Protein Transduction Domain of HIV-1 Tat Protein Promotes Efficient Delivery of DNA into Mammalian Cells*

Received for publication, November 26, 2000, and in revised form, May 7, 2001
Published, JBC Papers in Press, May 7, 2001
DOI 10.1074/jbc.M010625200

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The plasma membrane of mammalian cells is one of the tight barriers against gene transfer by synthetic delivery systems. Various agents have been used to facilitate gene transfer by destabilizing the endosomal membrane under acidic conditions, but their utility is limited, especially for gene transfer in vivo. In this article, we report that the protein transduction domain of human immunodeficiency virus type 1 Tat protein (Tat peptide) greatly facilitates gene transfer via membrane destabilization. We constructed recombinant λ phage particles displaying Tat peptide on their surfaces and carrying mammalian marker genes as part of their genomes (Tat-phage). We demonstrate that, when animal cells are briefly exposed to Tat-phage, significant expression of marker genes is induced with no harmful effects to the cells. In contrast, recombinant phage displaying other functional peptides, such as the integrin-binding domain or a nuclear localization signal, could not induce detectable marker gene expression. The expression of marker genes induced by Tat-phage is not affected by endosomotropic agents but is partially impaired by inhibitors of caveolae formation. These data suggest that Tat peptide will become a useful component of synthetic delivery vehicles that promote gene transfer independently of the classical endocytic pathway.

Recent progress in basic and clinical biomedical research has been largely dependent on the development of gene delivery technologies, including recombinant viruses (viral vectors) and other delivery strategies (nonviral vectors). However, none of the current technologies satisfies all of the requirements necessary for gene therapy. More than three-quarters of current gene therapy trials rely on various viral vectors, mainly because they deliver therapeutic genes much more efficiently and consistently than available nonviral vectors (1). Nevertheless, the development of a novel and efficient nonviral delivery system is an important goal, because recombinant viruses still have a number of disadvantages as practical tools for medical application (1).

Gene transfer into cultured mammalian cells using a synthetic carrier consists, in general, of the following steps: condensation of the DNA into a small complex, adsorption of the complex to the plasma membrane, traverse of the membrane by the complex, transport of the DNA into the nucleus, and unpacking the DNA in the nucleus (2). This schema also essentially describes the genome delivery of karyophilic recombinant viruses (3). Differences in the efficacy of viral and nonviral vectors reside partly in the mechanisms by which they deliver genes across the plasma membrane (2). Recombinant viruses deliver their genomes actively, either by membrane fusion or by membrane disruption, depending on their intrinsic machinery for infection. In contrast, most nonviral vehicles deliver their genes passively, relying on uptake into the vesicular compartments by endocytosis. A detailed understanding of the intracellular mechanisms of virus infection (nuclear transport of the viral genome and subsequent disassembly of the DNA-protein complex) has yet to be achieved (3). Therefore, most current efforts to refine nonviral delivery systems by mimicking viral functions focus on the first three stages.

The importance of membrane destabilization in nonviral gene delivery systems was first realized with the discovery that adenovirus particles greatly facilitate receptor-mediated gene delivery through disruption of the endosome (4). Since then, the roles of various chemical and biological agents have been examined in the facilitation of gene transfer by destabilization of the endosomal membrane under acidic conditions. These agents include endosomotropic agents, inactivated adenovirus particles, synthetic and natural amphiphilic peptides, cationic polymers, and synthetic neutral phospholipids (for a review, see Ref. 2). However, the utility of these agents is largely circumscribed, especially for in vivo application, by high toxicity and the instability of the complexes in the presence of serum proteins (2). Therefore, development of a novel and harmless
Efficient Gene Delivery by Tat Peptide-displaying Phage

Fig. 1. Characterization of the recombinant λ phage particle. A, structures of chimeric D proteins. B, protein analysis. Molecular weight markers are shown on the left. E, V, TAT-D, and D indicate the positions of the corresponding phage proteins. The arrowhead indicates electrophoresis origin. CBB, Coomassie Brilliant Blue staining; anti-D and anti-TAT, immunoblot analysis with anti-D or anti-human immunodeficiency virus TAT antibody, respectively; T, TAT-phage; VN, VN-phage; R, RGD-phage; W, wild type phage. C, EDTA sensitivity assay. Recombinant phage were incubated for 15 min at 37 °C in the presence of 10 mM MgCl₂ (open bars) or 1 mM EDTA (filled bars), and the residual phage titers were determined, as described under "Experimental Procedures." Phage titers were averaged from the results of duplicate experiments and are presented relative to control values. D and E, morphology of wild type phage (D) and TAT-phage (E), demonstrated by negative staining and electron microscopy.

agent that can facilitate the penetration of nucleic acids across the plasma membrane is a key to successful DNA delivery, even under the harsh conditions encountered in vivo.

Recently, some transcription factors, including the Tat protein of human immunodeficiency virus (5, 6), VP22 protein of herpes simplex virus (7), and antennapedia protein of Drosophila (8), have been shown to penetrate the plasma membrane directly from the cell surface. The peptide segments responsible for membrane penetration (the protein transduction domain (PTD)),1 consisting of 11–34 amino acid residues, were identi-

1 The abbreviations used are: PTD, protein transduction domain; DOTMA, N-[1-(2, 3-dioleoyloxy)propyl]-N,N,N-trimethyl-ammonium chloride; DOPE, dioleoyl phosphatidylethanolamine; DOTAP, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOPE, dioleoyl phosphatidylethanolamine; NLS, nuclear localization signal; GFP, green fluorescent protein; puromycin, puromycin; SV40, simian virus 40 (SV40) T antigen; SV40 T antigen (20) were displayed on

In this article, we examine the potential of the PTD of Tat protein (Tat peptide) (11) as an agent to stimulate gene transfer by membrane destabilization. We chose Tat peptide for this study because its function as a trans-element for membrane penetration has been analyzed in much greater detail than those of other PTDs (16). Furthermore, this peptide can facilitate the internalization of huge molecules, such as 45-nm dextran particles encapsulating magnetic beads (17). We demonstrate that Tat peptide displayed on the surface of λ phage greatly facilitates the transfer of marker genes encapsulated in the phage particles into mammalian cells, possibly via a nonendocytic pathway. These results strongly suggest that Tat peptide may become a useful component of synthetic gene delivery vehicles, applicable in the in vivo transfer of therapeutic genes.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant λ Phage Particles—The protein transduction domain (TAT peptide) (11), the integrating peptide (RGD peptide) (18), the heparin-binding domain of vitronectin (VN peptide) (19), and the nuclear localization signal (NLS) of simian virus 40 (SV40) T antigen (20) were displayed on λ phage heads, as described previously (21), except that we used our original lysogenic bacterial strains. λ D1180 (Dani15 del EcoRI–SalI cts857 min5 Sam100) was generated by crossing λ Dani15 (22) and λ gt11 (23), followed by deletion between unique EcoRI andSacI sites by linker oligonucleotides. The genome size of this phage is 78.5% of the wild type phage genome. The expression cassettes for enhanced green fluorescent protein (GFP) (CLONTECH Laboratories) and firefly luciferase (Promega Corp., Madison, WI) were constructed by inserting these cDNAs downstream of the cytomegalovirus immediate early promoter to produce pCMV-GFP and pCMV-luc. λ D1180 (GFP) and λ D1180 (luc) were prepared by inserting the expression cassettes for GFP and luciferase, respectively, into the unique EcoRI site of λ D1180. Lysogenic Escherichia coli were prepared by infecting TOP10 (Invitrogen BV, Groningen, The Netherlands) (originally appeared as DH10B (24)) with λ D1180 (or with its derivatives) at 32 °C, as described previously (25). Plasmids pTrc-D, pTAT-D, pTAT-RGD-D, pTAT-VN-D, and pTAT-NLS-D were constructed from pTrcHisA (Invitrogen), using the gene D fragment from λ phage, isolated by polymerase chain reaction, and synthetic oligonucleotides encoding the Tat, RGD, VN, and NLS sequences, respectively. Primary structures of these chimeric D proteins are shown in Fig. 1A. Tat and NLS peptides were displayed at the N terminus of D protein, whereas RGD and VN peptides were displayed at the C terminus of D protein, because we failed to produce the recombi-
nant phage displaying RGD and VN peptides at the N terminus of D protein.

Recombinant \( \lambda \) phage were prepared by inducing lysogenic \( E. \) coli carrying pTrc-D, pTrc-TAT-D, pTrc-RGD-D, pTrc-VN-D, and pTrc-NLS-D, at 45 °C for 15 min and then at 38 °C for 180 min (25). Phage particles were recovered from the bacteria with chloroform treatment and purified by two rounds of cesium chloride equilibrium centrifugation (25), followed by dialysis against H-SM buffer (10 mM Hepes-NaOH, 10 mM MgSO\(_4\), 100 mM NaCl, pH 7.5). All phage were titrated by plaque assay, using LE392 cells as host, at 37 °C (25). In the phage preparations used in this study, the number of infectious particles was identical to the number of DNA particles, as estimated from the amount of DNA and protein. For the experiments using recombinant DNA, physical and biological containment conformed to the guidelines of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Characterization of Recombinant \( \lambda \) Phage Particles—Purified \( \lambda \) phage (5.5 μg) was separated on 15% SDS-polyacrylamide gel electrophoresis and visualized by Coomassie Brilliant Blue staining or by immunoblotting with anti-D protein rabbit serum (1:1000) or anti-Tat protein rabbit serum (1:100), as described previously (26). For the EDTA sensitivity assay (27), 5 × 10\(^6\) plaque-forming units (pfu) of phage were incubated in buffer A (10 mM Tris-HCl, 10 mM MgCl\(_2\), pH 7.5), or in buffer B (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), for 15 min at 37 °C. The phage suspension was then diluted with H-SM buffer containing 0.1% gelatin, and the titer was determined as described above. Purified phage particles were examined under an electron microscope by negative staining, using 4% uranyl acetate.

Cell Culture and Transfection—All cell culture was performed at 37 °C under 5% CO\(_2\). COS-1, 293 (both obtained from RIKEN Cell Bank, Wako, Saitama, Japan), A431, and NIH3T3 cells were cultured in Eagle’s minimum essential medium (MEM), supplemented with 10% fetal calf serum. WI38/VA13/2RA (obtained from the Health Science Research Resource Bank, Tokyo, Japan) and HeLa cells were cultured in Dulbecco’s modified minimum essential medium (DMEM), supplemented with 10% fetal calf serum. A standard protocol for gene transfer into cultured cells was followed. Cells were seeded at 2.5 × 10\(^5\) cells/well, in 24-well plates, and cultured for 12 h. The cells were washed once with medium and incubated with 500 μl of medium containing recombinant phage (2.5 × 10\(^8\) pfu), or DNA (10 ng, 2.5 × 10\(^8\) copies) complexed with cationic lipid for 6 h at 37 °C. The cells were washed twice with medium and then cultured for 48 h before assaying for the expression of marker genes. Purified phage genomic DNA was complexed with cationic lipids (DOTMA/DOPE or DOTAP), according to the procedures recommended by the suppliers. Transfection with the DEAE-dextran-DNA complex has been described previously (25). Luciferase activity was evaluated using the Luciferase Assay System (Promega) and estimated in average relative light units with standard deviations. GFP was detected with fluorescence microscopy, using a GFP cube (Olympus Optical Co. Ltd., Tokyo, Japan). The cell nucleus was localized with fluorescence microscopy, using 4′,6-diamidino-2-phenylindole HCl and a WU cube (Olympus Optical Co. Ltd.), as described previously (28). Caveolin-1 was detected with fluorescence microscopy, using monoclonal anti-caveolin-1 and affinity-purified, fluorescein isothiocyanate-labeled anti-mouse IgG goat antibody (Organo Teknika Co., Durham, NC), as described previously (30).

In Vivo Gene Transfer—Eight-week-old female BALB/c mice were used in this study. Recombinant phage (8.5 × 10\(^8\) pfu) or plasmid pCMV-GFP (58 ng, 8.5 × 10\(^8\) copies), in 50 μl of H-SM buffer, were injected intrapericardially into the left lobe of a mouse liver, with a 27-gauge needle. After 48 h, the tissue was fixed by perfusion with 4% paraformaldehyde in phosphate-buffered saline, and a frozen section was examined for the expression of GFP by fluorescence microscopy, as described above. All animal experiments were performed according to the institutional guidelines for the care and use of laboratory animals.

RESULTS AND DISCUSSION

In this study, we examined the potential of Tat peptide to facilitate gene transfer into mammalian cells, by displaying it on \( \lambda \) phage particles that encapsulate marker genes. We employed the bacteriophage as a unique model for the development of a synthetic gene delivery system, because its size and structure resemble a condensed DNA-polymer complex. In addition, the peptide display systems established in \( \lambda \) phage (21, 31–33), filamentous phage (34), and T7 phage (35) offer unique opportunities for investigating the function of natural and artificial peptides in gene transfer.

Among these candidates, \( \lambda \) phage has several attractive characteristics for our experimental purpose. First, \( \lambda \) phage particles can display various peptides more flexibly and more abundantly than other types of phage, as chimeras with one of the two major head proteins (D protein, 420 copies/particle) (21, 32) or with a major tail protein (V protein, 200 copies/particle) (31, 33). D protein is particularly important as a fusion partner for peptide display, because a variety of protein segments, consisting of up to 244 amino acid residues (36), can be successfully displayed either at the N terminus or at the C terminus of D proteins (37). Most importantly, DNA packaging occurs totally independently of the assembly of D and V proteins into the \( \lambda \) phage particles (21, 31–33), which allows us to analyze the biological function of displayed peptides separately from DNA encapsulation.

Second, \( \lambda \) phage can encapsulate large duplex DNA fragments, of up to 50 kilobase pairs in size, in a small head of 55 nm diameter (38). The DNA is tightly packed in the proteinaceous shell, which consists of another major head protein (E protein), and is completely protected from destructive environmental nucleases (38). These characteristics make \( \lambda \) phage a more adequate model of a duplex DNA-polymer complex than the filamentous phage, because the latter encapsulates single-stranded circular genomic DNA in a long, proteinaceous sheath (890 × 7 nm). Furthermore, the \( \lambda \) phage particle is adaptable to large scale production, is physically stable, and can be purified under extremely stringent conditions.

For displaying the PTD of Tat protein (amino acid residues 43–60, Tat peptide) (10, 11) on the \( \lambda \) phage particle, we constructed a chimeric protein in which the Tat peptide was fused to the N terminus of the phase D protein (Tat-D) (Fig. 1A) (21). We then prepared the phage particles carrying Tat-D protein (Tat-phage) by simultaneously inducing the replication of the lysogenic phage genome with the defective D gene and the production of Tat-D protein in single bacterial cells. The recombinant phage also contained the expression cassette for either
protein with recombinant phage particles by determining the sensitivity of the phage to a chelating reagent. D protein is dispensable in the phage when the genome size is less than 82% that of wild type \(\lambda\) phage (48,514 bp) (27), as was the recombinant phage used in this study. However, D protein is essential in maintaining the phage structure when magnesium ions are depleted with a chelating reagent (1 mM EDTA) (27). We found that all of the recombinant phage carrying chimeric D proteins were as resistant to treatment with EDTA as wild type phage, whereas D-deficient phage were highly sensitive to the same treatment (Fig. 1C, filled bar). These results indicate that chimeric D proteins used in this study stabilize the phage particle through physical association with the phage head, like normal D protein. We further confirmed, by negative staining under electron microscopy, that Tat-phage has a structure indistinguishable from that of wild type phage (Fig. 1, D and E).

We then incubated cultured cells with these recombinant phage containing the luciferase gene, at 37 °C for 6 h, and determined the luciferase activity 48 h later (Table I) to estimate the potential of the phage particles to deliver the marker genes encapsulated in them. Naked DNA and wild type phage did not result in any luciferase activity, as expected. In contrast, Tat-phage produced significant luciferase activity in the absence of any special agent to assist their delivery into the cells (Table I).

Tat peptide has a net positive charge, which has been proposed to facilitate low affinity binding of Tat protein to the cell surface (39, 40). However, the observed enhanced gene delivery was not simply due to accelerated adsorption of the phage particle to the cell surface. For example, RGD-phage and VN-phage, which display cell surface binding ligands with high and low affinity, respectively, did not induce any luciferase activity under the standard conditions (10^4 phage particles/cell, 6-h incubation) (Table I). None of the control phage induced luciferase activity, even when the activity was determined 72 h after their exposure to the cells (data not shown). Furthermore, none of the control phage, except VN-phage, could induce luciferase activity when exposed to cells at higher doses (10^5 and 10^6 phage particles/cell). VN-phage could induce some luciferase activity at the highest dose tested (10^6 phage particles/cell), but the level of luciferase expression was below 1% of that induced by Tat-phage (data not shown).

We also examined the relative capacity of each cell line to bind Tat-phage, as described previously (19). We found that COS-1 cells, to which Tat-phage can deliver the marker gene most efficiently, bound more Tat-phage than other cell lines (data not shown). However, as the variation in the binding capacity was within severalfold among the cell lines examined, we conclude that the variation in marker gene expression is not simply the result of differences in the number of low affinity binding sites for Tat-phage.

We then examined the efficacy of Tat-phage-mediated gene transfer by evaluating the expression of the encapsulated GFP gene in situ. The number of cells expressing GFP increased in proportion to the dose of Tat-phage (Fig. 2). When Tat-phage were incubated with COS-1 cells for 6 h at doses of 10^6 and 10^5 phage particles/cell, we detected a very strong GFP signal in about 30 and 12% of the cells, respectively, 48 h after transfection (Fig. 2). Even under standard transfection conditions (10^4 phage particles/cell, 6-h incubation), we detected weak but significant GFP signal in about 12% of COS-1 cells 48 h after transfection (data not shown). In the case of wild phage, we could not detect any GFP signal even at the highest dose tested (10^6 phage particles/cell, 6-h incubation) (Fig. 2E). These observations contrast with results reported previously, when recombinant \(\lambda\) phage displaying the RGD peptide as a

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**Fig. 2. In situ detection of Tat-phage-mediated GFP gene expression in cultured cells.** COS-1 cells (2.5 × 10^6) were incubated for 6 h at 37 °C, with 500 μl of medium containing 2.5 × 10^10 pfu of Tat-phage (GFP) (A, B), 2.5 × 10^8 pfu of Tat-phage (GFP) (C and D), or 2.5 × 10^6 pfu of wild type phage (GFP) (E and F). After 48 h, the cells were counterstained with 4′,6-diamidino-2-phenylindole HCl and examined with fluorescence microscopy, using a GFPA cube (A, C, and E) or a WU cube (B, D, and F), as described under “Experimental Procedures.”
chimeric V protein were used (41). Although this phage was internalized efficiently by endocytosis after binding to the cell surface with high affinity, it could induce the expression of the marker gene in only 0.025% of COS-1 cells under similar incubation conditions (2 \( \times \) 10^4 phage particles/cell, 2-h incubation) to those used in this study (41). Furthermore, the efficiency of gene transfer into COS-1 cells by other targetable recombinant phage has been reported as 0.0001–0.002% cells/10^4 phage particles/cell/h (42–45), significantly less than the efficiency observed in this study.

In addition to its activity in assisting the penetration of cargo molecules, Tat peptide is known to actively localize to the nucleus (11), and the amino acid residues responsible for the nuclear localization of Tat protein have been identified in the Tat peptide (46). In a separate study, we found that phage particles carrying NLS-D (NLS-phage) can move actively into the nucleus when they are delivered into the cytoplasm, and this active transfer dramatically enhances the expression of marker genes encapsulated in the phage particles. These observations suggest that Tat-phage-mediated marker gene expression may partly be due to enhanced nuclear delivery of phage DNA. However, NLS-phage cannot induce the expression of marker genes by themselves (Table I), because they require the help of other delivery vehicles to penetrate the plasma membrane. Therefore, Tat-phage must first traverse the plasma membrane as a result of its intrinsic activity, whether or not the Tat peptide can enhance the nuclear delivery of phage genomic DNA.

The induction of marker gene expression by Tat-phage is completely blocked by anti-Tat protein polyclonal antibody (47), by dextran sulfate (48), and by heparin (48), all of which interfere with the function of Tat protein (Fig. 3B). From these data, we conclude that Tat peptide is actually involved in the enhanced delivery of marker genes, possibly through membrane destabilization. Dextran sulfate and heparin may obstruct the activity of Tat peptide by interacting with the cationic amino acid residues (arginine and lysine), which may be essential for membrane penetration (48).

We further characterized Tat-phage-mediated gene transfer using cultured cells. When we compared the efficiency of Tat-phage-mediated gene transfer with that of gene transfer mediated by cationic lipids (DOTMA/DOPE (49) and DOTAP (50)), we found that Tat-phage can induce luciferase activity at similar or superior levels to these popular transfection reagents (Table II). The facility of some cationic lipids in gene transfer is severely impaired by serum proteins, but that of Tat-phage is not affected and is even enhanced in some cell lines (such as COS-1 cells) that are sensitive to serum depletion (Table II, Fig. 3A).

The expression of the marker gene increased gradually as the cells were exposed to the phage for prolonged periods, of up to 6 h (Fig. 3A). This characteristic resembles that observed in cationic liposome-mediated transfection (51), which relies on nonspecific, low affinity adsorption of the DNA-lipid complex to the cell surface. In contrast, the recombinant viruses that bind actively to the cell surface by high affinity ligands generally only require a short exposure period (a few minutes to 1 h) for the transfer of genes (51). Therefore, our observations suggest that Tat-phage may also rely on low affinity adsorption to the cell surface, possibly through electrostatic interactions. Consistent

\[ \frac{\text{mg/ml fillipin (gray bars)}}{\text{or 25 mg/ml nystatin (filled bars). G–I, effects of inhibitors of caveolar formation on intracellular distribution of cavelolin-1}} \]

The cells were incubated in the absence (G) or presence of 2.5 \( \mu \text{g/ml fillipin (H)}} {\text{or 25 mg/ml nystatin (I) as described above and then examined with indirect immunofluorescence microscopy using anti-cavelolin-1 antibody, as described under “Experimental Procedures.”}} \]
with this supposition, no high affinity receptor has been identified, either for Tat protein or for Tat peptide (11, 48). The number of low affinity (or nonspecific) binding sites for Tat protein has been reported to be more than $10^7$/cell in HeLa cells (48).

Saturability is another general characteristic of receptor-mediated gene transfer. For example, transferrin-mediated gene transfer is saturated at a concentration of $1 \times 10^4$ DNA-transferrin complexes/cell (52), although more than $10^5$ high affinity transferrin receptors are present on the cell surface (53). In contrast, we found that the rate of Tat-phage-mediated gene transfer correlates linearly with the concentration of low affinity (or nonspecific) binding sites for Tat protein has been reported to be more than $10^7$/cell in HeLa cells (48).

Temperature-independent membrane penetration has been reported as another remarkable characteristic of PTD-mediated macromolecule transduction (10, 11). Therefore, we examined the rate of Tat-phage-mediated gene transfer at various temperatures. When we incubated the cells with Tat-phage at either 37 or 4 °C and then washed out the excess phage with medium, gene expression was induced, apparently independently of the incubation temperature (Fig. 3C). However, membrane destabilization by Tat peptide may proceed more slowly at the lower temperature, because stringent washing with medium containing dextran sulfate impaired gene transfer more effectively at 4 °C than at 37 °C (Fig. 3D).

We also characterized the route by which DNA penetrates the cell during Tat-phage-mediated gene transfer. Since Tat peptide-mediated protein transduction is considered to rely on a nonendocytic pathway (7, 10, 11, 48), we first examined the effects of endosomotropic reagents (chloroquine and monensin) on Tat-phage-mediated gene transfer. These reagents elevate the pH of vesicular compartments (54) and affect the efficiency of endocytosis-mediated gene transfer in either a stimulatory or an inhibitory manner, depending on the delivery vehicle. For example, gene transfer via receptor-mediated endocytosis (55) or mediated by DEAE-dextran (28) is markedly enhanced with these reagents, whereas gene transfer mediated by cationic lipid-DNA complexes (55) or by ligand-displaying phage (43) is severely impaired. We found that Tat-phage-mediated gene transfer is not affected by either of these reagents, whereas DEAE-dextran-mediated gene transfer is markedly enhanced (Fig. 3E), suggesting that the former may not rely on the endocytic pathway.

Next, we examined the effect of nystatin, an inhibitor of caveolae formation, on Tat-phage-mediated gene transfer. Caveolae are small (50–70 nm), uncoated invaginations of the plasma membrane (for a review, see Ref. 56). Recent studies have revealed that SV40 and some bacterial toxins penetrate into the cytoplasm through this domain (57, 58). Accordingly, infection by SV40 is inhibited to about 50% by nystatin (57).

In conclusion, we have demonstrated the potential of the protein transduction domain of Tat protein (Tat peptide) in facilitating the delivery of large fragments of duplex DNA into animal cells. As described above, we cannot explain this phenomenon simply as a consequence of the enhanced uptake of phage particles by endocytosis. Our observations that RGD-phage and VN-phage could not induce marker gene expression (Table I) and that endosomotropic agents do not significantly affect Tat-phage-mediated gene transfer (Fig. 3F) support this view. Rather, we propose that Tat-phage may penetrate directly into the cytoplasm, at least in part by destabilizing the caveolar membrane. Such membrane destabilization must be quite localized, since Tat-phage is not cytotoxic, even at the highest dose examined. The dense and uniform display of Tat peptide on the phage head (420 copies/head) may contribute to this local, but effective, membrane destabilization.

Another conceivable function of Tat peptide in phage-mediated gene transfer is to facilitate the transport of DNA to the nucleus, as a result of its intrinsic nuclear localization activity (60). Although this hypothesis is attractive, it remains unclear whether Tat-phage can move actively into the nucleus, by virtue of its intrinsic activity, once it is delivered into the cytoplasm. Even if Tat peptide plays some role in the nuclear transport of DNA, its primary function in gene transfer is to assist the phage particle to cross the plasma membrane, because nuclear localization activity alone is not sufficient to promote gene transfer, as demonstrated in this study using NLS-phage (Table I).

Because the role of Tat peptide in facilitating gene transfer differs from that of high affinity binding ligands, it should be possible to construct an ideal synthetic delivery system by combining Tat peptide with targetable high affinity ligands that bind the cell surface. Such systems may also be effective for delivering genes into tissue cells in situ, because Tat peptide can destabilize the cell membrane even in the presence of serum components, as shown in this study (Table II and Fig. 3A).

Indeed, we detected clear GFP signal at the site of injection when we injected $8.5 \times 10^6$ particles of Tat-phage carrying the GFP gene intraparenchymally into the mouse liver, while no clear GFP signal could be detected when wild type phage or purified DNA coding the GFP gene were injected (data not shown). Therefore, Tat-phage with high affinity-binding ligand displayed on its tail may become an efficient and self-sufficient nonviral vector for...
delivering genes in vitro. The Tat-phage system may also be useful for constructing cDNA libraries that can be transduced directly into cultured animal cells, for expression cloning (61). All of these possibilities remain as future challenges.

Acknowledgments—We thank Dr. Hisatoshi Shida (Hokkaido University) for providing anti-Tat antibody, Dr. Hiroyuki Mizuguchi (National Institute of Health Sciences) for antibody production, and Dr. Toyoshi Fujimoto (Nagoya University Medical School) for helpful suggestions.

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J. Biol. Chem. 2001, 276:26204-26210.
doi: 10.1074/jbc.M010625200 originally published online May 9, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010625200

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