Modulation of Alzheimer’s Disease related APP Trafficking via Protein Kinase C

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In Dedication to the faculty and staff at The University of Arizona

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I would like to thank Nicole Young, Sarah Ly, Glenn Sapp, Kristin Penunuri, Vanessa Nelson, and Nicole Konkowski, for their support and assistance with experiments on this project. I would like to thank Dr. Jonathan Valla, my mentor for this project, for his support and guidance throughout the years and especially with the thesis.
Abstract

Chronic phorbol ester treatment treatment of a neuroblastoma cell line, SH-SY5Y, was hypothesized to induce alterations in APP expression and trafficking such that the holoprotein will increasingly localize to mitochondria. Fluorescent immunocytochemistry and confocal microscopy was used primarily to visualize co-localization of Amyloid precursor protein (APP) to Translocase complex of the outer mitochondrial membrane (TOMM machinery) on the mitochondria. Co-localization experiments were inconclusive in showing that chronic phorbol 12-myristate 13-acetate (PMA) treatment affects APP trafficking to mitochondria. Due to those results, mitochondrial gradient fractionation experiments and subsequent western blots were started to determine if there was increased APP expression at the mitochondrial membrane and if protein kinase C (PKC) was activated by chronic PMA treatment.
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Introduction

a. Alzheimer’s Disease Background

Alzheimer’s disease (AD) is a progressive form of neurodegeneration that has been identified as the primary cause of cognitive decline in elderly individuals. There are two forms of AD present in patient populations. The first form, familial AD, makes up a minority of cases and is linked to inherited mutations in amyloid precursor protein (APP), presenilin-1 (PS1), or presenilin-2 (PS2) (Anandatheerthavarada et al 2003). The second arises from no known genetic mutation and has been termed sporadic AD (Sherrington et al, 1995), making up over 90% of AD cases. Although there are obviously different mechanisms in both forms of AD, the resulting neuropathology remains the same (Sherrington et al, 1995).

AD has been linked to abnormal mechanisms involving the cleavage of the amyloid precursor protein (APP) in neurons. APP is a plasma membrane protein that is primarily located in neurons. Its function is not known, but there are many theories suggesting its involvement with neuronal plasticity and neurogenesis (Vetrivel et al 2005). APP is ultimately cleaved by membrane associated enzymes, α-secretase and β-secretase, to produce β-amyloid and other peptide
fragments (Figure 1). β-amyloid is theorized to be a key component to 
the formation of plaques in the brain of AD patients. However, there 
has been recent evidence suggesting that the APP holoprotein may 
play a role in AD pathophysiology. The N-terminus of APP includes a 
partial mitochondrial targeting sequence, and, under unknown 
conditions in AD, APP arrests in the pore [of the mitochondrial 
translocase complex], blocking the import of other nuclear-encoded 
mitochondrial proteins and interfering with normal function 
(Anandatheerthavarada et al 2003). Because of this, it is thought that 
APP plays a key role in the pathogenesis of AD.
Figure 1: Proteolytic Processing of APP
The Aβ domain is shaded red and in panel A. Sites of cleavage by α, β, and γ-secretases are shown in panels B and C. Amyloidogenic processing of APP is carried out by sequential acting of β and γ-secretases (Vetrivel et al 2006).
b. APP Protein Trafficking

APP contains a specific targeting signal that is recognized by the translocase complex of the outer mitochondrial membrane (TOMM complex). Initially, the signal is recognized at the outer membrane by receptor proteins, identified as TOMM70, TOMM20, and TOMM22. The protein will move through the import pore, TOMM40, after that initial recognition. The translocase of the inner membrane (TIM) protein further targets proteins to different locations in the mitochondria. However, some evidence suggests that APP gets lodged in the TOMM machinery without fully entering the mitochondria in AD (Anandatheerthavarada et al., 2003), which provides a physical block in the mitochondrial import channels leading to reduced import of nuclear-encoded mitochondrial proteins (Devi et al 2006). The sequence that causes the arrest is a positively charged acidic region, different than the targeting signal to the mitochondria. The focus of this project was to replicate the mechanism leading to APP targeting and potential arrest in the translocase pore.
**Figure 2: A representation of APP.**
The mitochondrial targeting sequence is more upstream of the amino acid sequence that causes translocational arrest, consisting of a positively charged acidic region (Anandatheerthavarada et al. 2003)
Figure 3: Hypothesized models for the arrest of APP translocation into mitochondria via TOMM machinery

These are hypothesized models for the translocation of APP into the mitochondria. The acidic domain of APP is the targeting sequence that is initially recognized by the TOMM machinery, which most likely plays a role in arrest. In A and C, there is arrest. When the acidic domain is removed in B, APP is able to completely translocate. OM = outer membrane; TOMM = translocase of the outer membrane; IMS = intermembrane space; IM = inner membrane; N = amino terminus; C = carboxy terminus.
(Ananadatheeravadan 2007)
APP trafficking can be affected by a variety of factors, one being protein kinase C (PKC). Protein kinase C (PKC) is an enzyme that phosphorylates other proteins to manipulate their function. Phosphorylation is the addition of a phosphate group, usually to hydroxyl groups of particular amino acids on these proteins. PKC can be activated by a variety of signals, such as increasing levels of diacylglycerol (DAG) or calcium ions. PKC consists of two different domains, that work together to activate proteins involved with signal transduction pathways. The regulatory domain binds DAG, calcium ions, and other compounds such as phorbol esters. The catalytic domain binds ATP and substrate. This domain must be phosphorylated itself in order for PKC to be activated. When activated, PKC is translocated to the plasma membrane of cells where it will further act on other other proteins. PKC also has many isoforms, which differ by whether DAG or calcium ions are required for activation. In this particular study, PKC-α and PKC-ε will be analyzed. PKC-α requires DAG, calcium, and phospholipid for activation, while PKC-ε is activated by DAG only. In addition to these cofactors, PKC activation can be modulated by other exogenous molecules and proteins as well.
In particular, PKC can be upregulated when it undergoes a post-translational modification called myristoylation. In myristoylation, a myristoyl group is covalently attached to the alpha-amino group of an N-terminus of a newly translated protein via an amide bond.
Figure 4: Myristoylation Reaction

The enzyme N-myristoyl-transferase (Nmt) is what drives the reaction by transferring the myristoyl group from myristoyl-coenzyme A. The myristoyl group is enclosed in the green box.
The compound that completes the myristoylation is phorbol 12-myristate 13-acetate (PMA), a compound that is added exogenously in the protocol. PMA can myristoylate a particular protein kinase (either PKC\(\varepsilon\) or PKC\(\alpha\)), ultimately affecting the transport of APP to the mitochondria and its subsequent entrapment in the mitochondrial translocase, the TOMM proteins. The myristoylation of PKC affects the subsequent trafficking of APP to the mitochondria (da Cruz 2009) because it becomes activated via phosphorylation.

b. Objectives and Goals:

Our primary goal was to induce increased mitochondria-directed trafficking of APP, using PKC, that, when myristoylated and activated, is hypothesized to induce alterations in APP's normal secretory trafficking and overall expression.

Chronic PMA treatment of a standard neuroblastoma (SH-SY5Y) cell line was hypothesized to induce alterations in APP expression and trafficking such that the holoprotein will localize to the mitochondria, and we will be able to visualize this change with fluorescent immunocytochemistry and confocal microscopy. This high risk, high reward approach to testing our hypothesis ultimately did not succeed and we subsequently began additional assessments to test
each step in the induction of the trafficking changes: the activation of PKC, expression of APP, and isolation of a relatively pure mitochondrial fraction to probe for differing levels of APP. The ultimate goal was to induce APP-mitochondrial interactions in order to assess the consequences on mitochondrial function and viability.

c. Significance:

AD is the leading cause of dementia in individuals who are 60 years of age or older. As medicine and biomedical technology advances, illnesses that once claimed many lives are now easily treatable or quickly detected. Because of this, people are now living longer lives and are more vulnerable to developing AD; the leading risk factor for AD is age. More than 5 million Americans are believed to have AD and by 2050, as the U.S. population ages, this number could increase to 15 million. AD is also becoming more common worldwide, with an estimated 26 million people affected. This global figure is projected to grow to more than 106 million by 2050 (American Health Assistance Foundation). AD has a significant burden on the healthcare system because of its financial costs and emotional detriment to families. Currently, there are no cures for AD, even though the number of diagnosed cases continues to rise. The treatments that exist may
alleviate the burden temporarily in some, but the disease continues to relentlessly progress. Discovery of new mechanism of pathophysiology can provide much-needed targets for future therapeutic developments. By looking at this one specific aspect of APP, another puzzle piece can be added to the complex picture of AD.
Research Materials and Methods

a. Cell Culture:

The SH-SY5Y neuroblastoma cell line was used in these experiments, due to their ability to be differentiated into a neuronal phenotype. SH-SY5Y is a third generation cell line cloned from a metastatic neuroblastoma from a four year female (Ross et al., 1983). In each cell culture flask, cells would grow in approximately 15 milliliters of media, which consists of fetal bovine serum (FBS), penicillin-streptomycin (pen-strep), and Glutamax. Pen-strep is used to prevent growth of bacteria in the media, while FBS provides nutrients. The cells would grow in a 75 cm² flask incubated at 37°C in a 5% CO₂ atmosphere. Media changes would occur roughly twice a week.

b. Neuronal Differentiation:

In order to initiate differentiation, media was replaced with media containing 10 μM retinoic acid (RA) for four weeks once cells were 80-100% confluent. RA is stored in 1000X aliquots in dimethyl sulfoxide (DMSO) at 4 degrees C. RA is stable under these conditions for 3 months and will lose its efficacy afterwards (Kilonsky 2009). After four weeks, the media was replaced with standard media for two
days. For one week, the media was replaced with media containing 1 μM cytosine arabinoside, 10 μM fluorodeoxyuridine and 10 μM uridine for one week. Finally, for an additional two weeks, the cells are treated for media containing only 10 μM fluorodeoxyuridine and uridine. Cytosine arabinoside and fluorodeoxyuridine are mitotic inhibitors that disrupt nuclear DNA, which prevents cells from undergoing mitosis. When differentiated, cells appear like neurons with thin string like structures that appear from a central body (Figure 5).
Figure 5: SH-SY5Y Neuroblastoma Cell Line

In panel A, undifferentiated SH-SY5Y cells are shown. Panel B shows the cells once differentiation occurs (Wang 2009)
c. PMA Treatment:

Once cells are differentiated, the phorbol ester, 10 microliters of 10μM phorbol 12-myristate 13-acetate (PMA) or Phorbol 12,13-Dibutyrate (PDBu), are added for a 24 hour treatment under incubation in a 37°C environment. Phorbol ester treatment is used to induce the myristoylation reaction.

d. Immunocytochemistry:

For immunocytochemistry, confluent and differentiated SH-SY5Y were first grown on glass cover slips. Approximately 500 microliters of cells and media were taken from the flask and then placed into a sterile microwell plate containing a glass coverslip in it. The plate would be incubated for at least 24 hours to ensure that the grew and attached to the cover slip.

The cover slip would be gently rinsed with phosphate buffer solution (PBS). Cells were then fixed with 100 microliters of 2% paraformaldehyde (PFA) in a room temperature and humidified chamber for 30 minutes.

Cover slips were rinsed once again with PBS to remove any excess media or unattached cells. Cells were then permeabilized with
0.1% Triton X-100, a detergent that partially disrupts cell membranes. This will allow for antibodies that will be labeling APP and TOMM to penetrate the cell.

APP antibodies used are APP3EP specifically targeted the N terminus of APP because this portion remains intact after post translational cleavage by the variety of secretases. TOMM antibodies included anti-TOMM20 and anti-TOMM40.

Optimal dilutions of the antibodies were determined in pilot experiments. After washing cover slips again with PBS, incubation under a humidified setting occurs for 120 minutes at 37°C, Alexa Fluor 488 and anti-rabbit Alexa Fluor 594, secondary antibodies, were added at a dilution of 1:500 and incubated for 60 minutes. The coverslips were then onto slides with VectaShield/DAPI and nail polish and then visualized underneath the fluorescent microscope. DAPI staining was used to label the nuclei. When co-localization of APP and TOMM occurs, a yellow signal is seen under fluoroscopy.

e. Confocal Microscopy:

Once immunocytochemistry is complete, protein labeling is visualized underneath a Zeiss LSM-5 Pascal laser scanning confocal microscope. A constant, minimized pinhole diameter is used to capture
the slimmest optical section possible (≤1µm) for accurate co-localization. Confocal microscopy differs from standard fluorescent microscopy through the use of the pinhole to eliminate out of focus materials, establishing a focal plane or an “optical section.”

PMA treated cells were scanned under fluorescent confocal microscopy and the distribution of APP and TOMM signals were compared to vehicle-treated controls, with particular attention paid to potential co-localization of the two signals, indicating that APP may exist in close proximity to, and perhaps even in a complex with, TOMM.

*f. Follow up Western Blot Experiments:*

In the absence of apparent co-localization in the mitochondria of treated cells, we began to assess the individual parts of the hypothesis. In order to determine whether PKC was activated, more SH-SY5Y cells were treated with PMA at varying time intervals. The time intervals were 2 hours, 4 hours, 6 hours, 8 hours, and 12 hours (chronic PMA treatment). The treatment was done using the same method as stated above. After the designated time interval, the cells were harvested and frozen at -80°C. Using Western blots, we attempted to discern whether PKC are phosphorylated after the PMA treatment. An antibody that
labels phosphorylated PKC will allow the western blot to appropriately separate these proteins, confirming that the PMA treatment did actually activate PKC. Western blots are analytical experiments that use gel electrophoresis to detect proteins in a particular extract. Optimization of Western blotting protocols to probe for phosphorylated PKC isoforms was initiated but not completed.

**g. Mitochondrial Gradient Fractionation:**

Considerable effort was directed toward the establishment of cell fractionation protocols, so we could independently probe cytosolic and mitochondrial fractions for APP and its cleavage products, to determine if APP was indeed being trafficked to the mitochondria. APP can be present throughout the cell, mainly in the cytosol. It was essential to differentiate the APP present in the cytosol of the cell and APP associated specifically with the mitochondria.

After resuspending the cells that were grown in cell culture flasks, cells were chilled and pelleted. For the first wash, PBS is used and, in the second wash, HM buffer is used. In the final solution of cells, which is in HM buffer, protease inhibitor is added. Using a Dounce homogenizer, the cells are disrupted. The disrupted cells are added to a sucrose gradient in ultracentrifuge tubes, where the sucrose
solutions are 0.8, 1.2, 1.4, 1.6 M (Figure 6). The sucrose gradient is prepared using a 6 mL syringe and 4 inch needle, with heavier solutions at the bottom of the tube and the lighter solutions layered at the top. A sucrose gradient is used because large protein complexes precipitate to higher density sucrose layers and free proteins precipitate to lighter sucrose fractions. Mitochondria precipitate between the 1.2 M and 1.4 M layers in the sucrose gradient. (Taylor et al 2002).
A control tube is created, but instead of cells, HM buffer is present. The cells and gradient are centrifuged for 2 hours at 110,000 g (RPM = 38,664 rpm) at 4°C under vacuum settings. After centrifugation, layers were removed and labeled according to the density associated with each organelle.
Results

a. Cell Culture:

The SH-SY5Y cells grew to confluence within one week, mostly adherent to the surface of the flask. The cells looked like spherical bodies that would cover the majority of the cell plate surface, with some cells floating in suspension. This is the expected appearance of this cell line once it reaches confluence. There was no contamination with mold or bacteria in the flasks. dimethyl sulfoxide (DMSO).

b. Differentiation:

The SHSY5Y cells responded reliably to the differentiation treatment, taking on a neuronal morphology with dendritic-like processes and a thin and elongated soma.

c. Confocal Microscopy:

Reliable labeling of APP and TOMM was achieved (Figure x), with APP showing a ubiquitous punctate signal throughout the cytosol, and TOMM clearly illustrating the syncytial morphology of the mitochondrial network within the cells. APP appears as bright green signaling, as shown in the figure below. TOMM20 and TOMM40
signaling appears red in the figure below. DAPI successfully stained the nucleus, which appears blue in all studies completed.

Occasionally, potential co-localization of the two signals was seen (as yellow), but this appeared to be random or perhaps microsomal labeling, not mitochondrial.

No differences in morphology, intensity, or distribution of signal was seen between PMA- and vehicle-treated cells. Negative control slides showed no significant labeling, which suggests that nonspecific labeling by the antibodies was not present. Throughout the experiments, there were few to moderate amount of cells present on the slides analyzed. At times, some slides did not have enough cells to analyze the signaling. Initially, there were problems with mold contamination in the cell culture flasks that caused the yield to decrease. The hood vacuum systems were adjusted and the mold subsided, but the low cell counts still persisted. There were sufficient cells found in cell culture, so the problem must lie in plating cells to slides. There may have been too many washes with PBS that could have diluted the number of cells that could be plated on the cells.
Figure 7: APP and TOMM signaling in SH-SY5Y Cell Line
SH-SY5Y cells were differentiated with retinoic acid, treated with phorbol ester or vehicle, fixed on coverslips and probed with antibodies against TOMM20/40 (above left, green), APP N-terminus (above center, red), and stained with DAPI. This image shows the typical result of treatment, which did not differ from vehicle control. TOMM20/40 labeled the outer mitochondrial membrane and revealed the syncytial morphology of an intact mitochondrial network throughout the cells. N-terminus-specific APP labeling demonstrated a widespread, punctate distribution of APP and its potential cleavage products throughout the cytosol. Despite the widespread labeling of both probes, little indication of overlap between the two signals was found (which would be indicated by yellow), indicating that the phorbol ester treatments did not increase APP trafficking to the mitochondria as hypothesized.
Because the results above showed no co-localization, the strength of the antibody labeling at different concentrations was checked through immunocytochemistry experiments as well.
Table 1: TOMM 20/488 Staining

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<th>Concentration</th>
<th>No. of Cells Present</th>
<th>Strength of Labeling</th>
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</thead>
<tbody>
<tr>
<td>1:100</td>
<td>Good</td>
<td>Strong</td>
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<tr>
<td>1:200</td>
<td>Few</td>
<td>Moderate</td>
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<tr>
<td>0 (control)</td>
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<td>None</td>
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Table 2: TOMM 40/488 Staining

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<td>1:200</td>
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<td>None</td>
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Table 3: APP Staining

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<td>Good</td>
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**d. Western Blot Experiments:**

Initial blots of our PMA-treated cells were used to establish the appropriate parameters for the blot and probes (PKC, phospho-PKC and APP); little time remained for completion. Further, additional complications became apparent: multiple APP probes revealed a multitude of apparent bands, which, in later experiments, were determined to be genuine APP N-terminus-containing cleavage products, the result of the many possible cleavage events to which APP is subjected.

**e. Mitochondrial Gradient Fractionation:**

Our attempts to establish a reliable gradient fractionation protocol were not successful. Considerable trial and error ultimately yielded inconsistent and contaminated layers after ultracentrifugation. Mitochondrial pellets, when obtained, were often too small to proceed with further studies.

The results of the sucrose gradient are shown below in Figure 8. Samples from Layers 1-4 (least to most dense) of a prepared sucrose gradient. Upper band represents calreticulin; lower band represents TOMM20. Calreticulin is an ER-associated protein, while TOMM20 is present only on the outer mitochondrial membrane; considerable
presence of both in each fraction likely represents a failure of separation within the gradient. There is also little evidence of enrichment within any particular fraction. B-COP, a Golgi-associated protein, was also probed in this experiment, with similar results. Visualization here was via reaction product of alkaline phosphatase-conjugated secondary antibodies.
Figure 8: Western Blot of Sucrose Gradient Fractions
Discussion

a. Conclusions:

In Alzheimer’s Disease, APP trafficking is regulated by a number of mechanisms, many of which have yet to be studied. My hypothesis is that protein kinase C, specifically isoforms PKCε and PKCa, when activated regulates APP transport to the mitochondria. I used phorbol esters, which have been shown to activate protein kinases through myristoylation and thus result in increased APP expression and exit of APP from the secretory pathway to the cytosol, where it would be trafficked to the mitochondria.

From the standpoint of APP and TOMM co-localization, the immunocytochemistry experiments were inconclusive in showing that myristoylation of PKC affected the downstream co-localization of APP and TOMM. The immunocytochemistry experiments did not show a clear co-localization signal.

Because there was little co-localization signal, the strength of the antibodies were tested further. The ideal concentration of both TOMM antibodies was deemed to be 1:100 and, for APP, anything below 1:200. However, when ICC was further done with these concentrations, the co-localization signal still was not present. It is safe
to assume that the antibody and secondary antibodies are adequate because the individual APP and TOMM signaling was still present. The lack of co-localization is not due to problems with antibodies and their dilutions.

Although the antibodies are not the source of the problem, there are a number of reasons that could explain our inconclusive results. First, in order to determine whether the SH-SY5Y cells were differentiated, the morphological appearance was the sole tool used. However, if the cells were not fully differentiated, there may have been a variety of cell signaling pathways that were activated at this time to facilitate the change. Protein kinases are involved with differentiation and, for example, even can be manipulated and activated by oncogenes in certain malignant neuronal processes (Lacal 1990). If the cells were in the middle of differentiation, it is quite possible that PKC was involved in different signaling cascades, instead of traveling to the mitochondrial membrane; therefore, APP trafficking downstream would be lessened. Because the visualization of the cells was subjective, there is room for error and cells that were not completely differentiated may have been included in the study.
Second, the length of duration for the phorbol ester treatment also may not have been appropriate. The twenty-four hour treatment period may have been too long. The effect of PMA can actually peak at a certain time points depending on the cell line and have a reduced effect with longer treatments. In one study, peak PMA was found to be at 30 minutes in a Chinese hamster ovary cell line and then efficacy decreased thereafter (McGraw 1988). Co-localization of APP and TOMM has been shown to occur in SH-SY5Y cells after 10 hour PMA treatment (da Cruz 2009), but there have been no studies done with 24 hour PMA treatment. The efficacy of the PMA treatment could have decreased because the treatment was too long, thus reducing the activation of PKC and the subsequent increase in APP expression. In the next round of experiments, the PMA treatment will most likely be for a shorter interval, starting with 12 hours and advancing the time until co-localization is seen. Furthermore, in order to prove that co-localization does indeed occur once PMA activates PKC, future experiments must be done with antibodies that target activated PKC. Therefore, the possibility that PKC was never activated can be ruled out.
If future experiments continue to be inconclusive, there are a variety of other mechanisms that may explain why APP transport arrests as it enters the mitochondria via the TOMM proteins. As explained by Anandatheeravadan and colleagues, another possibility may be that a negatively charged acidic domain in APP alters its interaction with TOMM proteins. This domain offers electrostatic repulsion during the interaction with negatively charged TOMM22, resulting in the translocational arrest of APP (Anandatheeravadan 2007). In our hypothesis, it is implied the problem primarily lies with increased APP expression at the mitochondrial membrane due to abnormal signaling pathways. However, the problem may lie only in the structure of APP and its inability to transverse the TOMM machinery, and not increased APP expression. In that case, signaling pathways, which include PKC, that govern APP trafficking play a small role in the overall pathogenesis of APP translocational arrest.

When looking at the hypothesis, the question may have been too broad because it focused immediately on the downstream effects of myristoylation on PKC, assuming that phosphorylation of PKC occurs. Because of time constraints, this approach was deemed more reasonable because it could yield more concrete evidence supporting
APP trafficking. This project was riskier, but we expected to have more promising results. This was realized immediately after the immunocytochemistry experiments failed to yield any results. The mitochondrial gradient fractionation, further PMA treatments, and plan for future Western Blot experiments, can provide a more complete picture of how phorbol ester treatment can affect APP trafficking to the mitochondria.

**b. Future Direction:**

The studies completed must be repeated to substantiate the results and solidify that there indeed is no relationship between these two entities. There are a few changes I would make to the protocol to leave less room for error. When trying to determine when cells were ready for immunocytochemistry experiments, I would have more than one person visualize them underneath the microscope. It is still subjective, but if there is a consensus that the cells are truly differentiated, then it is less likely that the protein kinases may be involved in other pathways involved with differentiation.

Also, in regards to the fractionation experiments, because there was not enough pure mitochondrial pellets after multiple experiments, it was later deemed to be more reasonable to isolate mitochondria
using a magnetic system. In this system, after cell lysis, TOMM22 is targeted by Anti-TOMM22 MicroBeads to magnetically isolate functional human mitochondria. This approach is more expensive, but is less labor intensive and there is higher yield of intact mitochondria. Due to time constraints again, those experiments must be repeated more times to master the technique and ultimately yield a purified large mitochondrial pellet. Afterwards, western blot experiments can be completed to confirm the presence of APP within the mitochondrial membrane. The same primary antibody used to label APP for fluoroscopy could be used for the western blot experiments. If there is increased APP expression in mitochondria, the band corresponding to APP will be larger on the Western blots.

Confirmation of separation was persistently confounded by the presence of a massive and consistent artifact in the cytosolic eluents that would deform the bands during SDS-PAGE (Figure 9). Not until much later was it determined that these artifacts were likely due to a substantial percentage of BSA added to the proprietary buffers provided in the mitochondrial isolation kit (the manufacturer would not confirm this); successful fractionation was later confirmed after substituting custom-made buffers during the isolation. The arrow in
Figure 9 demonstrates the band that corresponds to successful fractionation, which is determined by size and charge (according to the protocol found with the magnetic bead kit).
Figure 9: Early results of mitochondrial/cytosolic fractionation using antibody-linked paramagnetic microbeads to capture mitochondria (Miltenyi Biotec)
The hypothesis was too broad and risky because it focused immediately on the downstream effects of myristoylation on PKC, but all parts of the hypothesis are addressed by the new experiments initiated. Although the overall results were inconclusive in showing increased APP expression due to PKC activation, the new experiments and changes in protocol discussed look promising and can shed more light on the complex pathogenesis of AD.
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