TAT-Mediated Protein Transduction into Mammalian Cells

Michelle Becker-Hapak, Sandra S. McAllister, and Steven F. Dowdy

Howard Hughes Medical Institute, Department of Pathology and Department of Medicine, Washington University School of Medicine, 4940 Parkview Place, Campus Box 8022, St. Louis, Missouri 63110

Manipulation of mammalian cells has been achieved by the transfection of expression vectors, microinjection, or diffusion of peptidyl mimetics. While these approaches have been somewhat successful, the classic manipulation methods are not easily regulated and can be laborious. One approach to circumvent these problems is the use of HIV TAT-mediated protein transduction. Although this technology was originally described in 1988, few improvements were reported in the subsequent 10 years. In the last few years, significant steps have been taken to advance this technology into a broadly applicable method that allows for the rapid introduction of full-length proteins into primary and transformed cells. The technology requires the synthesis of a fusion protein, linking the TAT transduction domain to the molecule of interest using a bacterial expression vector, followed by the purification of this fusion protein under either soluble or denaturing conditions. The purified fusion protein can be directly added to mammalian cell culture or injected in vivo. Protein transduction occurs in a concentration-dependent manner, achieving maximum intracellular concentrations in less than 5 min, with nearly equal intracellular concentrations between all cells in the transduced population. Full-length TAT fusion proteins have been used to address a number of biological questions, relating to cell cycle progression, apoptosis, and cellular architecture. Described here are the fundamental requirements for the creation, isolation, and utilization of TAT-fusion proteins to affect mammalian cells. A detailed protocol for production and transduction of TAT-Cdc42 into primary cells is given to illustrate the technique.

The transduction of proteins into cells was first described in 1988 independently by Green and Loewenstein (1) and Frankel and Pabo (2) with the discovery that the full-length (86-amino-acid) HIV Tat protein could cross cell membranes and transactivate a viral genome. In 1994, Fawell et al. (3) expanded on this observation by demonstrating that heterologous proteins chemically crosslinked to a 36-amino-acid domain of Tat were able to transduce into cells. While applicability of the Tat fusions was demonstrated by the chemical crosslinking of a 36-amino-acid domain of HIV Tat to a protein of choice (3), its widespread application could not be realized.

Subsequent to the Tat discovery, other transduction domains have been identified that reside in the Antennapedia (Antp) protein from Drosophila (4) and HSV VP22 protein from herpes simplex virus (HSV) (5). The exact mechanism of protein transduction across cellular membranes remains unclear in any of the domains studied. Attempts to answer some of these questions have reported that Tat peptides (6) and full-length fusion proteins (unpublished observation) transduce into cells at 4°C, which is indicative of a receptor-independent mechanism. Detailed studies exploring this issue are clearly needed.

Here we describe the methodology used to generate large quantities of transducible, full-length TAT-fusion proteins that can be applied to a broad spectrum of proteins independently of size or function. Briefly, TAT-fusion proteins are constructed in an expression vector, pTAT/pTAT-HA, that contains an amino-terminal, in-frame, 11 amino-acid, minimal transduction domain (residues 47–57 of HIV Tat) termed TAT. The fusion protein is expressed in bacteria and then isolated by sonication of the washed bacterial pellet in 8 M urea. The use of this denaturant achieves two goals. The first goal is to prepare usable proteins. The majority of hexahistidine (His6)-fused recombinant proteins are sequestered into inclusion bodies by the host bacteria.
and are therefore not easily isolated by standard aqueous methods. Sonication of the bacterial pellets in urea disaggregates the insoluble proteins, allowing for their enrichment and preparation into a usable, soluble form. The second goal is to prepare denatured proteins, which have a higher potential for biological effect than their counterparts prepared under soluble conditions (7). We hypothesized that, due to reduced structural constraints, higher-energy (ΔG), denatured proteins may transduce more efficiently into cells than lower-energy, correctly folded ones. Once inside the cell, transduced, denatured proteins, can be correctly refolded by chaperones (8) such as HSP90 (9). Indeed, an analysis of TAT-p27Kip1 protein revealed that this protein was urea denatured, the biological phenotypes were enhanced dramatically over protein prepared under soluble conditions (7). However, while preparing TAT-fusion proteins in this manner is advantageous in most situations and results in dramatic yield increases, it is clearly not an obligatory step.

TAT-Fusion proteins prepared by this method have been shown to transduce into a variety of both primary and transformed cell types, including peripheral blood lymphocytes (PBLs), diploid human fibroblasts, keratinocytes, bone marrow stem cells, osteoclasts, fibrosarcoma cells, leukemic T cells, osteosarcoma, glioma, hepatocellular carcinoma, renal carcinoma, NIH 3T3 cells, and all cells present in whole blood, including both nucleated and enucleated cells (10–13). Moreover, TAT-fusion proteins were shown to transduce into all cells and tissues present in mice, including those present across the blood-brain barrier (14, 15). To date, this strategy has resulted in the production of more than 60 full-length proteins with functional domains from 15 to 120 kDa. The fusions span a variety of functional classes, suggesting that many, if not most, proteins may be transduced into cells using this technology (7, 10–16).

SAFETY CONSIDERATIONS

TAT-Fusion proteins have been shown to cross several biological barriers such as the blood-brain barrier. They have also been shown to transduce virtually any cell type tested in vitro including cells classically thought untransducible such as bone marrow and osteoclasts. Because of these facts, it is important to consider the safe handling of the fusion proteins.

Although the TAT transduction domain was originally identified from HIV, no infectious materials are contained within this domain. The peptide or amino acid sequence attached to the TAT transduction domain must be weighed heavily in the safety of the transduction process. If the protein one wishes to transduce is known to be toxic or pro-apoptotic, then the TAT-fusion version will likely be more so. We recommend following NIH Biosafety Level (BSL) 2 guidelines for the safe handling of recombinant proteins. These safety guidelines can be accessed via the worldwide web at the following web site: http://bmbl.od.nih.gov/sec3bsl2.htm for BSL2.

General safety precautions should be taken when producing TAT-fusion proteins:

1. Purified fusion protein should be treated as a low-level radionuclide, such as 35S. Use plastic covered absorbent paper on benchtops, latex gloves, safety glasses, and a lab coat.
2. In case of spills involving concentrated protein, digest the proteins with a solution of 5% Terg-A-Zyme (VWR Scientific) at room temperature for approximately 10 min. The liquid should be adsorbed with damp paper towels and then wiped down again with 70% ethanol.
3. Any glassware containing the bacteria that express the fusion protein of interest should be decontaminated with a solution of 10% bleach, which should then be flushed down a sink with running water for at least 5 min.
4. When sonicating the bacterial pellets, use of a sonication cabinet is suggested to reduce the risk of aerosolized fusion protein.
5. Adhere to local guidelines on the disposal of solid biological waste.

ESSENTIAL COMPONENTS REQUIRED TO GENERATE TRANSDUCIBLE PROTEINS

A. Construction of Tat-Fusion cDNA using pTAT/pTAT-HA Plasmids.

Transducible proteins are generated by cloning the cDNA of interest into the pTAT or pTAT-HA bacterial expression vector. The pTAT-HA vector contains an ampicillin resistance marker, for selection after transformation; a T7 polymerase promoter; an N-terminal 6-histidine leader followed by the 11-amino-acid TAT domain (7), flanked by glycine residues for free bond rotation; a hemagglutinin (HA) tag; and a polylinker (Fig. 1A). The pTAT vector contains the same components with exception of the HA tag. To obtain a genetic N-terminal in-frame fusion with the TAT leader, the 5' UTRs of the cDNA must be deleted. Cloning into the NcoI restriction site and use of this site's ATG as the initiator methionine will yield an in-frame fusion with
the upstream leader. However, the plasmid was designed so that use of any of the restriction sites within the polylinker, except AegI, will yield an in-frame fusion. Although the plasmid contains a transcriptional termination sequence, to avoid adding nonspecific C-terminal amino acids, use of the stop codon from the cDNA of interest is suggested. The pTAT-HA-cDNA plasmid is then transformed into a bacterial strain that yields a high-copy plasmid number, such as DH5α (Life Technologies, Gaithersburg, MD). The individual clones can then be isolated and analyzed for the correct insert size by standard molecular biology techniques and finally the DNA sequence can be confirmed by automated DNA sequencing.

Analysis of Fusion Protein Expression

The pTAT-HA-cDNA plasmid is then transformed into a BL21(DE3)pLysS (Novagen, Madison, WI) bacterial strain. This strain has been optimized for recombinant protein expression by using Escherichia coli B that naturally lack Lon protease and OmpT protease. In a few cases, it may be advantageous to use other BL21 derivatives such as BL21 CodonPlus (Stratagene, Cedar Creek, TX) which further optimize recombinant protein expression because extra copies of rare E. coli tRNA genes have been added that overcome codon bias when genomes are AT or GC rich. Six to twelve independent isolates are grown as 1-ml overnight cultures in Luria broth (LB) (Sigma Chemical Co., St. Louis, MO), with 50 μU amp in the presence of 100 μM isopropylthiogalactoside (IPTG).

Troubleshooting. Optimization of culture conditions may be required if the fusion of interest does not produce protein in sufficient quantities. The necessity of IPTG induction is both construct and LB lot specific. The lactose content of LB among manufacturers and

FIG. 1. Characterization of pTAT vector and purification protocol. (A) pTAT expression vector. (B) Purification protocols. (C) Purification of TAT-HA-Cdc42 fusion protein over Ni-NTA resin. The crude lysate from a 1-L culture was applied to the resin in the presence of 10 mM imidazole, washed, and eluted with 100, 250, and 500 mM imidazole. Ten microliters of each fraction (St, start; Ft, flow through; W, wash), were resolved by SDS-PAGE and stained with Coomassie blue.
within lots can vary. Therefore, careful optimization of protein production is suggested by comparing LB from pRB, and TAT-Bid, are toxic to log-phase bacterial cells.

PepTib the bacterial suspension and resuspend the pellet fraction in 2x SDS sample buffer, then analyze by SDS-PAGE. Load two gels identically with the various clones plus one with a nonexpressing BL21(DE3)pLyS transformed with the empty pTAT/pTAT-HA vector as a negative control. One gel can then be stained with Coomassie blue to look for an overexpressing protein band with respect to the nonrecombinant expressing lysate. Note that not all TAT-fusion proteins are expressed at a substantial enough level to be viewed in this manner. Therefore, the second gel, if required, should be transferred to a filter and probed with anti-HA antibody (Berkley Antibody Co., Richmond, CA) or antibodies directed against the cDNA-encoded product.

We devised a denaturing protocol, using either 8 M urea or 6 M guanidine hydrochloride, to purify TAT-fusion proteins to increase both the transduction potential (7) and the yield by recovering all of the recombinant protein present in bacterial inclusion bodies. However, we have recently observed that several proteins produced superior results when purified under native conditions. In addition, although the protocol was originally designed for use with an FPLC (fast protein liquid chromatography) protein purification system, due to the use of single steps to perform buffer changes, commonly available resins combined with gravity flow columns can be substituted for FPLC columns. Moreover, the purification can be performed in a batch method.

Start a 100- to 200-ml LB amp overnight culture of highly expressing BL21(DE3)pLyS. The following morning, inoculate the entire volume into 1 liter of LB amp plus 100–500 μM IPTG and shake for 5 (or up to 10) h at 37°C. Isolate and wash the cell pellet in phosphate-buffered saline (PBS), then resuspend in 10–20 ml of Buffer Z and sonicate on ice 3 x 15 s pulses. (This sonication step may need further optimization for each sonication system.) Clarify the sonicate by centrifuging at 16,000g, 4°C for 10 min and save the supernatant fraction. Please note that some TAT-fusion proteins, including TAT-Caspase-3, TAT-pRB, and TAT-Bid, are toxic to log-phase bacterial cells. Therefore, we inoculate a 1-liter overnight culture and isolate the cell pellet immediately in the morning.

The clarified sonicate is equilibrated in 10–20 mM imidazole, then applied at room temperature to a pre-equilibrated 3- to 10-ml Ni-NTA (Qiagen, Valencia, CA) packed in an Econo column (Bio-Rad, Hercules, CA) in Buffer Z plus 10–20 mM imidazole. The low concentration of imidazole at this step is required to reduce the background contaminating proteins which may nonspecifically bind to the Ni-NTA resin. Although 20 mM imidazole is routinely used as a starting point, nonspecific binding can sometimes still be observed. If this problem persists, increase the imidazole concentration up to 30 mM. Allow the column to proceed by gravity or apply slight air pressure via syringe as required. Save some of the start (St) and flow-through (FT) for SDS-PAGE analysis. Wash the column with approximately 10 bed volumes of Buffer Z plus 10–20 mM imidazole. Elute the TAT-fusion protein by stepwise addition of 1 bed volume of Buffer Z containing 100 mM, 250 mM, 500 mM, and 1 M imidazole. Analyze the St, FT, and each column fraction by Coomassie blue staining of an SDS-polyacrylamide gel (Fig. 1B) and immunoblot analysis as outlined above. Pool the appropriate fractions together.

For TAT-fusion proteins purified under native conditions, sonicate in PBS or 20 mM Hepes, pH 7.2, 100 mM NaCl, in the presence of protease inhibitors. Apply to Ni-NTA as outlined above, elute with increasing imidazole concentrations, then simply desalt via a PD-10 column into the buffer or medium of choice.

Troubleshooting. If the TAT protein is detected primarily in the FT, decrease the imidazole concentration in loading Buffer Z; and if a high background of contaminating bacterial proteins are detected, increase the imidazole concentration of the loading buffer. In addition, due to the “head” binding nature of the Ni-NTA column, removing the urea (see below) on this column results in aggregation and precipitation of the protein on the column. If binding to Ni-NTA remains problematic, change Buffer Z from 8 M urea to 6 M guanidine-HCl (GuHCl) with 10–20 mM imidazole. Because 6 M GuHCl is a more stringent denaturant than 8 M urea, the protein becomes further unfolded and exposes the His6 purification leader. However, removal of GuHCl requires more steps than the 8 M urea protocol. Thus, we routinely use 8 M urea unless otherwise necessary.

If preparing the fusion protein under soluble conditions, some proteins may precipitate at high concentrations after elution from Ni-NTA. We suggest several possibilities to reduce this occurrence. First, centrifuge...
the eluate as soon as precipitation is noticed. Removal of the precipitating nuclei from the solution will reduce a further precipitation cascade. Second, immediate addition of glycerol at concentrations of 10–20% (v/v) can also reduce precipitation. Follow with the same concentration of glycerol into the desalting phase of the procedure in a PD-10 rapid buffer exchange procedure. (see next page)

SOLUBILIZATION OF DENATURED PURIFIED TAT-FUSION PROTEINS INTO AQUEOUS BUFFER

The major pitfall experienced when preparing TAT-fusion proteins is the high occurrence of precipitation of the unfolded proteins after placing them into an aqueous environment. To treat tissue culture cells and animal models with TAT-fusion proteins, the denaturant, 8 M urea or 6 M GuHCl, must be rapidly removed. Rapid removal of the denaturant achieves the goal of obtaining highly energetic (∆G) denatured TAT-fusion proteins that have a higher potential of yielding phenotypic effects than those prepared under slow folding (dialysis) techniques. Several choices for this procedure are described below. In our experience, use of Mono Q or S ion exchange chromatography yields superior results compared with removal by desalting columns. However, we have generated transducible TAT-fusion proteins by both of these approaches (Fig. 1B).

Ionic Exchange Chromatography (FPLC)

Although the purification of most proteins by ion exchange chromatography (Q and S resins, Amersham-Pharmacia, Uppsala, Sweden) is largely dependent on the isoelectric point (pl) of the fusion protein, it has been our experience that TAT-fusion proteins in 4 M urea will bind to the Mono Q or S resin independently of their predicted pl. Therefore, if the fusion protein of interest does not bind to the predicted resin, try the other.

For FPLC equipment that is maintained at 4°C, avoid crystallization of 8 M urea by diluting the pooled Ni-NTA fractions from above, 1:1 with 20 mM Hepes, pH 8.0 for Mono Q and pH 6.5 for Mono S. This will result in 50 mM NaCl and 4 M urea final concentrations. If the FPLC equipment is maintained at room temperature, dilute in Buffer Z with no NaCl to obtain a final 50 mM NaCl. Inject the sample into a 5/5 or 10/10 (preferred) Mono Q/S column equilibrated in Buffer A plus urea (4 or 8 M depending on temperature). Wash with ~20–50 ml Buffer A (no urea) and elute with a single 1–2 M NaCl step in Buffer A. Analyze St, Ft, and eluate fraction by SDS–PAGE as outlined above and pool appropriate fractions. The sample is then desalted on a PD-10 desalting column and analyzed by SDS–PAGE. See PD-10 procedure below.

By reducing the urea from 4 to 0 M in a single step, the denatured proteins are forced to become soluble in an aqueous environment. Due to the mixed population of protein configurations and biophysical properties, use of a single 1–2 M NaCl step is preferred to a linear NaCl gradient and will result in a sharper protein peak resolution and increased concentration.

Troubleshooting. If failure to bind or weak binding of protein is observed, try the other column type regardless of predicted pl. If strong binding is observed (i.e., no protein present in FT), but no or poor yield in eluate, several modifications to reduce the effective avidity of the protein to the resin can be used. As an example, if protein “X” binds the Q resin at 50 mM NaCl, but fails to elute, clean the column with HCl/NaOH (see manufacturer’s directions). Equilibrate the sample and column with 100 mM NaCl, inject sample, and elute as above. Check St, FT, and column fractions as outlined above on SDS–PAGE. Continue to increase NaCl concentration by 50 mM steps until recovery yield is 70–90% of input or protein is observed in FT. Alternatively, decrease (Q resin) or increase (S resin) the pH of Buffer A by steps of 0.5 pH unit until a small amount of protein is detected in the FT fraction. The goal is to decrease to avidity of the protein for the column in urea to the point of obtaining a reversible binding. We have used both of these strategies to isolate several proteins, including injecting samples at 200 mM NaCl/pH 7.0 into a Mono Q column. In addition, substitution of KCl for NaCl may be advantageous for some proteins.

Please note that due to the ionic nature of GuHCl, if it was used to denature and purify the TAT-fusion protein on Ni-NTA it must be placed into 8 M urea on a PD-10 prior to addition to the Q or S resin.

Ion Exchange by Gravity Flow Columns and Batch Preps

Due to the protocol design of using single elution steps, automated FPLC or HPLC is not required. Set up a 1- to 5-ml ion exchange column using 30-micron Resource Q or S resin (Amersham–Pharmacia, Uppsala, Sweden). The sample is diluted as above and, due to a low back pressure, is injected into the preequilibrated column via syringe. Wash the column with ~50 ml buffer A plus imidazole and elute TAT-fusion protein with a single step of 1–2 M NaCl. The sample is then desalted on a PD-10 desalting column, collected, and analyzed by SDS–PAGE. Troubleshoot as described above.
For batch preps, add 1–5 ml of Resource Q and S resin directly to the 10–20 ml of Ni-NTA eluate and place on a rotator at room temperature or 4°C for 15–60 min. Pellet beads in centrifuge at 2000–4000 g for 3–5 min, aspirate the supernatant fraction, and wash three times in 10–20 ml Buffer A. Elute the bound protein by adding 1–10 ml Buffer B and analyze as above. This method has the added benefit that by mixing equal amounts of Q and S resin, predetermination of resin binding by the protein is not required.

Troubleshooting. For some TAT-fusion proteins, we detect irreversible binding to the Q or S resin, even when using increased NaCl or KCl concentrations at the starting equilibrium, at 4°C. However, the same protocol performed at room temperature (20–24°C), instead of 4°C, can give higher yields.

Desalting/Buffer Exchange Column (PD-10 Column Procedure)

The theory behind rapid desalting is that passage of a denatured protein from 8 M urea or 6 M GuHCl through the interface into PBS forces the protein to rapidly hide its hydrophobic residues and become aqueously soluble. PD-10 desalting columns have a 1:1.4 dilution factor. Therefore, denatured proteins will be separated from each other, helping to avoid aggregation of the proteins and subsequent precipitation on the column. Although we have used this strategy to remove the urea from many TAT-fusion proteins, in general, it also produces protein preps that are more susceptible to precipitation and freeze-thaw problems. However, it is a rapid and inexpensive procedure that has proven useful for several proteins.

The PD-10 column is a prepacked Sephadex G-25 M resin that can be used to desalt or buffer exchange a protein preparation and is available from Amersham–Pharmacia. The column must be drained of its storage solution, then preequilibrated with 25 ml of the buffer of choice. We routinely equilibrate the PD-10 one of four different buffers: sterile PBS; 20 mM Hepes, 100 mM NaCl; 10 mM Tris–HCl, pH 7.5, 1 mM EDTA; or tissue culture medium without serum. PBS is the preferred buffer when the protein is to be injected into an animal, while tissue culture medium without serum is preferred when transducing tissue culture cells. Buffer exchange into the tissue culture medium allows one to add larger volumes of the fusion protein to the culture medium with little loss of nutrients due to dilution. Apply no more than 2.4 ml of protein from the Ni-NTA or ion-exchange columns onto the equilibrated PD-10 column. When the fusion protein has entered the gel bed, add more buffer to top out the column. Begin collecting fractions immediately on addition of the fusion protein onto the column. One-milliliter fractions are conveniently collected in microcentrifuge tubes. The protein will begin eluting in fraction 4. Analyze the ST, and each fraction collected, by SDS–PAGE. Pool the appropriate fractions.

Troubleshooting. The most common problem incurred when desalting directly from 8 M urea or 6 M GuHCl is protein precipitation on the PD-10 column. This can be alleviated by dilution of the protein or use of equilibrated columns in 10–20% glycerol and/or 1% BSA to help stabilize the protein. In addition, some proteins are more stable when desalted into 10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA.

Storage of the Purified Proteins

Determine the purified protein concentration by SDS–PAGE relative to a standard, such as BSA, and/or by protein concentration analysis if the protein appears pure by a Coomassie blue-stained SDS–PAGE. The purified TAT-fusion protein is then flash-frozen in 100- to 200-μl volumes in 10–20% glycerol and stored at −80°C. After freezing, test a vial for loss of protein due to freeze-thaw induced precipitation by thawing a vial, spinning at 16,000 g at 4°C for 10 min, and analyzing by SDS–PAGE. In addition, once a protein aliquot is thawed, it should never be refrozen. The length of time a fusion can be frozen in this manner will be protein and preparation specific. In our hands, frozen TAT-fusion proteins, such as TAT-E1A, are capable of retaining transduction potential on thawing when stored at −80°C for 2 years!

DETECTION OF PROTEIN TRANSDUCTION

The concentration and dosing schedule at which a TAT-fusion protein must be administered in vitro must be determined experimentally. Generally, it has been our experience that responses can be seen at 100–200 nM. Once a positive (or negative) signal is demonstrated, one can titer accordingly as required with a routine range of concentrations between 10 nM and 1 μM.

Protein transduction and localization can be achieved in one of two ways. The first method is by directly labeling the fusion protein with fluorescein isothiocyanate (FITC, Molecular Probes, Eugene, OR). Once conjugated to the basic residues of the fusion protein, the unconjugated material can be removed, and the labeled protein added to cells and analyzed by fluorescence-activated cell sorting (FACS) or direct visualization by confocal or fluorescent microscopy. A drawback to this
method is that its success depends heavily on a good labeling reaction. If the reaction is poor, the fluorescence intensity will be too low to detect.

The second method of visualization of the protein is the use of immunocytochemistry. The method uses a primary antibody specific for the fusion protein (anti-HA or anti-protein X) and a secondary antibody that is fluorescently labeled. This option is flexible, in that one may choose to colocalize other molecules or compartments of the cell with other fluorophores such as DAPI that will stain nuclei at a different excitation wavelength.

Troubleshooting. Keep in mind that protein transduction is a concentration-dependent process. Therefore, if the extracellular protein concentration is changed, i.e., slowly washing the cells, the intracellular FITC-labeled TAT-fusion protein will shift the equilibrium and can transduce back out of the cells. For FACS this is generally not an issue; however, for fluorescent confocal microscopy, this can make a significant difference in the ability to detect intracellular FITC-labeled TAT-fusion proteins.

Labeling TAT-Fusion Proteins with Fluorescein (FITC)

Twenty to fifty micrograms of TAT-fusion protein is placed in a 300-μl reaction mixture with the appropriate volume of 10X carbonate buffer, per the manufacturer’s instructions, for 2 h at room temperature. The nonconjugated FITC is then removed from the FITC-labeled TAT protein by either gel filtration chromatography or use of a PD-10 column equilibrated in PBS. When the PD-10 column is used, isolate 1-ml column fractions and check each fraction by protein assay and/or SDS–PAGE. If available, check for efficiency of FITC crosslinking by use of a fluorometer. Poorly labeled TAT-fusion proteins will result in a low signal-to-noise ratio. Therefore, if the amount of signal is not satisfactory, alter the labeling conditions by increasing/decreasing the ratio of protein to FITC in the labeling reaction.

To analyze transduction of fluorescently labeled TAT-fusion protein into cells, add 100–400 μl of purified FITC-labeled TAT-fusion protein to 10^2 to 10^6 cells in 0.5–1 ml of medium. Analyze for transduction by flow cytometry (FACS) analysis 10, 20, and 30 min after addition of the FITC-labeled TAT-fusion protein. In our hands, FITC-labeled TAT-fusion proteins rapidly transduced into cells, achieving maximum intracellular concentrations in less than 5 min. In addition, we note a narrow intracellular concentration range of the transduced protein within the population as supported by the narrow FACS peak width between control and transduced cells.

Visualization of TAT-Fusion Proteins by Immunocytochemistry

Intracellular localization of TAT-fusion proteins can also be visualized by immunocytochemistry using fluorophore-conjugated antibodies. An advantage of this method over the use of directly labeled proteins is that it does not rely on transduction of a fluorophore in addition to full-length protein. Furthermore, background fluorescence is abrogated with the use of epitope-specific antibodies. Cells are plated at the appropriate density into wells of commercially available glass or tissue culture-treated microscope slides, and allowed to adhere (typically overnight). Culture medium is then removed from each well using a glass pipet attached to a vacuum apparatus. It is important to vacuum the fluid from the same position in each well to minimize cell disruption. Cells are then incubated with fresh medium containing the specific TAT-fusion protein to be transduced. At the desired time point following transduction, cells are quickly washed with sterile PBS and fixed in 4% paraformaldehyde fix buffer, on ice for 10 min. All reagents throughout the procedure are applied directly to each chamber well using a transfer pipet, and removed using a clean glass pipet attached to a vacuum apparatus. If intracellular antigens are to be detected, then cells must be permeabilized with 100% ethanol for 10 min on ice following fixation. Cells are then washed three times with cold PBS. Nonspecific epitopes are blocked using the appropriate blocking agent diluted in PBS for 10 min at 42°C. Appropriate blocking agents include serum from the species in which the secondary antibody is generated and a 1% BSA solution. Cells are washed three times with cold PBS, and then incubated with the specific primary antibody for 15 min at 42°C. After being washed three times in cold PBS, the labeled secondary antibody is incubated on cells for 10 min at 42°C. At this point, the chamber gasket and adhesive are removed from the slide and
cells are rinsed with distilled water. Addition of commercially available reagents such as Slowfade (Molecular Probes) that diminish loss of fluorescence are recommended before the slide is mounted with a coverslip. Specific localization of the TAT-fusion protein can then be visualized via fluorescence or confocal microscopy.

Notes and troubleshooting. Select the primary antibody specific for the particular antigen under investigation. Choose appropriate controls such as nonimmune or preimmune sera, based on the species in which the antibody was generated. The secondary antibody is labeled with a fluorophore and is selected based on the species in which the primary antibody was generated. Fluorescently labeled secondary antibodies to specific IgG are commercially available. Antibody concentrations must be optimized to obtain the appropriate titer for specific epitope recognition while eliminating nonspecific binding. It is also very important to wash cells thoroughly between incubations to eliminate nonspecific background staining. Background can also be diminished by the addition of 0.1% BSA with the primary and secondary antibody incubations.

SPECIFIC METHODS FOR TAT±Cdc42

Construct Design

Human CDC42V12 (constitutively active) and CDC42N17 (dominant negative) plasmid cDNA was kindly provided by Channing Der (17). The coding region of each plasmid was polymerase chain reaction (PCR) amplified with the following primers: 5’-CGCCGCAGCATGGGCGGCGCATGACACATTAAAGTGTGTTGGTTGCGGC-3’, and 5’-CGCCGCGAATTCTCATAGCAGCACACCTGCGGC-3’. These primers added the NcoI and EcoRI restriction enzyme sites at the N and C termini, respectively. The amplimer identity was confirmed by restriction digest, then cloned into pTAT-HA using the same restriction sites. After standard screening methods, the DNA sequence was confirmed with a BigDye DNA sequencing kit (ABI, Foster City, CA).

Protein Production and Isolation

Plasmid DNA was transformed into BL-21 (DE3)-pLysS E. coli and screened for maximal protein production and protein identity by Western blotting using the anti-HA antibody (Berkley Antibody Co.). The highest-producing clone was inoculated into a 200-ml culture of LB plus 50 µg/ml ampicillin and incubated overnight at 225 rpm and 37°C for at least 16 h. The inoculum was placed into 1 liter of LB/ampicillin and incubated for 6 h at 225 rpm and 37°C. The cell suspension was harvested at 5000g for 10 min. The cell pellet was washed with PBS, then resuspended in 20 ml of Buffer Z. The suspension was sonicated with 4 cycles of 15 s on and 15 s off at 4°C. The lysed suspension was clarified by centrifugation at 16,000g for 10 min at 4°C. The clarified supernatant fraction was equilibrated with 10 mM imidazole and applied to a preequilibrated Ni-NTA resin with a 7.5-ml bed volume. The crude extract was allowed to bind to the resin by gravity flow until the entire extract had been adsorbed. The column was then washed with 10 bed volumes of Buffer Z with 10 mM imidazole. The enriched protein was then eluted by stepwise application of 100, 250, and 500 mM imidazole. TAT-Cdc42 variants eluted in the 100 mM imidazole fraction (Fig. 1C). The urea in the purified protein fraction must be optimized to obtain the appropriate titer for specific epitope recognition while eliminating nonspecific binding. It is also very important to wash cells thoroughly between incubations to eliminate nonspecific background staining. Background can also be diminished by the addition of 0.1% BSA with the primary and secondary antibody incubations.

Transduction into Primary Diploid Fibroblasts

SiFt’s were plated at 2.5 × 10⁴ cells/ml in an eight-chamber glass slide (Falcon) in Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal calf serum, 1X Pen/Strep, overnight at 37°C. After the cells had adhered to the glass, they were washed with fresh medium and then TAT-Cdc42V12 or TAT-Cdc42N17 was added to a final concentration of 50 nM. Protein was incubated on the cells for 10 min at 37°C, then washed two times with PBS. Cells were fixed with 4% paraformaldehyde prepared freshly in PBS, for 10 min on ice. After three washes with PBS, cells were permeabilized with 100% EtOH for 10 min on ice and washed three more times. The cells were stained with 200 ng/ml TRITC-conjugated phalloidin (Sigma Chemical Co., St. Louis, MO) for 1 h at room temperature in the dark. The gasket was then removed, and the slide washed in distilled H₂O and mounted with Slowfade reagent with a glass cover slip.

Microscopy

TRITC-labeled F-actin was detected on a Nikon Diaphot 300 microscope with an epifluorescence attachment using the 546-nm filter (Fig. 2). Images were obtained digitally under the 100X objective with a SpotRT camera (Diagnostic Instruments, Inc., St. Louis, MO). Images were analyzed and represented using Adobe Photoshop software.
TAT-MEDIATED PROTEIN TRANSDUCTION

DISCUSSION

This report has described and demonstrated the utility of TAT-fusion proteins. The reliance on time-consuming and low-efficiency transfection of DNA constructs is minimized. Nearly uniform distribution of the fusion protein within a culture is easily achievable, reducing the "background" unaffected cells normally seen in classic transfection experiments. Protein transduction can also reduce the need for laborious microinjection of molecules into individual cells. Once unattainable tight kinetics of activation cascades can be elucidated without the reliance on the synthesis or degradation of antibiotics. Consequently, experiments seeking to understand the kinetics of certain molecules, in certain pathways, are now resolvable in almost real time. The exogenous addition of effector molecule at specific time points within an experiment is achievable neither by transfection methods nor by use of the transfected VP-22 transduction domain that "feeds" proteins to surrounding cells.

There are some drawbacks and critical parameters of the TAT transduction system that should be considered. One of the primary caveats of the system is the solubility of the fusions. Purification of the fusion proteins in this method relies on the His_6 domain. While this domain is advantageous because of its small size, the preponderance of forming inclusion bodies in the bacteria is high and regaining solubility can be time consuming. Use of other affinity domains to enrich for the overexpressed proteins, such as maltose-binding protein and glutathione, has not yet been tried. Additionally, optimal transduction conditions must be determined independently. Each fusion protein will have a different half-life in serum under in vitro conditions. Therefore, if the expected phenotypic change is not observed, checking for the degradation of the fusion protein at various time points may be warranted. It may be also necessary to apply the fusion protein multiple times in a given time frame to overcome such difficulties. Another critical parameter is the careful design of the protein. If the native protein is known to be tightly associated at the N terminus, the TAT domain may be buried and transduce poorly. Therefore it may be necessary to change the spacing at the N terminus while maintaining the native conformation or consider constructing a C-terminal TAT fusion.

The obvious advantages of the system outweigh the disadvantages. We have successfully used the procedure described here to generate and transduce more than 60 TAT-fusion proteins ranging from 15 to 120 kDa into all cells assayed thus far, including primary cells, transformed cells, and tissues present in mice. Moreover, treatment of cells with TAT-Cdc42^V12 protein (constitutively active) results in the formation of filopodia in less than 10 min (Fig. 2), while the protein prepared in the same way but harboring a dominant negative mutation yielded no filopodia. These results are consistent with those reported by Nobes and Hall (18, 19) who obtained this phenotype within 12 min of microinjecting Cdc42^V12 into Swiss 3T3 cells. Our observation demonstrates that TAT can deliver functional protein with rapid kinetics of signal transduction. The fusion proteins tested have been shown to transduce into all primary cells that are normally highly resistant to other forms of manipulation to dissect both biological and in vivo biochemical processes. At this time, within our limited understanding of the mechanism of protein transduction (15), there appears to be no limit to the size of protein that can be transduced. Fusion proteins as small as 20 kDa easily transduce and are almost immediately biologically active, while proteins as high as 120 kDa transduce and are biologically active within 5 min (14).

The use of a genetic in-frame fusion combined with denaturation of the proteins achieves several goals, including isolation of the bulk of recombinant proteins that are usually present in inclusion bodies, increased efficiency of biological response, and ease of use. Once inside the cell, transduced denatured proteins appear to be rapidly refolded by chaperones (8), such as HSP90.
(9), and are capable of binding their cognate intracellular targets and performing biochemical functions, such as cell cycle arrest, cell migration, cytoskeletal rearrangement, protection from and induction of apoptosis, transcriptional inhibition and activation, and various enzymatic activities (7, 10–15). Indeed, every TAT-wild type fusion protein we have generated thus far has given us a biological or intracellular biochemical phenotype. Furthermore, due to the rapid rate of transduction, real-time kinetic experiments can be performed on primary cells that are not possible by any other means.

We conclude that transduction of full-length TAT-fusion proteins directly into primary or transformed cells has broad implications for manipulating intracellular processes in both experimental in vitro tissue culture systems and animal models.

**MATERIALS AND REAGENTS**

Buffers

Buffer Z = 8 M urea/100 mM NaCl/20 mM Hepes (pH 8.0)
Buffer A = 50 mM NaCl/20 mM Hepes (pH 8.0)
Buffer B = 1–2 M NaCl/20 mM Hepes (pH 8.0)
Phosphate-buffered saline (PBS)
4% Paraformaldehyde fix buffer (4% paraformaldehyde in PBS, pH 7.2; prepare fresh daily)
5 M Imidazole
6 M Guanidine–HCl
8 M Urea
Coomassie brilliant blue dye

Equipment

Sonicator
FPLC (optional)
SDS–PAGE and Protein Transfer units

Fluorescence-activated cell sorter (FACS)
Fluorescence microscope (preferably confocal)

**REFERENCES**