Biochemical and functional analysis of an adenovirus-based ligand complex for gene transfer

Krishna J. FISHER and James M. WILSON*

Institute for Human Gene Therapy, Department of Molecular and Cellular Engineering, University of Pennsylvania Medical Center and the Wistar Institute, Philadelphia, PA 19104, U.S.A.

Ligand-mediated approaches to gene transfer offer an alternative to viral vectors for both *in vivo* and *in vitro* applications. Although a significant percentage of the plasmid-based DNA complex is lost to lysosomal degradation following receptor-mediated endocytosis, simultaneous infection with adenovirus has been shown to increase the level of transgene expression [Curiel, Agarwal, Wagner and Cotten (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8850–8854; Wagner, Zatloukal, Cotten, Kirlappos, Mechtler, Curiel and Birnstiel (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6099–6103]. In this study we describe an adenovirus-based ligand complex where the plasmid DNA, polycation–ligand conjugate and adenovirus are contained within a single particle structure. At the core of the transfection particle is a replication-defective recombinant adenovirus encoding a cDNA minigene for human placenta alkaline phosphatase that was chemically modified with

poly(L-lysine) (Ad-pLys). Electron microscopy of an adenovirus-based ligand complex formed by successively adding plasmid DNA and an asialo-orosomucoid–poly(L-lysine) conjugate to Ad-pLys revealed structures that appeared as intact viral particles coated with a dense biomolecular layer. Adenovirus-based ligand complexes containing either a luciferase or β -galactosidase reporter plasmid were shown to efficiently deliver the plasmid transgene to cells that express the hepatic asialoglycoprotein receptor. Furthermore, the poly(L-lysine) modification greatly reduced the infectivity potential of the virus without causing a concomitant loss of augmented gene transfer. As an alternative to infectious virions, incomplete products of viral assembly were also considered as a source for endosomal activity. However, these defective virions were unable to significantly enhance plasmid transgene delivery.

INTRODUCTION

The development of a non-viral particle that efficiently delivers a plasmid-encoded transgene to a specific eukaryotic cell has important implications for somatic gene therapy. We and others have shown that a soluble DNA–protein complex composed of plasmid DNA and poly(L-lysine) tagged with a receptor ligand will successfully target cells that express the corresponding receptor (Wu et al., 1989; Zenke et al., 1990; Wilson et al., 1992). While this approach has demonstrated its potential for gene transfer both *in vitro* and *in vivo*, the normal vectorial movement of complex-containing receptosomes to the lysosomal compartment severely diminishes the efficiency of transgene expression. In an effort to circumvent the problem of lysosomal targeting, much attention has been focused on ways to disrupt the structural integrity of endocytotic vesicles, allowing a greater percentage of plasmid DNAs to be released into the cytoplasm. One method that has been used successfully to induce endosomal lysis is to combine a replication-defective adenovirus with the DNA–protein complex either in *trans* as free particles (Curiel et al., 1991) or in *cis* as an antibody–antigen (Curiel et al., 1992) or biotin–streptavidin bridge (Wagner et al., 1992). This has been shown to significantly augment the delivery of a transgene packaged within a polycation-based complex, presumably mediated by the endosome-disrupting properties associated with

adenoviral infection (FitzGerald et al., 1983; Seth et al., 1985; Yoshimura, 1985).

In the present study we describe an adenovirus-based ligand complex that is capable of delivering a plasmid transgene to cells that express hepatic asialo-orosomucoid (ASOR) receptors. While our transfection particle is similar to the adenoviral-linked systems described by Curiel et al. (1992) and Wagner et al. (1992) in utilizing the endosomal properties of adenovirus to enhance gene transfer, we have incorporated several novel aspects. First, the overall design departs from earlier models that envisage the adenovirus attached side-by-side to a preformed complex toroid, in favour of an arrangement that distributes the entire structure around the virion capsid. By adopting such a strategy, the need for a distinct DNA–polycation complex was eliminated. We arrived at the single particle concept because of our reservations that interactive linkages such as those between antibody and antigen or biotin and streptavidin would not be limited to a single union during complex formation, resulting in end-on-end concatamers. The potential for aggregates would be particularly problematic for *in vivo* studies, when restrictions imposed by the microcirculation become relevant. A second feature unique to the adenovirus-based ligand complex detailed here was the use of a recombinant virus containing an alkaline phosphatase minigene. When this was used in conjunction with a plasmid β -galactosidase reporter, both viral infectivity and plasmid gene transfer could be monitored.

Abbreviations used: Ad-biotin, adenovirus–biotin conjugate; Ad-pLys, adenovirus–poly(L-lysine) conjugate; ASOR, asialo-orosomucoid; ASOR-pLys, asialo-orosomucoid–poly(L-lysine) conjugate; BCIP, 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt; CMV, cytomegalovirus; FBS, fetal bovine serum; HBS, Hepes-buffered saline; NBT, Nitroblue Tetrazolium (chloride salt); NHS, *N*-hydroxysuccinimide; PVDF, poly(vinylidene difluoride); SMCC, *N*-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; TC-pLys, top component–poly(L-lysine conjugate).

* To whom correspondence should be addressed: Institute of Human Gene Therapy, University of Pennsylvania, 3601 Spruce Street, Room 204, Wistar Institute, Philadelphia, PA 19104-4268, U.S.A.

EXPERIMENTAL

In vitro cell transfections with DNA–protein complex and free adenovirus

The cell lines that were targeted for gene delivery, i.e. HUH7, HepG2 and HL, were selected based on their ability to internalize DNA–ASOR complexes via the hepatic asialoglycoprotein receptor system (Ashwell and Harford, 1982). HUH7 and HepG2 are both human hepatoma cell lines, and were grown in modified Eagle's medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C in an atmosphere of 5% CO₂. HL cells are clonally selected NIH 3T3 cells that express a recombinant human asialoglycoprotein receptor; these were maintained in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% calf serum and antibiotics as above. Human ASOR (Sigma) was prepared by exhaustive dialysis with insoluble neuraminidase (Boehringer Mannheim Corporation) and chemically coupled to poly(L-lysine) of average molecular mass of 68 kDa (Sigma) using 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide (Pierce) (Wu et al., 1989). The optimal ratio of the ASOR–poly(L-lysine) (ASOR–pLys) conjugate to DNA plasmid for complex formation was determined by an agarose gel retardation assay (Wu et al., 1989).

The general procedure that was followed for transfections with DNA–protein complex toroids was as follows. Complex was prepared by slowly adding drop-wise 1.5 µg of ASOR–pLys in 20 mM Hepes, pH 7.8, and 150 mM NaCl (HBS) to 0.5 µg of plasmid DNA in 75 µl of HBS, and incubating the mixture at room temperature for 30 min. The plasmids that were used, pCMVLuc and pCMVLacZ, contain cDNAs for firefly luciferase and bacterial β -galactosidase respectively, both of which are under the transcription control of an immediate early cytomegalovirus (CMV) enhancer/promoter (Boshart et al., 1985). Cells were seeded at 6×10^5 per 35 mm 6-well plate and allowed to attach for 20–24 h. Media supplemented with 2% serum (850 µl) was added to the complex and the entire volume was applied to a single 35 mm well from which the plating media had been removed. For transfections in the presence of free adenovirus or poly(L-lysine)-modified adenovirus, the virions were included immediately after the complex/media cocktail was added to the cells. The adenovirus used in these experiments will be described in a following section. In instances where the effects of chloroquine were to be determined, the complex/media cocktail was adjusted to 50 µM from a freshly made 10 mM stock. At 1 h after the addition of complex, media (1 ml) supplemented with 10% serum, 1 mM CaCl₂ and, where appropriate, 50 µM chloroquine was added to cells.

Following transfection (20 h) the cells were harvested and evaluated for transgene expression. For luciferase expression, an enzyme assay kit was used according to the instructions provided by the manufacturer (Promega). Briefly, cells were washed twice in PBS (pH 7.2) and lysed directly in the culture dish with $1 \times$ lysis buffer (25 mM Tris/phosphate buffer, pH 7.8, 2 mM EDTA, 2 mM dithiothreitol, 10% glycerol and 1% Triton X-100). The lysate was harvested and cell debris was removed by a 5 s spin in a microcentrifuge. Extract (5–10 µl) was added to 50 µl of luciferase assay reagent, mixed and placed into a Bio-Orbit 1251 Luminometer (Pharmacia). Photon emission was collected for an interval of 30 s in all experiments. For cytochemical analysis of β -galactosidase expression, cells were washed twice in PBS (pH 7.4) and fixed for 10 min at room temperature in 0.5% glutaraldehyde in PBS. Cells were washed twice for 15 min each in PBS containing 1.0 mM MgCl₂, followed by colour development in 1.0 mg/ml 5-bromo-4-chloro-3-indolyl β -

D-galactopyranoside (Boehringer Mannheim Corporation) dissolved in PBS containing 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, and 1 mM MgCl₂ for 2–3 h at 37 °C. After development of the β -galactosidase reaction, cells were processed for recombinant alkaline phosphatase activity by incubation at 65 °C for 30 min to destroy endogenous activity, washed twice in 100 mM Tris/HCl (pH 9.5), 0.1 M NaCl and 50 mM MgCl₂, and stained in the same buffer containing 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP; 0.165 mg/ml) and NitroBlue Tetrazolium chloride (NBT; 0.33 mg/ml) (Bethesda Research Laboratories) at 37 °C for 1 h. All light microscopy was performed using a Microphot-FXA Nikon microscope.

Poly(L-lysine) modification of adenovirus

The adenovirus used in this study, Ad.CBAP, was derived from a human adenovirus serotype 5 strain (sub 360). Briefly, the virus was made replication-defective by deleting sequences from the E1a:E1b region and replacing them with a cDNA encoding human placenta alkaline phosphatase. The alkaline phosphatase transgene expressed by Ad.CBAP is driven by CMV enhancer/ β -actin promoter chimeric transcriptional elements. Viral stocks were prepared by infecting subconfluent monolayer cultures of 293 cells seeded on 150 mm plates at a multiplicity of 5–10 plaque-forming units per cell. At 30–36 h post-infection, cells were harvested and extracts were prepared. Purification of viral particles was accomplished by two rounds of ultracentrifugation on a caesium chloride step gradient made from equal volumes of CsCl at 1.45 mg/ml and 1.20 mg/ml in 10 mM Tris/HCl buffer (pH 8.0). On completion of the first centrifugation, performed at 70000 *g* for 2 h at 5 °C, two distinct bands were retrieved, one sedimenting at a density of approx. 1.33 mg/ml and the second at 1.30 mg/ml. The denser of the two bands has been shown to contain complete infectious virus, while the less dense band is composed of intermediate, incompletely formed particles previously referred to as top components (Maizel et al., 1968). Both fractions were harvested and banded at 90000 *g* for 15 h at 5 °C. The final purified virus was stored at –20 °C in buffer containing 10 mM Tris/HCl (pH 8.0), 100 mM NaCl and 40% glycerol. Viral particle titres were determined by u.v.-absorbance measurements, where one absorbance unit at 260 nm (A_{260}) represents 1×10^{12} viral particles per ml. Top component A_{260} particle titres were adjusted according to the A_{280}/A_{260} ratio obtained with infectious particles due to their deficiency of nucleic acid. Infectious plaque-forming units were also determined by an agarose overlay assay.

Figure 1 shows a scheme detailing the two-step strategy used to conjugate poly(L-lysine) to adenovirus. Poly(L-lysine) was chemically coupled to purified adenovirus and viral top components through a heterobifunctional water-soluble cross-linking agent, sulpho-SMCC [sulpho-(*N*-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate) (Pierce). The conjugation reaction, which contained 0.5 mg (375 nmol) of sulpho-SMCC and 6×10^{12} A_{260} adenoviral particles in 3.0 ml of HBS, was incubated at 30 °C for 45 min with constant gentle shaking. This step involved formation of a peptide bond between the active *N*-hydroxysuccinimide (NHS) ester of sulpho-SMCC and a free amine (e.g. lysine) contributed by an adenovirus capsid protein, yielding a maleimide-activated viral particle. Unincorporated cross-linker was removed by gel filtration on a 1 cm $\times$ 15 cm Bio-Gel P6DG (Bio-Rad Laboratories) column equilibrated with 50 mM Tris/HCl buffer, pH 7.0, and 150 mM NaCl. Peak A_{260} fractions containing maleimide-activated adenovirus were combined and placed on ice. In the second step, poly(L-lysine) (270 kDa) at 10 mg/ml in 50 mM triethanolamine buffer

Figure 1 Scheme summarizing the synthesis of an adenovirus-based ligand complex

The upper portion of the diagram outlines the procedure for adenovirus poly(L-lysinylation), while the lower half shows the major steps involved in generating adenovirus-based transfection particles (see the Experimental section). Briefly, CsCl band-purified adenovirus was reacted with the heterobifunctional cross-linker sulphy-SMCC. The activated adenovirus is shown having the capsid protein fibre labelled with the nucleophilic maleimide moiety. In practice, other capsid polypeptides including hexon and penton base were also targeted. Free thiol groups were introduced on to poly(L-lysine), with the extent of thiolation determined to be approx. 3–4 mol of -SH/mol of polycation. The Ad-pLys conjugate was purified over a CsCl gradient to remove unincorporated poly(L-lysine), extensively dialysed against HBS and added to plasmid DNA, resulting in an adenovirus-based complex. From this core structure an adenovirus-based ligand complex was generated by adding an ASOR-pLys conjugate.

(pH 8.0), 150 mM NaCl and 1 mM EDTA was treated with a 4-fold molar excess of 2-iminothiolane/HCl (Traut's Reagent; Pierce) under N_2 ; the cyclic thioimidate reacts with the poly(L-lysine) primary amines resulting in a thiolated polycation. After a 45 min incubation at room temperature the reaction was

applied to a 1 cm $\times$ 15 cm Bio-Gel P6DG column equilibrated with 50 mM Tris/HCl buffer (pH 7.0), 150 mM NaCl and 2 mM EDTA to remove unincorporated Traut's Reagent. Peak A_{280} fractions containing thiolated poly(L-lysine) were combined and placed on ice. Quantification of free thiol groups was accomplished with Ellman's reagent [5,5'-dithio-bis-(2-nitrobenzoic acid)], revealing approximately 4 mol of -SH/mol of poly(L-lysine). The coupling reaction was initiated by adding 1×10^{12} A_{280} particles of maleimide-activated adenovirus/mg of thiolated poly(L-lysine) and incubating the mixture at 4 °C for 20 h; 2-mercaptoethylamine was added at the completion of the reaction and incubation carried out at room temperature for 20 min to block unreacted maleimide sites. Removal of unconjugated poly(L-lysine) was accomplished by ultracentrifugation through a CsCl step gradient composed of equal volumes of 1.45 mg/ml and 1.20 mg/ml CsCl in 10 mM Tris/HCl buffer (pH 8.0). Centrifugation was at 90000 g for 2 h at 5 °C. The opalescent band containing the adenovirus-poly(L-lysine) conjugate (Ad-pLys) or the top component-poly(L-lysine) conjugate (TC-pLys) was recovered and extensively dialysed against HBS.

Characterization of poly(L-lysine)-modified adenovirus

Gel mobility shift assays were conducted by adding dilutions of Ad-pLys in 15 μ l of HBS to 200 ng of pCMVLuc plasmid DNA in 15 μ l of HBS. After a 30 min incubation at room temperature the mixture was resolved on a 1.0% agarose gel and visualized with ethidium bromide. As a control, pCMVLuc (200 ng) was incubated at room temperature in the presence of 8×10^9 A_{280} particles of unmodified adenovirus. Ion-exchange chromatography of unmodified and poly(L-lysine)-conjugated adenovirus was performed through a 1.5 cm $\times$ 10 cm column packed with DE52 anion-exchange resin (Whatman) previously equilibrated with 50 mM Tris/HCl buffer (pH 8.0). The column was washed with 10 column volumes of buffer followed by a 100 ml linear salt gradient (0–0.5 M NaCl) in equilibration buffer. Fractions (2.0 ml) were monitored at 280 nm.

Biotinylation and Western blot analysis of adenoviral capsid proteins

CsCl band-purified adenoviruses or top components in HBS (2×10^{12} A_{280} particles) were incubated at room temperature for 1 h in the presence of 120 μ g of sulphy-NHS-long-chain-biotin (Pierce). The reaction was loaded on to a CsCl step gradient and centrifuged at 90000 g for 2 h at 5 °C to remove the unincorporated biotin analogue. Biotinylated particles (Ad-biotin) were recovered as an opalescent band, extensively dialysed against HBS and quantified according to absorbance at 260 nm. Samples of adenovirus, Ad-pLys and Ad-biotin were boiled for 2 min in the presence of 2% SDS, resolved on an SDS/12%-polyacrylamide gel, and the proteins electrophoretically transferred to an Immobilon poly(vinylidene difluoride) (PVDF) membrane (Millipore). A section of the blot containing one set of samples was stained for protein with Amido Black. The remaining blot containing samples of adenovirus and Ad-biotin was incubated at room temperature for 1 h in 20 mM Tris/HCl, pH 7.4, 150 mM NaCl and 0.05% Tween-20 (TBST) containing 3% BSA to block unoccupied binding sites. This was followed by a 1 h incubation at room temperature with a streptavidin-alkaline phosphatase conjugate (Gibco BRL) diluted 1:10000 in TBST containing 1% BSA. After several washes in TBST, the streptavidin-biotin complex was visualized with the chromogenic substrates BCIP and NBT dissolved in 100 mM Tris/HCl (pH 9.5), 0.1 M NaCl and 50 mM $MgCl_2$.

Figure 2 Cell-specific enhancement of ligand-directed gene transfer mediated by infectious adenoviral particles

The level of luciferase expression in three different cell lines, HL, HepG2 and HUH7, transfected with a DNA–polycation complex composed of 0.5 μ g of pCMVLuc and 1.5 μ g of ASOR-pLys is presented. Cells were simultaneously treated with 50 mM chloroquine (bars labelled +) or infected with 0, 1, 2, or 3 $\times 10^{10}$ Ad.CBAP particles/35 mm culture dish as indicated. In one assay, HUH7 cells received infectious Ad.CBAP virions together with defective viral top components (TC.CBAP). Luciferase expression is given in total light units generated by a confluent 35 mm culture dish (approx. 10^6 cells) 20 h post-transfection. Cell extracts were standardized to protein as measured by the Bradford assay.

In vitro cell transfections with adenovirus-coupled complex

Adenovirus-based complexes were formed by adding varying quantities of Ad-pLys or TC-pLys in 50 μ l HBS to 0.5 μ g pCMVLuc or pCMVLacZ also in 50 μ l HBS and allowing a complex to form at room temperature for 30 min (Fig. 1). Using this structure as a core, an adenovirus-based ligand complex could subsequently be generated by adding 0.5 μ g ASOR-pLys conjugate in 50 μ l HBS followed by a second 30 min room temperature incubation (Fig. 1). Transfections were initiated by adding complex to 35 mm culture dishes containing 10^6 cells in 1.0 ml MEM supplemented with 2% FBS and antibiotics. Following a 1 h incubation at 37 $^{\circ}$ C, 1.0 ml of MEM containing 10% FBS and antibiotics was added and the 37 $^{\circ}$ C incubation continued for an additional 19 h. Cells were washed twice with PBS and assayed for luciferase or co-stained for β -galactosidase and alkaline phosphatase activity as before.

RESULTS

Adenovirus-enhanced transgene delivery

The ability of free adenoviral particles to augment the nuclear delivery of an ASOR–ligand complex was tested in the context of two human hepatoma cell lines, HepG2 and HUH7, and a mouse fibroblast cell line (HL) that expresses ASOR receptor. Figure 2 summarizes the results of transfection experiments using the luciferase reporter gene. The hepatoma cell lines produced the greatest increase in recombinant activity when co-infected with adenovirus, whereas HL cells were observed to be relatively unresponsive to the inclusion of virions. This latter finding cannot be attributed to deficient levels of ASOR receptor on the surface of HL cells or a promoter effect, since transfection with DNA–protein complex in the presence of chloroquine produced high levels of luciferase expression. A more plausible

explanation is that the number of adenovirus receptors on HL cells is low compared with HepG2 and HUH7 cells. Indeed, the adenovirus used in these experiments, Ad.CBAP, was found to be significantly more tropic for the hepatic cell lines, based on its ability to transduce alkaline phosphatase. This was evident from cells that received a simultaneous dose of Ad.CBAP and an ASOR ligand complex containing a β -galactosidase minigene (Figure 3). Under these conditions, transfection with complex alone resulted in few LacZ-plasmid-expressing HUH7 (Figure 3a) or HL (Figure 3c) cells. When HUH7 cells were co-infected with 1×10^{10} A_{260} adenoviral particles, virtually all cells expressed alkaline phosphatase whereas a few percent of these same cells also exhibited β -galactosidase activity, suggesting adenovirus-enhanced plasmid transgene delivery (results not shown). Increasing the multiplicity of infection 2-fold resulted in a greater number of HUH7 cells transfected with the LacZ plasmid reporter; however, there was a concomitant increase in viral-induced cell death (Figure 3b). In contrast, HL cells given 2×10^{10} A_{260} adenoviral particles/ 10^6 cells displayed alkaline phosphatase activity in a minority of cells, a degree of infection that was not sufficient to augment plasmid gene transfer as shown by the lack of β -galactosidase-positive cells (Figure 3d). We also tested unmodified defective viral top particles (i.e. top components) and found that they do not facilitate in *trans* the endosomal release of complex toroids (results not shown). In fact, HUH7 cells infected with infectious Ad.CBAP and a 2-fold excess of TC.CBAP displayed a reduced level of plasmid transgene expression, suggesting that viral top components are able to effectively compete for receptor binding sites (Figure 2).

Biochemical analysis of poly(L-lysine)-modified adenovirus

Our design for an adenovirus-coupled ligand complex called for the synthesis of a poly(L-lysine)-modified viral particle using the heterobifunctional reagent sulfo-SMCC (Figure 1). Poly(L-lysine) was conjugated to both infectious virus (Ad-pLys) and to an incompletely assembled form of the virus, called top component, that migrates higher on the caesium gradient (TC-pLys). The success of adenovirus poly(L-lysinylation) was measured according to several criteria, the first of which was ion-exchange chromatography. Unmodified adenovirus injected on to an anion-exchange column at pH 8.0 bound to the support matrix, in agreement with previous reports that the viral capsid shell displays a net negative charge (Figure 4a) (Wilcox and Ginsberg, 1961). The virus was recovered from the column as a single peak during a linear salt gradient. This result was in contrast to Ad-pLys, which emerged from the column in the void volume as a positively charged species (Figure 4b). While the recovery of the Ad-pLys peak from the column appeared to be quantitative, a minor peak eluted during the salt gradient suggesting residual quantities of unmodified virus. The second analysis of the conjugate was based on binding of Ad-pLys to DNA. A gel mobility shift assay revealed a progressive retardation in the migration of supercoiled plasmid DNA through an agarose gel in response to incremental increases in Ad-pLys (Figure 5). At 3.8×10^{10} A_{260} particles/ μ g of plasmid DNA, virtually all of the nucleic acid present in the assay reaction was complexed with poly(L-lysine)-modified adenovirus. This interaction was specific for Ad-pLys, since a control sample containing 4.0×10^{10} A_{260} unmodified adenoviral particles/ μ g of plasmid had no effect on the migration of plasmid DNA. A similar gel retardation assay using TC-pLys indicated that poly(L-lysine) was less efficiently conjugated to viral top components, since a complete shift of plasmid did not occur until a concentration of 6×10^{10} A_{260}

Figure 3 Cytochemical detection of reporter genes following ligand-directed gene transfer

HUH7 and HL cells were transfected with a DNA–polycation complex prepared with 0.5 μ g of pCMVLacZ and 1.5 μ g of ASOR-pLys. At 24 h post-transfection, cells were fixed and stained for plasmid-encoded β -galactosidase, followed by histochemical staining for viral-encoded alkaline phosphatase activity as described in the Experimental section. Cells were transfected with complex alone (**a**, HUH7; **c**, HL) or in the presence of 2×10^{10} AD.CBAP particles (**b**, HUH7; **d**, HL).

particles/ μ g of DNA was achieved (results not shown). The final analysis of Ad-pLys was a direct amino acid composition determination of the purified preparation. The integrated mol% of lysine residues increased 3-fold, from 4.0 for unmodified adenovirus to 12.0 for Ad-pLys.

Identification of poly(L-lysine)-modified capsid proteins

In an effort to delineate the viral proteins that were modified with poly(L-lysine) during the conjugation reaction, equivalent samples of adenovirus, Ad-pLys and TC-pLys were analysed by Western blotting. Proteins that were modified with at least one poly(L-lysine) molecule would be expected to attain a sufficient increase in both positive charge and molecular mass to prevent them from entering the resolving gel; a relative decrease in protein band intensity was used as a measure of conjugation. This analysis revealed a significant loss of the major capsid polypeptides hexon (II, 120 kDa), penton base (III, 85 kDa) and fibre (IV, 62 kDa) from the Ad-pLys sample (Figure 6, lane D) relative to the unmodified virus (Figure 6, lane C). The same capsid proteins were diminished in TC-pLys (Figure 6, lane A) compared with a sample of unmodified top components (Figure 6, lane B); however, the degree to which hexon and penton base were labelled was clearly lower than was observed with Ad-pLys. With both Ad-pLys and TC-pLys the decrease in capsid band intensities was accompanied by the presence of high-molecular-mass protein trapped in the well of the stacking gel (results not

shown). A second obvious difference between unconjugated forms of infectious virus and top components was the accumulation of post-translational precursors in top components relative to several core proteins (Edvardsson et al., 1976).

An indirect approach for identifying poly(L-lysine)-modified viral proteins involved labelling adenoviral particles with a biotin analogue that contains an NHS ester as its coupling group. We predicted that the same viral proteins that were susceptible to poly(L-lysinylation) should be targets for biotinylation, because the labelling reaction utilizes the same chemistry that was used to maleimide-activate adenovirus with sulpho-SMCC. A Western blot of Ad-biotin (Figure 6, lanes F and G) and TC-biotin (Figure 6, lanes H and I) again revealed hexon (II), penton-base (III) and fibre (IV) as target polypeptides followed treatment with a streptavidin–alkaline phosphatase conjugate. A third capsid protein, hexon-associated (VI, 27 kDa), was also labelled with biotin, implicating it as a possible substrate for poly(L-lysine) modification. Post-translational precursors to hexon-associated and viral core polypeptide VIII were additionally labelled in viral top components.

Functional analysis of poly(L-lysine)-modified adenovirus and viral top components

Having synthesized a poly(L-lysine)-modified version of the recombinant virus Ad.CBAP and verified its ability to complex with nucleic acid, transfection particles were prepared. The

Figure 4 Efficiency of adenoviral poly(L-lysine)ylation measured by ion-exchange chromatography

Samples of adenovirus before (a; 3.0×10^{12} A_{260} particles in 0.5 ml) and after (b; 1.5×10^{12} A_{260} particles in 0.5 ml) modification with poly(L-lysine) were independently injected onto a 1.5 cm $\times$ 10 cm DE-52 anion exchange column. The column was washed with 2 vol. of equilibration buffer followed by a 100 ml linear salt gradient from 0 to 0.5 M NaCl. Eluant was collected in 2 ml fractions and monitored at 260 nm.

Figure 5 Gel mobility shift assay to determine the plasmid DNA binding capacity of poly(L-lysine)ylated adenovirus

Various quantities of AD-pLys in 15 μ l of HBS was added to 200 ng of plasmid DNA, also in 15 μ l of HBS. After a 30 min incubation at room temperature, the samples were applied to a 1.0% agarose gel and electrophoresed. The resulting ethidium bromide-stained gel shows the migration of supercoiled plasmid DNA (band labelled Free) which was shifted to a position directly below the sample origin once it formed an electrostatic complex with the Ad-pLys conjugate (band labelled Complex). Between 2.2×10^{10} and 4.0×10^{10} Ad-pLys A_{260} particles/ μ g of plasmid DNA was used, as indicated. The lane marked zero contains a sample of plasmid DNA incubated with unmodified adenovirus at 4.0×10^{10} A_{260} particles/ μ g of plasmid DNA.

strategy was to first generate a core adenovirus-based complex by coating Ad-pLys with plasmid DNA, followed by the addition of an ASOR-pLys conjugate which would bind to exposed portions of DNA (Figure 1). With the aid of electron

Figure 6 Western blot analysis of adenovirus and viral top components modified with either poly(L-lysine) or biotin

Samples of adenovirus and viral top components were resolved on an SDS/12% polyacrylamide gel and electrophoretically transferred to a PVDF membrane. The blot was cut in half and stained for protein with Amido Black (a) or biotinylated proteins (b) using a streptavidin-alkaline phosphatase conjugate as described in the Experimental section. The samples that were loaded on to the gel included unmodified adenovirus (lanes C and E), unmodified top components (lanes B and J), poly(L-lysine)ylated adenovirus (lane D), poly(L-lysine)ylated top components (lane A), biotinylated adenovirus (lanes F and G) and biotinylated top components (lanes H and I). In each of the four sample lanes stained for protein, 5.5×10^{10} A_{260} particles were loaded on to the gel. Two different quantities of biotinylated viral particles were resolved on the gel, 4.7×10^8 A_{260} particles (lanes F and I) or 2.4×10^9 A_{260} particles (lanes G and H). Viral encoded polypeptides are designated with roman numerals according to Maizel et al. (1968): II, hexon; III, penton base; IIIa, penton-associated; IV, fibre; VI, hexon-associated; V, core; VI, core. Several proteins were found to be enriched in the top component sample: pVI, hexon-associated precursor; pVIII, core polypeptide precursor; and proteins of 52, 46 and 36 kDa.

Figure 7 Electron microscopy of adenovirus-based transfection particles

Samples of unmodified adenovirus (a) and adenovirus-based ligand complex (b and c) were prepared on grids and viewed with an electron microscope. The adenovirus-based particles were prepared by adding either 1.2×10^{10} (b) or 2.0×10^{10} (c) A_{260} particles of Ad-pLys to 0.5 μ g of pCMVLuc. After 30 min at room temperature the mixtures were added to 0.5 μ g of ASOR-pLys conjugate and incubated for an additional 30 min.

microscopy, the image of an intermediate in the formation of an adenovirus-based ligand complex was captured (Figure 7b). The structure was prepared at an Ad-pLys/plasmid ratio (2.4×10^{10} A_{260} particles of Ad-pLys/ μ g of plasmid DNA) that was not

Figure 8 Enhanced plasmid transgene delivery mediated by an adenovirus-based ligand complex

The Figure shows the level of luciferase expression in HUH7 and HL cells 20 h post-transfection with $0.5 \mu\text{g}$ of pCMVLuc. One of three different modes of plasmid delivery was used: DNA-protein complex ('Complex'), adenovirus-based ligand complex ('Ad-based'), or top component-based ligand complex ('TC-based'). DNA-protein complex toroids were composed of the reporter construct pCMVLuc and $1.5 \mu\text{g}$ of ASOR-pLys. Immediately following the addition of complex to cells, the medium was supplemented with $(0, 1.0, 1.5 \text{ or } 2.0) \times 10^{10}$ A_{260} particles of Ad-pLys as indicated by the row labelled Free Ad-pLys. Adenovirus-based transfection particles were prepared with $(1.0, 1.5, 2.0, \text{ and } 3.0) \times 10^{10}$ A_{260} particles of Ad-pLys as described in the Experimental section. The bar labelled with an asterisk shows cells that received the core adenovirus-based complex minus the ASOR-pLys conjugate (see Figure 1). Top component-based ligand complex was formed at $(1.0, 1.5 \text{ and } 2.0) \times 10^{10}$ A_{260} particles of Ad-pLys as described in the Experimental section. The bar labelled 'A' identifies cells that were transfected with adenovirus-based ligand complex in the presence of $25 \mu\text{g}$ of unconjugated ASOR.

sufficient to counter the net charge contributed by the nucleic acid according to the gel shift assay shown in Figure 5. As a result, excess plasmid formed loose associations with ASOR-pLys, which appeared as string-like material projecting outward from the virion capsid (Figure 7b). The generation of a fully coated adenovirus-based ligand complex was accomplished by increasing the ratio of Ad-pLys to plasmid (4.0×10^{10} A_{260} particles of Ad-pLys/ μg of plasmid DNA; see Figure 5) such that virtually all of the nucleic acid was bound by the poly(L-lysine)ated adenovirus before the addition of ASOR-pLys. The plasmid/ASOR-pLys layer that surrounded the mature transfection particle (Figure 7c) was clearly evident when compared with unmodified adenovirus (Figure 7a).

The adenovirus-based ligand complex was used to transfect HUH7 and HL cells with a luciferase reporter gene (Figure 8). Both cell types displayed recombinant luciferase expression over the tested range of $(1.0\text{--}2.0) \times 10^{10}$ A_{260} particles of Ad-pLys/ $0.5 \mu\text{g}$ of plasmid DNA, with the highest levels occurring when the plasmid DNA was completely complexed with Ad-pLys according to a gel shift assay (Figure 8). Beyond this optimal ratio (e.g. 3.0×10^{10} A_{260} particles of Ad-pLys/ $0.5 \mu\text{g}$ of plasmid DNA), transgene expression fell when tested on HUH7 cells. This decrease was probably due to an observed increased tendency of the adenovirus-based transfection particles to aggregate at high Ad-pLys/plasmid ratios as seen by electron microscopy (results not shown). An ASOR receptor competition experiment was performed with HUH7 cells and resulted in a maximum decrease in transgene expression of 50% (Figure 8). One possible explanation for incomplete inhibition with ASOR is that the transfection particles can be endocytosed via a second pathway, i.e. adenoviral receptors. Indeed, an adenovirus-based complex formed at 4.0×10^{10} A_{260} particles of Ad-pLys/ μg of plasmid DNA, without ASOR-pLys, was found to generate high levels of gene transfer, yet lower than those produced with the same core tagged with the ASOR ligand (Figure 8). The ability

of the adenovirus-based transfection particle to enter the cell through the adenovirus receptor probably contributes to the higher levels of gene transfer noted in HUH7 cells, as they are more tropic for adenovirus than HL cells (Figure 3).

The degree of luciferase expression seen with the optimal adenovirus-based ligand complex (4.0×10^{10} A_{260} particles of Ad-pLys/ μg of plasmid DNA) was 4-fold greater in HUH7 cells and 200-fold greater in HL cells when compared with the highest levels obtained in cells exposed to a mixture of complex toroids and adenovirus (Figures 2 and 8). This comparison does not accurately reflect the degree of augmentation achieved with the adenovirus-based system, since the viruses in the two protocols were not functionally equivalent in terms of enhancing endosomal escape in *trans*. Adenovirus purified through a CsCl gradient typically contains a 50-fold greater number of viral particles than infectious plaque-forming units. The plaque-forming-units/particle ratio decreased from 1:50 to 1:10⁶ following conjugation to poly(L-lysine) molecules. The reduced infectivity of Ad-pLys was also shown by histochemical staining for the adenovirus reporter transgene (results not shown). Of importance to this study is the fact that coupling poly(L-lysine) to adenovirus also diminished its ability to provide endosomal escape in *trans*. Transfections performed with a mixture of toroids and free Ad-pLys were 1000-fold lower in HUH7 cells and 600-fold lower in HL cells when compared with transfections with toroids and free adenovirus (Figure 8). This further underscores the relative efficiency of the adenovirus-based complex. A direct comparison of transfections conducted with toroids mixed with Ad-pLys and transfections performed with the fully formed adenovirus-based complex indicates that incorporation of the plasmid DNA on to the Ad-pLys improved transfection efficiency by three to four orders of magnitude in HUH7 and HL cells.

The same methodology used to generate a ligand complex with Ad-pLys was applied to TC-pLys. However, because of the lower degree of poly(L-lysine) conjugation that was achieved

Figure 9 Efficiency of the adenovirus-based ligand complex demonstrated by cytochemical localization of plasmid and viral reporter cDNAs

Adenovirus-based ligand complex was prepared at a ratio of 4.0×10^{10} A_{260} particles of Ad-pLys/ μg of pCMVLacZ, as described in the Experimental section. An aliquot of the complex containing $0.5 \mu\text{g}$ of the plasmid reporter was added to HUH7 (b) and HL (d) cells. Alternatively, cells were transfected with DNA-protein complex composed of $0.5 \mu\text{g}$ of pCMVLacZ and $1.5 \mu\text{g}$ of ASOR-pLys (a, HUH7; c, HL). Cells were fixed 20 h post-transfection and stained for plasmid-encoded β -galactosidase and recombinant viral alkaline phosphatase. Cells were identified as being transduced with the minigene encoding alkaline phosphatase (small arrowhead), β -galactosidase (large arrowhead), or both (solid arrow).

with these defective virions (Figure 6), a greater number of conjugate particles was needed to complex plasmid DNA; the extent of positive charge contributed by 6.0×10^{10} A_{260} particles of TC-pLys was sufficient to completely shift $1 \mu\text{g}$ of plasmid DNA (results not shown), compared with 3.8×10^{10} A_{260} particles required for Ad-pLys (Figure 5). With luciferase as the plasmid reporter, TC-based transfection particles generated only a slight increase in gene transfer when added to HUH7 cells (Figure 8). The highest level using TC-pLys as the complex core was four orders of magnitude lower than that with the optimal Ad-pLys transfection particle. This result strongly suggests that the factor(s) responsible for adenovirus-mediated endosomal lysis are either deficient or not functional in viral top components.

In addition to gaining a quantitative measure of gene transfer mediated by Ad-based ligand complexes, we also considered the transfection efficiency in terms of the number of infectious alkaline phosphatase-transducing virions needed to reach a given level of plasmid transgene expression. This was accomplished by replacing the luciferase expression vector with a LacZ reporter construct. HUH7 and HL cells that were transfected with adenovirus-based ligand complex displayed a greater degree of β -galactosidase expression than cells transfected with complex alone (compare Figures 9a and 9b for HUH7 and Figures 9c and 9d for HL). TC-pLys-driven complex particles were similarly used to transfect HUH7 cells, resulting in sporadic expression of the plasmid β -galactosidase cDNA (results not shown). The cells

were subsequently stained for the presence of viral-encoded recombinant alkaline phosphatase to give a relative measure of Ad-pLys infectivity. Few HUH7 cells were detected that expressed only alkaline phosphatase, while virtually none were evident among the transfected HL cells (Figures 9b and 9d). This finding was therefore substantially different compared with that in cells treated with a mixture of complex toroids and free unmodified adenovirus, where high levels of adenoviral infection, as shown by alkaline phosphatase expression, were necessary to enhance plasmid-mediated expression (Figure 3). Furthermore, the reduced background infectivity associated with the adenovirus-based ligand complex significantly lowered viral-induced cell death (compare Figures 3b and 3d with Figures 9b and 9d). Of the cells that expressed plasmid-encoded β -galactosidase, approx. 50% were co-transduced with the viral alkaline phosphatase reporter (Figure 9). The intense dark stain deposited by these 'duel-hit' cells enabled them to be distinguished from those that expressed only β -galactosidase (Figure 9).

DISCUSSION

Eukaryotic viruses have evolved efficient mechanisms for packaging, delivering and expressing nucleic acids that lend themselves as powerful tools for gene therapy. Unfortunately, many of the very attributes that make viruses attractive gene transfer vehicles

additionally restrict their utility, not to mention safety issues that arise over creating a viral-induced pathogenic condition. For instance, retroviruses are able to efficiently transfer genes into cells and integrate the recombinant gene into chromosome DNA (Miller, 1992). However, this requires that the recipient cell is in an active mitotic state, thereby preventing use in quiescent cells. Furthermore, because the biology of retroviral integration is random, there are justifiable concerns regarding transcriptional activation or inactivation of nearby critical loci. An alternative approach for targeted gene transfer *in vivo* that addresses the weaknesses of viral substrates is based on receptor-mediated endocytosis of non-viral constructs. The general concept is to attach DNA containing a therapeutic gene to a polypeptide ligand that binds a receptor specifically expressed on the desired target cell. This complex of DNA and protein is internalized by receptor-mediated endocytosis and the DNA is transported to the nucleus where it is expressed. Because this technology can be modified to include virtually any receptor ligand, a host of cell types can be targeted for gene transfer. There is similar flexibility regarding the input nucleic acid since the formation of the soluble transfection particle is relatively independent of size or sequence. Finally, the ideal DNA-protein complex would contain only components that present no health risk to the recipient.

The primary limiting factor with ligand-mediated approaches is the efficiency with which the internalized complex is diverted from lysosomes and released from endosomal vesicles. Therefore, incorporating an endosomal-lysing agent into the DNA-protein complex should greatly facilitate the level of gene transfer. This has been demonstrated with chemicals such as chloroquine that neutralize the pH of acidic compartments and exert their effect by either destabilizing the integrity of membrane-bound vesicles or lowering the activity of acid hydrolases in lysosomes (Zenke et al., 1990). A more physiological approach involves taking advantage of the well documented membrane permeabilizing characteristics associated with adenoviral infection. Adenoviruses have evolved a mechanism of cell entry that utilizes receptor-mediated endocytosis followed by vesicle rupture, thereby releasing the luminal contents into the cytoplasm (Seth et al., 1986). The precise mechanisms by which adenoviruses promote endosomal lysis remains one of the many unanswered questions surrounding its life cycle. There is evidence that this may involve complex interactions between determinants buried in the viral capsid that become uncovered in response to the acidic pH of receptosomes (Seth et al., 1984b). Data have been presented suggesting that adenoviral infection is associated with prolific alterations in specific cytoskeletal elements (Defer et al., 1990). It is, therefore, possible that these changes perturb the normal vectorial movement of endocytotic vesicles along the cytoskeletal network. The major drawback to adenoviral-mediated augmentation of DNA-protein complex is the dependence on an intact virion. This strategy is, however, an important conceptual breakthrough providing an intermediary link towards a fully non-viral transfection particle.

The success of transfection protocols that rely on free adenoviral particles to augment DNA-protein complex gene transfer have been shown previously to be extremely cell-type-dependent (Curiel et al., 1991). Cell-type-specific responses to adenoviral-enhanced expression were observed in the present study using both luciferase and LacZ plasmid transgenes (Figures 2 and 3). With the latter reporter it was possible to extend the results further by additionally staining transfected cells for alkaline phosphatase to determine the extent of viral infection. This approach revealed that cells did not experience enhanced plasmic β -galactosidase expression until virtually the entire monolayer stained positive for the adenovirus alkaline phosphatase transgene. The apparent

correlation between a cell's tropism for adenovirus and plasmid gene transfer probably reflects (1) the number and distribution of adenoviral receptors relative to ASOR receptors on the cell surface, and (2) the probability of chance occurrence that the two receptors with their respective ligands occupy a common endocytotic vesicle. Consequently, transfections performed with a mixture of complex toroids and adenovirus are most productive with cells that are readily infected by the virus. This is not to suggest, however, that the delivery and expression of the viral genome is required in order to take advantage of the endosomal lysing properties of adenovirus. Cotten et al. (1992) showed that the two events could be separated by inactivating the adenoviral genome with u.v. light or 8-methoxypsoralen. These treatments effectively reduced the titre of adenoviral stocks by as much as five orders of magnitude, yet had little impact on virus-mediated enhanced expression of a plasmid-encoded transgene.

Conditions that disrupt the structure of the adenovirus, such as heat inactivation, ablate its endosomal-lysing capability (Defer et al., 1990; Curiel et al., 1991). Such denaturing conditions probably perturb the native conformation of the capsid organization, thereby destroying signals that regulate various virus-cell interactions. This presented a particular concern for the present study since poly(L-lysine) modification of adenovirus would undoubtedly affect the structure of the virion capsid and possibly destroy the endosomal-lysing properties that were of interest. Initially this appeared to be the case, since a functional survey of the modified adenovirus revealed an exponential drop in both titre and its ability to enhance *trans* the delivery of a DNA-protein complex toroid (Figure 8). The concurrent loss of viral infectivity and *trans* endosomal lysis was therefore similar to what has been reported for heat-inactivated adenovirus. This was not altogether surprising, however, since Western blot analysis of the proteins from Ad-pLys revealed that hexon, penton base, fibre, and possibly hexon-associated were all labelled with the polycation (Figure 6). In fact, the degree of conjugation, particularly for hexon, penton base and fibre, appeared to be quite significant based on the residual quantity of unmodified protein that migrated into the resolving portion of the gel during electrophoresis. Because fibre is believed to be the primary recognition determinant for cell surface receptors (Philipson et al., 1968), defective binding and uptake is one possible explanation for the crippling effects caused by poly(L-lysine)lation.

Although it was difficult to conclude whether the silencing of adenoviral *trans* endosomal lysis was due to poly(L-lysine) interfering with virus-cell recognition or direct modification of proteins involved in the disruption of receptosomes, the latter appeared to be less relevant since it was possible to reconstitute this activity using Ad-pLys in a *cis* configuration with the adenovirus-based ligand complex (Figures 8 and 9). The high efficiency of the single particle transfection system was best demonstrated in the context of adenoviral infection. Unlike transfections with complex toroids in the presence of free virus (Figure 3), viral infectivity was largely restricted to cells that were successfully targeted by the adenovirus-based ligand complex and therefore co-transduced with the plasmid β -galactosidase reporter (Figure 9). This had the added benefit of lowering viral-induced cell death to a level that was virtually undetectable. Interestingly, a significant percentage of cells showed no evidence of viral transduction, yet expressed the plasmid transgene. This finding was somewhat unexpected and suggested that the delivery of the transfection particle could occur independently of viral infection (Figure 9). The apparent lack of continuity between cell entry and adenoviral gene expression is difficult to rationalize without a better understanding of the entire sequence of events that regulate adenoviral infection. Derivatization of adenoviral

nucleic acids or proteins involved in nuclear delivery and expression by the highly nucleophilic SMCC linker can be offered as one mechanism that may account for the absence of virus transgene expression.

In light of the observation that viral gene expression was not necessarily an obligatory step in endosomal analysis, we focused on an alternative adenovirus-related substrate that could be incorporated into the transfection particle, i.e. the top components. These defective particles were considered because they are largely void of nucleic acid, further lowering their infectivity potential, and because they contain a full complement of structural capsid proteins (Figure 6) (Edvardsson et al., 1976). This is important because protein(s) that promote endosomal analysis are likely to reside on or near the surface of the virion capsid where they are more accessible and able to interact with the cell, therefore implicating hexon, penton base and fibre among others. For instance, antibodies against the capsid polypeptide penton base have been shown to block adenoviral-enhanced delivery of an epidermal growth factor conjugate, with no apparent impact on virus uptake (Seth et al., 1984a). Here we demonstrated that the top component-based ligand complex produced only a slight augmentation in plasmid gene transfer, well below what was recorded for the adenovirus-based version (Figure 8). The question thus arises as to why top components do not promote efficient plasmid gene transfer. Again, comparing the top component protein profile with that from infectious virions it is evident that several of the core proteins are primarily in their precursor form (Figure 6) (Edvardsson et al., 1976). If these proteins are involved in vesicle disruption, their failure to reach a fully processed state may prevent expression of the activity. An alternative theory is that the factor(s) that confer endosomal analysis are present, but are not organized in the necessary native conformation. We have examined viral top components by electron microscopy, and while they maintain an icosahedral geometry, the structure appears to lack tertiary dimension and appears collapsed (results not shown). This probably results from the absence of a mature nucleic acid/protein core that normally acts as a supporting framework over which the protein capsid shell is built. That top components present a different conformational symmetry may also be reflected in the lower degree with which the capsid proteins hexon and penton base were labelled with poly(L-lysine) (Figure 6). If the protein(s) responsible for adenoviral-mediated endosomal analysis are indeed accounted for in top components, this raises additional questions regarding the efficacy of reconstituting the endosomal analysis activity in the context of a purified viral proteins. The negative results with top components may indicate that the activity is dependent on the entire virion structure in a manner comparable with how the active site of an enzyme is properly aligned and functional only when supported by surrounding ancillary sequences. It

remains to be established whether adenoviral endosomal analysis protein(s) can be isolated while maintaining a level of activity suitable for use in the context of a DNA-protein complex.

In summary, the results presented here and by others (Curiel et al., 1991, 1992; Wagner et al., 1992) using intact virions demonstrate an important principle, i.e. that ligand-mediated approaches to gene transfer offer a potentially powerful alternative to viral substrates given the presence of an efficient endosomal analysis agent. For this to be useful for *in vivo* gene therapy, it will be critical to simplify the synthetic construct so that it does not contain an intact virion. It will be important to continue to explore other endosomal analysis factors that can be conjugated to a DNA-protein complex, with the focus being on simplicity and absence of potentially infectious agents.

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