

# Establishment of an assay to determine adenovirus-induced endosome rupture required for receptor-mediated gene delivery

Research Article

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## Summary

Successful human gene therapy requires methods to transfer recombinant genes to cells efficiently. One possibility is to use adenoviral-based vectors. The entry route of adenovirus involves endocytic uptake, penetration of modified viral particles into the cytoplasm by endosome rupture, transport to the nuclear pore complex, disassembly of modified particles and import of the DNA into the nucleus. Since endosome rupture is a rate-limiting step in foreign gene expression, we developed a two-step assay to quantitative virus-mediated membrane rupture. Following endosome labeling of HeLa cells with a pH-sensitive (FITC-dextran) and pH-insensitive (Cy5-dextran) fluid-phase marker in the absence or presence of replication-defective adenovirus type 5 (Ad5), first, the pH of labeled compartments was determined by flow-cytometry of cell suspensions. When compared to control cells, the pH of labeled compartments was elevated by co-internalization of Ad5 indicating endosome lysis and penetration of the marker into the pH-neutral cytoplasm. Second, single-organelle flow analysis (SOFA) of cell homogenates of the same cells was applied to quantitate the amount of labeled as well as unlabeled vesicles in the presence of Ad5. Our results demonstrate that adenovirus internalized for 10 min into HeLa cells destroys about 30% of endosomal compartments. This assay can be applied to rapidly screen various gene delivery systems for their ability to disrupt endosomal membranes and to enter the cytoplasm.

## I. Introduction

Adenovirus (Ad) is widely used as a vehicle to deliver foreign DNA into the cell of interest. This is due to the broad expression of adenoviral receptors on human cells and the particular mechanism of uncoating of this virus. 47 serotypes of adenoviruses have been characterized and classified into 6 subgroups (A - F). Adenovirus is a non-enveloped virus with an icosahedral capsid shell and protruding fibers anchored to the penton base proteins. The linear double stranded DNA is attached to four core proteins. In contrast to other DNA viruses, adenovirus

infects non-dividing cells and replicates in the nucleus but rarely integrates into the host genome. Expression of foreign genes can be achieved by direct DNA insertion into the viral genome or conjugation to the virus. Moreover, replication deficient adenoviruses have been generated that can be propagated in cell lines expressing the complementing viral proteins (Graham et al., 1977; Jones and Shenk, 1979). Recombinant adenoviruses have also been used for gene transfer (Fujita et al., 1995).

So far, the internalization pathway and mechanism of uncoating of adenoviruses of subgroup C (e.g. Ad2 and

Ad5) are fairly well characterized, although not fully understood at the molecular level. After binding to primary cellular receptors (MHC-class I complex and coxsackievirus-adenovirus receptor) via the distal portion of its fiber protein (Bai et al., 1994; Bergelson et al., 1997; Hong et al., 1997), subsequent internalization of the virus requires binding of five RGD motifs in the penton base to  $\alpha_v\beta$ -integrins (Wickham et al., 1993; Nemerow et al., 1994). Following endocytosis via clathrin coated pits and vesicles (Wang et al., 1998), adenovirus undergoes a series of modifications of its capsid proteins in the low pH environment of endosomes that ultimately results in rupture of the endosomal membrane (Greber et al., 1993). The low pH-dependent, virus-induced endosome lysis also requires the presence of  $\alpha_v\beta$ -integrins (Wickham et al., 1994). During endocytosis and penetration into the cytoplasm, capsid proteins are degraded or dissociated and finally the DNA core is freed from the hexon (Greber et al., 1993; Greber et al., 1996). Binding of the DNA core to the nuclear pore complex results in its disassembly and import of the viral genome into the nucleus (Greber and Kasamatsu, 1996; Greber et al., 1997). Thus, the capacity of adenovirus to rupture the endosome and the selective targeting of viral DNA to the nucleus provides a powerful vehicle for gene transfer (Curiel et al., 1991; Wagner et al., 1992; Wagner, 1998).

Adenovirus has long been known to increase the permeability of the plasma or endosomal membrane at low pH (Seth et al., 1985; Seth, 1994). In this process, co-internalized macromolecules can gain access to the cytoplasm. Using an in vitro assay (a so-called endosome leakage assay), we have recently shown that adenovirus leads to release of small and large molecules from isolated endosomes when incubated in low pH buffer (Prchla et al., 1995). Although this in vitro assay allows determination of conditions required for endosomal content release, it is laborious and time consuming.

Since efficient gene transfer primarily depends on endosome rupture, we sought to establish a rapid assay to determine the endosomolytic activity of adenovirus in vivo. Macromolecules and viruses taken up by endocytosis are exposed to the low pH environment of the endosomes due to the activity of the vacuolar proton ATPase (Mellman et al., 1986; Mukherjee et al., 1997). Therefore, we took advantage of the pH-dependence of FITC-derivatives to selectively label endosomes and measured endosomal pH using flow cytometry and the dual fluorescence (dual fluorochrome) ratio method (Murphy et al., 1984; Cain and Murphy, 1986). Flow cytometry can also be applied to resolve distinct fluorescent endocytic vesicles in cell homogenates, a method termed single-organelle flow analysis (SOFA) (Murphy, 1985, 1990; Murphy et al., 1989; Wilson and Murphy, 1989).

In the present study we co-internalized replication-defective adenovirus type 5 (Ad5) together with pH-sensitive (FITC) and pH-insensitive (Cy5) derivatives of the fluid-phase marker dextran into HeLa cells. The pH of labeled compartments was determined by flow cytometry and, in addition, the integrity of total vesicles and fluorescent endosomes was evaluated by SOFA. Using these techniques, we here demonstrate that Ad5 elevates the pH of labeled compartments, suggesting endosome lysis and access of the dextran into the pH neutral cytoplasm. This conclusion was verified by SOFA, in that a reduction in the number of labeled as well as unlabeled vesicles in the presence of Ad5 was observed. The results confirm the utility of these flow cytometric methods for monitoring adenovirus-induced endosome lysis.

## II. Results

### A. Flow cytometry of HeLa cell endosomes

Fluid-phase markers are non-specifically internalized into cells and can therefore be used to label all endocytic vesicles, depending on the internalization conditions applied (time, temperature). Furthermore, fluid-phase markers do not bind to cellular membranes and are released into the cytoplasm when endosomes are lysed (Yoshimura, 1985; Defer et al., 1990). Under control conditions, internalized markers will be exposed to the low pH environment of intact endosomes whereas in the presence of membrane disrupting agents they will be released into the pH-neutral cytoplasm (see **Fig. 1**).

**Figure 1.** Receptor mediated adenovirus entry and virus-induced endosome rupture.

**Figure 2.** Experimental set-up for FACS and SOFA analysis of HeLa cells infected with adenovirus type 5 (Ad5) in the presence of FITC- and Cy5-dextran.

We therefore internalized a pH-sensitive (FITC) and insensitive (Cy5) derivative of the fluid-phase marker dextran (MW 70 kD) in the presence or absence of Ad5 (1000 particles / cell) into HeLa cells for 10 min at 34°C, followed by a 10 min chase in marker-free medium (for experimental set-up see **Fig. 2**). Under this condition primarily late endocytic compartments will be labeled with the marker (Schober et al., 1998). Flow cytometry of cell suspensions was then used to determine the amount of internalized marker (reflected by Cy5 fluorescence) and endosomal pH (reflected by the ratio of FITC and Cy5 fluorescence). The total amount of marker internalized was found to be stimulated by adenovirus by 40% when compared to controls.

For pH measurements, a standard curve was generated for each sample by measuring the FITC/Cy5 ratio as a function of external pH. As shown in **Fig. 3**, increasing the pH of the external medium (in the presence of permeant ions) from 4.5 to 7.5 results in a linear increase in the FITC/Cy5 ratio. Based on this calibration curve, an average endosomal pH of 6.3 +/- 0.1 was obtained when marker was internalized in the absence of Ad5 (**Fig. 4**). When Ad5 was co-internalized with the dextran, the pH of labeled compartments was increased to 7.3 +/- 0.1, suggesting virus-induced endosome rupture and release of internalized marker into the pH neutral cytoplasm.

**Figure 3.** Normalized pH calibration curves of FITC/Cy5-dextran labeled endosomes obtained by flow cytometry of cell suspensions and post-nuclear supernatants (PNS), respectively. HeLa cell endosomes were labeled as described in **Fig. 2**. Cell suspensions or PNS were incubated with pH buffers containing permeant anions and azide to deplete cellular ATP and nigericin to equilibrate internal with external pH.

### **B. Single-organelle flow analysis (SOFA) of HeLa cells infected with adenovirus**

To verify whether the increase of the "endosomal" pH of labeled compartments in the presence of adenovirus is due to loss of internalized fluid-phase marker from acidic organelles such as endosomes, we applied single-organelle

flow analysis (SOFA). When a cell homogenate or post-nuclear supernatant (PNS) is subjected to SOFA, the following parameters can be analyzed at the same time: forward scatter (FS) and side scatter (SS), both of which are related to size and optical density, and FITC- and Cy5-fluorescence (Wilson and Murphy, 1989). Using these parameters the following information can be obtained: (i) size distribution and number of vesicles of a certain size; (ii) the degree of co-localization of two distinct fluorescent markers; and (iii) the internal pH of individual vesicles. Our goal was to determine (for cells treated and untreated with adenovirus) the number of total endocytic vesicles (unlabeled and fluorescent), the number of fluorescent endosomes, and the average pH of intact endosomes.

When measuring the properties of subcellular organelles by SOFA, a criterion must be chosen to define which objects in an organelle suspension are to be analyzed. This is normally accomplished by using a threshold on a light scattering parameter (FS or SS), since both fluorescent and

previously (Wilson and Murphy, 1989), we chose a threshold value of SS just above the maximum value observed when sheath fluid without sample was analyzed.

As an illustration of the SOFA method, all endocytic compartments (endosomes, lysosomes, recycling vesicles) of HeLa cells were labeled by continuous internalization of FITC- and Cy5-dextran for 2 h at 34°C. Thereafter, cells were rapidly cooled, washed and homogenized with a ball-bearing homogenizer ensuring minimal destruction of vesicles during homogenization (Balch and Rothman, 1985). Nuclei and unbroken cells were removed by centrifugation and the resulting PNS was subjected to SOFA (see Materials and Methods). For control purposes, a PNS was prepared from unlabeled cells and also analyzed by SOFA. To differentiate large vesicles (such as late endosomes) from small vesicles, an analysis window was created with a lower FS value just above the maximum observed for sheath fluid alone (as above for SS). As depicted in **Fig. 5** and **Table 1**, about 29% of the total events (objects) detected in both unlabeled (**Fig. 5A**) and labeled (**Fig. 5E**) samples were in this large vesicle window, while about 61% of all events fell in a corresponding window for small vesicles. In histograms displaying the fluorescence parameters, a region defining events positive for FITC and/or Cy5 fluorescence was created to exclude essentially all events from unlabeled cell homogenates. When either large or small vesicles from **Fig. 5A** were depicted in dual fluorescence histograms, a minute number (0.2%) of vesicles from unlabeled cells were detected in the fluorescence-positive region (**Fig. 5B** and **F**). This confirmed that the region was appropriately defined. (**Fig. 5C**). In the PNS of FITC/Cy5-dextran labeled HeLa cells, 64% of all vesicles counted (small and large) were fluorescent (**Fig. 5G, Table 1**). However, whereas the majority (83%) of the large vesicles contained FITC/Cy5-dextran (**Fig. 5F**), only 47% of the small vesicles were fluorescent (**Fig. 5H, Table 1**). Thus, nearly all large vesicles can be defined as endocytic vesicles due to labeling with internalized fluorescent fluid-phase marker. Consequently, the number of vesicles in this population was used as one indication of endosome disruption by adenovirus.

**Figure 4.** Influence of adenovirus on the pH of labeled compartments of HeLa cells. HeLa cell endosomes were labeled with FITC/Cy5-dextran without or with Ad5 as described in **Fig. 2**. Cell suspensions were analyzed by flow cytometry and the internal pH was calculated using the pH calibration curve shown in **Fig. 3**. Values depicted are the mean  $\pm$  SD from 3 independent experiments.

non-fluorescent objects can be detected in this fashion. In order to reproducibly measure endocytic vesicles, a method for choosing a consistent threshold is needed. As described

Having defined the flow-cytometer settings to analyze endosomes, FITC- and Cy5-dextran was internalized into HeLa cells in the absence or presence of Ad5 (10 min pulse, 10 min chase) as in **Fig. 2**. Cell homogenates were prepared, centrifuged at low speed and the resulting supernatant (PNS) was subjected to SOFA. As shown in **Fig. 6A**, the total number of large vesicles was reduced by co-internalization of Ad5 to 69% of controls. Further support for endosome lysis due to adenovirus entry is

**Figure 5.** Selection of gates to define small, large and fluorescent vesicles for SOFA. All endocytic compartments in HeLa cells were labeled by continuous internalization with 2 mg / ml FITC-dextran and 0.1 mg / ml Cy5-dextran for 2 h at 37°C. A post-nuclear supernatant was prepared as described in Materials and Methods and subjected to SOFA (**E-H**). Results for a post-nuclear supernatant from unlabeled HeLa cells are also shown (**A-D**).

**Table 1.** Quantitation of large, small and fluorescent vesicles in post nuclear supernatants analyzed by SOFA. The data presented in **Fig. 5** are summarized and expressed as % of total events counted (**Fig. 5B, D, F, H**). In addition, when endosomes had been labeled with FITC/Cy5-dextran (**Fig. 5E, F, H**) fluorescent vesicles were also normalized to the amount observed in the respective FS gate for large and small vesicles.

**Figure 7.** Influence of adenovirus on the internal pH of residual intact endosomes. The internal pH of large fluorescent endosomes for the samples in **Fig. 6B** was calculated using the pH calibration curve shown in **Fig. 3**.

provided when bona fide endosomes, i.e. fluorescent vesicles, are analyzed. In Ad5-infected cells, the number of FITC/Cy5-positive endosomes was decreased to 73% of controls (**Fig. 6B**). This demonstrates that quantitation of large vesicles by SOFA can be used to reflect endosome rupture.

Finally, the influence of adenovirus on the average internal pH of residual intact endosomes was determined. Endosomes maintain their low intravesicular pH after cell homogenization at 4°C for up to 20 h in the absence of highly permeant ions (Fuchs et al., 1989; Wilson and Murphy, 1989). A pH calibration curve similar to that obtained for whole cells was created for endosomes in the PNS analyzed by SOFA (**Fig. 3**). Using this calibration curve, an average internal pH of 6.0 +/- 0.1 was found for large vesicles (endosomes) from control cells (**Fig. 7**), while co-internalization of Ad5 slightly decreased the pH of residual intact endosomes (5.5 +/- 0.2). This confirms that the increase in pH of labeled compartments observed in cell suspensions by flow cytometry is indeed due to release of internalized marker into the cytoplasm, rather than to alteration of endosomal pH per se.

**Figure 6.** Adenovirus internalization reduces the number of large vesicles (**A**) and fluorescent endosomes (**B**). FITC/Cy5-dextran was internalized into HeLa cells in the absence or presence of Ad5 as described in **Fig. 2**. A PNS was prepared and analyzed by SOFA. (**A**) The number of total large vesicles was determined based on FS and SS histograms (as in **Fig. 5A**). (**B**) The number of large fluorescent endosomes in scatter-gated histograms of FITC and Cy5 fluorescence is shown (as in **Fig. 5F**). Data are expressed as percent of the corresponding values obtained in the absence of virus.

### III. Discussion

We here demonstrate that flow cytometry is a rapid and sensitive technique that can be used to analyze the endosome-disrupting potential of adenovirus. The two-step analysis involves 1/ determination of endosomal pH of labeled compartments by flow cytometry of cell suspensions, and 2/ SOFA of cell homogenates of the same

cells. The first analysis indicates the potential endosome leakage induced by the virus that it then verified and quantified by SOFA. Our results show that short co-internalization (10 min) of Ad5 and fluid-phase marker results in rupture of about 30 % of endocytic vesicles.

Adenoviruses of subgroup B and C have been shown to increase the rate of fluid-phase uptake and in addition to permeabilize the plasma or endosomal membrane for small and large molecules (Yoshimura, 1985; Otero and Carrasco, 1987). In particular, adenoviruses type 2 and 5 (subgroup C) are known to enter the cytoplasm by endosome lysis. Comparison of the data presented in this investigation with previous studies are difficult, because in former studies large quantities of adenovirus (2000 - 5000 particles / cell) were used and the internalization conditions applied resulted in labeling of early and late endosomes as well as of lysosomes (Defer et al., 1990). Furthermore, permeabilization of the plasma membrane could not be differentiated from endosome rupture. Adenovirus-mediated enhancement of cytoplasmic delivery has mainly been analyzed using toxins or toxin-conjugates that inhibit protein synthesis (Seth, 1994). So far, adenovirus-induced endosome rupture *in vivo* (i.e. the number of endosomes lysed) has not been quantitated.

Our results show that relatively small amounts of Ad5 (1000 particles / cell) when co-internalized for 10 min with the fluid-phase marker dextran and chased for an additional 10 min (to label late endosomes but not lysosomes (Prchla et al., 1994)) stimulated dextran uptake by 40%. This is in good agreement with published data (Defer et al., 1990). Under the same conditions, the virus destroyed about 30% of all endocytic compartments. Since the number of large vesicles in FS/SS histograms primarily reflect endocytic compartments (**Fig. 5** and **Table 1**), the endosomolytic activity of a given agent can be rapidly analyzed without prior endosome labeling with fluorescent tracers. Thus, this system offers the advantage to rapidly screen DNA-delivery systems such as low pH activated liposomes for their ability to lyse endosomes and to enter the cytosol.

Using adenovirus as gene delivery system one has to bear in mind that destruction of endosomes will also affect subsequent endocytic uptake of nutrients, hormones, and growth factors as well as signaling events from endosomes. So far, it is unknown how rapidly the endosomal system is regenerated after adenovirus internalization. Presumably, this may depend on whether for the particular cell of interest, transport to lysosomes occurs by endosome maturation or by carrier vesicles (Murphy, 1993; Gruenberg and Maxfield, 1995). We intend to apply SOFA to investigate the recovery of the endocytic system after adenovirus infection.

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## IV. Materials and Methods

### A. Chemicals

All chemicals were obtained from Sigma unless specified. Fluorescein isothiocyanate-conjugated (FITC)-dextran (FD 70) was extensively dialyzed against Tris-buffered saline pH 7.4 (TBS) and finally against phosphate buffered saline (PBS) before use. Cy5.18-OSu (Cy5) was obtained from Amersham (UK) and coupled to dextran (M<sub>w</sub> 70 kD) as described (Rybak and Murphy, 1998).

### B. Cell culture and virus propagation

HeLa cells (Wisconsin strain, kindly provided by R. Rueckert, University of Wisconsin) were grown in monolayers in MEM-Eagle (GIBCO) containing heat-inactivated 10% fetal calf serum; in suspension culture Joklik's MEM (GIBCO) supplemented with 7% horse serum was used. Adenovirus serotype 5 mutant dl 312 (Ad5), a replication incompetent strain deleted in the E1a region was propagated in 293 cells (Graham et al., 1977; Jones and Shenk, 1979).

### C. Endosome labeling for flow cytometry

HeLa suspension cells (2x10<sup>7</sup>) were preincubated in 2 ml DMEM containing 10% FCS for 30 min at 37°C. For labeling of all endocytic compartments, HeLa cells were incubated in 2 ml fresh medium with serum containing 2 mg / ml FITC-dextran and 0.1 mg / ml Cy5-dextran for 2 h at 37°C. To determine the influence of adenovirus on endosomal pH and endosome integrity, endosomes were labeled by incubation of HeLa cells in 2 ml DMEM containing 10% FCS, 6 mg / ml FITC-dextran, and 1 mg / ml Cy5-dextran without or with Ad5 (MOI of 1000) for 10 min at 34°C followed by a 10 min chase in DMEM in the absence or presence of Ad5. Internalization was halted by addition of ice-cold PBS (pH 7.4), pelleting the cells and washing the pellet twice with 30 ml ice cold PBS. The final cell pellet was resuspended in 2 ml PBS and divided into 7 aliquots. One aliquot (further diluted with PBS to 500 µl) was analyzed immediately by flow cytometry, the remaining aliquots were used for generation of the pH calibration curve (see below).

### D. Preparation of post-nuclear supernatant (PNS) for SOFA

All manipulations were carried out at 4°C. Following endosome labeling, the cells were washed twice with 50 ml PBS and pelleted. The cell pellet was resuspended in 4 vol. PBS and homogenized with a ball-bearing homogenizer (Balch and Rothman, 1985). The resulting homogenate was centrifuged for 15 min at 4300 g (Rotixa/RP, Hettich) to obtain the post-nuclear supernatant (PNS). The PNS was diluted 1:5 with PBS (pH 7.4) and immediately subjected to SOFA and generation of the pH standard curve, respectively (Murphy et al., 1989; Wilson and Murphy, 1989; Murphy, 1990).

### D. Generation of pH standard curves and

### calculation of internal pH for flow cytometry and SOFA

50  $\mu$ l aliquots of cells or PNS were resuspended in 250  $\mu$ l of buffers of various pH. Buffers (pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5) were obtained by mixing 50 mM HEPES with 50 mM MES (both containing 50 mM NaCl, 30 mM ammonium acetate, 40 mM sodium azide and 1  $\mu$ M nigericin) accordingly. The samples were left on ice for 5 min for ATP-depletion and for equilibration of intravesicular pH.

### E. Calculation of the pH of labeled compartments

The mean fluorescence value for each fluorochrome of experimental samples (8 parallels) and samples of the pH standard curve (duplicates) was calculated and the corresponding mean autofluorescence of unlabeled cells was subtracted from each. The ratio of the resulting average FITC and Cy5 values was calculated for each condition and normalized to the value obtained for that sample after clamping at pH 7.5. Finally, the average pH of labeled compartments was determined using the pH calibration curve.

### F. Flow cytometry

A dual laser FACS Calibur (Becton Dickinson Immunocytometry Systems) equipped with argon-ion and red-diode lasers was used. FITC-fluorescence (488 nm excitation) was measured using a 530 nm band pass filter (30 nm band width) and Cy5-fluorescence (635 nm excitation) was measured using a 661 nm band pass filter (16 nm band width). Forward light scatter and 90° (side)-scatter, along with both fluorescence values, were collected in list mode using 256-channel resolution. For flow cytometry of cell suspensions, data for 10.000 cells were collected, while 100.000 events per sample were collected for SOFA. The following threshold parameters were defined for SOFA (see also Results and Fig. 5): 1/ Forward scatter (FS) and side scatter (SS): As described (Wilson and Murphy, 1989), a threshold value of SS just above the maximum value observed when sheath fluid without sample was analyzed was chosen. To differentiate large vesicles (such as late endosomes) from small vesicles, an analysis window was created with a lower FS value just above the maximum observed for sheath fluid alone (as above for SS). 2/ FITC and Cy5 fluorescence: In dual fluorescence histograms, threshold parameters were set after analyzing the PNS of unlabeled cells. Thus, a region was defined for FITC and/or Cy5 fluorescence positive events.

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