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Role of endocytosis in the transfection of L929 fibroblasts by polyethylenimine/DNA complexes

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Abstract

Polyethylenimine (PEI) is one of the most efficient nonviral vectors for gene therapy. The aim of this study was to investigate the role of endocytosis in the transfection of synchronized L929 fibroblasts by PEI/DNA complexes. This was performed by confocal microscopy and flow cytometry, using the endocytosis marker FM4-64 and PEI/DNA complexes labeled either with the DNA intercalator YOYO-1, or with fluorescein covalently linked to PEI. Endocytosis appeared as the major if not the sole mode of entry of the PEI/DNA complexes into the L929 cells. The complexes followed a typical fluid phase endocytosis pathway and were efficiently taken up in less than 10 min in endosomes that did not exceed 200 nm in diameter. Later, the localization of the complexes became perinuclear and fusion between late endosomes was shown to occur. Comparison with the intracellular trafficking of the same complexes in EA.hy 926 cells (W.T. Godbey, K. Wu, A.G. Mikos, Proc. Natl. Acad. Sci. USA 96 (1999)) revealed that endocytosis of PEI/DNA complexes is strongly cell-dependent. In L929 cells, escape of the complexes from the endosomes is a major barrier for transfection. This limited the number of transfected cells to a few percent, even though an internalization of PEI/DNA complexes was observed in most cells. In addition, the entry of the complexes into the nucleus apparently required a mitosis and did not involve the lipids of the endosome membrane. This entry seems to be a short-lived event that involves only a few complexes.

Keywords: Confocal microscopy; Endocytosis; Transfection; Polyethylenimine

1. Introduction

Polyethylenimines (PEI) are among the most efficient nonviral vectors developed for gene therapy, both in vitro [1–4] and in vivo [1,5–7]. PEI molecules exist either as a linear or a branched form. The latter form seems to be more efficient than the former and is thus the standard form used for gene delivery [8]. Since every third atom of the PEI backbone is an amino nitrogen, they are the organic molecules with the highest cationic charge density potential [1]. Therefore, PEI molecules exhibit considerable buffer capacity over almost the entire pH range [9,10]. Moreover, the transfection efficiency depends both on the average molecular weight and the polydispersity of PEI molecules [4,8,11–13]. Both in vitro and in vivo, the highest efficiencies are obtained with PEI of 25 or 50 kDa [4,11] albeit particles with poly-

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disperse PEI of high average molecular weight are efficient too [13]. Optimal internalization and in vivo diffusion require particles of small size (<100 nm). This requirement is achieved by adjusting the molar ratio of PEI nitrogen atoms to DNA phosphate (N/P) above two [10] or six [14], depending on the ionic strength of the buffer. Indeed, small particles are only obtained under conditions in which their surface charge, characterized by the ζ potential, is strongly positive in order to obtain strong repulsive interactions for preventing aggregation. Optimized transfection efficiency is obtained for a N/P ratio of about 10 [1].

Significant progress in the understanding of the transfection mechanism by PEI/DNA complexes has been recently achieved with the examination by confocal microscopy of the intracellular path of these complexes in EA.hy 926 cells [15]. After attachment to the cell surface, the complexes migrate into clumps that are endocytosed. Then, endosomes grow and, subsequently, some of them undergo a lytic process and release the complexes into the cytoplasm. Finally, complexes are transported by an ill-defined mechanism to the nucleus, allowing transcription and further translation of the plasmid-encoded gene. However, it remains questionable whether or not this pathway may apply to all cell types, since it has been reported that intracellular barriers to plasmid trafficking quantitatively vary with cell type [16].

Since endocytosis plays a critical role in the intracellular path of the PEI/DNA complexes, our purpose was to further investigate by confocal microscopy and flow cytometry the intracellular pathway of these complexes in L929 fibroblasts. These cells were of special interest because they have strong endocytotic activities and their endocytosis mechanism has been extensively studied [17,18]. Moreover, since transfection efficiency strongly depends on the cell cycle stage at the time of transfection [19], synchronized cells were used for this study. Using FM4-64, a fluorescent marker of endocytosis [20–22], together with fluorescently labeled PEI/DNA complexes, we planned to address the following questions. (a) Is endocytosis the sole mode of entry of the complexes into the cells? (b) Does the involved endocytosis display specific features like in receptor-mediated endocytosis and is this pathway affected by the uptake of PEI/DNA complexes? (c) Is DNA protected by PEI from the DNases in the lysosomes? (d) Are there some qualitative differences between the intracellular path of PEI/DNA complexes in L929 fibroblasts and EA.hy.926 cells? (e) Do the membrane components of endosomes play an active role in the delivery and uptake of the PEI/DNA complexes into the nucleus?

2. Materials and methods

2.1. Cell cultures

Mouse fibroblasts from the L929 strain (American Tissue Collection, Rockville, MD, USA) were cultured as monolayers in 75 cm^2 culture flasks (Costar) in Dulbecco's modified Eagle's medium (4.5 g/l glucose) (Seromed) supplemented with 10% fetal calf serum (Biowhittaker), 1 mM sodium pyruvate (Seromed), 2 mM L-glutamine (Seromed) and antibiotics (penicillin 50 IU/ml, streptomycin 50 μg/ml) (Seromed) in a 8% CO_2 atmosphere, at 37°C.

To synchronize L929 cells, the procedure of Coupin et al. [17] was used. Since L929 cells display contact inhibition at confluence and stop growing, the cells were grown to confluence. In these conditions, most of the cells are arrested at the end of the G_1 phase. These cells were then seeded at low density, enabling them to synchronously enter the S phase. Their viability ($>95\%$) was assessed by Trypan blue exclusion.

2.2. Polycations and plasmids

Branched PEI (25 kDa) and its labeled (FITC) counterpart (a kind gift from J.P. Behr, Illkirch, France) were used as 100 or 60 mM monomer aqueous stock solutions at pH 7.5, respectively.

The plasmid pCMV-Luc, coding for the *Photinus pyralis* luciferase gene and the plasmid pEGFP-C3 vector (Clontech), coding for the enhanced green fluorescent protein were amplified to sufficient quantities by standard molecular biology techniques, including harvesting and purification via Jetstar kits (Bioprobe). The rhodamine-labeled pGeneGrip plasmid was obtained from Gene Therapy Systems (San Diego, CA, USA).

2.3. Fluorescent labels

The membrane marker of endocytosis, FM4-64 or *N*-(3-triethylammoniumpropyl)-4-[6-(4-diethylamino)phenyl)hexatrienyl]pyridinium dibromide, is a vital plasma membrane stain with high solubility in water (Molecular Probes). The dye was added to cells to a final concentration of 4 μM from a 0.4 mM stock in PBS-Dulbecco (Seromed). The bis-intercalating agent YOYO-1 (491/509) from Molecular Probes was added at a ratio of one dye for 300 bases of the plasmid DNA.

2.4. Cell transfection

After trypsination, the synchronized cells were seeded 4 h (a time required to obtain again adherent cells) before transfection in a four-well chambered coverglass (Lab-Tek, Napperville, USA) at a density of 10^5 cells per well (for microscopy observations) or in 35 mm petri dishes (Costar, D. Dutscher) at a density of 2×10^6 cells per dish (for flow cytometric analysis). All experiments were done in triplicate. Prior to transfection, cells were rinsed and supplemented with 1 ml of fresh serum-free cultured medium.

Two micrograms of the desired plasmid were diluted into 50 μl of 25 mM HEPES/150 mM NaCl buffer at pH 7.4. PEI was then added to the plasmid at 10 equivalents (10 amino groups per phosphate group). The final solution was vortexed, spun down, and incubated for 20 min. The sizes of the PEI/DNA complexes were controlled by quasielastic light scattering using a Zetamaster 3000 (Malvern Instruments, Paris, France). The complexes were then added to 10^5 cells and the cell supernatant was homogenized by gentle horizontal hand rotation. After 2 h, the medium was complemented with 10% fetal calf serum.

Transfection protocol and measurement of luciferase gene expression were performed as described [23].

2.5. Flow cytometry

After incubation with the membrane FM4-64 probe (4 μM) or the labeled PEI/DNA complexes for a given time, the L929 cells in a petri dish were washed five times with PBS-Dulbecco (Seromed) and then carefully suspended in 1 ml PBS with a cell lifter (Costar). After homogenization, the cell suspension was transferred to a 1.5 ml microtube (Eppendorf) and centrifuged for 15 s at $5600\times g$ in a mini-centrifuge. The pellet was incubated with 0.5 ml Cell-fix (Becton-Dickinson) at 10%, overnight at 4°C, for fixation. Finally, the fixed cells were analyzed with a Becton-Dickinson FACStar Plus cell sorter equipped with a 488 nm excitation argon laser and an emission filter at 630 ± 11 nm.

2.6. Confocal laser scanning microscopy

A Bio-Rad MRC 1024ES confocal microscope, with a Nikon Eclipse TE 300 inverted microscope, was used for the optical sectioning of cells. An argon/krypton mixed gas laser with excitation lines at 488, 568 and 647 nm was used to illuminate a Nikon 60×1.2 NA water immersion objective. The laser power was reduced to less than 10% of its initial power with a neutral density filter wheel. Emitted light was detected with two photomultipliers through selected band pass filters. Measurements were directly performed on the four-well chambered coverglass (Lab-Tek) at room temperature. Confocal sections were taken every 0.2 μm. Digital image recording was performed using the LaserSharp 2.3 software (Bio-Rad).

Excitation of fluorescein-labeled PEI, YOYO-1 or green fluorescent protein was achieved using the 488 nm excitation line, with the resulting fluorescent wavelengths observed using a 506–538 nm band pass filter. Excitation of FM4-64 was achieved with the 568 nm excitation line, with the resulting fluorescence observed using a 664–696 nm band pass filter.

3. Results

3.1. FM4-64 uptake studies

As a first step to investigate the role of endocytosis in the transfection mechanism of L929 fibroblasts by PEI/DNA complexes, we analyzed the endocytotic activity of these cells by examining the uptake of the marker of membrane endocytosis, FM4-64, in the absence of PEI/DNA complexes. FM4-64 is a nontoxic, water-soluble dye that does not fluoresce

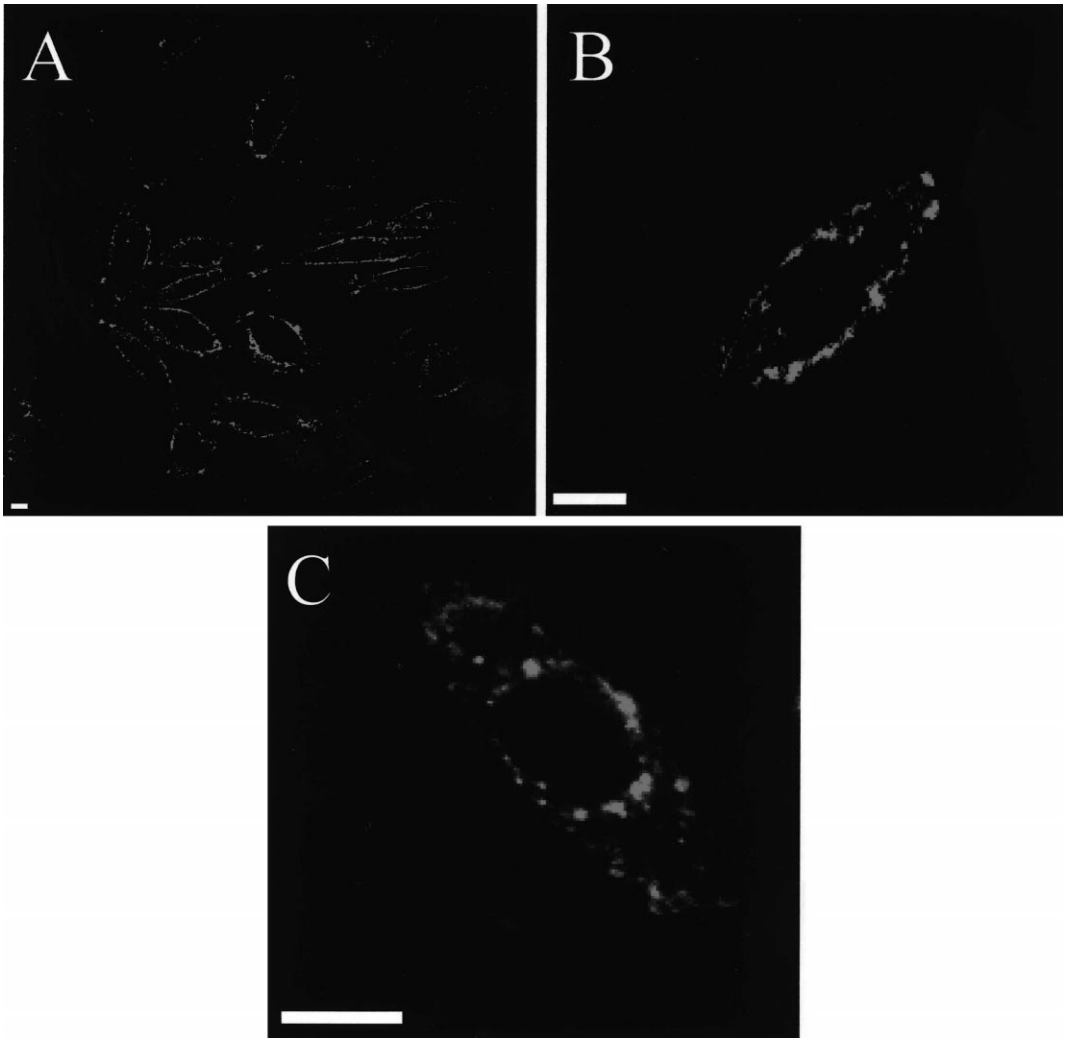


Fig. 1. FM4-64 uptake by L929 fibroblasts. The dye was added to cells to a final concentration of 4 μM and incubated at 37°C for either 5 (A), 20 (B) or 60 (C) min. Confocal images were obtained as described in Section 2. Each bar represents 10 μm.

in water but emits an intense red fluorescence when inserted into the membrane [20,21]. According to its amphiphilic nature, this dye is believed to insert into the outer leaflet membrane with its positive charge pointing toward the aqueous medium.

Five minutes after exposure to FM4-64, the fibroblasts exhibit a bright and uniform fluorescent staining of the plasma membrane (Fig. 1A) that suggests a strong and fast association of the dye to the membrane. Small fluorescent dots that may correspond to early endosomes also appeared in the cytoplasm. As expected, no fluorescence could be observed in the medium. At 20 min after exposure to the dye (Fig. 1B), numerous fluorescent dots are observable in the whole cytoplasm, suggesting a continuous formation of labeled endosomes. One hour after exposure to FM4-64 (Fig. 1C), the cells exhibited large and strongly fluorescent particles that are concentrated around the nucleus. These probably correspond to late endosomes resulting from the evolution and fusion of early endosomes. Only limited further changes were observed with longer incubation times.

To get additional information, the mean fluorescence intensity of FM4-64-labeled fibroblasts was measured by flow cytometry after various incubation times with the dye (Fig. 2). The rapid internalization of FM4-64 was confirmed since a significant amount of dye is taken up in less than 5 min. The kinetics of internalization are clearly biphasic with a rapid initial increase in fluorescence intensity followed by a

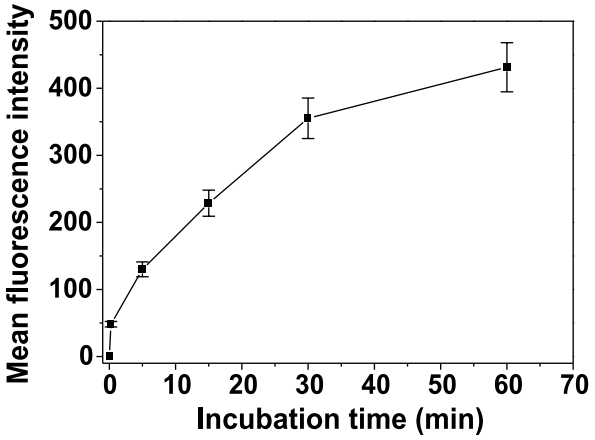


Fig. 2. Evolution of FM4-64 intracellular fluorescence intensity. The L929 fibroblasts were incubated at 37°C with FM4-64 for the indicated times, then fixed and analyzed by flow cytometry. The fluorescence intensities (■; arbitrary units) are corrected for cell autofluorescence and correspond to the mean of three independent measurements.

slower increase. This kinetic behavior is very similar to that of the TMA-DPH marker in the same cells [17] and further validates the use of FM4-64 as an endocytosis marker. By analogy with TMA-DPH, we infer that the biphasic behavior of FM4-64 may result from a balance between endocytosis and membrane recycling [17].

3.2. Endocytosis of PEI/DNA complexes

The complexes were prepared at a ratio of PEI amino groups to DNA phosphate groups of 10, that has been shown to lead to an optimal transfection efficiency [1]. By quasielastic light scattering, we assessed that the particles were monodisperse with an average diameter of 120($\pm$30) nm, consistent with the literature values [10].

The intracellular path of the PEI/DNA complexes was followed either with the fluorescein-labeled PEI, the DNA bis-intercalating agent YOYO-1, or the use of a rhodamine-labeled plasmid. Very similar results were obtained with the various labels. In contrast to FM4-64, no fluorescence staining of the plasma membrane was observable at short times ($<$10 min) after administration of the labeled complexes to cells. However, discrete patches of fluorescence readily appeared inside the cells, close to the plasma membrane (Fig. 3A). The diameter of these patches was estimated to be about 200 nm. A more accurate measurement could not be achieved since the theoretical lateral resolution of a confocal microscope with a 488 nm excitation wavelength and an objective with a 1.2 numerical aperture is 180 nm. In sharp contrast to previous observations on EA.hy 926 cells [15], no micrometric fluorescent clumps were observable on the outer surface of the plasma membrane of L929 cells. The complexes seem thus to be taken up by L929 cells in their initial form and no aggregation occurs. Accordingly, some mechanistic differences may exist in the internalization of the complexes in the two cell types.

As time progressed, the fluorescence patches increased in size and number, and accumulated around the nucleus (Fig. 3B,C). These features were similar to those observed with FM4-64 (Fig. 1) and suggested that PEI/DNA complexes follow an endocytosis pathway. Noticeably, the fluorescence of YOYO-labeled complexes was not significantly altered in the endosomes, even after 3 days post transfection (data not shown). Since YOYO-1 is only fluorescent when it is intercalated in DNA, it is suggested that DNA is not strongly digested by the DNases present in late endosomes.

To further investigate the endocytosis pathway of PEI/DNA complexes, FM4-64 and YOYO-labeled PEI/DNA complexes were administered simultaneously. The two dyes could be followed simultaneously since the red fluorescence of FM4-64 could be easily distinguished from the green fluorescence of YOYO-1 or PEI-FITC. Colocalization of FM4-64 and PEI/DNA complexes would yield yellow spots (a combination of red and green). One hour after administration, numerous discrete yellow patches appeared under the membrane (Fig. 4A). These patches indicated that PEI/DNA particles were taken up in early endosomes. Several red patches were also visible, suggesting that some endosomes internalized external medium that did not contain any PEI/DNA particles. This suggests that the concentration of complexes does not saturate the endocytosis process, a favorable feature for enabling the cells to internalize the components necessary for their metabolism. In contrast, green fluorescence corresponding to isolated PEI/DNA complexes represented less than 10% of the observed particles. This conclusion could be extended to any observation time and strongly suggested that the PEI/DNA com-

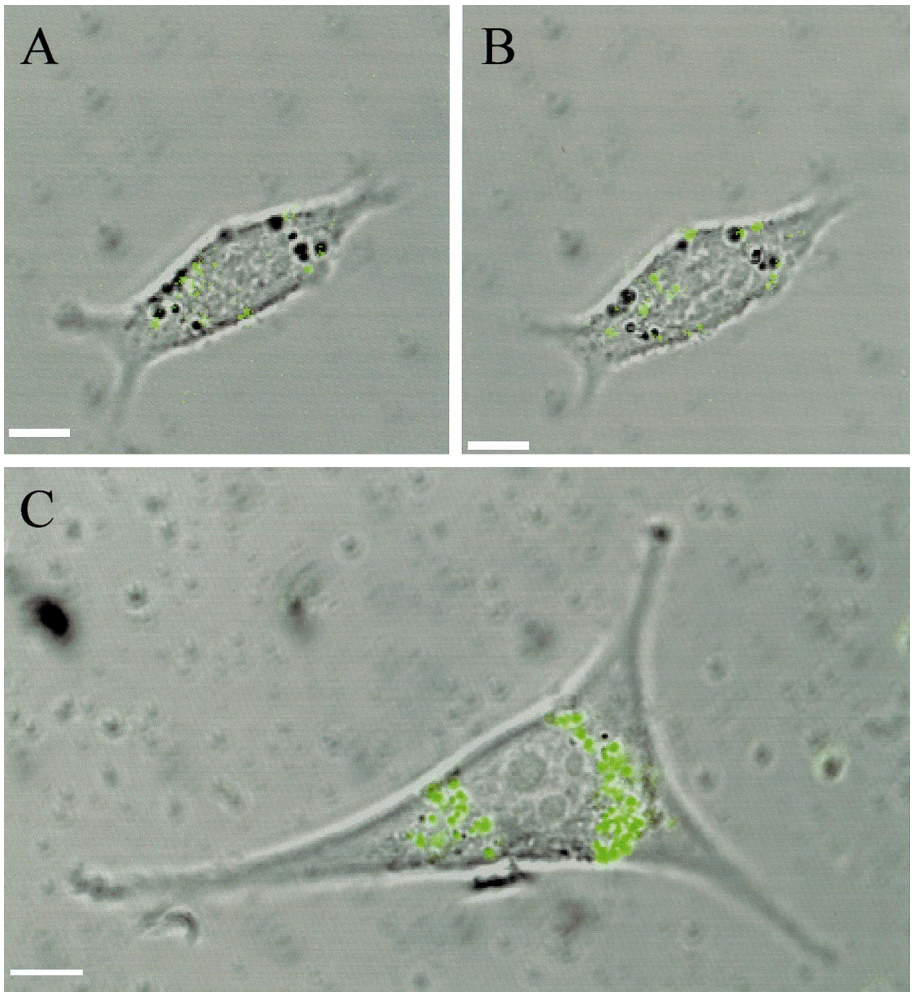


Fig. 3. Internalization of PEI/DNA complexes by L929 fibroblasts. The complexes were labeled with YOYO-1. The pictures correspond to the overlay of light transmission and fluorescence confocal images. The pictures taken at 30 min (A), 1 (B) and 15 (C) h post transfection are representative of more than 90% of the observed cells. Scale bars represent 10 μm.

plexes were essentially taken up by endocytosis. Moreover, the absence of green fluorescence further suggested that no massive release of PEI/DNA complexes from the endosomes into the cytoplasm occurred.

As time progressed, the yellow spots grew with time and accumulated around the nucleus (Fig. 4B), suggesting that the complexes were conveyed into late endosomes and finally accumulated in lysosomes. This indicates PEI/DNA complexes and FM4-64 may follow the same intracellular pathway. To confirm this, a sequential administration of FM4-64 and PEI/DNA complexes was performed. FM4-64 was incubated first during 20 min with the cells. The cells were then washed and incubated with free medium. After 2 h, labeled PEI/DNA complexes were added. Ten minutes after addition, the complexes could be observed inside the cells as discrete green patches close to the plasma membrane (Fig. 5A). In contrast, FM4-64 was essentially located in late endosomes around the nucleus and no yellow spots indicating the colocalization of the two dyes could be observed. However, 1 h after addition of the complexes, some yellow spots appeared in the perinuclear region (Fig. 5B). This suggests that the late endosomes that contain PEI/DNA complexes were able to fuse with FM4-64-labeled late endosomes. This process was not complete at that time since numerous endosomes still contained only either green or red fluorescence. In contrast, 24 h after addition of

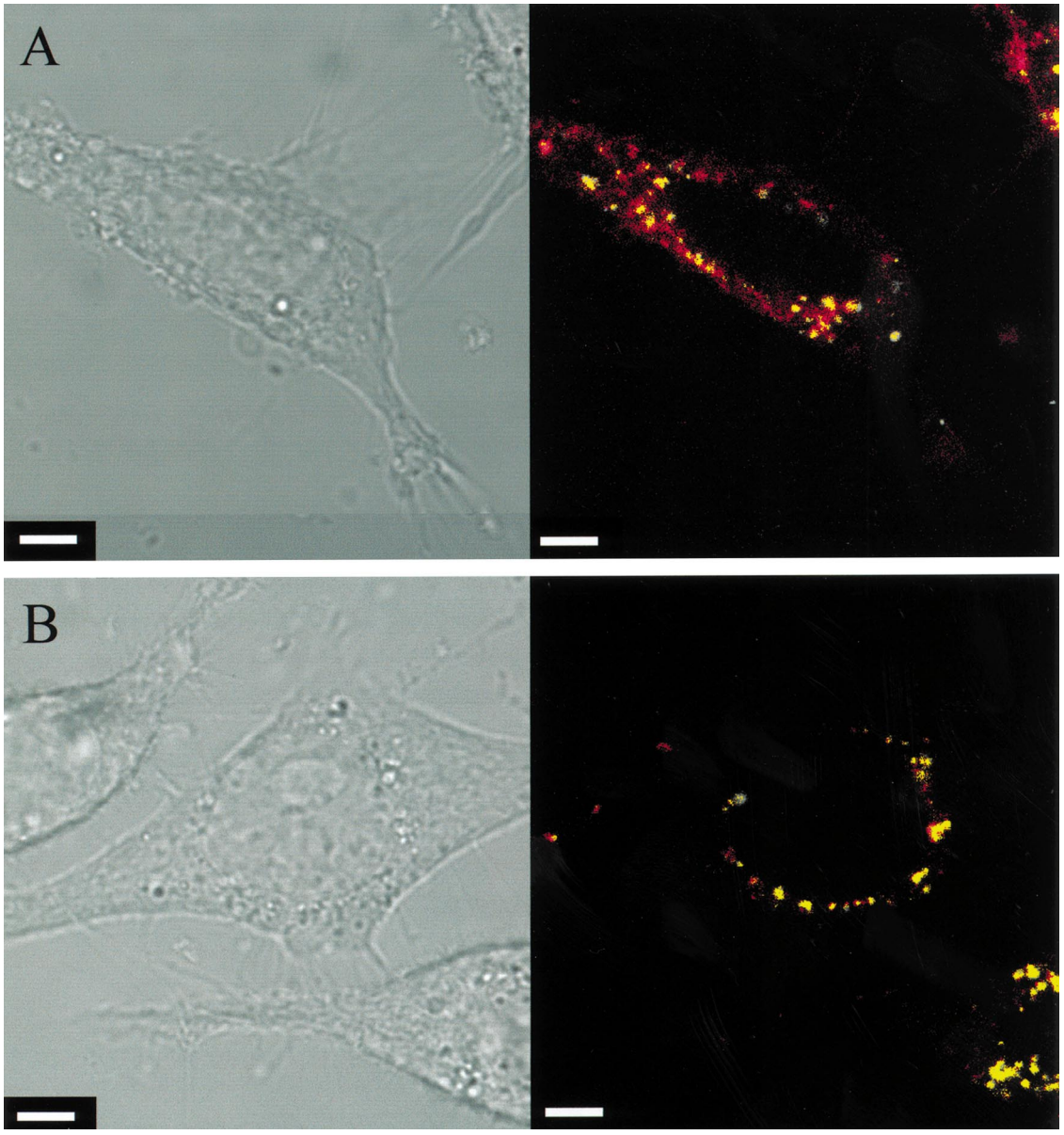
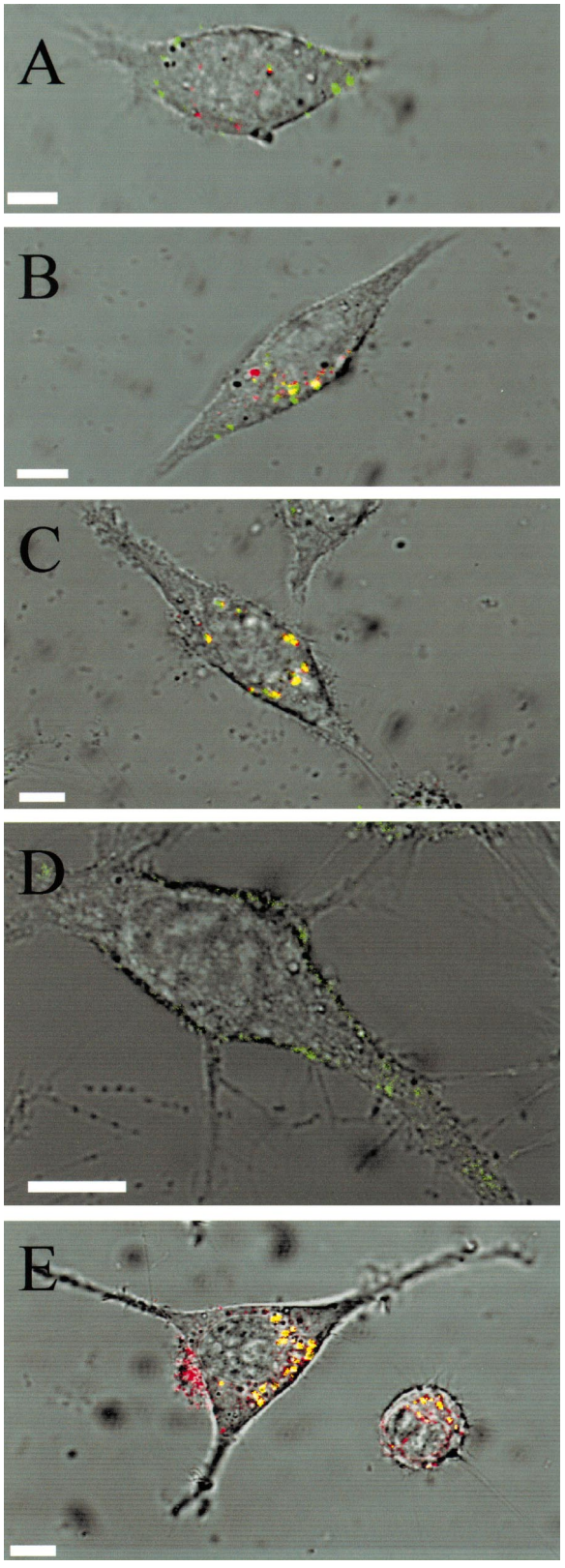


Fig. 4. Colocalization of YOYO-labeled PEI/DNA complexes and FM4-64 after simultaneous addition to L929 fibroblasts. Confocal images taken at 90 min (A) and 24 h (B) post transfection are representative of more than 90% of the observed cells. Due to the difference in brightness of the two dyes, the pictures in A and B correspond to the superimposition of the computer-generated colocalization picture (yellow spots) with the green and red confocal images. Red spots correspond to areas containing only FM4-64. Green spots that would correspond to areas containing only labeled complexes are not observed. The left panels are the light transmission images corresponding to the same cells. Scale bars represent 10 μm.

the complexes, most of the late endosomes were yellow (Fig. 5C), confirming that PEI/DNA complexes follow the same intracellular pathway as FM4-64.

In a final step, the intracellular trafficking of PEI/DNA complexes was compared with that of the plasmid or PEI administrated individually to L929 cells. In the case of the plasmid, the green fluorescence of the bound YOYO was homogeneously distributed on the cell surface but was not able to enter the cells (Fig. 5D). In the case of PEI-FITC, a simultaneous administration with FM4-64 led to numerous yellow patches but no green intracellular fluorescence (corresponding to PEI molecules able to enter independently of FM4-64) (Fig. 5E). This strongly suggests



that PEI molecules are essentially internalized by endocytosis and accumulate in late endosomes and lysosomes. In fact, no major differences could be observed between free and bound PEI.

3.3. Internalization of PEI/DNA complexes into the nucleus and gene expression

In contrast to EA.hy 926 cells [15], no labeled PEI/DNA complexes could be detected in the nucleus of transfected L929 cells. This is illustrated in the z-sections of a L929 cell which reveal that the interior of the nucleus is devoid of fluorescence (Fig. 6B,C) whereas fluorescent spots are located on the outer surface of the nucleus (Fig. 6D).

To check then if L929 fibroblasts were resistant to transfection, the cells were transfected with a plasmid coding for the luciferase gene. The expression of the reporter gene was quantified by measuring the light emission with a luminometer. A mean luminescence of 10^8 light units per mg of protein was measured at 24 h post transfection (data not shown). This value is in the range of that measured for easily transfectable cells like 3T3 and COS7 cells [1] and is not affected by the presence of YOYO or labeled PEI. To further quantify the transfection efficiency and, notably, the percentage of transfected cells, L929 cells were transfected with a GFP-coding plasmid. At various times after transfection, the percentage of cells expressing GFP was determined by flow cytometry. No expression could be observed before 15 h, which corresponds to the time required for the synchronized cells to enter mitosis [17]. This suggests that a cell division may be necessary for the internalization of PEI/DNA

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Fig. 5. Colocalization of FM4-64 and PEI/DNA complexes after sequential addition to L929 fibroblasts. FM4-64 was incubated first during 20 min with L929 fibroblasts. The cells were then washed and incubated with free medium. After 2 h, labeled PEI/DNA complexes were added. The pictures, corresponding to the overlay of light transmission and fluorescence confocal images, were taken at 10 min (A), 1 h (B) and 24 h (C) post transfection and are representative of more than 90% of the observed cells. Scale bars represent 10 μm. As a control, YOYO-labeled plasmid without PEI (D) or FITC-labeled PEI without DNA (E) was added to L929 cells. Pictures were taken at 1 h post transfection. In E, FM4-64 was also added to the cells.

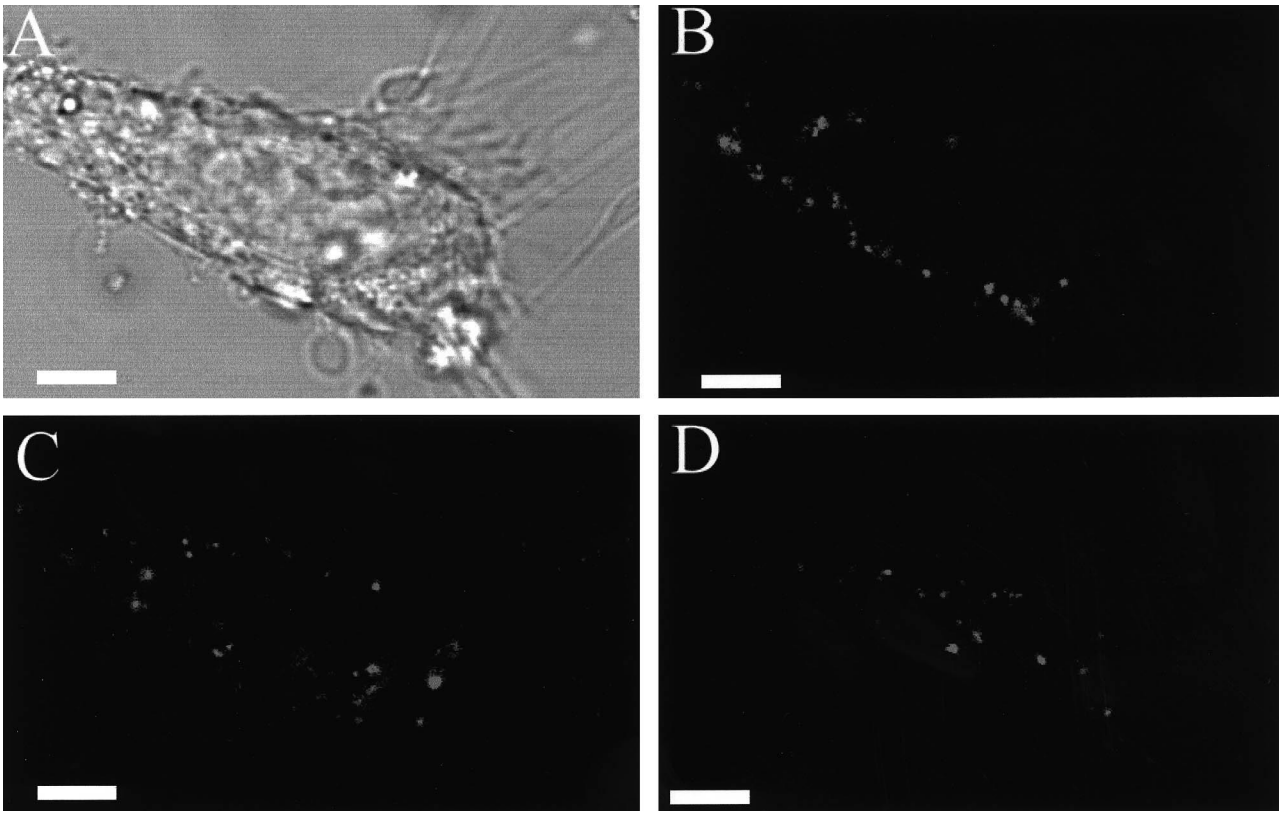

Fig. 6. z-Sections of L929 fibroblasts incubated with PEI/DNA complexes. YOYO-labeled complexes were administered as in Fig. 3. At 90 min post transfection, z-steps of a L929 cell were performed. The optical sections in B–D are at 2, 3.6 and 5.2 μm from the bottom of the cell, respectively. In B and C, the optical sections pass through the nucleus whereas in D, it is above the nucleus. The transmission image of the observed cell is given in A. Scale bars represent 5 μm.

complexes into the nucleus. The frequency of transfected L929 cells was rather low and did not exceed 5%, 24 h after administration of the PEI/DNA complexes (data not shown). This poor percentage was confirmed by direct observation of transfected cells by confocal microscopy, since only a few cells exhibit the green fluorescence of GFP (Fig. 7A). The GFP expression led to a diffuse green fluorescence all over the cell, including the nucleus. Interestingly, despite the overlap in the fluorescence emission of FITC and GFP, the diffuse fluorescence of GFP can be easily differentiated from the granular fluorescence of PEI-FITC/DNA complexes allowing multi-labeling assays to be performed (Fig. 7B). In contrast to the limited number of GFP-expressing cells, most cells were able to take up labeled PEI/DNA complexes. At 1 day post transfection, the percentage of cells labeled with PEI/DNA complexes was found by flow cytometry to be higher than 95%. Taken together, our data suggest that no obvious correlation exists between the presence of intracellular complexes and the expression of GFP.

4. Discussion

The present study sheds light on the role of endocytosis in the transfection of L929 cells by PEI/DNA complexes. Using the endocytosis marker FM4-64 and labeled complexes, we found that the fluorescence of the complexes was colocalized with that of FM4-64. This suggests that endocytosis is the main mechanism for the entry of PEI/DNA complexes into L929 cells. Moreover, the absence of staining of the plasma membrane with the labeled complexes further suggests that the internalization of the complexes proceeds by a fluid phase rather than a membrane endocytosis (for a review, see [24–26]). An internal-

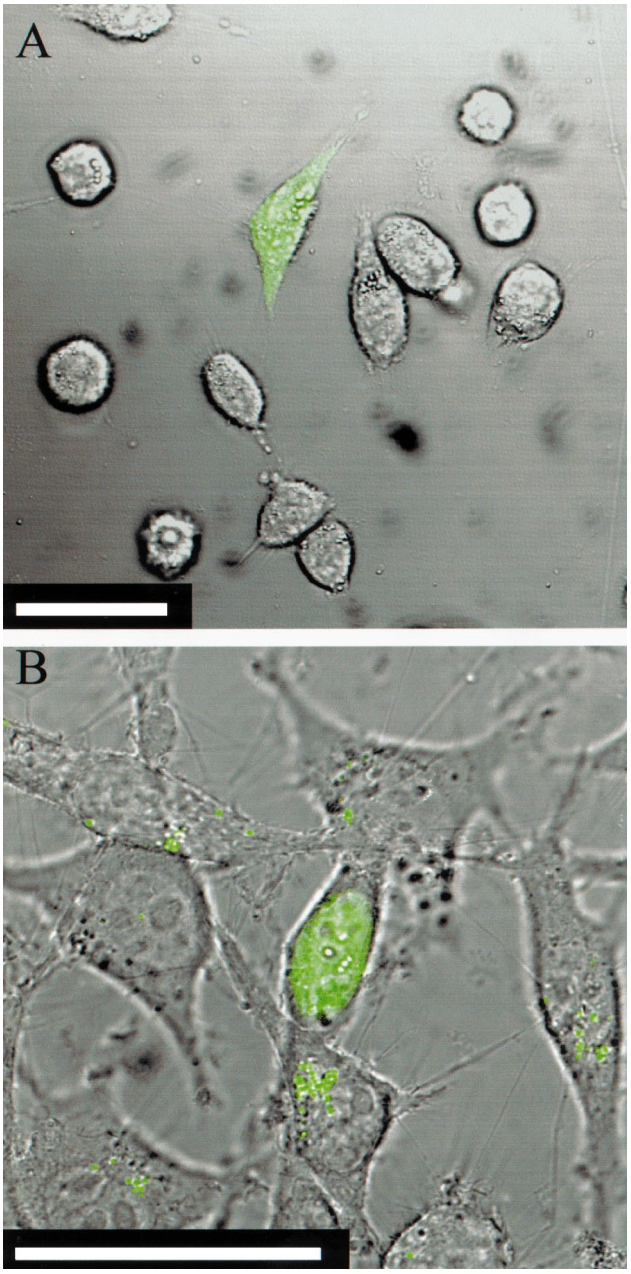

Fig. 7. Transfection of L929 fibroblasts with a GFP-coding plasmid complexed with PEI. The cells were incubated with a GFP-coding plasmid complexed with either unlabeled (A) or FITC-labeled PEI (B). At 24 h post transfection, a positive transfection is noted by the expression of the diffuse fluorescence of the green fluorescent protein (A) that could be easily differentiated from the fluorescence of the FITC-labeled complexes (B). Scale bars represent 50 μm.
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ization of PEI/DNA complexes by endocytosis has also been deduced in EA.hy 926 cells, albeit with a somewhat less direct approach [15]. However, several features clearly distinguished the two cell types. Indeed, the PEI/DNA complexes migrate to particular areas on EA.hy 926 cell surfaces giving large aggregates that are further taken up in vesicles of several microns. Moreover, this aggregation step is rather slow since intracellular aggregates appear only 30 min post transfection and increase in number over 1–2 h. In contrast, an efficient uptake of the same complexes by submicrometric endosomes was observed in less than 10 min all over the L929 cell and no aggregation occurred in full agreement with an internalization of PEI/DNA complexes by fluid phase endocytosis [17,24]. In EA.hy 926 cells, the dimensions of the observed compartments are reminiscent of macropinocytosis, a form of endocytosis frequently encountered in tumor cells [27] and involving compartments as large as 5 μm in diameter [28,29]. Moreover, in keeping with the data of Godbey et al. [15], macropinosomes have been reported to change from an early endosome-like organelle to a late endosome-like organelle, and finally to merge into the lysosomal compartment [30].

In addition to the dependence on the cell type, the entry mode of DNA into cells may also depend on the nature of the nonviral vector. For example, though endocytosis may also be the major mode of entry for cationic lipid/DNA complexes [31,32], an entry of the complexes via direct membrane disruption is also possible [31]. Moreover, endocytosis seems to be rather slow in this case since 30 min after administration, less than 5% of the cells contain significant amounts of complexes [32]. In contrast, the efficient uptake of PEI/DNA complexes by L929 cells indicates that the entry of these complexes by endocytosis is obviously not a limiting step in these cells.

After entry of the complexes in the cells, the complex-containing compartments evolved from the peripheral to the perinuclear region in a few hours. Moreover, either by simultaneous or sequential administration, the complexes and FM4-64 were found to accumulate in the same perinuclear compartments. Since fluid phase endocytosis and membrane endocytosis have overlapping intracellular pathways, the similarities in the intracellular pathway of the PEI/DNA complexes with that of FM4-64 are in further

keeping with an internalization of the complexes by fluid phase endocytosis. Moreover, these similarities further suggest that the uptake of the complexes does not affect the mechanism of endocytosis itself.

PEI/DNA complexes remained largely sequestered in late endosomes or lysosomes, a feature that may constitute a major barrier to transfection in L929 cells. In contrast to EA.hy 926 cells [15], no evidence of endosome disruption and/or delivery of PEI/DNA complexes into the cytoplasm could be observed. This latter effect has been linked to the 'proton sponge effect' of PEI, that allows a massive proton accumulation followed by passive chloride influx. This leads in turn to osmotic swelling and subsequent endosome disruption [1]. Accordingly, we infer that osmotic swelling may depend on the cell type (a feature that may be related to differences in the nature and activity of the proton pumps) and be more difficult to achieve in L929 than in EA.hy 926 cells.

The absence of a significant fluorescence decrease of the YOYO-labeled PEI/DNA complexes in the final endosomal compartments even after several days suggests that PEI protects DNA from the lysosomal nucleases. This protective effect may be related to the buffering activity of PEI which may raise the lysosomal pH and inactivate the degradative enzymes [13]. This effect may also partly be related to a mechanic effect of the binding of PEI to DNA, that does not favor the enzymatic attack of DNases.

The most intriguing step is finally the entry of the complexes into the nucleus. This step is probably inefficient since only a small percentage of the cells that had taken up GFP-coding plasmids were able to express the GFP protein. Similar small percentages have already been reported (for a review, see [4]) and found to vary with the cell type, the molecular weight of PEI and the final size of DNA complexes [8,13,14,19].

An additional intriguing feature is the absence of observable free PEI/DNA complexes in the cytoplasm or the nucleus of L929 cells. Similar statements have been made for the transfection of MRC5 cells with DNA/lipopolyamine complexes [33] or COS cells with lipoplexes [32]. This is in sharp contrast with EA.hy 926 cells where labeled complexes are easily observed in the nucleus [13,15]. To reconcile the absence of observable complexes in L929 cytoplasm or nucleus with the transfection results, we speculate that the entry of the complexes in L929 nuclei may be a short-lived event involving a small number of complexes.

In regard to the mechanism of entry of the PEI/DNA complexes into the nucleus, it has been previously suggested that after osmotic swelling and endosome disruption, the complexes may remain adhered to fragments of the endosome membrane [15]. This phospholipid coating would then allow the fusion of the complexes with the nuclear membrane. Our FM4-64 data do not support this hypothesis, since no transfer of this dye into the nuclear membrane could be observed (Fig. 4). In contrast, since no protein expression occurred before L929 cells are entering mitosis, it is suggested that the mechanism of entry of the complexes into the nucleus may require the disruption of the nuclear membrane. A similar conclusion has been reached for K562 cells [19] but not for COS7 [16] or postmitotic cells [34], suggesting that this step also depended on the cell type.

Another interesting point concerns the absence of correlation between the percentage of transfected cells and the level of transgene expression. While only a small percentage of cells could be transfected, the level of expression measured from luciferase assays is very high. This suggests that once the transgene is in the L929 nucleus, it is expressed at a high level.

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