Spring 4-1-2005

Function and Role of Synaptotagmin Proteins in Yeast

Aaron J. Yang

INTRODUCTION

Background

Since the discovery that calcium ions trigger exocytosis of vesicle contents such as neurotransmitters, a family of proteins known as synaptotagmins has been studied as calcium sensors that couple a rise in calcium with synaptic vesicle formation and release. A membrane protein on synaptic vesicles, the synaptotagmin proteins bind calcium ions through two calcium binding domains; C2A and C2B. To date, there have been 13 identified isoforms that may be organized into six different classes. Of all the variants, Synaptotagmin 1 (Syt 1) has been studied most extensively due to its abundance and prominent function at various stages in synaptic vesicle formation (Tucker and Chapman 2002). In addition, recent studies have concluded that Synaptotagmin 1 is the only isoform demonstrated to control synaptic vesicle fusion (Yoshihara and Montana 2004).

The C2 domain is a phospholipid and calcium binding motif that is found in a variety of plant and animal proteins. Following synaptic calcium influx, these domains are polarized in that calcium binds to the top loops of the domain. The C2A domain is able to ligate three calcium ions through five aspartate and one serine residue while the C2B domain ligates only two calcium ions due to its lack of a binding site (**Figure 1**). These domains have also been shown to interact with the SNARE complex; which is known to mediate membrane fusion in exocytosis (Sudhof 2001).

INSERT FIGURE 1 HERE

The assembly of the SNARE complex is known to drive vesicular fusion though exocytosis (Chapman 2002). **Figure 2** shows the general schematics of calcium triggered exocytosis and the involvement of calcium sensing proteins. Previous studies have indicated that synaptotagmins play an important role in regulating calcium-dependent exocytosis by interacting with the SNARE proteins syntaxin and SNAP-25 in the synaptic region (Sudhof 2001). At low cytosolic calcium levels, synaptotagmin binds to the plasma membrane proteins neurexin, syntaxin, and SNAP 25; acting as a "clamp" in which the binding of other essential fusion proteins to the neurexin-syntaxin complex is been blocked (**Figure 3**). As a result, vesicle fusion is prevented in the presence of low calcium ions. However, when the cytosolic calcium levels are high, synaptotagmin binds to the calcium ions through the C2 binding domains and is displaced from the SNARE complex. Subsequently, other proteins are able to bind to the complex initiating docking and fusion of the membrane. In response to calcium binding, the synapotagmin protein is therefore critical for the fusion of the docked synaptic vesicle with the plasma membrane (Sudhof 2001).

Previous experiments have shown that synaptotagmins function in a variety of membrane-trafficking events such as exocytosis. However, recent experiments have shown that transfection of truncated synaptotagmins led to the inhibition of endocytosis by preventing clathrin coat assembly. As a result, synaptotagmins have been seen to play a role in both vesicle formation and release (Sudhof 2001).

INSERT FIGURE 2 HERE

INSERT FIGURE 3 HERE

In the yeast genome sequence, three homologous genes have been identified that encode a transmembrane domain near the N-terminal region and calcium binding C2 domains with organization similar to that of mammalian synaptotagmin genes. Western blots have revealed high-molecular weight bands at 130 kDa and smaller fragments at 46 kDa and 39 kDa (Creutz et al. 2004). As mentioned before, mammalian synaptotagmins are known to have two C2 domains while the yeast homologs have three calcium binding domains; C2A, C2B, and C2C. Previous experiments that utilized the techniques of combinatorial disruption of the multiple C2 regions concluded that the domains functioned as multimers in membrane trafficking.

BLAST searches of the amino acid sequence of C2 domains in the yeast genome revealed three open reading frames; each with three C2 domains. The corresponding genes were revealed to be YOR086c, YNL087w, and YML072c. In comparison with the mammalian synaptotagmin gene, YOR086c and YNL087w have 55% identity to one another in the region of overlap between the two genes while YML072c has only 28-30% identity to the first two C2 domains in the regions of overlap. In addition, YML072c is larger at 1545 residues in comparison to YOR086c and YNL072c (1186 and 1178 residues). The transmembrane domain is present near the N terminus as was observed in the synaptotagmin proteins in mammalian systems (Creutz et al. 2004).

The question regarding the presence of a third C2 domain in yeast has yet to be fully answered and the interaction between the C2 domains has to be further analyzed. As mentioned before, the synaptotagmin proteins play an important role

in vesicular trafficking and exocytosis. Through homologous recombination using DNA cassettes with selectable markers and PCR amplification, gene deletions of the three homologs can be created and characterized for their phenotypes. As the focus of this research, double and triple mutants have been tested under various conditions and assays for growth defects. Cell-cell fusion defects in the mating process have been tested as this step is likely to be dependent on vesicle trafficking. In addition, strains have been tested for their response to alpha factor pheromone as this is another important process during mating that is dependent on the proper membrane docking and fusion of vesicles. Through a variety of experiments, the goal of my research was to gain a further understanding of the function and role of the synaptotagmin homologs in yeast and perhaps open the door for others to further study the important roles of these proteins in higher organisms.

MATERIALS AND METHODS

Yeast Strains

The yeast strains constructed and used in this study are listed in **Table 1**. The strains that contained the single deletion were obtained from a stock collection in Dr. Erdman's laboratory. The stocks, in 50% glycerol, were frozen and stored in a -80°C freezer. The strains were then streaked and grown on YPAD plates which were stored overnight for optimal growth in a 30°C incubator. Sterile toothpicks were used to transfer the stocked strains onto the YPAD plates.

Following the growth of the strains on the YPAD plates, they were stored for future use in the 4°C refrigerator. The double and triple mutants were all derived from strains yor086c, yml072c, and ynl087w. These strains were generally stored on YPAD plates in the 4°C refrigerator (Adams et al. 1997).

YPAD Media and Plates

Media for petri plates were prepared in 2-liter flasks, with each flask containing 1 liter of medium. Each flask was sufficient enough for about 30-40 plates. The components were: 10g yeast extract, 20g bacto peptone, 20g dextrose, and 30mls 10MM adenine to 800ml of distilled water. The mixture was brought up to 1L with ddH20. The mixture was stirred for approximately 15 minutes. Twenty grams of agar was added only for plates. Following the stir, the mixture was autoclaved for 25 minutes on the liquid cycle followed by a cool down time of 25 more minutes. The hot media was cooled to touch and poured onto the petri

Table 1

plates. The liquid media was aliquoted to various flasks and stored at room temperature until further use.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed using 0.8% agarose in 1X TAE buffer solution (40 mM Tris (pH 8.0), 20 mM acetic acid, 1 mM EDTA) along with 10ug/ml ethidium bromide. The protein bands that formed on the gel were visualized using an UV illuminator following the electrophoresis.

PCR Screening of Genomic Target Sequences

Two milliliters of each transformant was grown overnight. Genomic DNA was prepared following a Genomic Miniprep protocol by a DTAB (Dodecyltrimethylammonium bromide) extraction procedure. Final volume was set to 50µl. The final volume of the PCR mixture was set at 30µl and the mixture was placed into 200µl PCR reaction tubes. The mixture was as follows: first 23.75 µl of double distilled water was added to the tube followed by 0.5 µl of dNTPs at 10mM each. 1.5µl of genomic DNA was added to the mixture followed by .5µl of each of the primers. The last step included the addition of .25µl of Taq polymerase followed by centrifugation for 15 seconds. The tube was placed into the PCR machine and the program was as follows: 95°C for 4 minutes followed by 32 cycles of amplification (94°C for 1 minute, 53°C for 2 minutes, 72°C for 3 minutes) and completed with an extension time of 5 minutes at 72°C. The samples were held at 4°C until they were loaded onto the agarose gels.

Yeast Genomic Miniprep

A strain of interest was collected onto a sterile toothpick and swirled into 2ml of YPD media in a glass tube. The tubes were placed onto a roller and kept at 30°C overnight. The liquid culture was then poured into an 1.5ml microcentrifuge tube followed by pelleting and removal of the supernatant. The pellets were spheroplasted at 37°C for 60 minutes in 250µl fresh SEM (2ml stock: 1ml of 2M Sorbital, 100 µl of 1M NaPO4 pH 6.8, 400µl of .5M EDTA, 2µl of .1% betamercaptoethanol, 60µl of 10mg/ml Zymolyase, and 440 µl of ddH20). 500µl of DTAB lysis buffer was added and the tube was heated at 65°C for 10 minutes. The tubes were allowed to cool for a few minutes and then extracted with 500µl chloroform, spun for 4 min., and 600µl of aqueous phase was collected in another eppendorf tube. 750µl of ddH20 was added along with 45µl of 5% CTAB (Cetyltrimethylammonium bromide) in 0.4M NaCl. This mixture was allowed to sit for 30 minutes at room temperature. The tubes were mixed to precipitate the DNA and spun down for 5 minutes. The supernatant was aspirated and the pellet was resuspended in 300µl 1.2M NaCl to exchange detergent and precipitated with 750µl EtOH. The pellet was finally rinsed and dried and 50µl of TE+RNase A was added to dissolve the pellet.

Tetrad Dissection

Before the tetrad dissection of the spores of interest can be performed, the spores must be previously prepared by a series of steps. First, the strain of interest is streaked from the stock collection onto a YPAD plate using a sterile toothpick. The plate is allowed to incubate overnight in the 30°C incubator. The following

day, the patches were then streaked onto GNA pre-sporulation plates and left at room temperature overnight. The patches were then inoculated into 2.5mls of 2% KAc sporulation medium. The tubes were placed in a room temperature roller for 5 days. Following that time, the culture was centrifuged in tubes and the aqueous solution was discarded. Five hundred microliters of ddH20 were added, vortexed, centrifuged, and the liquid was removed again. Another 500µl of sterile ddH20 were added and the tubes were stored at the 4°C refrigerator until it was time to perform the tetrad dissection. In order to perform the tetrad dissection, 20µl of the mixture stored in the 4°C refrigerator was added to 30µl of ddH20 in a 1.5ml centrifuge tube. Five microliters of zymolyase (10mg/ml) was added to the centrifuge tube and vortexed. The tube was then placed in a 30°C incubator for 13 minutes. Six microliters of the mixture in the tube was spread in a straight line across a YPAD plate right through the middle of the plate. Using the Nikon compound microscope in Dr. Erdman's lab, each spore from a tetrad was separated by placing the needle so that it gently touches the tetrad on the plate (**Figure 4a**). By gentling tapping the needle up and down against the tetrad, the tetrads were broken apart and each spore was distributed across the plate in a row until the tetrad was completely separated (**Figure 4b**). The plates containing the dissected spores were plated with different nutritional or drug resistant markers and tested on the segregation that followed on the plates. The plates were placed in the 30°C incubator for about 2 days until the dissected spores grew and could be collected as vegetative strains.

Insert figure 4

High Efficiency Transformation

5ml of liquid YPAD medium was inoculated by the strain of interest to be transformed. The culture tube was then placed in a roller in the 30°C incubator overnight. The optical density of the overnight culture was observed using a spectrophotometer (set at 600 ηm in Absorbance mode) and 50ml of YPAD was inoculated to a cell density of 5 X 10^6 cells/ml culture. The culture was then incubated at 30°C on a shaker in 500ml flasks at 200rpm until the cells reached a density of 2 X 10^7 cells/ml. The incubation time was about 6 hours. The culture was harvested in sterile 25ml centrifuge tubes at 2500rpm for 10 minutes. The medium was poured off and the cells were resuspended in 25ml of ddH20 and centrifuged again. The water was poured off and the cells were resuspended in 1ml of 100mM lithium acetate (LiAc). The suspension was transferred to a sterile 1.5 eppendorf tube and pelleted at top speed for 5 seconds. The LiAc was removed with a micropipette and the cells were resuspended to a final volume of 500µl (2 X 10^9 cells/ml), which is about 400µl of 100mM LiAc. (The excess was frozen by the following method: 100µl aliquots were pelleted and the LiAc was removed. The cells were then resuspended in 30µl of 50% glycerol and 70µl of YPAD.) The cell suspension was vortexed and 50µl samples were pipetted into labeled 1.5ml microcentrifuge tubes. The cells were pelleted and the LiAc was removed. The basic "transformation mix" consisted of the following reagents, added in the following order: 240µl of PEG (50%w/v), 36µl of 1M LiAc, 5µl of single stranded carrier DNA (2mg/ml), and 50µl of ddH20 and plasmid DNA. The tube containing the mix was vortexed vigorously until the cell pellet was

completely mixed and then it was incubated at 30°C for 30 minutes. Then the tube was heat shocked for 20-25 minutes in a water bath at 42°C. The tube was then spun down at full speed for 15 seconds and the transformation mix was removed. Two hundred microliters of ddH20 was pipetted into each tube and the pellet was resuspended by gently pipetting up and down. The aliquots of the transformation mix were plated onto selective plates and stored overnight in a 30°C incubator until colonies were visible (2-3 days).

Double Mutant Construction

Crosses of MAT a and MAT alpha strains of yor086c::KAN^R, ynl087w::KAN^R, and yml072c::KAN^R were made on YPAD plates. The strains, which each contain a specific deletion that encompasses the entire coding region of each of the synaptotagmin homologs, were obtained from the freezer collection in Dr. Erdman's lab. The MAT a and MAT alpha cells were collected using a sterile toothpick and crossed in a drop of liquid YPAD medium on a YPAD plate. The patches of the crossed cells on the YPAD plate were incubated overnight in the 30°C incubator. The grown patches were then streaked onto SC –met/-lys plates and incubated overnight. Growth on the selective plates confirmed that the crossed strains were lys2Δ/LYS2+ met15Δ/MET15+. The patches were then re-streaked onto GNA pre-sporulation plates and left at room temperature overnight. The patches were then inoculated into 2.5mls of 2% KAc sporulation medium. The tubes were placed in a room temperature roller for 5-7 days. Following that time, the culture was centrifuged in tubes and the aqueous solution was discarded. Five hundred microliters of ddH20 were added, vortexed, centrifuged, and the

liquid was removed again. Another 500µl of sterile ddH20 were added and the tubes were stored at the 4°C refrigerator until it was time to perform the tetrad dissection (see above). Tetrad dissection and genotyping of the progeny spores was performed to find the spore that contained the double deletion. The dissection plates were pressed onto velvets and Kanamyacin resistant plates (a 2:2 segregation ratio should be observed by growth on the plate) as well as SC-met and SC-lys plates were pressed onto the velvets. The replicated plates were incubated overnight in the 30°C incubator and observed the next day for the following patterns: growth on the KanR plates (indicates resistance to Kanamyacin); growth on SC-lys plates and growth on SC-met plates. MAT a cells were selected with the genotype (met-/LYS+) while MAT alpha cells were selected with the genotype (MET+/lys-). After collecting and recording the data, the strains that reflected KanR + and the correct genotypes for the MAT a and MAT alpha cells were collected using sterile toothpicks and streaking the strains onto a new YPAD plate. Following the growth of the strains on the new YPAD plate, 2ml of YPAD media were inoculated by the collected strains and put on the 30°C roller overnight to confirm the correct mating type. 1ml of YPAD was put into a new tube along with 500µl of the grown culture and 500µl of wild type culture of either MAT a or MAT alpha cells depending on the recorded results from the SC-lys and SC-met plates. The mating type was confirmed by observing clumping of MAT a or MAT alpha cells. The data was collected and the strains that reflected the correct mating type were collected. Glycerol stocks of the strains were prepared by the following: 2ml of YPAD were inoculated by the specific

strain and placed on the 30°C roller overnight. The following day, 750µl of culture were collected in an eppendorf tube and 750µl of sterile 50% glycerol was added to the tube. The tubes were vortexed and stored in the -80°C freezer in Dr. Erdman's lab. Genomic DNA of the collected strains was prepared followed by a PCR reaction using the corresponding upstream and downstream primers. The PCR products were then tested for the presence of the double deletion by running gel electrophoresis.

Triple Mutant Construction

A PCR reaction was set up to amplify the URA3 marker using pRS306 and YOR086c upstream and downstream primers (see previously for PCR protocol). A 50µl reaction was set up using the New England Biolabs Taq polymerase. 5ml of YPAD liquid media were inoculated with the strain YAY34 double mutant (**Table 1**) and transformed by following the High Efficiency Transformation Method mentioned previously. Ten microliters of PCR reaction were used per transformation. Following the transformation, the colonies were collected from the plates, patched onto SC-URA, and observed for growth to confirm the successful insertion of the URA cassette into the genome. Genomic DNA of the candidate colonies were made and a PCR reaction was set up using checking primers. This was followed by gel electrophoresis in order to confirm the successful integration of the third and final cassette (yoro86c::URA3) through homologous recombination (**Figure 5a)**.

Insert figure 5

Growth Assays by Serial Dilutions

4ml of liquid YPAD were inoculated with the strain of interest. The culture tubes were placed in the roller at room temperature overnight. The optical density at A_{600} of the culture was determined using a spectrophotometer. Sterile water was added to bring the final volume of the culture to 1ml. Each culture was diluted by the addition of 100µl of previous culture each time until the final concentration came to .001 of the normal starting concentration (**Figure 5b**). Plastic boxes that contained wells were used to carry on the dilutions. Finally, a sterile metal pinner was dipped into the boxed wells and the cells were pinned onto plates that contained various media (i.e. 100mM $CaCl_2$, YPAD) and incubated at 39°C overnight for optimal growth conditions. Growth phenotypes of the strains were observed the following day.

Scoring Mating Cell Morphology and Fusion Defects

MAT a and MAT alpha cells of wild type and double mutant strains were grown in culture tubes overnight to early log phase. The cells were diluted to an optical density between 0.05 and 0.1. The cells were then allowed to grow up to an optical density of 0.4. The numbers of cells were counted using a hemacytometer and compound microscope. For fusion defects and morphology scoring, an equal number of a and alpha cells were used; which was set at 5×10^6 cells. The strains were mixed to mate in a fresh tube and fresh media was added to reach a final volume of 5ml. The tubes were vortexed to mix the components and mated on filters for 4 hours. The cells were then harvested by washing the cells into eppendorf tubes from the filter. The cells were then fixed with 0.1 volume

37% formaldehyde at room temperature overnight. The cells were pelleted and washed with 1.0 ml 1xPBS, 1M Sorbitol. The cells were resuspended in 1.0ml 1xPBS, 1M Sorbitol and stored at 4°C. Cell fusion defects and morphology were scored using the Nikon TE-300 microscope. The microscope was set up for DIC (Differential Interference Contrast) microscopy using a 100x objective.

Alpha Factor Pheromone Response Assay

MAT a strains of interest were inoculated in 4ml of YPAD in culture tubes. The tubes were placed on a roller in the 30°C incubator overnight. The number of cells in the culture was counted using the hemacytometer and compound microscope. The cells were brought to a final concentration of 5×10^2 cells/ml. 4ml of molten agar were poured into tubes and placed to warm in a heating block set at 42°C. The equilibrated cultures were pipetted into the tubes in the heating block. Immediately after adding the culture to the molten agar, the tubes were vortexed and the agar was poured onto warm YPAD plates and cooled. Following the cooling step, 4 paper discs were placed on the plates. Varying concentrations of alpha factor pheromone (5ug/µl) were added to the paper discs: 0.5ug, 1ug, 2.5ug, and 5ug. The plates were incubated at room temperature for a few days during which the halo sizes of growth inhibition due to alpha factor response were observed and measured.

BAR1 Gene Deletion

Freezer strain YSE7, containing a bar1::HIS3 allele, was obtained from Dr. Erdman's yeast collection. Genomic DNA was prepared following the previously mentioned protocol. The PCR reaction was performed with bar primers ordered

from MWG-Biotech Company. Gel electrophoresis was performed following the PCR reaction. Through the high efficiency transformation method (mentioned previously), the MAT a wild type and mutant strains were transformed. Through homologous recombination using the DNA cassettes (HIS3 flanked by BAR1 sequences in this case), the PCR product effectively replaced the BAR1 gene through the transformation method. The colonies were grown on SC–his selective plates for a few days in the 30°C incubator until the colonies could be effectively collected and screened. The BAR1 gene replacement (bar1::HIS3) was confirmed by a PCR reaction followed by gel electrophoresis using the corresponding checking primers.

RESULTS

Construction of Strains Lacking Different Synaptotagmin Homologs

In order to identify potential C2 domain containing proteins encoded in the yeast genome, BLAST searches were used to compare amino acid sequences of C2 domains of mammalian synaptotagmin genes with predicted protein sequences encoded in the yeast genome. The three open reading frames that each contained three C2 domains in the yeast genome were YOR086c, YML072c, and YNL087w. Creutz and colleagues observed that YOR086c and YNL087w were the most similar, with a 55% identity in the region of similarity. YML072c was observed to have only 28-30% identity to the other two genes in the region of similarity. In all cases, the proteins contained a highly conserved transmembrane domain in their N-terminal regions (Creutz et al. 2004).

Consequently, in order to observe the knockout phenotypes and genetic interactions of the synaptotagmin homolog mutants, double mutants were first constructed by homologous recombination. It has been previously observed that single mutant knockouts were viable and did not yield any significant observable phenotypes in comparison to wild-type strains. As a result, double mutant constructs were created in order to further analyze the phenotypes of the synaptotagmin homolog genes. Single mutant constructs were obtained from the freezer stock that contained the respective *yor086cΔ*, *yml072cΔ*, and *ynl087wΔ* mutants replaced by kanamycin resistance cassettes. These were crossed to produce diploid strains and double mutants obtained among their meiotic progeny following sporulation and tetrad dissection (See **Figure 6** for general schematics).

Figure 6

All deletions were verified by PCR followed by gel electrophoresis (**Figure 7**). Primers used corresponding to sequences outside the disruption sites were employed to confirm the presence of the kanamycin resistance replacement allele of each gene. MAT a and MAT alpha strains of each of the combination of disruptions were created and organized into the chart seen in **Table 1**.

Phenotypic Analyses of Strains Lacking Different Pairs of Synaptotagmins

Each of the single and double mutant constructs were able to grow on YPAD plates. The strains also were able to grow in liquid culture. The strains were all able to mate and diploid mutants sporulated normally. However, upon further observation of the growth kinetics through various assays and microscopic analyses, some of the double mutant strains exhibited growth and cell-cell fusion defects in comparison to wild-type strains as described below.

To more carefully analyze the growth phenotypes of the single and double mutant strains, serial dilutions were made in increments of 10. By making a 1:10 dilution each time, a final concentration of .001 of the original concentration was observed (refer to **Figure 5b**). The cells were pinned onto YPAD plates as well as 100mM $CaCl^2$/YPAD plates. Both plates were incubated at 30°C for growth and observed the next day.

In observing the plates that contained only the YPAD medium (**Figure 8**), growth rates of single mutants were similar to those of wild-type cells under the same conditions. However, in the double mutant constructs, it is apparent that some of the double mutants exhibited growth defects and grew at a slower rate

Figure 7

Figure 8

than the single mutants and wild-type strains. Strains MAT a YAY34 (*ynl087w::KanMX yml072c::KanMX)* and MAT alpha YAY35 (*ynl087w::KanMX yml072c::KanMX*) displayed slow growth kinetics at elevated growth temperatures; especially YAY34 in which shows almost no growth at 0.001 dilution.

In high concentrations of calcium (100mM), we observed that again, the double mutant YAY34 (*ynl087w::KanMX yml072c::KanMX*) exhibited a much slower growth compared to single mutants and wild-type strains (**Figure 9**). Some of the other double mutants also displayed slightly slower growth kinetics compared to the single mutants and wild-type strains. In fact, YAY34 shows very little growth at 0.1 dilution and almost no growth at 0.01 dilution. Compared to what we observed on the YPAD plates, the growth of YAY34 is almost completely blocked at 0.01 dilution. As for the other double mutant strains, it was observed that they grew reasonably at the same rates compared to the wild-type and single mutant constructs.

Evaluating Synaptotagmin Mutants for Defects in the Mating Process

Although the different mutants we constructed were able to mate reasonably well, such assays are not sensitive enough to determine whether these strains might possess more subtle defects in the mating process. Thus, in order to examine whether the synaptotagmin homologs played a role in cell-cell fusion during mating, fusion defects and mating morphology were observed and scored using microscopic analysis. **Figure 10a** shows the comparison of wild-type mating morphology in which the mating cells completely fuse together.

Figure 9

Figure 10

On the other hand, defects in cell-cell fusion cause incomplete fusion in which a cell wall is observed between the fusing cells. As a result, it may be observed that there is not a complete transfer of cytoplasmic material between the mating cells due to the intervening cell wall. Using a microscope set up for DIC optics using a 100x objective, the double mutant strains were scored for such cell-cell fusion defects in comparison to wild-type strains.

From 80 wild-type zygotes observed under the microscope, there were no fusion defects observed. However, when observing the zygotes formed from matings involving partners that were double mutants, double mutant cell-cell fusion defects were observed in approximately 8.7% of the zygotes. For the bilateral mating of *yor086cΔ* and *ynl087wΔ* strains, for every 75 normal zygotes observed, 9 fusion defects were counted. For the bilateral mating of the *yor086cΔ yml072cΔ* strains, there were 6 fusion defects for 75 normal zygotes and for the *ynl087wΔ yml072cΔ* strains, there were 5 fusion defects for 80 normal zygotes (**Figure 10b**).

As a result, we can clearly observe by scoring the fusion defects among the zygotes that the double mutant strains displayed increases in cell fusion defects relative to wild-type cells during the mating process. Interestingly, this step of the mating process is known to be dependent on vesicle trafficking and from these observations, it is also likely that this vesicle fusion occurs in a calcium-dependent manner.

Construction and Phenotypic Analysis of a Strain Lacking Any Synaptotagmins

As mentioned before, synaptotagmin like proteins are known to be present in the yeast genome. As a result, a third deletion strain was constructed by homologous recombination using DNA cassettes with selectable markers and PCR amplification. Double mutant strain YAY34, which already contains the ynl087w and yml072c knockouts by the Kanamyacin cassettes (**Table 1**), was transformed with the URA3 marker flanked by yor086c sequences; which was prepared by PCR amplification. Through homologous recombination, the URA3 marker successfully replaced the yor086c gene creating a triple deletion strain. This was confirmed by PCR followed by gel electrophoresis using the correct upstream and downstream primers corresponding to sequences outside of the disruption sites (**Figure 11**). The triple mutants were viable and exhibited normal growth in liquid culture as well as on YPAD plates.

Alpha Factor Mating Assay

Budding yeast respond to mating pheromones by arresting their cell cycle division at G1. The cells are then able to differentiate into a type of cell capable of finding and fusing with mating partners (Erdman and Snyder 2001). Cell surface growth (forming mating projections) occurs when MAT a cells are exposed to the mating pheromone alpha factor which is only secreted by MAT alpha cells. Thus the life cycle of these organisms depends on the reception of extracellular signals followed by cell differentiation. Reception of the pheromone signal occurs through receptors present in the cell membrane. Such receptors are delivered to and retrieved from the membrane largely through vesicle trafficking.

Figure 11

In order to observe whether the triple mutant synaptotagmin homolog strain showed any observable phenotypes (in contrast to wild-type) in response to alpha factor, a halo mating assay was performed. MAT a strains YAY36 and 430a (wild-type) strain were plated onto YPAD plates. Four paper discs were placed on each plate with each disc containing increasing amounts of alpha factor (5ug/µl). The concentrations were: 0.5ug, 1ug, 2.5ug, and 5ug. The plates were allowed to grow at room temperature for a few days and the halo sizes were observed.

Observing **Figure 12**, halo sizes for the 430a wild-type strain were slightly larger in comparison to the triple mutant strain. The same result was observed throughout many repeat experiments (n=10). From this experiment, it was concluded that the triple mutant strain showed a reduced responsiveness to the alpha factor mating signal and as a result, the halo sizes were smaller in comparison to the triple mutant strain (See **Table 2**).

Bar1 Gene Deletion

The expression of the BAR1 gene in yeast is induced by the alpha factor pheromone. Produced only by MAT a cells, BAR1 inhibits the activity of alpha factor on MAT a cells. This protease is secreted into the periplasmic space of MAT a cells and cleaves and inactivates alpha factor allowing cells to recover from alpha factor induced cell cycle arrest (hence the name is derived from the BARrier effect). Mutations in the BAR1 gene lead to increased supersensitivity of MAT a BAR1 cells to the G1 arrest induced by the alpha factor (Manney 1983). We took advantage of the increased sensitivity to alpha factor conferred by the loss of BAR1 to help confirm the reduction in alpha factor sensitivity of the triple

Figure 12

Table 2

mutant strain. To further analyze the response to alpha factor of the synaptotagmin-less strain, a replacement of the BAR1 gene with HIS3 was performed in both the parental 430a (wild type) and YAY36 (*ynl087wΔ yml072cΔ yor086cΔ*) strains, followed by the same halo inhibition assay as described above.

In observing **Figure 13**, the first thing that is noticed (in comparison to the alpha factor halo mating assay without the BAR1 deletion) is the increased sizes of the halos. This is due to the fact that the MAT a strains have become hypersensitive to the pheromone inducing G1 arrest by the alpha factor. When comparing the halo sizes between the wild-type strain 430a and YAY36 lacking BAR1, we can slightly observe that the halos for the wild-type are larger in comparison to the triple mutant. Though the difference in halo size between the wild-type and triple mutant is very small, the observation that the halos for the wild-type are slightly larger support the data that was previously observed before the BAR1 gene deletion.

Figure 13

DISCUSSION

Through BLAST searches and by analyzing the synaptotagmin homolog genes in yeast, we have revealed the presence of three C2 domain encoding genes: YOR086c, YML072c, and YNL087w. By eliminating these C2 domain proteins encoded in the yeast genome, we have observed that the mutant strains, double and triple, are still viable under normal laboratory conditions.

However, upon further observation of growth kinetics by serial dilutions, it was observed that double mutant strains, especially YAY34 and YAY35, exhibited growth defects and grew at a slower rate compared to wild-type strains. With the knowledge that YAY34 and YAY35 are both the MAT a and MAT alpha strains of the ynl087w and yml072c deletions (from **Table 1**), it was unexpected to observe that these two C2 domains have only 28-30% identity in regions of overlap in comparison to YOR086c and YNL087w, which has 55% identity in region of overlap. In either case, it would be necessary to observe the growth kinetics of the triple mutants in order to further analyze and determine whether the synaptotagmin homolog genes function as multimers as well as observe the interactions between multiple C2 domains. However, in a study released by Creutz and colleagues (2004), they observed that the synaptotagmin homolog proteins functioned as heterodimers. Interestingly, through two-hybrid screens to observe homolog affinity, they discovered that one of the dimer partners had to be YNL087w (Creutz et al. 2004). As a result, this may partially explain the growth defects that were observed in the serial dilutions. Nevertheless, I believe that a further analysis of the triple mutants is necessary in order to fully

understand the genetic and physiological interactions of the homolog synaptotagmin encoding genes.

In observing the cell morphology of the double mutants as well as scoring the fusion defects during cell-cell fusion, it not surprising to observe fusion defects in the double mutants as cell-cell fusion during the mating process is highly dependent on vesicle trafficking. Altered vacuole morphology was observed in other experiments conducted on yeast by Creutz and colleagues. In their study, they observed that instead of the multi-lobed vacuoles of wild-type cells, the mutant homolog genes had an enlarged vacuole consisting of a single compartment (Creutz et al. 2004). As a result of the studies, it is concluded that the synaptotagmin homolog genes play an important function in guiding cell-cell fusion during the mating process.

Finally, in characterizing the phenotypes of the triple mutants in response to alpha factor pheromones, it was observed that the mutant strains exhibited decreased ability to respond to alpha factor as observed by the smaller sizes of the halos in comparison to wild-type strains. One of the possible explanations may be that the inactivation of synaptotagmin homologue genes may lead to aberrant regulation of transcription of another gene, Bar or a gene controlling it, that may alter the degradation and inactivation of alpha factor either directly or indirectly. Another possibility is that since the synaptotagmin genes are crucial for vesicle docking and release (endocytosis and exocytosis), mutations or deletions in these genes might not allow proper vesicle docking and thus the level of alpha factor receptor on MAT a cells may be reduced. We favor the latter hypothesis as it is

known that vesicle transport is crucial for transport from the endoplasmic reticulum to the Golgi apparatus and finally to the plasma membrane. Thus, loss of function of one or more of the synaptotagmin proteins might affect transport of vesicles and their contents, including cell surface resident pheromone receptors, to the plasma membrane. As a result, a reduced pool of pheromone receptor on the cell surface due to a reduction in vesicle trafficking may explain the lack of response from mutant cells to alpha factor. In addition, absence of the synaptotagmins may have also affected the endocytic process, allowing receptors to be trafficked to and degraded by lysosomes instead of being recycled back to the plasma membrane surface. This would also lead to the reduction of cell surface receptors and response to alpha factor pheromone.

As the function of synaptotagmins becomes better understood in regards to their roles in membrane fusion and trafficking, research on these genes has been steadily growing. As mentioned previously, there are many isoforms of the synaptotagmin genes and there are at least 14 mammalian variants (Creutz et al. 2004). Different forms have been found to be localized on the synaptic vesicles as well as the plasma membrane. For example, it has been studied that Syts 3, 6, and 7 have been localized to the plasma membrane (Sudhof 2001). In addition, though Syt 1 is known to be the only isoform demonstrated to control synaptic vesicle fusion, Syts 4, 7, 12, and 14 have been identified in the Drosophila genome and it has been observed in that organism that the synaptotagmins are differentially distributed to different subcellular compartments. For example, it was shown that Syt 4 localized to the postsynaptic compartment. As a result, the studies

concluded that calcium-dependent membrane trafficking functions on both sides of the synapse (Adolfsen et al. 2004).

As far as other research on synaptotagmin homologs in yeast is concerned, there are some noteworthy experiments that have been conducted. In one study, rat Synaptotagmin 2 was expressed in several temperature sensitive secretory mutant yeast strains that were defective in Golgi to plasma membrane vesicle transport (Damer and Creutz 1996). What they observed was that Syt 2 expression partially rescued the growth defect in one particular mutant, Sec15. As a result, they concluded that the protein complex of Sec15 and Syt 2 functioned in a late stage of exocytosis in yeast and that the synaptotagmin is able to regulate the function of this complex in cells. As mentioned before, one other experiment that has been crucial in understanding the interactions of multiple C2 domains was recently published by Creutz and colleagues. Through two-hybrid screening, they discovered that the C2 domains interact with one another by forming heterodimers in order to carry out their biological functions. More importantly, they stated that one of the partners of the dimer must be YNL087w (Creutz et al. 2004). The proposed model for the dimerization of the homolog strains has been proven to be essential in characterizing and studying the interactions of the synaptotagmin homolog genes.

As my research on the synaptotagmin homolog genes in yeast concludes, there are some unfinished tasks that must be addressed in order to further understand the role of these genes in vesicular fusion and trafficking. As mentioned before, serial dilutions of the triple mutants must be observed; as well

as further investigation of other observable phenotypes such as budding and mating morphology. However, looking at the bigger picture, the synaptotagmin gene family has a variety of isoforms that are localized to various subcompartments throughout the cell. I believe that my research serves as a basic foundation for the future research that will be conducted to help reveal the important roles and functions of the synaptotagmin gene family in regulating cell to cell signaling.

SOURCES CITED

Adams, A., D. Gottschling, C. Kaiser, and T. Stearns. Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, 1997

Adolfsen B., M. Saraswati, M. Yoshihara, and J. T. Littleton. (2004). Synaptotagmins are trafficked to distinct subcellular domains including the postsynaptic compartment. *J. Cell. Biol.* 166: 249-260

Chapman E. R., (2002). Synaptotagmin: A Calcium Sensor That Triggers Exocytosis? *Nat. Rev. Mol. Cell Biol.* 3: 498-508

Creutz C. E., S. L. Snyder, and T. A. Schulz. (2004). Characterization of the yeast tricalbins: membrane-bound multi-C2-domain proteins that form complexes involved in membrane trafficking. *Cell. Mol. Life Sci.* 61: 1208-1220

Damer C. K., and C. E. Creutz. (1996). Synaptotagmin II expression partially rescues the growth defect of the yeast sec15 secretory mutant. *Biol. Cell* 88: 55-63

Erdman, S. E. and M. Snyder. (2001). A Filamentous Growth Response Mediated by the Yeast Mating Pathway. *Genetics* 159: 919-928

Giaever, G. Yeast Knock Outs. (2002). *Open Biosystems*. http://www.openbiosystems.com/yeast_knock_outs.php

Guthrie, C., and G. R. Fink. Guide to Yeast Genetics and Molecular Biology. London: Academic Press, INC. 1991.

Manney T. R. (1983). Expression of the BAR1 gene in Saccharomyces cerevisiae: induction by the alpha mating pheromone of an activity associated with a secreted protein. *J. Bacteriol* 155: 291-301

Molecular Cell Biology. New York: W. H. Freeman and Company, 2001.

Sudhof, T. C. (2001). Synaptotagmins: Why So Many? *Journal of Biological Chemistry* 227: 7629-7632

Sudhof, T.C. (1995). The synaptic vesicle: a cascade of protein-protein interactions. *Nature*. 6533: 645-653

Sudhof, T.C., and R. Jahn. (1991). Proteins of synaptic vesicles involved in exocytosis and membrane recycling. *Neuron*. 6: 665-677

Tucker W. C., and E. R. Chapman. (2002). Role of synaptotagmin in Ca2+-triggered exocytosis. *Biochem. J.* 366: 1-13

Yoshihara M., and E. S. Montana. (2004). The synaptotagmins: calcium sensors for vesicular trafficking. *Neuroscientist*. 10: 566-574