Published in final edited form as:

Biochemistry. 2010 November 9; 49(44): 9470–9479. doi:10.1021/bi101057v.

TAT Fusion Protein Transduction into Isolated Mitochondria Is Accelerated by Sodium Channel Inhibitors†

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Abstract

Stringent control of ion and protein transport across the mitochondrial membranes is required to maintain mitochondrial function and biogenesis. In particular, the inner mitochondrial membrane is generally impermeable to proteins entering the matrix except via tightly regulated protein import mechanisms. Recently, cell penetrant peptides have been shown to move across the inner mitochondrial membrane in a manner suggesting an independent mechanism. HIV-1 transactivator of transcription (TAT) is an arginine-rich cell penetrant peptide, ⁴⁷YGRKKRRQRRR⁵⁷, which can transduce full-length proteins not only across the cell membrane but also into intracellular organelles. In this study, we investigated the ability of a TAT-containing protein to move into the mitochondrial matrix. Using a novel FACS assay for isolated, purified mitochondria, we show that TAT can deliver a modified fluorescent protein, mMDH-GFP, to the matrix of mitochondria and it is subsequently processed by the matrix peptidases. In addition, transduction of TAT-mMDH-GFP into mitochondria is independent of canonical protein import pathways as well as mitochondrial membrane potential. In direct contrast to published reports regarding the cell membrane where the sodium channel inhibitor, amiloride, blocks endocytosis and inhibits TAT transduction, TAT transduction into mitochondria is markedly increased by this same sodium channel inhibitor. These results confirm that the cell penetrant peptide, TAT, can readily transduce a protein cargo into the mitochondrial matrix. These results also demonstrate a novel role for mitochondrial sodium channels in mediating TAT transduction into mitochondria that is independent of endocytotic mechanisms. The mechanism of TAT transduction into mitochondria therefore is distinctly different from transduction across the cell membrane.

Over the past 10 years, multiple studies have shown that protein transduction domains (PTDs), or cell penetrating peptides, are capable of delivering large molecules such as oligonucleotides, peptides, full-length proteins (1), nanoparticles (2), bacteriophages (3), and liposomes (4) across cellular membranes. The transactivator of transcription (TAT)¹ peptide derived from HIV contains a positively charged arginine-rich peptide (residues 47–57), YGRKKRRQRRR (5), that is among the most commonly used and widely studied PTD. Previous reports have examined the mechanism of TAT-mediated protein transduction across the plasma membrane and have strongly implicated endocytosis as the mechanism for TAT transduction. In support of this, drugs known to block endocytosis inhibit TAT

[†]Support for this project was provided in part by the Friedreich's Ataxia Research Alliance and the National Institutes of Health, 1P01HL 085098 and R21 NS052198.

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¹Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; eGFP, enhanced green fluorescent protein; EIPA, ethylisopropylamiloride; ENaC, epithelial sodium channel; FACS, fluorescent-activated cell sorting; GFP, green fluorescent protein; M β CD, methyl- β -cyclodextrin; MGA, malate/glutamate/ADP; mMDH, mitochondrial malate dehydrogenase; NCX, Na⁺/Ca²⁺ exchanger; NHE, Na⁺/H⁺ exchanger; PBS, phosphate-buffered saline; ROT, rotenone; TAT, transactivator of transcription; TMG, TAT-mMDH-GFP fusion protein.

transduction into cells (6,7). These have also demonstrated that TAT fusion proteins transduce via lipid raft macropinocytosis in T cells (8,9), whereas in CHO cells and HeLa cells, clathrin-dependent endocytosis is the mode of transduction (10,11). In Cos-1 and NIH 3T3 cells, it was shown that caveolar-mediated endocytosis is an important pathway for TAT transduction (12,13). Duchardt et al. (14) showed that TAT-mediated protein transduction in HeLa cells is a concentration-dependent process. At lower concentrations, transduction occurs by caveolae/lipid-raft-mediated endocytosis and macropinocytosis, whereas at higher concentrations it is a clathrin-mediated endocytosis.

Previously, we had shown that TAT-mediated protein transduction causes membrane rearrangement as evidenced by a phosphatidyl serine flip within the plasma membrane. Transduction by TAT and the phosphatidylserine flip were blocked by positively charged polylysine (15), indicating that a negative charge on the cell membrane was essential to mediate transduction. It was also shown that the presence of heparin sulfate proteoglycans on the plasmamembrane facilitated the uptake of TAT mediated cargo into the cells (9,16,17). From these data, the hypothesis has been advanced that interaction of the arginine-rich peptides with the membrane-associated proteoglycans rapidly activates intracellular signals inducing actin organization and macropinocytosis (9,18). This dependence on proteoglycans in the plasma membrane and the multiple modes of endocytosis in cells collectively suggests that TAT-mediated protein transduction across the plasma membrane is an energy-dependent process (17,18). Finally, although endocytosis has become widely accepted as a primary mechanism for cellular uptake of arginine-rich peptides, there is a substantial evidence for alternative mechanisms that enable translocation through the membranes in an energy- and endocytic-independent manner (18–20).

On the basis of these earlier studies, we (21) and others (22) have shown that TAT will also transduce into mitochondria, which are not known to employ any endocytotic mechanisms. Studies supporting this have shown that a TAT fusion protein with an internal mitochondrial targeting sequence from malate dehydrogenase, TAT-mMDH-GFP (TMG), would localize to mitochondria, be processed by the mitochondrial processing peptidase, and remain in mitochondria *in vivo* and *in vitro* (21,23). Furthermore, we have shown that TAT transduction into mitochondria is a receptor- and energy-independent process (21) for cells in culture. However, neither endocytosis nor lipid rafts (24) have been demonstrated for mitochondria leaving the process of TAT transduction into mitochondria in doubt. Whereas the mitochondrial outer membrane is similar to the plasma membrane in terms of protein to lipid constitution (1:1), there are no proteoglycans present on the surface of mitochondria although the phospholipid, cardiolipin, imparts a net negative charge to the membrane. The inner mitochondrial membrane displays a higher protein to lipid ratio (3:1) compared to the plasma and outer mitochondria membranes (25). Based on the current understanding of cell penetrant peptide transport across membranes, there have been challenges to the notion that cell penetrant peptide transduction into the matrix of mitochondria occurs (26,27). Thus, in order to better understand the mechanisms that allow for TAT-mediated protein transduction into mitochondria, we tested the hypothesis that TAT transduction into mitochondria could be blocked using endocytosis inhibitors. In distinct contrast to TAT transduction across the cell membrane, we found that TAT transduction into mitochondria was not inhibited by compounds known to block endocytosis, thus confirming TAT-mediated transduction and suggesting an alternative endocytosis-independent mechanism. Surprisingly, compounds known to inhibit endocytosis via blocking the function of sodium channels, such as amiloride, markedly increased TAT transduction into mitochondria. We also confirmed that TAT transduction into the mitochondrial matrix occurs via an energy-independent pathway. Together, these data reveal novel mechanisms employed by cell penetrating peptides that circumvent the differences in membrane composition to deliver cargo intracellularly as well as intercellularly.

MATERIALS AND METHODS

Reagents

Metrizamide and all other endocytosis inhibitors (dansylcadaverine, nystatin, methyl- β -cyclodextrin, amiloride, cytochalasin D, cytochalasin B, colchicin, benzamil, dimethylamiloride, ethylisopropylamiloride (5-(*N*-ethyl-*N*-isopropyl)-amiloride), and dichlorobenzamil) were obtained from Sigma Chemicals. All of the endocytosis inhibitors were dissolved in DMSO. Anti-GFP HRP conjugate (120-002-105) was obtained from Miltenyi biotec, and anti-complex II subunit primary (MS-203) was obtained from MitoSciences. Malate, glutamate, succinate, ADP, ATP, rotenone, and CCCP were from Sigma Chemicals. Rhodamine-123, luciferin, and luciferase were obtained from Roche. The SuperSignal West Pico kit for Western blotting was purchased from Thermo Scientific.

Purification of TMG

TMG was expressed and purified as previously described (23) with modifications. Briefly, TMG was expressed in BL21 DE3 pLys S cells. A 2 L broth of LB media with ampicillin (100 μ g/mL) and chloramphenicol (40 μ g/mL) was seeded and incubated overnight at 16 °C with 250 μ M IPTG. After sedimentation, the cell paste was resuspended in buffer A (PBS, pH 7.5, with an additional 500 mM NaCl), 10 mM imidazole, and protease inhibitor cocktail (Sigma P8849) and sonicated for 15 min on ice in a rosette cooling cell. The lysate was centrifuged at 23500g for 20 min at 4 °C, and the supernatant was subjected to affinity purification by passing over a 5 mL His Trap HP column (GE Healthcare) on an AKTA Basic UPC100 FPLC chromatography system (Amersham Biosciences Corp.). After the column was washed with 10% buffer B (PBS, pH 7.5, with an additional 500 mM NaCl, 500 mM imidazole) until the flow-through returned to baseline, the TMG protein was eluted as a single step with 250 mM imidazole. The fractions were pooled and concentrated in an Amicon Ultra-15 centrifugal filter unit with a 10000 MW cutoff to a volume of 2.5 mL. This was desalted on a PD-10 column into PBS (pH 7.5) with 7% glycerol. The eluted protein purity was checked on a 15% SDS-PAGE with Coomassie R-250 staining, concentrated to 1 mg/mL, and stored at -80 °C in aliquots until further use.

Preparation of Mitochondria

Mitochondria were freshly isolated from mouse liver on the day of the experiment using previously published methods (21). Briefly, livers were homogenized and subjected to three rounds of differential centrifugation to recover isolated mitochondria in the pellet of the final spin. Mitochondrial protein concentration was determined by bicinchoninic acid (BCA) assay (Pierce Chemical Co., Rockford, IL) and brought to a final concentration of 10 mg/mL with mitochondrial isolation buffer (MIB, 220 mM mannitol, 70 mM sucrose, 5 mM Tris, pH 7.5). Mitochondria were further purified to reduce ER contamination using metrizamide-Percoll density gradient centrifugation as described previously (28). Final preparations were brought to a protein concentration between 5 or 15 mg/mL in MIB and used immediately for the subsequent experiments.

Rhodamine-123 Assay for Analysis of $\Delta\Psi_m$

Mitochondria were washed with isolation buffer containing no BSA and then assayed for protein concentration using the BCA assay. During the protein assay incubation, mitochondria were sedimented (2 min at 10000g) at 4 °C in isolation buffer to avoid exposure to air and were tested for $\Delta\Psi_m$. Mitochondria (60 μ g) were resuspended in 200 μ L isolation buffer containing 0.1% BSA and added to a 96-well clear-bottom plate. Rhodamine-123 (Rh123, 300 nM) was added to each sample just before reading. Samples were read in a SpectraMax 340 PC spectrofluorometer at 25 °C. Readings were taken every

45 s at 485 ± 20 nm excitation and 528 ± 20 nm emission wavelengths. Baseline readings were measured for 5 min before the addition of any substrates or treatments. Malate (7 mM), glutamate (7 mM), and 100 μ M ADP were added, and fluorescence was read for 10 min. The mitochondrial complex I inhibitor rotenone (5 μ M) was added, and samples were monitored for 10 min followed by the addition of succinate (7 mM) and ADP (100 μ M) with another 10 min of fluorescence readings. Finally, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (50 μ M) was added, and fluorescence was monitored for the last 10 min. Water or DMSO was used as vehicle control ($n=3$).

ATP Production Assay

Mitochondria were assessed for ATP production. After determining protein concentration, 250 μ g aliquots of mitochondria were sedimented as above and placed on ice until ready for use. For the ATP production assay, mitochondria were resuspended in 185 μ L of respiration buffer (20 mM HEPES–KOH, pH 7.4, 80 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose, 1 mM glutamate, and 1 mM malate). Buffer B (0.5 M Tris–acetate, pH 7.75, 0.8 mM D-luciferin, 20 μ g/mL luciferase), 10 μ L, was immediately added to the reaction, and sample luminescence was read in standard mode on a L max luminometer (Molecular Devices) for 4 s with no delay. ADP (0.1 mM) was then added to the sample, and the luminescence was read every 4 s for a total of 60 s. Buffer background measurements were taken using buffers containing all substrates, and these measurements were subtracted from the mitochondrial readings. ATP standards were read using the same conditions and used to extrapolate ATP generation rates for each sample ($n=3$).

Endocytosis Inhibitor Experiments

Isolated mitochondria (50 μ g) were pretreated with or without specified endocytosis inhibitors for 5 min at either 4 or 22 °C, followed by incubation with TMG for 15 min at either 4 or 22 °C. Where indicated, samples were incubated with either proteinase K (50 μ g/mL) or without proteinase K, at 4 °C for 10 min. The reactions were immediately separated by centrifugation at 10000g at 4 °C for 10 min. The mitochondrial pellets were then subjected to either SDS–PAGE and Western blotting or flow cytometry to quantify the transduced TMG.

Western Blot Analysis

All samples were separated by 15% PAGE and transferred to PVDF membranes (Immobilon-P). Membranes were probed with anti-GFPHRP conjugate antibody (1:5000 dilution) or anti-complex II subunit primary antibody (1:5000 dilution) and goat anti-mouse HRP secondary antibody (1:5000 dilution). Membranes were developed with SuperSignal West Pico luminol/enhancer and stable peroxide solution.

Flow Cytometry Analysis of Mitochondria

Mitochondria from these experiments were suspended in 100 μ L of PBS, then added to 400 μ L of Isoton II diluent (Beckman Coulter isotonic saline solution), and subjected to flow cytometry on a BD FACS-Calibur flow cytometer (BD Biosciences) using voltage channel E02. The GFP positive mitochondria were measured on the FL1 channel by comparing with respective controls. Data were collected for 10000 counts.

Membrane Permeability Transition Experiments

Pore opening was measured by swelling and shrinking of mitochondria (29), where the light scattering of 250 μ g of mitochondria in a 1 mL volume was measured at 540 nm through a 1 cm path. All swelling assays were performed in 120 mM KCl, 10 mM Tris, pH 7.6, and 5

mM KH_2PO_4 , and the absorbance change was measured per minute for 10 min in the presence of amiloride and/or 1 mM CaCl_2 .

Statistical Analysis

Results are expressed as the mean $\pm$ SE. Comparisons among groups were made by Student's *t* test. *AP* value of 0.05 or less was considered as statistically significant.

RESULTS

TMG Protein Transduction into Mitochondria Is Increased by the Endocytosis Inhibitor, Amiloride

In order to test the hypothesis that TAT transduction into mitochondria is mediated by endocytosis, freshly isolated mitochondria from mouse liver were incubated with TMG. These mitochondria had been pretreated with a panel of known inhibitors of endocytosis and macropinocytosis prior to incubation with TMG. Mitochondria were treated with 0.5 mM dansylcadaverine (30), 5 $\mu\text{g/mL}$ nystatin (30), 10 mM methyl- β -cyclodextrin ($\text{M}\beta\text{CD}$) (30), 5 mM amiloride (1), 20 μM cytochalasin D (31), 100 μM cytochalasin B (31), and 5 mM colchicin (31). Following this, proteinase K was added to the reaction to digest any TMG remaining in either the supernatant or adherent to the outer mitochondrial membrane, and the reaction contents were separated by centrifugation at 4 $^\circ\text{C}$. The pellet and supernatant were then separated by SDS-PAGE and transferred to membranes for Western blotting.

Shown in Figure 1 are representative Western blots from transduction of TMG into isolated mitochondria ($n>5$ repetitions for each experiment). In Figure 1A, Western blotting for GFP shows that none of the traditional endocytosis inhibitors could prevent protein transduction in mitochondria as they do for TAT transduction across the cell membranes (6,8). TMG has a predicted M_r of 34.8 kDa, and a band is seen at ~ 35 kDa corresponding to full-length TMG in lane 1 (Figure 1A, top panel). A second immunodetected band for GFP migrates faster at approximately 27kDa and is at the predicted M_r of processed GFP after removal of the TAT-mMDH moiety by the mitochondrial processing peptidase. Both bands are protected from proteinase K digestion, indicating they have translocated into the mitochondrial matrix. The supernatants containing the cytosolic and nuclear cell proteins from the mitochondrial preparations did not show any signal for GFP, indicating that all extra mitochondrial TMG had been successfully digested prior to mitochondria protein analysis (Figure 1A, bottom panel). Among the inhibitors employed, only $\text{M}\beta\text{CD}$, a clathrin- and caveolin-mediated endocytosis inhibitor, had any effect on inhibiting transduction into mitochondria, and this effect was mild as compared to the control lane of TMG alone (Figure 1A, upper panel). On the other hand, neither dansylcadaverin, a clathrin-mediated endocytosis inhibitor, nor nystatin, a caveolin-mediated endocytosis inhibitor, inhibited mitochondrial transduction of TMG.

The immunoblot signals were quantified, and a representative bar graph for the Western blot is shown in Figure 1B. Strikingly, amiloride, a macropinocytosis inhibitor, demonstrated a clear increase of mitochondrial TMG transduction. GFP immunodetection of both precursor and processed TMG was significantly increased, and both were protected from proteinase K digestion. This result of increased transduction across mitochondrial membranes is in contrast to reports (8) that TAT-mediated protein transduction in mouse reporter T cells is mediated by lipid raft macropinocytosis and is inhibited by amiloride at the plasma membrane. In contrast, $\text{M}\beta\text{CD}$ caused a modest decrease in TMG transduction when compared to TMG transduction alone. The remaining agents had no significant effect on transduction of TMG into mitochondria. As a control, transduction experiments done in the

absence or presence of DMSO did not show any difference in TMG transduction into mitochondria.

In a similar set of experiments without using proteinase K after incubation with TMG (Figure 1C, upper panel), no significant difference from the set with proteinase K treatment was observed. A representative bar graph for these Western blot data is shown in Figure 1D. Again, amiloride increased TMG transduction and $M\beta CD$ slightly reduced transduction as compared to no inhibitor treatment. As expected, the supernatant panel (Figure 1C, lower panel) showed non transduced TMG. Interestingly, there was essentially no TMG present in the supernatant of amiloride-treated mitochondria, indicating that almost all of the TMG had been transduced into the mitochondria. The absence of TMG processing in the supernatant also indicates that the mitochondria were intact and processing of TMG in panel 1A was not the result of the mitochondrial processing peptidase leaking from damaged mitochondria. Taken together, these findings indicate that endocytosis is not an operative mechanism for protein transduction into the mitochondrial compartments.

Flow Cytometry Analysis of Mitochondria Indicates That Amiloride Increases the Transduction of TMG

In order to more precisely quantify transduction of fluorescent proteins into mitochondria, we developed a flow cytometry-based technique to measure transduction of TMG into isolated mitochondria. This was validated using Western blotting, i.e., performing simultaneous transduction experiments in mitochondria with the same spectrum of endocytosis inhibitors as in Figure 1 and comparing Western blotting with flow cytometry. In Figure 2A, TMG transduction was quantified by dot plot analysis. Figure 2A is a representative graph of five separate repetitions of this experiment. Gating was set to contain all of the control mitochondria alone (panel Data.011 of Figure 2A). The addition of TMG alone (panel Data.012) shifted signal intensity substantially and is comparable to lane 3 of Figure 1A and the TMG bar of Figure 1B. Addition of $M\beta CD$ decreased transduction as shown by the third panel in 2A (panel Data.013), and is comparable to lane 6 of Figure 1A and the $M\beta CD$ bar of Figure 1B. In contrast, addition of amiloride increased transduction by nearly 100% (panel Data.014 of Figure 2A) over TMG alone and is comparable to lane 7 of Figure 1A and the amiloride bar of Figure 1B. Quantifying the results of Figure 2A as a bar graph in Figure 2B shows that amiloride significantly increases transduction of TMG into mitochondria to ~180% of control (TMG alone). Addition of $M\beta CD$ decreases transduction of TMG by ~20%, which is significant but substantially less change than the positive effect of amiloride. None of the other endocytosis inhibitors that are specific for phagocytosis, pinocytosis, clathrin-mediated endocytosis, or caveolin-mediated endocytosis significantly altered the transduction of TMG into mitochondria (Figure 2B).

TMG Transduction Increases in a Dose-Dependent Manner

At a concentration of 2 μg of TMG per reaction, transduction of ~75% of the mitochondria was observed (Figure 2A, panel Data.012). In order to quantify the effect of amiloride-mediated increase in protein transduction, a lower concentration of TMG needed to be identified. Mitochondria were incubated with increasing amounts of TMG at 0.2, 0.5, 1, and 2 μg of TMG per reaction, and 9%, 14%, 28%, and 73%, respectively, of mitochondria undergoing protein transduction was observed (Figure 3A). Based on these data, a concentration of 0.5 μg of TMG per 50 μg of mitochondria was used for the amiloride dose escalation experiments because this low dose of TMG allowed clear interpretation of the effect of amiloride on transduction.

Amiloride Increases TMG Transduction into Mitochondria in a Dose-Dependent Manner

To determine if the response to amiloride was dose dependent, mitochondria were pretreated with increasing amounts of amiloride from 0.5 to 5 mM before incubating them with TMG. FACS analysis showed that amiloride did not significantly increase transduction at a lower concentration of 0.5 mM (Figure 3B, panel Data.013). However, higher doses of 1 mM increased transduction significantly, and transduction levels were 5–6-fold higher at 5 mM (Figure 3B, panels Data.014 and Data.015, respectively) when compared with TMG alone or TMG+ 0.5 mM amiloride. These data suggest that a minimum concentration of 1 mM amiloride is required to stimulate TMG transduction into isolated mitochondria under these conditions. Among other possibilities, these data strongly support the hypothesis that transduction of TMG into mitochondria is sensitive to inhibition of the sodium channel(s) in the mitochondrial membrane(s).

To exclude the possibility that amiloride increased the membrane permeability transition pore formation allowing movement of TMG into the matrix, we determined if amiloride altered the membrane permeability transition in mitochondria. CaCl_2 at 1 mM was used as a positive control and clearly increased the membrane permeability transition at 22 °C (Figure 3C). In contrast, no mitochondrial swelling was observed in mitochondria treated with amiloride alone. Amiloride plus CaCl_2 was not significantly different from CaCl_2 alone. This result demonstrates that amiloride does not cause membrane permeability transition in mitochondria as a mechanism for transduction of TMG into the mitochondrial matrix.

Amiloride Analogues, Dichlorobenzamil and Ethylisopropylamiloride, Also Increase TMG Transduction into Mitochondria

Amiloride not only inhibits macropinocytosis at the cell surface, but is also a sodium channel blocker and inhibits three types of sodium channels: the epithelial sodium channel (ENaC), the Na^+/H^+ exchanger (NHE), and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) (32). All three NCX sodium channels (NCX1, NCX2, NCX3) (33,34) and an NHE isoform (35,36) have been localized in the mitochondrial inner membrane although the NCX has not been molecularly identified. In order to explore the amiloride-induced increase in TAT-mediated transduction into mitochondria, amiloride analogues, which are specific for one or two types of sodium channels (32), were used in mitochondrial protein transduction experiments.

Dichlorobenzamil, which is a NCX specific blocker (32), significantly increased TMG transduction into mitochondria as measured by both flow cytometry dot plot analysis (Figure 4A) and Western blot results with anti-GFP antibody (Figure 4B). This effect was significantly greater than amiloride. Compared with TMG alone, dichlorobenzamil pretreatment increased TMG transduction ~250%, and this effect was found at a 10-fold lower (500 μM) concentration than amiloride (5 mM). Ethylisopropylamiloride (EIPA, or 5-(*N*-ethyl-*N*-isopropyl)amiloride), which is a NHE1 blocker (32), also increased transduction but not to the same degree as dichlorobenzamil (Figure 4A,B). Similar to EIPA, benzamil (an ENaC and NHE1 blocker) significantly increased transduction at 500 μM but not at lower doses (Figure 4C,D), and dimethylamiloride (NHE1 and NCX blocker) did not increase TMG transduction significantly (Figure 4C, D). These data suggest that the NCX is involved in TAT-mediated transduction into mitochondria.

TMG Transduction in Mitochondria Is an Energy-Independent Process

In order to determine if TAT-mediated transduction in mitochondria is an energy-dependent process, two sets of experiments were performed. In the first one, transduction studies were done at either 22 or 4 °C, and TMG transduction was examined by Western blot analysis. Mitochondrial respiration is markedly decreased at 4 °C (37). As before, proteinase K was used to ensure that the TMG signal represented internalized protein and not signal from

adherent (on outer membrane) protein. These experiments were analyzed by Western blotting and show that TMG transduced efficiently into mitochondria in a dose-dependent manner at both temperatures, indicating there is no dependence on temperature (Figure 5A). At low temperatures (4 °C) the mitochondrial processing peptidase is less active, and thus, there is less processing of the full-length TMG to the smaller GFP when compared to 22 °C.

To examine energy dependence of TMG transduction into mitochondria, mitochondria were pretreated with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). CCCP is a proton ionophore that depolarizes mitochondria and rapidly depletes ATP (38). This is particularly important in mitochondria because canonical protein import into mitochondria via the import apparatus is energy dependent (39–41). Mitochondria were pretreated with 50 μ M CCCP or buffer (control) and incubated with TMG at 22 °C. Either amiloride or dichlorobenzamil was added in separate experiments, and transduction was measured by flow cytometry. The results clearly show that CCCP did not inhibit TAT-mediated transduction into mitochondria with TMG alone or with amiloride or dichlorobenzamil (Figure 5B). These two experiments show that TMG transduction into mitochondria is not an energy-dependent process and is not inhibited by low temperature. They also demonstrate that transduction of TAT-mMDH-GFP into mitochondria does not depend on the mitochondrial import machinery, which is an energy-dependent process specifically recognizing the mitochondrial targeting sequence (mMDH in this case) (39).

In order to assess the integrity and function of the mitochondria used in these experiments, and the effect of CCCP on mitochondria, the mitochondrial membrane potential ($\Delta\Psi_m$) was measured using a Rhodamine-123 assay (42,43). In the presence of ADP, malate, and glutamate (MGA) $\Delta\Psi_m$ is increased, and fluorescence decreases in this assay (Figure 5C, MGA lane). With the addition of rotenone, complex I is inhibited, and the $\Delta\Psi_m$ is decreased. Thus, fluorescence increases (Figure 5C, ROT lane). As predicted, addition of succinate and ADP increases $\Delta\Psi_m$ again as evidenced by a drop in fluorescence (lane S/A). Finally, when the mitochondria are exposed to CCCP, the membrane is completely depolarized, and fluorescence peaks (lane CCCP). In a separate set of experiments, ATP production in mitochondria was measured in the absence and presence of CCCP using malate and glutamate as substrates (Figure 5D). As predicted, CCCP treatment effectively ablated ATP production when compared to untreated mitochondria, rendering them incapable of generating significant ATP. However, ATP depletion did not inhibit TMG transduction into mitochondria in the presence of either amiloride or benzamil, indicating that the transduction process does not require energy (Figure 5B). These two experiments show that the mitochondria used in these experiments were well coupled and functionally intact.

DISCUSSION

Multiple previous reports (8,10,13) have suggested that TAT-mediated protein transduction across the plasma membrane is mainly regulated by one or more of the energy-dependent endocytosis processes: macropinocytosis or caveolin- and/or clathrin-mediated endocytosis. Here, we show that TAT-based protein transduction across mouse liver mitochondrial membranes is unique from TAT-induced transduction across the plasma membrane and that TAT (and attached cargo) indeed reaches the mitochondrial matrix.

There are three key findings from these experiments. First, TAT transduction into mitochondria is confirmed and is independent of energy status of the mitochondria. TMG is transduced into mitochondria at both room temperature and 4 °C, which inhibits receptor-mediated import of mitochondrial proteins. In addition, depolarization and ATP depletion of mitochondria did not inhibit TMG transduction into mitochondria. This is important because movement of the presequence of properly targeted mitochondrial matrix proteins across the

membranes is driven by $\Delta\Psi_m$ (44). This confirms that TAT transduction into mitochondria is an energy- and receptor-independent process. This result is consistent with our previous report (21) showing that TMG transduction into mitochondria is a receptor-independent process and, thus, is not imported into mitochondria via the TOM/TIM pathway (45). It is also consistent with our earlier report that transduction of TAT proteins into mitochondria is independent of energy status of the mitochondria (21) and does not depend on the presence of a mitochondrial targeting sequence. Nakase et al. (46) have shown that arginine-rich peptides can translocate through the plasma membrane into cells at 4 °C (18), suggesting that energy-independent TAT-mediated protein transduction is also possible across the plasma membrane. The findings presented here differ significantly from the energy requirements for TAT-based protein transduction across the plasmamembrane and strongly suggest a novel mechanism of TAT-mediated protein transduction into mitochondria, which is an energy-independent process.

Second, TAT transduction into mitochondria is not significantly mediated by endocytosis. Except for an observed 20% reduction in transduction by M β CD treatment, none of the previously described endocytosis or macropinocytosis inhibitors (amiloride, wortmannin, and cytochalasin D), clathrin-mediated endocytosis inhibitors (dansylcadaverine), and caveolin-mediated endocytosis inhibitors (nystatin) significantly inhibited TMG transduction into mitochondria. These findings show that TAT-mediated protein transduction into mitochondria occurs through a different mechanism than the plasma membrane. However, as with the plasma membrane, the precise mechanism of TAT transduction into mitochondria cannot be clearly identified.

Third, sodium channels clearly play a major role in mediating TAT protein transduction in mitochondria as evidenced by increased transduction with the different sodium channel inhibitors amiloride and its analogues dichlorobenzamil and EIPA. Dichlorobenzamil is specific for the NCX channel (47) and, like amiloride, was most effective at a higher dose (500 μ M). Both benzamil and amiloride inhibit the ENaC channel at low doses (48), which had no effect on transduction in these experiments. At intermediate doses (5.3–50 μ M) amiloride inhibits the NHE1 channel (49), which had little effect on transduction, whereas at high doses (1 mM) it effectively inhibits the NCX channel (48). EIPA is also specific for the NHE1 channel and had a moderate effect in these experiments. Thus, it is most likely that an NCX channel in the mitochondrial membrane(s) is responsible for accelerated transduction of TMG seen in these experiments, although the contribution of the NHE1 cannot be ruled out given the results with EIPA at higher doses. In contrast, those agents that inhibit clathrin- and caveolin-mediated endocytosis at the plasma membrane had no effect on TAT transduction into mitochondria. These findings underscore a significant difference between TAT fusion protein transduction across the plasma and mitochondrial membranes: specific inhibitors of endocytosis at the plasma membrane inhibit TAT transduction into the cell, whereas the same inhibitors markedly accelerate TAT transduction into the mitochondrial matrix. The exact mechanism underlying this difference is unknown, in part because the molecular identity of the mitochondrial sodium channels, such as NCX_m, remains unknown (33,34).

Amiloride is a guanidinium compound containing a pyrazine derivative with effects on multiple sodium channels. Amiloride and its analogues also inhibit other sodium channels, such as the Na⁺/H⁺ exchanger (NHE) and the NCX (32). The mitochondrial inner membrane has been reported to contain both NHE and NCX channels (33,35). Thus, the increase in TMG transduction into mitochondria by amiloride and its analogues raises the interesting possibility of exploiting these pathways to maximize TAT-mediated protein and cargo delivery to test novel hypotheses of mitochondrial function *in vivo* that are difficult to test without a transgenic approach. Increasing transduction of cell penetrant peptides as a class

may also provide a novel therapeutic approach for mitochondrial diseases that does not exist today.

Analysis of isolated mitochondria by flow cytometry is not entirely a new technique. For example, it has been used on mitochondria isolated from mouse liver to measure membrane potential using the fluorescent dye, Rhodamine-123. The ability of mitochondria to take up this dye depends on the membrane potential ($\Delta\Psi_m$) and is the basis for its use in Figure 5C (50). Subsequently, a significant number of groups have used this technique with Rhodamine-123, but also with other dyes, such as safranin and dihydrorhodamine 123, as the fluorescent marker (51). Here, we have used a fluorescent protein marker, GFP within the TMG, as the reporting marker to quantify TAT-mediated transduction in mitochondria. To our knowledge, using FACS analysis to measure uptake of a fluorescent protein in isolated mitochondria has not been reported. Comparing data obtained by FACS analysis of GFP uptake into isolated mitochondria (Figure 2B) with the signal intensity obtained from Western blotting using antibody directed against GFP (Figure 1A and B) clearly shows that FACS accurately reflects the uptake of a fluorescent protein into mitochondria. Thus, FACS is not only sensitive when compared to Western blotting for measuring fluorescent protein transduction in mitochondria but also faster in terms of obtaining quantitative results when compared with analysis by Western blotting.

In summary, these findings confirm that TAT can transduce into the matrix of mitochondria. Those specific agents reported to block endocytosis and TAT transduction across the plasma membrane paradoxically increase TAT-mediated protein transduction into mitochondria. This strongly suggests that the transduction mechanism for TAT peptides into mitochondria is novel and differs significantly from the mechanisms proposed for transduction of TAT fusion proteins across the cell membranes.

Acknowledgments

The authors gratefully acknowledge the comments and review by Dr. Anthony Firulli.

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Figure 1.

TAT-mMDH-GFP (TMG) protein transduction into mitochondria under endocytosis inhibitors. (A) 50 μ g of mitochondria was pretreated without inhibitors (control) or with the endocytosis inhibitors dansylcadaverine (0.5 mM), nystatin (5 μ g/mL), methyl- β -cyclodextrin (10 mM, M β CD), amiloride (5 mM), cytochalasin D (20 μ M), cytochalasin B (100 μ M), and colchicin (5 mM). This was followed by the application of TMG (2 μ g) at 4 $^{\circ}$ C. Proteinase K was added at a final concentration of 50 μ g/mL for 10 min at 4 $^{\circ}$ C, and mitochondria and supernatant were collected and subjected to Western blot with anti-GFP-HRP antibody. The internal control for mitochondrial protein loading was complex II (anti-complex II antibody). (+) and (–) indicate the addition or absence of TMG or mitochondria. (B) The immunoblot signals were quantified by scanning the representative Western blot in panel A. The transduction signal from TMG was set at 100%, and remaining conditions were referenced to the TMG signal. (C) The experiment in panel A was repeated except without proteinase K treatment after incubation with TMG. As before, mitochondria and supernatant were subjected to Western blot with anti-GFP-HRP antibody, and complex II was used as a loading control. (D) Quantified signal from immunoblot of panel C.

Figure 2.

Flow cytometry analysis of isolated mitochondria shows that amiloride increases the transduction of TAT-mMDH-GFP (TMG). (A) Representative dot plot analysis of mitochondria alone (50 μ g of mitochondrial protein and in all panels, panel Data.011), TMG (2 μ g) transduction in mitochondria (panel Data.012), or with the endocytosis inhibitors methyl- β -cyclodextrin (10 mM, M β CD, panel Data.013), or amiloride (5mM, panel Data.014). All mitochondria transduction experiments were performed at 22 $^{\circ}$ C and treated with proteinase K, 50 μ g/mL at 4 $^{\circ}$ C, as in Figure 1 prior to analysis. (+) indicates addition of TMG, M β CD, or amiloride. (B) Flow cytometry results of TMG transduction (1.5 μ g) into mitochondria at 22 $^{\circ}$ C under the different endocytosis inhibitors shown in Figure 1A and quantified by dot plot analysis ($n=5$ for each condition). The signal from TMG transduction alone into mitochondria is set at 100%, and all other signals are referenced to this value.

Figure 3.

Amiloride increases TAT-mMDH-GFP (TMG) transduction in mitochondria in a dose-dependent manner. (A) Dot plot analysis of mitochondria (50 µg) that were incubated with 0.2, 0.5, 1, and 2 µg of TMG at 22 °C and subjected to flow cytometry ($n=2$ for each condition). These data were used to identify the TMG dose for endocytosis inhibitors (panel B). (+) indicates addition of TMG or amiloride. (B) Dot plot analysis of mitochondria (50 µg) that were pretreated with 0.5, 1, or 5mM amiloride, then incubated with 1 µg of TMG at 22 °C, and subjected to flow cytometry ($n=5$ for each condition). The average percent of transduced mitochondria is shown within each dot plot. All mitochondria transduction experiments were treated with proteinase K, 50 µg/mL, as in Figure 1 prior to analysis. (C) Membrane permeability transition in mitochondria was measured by light transmission at 540 nm with either 1mM CaCl_2 and/or 5mM amiloride with 50 µg of mitochondria. $N=3$ for each condition.

Figure 4.

TAT-mMDH-GFP (TMG) transduction into mitochondria with amiloride analogues. (A) TMG (1 μ g) was incubated with mitochondria (50 μ g) at 22 $^{\circ}$ C, which had been pretreated with either EIPA or dichlorobenzamil at concentrations of 5, 50, or 500 μ M, and subjected to flow cytometry with quantification by dot plot analysis ($n=5$ for each condition). All mitochondria transduction experiments were treated with proteinase K, 50 μ g/mL, as in Figure 1 prior to analysis. Amiloride (0.5 and 5 mM) served as a positive control ($N=5$ for each condition). The percentage of mitochondria generating a positive signal was compared with TMG transduction alone, which was set at 100%. (B) Western blot analysis of TMG transduction into mitochondria. The experiment of panel A was repeated and subjected to probing with anti-GFP-HRP by Western blot. The internal control for mitochondrial protein loading was complex II. The TMG carat refers to full-length TMG (not processed), and the GFP carat indicates TMG after processing by mitochondrial processing peptidase. Bars above the lanes indicate increasing amount of drug as listed in panel A. (+) or (-) indicates the addition or absence of TMG, mitochondria, EIPA, dichlorobenzamil, or amiloride. (C) TMG was incubated with 50 μ g of mitochondria at 22 $^{\circ}$ C, which were pretreated with benzamil and dimethylamiloride at concentrations of 5, 50, or 500 μ M, treated with proteinase K, and subjected to flow cytometry with quantification by dot plot analysis ($n=5$ for each condition). (D) Anti-GFP-HRP Western blot results of same experiment as in panel C. Internal control for mitochondrial protein loading was complex II. Data in both (A, B) and (C, D) have amiloride (0.5 and 5mM) as the positive control. (+) or (-) indicates the addition or absence of TMG, mitochondria, benzamil, dimethylamiloride, or amiloride.

Figure 5.

TAT-mMDH-GFP(TMG) transduction in mitochondria is an energy-independent process. (A) Mitochondria (50 μg) were incubated with different doses of TMG(1, 2, and 4 μg) at 4 or 22 °C for 15 min. After treatment with proteinase K(50 μg/mL), mitochondria were separated by centrifugation at 4 °C, and the supernatant and pellet were subjected to Western blotting and probed with anti-GFP-HRP. Internal control for mitochondrial protein loading was complex II. (+) or (-) indicates the addition or absence of TMG and/or mitochondria. (B) Mitochondria were incubated at 22 °C with 1 μg of purified enhanced GFP (eGFP), or TMG, with (gray□=CCCP) or without (■=control) pretreatment of 50 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), in the presence or absence of amiloride (5 mM) or dichlorobenzamil (0.5 mM), and subjected to flow cytometry with quantification by dot plot analysis (*n*=5 for each condition). The Y-axis is measured in % of counts with positive signal. All conditions are referenced to TMG transduction, which is set at 100%. (C) The membrane potential ($\Delta\Psi_m$) of isolated mitochondria was measured using fluorescence of Rhodamine 123. $\Delta\Psi_m$ results are shown as “%initial reading”, where the baseline Rh123 fluorescence reading is set at 100%. $\Delta\Psi_m$ was examined in response to the sequential addition of malate/glutamate/ADP (MGA), rotenone (ROT), succinate/ADP (S/A), and carbonyl cyanide *m*-chlorophenylhydrazone(CCCP) to the mitochondrial suspensions. Summary graphs show the mean values (*n*=3) of $\Delta\Psi_m$ in mitochondria. (D) The mitochondrial respiration rate was measured. The rate of ATP production is expressed as n mol min⁻¹ mg⁻¹ of mitochondrial protein in the mitochondrial samples with and without treatment of CCCP (10 μM) using malate and glutamate as substrates in the presence of ADP (*n*= 3).