Improved neurotensin-vector-mediated gene transfer by the coupling of hemagglutinin HA2 fusogenic peptide and Vp1 SV40 nuclear localization signal

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Abstract

Recently we reported that neurotensin-SPDP-poly-L-lysine (NT-vector) is able to bind plasmid DNA (NT-polyplex) and polyfect cells expressing the high-affinity neurotensin receptor (NTRH) although with low transfecting efficiency: in vitro, 6.5±1.5%, and in vivo, 5±4%. In this work, we attempted to increase the transfecting efficiency by integrating the hemagglutinin HA2 fusogenic peptide and the Vp1 nuclear localization signal of SV40 to the NT-polyplex (fusogenic-karyophilic-NT-polyplex). Confocal microscopy and flow cytometry analysis showed that the fusogenic-karyophilic-NT-polyplex produced mostly nuclear localization of the plasmid DNA in NTRH-bearing N1E-115 cells. About 50% of N1E-115 cells internalized and expressed the reporter gene when the plasmid DNA was transferred by the fusogenic-karyophilic-NT-polyplex. Although to a less extent, the addition of each viral peptide separately to NT-polyplex (fusogenic-NT-polyplex or karyophilic-NT-polyplex) improved polyfection. Fusogenic-NT-polyplex produced 22.41±5.96% of internalization and 20.35±0.82% of expression in N1E-115 cells, whereas karyophilic-NT-polyplex yielded 13.75±3.88% and 10.94±2.04%, respectively. Basal internalization and expression were detected in N1E-115 cells in the presence of 100 nM SR-48692 and in NTRH-lacking cells. The fusogenic-karyophilic-NT-polyplex was microinjected into the substantia nigra to test its ability for gene transfer in vivo. Fusogenic-karyophilic-NT-polyplex internalization was observed within dopamine neurons only. Reporter gene expression was observed in a high proportion of dopamine neurons up to 60 days after NT-polyfection. Both internalization and expression were prevented by SR-48692. Our results show that the fusogenic-karyophilic-NT-polyplex is a highly efficient and specific gene vector and encourage its use to transfer gene of physiological interest to NTRH-bearing neurons.

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1. Introduction

Taking advantage of the internalization properties of neurotensin (NT), we have recently developed a gene vector by crosslinking NT with poly-L-lysine [31], capable of transferring plasmid DNA (NT-polyplex) to NTRH-expressing NIE-115 cells [30]. The NT-vector is also able to transfer reporter genes to dopamine neurons of the pars compacta of the rat substantia nigra [1], one of the nucleus with high density of NT receptors [5,6,28]. Since NT bypasses the lysosomal compartment [9], the NT moiety of the gene vector must have provided the escape of plasmid DNA from endosomes, thus assuring an effective gene transfer to dopamine neurons. Nevertheless, the polyfection efficiency of the NT-vector is low compared with that of retroviral vectors [38].
It has been reported that receptor-mediated gene transfer vectors confront various barriers that turn them inefficient and unattractive for polyfection of physiological genes in vivo. The first limiting barrier to nonviral vectors appears to be their inactivation in acidic endosomal vesicles [40] and their degradation in the lysosomal compartment [34]. In addition, the low efficiency of endogenous transporting mechanisms of exogenous DNA to the nucleus could represent a second barrier to receptor-mediated gene transfer systems [10,50]. To bypass the first barier, diverse approaches have been successfully used together with receptor-mediated gene transfer systems, including hepatectomy-induced liver regeneration after the injection of the asialoglicoprotein-polyplex [48], replication-defective adenovirus to induce disruption of DNA-containing endosomes [15,45], histidylation of polylysine [33] to destabilize endosome membrane and chloroquine to neutralize the acidic pH of endosomes [10].

Several viruses have developed successful strategies to bypass the lysosomal compartment and to target the viral genetic material to the nucleus. An adenoviral protein capable of fusing the lipid bilayer of endocytic vesicles produces the release of the viral particle to the cytoplasm [24]. For this reason, adenoviruses have been used to enhance gene expression in polyfected cells [15,45]. A 22-amino-acid long peptide (GLFEAIÆFIEGGWEG-LIEGC) from the amino-terminus of influenza virus hemagglutinin HA2 capable of fusing lipid bilayer of endocytic vesicles [42] significantly increases receptor-mediated reporter gene expression [32,33,44]. Once the virus is released to the cytoplasm, some viral proteins possessing karyophilic determinants also known as nuclear localization signal (NLS) target virus genome to the host cell nucleus (for review see [37]). A NLS of the SV40 major capsid protein Vp1 [23] is responsible for nuclear targeting of Vp1 and virions as well [22]. Further analysis of Vp1 NLS has shown that one of its 19-amino-acid long (MAPTKRKGSCPGAAPNKPK) peptide mutants has a potent nuclear transport activity [23].

This work was performed to test if the coupling of a hemagglutinin HA2-derived fusogenic peptide to the NT-vector and the binding of Vp1 NLS of SV40 [23] to plasmid DNA (fusogenic-karyophilic-NT-polyplex) significantly improves polyfection efficiency in vitro. Once known the optimal conditions in vitro, the fusogenic-karyophilic-NT-polyplex was challenged to transfer pGreen Lantern 1 in vivo to dopaminergic neurons of the substantia nigra. Our results indicate that the incorporation of at least two viral peptides to a nonviral vector were sufficient to overcome two major barriers to receptor mediated-endocytosis gene transfer systems.

2. Materials and methods

2.1. Synthesis of the fusogenic-NT-vector

To obtain the fusogenic-NT-vector, both the HA2-derived fusogenic peptide (GLFEAIÆFIEGGWEG-LIEGC) and NT were crosslinked with poly-L-lysine (mean molecular weight 48 000 Da) using N-succinimidyl-3-(2-pyridyldithio) propionate (LC-SPDP) following the method that we described previously [31]. Briefly, in separate assays 3 mM NT, 3 mM fusogenic peptide and 0.43 mM poly-L-lysine were crosslinked with 6 mM LC-SPDP each. NT–SPDP and fusogenic peptide–SPDP were purified by gel filtration and concentrated to 0.5 ml each. Poly-L-lysine–SPDP was additionally reduced to poly-L-lysine–SPDP-SH with 50 mM dithiothreitol, purified by gel filtration and concentrated to 1 ml. Concentrated SPDP derivatives were incubated for 36 h at room temperature under continuous shaking. The resulting conjugates were purified in a Biogel A1.5 m column using 2 M guanidine in 10 mM Hepes, pH 7.4 as mobile phase. The fractions containing (fusogenic peptide–SPDP)–NT–SPDP–poly-L-lysine (the fusogenic-NT-vector) were pooled and concentrated to 1 ml by using an ultrafiltration cell. The fusogenic-NT-vector was further dialyzed against phosphate-buffered saline (PBS) solution, pH 7.4 and sterilized by filtration using a 0.22-µm filter.

2.2. Formation of the fusogenic-karyophilic-NT-polyplex

The mutant Vp1 NLS peptide (MAPTKRKGSCPGAAPNKPK; purity >90%; Synpep) was electrostatically bound to plasmid DNA to form the karyophilic-plasmid DNA. The peptide and the plasmid DNA were dissolved in serum-free Dulbecco’s modified eagle medium (DMEM). Similar amounts of 6 nM plasmid DNA were incubated with increasing amounts of the karyophilic peptide (2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 25.0 µM) for 30 min at room temperature and then subjected to 0.8% agarose gel electrophoresis as we described previously [31].

Since 15 µM karyophilic peptide solution does not saturate the anionic charges of DNA, this concentration was selected to form the fusogenic-karyophilic-NT-polyplex. Complexes were formed at increasing (2, 4, 6, 8, 10, 12, 14, 16, 18 and 20) molar ratios by adding 0.6 ml of fusogenic-NT-vector dropwise to 1.4 ml of karyophilic-plasmid DNA solution (15 µM:6 nM). Reaction mixtures were incubated for 30 min at room temperature and subjected to 0.8% agarose gel electrophoresis as we described previously [31].

2.3. NT-polyfection of cultured cells with pGreen Lantern 1

Neuroblastoma N1E-115 and COS7 cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin–streptomycin (100 µg/ml of each) and amphotericin (0.25 µg/ml). Cell cultures were kept at
37 °C under a 5% CO₂ atmosphere. Internalization and gene expression were assayed as described previously [30,31]. Twenty-four hours before the expression and internalization assays, cells were seeded in 4-well plates at 50 and 80% confluence, respectively. For internalization assay, cells were exposed to 1 μM calcein AM for 20 min and then incubated with propidium iodide labeled NT-polyplexes (6 nM cDNA) for 30 min. Cells were washed with PBS, fixed with 4% paraformaldehyde and mounted with anti-quenching medium (Vectashield, Vector Labs., Burlingame, CA, USA) for confocal microscopy analysis. For expression assays, cells were exposed to different NT-polyplexes (6 nM cDNA) for 30 min, washed with PBS and further incubated in serum-antibiotic-supplemented DMEM for 48 h. Cells were washed with PBS and fixed with 4% paraformaldehyde. After counterstaining with 1 μM propidium iodide, cells were mounted with Vectashield for confocal microscopy analysis. Blocking assays were carried out using either 100 nM SR-48692, a specific nonpeptide antagonist of NT receptors [19] or 0.45 M sucrose solution, a nonspecific inhibitor of receptor-mediated endocytosis [21]. Cells were analyzed in a confocal imaging system equipped with a krypton–argon laser beam (Bio-Rad MRC-600, Watford, UK) as described [1,30,31]. The fluorescence was detected with a 60× oil-immersion objective at excitation/emission wavelengths of 488/522 nm (green channel) and 568/585 nm (red channel). Ten to twenty consecutive optical sections at 1-μm intervals were obtained in the z-series. The resulting images were projected in a bidimensional plane and were overlapped on the screen monitor using green for calcein and the green fluorescent protein (GFP) (the product of reporter gene pGreen Lantern-1) and red for propidium iodide.

2.4. Flow cytometry

Quantitative analysis of internalization and expression assays was achieved using FACSsort equipment (Becton Dickinson, San Jose, CA, USA). Internalization was evaluated through the fluorescence of propidium iodide-labeled NT-polyplexes, whereas expression was evaluated through the GFP fluorescence. Upon completion of internalization or expression assay, cells were trypsinized, suspended in PBS and immediately analyzed in the FACSsort equipment. Populations of 10⁴ cells were excited at either 488 or 568 nm and the fluorescence of GFP and of propidium iodide was detected at 522 and 585 nm, respectively. The dotplot was obtained by semilog plotting side scatter, a parameter of cell granularity, versus the logarithm of fluorescence intensity, which was divided in three regions: R1 (10⁰–10¹), R2 (10¹–10²) and R3 (10²–10³). Fluorescence values in R1 and R2 were considered as basal values for all experimental conditions.

Internalization and expression assay controls were cells exposed to propidium iodide-labeled plasmid DNA.

2.5. Animals

Experiments were conducted on male Wistar rats (weighing 230–250 g at the onset of experiment) bred in our facilities. Animals were maintained under constant room temperature (23°C) and light–dark cycle (12:12 h light–dark); with food and water ad libitum. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of the Mexican Council for Animal Care as approved by the Cinvestav Animal Care Committee. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques.

2.6. Surgical procedures

Each rat was anesthetized by an intraperitoneal injection of chloral hydrate (350 mg/kg) and placed in a stereotaxic instrument (David Kopf) with the incisor bar 3.3 mm below the interaural line. Kelorphan (50 mM), an in vivo endopeptidase inhibitor, was used to protect the NT moiety of polyplex against the enzymatic cleavage [8]. After cranial trepanation, 2 μl of fusogenic-karyophilic-NT-polyplex (6 nM with respect to DNA) containing kelorphan were microinjected into the dorsal border of substantia nigra compacta at a rate of 0.1 μl/min. The coordinates were AP –4.9 from bregma; L +2.0 from midline, VD –6.0 from the cortex surface [1]. In addition, a local administration of 2 μl of 50 mM kelorphan preceded the fusogenic-karyophilic-NT-polyplex administration. To visualize the polyplex internalization, propidium iodide (10 μM) was added to the polyplex solution to label the plasmid DNA (pGreen Lantern-1). To block the receptor-mediated endocytosis of fusogenic-karyophilic-NT-polyplex, 2 μl of 1 μM SR-48692 containing kelorphan was injected 10 min before and concurrently with the polyplex. A stock solution of 10⁻² M SR-48692 in dimethylsulfoxide was subsequently diluted with PBS to yield a final concentration of 10⁻⁶ M, following the manufacturer specification. After surgery, all animals were injected with benzathine penicillin (300 000 I.U./kg, i.m.) to prevent infection.

2.7. Immunofluorescence

Internalization was assessed 4 h after the injection of the fusogenic-karyophilic-NT-polyplex. Gene expression was verified from 48 h up to 60 days after polyfection. For immunofluorescence observations, rats were deeply anesthetized with chloral hydrate and perfused through the ascending aorta with 100 ml of PBS, followed by 150 ml of 4% paraformaldehyde in PBS. The brain was then removed and maintained in the fixative for 48 h at 4°C. After overnight incubation in PBS containing 10% sucrose at 4°C, the brain was frozen and sectioned in 30-μm slices on the sagittal plane using a Leitz cryostat. Slices were
individually collected in a 24-wells plate containing PBS, and used for fluorescent immunolabeling of dopamine neurons and astroglial cells. Slices were incubated with 10% IgG-free bovine serum albumin in PBS–Triton-X-100 (0.2%) for 20 min at room temperature. The primary antibodies were mouse anti-tyrosine hydroxylase (TH) monoclonal antibody (1:20 dilution; Boehringer Mannheim) and rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (1:400 dilution; Dako). The secondary antibodies were fluorescein (FITC)—or rhodamine (Rho)—goat anti-mouse IgG (1:100 dilution; Pierce), and FITC (Vector Labs.) or Rho (Pierce) goat anti-rabbit IgG (1:60 dilution). Slices were mounted on glass slides using vectashield and then scanned in a confocal imaging system. The fluorescence was detected with either 20× or 60× oil-immersion objectives at excitation/emission wavelengths of 488/522 nm (green channel) and 568/585 nm (red channel). Ten to twenty consecutive 1-μm optical sections were obtained in the z-series. The resulting images were projected in a bidimensional plane and were overlapped on the screen monitor using the color green for FITC and GFP, and red for propidium iodide and rhodamine. Negative controls were obtained by omitting the primary antibody, and replacing it by an irrelevant antibody of the same IgG subclass.

2.8. Efficiency determination and statistical analysis

All values are the mean±S.E.M. obtained from four independent duplicated experiments. For statistical analysis, ANOVA with Student’s t test was used to compare the groups. In all cases, a difference was considered significant when P was <0.05.

2.9. Chemicals

Both the HA2-derived fusogenic peptide (GLFEAIAEIEGGWEGGLIEGCAKKK) and the peptide derived from the SV40 major capsid protein Vp1 NLS (MAPTKRKSGCPGAPNKPK), >90% purity, were synthesized by Synpep. Mouse anti-TH monoclonal antibody was purchased from Roche Diagnostics (Indianapolis, IN, USA) and rabbit anti-GFAP polyclonal antibody was from Dako (Carpinteria, CA, USA). FITC-goat anti-rabbit IgG and Vectashield was purchased from Vector Labs. FITC-and Rho-goat anti-mouse IgG, Rho-goat anti-rabbit IgG, LC-SPDP and dithiothreitol (DTT) were purchased from Pierce Chemical (Rockford, IL, USA). Poly-L-lysine hydrochloride (48 000 Da), ethidium bromide, propidium iodide, dimethyl sulfoxide, EDTA disodium salt, agarose, HEPES, guanidine, paraformaldehyde and NT were purchased from Sigma (St. Louis, MO, USA). pGreen Lantern-1, PBS (Ca²⁺ and Mg²⁺ free), DMEM, fetal bovine serum, trypsin–EDTA, HEPES buffer, sodium bicarbonate and antibiotic–antimicrobial solutions were obtained from Gibco-BRL (Grand Island, NY, USA). Calcein AM was purchased from Molecular Probes (Eugene, OR, USA). Econo-Pac 10DG, Sephadex G-10 and Biogel A 1.5

Fig. 1. DNA retardation microassays. (A) Plasmid DNA retardation by the electrostatic binding of SV40 Vp1 NLS (karyophilic peptide). The numbers indicate the concentration of karyophilic peptide tested for binding 6 nM plasmid DNA. (B) Retardation microassay showing that 15 μM karyophilic peptide does not interfere with the coupling between fusogenic-NT-vector and plasmid DNA to form the fusogenic-karyophilic-NT-polyplex at 1:8 molar ratio. (a) Electrophoretic migration of 6 nM plasmid DNA (pGreen Lantern 1); (b) migration of 6 nM plasmid DNA after incubation with 15 μM karyophilic peptide; (c) migration of fusogenic-NT-polyplex resulted from incubation of 6 nM plasmid DNA with fusogenic-NT-vector at molar ratio of 1:8; (d) migration of fusogenic-karyophilic-NT-polyplex resulted from incubation of plasmid DNA–karyophilic peptide (6 nM:15 μM) with fusogenic-NT-vector at 1:8 molar ratio.
columns were obtained from Bio-Rad (Richmond, CA, USA). All other chemicals were of analytical reagent grade quality and obtained from usual commercial sources.

3. Results

3.1. Formation of fusogenic-NT-polyplex and fusogenic-karyophilic-NT-polyplex

The karyophilic peptide was able to bind plasmid DNA in an electrostatic manner (Fig. 1A). After incubation of identical aliquots of 6 nM plasmid DNA with increasing concentrations of karyophilic peptide (2.5–25 μM), the electrophoresis showed an increasing retardation of DNA migration up to the total retention of DNA by the 25-μM concentration (Fig. 1A).

The retardation microassay showed that the optimal molar ratio between plasmid DNA and fusogenic-NT–SPDP–poly-L-lysine was 1:8 (Fig. 1B, c). The 15-μM concentration of karyophilic peptide did not impede the electrostatic binding of plasmid DNA with the poly-L-lysine.

Fig. 2. Fusogenic and karyophilic peptides enhance NT-polyplex internalization in NTRH-bearing cells. Internalization of propidium iodide-labeled fusogenic-karyophilic-NT-polyplex in N1E-115 cells (A and B). Effect of 0.45 M sucrose on fusogenic-karyophilic-NT-polyplex internalization by N1E-115 cells (C and D). Effect of 100 nM SR-48692 on fusogenic-karyophilic-NT-polyplex internalization by N1E-115 cells (E and F). Lacking of internalization of the fusogenic-karyophilic-NT-polyplex by COS7 cells (G and H). Cells were counterstained with calcein AM (A, C, E and G) 20 min before incubation with the fusogenic-karyophilic-NT-polyplex labeled with propidium iodide (B, D, F and H). Fluorescence was detected with a confocal microscope. Images A–D are 1-μm thick horizontal sections through the cell nucleus, and images E–H are projections of the corresponding z-series of horizontal sections. Scale bars, 20 μm.
lysine moiety of the vector (Fig. 1B,d), thus resulting in the fusogenic-karyophilic-NT-polyplex.

3.2. Fusogenic and karyophilic peptides in the NT-polyplex enhance plasmid DNA internalization in vitro

Confocal microscopy analysis revealed that the fusogenic-karyophilic-NT-polyplex produced mostly nuclear localization of propidium iodide-label plasmid DNA (Fig. 2B) of calcein-counterstained N1E-115 cells (Fig. 2A). In the presence of 0.45 M sucrose, a nonspecific inhibitor of receptor-mediated endocytosis [21], propidium iodide-labeled fusogenic-karyophilic-NT-polyplex (Fig. 2D) was observed only in the perimeter of N1E-115 cells (Fig. 2C). In the presence of 100 nM SR-48692, a selective non-peptide antagonist of NT receptors [19], propidium iodide-labeled fusogenic-karyophilic-NT-polyplex (Fig. 2F) was not observed in N1E-115 cells (Fig. 2E). COS7 cells lacking NTRH-receptors were unable to internalize the fusogenic-karyophilic-NT-polyplex (Fig. 2G and H).

Quantification of internalization of propidium iodide-labeled NT-polyplexes was assessed by flow cytometry in a variety of conditions. For statistical analysis, only values in R3 of the dotplots were taken into account since they were two orders of magnitude higher than basal values found in R1 (Fig. 3A). Karyophilic peptide bound to plasmid DNA in the NT-polyplex (karyophilic-NT-polyplex) produced internalization of the labeled DNA in 13.75±3.88% of N1E-115 cells (Fig. 3D). The fusogenic-NT-polyplex accounted for 22.41±5.96% of N1E-115 cells internalizing the labeled DNA (Fig. 3E). Fusogenic-karyophilic-NT-polyplex significantly increased up to 48.44±7.18% the proportion of N1E-115 cells internalizing the labeled DNA (Fig. 3F). The presence of 100 nM SR-48692 reverted the effect of fusogenic-karyophilic-NT-polyplex in N1E-115 cells (Fig. 3B). Low fluorescence values of COS7 cells exposed to the propidium iodide-labeled fusogenic-karyophilic-NT-polyplex were localized in R3, indicating basal nonspecific internalization (Fig. 3C).

3.3. Fusogenic and karyophilic peptides in the NT-polyplex enhance reporter gene expression in vitro

In agreement with the internalization assays, the

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**Fig. 3.** Flow cytometry analysis of internalization of different propidium iodide-labeled NT-polyplexes. (A) Basal fluorescence in N1E-115 cells. (B) Blockade by 100 nM SR-48692 of fusogenic-karyophilic-NT-polyplex internalization in N1E-115 cells. (C) Lack of uptake of the fusogenic-karyophilic-NT-polyplex by COS7 cells. (D) Internalization of karyophilic-NT-polyplex in N1E-115 cells. (E) Internalization of fusogenic-NT-polyplex in N1E-115 cells. (F) Internalization of fusogenic-karyophilic-NT-polyplex in N1E-115 cells. Populations of 10^3 cells emitting red fluorescence of propidium iodide were distributed in three arbitrary regions (R1, R2 and R3) according to their fluorescence intensity. All values are expressed as the mean±S.E.M. from four independent experiments performed in duplicate and only R3 values were considered for statistical analysis. *, significantly different from R3 in (D); **, significantly different from R3 in (E).
fusogenic-karyophilic-NT-polplex produced qualitatively higher expression of GFP (Fig. 4A) in propidium iodide-counterstained N1E-115 cells (Fig. 4B). SR-48692 (100 nM) produced basal GFP expression in N1E-115 cells exposed to the fusogenic-karyophilic-NT-polplex (Fig. 4C and D). By confocal microscopy, no expression of reporter gene was observed in COS7 cells lacking NT receptors when incubated with the fusogenic-karyophilic-NT-polplex (Fig. 4E and F).

Quantification of pGreen Lantern 1 polyfection was assessed by flow cytometry under a variety of conditions. Only values in R3 of the dotplots were taken into account for statistical analysis, since basal values were found in R1 (Fig. 5A). The karyophilic-NT-polplex led to GFP expression in 10.94±2.06% of N1E-115 cells (Fig. 5D). Fusogenic-NT-polyfection resulted in a significant increase in the proportion (20.35±0.82%) of N1E-115 cells expressing GFP (Fig. 5E) as compared with that achieved with the karyophilic NT-polplex. Fusogenic-NT-polyfection further increased the proportion (48.93±3.24%) of N1E-115 cells expressing GFP (Fig. 5F). SR-48692 (100 nM) in the incubation medium prevented the fusogenic-karyophilic-NT-polplex-mediated GFP expression in N1E-115 cells (Fig. 5B). COS7 cells lacking NT-receptors when incubated with the fusogenic-karyophilic-NT-polplex at optimal ratio revealed basal nonspecific expression of reporter gene (Fig. 5C).

3.4. Fusogenic-karyophilic-NT-polplex enhances plasmid DNA transfer to dopamine neurons

Since the fusogenic-karyophilic-NT-polplex resulted in the most effective transfer system in vitro, such a polplex was challenged for transferring pGreen Lantern 1 to substantia nigra pars compacta of Wistar rats. The polplex was labeled with propidium iodide to be localized by confocal microscopy in the nigral cell population immunostained against either TH or GFAP, well-known markers of dopamine neurons.
Fig. 5. Flow cytometry analysis of GFP expression when pGreen Lantern 1 was delivered by different NT-polyplexes. (A) Basal fluorescence in N1E-115 cells. (B) Lack of GFP expression in N1E-115 cells exposed to the fusogenic-karyophilic-NT-polyplex in the presence of 100 nM SR-48692. (C) Lack of GFP expression in COS7 exposed to fusogenic-karyophilic-NT-polyplex. (D) Karyophilic-NT-polyfection of pGreen Lantern 1 in N1E-115 cells. (E) Fusogenic-NT-polyfection of pGreen Lantern 1 in N1E-115 cells. (F) Fusogenic-karyophilic-NT-polyfection of pGreen Lantern 1 in N1E-115 cells. Populations of 10^3 cells emitting the GFP fluorescence were distributed in three arbitrary regions (R1, R2 and R3) according to their fluorescence intensity. All values are expressed as the mean ± S.E.M. from four independent experiments performed in duplicate and only R3 values were considered for statistical analysis. *, significantly different from R3 in (D); **, significantly different from R3 in (E).

3.5. Fusogenic-karyophilic-NT-polyplex enhances transgene expression in dopamine neurons

The plasmid pGreen Lantern 1 encoding GFP was used to show qualitatively the fusogenic-karyophilic-NT-polyfection efficiency in vivo. Forty-eight hours after the local microinjection of the polyplex, the propidium iodide label (Fig. 6B) was localized in a high proportion of TH-immunopositive cells (Fig. 6A) but not in GFAP-immunopositive cells (Fig. 6E and F), showing that nigral dopamine neurons are capable of internalizing the fusogenic-karyophilic-NT-polyplex. Such internalization was totally blocked by 1 μM SR-48692 (Fig. 6C and D).

4. Discussion

Targeted gene delivery is the striking feature that makes receptor-mediated gene transfer vectors of great experimental and therapeutic potential [26,29,46,47,49]. Unfortunately, low efficiency has delayed its use in protocols in vivo. Compared with viral vectors, receptor-mediated gene transfer vectors require additional commands to bypass the acidic endosomal vesicles and to overcome the nuclear membrane barrier. Accordingly, our results showed that the integration of fusogenic and karyophilic peptides in the NT-polyplex resulted in a significant increase in the percentage of cells internalizing the plasmid DNA and expressing the transgene in vitro, without losing specificity. As compared with the NT-polyplex [1], the fusogenic-karyophilic-NT polyplex was a more efficient gene transfer system able to extend the transgene expression in vivo.

Procedures such as addition to culture medium of replication-defective adenovirus [15], fusogenic peptides [32] or pH neutralizing drugs [33] have been used in vitro together with nonviral vectors to avoid their inactivation in acidic endosomal vesicles and the subsequent cDNA...
Fig. 6. Internalization of fusogenic-karyophilic-NT-polyplex by nigrostriatal dopamine neurons. FITC-immunostaining against TH (A and C) and GFAP (E) were observed by confocal microscopy at 488/522 nm, Ex/Em. Propidium iodide-labeled fusogenic-karyophilic-NT-polyplex was observed at 568/585 nm, Ex/Em (B, D and F). (A and B) Internalization of fusogenic-karyophilic-NT-polyplex in dopamine neurons. (C and D) Lack of internalization of fusogenic-karyophilic-NT-polyplex in dopamine neurons when exposed to 1 mM SR-48692. (E and F) Lack of internalization of fusogenic-karyophilic-NT-polyplex in glial cells. Scale bars, 30 μm.

degradation in lysosomes. We recently showed that a nonlysosomal endocytosis pathway such as that of NT [9,17] is effective and reliable for gene transfer in vitro [30] and in vivo [1]. However, the efficiency as low as 8% in vitro and 5% in vivo suggests that NT is not capable of cotransporting sufficient genetic material, probably due to either the dissociation or precipitation of the NT-polyplex as consequence of endosomal acidic pH. Our results clearly demonstrated that the coupling of hemagglutinin HA2-derived fusogenic peptide to the poly-L-lysine moiety of the NT-vector improved both the nuclear localization of plasmid DNA and the subsequent reporter gene expression. The improvement of NT-polyfection due to the fusogenic peptide might have resulted from increased amount of exogenous DNA in the cytoplasm following the endosomal membrane disruption. The fusogenicity and disruption of the endocytic vesicle is a consequence of α-helix tertiary structure in the fusogenic peptide triggered by pH 6.0, an acidity found in early endosomes [7,13,27,35,36]. The absence of gene transfer in NTRH-lacking COS7 cells and in N1E-115 cells incubated with SR-48692 further confirms that the fusogenic peptide is inactive at neutral pH such as that of the extracellular medium. That characteristic allows NT-vector to conserve its transfecting specificity, lacking in amphiphilic cationic peptide-based vectors [18]. Our results agree with the finding that the addition of fusogenic peptides improves transfection efficiency of receptor-mediated gene transfer vectors [43]. Contradictory results have been reported about the fusogenic peptide-induced improvement of gene transfer to cultured cells via the galactose receptor [12,20,25]. Nevertheless, the successful results in vitro with other nonpeptide fusogenic agents such as histidylated polylysine [33] supports the idea that the early rescue of polyplex is a key factor to improve receptor-mediated gene transfer efficiency.

It has been recently shown that the covalent linking of the simian virus SV40 large tumor antigen NLS to polylysine enhances the polyplex nuclear targeting and the subsequent reporter gene expression [10,11]. However, this approach requires the use of chloroquine suggesting that lysosomal avoidance and nuclear targeting enhancement
should occur simultaneously to increase polyfection efficiency. Of the three kinds of characterized NLS, we selected the Vp1 NLS exhibiting basic properties [23] in an attempt to achieve two goals: spontaneous binding to plasmid DNA and potent karyophilic force. Retention microassays showed a strong electrostatic binding of NLS to plasmid DNA, stable under an 80-V electrophoretic field. At a 15-μM concentration, NLS did not saturate the anionic DNA charges, thus allowing its electrostatic binding to the NT-vector. In contrast with irreversible chemical linking of the NLS-peptide to cDNA [10,11,51], electrostatic binding is a simple, reproducible and fast procedure. Although less effective, the karyophilic peptide alone enhanced plasmid DNA internalization, from 8±1 [30] to 13.75±3.88%, and transgene expression, from 6.5±1.5% [30] to 10.94±2.06%. The potent karyophilic activity of the Vp1 NLS, capable of the nuclear targeting of large protein complexes such as virions [22], could account for the increase of both nuclear internalization and reporter gene expression. However, addition of fusogenic peptide to NT-polyplex yielded even better results than addition of the karyophilic peptide alone. This finding strongly supports the idea that the major barrier to receptor-mediated gene transfer systems is acidification of endosomal vesicles [14,40]. In gene transfer systems different from those based on receptor-mediated endocytosis, covalent bond of karyophilic peptides to DNA has yield contradictory results regarding to the improvement of gene transfer efficiency [39,41].

The fusogenic and karyophilic peptides present together in the NT-polyplex maximized internalization and gene expression in vitro (up to 50% in both cases). Flow cytometry analysis suggested that the individual peptides act synergistically to improve the receptor-mediated gene transfer. These results support the idea that the early rescue of polyplexes from acidic endosomal vesicles and the nuclear targeting of plasmid DNA must occur simultaneously to improve polyfection efficiency. Recently, a recombinant strategy consisting in the integration of multifunctional domains in a single polypeptide chain has

Fig. 7. GFP expression in nigrostriatal dopamine neurons when pGreen Lantern 1 was transferred by the fusogenic-karyophilic-NT-polyplex. Confocal microscopy was used to identify both GFP expression at 488/522 nm, Ex/Em (A, C and E) and Rho-immunostaining of dopamine neurons at 568/585 nm, Ex/Em (B, D and F). (A and B) GFP expression in TH-positive neurons 48 h after polyfection. (C and D) Absence of GFP expression in TH-positive neurons when exposed to 1 mM SR-48692. (E and F) GFP expression 2 months after polyfection. Scale bars, 20 μm.
been proven to be functional for gene transfer [2,3]. Those authors have included in a single recombinant protein three functions involved in gene transfer: (1) receptor recognition, (2) DNA condensation and (3) nuclear targeting. The addition of a fusogenic domain in the recombinant nonviral vector could result in a more efficient gene transfer system.

Two relevant targets for the fusogenic-karyophilic-NT-polypelex are dopamine neurons of the substantia nigra and of the ventral tegmental area known to express NTRH [5,6,28]. Although with low efficiency, the original NT-vector was capable of polyfecting dopamine neurons in vivo, thus showing the feasibility of targeted gene delivery to the central nervous system [1]. The fusogenic-karyophilic-NT-polypelex either injected in the substantia nigra or in the striatum (data not shown) was able to transfer reporter gene to dopamine neurons with high efficiency without losing specificity. The retrograde transport of fusogenic-karyophilic-NT-polypelex from the substantia nigra represents an attractive route of polyfection for a putative therapeutic approach of Parkinson’s disease in order to avoid additional injury to dopamine neurons. Other alternative routes could be the injection of NT-polypelex into either the lateral ventricle or the striatum to the substantia nigra known to degenerate in Parkinson’s disease [4]. It now seems logical to test the NT-vector improved by the viral peptides to transfer genes of physiological interest to dopamine neurons in a parkinsonian animal model. The fusogenic-karyophilic strategy could also be of use to increase the efficiency of other vectors of receptor-mediated gene transfer systems.

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