# Phosphatidylcholine Transfer Activity of the START Domain of Them1

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# Dedication

This thesis is dedicated to my parents, who have encouraged and supported me in all of my academic and personal pursuits throughout my high school experience

# Contents:

**Phosphatidylcholine Transfer Activity of the START Domain of Them1 ..... 1**

**Contents:..... 3**

**Acknowledgements..... 5**

**Abstract..... 6**

**Introduction: ..... 7**

*Biology Fundamentals: ..... 7*

*PC-TP: ..... 7*

*Them1:..... 8*

*Overview..... 9*

**Methods.....11**

*Bacterial expression of histag recombinant PC-TP and Them1 START domain proteins: ..... 11*

*Preparing Protein Samples for Fluorescence Assay: ..... 14*

*Testing phosphatidylcholine transfer activity due to the Them1 START domain using a fluorescence assay:..... 16*

*Verification of Protein Expression:..... 21*

**Results:.....23**

*SDS-PAGE:..... 23*

*Experiment 1:..... 24*

*Experiment 2:..... 26*

*Experiment 3:..... 27*

**Discussion .....30**

**Bibliography .....34**

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# Abstract

Phosphatidylcholine Transfer Protein (PC-TP), also known as StarD2, is a member of the steroidogenic acute regulatory (Star)-related transfer (START) domain superfamily of proteins. It is expressed primarily in liver cells. PC-TP was discovered based on its ability to transfer phosphatidylcholines, a phospholipid molecule, between cell membranes. Using a fluorescence assay, the functionality of PC-TP can be readily tested. In this assay, donor membrane vesicles contain fluorescent-labeled phosphatidylcholines together with nearby quencher molecules that absorb the light emitted when fluorescence is excited. When the PC-TP binds to fluorescent phosphatidylcholine molecules and transfers them to the acceptor vesicles that do not contain quenchers, the phosphatidylcholines fluoresce.

In this research, the START domain of protein named thioesterase superfamily member 1 (Them1) was studied. Them1 is a larger protein that is expressed mainly in brown fat tissue and contains a domain (amino acid sequence) that is predicted to resemble PC-TP in structure. Based upon the predicted similarity, the START domain of Them1 was anticipated to transfer phosphatidylcholines. To test this hypothesis, *Escherichia coli* was engineered to express the Them1 START domain at high levels using recombinant DNA techniques. After verifying high-level expression of correct region of the Them1 protein, bacterial homogenates were prepared. Because *Eschericia coli* do not normally express proteins that transfer phosphatidylcholines, purification of the Them1 START domain was not required in order to test its activity. Instead, the bacterial homogenates were tested for activity using the fluorescence assay. In contrast to what had been predicted, the START domain of Them1 did not transfer phosphatidylcholines at an appreciable rate. This finding indicates that structurally similar START domain proteins most likely bind different lipids. The identification of the natural ligand for the START domain of Them1 will most likely provide important insights into the true biological function of Them1.

# Introduction:

## *Biology Fundamentals:*

Biology studies and explains the fundamentals of life by testing hypothesis on biological structures, components, and pathways. The primary biological structure in all life forms is the cell. Humans have on average 50 trillion cells in their body. Cells perform all the necessary functions for its organism to survive, including respiration and metabolism. Each cell contains many components and organelles to help it perform its function. Central to the cell is the nucleus. The nucleus contains the genetic makeup for the entire organism in the form of DNA (deoxyribonucleic acid). DNA is a double helix molecule held together by nitrogen bases. Different arrangements of the four nitrogen bases code for different protein products. In order to produce these proteins, a segment of DNA is transcribed into RNA (ribonucleic acid) which is then translated into the protein by combining amino acids. Proteins perform many vital functions for the cell, such as producing other cellular components and facilitating chemical reactions. In this experiment, DNA was engineered to produce certain proteins to be studied. Another key component of the cell is the cell membrane, which protects the cell components and organelles from outside dangers. It is made up of a lipid bilayer lined with cholesterol molecules. Certain phospholipids in the cell membrane are studied in this experiment.

## *PC-TP:*

Phosphatidylcholine transfer protein (PC-TP), also known as STARD2, is a member of the steroidogenic acute regulatory (Star)-related transfer (START) domain superfamily of proteins. It is a specific lipid-binding protein that promotes the intermembrane transfer activity of the phospholipid molecule phosphatidylcholine (Wirtz, 1991). This phenomenon is only observed *in vitro*; its *in vivo* function is not understood (Kanno, 2007). PC-TP is thought to transfer

phosphatidylcholines between mitochondrial layers when high-density lipoproteins (HDL) cause an efflux of phospholipids (Alpy, 2005). It has been observed in mice lacking the PC-TP gene that cells are insulin sensitized and atherosclerosis is reduced(Kanno, 2007).

The gene that codes for PC-TP, *Pctp*, is made up of six exons and is found on human chromosome 17 and mouse chromosome 11 (Soccio, 2003; Cohen, 1999). The structure of PC-TP consists of one START domain containing a nine stranded β sheet with four α-helices surrounding it (Kanno, 2007). The molecular weight of the protein is 24.7 kiloDaltons (Kanno, 2007). PC-TP is expressed in a wide range of organs, primarily in the liver (Cohen, 1999).

## *Them1:*

Thioesterase superfamily member 1 (Them1), also known as Brown fat inducible thioesterase (BFIT) (Adams, 2001), STARD14 (Alpy, 2005), and Thioesterase adipose-associated (THEA1) (Soccio, 2003), is a larger protein than PC-TP. Like PC-TP, it is a member of the steroidogenic acute regulatory (Star)-related transfer (START) domain superfamily of proteins. More specifically, it is a member of the thioesterase START group. It is made up of one C-terminal START domain and two N-terminal thioesterase groups (acyl-CoA thioesterase domains) as illustrated in figure 1 (Alpy, 2005; Soccio, 2003). Them1 is located on mouse chromosome 4 and human chromosome 1. The gene coding for Them1 has a 607 amino acid open reading frame, and the molecular weight is 67 kiloDaltons (Adams, 2001). Them1 is primarily expressed in brown adipose tissue (BAT)/brown fat, common in infants and hibernating animals to keep them warm (Adams, 2001). In mice, expression can be induced by cold exposure (Adams, 2001).

By using recombinant DNA techniques, the START domain of Them1 can be studied *in vitro*. The nucleotide sequence of the START domain of Them1 and an

ampicillin resistance coding gene can be cloned into a plasmid with a polyhistadine tag and then transfected into *Escherichia coli* cells (Figure 2, next page). This process causes the START domain to be expressed without the two thioesterase domains. Because PC-TP as a whole and this domain of Them1 are both START domains, the amino acid sequence of Them1's START domain has been predicted to resemble PC-TP in structure. The molecular weight of this START domain protein is 25kDa, similar to PC-TP. Including the polyhistidine tag, the molecular weight of the protein amounts to 26kDa.

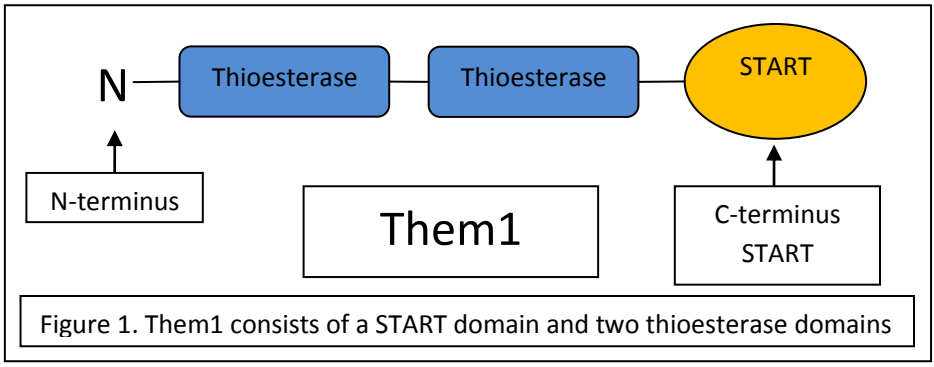


Figure 1. Them1 consists of a START domain and two thioesterase domains

## *Overview*

Based on this information, the phosphatidylcholine transfer activity of the START domain of Them1 was studied. *E. coli* cells were engineered to express the START domain of Them1. The protein was then put in a fluorescence assay to determine whether it facilitated the rapid exchange of phosphatidylcholines between membranes. It was found that Them1 START protein does not appreciably transfer phosphatidylcholines, suggesting that the biological function of the protein does not require it to transfer phosphatidylcholines or that it may bind phosphatidylcholines or another phospholipid molecule.

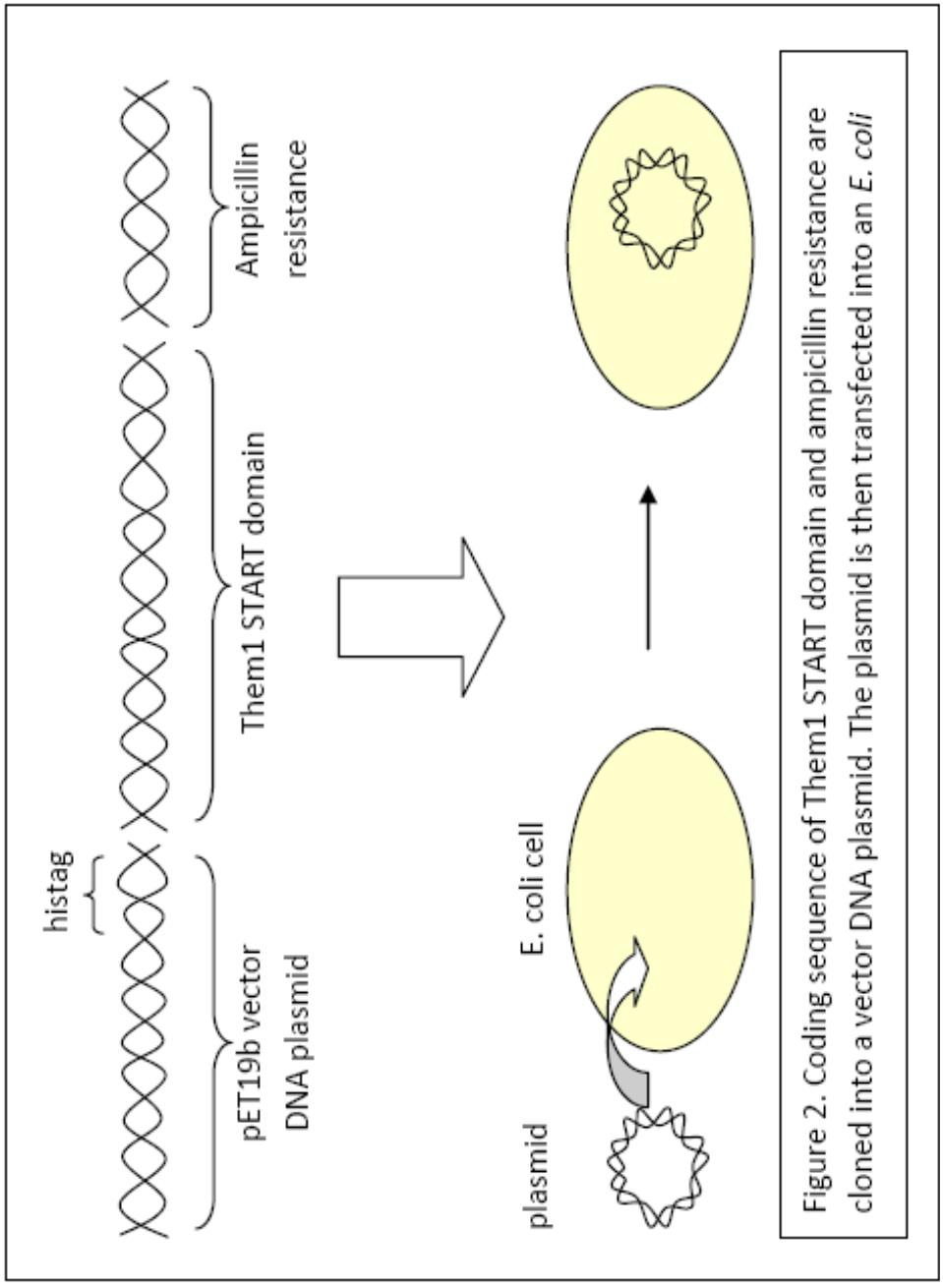


Figure 2. Coding sequence of Them1 START domain and ampicillin resistance are cloned into a vector DNA plasmid. The plasmid is then transfected into an *E. coli*

# Methods

## *Bacterial expression of histag recombinant PC-TP and Them1 START domain proteins:*

The Them1 START domain open reading frame (Adams, 2001) and the PC-TP open reading frame were cloned into separate Novagen (Darmstadt, Germany) pET19b vector DNA plasmids with an ampicillin resistance coding gene (Kanno, 2007, JBC). These plasmids were then transfected into *Escherichia coli* BL21 (DE3). To create a starter culture, a sterile loop was used to add a cell colony sample to 5mL of Luria-Bertani broth (LB) with ampicillin in a 14mL BD Falcon (Mississauga, ON Canada) round bottom test tube. The ampicillin prevents all cells, except cells with ampicillin resistance, from growing in the LB. By lightly touching the sterile loop to one colony of the cells in the glycerol stock or agar plate, a few cells affixed to the loop. When the loop was submerged into the LB growth medium, the cells detached from the loop into the LB. To perform any experiment on a viable culture, more cells were required. In order to cause the cells to multiply, samples in the test tubes were shaken at 250RPM overnight at 37°C. These conditions ensured that the cells would divide at high rate.

Next, experimental cultures were to be created from the starter culture. Accordingly, 60μL of each starter culture was added to 6mL of LB with ampicillin in a round bottom test tube. The test tubes were incubated at 37°C 250RPM until cultures reached an optical density (OD) of 0.650 at a 600nm wavelength. Optical density is a measure, between zero and one, of light transmittance at a certain wavelength through a medium. A measurement of zero indicates that 100% of the light is transmitted through the medium, or conversely, 0% is absorbed by the medium. In this case, the optical density of the cultures in the test tubes was measured. This was done using a Molecular Devices (Sunnyvale, CA) SpectraMax M5 Microplate Reader. Because LB alone has a yellow tint that may absorb light, it

affects the OD reading. In order to compensate for this effect, the plate reader was zeroed on a sample of LB with ampicillin. Measurements of the cell cultures were taken after 2h and then every 0.5h after that until $OD_{600}$ equaled 0.600. After this time, measurements were taken more frequently until $OD_{600}$ was 0.650. An $OD_{600}$ of 0.650 was chosen because the growth rate of E. coli is maximal at this level; after this, division rate begins to decrease. Once the $OD_{600}$ of 0.650 was reached, 2mL of each sample were transferred to new test tubes as controls. To induce protein expressions, 4μL of 1mM isopropyl β-D-thiogalactopyranoside (IPTG) was added to each cell culture test tube, which had 4mL remaining after the removal of the control sample. IPTG triggers the transcription of the lac operon. In this instance, the IPTG will cause transcription of either PC-TP or the Them1 START domain. The two samples with the IPTG and the two control cultures were shaken overnight at 25°C 250RPM, causing the production of the protein in the experimental cultures.

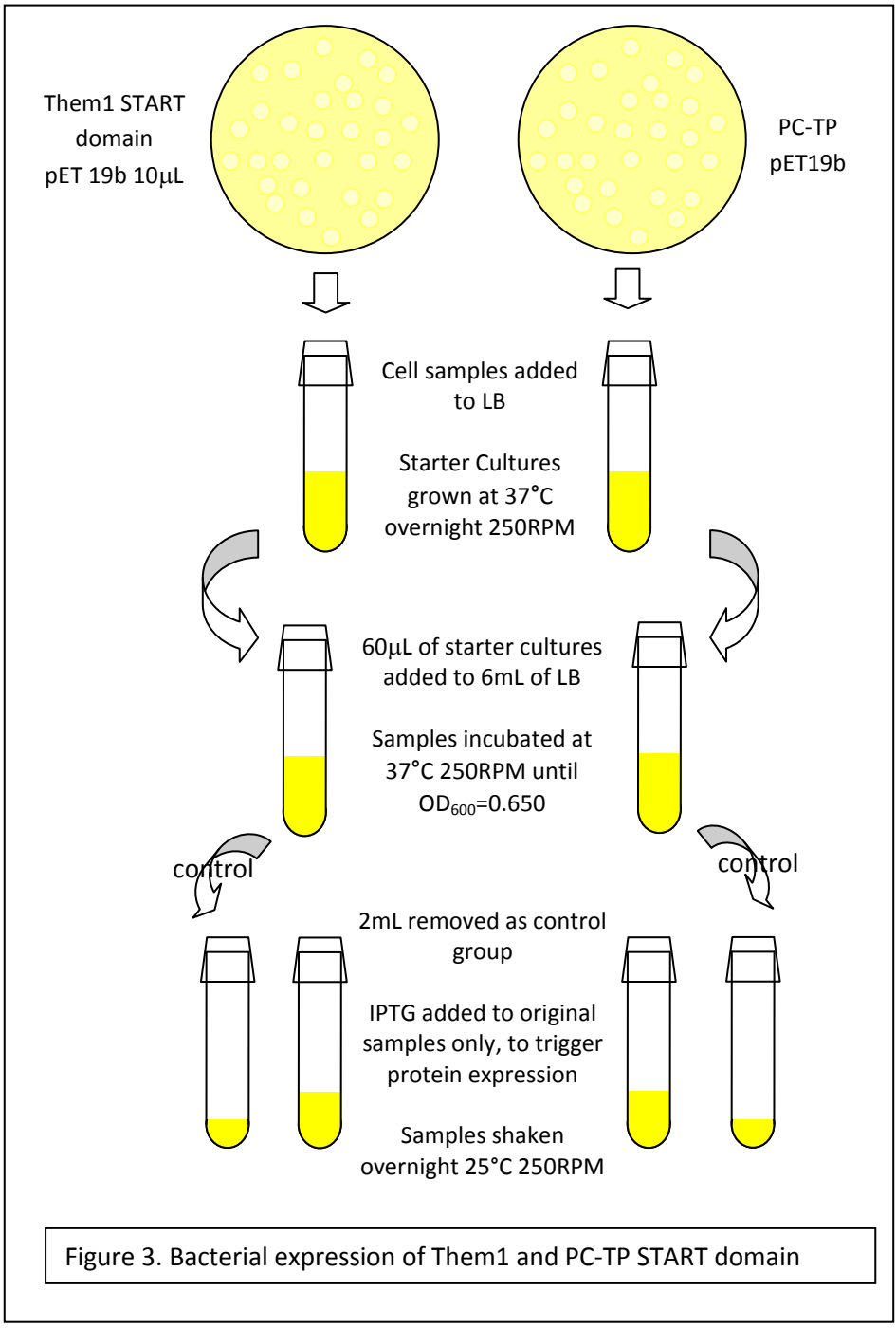


Figure 3. Bacterial expression of Them1 and PC-TP START domain

## *Preparing Protein Samples for Fluorescence Assay:*

In order to test the PC-TP and Them1 START domain samples, the cells containing the proteins were lysed. Lysing bursts open the cell to expose the proteins to the experimental conditions. First, one half of a Roche Applied Science (Indianapolis, IN) mini EDTA-free protease inhibitor tablet was added to each sample tube, including the controls. These tablets prevent the proteins from degrading after the cell has been lysed. The samples were then sonicated on ice three times with an ultrasonic probe for 45 seconds to disrupt the cell membranes. To pellet the unwanted cell components, each sample was centrifuged at 4,000 RPM for 30 min. As a result, proteins remained in the supernatant, whereas the cell membrane and other components were forced to the bottom of the test tube. The lysate supernatants were then transferred into new test tubes.

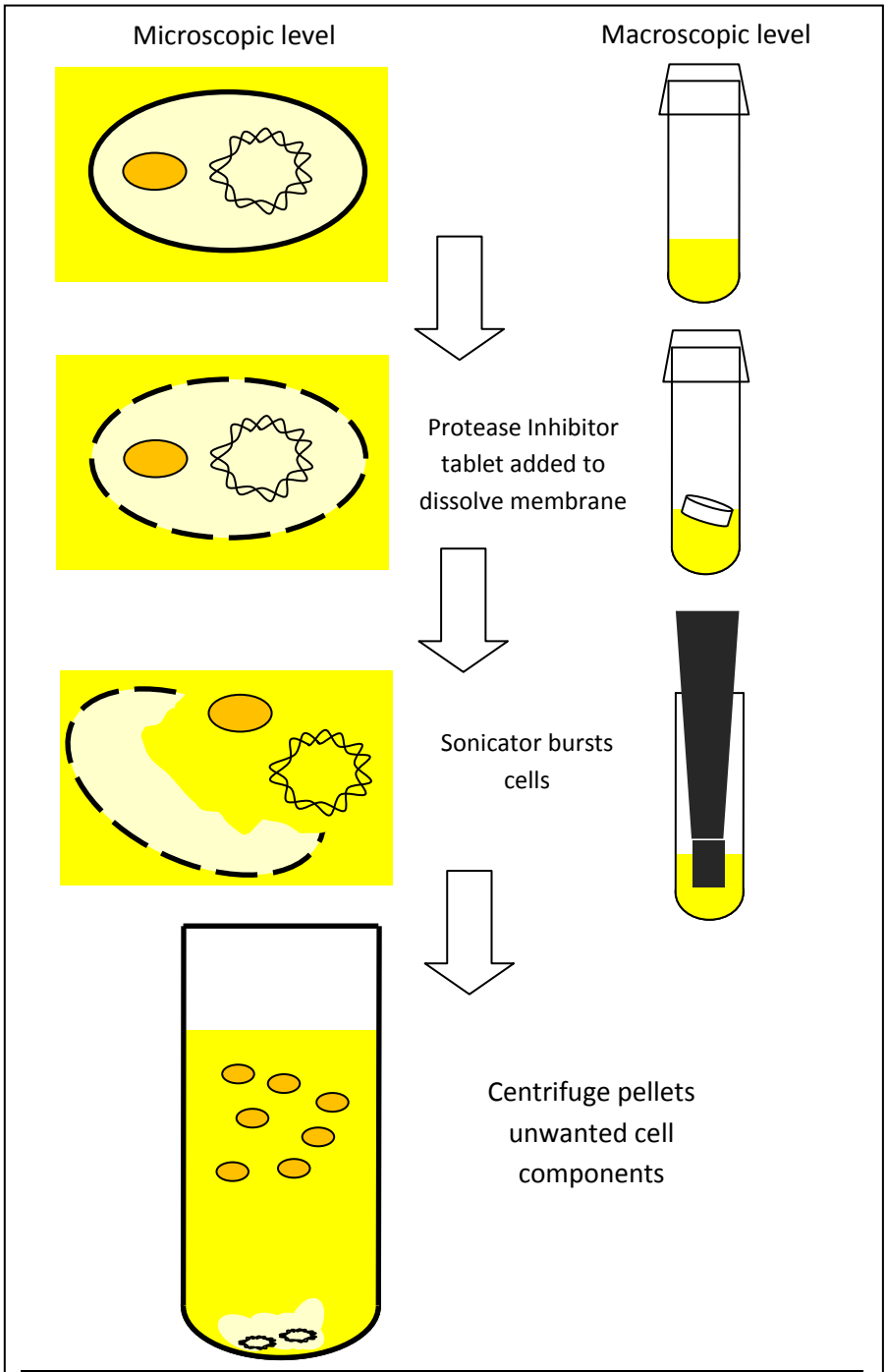


Figure 4. Preparing Them1 START domain protein samples for assay

## *Testing phosphatidylcholine transfer activity due to the Them1 START domain using a fluorescence assay:*

Using a fluorescence assay, the phosphatidylcholine transfer activity promoted by the Them1 START domain was tested (Nichols, 1982). This assay contains three components: donor membrane vesicles, acceptor membrane vesicles, and protein. The donor membrane vesicles contain fluorescent-labeled phosphatidylcholines and quencher molecules that absorb the light emitted from the excited nearby phosphatidylcholines. Accordingly, no fluorescence from the phosphatidylcholines in the donor vesicles would be detected because the light is absorbed by the quencher molecules. The acceptor membrane vesicles contain phosphatidylcholines that are not fluorescent-labeled. Because phosphatidylcholines are not soluble molecules outside of a membrane, they will not transfer spontaneously. However, in this assay phosphatidylcholine analogs were used that occasionally spontaneously exchange between vesicles. This arrangement allows the third component, PC-TP or a similar protein, to be tested for the ability to transfer phosphatidylcholines between the acceptor and donor vesicles. When the PC-TP binds to the fluorescent-labeled phosphatidylcholines and transfers them to the acceptor vesicles that do not contain quencher molecules, the emitted light will not be absorbed. As a result, the fluorescence will be detected. Consequently, the transfer activity caused by PC-TP, or any protein used in the assay, can be tested by measuring the fluorescent light emitted. A protein that readily transfers phosphatidylcholines will cause light to be emitted in the assay. Conversely, a protein that does not readily transfer phosphatidylcholines will not cause considerable fluorescence to be detected. Fluorescence emission is detected when a suitable molecule is excited by light at a characteristic wavelength. In this assay, the fluorescent-labeled phosphatidylcholines fluoresced when exposed to light at 475nm. By using a specially-designed spectrophotometer, this level of this fluorescence can be quantified.

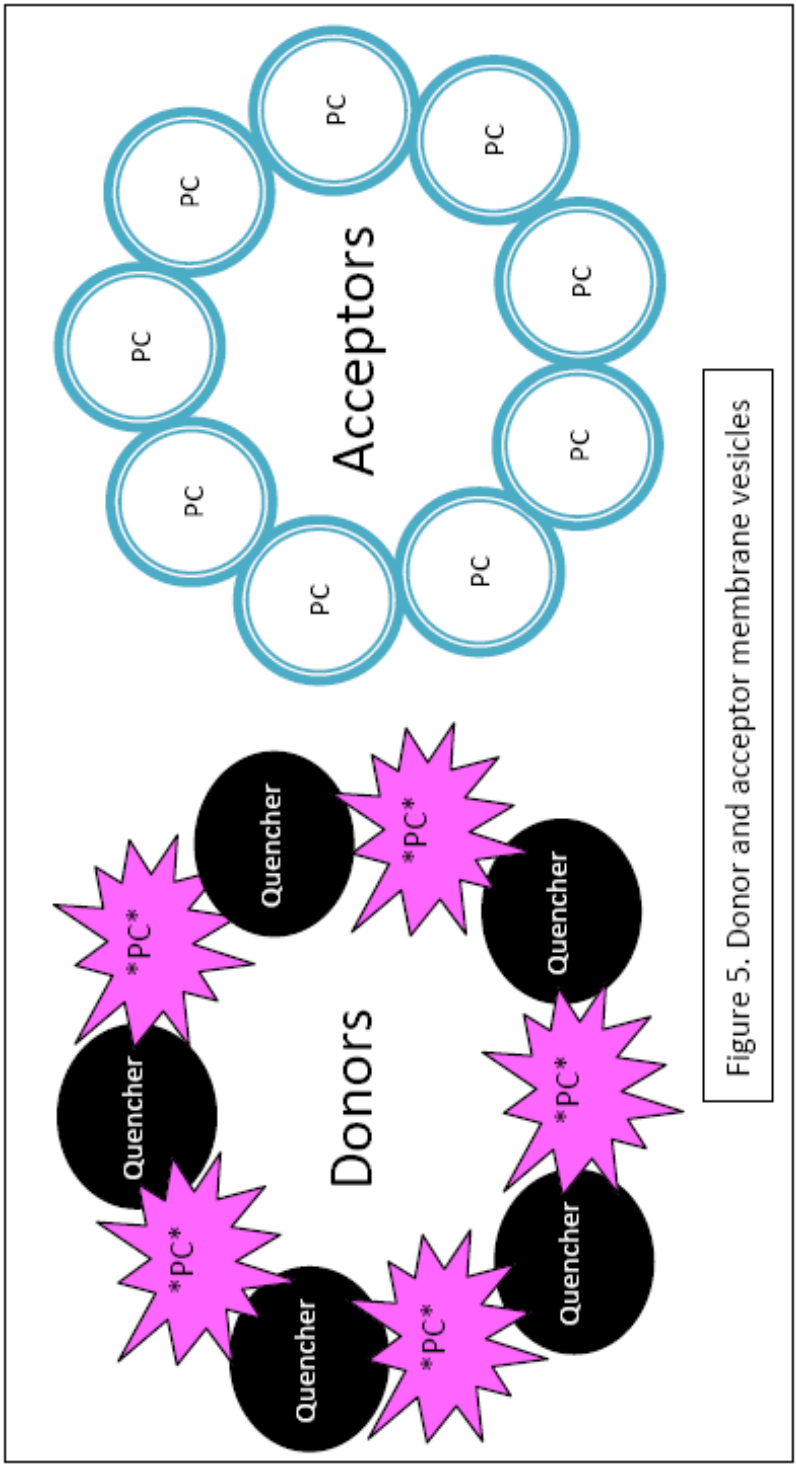


Figure 5. Donor and acceptor membrane vesicles

Samples for the assay were tested in a 96-well microplate. First, 45μL of acceptor vesicles were added to each well using a micropipette. In rows A and B, 35μL of assay buffer were added as the negative control. Using buffer, instead of PC-TP or Them1 START domain, reveals transfer of phosphatidylcholines when no protein is present to facilitate it. In rows C and D, 35μL of the Them1 START domain lysate was added. 35μL of PC-TP lysate were added to rows E and F as the positive control. 35μL of a purified PC-TP were added to rows G and H, serving as duplicate positive controls. The purified PC-TP was free of LB and any other components in the supernatant of the PC-TP lysate from *E. coli*. 20μL of donor vesicles were then added to all wells, bringing the total volume to 100μL. This allowed the proteins present to initiate transfer of phosphatidylcholines between the donor and acceptor vesicles. Immediately after the addition of the donor vesicles, the microplate was inserted into the Molecular Devices SpectraMax M5 Microplate Reader. The microplate was shaken for 2 seconds prior to the each measurement (Kanno, 2007). The fluorescence in each well was read at ten second intervals for 10 minutes. The excitation wavelength was 475nm and the emission wavelength was 535nm (Kanno, 2007).

The assay was then repeated, but an increased concentration of Them1 START domain lysate was used. This experiment was intended to determine whether the concentration of the experimental group affected the transfer rate of the phosphatidylcholines. Two columns in the microplate were used. 45μL of acceptor vesicles were added to all of the wells. In row A, 35μL of buffer were added to each well. In row B, various concentrations of buffer were put in to each column. 45μL were added to the first column and 60μL to the second. In rows C and D, 45μL of the lysate containing Them1 START domain was added to the first column and 60μL to the second. In row E, 35μL of lysate containing PC-TP was added to each well. Row F contained 45μL of PC-TP lysate in the first column and 60μL in the

second. Rows G and H mirrored rows E and F, respectively, substituting purified PC-TP for PC-TP lysate. 20μL of donor vesicles were added to each well, and the microplate was immediately put into the microplate reader. The fluorescence was measured under the same conditions as in the first experiment.

A third experiment was performed to ensure that the desired protein in the lysate supernatant, PC-TP or the START domain of Them1, instead of some other component, was permitting the transfer activity of the PCs. In this trial, the Them1 START domain and PC-TP lysates were compared to their respective control homogenates. These controls were the cell cultures that did not have IPTG added to them. Thus, the controls should not express either PC-TP or the Them1 START domain. In this trial, two columns of the microplate were used. 45μL of acceptor vesicles were added to all wells. In rows A and B, 35μL of assay buffer were added. In the first column of rows C and D, 35μL of Them1 START domain lysate were added. In the second column of rows C and D, 35μL of PC-TP lysate were added. In rows E and F, 35μL of Them1 START domain control were added to the first column, and 35μL of PC-TP control were added to the second column. 35μL of purified PC-TP were added to rows G and H. 20μL of donor vesicles were added to all well and the microplate was put into the microplate reader. Fluorescence was measured under the same conditions as in the previous two experiments.

(a)

| Assay Experiment 1 | |
|---|---|
| A (Buffer) | 35μL |
| B (Buffer) | 35μL |
| C (Them1 START Domain Lysate) | 35μL |
| D (Them1 START Domain Lysate) | 35μL |
| E (PC-TP Lysate) | 35μL |
| F (PC-TP Lysate) | 35μL |
| G (Purified PC-TP) | 35μL |
| H (Purified PC-TP) | 35μL |
| All wells used 20μL of donor vesicles and 45μL of acceptor vesicles | |

Table 1. Assay microplate well sample concentrations in (a) experiment 1, (b) experiment 2, and (c) experiment 3.

(b)

| Assay Experiment 2 | | |
|---|---|---|
| | 1 | 2 |
| A (Buffer) | 35μL | 35μL |
| B (Buffer) | 45μL | 60μL |
| C (Them1 START Domain Lysate) | 45μL | 60μL |
| D (Them1 START Domain Lysate) | 45μL | 60μL |
| E (PC-TP Lysate) | 35μL | 35μL |
| F (PC-TP Lysate) | 45μL | 60μL |
| G (Purified PC-TP) | 35μL | 35μL |
| H (Purified PC-TP) | 45μL | 60μL |
| All wells used 20μL of donor vesicles and 45μL of acceptor vesicles | | |

(c)

| Assay Experiment 3 | | |
|---|---|---|
| | 1 (35μL) | 2 (35μL) |
| A | Buffer | Buffer |
| B | Buffer | Buffer |
| C | Them1 START Domain Lysate | PC-TP Lysate |
| D | Them1 START Domain Lysate | PC-TP Lysate |
| E | Them1 START Domain Control | PC-TP Control |
| F | Them1 START Domain Control | PC-TP Control |
| G | Purified PC-TP | Purified PC-TP |
| H | Purified PC-TP | Purified PC-TP |
| All wells used 20μL of donor vesicles and 45μL of acceptor vesicles | | |

## *Verification of Protein Expression:*

To ensure the validity of the fluorescence assay, the presence of PC-TP and Them1 START domain in the lysate needed to be confirmed. The proteins were separated and visualized using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis utilizes the negative charge of denatured protein to separate components of various weights. By running a current through the gel, having the positive electrode at the bottom of the gel and the negative electrode at the top of the gel where the protein samples are loaded, the proteins will experience a force pulling them to the bottom of the gel. Because of their weight, lighter and shorter proteins will travel faster through the gel than heavier and longer proteins. Accordingly, the sample components are separated by molecular weight.

First, a gel was prepared for the electrophoresis. Before running the electrophoresis, the samples were prepared. The four samples to be used were the PC-TP lysate, the PC-TP control, the Them1 START domain lysate, and the Them1 START domain control. 25μL of each lysate were added to an eppendorf tube. 5μL of sample buffer were added to each tube. The sample buffer denatures the protein samples. Each test tube containing the samples was centrifuged, then vortexed. Samples were then heated to 60°C for 10 minutes. Denaturation causes the proteins to lose much of their complex shape and coats them in negative charge, allowing for a more effective electrophoresis result. All four samples were then loaded into the wells of the gel using a micropipette. Protein ladder was loaded into the first well. The ladder contains different protein standards of varying weights that will spread out in the gel during electrophoresis. By comparing the location of sample components in the gel to the location of bands created by the protein in the ladder, the molecular weight of the components can be determined. The gel electrophoresis was run for 90 minutes at 150V, 3A, and 300W. The polyacrylamide gel was then

immersed in coomassie stain for 30 minutes and destained overnight, in order to make visible the protein bands.

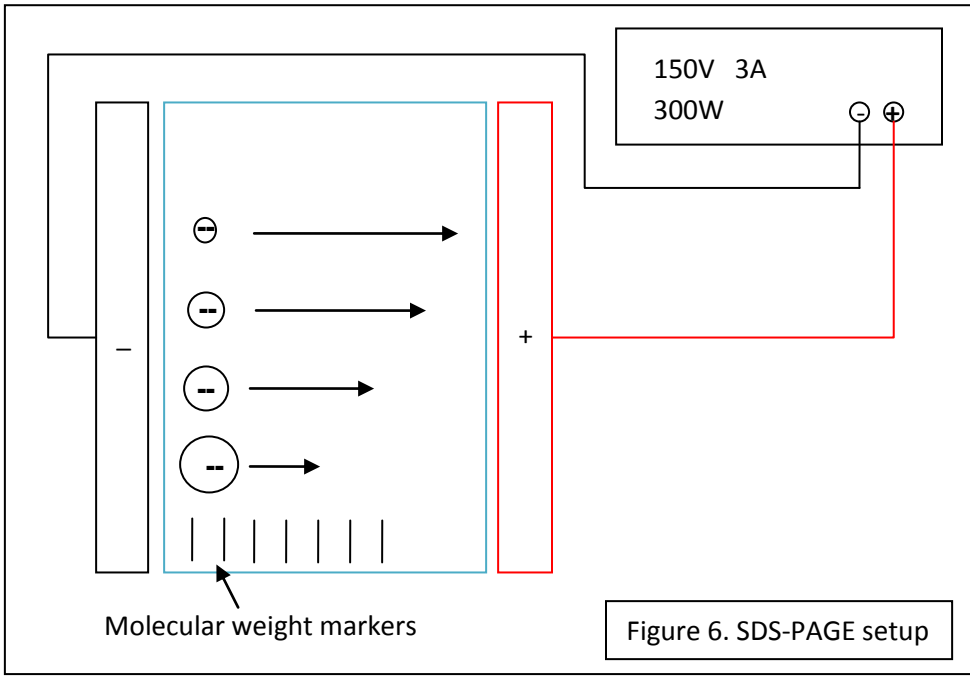


Figure 6. SDS-PAGE setup

# Results:

## *SDS-PAGE:*

The protein standard ladder was loaded into the first well of the gel. This is visualized by the blue and orange bands that represent different molecular weights. PC-TP control sample is in the second column. Evident by the many bands at various molecular weights, all of the unwanted cell components are still in the sample. A band is visible at 26kDa, suggesting that some PC-TP was expressed without added IPTG. However, it is unlikely to affect the results of the experiment. In the third column of the gel is the lysate of PC-TP. In this column, there is a slightly darker band at the 26kDa marker, consistent with PC-TP. Them1 START domain control samples were loading into fourth column. Here, like in column 2, all of the unwanted cell components are still in the sample. There is no evidence in this column for a 26kDa protein expressed at a rate much greater than other proteins, indicating that Them1 START domain was not overexpressed in this sample. In the fifth column was lysate of Them1 START domain lysate. A band on the fifth column at the 26kDa marker indicates the presense of a 26kDa protein, consistent with Them1 START domain. This pattern of only seeing the Them1 START domain occurs because the sonicator and centrifuge removed most unwanted cell components from the cell sample.

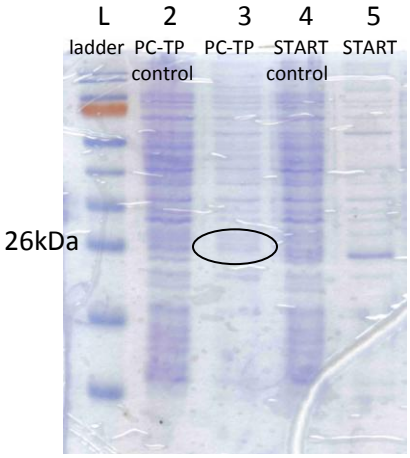


Figure 13. SDS-PAGE results

## *Experiment 1:*

Flourescence intensity was measured in each microplate well every 10 sec for a duration of 10 min (Figure 7). The flourescence intensity in the wells with sample buffer increased slightly with time in a linear fashion. In the wells with Them1 START domain, the rate of change of the fluorescence resembled that of the samples with buffer, but for the first 45 sec only, the fluorescence increased in a curvilinear fashion. Because the LB has a yellow tint, the initial flourescence was much higher. The fluorescence of the samples with PC-TP lysate increased with time in a logarithmic fashion, at a rate much greater than the buffer or Them1 START domain samples. Because it was immersed in yellow LB, its starting flourescence was the same as the wells with Them1 START domain. The fluorescence in the wells with purified PC-TP began at approximately the same level as the wells with buffer. However, the fluorescence of pure PC-TP samples increased at a much greater rate than the buffer samples. To better compare transfer activity and fluorescence levels in the samples in the assay, all values were adjusted so that the fluorescence was set to zero for the first measurement (Figure 8).

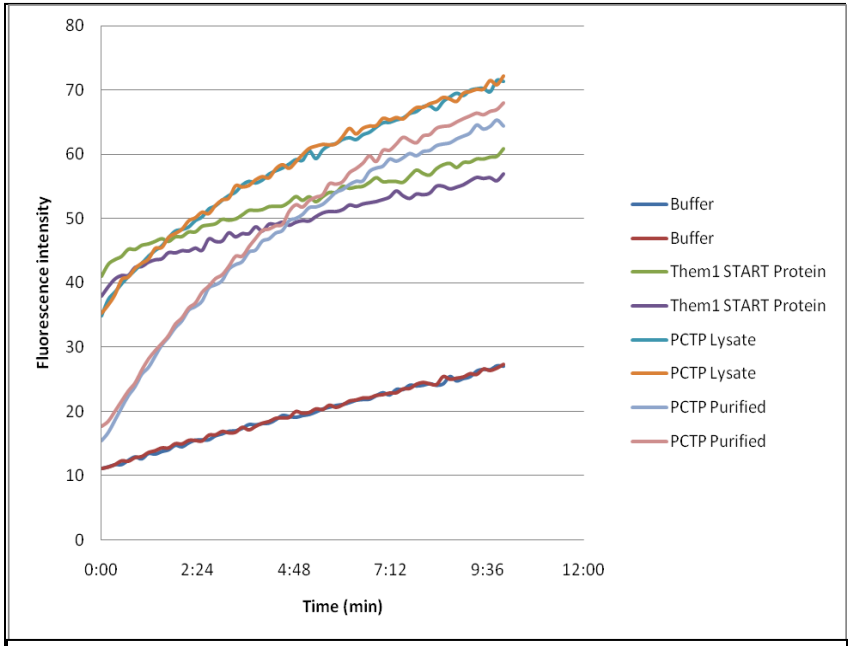


Figure 7. Phosphatidylcholine transfer activity promoted by the Them1 START domain lysate. Fluorescence intensity is plotted as a function of time. The fluorescence units are arbitrary.

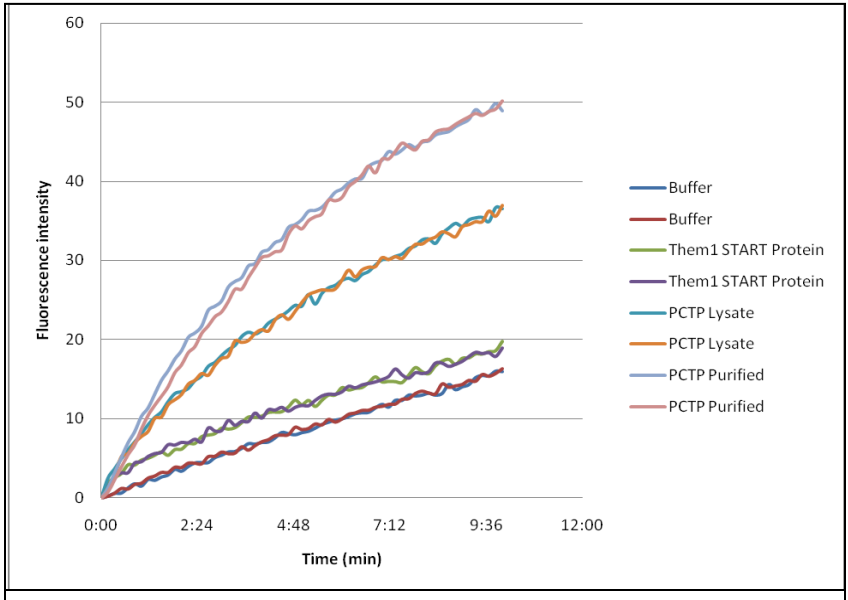


Figure 8. Phosphatidylcholine transfer activity promoted by the Them1 START domain lysate. Fluorescence intensity is plotted as a function of time and adjusted to zero at time=0.

## *Experiment 2:*

The fluorescence in the wells with buffer and the wells with Them1 START domain lysate increased at the same linear rate, regardless of the concentration. The PC-TP lysate and purified PC-TP samples' fluorescence intensities increased at a much greater rate than that of the buffer or Them1 START domain samples. The fluorescence intensity of the purified PC-TP samples increased at a slighter greater rate than that of the PC-TP lysates. However, each showed a greater rate when the concentration was increased.

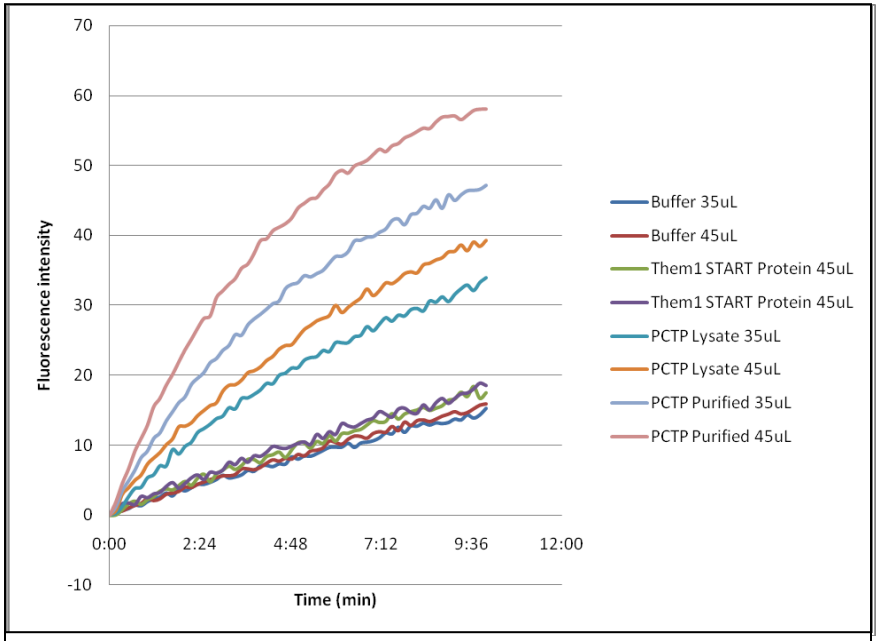


Figure 9. Phosphatidylcholine transfer activity promoted by Them1 START domain lysate at a concentration of 45µL

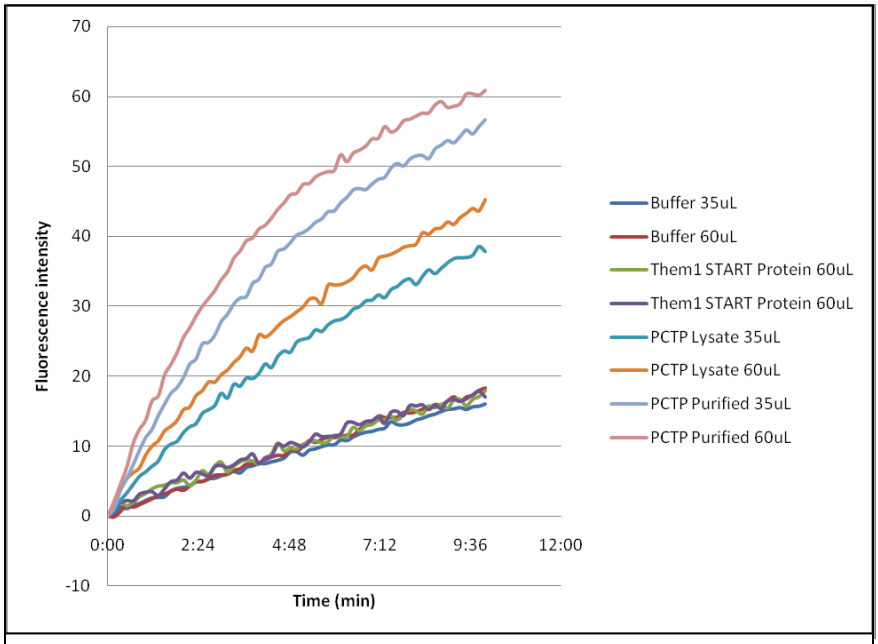


Figure 10. Phosphatidylcholine transfer activity promoted by Them1 START domain lysate at a concentration of 60µL

## *Experiment 3:*

The fluorescence intensity of the Them1 START domain lysate and the Them1 START domain control wells increased at approximately the same linear value as the wells with buffer. The fluorescence intensity in the wells with purified PC-TP increased at a much greater rate than any other well (Figure 11).

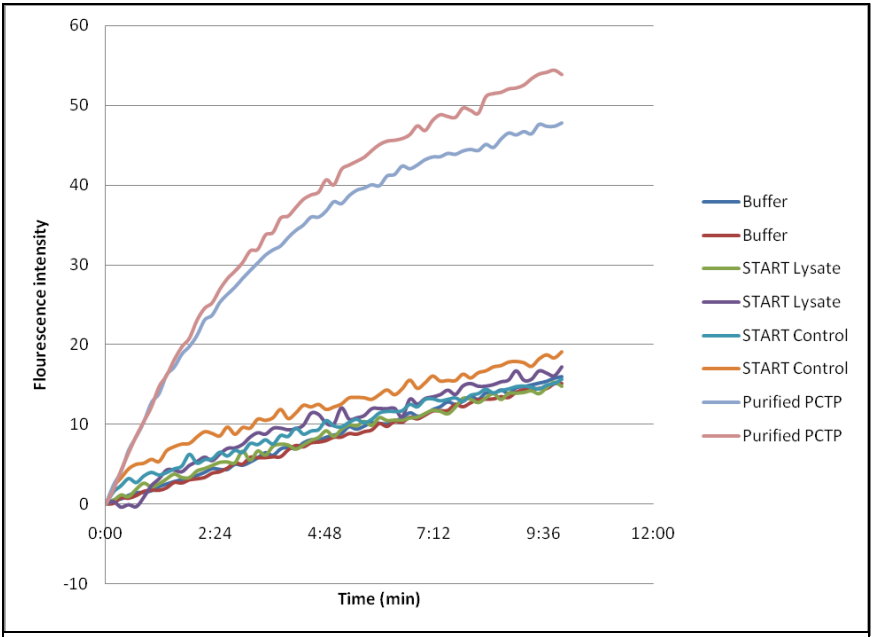


Figure 11. Phosphatidylcholine transfer activity promoted by expressed Them1 START domain lysate compared with unexpressed lysate. All affected values are adjusted to account for the fluorescence of LB.

In the PC-TP column of the microplate, the fluorescence intensity in the wells with buffer increased at the same rate as in all other trials (Figure 12). The fluorescence intensity of the PC-TP control wells increased at a slightly greater rate, though still linear in fashion. In the wells with PC-TP lysate, the fluorescence increased linearly, at a rate slightly greater than that of the PC-TP control. As in the other experiments, the intensities for purified PC-TP increased at the greatest rate.

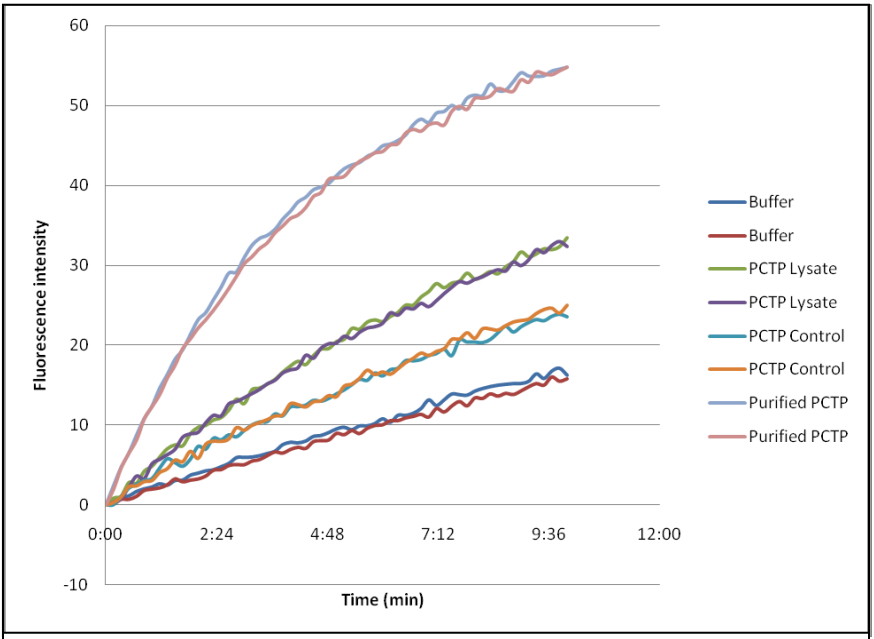


Figure 12. Phosphatidylcholine transfer activity promoted by expressed PC-TP lysate compared with unexpressed lysate

# Discussion

Over time, fluorescent analogs of phosphatidylcholines will slowly transfer between acceptor and donor vesicles due to spontaneous exchange. The linear increase of the fluorescence in the wells with buffer is due to this spontaneous exchange. At the beginning of the first assay experiment, the wells with Them1 START domain fluoresced at a rate greater than the wells with buffer (Figure 8). This could indicate that the Them1 START domain bound to the phosphatidylcholines and promoted their exchange between donor and acceptor vesicles at a rate higher than that of spontaneous exchange. However, after the first 45 seconds, these wells fluoresced at the same extent as the buffer wells. This suggests that after 45 seconds, the Them1 START domain was no longer facilitating the transfer of phosphatidylcholines. Compared to the fluorescence emitted from the wells with the positive controls (PC-TP lysate and purified PC-TP), the Them1 START domain wells did not appreciably fluoresce. Accordingly, the Them1 START domain did not transfer PCs at an appreciable rate in this trial.

The second experiment, using increased concentrations of the control and experimental groups, only corroborated the results in the first trial. When PC-TP lysate and purified PC-TP were added to the wells at an increased concentration, more fluorescence was measured, indicating that PC-TP concentration is proportional to phosphatidylcholine transfer activity (Figures 9 and 10). More PC-TP in the assay mixture led to greater exchange rate of phosphatidylcholines between donor and acceptor vesicles. This phenomenon was observed when the PC-TP samples were increased to both 45μL and 60μL from the original 35μL concentration. The Them1 START domain in this trial, unlike the PC-TP, showed no relation to phosphatidylcholine transfer activity. When the concentration of the Them1 START domain protein was increased to 45μL and then to 60μL, there was no effect on the fluorescence. In both cases, the fluorescence increased at nearly the same rate as the negative control group wells with buffer. Moreover, in this trial, the

fluorescence in the wells with Them1 START domain sample did not increase at a greater rate for the first 45 seconds than for the rest of the measured time as it did in the first trial. Taking into account spontaneous phosphatidylcholine exchange, these data indicated that Them1 START domain protein did not at all facilitate the transfer of phosphatidylcholine during any part of the assay.

The third experiment ensured that the experimental group protein, either PC-TP or the Them1 START domain, and not some other component in the lysate, was affecting phosphatidylcholine transfer activity. In the wells comparing the effect of expressed Them1 START domain lysate with unexpressed Them1 START domain lysate control, the measured fluorescence matched that of the wells with buffer (Figure 11). This measurement confirms that neither Them1 START domain nor any other component in the lysate influenced the transfer rate of phosphatidylcholines. In the wells comparing unexpressed vs. expressed PC-TP lysate (Figure 12), the unexpressed PC-TP sample fluoresced at the rate slightly greater than that in the wells with buffer. This unexpected fluorescence suggests that some PC-TP was expressed in the *E. coli* cells without the presence of IPTG. However, this phenomenon often occurs (Kanno, 2007, JBC). The wells in the assay with expressed PC-TP lysate fluoresced at a greater rate than the unexpressed lysate, indicating more rapid phosphatidylcholine exchange between vesicles. Although some PC-TP was expressed in the control lysate, the difference in measured fluorescence between the expressed and unexpressed samples indicates that cellular components in the lysate did not appreciably promote phosphatidylcholine transfer activity.

The lipid specificity of other START domain proteins may shed light on that of Them1 START protein. Two members of the PCTP group, PC-TP and STARD10, both transfer phosphatidylcholines (Wirtz, 1991; Oliayioye, 2005). STARD11, another member of the PCTP group, has been found to be a non-vesicular ceramide-carrier protein that transfers ceramide from the endoplasmic reticulum to

the Golgi apparatus (Hanada, 2003). Members of the STARD1 group have been found to be involved in the transfer of cholesterol from the inner to outer mitochondrial membrane (Tsujishita and Hurley, 2000). STARD5, part of the STARD4 group, binds cholesterol and 25-hydroxycholesterol in cholesterol metabolism (Rodriguez-Agudo, 2005). The lipid specificity of the two proteins in the Thioesterase START group, Them1 and STARD15/CACH, is not known. It is possible that these proteins may bind or transfer ligands common to the other START domain proteins.

The assay performed in this experiment tests only for phosphatidylcholine transfer activity; it does not test for phosphatidylcholine binding. It is possible that Them1 START domain binds to phosphatidylcholines but does not transfer the lipid. This phenomenon could be explained by the presence of the two thioesterase groups on the complete Them1 protein. It has been found that PC-TP transports phosphatidylcholines to a Them2 protein complex (Kanno, 2007, JBC). PC-TP's biological function may be to bring phosphatidylcholines to the thioesterase, causing the need for transfer activity. In this case, the Them1 protein would not be required to transfer phosphatidylcholines because the START group is already attached to the thioesterase; it would only need to bind them.

There are several ways to determine whether the Them1 START domain protein binds phosphatidylcholines. Them1 protein could be purified from brown fat tissue in rats. Its ligand could then be identified using mass spectrometry. Alternatively, the recombinant Them1 START protein lysate could be added to brown fat, allowing it to interact with lipids present in the tissue. The protein could then be extracted and its ligand identified with mass spectrometry. If the identified ligand is phosphatidylcholine, then it can be concluded that Them1 START protein binds phosphatidylcholines but does not transfer them. If the identified ligand is not phosphatidylcholine, then an assay can be created to determine whether Them1 START protein binds other lipids. Lipids found in brown fat, where Them1 is

expressed *in vivo*, and lipids bound by other START domain proteins, such as cholesterol and ceramide, would be likely candidates.

This study determined that Them1 START domain likely binds to phosphatidylcholines to some degree, though it does not promote appreciable transfer activity. While the assay established that Them1 START domain does not facilitate this transfer activity, there were many limitations to the study and sources of error. The Them1 START domain may have transferred phosphatidylcholines from donor vesicles to acceptor vesicles but also acted as quenchers themselves, thereby causing the spectrophotometer to detect no fluorescence. In effect, it is possible that the Them1 START domain absorbs the light emitted from the fluorescent-labeled phosphatidylcholines. Additionally, phosphatidylcholines were the only lipid molecule tested for transfer activity in the assay. Because Them1 START domain resembles PC-TP in structure, it is likely to bind another lipid molecule. Further investigations could reveal the true function of the Them1 START domain.

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