AWARD NUMBER:
W81XWH-18-1-0515

TITLE:
Investigating the Oligomerization of TorsinA as a Means to Develop DYT1 Dystonia Therapeutics

PRINCIPAL INVESTIGATOR:
Thomas U. Schwartz

CONTRACTING ORGANIZATION:
Massachusetts Institute of Technology

Cambridge, MA 02139-4301

REPORT DATE: September 2019

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
Sept 2019	Annual	15 Aug 2018 - 14 Aug 2019

4. TITLE AND SUBTITLE

Investigating the Oligomerization of TorsinA as a Means to Develop DYT1 Dystonia Therapeutics

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-18-1-0515

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Thomas U. Schwartz

E-Mail:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Massachusetts Institute of
Technology (MIT)
77 Massachusetts Avenue
Cambridge, MA 02139-4307

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Dystonia is movement disorder than manifests itself in repetitive, involuntary muscle contractions, affecting parts (focal) or the entire (general) human body. A glutamate deletion (deltaE) in the enzyme TorsinA triggers the most common form of generalized dystonia, Toxins and traumatic brain injury can also trigger dystonia. The molecular mechanism of the disease is unclear. In this project we are examining the three-dimensional structure of TorsinA, particularly its filamentous form, and To develop we are establishing assays to screen for effector molecules that will rescue the enzymatic activity of TorsinAdeltaE. In this progress report we lay out the advances that have been made in the first year of the funding period. We have published the filamentous structure of TorsinA, which was our first specific Aim. We are now engaged in improving the resolution of the published structure, and we are trying to establish a membrane-bound form of the protein. To develop the functional assays, we are in the process of establishing a procedure to produce milligram quantities of TorsinA at high purity.

15. SUBJECT TERMS
Protein expression, purification, structural analysis, dystonia

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

1. Introduction

Dystonia is a movement disorder than manifests itself in repetitive, involuntary muscle contractions, affecting parts (focal) or the entire (general) human body. The glutamate deletion at position 302 (deltaE) in the enzyme TorsinA triggers the most common heritable form of generalized dystonia, DYT1. Toxins and traumatic brain injury can also trigger dystonia. The molecular mechanism of the disease is unclear. DYT1 likely provides the most tractable form of the disease, due to the direct causality between the mutation and the disease. In this project we are examining the three-dimensional structure of TorsinA and use that information to establish functional assays that can hopefully lead to drugs against this incurable disease in the mid- to long-term. TorsinA belongs to the AAA+ ATPase family of proteins. These proteins are known to enable the remodeling or degradation of their substrates (proteins or nucleic acids), catalyzed by ATP hydrolysis, chemical energy that is used to generate mechanical force. TorsinA differs from canonical AAA+ ATPases in two fundamental and surprising ways – first, the protein does not self-activate but requires an activating protein, LAP1 or LULL1, to trigger ATP hydrolysis. Second, the protein does not form hexameric rings but rather filamentous assemblies. From our prior analysis we know that the deltaE mutation prevents ATPase activation by LAP1/LULL1. Our preliminary data, when applying for this grant, also suggested that the deltaE mutation prevented filament formation. Here I describe our progress within year 1 of the funding period.

2. Keywords

Dystonia, TorsinA, DYT1, AAA+ ATPase, protein expression, protein purification, structural biology, cryo-electron microscopy, X-ray crystallography

3. Accomplishments

We structured this grant application around three specific aims. Here I describe progress in year 1 on those three aims.

Specific Aim 1
To determine the three-dimensional structure of TorsinA in its filamentous form

We have determined the 3D structure of the filamentous form of TorsinA at 4.4 Å and published it (Demircioglu et al., Nat. Comm., 2019). The structure shows that TorsinA is ATP-bound in the filamentous form, and that it has 8.5 molecules per helical turn. The structure was solved by helical reconstruction using micrographs obtained by cryo-electron microscopy. We have undertaken a mutational analysis of TorsinA in order to figure out which residues are important for filament assembly. Importantly, the deltaE mutation also does not form filaments. We seek to exploit this behavior for our planned functional analysis. (Specific Aim3). The filamentous structure that we observe can be modulated when we incubate the protein with liposomes. In the presence of liposomes, we observe tubulations or protrusions emanating from the liposomes. These protrusions are protein-coated, but, importantly, they have a larger diameter than the membrane-free TorsinA assemblies. Therefore, we hypothesize that Torsin can form filaments of different diameters, raising the interesting question about which adjustments the protein has to make in order to form filaments of different thickness. Our cryo-EM structure reaches a resolution of 4.4 Å, which is not sufficient to see atomic details. Consequently, the atomic details of filament formation / regulation and an exact view of the bound nucleotide are still unclear. To achieve higher resolution, we attempt to solve crystal structures of paired Torsins. The idea here is to crystallize a pair of TorsinA molecules and to visualize their interaction at high resolution. To do this, we need to prohibit filament formation. To enforce a TorsinA dimer, we generated a fused TorsinA-TorsinA construct, in which a flexible glycine-serine linker tethers two proteins together. To prevent further polymerization, we introduced mutations on the backside of the C-terminal TorsinA molecule in the pair, which we have shown to ablate TorsinA-TorsinA interaction. With this construct we are now in the position to pursue X-ray crystallographic studies, with the goal to possibly get a structure of the TorsinA-TorsinA interface at atomic (<1.3Å) or near-atomic resolution (~1.3-2.3Å). These experiments

are ongoing. There are a number of constructs that we are also testing. For example, sealing the backside of the fused TorsinA dimer could also be achieved by adding a LAP1 or LULL1 molecule. We are testing these options.

Another important research direction is to establish a procedure that produces large, milligram quantities of TorsinA. This is acutely necessary for establishing the functional assays we have in mind (aim 3). We are pursuing this task aggressively, exploring different strategies in parallel. In the past, we have exclusively expressed and purified recombinant TorsinA from bacterial cells. Specifically, we used an N-terminal truncation to produce the largest quantities of protein (residues 51-332). This protein tends to aggregate, cannot be stored well, and it is often somewhat contaminated with GroEL, an E. coli chaperone. For our structural studies so far, this protein preparation was of sufficient quality. Now, since GroEL is an ATPase itself, it is critically important to eliminate it before we can use our TorsinA preparation for ATPase activity assays. To solve this problem, we are going in two directions. First, in keeping with bacteria as our expression host, we are testing whether any of the human TorsinA homologs, i.e. TorsinB, Torsin2A, Torsin3A, or Torsin4 is easier to produce. There is no option to predict the outcome, so this needs to be tested experimentally. These experiments are ongoing. We are also trying a number of homologs from other species to understand whether one of them may just produce better and more protein. While, in the end, we will need human TorsinA, such tests with homologs may give us the hint on how the human TorsinA may need to be mutated locally such that it becomes more soluble and less aggregation-prone. There are numerous examples in the literature and in our own experience showing how just a single surface residue mutation can dramatically affect protein solubility and purity. Alternatively, we are also testing whether eukaryotic expression systems may be more suitable for TorsinA production. To this end, we are testing baculovirus infected insect cells as an expression system. So far, we have tested full-length TorsinA, C-terminally tagged with a His-tag. It expresses and we can purify it, however, the majority of the protein is insoluble. Curiously, a fraction of the protein is glycosylated (as determined by SDS-PAGE analysis and PNGase treatment) and this form remains soluble. We mapped two surface-exposed asparagine residues to be phosphorylated. A variant that we are now testing has these asparagines replaced with the more soluble aspartate sidechain, testing whether we will be able to shift more of the protein into the soluble fraction. Another approach we are testing is to express TorsinA in insect cells without signal peptide. This would keep the protein in the

cytoplasm rather than targeting it to the endoplasmic reticulum, which may affect protein production and solubility as well. In summary, we are pursuing a wide panel of approaches in order to improve recombinant Torsin production. This is critical for establishing our functional assay and for the wider Torsin/DYT1 research community.

Specific Aim 2

To determine the three-dimensional structure of TorsinA in its filamentous, membrane-bound form

The membrane-bound filamentous form of TorsinA poses a new challenge with regard to its structural characterization by cryo-electron microscopy. In comparison to the unbound form, the protein-coated membrane protrusions we observed on liposomes are less uniform. The protrusions are not straight, but appear somewhat askew. For helical reconstruction, it is important to have a regular, repetitive structure, in order to exploit the power of averaging for gain in resolution. At the current stage, the filamentous protrusion that we can produce are not suitable for cryo-EM reconstructions. To improve the situation, we are experimenting with generating membrane nanotubes (MNTs) directly, rather than relying on extruding them from spherical liposomes. This way we can control the composition better, and also diameter and length. MNTs can be produced without the irregularities that we believe are inherent to the liposome extruded structures. The work on generating MNTs and to assemble TorsinA onto it has just begun. We believe that within the next 3-6 months we will know how feasible this approach may be.

Specific Aim 3

To develop assays to rescue activation and oligomerization of TorsinA dystonia mutants using small molecules

We have established that TorsinAdeltaE compromises Torsin function in at least two ways. First, we have shown that TorsinAdeltaE cannot be properly activated by LAP1 or LULL1, thereby rendering the enzyme inactive. Since the deltaE mutation only results in a small surface variation on TorsinA, albeit with significant consequences, we argue that small molecules may exist to partially rescue the functionality of TorsinAdeltaE. In a first

experiment we plan to couple a nanobody stabilized TorsinA-LULL1 and a TorsinAdeltaE-LULL1 complex onto an affinity column and incubate both complexes with a DNA-encoded drug library. We then seek to identify the drugs that selectively bind the deltaE variant. The screening is done by deep-sequencing the two DNA-encoded library pools We collaborate with David Liu's lab at the Broad Institute on this project. At this point we have performed pilot experiments to ensure that the principal approach works. The current bottleneck is the availability of the most suitable DNA-encoded library. The Liu lab is engaged in constant optimization of their libraries. We are currently waiting for the next version of the library to be accessible, at which point we are ready to perform the experiment immediately. We expect this to happen in the next 6 months.

The second assay we are developing is to monitor ATPase activity in a two-component system, i.e. with both TorsinAdeltaE and its activator present in the test tube. We seek to identify small molecules that rescue the activation. As a readout we are planning to use a colorimetric ATPase assay based on the production of a green complex that forms between malachite green, molybdate, and free orthophosphate. We have established that this assay principally works in our hands. The protein prep we used so far was, however, contaminated with GroEL (see Aim 1 accomplishments above) which results in an unacceptably high background (because GroEL is itself an ATPase). We are actively working on eliminating this problem, as detailed above. As soon as the protein is ready, i.e. free of a contaminant with ATPase activity, we can setup the drug screen immediately.

The third assay we have planned will probe restoration of filament formation of TorsinAdeltaE in the presence of small molecules. For this assay we need to measure protein oligomerization in a high-throughput fashion. This is currently the least developed element of the proposal. In a first experiment we will use dynamic light scattering to probe for restoration of oligomerization of TorsinA deltaE. DLS is very sensitive to detecting small amounts of oligomeric species, but protein aggregation generates an even bigger signal. We recently acquired a brand-new technology, called mass photometry. Here we can also test for oligomerization in a small volume at low micromolecular concentrations. We will compare and contrast these approaches and will work on establishing a medium-to-high throughput approach. With the proper

protein supplied from our production pipeline (see Aim 1) we should be able to make substantial progress on this aim in the coming year.

4. Impact

At this early stage of the project, the biggest impact so far has been the publication of the filamentous form of TorsinA (Demircioglu et al., Nat. Comms. 2019). We have received a number of responses from the field as they welcomed this intriguing finding. As with any publication, the true impact to the field will establish itself over time.

5. Changes/Problems

Nothing to report.

6. Publications

Demircioglu, F.E., Zheng, W., McQuown, A.J., Maier, N.K., Watson, N., Cheeseman, I.M., Denic, V., Egelman, E.H., Schwartz, T.U. (2019). The AAA + ATPase TorsinA polymerizes into hollow helical tubes with 8.5 subunits per turn. Nature Communications. doi:10.1038/s41467-019-11194-w

7. Participants

Thomas U. Schwartz, Ph.D.
Principal Investigator
ORCID 0000-0001-8012-1512
Nearest person month worked: 12
Contribution to project: Oversees all projects and directs research
Funding Support: Receives 9-month salary from MIT. Receives additional summer salary from NIH sponsored research.

F. Esra Demircioglu, Ph.D.
Postdoctoral Associate
Nearest person month worked: 12
Contribution to project: Performed all tasks associated with the structural characterization of TorsinA

Xun Bao, Ph.D.
Postdoctoral Associate
Nearest person month worked: 5
Contribution to project: Xun Bao is primarily involved with all aspects of protein expression and purification, as well as establishing the functional assays.

Joshua David
Research Technician
Nearest person month worked: 8
Contribution to project: Helps in generating reagents, organizing the lab, and ordering reagents.