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

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14. ABSTRACT
Dystonia is a 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 drug candidates 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 second 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. To develop the functional assays, we are in the process of establishing a procedure to produce milligram quantities of TorsinA at high purity. Further, we have developed a new cell-based assay for drug screening.

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

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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 2 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 2 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 at the beginning of the funding period (Demircioglu et al., Nat. Comm., 2019). 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 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). While we have purified the proteins, we were not able to obtain crystals yet. We can test further experimental conditions, but there is a limit to the efficacy of this approach. Therefore, we will try to identify nanobodies to serve as crystallization chaperones. We had great success with this approach in the past and are very familiar with the technology. 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 were testing over the past year whether any of the human TorsinA homologs, i.e. TorsinB, Torsin2A, Torsin3A, or Torsin4 may be easier to produce. Unfortunately, in our hands none of th TorsinA homologs was produced in higher yield. 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 glycosylated. Using a asparagine-to-aspartate mutation we were able to drive most expressed protein into the soluble fraction. Excitingly, we can now produce milligram quantities of TorsinA, which is also well behaved as seen by chromatographic analysis. We are currently testing a number of truncation variants of TorsinA in the baculovirus expression system in order to have functionally relevant protein fragments for biochemical assays. In summary, we were able to establish a eukaryotic expression system for the production of milligram quntities of pure and functional TorsinA. 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 were experimenting with generating membrane nanotubes (MNTs) directly, rather than relying on extruding them from spherical liposomes. This way we hoped to 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. Work over the past 9 months has shown that this is a difficult approach. Also, since we do not consistently see TorsinA filaments in all our preparation, notably much fewer in the insect cell expression system when compared to the bacterial expression system, we are at this point not sure whether there is too much merit in chasing a membrane-bound filamentous form of TorsinA. Specific Aim 3 is now the main focus, since it should most directly lead to drug leads, which would ultimately be the most consequential outcome of this study.

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. Due to the COVID-induced complete shutdown of our institute, from March 2020 until the partial opening end of June 2020, the Liu lab has lost critical time to assess their newest DNA-encoded library. We should still be able to screen for drugs in the first quarter of 2021.

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). With the baculovirus expression system yielding much purer TorsinA, we are now in the position to run a drug screen through this platform.

Over the past year we have developed yet another assay platform, modified from the so-called HiBiT system by Promega. Here, we transiently transfect HEK293 or HeLa cells with TorsinAdeltaE tagged with LgBiT and LAP1 tagged with HiBiT. When both proteins interact, LgBiT and HiBiT complement to yield a luminescence signal. This assay is highly sensitive and can be carried out in cells. It is perfectly suited for high-throughput (HT) approaches. We have tested the system with TorsinA and TorsinAdeltaE and can see the expected drop in the signal for TorsinAdeltaE which interacts poorly with LAP1. Therefore, we reason that it should be straightforward to now use these cells in a HT format to test a compound library for drug candidates that improve the TorsinAdeltaE-LAP1 signal. The sensitivity of this assay, paired with the use of proteins expressed in human cell should be highly beneficial for our drug screening approach. Thus, we are very excited about the prospect of using this new cell-based approach.

4. Impact

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. Second, over the past year, we have found a way to significantly improve the yield of TorsinA for structural and functional studies. Third, we have established the HiBiT luminescence detection method as a means to test TorsinA-LAP1 interaction in cell lines. This will give us the possibility to do

drug screens in a high-throughput manner and we will start these screens in the coming months.

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

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Nearest person month worked: 12

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Funding Support: Receives 9-month salary from MIT. Receives additional summer salary from NIH sponsored research.

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Nearest person month worked: 4

Contribution to project: Performed all tasks associated with the structural characterization of TorsinA

Xun Bao, Ph.D.

Postdoctoral Associate

Nearest person month worked: 11

Contribution to project: Xun Bao is primarily involved with all aspects of protein expression and purification, as well as establishing the functional assays.

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Nearest person month worked: 11

Contribution to project: Helps in generating reagents, organizing the lab, and ordering reagents.

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Nearest person month worked: 6

Contribution to project: Structural characterization of TorsinA