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In Vivo Applications of Cell-Penetrating Zinc-Finger Transcription Factors

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Abstract

Artificial transcription factors based on zinc finger, TALE, and CRISPR/Cas9 programmable DNA-binding platforms have been widely used to regulate the expression of specific genes in cultured cells, but their delivery into organs such as the brain represents a critical challenge to apply such tools in live animals. In previous work, we developed a zinc-finger-based artificial transcription factor harboring a cell-penetrating peptide (CPP) that could be injected systemically, cross the blood–brain barrier, and alter expression of a specific gene in the brain of an adult mouse. Importantly, our mode of delivery produced widespread distribution throughout the brain. Here we describe methods for the production and purification of the factor, testing CPP activity in cells, and testing CPP activity in mice.

Keywords

Engineered zinc-finger protein; Cell-penetrating peptide; TAT peptide; Animal models; Artificial transcription factors

1 Introduction

Artificial transcription factors (ATFs) are based on the attachment of transcriptional effector domains to programmable DNA-binding platforms such as zinc fingers (ZFs), transcription activator-like effectors (TALEs), or catalytically inactive clustered regularly interspaced short palindromic repeats/dead Cas9 (CRISPR/dCas9). These tools are capable of activating or repressing specific genes as has been described extensively [1–4]. Widespread delivery of these gene regulators to the organs such as brain remains a significant challenge for the study and treatment of medical conditions.

We previously reported the systemic delivery of a purified ATF protein as a potential therapeutic approach for the treatment of Angelman syndrome (AS) [5]. AS is a rare neurological genetic disorder caused by loss or mutation of the maternal copy of *UBE3A* in the brain. Due to brain-specific genetic imprinting at this locus the paternal copy of *UBE3A* is silenced, resulting in the complete loss of *UBE3A* expression in brain neurons of patients.

Competing Interests: The authors declare no competing interests.

The ATF was designed to unsilence the paternal *UBE3A* by inhibiting an antisense transcript that is responsible for the silencing. However, as with many gene therapy methods, a significant challenge to actual therapeutic use is an efficient means of delivering the ATF to the many neurons in the brain.

One method for protein delivery into cells is the use of cell-penetrating peptides (CPPs). These short peptides are positively charged sequences that can be added to various proteins to facilitate their translocation across cellular membranes, usually by hijacking the normal process of receptor-mediated endocytosis [6, 7]. The cationic class of CPPs contains clusters of arginine and lysine residues, such as Antp (RQIKIWFQNRRMKWKK), derived from the third helix of the Antennapedia protein homeodomain from *Drosophila* [8], and Tatp (GRKKRRQRRR), derived from HIV-1 TAT transcription-activating protein [9]. Successful delivery using these CPPs had been shown both in vitro and in vivo with several full-length proteins that retain their biological activity [6, 7, 10–12], including engineered ZF proteins [13–15].

For our AS studies, we combined the ATF with a CPP in a construct composed of an N-terminal maltose-binding protein for purification, the CPP consisting of the 10 aa transduction domain of the HIV transactivator protein (TAT, residues 48–57), an mCherry red fluorescent protein to aid in protein solubility and visualization, an HA epitope tag for detection, an SV40 nuclear localization signal to ensure nuclear delivery, an engineered zinc-finger protein (designated “S1”), and a KRAB transcriptional repression domain (Fig. 1) [5]. The purified fusion protein was able to enter cells both in vitro (Fig. 2) and in vivo (Fig. 3). In both cases, a protein containing all the components except the TAT CPP was still able to enter cells, but with considerably reduced efficiency. In mice, we observed that intraperitoneal (i.p.) or subcutaneous (s.c.) injection of the purified protein at 160–200 mg/kg was able to cross the BBB and distribute widely throughout the brain. Importantly, significant activation of *Ube3a* expression in the brain was observed after a 4-week treatment period.

In principle, a CPP could be used to deliver ATFs designed to target other promoters or DNA elements in the brain or other organs by using a different ZF protein, TALEs or dCas9. Exchanging the KRAB transcriptional repression domain with an activation domain (e.g., VP64 or p300) or epigenetic modifiers (e.g., DNMT3A or G9A) could produce tools for activating gene expression and altering epigenetic information, respectively. Cellular internalization of CPPs has been observed for a large number of cell types and tissues, although the efficiencies vary depending on the CPP, the cargo, and the target cell type [16, 17]. In fact, it has recently been shown that since ZFs carry a net positive charge they can penetrate cells in the absence of additional peptides [18, 19]. It is therefore important to test CPPs empirically to determine their utility for a particular application. The method that follows can be used to quickly determine if a CPP increases cellular uptake more than a zinc-finger protein alone, in vitro (Subheading 2) and in vivo (Subheading 3). Although the mCherry fluorescence could be directly visualized in mouse tissues, an antibody staining protocol is provided in Subheading 3 that could be used for weaker fluorophores.

2 Materials

2.1 Expression and Purification of CPP-ZF ATFs in Bacteria

1. Construct a prokaryotic expression vector expressing a CPP-ZF ATF. There are a number of possibilities, but a good starting point might be to use TAT-S1 ATF construct described in our published study. A modified pMAL-c2X (New England Biolabs, Ipswich, MA) with an expression cassette containing (1) an N-terminal maltose-binding protein (MBP) for purification, (2) a TEV1 protease cleavage site, (3) a cell-penetrating peptide consisting of the 10 aa transduction domain of the HIV-transactivator protein (TAT, residues 48–57), (4) mCherry red fluorescent protein to aid in protein solubility and visualization, (5) an HA epitope tag for detection, (6) an SV40 nuclear localization signal to ensure nuclear delivery, (7) a six-finger zinc-finger DNA-binding protein designated “S1,” and (8) a KRAB transcriptional repression domain. “S1 (no TAT)” and “S1 no mCherry” do not contain the TAT or mCherry components, respectively. Vectors expressing these constructs are available from the authors upon request. The complete sequence of the full-length ATF TAT S1 protein is provided in Fig. 1 (*see Note 1*).
2. Chemically competent NEB5 α *E. coli* bacteria (New England Biolabs): Store at -80°C (*see Note 2*).
3. Carbenicillin antibiotic stock at 100 mg/mL in H₂O: Keep stock at -20°C .
4. 10 cm Plates of Luria Broth (LB) agar (*see Note 3*) supplemented with carbenicillin at 50 $\mu\text{g/mL}$: Prepare no more than 1 month in advance and store at 4°C .
5. 1 $\times$ Luria Broth medium (*see Note 3*).
6. Isopropyl β -D-1-thiogalactopyranoside (IPTG) stock at 0.5 M in H₂O: Keep stock at -20°C .
7. Zinc chloride (ZnCl₂) stock at 1 M in H₂O: Store at room temperature.
8. Zinc buffer A (ZBA): 10 mM Tris base, 90 mM KCl, 1 mM MgCl₂, 100 μM ZnCl₂. Adjust pH to 8.5 using HCl (*see Note 4*). Store at room temperature.
9. Bacterial culture shakers at both 37 and 4°C .
10. Centrifuge for bacterial cultures.
11. Microfluidizer (Microfluidics model M-110Y).
12. Chromatography columns (~100 mL volume) with valves.
13. Amylose resin (New England Biolabs, E8021L).
14. Maltose.
15. Dithiothreitol (DTT) stock at 1 M in H₂O: Aliquot in 0.5–2 mL volumes and store at -20°C .

16. Elution buffer: ZBA, 1 M maltose.
17. Centricon Plus-70 spin concentrators (Millipore, Billerica, MA, UFC710008).
18. Refrigerated tabletop centrifuge.
19. Nalgene Rapid-Flow sterile disposable filter units, 0.2 μ m (Thermo Fisher).
20. HEK-Blue LPS Detection Kit (InvivoGen, San Diego, CA, rep-lps2).
21. 4–20% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad, Hercules, CA, 4561096).
22. Apparatus and materials for performing Coomassie-stained SDS-PAGE.
23. Nanodrop UV spectrophotometer, or equivalent.
24. Glycerol, sterilized by autoclave.

2.2 Testing CPP Activity in Cell Culture

1. Purified ATF protein in elution buffer, 30% glycerol, 5 mM DTT.
2. The human cell line HEK293 (ATCC CRL-1573, *see* **Note 5**).
3. Water-jacketed CO₂ 37 °C incubator.
4. Class II biosafety cabinet.
5. Six-well polystyrene tissue culture plates.
6. 75 cm² Tissue culture flasks.
7. DMEM medium.
8. Bovine calf serum: 50 mL Aliquots are stored at –20 °C.
9. Pen–Strep contains 100 U/mL of penicillin and 100 μ g/mL streptomycin: 5 mL Aliquots are stored at –20 °C.
10. DMEM complete medium: 1 L DMEM medium supplemented with 50 mL bovine calf serum (10% final) and 5 mL of Pen–Strep. Store at 4 °C.
11. Phosphate-buffered saline, sterile for tissue culture.
12. Trypsin-EDTA.
13. Hemocytometer for counting cells.
14. 10% bleach solution.
15. Zeiss Axiovert 135 inverted epifluorescent microscope with filters for mCherry imaging (Filter Set 15, Excitation: 546 nm; Emission: LP 590 nm) and a digital camera.

2.3 Testing CPP Activity in Mice

1. Purified ATF protein in elution buffer, 30% glycerol, 5 mM DTT.
2. C57BL/6 mice of either sex, approximately 8 weeks of age.

3. One milliliter syringe with 25-gauge hypodermic needle.
4. Isoflurane and appropriate apparatus.
5. CO₂ chamber or other apparatus for humane euthanasia.
6. TBS: 50 mM Tris–Cl, pH 7.5, 150 mM NaCl.
7. Buffered paraformaldehyde: 10% (v/v) in PBS.
8. 30% Sucrose in water.
9. Tissue-Tek CRYO-OCT Compound (Thermo Fisher 14-373-65).
10. Glass microscopy slides.
11. Glass coverslips.
12. Leica CM 1850 UV cryotome (Nussloch, Germany).
13. Superblock (Thermo Fisher).
14. Goat serum.
15. Triton X.
16. Apex Antibody labeling kit with Alexa Fluor 555.
17. Anti-HA primary antibody: Pre-label with the Apex Antibody labeling kit.
18. 4',6-Diamidino-2-phenylindole (DAPI).
19. Prolong Gold (Thermo Fisher, P10144).
20. Leica DM6000B epifluorescent microscope with deconvolution software and filter cubes to image mCherry (TxRed 4040B, excitation: 562 nm; emission: 624 nm) and DAPI (DAPI 5060B, excitation: 377; emission 447).

3 Methods

3.1 Expression and Purification of CPP-ZF ATFs in Bacteria

1. Transform the vector into NEB5 α *E. coli* by standard heat-shock methods.
2. Plate the transformed bacteria on LB agar supplemented with 50 μ g/mL carbenicillin and incubate overnight.
3. Pick a single colony to inoculate into 5 mL of LB medium supplemented with 50 μ g/mL carbenicillin. Incubate overnight with shaking at 37 °C.
4. Inoculate the 5 mL overnight cultures into 800 mL of LB medium supplemented with 50 μ g/mL carbenicillin. Incubate with shaking overnight at 37 °C.
5. At optical density ~1.0, induce protein expression by moving the culture to 4 °C (see **Note 6**) and adding IPTG to 0.75 mM and 1 mL of zinc chloride. Shake gently at 4 °C for 4 days.
6. To release the protein from the bacteria, pellet by centrifugation at $1663 \times g$ (3200 rpm) for 15 min in a refrigerated tabletop centrifuge. Resuspend the

culture in 30 mL of cold ZBA (*see Note 7*). Apply the suspension to a microfluidizer according to the manufacturer's instructions (*see Note 8*). Keep the lysate on ice.

7. Pellet the insoluble fraction by centrifugation at $1663 \times g$ for 15 min in a refrigerated tabletop centrifuge. Keep supernatant for column purification and discard pellet.
8. Prepare gravity flow amylose resin purification columns by applying enough amylose resin to produce a 30 mL compact bed in the columns (*see Note 9*). All steps can be performed at room temperature. Wash the columns twice with four-column volumes of deionized water, and then one-column volume of ZBA. Finally, apply the microfluidized supernatants to the columns. The initial drip rate should be approximately two drops/s, but will decrease as the solution in the column decreases.
9. Dissociate and elute the protein in ~150 mL of elution buffer supplemented with 5 mM DTT (add DTT just before use). Keep eluted samples on ice.
10. Concentrate the eluate to 16 mg/mL using a Centricon Plus-70 in a refrigerated centrifuge at $2000 \times g$ for ~60 min (depending on the initial concentration) at 4 °C. For maximum tolerated dose studies, additional higher concentrations may be desired.
11. Sterilize the protein sample using Nalgene Rapid-Flow sterile disposable filter units to remove any residual bacteria. Purified, sterilized protein samples are routinely checked by an endotoxin kit (e.g., HEK-Blue LPS Detection Kit) to assure that no detectable endotoxins are present.
12. Measure protein concentration using a Nanodrop UV spectrophotometer at A280, blanking with elution buffer. The procedure typically yields 4 g total protein/L of culture for S1-KRAB, but values will likely change for other ATFs. Evaluate protein integrity by SDS polyacrylamide gel electrophoresis using a 4–20% TGX precast protein gel followed by staining with Coomassie blue. Usually there is a 44 kD band corresponding to free MBP. Protein concentrations for injections refer to the full-length + free-MBP band intensities, of which only half was considered to be the 100 kD full-length protein.
13. For storage of the proteins, add glycerol to 30% and DTT to 5 mM. This typically decreases the concentration from 16 to 12 mg/mL total protein. Store at –20 °C (*see Note 10*). Note that it is difficult to measure the protein concentration after addition of glycerol, so concentration is measured in the previous step.

3.2 Testing CPP Activity in Cell Culture

1. Obtain prior approval from the Institutional Biosafety Committee of the investigator's institution before any work using human cells is performed.

Day 0: Seed the cells in the six-well plates

2. Prepare the HEK293 cells to seed on six-well plates. Aspirate the medium from the cells growing in a 75 cm² flask.
3. Wash the cells with 5 mL of PBS. Swirl the PBS around to wash away remaining medium, and then aspirate the PBS.
4. Add 2 mL of trypsin-EDTA and swirl the flask to ensure that the entire surface is covered. Incubate at room temperature for 1 min.
5. Resuspend the cells using 8 mL of DMEM complete medium.
6. Place 15 µL of the above suspension onto a hemocytometer. Count the number of cells using an inverted microscope. After use, decontaminate the hemocytometer in 10% final bleach solution.
7. Add 400,000–600,000 cells to each well.
8. Incubate for 24 h in a 37 °C CO₂ incubator before protein transduction.

Day 1: Protein transduction

9. The cells should be about 60% confluent at the time of protein transduction. Replace the complete medium with 2 mL DMEM without supplements.
10. Add ATF proteins directly to the wells. Varying concentrations should be used. The results shown in Fig. 2 required 100 µL of the 12 mg/mL purified protein, for a final concentration of about 11 nM per well (see **Note 11**).
11. Incubate for 2 h in a 37 °C incubator.
12. Add an additional 2 mL of DMEM complete medium.
13. Incubate the cells in a 37 °C incubator for 24 h.

Day 2: Imaging

14. To prepare the cells for imaging, remove the media from wells.
15. Wash twice with 2 mL of PBS to remove remaining red indicator dye from the DMEM complete medium. Image in 2 mL of PBS.
16. Image using a Zeiss Axiovert 135 inverted epifluorescent microscope with filters for mCherry imaging. Example images are shown in Fig. 2.

3.3 Testing CPP Activity in Mice**ATF injections**

1. Obtain prior approval from the Institutional Animal Care and Use Committee of the investigator's institution before any work on animals is performed.
2. Mice should be anesthetized with 4% isoflurane before injection (see **Notes 12**). Inject three mice with TAT-S1 ATF at a dose of 160–200 mg/kg *s.c.* (see **Note 13**). Also inject three mice with elution buffer, 30% glycerol, and 5 mM DTT

["Mock"] as a negative control. When mice are ambulatory, they can be returned to their cages.

3. Repeat **step 2** for 2 consecutive days, for a total of three injection days.
4. Humanly euthanize mice on the fourth day. Harvest brain or other organs to be studied. Tissues that are not used immediately for immunohistochemistry should be flash frozen.

Preparation of tissue sections

5. Wash one brain hemisphere in 1× TBS, and then fix in 10% buffered paraformaldehyde overnight.
6. Place the tissue in 30% sucrose for 3 days.
7. Following brain saturation, freeze the tissue in Tissue-Tek CRYO-OCT Compound, and then section on a Leica cryotome in 50 µm thick sections. Transfer the sections to glass microscope slides.

Immunohistochemistry (if fluorescence cannot be observed from tissues directly)

8. Block using Superblock for 1 h, followed by 10% goat serum and 0.3% Triton X in 1× TBS for 2 h.
9. Aspirate slides, and then stain with primary anti-HA (that had been directly labeled with the Apex Antibody labeling kit) at 1:150 in 5% goat serum and 0.15% Triton X.
10. Incubate at 4 °C overnight.
11. Wash slides three times with 1× TBS.
12. Stain with DAPI for 10 min.
13. Wash three times with 1× TBS.

Imaging

14. Mount with coverslip using Prolong Gold.
15. Image using a Leica DM6000B epifluorescent microscope using filter cubes for imaging DAPI and mCherry. Example images are shown in Fig. 3.

4 Notes

1. The expression and purification methods described here will likely require significant optimization for any new type of zinc-finger protein or effector domain used. Also, at the time of this writing, it is not clear if protein domains such as MBP and mCherry are required for full function of the ATF.

2. BL21 Star cells could also be used for protein expression. The ATF S1-KRAB seems to express equally well in both BL21 Star and NEB5a cells. However, others, such as TALE proteins, express much better in BL21.
3. Any source of LB medium is usually acceptable. However, we have found for some ATFs (not S1-KRAB) that LB from some vendors produced a dramatic reduction in yield, which was restored by using LB from VWR.
4. A good guide to the appropriate pH for the ZBA is the protein's isoelectric point, which can be calculated by any of several online services (e.g., <http://web.expasy.org/protparam/>). Some optimization may be necessary.
5. For safety, human cells should always be handled using the philosophy of Universal Precautions, as if they were contaminated with a blood-borne pathogen such as HIV or hepatitis virus B. This includes biosafety level 2 conditions, such as appropriate personal protective equipment and a biosafety cabinet.
6. The cold-temperature induction was critical to obtaining high yields of the TAT-S1 ATF protein. Induction at room temperature or 37 °C was far less efficient. This unusual requirement was fortuitously observed when several standard induction conditions were tried and proved unsatisfactory. Concurrently, a culture that had been accidentally left at 4 °C for several days turned noticeably pink. This was an indication that the protein was being expressed, at least the mCherry domain. Experimental refinement of methods resulted in the reported production protocol. However, versions of the ATF that contained other effector domains required induction at an optical density of 0.6, and incubation for only 4 h at 37 °C.
7. Protease inhibitors are not used when purifying the ATF S1-KRAB. They are sometimes used with other factors, especially if degradation appears as a significant issue. However, we have generally been cautious out of concern for undesired effects of residual protease inhibitors in animals.
8. Use of the microfluidizer was found to be critical for obtaining full-length protein. Sonication and freeze/thaw techniques produced fragmented proteins.
9. The amylose resin can be reused up to 14 times by washing. In some cases, yield seemed to increase using resin that had been used and washed.
10. Proteins were originally stored at –80 °C, but later studies showed less protein fragmentation due to freeze/thaw when the ATF was stored at –20 °C.
11. Some optimization of the protein concentration applied to the cells may be necessary. It is useful to test a range of concentrations initially.
12. In addition to avoiding accidental autoinoculation by trying to inject a moving mouse, anesthesia before injection also provides a transient immunosuppression that prevents an acute immune response to the protein when the bolus is injected. Anesthesia is thus highly recommended.

13. A power calculation would be performed first to determine how many mice would be required to observe the molecular phenotype (i.e., the change in Ube3a expression). This calculation requires an estimation of the variance in the live fluorescence assay. Since the variance may differ for different proteins, testing three mice here can provide information on the variance that can be used to make a more informed calculation for a sufficiently powered experiment. In our experience, three mice per group are typically sufficient.

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Fig. 1.
 Design of TAT-S1 ATF. (a) Diagram of TAT-S1 ATF indicating individual protein domains.
 (b) Protein sequence of TAT-S1 ATF. Individual domains are indicated. Intervening
 sequences are shown as grey sequences in parenthesis

Fig. 2. TAT-S1 ATF in cultured HEK293 cells. mCherry fluorescent images of cells transduced with 11 μ M TAT-S1 ATF, S1 ATF with no TAT, or TAT-S1 ATF with no mCherry

Fig. 3. TAT-S1 ATF in mouse brain. Sectioned tissues (50 μm) were harvested after 3 days of injections (160–200 mg/kg s.c.) with purified TAT-S1 ATF, or elution buffer (Mock) that contains no ATF as a negative control. Top panel, mCherry fluorescent images of mouse brain cortex; bottom panel, bright field