A FLUORESCENCE-BASED REPORTER OF ARGINYLTRANSFERASE 1 (ATE1)

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Abstract

Arginyltransferase 1 (ATE1) is an enzyme that catalyzes the transfer of arginine onto protein fragments with acidic N-termini. This is an essential step in the degradation of these fragments by the N-degron pathway of the ubiquitin proteasome system. Previous studies have shown that arginylation is required for the removal of specific fragments associated neurodegeneration, and that the loss of ATE1 activity leads to neurological problems. Interestingly, reduced ATE1 activity was also associated with fat loss and resistance to diet-induced obesity. Thus, the modulation of ATE1 holds promise for treating these increasingly common human diseases. To this end, we synthesized a cell-based reporter that employs direct fluorescence to monitor ATE1 activity. Using confocal microscopy and immunoblot analysis, we show that this reporter provides a robust readout of ATE1 activity in vivo. This reporter will be useful in screening approaches aimed to identify modulators of ATE1, which may ultimately have therapeutic potential.

Keywords: arginylation, ubiquitin proteasome system, N-degron pathway, fluorescence microscopy

Introduction

The temporal and spatial regulation of protein function often occurs by way of non-processive protein cleavage. This generates protein fragments that, if not removed, can lead to cellular toxicity due to aberrant functions or aggregation. The removal of damaged proteins is carried out largely by the ubiquitin-proteasome system (UPS) which marks proteins for degradation by the proteasome through the covalent attachment of ubiquitin (Ub) (1). A specific UPS pathway that degrades protein fragments is called the N-degron pathway (formerly called the N-end rule pathway). In this pathway, protein fragments bearing N-terminal basic (e.g. Arg, Lys, or His) or bulky hydrophobic amino acids (e.g. Phe, Lue, Trp, Tyr, or Ile) are recognized by the ubiquitin recognin box (UBR) family of Ub-ligases. These ligases facilitate the poly-ubiquitylation and proteasomal degradation of the fragments. N-terminal Asn, Gln, Asp, Glu, and Cys are also destabilizing, but they require enzymatic modifications. These modifications include deamidation of Asn and Gln by NTAN1 and NTAQ1, respectively, and N-terminal arginylation of Asp, Glu, and oxidized Cys, carried out solely by the *Ate1*-encoded arginyltransferase 1 (ATE1) (2-4).

There is mounting evidence demonstrating the role of arginylation in a number of physiological processes. It was found that deletion of the *Ate1* gene in mice resulted in embryonic lethality resulting from cardiovascular defects (5). Subsequent studies using conditional knockout mouse strains found that the loss of post-natal *Ate1* gene function resulted in a marked loss of fat and resistance to high-fat diet-induced obesity (4). There is also evidence that arginylation plays a role in nervous system development as well as in maintaining brain function and preventing neurodegeneration (4, 6). We found that the N-degron pathway can degrade specific fragments of aggregation-prone proteins associated with amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), Alzheimer's disease, and Parkinson's disease (7). We also found that in the absence of ATE1, C-terminal fragments of the TAR DNA-binding protein-43 (TDP43) form intracellular aggregates similar to those identified in patients with ALS and FTD (8). Given these findings, we expect ATE1 to be an effective therapeutic target for both obesity and neurodegeneration.

There is great interest in discovering pharmacological modulators of the UPS. Indeed, inhibitors of the proteasome have shown success in treating multiple myeloma and breast cancer (9-12). As the proteasome is the final step in the UPS-mediated degradation of a large variety of proteins, inhibitors of specific upstream steps are expected to have fewer off-target effects and greater specificity. For example, inhibitors of ATE1 would stabilize only a subset of proteins degraded by the N-degron pathway and would leave the bulk of UPS-mediated degradation unperturbed. Several natural and synthetic inhibitors of UBR proteins and ATE1 have been described; however, they were either identified through *in vitro* screening systems and do not work well *in vivo,* or they have low specificity (13-22). For example, tannic acid, a naturally occurring plant-derived polyphenol found in tea and red wine, was shown to inhibit ATE1 activity (23). However, tannic acid causes a wide range of cellular effects and has a growing list of molecular targets (24). As such, there is a need for *in vivo* screening methods to identify compounds capable of penetrating cells and with regulatory function restricted to specific steps in protein degradation. To that end, we have developed a highly sensitive, cell-based, dual-fluorescent reporter that provides a "digital" signal of ATE1 activity *in vivo*. This reporter can be employed in the search for pharmacological or genetic modulators of ATE1 that may have therapeutic benefit in the treatment of human diseases such as obesity and neurodegeneration.

Materials and methods

Synthesis of a dual fluorescent reporter for ATE1. To generate the URT-based plasmid pYK17 encoding mCherry-Ub-Ndeg-GFP, the Q5 site directed mutagenesis kit (New England Biolabs) was used according to the manufacturer's protocol to remove the cDNA encoding human TDP43 amino acids 264 to 414 from pYK08 encoding mCherry-Ub-TDP43^{247}-eGFP. Briefly, pYK08 was amplified (25 cycles) using primers, 5-GGTTCAATGGTGAGCAAGGGC-3 (forward primer) and 5-CTTAGGTTCGGCATTGGATATATG AACGC-3 (reverse primer), then 1µl of the PCR product was incubated in Kinase-DpnI-Ligase (KDL) reaction for 10 min at room temperature to remove the pYK08 template and ligate pYK17. NEB5α cells (New England Biolabs) were transformed with the ligation mix and clones were selected on LB medium agar plates supplemented with 100ug/ml ampicillin.

Cell culture and transfection. Wild type and CRISPR/Cas9-mediated *Ate1*-lacking (8) neuroblastoma cells (Neuro2a) cells were maintained at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (Corning) containing 10% fetal bovine serum, 100U/ml penicillin, 0.1U/ml streptomycin and 20mM glutamine. At 80% confluency, cells were passaged and plated to poly-D-lysine coated plates for experiments. For transient transfection of pYK17, cells at ~75% confluency were transfected using BioT (Bioland Scientific) according to manufacturer's protocol. For proteasome inhibition, cells were treated with 10 µM MG132 (Caymen Chemical) for 6 hours before lysis.

Lysate preparation and immunoblot analysis. Cells were harvested and lysed in tissue lysis buffer (TLB) (50 mM HEPES, 10% glycerol, 0.05% NP-40, 150 mM NaCl, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride containing the complete protease inhibitor mixture (Thermo Scientific)) by freezing-thawing. The lysate was then centrifuged at 13,000 rpm for 20 min at 4°C, and the supernatant was collected. Protein concentrations were determined by using the Bio-Rad protein assay reagent according to the manufacturer's protocol. For immunoblotting, sample protein concentrations were normalized, heated to 95°C for 5 min in 2x Laemmli buffer, separated on 4-to-12% gradient NuPage Bis-Tris premade gels (Invitrogen), and transferred onto a methanol-activated PVDF membrane (Bio-Rad) in Towbin buffer (25 mM Tris, 192 mM glycine, and 20% methanol). Membranes were blocked in 5% milk in phosphate buffered saline (PBS) containing 0.1% Tween 20 at room temperature for 1 h. Membranes were then incubated with an anti-GFP (1:4000 dilution; Abcam) or anti-GADPH (Santa Cruz Biotech) antibody for 4 hours, washed three times in PBS containing 0.1% Tween 20 for 5 min, and then incubated with secondary antibodies (1:7,000 dilution) for 1 h at room temperature. Thereafter, blots were washed three times with PBS containing 0.1% Tween 20 and twice in PBS and developed by using the Licor Odyssey CLx system.

Immunocytochemistry. Cells were fixed in 4% formaldehyde and washed three times with PBS and mounted in 4,6-diamidono-2-phenylindole (DAPI)-containing Vectashield mounting medium (Vector Laboratories). Fluorescent cells were imaged and quantified using a Nikon A1 confocal microscope and Nikon instrument software elements AR-3.2 imaging software.

Results

To develop a fluorescent reporter highly sensitive to ATE1 activity, we generated an unstable green fluorescent protein (GFP) that is degraded in an ATE1-dependent manner. For this, we exploited TDP43^{247}, a specific aggregation-prone fragment of human TDP43 (amino acids 247 - 414) that was shown to be deposited in the brains of patients with FTD (25). We previously reported that TDP43^{247} was ubiquitylated and degradation by the N-degron pathway in an ATE1-dependent manner (7, 8). Initially, we fused GFP to the C-terminus of TDP43^{247} to generate TDP43^{247}-GFP (Figure 1A). Through immunoblot analysis using an anti-GFP antibody, TDP43^{247}-GFP could not be detected in extracts from wild type Neuro2a cells unless they were treated with the proteasome inhibitor MG132 (Figure 1A). On the other hand, proteasome inhibition was not required to detect TDP43^{247}-GFP in extracts from Neuro2a cells that had undergone CRISPR/Cas9-mediated Ate1-knockout (ATE-KO). This result indicated that, similar to TDP43^{247}, TDP43^{247}-GFP was degraded by the N-degron pathway in a manner that required ATE1. Confocal fluorescence microscopy of TDP43^{247}-GFP in ATE1-KO cells revealed that it readily forms aggregates, precluding its usefulness as a soluble reporter (Figure 1B).

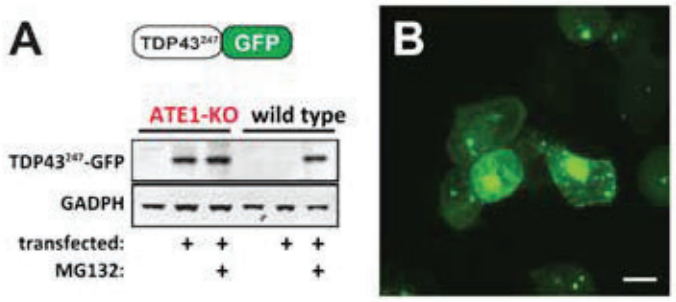

Figure 1. TDP43^{247}-GFP is sensitive to ATE1 but aggregates in cells. **A)** *Upper*, schematic of TDP43^{247}-GFP fusion protein. *Lower*, SDS-PAGE and immunoblotting using an anti-GFP antibody to detect steady-state levels of TDP43^{247}-GFP expressed in wild type and ATE1-knockout (ATE1KO) Neuro2a cells treated in the presence or absence of the proteasome inhibitor, MG132. Note that TDP43^{247}-GFP is detected in wild type cells only in the presence of MG132, whereas MG132 is not required for its detection in ATE1-KO cells. Lower panel shows anti-GADPH immunoblot.
B) Aggregation of TDP43^{247}-GFP in ATE1-KO cells produces insoluble fluorescent aggregates. Bar, 10 µm.

In subsequent efforts, we replaced TDP43^{247} with the minimal TDP43 sequence (amino acids 247-DLIIKGISVHISNAEPK-263) needed for ATE1-dependent degradation. This sequence is referred to as the N-terminal degradation signal or "Ndeg" (Figure 2A). The ubiquitin reference technique (26) was used to express Ndeg-GFP bearing N-terminal Asp, the natural N-terminal amino acid of TDP43^{247} that is arginylated by ATE1 (Figure 2A). For this, the cDNA encoding mCherry-Ub was cloned upstream and in-frame with Ndeg-GFP. The URT-based fusion was expressed as a single transcript, which was co-translationally cleaved by intracellular deubiquitylases (DUBs). This produces, at initially equimolar ratio, Ndeg-

GFP, which is unstable in the presence of ATE1, and the stable "reference" protein, mCherry-Ub, which marks transfected cells. Ratiometric fluorescence of GFP to mCherry allows for a quantitative measurement of ATE1 activity.

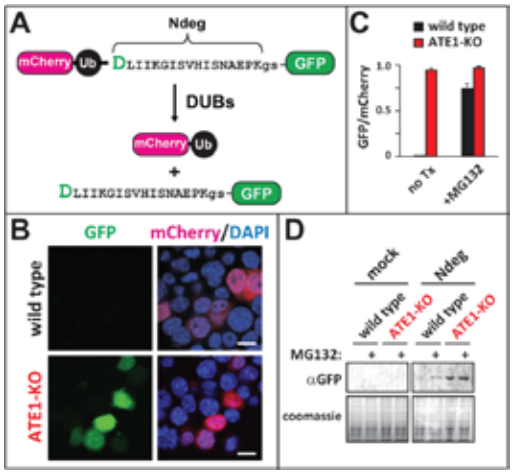

Figure 2. Ndeg-GFP is a soluble and robust reporter for ATE1 activity. **A)** mCherry-based URT expression of Ndeg-GFP. Co-translational cleavage of mCherry-Ub-Ndeg-GFP by intracellular deubiquitylases (DUBs) produces Ndeg-GFP, which is unstable in the presence of ATE, and mCherry-Ub, a stable internal reference, at initial equimolar ratio. Ratiometric fluorescence allows for the quantitative measurement of ATE1 activity. **B)** Ndeg-GFP is degraded in WT cells but detected as diffuse fluorescence in ATE1-KO cells. Bars, 10 μm. **C)** The ratio of Ndeg-GFP-positive cells divided by mCherry-positive cells in the presence (+MG132) or absence (no Tx) of MG132. **D)** SDS-PAGE and immunoblotting using an anti-GFP antibody to detect steady-state levels of Ndeg-GFP expressed in wild type and ATE1-KO Neuro2a cells treated with a proteasome inhibitor MG132. Samples were in duplicates. The lower panels show coomassie staining of the membrane to indicate equal loading.

To determine the *in vivo* aggregation propensity and degradation of Ndeg-GFP, we examined its fluorescence in wild type and ATE1-KO Neuro2a cells using confocal microscopy. Similar to TDP43^{247}-GFP, Ndeg-GFP was not detected in transfected wild type cells but were easily detected in transfected ATE1-KO cells (transfected cells were marked by mCherry) (Figure 2B). In contrast to TDP43^{247}-GFP, Ndeg-GFP was detected as diffuse fluorescence (compare Figure 2B to Figure 1B). These results indicate that Ndeg-GFP is a soluble reporter for ATE1 activity.

In order to determine the sensitivity of the Ndeg-GFP reporter, we counted the number of mCherry-positive wild type and ATE1-KO Neuro2a cells in which green fluorescence was detected. Signifying its robustness, Ndeg-GFP was detected in 95.4% of mCherry-positive ATE1-KO but was undetected in mCherry-positive wild type cells (Figure 2C). When treated with the proteasome inhibitor MG132, Ndeg-GFP was detected in 100% of mCherry-positive ATE1-KO and in 75.5% of mCherry-positive wild type cells (Figure 2C). This result indicates that Ndeg-GFP is degraded by the UPS and ATE1 is required.

To examine Ndeg-GFP expression using another method, we separated cell extracts from transfected wild type and ATE1-KO Neuro2a cells by SDS-PAGE and carried out immunoblot analysis using an anti-GFP antibody. Similar to TDP43^{247}-GFP, Ndeg-GFP expression was only detected in extracts from wild type cells treated with the proteasome inhibitor, MG132 (Figure 2D). One the other hand, proteasome inhibition was not required for detection of Ndeg-GFP in extracts from ATE-KO cells. Together, the above results demonstrate that fluorescence produced by Ndeg-GFP is inversely related to ATE1 activity; therefore, Ndeg-GFP functions as a robust reporter of ATE1 in cells.

Discussion

To develop a fluorescent reporter capable of detecting ATE1 activity *in vivo*, we generated Ndeg-GFP, a variant of GFP that is unstable in the presence of ATE1. Ndeg is the minimal TDP43 sequence (amino acids 247 - 263) required for arginylation and degradation by the N-degron (8). Although substrate recognition by ATE1 and UBRs occur largely through the first two N-terminal residues (27, 28), Ndeg extends seventeen residues to Lys263, which is the preferred site of poly-Ub attachment (8). In order to express Ndeg-GFP, bearing N-terminal Asp (as opposed to N-terminal Met), and a separate fluorescent reporter with different excitation and emission spectra, we expressed it in a linear fusion with mCherry-Ub using the ubiquitin reference technique (Figure 2A) (26). Whereas the stability of Ndeg-GFP is determined by ATE1, mCherry-Ub is stable and can be used to mark cells transfected with the reporter. This design also enables ratiometric fluorescence, which reduces false positives by normalizing off-target effects such as those on transcription, translation, or cell fitness. We envision this reporter will be useful in unbiased screening applications using either pooled or arrayed platforms. For example, stable cell lines expressing Ndeg-GFP can be used in genetic screens (e.g. CRISPR/Cas9 knockout screens) or in fully automated, high-throughput compound library screens for modulators with *in vivo* efficacy. Activators of ATE1 are predicted to decrease the GFP/mCherry ratio, whereas inhibitors should increase the ratio. In addition, this reporter can be used in experiments to determine if ATE1 is regulated in a context-specific manner (e.g. cell cycle or stress).

The expression level of the reporter as well as the cell type used in specific screening applications should be tailored to the desired outcomes. For example, a screen for inhibitors of ATE1 should be carried out with Ndeg-GFP expressed from a relatively weak promoter (so as not to exhaust degradation machinery) and in cells that have robust N-degron activity. In this report, the URT-based mCherry-Ub-Ndeg-GFP construct was expressed using the mouse prion promoter as it generates adequate levels of mCherry-Ub and Ndeg-GFP for easy detection, but does not exhaust degradation machinery in Neuro2a cells. Clonally derived, stable cell lines using this reporter should be validated on an individual basis by comparing fluorescence ratios in the presence and absence of proteasomal inhibition to determine the sensitivity of the reporter cell line.

The design of this reporter also incorporates additional features that are beneficial in screening applications. Due to the hierarchical nature of the N-degron pathway (29), otherwise identical Ndeg-GFP reporters bearing alternative N-terminal amino acids can be used to target alternative

steps in the N-degron pathway or in orthogonal screens to validate modulators and help determine their "mode of action". Here, we use Ndeg-GFP bearing N-terminal Asp (Asp-Ndeg-GFP) so that its degradation would depend on ATE1. Asn-Ndeg-GFP or Gln-Ndeg-GFP, on the other hand, can be used to identify modulators of NTAN1 or NTAQ1, respectively. Modulators that influence the stability of Leu-Ndeg-GFP, for example, will likely regulate the N-degron pathway downstream of ATE1.

Summing up, we have generated and validated a robust, cell-based, dual-fluorescent reporter to identify modulators of ATE1. Downstream applications of this reporter include its use in the search for inhibitors and activators of ATE1, which may ultimately have therapeutic potential to treat human diseases such as obesity and neurodegeneration.

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