Nuclear targeting peptide scaffolds for lipofection of nondividing mammalian cells

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Lipofection of nondividing cells is inefficient because much of the transfected DNA is retained in endosomes, and that which escapes to the cytoplasm enters the nucleus at low rates. To improve the final rate-limiting step of nuclear import, we conjugated a nonclassical nuclear localization signal (NLS) containing the M9 sequence of heterogeneous nuclear ribonucleoprotein (hnRNP) A1, to a cationic peptide scaffold derived from a scrambled sequence of the SV40 T-antigen consensus NLS (ScT). The ScT was added to improve DNA binding of the M9 sequence. Lipofection of confluent endothelium with plasmid complexed with the M9–ScT conjugate resulted in 83% transfection and a 63-fold increase in marker gene expression. The M9–ScT conjugate localized fluorescent plasmid into the nucleus of permeabilized cells, and addition of the nuclear pore blocker wheat germ agglutinin prevented nuclear import. This method of gene transfer may lead to viral- and lipid-free transfection of nondividing cells.

Keywords: Gene transfer, endothelium, lipofection, nuclear import

Compared with adenoviral vectors, which provide nearly 100% transduction of endothelial cells in vitro and in vivo[1–7], the efficiency of cationic lipid-mediated gene transfer to endothelial cells is low. The efficiency of adenovirus transduction can be attributed to the efficiency of its receptor recognition, endosome escape, and nuclear pore targeting[8–14]. During lipofection, internalization of DNA–lipid complexes is efficient (>10,000 plasmids per cell)[15–17], and addition of endosome disruptors such as chloroquine and dioleoyl phosphatidyl ethanolamine (DOPE) can increase expression[3,16,18,19]. However, adding replication-deficient adenovirus to lipid–DNA complexes during transfection of subconfluent human or bovine endothelial cells provides only a modest increase in transfection efficiency, from a few percent to a level of 20–35%[20,21]. A level of 20% transfection of actively dividing, subconfluent cells can be reached by protecting DNA from cytoplasmic DNAses with histone[3,22]. Together these studies indicate that even with endosome escape facilitated by adenovirus[20,21], transport of cytoplasmic plasmid to the nucleus remains an important limiting step in nonviral gene transfer to nondividing cells.

Limitations in nuclear import have been reported previously[16,23,24]. Plasmid DNA injected directly into the nucleus of mouse thymidine kinase-negative L cells resulted in over 50–100% of the cells expressing thymidine kinase activity, compared with 0% when plasmid was injected into the cytoplasm[23]. Radioactively labeled plasmid was shown to be efficiently internalized in the cells, but <1% of plasmid was found in the nuclear fraction[16]. In addition, when lipofected plasmid was transcribed from the cytoplasm using a vaccinia virus system, nearly 98% of COS-1 cells were shown to contain plasmid in the cytoplasmic compartment. In contrast, when plasmid was transcribed in the nucleus using a cytomegalovirus (CMV) promoter system, only 10% of the cells expressed β-galactosidase[24].

Molecules larger than 30–60 kDa require active transport into the nucleus via nuclear localization signals (NLS)[25]. Two types of NLS have been identified: the basic amino acid sequence KKKKK′K found on histones and SV40 T antigen; and the bipartite signal found on nucleoplasmin[25]. Complexing histones to DNA by charge interactions before lipofection leads to modest increases in transfection efficiency in endothelial cells[3] and in other cell types[22], but does not provide unusually high-efficiency nuclear import of plasmid. Recently, Sebestyen *et al.*[26] covalently attached SV40 T-antigen consensus NLS to plasmid. They observed that increasing the amount of NLS peptide to 101 peptides per 1 kb of plasmid increases the amount of plasmid delivered to the nucleus of permeabilized cells. However, the method resulted in only a fourfold increase in expression after lipofection, similar to increases observed by complexing plasmids with histones[3,22], SV40 T-antigen consensus NLS peptide or its scrambled variants[27], or recombinant histones with SV40 T-antigen consensus NLS[26]. Conjugating a single classical NLS signal to plasmid results in over 1,000-fold increase in dividing HeLa and 3T3 cells but only a 10- to 30-fold increase in nondividing cells such as macrophages, dorsal root ganglion neurons, or rapidly dividing rat hepatocytes[28].

The heterogeneous nuclear ribonucleoprotein (hnRNP) A1 contains a nonclassical NLS termed M9 (ref. 29). This 38-residue sequence confers nuclear import and export activity to β-galactosidase and pyruvate kinase, two proteins that are normally excluded from the nucleus[29]. The M9 import mechanism requires an endogenous carrier protein, transportin[30], and is not affected by addition of excess of classical NLS[31]. Transportin binds to the M9 sequence of hnRNP A1 and transports it to the nuclear pore, where the complex is translocated across the nuclear pore by a GTPase. In the nucleus, transportin detaches from hnRNP A1 and translocates to the cytoplasm[30]. In this study, we have chemically conjugated the M9 sequence of hnRNP A1 to a 13-residue cationic peptide (Fig. 1) to enhance its ability to bind DNA, and examined whether it would mediate efficient lipofection of confluent, nondividing, bovine aortic endothelial cells.

Results

When highly confluent bovine aortic endothelial cells (BAECs) were lipofected with rhodamine-conjugated plasmid, punctate staining in the cytoplasm of every cell (Fig. 2A) demonstrated that uptake and

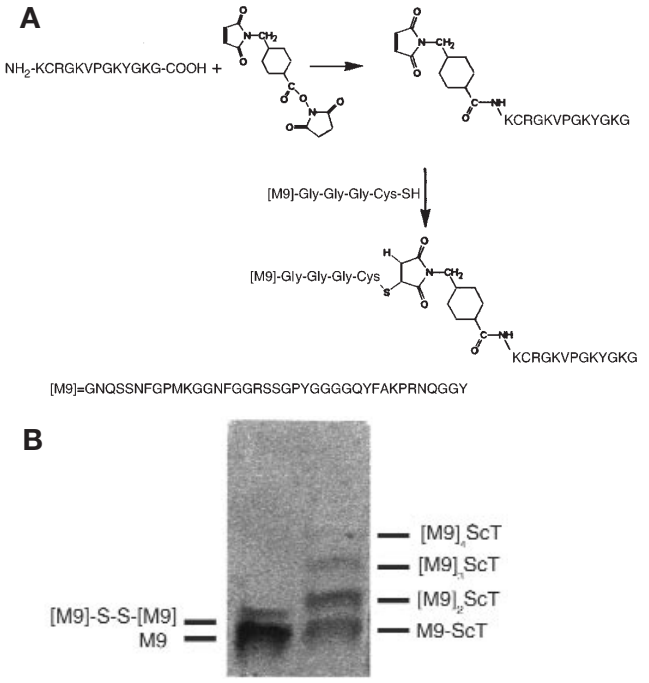

Figure 1. (A) Two-step reaction to conjugate a cationic peptide (ScT) to the M9 sequence of hnRNP A1. (B) SDS–PAGE was conducted on the pure M9 (lane 1) and reaction products after conjugation (lane 2). The pure peptide in lane 1 was havily loaded to detect the M9 dimer, which was reducible by β-mercaptoethanol.

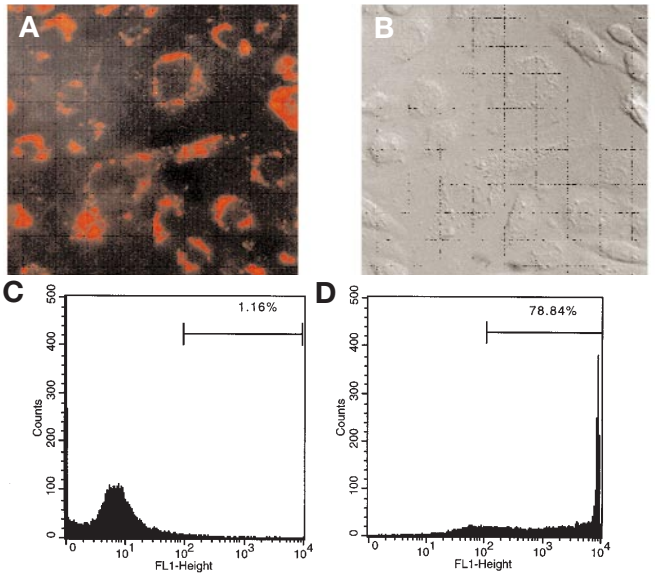

Figure 2. Endosomal escape and detection of lipofected plasmid in the cytoplasm. Confluent BAECs were lipofected with 1 μg rhodamine–pCMVβgal for 16 h and visualized by epifluorescence (A) and differential interference contrast (B) microscopy. Confluent BAECs were lipofected with 1 μg pTMβgal alone (C) or with 1 μg pTMβgal with 100 U T7 RNA polymerase (D) for 20 h and analyzed by flow cytometry using the β-galactosidase fluorogenic substrate FDG. Percentage transfection was defined by percentage of cells having fluorescence ≥179 100 f.u. (10 times above endogenous galactosidase activity and FDG autofluorescence).

internalization of lipofected plasmid were extremely efficient. However, most of the plasmid remained in endosomes at 16 h postlipofection and did not reach the nucleus, as shown by lack of nuclear fluorescence. To determine the amount of plasmid that escaped from the endosomes, confluent BAECs were transfected for 20 h with pTMβgal and T7 RNA polymerase to allow for cytoplasmic transcription of escaped plasmid. This assay showed that (Fig. 2C and D) nearly 80% of the cells expressed high levels of β-galactosidase, thereby confirming that significant amounts of plasmid enter the cytoplasm of lipofected BAECs. Without addition of the T7 RNA polymerase, the flow cytometry signal reflected the level of endogenous activity and fluorescein di—D-galactopyranoside (FDG) autofluoresence detected in untransfected cells (Fig. 2C). Bromochloroindolylgalactoside (X-gal) staining of confluent BAECs lipofected with pTMβgal/T7 RNA polymerase demonstrated that over half of the cells were intensely blue and the other half were moderately blue (A.S., data not shown).

These experiments demonstrated the existence of a significant cytoplasmic pool of plasmid. However, this pool is not efficiently imported into the nucleus of nondividing cells, as shown by lipofection of highly confluent BAEC monolayers with pCMVβgal alone, which resulted in <1% of the cells staining X-gal positive (Fig. 3A).

The M9 sequence mediates importation in the nucleus but is not highly cationic and therefore does not bind DNA strongly. In order to improve its DNA binding, we crosslinked the M9 sequence to scrambled SV40 T antigen (ScT), which has five positively charged amino acids. At least one M9 sequence was attached to the ScT as shown by SDS–PAGE (as in Fig 1B). In contrast to low transfection efficiency of plasmid alone (Fig. 3A), when M9–ScT was bound to the plasmid, nearly all the cells displayed X-gal staining to varying extent (Fig. 3B).

To explore the role of the various constituents of M9–ScT that confer this improved gene transfer, confluent BAECs were lipofected with pCMVβgal complexed to various combinations of peptides, and analyzed by flow cytometry (Fig. 4). The majority of untransfected cells (Fig. 4A) had a fluorescence of <10 fluorescence units (f.u.) due to endogenous galactosidase activity and FDG autofluorescence. When confluent BAECs were transfected with pCMVβgal alone, the majority of cells displayed a signal above 10 f.u., demonstrating low-level expression (possibly because of uptake of β-galactosidase in the conditioned media) that was well below the sensitivity of X-gal staining. Only 5.27% of cells lipofected with plasmid alone had fluorescence above 100 f.u. In contrast, when the plasmid was precomplexed with M9 peptide alone, this level increased to 55% (Fig. 4C). Adding M9 and ST to the plasmid without chemical conjugation resulted in a transfection efficiency of 76.4%. The increase observed with M9 alone or M9 + ScT (unconjugated) was obtained in the absence of the nonpeptide bifunctional crosslinker. However, when M9 was conjugated to ScT, the highest transfection efficiency of 83% was achieved (Fig. 4E), providing a 15.7-fold increase in the percent positive cells (with $\geq 10^2$ f.u. measured by flow cytometry), as compared with lipofection with plasmid alone.

Total β-galactosidase expression in the cells was measured fluo-

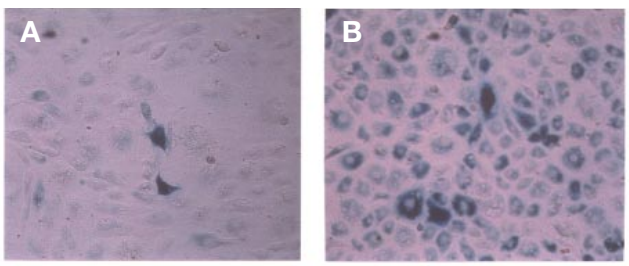

Figure 3. The effect of M9 sequence of hnRNP on transfection efficiency of confluent BAECs. Cells were lipofected for 48 h with pCMVβgal alone (A) or plasmid pCMVβgal + M9-ScT (B), and stained with X-gal. Results are representative of experiments conducted with three independent passages of BAECs.

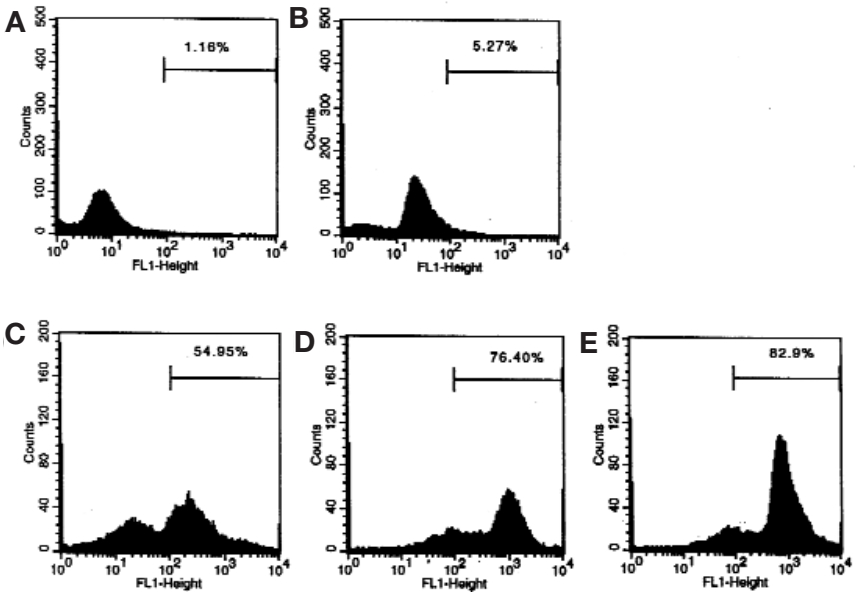

Figure 4. Lipofection of confluent BAECs with pCMVβgal in the presence or absence of peptides. Untransfected BAECs (A) were used as a measure of endogenous galactosidase activity and FDG autofluorescence. Confluent BAECs were transfected with 1 μg plasmid alone (B), 1 μg plasmid + 60 μg M9 peptide (C), 1 μg plasmid + 30 μg M9 + 30 μg ScT (unconjugated) (D), or 1 μg plasmid + 60 μg M9-ScT conjugate (E). Similar results were obtained in an independent experiment. Percentage transfection was defined in each panel by the percentage of cells with ≥ 100 f.u.

rometrically (Fig. 5). Complexing the plasmid with M9 peptide alone provided an 18.3-fold increase in expression, an increase that was not simply due to a condensation effect, as ScT (which condenses plasmid effectively by electrostatic interactions, as observed by an ethidium bromide fluorescence release assay; A.S. data not shown) provided only a fourfold increase in β-galactosidase expression. A similar low level of β-galactosidase expression was observed when the plasmid was complexed with SV40 T-antigen consensus NLS, or with polylysine (13-residue peptide), indicating that classical NLS (or sequences with equivalent charge density and size) were markedly less efficient than M9 NLS in targeting the plasmid to the nucleus. Transfection of confluent BAECs with plasmid precomplexed with M9–ScT conjugate resulted in a 63-fold enhancement in expression over transfection with plasmid alone, whereas M9 + ScT (unconjugated) complexed to plasmid resulted in a 47-fold increase.

To determine whether the enhanced gene expression observed with M9–ScT depended on the M9 sequence, we used a scrambled sequence of M9 (ScM9) conjugated to ScT. The ScM9–ScT conjugate (from 5 to 50 μg ScM9–ScT per microgram of plasmid) provided only a fourfold to eightfold enhancement over lipofection, which was not dose dependent and was likely due to the cationic charge of the ScT scaffold. We have observed similar enhancements in expression when confluent BAECs were lipofected with ScT (as seen in Fig. 5), SV40 T-antigen consensus NLS, and two other mutations of the SV40 NLS.

To investigate the nuclear localization of plasmid by M9-ScT, confluent BAECs were permeabilized and incubated for 30 min in a nuclear import buffer containing fluorescent plasmid with and without M9–ScT. The cells incubated with plasmid alone displayed cytoplasmic and perinuclear staining but no nuclear staining (Fig. 6A). This demonstrates that the nuclei of permeabilized cells (which have a diluted cytoplasm) offered significant resistance to the import of plasmid, even when high cytoplasmic levels of the plasmid were maintained in the assay. However, when cells were incubated with plasmid complexed with M9–ScT, the amount of plasmid in the nucleus increased markedly (Fig. 6C and D). In contrast, M9–ScT conjugate was not observed in the nuclei of cells pretreated with wheat germ agglutinin, a known blocker of nuclear pores (Fig. 6B).

Discussion

Nonviral gene transfer to vascular cells such as endothelium is extremely inefficient when compared with adenoviral gene transfer[1,2,4–7]. Recent improvements of lipofection protocols for *subconfluent* epithelium tend to plateau at about 30% transfection efficiency: $\alpha_5\beta_1$ integrin-targeting peptide results in 25% transfection of corneal endothelium[32]; replication-deficient adenovirus-complexed plasmid results in 20% transfection of human umbilical vein endothelial cells (HUVEC)[20] and 25–35% transfection of BAECs[33]; histone complexation of plasmid results in 20% transfection of BAECs[3]; adenovirus fiber added to plasmid results in 30% transfection of BAEC[34]; and plasmid condensed with recombinant histone H1 containing SV40 T-antigen NLS resulted in 10–30% transfection of COS-7 or NIH3T3 (ref. 22). It is not known whether the benefits of these protocols are achieved when using confluent cells at the time of transfection. The 30% plateau likely represents the persistence and elevated level of intact cytoplasmic plasmid available to accomplish gene transfer in cells dividing at times one to two days after transfection. An unprotected plasmid with short half-life in the cytoplasm would transfect only the cells dividing in the first few hours after the lipofection.

In nondividing cells, the frequency of nuclear import events may increase with elevated cytoplasmic plasmid levels, particularly if the probability of plasmid encounter with the nuclear pore entrance is increased. However, inefficient nuclear targeting because of cytoplasmic sequestration and inefficient transit of plasmid across the pore likely remain important rate limits during nonviral gene transfer[16,17,23,24]. Conjugation of classical NLS to plasmid may alleviate this

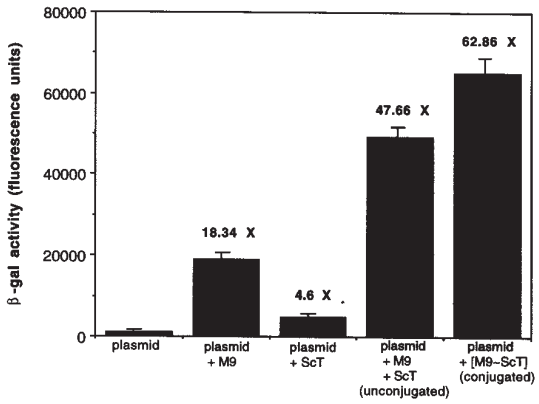

Figure 5. Total expression of β-galactosidase (β-gal) activity after complexation of pCMVβgal with peptides containing the M9 sequence. In all samples, cell counts were kept at 10^7 cells/ml. Similar results were obtained in an independent experiment.

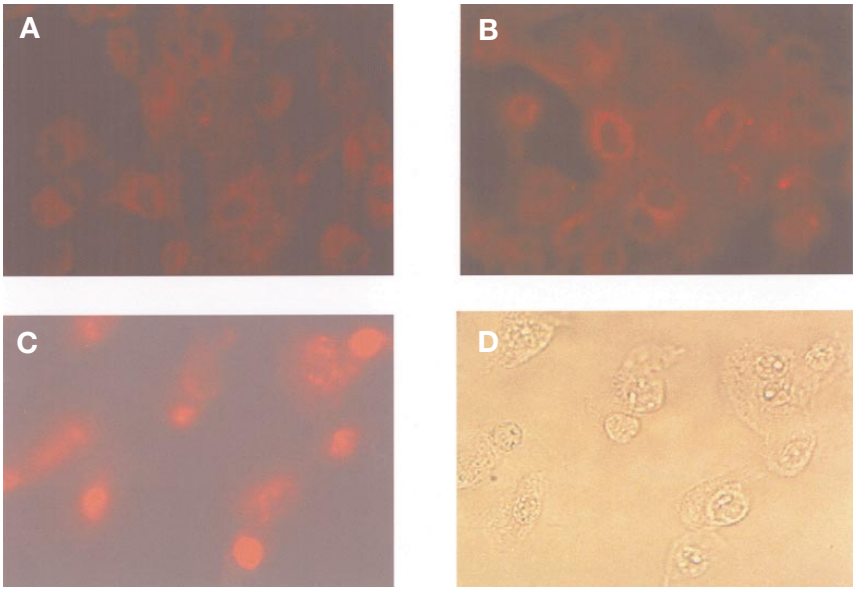

Figure 6. Localization of rhodamine–plasmid complexed with M9–ScT in digitonin-permeabilized BAECs. Cells were incubated with rhodamine–plasmid (1 μg) (A), wheat germ agglutinin (40 μg/ml) followed by rhodamine plasmid (1 μg) + 60 μg M9–ScT (B); and rhodamine plasmid (1 μg) + 60 μg M9–ScT (C). Light microscopy of cells in (C) is shown in (D). Results are representative of two independent experiments.

problem. Recent studies, however, have shown that plasmids linked to histones[3,22] or SV40 T antigen[26], either covalently or by charge interactions, are unable to cross nuclear pore of intact cells to effect fully efficient gene transfer.

Import and export of endogenous ribonucleic acid across the nuclear pore by hnRNP represents a new approach for achieving highly efficient nonviral gene transfer. The M9 epitope of human hnRNP A1 is a small, potentially nonimmunogenic sequence with no homology with known fusigenic epitopes, consistent with the fact that hnRNP A1 is a soluble intracellular protein. In experiments with lipofecting confluent BAECs with M9–ScT/rhodamine plasmid, the majority of fluorescence was endosomal after 20 h (as in Fig. 2A), indicating that the M9 sequence was not fusigenically active to mediate endosome escape.

By flow cytometry, confluent BAECs transfected with plasmid alone resulted in 5% of the cells testing positive, whereas 83% of cells were positive when the plasmid was complexed with M9–ScT. M9-ScT caused a 63-fold increase in total β-galactosidase expression compared with transfection with plasmid alone. In contrast, complexing the plasmid with a cationic peptide alone that aids in plasmid condensation and protection against DNases provided only a modest fourfold enhancement in expression (Fig. 5). These striking differences were seen in replicate cultures of confluent cells with the same cell density at the time of transfection or at the time of assay.

At present, we do not know the limitations in plasmid size that allow M9-assisted expression. The current studies with conjugated synthetic peptides demonstrate the feasibility of testing recombinantly expressed peptides that contain the M9 sequence and DNA binding sequences. Synthetic peptides are expensive, but recombinant peptides would represent a cost-effective approach for gene transfer. Along with the M9 NLS, peptides containing fusigenic or receptor-targeting sequences may help reduce the need for lipids during transfection of nondividing cells.

Experimental protocol

Cell culture and transfection. Bovine aortic endothelial cells (Cell Systems Corporation, Kirkland, WA) were grown to confluence, passed at a high-density 1:1 split to 24-well culture plates, and then grown to dense confluence (>100,000/cm^2) for two days before lipofection. Growth medium was Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated newborn calf serum, 0.30 mg/ml of glutamine, 150 U/ml penicillin, and 0.15 mg/ml streptomycin (Gibco-BRL, Grand Island, NY). Lipofectamine reagent (Gibco-BRL) containing 3:1 (wt:wt) mixture of polycationic lipid 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1 propanaminium trifluoroacetate (DOSPA) and the neutral lipid DOPE was used in all transfections. The use of DNA and lipofectamine for lipofection of confluent BAECs followed our previous work[3] in which a weight ratio of 10:1 lipofectamine to plasmid was optimal. For each transfection, 5 μl of lipofectamine (2 mg/ml) and 1 μg of plasmid (with or without peptides) were diluted into 50 μl of serum-free medium (OPTIMEM Reduced Serum Medium, Gibco-BRL) and incubated for 45 min. A mammalian expression vector pCMVβgal (7.2 kbp) was obtained from Clontech Laboratories (Palo Alto, CA) for expression of β-galactosidase driven by a CMV promoter. In some experiments, peptide (60–100 μg) was added to 1 μg plasmid and incubated for 15 min to allow for complexation before adding the lipid. Then, 150 μl of OPTIMEM media was added to 50 μl lipid/plasmid ± peptide mixture, which was then overlaid on the cells (200 μl per 2 cm^2 well). The cells were incubated for 2 h at 37°C, aspirated, fed 4% serum in OPTIMEM media, and maintained for two days before assay. During the transfections, cells were frequently monitored for viability and adhesion. No loss in cell viability was observed after one week postlipofection with plasmid–protein complexes. The plasmid pCMVbgal was labeled with rhodamine using the Label IT reagent (Panvera Corporation, Madison, WI) following the manufacturer's instructions. Briefly, the plasmid (1 μg) was diluted to a final concentration of 0.1 μg/ μl, and 10 μl of the Label It reagent was added. The labeling reaction proceeded for 1 h (37°C) followed by gel filtration with a Sephadex G25 spin column to remove unincorporated fluorescence.

β-galactosidase assays. To measure percent transfection, cells were fixed with 1 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS) and stained with 1 mg/ml X-gal (Sigma, St. Louis, MO), 5 mM K_3FeCN_6, 5 mM K_4FeCN_6, and 2 mM $MgCl_2$ for 4 h. The percentage of cells stained blue was counted in four random fields of view for each transfected monolayer and averaged. For flow cytometry and total β-galactosidase expression, trypsinized cells were pelleted and resuspended at 10^7 cells/ml in incubation buffer containing 4% newborn calf serum in PBS with 10 mM HEPES (pH 7.2). The fluorogenic substrate FDG (Molecular Probes, Eugene, OR) is cleaved by β-galactosidase to fluorescein monogalactoside and then to fluorescein. A 100 μl cell suspension at 37°C and 100 μl of 2 mM FDG were mixed and incubated at 37°C for 1 min. The FDG loading was stopped by adding 1.8 ml of ice-cold incubation buffer containing 1.5 mM propidium iodide and 1 mM phenylethyl β-D-thiogalactopyranoside. Total fluorescence (excitation/emission: 494/520 nm) was measured in an SLM-Amico fluorimeter (Spectronics Corp., Westbury, NY). Background enzyme activity and autofluorescence were subtracted using untransfected cells. The cells were also analyzed using a Becton-Dickinson FACScan flow cytometer (University of Pennsylvania, Department of Pathology). The BAECs cells prepared without FDG were used to compensate for autofluorescence. A threshold of 10^2 f.u. corresponding to a signal 10 times above background was used to define successful transfection. This threshold provided a conservative estimate of percentage transfection and a reasonable correlation with the percentage transfection determination by X-gal staining, which is less sensitive than flow cytometry.

Cytoplasmic transcription assay. The T7 RNA pol transcription assay[35] was used to detect plasmid in the cytoplasm after lipofection. The plasmid

pTMβgal (1 μg) (kind gift of Dr. Michael Welsh, Howard Hughes Medical Institute, Iowa City, IA), which expresses β-galactosidase under the control of a T7 promoter, was incubated with 100 U of T7 RNA polymerase (Promega Corp., Madison, WI) in 50 μl of serum-free OPTIMEM medium. Pure lipofectamine (5 μl) was added to the pTMβgal/T7 RNA polymerase complex, incubated for 45 min, and then diluted to 200 μl with OPTIMEM media. The cells were lipofected for 2 h at 37°C. After 2 h, the mixture was aspirated, 4% serum in OPTIMEM media was added, and the cells were maintained for 20 h before assay by flow cytometry.

Peptides for transfection. The M9 sequence of hnRNP A1 with GGC at the C terminus for conjugation, GNQSSNFGPMKGGNFGGRSSGPYGGGGQY-FAKPRNQGGYGGC (4,160 Da) was chemically synthesized (Research Genetics, Huntsville, AL). The synthetic M9 peptide was crosslinked to a scrambled sequence of the SV40 T-antigen consensus NLS peptide (ScT = KCRGKVPGKYGKG, 1,312 Da) using succinimidyl 4-(*N*-maleimidomethyl) cyclohexane 1-carboxylate bifunctional crosslinker (SMCC; Pierce Co., Rockford, IL). The reaction scheme (Fig. 1A) is a two-step reaction in which the primary amine of ScT reacts with the *N*-hydroxy succinimide ester moiety of the SMCC followed by a maleimide [?AUTHOR:? spelling?] reaction with the C-terminal cysteine thiol of the M9 peptide. The ScT (100 μg) was incubated 2 h at 22°C with 10 mM SMCC (10-fold molar excess crosslinker) in 0.1 M PBS, pH 7.2, containing 10% dimethylsulfoxide in a final volume of 100 μl. Unreacted SMCC was removed by Sephadex G-25 gel filtration. A total of 150–300 μg of M9 in 100 μl was reduced with the immobilized reducing agent tris[2-carboxyethylphosphine] hydrochloride (Pierce Co.). The reduced M9 peptide was reacted at 4°C with the activated ScT, and the final product was freeze-dried. Analysis of the conjugation reaction by SDS–PAGE on a Tris-tricine gel (Fig. 1B) showed a major reaction product at 5,300 and smaller bands at 8,000 and 12,000 Da corresponding to M9 conjugated with ScT (M9–ScT) with smaller detectable quantities of $[M9]_2$-ScT and $[M9]_3$-ScT species with $[M9]_4$-ScT occasionally detected in some reactions. The higher band in lane 1 (Fig. 1B) was a dimer of M9 due to disulfide bond formation, and was sensitive to reduction by β-mercaptoethanol.

To optimize the amount of M9–ScT, BAEC cells were lipofected with increasing amounts of M9–ScT complexed to 1 μg of pCMVβgal, and the total expression was determined using fluoresence assay of β-galactosidase activity. The expression increased 4.3-, 19.9-, and 60.1-fold over lipofection by addition of 10, 20, and 50 μg of M9–ScT, respectively. A level of M9-ScT in excess of 100 μg was found to be mildly cytotoxic after 48 h, potentially because of the inhibition of endogenous hnRNP A1 transport as a scrambled M9 sequence (ScM9–ScT) lacked toxicity at 100 μg dose.

Nuclear import assay. The nuclear import assay followed the method of Adam and colleagues[36]. The BAECs were washed in ice-cold import buffer (20 mM HEPES, pH 7.3, 110 mM $KC_2H_3O_2$, 5 mM $NaC_2H_3O_2$, 2 mM $MgC_2H_3O_2$, 0.5 mM EGTA, 2 mM DL-dithiothreitol [DTT] and 1 μg/ml each of aprotinin, leupeptin, and pepstatin). The cells were permeabilized by incubation of cells with import buffer containing 40 μg/ml digitonin for 5 min and then washed with import buffer. A total of 10 μl of rhodamine labeled plasmid (0.1 μg/μl) was mixed with 40 μl complete import buffer (50% [v/v] rabbit reticulolysate [Promega Corp.]; 20 mM HEPES, pH 7.3; 110 mM $KC_2H_3O_2$; 5 mM $NaC_2H_3O_2$; 2 mM $MgC_2H_3O_2$; 0.5 mM EGTA; 2 mM DTT; 1 μg/ml each of aprotinin, leupeptin, and pepstatin; 5 mM ATP; 5 mM creatine phosphate; and 20 U/ml creatine phosphokinase). Cells were incubated at 30°C for 30 min with a solution containing 10 μl of rhodamine–plasmid (0.1 μg/μl) with or without 5 μl of M9–ScT conjugate (12 μg/μl), and 35 μl of complete import buffer. In import inhibition experiments, cells were preincubated with wheat germ agglutinin (40 μg/ml) in import buffer for 20 min before adding rhodamine–plasmid with or without peptide. The coverslips were rinsed with import buffer, mounted on a slide, and then observed by phase contrast and epifluorescence microscopy using an inverted Zeiss Axiophot microscope equipped with a 63× planapo (NA 1.4) lens.

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