVLuc-NLS was purified by gel permeation.

Transfection with Small Amounts of DNA. The reporter gene construct was obtained by chemical/enzymatic synthesis and purified by PAGE/gel permeation, instead of being amplified in bacteria. Only limited amounts of DNA could, as expected, be transfected. In this case, the oligonucleotide-NLS was purified over 10 sec (Mediators, Wien, Austria). Results were expressed as light units per mg of cell protein (BCA assay, Pierce).
followed with fluorescent tags and fluorescence.

Nuclear import of DNA and oligonucleotides has mainly been put forward any hypothesis concerning the mechanism. Comparative experiments between CMVLuc and two other cell types (Table 1, entry 4, and Fig. 4), thus putting our ultimate goal to obtaining an effective transfection with less than 200 ng of DNA. As a preliminary test, we looked at the relevance to cap the reporter gene at both ends, thus avoiding free DNA ends that are prone to exonuclease-mediated DNA degradation and to eliciting a DNA repair response. To this end, the uncapped, hemicapped, and fully capped CMVLuc genes (200 ng) were transfected into hepatocytes. Luciferase activities (Table 1) showed both un- and hemicapped molecules to give 25-fold less gene expression than the capped one thus justifying the choice of our chemical strategy.

The NLS Peptide Allows Effective Transfection with Minute Quantities of DNA. 3T3 cells were transfected with decreasing amounts of DNA—the NLS-bearing capped gene (CMVLuc-NLS), the capped gene (CMVLuc), and the corresponding mass-corrected amount of plasmid DNA (pCMVLuc). Although efficacy very much decreased for “20-ng” plasmid DNA (in fact 38 ng), as expected (see above), transfection levels remained remarkably high and constant over the range of 200–10 ng of DNA with CMVLuc-NLS (Fig. 4).

The capped gene lacking the NLS peptide showed 100- to 1,000-fold more expression when the NLS peptide was present on the gene. Similar conclusions were obtained, by using a cationic lipid (Transfectam, vector nitrogens over DNA phosphate, N/P = 6) or to a cationic polymer (25-kDa PEI, N/P = 10) in the absence of serum.

brought transfection down to the level obtained with the capped gene having no peptide at all, confirming the functionality of the NLS—importin α interaction.

NLS Peptide-Mediated Transfection Enhancement Is a General Phenomenon. A series of comparative experiments was undertaken with various cell types. Transfection enhancement due to the presence of the NLS peptide was always observed (Table 2). However, enhancement factors were spread widely with cell type (10- to 1,000-fold), with no obvious relation with tissue origin nor cell type (primary vs. transformed cells). Nondividing primary cells such as human monocyte-derived macrophages and rat dorsal root ganglia neurons showed less impressive enhancement than 3T3 and HeLa cells. However, similar values were also seen for the easily transfected and fast dividing rat hepatocyte-derived cell line BNL CL.2.

Improved Transfection Involves the Cellular Nuclear Import Machinery. Comparative experiments between CMVLuc and CMVLuc-NLS, although interesting, do not really allow one put forward any hypothesis concerning the mechanism. Nuclear import of DNA and oligonucleotides has mainly been followed with fluorescent tags and fluorescence in situ hybridization, in microinjected or digitonin-permeabilized cells. Proof of active import relied on inhibition experiments with energy-decoupling molecules, nuclear pore-binding agglutinins, or mutations in the signal peptide sequence. Gene expression is an unambiguous proof of nuclear localization and transfection can be regarded as a straightforward way of introducing DNA into intact cells. One lysine to threonine mutation abolishes nuclear import (24). We therefore synthesized a capped gene (CMVLuc-mNLS) with a mutated PKTKRKVEFDPYC sequence. Comparative transfection experiments with HeLa cells are shown in Fig. 5. Irrespective of the amount of DNA used, the lysine to threonine mutation

*Table 1. Free DNA ends decrease efficacy

<table>
<thead>
<tr>
<th>DNA form</th>
<th>Symbol</th>
<th>Transfection efficiency, RLU/mg of protein</th>
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<tbody>
<tr>
<td>Uncapped CMVLuc</td>
<td>⚫</td>
<td>1 ± 0.4 × 10⁴</td>
</tr>
<tr>
<td>Hemi-capped CMVLuc</td>
<td>⚫</td>
<td>9 ± 2 × 10⁴</td>
</tr>
<tr>
<td>CMVLuc</td>
<td>⚫</td>
<td>2.5 ± 0.6 × 10⁶</td>
</tr>
<tr>
<td>Circular pCMVLuc plasmid</td>
<td>⚫</td>
<td>2.5 ± 0.2 × 10⁸</td>
</tr>
</tbody>
</table>

BNL CL.2 hepatocytes were transfected with 200 ng of DNA per well. Complexes were formed with 25-kDa PEI (N/P = 10) in 150 mM NaCl and in the absence of serum. RLU, relative light units. *Transfection with 20 ng of plasmid.

Fig. 4. NLS peptide promotes high and sustained transfection levels down to 10 ng DNA. 3T3 cells were transfected with decreasing amounts of DNA complexed in 150 mM NaCl to a cationic lipid (Transfectam, vector nitrogens over DNA phosphate, N/P = 6) or to a cationic polymer (25-kDa PEI, N/P = 10) in the absence of serum.

Fig. 5. Sustained luciferase expression levels are due to the nuclear localization peptide. HeLa cells were transfected with various amounts of DNA complexed to ExGen500 PEI (N/P = 5 in a 5% glucose solution) in the presence of 10% serum.
The general experimental setup for transfection included 96-well microtiter plates and no serum for 2 h after addition of the DNA–vector complexes to the cells. Several experiments were also performed on a larger scale (24-well plates) or in the presence of 10% serum during transfection. The results obtained for HeLa and 3T3 cells (Table 2) confirmed the general conclusions derived with the 96-well setup.

**Table 2. Average enhancement of transfection with CMVLuc-NLS**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Enhancement factor*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNL CL.2</td>
<td>10</td>
</tr>
<tr>
<td>HeLa</td>
<td>400^f–1000</td>
</tr>
<tr>
<td>Murine DRG neurons</td>
<td>200^f–300</td>
</tr>
<tr>
<td>Human macrophages</td>
<td>30</td>
</tr>
<tr>
<td>HeLa</td>
<td>10</td>
</tr>
</tbody>
</table>

Values refer to transfection with 10–20 ng of DNA per well, in the absence of serum. DRG, dorsal root ganglion.

*Estimated spread is 1±30%.
†In the presence of 10% serum during transfection.
‡In 24-well plates using 200 ng of DNA in the presence of 10% serum.

The general experimental setup for transfection included 96-well microtiter plates and no serum for 2 h after addition of the DNA–vector complexes to the cells. Several experiments were also performed on a larger scale (24-well plates) or in the presence of 10% serum during transfection. The results obtained for HeLa and 3T3 cells (Table 2) confirmed the general conclusions derived with the 96-well setup.

**Time Course of the Transgene Expression.** Active transport of the reporter gene via the nuclear import machinery could result in faster appearing expression. More interesting, a lower intracellular barrier to gene delivery means less DNA–vector complexes required to effectively transfect cells in vitro. The kinetics of gene expression was followed in detail up to 24 h (Fig. 6) and then daily up to 3 days. Again, transfection with CMVLuc-NLS was much more effective than with CMVLuc-mNLS. Remarkably (and reproducibly), NLS-driven transfection reached its plateau and more sustained expression. The kinetics of gene expression were shifted from the microgram to the nanogram range (see also ref. 17). Although the improvement (one to three orders of magnitude) varies with cell type (showing that the nuclear membrane is not the only possible barrier), even the modest

![Fig. 6](Image) Reporter protein activity appears faster with CMVLuc-NLS. HeLa cells were transfected with 10 ng of DNA complexed to ExGen 500 PEI (N/P = 5 in 5% glucose solution) in the absence of serum. Cells were lysed and luciferase activity was measured at the indicated time after transfection. The absence of error bars indicates that the SEM is smaller than the label on the graph.

![Fig. 7](Image) Hypothetical scheme of foreign DNA entering the nucleus. The vectorial movement is driven by DNA binding to chromatin-forming basic proteins. A single NLS peptide helps initial threading, whereas too many signals may inhibit full translocation.

**DISCUSSION**

The goal of this work was to improve nonviral plasmid-mediated gene delivery. When fully counterion-condensed, a single plasmid molecule collapses into a sphere of about 25 nm (26) that is already at the size-exclusion limit for signal-mediated nuclear import (4, 27). Plasmid condensation with cationic lipids or polymers generally leads to even larger multimolecular aggregates that reach the cytoplasm after binding to cell-surface anionic proteoglycans (9, 28) and eventual escape from the formed vacuoles (8, 9, 30). Because the particles are too large to cross an intact nuclear membrane, transfection of resting cells can reasonably only be accounted for by uncomplexed DNA that has been released by exchange with phosphatidylinerine (31) or heparan sulfate (9) present in the vacuolar membrane. Any molecular information (e.g., NLS) borne by the cationic vector will thus be lost before reaching the nuclear membrane, hence its ineffectiveness.

In sharp contrast to a condensed DNA molecule, a free hydrated DNA double helix (3 nm) is thin enough to enter the nuclear pore by diffusion, i.e., with no energy or signal requirement. Restricted intracellular motion of DNA [the cytoplasm is equivalent to 13% dextran (32), and a rather low nuclear pore coverage (<10% of the membrane surface (33)], however, give this event a low probability to occur: the nuclear membrane has been recognized as a major barrier to gene delivery (8, 9). Quantitative cytoplasmic microinjection of DNA (25, 34, 35) indeed led to less than 0.1% of the DNA being expressed. The probability for DNA to find and subsequently enter a nuclear pore can be increased by a bound karyophilic signal peptide able to dock DNA to the nuclear pore filaments and help an initial part of the molecule to cross the membrane. The DNA–karyophilic signal should, however, be stable, hence the chemical strategy we chose to use in this study.

The major achievement of our approach is that the amount of DNA required to effectively transfet cells in vitro has now shifted from the microgram to the nanogram range (see also ref. 17). Although the improvement (one to three orders of magnitude) varies with cell type (showing that the nuclear membrane is not the only possible barrier), even the modest
enhancements observed for primary cell cultures look interesting (1,000%) when not presented on a logarithmic scale. Is this exciting result really a consequence of the involvement of the nuclear import machinery? We choose to tackle the academic aspect of this work by using the lysine to threonine mutation, which indeed abolished the beneficial effect brought about by the DNA-bound NLS sequence.

Taking our hypothesis further, the last unanswered question deals with threading of the rest of the plasmid molecule (Fig. 7). Random-walk diffusion of a micrometer-long molecule would be inefficient and slow. Fortunately, naked DNA will not remain free in the nucleus: histones (and eventually basic nuclear matrix proteins) indeed quickly assemble transfected DNA into chromatin-like structures (36, 37), thus providing a mechanism for pulling and condensing the filamentous molecule into the nucleus. Following this line of thinking, many NLS signals distributed along the DNA may actually inhibit nuclear entry if the nucleic acid is longer than the distance separating adjacent pores (Fig. 7). A straightforward calculation that uses the pore density in HeLa cells (33) shows this to become probable above 1 kbp.

The present work is a proof of principle rather than a new gene delivery technology. However, it will hopefully provide the impetus for searching more versatile solutions to the link between a plasmid and a NLS peptide, such as triple-helix formation (38, 39) or strand-invading (40, 41) oligonucleotide-NLS conjugates.

We thank Dr. Olivier Feugeas and Dr. Anne Feltz for providing the monoeytes and neurons, respectively. We are grateful to Dr. Robert P. P. Fuchs and Dr. M. Bichara for providing technical facilities and for helpful discussions. This work was supported by grants from the Association Franc¸aise contre les Myopathies, the Association pour la Recherche contre le Cancer, and the Ligue contre le Cancer. M.A.Z. received a fellowship from Transgene SA.