Transactivator of Transcription Fusion Protein Transduction Causes Membrane Inversion*

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The transactivator of transcription (TAT) protein transduction domain is an 11-amino acid positively charged peptide that has been shown to pull diverse molecules across cell membranes in vitro and in vivo. Fusion proteins constructed with TAT rapidly enter and exit cells and have been shown to cross intracellular membranes as well. Electrostatic interactions between TAT and the cell membrane have been implicated as a part of the mechanism of transduction. Here, we report that TAT transduction causes membrane phospholipid rearrangement as evidenced by detection of phosphatidylserine on the outer surface of the cell membrane. Furthermore, these rearrangements can be blocked by positively charged polylysine, further implicating electrostatic interactions as a part of the mechanism. Neither apoptosis nor necrosis is induced in these cells after exposure to TAT. We conclude that the process of TAT-GFP transduction causes phosphatidylserine to translocate from the inner to the outer leaflet of the plasma membrane. These results provide insight into the mechanism of TAT protein transduction domain transduction.

Protein transduction domains (PTDs) have been shown to transfer a wide range of cargos across the cell membrane, including large proteins (1), polyamionic oligonucleotides (2), liposomes (3, 4), and even metallic beads (5, 6). The exact mechanism that these short, positively charged PTDs use to transport such diverse molecules into cells has been the subject of much recent investigation. The process is not receptor-mediated, but there have been conflicting reports about whether transduction is temperature-dependent (7) or -independent (8–10). Likewise, different groups have found TAT movement to be independent of endocytosis and caveolae formation (11, 12), whereas others have shown that transduction is blocked by inhibitors of endocytosis (7, 10). Treatment of cells with drugs that inhibit cellular transport, such as brefeldin A (inhibits Golgi transport), or metabolic processes, such as rotenone (inhibits mitochondrial respiratory chain), have also been shown to have no effect on transduction of PTDs (11, 13). Torchilin et al. (4) confirmed that neither the respiratory chain nor the cytoskeleton is involved by showing that uptake of TAT attached to liposomes occurred at low temperatures and in the presence of sodium azide or iodoacetamine, respectively. Following fluorescently labeled Antennapedia peptide by microscopy suggested that only peptide interaction with membrane lipids is required and no pore formation occurs (14). Many studies have shown that electrostatic interactions of TAT and other PTDs with the negative charges of the membrane are important for transduction (9, 15–17). Even though TAT interacts with negatively charged membrane phospholipids (18), it has also been shown that TAT has a higher affinity for negatively charged membrane sugars, such as glycosaminoglycans (18). Recent data concerning TAT fusion protein transduction suggest that PTDs may use lipid rafts to get into cells (7, 12). These studies showed that electrostatic interactions between TAT and negatively charged cell-surface constituents were necessary before internalization and depletion or sequestering cholesterol in these cultured cells blocked nearly all TAT-mediated uptake. Previously we had shown that TAT fusion proteins not only transduce across the cell membrane but will also cross both mitochondrial membranes to enter the matrix and that transduction does not involve classical mitochondrial import mechanisms (19, 20). Therefore, TAT and other PTDs cross membranes in a novel way that, to date, has not been elucidated.

To gain further insight into the mechanism of TAT transduction into and out of cells, we examined the state of phospholipid asymmetry by tracking the movement of phosphatidylserine (PS) during TAT transduction. Here, we report that TAT fusion proteins interact specifically with negatively charged components of the cell membrane and cause membrane inversion during transduction. These findings indicate that PTD transduction across membranes involves membrane lipid movement and rearrangement, thereby providing further insight into their mechanism.

EXPERIMENTAL PROCEDURES

Materials—Rat tail collagen, nerve growth factor, poly-L-lysine hydrobromide, hydrogen peroxide, neutral red, Sephadex-G25, and Triton X-100 were obtained from Sigma. Treated cell culture flasks, Lab-Tek II chambered 1.5 cover glass dishes, and polycarbonate membranes were from Fisher. Dialysis membrane was from Spectrum Laboratories Inc. Caspase-3 assay kit, Alexafluor-568 Annexin-V, and calcein were from Molecular Probes. TAT-GFP was purified as previously described (19). General Cell Culture Care—PC12 cells were grown in RMPI 160 medium supplemented with 10% heat-inactivated horse serum and 5% fetal calf serum in 75-cm² treated cell culture flasks that were treated with 50 µg/ml rat tail collagen. Cells were differentiated by plating them with RMPI 1640 medium containing 1% horse serum, supplemented with 100 ng/ml nerve growth factor for a minimum of 48 h.

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§ The abbreviations used are: PTD, protein transduction domain; TAT, transactivator of transcription; PS, phosphatidylserine; GFP, green fluorescent protein.

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Polypeptide Pretreatment—Poly-lysine hydrobromide (1,000–4,000 Da molecular mass) was reconstituted in 1× sterile filtered phosphate-buffered saline and dialyzed using Spectra/Por CE membrane (molecular mass cutoff 500 Da) against three changes of 1× phosphate-buffered saline to remove the bromide.

Differentiated PC12 cells were plated in triplicate and incubated for 1 h with either 0.295 or 2.95 mM polylysine solutions and then 20 min with 0.01 mg/ml (29.5 μM) TAT-GFP. The cells were washed with 1× phosphate-buffered saline and analyzed.

Cell Viability—To determine the viability of cells, differentiated PC12 were incubated with 0, 150, 300, 500, and 1000 μM hydrogen peroxide (H2O2) for 12 h or either 0.295 or 2.95 mM polylysine for 1 h. At each time point, cells were assayed for viability using a neutral red assay as described by Fautz et al. (21).

Caspase Activation—Differentiated PC12 cells were either left untreated, treated with 0.01 mg/ml TAT-GFP for 1 h, or induced to undergo apoptosis with 300 μM H2O2 for 12 h. After the appropriate time, cells were harvested by centrifugation, washed once in phosphate-buffered saline, and resuspended in 50 μl of cell lysis buffer. The cell suspensions were subjected to a freeze-thaw cycle and transferred into individual wells of a 96-well flat bottomed tissue culture plate. Z-DEVD-AMC substrate (Molecular Probes caspase kit) was added to give a 10-μM final concentration. Plates were incubated for 30 min at room temperature and directly read in a Fmax fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) using the SOFTmax computer program.

RESULTS

TAT Transduction Causes Phosphatidylserine in the Cell Membrane to Translocate—Our results, along with those from the literature, suggest that the TAT PTD interacts specifically with negatively charged membrane phospholipids. This lead us to hypothesize that as TAT fusion proteins enter and exit the cell these interactions with membrane phospholipids would cause rearrangement of the membrane in the process. To test our hypothesis, we tracked phospholipid rearrangements during TAT transduction, using Annexin-V conjugated to a fluorescent dye. Annexin is specific for PS and only comes into contact with PS when it translocates to the outer surface during apoptosis (22, 23). Therefore, annexin binding was used as a marker for phospholipid movement. As a positive control for annexin staining we established a PC12 model of oxidative stress to induce apoptosis. Based on the literature (24), differentiated PC12 cells were exposed to 0–1,000 μM H2O2 for 12 h. Consistent with prior studies, we found that 300 μM H2O2 reproducibly kills -40% of the cells via apoptosis, which was evident by a significant increase in caspase-3 activation over controls (3.03 ± 0.082 mean fluorescence units versus 1.12 ± 0.006 for controls; Fig. 1A). We then treated differentiated PC12 cells with 300 μM H2O2 for 12 h or 0.01 mg/ml TAT-GFP for 1 h and then stained the cells with Annexin-V. The 300 μM H2O2-treated cells had a strong Annexin-V signal (Fig. 1B) and showed obvious signs of apoptosis, whereas untreated cells did not have a detectable Annexin-V signal (Fig. 1C). However, TAT-GFP-treated cells had strong Annexin-V fluorescence around their perimeters (Fig. 1, D and E), corresponding to staining localized to the outer cell membrane. The positive annexin signal was not because of apoptosis, because TAT-GFP-treated cells did not have significant caspase-3 activation (1.36 ± 0.005 mean fluorescence units versus 1.12 ± 0.006 for controls; Fig. 1A) nor did they appear apoptotic by light microscopy. Furthermore, we have previously shown that neither increasing concentrations nor long term exposure of our TAT fusion proteins caused a significant difference in cell viability between treated cells as compared with control, untreated cells (19).

Polypeptide Blocks TAT Transduction and the Subsequent PS Flip—Polypeylene has been shown to partially block TAT transduction (9). This suggested that the ability of polypeylene to inhibit uptake of PTDs reflects the importance of charge interactions in peptide-mediated internalization, because the posi-
PC12 cells were pretreated with poly-DL-lysine (100- and 1000-fold excess over TAT-GFP, respectively) for 1 h and analyzed for viability, PS flip, and the ability to prevent TAT-GFP transduction. A, cell viability is not affected by polylysine treatment. B, polylysine treatment does not cause an Annexin-V signal. C, pretreating with polylysine prevents TAT-GFP entering into cells. D, no Annexin-V signal is detected in cells pretreated with polylysine prior to TAT-GFP incubation. Micrographs are illustrative of at least two separate experiments. The micron bar represents 20 microns.

Fig. 2. Polylysine blocks TAT transduction and the PS flip. Differentiated PC12 cells were pretreated with poly-DL-lysine (100- and 1000-fold excess over TAT-GFP, respectively) for 1 h and analyzed for viability, PS flip, and the ability to prevent TAT-GFP transduction. A, cell viability is not affected by polylysine treatment. B, polylysine treatment does not cause an Annexin-V signal. C, pretreating with polylysine prevents TAT-GFP entering into cells. D, no Annexin-V signal is detected in cells pretreated with polylysine prior to TAT-GFP incubation. Micrographs are illustrative of at least two separate experiments. The micron bar represents 20 microns.

It has been proposed that TAT fusion proteins transduce across membranes through electrostatic interactions with cell membranes (8, 9, 12, 25–27). We hypothesized that TAT transduction into and out of cells could involve specific membrane phospholipid interactions causing rearrangement of the membrane during transduction. Evidence of this would come from tracking membrane phospholipid components during TAT fusion protein transduction. We chose to track the inner membrane phospholipid PS during TAT-GFP transduction, using Annexin-V. Upon incubation with TAT-GFP a membrane-localized Annexin-V signal was detected (Fig. 1, B and C). This signal was not caused by the initiation of apoptosis by TAT-GFP because incubation with TAT-GFP does not cause toxicity (19) or caspase-3 induction. We do not believe that this PS exposure to the outer leaflet is permanent, because cell death does not occur in vitro via apoptosis. Furthermore, we did not observe abnormal cell/tissue damage in injected animals nor did Schwarze et al. (1), who injected animals every day for 14 days with a high dose of TAT-β-galactosidase. Finally, several mechanisms have been proposed for the maintenance of phospholipid membrane asymmetry (28) that could allow adjustment of the PS position after transduction to return the membrane to its normal state after TAT transduction.

To further verify that TAT-GFP was directly responsible for the PS flip observed, cells were pretreated with polylysine. Previously, it had been shown that pretreating cells with 100-fold excess of a polylysine 12-mer was able to partially inhibit uptake of the TAT PTD (3). Pretreatment of cells with 100- or 1000-fold excess polylysine over TAT-GFP fully inhibits TAT-GFP transduction (Fig. 2B), and these cells then did not bind Annexin-V (Fig. 2C). Therefore, the translocation of PS we observed was a direct consequence of TAT-GFP transduction, supporting our hypotheses.

These data also show that TAT-GFP does not disrupt the membrane by forming pores or causing necrotic changes that would allow annexin to bind with PS on the inner leaflet of the cell membrane. The estimated molecular mass of Annexin-V is ~35–36 kDa, making it too large to cross the plasma membrane as an isolated protein (29, 30). Formation of a pore large enough to allow passage would also cause cell death, which we, and others, have shown does not occur even after prolonged exposure to TAT-GFP (20). Necrotic changes were not present in these cells in culture, making it unlikely that Annexin-V diffuses through a disrupted membrane. Other authors (31) have shown that incubation of TAT peptides does not increase the permeability of the cell membrane to small dyes, such as propidium iodide, indicating that the cell membrane is not disrupted by necrosis or pore formation. It is unlikely that TAT-GFP pulls the annexin into the cells because in these experiments the TAT-GFP is removed from the medium prior to the addition of annexin conjugate. Other investigators (32) have also found that TAT-mediated transduction does not promote uptake of unrelated, or non-conjugated, peptides present in the incubation medium. Taken together, these data indicate that the cell membrane is not disrupted, and thus it is unlikely that annexin gains access to the inner leaflet of the cell membrane where PS normally resides. We conclude that the process...
of TAT-GFP transduction causes PS to translocate from the inner to the outer leaflet of the plasma membrane.

Earlier data from our laboratory demonstrated that TAT fusion proteins cross the mitochondrial membranes independent of outer mitochondrial membrane receptors or an intact membrane potential (20). If transduction does not involve the regular protein import mechanisms into mitochondria, then some other interaction must be implicated. It seems reasonable that TAT would cross all membranes in a similar way. Therefore, we believe that the same mechanism used to cross mitochondrial membranes is also used to cross cell membranes, thereby ruling out endocytosis or the use of caveolae, because these mechanisms have never been identified for crossing mitochondrial membranes.

In summary, these data show that TAT fusion proteins interact with membrane phospholipids to cause a flip in PS during transduction. Negatively charged phospholipids play a critical role in this process, and if the negative charges of the cell membrane are shielded from TAT, transduction does not occur nor does the PS flip. The fact that PS is translocated from the inner to outer cell membrane during transduction, which is not a consequence of apoptosis, suggests a novel mechanism for PTD transduction that will be of interest to all researchers in this field.

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