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Palmitoylation as a Regulator of MAGUK Proteins Postsynaptic Localization

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Abstract (Santiago)

Synaptic plasticity is the ability of the brain to make changes and the changes occur at synapses. To achieve the complicated functions, a good number of proteins are present at synapse and are called synaptic proteins. To stabilize these proteins at synapses, proteins are modified through posttranslational modifications (PTMs). The most studied PTMs include phosphorylation, acetylation, ubiquitination, glycosylation, palmitoylation, etc. Palmitoylation is a type of lipid modification and has received more attention recently for its contribution to protein trafficking, localization, and interaction in various synaptic plasticity. The membrane-associated guanylate kinase (MAGUK) family includes PSD-95, PSD-93 (also known as chapsyn-110), SAP102, and SAP97. They are present in the synapses and regulate the localization of synaptic proteins. Palmitoylation of PSD-95 has been demonstrated to involve in postsynaptic localization of PSD-95. SAP97 and PSD-93 contain palmitoylation sites that are very similar to PSD-95. However, the palmitoylation of SAP102 has not been fully studied. The proteomic analyses suggest the potential presence of palmitoylation of SAP102. In this study, we used human embryonic kidney 293 cells (HEK 293) to express SAP102. SAP102 palmitoylation was assayed using acyl-biotinyl exchange (ABE) method. The basal palmitoylation level of SAP102 and how the palmitoylation regulates SAP102 intracellular trafficking will provide a deeper understanding of SAP102 protein structure and how the protein plays a role at synapses.

Introduction: (Rozena)

Membrane-associated guanylate kinase (MAGUK) scaffolds are important components of the excitatory synapse's postsynaptic density (PSD) region. They play a role in structural and functional synaptic plasticity and interact with various proteins including receptors, adhesion, cytoskeletal, and signaling molecules. Of these MAGUKs, synapse-associated protein (SAP102) is a dominant scaffold of the fetal brain. Clinically, mutations in SAP102 are identified in nonsyndromic X-linked mental retardation and are associated with increased long dendritic filopodia. It's suggested to be involved in experience-dependent cortical development and is formed in early developmental stages. SAP102 knockdown has also been shown to reduce dendritic expression of the EphB signaling pathway, involved in actin reorganization, and decreased synapse formation and glutamate receptor trafficking in young cortical neurons.

(Murata et al., 2013) Studies have also shown that the increase in SAP102 and its mRNA concentration parallel the growth of newly formed synapses in the developing rate cerebral cortex as well. It also structurally resembled a tumor suppressor gene essential for the appropriate assembly of septate junctions in the drosophila fly and was able to rescue neuromuscular junctions in mutant phenotypes of the gene. All these factors suggest a critical role in synaptogenesis. (Muller et al., 1996) (Murata et al., 2013)

SAP102 and PSD95 in the cortical cytomatrix located at the PSD, where receptors are localized according to neurotransmitter release sites, have been shown to bind subunits of the postsynaptic n-methyl-d-aspartate (NMDA) receptor. (Muller et al., 996) These ligand-gated ionotropic glutamate receptors are involved in fast synaptic transmission and synaptic plasticity at excitatory synapses in the central nervous system and SAP102 may be involved in NMDA receptor clustering and immobilization. (Lau et al., 1996) Studies show that SAP102 knockdown decreased NMDA receptor-mediated excitatory postsynaptic currents during early development suggesting a crucial role in NMDA receptor trafficking during synaptogenesis. (Wei et al., 2018)

The MAGUKs including SAP102 and PSD 95 link NMDA receptors to the actin cytoskeleton and rely on posttranslational modifications for their functions. PSD95 requires palmitoylation for synaptic localization and phosphorylation increases its synaptic accumulation. SAP102 is enhanced by phosphorylation but has not been shown to be palmitoylated. (Chen et al., 2011; Wei et al., 2018) Palmitoylation, the reversible attachment of palmitate to cysteine residues, impacts protein dynamics including PSD 95's involvement in synaptic plasticity and we theorize it to affect SAP102 due to potential palmitoylation sites found by proteomic analysis. (Matt et al, 2019) In our study, we use human embryonic kidney 293 (HEK 293) cells to express SAP102 and asses palmitoylation using the acyl-biotinyl exchange method.

Methods: (Santiago)

Bacteria transformation

In order to express SAP102 in HEK 293 cells, we amplified SAP102 plasmid in BL21 competent cells. We created a mixture of LB agar powder and water, which served as the base. We autoclaved it and, after it had cooled, added a carefully calculated concentration of ampicillin in order to prevent other bacteria from growing. The final mixture was then poured over a 100 mm dish and allowed to solidify. Next, we incorporated a transformation process which involved introducing the SAP102 plasmids into the bacterial environment. After thawing out and centrifuging the frozen BL21 cells, we pipetted the plasmids into the competent cells. After the introduction, the next step was to run the culture through a series of environmental changes that optimized both the bacteria and plasmids for effective transformation. The culture was flicked several times to ensure a homogenous mixture and incubated in ice. It then ran through an

incubation period of heat shocking followed by more ice immersion. The culture was then introduced to SOC medium and incubated inside of an incubator shaking series. This was conducted for an hour at a steady temperature of 37 degrees Celsius. Finally, the plasmid / bacterial culture was split into agar plates of various concentrations to allow a fair comparison of growth. These plates were incubated at 37 oC overnight.

Plasmid amplification and extraction

The following day we would obtain 14 ml tubes with our LB / Ampicillin mixture and inoculated a single bacteria colony with a pipette tip and placing it inside the 14 ml tubes. These were then placed in a shaking incubator overnight. The next day we began our DNA extraction. The process of extracting and isolating our culture's DNA was performed according to manufacturer's protocol. For example, the initial process involved adding 250 microliters of R3 into our tube of glycerol / liquid culture, which was then resuspended and added to 1.5 ml tube. 250 microliters of L7 was added and the tubes were inverted several times before being incubated at room temperature for five minutes. 350 microliters of N4 were added and inverted 10 more times before being centrifuged at 12,000 rpm for 10 minutes. Our liquid cultures were subjected to further solutions and centrifugations until we were eventually draining out the last of our flowthrough and observing our DNA extraction. In order to ensure the DNA concentration was sufficient, we ran a microliter of our sample through a Nanodrop program, which calculated the concentration of our product.

Cell culture

Cells were cultured in DMEM, supplemented with 10% FBS and ampicillin/streptomycin. Because we were constantly needing healthy bacteria, we often split the cells. This was done with careful consideration. After looking at the plate through the microscope, we predicted the growth percentage and determined how many dishes we would split it into. We then completed a series of steps that involved detaching the cells from the plates with trypsin, resuspending them in complete media, and centrifuging to assure a cell pellet. After the centrifugation process, we would vacuum the supernatant and either resuspend and divide into plates with fresh complete media, or mix in freezing media and store in the cryotank for future use.

Cell transfection

Although there was proper SAP102 growth within the bacterial cultures, the overall purpose of this experiment was to determine the level of palmitoylation that occurs within mammalian cells. In addition to the transformation process conducted on the bacterial lineage, our SAP102 plasmid was also introduced to Human Embryonic Kidney 293. The process of incorporating our plasmid into the mammalian cells is known as transfection. With transfection, the HEK293 cells will take up the plasmids and merge the genetic information with their own. From here, through a series of splitting and freezing our cells, our experiment will have an adequate amount of cells needed to conduct the Western Blot. This protocol began with observing the previously incubated dishes

under the microscope. If they were confluent, we removed the old medium, added fresh CM, and incubated them again for half an hour. During this time, we prepared our solutions of SAP102 plasmid and the LipoD293. This was done by mixing them separately into 1 ml of DMEM and vortexing the until properly dissociated. From here, the Lipo was added into the DNA and vortexed immediately. It was crucial that the Lipo was added to the DNA, otherwise we would not yield accurate results. After the dishes had finished their incubation period with the new medium, we added 500 microliters of our solution to each of the dishes, which were then incubated until further notice. If this was completely correctly, our incubated HEK293 dishes will have the SAP102 genetic information incorporated. With this gathered, we can conduct the necessary DNA extractions and Western Blots needed to measure the level of palmitoylation SAP102 undergoes in mammalian cells.

Western bloting

Western blots were completed several times over the course of our research in order to ensure the desired SAP102 protein was being expressed. In order to complete the western blot, however, we first had to construct our SDS. This involved constructing the gel electrophoresis and setting up the wells with appropriate levels of our samples. The first and last wells were filled with our ladder, demonstrating a base for us to compare our samples to. The remaining wells consisted of increasing sample loads, ensuring we had many opportunities to identify our protein expression. Running buffer was added to the gel electrophoresis and our SDS-PAGE ran for an hour and ten minutes. Afterwards, we obtained filter paper for our gel and we transferred our proteins to the nitrocellulose membrane (NC membrane). The process ran for another hour and forty five minutes, which yielded our samples embedded into the NC membrane. The NC membrane was placed inside a western blot box, soaked in TBST and washed. Finally, the NC membrane was rinsed and incubated with our primary antibody. This would then be stored in the 4 degrees Celsius cold room until the follow day, at which it was washed several more times and incubated with the secondary antibody. This served as the solution to which our NC membrane were incubated on the shaker for an hour at room temperature. The NC membrane was then washed with TBST and the protein bands were detected using a chemiluminescent (ECL).

Results & Discussion: (Rozena)

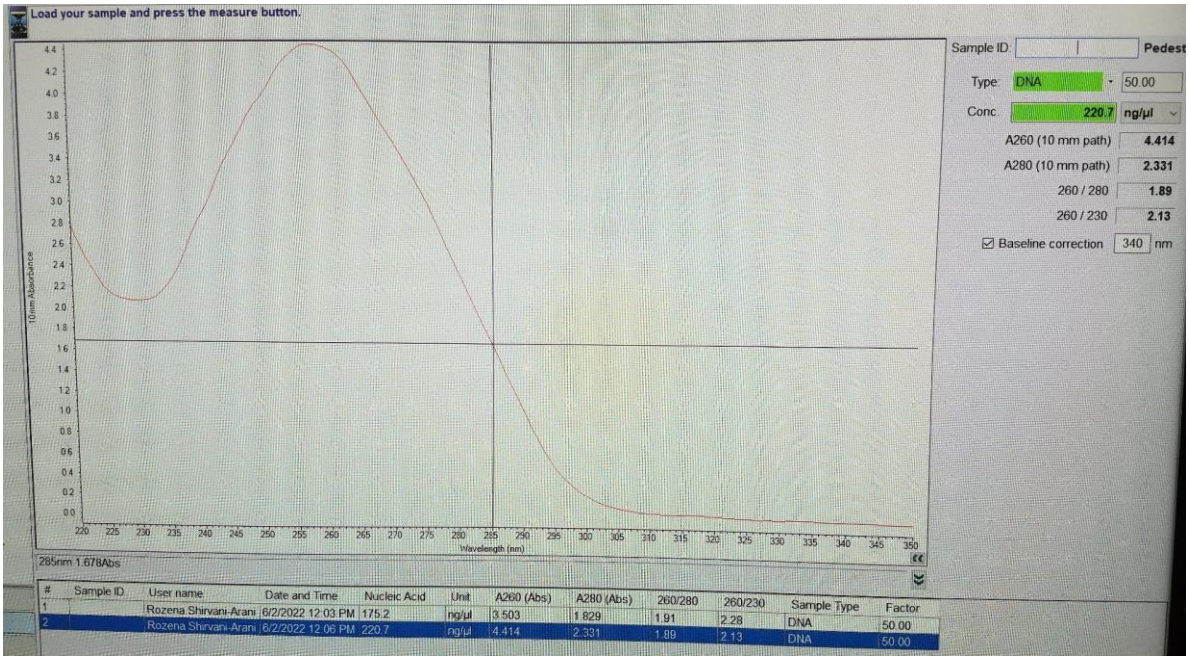

Figure 1. DNA extraction results

After SAP 102 was transformed and grown on colonies, inoculated a liquid bacterial culture at 37oC overnight. DNA extraction of the plasmid was done in preparation for future experiments. This was repeated several times to collect a sufficient amount of concentrated DNA. In some cases, the overnight culture was unable to shake at an appropriate speed due to the machine being in use which resulted in low levels of bacteria growth and DNA concentrations. However, most preparations were successful with a concentration of DNA ranging from 125- 220 ng/ul which is to be expected. An example of the resulting DNA analysis from the nanodrop machine is shown in Figure 1 where the wavelength curve and concentration levels were within normal levels.

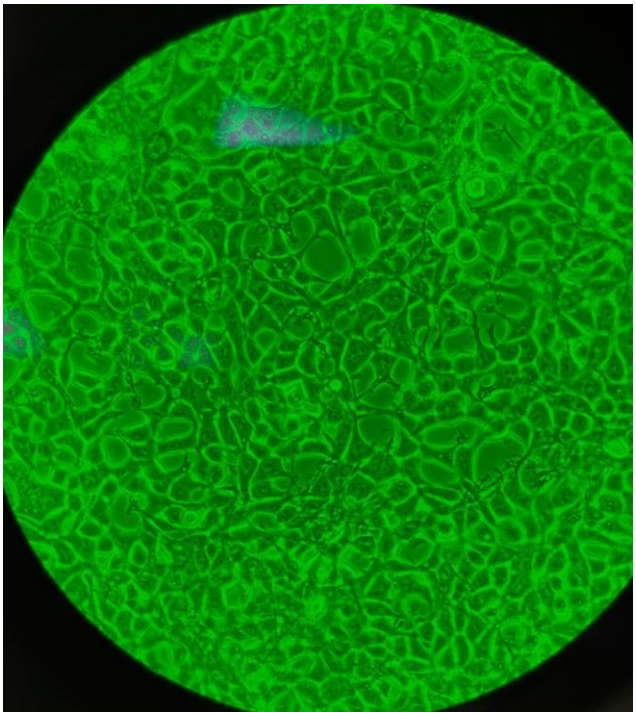

Figure 2. Cell Culture of HEK 293

The cell cultures using HEK 293 cells were successfully grown, split, and frozen for later use. We had a 90% confluency of cells shown in figure 2 and will be used in later transfection protocols with the SAP102 plasmid.

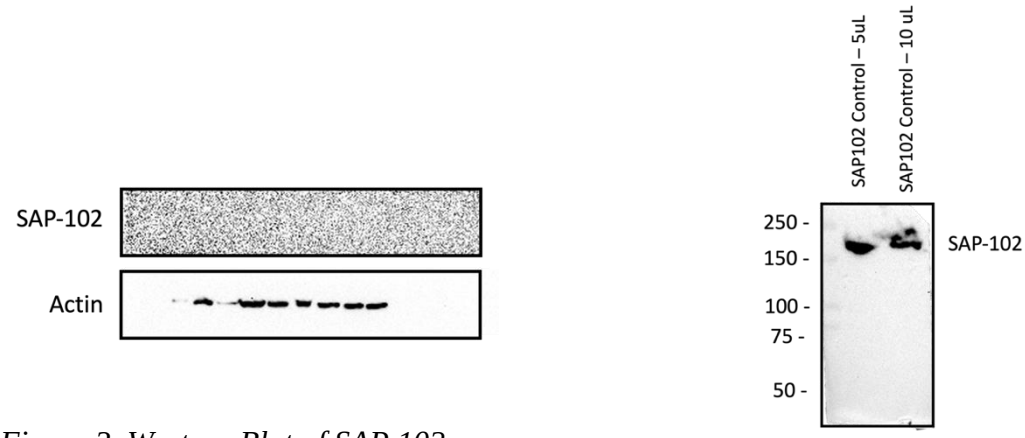

Figure 3. Western Blot of SAP 102

The Western blot was repeated twice to assess the expression of SAP 102 in our plasmid. Not only was it not expressed in the pulldown samples, where palmitoylated SAP 102 would be isolated, but also was not in the input samples, where all total SAP 102 proteins are present Figure 3A . Actin was expressed in the input samples as expected. Due to the lack of expression of the SAP 102 protein in our plasmid which was constructed specifically to carry the SAP 102

DNA, we went back to review the plasmid's information. as shown in Figure 3B, the initial western blot analysis done earlier in the lab has shown clear bands in WB analysis. However, the positions of the protein bands were much higher (above 150 kDa) than what was predicted (102 kDa). This would explain why it was not expressed in our western blot as our antibodies targeted SAP 102 and there was no SAP 102 protein expressed in our cells. In light of this, we purchased new SAP 102 plasmid and will re-transfect HEK 293 with the new plasmid and continue to study its interaction with palmitoylation when possible.

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