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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 3 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 3 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 first attempted to solve crystal structures of paired Torsins. The idea was 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. While we have purified the proteins, we were not able to obtain crystals, unfortunately. Victoria Hernandez, a graduate student in the lab, works on finding the elusive TorsinA substrate and performed a tailored pull-down assay. In her yet unpublished results, she finds the TorsinA-homologs TorsinB and Torsin2 specifically co-purifying with TorsinA. Therefore, we speculate that they form heterooligomeric assemblies with TorsinA. We hypothesis, that these heterooligomeric species are potentially more important physiologically, and so we will expand our tethering approach to include heterodimeric Torsin-Torsin fusions.
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). Using baculovirus-infected insect cells, 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. We are also pursuing the bacterial

expression of the *C. elegans* homolog OOC-5, which has been reported to be easier to purify. Although rather a side project, it could be highly beneficial if successful. Structural characterization could help with understanding the phylogeny of the protein, and could help in narrowing down the functionally important regions.

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. The filamentous protrusion that we can produce, however 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. Performed in year 2, this approached proofed to be challenging. In the meantime, our cryo-EM facility was expanded to now include a cryo-focused-ion-beam (cryo-FIB) mill. With this instrument, we can generate thin lamellae from frozen cells for cryo-tomographic image reconstruction. As we can produce cells with long, TorsinA decorated membrane extrusions, we aim to now study them directly using the cryo-FIB milling technology. At the current stage, we believe that this gives us the best opportunity to get a better understanding of the membrane-bound filamentous form. While this technology will not yield atomic resolution, it should give us a much improved picture of the organization of the lipid protrusions and the Torsin coat in the unperturbed cellular context.

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 planned 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 sought to identify the drugs that selectively bind the deltaE variant. The screening would be 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 bottleneck still is the availability of the most suitable DNA-encoded library. The Liu lab is engaged in 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 within 2021.

Over the past 1.5 years we have developed an alterntive assay platform, modified from the so-called HiBiT system by Promega. Here, we first transiently transfected 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. The results using transient transfection were not reproducible to the standards we hoped for. Therefore, we decided to generate stable cell lines with CRISPR-modified TorsinA/TorsinAdeltaE and LAP1 proteins. As anticipated, the cell lines provide much more stable results. We have now started to screen large compound libraries using the HiBit system in conjunction with the stable cell lines. The Koch Institute for Cancer Research at MIT has a core facility for drug screening that we collaborate with. We are very excited that we now have established a highly sensitive drug screening platform. We anticipate to find drug candidates within

the next few weeks and months. Hits will be tested in biochemical assays to quantify ATPase activity enhancement and affinity. Structural characterization by X-ray crystallography will be performed for all promising hits.

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 now gives us the possibility to do drug screens in a high-throughput manner, which we currently perform with high intensity.

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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