doi:10.1006/mthe.2001.0279, available online at http://www.idealibrary.com on IDEAL

Intracellular Delivery of a Tat-eGFP Fusion Protein into Muscle Cells

Nicolas J. Caron,* Yvan Torrente,*,† Geoffrey Camirand,* Mathieu Bujold,* Pierre Chapdelaine,* Karine Leriche,* Nereo Bresolin,†,‡ and Jacques P. Tremblay*,[1]

*Unité de Recherche en Génétique Humaine, Centre de Recherche du Centre Hospitalier de l'Université Laval, Université Laval, Ste-Foy, Quebec, Canada G1V 4G2
†Centro Dino Ferrari, Institute of Clinical Neurology, University of Milan, 20122 Milan, Italy
‡IRCCS Ospedale Maggiore Policinico and IRCCS Eugenio Medale, 20038 Bosisio Parini, Italy

Received for publication December 13, 2000; accepted in revised form February 2, 2001; published online March 9, 2001.

The Tat protein from HIV-1, when fused with heterologous proteins or peptides, can traverse biological membranes in a process called "protein transduction," delivering its cargo into cells. A Tat-eGFP fusion protein was purified from bacteria to study the transduction kinetics of Tat fusion proteins into cultured myoblasts and in the muscle tissue. Correctly folded Tat-eGFP reaches a maximum intracellular level in nearly 30 min, while its endogenous fluorescence is first detected only after 14 h. The nuclear localization signal from the basic domain of Tat was not sufficient to confer nuclear localization to Tat-eGFP, suggesting that the nuclear import pathway used by the exogenously added Tat-eGFP might be sensitive to the folding state of eGFP. In mice, the direct delivery to the muscle tissue using subcutaneous injections or the intra-arterial pathway led to few positive fibers in the muscle periphery or surrounding the blood vessels. Muscles injected with Tat-eGFP showed intense labeling of the extracellular matrix (ECM), suggesting that, although Tat fusion proteins can transduce muscle fibers, their binding by components of the ECM surrounding myofibers could interfere with the intracellular transduction process.

Key Words: **Tat; protein transduction domain; skeletal muscle; eGFP; internalization; extracellular matrix; cationic peptides.**

INTRODUCTION

Direct viral-mediated and *ex vivo* cell-mediated gene therapy have been studied for years for the potential treatment of genetic diseases. The success of gene therapy protocols is often limited by toxicity and immunological problems. Recent protocols for cell-mediated gene therapy often require, in addition to a corrective therapeutic gene, the delivery of several transgenes to immortalize, increase the life span of, or switch the differentiation program of cells isolated from the patients, before they can be transplanted back into the host. Previously, the potential for the therapeutic use of proteins to supplement the product of mutated genes was limited by the impermeable nature of the cell membrane to such macromolecules. However, reports in the past decade showed that some proteins could bypass the molecular cutoff imposed by the cell membrane, opening new possibilities in the field of molecular therapy.

Small regions of proteins and synthetic peptides, called protein transduction domains (PTDs),[2] were recently identified in *Drosophila* (ANTP), HSV-1 (VP22), and HIV-1 (Tat) (1–4). Even fused with heterologous proteins or peptides, PTDs can traverse biological membranes efficiently in a process termed "protein transduction," delivering their cargo into the cell. Cellular uptake of the Tat protein was first reported by two groups who showed that the exogenously added Tat protein from HIV-1 could enter cells and subsequently *trans*-activate the HIV-1 LTR promoter (5, 6). In 1991, Frankel suggested that Tat might prove a useful vehicle to deliver proteins or peptides into cells (7). Later, antibodies (8) and enzymes (HRP and β-galactosidase) (9) were shown to transduce into cells when crosslinked to a Tat peptide. The study of several Tat-derived peptides showed that residues 47 to 57 from

[1] To whom correspondence and reprint requests should be addressed at the Unité de Recherche en Génétique Humaine, Centre de Recherche du CHUL, 2705 Boulevard Laurier, Ste-Foy, Quebec, Canada G1V 4G2. Fax: (418) 654-2207. E-mail: Jacques-P.Tremblay@crchul.ulaval.ca.

[2] Abbreviations used: TA, tibialis anterior; PTD, protein transduction domain; eGFP, enhanced green fluorescent protein; ECM, extracellular matrix; NLS, nuclear localization signal; HS, heparan sulfate.

1525-0016/01 $35.00

the basic domain were responsible for the functional internalization into cells (2, 10, 11).

While studies aimed at better understanding the transducing capabilities exhibited by the HIV-1 Tat protein, the first convenient method to apply the protein delivery potential of Tat was developed by the group of Dowdy (12, 13). In this system, in-frame polyhistidine-Tat fusion proteins are purified from a bacterial lysate under denaturing conditions through a series of affinity, ion-exchange, and desalt columns. Isolated denatured Tat fusion proteins are made soluble in an aqueous buffer and added directly to the cell culture medium. While this strategy was used before, the exact transduction mechanism by which Tat fusion proteins cross lipid bilayers is still unknown. Tat PTD fusion proteins can be transduced into cells simply by adding them to the cell culture medium (11). After their exogenous application to cultured cells, Tat fusion proteins, purified under denaturing conditions, are internalized in a rapid, concentration-dependent manner to achieve maximum intracellular concentration in a matter of minutes and are also found in the nucleus (13, 14). The internalized proteins are then correctly refolded in the cytoplasm, perhaps by intracellular chaperones. The use of denaturing conditions for the purification of Tat fusion proteins is necessary to solubilize proteins from bacterial inclusion bodies and is also involved in increasing transduction efficiency and speed (13). Two previous studies examined the possibility of direct delivery of Tat proteins in animals. Results published by Fawell using a folded Tat-conjugated β-galactosidase protein injected intravenously showed strong activity in the spleen, heart, and liver but low staining into skeletal muscles and lungs (9). Schwarze reported that the intraperitoneal delivery of the denatured Tat-β-galactosidase fusion protein allowed the uptake into many tissues in mice, including the heart, the liver, and the kidneys, while skeletal muscles and the brain showed weaker signals (14).

In the present work, we have purified a Tat-eGFP fusion protein from bacteria to study the transduction kinetics of Tat fusion proteins into cultured myoblasts and into the muscle tissue. The aim of our study was to examine the potential of this new technology to deliver proteins into cultured cells and into the muscle tissue, to better understand the power and limits of Tat-mediated transduction. We report that correctly folded Tat-eGFP labeled with rhodamine can transduce C_2C_{12} myoblasts to reach a maximum intracellular level in nearly 30 min, while its endogenous green fluorescence was first detected only after 14 h. The Tat basic domain nuclear localization signal (NLS) was not sufficient to confer nuclear localization to the Tat-eGFP fusion protein in the first 18 h of treatment, suggesting that the nuclear import pathway used by the exogenously added Tat fusion proteins might be impaired by the folded structure of Tat-eGFP. In mice, the direct delivery into muscles using subdermal injections or the intra-arterial pathway led to a few positive fibers in the muscle periphery or surrounding the blood vessels. Muscles injected with the Tat-eGFP fusion protein showed intense labeling of the extracellular matrix (ECM), suggesting that, although Tat fusion proteins can transduce muscle fibers, their binding by components of the extracellular matrix surrounding myofibers could interfere with the intracellular transduction process.

MATERIAL AND METHODS

Fusion protein purification. The pTat-eGFP expression vector was constructed by inserting eGFP from pIRES-eGFP (Clontech) into the pTAT-HA vector (gift from Dr. Steven F. Dowdy) to produce an in-frame fusion protein. The pTAT-HA vector has an N-terminal 6-histidine leader followed by the 11-amino-acid TAT protein transduction domain flanked by glycine residues (GYGRKKRRQRRRG), a hemagglutinin tag (GYPYDVPDYAG) flanked by glycine residues, and a polylinker (13). The peGFP control vector was generated by *Bam*HI digestion and religation of pTat-eGFP, resulting in the deletion of 51 bp containing the Tat PTD. HIS-tagged fusion proteins were purified from BL21(DE3)pLysS (Novagen) as previously described (13) with only minor modifications. BL21 cells from overnight culture were spun down and then sonicated in buffer Z (8 M urea, 100 mM NaCl, 20 mM Hepes, pH 8.0). Centrifugation-cleared cell lysates were adjusted to 10 mM imidazole, loaded into a 3-ml Ni-NTA agarose column (Qiagen), washed with buffer Z + 10 mM imidazole, and eluted with buffer Z + 250 mM imidazole. Fluorescent fractions were then applied to a 3-ml Q Sepharose column (Bio-Rad) in 4 M urea, 50 mM NaCl, 20 mM Hepes (pH 9.5) and eluted in 1 M NaCl, 20 mM Hepes, pH 8.0. Eluted proteins were then desalted on a PD-10 column in PBS, aliquoted, flash frozen in 10% glycerin, and stored at −80°C. Tat-E7 was purified from BL21 cells containing the pTat-E7 expression vector as previously published (15). Rhodamine-labeled fusion proteins were generated by NHS-rhodamine labeling according to the manufacturer's protocol (Pierce), followed by gel filtration on a PD-10 column. Purified fusion protein concentrations were determined by Coomassie blue staining of SDS–PAGE compared to BSA standards.

Cells and cell culture. C_2C_{12} mouse myoblasts, NIH/3T3 mouse fibroblasts, mouse primary myoblast cultures, and human skin fibroblasts (graciously provided by Dr. François Auger, Hopital St-Sacrement, Quebec, Canada) were grown in DMEM HG medium supplemented with 10% FBS, 10,000 U/ml penicillin/streptomycin in 5% CO_2 at 37°C. Primary myoblast cultures were established from muscle biopsies of newborn BALB/C mice as previously described (16). Mouse myotubes were obtained by inducing differentiation of mouse primary myoblast cultures in DMEM with lowered 2% NCS for 4 days. Human myoblasts were obtained from the biopsy of a 15-year-old patient suffering from Duchenne muscular dystrophy (DMD) as previously described (17) and grown in MCDB120 containing 15% FBS, 0.5 mg/ml BSA, 0.39 μg/ml dexamethasone, 5 μg/ml insulin, and 10 ng/ml bFGF (18, 19).

In vitro intracellular transduction assays. Intracellular transduction assays were performed in 35-mm petri dishes (Fisher) and 24-well plates (Costar). Cells treated with eGFP, Tat-eGFP, rhodamine labeled Tat-eGFP, and Tat-E7 at various concentrations were examined directly or were quickly rinsed once for approximately 5 s in PBS after different time intervals and fixed in 4% paraformaldehyde for 5 min, rinsed twice in PBS, and mounted in PBS/glycerol (1/1) under coverslips before their observation. To study the time course of TAT-eGFP transduction into muscle cells, rhodamine-labeled Tat-eGFP (20 μg/ml) was added to the culture medium of C_2C_{12} cells in 96-well plates (Corning) for various time intervals at 37°C. Following the cell fixation with 4% paraformaldehyde and PBS rinsing, the fluorescence was read with a cytofluorometer (Cytofluor 2350; Perseptive Biosystems; EX, 530 nm; EM, 590 nm).

Animal studies. Animals were deeply anesthetized with 0.15 ml of ketamine/xylazine (10 mg/ml) prior to all surgeries. Intramuscular injections of recombinant proteins were performed in 12 C57BL/10J mdx/mdx mice. Fifty microliters of Tat-eGFP or control eGFP in 10% glycerin/PBS (500 μg/ml) was injected slowly through the skin into the belly of the TA muscle along the longitudinal axis. U-100 0.5-cc insulin syringes (Becton–Dickinson) were used for these injections, applying low pressure as previously described (20).

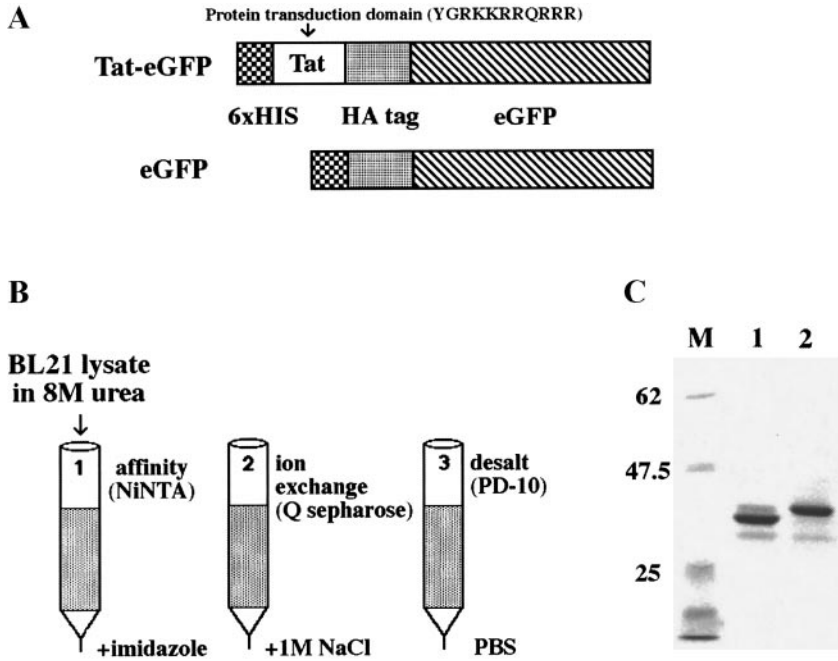

FIG. 1. Schematic representation of Tat fusion protein constructs and purification strategy. (A) Diagram of Tat-eGFP recombinant protein and control used for protein transduction assays and (B) HIS-tagged recombinant protein purification procedures. Overnight bacterial cultures of pTat-eGFP and peGFP were sonicated in 8 M urea and spun, before their purification over a Ni-NTA column, followed by a Q Sepharose column and a gel filtration step. (C) Yield from protein purification steps. Fluorescent fractions were analyzed by SDS–PAGE stained with Coomassie blue. Lane 1, eGFP (34 kDa); lane 2, Tat-eGFP (35 kDa); M, molecular weight markers.

The subcutaneous delivery of Tat fusion proteins was performed in 10 BALB/C mice. In short, a pouch of highly concentrated fusion proteins was created beneath the skin, in direct contact with the leg muscles. To do this, a very small incision was performed through the skin above the proximal region of the TA muscle, near the knee. The skin was then carefully detached from the leg muscles using rounded-tip forceps, to create a void. In some cases, the aponeuroses (the thin membrane covering muscles and bones) from the lower leg was very carefully removed using fine-tip forceps. A 50-μl volume, of Tat-eGFP or control eGFP (800 μg/ml), was injected into the void created under the skin using a Pipetman P200 (Gilson). Animals exhibiting significant leakage were rejected. Mice were sacrificed 16 h after treatment.

Fusion proteins were also delivered in the muscle limbs of four female BALB/C mice using the artery route as previously described (21). Surgical exposure of the right iliac artery was performed by opening the abdomen followed by the peritoneal cavity and then displacing the bowels. Injections of 200 μl rhodamine-labeled fusion proteins in 10% glycerin/PBS (250 μg/ml) were performed in mice with a 0.20-mm-gauge needle (Becton–Dickinson) caudally inserted into the iliac artery. There was no visible damage to the vessel wall during or after the operation. The abdomen was then sutured. Mice were sacrificed after 2 h. All animals were housed in microisolator cages with sterile water and food and maintained in accordance with the guidelines set by the Canadian Council of Animal Care.

Muscle necropsy and histology. Mice were sacrificed by cervical dislocation after being deeply anesthetized with 0.3 ml of ketamine/xylazine (10 mg/ml). Muscle biopsies were dissected out and transversely oriented in cryomatrix embedding medium (Shandon) to be immediately frozen in liquid nitrogen. Thin frozen sections (14 μm) were cut with a cryomicrotome at −24°C, placed on gelatin-coated slides, and kept frozen at −80°C. Sections were fixed for 5 min with 4% paraformaldehyde/PBS, rinsed twice with PBS, and mounted under coverslips. Hematoxylin–eosin and alizarin red staining were performed as previously described (22).

RESULTS

We selected eGFP as a study tool because its fluorescence can be observed only when it is correctly folded. The use of rhodamine-labeled eGFP as a cargo in a Tat fusion protein made possible the live observation of the translocation and the refolding processes. GFP and its variants like eGFP are very resistant to denaturation. GFP is not unfolded with 8 M urea, its denaturation requires treatment with 6 M guanidium hydrochloride at 90°C or a pH lower than 4 or greater then 12 (23). The eGFP protein has a newly characterized folding structure named β-can. On the outside, 11 antiparallel β strands form a compact cylinder, surrounding an α helix and the chromophore. The β-can protein structure of eGFP is responsible for its resiliency to denaturation, but the N-terminal portion of Tat-eGFP, encompassing the 6-histidine leader and Tat PTD, is free and therefore not protected. The fusion proteins used in this work (Fig. 1A) kept its fluorescence during the entire protein purification process (Fig. 1B), producing more than 95% pure yields (Fig. 1C) ranging from 2.5 to 6 mg/L of LB bacterial growth medium at a final concentration of 0.5 to 2 mg/ml.

Intracellular Localization and Time Course of Tat-eGFP Transduction in Cultured Cell Lines

Samples of the Tat-eGFP fusion protein and eGFP control were applied to C_2C_{12} and NIH/3T3 cells at concentrations ranging from 15 to 300 μg/ml to assess their

intracellular transduction capacity on cultured mouse myoblasts and fibroblasts. When cells were treated with Tat-eGFP at a concentration of 150 μg/ml (Figs. 2A and 2B), intracellular green fluorescence from the refolded protein was detected in the cytoplasm starting at 14 h, increasing progressively until approximately 24 h. C_2C_{12} and NiH/3T3 cells treated with Tat-eGFP concentrations in excess of 25 μg/ml consistently revealed cytoplasmic green fluorescence, without nuclear localization. Even at the highest concentration tested (300 μg/ml), no fluorescence was detected in cells treated with control eGFP (Figs. 2C and 2D). The rhodamine-labeled Tat-eGFP fusion protein was rapidly taken up by 100% of cells (Figs. 2E and 2F), reaching a maximum intracellular concentration in nearly 30 min (Fig. 3) in C_2C_{12} myoblasts. Cells treated with rhodamine-labeled Tat-eGFP mainly showed perinuclear cytoplasmic fluorescence after 24 h, without nuclear localization in the majority of cells (Fig. 2G). Less than 1% of cells exhibited a low level nuclear rhodamine fluorescence after 24 h. Rhodamine-labeled Tat-E7 (15), a fusion protein that does not resist denaturing conditions, was applied to cells to compare with Tat-eGFP. In contrast with Tat-eGFP, Tat-E7 localized in less than 1 h to the nucleus (Figs. 2H to 2J). No significant toxicity was observed in all cell types studied, even when using concentrations of Tat fusion proteins in excess of 300 μg/ml.

Intracellular Transduction of Tat-eGFP Cultured Primary Cells

To determine if Tat-eGFP could be internalized in primary cells, mouse primary myoblast cultures, human DMD myoblasts, and human skin fibroblasts were treated with rhodamine-labeled Tat-eGFP (75 μg/ml). Cells isolated from the muscle tissue of newborn mice were grown under conditions permitting fusion for 3 days to give rise to multinucleated myotubes. As was the case with C_2C_{12} and NIH/3T3 cells, primary cells internalized rhodamine-labeled Tat-eGFP rapidly in less than 30 min before leveling off in approximately 4 h. Rhodamine-labeled Tat-eGFP was internalized less efficiently into myotubes than in undifferentiated mononucleated cells (Figs. 4A and 4B). Highly contrasted, round cells consistently showed the most intense fluorescent labeling. Cultured human muscle cells and human skin fibroblasts can also be very efficiently transduced by a Tat-eGFP fusion protein. Nearly 100% of DMD human myoblasts (Fig. 4C) and human skin fibroblasts (Fig. 4D) showed internalization of rhodamine-labeled Tat-eGFP.

Tat-eGFP Is Found Associated with the ECM Following Intramuscular Injection

To examine the intramuscular distribution and the potential for Tat fusion protein to be delivered to the muscle tissue, we first performed intramuscular 25-μg injections of Tat-eGFP and control eGFP in TA muscles of C57BL/10J mdx/mdx mice (the mouse model of Duchenne muscular dystrophy). Intense eGFP fluorescence inside myofibers was rapidly (5 min) detected, for both Tat-eGFP and control eGFP (results not shown). Further investigation revealed massive extracellular calcium entry into myofibers found in the vicinity of the injection sites as indicated by alizarin red staining, indicating that the fluorescent myofibers at the injection sites were mechanically damaged or pressure perfused (results not shown). After 4 h, eGFP fluorescence was partially washed out of these mechanically damaged fibers and was mainly associated with the ECM surrounding muscle fibers in the case of Tat-eGFP (Fig. 5A). After 18 h, myofibers in the vicinity of the Tat-eGFP injection site still showed strong ECM-associated fluorescence, while there was total clearance from inside myofibers at the site of injection (Fig. 5B). Also, the ECM-associated fluorescence was significantly stronger in C57BL/10J mdx/mdx muscles injected with Tat-eGFP (Fig. 5B) than in muscles injected with control eGFP (Fig. 5C). Some muscle fibers outside damaged areas (as indicated by the lack of alizarin red staining) in Tat-eGFP-injected muscles exhibited weak intracellular fluorescence, indicating that some muscle fibers might have been transduced by the Tat fusion protein. After 2 days, eGFP fluorescence could still be observed in muscles injected with Tat-eGFP while no fluorescence remained attached to the ECM in control muscles injected with eGFP.

Subcutaneous and Intra-arterial Delivery to the Muscle Fibers

Light injuries, not leading to fiber necrosis, could explain the intracellular fluorescence in some muscle fibers outside damaged areas in Tat-eGFP-injected muscles. We, thus, proceeded to deliver fusion proteins to the muscles without creating trauma. Subcutaneous injections were performed, leading to the accumulation of liquid pockets between the skin and the lower leg muscles (gastrocnemius, soleus, and TA). Such interventions led only to the labeling of mononucleated cells embedded into the conjunctive tissue surrounding the leg muscles (Fig. 5D). The removal of the aponeuroses before the subcutaneous injection was necessary to obtain fluorescently labeled muscle fibers in the superficial layers of the TA (Fig. 5E), while myofibers from muscles treated with eGFP showed no significant fluorescence (Fig. 5F). In some cases, several external muscle fiber layers showed intracellular fluorescence (gradually decreasing toward the inside of the muscle) after the subdermal injection of Tat-eGFP, as revealed by longitudinal cryosections from a gastrocnemius muscle (Fig. 5G). To exclude the possibility that the microsurgery consisting in cutting the aponeuroses could produce muscle fiber injury, leading to mechanical perfusion, we then proceeded to inject Tat-eGFP through the right iliac artery. Such direct delivery to the leg completely eliminated the possibility of undesired injuries to muscle fibers of the lower leg, since the injection and the surgery took place several centimeters away from the muscles of interest. The delivery of rhodamine-labeled Tat-eGFP (50 μg) through the iliac artery led to a few red fluorescent myo-

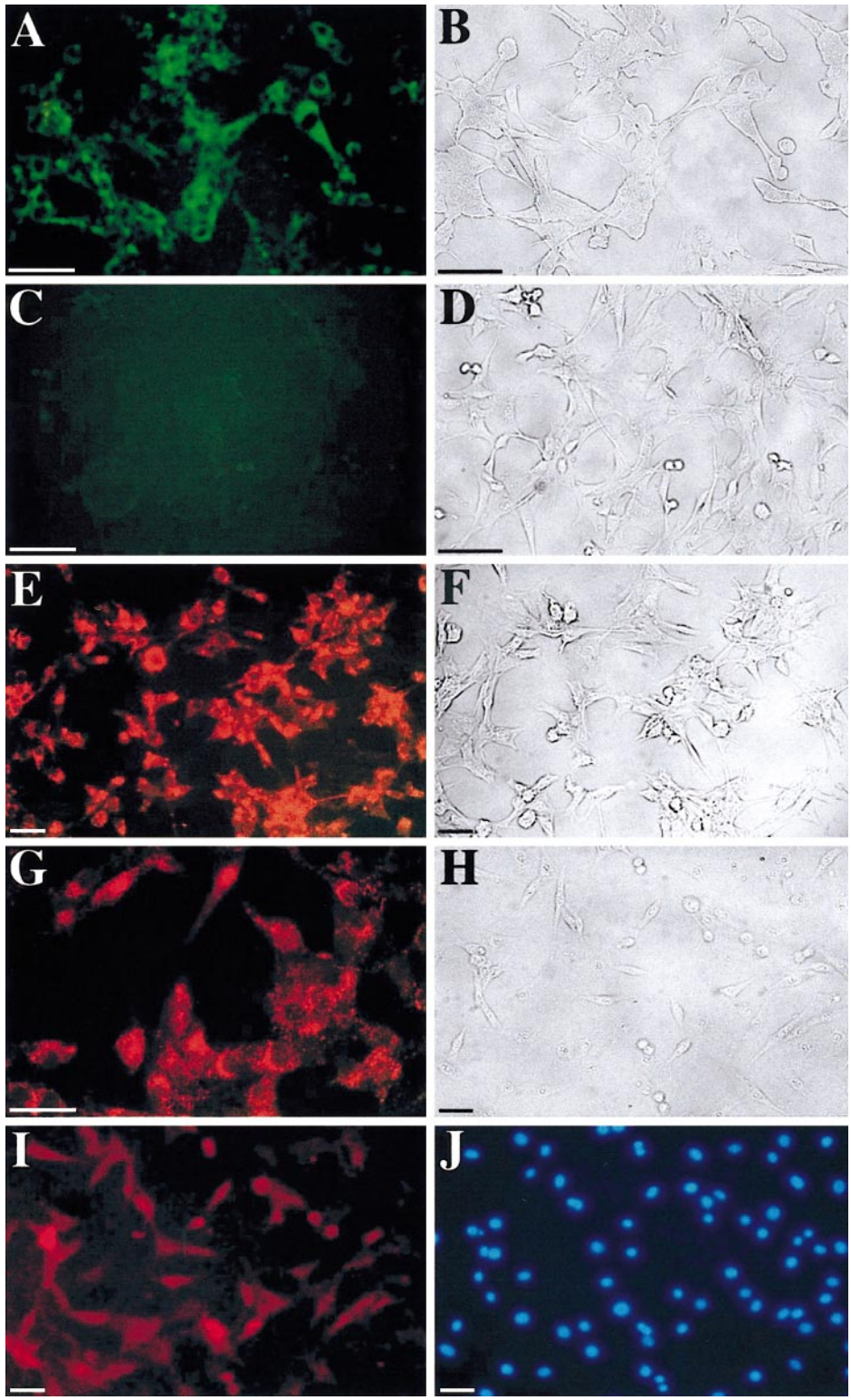

FIG. 2. Intracellular delivery of Tat-eGFP in NIH/3T3 fibroblasts and C_2C_{12} myoblasts. Fluorescence micrographs of NIH/3T3 treated with (A) Tat-eGFP (150 μg/ml) or (C) eGFP (150 μg/ml) for 18 h and corresponding phase-contrast images (B and D). (E and G) C_2C_{12} treated for 4 h with rhodamine-labeled Tat-eGFP (40 μg/ml) and corresponding phase-contrast image (F). (I) NIH/3T3 treated with rhodamine-labeled Tat-E7 (15 μg/ml), corresponding phase contrast (H) and DAPI staining (J). After the treatment, cells were rinsed quickly in PBS and fixed for 10 min in 4% paraformaldehyde before they were mounted and examined. Bars, 50 μm.

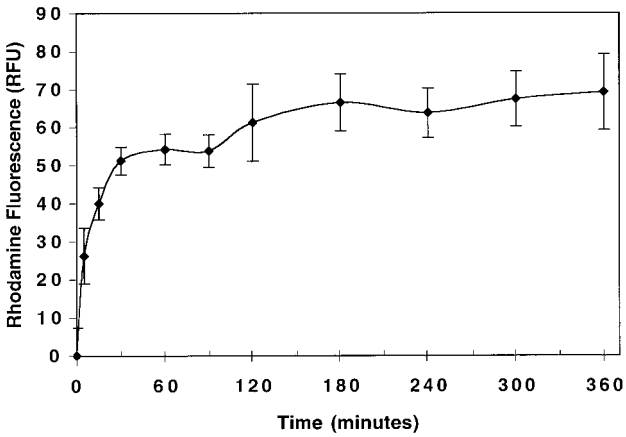

FIG. 3. Rapid uptake of rhodamine-labeled Tat-eGFP in C_2C_{12} myoblasts. To study the time course of TAT-eGFP transduction into cells, rhodamine-labeled Tat-eGFP (20 μg/ml) was added to the culture medium of C_2C_{12} cells in 96-well plates for various time intervals at 37°C. The cells were rinsed quickly in PBS and fixed for 10 min in 4% paraformaldehyde and read with a cytofluorometer. The intensity is expressed as arbitrary units, dots correspond to means and bars to standard errors of five duplicate wells.

fibers locally surrounding blood vessels (Fig. 5H) in the quadriceps and gastrocnemius.

DISCUSSION

Several proteins can traverse biological membranes by protein transduction. Small regions of proteins, 10 to 16 residues long, are responsible for this phenomenon (11). It was previously shown that fusion proteins containing the protein transduction domain from the HIV-1 Tat protein could transduce through the cell membrane to deliver their cargo into the cell body (2). After their exogenous application to cultured cells, Tat fusion proteins purified under denaturing conditions are rapidly internalized in a matter of minutes and are also located in the nucleus. In addition, their refolding into the host cell makes possible the observation of *in vivo* biological effects resulting from their interactions with nuclear proteins (11, 13). While previous studies were conducted using cargo proteins sensitive to denaturation in 8 M urea, the eGFP β-can structure of our Tat-eGFP fusion protein resisted the denaturing steps of our purification protocol. Intracellular transduction assays conducted with this folded fluorescent cargo resulted in a delay in reaching maximum intracellular levels (approximately 30 min) compared with previous studies using chemically synthesized Tat peptides and denatured Tat fusion proteins that all attained their peek in less than 15 min (13, 14, 24, 25).

Our data, showing intracellular transduction of native Tat-eGFP in several cell types, illustrate that a folded Tat fusion protein can decrease the efficiency and speed of the internalization process compared with completely denatured fusion proteins (13, 14, 25), but that unfolding prior to the application to cells is not necessary. The fluorescent Tat-eGFP fusion protein is unfolded, at least partially to lose its fluorescence, during the translocation process and is then slowly renatured in a matter of hours to reach detectable levels of fluorescence after 14 h. The apparent discrepancy between our results and those obtained by

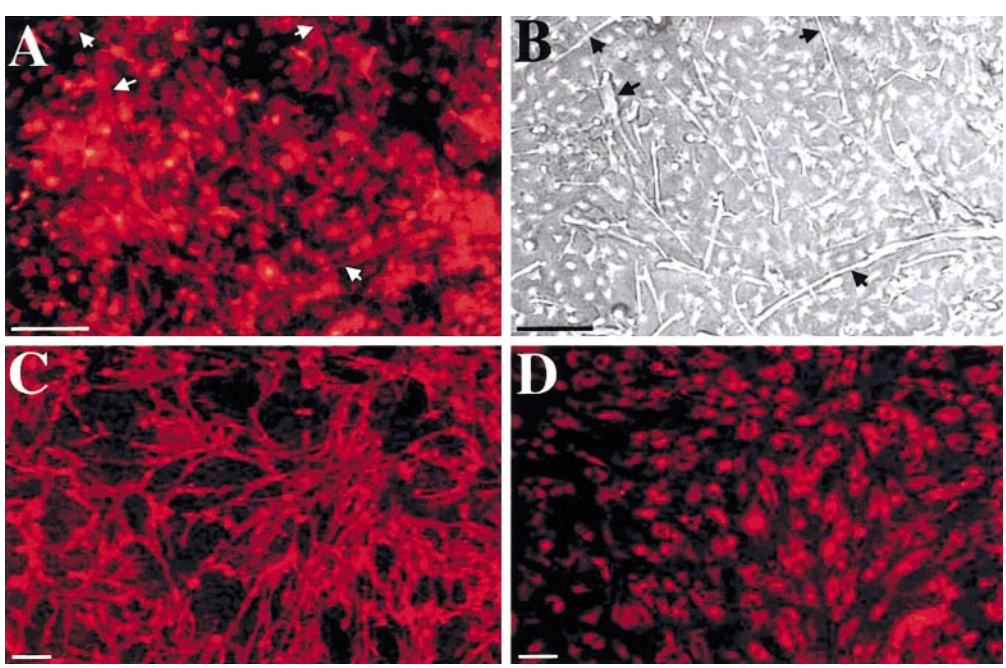

FIG. 4. Intracellular delivery of Tat-eGFP in cultured primary cells. Fluorescence micrographs of cells treated with 75 μg/ml rhodamine-labeled Tat-eGFP. After 4 h, cells were rinsed quickly in PBS and fixed for 10 min in 4% paraformaldehyde before they were mounted and examined. (A) Rhodamine fluorescence in differentiated myogenic cells isolated from newborn BALB/C mice and (B) corresponding phase contrast, (C) rhodamine-labeled proliferating DMD myoblasts isolated from a 15-year-old patient and (D) proliferating human skin fibroblasts. Arrowheads point toward myotubes. Bars, 100 μm.

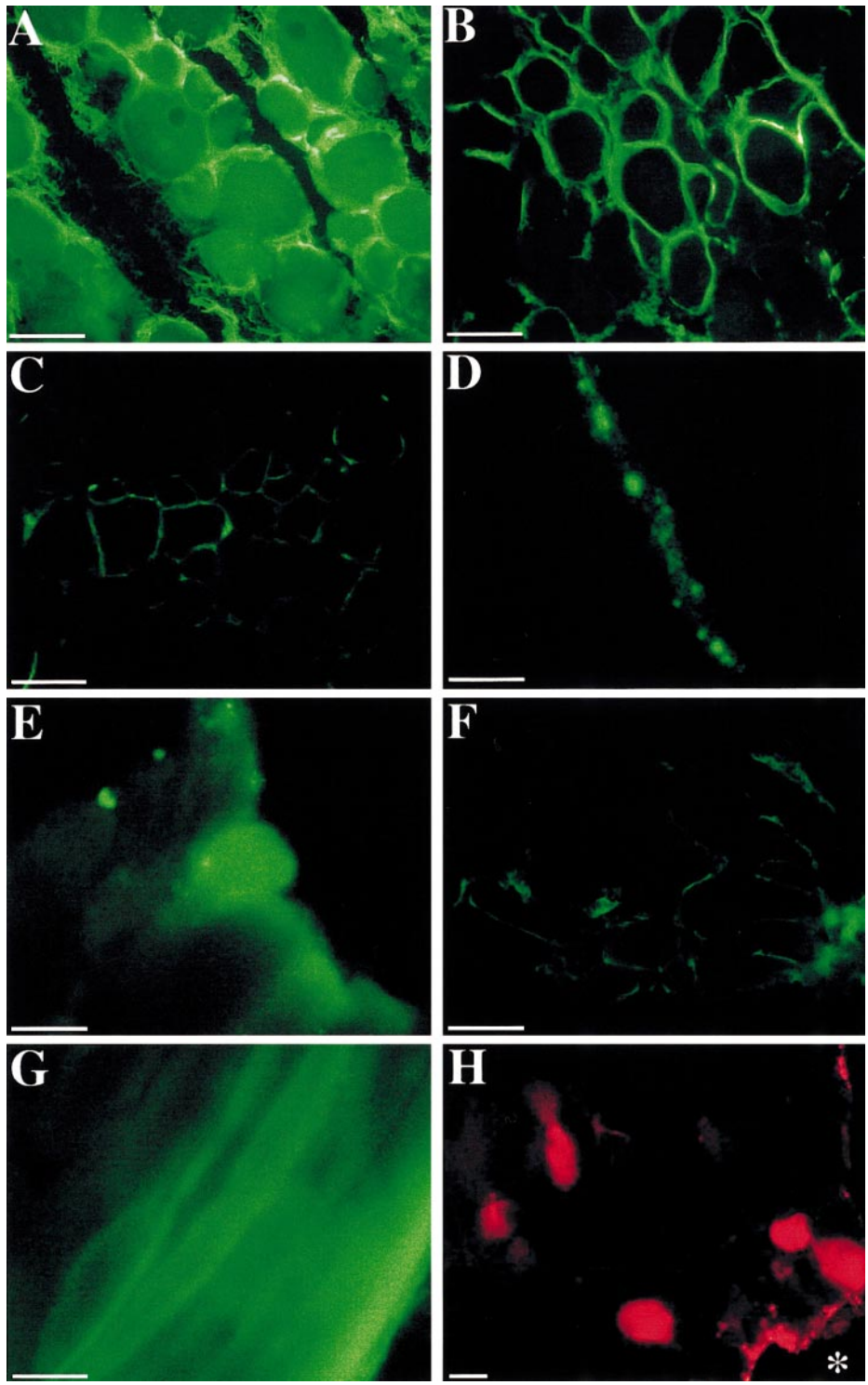

FIG. 5. Intracellular localization of Tat-eGFP in myofibers and retention in the extracellular matrix. Fluorescence micrograph sections of TA muscles. (A) The injection site of a TA muscle of a C57BL/10J mdx/mdx mouse injected with Tat-eGFP (25 μg) after 4 h. ECM surrounding myofibers near the Tat-eGFP (B) injection site or control eGFP (C) after 18 h. Fluorescence micrograph sections of whole leg muscles of BALB/C mice 18 h following the subdermal delivery of Tat-eGFP (D, E, and G) or control eGFP (F). (D) Mononuclear cells inside the conjunctive tissue surrounding a TA muscle with intact aponeuroses. (E and F) Myofibers from the periphery of a TA muscle (on the left) with injured aponeuroses. (G) Longitudinal myofibers from the soleus muscle. (H) TA muscle fibers 2 h following the intra-arterial delivery of rhodamine-labeled Tat-eGFP (50 μg). The star shows a blood vessel. Bars, 50 μm.

the group of Dowdy, stating that mammalian cells appear to be unable to refold GFP into a fluorescent conformation (11), might be explained by the fact that the eGFP variant used in our study is 35 times more fluorescent than its parental counterpart, wild-type GFP (26). To analyze the ability of the Tat-eGFP fusion protein to transduce into cells prior to its intracellular refolding, it was conjugated with rhodamine and added to C_2C_{12}, NiH/3T3, or human fibroblasts. Our experiments revealed that contrary to PTD-5 (RRQRRTSKLMKR) (3), the NLS (RKKRRQRRR), part of Tat PTD, was not sufficient to confer nuclear localization to eGFP following its intracellular transduction. The absence of a nuclear localization is not entirely surprising since previous work showed that the exogenous application of folded Tat protein did not result in nuclear localization (1, 27). In addition, the nuclear localization pathway taken by the Tat protein was shown to be sensitive to the folding state of Tat. Indeed, Bonifaci *et al.* showed that the pretreatment of cells with MTX, an analogue of methotrexate that stabilizes the three-dimensional structure of DHFR, blocked the nuclear localization of the Tat-DHFR fusion protein (28). This last case shares striking similarities with our denaturation-resistant Tat-eGFP fusion protein which is internalized more slowly than many other proteins. Recent reports also suggested that the Tat NLS might be part of a novel class of basic NLS that function independent of Importin α by binding directly to Importin β (25, 29). The nuclear import pathway used by the exogenously added Tat protein might be more sensitive to the folding state of the proteins to be imported than the classical Importin α-dependent pathway.

As discussed in the Introduction, previous attempts at the *in vivo* delivery of Tat fusion proteins into skeletal muscle cells gave poor staining results compared with other well-vascularized organs and mostly when compared with the intense staining observed throughout the heart muscle (9, 14). Our attempts to deliver Tat-eGFP to muscle fibers through intra-arterial and subdermal injections were successful, but gave rise to a limited number of fibers of low level fluorescence intensity located only in the periphery of blood vessels or at the surface of the muscle. The number of fluorescent muscle fibers was very small (less than 1%), indicating that the transduction of muscle fibers using intra-arterial injection of concentrated Tat fusion proteins is possible, but not very efficient, in the case of Tat-eGFP. Twenty-four hours after intramuscular injections in C57BL/10J mdx/mdx mice, most of the Tat-eGFP fluorescence was found associated with the ECM. In agreement with these results, myotubes obtained from primary mouse myoblast cultures, ensheathed by an ECM-rich basement membrane, failed to be transduced by Tat-eGFP as efficiently as some adjacent mononuclear cells not embedded beneath a basal lamina. Our data suggest that Tat PTD shows an affinity for components of the ECM. Previous studies demonstrated that Tat shared many characteristics with growth factors (10) and that it is, in fact, a heparin binding protein that interacts with heparan sulfate (HS), proteoglycans of the cell surface, and ECM (30). Heparin was found to bind specifically to recombinant HIV-1 GST-Tat fusion protein, and like HS, can inhibit HIV-LTR *trans*-activation induced by extracellular GST-Tat (31). Residues 49 to 57 of Tat, part of our fusion protein, are responsible for its affinity to heparin/HS (30). Interestingly, HS-proteoglycans are abundant in the muscle tissue. The basal lamina surrounding muscle fibers is composed of laminin, fibronectin, collagen, and HS-containing proteoglycan (32). Clonal mouse myoblasts were shown to synthesize and secrete collagen and other extracellular proteins and incorporate these macromolecules in an insoluble and organized matrix (33–35). Yet, we showed that C_2C_{12} cells rapidly internalized Tat-eGFP. We thus hypothesize that the association between Tat PTD and the ECM surrounding myofibers interferes with the intracellular transduction process in the case of Tat-eGFP or that this retention in the conjunctive tissue diminishes its local bioavailability to the cell membrane of myofibers.

In conclusion, we have focused our efforts on the use of Tat fusion proteins for protein replacement therapy in muscle cells and muscle tissue. If the poor efficiency of Tat-mediated transduction in muscle fibers is due to its retention to the ECM, it would be important to determine whether recently designed PTDs derived from known sequences such as polyarginine (4) or PTD-5 (3) also interact so strongly with components of the ECM. Although we could not deliver Tat fusion proteins in a high percentage of muscle fibers using noninvasive techniques, our experiments showed that Tat fusion proteins could be used to deliver proteins in human primary cultured DMD myoblasts. This could prove to be interesting since primary myoblasts demonstrate a poor efficiency of transfection and infection in culture, where the goal is to maximize delivery of therapeutic molecules. In addition, several *ex vivo* gene therapy protocols require genetic engineering to correct a particular defect, increase the life span of the targeted cells, or overexpress a therapeutically significant gene, thus necessitating the use of several different vectors at high dosage. Among the proteins that could be delivered to cells, there are proteins to transiently immortalize, increase the life span, or switch the differentiation program of cells. Such tools could significantly help cell-mediated gene therapies.

ACKNOWLEDGMENTS

We thank Dr. Steven F. Dowdy and his team for pTAT-HA, pTAT-E7, and their helpful comments. We also thank Dr. El Bachir Affar for helpful comments on cytofluorometry and Philippe Girard, Marlyne Goulet, and Brigitte Roy for their technical assistance. This work was supported in part by l'Association Française contre les Myopathies (AFM), the Muscular Dystrophy Association (MDA), and a graduate research scholarship from La Fondation de l'Université Laval (N.J.C.).

REFERENCES

[1] Elliott, G., and O'Hare, P. (1997). Intercellular trafficking and protein delivery by a herpesvirus structural protein. *Cell* **88:** 223–233.

[2] Schwarze, S. R., and Dowdy, S. F. (2000). In vivo protein transduction: Intracellular delivery of biologically active proteins, compounds and DNA. *Trends Pharmacol. Sci.* **21:** 45–48.

[3] Mi, Z., Mai, J., Lu, X., and Robbins, P. D. (2000). Characterization of a class of cationic peptides able to facilitate efficient protein transduction in vitro and in vivo. *Mol. Ther.* **2:** 339–347, doi:10.1006/mthe.2000.0137.

[4] Wender, P. A., Mitchell, D. J., Pattabiraman, K., Pelkey, E. T., Steinman, L., and Rothbard, J. B. (2000). The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: Peptoid molecular transporters. *Proc. Natl. Acad. Sci. USA* **97:** 13003–13008.

[5] Frankel, A. D., and Pabo, C. O. (1988). Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* **55:** 1189–1193.

[6] Green, M., and Loewenstein, P. M. (1988). Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. *Cell* **55:** 1179–1188.

[7] Mann, D. A., and Frankel, A. D. (1991). Endocytosis and targeting of exogenous HIV-1 Tat protein. *EMBO J.* **10:** 1733–1739.

[8] Anderson, D. C., Nichols, E., Manger, R., Woodle, D., Barry, M., and Fritzberg, A. R. (1993). Tumor cell retention of antibody Fab fragments is enhanced by an attached HIV TAT protein-derived peptide. *Biochem. Biophys. Res. Commun.* **194:** 876–884.

[9] Fawell, S., *et al.* (1994). Tat-mediated delivery of heterologous proteins into cells. *Proc. Natl. Acad. Sci. USA* **91:** 664–668.

[10] Jeang, K. T., Xiao, H., and Rich, E. A. (1999). Multifaceted activities of the HIV-1 transactivator of transcription, Tat. *J. Biol. Chem.* **274:** 28837–28840.

[11] Schwarze, S. R., Hruska, K. A., and Dowdy, S. F. (2000). Protein transduction: Unrestricted delivery into all cells? *Trends Cell Biol.* **10:** 290–295.

[12] Ezhevsky, S. A., Nagahara, H., Vocero-Akbani, A. M., Gius, D. R., Wei, M. C., and Dowdy, S. F. (1997). Hypo-phosphorylation of the retinoblastoma protein (pRb) by cyclin D:Cdk4/6 complexes results in active pRb. *Proc. Natl. Acad. Sci. USA* **94:** 10699–10704.

[13] Nagahara, H., *et al.* (1998). Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. *Nat. Med.* **4:** 1449–1452.

[14] Schwarze, S. R., Ho, A., Vocero-Akbani, A., and Dowdy, S. F. (1999). In vivo protein transduction: Delivery of a biologically active protein into the mouse. *Science* **285:** 1569–1572.

[15] Lissy, N. A., Van Dyk, L. F., Becker-Hapak, M., Vocero-Akbani, A., Mendler, J. H., and Dowdy, S. F. (1998). TCR antigen-induced cell death occurs from a late G1 phase cell cycle check point. *Immunity* **8:** 57–65.

[16] Kinoshita, I., Huard, J., and Tremblay, J. P. (1994). Utilization of myoblasts from transgenic mice to evaluate the efficacy of myoblast transplantation. *Muscle Nerve* **17:** 975–980.

[17] Tremblay, J. P., *et al.* (1993). Results of a triple blind clinical study of myoblast transplantations without immunosuppressive treatment in young boys with Duchenne muscular dystrophy. *Cell Transplant.* **2:** 99–112.

[18] Ham, R. G., St. Clair, J. A., Webster, C., and Blau, H. M. (1988). Improved media for normal human muscle satellite cells: Serum-free clonal growth and enhanced growth with low serum. *In Vitro Cell Dev. Biol.* **24:** 833–844.

[19] Huard, J., Roy, R., Guérette, B., Verreault, S., Tremblay, G., and Tremblay, J. P. (1994). Human myoblast transplantation in immunodeficient and immunosuppressed mice: Evidence of rejection. *Muscle Nerve* **17:** 224–234.

[20] Caron, N. J., Asselin, I., Morel, G., and Tremblay, J. P. (1999). Increased myogenic potential and fusion of matrilysin-expressing myoblasts transplanted in mice. *Cell Transplant.* **8:** 465–476.

[21] Torrente, Y., *et al.* (1999). Extracorporeal circulation as a new experimental pathway for myoblast implantation in mdx mice. *Cell Transplant.* **8:** 247–258.

[22] Dubowitz, V. (1985). *Muscle Biopsy: A Practical Approach,* 2nd ed. Baillière, Tindall, London.

[23] Yang, F., Moss, L. G., and Phillips, G. N., Jr. (1996). The molecular structure of green fluorescent protein. *Nat. Biotechnol.* **14:** 1246–1251.

[24] Vives, E., Brodin, P., and Lebleu, B. (1997). A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J. Biol. Chem.* **272:** 16010–16017.

[25] Truant, R., and Cullen, B. R. (1999). The arginine-rich domains present in human immunodeficiency virus type 1 Tat and Rev function as direct importin beta-dependent nuclear localization signals. *Mol. Cell. Biol.* **19:** 1210–1217.

[26] Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173:** 33–38.

[27] Ensoli, B., *et al.* (1993). Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. *J. Virol.* **67:** 277–287.

[28] Bonifaci, N., Sitia, R., and Rubartelli, A. (1995). Nuclear translocation of an exogenous fusion protein containing HIV Tat requires unfolding. *AIDS* **9:** 995–1000.

[29] Efthymiadis, A., Briggs, L. J., and Jans, D. A. (1998). The HIV-1 Tat nuclear localization sequence confers novel nuclear import properties. *J. Biol. Chem.* **273:** 1623–1628.

[30] Rusnati, M., *et al.* (1998). The basic domain in HIV-1 Tat protein as a target for polysulfonated heparin-mimicking extracellular Tat antagonists. *J. Biol. Chem.* **273:** 16027–16037.

[31] Rusnati, M., *et al.* (1997). Interaction of HIV-1 Tat protein with heparin. Role of the backbone structure, sulfation, and size. *J. Biol. Chem.* **272:** 11313–11320.

[32] Sanes, J. (1994). The extracellular matrix. In *Myology: Basic and Clinical* (A. Engel and C. Franzini-Armstrong, Eds.). McGraw–Hill, New York.

[33] Beach, R. L., Rao, J. S., and Festoff, B. W. (1985). Extracellular-matrix synthesis by skeletal muscle in culture. Major secreted collagenous proteins of clonal myoblasts. *Biochem. J.* **225:** 619–627.

[34] Beach, R. L., Burton, W. V., Hendricks, W. J., and Festoff, B. W. (1982). Extracellular matrix synthesis by skeletal muscle in culture. Proteins and effect of enzyme degradation. *J. Biol. Chem.* **257:** 11437–11442.

[35] Rao, J. S., Beach, R. L., and Festoff, B. W. (1985). Extracellular matrix (ECM) synthesis in muscle cell cultures: Quantitative and qualitative studies during myogenesis. *Biochem. Biophys. Res. Commun.* **130:** 440–446.