Synthetic Protein Transduction Domains: Enhanced Transduction Potential in vitro and in vivo

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Abstract

The protein transduction domain (PTD) embedded in the HIV TAT protein (amino acids 47–57) has been shown to successfully mediate the introduction of heterologous peptides and proteins in excess of M r 100,000 into mammalian cells in vitro and in vivo. We report here that the modeled structure of the TAT PTD is a strong amphipathic helix. On the basis of this information, we synthesized a series of synthetic PTDs that strengthen the α-helical content and optimize the placement of arginine residues. Several PTD peptides possessed significantly enhanced protein transduction potential compared with TAT in vitro and in vivo. These optimized PTDs have the potential to deliver both existing and novel anticancer therapeutics.

Introduction

A severe limitation of cancer therapeutics is the problem of transporting pharmacologically relevant compounds, peptidyl mimetics, antisense oligonucleotides, and proteins in excess of M r 700 into cells (1, 2). Overcoming this bioavailability problem would not only enhance the effectiveness of existing therapeutics but also broaden the scope of viable cancer therapeutic strategies, such as reconstitution of tumor suppressor protein activity in tumor cells. In 1988, Green and Lowenstein (3) and Frankel and Pabo (4) independently uncovered the ability of HIV TAT protein to cross cell membranes. Subsequent studies have shown that TAT is capable of mediating the transduction of heterologous peptides and proteins in a concentration-dependent and receptor-, transporter-, and endocytic-independent manner into 100% of targeted cells (5–11). Given the therapeutic potential of this technology for the treatment of cancer, improving the effectiveness of the TAT PTD3 would significantly increase the bioavailability and lower the required doses of existing and novel therapeutics. The ability of the TAT protein to transduce into cells has no known biological function for HIV; therefore, it is likely that the TAT PTD has not undergone evolutionary selective pressures for protein transduction, suggesting that the protein transduction potential may be synthetically optimized. We report here that the modeled structure of the TAT PTD has a strong α-helical character with a face of basically charged Arg residues. We enhanced these structural motifs in a series of nonnaturally occurring PTD peptides and show that these novel domains demonstrate a significant enhancement of protein transduction potential into cells both in vitro and in vivo.

Materials and Methods

Cell Culture and Flow Cytometry Analysis. Human Jurkat T cells were maintained in RPMI plus 5% fetal bovine serum, penicillin, and streptomycin in 5% CO 2 at 37°C as described previously (6). Jurkat cells (1 × 10 6/ml) were treated with FITC-labeled peptides at 37°C, and 10,000 cells were assayed by flow cytometry (FACS; Becton Dickinson) at indicated times. For kinetic FACS analysis, Jurkat T cells (1 × 10 6/0.7 ml) were placed in a polypolyethylene FACS tube in an ice bath, and the cellular autofluorescence was normalized. Ten μl of FITC-labeled peptides or control FITC were then injected directly into the tube during continuous FACS analysis. Fluorescent confocal microscopy was performed on treated Jurkat T cells fixed in 4% paraformaldehyde. PTD Peptides. Peptides were synthesized containing an NH 2-terminal synthetic FITC-Gly residue that resulted in a near 100% coupling of FITC to the synthesized peptide. The FITC-Gly NH 2-terminal residue was followed by three times with Gly residues and then with the 11-amino acid TAT (residues 47–57) or 11-amino acid synthetic PTD listed in Fig. 2. After synthesis and high-performance liquid chromatography purification, all of the peptides were resuspended in water. Because of the NH 2-terminal FITC-Gly coupling efficiency (>99%) to all of the peptides during synthesis, peptide concentrations were normalized by fluorescence values from a fluorometer.

Modeled TAT 47–57 Structure. Structural predictions of the TAT (residues 47–57) PTD were made using the LINUS program (12). The molecular surface of the predicted structure was then calculated and displayed using the GRASP program (13).

Mice. C57BL/6 mice (4–8 weeks old; ~20–30 g) were injected i.p. with 0.6 μmol of TAT-FITC peptide, PTD-FITC peptides, or control-free FITC in 300–500 μl PBS. Blood was isolated from the orbital artery at indicated time points and was analyzed by FACS.

Results and Discussion

Modeled Structure of the TAT Transduction Domain. The region of HIV TAT encompassing amino acids 47–57 (Fig. 1A) has previously been shown to transduce across cellular lipid bilayer membranes in a receptor-independent fashion (5). However, the sequence or structural requirements for transduction mediated by TAT remain unknown (14–16). We modeled the molecular structure of the TAT domain using the LINUS (12) protein structure and GRASP (13) molecular surface predictive programs and found that this region of TAT has strong α-helical characteristics (light blue ribbon backbone; Fig. 1B). Strikingly, rotation of the helix by two 90° right-handed rotations reveals that the domain is an amphipathic helix. Basically, charged Arg residues (dark blue) line one face of the predicted α helix, whereas hydrophobic residues line the opposite face (Fig. 1C).

Synthetic PTDs. On the basis of the structural motifs uncovered in the above analysis, we synthesized a series of synthetic PTD peptides (Fig. 2) that could be generally grouped into two categories: (a) peptides with optimized Arg alignment on one face of the helix and strengthened α-helical character conferred by Ala residue substitutions [Ala possesses the highest α-helical stabilizing value (~0.77 kcal/mol) among all amino acids (Ref. 17; PTDs 3–6)] and (b) peptides consisting of an Arg cylinder (PTDs 7 and 8). Group I PTD peptides are further delineated by molecules containing an optimized...
Arg face and a reduction in the extent of Arg around the helical cylinder (Fig. 2). Each PTD peptide was synthesized with an NH₂-terminal FITC-Gly residue followed by a Gly-Gly-Gly motif. Because of a >99% coupling efficiency during peptide synthesis, direct quantitative comparisons could be made between each PTD peptide on the basis of relative fluorescent values. Before use on cells, all of the peptide concentrations were normalized by fluorometric analysis and diluted accordingly.

Fig. 1. Predicted TAT PTD structure. A, primary sequence of minimal HIV TAT PTD (amino acids 47–57; single amino acid code). B, modeled molecular surface and peptidyl bond of the 11-amino acid HIV TAT PTD using the LINUS and GRASP programs (see “Materials and Methods”). Orientation: N', NH₂ terminus; C[preime], COOH-terminus. Rotations are 90° and 180° along vertical axis of helix. Dark blue, basic surface regions of Arg residues; light blue ribbon, helical peptidyl backbone. C, α-helical wheel of TAT transduction domain sequence.

Fig. 2. Predicted α-helical wheels of TAT and nonnaturally occurring PTD (single-letter amino acid code). Numbering, sequential amino acid position; values in parentheses, fold change of FITC emission normalized to TAT peptide (1×) (see Fig. 3A).
SYNTHETIC TRANSDUCTION DOMAINS

To analyze the transduction potential of each peptide, Jurkat T cells were treated with normalized TAT and PTD peptides for 30 min at 37°C. Peptide-treated cells were then analyzed by flow cytometry (FACS) analysis and compared with the autofluorescence of untreated cells and control cells treated with an equal molar amount of free FITC (Fig. 3A). Consistent with previous reports (6–10), ~100% of cells in the population were transduced by TAT peptide. In addition, data from FACS and fluorescent confocal microscopy analysis suggested that all of the cells in the treated population have a near identical intracellular concentration of TAT peptide (Fig. 3, A and B).

Comparison of the transduction potential of PTD peptides to TAT peptide by FACS analysis revealed multiple levels of increased transduction potential. Substitution of α-helical-promoting Ala residues at three positions opposite the Arg face, while maintaining five Arg residues (PTD-3), resulted in a significant increase (5×) in transduction potential compared with TAT peptide (Fig. 3A). Additional substitutions with Ala residues, while maintaining the Arg content and distribution of PTD-3, yielded the PTD-5 peptide that resulted in additional enhancement of the transduction potential (8×; Fig. 3A). Strikingly, additional strengthening the putative α helix with Ala residues while limiting the Arg content to three closely aligned residues down the face of the helix (PTD-4) provided the most dramatic improvement in transduction potential relative to the original TAT sequence (33×; Fig. 3A). In contrast, PTD peptides containing entire cylinders of nine or seven Arg residues, PTD-7 and PTD-8, resulted in near identical transduction potentials compared with TAT peptide (Fig. 2). Fluorescent confocal microscopy of treated cells confirmed the rank order of transduction potentials and demonstrated the presence of PTD and TAT peptides within the cytoplasm and nucleus of cells, whereas control FITC was merely bound to the cellular membrane (Fig. 3B). Thus, the dramatic enhancements in transduction potential of PTD peptides compared with TAT peptide resulting from a tightening of the Arg residue alignment and additional strengthening of the putative α helix with Ala residues validate the significance of the modeled TAT PTD structure.

We next investigated the kinetics of protein transduction into cells. Our previous work had shown that cells treated with transducing peptides and proteins achieved maximum intracellular concentration in <10 min (6, 7). Therefore, we performed a kinetic (real time) FACS analysis of cells treated with either PTD-4 peptide or TAT peptide (Fig. 3C). The determination of baseline autofluorescence of Jurkat T cells (1 × 10⁶ cells/0.7 ml) was followed by injection of 10 µl of PTD-4 peptide or TAT peptide into the tube during continuous FACS analysis. Notably, both PTD-4- and TAT peptide-treated cells reached maximum intracellular concentration in <30 s in an apparent first-order rate constant for transduction. However, consistent with the above histogram analysis (Fig. 3A) and confocal microscopy, PTD-4 peptide achieved a significant increase in intracellular concentration compared with TAT peptide (Fig. 3C). Thus, PTD peptides transduced into ~100% cells in an extremely rapid fashion, with significant enhancement of transduction potential compared with TAT peptide.

Comparison of PTD-4 and TAT Peptides in Vivo. We next assayed the in vitro ability of PTD-4 peptide to transduce into cells in a mouse model. We reasoned that transducing peptides could be delivered via an i.p. injection and be taken up by several mechanisms, including the lymphatic system, which drains the peritoneal cavity, direct transduction across the peritoneum into the bloodstream, and direct transduction into organs present in the peritoneal cavity. C57BL/6 mice were i.p. injected with 0.6 nmol of PTD-4 peptide, TAT peptide, or control-free FITC in 500 µl PBS. Whole blood was isolated 30 min post-i.p. injection and analyzed by FACS (Fig. 4). Both PTD-4 peptide- and TAT-peptide-treated mice demonstrated transduction into ~100% of cells present in whole blood compared with untreated control mice. No additional increases were observed with either PTD-4 or TAT peptides at 60 min post-i.p. injection (data not shown). Consistent with the enhanced in vitro transduction potential above, PTD-4 peptide showed a significant enhancement of transduction potential in vivo.
increase (5×) in whole blood cell intracellular accumulation relative to TAT peptide by FACS (Fig. 4). Control FITC-injected mice showed no increase in background fluorescence of blood cells. Thus, PTD-4 peptide has an increased transduction potential both in vitro and in vivo.

Here we demonstrate, both in vitro and in vivo, that optimization of Arg residues plus substitution with α-helical-promoting residues (Ala) resulted in significant enhancements of protein transduction potential. Remarkably, these observations are entirely consistent with the modeled α-helical structure of the TAT domain. Importantly, fluorescein (FITC) is a M550 compound with no bioavailability in vitro or in vivo (see Figs. 3 and 4). Thus, FITC serves as a model, undeliverable drug. However, covalent linkage of FITC to a PTD results in transduction of FITC into ~100% of cells in culture and into most, if not all, tissues in mouse models (9). The optimized PTDs described here lay down a foundation for facilitating the effective delivery of existing drugs, especially compounds that are specific for a given intracellular target but have poor bioavailability properties, and for the development and delivery of entirely novel anticancer therapeutics.

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References

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