Evaluation of Strategies for the Intracellular Delivery of Proteins

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Purpose. The intracellular delivery of functionally active proteins represents an important emerging strategy for laboratory investigation and therapeutic applications. Although a number of promising approaches for protein delivery have been developed, thus far there has been no attempt to compare the merits of the various delivery technologies. This issue is addressed in the current study.

Methods. In this study we utilize a sensitive luciferase reporter gene assay to provide unambiguous and quantitative evaluation of several strategies for the intracellular delivery of a biologically active protein comprised of the Gal4 DNA binding domain and the VP16 transactivating domain.

Results. Both a cationic lipid supramolecular complex and a polymeric complex were able to effectively deliver the chimeric transcription factor to cultured cells. In addition, protein chimeras containing the Tat cell penetrating peptide, but not those containing the VP22 peptide, were somewhat effective in delivery.

Conclusions. Both supramolecular protein-carrier complexes and protein chimeras with certain cell penetrating peptides can support intracellular delivery of proteins. In the cell culture setting the supramolecular complexes are more effective, but their large size may present problems for *in vivo* applications.

KEY WORDS: protein; delivery; transcription factor; Tat; transport; cell penetrating peptides; transfection.

INTRODUCTION

The most widely utilized approach for intracellular delivery of proteins has been to link the "cargo" protein with peptide sequences that have the ability to penetrate membranes. These sequences are usually termed "cell penetrating peptides" or "protein transduction domains" (1,2). Protein delivery has been accomplished both by chemically joining the delivery peptide to the cargo protein, and by forming chimeric proteins containing the delivery sequence and the cargo protein.

A substantial amount of work has been done using a 10–12 amino acid domain derived from the HIV Tat protein (1). This peptide itself has been shown to enter cells by a nonreceptor dependent, nonenergy dependent mechanism that presumably involves passive permeation across the plasma membrane (3). The Tat peptide sequence has been incorporated into a number of chimeric proteins that were subsequently expressed in bacteria, purified, and presented to mammalian cells in culture. Some very striking results have been attained with this approach, using microscopy as well as various functional assays to evaluate intracellular delivery (4–6). Perhaps most impressive was a report indicating that a Tat-β-galactosidase chimera could be delivered *in vivo*, with uptake into various organs, including the brain (7).

Another series of studies concerns a herpes virus-derived protein termed VP22. This protein was reported to have the ability to be secreted from cells where it is expressed and to be taken up by other cells; a region of approximately 40 amino acids in the C-terminal of VP22 was implicated in this activity (8). VP22 was used to generate chimeric proteins that have been observed to spread from one cell to another (8–10). The VP22 sequence apparently may be linked by either N-terminal or C-terminal to the cargo protein (9). Although there have been reports of direct uptake of VP22 chimeric proteins from crude lysates (8), no work has been reported using purified protein, and most of the observations involve intercellular trafficking by coculture of VP22 producing cells with other cells. In contrast to the several positive reports concerning VP22-mediated protein delivery, one study failed to detect intercellular trafficking of GFP-VP22 chimeras (11).

Another short peptide with delivery capabilities is a sequence from the *drosophila* Antennapedia transcription factor (2). This peptide is taken up by cells in a non-receptor dependent manner (12). Various versions of the Antennapedia peptide have been successfully used to deliver oligonucleotides (13,14), as well as other peptides (15); however, the Antennapedia peptide does not seem to be very effective for delivering large proteins. Other studies have used hydrophobic cell penetrating peptides based on signal sequences to deliver both peptides and proteins (16–18). Other protein delivery modules include those based on the transduction domains of bacterial or plant toxins (19).

A different strategy for protein delivery is akin to that widely used for DNA transfections. That is, the cargo protein is complexed with a supramolecular delivery agent, which then carries the protein into the cell. Although a number of standard commercial DNA transfection agents have been tried for protein delivery, in most cases they perform poorly. However, two agents have recently been described that seem to be quite effective. BioPORTER® is a cationic lipid preparation comprised of a novel trifluoroacetylated lipopolyamine and dioleoyl phosphatidylethanolamine. This reagent has been used to deliver β-galactosidase and several caspases into cells (20). Another agent, TransIT®, is a histone-based polyamine that has been used to deliver β-galactosidase (21).

Although the recent literature on intracellular delivery of exogenous peptides and proteins seems promising, it is also problematic. One important issue is that no direct comparisons have been made among the various delivery strategies. A second issue is that the methods used thus far to evaluate protein delivery are subject to uncertainties; thus, some of the assays used may not discriminate between true protein delivery to healthy cells and uptake by damaged or dying cells having impaired membrane permeation properties. In this report we describe an assay for intracellular protein delivery that we believe to be both quantitative and unambiguous. In this assay, mammalian cells are transfected with a reporter gene that expresses luciferase driven by a promoter that is a target for the yeast Gal4 DNA binding domain (Gal4 DBD). After recovery the cells are exposed to exogenous chimeric proteins that contain the Gal4 DNA-binding domain linked to the VP16 transactivating domain; these chimeric proteins may also include any one of several "cell penetrating pep-

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tides." In this assay, luciferase is induced only if there is successful intracellular delivery of the exogenous protein chimera to viable, fully functioning cells. Using this assay, we have evaluated four different protein delivery approaches, these being the Tat and VP22 delivery peptides, and the BioPORTER and TransIT delivery complexes.

METHODS AND MATERIALS

Expression Vector Construction

The plasmid pDual GC (Stratagene, La Jolla, CA), which has the ability to express proteins in both *E. coli* and mammalian cells, was used as a vector. Using a "seamless" cloning kit (Stratagene), several expression vectors were constructed; these were named pHGV, pHTGV, pHNGV, and pHNGV-VP22$C_{159\text{-}301}$. These plasmids each contain a sequence encoding an HA epitope at the amino terminal, a chimeric transcription activating module Gal4-VP16 in the middle, and hexahistidine at the carboxyl terminal. In some cases sequences representing the Tat (pHTGV) or VP22 (pHNGV-VP22$C_{159\text{-}301}$) cell penetrating peptide modules were inserted into the vectors. Plasmid pHNGV contains a nuclear localization sequence from SV40. The Gal4-VP16 insert was polymerase chain reaction (PCR) amplified from vector pJL11 (a vector where VP16 activation domain was cloned in frame with Gal4 DNA binding domain into pFA2-elk1 [Stratagene]). The template for PCR amplification of VP22$C_{159\text{-}301}$ was a VP22 plasmid pUL49ep kindly provided by Dr. Peter O'Hare. In pVP22-HNGV the HNGV coding sequence was cloned into a mammalian expression vector pVP22 Myc-His Topo from InVitrogen; it encodes the full VP22 sequence plus HNGV. The protein coding region of each vector was sequenced by the UNC DNA Sequencing Core Facility. The reporter plasmid pFR-Luc has the luciferase gene driven by five tandem repeats of the yeast Gal4 binding site (Stratagene).

Cotransfection and Luciferase Assay

Superfect reagent (Qiagen, Valencia, CA) was used to transfect HEK293 cells in 24-well plates with 0.25 μg of each test plasmid (e.g., pHTGV) and the reporter plasmid pFR-Luc. Twenty-four hours after transfection the cells were harvested for luciferase assay following the manufacturer's protocol (Promega, Madison, WI). The protein concentrations were determined using a coomassie blue assay (Pierce, Iselin, NJ) with bovine albumin as a standard. The luciferase activity was reported as relative light units (RLU) per microgram protein sample, and presented as mean ± standard deviation from three independent experiments.

Coculture and Cell Extract Studies

For coculture assays HEK293 cells were split into 24-well culture plates at a density of 0.5×10^5/well and grown overnight. The cells were transfected with either protein expressing test plasmid (e.g., pHTGV) or reporter plasmid pFR-Luc using Superfect and grown overnight. The next day the cells were washed with PBS, trypsinized, and resuspended in Dulbecco MEM (DMEM) media containing 10% fetal bovine serum. Cells containing the reporter plasmid and those containing the plasmid expressing the protein chimera to be tested were mixed at a ratio of 4:1 and replated. After 24 h the cells were harvested and luciferase activity was measured. For cell extract studies, cell growth and transfection was the same as for coculture; 24 h after transfection the cells transfected with test plasmids were scraped off the plate and centrifuged at 1000 rpm for 5 min. The cells were snap frozen in dry ice/ethanol, thawed, and lysed with 100 μl/sample of lysis buffer (25 mM Tris Cl, pH 8, 400 mM NaCl, 1 mM DTT, and 1 mM EDTA) for 5 min. The supernatants were collected after centrifugation at 14,000 rpm for 10 m, diluted in DMEM and added directly to the cells containing the pFR-Luc reporter. The medium was replaced after 1 h incubation, and the luciferase assay was performed 18 h later.

Protein Purification

Transformed BL21 DE3 Tuner *E. coli* (Novagen, Madison, WI) were grown in LB media at 37°C in a shaker incubator. At OD_{600nm} of 0.7–0.9, IPTG was added to a final concentration of 0.2 mM to induce recombinant protein production. Several hours later cells were pelleted by centrifugation and resuspended in a lysis buffer containing 25 mM Tris pH 8, 150 mM NaCl, 100 μg/ml of lysozyme and protease inhibitor cocktail without EDTA (Novagen). The cells were broken by three passes through a French press. The samples were centrifuged at 100,000 g for 45 m, and the supernatant was used for nickel ion chromatography in a fast protein liquid chromatography (FPLC) system (Amersham Pharmacia, Piscataway, NJ). The hexahistidine-tagged recombinant protein was eluted with buffer containing 25 mM Tris pH 8, 150 mM NaCl, and 400 mM imidazole. The eluted protein was dialyzed against a buffer containing 25 mM Tris pH 8, 20 mM NaCl, 2 mM EDTA, and 1 mM DTT for at least 2 h, and was further purified on a high trap Q ion exchange FPLC column. Fractions containing pure recombinant protein were concentrated with a spin column concentrator (Pierce, Iselin, NJ). After glycerol was added to a final concentration of 10%, the protein was filter sterilized. The protein concentration was measured by a coomassie blue assay using bovine albumin as reference protein. Aliquots of the proteins were analyzed by SDS-PAGE to estimate purity.

Protein Delivery Studies

HEK293 cells were split into 24-well culture plate at a density of 0.5×10^5/well and grown overnight at DMEM media with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). The reporter plasmid pFR-Luc (Stratagene) was transfected into the cells with Superfect transfection reagent. After a recovery period the cells were rinsed and grown overnight in medium with 10% fetal bovine serum. The cells were washed and placed in 250 μl serum free OptiMEM (Invitrogen). Thereafter, the bacterially expressed chimeric proteins were introduced into the media. Incubations were conducted for different periods of time after the addition of various concentrations of protein, as detailed in the figure legends. In studies with TransIT LT1® (Mirus, Madison, WI) and BioPORTER (Gene Therapy Systems, San Diego, CA) the bacterially expressed chimeric proteins were complexed with the reagent and applied to cells containing the FR-Luc reporter, essentially following the manufacturers' recommendations. Luciferase activity was measured after 16 h.

Cytotoxicity Studies

HEK293 cells were incubated in triplicate wells of 24-well plates with the various chimeric proteins or with TransIT

LT1 or BioPORTER transfection reagents. The incubation conditions were the same as those described above in the assays for protein delivery. At the end of the incubation period the viable cells were recovered by trypsinization and cell numbers were determined using a particle counter.

RESULTS

Description of the Assay System

The basic concept of the assay system is depicted in Fig. 1A. Activation of a reporter gene is used to evaluate the delivery of an exogenously produced chimeric transcription factor from the culture medium to the nucleus of the "reporter cell." Because only healthy cells with intact membranes can engage in transcription and translation, and thus express the reporter gene, this assay unambiguously evaluates delivery of exogenous proteins to intact cells.

Vectors for Chimeric Transactivating Proteins

We prepared vectors for expression of several chimeric proteins incorporating the Gal4-VP16 transcription activator. In some cases the chimeras included the Tat or VP22 delivery moieties, while in all cases, a hexahistidine sequence and a hemagglutinin epitope tag were included for purposes of purification and identification. We used the pDual GC shuttle vector system that is capable of protein expression both in bacteria and mammalian cells. The various chimeras are depicted in Fig. 1B. HGV contains only the Gal4 DBD -VP16 transactivating module and the HA and hexahistidine tags. HTGV adds the Tat delivery sequence between the HA tag and the Gal4 DBD. HNGV has a nuclear localization (NLS) sequence between the HA tag and the Gal4 DBD. HNGV-$VP22C_{159\text{-}301}$ (also termed HNGV-VP22C) is a chimeric protein with the VP22 carboxyl terminal domain added to the carboxyl end of HNGV. VP22-HNGV is a chimeric protein with full length VP22 fused to the amino terminal of HNGV.

Coexpression of the Chimeric Transactivating Proteins in Cells Containing the Reporter Gene

HEK293 cells were cotranfected with the Gal4-luciferase reporter vector (pFR-Luc) and with one of the vectors de-

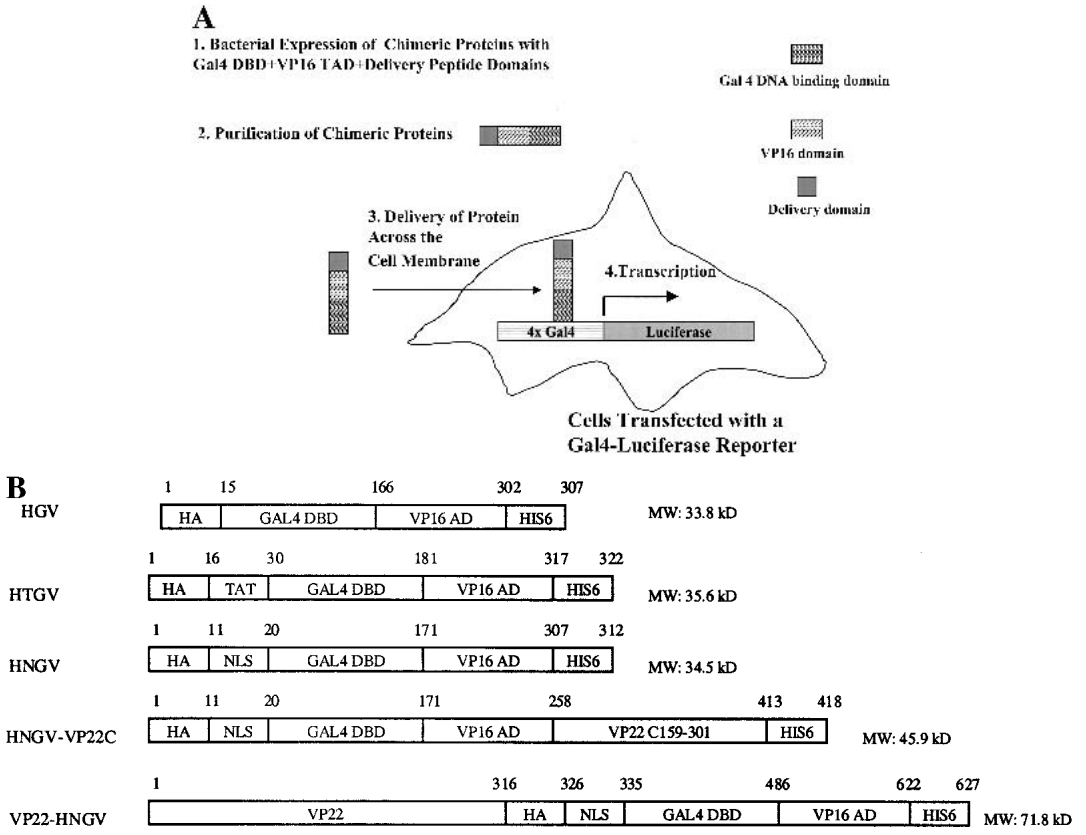


Fig. 1. (A) Diagram of the assay system. The "reporter" cells are transfected with a luciferase gene that is regulated by upstream sites that bind the yeast Gal4 DNA-binding domain. Chimeric transcriptional activating proteins comprised of the Gal4 DNA-binding domain and the VP16 transactivating domain with or without a "cell penetrating peptide" module are expressed in bacteria. The expressed proteins are purified and then added to the medium of the reporter cells. When a chimeric protein penetrates into the nucleus of the reporter cell the luciferase gene is induced. Thus, the assay unambiguously tests the ability of the "cell penetrating peptide" (or other delivery agent) to promote the delivery of the chimeric transcription factor to a healthy cell capable of gene expression. (B) Diagram of the coding sequences of the expression vectors for chimeric transactivating proteins. The numbers indicate the amino acids the sequence encodes. The different regions are represented as boxes labeled with letters indicating protein epitopes or domains: HA, hemagglutinin epitope; GAL4 DBD, Gal 4 DNA binding domain from *Saccharomyces cerevisiae*; VP16 AD, VP16 transcription activation domain; HIS6, hexahistidine epitope; TAT, cell penetrating peptide sequence from HIV Tat protein; NLS, nuclear localization sequence from simian virus 40 large T antigen; $VP22\ C_{159\text{-}301}$, VP22 protein C-terminal sequence 159-301; VP22, VP22 complete coding sequence. Expected molecular masses are given in kilodaltons.

scribed above. Thus, each chimeric protein was expressed in the same cells that contained the reporter, providing a basis for direct comparison of the transcriptional activity of the various proteins. As seen in Fig. 2A, significant amounts of each protein were expressed, as detected by western blotting. As seen in Fig. 2B, each protein effectively activated the Gal4-luciferase reporter. This demonstrates that the inclusion of the Tat or VP22 delivery sequences did not alter the transcriptional efficiency of the Gal4-VP16 module in terms of its ability to induce the reporter gene.

Delivery of Chimeric Transactivating Proteins by Cellular Coculture

As an initial step in evaluating the intracellular delivery efficacy of the chimeric proteins described in Fig. 2, we used a coculture format. Both VP22 and Tat have been reported to be able to transfer from one cell to another in coculture assays (1,8). Thus, separate cell populations were transfected with the Gal4-luciferase reporter or with one of the vectors expressing a chimeric protein. After recovery the cells were harvested, mixed, replated, and eventually assayed for luciferase activity. As seen in Fig. 3A, all of the vectors expressing chimeric transcriptional activating proteins induced luciferase expression above that seen with the empty vector control; however, the effects were modest. The strongest signals were attained with pHTGV, suggesting that the presence of the Tat sequence can enhance cell to cell protein transfer. Surprisingly, however, there was only a very limited effect of the two VP22 expressing plasmids. We also examined the effects of crude extracts from the protein producing cells on luciferase

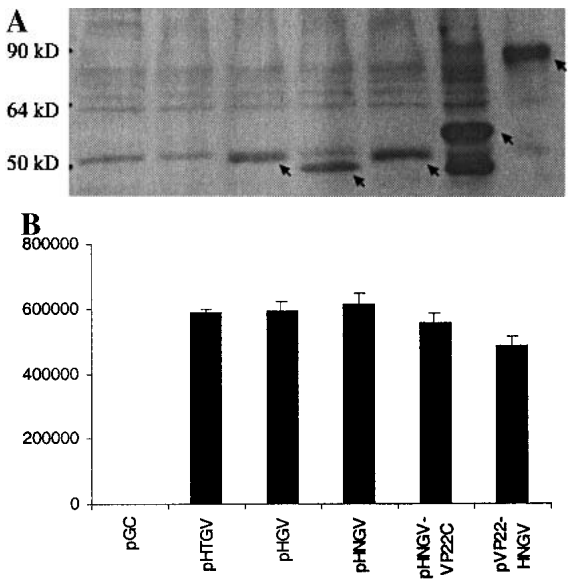


Fig. 2. Cotransfection using plasmids expressing different chimeric proteins. The test plasmids expressing the chimeric proteins described in Fig. 1 were transfected together with the reporter plasmid pFR-Luc into HEK293 cells. Western blotting with anti-HA antibody was performed (A) and luciferase activity was measured (B) at 24 h after transfection. In (A): 1, no test plasmid; 2, pGC vector control; 3, pHTGV; 4, pHGV; 5, pHNGV; 6, pHNGV-VP22C; 7, pVP22-HNGV. The arrowheads indicate the expressed protein. In (B): Luciferase activity (ordinate) was measured as relative light units (RLU) per microgram protein and presented as mean ± standard deviation of three independent experiments.

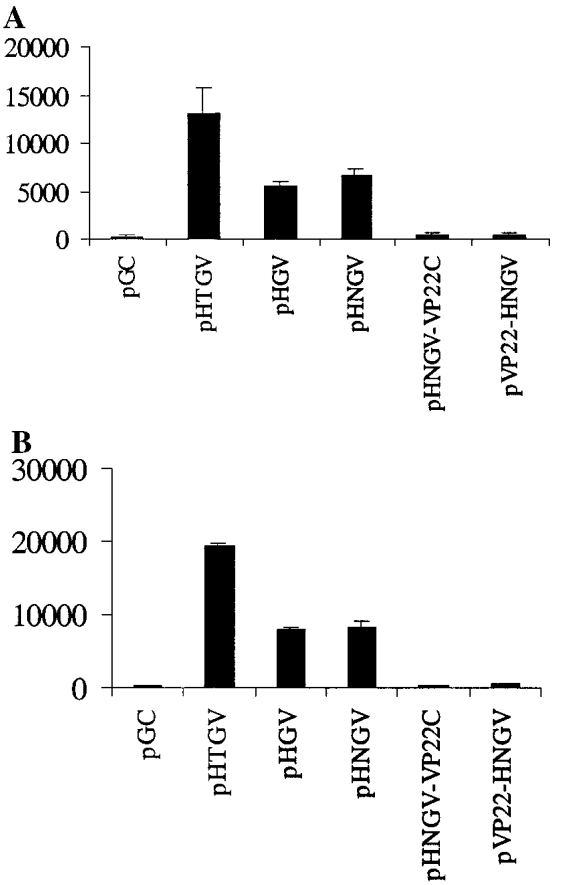


Fig. 3. (A) Coculture experiments. Separate pools of HEK 293 cells were transfected with either test plasmids expressing the chimeric proteins described in Fig. 1, or the pFR-Luc reporter plasmid. The two population of cells were mixed in a 1:4 ratio (protein-expressing cells: reporter cells), and replated in 24-well culture plates. Luciferase activity (ordinate) was measured 18 h later as RLU per microgram protein and presented as mean ± standard deviation of three independent experiments. pGC represents the empty vector control. (B) Cell extract experiments. HEK 293 cells were transfected with either plasmids expressing the chimeric proteins described in Fig. 1, or the pFR-Luc reporter plasmid. The cells expressing chimeric protein were scraped off the culture dish, snap-frozen, and lysed in 100 μl lysis buffer per well. The lysate supernatants were added to cells transfected with pFR-Luc reporter plasmid. One hour later the media were replaced with DMEM media containing 10%FBS. Luciferase activity (ordinate) was measured 18 h later as RLU per microgram protein and is presented as mean ± standard deviation of three independent experiments.

expression in the reporter cells (Fig. 3B). Once again, a modest but distinct effect was seen with extracts from the cells producing HTGV, whereas extracts from the two sets of cells expressing VP22 containing proteins were barely above background. The extracts used here were prepared in a manner similar to those used initially to demonstrate intracellular delivery of VP22 (8).

Purification of Bacterially Expressed Chimeric Transactivating Proteins

To pursue protein delivery studies in a more precise manner, we undertook to purify the various chimeric proteins after their expression in bacteria. Figure 4A depicts the pu-

rification of HTGV by a combination of metal ion affinity chromatography and ion exchange chromatography. As seen, the HTGV protein appeared in the soluble portion of the bacterial lysate at only moderate levels; however, it could be readily purified to approximately 95% homogeneity, as estimated by SDS gel electrophoresis and densitometry. Similar results were obtained for the HGV, HNGV, and HNGV-VP22C$_{159\text{-}301}$ proteins (not shown).

Delivery of Exogenous Chimeric Transactivating Proteins

The purified, bacterially expressed proteins were evaluated for their ability to activate luciferase expression by adding the protein to the medium of cells previously transfected with the Gal4-luciferase reporter. This provided a direct measure of the ability of the functional protein to enter viable cells, because the various proteins had similar inherent abilities to activate transcription, as shown above. Figure 4B illustrates the effect of exposing reporter cells to HTGV or HGV for various periods of time. Detectable expression of luciferase was attained within 2–3 h, whereas a maximum was reached at about 18–20 h. Because the expression of significant amounts of luciferase takes some time, this suggests that the HTGV protein rapidly entered the cells. Somewhat surprisingly, HGV, which lacks a delivery peptide, also had a significant effect on luciferase induction. The gradual late decline in luciferase activity may be due to degradation of the chimeric proteins by various proteases both within the cell and in the culture medium. Figure 4C illustrates a concentration-response curve for HTGV. As seen, although detectable responses were observed on exposure to submicromolar amounts of HTGV protein, the response continued to increase with doses up to 5 μM, suggesting that saturation of the reporter gene promoter sites was not reached. It should be noted that, as in 4B above, significant responses were observed using HGV, which lacks the Tat sequence. This was especially true at higher concentrations, suggesting that some degree of protein delivery can take place via constitutive cellular processes. We did not detect any signs of toxicity at the exogenous protein concentrations used in these experiments; the cells retained their normal morphology throughout, and no obvious cell loss was observed. In parallel experiments possible toxicity was monitored by counting viable cells released by trypsinization, using a particle counter. The viability of the cells treated with the highest amounts HTGV or HGV used in Fig. 4 were greater than 95% vs. untreated controls.

Comparison of Strategies for the Delivery of Exogenous Chimeric Transactivating Proteins

An approach similar to that shown in Fig. 4 was employed to compare the delivery effectiveness of the four bacterially expressed protein chimeras HGV, HNGV, HTGV, and HNGV-VP22C. In these experiments two types of controls were used: (a) the cells were exposed to BSA, which cannot activate transcription; (b) the cells were exposed to HGV, which lacks a delivery sequence but which has the Gal4-VP16 transcriptional activating module. As seen in Fig. 5A, exposure of the reporter cells to the HTGV protein resulted in a substantial induction of luciferase activity. A more modest effect was observed with HNGV, whereas the HNGV-VP22C protein displayed an effect that was above the BSA control, but slightly less than the HGV control. Thus, this set of experiments indicated that the Tat sequence was capable of enhancing intracellular delivery of the Gal4-VP16 module, whereas the VP22 sequence did not seem to provide any additional effect above constitutive cellular uptake processes.

We also examined the ability of the BioPORTER and TransIT transfection reagents to attain intracellular delivery of proteins. The proteins were complexed with the reagents and then the complexes were incubated with cells previously transfected with the pFR-Luc reporter. As seen in Figs. 5B and 5C, both of these reagents achieved very significant intracellular delivery of each of the proteins tested, as indicated by activation of the reporter gene. The fact that bacterially expressed HTGV, HNGV, HGV, and HNGV-VP22C all displayed strong activation of the pFR-Luc reporter clearly indicates that each of these proteins can function effectively as a transcriptional activator if it is delivered to the cell interior. Figure 5D shows a comparison of the different delivery systems, namely peptide-mediated, BioPORTER mediated, and TransIT mediated protein delivery. As seen, when tested using the same amount of protein, the complexation agents were approximately 10–20 times as effective as the Tat sequence in delivery of HTGV, and also effective in delivering HNGV, HGV, and HNGV-VP22C. In parallel experiments possible toxicity was monitored by counting viable cells released by trypsinization, using a particle counter. The viability of the BioPORTER and TransIT treated cells ranged from 90–95%of that of untreated controls.

DISCUSSION

Two general approaches have been employed for the intracellular delivery of proteins. One approach is similar to that used for transfection of DNA, in that a non-covalent complex is formed between the substance to be delivered and a delivery agent. The second approach involves molecular engineering of the protein to be delivered so as to incorporate a "cell penetrating peptide" that is designed to carry the protein into the cell.

In the first case the resultant complex is usually quite large, being particulate rather than molecular in scale. This can present a serious liability in terms of projected *in vivo* uses, because large particles will likely be rapidly cleared from the circulation by the phagocytic cells of the reticuloendothelial system (22). By contrast, a very useful aspect of this approach for laboratory studies is its ease and rapidity. The delivery complexes are formed by simply intermixing the "cargo" with the delivery agent, and the mixture is added to cells; no "reengineering" of the cargo protein is needed. It seems likely that this type of agent can be used flexibly for the delivery of a variety of proteins, as long as complex formation can occur efficiently between the polycationic delivery agent and the protein (20). The mechanism involved in intracellular delivery of proteins by these agents has not been studied extensively, but is likely to be similar to the lipid bilayer destabilization mechanisms involved in DNA delivery by standard transfection reagents (23).

The second approach for intracellular delivery of proteins involves the creation of protein chimeras that include a delivery module, often termed a "cell penetrating peptide" (2). To date the most widely studied protein delivery modules

are a 10–12 amino acid peptide from the HIV Tat protein and a longer polypeptide from the herpes simplex virus VP22 protein (1). A positive aspect of such chimeric proteins is that they are macromolecules, rather than particles, and thus they should be able to widely distribute in the body subsequent to *in vivo* administration. One negative aspect is that their creation involves a degree of protein engineering, a corollary being that the introduction of the delivery module might affect the overall functioning of the protein. A more serious problem with this approach is that virtually nothing is known about the mechanisms involved in the protein delivery process. There have been extensive studies of cellular entry of the Tat and Antennapedia peptides themselves (3,12). A mechanism has been proposed that involves interaction of the cationic peptide with the bilayer membrane, formation of inverted micelles, followed by delivery to the cytoplasm (24). However, it seems very risky to assume that mechanisms that pertain to the delivery of a 10–20 amino acid peptide, will also pertain to the delivery of proteins containing hundreds of amino acids. In the case of VP22 the mechanism of the putative trans-membrane penetration seems to be completely unknown. Another important issue is the generality of the delivery module approach. Although several different types of protein have apparently been successfully delivered using both the Tat and the VP22 sequences, it is not clear that these approaches will be applicable to all proteins. Part of the literature on Tat-mediated delivery suggested that proteins are best delivered in the unfolded state and are refolded within the cell (25). If this is the case, the universality of this approach is in doubt because some proteins may be very difficult to refold after denaturation. Thus, important questions remain to be addressed before chimeric proteins containing "cell penetrating peptide" delivery modules can be used with confidence.

In the work described here we have used a reporter gene assay to evaluate different strategies for intracellular delivery of proteins. Proteins containing a Gal4-VP16 transcriptional activating module, with or without Tat or VP22 "cell penetrating peptide" delivery modules were incubated with cells containing a Gal4-luciferase reporter gene. This assay has a low background, it can be used for quantitative comparisons, and its interpretation is unambiguous, because it is only when a functional transactivating protein reaches the nucleus of a viable, metabolically active cell that expression of luciferase ensues. Many of the results obtained using this assay agree with prior literature on protein delivery, whereas other aspects of our studies seem to conflict with some previous work.

Studies using the BioPORTER and TransIT transfection agents confirm their ability to effectively deliver proteins to cells in culture. These experiments also demonstrated that the chimeric transactivating proteins that we produced in bacteria were fully functional when they were adequately delivered to cells.

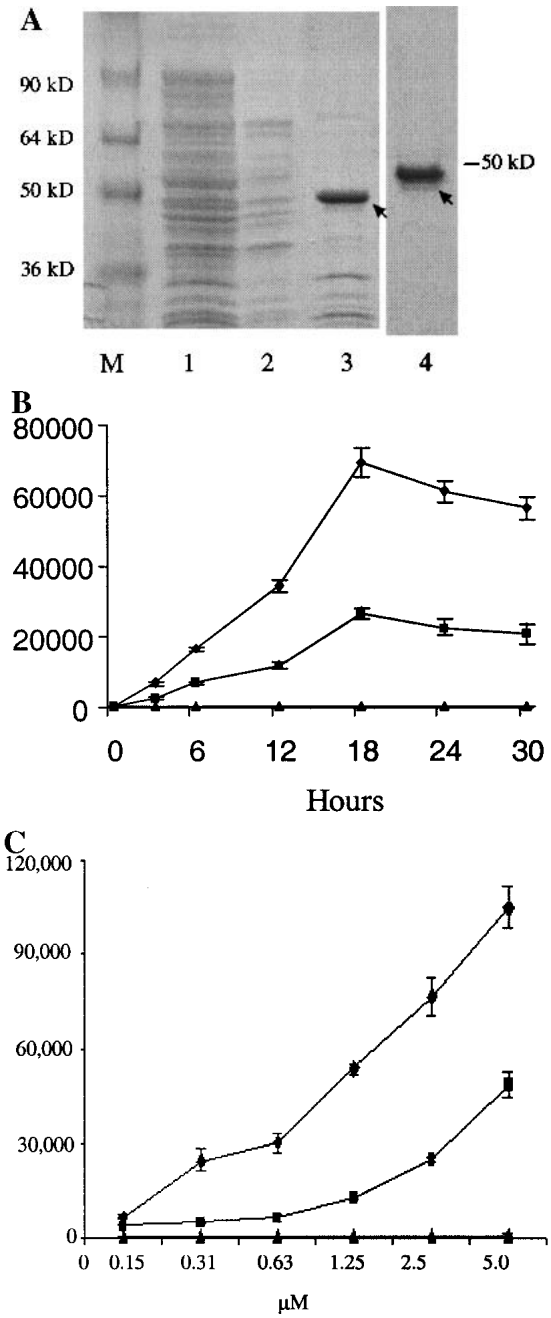
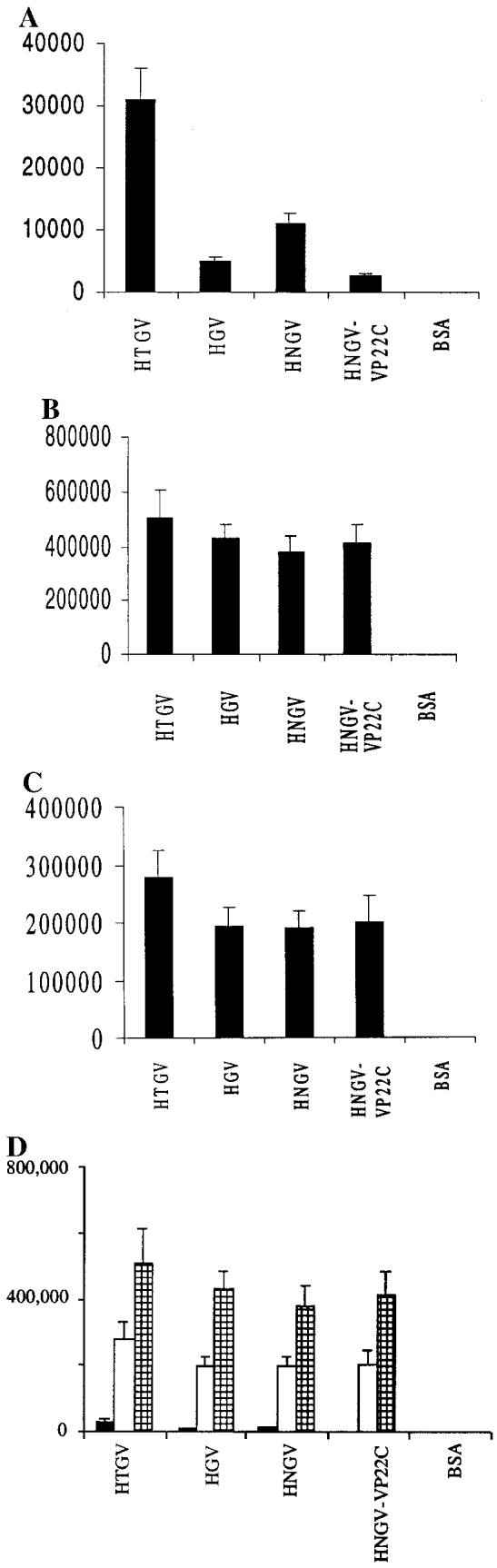


Fig. 4. Purification and intracellular delivery of chimeric transactivating proteins. A.SDS-PAGE of samples taken during the purification of HTGV. Proteins were visualized by coomassie blue staining. M, molecular weight markers; 1, supernatant of lysed bacteria after ultracentrifugation; 2, nickel column wash with 20 mM imidazole buffer; 3, nickel column elution with 400 mM imidazole buffer; 4, protein after anion exchange chromatography, spin column concentration, and filter sterilization. The positions of HTGV on the gels is indicated by arrowheads. In the experiments below purified bacterial-expressed protein was incubated with cells containing the pFr-Luc reporter. (B) Time course of response to HTGV. HEK 293 cells transfected with the pFR-Luc reporter plasmid were treated in serum-free OptiMEM with purified bacterially expressed HTGV (diamond symbols), HGV (square symbols),or with BSA (triangle symbols) as a control, at a concentration of 0.3uM. At different time points the cells were trypsinized, centrifuged, and stored as frozen pellets. Luciferase activity (ordinate) was measured and reported as RLU per microgram cell protein. (C) Concentration-response curve for HTGV. HEK 293 cells transfected with the pFR-Luc reporter plasmid were treated in serum-free OptiMEM with purified bacterially expressed HTGV (diamond symbols), HGV (square symbols), or BSA (as a control) (triangles) at various concentrations from 0.15 μM to 5 μM. Luciferase activity (ordinate) was measured 18 h later, reported as RLU per microgram of cell protein, and presented as mean ± standard deviation of three independent experiments.



Studies using the Tat-containing chimera HTGV agreed with prior results from others (1,6) indicating that the Tat sequence can enhance the intracellular delivery of large proteins. However, the delivery was not nearly as effective as that attained with BioPORTER or TransIT. Further, studies comparing HTGV with HGV (which lacks the Tat sequence) showed that some delivery of active protein took place due to normal cellular activity, presumably some form of endocytosis followed by intracytoplasmic release. Another interesting point concerns the HNGV protein, which contains a polybasic nuclear localization sequence in place of the Tat sequence. HNGV also showed delivery above that seen in the HGV control; thus, the Tat sequence is not unique, and other polybasic sequences may also enhance protein delivery. This is consistent with recent observations using short synthetic peptides, where improved versions of the Tat sequence (26) or arginine-rich sequences (27) were found to enter cells.

The most surprising aspect of our studies concerns the lack of effect of the VP22 sequence. We were unable to attain significant intracellular delivery of VP22 containing chimeras. This was true for experiments where the HNGV-VP22C protein was purified from bacteria and incubated with reporter cells; it was also true when we attempted coculture of cells expressing HNGV-VP22C or VP22-HNGV with cells containing the reporter gene. It seems that VP22 is unable to deliver the Gal4-VP16 module, although it has been reported to deliver other transcriptional activators such as P53 (9). It is interesting to note that other workers have also reported the inability of VP22 to promote intracellular delivery of proteins, using a quite different approach (11). Perhaps the delivery ability of the VP22 sequence is highly dependent on the nature of the "cargo" protein or on the cell type under study.

The lack of effect of VP22 and the relatively modest effects attained with the Tat-sequence in our system suggest that these delivery modules are not universally applicable to all proteins. In particular, because Tat-mediated delivery has sometimes been associated with denaturation–renaturation events, the highly ordered structure of the Gal4 DBD (28) may hinder delivery of the Gal4-VP16 module used here. The

Fig. 5. Comparison of intracellular delivery of various chimeric transactivating proteins. HEK 293 cells transfected with the pFR-Luc reporter plasmid were treated with purified bacterially expressed chimeric proteins in serum free OptiMEM using 3 μg protein per well (approximately 0.3 ml medium). The proteins HTGV, HGV, HNGV, HNGV-VP22C$_{159\text{-}301}$, or BSA control were tested. After 18 h luciferase activity (ordinate) was measured, calculated as RLU per microgram cell protein, and presented as mean ± standard deviation of three independent experiments. Use of 3 μg of protein corresponds to the following molar amounts: HTGV (83 picomoles); HGV (88 picomoles); HNGV (86 picomoles); HNGV-VP22C (66 picomoles). (A) Effect of free soluble proteins. The purified test proteins were added directly to the medium. (B) Effect of proteins complexed with BioPORTER. The test proteins were complexed with BioPORTER according to the manufacturer's directions prior to incubation with cells. (C) Effect of proteins complexed with TransIT LT1. The test proteins were complexed with TransIT LT1 according to the manufacturer's directions prior to incubation with cells. (D) A comparison of delivery of free and complexed transactivating proteins. The results for (A) through (C) are summated on the same scale. Free protein, black bars; protein complexed with BioPORTER, open bars; protein complexed with TransIT, hatched bars.

BioPORTER and TransIT transfection complexes seem to provide the most effective and generally applicable mode of protein delivery for cell culture. However, the inability of BioPORTER to function in the presence of serum proteins (according to the manufacturer), and the relatively large size of both types of complex, militate against their potential for *in vivo* delivery applications.

As mentioned in the Introduction, several additional "cell penetrating peptides" have been described, including ones based on hydrophobic signal sequences, or on other types of cationic sequences such as the Antennepedia derived peptides, transportan, or poly-arginine (29); it will be of interest to evaluate these moieties in the stringent assay system described here. In addition, it seems possible that other "cell penetrating peptides" exist, or can be designed, that are more effective than currently available ones, that may be able to deliver a wider variety of proteins, and that may find use in the *in vivo* setting. The assay system described here should be a powerful tool in seeking such molecules. In particular, the ability to detect protein delivery in a coculture setting may allow the development of efficient screens for delivery peptides.

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