Characterization of Ecm29: A 26S Proteasome Interacting Protein

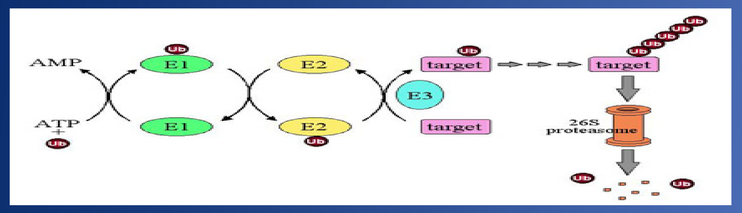
Ajinkya K. Nene, Frankie R. Gonzales, Dr. Patrick Gentry

Department of Biological Sciences, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093

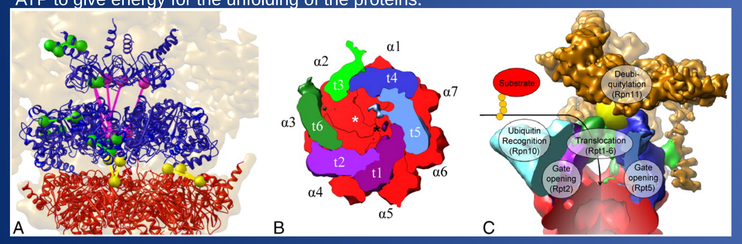
ABSTRACT

This investigation seeks to characterize the Extra-Cellular Matrix 29 protein, which has been found to interact with the 26s proteasome. The investigation mainly seeks to characterize the protein in the context of neurons. Different Isoforms of the protein were first identified by running lysates through a gel and by conducting an Immunoprecipitation. The IP gave us 3 isoforms of the Ecm29 protein, The smallest being between 40 – 50 kD. It was seen that Ecm29 was being degraded at a constant rate by conducting a Cyclohexamide Treatment. A Untreated, Bicuculline, Tetrodotoxin treatment was used, which showed that the Ecm29 wasn't affected by the strength of action potentials. With these same mouse neuron cells, a neuron staining was done. The neuron staining showed Ecm29 in the nucleus of neurites while absent in mature neurons. The dendrites were also lighting up. Probing for rpt6, a subunit of the proteasome, and gankyrin, a regulatory cap interacting protein, was done, with both proteins showing up in the IP and lysates.

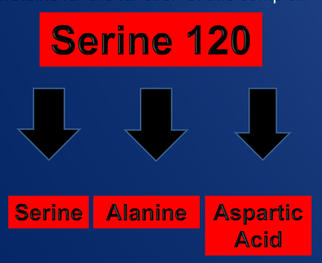
Background Information



Proteins targeted for the proteasome must have ubiquitin attached to them in a specific way. The ubiquitin is transferred through two pathways, E1 ligase to E2 ligase which transfers it to the E3 ligase, which joins the target protein and attaches ubiquitin. In the second method, the E2 and E3 ligase form a protein complex that attaches ubiquitin to the target protein. The regulatory cap recognizes certain sequences like lyseine-48 poly-ubiquitinated chains and lets them enter the 20s core particle. The ATPase's break down ATP to give energy for the unfolding of the proteins.



The 26s proteasome, pictured above, is a protein complex that degrades other protein through the use of the Ubiquitin-Proteasome System. It consists of a 2 19s regulatory caps and a 20s core. The 19s regulatory cap functions to register which proteins need to be degraded by identifying the ubiquitination signature. It consists of many subunits including rpn10 (recognition), rpt6 (ATPase/gate-opening), rpt2(ATPase/gate-keeper), etc. These subunits function to regulate the proteasome, and in turn are extremely important proteins for the function of this complex



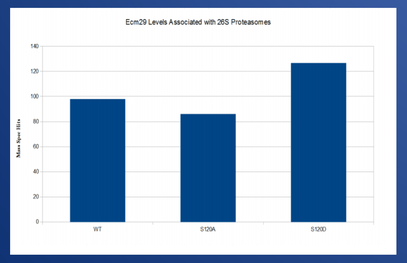


Figure 1: The data received by a Mass Spec analysis of the interacting/subunit proteins with the proteasome complex yielded interesting results for ecm29, for different rpt6 mutants. The Wild-Type proteasome functions as a regular Serine 120 residue. The Alanine(S120A) substitution acts as if it is anti-phosphorylated. Aspartic Acid(S120D) mimics being phosphorylated. Seeing that emc29 levels are affected by the levels of phosphorylation of rpt6, gives the need to characterize this protein. This characterization will shed more light on this protein.

Ecm29: Conservation between the 3 species

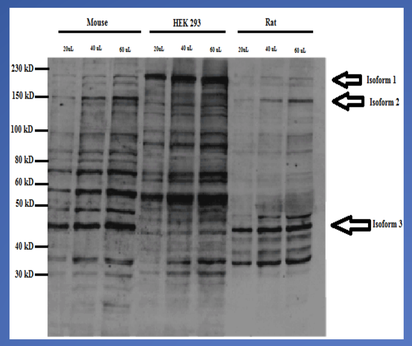


Figure 2: Mouse, HEK 293, and Rat lysates ran with Ecm29 being probed for. Increasing concentrations of 20, 40, 60 uL

Immunoprecipitation of Ecm29

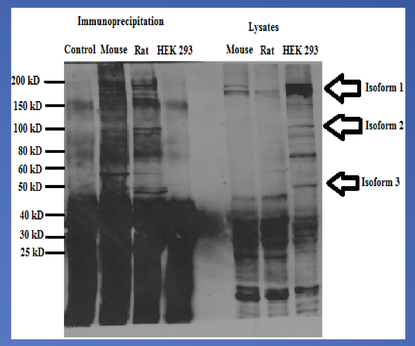


Figure 3: Immunoprecipitation of Ecm29. On the left is IP with the control rabbit serum, mouse brain, rat brain, and HEK 293. The lysates are run on the right to compare bands of Ecm29.

Untreated, Bicuculline, Tetrodotoxin Treatment

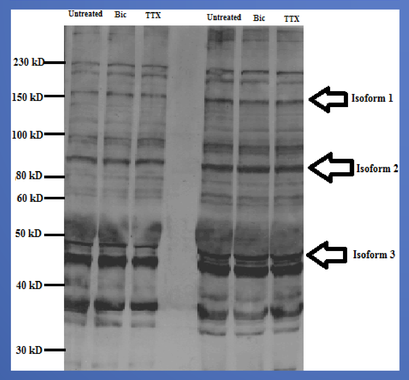


Figure 4: Used mouse neurons. Bicuculline inhibits the inhibitory action of GABA receptors. Tetrodotoxin blocks action potentials through the inhibiting voltage-gated channels. This treatment shows Ecm29 levels based on action potentials.

Cyclohexamide Treatment/ Protein Half-Life Experiment

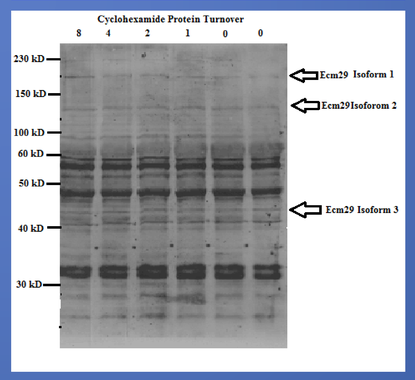


Figure 5: Cyclohexamide inhibits protein synthesis by inhibiting the translocation step. 8, 4, 2, 1, 0, 0 hour time points were used to see the rate of degradation of Ecm29

Neuron Staining Images using Fluorophore for Ecm29

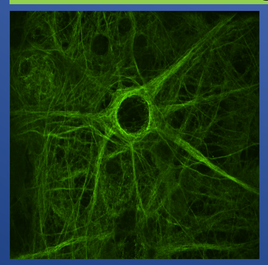


Figure 6: The neuron pictured above has no Ecm29 in its nucleus (Mouse Neuron)

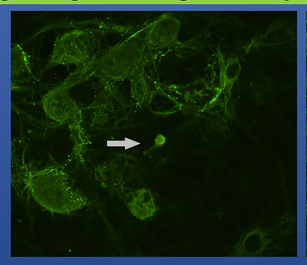


Figure 7: The neurite pictured above has Ecm29 concentrated in its nucleus (Mouse Neuron)

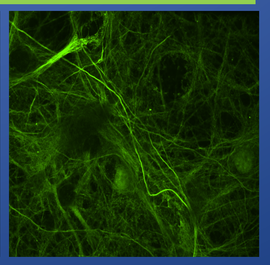


Figure 8: The dendrite pictured above is lighting up due to Ecm29 presence (Mouse Neuron)

Probing for Ecm29 Interacting Protens

Rpt6

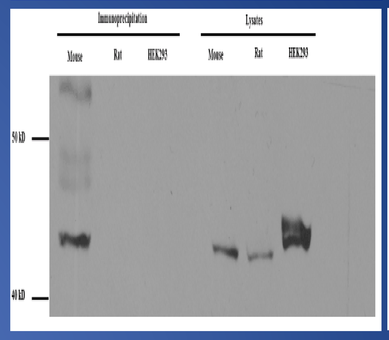


Figure 9: Using a rabbit antibody, the Ecm29 IP was stripped of antibody in a block solution, and probing for rpt6 was done. rpt6 showing means that it interacts with ecm29

Gankyrin

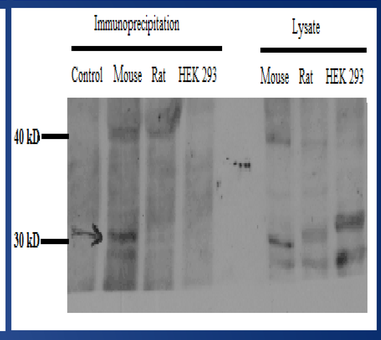


Figure 9: Gankyrin is a protein that helps assemble the proteasome. Using the same method as (5), gankyrin was probed for. It showed up around 30 kD.

CONCLUSIONS

- (2)Ecm29 was conserved across three species, rat, brain, and HEK 293 possibly because the proteasomes of these three species are also conserved.
- (5)The Cyclohexamide Treatment showed that the Ecm29 was being degraded at a constant rate.
- (3)The Immunoprecipitation of the Ecm29 showed 3 isoforms of the proteins between the three species.
- (4)The Untreated, Bicuculline, Tetrodotoxin Treatment showed that Ecm29 levels are not changed by action potentials in the neuron.
- (6,7)The neuron staining images showed the Ecm29 is concentrated in the nucleus of neurites, while lacking from the nucleus and in mature neurons. Because most proteasome assembly takes place during the neurite stage, it makes sense that the Ecm29 would be concentrated in the nucleus. As the neuron matures, the proteasomes move into the axon where they regulate synaptic plasticity, and possibly so does Ecm29.
- (8) The dendrites are lighting up very brightly, showing that Ecm29 is concentrated in these areas. This may show it still works with the proteasome post-assembly. Still, because the antibody was a bit dirty due to the HEAT motif, this can't be said with complete certainty.
- (9) This figure shows that Ecm29 interacts with the Rpt6 subunit. Rpt6 is a gatekeeper ATPase subunit[1]. Rpt6 is important for assembly also[1].
- (10) This figure shows that Ecm29 could possibly interact with Gankyrin. Gankyrin, or Nas6, is a protein that interacts with the rpt6 protein also. It is a chaperone protein and helps in assembly of the protein. A possible partnership between Ecm29 and Gankyrin hasn't been studied yet.

FUTURE DIRECTIONS

- Use constructs specifically targeted for Ecm29 rather than the rabbit antibody.
- Observe the effects on the proteasome after overexpression of Ecm29
- Look at the differences in Ecm29 for free cap and 26s Proteasome, and also during action potentials. Look at more Ecm29 trafficking.
- Investigate interactions between Gankyrin and Ecm29.

ACNOWLEDGMENTS

I would like to thank my mentor, Frankie Gonzales for being a great teacher and instructor. I would like to thank Dr. Patrick Gentry for his supportive guidance as I did this project. And finally, I would like to thank Dr. Komives for giving me this opportunity.

REFERENCES

Lander, Gabriel C., Eric Estrin, and Andreas Matrin. "Complete Subunit Architecture of the Proteasome Regulatory Particle." *Nature* 191st ser. 482.162 (2012): n. pag. Print. [1]