2008-01-01

Giardia Lamblia: Genomic And Molecular Analyses Of Flippase

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Dedication

I dedicate this work to my three boys, my wonderful husband, my mom, my grandmother and my sister. They stood behind me and never let me forget that anything is possible. To my beloved grandmother, who has joined our heavenly father, this is for you grandma……we did it. Without their support this would not have been possible. I love you all very much…….Thank you.

GIARDIA LAMBLIA: GENOMIC AND MOLECULAR ANALYSES OF FLIPPASE

by

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THESIS

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

In Partial Fulfillment

Of the Requirements

For the Degree of

MASTER OF SCIENCE

Department of Biology

THE UNIVERSITY OF TEXAS AT EL PASO

December 2008

Acknowledgements

First and for most I would like to thank my mentor Dr. S. Aley and my board members Dr. S. Das and Dr. C. Dirk. I would also like to thank my colleagues Jamie Chapoy, Chris Bond, Dr. Armando Varela, and Yuni Hernandez for all the endless hours of help.

Abstract

Giardia lamblia, is an intestinal protozoan parasite responsible as a major cause of water-borne intestinal infection in humans (giardiasis). It has been proposed earlier that *Giardia* has limited lipid synthesis ability and therefore relies on preformed lipid molecules from outside sources. It has also been proposed that lipids are taken up through passive and active transport, and giardial flippase play a significant role in this process. This study describes the identification and characterization of a putative homologue of flippase gene from *Giardia*. Flippase is an enzyme that functions as a trans-bilayer transport system facilitating the flip flop diffusion of phospholipids that are generated and or modified within the cell to the outer face of the lipid bilayer. Flippase can also actively transport aminophospholipids from the outer membrane to the inner monolayer Additionally, eukaryotic flippase has been implicated in the movement of synthesized membrane lipids among organelles.

Beginning with the putative gene sequence from the *Giardia* genome, this study verifies the expression of the gene in trophozoites, expresses recombinant proteins for immunologic studies, and constructs a knockout flippase mutant to test for functional dependence of *Giardia* on this gene. I speculate that flippase plays a potentially vital role in phospholipid transport and provides potential for novel chemotherapeutic treatments against *G. lamblia.*

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Chapter 1: Introduction

Giardiasis, a clinical syndrome caused by the intestinal protozoan *Giardia lamblia*, is a reemerging waterborne infectious illness worldwide [5]. *G. lamblia* has two well characterized stages, trophozoite and cyst. During an active infection, the trophozoites are found to inhabit the upper region of the small intestine, while the cyst stage is often recovered from the stool [1]. Cysts are ovoid, smooth and colorless, highly effective, and resistant to chloride and other environmental stressors. Cyst ingestion initiates the infection. Once the cyst enters the acidic environment of the stomach, the decrease in pH and proteases cues the process of excystation [4]. Trophozoites are released from the cyst, and either attach to the intestinal epithelium by suctioning to it or swim freely within the intestinal lumen [1,4]. In this stage, the organism possesses a circular sucking disk, on its anterior side which enables the trophozoite to adhere to the intestinal wall [1]. This action causes a physical blockage of the microvilla and results in malabsorption of nutrients, most notably fats, and hampers the ability of the small intestine to secrete digestive enzymes [5]. The trophozoite replicates through binary fission in the upper portion of the small intestines [1, 4]. Free-swimming trophozoites have the ability to completely detach themselves and make their way into the fecal material and be eliminated from the body. During the trophozoite's progress through the intestinal tract, encystation (or cyst formation) takes place [1, 4]. Although the encystation process is not clearly understood yet, is it believed to be triggered by drastic changes in the environment of the parasite such as a change in pH, concentrations of dietary lipids, bile salts and other small intestinal components [4]. Not all trophozoites encyst, and the host will shed both dying trophozoites and infectious cysts in the feces.

The illness caused by this parasite is of great medical importance in developing countries where approximately 30% of individuals are infected, and of those, 20% are children [2]. In industrialized

countries like the United States, the prevalence of infection is lower in the general population but is still very high in children that attend daycare centers, where there is about a 35% rate of incidence [3].

G. lamblia is also considered an early-diverging eukaryote, possessing some of the characteristics of prokaryotes such as the lack of introns, mitochondria, lysosomes, well-evolved Golgi complex and peroxisomes. It has two symmetrical nuclei, and four pairs of flagella [4]. For this and other reasons, *G. lamblia,* serves as an appropriate model for investigating the origins of gene organization and regulation [figure 5].

In order for this organism to flourish it must be grown in complex media that supplies bile and serum or serum fractions that are enriched with lipoproteins. Blair and Weller demonstrated that *G. lamblia* depleted all fatty acids and cholesterol from its growth media indicating that the lipids supplied in the medium were taken up by the parasite [4, 15]. This data further supported the proposal that *G. lamblia* acquires its supply of lipids from its environment.

The extraction of lipids from the environment and their incorporation into its phospholipid bilayer is an active process requiring a specific mechanism to compete this procedure. Within all living organisms the maintenance of the transbilayer lipid asymmetry is essential for normal membrane function [6, 7]. In order to maintain cellular membrane integrity, manufactured phospholipids within the cell need to be transported and properly positioned into the outer layer of the membrane. Transmembrane lipid transporters, such as a flippase, are responsible for the movement and positioning of these phospholipids. If the cellular membrane is disrupted, the organism will not function properly, resulting in cell death. Maintenance of the lipid bilayer is therefore, essential for survival [6, 7]. Several mechanisms have been identified in other eukaryotes and prokaryotes that perform such a function [6].

The enzyme that performs this function in other organisms is referred to as the flippase enzyme [figure 2]. In eukaryotic cells, flippase works by bringing in lipids from the cytosolic leaflet in a “flip-flop” motion across biogenic membranes [6,4,11]. A balance is maintained between the incorporation

and synthesis of new lipids. This balance is reached when the availability of lipids either increases or decreases and this fluctuation will affect the uptake of lipids from one side of the membrane to the other. In eukaryotes, but apparently not in prokaryotes, the flippase enzyme is ATP-dependent [6,27,28].

A study conducted by Kornberg and McConnell [26,27] showed that removing membrane proteins that exhibited flippase activity resulted in a drastic reduction in the incorporation of lipids into the inner mitochondrial membrane. Bishop and Bell [29,30], used a hydrophilic phospholipid analog, di-C_4-PC, to examine the function of uptake of phospholipids by the flippase enzyme within rat liver microsomes [6]. The membrane was then treated with sulfhydryl reagent N-ethylmaleimide (NEM) and trinitrobenzene sulfonic acid (TNBS). This treatment denatured the flippase protein, and allowing investigators to see if the translocation and incorporation of the radiolabeled lipid across the membrane were interrupted. The results of these experiments indicated that the translocation of phospholipids was affected by the protease activity of the reagents used, thereby indicating that the mechanism of translocation does indeed involve a protein [6,26,29,30]. This further supported the notion that flippase plays a role in lipid transport and is sensitive to the action of proteases. This sensitivity also leads to the proposal that the flippase enzyme is indeed a protein that is imbedded into the cellular membrane.

A similar mechanism has been identified in bacteria. Bacterial cells produce phospholipids in the cytoplasmic side of their plasma membrane. These phospholipids must be transported to the other side of the plasma membrane in order for normal membrane growth to occur [6,4]. The translocation is most likely protein-mediated and does not show specificity for head group and seems to be ATP-independent. However, these processes in bacteria do show great similarity to the eukaryotic flippase in function and structure [6,7,15]. These studies showed that bacterial flippase is sensitive to proteases, transport is irrespective of the phospholipid head group, and *in vitro* transport of lipids are ATP-independent [26,27,6].

The current study uses the genome of *G. lamblia* to search for potential lipid synthesis and transport enzymes in this organism [14]. These studies identify a putative flippase homology with eukaryotic characteristics. This putative enzyme seems to contain open reading frames of approximately 3500bp. This study focuses on the function and association of this putative flippase enzyme with the translocation of phospholipids into the *G. lamblia* plasma membrane.

Chapter 2: Materials and Methods

2.1 Materials

Organism and cell culture: *Giardia lamblia* trophozoites (strain WB, ATCC No. 30597) were cultivated following the method of Diamond et al. (1978-ref) using TYI-S-33 medium supplemented with adult bovine serum and bovine bile (Keister, 1983-ref). The antibiotic piperacillin (50µg/ml) was added during the routine and harvested by centrifugation at 1050 x g for 10 min at 4^{o}C, washed and counted using emocytometer under phase-contrast optics by light microscopy.

DNA extraction and purification: Genomic DNA was extracted and purified from cultured trophozoites using a High Pure PCR Template Preparation Kit obtained from Roche Diagnostics (Indianapolis, IN). Cells were lysed during a short incubation with proteinase-K in the presence of guanidine hydrochloride, which immediately inactivates all nucleases. Cellular nucleic acids (NA) were selectively bound to glass fiber fleece in a special centrifuge tube. The NA remains bound while a series of rapid "wash-and-spin" steps removes small molecule contaminants. Finally, low salt elution was used to remove the NA from the glass fiber fleece. Precipitation, organic solvents, or extensive handling of NA was avoided (Roche manual chapter 1).

Primer Construction and Polymerase Chain Reaction (PCR): Primers were constructed to amplify the entire putative flippase sequence from genomic DNA. Oligos were extended to profile unique restriction sites for cloning and ordered from MWG Biotech (Cork, Ireland) as depicted in Table-1. Primers were reconstituted according to manufacturer's recommendation.- PCR master mix was used in combination with primers, template, and sterile water: 12.5µl of PCR mix, 2µl of primer 1, 2µl of primer 2, 5µl genomic DNA, and 3.5µl sterile water; to a total volume of 25µl. PCR master mix was purchased from Promega. PCR products were cloned using a TOPO TA cloning kit from Invitrogen Corporation, (Grand Island, NY). PCR was conducted using a program consisting of an initial cycle of

94°C for 2 minutes, followed by 35 cycles consisting of 94°C for 1 minute, 45-72 °C for 2 minutes depending upon primer annealing temperatures, then increased in later rounds, for 2 minutes and then 72°C for 3 minutes. The last cycle consisted of a hold at 4°C. Samples were pipetted into a 0.75% SeaKem Genetic Technology Grade (GTG) agarose gel. Gel was prepared by mixing 50ml of Tris-Acetate-Ethylenediaminetetra acetic acid (TAE) buffer with 0.35g of agarose. This mixture was heated using a microwave oven which ran on 2.45 gHz, until all agarose dissolved. After agarose cooled slightly, 5µl of 10mg/ml ethidium bromide (EtBr) was added. Gel was poured into a BioRad casting tray gel electrophoresis apparatus. A loading comb was placed into the agarose gel in designated slots and allowed to cool completely and harden. Samples were run at 30V for 15 minutes and then 60V for 2 hours.

DNA Purification from Agarose Gel: A DNA band matching the predicted flippase gene size of ~ 3500bp was cut out of gel using an industrial razor blade. Gel slices were placed in tared 1.5ml microfuge tubes and weighted. Weights were used in preparation for DNA extraction. Extraction of DNA was done using a QIAquick gel extraction kit manufactured by Qiagen (Hilden, Germany). After using the kit to purify the DNA bands 5µl of purified samples were pipetted into a 0.75% agarose gel and run for 15 minutes at 30V and then at 60V for 2 hours to confirm presence of DNA.

2.2 Methods

Ligation Method and Vectors Used. Restriction enzymes compatible with amplicons and vectors restriction sites were used to cut both the vectors and the PCR products, [detailed in Table 1]. The restriction enzymes used were purchased from New England Biolabs (Ipswich, MA). The two vectors that were used for this study were, PICA (3382bp) and AU1 (3789bp) *Giardia* vectors. Several ligation reactions were set up according to protocol. Samples constructed using TOPO TA cloning kit and were cloned into pCR 2.1-TOPO vector, according to kit protocol. Samples other than those constructed using the TOPO TA kit were left in a 15^{o}C water bath in their respective reaction mix for 16 hours.

Transformation and Selection: Ligation mixtures were transformed into JM109 competent cells. Competent cells were incubated at 37^{o}C in PSI broth for 2 hours. PSI broth contains 2% tryptone, 0.5% yeast extract, 10mM NaCl, 20mM $MgCl_2$, and pH is adjusted to 7.6 using 1 (M) KOH. PSI broth contains LB media, 4mM magnesium sulfate ($MgSO_4$) and 10mM potassium chloride (KCl). LB media consists of 10g trytone, 5g yeast extract, 10g NaCl, 15g of agar-B and suspend in 800ml DDI water (1L). Media-cell mixtures were spread onto agar plates that contained antibiotics that corresponded to selective markers on the vectors used. Plates were left at 37^{o}C for 16 to 20 hours; selection of specific cones was based on selectable markers present such as blue white selection. The blue-white selection protocol is: first prepare LB plates as described above, to include dry plate using UV light for approximately 30min. Second, add 40µl eta X-gal, 5-bromo-4-cholor-3-indolyl-beta-D-galacto-pyranoside, allow plates to dry. Third add 40µl 100mM IPTG, isopropyl-beta-D-thiogalactopyranoside, to plates and allow them to dry. Fourth spread colonies evenly on the plates using a sterile spatula. Finally incubate at 37 degrees Celsius. The colonies appearing white contain the insert. The blue colonies have a fully non-interrupted beta-gal gene.

Northern Blot: Selection of Flippase mRNA using extraction protocol. Flippase mRNA was isolated from the trophozoites and loaded onto a specific RNA gel. The samples were transferred onto a nitrocellulose filter and exposed to a radiolabeled probe (^{32}P), acquired from NEN^{TM} Life Science Products (Ipswich, MA). This probe works by recognizing and adhering to the targeted flippase mRNA thereby flagging the sequence. Exposure of this filter to a phosphorus screen will reveal if the mRNA is present.

Culture of AU1 colonies: Colonies containing AU1 vector were selected using sterile toothpicks and put into several 50ml conical tubes containing 5ml of LB broth and corresponding antibiotics. The conical tubes were placed into a 37^{o}C incubator and left shaking at 200 rpm for 16 to 20 hours. LB broth (95 ml) was added to several 200ml flasks and 5 ml of the previous solution from the 50 ml conical tubes were added. These samples were incubated at 37^{o}C at 200 rpm until all samples reached or exceeded 0.6 OD. An initial 2 ml sample was taken from all flasks; the 2 ml samples were pelleted and dried. Then 500µl of IPTG was added to the flask and the samples were placed back into the incubator. Every 30 minutes a 2ml sample was taken, pelletted, and dried. This was done until the total time reached 240 minutes.

Western Blot: Samples previously prepared using standard expression procedures were prepared and run on a prepared SDS-polyacrylamide gel (PAGE). Proteins were transferred onto a Nytran transfer membrane using a Mini Trans-Blot Electophoretic Transfer Cell BioRad (Hercules, CA) before carrying out the immunoblot analyses.

Epitope Tagging. The flippase gene expression clone includes approximately 3500 bp at the 5' end of coding site and its own natural promoter. The plasmid used for epitope tagging was the AUI vector. The PCR products previously cloned which contain the flippase gene and its promoter was cloned into a TA-cloning vector; this was done using a TA-cloning kit that was purchased from Invitrogen. Using the cloning vector pGEM T-Easy, Promega (Madison,WI). The flippase gene was

ligated in the correct orientation into the pGEM vector using blue-white selection (described previously) and transformed into JM109 competent cells. The cells were streaked out onto ampicillin (50 mg/ml) containing LB plates and incubated for 16 to 20 hours. Materials used to make LB plates were purchased from Fisher Scientific (New York City, NY), utilizing the previously described protocol. Colonies were collected with aseptic technique and transferred into several 50ml conical tubes. These tubes contained 5 ml of LB media and 5µl of 50mg/ml of amplicillin, from SIGMA (St. Louis, MO). After approximately 20 hours of growth DNA was extracted from colonies using a QIAprep Spin Miniprep Kit purchased from Qiagen (Hilden, Germany), as described previously. Sequencing of the extracted DNA was done to insure that the gene was present. The Flippase gene was cut out of the TOPO vector and inserted into the epitope tagged vector, AUI. The epitope tag was fused on to the N-terminal region. Dephosphorylation of the vector was achieved using shrimp alkaline phosphatase that was purchased from Promega (Madison, WI). This was necessary to prevent re-ligation of the vector with itself. The ratio of plasmid DNA to insert DNA was a 2:1 concentration. Ligation of the flippase into the vector was done according to protocol described previously. To check if the ligation reaction was successful, a standard PCR reaction using specific primers for the expected size was performed. Bands present were compared to expected size of vector-insert; this indicates whether the reaction was a success. Primers were purchased from MWG (High Point, NC).

Successful ligations were transformed into JM109 competent cells through standard heat shock method and colonies were selected on amplicillin plates. Recipient cells where checked to confirm that the right size insert is present and the insert is in frame with the epitope tag. Once the right size insert was identified, a 100ml culture was inoculated with the correct colonies. From these colonies a Maxiprep extraction was performed with a kit purchased from Qiagen (Hilden, Germany).

Transfection: Approximately 50 µl of Epitope tagged flippase was transfected into *G. lamblia* using a DNA-transfection system developed by Yee and Nash [9]. This system uses the puromycin-*N*-acetyltransferase (*pac*) gene for its selection of transfected parasites (8, 9).

Antipeptide Antibody Generation: Rabbit Antibodies were constructed to recognize specific sequences of the Flippase enzyme. One site was located at the 3' end and the other located at the 5' end. The same company constructed a mouse anti-rabbit antibody, IgG, that recognized the constant region of the initial antibody. The mouse anti-rabbit antibody was also coupled to HRP (horse radish peroxidase). Antibodies were purchased from Biomedia, Beaufort, SC.

Bioinformatic Characterization and Homology. Predicted giardial genes were obtained from the Giardia genome project [14,16] and compared to known protein sequences using BLASTP [20], Pfam [21], and COG[22] motif similarities. All potential giardial flippase genes were used to probe the SWISPROT [23] database to obtain a base set of putative flippase homologs across all species. All the identified homologs were aligned using Clustal X [24] and from the multiple sequence alignment. Neighbor Joining phylogenetic trees (1000 iterations) were constructed and displayed using ClustalX and Phylip [25].

RNAi: RNA interference uses antisense RNA to interfere with specific gene functions. This was done with the introduction of homologous dsRNA that specifically targeted our flippase enzyme [12]. There are, however, conflicting reports as to whether this system works in *G. lamblia*. Because of the relative ease of preparation we first tried to use RNAi to knock out the putative flippase enzyme. Using preparation kits from Ambion and following protocol appropriately. RNAi was unsuccessful and an alternative method was used.

Hammerhead Enzymes. The hammerhead ribozymes are small RNA molecules that have catalytic activity. These enzymes have the added ability to undergo self-cleavage to produce two RNA products. These two RNA molecules can act to inhibit specific mRNA and, thereby affect the overall

activity of a cell with regard to the specific gene targeted [10]. In the experiment we constructed cDNA molecules that encode the hammerhead ribozyme enzyme that is specific for the flippase enzyme. The mode of entry of the hammerhead constrict was via transfection by cloning the hammerhead enzyme into *G. lamblia* virus (GLV) genome. The actual transfection occurred through electroporation. Selection of successful transfected cells was accomplished by the exposure of the transfected trophozoites to serial dilution of puromycin. The protocol that we used to knockout the flippase enzyme using the hammerhead ribozyme was developed by C. C. Wang [13].

Chapter 3: Hypothesis:

This study tested the hypothesis that a flippase enzyme does exist in Giardia lamblia and that it plays a vital role in transporting and properly positioning phospholipids within the membrane lipid bilayer. Through a series of experiments this study will characterize this putative flippase enzyme within Giardia lamblia.

Chapter 4: Specific Aims

4.1- Specific Aim One: Bio-informatic characterization.

Giardia lamblia genes have been shown to have commonalties with structural and metabolic features from bacteria, archaea, and eukaryotes. BLASTP, PFAM, and COG comparisons of the putative Giardia lamblia flippase proteins to other putative flippase homologs and motifs can help us to refine functional aspects of the enzyme. In addition, construction of a phylogentic tree using a representative set of related transporters should reveal the evolutionary origins of the gene and could further refine functional studies.

4.2 -Specific Aim Two: Clone putative flippase enzyme.

In order to proceed with molecular biology studies of modified giardial-flippase in situ, the gene must first be isolated and cloned into a suitable vector. Based on known sequence, targeted genes can be amplified by Polymerase Chain Reaction and cloned into a dual host (E. coli and G. lamblia) vector.

4.3-Specific Aim Three: Expression of the recombinant flippase enzyme in bacteria and epitope tagging.

Using the cloned giardial flippase gene, an epitope tagged version of the gene, including its native promoter, can be constructed and transfected into giardia trophozoites. Expression of this product can be assessed by both mRNA detection and quantization and by immunoassay of the epitope tag. Determination of epitope tags will also demonstrate the location and organellar association of the cloned enzyme.

4.4-Specific Aim Four: Down regulation of the flippase mRNA in trophozoites.

Using the cloned gene as starting material, we can construct a mRNA specific hammerhead ribozyme. The specific ribozyme can then be transfected into giardial trophzoites in an attempt to decrease expression of the flippase gene. If the resulting transfection is viable, morphology of the transformed cells can provide indirect evidence of the function of the targeted enzyme.

Chapter 5: Results and Discussion

5.1 Specific Aim One: Bio-informatic characterization.

This project was executed during the initial phases of the total genome sequencing project of Giardia lamblia strain WB clone C6, now completed. During this time, through our collaboration in this project, we had access to early and preliminary data from the project to allow us to identify and clone key enzymatic activities. With the completed genome now available, I have reformed my initial analysis to allow for a more complete picture of the flippase enzyme in giardia.

Flippase, or phospholipid translocase enzymes are membrane associated proteins that can move phospholipids and other lipids from one side of the membrane leaflet to the other side. As such, they are a key element in the uptake of lipids from the external milieu into the cytoplasmic side of the cell. They may also have roles in the transfer of lipids among internal membranes.

Prokaryotes and higher eukaryotes are fundamentally different in that the eukaryotic enzyme includes an ATPase activity, allowing cellular energy to assist in the movement of materials across membranes. Because giardial genes can have characteristics of both prokaryotes and eukaryotes, our first question was whether the giardia enzyme is more closely related to prokaryotes or eukaryotes.

BLASTP, Pfam, and COG comparisons now identify four giardial genes with at least some homology to known flippase genes (Table 4,5,6,7,& 8). While subsequent studies in this thesis center on sequence #1 (Table 5,6), it is important to note that each of these putative homologs clearly contains an ATPase region, indicating that all potential flippases are of eukaryotic origin.

Flippases are only one part of the eukaryotic ATPase transporter family. Other membrane transport proteins, such as cation transporters, are also within this family. To determine which families are more closely related to flippases and to try to refine the evolutionary origins of giardia flippase, we assembled an unrooted tree of a representative set of flippase related homologs.

Each of the four potential giardia flippases was compared with the SWISSPROT database using BLASTP, and all potential homologs (eval <10E-5 for any one of the four giardia proteins) were pooled and assembled into a multisequence alignment using CLUSTALX. This alignment revealed areas of high similarity among putative flippase sequences from a broad sampling of organisms (Figure 3).

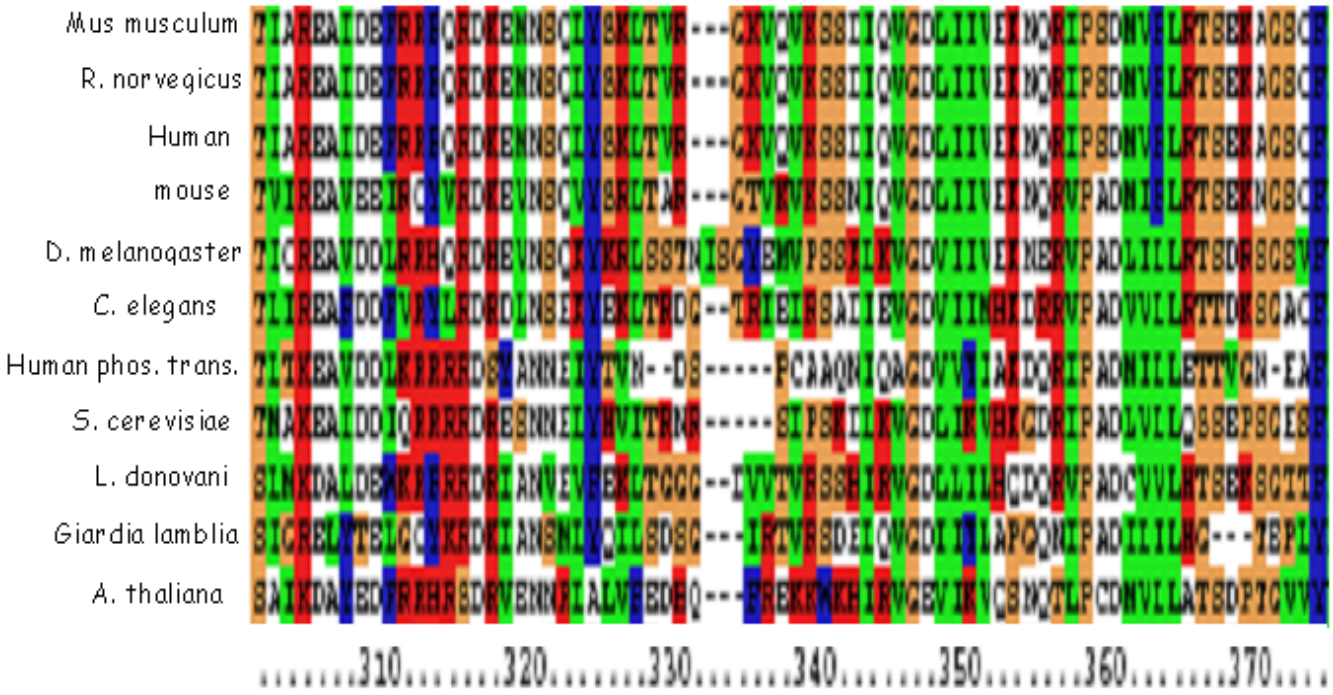

Figure 3: multiple sequenced alignments. A sequence alignment done using a section of 70 A.A by ClustalX reveals some similarities between the putative Giardia lamblia flippase enzyme and other higher eukaryotes such as human and mouse. By being able to match a flippase protein sequence to that of Giardia it gives insight into the possibility of a flippase site within its genome.

In particular, these studies helped identify how similar our enzyme was to other functioning systems and whether the enzyme could indeed require an ATP-based energy system to function. This assumption was based on the similarities of G. lamblia to already existing ATP-dependant flippase enzymes of the other organisms, such as the flippase enzyme identified in C. elegans which is ATP-

dependent and has an approximate 40% homology to G. lamblia (Weng et al., 1999). Additionally, the other homologous matches helped to uncover whether our putative enzyme recovered from the G. lamblia genome could play a functional role in phospholipid trafficking as well as provide a possible starting point for testing it's functionality.

To better understand the evolutionary, and potentially functional, relationships among the transporter enzymes, a phylogenic tree was constructed involving all the potential homologues identified from SWISSPROT. This unrooted tree, based on 1000 iterations of the Neighbor-joining algorithm, was constructed and viewed by a combination of CLUSTALX and Phylip (Illustration 1). Several points can be made from this construction. First, most of the sequences cluster in a reasonably orderly manner, lending credence to the validity of the sorting. There are broad clusters of invertebrates, vertebrates, and fungi, with dense clusters focused on model organisms such as Rattus, Mus, or Drosophila. Second, there are clearly several paralogs, related genes with separate evolutionary paths, represented in this tree. For example, there are two clear clusters of fungi related genes that are widely separate from each other. Thirdly, there are two regions of the tree with deeply or basally separated branches: In the middle of the wheel are widely spaced individual or singly branched arms, indicating distant linkage to its neighbors. Then, at the end of the tree are a series of sparse branches, generally divergent from the main cluster of ATPase transporters. These latter, highly divergent branchings, include samples from a wide range of organisms and presumably represent either a rare, specialized evolutionary tree or a series of related sequences that align poorly.

While the tree is well organized at the level of organisms, it is less well clustered at the level of enzyme functionality. In particular, flippase or phosphlipid transporters are found annotated throughout the tree, interspersed with cation transporters or uncharacterized ATPase activities. There are at least two possible interpretations of this distribution. One is that the changes in transport specificity are highly variable and not well defined, such that multiple sequence alignments de-emphasize these areas.

This line of reasoning is supported by the general signal for transporters detected by COG or Pfam comparisons. Alternatively, poor annotation could be a major factor in the lack of segregation. Most of the annotations given are based solely on sequence similarity with a gene that was also annotated solely by sequence similarity with another gene. Distinguishing and correcting either or both of these errors is beyond the scope of this project.

Perhaps the most intriguing observation on this tree is the distribution of the four putative giardial flippases. Three of these are in the deep branching part of the tree, with the remaining sequence in the divergent or mismatched end of the unrooted tree. The branches labeled 1, 3a and 3d represent those putative giardial flippases. The location of those branches further bolsters the contention that those putative flippase enzymes do indeed belong within the same family as already established flippases of higher eukaryotes.

The take home lesson here is that, once again, giardial genes are distant from almost all other species, minimizing the amount of information that can be gleaned by comparison with "near" neighbors. With the key flippase, numbered 3 on the tree, which is the focus of the remainder of this thesis, there is only one close homolog, and that is a flippase of the equally enigmatic *Trichomonas vaginalis*.

5.2- Specific Aim Two: Clone putative flippase enzyme.

Now that we have established the presence of our putative flippase enzyme gene, the exact location needed to be identified. In order to pin point the location of the gene the DNA was purified and several primers were constructed using the data gained from the previous alignment studies (Table 1). Using several rounds of PCR the primers were allowed to react by annealing at specific sites within the DNA. The process allowed for amplification in quantity of our putative flippase enzyme. The putative flippase enzyme measures approximately 3500bp.

The gene was successfully removed, purified, and concentrated. Since the primers used were constructed not only to recognize the gene but were also embedded with a restriction site on the 3' and

5’ end of the gene (Table 1). The reason the amplicons were constructed with specific restriction enzymes were to provide annealing ends that corresponded with sites on the vector used, AU1, cloning proposes. The choice vector has been previously identified to have been successfully transfected into G. lamblia while maintaining full functionality once inside the cell. In addition the vector contained a specific antibiotic resistance sites which were used for purification proposes, discussed below.

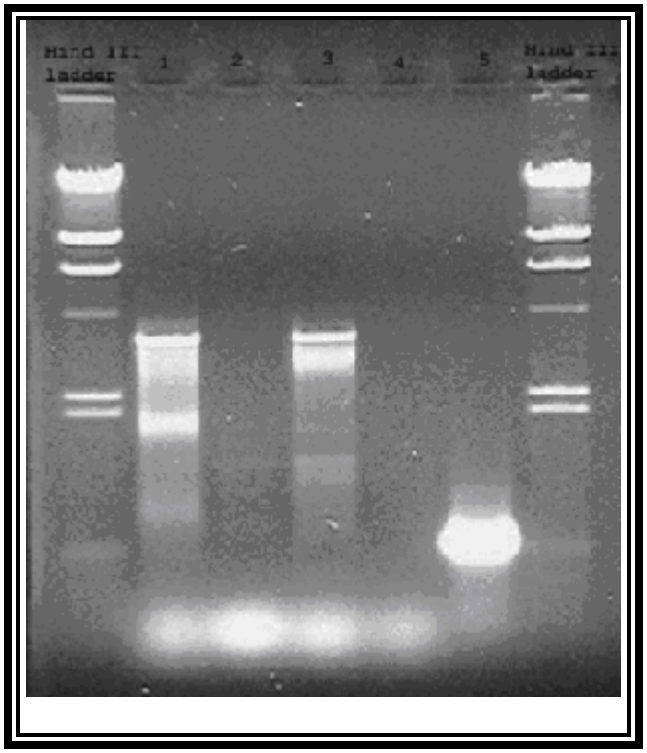

Figure 1: In this figure the bands in lanes one and three represent the amplification of the flippase enzyme using the primers previously constructed. Control is found in lane 5. In the first and last lanes are a Hind III ladder.

In order to ligate the PCR product into the vectors, both the vector and product were incubated with the restriction enzymes that corresponded with the embedded restriction enzyme sites. The AU1 vectors were incubated with the same corresponding restriction enzymes. The restriction enzymes act as molecular scissors and cut both the vector and PCR produces at their recognition sites. To prevent the vector from re-ligating to each other their ends were dephosphorylated, while the PCR products were

kept intact. After the incubation of the PCR products with their corresponding vector in a specific ligation mix, according to protocol as listed in the M&M section. The ligation mix was transformed into competent JM-109 cells. The colonies were grown on specific media that contained not only the corresponding sensitive selection antibiotics but also contained large amounts of galactose. The reason for the addition of galactose was to assist in the identification of successful ligation of the flippase enzyme. The AU1 vector has an intact beta-galacatasidase enzyme site embedded at the point where the restriction enzymes are located. When the restriction enzymes cut and proper ligation of the choice gene occurs the beta-galacatasidase gene is interrupted. At this point a blue/white selection can be made using the substrate, X-gal. Blue colonies indicate that the beta-galacatasidase is functioning and therefore the gene was not incorporated. The reason for the white colonies indicate that the gene was embedded at the proper site the activity of this gene was interrupted and therefore was unable to process the conversion of lactose into its appropriate breakdown products, glucose and galactose. The breakdown products were not present and could not interact with the substrates, X-gal. Due to the lack of these products the cells were unable to show the characteristic blue change color (picture not shown).

In addition, the successfully ligated flippase enzyme vectors would upon incubation on this media survive the exposure to an antibiotic, ampicillin, due to the presence of the resistance gene to that specific antibiotic on the vector. The coloration change and survival after exposure to ampicillin further insured that the colonies picked did contain the gene. The successful JM-109 cells that contained the putative flippase enzyme were grown in a controlled environment, and from the colonies both RNA and DNA were extracted from the cells.

Total RNA was purified and using primers specific for the corresponding G. lamblia flippase enzyme, was run through a series of RT-PCR specific cycles (Table 2). In addition to the total RNA extraction the samples were further processed using a messenger RNA kit to further purify the sample and only remove the messenger RNA. Both samples were placed into an RNA sensitive agarose gel and

monitored. Throughout the whole process extra precautions were taken to insure the integrality of the RNA remained intact. The results of the RNA on agarose gel further supported the notion that there is indeed a flippase enzyme within the G. lamblia genome (figure 4).

5.3- Specific Aim Three: Expression of the recombinant flippase enzyme in bacteria and epitope tagging.

My next step was to provide evidence that the gene is in fact present as a folded and functioning protein. In order to visualize whether the putative G. lamblia flippase enzyme was present in a protein form, protein purification and western blotting was performed. Colonies were chosen based on growth and appearance and transferred into several fifty-milliliter conical tubes and grown in an incubator for approximately twenty hours. Antibiotics were used as a selection device, since our vectors contained ampicillin resistant genes. Five milliliters of these cells were transferred into ninety-five milliliters of media and returned to the incubator until the OD reading was at or above 0.6. At this time, the samples were centrifuged, and supernatant suctioned out. According to Qiagen protocol a certain amount of the inducer isopropyl-beta-D-galactoside (IPTG) was added to the flasks. IPTG works by inducting the cells to enter a high state of metabolism where the overall levels of protein catabolism are increased. At intervals of thirty-minutes up to two hundred and forty minutes, a two-milliliter sample was taken, centrifuged, and supernatant was removed. The total proteins were removed at specific time frames and were run onto an SDS-PAGE protein gel. The gel was placed into a solution that contained a specific dye, Coomassie yellow dye, the dye reacted to the protein produce, flippase protein, and under a limited spectrum of light the protein fluorescents. The presence of this band again further cemented the presence of a flippase enzyme. The gel was than transferred onto a nitrocellulose filter, using the western blot procedure.

In addition the AU1 vector was constructed with an epitope tag. During the cloning process the putative flippase enzyme was cloned in the forward orientation in front of the epitope tag. The forward

orientation was selected for by the presence of two separate restriction enzyme recognition sites that corresponded to both the AU1 vector and the putative flippase enzyme. The location of the restriction enzyme sites insured that the putative flippase enzyme was indeed ligated into the forward orientation. The presence of the tagged flippase enzyme was determined using the anti-rabbit antibodies and mouse IgG antibodies. At a later stage we will use a series of Confocal microscopy for the visualization the flippase enzyme within the phospholipid bilayer.

Following protein purification and expression, the purified AU1 vector containing the flippase protein was transfected into G. lamblia using the DNA-transfection method described earlier. After the procedure was complete the colonies were transferred into appropriate media along with the antibiotic, puromycin. The vectors transfected also contain a Puromycin-resistant site this was done in order to further purify the samples and promote only the growth of the G. lamblia that had properly untaken the vector-epitope product.

After several generations of growth the cells where prepared for Confocal microscopy. Prior to confocal microscopy various steps were taken to prepare the cells for visualization. The cells were first exposed and incubated with the anti-rabbit antibody that recognized the epitope tagged enzyme. After the initial incubation a second antibody, mouse anti-rabbit antibody that recognized the primary antibody was incubated and allowed to react with the cells. The mixture was rinsed in order to remove all the excess antibodies. The next step was addition of various dyes that were specific to the secondary antibody and the nucleus. Rhodamine was used for the identification of the flippase enzyme depending on the excitation spectrum it would either emit green or yellow fluorescents. Another dye was used to adhere to specific nuclear proteins, DAPI, this dye gave the nuclear products a blue coloration. This was done in order to assist with the separation of the nuclear membrane and the phospholipid bilayer visualization and help with localization of the different cellular compartments.

The samples were then placed into a Confocal Microscope, the exposure of specific light rays to the samples resulted in excitation of tagged enzymes and a color change was seen. Various pictures were taken of both excited cells and non-excited cells (figure 5 & 6). The non-excitation cell-preparations showed healthy appearing G. lamblia trophozoites. This supported the notion that the cells were not injured during the transfection and were successfully growing (figure 5).

The most profound finding was the localization of the tagged flippase enzyme to the lipid bilayer membrane (figure 6 & 8). The finding clearly shows the enzyme does indeed embed into the outer phospholipid layer. The internal membrane showed very little placement of the flippase enzyme. The nuclear structures also showed very little localization of the flippase within its membrane. Rather the enzyme seems to be more concentrated on the outer leaflet.

Additionally, several comparative studies using the same cells were conducted between both excitation and nonexcitation conditions in order to establish that the cells were not injured but rather intact and the enzyme placement occurred (figure 6). These findings were very exciting and further fueled the proposed hypothesis. Many more confocal studies were conducted; the repeated studies supported the initial finding.

5.4- Specific Aim Four: Down regulation of the flippase mRNA in trophozoites.

The final aspect of our study was to knock down the function of the flippase enzyme and visualize whether our cells livelihood was affected. We used two different attempts; one being RNAi and the other being the hammerhead enzyme. Initially RNAi was employed, however this method failed. This method was used and documented by Firelab at the Carnegie Institution of Washington to be successful, but in our experiment it failed repeatedly.

We then moved on to the next option. This method involved using a hammerhead ribozyme protocol of Wang, et al (10). The hammerhead enzyme used was constructed to recognize a 15bp sequence particular 5’ and 7bp at the 3’ end of our G. lamblia putative flippase enzyme mRNA. This

enzyme has the ability to split into two catalytically active subunits. Both act by binding to the specific mRNA subunits and prevent the messenger RNA from being transcribed (table 3). This process will down-regulate the putative flippase enzyme and resulted in a non-functional flippase enzyme. In the presence of this non-functional enzyme the kinetics of lipid trafficking were interrupted and the cells' survival rate was affected. The morphological changes were identified using Confocal microscopy (figure 7). The cells were grossly rotund, bloated, and ill looking. On microscopic examination the cells were not only bloated in appearance but visual distortions in shape and movement were noted. In addition, cells exposed to the hammerhead enzyme did not survive in culture, as compared to a non-exposed control. RT-PCR results revealed a decline in cell population.

The results of this experiment further expanded our knowledge of the specific role our putative enzyme plays in the survival and proliferation of Giardia lamblia.

Chapter 6: Conclusion

The use of bioinformatics expanded this study's capability to compare and contrast well established flippase enzyme sequences with our putative flippase enzyme. Programs such as ClustalX, COG, SWISSPROT, and pylogenic tree program gave us the insight into the extensive similarities between the putative flippase enzyme in *G. lamblia* and various other established flippase enzymes of multiple eukaryotic organisms. After comparison of the various organisms we used that data to assemble various sets of primers to target the sequence discovered within the *Giardia lamblia* genome.

Once the targeting and amplification of the putative flippase enzyme was complete, the functional capacity of the enzyme was tested. Several rounds of confocal microscopy allowed for visual appreciation of the flippase enzyme. With these multiple rounds of confocal microscopy it was indeed revealed that the enzyme did indeed localize to the inner leaflet of the plasma membrane.

In order to prove whether this enzyme was vital for the survival of *Giardia lamblia*, the enzyme's function was affected in such way as to lower its ability to transport and incorporate lipids into its phospholipid bilayer. This knocking out of the flippase enzyme could have been done by the use of two different enzymatic reactions that would in essence work to differently to interrupt the function of the *G. lamblia* flippase enzyme. The first consisted of the use of RNAi, after various rounds it was determined that this mechanism didn't work for our purpose. The second option was the use of the hammerhead ribozyme.

With the construction of a specific hammerhead ribozyme and successful transportation into Giardia using a giardial virus the cells were infected and incubated. Cells were viewed under the light microscope prior to the preparation for confocal microscopy. Cells were grossly bloated and their movements were restricted under confocal microscopy the cells were rotund, inflamed, and ill appearing. When the cells were allowed to grow in culture and compared to cells which were not infected, the infected cells didn't survive. According to the hypothesis it is believed that the *G. lamblia*

flippase enzyme is vital for the transport and incorporation of various phospholipids into the lipid bilayer. As explained earlier this parasite is unable to produce its own phospholipids denovo, and it is believed that the flippase enzyme identified functions in both transport and incorporation of these needed lipids. To date there have been three other potential "flippase" enzymes identified (Table 5). For this study all the focus was placed onto the first putative flippase enzyme sequence identified. The identification of this sequence was due to the involvement of our laboratory with the Giardia lamblia genome project we had privy to early unpublished data. Our experiment indicated that the flippase enzyme was indeed localized to the phospholipid bilayer and when the activity was knocked down it affected not only the normal features but the survivability of the cells.

Tables and Figures

Tables

TABLE 1. PCR primers used to recognize and amplify the flippase enzyme. The primers were embedded with certain enzyme restriction sites. Both AU1 and PICA vectors were used in order to expand our pool of available vector-gene products.

Construct	Primers	Sequence	Restriction enzyme
Au1 vector (left)	GFliAu1F01	ATCCCTCTGCTT**GAATTC**ATTGAG	EcoR1
Au1 vector (right)	GFliAu1R01	ACCAGTG**GAATTC**CGAGAAGGACAG	EcoR1
Au1 vector 2nd (left)	GFliAu1F02	TTAAGCT**GAATTC**CTGTTGC	EcoR1
Au1 vector 2nd (right)	GFliAu1R02	CTT**GAATTC**GTCATAAGGATGTAG	EcoR1
PICA vector (left)	GFlipPICAF01	TATCCCTCT**GGTACC**TTCTATTGAG	Kpn I
PICA vector (right)	GFlipPICAR01	ACCA**GCGGCCGC**ACGAGAAGGACAG	Not I
PICA vector 2nd (lf)	GFlipPICAF02	TTAAGCTGATG**GGTACC**TGC	Kpn I
PICA vector 2nd (rt)	GFlipPICAR02	GCTT**GCGGCCGC**CATAAGGATGTAG	Not I

TABLE 2. RT-PCR primers used to recognize and amplify messenger RNA of the flippase enzyme. The first two primers were constructed to amplify the first half of the sequence whereas the second two amplified the second half of the sequence.

Construction	Primers	Sequence	Produce size
Au1 vector (left)	mRNA01	atgggctgttgctgttgtc	1520 bp
Au1 vector (right)	mRNA02	cctctagcatgcattcctct	1520 bp
Au1 vector (left)	mRNA03	tgaagtggctatcatcagagg	2361 bp
Au1 vector (right)	mRNA04	cttacgagggcacgtagagc	2361 bp

TABLE 3. Hammerhead Ribozyme primer construction.

Construction	Primers	Sequence	Restriction Sites
Hammerhead plasmid (right)	Flip-ribo01	cctctagcatgcattcctctg**ctgatgagtccgtga**	Xho I
Hammerhead plasmid (left)	Flip-ribo02	**ggacgaa**tgaagtggctatcatcagagg	Xho I

TABLE 4. Amino Acid sequence of flippase enzyme

A.A. length 1284
Predicted Protein Sequence MGCCCCRPIEGEEEDITLRFRGLAVNYGGRKFLPNVIKTRTYNWISFPFIVLLLQFKPFF SQFFTAVALLQIFRALKVGLAFTYYLPLIFIFLVSIGRELYTELGCYKRDKIANSMLYQI LSDSGIRTVRSDELQVGDIIYLAPGQNIPADILILHGTEPLYLKTTNLDGETDYKSRTAC FDTTDIYSTDYKDILRKKLDSHILQYEPPSSQLYKFNGKFVLLSAPQDVSMSPVMPISLA NTAWMNCTLAKGSVTGLVIYTGKQVRIMLGRKKPKSKSGKLDVDINLYVKGLFVLCIFLA FVLTMAGSVYSLVTFIRWIIILNAIIPLAMKVSLEYGKFTMAGTISYDPEIPGVRVQNSN LVEELGLINHIFSDKTGTLTKNDMWLHEVVGSNKNVTDGCETMLHALMTCHNVELVTERC RSIKVTAKNGIVQHSDQDSEEDQAKQLVPQKTNTRIVLEEEKVIEGFGEAVSLCFTKHTT NEYLLLNNYIGASPDEVAIIRGMHARGHILKEKMHNRIQYSSGTEDYEFELIHVFPFTSE TRRMSVIVRDRETQVVYLFVKGADTAIEVISKPCDWMRNVVDQLATTGRRTLVYAYRILS ADDLDAFQRAWMSAAEDINSRQDKLDQAEEDLLVNLDVLCVTGIEDQLQDNVKDTIQSLK RAGIKFWMLTGDKIITSLSIAQACSILPNDVISKHTDFCSRVSEIGNSTSRCVELDAQTG DVQHSLHIVGENTSSSAMQLASVASNPITMSPLDPVLITTTCAQERSHSCSSPKSGLRSP LHHRTNRALLRHPLKKSGLRNKKLANIKESAYVRKSQGVFFLTEELSLDRLLQTLHDVLA VVETQKNIAIIIDGGAIDALLGTYPTNYFYRYRIHPITGSLLPLPLILKAWRYFVRFLYV YFTGKAKSGKGSLGPDAKIRELFAEVTCRAETVICCRCTPGQKALVSEIITTYSGKQSLG IGDGANDVPLIQLCAVGVGIAGKEGTQAANSADFVINEFQGLKKLLLWHGRNSYIGSAQM CQFIMQRGITQTLLQIWFSMLYYFVTLVIYTGVLLLGFATFFTMLPPFNYYINEDIDYKL VMQYPEVYRYTSSGRLISFKTFCLWLLASIVISFMIFVSLVCVLPWDVIYTEFVLVSFAA LLINQLLLISFTSHRWSVLLVITLVGSYASFYIVQTIYPDIFPLSFLYSFSFTWSSLYIA FSAALPIYIGSLLVKHLSVPPVIKKLRKLRAPADCACCAKKSNCACTKSPIHYYSSTGTY YFNLTDKAANVLTCRYCSTCPRKC

TABLE 5. DNA sequence of flippase enzyme

N.A. sequence, 3855pb
ATGGGCTGTTGCTGTTGTCGTCCCATCGAGGGCGAAGAGGAGGACATCACCCTTCGCTTC
CGCGGCCTGGCCGTTAATTATGGGGGCAGAAAATTCTTGCCAAATGTCATTAAAACGCGC
ACATATAATTGGATTAGCTTTCCGTTTATAGTTCTTCTCCTTCAGTTTAAACCGTTTTTT
AGTCAGTTCTTCACTGCTGTAGCCCTGCTGCAGATATTCCGGGCCCTGAAAGTGGGCCTG
GCCTTCACATACTACCTTCCACTCATATTTATTTTCCTCGTTAGCATCGGGAGGGAATTA
TACACCGAGCTGGGTTGCTACAAGAGAGATAAGATAGCTAACTCCATGCTCTATCAGATT
CTTTCTGACAGCGGAATTAGAACTGTCCGCTCAGACGAGTTACAAGTTGGAGACATTATC
TACTTGGCACCAGGGCAGAATATTCCCGCAGATATTTTGATACTGCACGGCACTGAGCCT
CTGTACTTGAAAACAACCAACTTGGACGGAGAGACGGACTATAAGTCTCGTACTGCGTGC
TTTGATACTACAGACATCTATAGTACCGACTACAAGGACATACTTCGGAAGAAGTTGGAC
TCGCACATATTGCAGTATGAACCGCCAAGCTCCCAACTCTACAAGTTCAACGGGAAGTTT
GTTCTGTTATCGGCACCTCAAGATGTCTCTATGTCACCCGTCATGCCCATTTCACTAGCA
AATACTGCGTGGATGAATTGTACTCTTGCGAAGGGGTCAGTGACGGGGCTTGTCATCTAC
ACCGGAAAGCAAGTCCGGATTATGCTGGGCCGAAAGAAACCCAAGAGCAAGAGTGGGAAG
TTAGATGTGGATATCAACTTGTATGTCAAGGGCCTCTTCGTGCTTTGCATCTTCTTGGCC
TTTGTGTTGACTATGGCAGGGTCTGTTTATTCTCTAGTAACGTTCATACGGTGGATAATC
ATTCTTAACGCTATCATTCCTCTCGCTATGAAGGTGTCTCTCGAGTACGGCAAGTTTACT
ATGGCGGGGACTATCAGCTACGACCCCGAAATACCAGGTGTCAGGGTACAAAACAGTAAT
CTAGTAGAAGAGCTGGGGCTTATTAACCATATATTTAGTGACAAAACAGGCACGTTGACA
AAGAACGATATGTGGCTTCACGAGGTCGTTGGGAGTAATAAGAACGTAACGGATGGATGT
GAAACTATGCTTCACGCTCTTATGACATGCCACAATGTTGAGCTGGTCACTGAGAGATGT
CGGAGCATTAAAGTAACAGCAAAGAATGGCATCGTACAGCATAGCGATCAGGACTCTGAG
GAAGATCAAGCAAAGCAGCTTGTGCCTCAAAAGACGAACACTCGCATAGTGCTAGAAGAG
GAAAAAGTGATCGAGGGATTTGGTGAAGCTGTTTCGCTGTGCTTCACTAAGCACACCACT
AATGAATACCTGCTTCTTAACAATTATATAGGAGCATCCCCTGATGAAGTGGCTATCATC
AGAGGAATGCATGCTAGAGGACACATTTTAAAAGAAAAAATGCACAATCGCATCCAGTAT
AGTTCTGGCACTGAGGACTATGAGTTTGAGCTTATTCATGTGTTTCCATTTACCTCGGAA
ACAAGGCGCATGTCGGTCATTGTTAGGGATAGAGAAACTCAAGTTGTCTACCTCTTTGTC
AAAGGCGCTGATACTGCCATAGAGGTTATTTCTAAACCGTGCGATTGGATGCGCAACGTT
GTTGATCAGCTTGCGACCACCGGGCGACGTACTCTAGTGTATGCATATAGGATTCTTTCT
GCGGATGACTTAGATGCCTTTCAGCGTGCATGGATGAGTGCCGCGGAGGACATCAATTCG
AGACAGGATAAGCTAGATCAAGCAGAGGAGGACCTTTTAGTTAATTTAGACGTTTTGTGT
GTAACGGGGATAGAAGATCAACTCCAAGACAACGTGAAGGACACTATTCAGTCTCTGAAG
AGGGCAGGAATTAAGTTCTGGATGTTAACGGGTGATAAGATCATAACGTCCCTGAGCATT
GCTCAAGCATGCAGTATTCTTCCTAATGATGTGATCAGCAAGCATACAGACTTTTGCAGT
CGTGTATCTGAGATAGGAAATAGTACTTCCCGGTGCGTAGAGCTTGACGCGCAAACTGGG
GATGTACAGCACAGCCTTCATATTGTGGGCGAAAATACATCTAGTTCAGCTATGCAGTTG
GCAAGCGTGGCCTCAAACCCGATCACTATGTCTCCCCTAGACCCTGTACTCATCACCACA
ACATGCGCGCAAGAACGGAGCCACAGCTGTTCGTCCCCTAAAAGTGGTCTGCGGTCTCCA
CTGCACCATAGGACTAATAGGGCATTATTAAGACATCCACTTAAGAAAAGTGGATTACGG
AATAAGAAGCTTGCAAATATCAAAGAGTCAGCATATGTGCGTAAGTCCCAGGGAGTGTTT
TTCCTCACAGAAGAGCTTTCGTTGGATCGCTTGCTTCAAACACTTCACGATGTGCTGGCA
GTCGTGGAGACTCAGAAGAACATAGCAATCATCATTGACGGGGGTGCTATAGATGCTCTT
TTAGGCACCTATCCAACTAACTACTTCTACCGCTACCGGATACACCCTATTACTGGATCC
CTCCTGCCGCTGCCACTCATACTAAAGGCCTGGCGATACTTTGTGCGCTTCCTCTATGTC
TACTTCACTGGAAAAGCGAAGTCTGGAAAGGGGAGTTTGGGGCCAGATGCCAAGATACGG
GAGCTCTTCGCCGAAGTTACTTGCCGTGCCGAAACGGTGATCTGCTGCCGCTGCACCCCG
GGACAGAAAGCCTTGGTGTCAGAAATAATAACGACTTATAGTGGGAAGCAGTCGCTAGGC
ATTGGGGATGGTGCCAATGATGTTCCCCTAATCCAGCTCTGCGCTGTAGGAGTCGGTATA
GCAGGAAAGGAGGGGACACAAGCAGCAAACTCGGCAGACTTCGTCATCAATGAGTTTCAA
GGACTGAAAAAGCTTCTCCTGTGGCACGGGAGAAATAGTTACATAGGGAGTGCACAAATG
TGTCAGTTCATCATGCAGCGAGGCATAACGCAAACGCTCCTTCAAATATGGTTCAGTATG
CTTTATTATTTCGTAACTCTAGTAATATACACAGGCGTCTTATTGCTGGGATTTGCCACA
TTCTTTACAATGCTTCCTCCGTTCAACTATTATATTAACGAGGACATAGATTATAAGCTC
GTTATGCAGTATCCAGAGGTCTATAGATATACGTCGTCTGGAAGACTGATTTCCTTCAAG
ACCTTCTGCCTCTGGCTCCTTGCGTCCATCGTGATTTCGTTTATGATCTTTGTATCTTTG
GTCTGTGTTCTTCCGTGGGATGTGATATATACCGAGTTTGTTCTGGTGTCCTTCGCCGCT
CTTCTCATTAACCAGCTCCTTCTAATCTCCTTCACGTCTCATCGGTGGTCTGTCCTTCTC
GTAATTACACTGGTGGGTAGTTACGCCTCATTCTACATCGTGCAAACAATATATCCTGAC
ATATTTCCCCTTTCCTTTCTCTACTCCTTTTCTTTTACTTGGAGTTCGCTGTATATCGCCTTCAGTGCAGCTCTACCAA
TCTACATTGGCTCTCTTCTGGTAAAACACTTAAGTGTACCTCCTGTGATCAAAAAGCTTAGGAAGTTAAGAGCTCCCGCT
GACTGTGCGTGTTGTGCTAAAAAGAGCAATTGTGCATGCACCAAGTCCCCAATCCACTACTACAGTTCCACG
GGCACATACTACTTTAATTTGACAGATAAGGCTGCGAACGTACTCACATGTCGATACTGCTCTACGTGCCCTCGTAAGTGCTAG

TABLE 6. Amino Acid sequence of alternative flippase enzymes, ATPase, locus XP_001703950

A.A. length 940

Predicted Protein Sequence

MTCHNVELFR EDQDSPEQDT ALRPRRRRQP RTCSASTSQS SEQRVSQEPT SFLDASISEA
IGLEDRSQPV QTLSLSDSPT ELLFTRREID ENLILNRYIG SSPDEVAIIR SMHARGYILE
EKSHNLVRYT ASTGSQKFEL LHTFPFTSET KRMSVVTHHI ATGTTFIFVK GADTTIEASA
KYCPWLRNTV DALAGLGRRT LVYAYRTLDD DDLSNFHSSI TAASADLANR DSILVKCEET
LLTGVEVLCV TGIEDQLQLN VKATIQDLKR AGIKFWMLTG DKVVTCLSIA QSCGLLPNTA
VKKHTSKLRA SENTAPLTTQ SLHPEACKYP TTPSLDAVSI PTRSEIEVGQ EDKTPMAILR
RAIRDDPNSF MSVTSRREKR LNRKKRPGSL VLASTCYGRL SEHIYFLTEE LTPVEIVSVL
KELSQMVSFT HEVTIVIDGS ALEKLLGNYP VDYFYTHAIH PITGNLLPLH PCVRIWRRFT
RWLLIYLTGH PRRGKGSLGP DAETRELFAS IASKAETVLC CRCTPTQKAL VAGLISVYTG
SKSLGIGDGA NDVPLIETCS VGIGLRGKEG NQAANAADYV MTEFRGLKKL LLWYGKCSYV
GSAQICQFIV QRGVALCLLQ ICFTCLYFFT SLVLHTGVLL LGYVTLFTMF PPFNYYINED
VDYQLVLKYP EIYRITSRGS ILNLKTFFLW TFSGFIIAVV VFLLAYIFSS FPISVSGFTF
FSFAILLWSE LCLILVTTHR FSLMLLVALL LSYLMLFLVV AIYPGSLTRS EVFSLDMFWR
LCVTNAISAV LLFVGERIVR RFSKPNVVRI LRSMRKPVYC ATCKVPRCCT RESQQNVAVI
GKPSFNQDHP GLSRSVLRDR SKPMYRAGSG IKTESESGSG WPSGMVVTSR SYRRPSSSSY
VRLRGIYILS STGRHFFNMP ESVFNILTLR MCRRCVRINS

TABLE 7. Amino Acid sequence of alternative flippase enzymes, Phospholipid-transporting ATPase IA; locus XP_001705456

A.A. length 1309

Predicted Protein Sequence

MIADHPATCY TCDANATVAR QVVPIYEDLA ACEQGGNVKA SRERGKKRKQ GTVHKVRINY
YPLSQRSSNK FRSSPYTPLN FVPLFLKAQF SRPSNVIFVG IMILNYMPGI TIVSKATAVI
PVVFILATSL LKDLIELGIR IKNDKVINKA EYGEHISGGK VCSTVEAQNI NPGDIFKITA
GQTAPCDVLV LASDKNVVYF SQSSLTGEQN LVQKNPIYTS TDIISSQFIG DVEFSFYQGK
VFGCYTGPAV EENTQGYVST APDLHAAPVS VCIEDVQAIQ RARERRYRNA AMSTHHIQMN
SVVKRASMGK RPIQPEPIVH SILLNQGNVG FKGTAATTDY YALALATGEN CMSTYTAHKR
ESRISNGAKR LQLIIIVQCS LVLVFVISFS SVVTERYQGY IERYPYLEIA DQFASKAGAF
FISFVSYIIL FSYALPICIF VTIELLNILN RLFVRSDLNL IHLYGSCTVN NDKVLADLSR
ITHIFTDKTG TLTRNQFTYH SFLGVDECRR LSTESILKSI YALGENRAND ADLNVFYGNY
ASQTPQHTVD TIQNRVLQLV ALGLCNSLIP VKSNGRLEYF GESVEETCYI RYLSTNGILS
IVEKTESTVR LALSRSFIET GNVSSARIQT VRLSNTFESV ISDDYSIDKY VLLDFELLKV
YEFTPSLKRM SVICCQKASH ETTSCSMYVV NGMRPFLITK GSHVVMGQLM NRIPMAEGEQ
STEQPQSLSI ENIITKNLAD KRCFVFAFKP FPTLPNVKTE PQQSVEQGSN FLGISVIEDE
LAPNVLESVK KLRFAGIKIN IVTGDSIETT IETAARTGII DVDTNKVIIT RMDEIAVRKA
ELQDEEDQRY CLVVSGEVIN EVFPNLERRA QINAFLRKGK LPKKFARQYF KDTGVECSHE
PKVSLDLMYL ISQANACLFC SMSPESKKVV IQYHSVYISL QKTRIKLWCR GKSPSVGASL
AIGDGQNDLQ MIDAADVSVG VRGREGLYVA NNADVSVPSF STLVRLILVH GVLIEQRMRM
TIFYNLYKNT MLAIICGFYS GESLFSSVLI INDFLSLMYN VILNFIPIFI YALSEQHVKP
RYLENFPTIY RTNCQPWRYW FEFVTFYVSG IYMAVIIYFC TAFMFGNSAI LGTSGRVADT
TVFSFIIITV ITFVSLARLM IASNYYSTAF AWSIILSIAL YYFTLVGINY TFFFTQYFFN
TLTVASASLS YYLQCLVMII FCLVPDIIYS TLSRLYINPD PNSLLTHYFK KETKGLGKRK
DIQLELRYKR CLEEVIRSIQ TTAKSSKEQS QNAIFCTESL EVDVKQEYN

TABLE 8 Amino Acid sequence of alternative flippase enzymes, Phospholipid-transporting ATPase IA; locus XP_001710085

A.A. length 1309

Predicted Protein Sequence

MARPKCCKCN SLCCKRSSKR TSSASGDKQN AILFNFFDEG IAKTNHISTC KYTWYNFIFK
FLIEQYQRPN NIFFLTVLLC NSIPGLAIVG FGTLITPVIF ILVISAVREI IEDIGRSRSD
KISNSKVYKI VRKGEVLSLP ASKISQGDLV IIRDGDEVPA DCLLMISSSQ TKTAMVNTAS
LDGESNLKPR NVILTHLSPY NNELELLSFL RTLWGRITAY PPNSNLTLFT GKVSYHWSPY
GIGFHDPGAR AGTANDFAAR LAIKSMALYA PPPAVAGGYS GVNKTPGNLK SMASPFSAVY
VSARYPSYQP SSPSAQDTVP GTGPLGVRAD NLLSTAGISN IAENSEDGDS ILNDASVGNS
SHTFVKPTSG CAGSLAIDLD LAVTSSEPTE VAKDLTSSHG FSIDKLDSVV PRVTSISSKA
SRDSPVDQAG RDMATRSSIT TTPRTGFAAS VAQACDRANG ELPLTETLDL VSAFPLTNLA
AGPNYTTDNT HKLLHFNTDN TLLKGMKLQN TELAVGAVIY TAYDTRLMMN QTKPPLKLSH
VQKRLDRVLL LEILFLVAGV IICGAIAAAT NGDYLTRYPY AGFPNTKKFG TFLMRGMSYA
VLFSYALPMS LFVSLELVRL VQGIFIEMDT RLRKAELLPQ LDKRAYDPLP PAKVHCVVKA
SALIEDLAEV DIIFSDKTGT LTSNEMIFHS FYTLFDGVQC VYDTVIAAGK LELCTISHST
LVLEAKEEKN HVSDLMTYAL ALCNTIIVSD KGGTLVYQGE SPDEIAFAKA AAAFRLVITG
REAEYVRYNY SFSLNEDSVA PRQVSVPVTY TFPVVVPFTS ARKRMSIVLC EEGIEPSEHF
TSMIAPYLVN GSPCPTPDPK DVVCFPRFSY SRSKRRVFFT PIQLCTVPGN PFLLSKGADS
FLLPYCKPIL GENPMNLPPI KAAIDNFSCE GLRTLVWAVR ELSPGFLDNF LKTWREVSIK
PDSDPEYISH TKRLERDLTF LAASAIEDRL ADMVPETLAT LLSAGIKVWM LTGDKTQTAV
NIARSSNLAP AESEWLYLTH EEVTLMEEKL KQGEVVAPPI SEFVLEKLEK SPTKNKLLAL
ITLLDAFEKR VSPTVIKERR KLIHRRVEDA AEYNTYDTSE HPQDRKPSGA QQPTSDVSSV
KQKFTKFGRQ LKDAFSFTEL YRKKKEYKTT VPFTTVIDSD VFKLIYMNNL HDFLLSVTMH
STAVVCCRLS PEEKALIVTM TRNRDKYITT LAIGDGANDC PMIKSANIGV GIAGNEGLHA
SNSSDFSLPE FKYLRRLLFV HGHYTHLRNS ELIEYCIYKN LILVFVNGLF SGQCLFTSQI
LFNDMMVTMY NIVLTFFPIF IYALTEHDIK DRVLEVHPRV YSVFEKKPET NIKSIGLRIL
IAFYHSLCMY FLTRFAISYN VTEMNGHNID LTLFGYYLLN ELIFIVTIKI CLQTKYWSWA
TLIALIFTVT VFVILLLLTN YTTIITETLL NTMSFASHTL HFWVTVSSVV FAALLPDVMY
TFIRRFYFPN SHDLLYHQYR HLKRNCLGHA LDVGRDIERV EKMDNCLEEV QARLRQSDQV

Figures

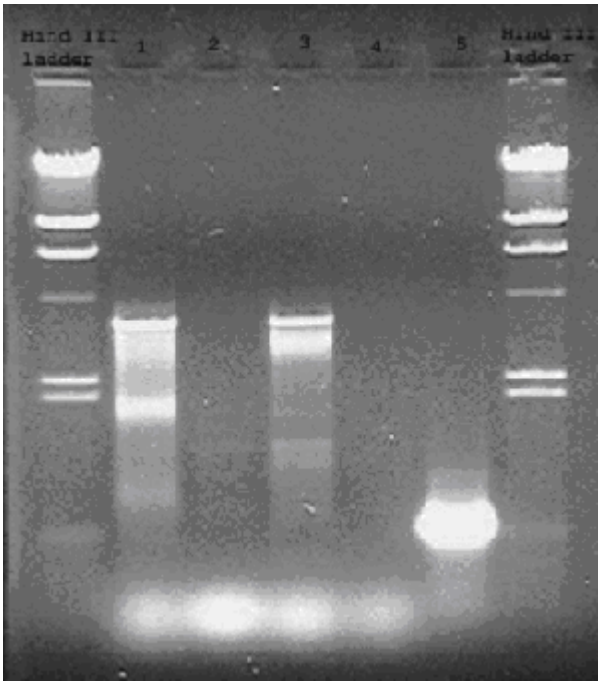

Figure 1: **Flippase PCR.** In this figure the bands in lanes one and three represent the amplification of the flippase enzyme using the primers previously constructed. Control is found in lane 5. In the first and last lanes are a Hind III ladder.

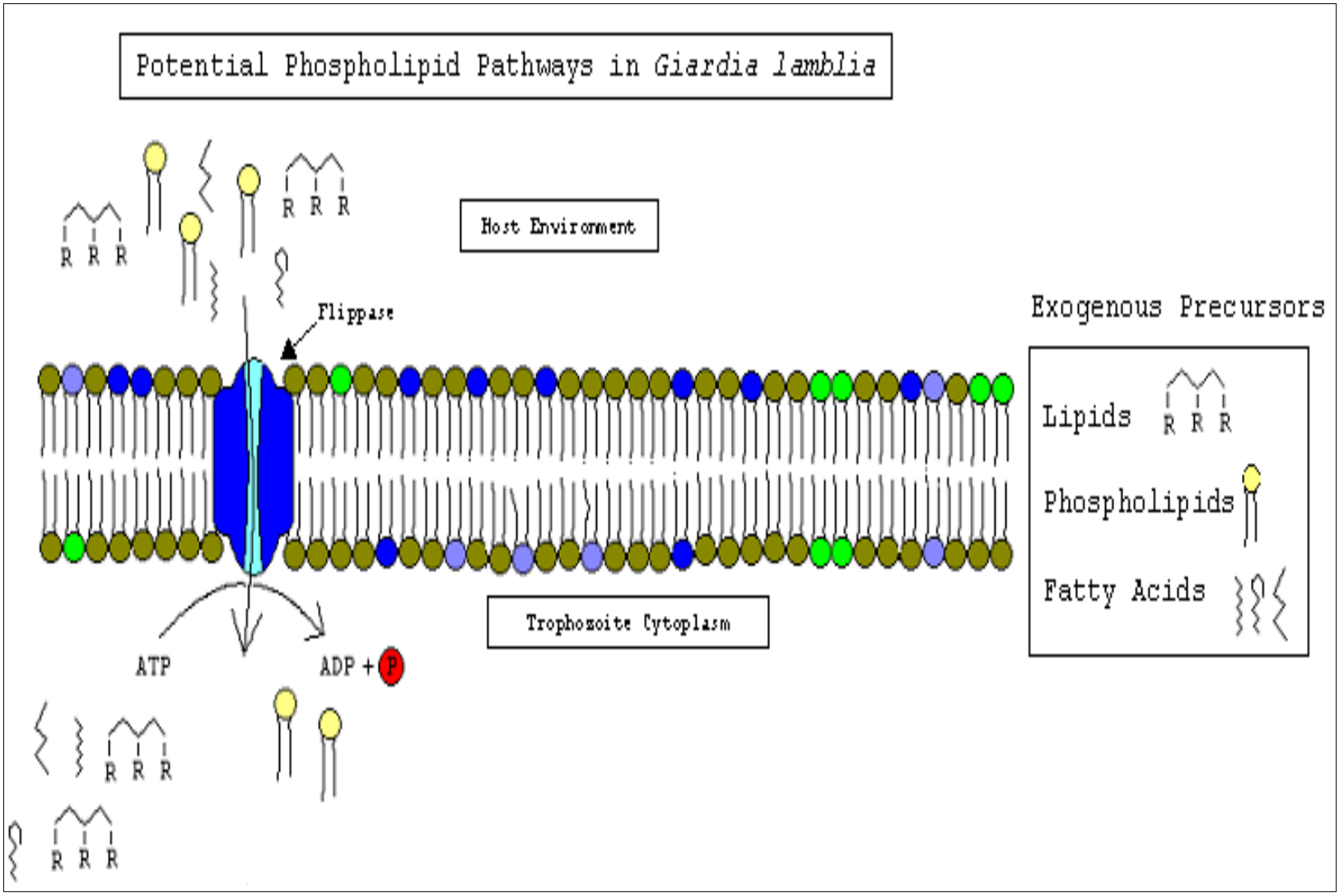

Figure 2: **Flippase model.** Flippase involvement in the proposed lipid-remodeling pathway in ***Giardia lamblia.*** Comparisons and alignments between the putative *G. lamblia* Flippase enzyme with other know eukaryotic flippase were used to construct the diagram. It gives us an idea where and how this enzyme may function.

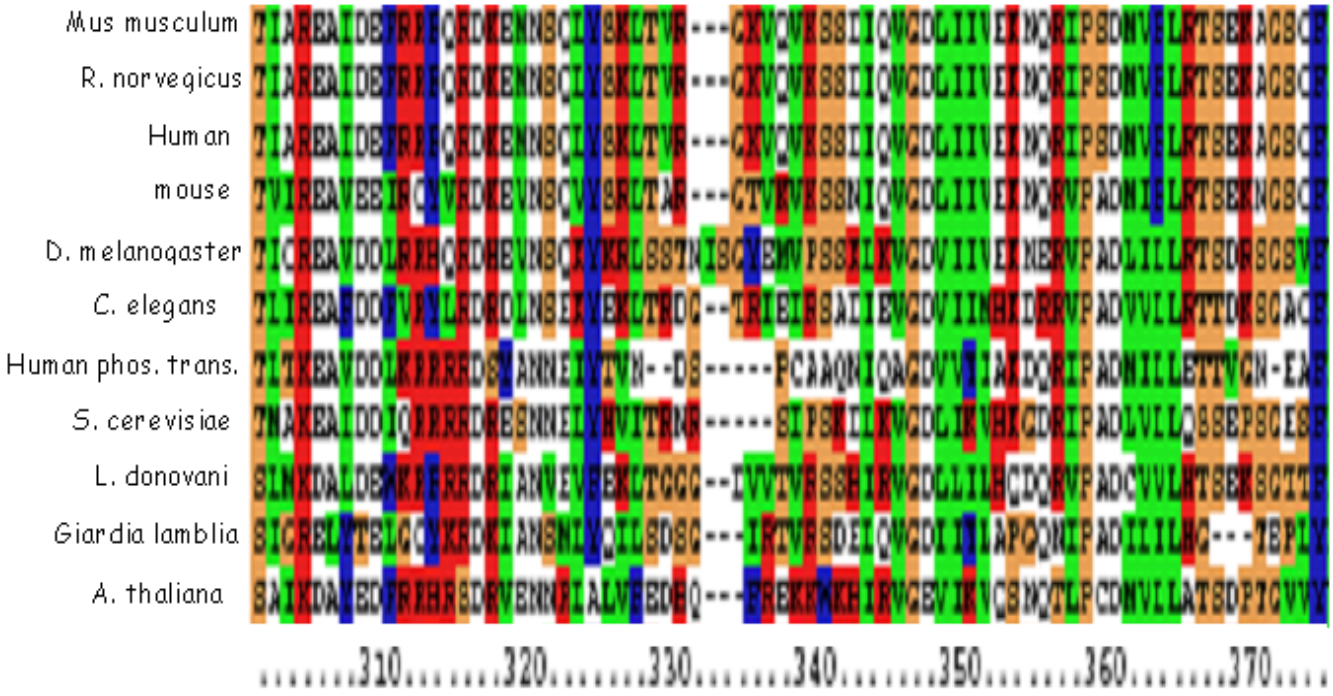

Figure 3: **Multiple sequenced alignments.** A sequence alignment using a section of 70 A.A by ClustalX reveals some similarities between the putative *Giardia lamblia* flippase enzyme and other higher eukaryotes such as human and mouse. By being able to match a flippase protein sequence to that of *Giardia* it gives insight into the possibility of a flippase site within its genome.

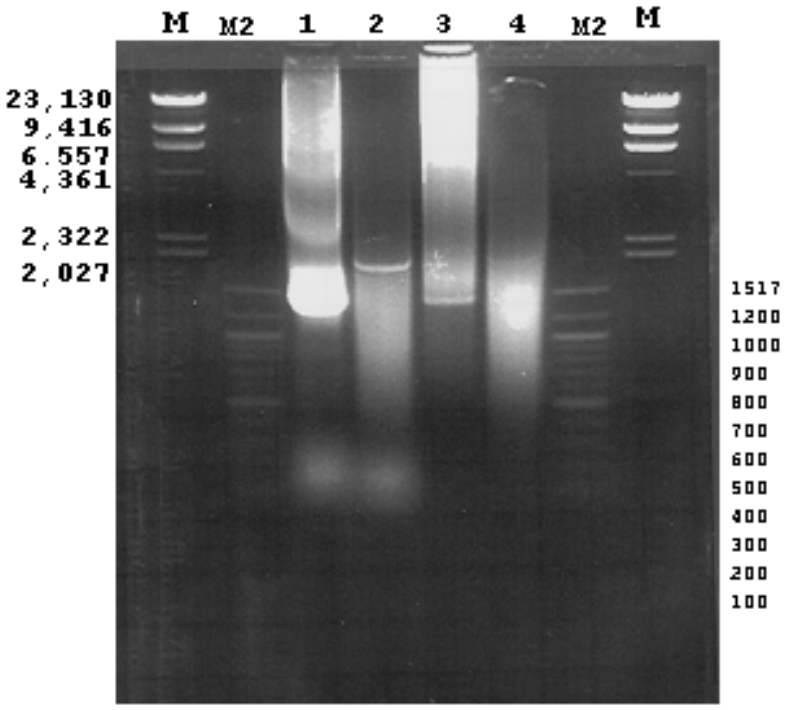

Figure 4: **RT-PCR of Flippase gene from *Giardia lamblia.*** Using two sets of primers the mRNA was amplified in both total RNA and total mRNA. Lanes marked with 'M' are the l-HIND III marker ladders and lanes marked with 'M2' contain the 100pb marker ladder. Lanes 1 contain total RNA, Lane 2 contained PCR amplification of flippase enzyme from total RNA, lanes 3 contained only mRNA, and lane 4 contained PCR amplification of flippase enzyme using mRNA. In both instances the flippase message was identified.

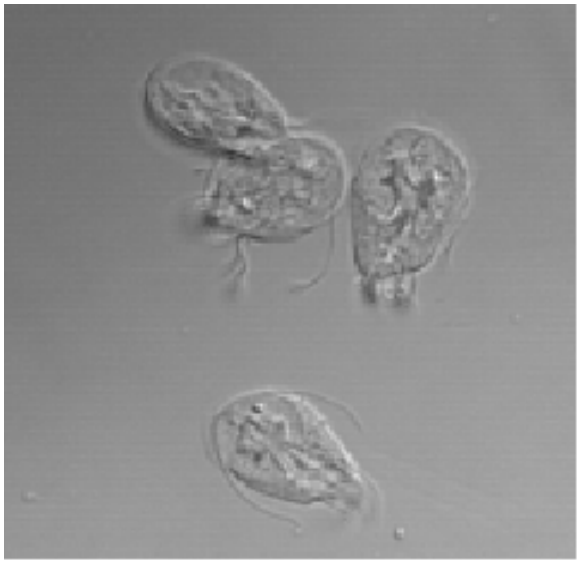

Figure 5: **DIC confocal image of C6 *Giardia lamblia*.** This image clearly shows the trophozoite stage, double nuclei, 8 discernable flagella, and characteristic “pear shaped” body.

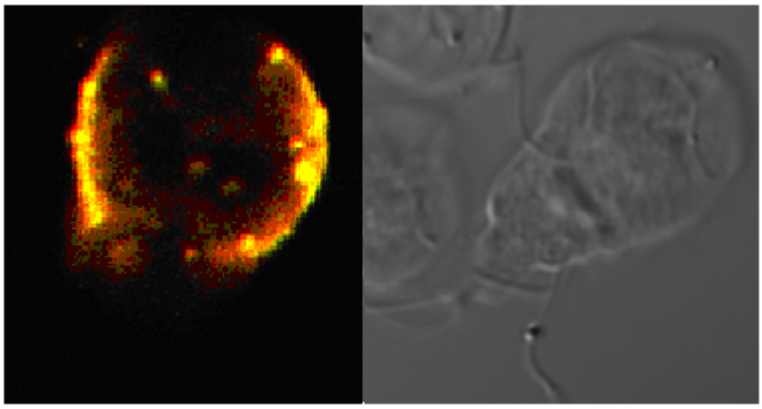

Figure 6: **Comparison of both confocal imaging and DIC** of the same G. lamblia trophozoite to show the localization of the flippase enzyme by its florescent tagged α–anti-monoclonal site. The magnification of the florescent image was at 6x its normal size were as the DIC was magnified at 5x its normal size.

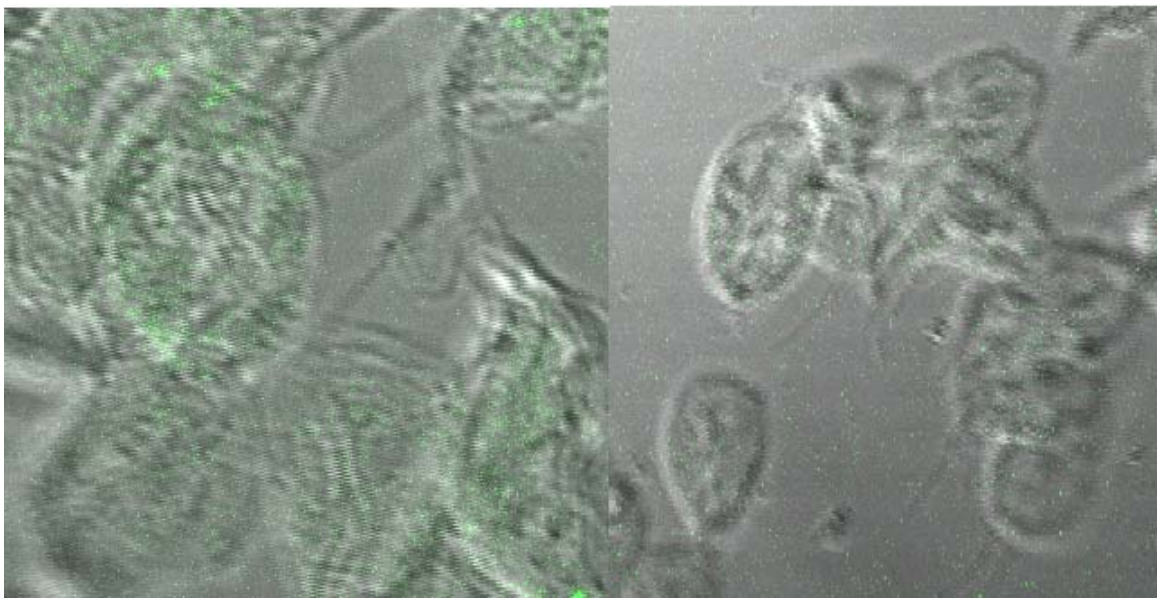

Figure 7: **Confocal of G. lamblia** infected with the hammerhead ribozyme. Cells are globular and distorted in shape.

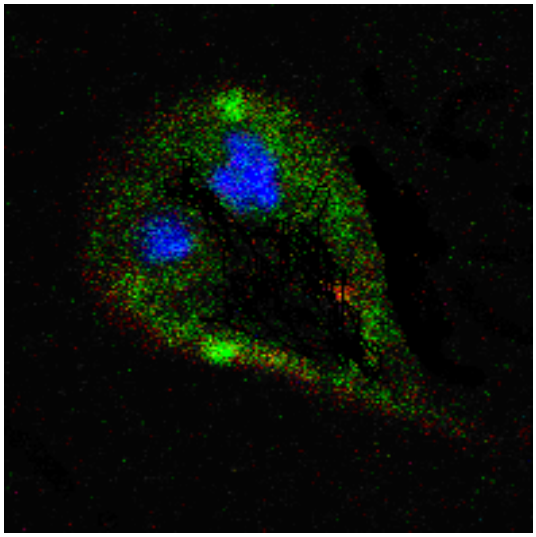

Figure 8: Demarcation of the localization of flippase enzyme. Two distinct monoclonal fluorescent dyes, Dapi and Rhodamine, were used to visualize whether the flippase enzyme had been incorporated into the lipid bilayer of *G. lamblia.* Dapi (blue) fluorescent dye was used to demarcate the nuclei, whereas Rhodamine (green) was "tagged" on to the anti-α-monoclonal site, which is attached to the flippase enzyme. The tagged flippase enzyme was cloned into an AU1 vector making it possible for the enzyme to be transfected into the trophozoite. Once incorporated, the vector amplified the enzyme via many rounds of replication making it available to the cell. Its availability increased the utilization of the flippase enzyme by the cell so that it could be properly processed and incorporated into its site of activity. Using Rhodamine to locate the enzyme it became apparent by the clearly demarcated green coloration that the enzyme was localized to the periphery of the parasite's outer lipid bilayer. This evidence further supposes the notion that our putative enzyme does indeed incorporate into the outer lipid bilayer and could perhaps have a role with lipid remodeling.

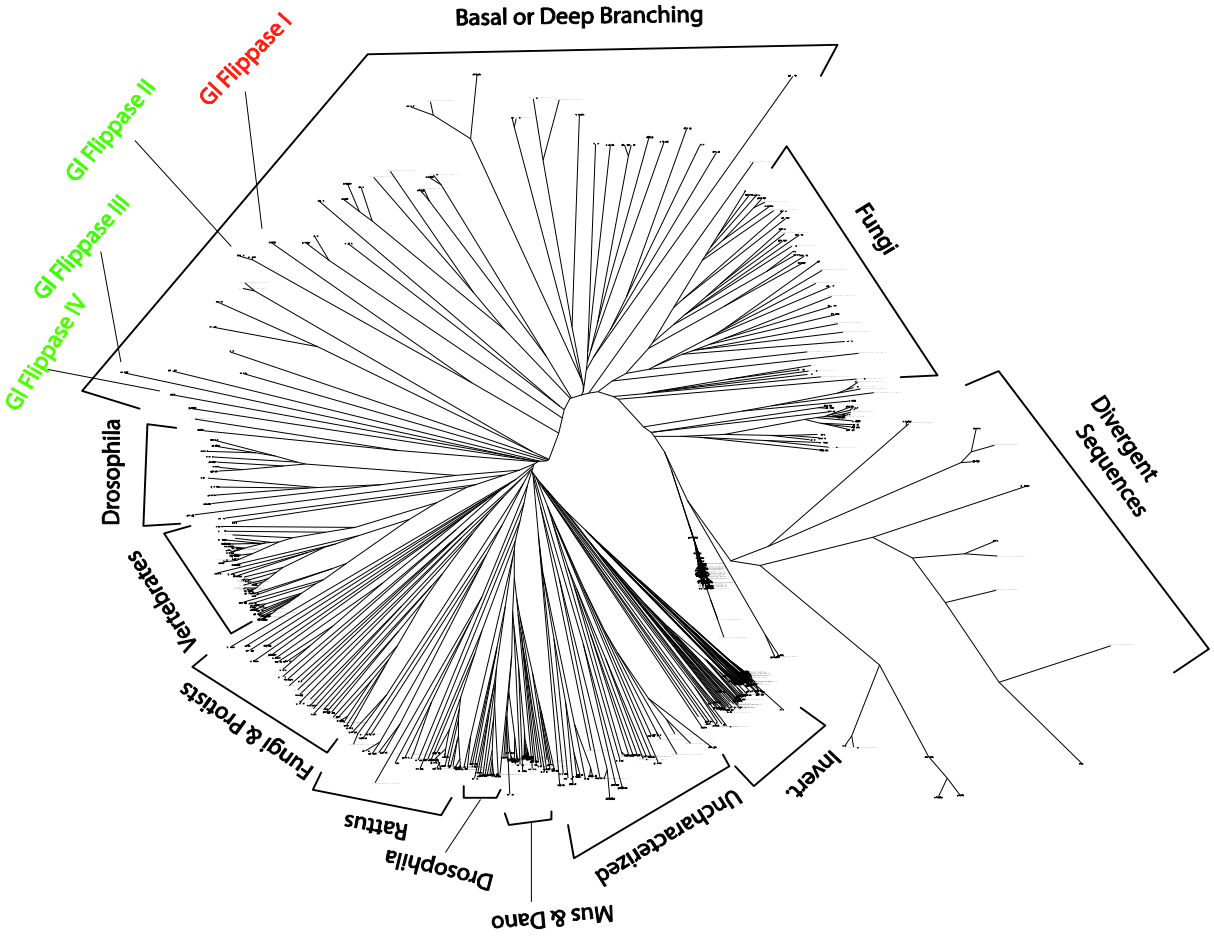

Illustration 1: Phylogenic tree. Unrooted Phylogenic Neighbor-Joining Tree of a broad range of homologs identified in the SWISSPROT database. The three additional *Giardia lamblia* flippases identified are notated in green, while our flippase in notated in red.

Table of abbreviation

1. *G. lamblia*- Giardia lamblia, flagellate parasitic protozoan
2. NA- Nuclei acids
3. Oligos- many nucleotide bases construction together to form a primer, Oligonucleotides.
4. DNA- Deoxyribonucleic acid is a nucleic acid that contains the genetic makeup for all biological entities of most cellular form of life.
5. RNA- Ribionucleic acid are nucleic acids combined in a single chain containing encoded material for the construction of proteins.
6. ATP- Adenosine triphosphate, main energy storage molecule in the cell.
7. PCR- polymerase chain reaction, used to amplify sections of DNA
8. TOPO TA- cloning kit using the TOP-10 cell lines and topoisomerase for the directed insertion of *tag* polymerase-amplified PCR products.
9. PICA- vector used for cloning
10. AU1- recombinant vector used for cloning
11. UV- ultraviolet light
12. IPTG- isopropyl-beta-D-thiogalactopyranoside, inducing reagent
13. JM109- strain of competent cells
14. cDNA- DNA constructed directly from messager RNA
15. RT-PCR- reversed transcription polymerase chain reaction
16. COG- Clusters of Orthologous Groups of proteins

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Vita

Diana L. Villazana was born in El Paso, Texas. She is the third child of Robert Villazana and Virginia Franco Villazana. She graduated from Burges High School in El Paso, Texas, in the spring of 1992. She began her pursuit of higher education in 1998 by attending El Paso Community College. She graduated in 2001 with honors from El Paso Community College with an Associate of Science degree. Upon transfer to the University of Texas at El Paso, she began her research in microbiology with Dr. Steven Aley. She completed her Bachelor of Science degree in Biology with Honors in 2003. She continued working in Biology while pursuing an MS in the same field. She participated in summer research in 2002, working with several of the Fulani tribes in Nigeria. During this time she also supported a military spouse and is the mother of three young boys. She has collaborated on several research projects that have resulted in publication in various scientific journals. In 2005 she was accepted into medical school at The University Of North Texas Health Science Center-Texas College of Osteopathic Medicine. She will graduate in May 2009 with her medical doctorate degree and enter a residency in General Surgery.

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