IDENTIFICATION OF THREE *CHLAMYDOMONAS REINHARDTII* GENES ENCODING ACTIN-ASSOCIATED PROTEINS

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INTRODUCTION

Actin and its associated proteins are a critical component of the fertilization process and other cellular events (Wilson *et al.,* 1998). Highly conserved among plant and animal species, the abundance and versatility of actin contribute to its functional importance in the eukaryotic cell. Actin exists as a globular protein that associates with other actin subunits to create a helical actin filament. The specific arrangement and location of these actin filaments determines the cytoskeletal shape and movement of the cell (Bray, 1992; Winder and Ayscough, 2005). Although actin filaments are present throughout the cell, they are highly concentrated within the cell cortex below the plasma membrane. Here, actin and actin-associated proteins create the cortical cytoskeleton, which is involved in many cellular activities including signaling, cytokinesis, and the formation of cellular processes (Bretscher, 1991).

Actin Polymerization

The capability of actin filaments to polymerize and depolymerize in response to various cellular signals contributes to the functionality of actin. Actin polymerization enables the formation of cellular surface projections such as filopodia and microvilli. According to Bray (1992), the addition of actin subunits to growing actin filaments occurs at different rates on opposing ends of the filament. The *plus end* elongates 3-4 times faster than the slower growing, *minus end*. The process of actin polymerization and the stability of actin filaments are regulated by various actin-associated proteins. These proteins perform a variety of functions including capping, bundling, contracting, cross-linking, and severing actin filaments (Bray, 1992; Winder and Ayscough, 2005).

Actin Cytoskeleton

The actin cytoskeleton is involved in a variety of cellular functions including fertilization, cellular movement, division, and formation of cellular protrusions (Winder and Ayscough, 2005). However, the roles actin and its associated proteins play in the fertilization process are not completely understood. Actin is an integral component of gamete binding and fusion during fertilization. The objective of this study is to identify actin-associated proteins and ultimately determine their potential role in fertilization.

Role of Actin in Fertilization

Fertilization is characterized by a two-step process consisting of an initial binding of sperm to the egg surface following by the fusion of gamete plasma membranes (Mengerink and Vacquier, 2001). During the initial phase of fertilization, gametes adhere to each other by specific receptors on the gamete surfaces. This adhesion typically leads to activation of the gametes, preparing them for the second phase – cell fusion. In many species, this initial binding event and subsequent gamete activation induces the sperm acrosomal reaction. The acrosomal reaction involves fusion between the acrosomal and sperm plasma membranes and serves two main functions (Ramalho-Santos *et al.,* 2002). The first is the release of digestive enzymes which enable sperm penetration through the thick extracellular layers surrounding the egg allowing it to reach the egg plasma membrane. The second is the exposure of a previously hidden membrane, the inner acrosomal membrane, which is the site or fusion between sperm and egg plasma membranes (Yanagimachi, 1988; Kaji and Kudo, 2004).

Frequently associated with the exposure of the inner acrosomal membrane is the production of an acrosomal filament, a core of actin filaments forming a microvillus-like

structure at the tip of the sperm. Sperm acrosomal filaments are either pre-formed prior to activation or actively assembled upon gamete activation. In some invertebrate marine species such as *Mytilus*, the pre-formed acrosomal filament contains short actin filaments present in the unactivated sperm. After initial contact with the egg, the acrosomal process is extended out from the sperm head (Yanagimachi, 1988). In other species such as sea urchins and horseshoe crabs, the acrosomal filament is rapidly assembled following gamete activation from monomeric actin (Foltz and Lennarz, 1993; Wilson and Snell, 1998). In both cases, the acrosomal filament is believed to aid in bringing the inner acrosomal membrane in close proximity to the egg plasma membrane to facilitate membrane fusion (Primakoff and Myles, 2002).

Chlamydomonas mt+ gametes undergo a process analogous to the acrosome reaction seen in other species (Wilson *et al.,* 1997). Prior to gamete activation, no apparent actin filaments are associated with the eventual fusogenic plasma membrane. Upon gamete activation, an acrosomal filament-like structure is formed from polymerizing actin. This microvillus-like structure is termed the fertilization tube and similar to the acrosomal filament, it is the site of cell-cell fusion (Friedmann *et al.,* 1968; Goodenough *et al.,* 1982). Similar to acrosomal filaments, the fertilization tubule contains a core of actin filaments created by rapid actin polymerization (Detmers *et al.,* 1983; Wilson *et al.,* 1997).

Actin is also a major component of the egg during fertilization. Underlying the plasma membrane in the egg cortex is a network of fine actin filaments. Extending outward from this network is a dense array of short, actin-filled projections termed microvilli (Bray, 1992; Kaji and Kudo, 2004). Except for the region above the meiotic

spindle, the entire egg surface characterized by numerous microvilli. Fertilization studies reveal that sperm only bind to these microvillus-rich areas on the egg surface and that cell-cell fusion occurs between the tip of the acrosomal filament and the egg microvilli (Yanagimachi, 1988; Foltz and Lennarz, 1992; Wilson *et al.,* 1997). In the classical fertilization model of the sea urchin, egg microvilli are relatively short in length (0.3 μm). In response to fertilization, the activated microvilli elongate to approximately 1.0 μm. (Bray, 1992). In *Chlamydomonas*, the microvillus-like fertilization tube elongates to approximately 3μm in length upon activation (Friedmann *et al.,* 1968; Wilson *et al.,* 1997).

Role of Actin in other Cellular Events

Actin and actin-associated proteins are also involved in other cellular events such as cell movement, cell division, and cytoskeletal shape (Bray, 1992; Winder and Ayscough, 2005). Cell motility requires the specific orientation of actin filaments at the leading edge of the cell (Condeelis, 2001; Roberts and Stewart, 2000). Actin filaments are arranged in a head-to-tail association to create a distinct polarity (Pollard and Borisy, 2003). The *plus end* of the actin filament is positioned toward the cell membrane at the tip of the leading edge of the cell while the *minus end* is oriented towards the cell interior. Repeated cycles of actin polymerization elongate the actin filaments and push the cell membrane outward to achieve cell movement while disassociation at the pointed end recycles actin monomers for future polymerization cycles (Welch *et al.,* 1997; Roberts and Stewart, 2000).

Eukaryotic cell division is dependent upon the formation of the contractile ring, a filamentous actin structure. The contractile ring forms during late mitosis and contains

actin filaments oriented parallel to the plasma membrane. As it compresses cellular cytoplasm, the contractile ring maintains its original thickness by consistently depolymerizing actin filaments. Actin-binding proteins such as α-actinin, filamin, and Myosin-II are the key components of the contractile ring (Bray, 1992).

Actin in *Chlamydomonas*

In the unicellular algae *Chlamydomonas,* actin is a key component of several important cellular structures. Upon gamete activation, the plus mating type (*mt*+) forms the microvillus-like fertilization tube that contains a core of actin filaments (Misamore *et al.,* 2003). To date only actin and the FUS1 protein have been identified as localizing specifically to the fertilization tube. Epifluorescent staining of *mt*+ activated cells with phallacidin distinctly labels the actin-rich fertilization tube (Misamore *et al.,* 2003).

Actin is also a component of the inner-arm dynein of *Chlamydomonas* flagella. The presence of actin has been discovered in six of the seven total flagellar inner-arm dynein subspecies present in *Chlamydomonas* (Ohara *et al.,* 1998). However, immunofluorescent staining of *Chlamydomonas* wild-type flagella with phallacidin shows no detectable actin filaments.

Chlamydomonas mutant Ida5 strains contain a mutation in the gene for conventional actin, which results in the absence of four of the seven flagellar dyneins (Kato-Minoura *et al.,* 1998; Hayashi *et al.,* 2001). Ida5 *mt*+ gametes do not produce the actin-rich fertilization tube, which results in low mating ability. *Chlamydomonas* also contains a second actin-like protein termed the novel actin-like protein (NAP). NAP, which shares a 64% homology with conventional actin, is scarcely present in wild type cells but profusely present in Ida5 cells (Kato-Minoura *et al.,* 1998; Hayashi *et al.,* 2001).

Transformation of Ida5 mutants with the cloned actin gene rescues the mutant phenotype. These results indicate that the inability of Ida5 to form a fertilization tube and the loss of flagellar dynein is directly associated with the absence of the conventional actin gene (Ohara *et al.,* 1998).

Chlamydomonas **as a Model Organism**

Chlamydomonas has been successfully used as a model organism to study numerous cellular processes including fertilization (Ferris *et al.,* 1996). Because the entire genome has been sequenced, *Chlamydomonas* is an excellent organism to use for genetic analysis. The latest version of the *Chlamydomonas* genetic database was constructed using the whole genome shotgun strategy at the Joint Genome Institute (JGI) of the U.S. Department of Energy and released online in January 2006 at http://genome.jgi-psf.org/Chlre3/Chlre3.home.html. The shotgun method is the most common genome sequencing strategy and consists of the random severing of long segments of DNA into multiple shorter segments for genetic analysis. These shorter DNA segments are sequenced individually and then assembled into chromosomes via computer analysis programs.

Chlamydomonas has a fairly small genome comprised of 17 chromosomes, approximately 15,256 predicted genes, and is 100 megabases (MB) in length. Table 1 illustrates the genomic comparison between *Chlamydomonas* and other model organisms.

Table 1. Genomic Comparison Among Model Eukaryotic Organisms

Species	Genome Size (MB)	Estimated Gene Number	Chromosome Number
Chlamydomonas reinhardtii	100	15,256	17
Saccharomyces cerevisiae	12	5,800	16
Arabidopsis thaliana	125	31,407	5
Caenorhabditis elegans	100	20,000	6
Drosophila melanogaster	180	13,600	7
Homo sapiens	3200	32,000	23

Table 1 illustrates comparisons between the gnomes of six model organisms. Information was obtained from the NCBI website located at http://www.ncbi.nlm.nih.gov/genomes/leuks.cgi.

When compared to other model organisms, *Chlamydomonas* has an average size genome that is similar to *Arabidopsis thaliana* and *Caenorhabditis elegans*. Based on the data provided in Table 1, *Chlamydomonas* is second only to *Homo sapiens* in highest number of chromosomes, although average in total number of genes when compared to the other organisms. The frequent refinement of the *Chlamydomonas* genome continues to enhance its usefulness as a model organism in molecular and cellular biology.

Another genetic analysis tool available to the *Chlamydomonas* community is the expressed sequence tag (EST) database. ESTs are short segments (300-400 nucleotides) of cDNA generated by reverse transcribing mRNA from a cell at a certain point in time (Boguski, 1995). ESTs provide information as to gene expression within an organism based on the selected segments of mRNA. These ESTs are randomly selected from a cDNA library. According to the *Chlamydomonas* center at Duke University, *Chlamydomonas* cDNA libraries contain cDNA from cells grown under varying conditions such as light/dark cycles, selectable media, and developmental stages. The gametogenesis cDNA library contains cDNA from *mt*+ and *mt*- cells at various time points during gametogenesis and zygote formation following fertilization

(www.chlamy.org/libraries.html). According to the NCBI EST database (www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html) updated on January 20, 2006, *Chlamydomonas* has 167,641 EST sequences compared to 421,027 EST sequences from *Arabidopsis thaliana* and 7,596,977 from *Homo sapiens*. Although ESTs have a 3% error rate due to the partiality of cDNA sequencing, they have greatly contributed to the discovery of new genes and have allowed gene expression analysis at various cellular stages (Boguski *et al.,* 1993).

Another advantage to using *Chlamydomonas* as a fertilization model organism exists within its strategy for reproduction. Throughout most of its life cycle, *Chlamydomonas* exists as vegetative cells that reproduce asexually. During unfavorable conditions such as when nutrients are scarce, *Chlamydomonas* undergo gametogenesis to create two types of gametes, mating type plus (*mt*+) and mating type minus (*mt*-). When gametes of opposite mating type are mixed together, initial binding occurs via flagellar adhesion between the *mt*+ and *mt*- gametes (Goodenough *et al.,* 1995). This process initiates gamete activation and causes the *mt*+ gametes to erect an actin filled microvillus structure termed the fertilization tube. Activated *mt*– gametes produce a less prominent, dome-shaped mating structure (Wilson *et al.*, 1997). Gamete fusion occurs when the two mating structures adhere to one another and their plasma membranes fuse (Goodenough *et al.*, 1995).

Chlamydomonas Fertilization Tubule Resembles a Microvillus

The fertilization tube produced upon activation of the *mt*+ gametes is structurally and functionally very similar to a microvillus. A microvillus is a projection emanating from the cell surface that contains a core of actin filaments (Bray, 1992; Bretscher, 1991).

Microvilli contain many proteins that serve various functions such as assembly of the actin core, cross-linking the actin filaments together, and fusing the microvillus to other structures (Bretscher, 1991; Pollard and Earnshaw, 2002; Bray, 1992). Microvilli are commonly located in areas of high absorption and secretion, such as cells lining the intestine (Bray, 1992). They are also present at the site of cell-cell fusion, such as the fusion of trophoblast cells to create the syncytiotrophoblast during placental formation (Gilbert, 2000). Furthermore, microvilli are involved in the fusion of undifferentiated muscle cells to create skeletal muscle fibers, a process that is essential for muscle development, growth, and repair (Fisher-Lougheed *et al.,* 2001).

Actin-Associated Proteins Present in Typical Microvilli

Proteins associated specifically with microvilli are actin-associated proteins that interact with the actin filaments. Generally, most actin-associated proteins bind to actin and either function in stabilizing, polymerizing, or depolymerizing the actin filament (Puius *et al.,* 1998; Winder and Ayscough, 2005). Profilin is an actin-binding protein that functions in recruiting actin subunits for actin polymerization (Staiger *et al.,* 1997). Cofilin, an actin depolymerizing protein, destabilizes actin filaments by binding to the filament and promoting actin disassociation at the *plus end*. This severing of filamentous actin drives actin turnover within the cell (Winder *et al.,* 2005). Other proteins such as villin, fimbrin, and fascin, function in stabilizing the structure by crosslinking the actin filaments together (Puius *et al.,* 1998; Fath and Burgess, 1995). Proteins such as ezrin, radixin, and moesin link the actin filaments to the plasma membrane (Mangeat *et al.,* 1999; Tsukita *et al.,* 1997). Similar to other myosin motor proteins, myosin-I contains an actin-binding region in its single heavy chain in the highly conserved motor domain.

Myosin-I functions by laterally joining the actin filaments of the microvillus to the surrounding plasma membrane (Footer and Bretscher, 1994).

The purpose of this study was to identify the presence of actin-associated proteins specific to microvilli in *Chlamydomonas* and to determine their possible role in the fertilization process. Various molecular techniques were performed to determine the presence and possible localization of selected actin-associated proteins in the following *Chlamydomonas* cell types: *mt*+ and *mt*- vegetative cells and *mt*+ and *mt*- gametes in both the unactivated and activated states. Because the fertilization tube of *mt*+ gametes resembles a microvillus, we hypothesize that actin-associated proteins present in microvilli may localize to this structure in *Chlamydomonas*.

MATERIALS AND METHODS

Protein Selection

Actin-associated proteins known to be associated with microvilli across broad taxa were identified through an extensive literature search. These candidate proteins were selected based on the following criteria: (1) Actin-associated protein known to be present in basic microvilli (2) Presence of a unique, highly conserved domain that illustrated sequence similarity to regions in the *Chlamydomonas* genome (3) Availability of taxonomically-broad antibodies to the known candidate proteins.

Based on these criteria the following nine candidate proteins were selected for further study:

Ezrin	Radixin	Moesin
Villin	Fimbrin	Fascin
Myosin-I	Profilin	Cofilin

Conserved Domain Searching

Conserved domains were identified for all candidate proteins using the conserved domain search tool at the National Center for Biotechnology Information (NCBI) website located at http://www.ncbi.nih.gov/index.html. A complete list of all conserved domains for all candidate proteins is located in Appendix 2. Conserved domains for each candidate protein were selected and viewed in FASTA format, the required sequence format for BLAST analysis, to determine their presence across broad taxa. The conserved domain protein sequence was searched against the *Chlamydomonas* genome (http://genome.jgi-psf.org) by performing a TBLASTN search with an expect value of 10 to obtain a reasonable number of significant matches. A TBLASTN search compares a protein query sequence against a nucleotide sequence database translated in all six reading frames.

Graphical BLAST alignment results, termed hits, were displayed and represented by segments of colored arrows. Hits are color-coded and ranked based on their BLAST score. A BLAST scoring color key located above the BLAST alignment box displayed the color-coded range of BLAST scores. Hits indicated the location of scaffolds, specific regions in the *Chlamydomonas* genome, which matched segments of the conserved domain sequence. The scaffold number located to the left of the arrow provided the exact position of the sequence match within the *Chlamydomonas* genome.

The hit with the highest BLAST score was selected (colored arrow). An alignment hit report was generated which contained data about the hit and produced a pairwise alignment of the two protein sequences. The significance of the matching amino acids was determined by identifying the percent homology between the two sequences

and the diversity of amino acid matches. For example, a low complexity sequence contains numerous repeats of glutamic acid (E) and alanine (A) amino acid matches, which are observable through visual inspection of the alignment. These low complexity sequence alignments are less significant than an alignment containing a variety of amino acids such as lysine (K), methionine (M), and proline (P).

The scaffold genomic sequence for the highest scoring hit was obtained by selecting the scaffold sequence from the alignment hit report. The resulting nucleotide sequence of this scaffold was searched against the NCBI protein database (http://www.ncbi.nlm.nih.gov/blast/) by performing a BLASTX search to determine if the *Chlamydomonas* protein sequence match resembled other similar proteins across broad taxa. A BLASTX search compares a nucleotide query sequence translated in all reading frames against a protein sequence database.

Once it was confirmed that the candidate protein sequence match in *Chlamydomonas* resembled similar proteins in other organisms, the same scaffold nucleotide sequence containing the protein match was searched against the expressed sequence tag database of *Chlamydomonas*. This was accomplished by performing a TBLASTX search of the NCBI database (http://www.ncbi.nlm.nih.gov/blast/) and limiting the search to the EST database of *Chlamydomonas*. TBLASTX compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database. Alignments produced by this search would indicate gene expression of this sequence in *Chlamydomonas*. The nucleotide sequence of the hit with the highest BLAST score was selected. A BLASTX of this sequence was performed

against the NCBI database to determine if the *Chlamydomonas* EST result resembled other candidate proteins across broad taxa.

The process described above was performed on all conserved domains listed for each of the candidate proteins. Genomic DNA sequences were obtained by selecting the genome browser link after the nucleotide sequence of the scaffold match was selected.

Cell Maintenance

Chlamydomonas strains 6145 (wild type *mt*-), 21gr (wild type *mt*+), Ida5 *mt*+ (actin mutant), and Ida5 *mt*- (actin mutant) (available from *Chlamydomonas* Genetics Center, Duke University) were utilized. Cells were maintained as asexually reproducing vegetative cells in 150ml liquid culture of M media (Sager and Granick, 1954) at 22°C on a 14:10 light/dark cycle and constant aeration. For long-term cultures, cells were plated on a 2% agar medium and stored at 12°C.

Gametogenesis was induced by transferring vegetative cells to a nitrogen free medium (M-N media) overnight in direct light with aeration as described by Snell (1976a). Transfer was accomplished by pelleting cells at 1700 x g for 4 min using a Beckman Coulter Allegra 6R centrifuge containing a GH 3.8 rotor. The supernatant (M medium) was discarded and cell pellets were resuspended in M-N medium overnight. After inducing gametogenesis, gamete quality was determined by (1) measuring negative-phototaxic response by placing cells in direct light for 10 min and (2) observing cellular agglutination and zygote formation during fertilization tests.

Gametes were activated by adding 50 µl of 15mM dibutyryl cAMP and 5µl of 0.15mM freshly prepared papaverine to 500µl cells in direct light and bubbled vigorously for 45 min (Pasquale and Goodenough, 1987). Gamete activation was verified using the

cell wall loss assay as described by Wilson *et al.* (1997) in which 1ml cell wall loss buffer (0.075% Triton X-100 in 0.5 mM EDTA) was added to 10µl of activated cells in a microfuge tube. Cells were vortexed briefly for 5 s using a minivortexer (Fisher Scientific, Pittsburgh, PA) and then centrifuged for 15 s at 16,060 x g using an accuSpin micro centrifuge (Fisher Scientific, Pittsburgh, PA) to pellet cells. Activated cells that had lost their cell walls were solubilized by the detergent and released their chorophyll into the supernatant, creating a white pellet with a green supernatent. Unactivated cells with intact cell walls were resistant to the detergent, thus producing a green pellet and clear supernatent.

Cell Counts

To determine the number of cells per ml, 900µl M-N media, 50µl of 5% gluteraldehyde in 1x10mM phosphate buffered media (1.7mM sodium citrate, 0.036mM ferric chloride, 0.361mM calcium chloride, 1.22mM magnesium sulfate, 7.35mM potassium phosphate and 5.74mM dipotassium phosphate, pH 7.1-7.3) and 50µl cells were added to a 1.5ml microfuge tube. Cells were loaded onto a bright-line hemacytometer (Hausser Scientific, Horsham, PA) and cell totals determined by counting all cells located within the five diagonally spaced inner squares of the hemacytometer grid.

Antibodies

A search of commercial vendors for α-villin, α-fimbrin, and α-Myosin-I antibodies was performed. The targeted amino acid sequence and genbank sequence identification number were obtained from the manufacturer. A TBLASTN search against the *Chlamydomonas* genome was performed using each antibody amino acid sequence to

determine the degree of recognition between the commercial antibody and *Chlamydomonas*. The following antibodies were obtained: Anti-ezrin and anti-villin polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and Anti-Myosin-I polyclonal antibody (Orbigen Inc., San Diego, CA). No commercial fimbrin antibodies were available.

Immunofluorescence Antibodies

Anti-ezrin and anti-villin polyclonal antibodies were diluted 1:100 in immunofluorescence blocking buffer (1% cold water fish gelatin, 0.1% BSA, 5% glycerol, 30mM NaCl, 2mM sodium phosphate buffer, pH 7.3) (Misamore *et al.,* 2003). Anti-Myosin-I polyclonal antibody was diluted 1:100 in immunofluorescence blocking buffer. To lower background fluorescence, all secondary antibodies were absorbed against mt- cells to reduce nonspecific bindng. The following secondary antibodies were used at a concentration of 1:2000 in PBS: Alexa Fluor 488 donkey anti-goat IgG (H+L) polyclonal secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG (H+L) polyclonal secondary antibody and Alexa Fluor 488 goat anti-chicken IgG (H+L) polyclonal secondary antibody (Invitrogen, Corp., Carlsbad, CA).

Immunoblotting Antibodies

Anti-villin and anti-Myosin-I polyclonal antibodies were diluted 1:1,000 in 3% milk-TBST. Donkey anti-goat IgG-HRP secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), goat anti-rabbit IgG-HRP secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and goat anti-chicken IgG (H+L)-HRP secondary antibody (Southern Biotech, Birmingham, AL) were diluted 1:10,000 in 3% milk-TBST. Both primary and secondary antibodies were prepared fresh for each experiment. Mouse

kidney lysate (Santa Cruz Biotechnology Inc., Santa Cruz, CA) served as a villin and myosin-I immunoblotting control.

Immunofluorescence Sample Preparation

Immunofluorescence samples were prepared by fixing cells in 4% paraformaldehyde/0.1% gluteraldehyde in 50mM phosphate buffered medium (1.7mM sodium citrate, 0.036mM ferric chloride, 0.361mM calcium chloride, 1.22mM magnesium sulfate, 7.35mM potassium phosphate and 5.74mM dipotassium phosphate, pH 7.1-7.3) for 15 min. Samples were washed three times in 1% glycine-PBS and re-suspended in 50mM phosphate buffered medium. Samples containing 7µl cells were applied to 8-well glass slides pre-coated with 0.05% polyethylenimine.

To permeabilize cells, slides were immersed in 80% acetone for 6 min at -20°C followed by immediate immersion in 100% acetone for 6 min at -20°C. After the slides were completely dry, samples were blocked for 30 min at 37°C with 20µl/well of immunofluorescence blocking buffer. Excess blocking buffer was siphoned off and 20µl of primary antibody was applied to each sample and incubated at 37°C for 60 min. Samples were washed three times in phosphate-buffered saline.

To label actin, Alexa Fluor 546-Phalloidin (Invitrogen, Corp., Carlsbad, CA) was first evaporated and then resuspended in secondary antibody at a concentration of approximately 1U/20ul. Samples were incubated in the secondary antibody/Phalloidin solution for 30 min at 37°C. Samples were washed three times in PBS. Slides were coated with Fluoromount-G mounting medium (Southern Biotechnology Associates, Inc., Birmingham, AL), covered with Corning No. 1.5 (24x50mm) coverslips and sealed with nail polish.

Microscopy

Cell agglutination and zygote formation was observed using a Zeiss Axiostar Plus microscope (Carl Zeiss Inc., New York, NY) equipped with a 40x phase contrast objective. Cell counts were performed using the 20x objective on the Zeiss Axiostar Plus microscope. Fluorescent labeling was observed using a Zeiss Axiovert 200 microscope (Carl Zeiss Inc., New York, NY) containing FITC/GFP and TRITC/Rhodamine barrier filters. Images were captured using a Zeiss AxioCam MRm camera and compiled using Zeiss Axiovision version 4.3 software (Carl Zeiss Inc., New York, NY).

Immunoblotting

Samples were prepared by mixing 1 x 10^8 cells in 250µl 1x SDS sample buffer (62.5mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.1M dithiothreitol, and 0.025% bromophenol blue) and boiled for 4 min. Samples were sonicated for 7 s each and stored at -20°C. Immunoblotting mini-gels (10% separating gel/3% stacking gel) were prepared following standard SDS-Page protocol. Generally, each well was loaded with 15-30µl of samples containing approximately 2 x 10^7 cells in 50µl sample buffer. Kaleidoscope prestained standards (Bio-Rad Laboratories, Inc., Hercules, CA) were used as standard molecular weight markers. Approximately 20µl of the positive control (mouse kidney lysate) was used in each gel. Electrophoresis was preformed using a Bio Rad minigel apparatus immersed in electrode buffer (25mM Tris-Base, 192mM glycine, and 0.1% SDS, pH 8.0) for 2-2.5 hours at 100 volts. Proteins were transferred onto Immobilon-P transfer PVDF membranes containing 0.45µm pores (Millipore Corp., Billerica, MA). The membranes were pre-soaked in methanol for 5 min prior to protein transfer. Proteins

were transferred overnight at 36 V at 4°C in transfer buffer (25mM Tris-Base, 192mM glycine, and 20% methanol.)

Membranes were rinsed twice in TBST (20mM Tris, pH 7.6, 137mM NaCl, and 0.05% Tween 20) and blocked for 45 min in 5% milk-TBST blocking buffer. Primary antibody in 3% milk-TBST (1:1,000) was applied for one hour. After rinsing membranes three times in TBST, secondary antibody in 3% milk-TBST (1:10,000) was applied for 30 min. Membranes were rinsed three times in TBST and soaked in SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL) for 1 min. Membranes were imaged using Hyperfilm (Amersham Biosciences, Buckinghamshire, UK) and developed using a standard X-ray film developer.

Genetic Analysis

Bacterial artificial chromosomes (BAC) containing the desired villin, fimbrin, and myosin genes were identified on the *Chlamydomonas* center website (www.chlamy.org.) At Clemson University Genomics Institute, BAC clones were constructed with the pBeloBAC11 + NIT8 vector and DNA was cut with the HindIII enzyme with an average insert size of 75 kB. The BAC clones contained a selectable marker for Chloramphenicol. Plasmids containing the BAC DNA were transfected into *E.coli* cells using the HindIII enzyme and shipped on an agar slant. The purchased BAC clones containing the specified hypothetical *Chlamydomonas* genes are listed in Table 2.

Table 2: BAC Identification Numbers

BAC ID	*Gene*
35J9	hcVillin
2I18	hcVillin
14F9	hcFimbrin
2C21	hcMyosin

Table 2 identifies the BAC ID numbers assigned to each specified gene upon assembly at the Clemson University Genomics Institute. BAC ID numbers indicate the plate, row, and well number of each colony culture.

Primers for villin, fimbrin, and Myosin-I were designed and purchased from Integrated DNA Technologies, Inc., Coralville, IA and diluted to 100μM concentrations. Primer sequences and PCR products for villin, fimbrin, and Myosin-I are illustrated in Table 2. To purify plasmid DNA from bacteria, a standard QIAprep Spin Miniprep Kit(50) (Qiagen Inc., Valencia, CA) was used. Plasmid DNA was subjected to PCR in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the following cycles: 5 min at 94°C, 45 s at 58°C, 30 cycles of 30 s at 72°C, and finally 5 min at 72°C. PCR products were subjected to gel electrophoresis using a 1.4% agarose gel at 100 volts for 60 min. Gels were imaged using a Kodak Gel Logic200 Imaging System (Kodak Scientific Imaging Systems, New Haven, CT).

Reverse Transcriptase-PCR

Purified *Chlamydomonas* mRNA templates from *mt*+ vegetative cells, *mt*– vegetative cells, *mt*+ gametes, and *mt*– gametes were obtained from the laboratory of Dr. William J. Snell at UTSW Medical Center. To synthesize single-stranded cDNA from *Chlamydomonas* mRNA, reverse transcriptase polymerase chain reaction (RT-PCR) was performed (1st Strand cDNA Synthesis Kit for RT-PCR, Roche Applied Science, Indianapolis, IN) using $p(dN)_6$ random primers and avian myeloblastis virus reverse

transcriptase (AMV-RT). The resulting single-stranded cDNA was amplified through PCR using sequence specific primers (Table 1) and Taq polymerase (Roche Applied Science, Indianapolis, IN). The amplification process was performed in an Eppendorf Mastercyler with the following cycles: 10 min at 25°C, 60 min at 42°C, 5 min at 99°C, and finally 5 min at 4°C. The resulting single-stranded cDNA was amplified using a standard PCR reaction using sequence specific primers. PCR products were subjected to electrophoresis on a 1.4% agarose gel at 100 volts for 60 min and imaged using a Kodak Gel Logic200 Imaging System.

Table 3. PCR Primer Sequences

Gene	PCR Primers
hcVillin	(+) 5'-ATGACGCAAGAGGCGACTGC-3'
	(-) 5'-TGACTCCTCCTCCTCGCCCTTA-3'
hcFimbrin	(+) 5'-AGCAGTGTCCGCTTCTGCCA-3'
	(-) 5'-ACCAGCTTGTTATGCTCCGACAAC-3'
hcMyosin-I	(+) 5'-AGGCATGGATCAAAGGAGAGGTCA-3'
	(-) 5'-CGCGGTATTGGTTCATCATGTGCT-3'

Table 3 illustrates the upstream (+) and downstream (-) primers used for hcVillin, hcFimbrin, and hcMyosin-I PCR reactions.

RESULTS

Database Analysis Results of Villin, Fimbrin, and Myosin-I BLAST Searches

The conserved domain families and sequences for all candidate proteins searched against the *Chlamydomonas* genomic database can be viewed in Appendix 2. Those producing the most significant results will be further discussed. Performing a TBLASTN query of the conserved domains of all candidate proteins against the *Chlamydomonas*

genomic database resulted in significant sequence matches for the following candidate proteins: villin, fimbrin, and Myosin-I.

Villin BLAST Results

The first candidate actin-associated protein containing a conserved domain that produced a significant match in the *Chlamydomonas* genome was the actin-bundling protein, villin. The villin headpiece domain from the smart00153 family was searched against the *Chlamydomonas* genomic database by performing a TBLASTN search. Figure 1.1 illustrates the graphical alignment results from the TBLASTN search of the villin headpiece domain.

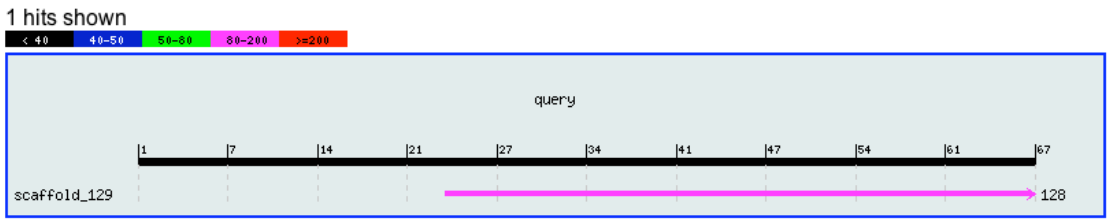


Figure 1.1: *Chlamydomonas* TBLASTN Results of Villin Conserved Domain

Figure 1.1 illustrates the best hit result generated through a TBLASTN search of the conserved villin headpiece domain against the *Chlamydomonas* genome. Because this hit has a BLAST score between 80-200, it is colored pink. The arrow represents amino acids in *Chlamydomonas* that align with the conserved domain query sequence.

The BLAST alignment box pictured above in figure 1.1 provides information about the alignment results generated from the TBLASTN search in the *Chlamydomonas* database. The selected conserved domain query sequence for villin containing 67 amino acids is shown in black. The hit, represented by the pink arrow, indicates the region within the *Chlamydomonas* genome where the amino acid match occurs. Because this hit has a BLAST score of 128, it falls within the range of 80-200 and is shaded pink, the second highest rank of sequence matches. Scaffold 129 indicates the specific region within the genome where this sequence match exists.

Figure 1.2 illustrates the pairwise alignment of both the query conserved domain sequence and the matching *Chlamydomonas* scaffold 129 sequence.

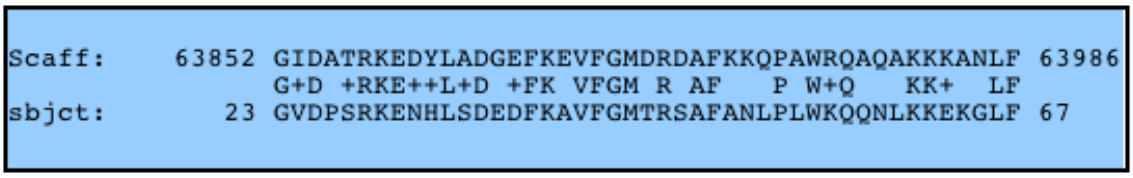


Figure 1.2: Pairwise Alignment of Villin Conserved Domain Sequence and *Chlamydomonas* scaffold 129 Sequence

Figure 1.2 aligns the villin conserved domain query sequence, represented as the subject sequence, with the scaffold region in *Chlamydomonas* producing a pairwise alignment. Scaffold numbers represent the location within the genome where the amino acid match occurs. The subject numbers indicate that amino acids 23-67 of the conserved domain query sequence align with the indicated *Chlamydomonas* scaffold.

In figure 1.2, the subject sequence is the villin conserved domain amino acid sequence while the scaffold sequence represents the *Chlamydomonas* sequence match. While the entire villin conserved domain sequence is 67 amino acids, the match with Chlamydomonas only occurs between amino acids 23 through 67. There is a 51% homology between the two sequences based on the 23 positive amino acid identities out of a total sequence length of 45 amino acids.

The nucleotide sequence of this region on scaffold 129 where the villin conserved domain sequence match occurs was obtained from the *Chlamydomonas* website. This sequence is illustrated in Figure 1.3.

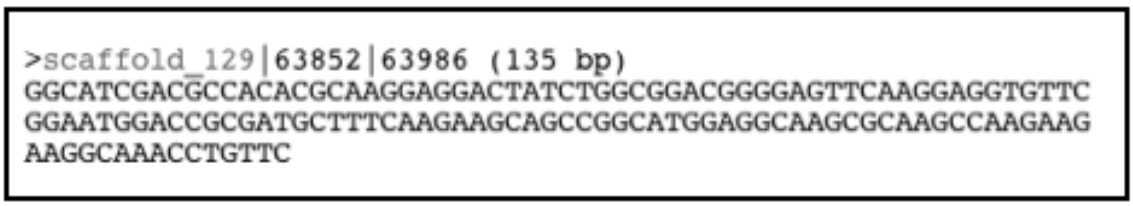
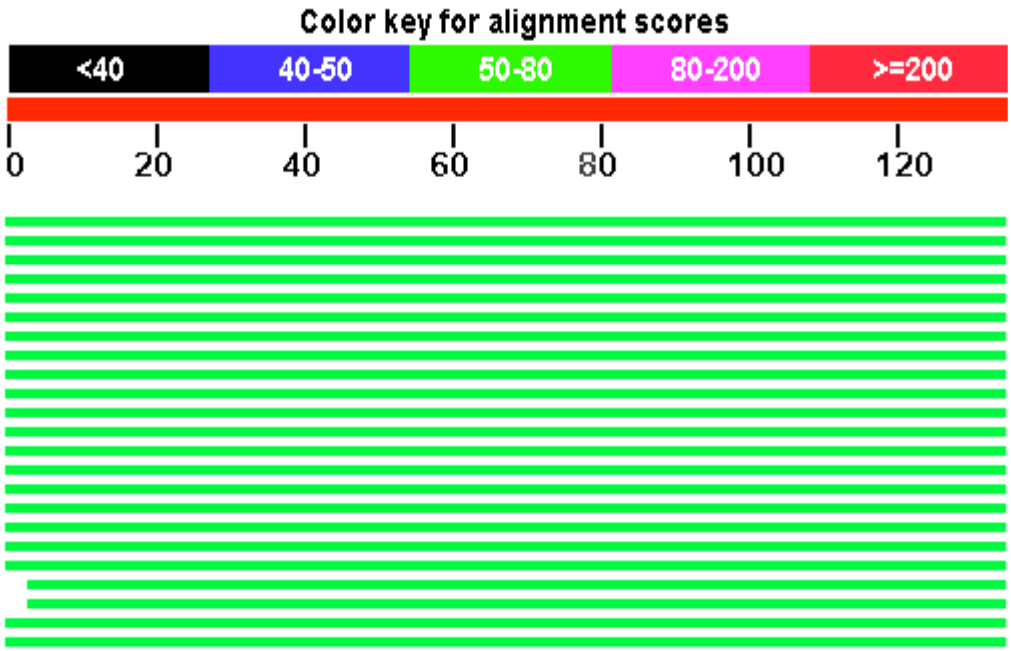


Figure 1.3: Scaffold 129 Nucleotide Sequence

Figure 1.3 displays the nucleotide sequence of bases 63852-63986 of *Chlamydomonas* scaffold 129, the region producing a pairwise alignment with the villin conserved domain sequence as illustrated in figure 1.2.

The nucleotide sequence displayed in figure 1.3 is 135 base pairs long and is located from bases 63852-63986 within scaffold 129 of the *Chlamydomonas* genome. This nucleotide sequence from scaffold 129 was used in a BLASTX search to determine if the *Chlamydomonas* villin sequence match resembles other villin proteins across broad taxa. Figure 1.4 graphically displays the BLASTX results for the scaffold 129 nucleotide sequence.

Figure 1.4 lists the protein matches from other organisms based on the BLAST score listed to the right of the entry. The alignment score color key in the top of figure 1.4 displays colored lines based on the BLAST score of each entry listed below. The top match was a villin 3 protein in *Arabidopsis thaliana* and had a BLAST score of 55.8. Subsequent matches also listed similarity to villin proteins across broad taxa, indicating a resemblance of this hypothetical *Chlamydomonas* villin (hcVillin) to other villin proteins.

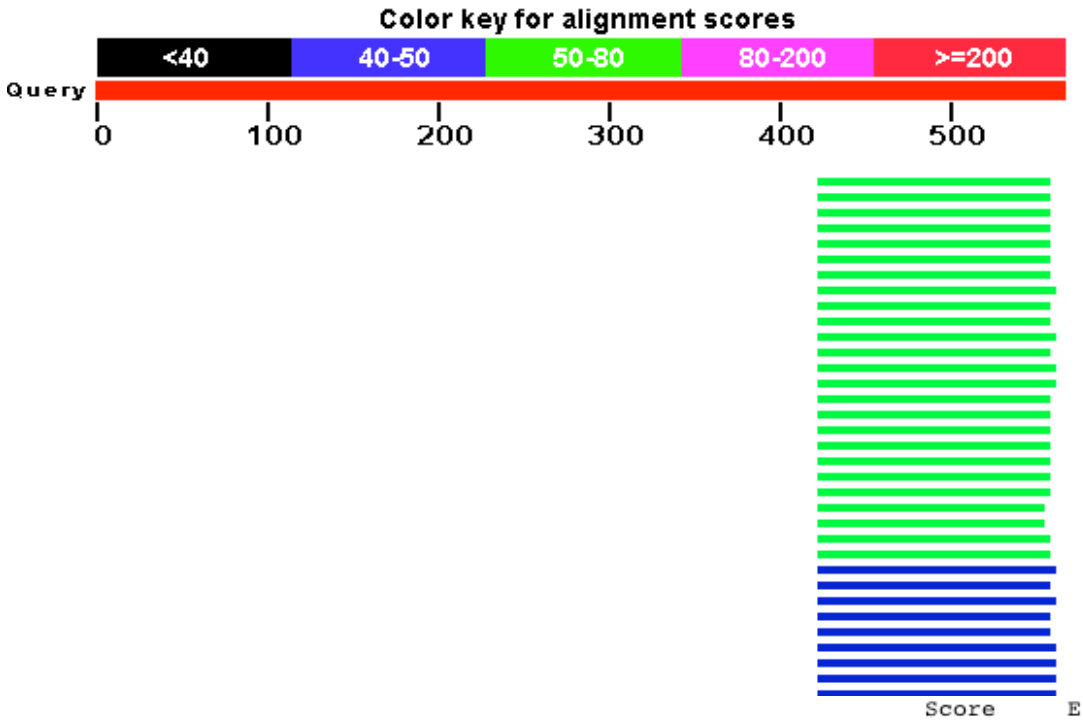


gi|18410709|ref|NP_567048.1|	VLN3 (VILLIN 3); actin binding [Ara	55.8
gi|6735320|emb|CAB68147.1|	villin 3 fragment [Arabidopsis tha...	55.8
gi|3415117|gb|AAC31607.1|	villin 3 [Arabidopsis thaliana] >gi...	55.8
gi|45382125|ref|NP_990773.1|	villin 1 [Gallus gallus] >gi|138...	53.9
gi|75765644|pdb|1YU5|X	Chain X, Crystal Structure Of The Head...	53.9
gi|6730521|pdb|1QQV|A	Chain A, Solution Structure Of The Head...	53.9
gi|47223390|emb|CAG04251.1|	unnamed protein product [Tetraodon n	53.5
gi|57283139|emb|CAE17317.1|	villin 2 [Nicotiana tabacum]	52.0
gi|32766461|gb|AAH54960.1|	Vil1-prov protein [Xenopus laevis]	52.0
gi|75765646|pdb|1YU8|X	Chain X, Crystal Structure Of The R37a...	51.6
gi|18405794|ref|NP_565958.1|	VLN2 (VILLIN 2); actin binding [...	51.2
gi|3415115|gb|AAC31606.1|	villin 2 [Arabidopsis thaliana]	51.2
gi|25295744|pir||E84845	probable villin 2 [imported] - Arabidops	51.2
gi|47220696|emb|CAG11765.1|	unnamed protein product [Tetraodon n	50.8
gi|18256316|gb|AAH21808.1|	Villin-like [Mus musculus] >gi|239...	50.8
gi|18490748|gb|AAH22664.1|	Vill protein [Mus musculus]	50.8
gi|50732191|ref|XP_418521.1|	PREDICTED: similar to MGC52940 prot	50.8
gi|15487264|emb|CAC69079.1|	villin-like protein [Mus musculus...	50.8
gi|26390015|dbj|BAC25828.1|	unnamed protein product [Mus musculu	50.8
gi|50942753|ref|XP_480904.1|	putative villin [Oryza sativa (j...	50.4

Figure 1.4: BLASTX Results for Scaffold 129-Villin Match

Figure 1.4 displays the results of a BLASTX search comparing the nucleotide sequence of scaffold 129, which produced the villin match, to all organisms in the NCBI database. This search was performed to determine if the *Chlamydomonas* villin sequence match resembled other villin proteins across broad taxa. The colored arrows correspond with the BLAST scores of the protein matches listed below. These results indicate similarity of the *Chlamydomonas* hypothetical villin protein to other villin proteins in other organisms.

The next phase of database analysis involved performing a TBLASTX search limited to the EST database of *Chlamydomonas* to determine if the mRNA of hcVillin is actively transcribed in the EST database. The nucleotide sequence of scaffold 129 (Figure 1.3) was the query sequence used in the search. The top listed *Chlamydomonas* EST gene was genbank index number 16438573, which had the highest BLAST score of 113. The nucleotide sequence of this *Chlamydomonas* EST gene was selected and a BLASTX search was performed using this sequence to determine sequence similarity across broad taxa. Results of the BLASTX search are illustrated in figure 1.5. The results shown in figure 1.5 indicate a similarity between the *Chlamydomonas* hcVillin EST gene and other villin-like proteins across broad taxa.



Sequences producing significant alignments:		Score (Bits)	E Value
gi|3415117|gb|AAC31607.1|	villin 3 [Arabidopsis thaliana] >gi...	55.8	9e-07
gi|18410709|ref|NP_567048.1|	VLN3 (VILLIN 3); actin binding [Ara	55.8	9e-07
gi|6735320|emb|CAB68147.1|	villin 3 fragment [Arabidopsis tha...	55.8	9e-07
gi|138530|sp|P02640|VILI_CHICK	Villin-1 >gi|45382125|ref|NP_9...	53.9	3e-06
gi|75765644|pdb|1YU5|X	Chain X, Crystal Structure Of The Head...	53.9	3e-06
gi|6730521|pdb|1QQV|A	Chain A, Solution Structure Of The Head...	53.9	3e-06
gi|47223390|emb|CAG04251.1|	unnamed protein product [Tetraodon n	53.5	4e-06
gi|47220696|emb|CAG11765.1|	unnamed protein product [Tetraodon n	53.1	6e-06
gi|32766461|gb|AAH54960.1|	Vill-prov protein [Xenopus laevis]	52.0	1e-05
gi|57283139|emb|CAE17317.1|	villin 2 [Nicotiana tabacum]	52.0	1e-05
gi|50732191|ref|XP_418521.1|	PREDICTED: similar to MGC52940 prot	51.6	2e-05
gi|75765646|pdb|1YU8|X	Chain X, Crystal Structure Of The R37a...	51.6	2e-05
gi|49256072|gb|AAH74148.1|	LOC398504 protein [Xenopus laevis]	51.2	2e-05
gi|28278754|gb|AAH44966.1|	LOC398504 protein [Xenopus laevis]	51.2	2e-05
gi|3415115|gb|AAC31606.1|	villin 2 [Arabidopsis thaliana]	51.2	2e-05
gi|25295744|pir||E84845	probable villin 2 [imported] - Arabidops	51.2	2e-05
gi|18405794|ref|NP_565958.1|	VLN2 (VILLIN 2); actin binding [...	51.2	2e-05
gi|18256316|gb|AAH21808.1|	Villin-like [Mus musculus] >gi|239...	50.8	3e-05
gi|18490748|gb|AAH22664.1|	Vill protein [Mus musculus]	50.8	3e-05
gi|15487264|emb|CAC69079.1|	villin-like protein [Mus musculus...	50.8	3e-05
gi|26390015|dbj|BAC25828.1|	unnamed protein product [Mus musculu	50.8	3e-05
gi|50942753|ref|XP_480904.1|	putative villin [Oryza sativa (j...	50.4	4e-05

Figure 1.5: BLASTX Results of *Chlamydomonas* hcVillin EST Sequence

Figure 1.5 displays the results of a BLASTX search comparing the nucleotide sequence of *Chlamydomonas* gene 16438573, the highest scoring EST gene containing the hcVillin sequence. This search was performed to determine if the *Chlamydomonas* hcvillin EST gene resembled other villin proteins across broad taxa. The colored arrows correspond with the BLAST scores of the protein matches listed below. These results indicate similarity of the *Chlamydomonas* hcvillin EST gene to other villin proteins in other organisms.

Fimbrin BLAST Results

The second candidate protein containing a conserved domain which produced a significant match in the *Chlamydomonas* genome was the actin-binding protein, fimbrin. The fimbrin conserved domain sequence, containing 642 amino acids from the cd00014 family, was searched against the *Chlamydomonas* genome for sequence similarity. Figure 2.1 illustrates the alignment results for the TBLASTN search of the fimbrin conserved domain sequence.

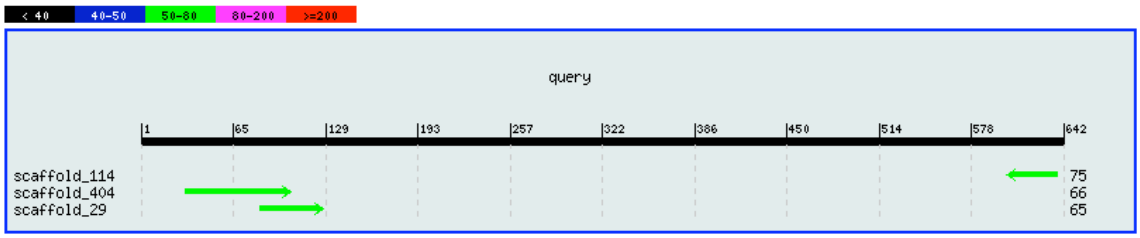


Figure 2.1 *Chlamydomonas* TBLASTN Results of Fimbrin Conserved Domain

Figure 2.1 illustrates the best hit results generated through a TBLASTN search of the fimbrin conserved domain against the *Chlamydomonas* genome. Because these hits have a BLAST score between 50-80, it is colored green. Colored arrows represent amino acids in *Chlamydomonas* that align with the conserved domain query sequence.

The selected conserved domain query sequence for fimbrin is represented by the black line. The multiple hits, as produced by selecting an expect value of 10, are ranked based on their BLAST score and represented by colored arrows. The best hit has a score of 75 and is represented as a short arrow near the end of the query sequence in scaffold 114 of the *Chlamydomonas* genome.

Figure 2.2 illustrates the pairwise alignment of both the query conserved domain sequence and the matching *Chlamydomonas* scaffold 114 sequence.

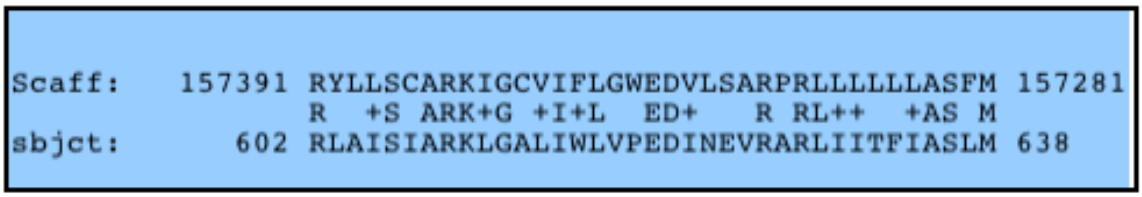


Figure 2.2: Pairwise Alignment of Fimbrin Conserved Domain Sequence and *Chlamydomonas* Scaffold 114 Sequence

Figure 2.2 aligns the fimbrin conserved domain query sequence, represented as the subject sequence, with the scaffold region in *Chlamydomonas* producing a pairwise alignment. Scaffold numbers represent the location within the genome where the amino acid match occurs. The subject numbers indicate that amino acids 602-638 of the conserved domain query sequence align with the indicated *Chlamydomonas* scaffold.

The subject sequence illustrated in figure 2.2 represents the fimbrin conserved domain amino acid sequence while the scaffold sequence represents the *Chlamydomonas* sequence match in scaffold 114. The sequence match occurs between amino acids 602-638 of the total 642 amino acid query sequence. There is a 43% homology between the two sequences based on the 16 positive amino acid identities out of a total sequence length of 37 amino acids.

The nucleotide sequence of this region on scaffold 114 where the fimbrin conserved domain sequence match occurs was obtained from the *Chlamydomonas* genome browser. This nucleotide sequence is illustrated in Figure 2.3.

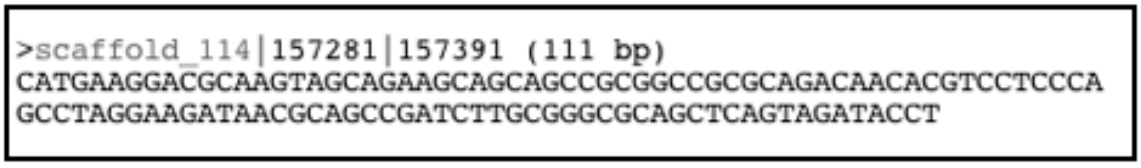
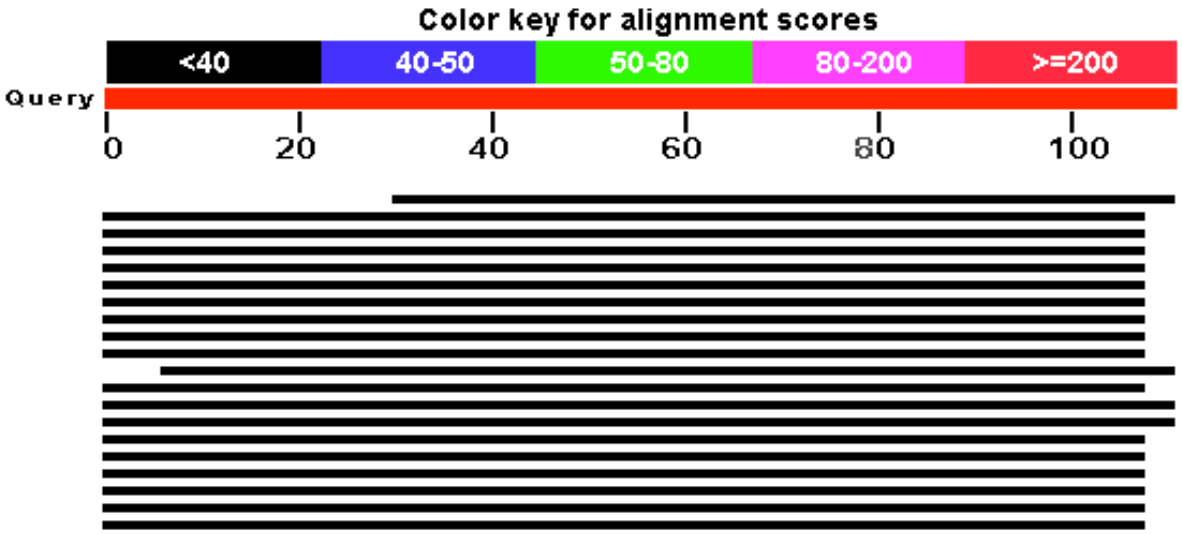


Figure 2.3: Scaffold 114 Nucleotide Sequence

Figure 2.3 displays the nucleotide sequence of bases 157281-157391 of *Chlamydomonas* scaffold 114, the region producing a pairwise alignment with the fimbrin conserved domain sequence as illustrated in figure 2.2.

The nucleotide sequence displayed in figure 2.3 is 111 base pairs long and is located from base position 157281-157391 within scaffold 114 of the *Chlamydomonas* genome. This nucleotide sequence from scaffold 114 was used in a BLASTX search to determine if the *Chlamydomonas* fimbrin sequence match resembles other fimbrin proteins across broad taxa. Figure 2.4 graphically displays the BLASTX results for the scaffold 114 nucleotide sequence.



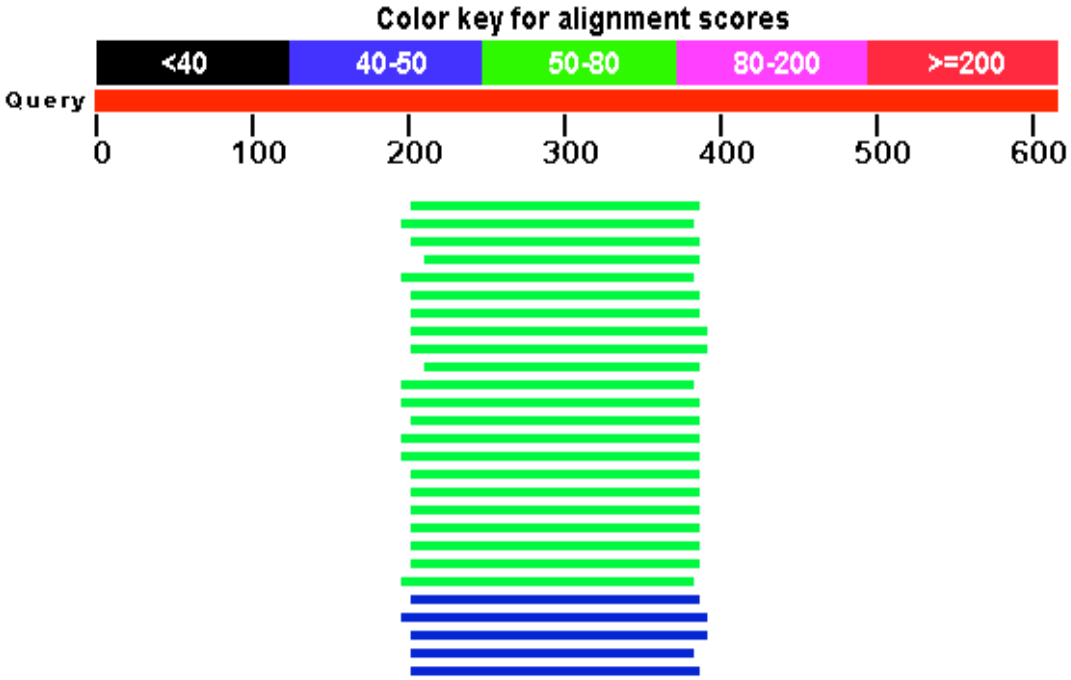
gi|66816913|ref|XP_642432.1|	hypothetical protein DDB0205524 ...	38.1
gi|15224051|ref|NP_178552.1|	unknown protein [Arabidopsis tha...	35.0
gi|56201784|dbj|BAD73234.1|	putative plastin 3 [Oryza sativa (ja	34.3
gi|50909921|ref|XP_466449.1|	putative fimbrin 1 [Oryza sativa...	34.3
gi|34914666|ref|NP_918680.1|	fimbrin-like protein (actin bind...	34.3
gi|62319675|dbj|BAD95202.1|	fimbrin [Arabidopsis thaliana]	33.1
gi|51971889|dbj|BAD44609.1|	fimbrin 2 [Arabidopsis thaliana]	33.1
gi|2737926|gb|AAC49919.1|	fimbrin-like protein AtFim2 [Arabidops	33.1
gi|1575595|gb|AAC49813.1|	fimbrin/plastin-like [Triticum aestivu	33.1
gi|15238983|ref|NP_199657.1|	unknown protein [Arabidopsis tha...	33.1
gi|6682933|dbj|BAA88953.1|	fimbrin [Tetrahymena thermophila]	33.1
gi|15238586|ref|NP_198420.1|	unknown protein [Arabidopsis tha...	33.1
gi|4539245|emb|CAB39801.1|	SPBC1778.06c [Schizosaccharomyces ...	33.1
gi|50513408|pdb|1RT8|A	Chain A, Crystal Structure Of The Acti...	33.1
gi|50912181|ref|XP_467498.1|	putative fimbrin [Oryza sativa (...	32.7
gi|15240502|ref|NP_200351.1|	calcium ion binding [Arabidopsis...	32.0
gi|15236837|ref|NP_194400.1|	ATFIM1; actin binding / calcium ...	32.0
gi|2905893|gb|AAC39359.1|	fimbrin-like protein [Arabidopsis thal	32.0
gi|2811224|gb|AAB97843.1|	fimbrin 1 [Arabidopsis thaliana]	32.0
gi|50513334|pdb|1PXY|B	Chain B, Crystal Structure Of The Acti...	32.0

Figure 2.4: BLASTX Results of Scaffold 114-Fimbrin Match

Figure 2.4 displays the results of a BLASTX search comparing the nucleotide sequence of scaffold 114, which produced the fimbrin match, to all organisms in the NCBI database. This search was performed to determine if the *Chlamydomonas* villin sequence match resembled other fimbrin proteins across broad taxa. The colored arrows correspond with the BLAST scores of the protein matches listed below. These results indicate similarity of the *Chlamydomonas* hypothetical fimbrin protein to other fimbrin proteins in other organisms.

Figure 2.4 displays the protein matches from other organisms based on the BLAST score listed to the right of the entry. The alignment score color key located in the top of figure 2.4 displays colored lines based on the BLAST score of each entry listed below. The entry with the highest BLAST score of 34.3 was a putative fimbrin protein in *Oryza sativa*. Subsequent matches also listed similarity to fimbrin proteins across broad taxa, indicating a resemblance of the hypothetical *Chlamydomonas* fimbrin (hcFimbrin) to other fimbrin proteins.

To determine if this hcFimbrin was present in the gametic EST library, a TBLASTN search was performed. This search was limited to the EST database of *Chlamydomonas* using the scaffold 114 nucleotide sequence representing hcFimbrin pictured in figure 2.3. The *Chlamydomonas* EST TBLASTX results are illustrated in figure 2.5. The top listed sequence was genbank index number 15369428, which had the highest BLAST score of 47.8. The nucleotide sequence of this *Chlamydomonas* EST gene was selected and a BLASTX was performed using this sequence to determine sequence similarity across broad taxa. Results of the BLASTX search are illustrated in figure 2.6. The sequences listed in figure 2.6 demonstrate a similarity between the *Chlamydomonas* hcFimbrin EST gene and other fimbrin-like proteins across broad taxa.



Sequences producing significant alignments:		Score (Bits)
gi|50912181|ref|XP_467498.1|	putative fimbrin [Oryza sativa (...	59.7
gi|39585979|emb|CAE68269.1|	Hypothetical protein CBG13946 [Caeno	56.6
gi|1575595|gb|AAC49813.1|	fimbrin/plastin-like [Triticum aestivu	55.8
gi|15224051|ref|NP_178552.1|	unknown protein [Arabidopsis tha...	54.7
gi|47271164|gb|AAK68402.2|	Hypothetical protein Y104H12BR.1 [...	54.3
gi|34914666|ref|NP_918680.1|	fimbrin-like protein (actin bind...	53.9
gi|56201784|dbj|BAD73234.1|	putative plastin 3 [Oryza sativa (ja	53.9
gi|6682933|dbj|BAA88953.1|	fimbrin [Tetrahymena thermophila]	53.9
gi|89301499|gb|EAR99487.1|	fimbrin-like 71 K protein [Tetrahymen	53.9
gi|15238586|ref|NP_198420.1|	unknown protein [Arabidopsis tha...	53.5
gi|13775565|gb|AAK39373.1|	Hypothetical protein Y73B3B.1 [Cae...	53.1
gi|51971889|dbj|BAD44609.1|	fimbrin 2 [Arabidopsis thaliana]	53.1
gi|50909921|ref|XP_466449.1|	putative fimbrin 1 [Oryza sativa...	53.1
gi|2737926|gb|AAC49919.1|	fimbrin-like protein AtFim2 [Arabidops	53.1
gi|15238983|ref|NP_199657.1|	unknown protein [Arabidopsis tha...	53.1
gi|15240502|ref|NP_200351.1|	calcium ion binding [Arabidopsis...	52.0
gi|15236837|ref|NP_194400.1|	ATFIM1; actin binding / calcium ...	51.6
gi|2905893|gb|AAC39359.1|	fimbrin-like protein [Arabidopsis thal	51.6
gi|2811230|gb|AAB97846.1|	fimbrin 1 [Arabidopsis thaliana]	51.6
gi|2811224|gb|AAB97843.1|	fimbrin 1 [Arabidopsis thaliana]	51.6

Figure 2.5: BLASTX Results of *Chlamydomonas* hcFimbrin EST Sequence

Figure 2.5 displays the results of a BLASTX search comparing the nucleotide sequence of *Chlamydomonas* gene 15369428, the highest scoring EST gene containing the hcFimbrin sequence. This search was performed to determine if the *Chlamydomonas* hcvillin EST sequence resembled other fimbrin proteins across broad taxa. The colored arrows correspond with the BLAST scores of the protein matches listed below. These results indicate a similarity between the *Chlamydomonas* hcFimbrin EST sequence and other villin-like proteins in other organisms.

Myosin-I BLAST Results

The third candidate protein containing a conserved domain which produced a significant match in the *Chlamydomonas* genome was the brush border Myosin-I protein. The Myosin-I conserved domain sequence containing 697 amino acids from the cd0138 family was searched against the *Chlamydomonas* genomic database for sequence similarity. Figure 3.1 illustrates the alignment results for the TBLASTN search of the Myosin-I conserved domain sequence.

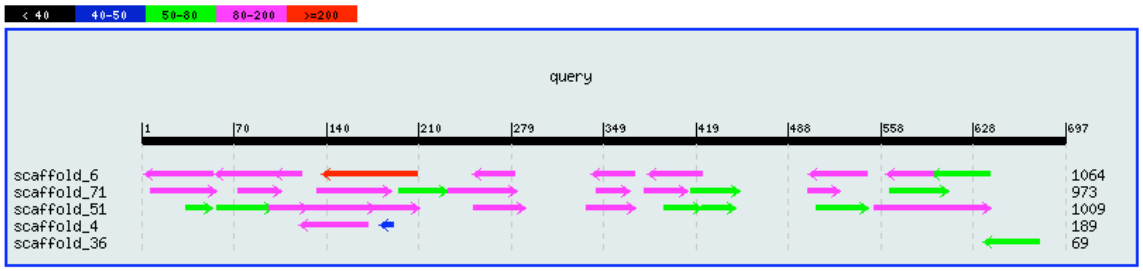


Figure 3.1: *Chlamydomonas* TBLASTN Results of Myosin-I Conserved Domain

Figure 3.1 illustrates the best hit results generated through a TBLASTN search of the Myosin-I conserved domain against the *Chlamydomonas* genome. Because these hits have different BLAST scores, the different colored arrows reflect those different scores. The arrows represent amino acids in *Chlamydomonas* that align with the Myosin-I conserved domain query sequence.

In figure 3.1, the Myosin-I conserved domain query sequence is represented by the black line. Multiple hits were produced at various regions of the sequence among five *Chlamydomonas* scaffolds by selecting an expect value of 10. The region in scaffold 6 represented with a red arrow produced the best hit with a BLAST score of 231, the highest rank of similarity.

Figure 3.2 illustrates the pairwise alignment of both the Myosin-I query conserved domain sequence and the matching *Chlamydomonas* scaffold 6 sequence.

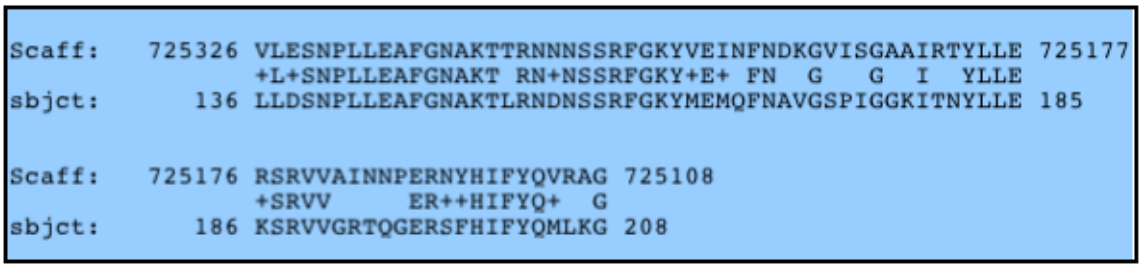


Figure 3.2: Pairwise Alignment of Myosin-I Conserved Domain Sequence and *Chlamydomonas* Scaffold 6 Sequence

Figure 3.2 aligns the Myosin-I conserved domain query sequence, represented as the subject sequence, with the scaffold region in *Chlamydomonas* producing a pairwise alignment. Scaffold numbers represent the location within the genome where the amino acid match occurs. The subject numbers indicate that amino acids 136-208 of the conserved domain query sequence align with the indicated *Chlamydomonas* scaffold.

The subject sequence illustrated in figure 3.2 represents the Myosin-I conserved domain amino acid sequence while the scaffold sequence represents the *Chlamydomonas* sequence match in scaffold 6. The sequence match occurs between amino acids 136-208 of the total 697 amino acid query sequence. There is a 63% homology between the two sequences based on the 46 positive amino acid identities out of a total sequence length of 73 amino acids.

The nucleotide sequence of this region on scaffold 6 where the Myosin-I conserved domain sequence match occurs was obtained from the *Chlamydomonas* website. This sequence is illustrated in Figure 3.3.

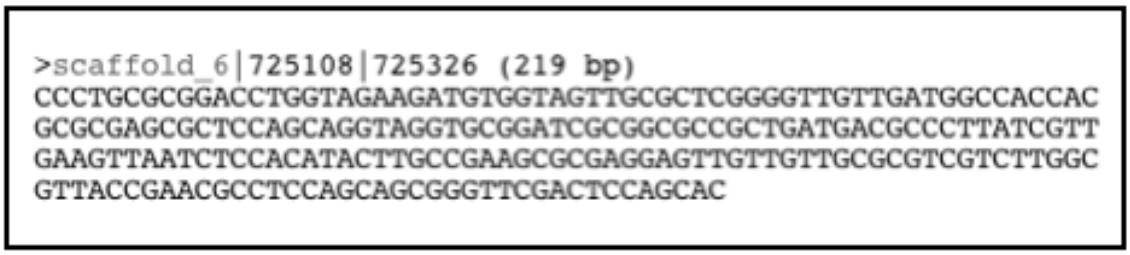
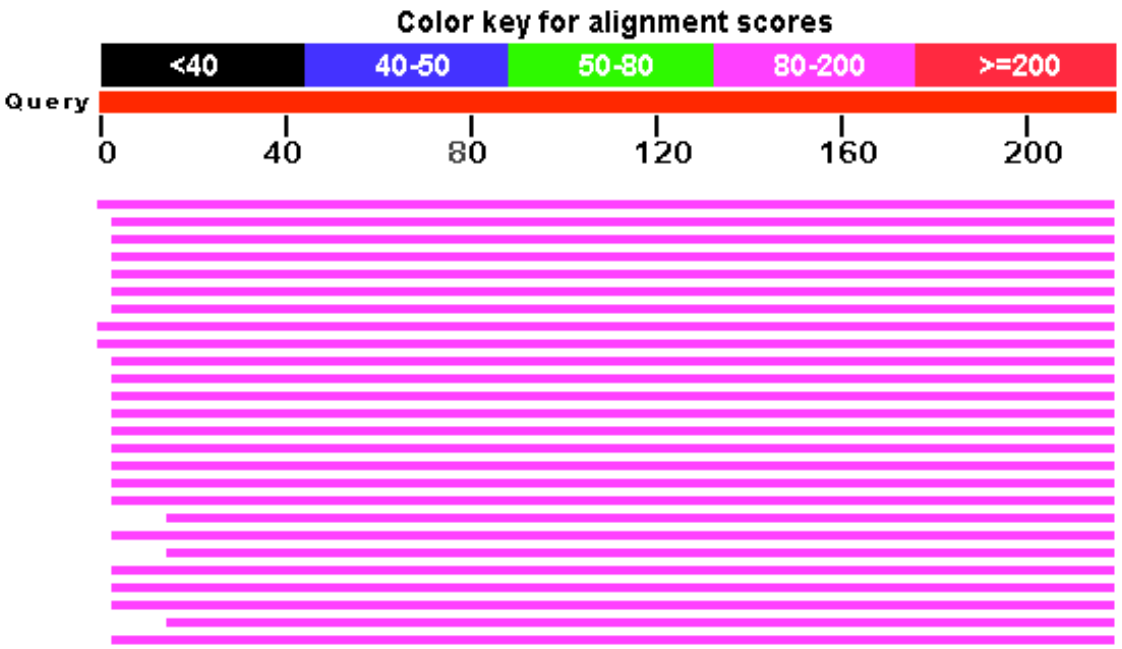


Figure 3.3: Scaffold 6 Nucleotide Sequence

Figure 3.3 displays the nucleotide sequence of bases 725108-725326 of *Chlamydomonas* scaffold 6, the region producing a pairwise alignment with the Myosin-I conserved domain sequence as illustrated in figure 3.2.

The nucleotide sequence displayed in figure 3.3 is 219 base pairs long and is located from base position 725108-725326 within scaffold 6 of the *Chlamydomonas* genome. This nucleotide sequence from scaffold 129 was used in a BLASTX search to determine if the *Chlamydomonas* Myosin-I sequence match resembles other Myosin proteins across broad taxa.

Figure 3.4 illustrates the BLASTX results for the scaffold 6 nucleotide sequence. The protein matches from other organisms are ranked by their BLAST score displayed at the right of each entry. At the top of the figure, the alignment score color key displays colored lines correlating to the BLAST score of each entry listed below. The entry with the highest BLAST score of 142 was the myosin heavy chain, a major component of all myosin proteins. Subsequent matches also listed similarity to other myosin proteins across broad taxa, indicating a resemblance of the hypothetical *Chlamydomonas* Myosin-I (hcMyosin-I) to other myosin proteins.



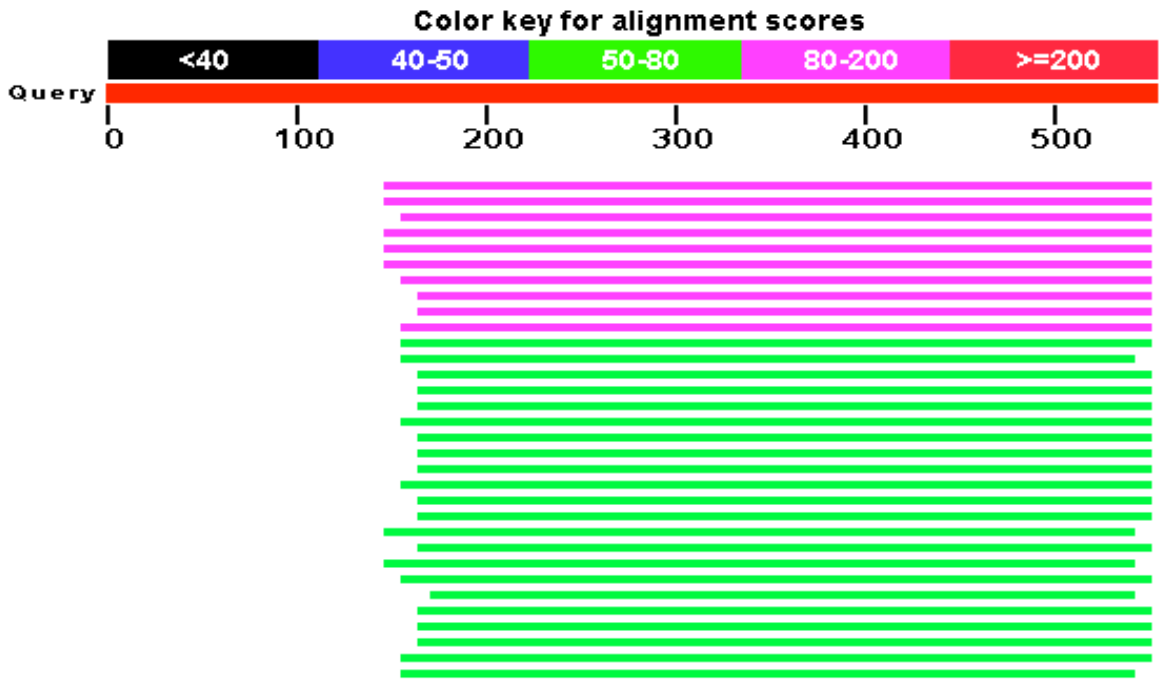
Sequences producing significant alignments: Score (Bits)

gi|3342148|gb|AAC27525.1|	myosin heavy chain [Chlamydomonas rein	142
gi|56603657|dbj|BAD80749.1|	myosin class 11-2 [Adiantum capillus	119
gi|50913341|ref|XP_468078.1|	putative myosin heavy chain [Ory...	117
gi|499047|emb|CAA84066.1|	myosin [Arabidopsis thaliana]	116
gi|7270268|emb|CAB80037.1|	myosin-like protein [Arabidopsis t...	116
gi|50920299|ref|XP_470510.1|	putative myosin heavy chain [Ory...	116
gi|15240028|ref|NP_199203.1|	MYA2 [Arabidopsis thaliana] >gi|...	116
gi|9453839|dbj|BAB03273.1|	myosin [Chara corallina]	116
gi|6472600|dbj|BAA87057.1|	unconventional myosin heavy chain [Ch	116
gi|42567348|ref|NP_195046.3|	XI-I; motor/ protein binding [Arabi	116
gi|29170491|dbj|BAC66162.1|	myosin XI [Nicotiana tabacum]	116
gi|37533058|ref|NP_920831.1|	putative myosin heavy chain [Ory...	115
gi|56603655|dbj|BAD80748.1|	myosin class 11-1 [Adiantum capillus	115
gi|4960051|gb|AAD34597.1|	myosin XI [Zea mays]	115
gi|78708250|gb|ABB47225.1|	Myosin head, putative [Oryza sativa (	115
gi|13384201|gb|AAK21311.1|	myosin subfamily XI heavy chain [Petr	115
gi|7243765|gb|AAF43440.1|	unconventional myosin XI [Vallisneria	115
gi|2444178|gb|AAB71528.1|	unconventional myosin [Helianthus annu	115
gi|34910550|ref|NP_916622.1|	putative myosin heavy chain [Ory...	115
gi|20196856|gb|AAM14807.1|	putative myosin heavy chain [Arabidop	115
gi|56201391|dbj|BAD72949.1|	myosin XI [Nicotiana tabacum]	115
gi|42569586|ref|NP_180882.2|	XID; motor/ protein binding [Arabid	115
gi|7269726|emb|CAB81459.1|	myosin heavy chain-like protein (f...	114
gi|4218127|emb|CAA22981.1|	myosin heavy chain-like protein (f...	114

Figure 3.4: BLASTX Results of Scaffold 6-Myosin-I Match

Figure 3.4 displays the results of a BLASTX search comparing the nucleotide sequence of scaffold 6, which produced the Myosin-I match, to all organisms in the NCBI database. This search was performed to determine if the *Chlamydomonas* Myosin-I sequence match resembled other myosin proteins across broad taxa. The colored arrows correspond with the BLAST scores of the protein

The next phase of database analysis involved performing a TBLASTX search limited to the EST database of *Chlamydomonas* to determine if hcMyosin-I was present in the gametogenesis EST library. The nucleotide sequence of scaffold 6 (Figure 3.3) was used in this search. The *Chlamydomonas* EST TBLASTX results are illustrated in figure 3.5. The top listed sequence was Genbank index number 6546280, which had the highest BLAST score of 23.5. The nucleotide sequence of this *Chlamydomonas* EST gene was selected and a BLASTX was performed using this sequence to determine sequence similarity across broad taxa. Results of the BLASTX search are illustrated in figure 3.5. Although, these results do not indicate a similarity between the *Chlamydomonas* hcMyosin-I EST sequence and other myosin-like proteins in other organisms, they do confirm the active transcription of hcMyosin-I mRNA.



Sequences producing significant alignments:		Score (Bits)
gi|56412219|gb|AAV88604.1|	voltage dependent anion channel pr...	91.3
gi|29367429|gb|AAO72587.1|	porin-like protein [Oryza sativa (jap	90.1
gi|50899632|ref|XP_450604.1|	voltage-dependent anion channel ...	89.7
gi|60279272|emb|CAA63968.1|	pom30 [Solanum tuberosum]	88.2
gi|77999247|gb|ABB16970.1|	POM30-like protein [Solanum tuberosum	88.2
gi|36957216|gb|AAQ87022.1|	VDAC2.1 [Lotus corniculatus var. japo	83.2
gi|5929928|gb|AAD56651.1|	voltage-dependent anion channel protei	81.6
gi|44894747|gb|AAS48868.1|	voltage-dependent anion-selective ...	81.3
gi|42601787|gb|AAS21632.1|	voltage-dependent anion-selective ...	81.3
gi|5031279|gb|AAD38145.1|	porin [Prunus armeniaca]	81.3
gi|5929930|gb|AAD56652.1|	voltage-dependent anion channel protei	79.7
gi|1172553|sp|P46274|VDAC1_WHEAT	Outer mitochondrial membrane...	79.7
gi|36957170|gb|AAQ87019.1|	VDAC1.1 [Lotus corniculatus var. japo	79.3
gi|515360|emb|CAA56600.1|	36kDA porin II [Solanum tuberosum] ...	79.0
gi|515358|emb|CAA56601.1|	36kDa porin I [Solanum tuberosum] >...	79.0
gi|83283993|gb|ABC01904.1|	34 kDa outer mitochondrial membran...	78.6
gi|83032228|gb|ABB97031.1|	unknown [Brassica rapa]	78.6
gi|56693621|gb|AAW22621.1|	outer mitochondrial membrane protein	77.8

Figure 3.5: BLASTX Results of *Chlamydomonas* hcMyosin-I EST Sequence

Figure 3.5 displays the results of a BLASTX search comparing the nucleotide sequence of *Chlamydomonas* gene 15701117, the highest scoring EST gene containing the hcMyosin-I sequence. This search was performed to determine if the *Chlamydomonas* hcMyosin-I EST sequence resembled other myosin proteins across broad taxa. The colored arrows correspond with the BLAST scores of the protein matches listed below. Although, these results do not indicate a similarity between the *Chlamydomonas* hcMyosin-I EST sequence and other myosin-like proteins in other organisms, they do confirm the active transcription of hcMyosin-I mRNA.

Commercial Antibody Sequence Alignments in *Chlamydomonas*

Commercial antibodies were purchased against human villin and human Myosin-I proteins. No commercial antibodies were available for fimbrin. A TBLASTN search using the targeted amino acid sequences of villin and Myosin-I antibodies was performed.

For this study, the selected anti-villin antibody was produced using the human villin protein sequence. The antigenic region of human villin consists of 28 amino acids. The pairwise alignment between the antibody target sequence and the matching scaffold region in *Chlamydomonas* is shown in figure 4.

```
Scaff:    63903 EVFGMDRDAFKKQPAWRQAQAKKKANLF 63986
                + FGM   AF   P W+Q   KK+  LF
sbjct:        1 QAFGMTPAAFSALPRWKQQNLKKEKGLF 28
```

Figure 4.1: Anti-Villin Pairwise Alignment in *Chlamydomonas*

Figure 4.1: Pairwise alignment from a TBLASTN search against the *Chlamydomonas* genome using the 28 antigenic amino acids from the commercially obtained anti-human villin antibody. The antigenic region of human villin is represented as the 28 amino acid subject sequence, while the region in *Chlamydomonas* producing a pairwise alignment is listed as the scaffold sequence. Scaffold numbers represent the location within the genome where the amino acid match occurs.

The pairwise alignment illustrated in figure 4.1 indicates 12 positive amino acid matches out of 28, thus producing a 42% homology between the anti-villin sequence and *Chlamydomonas*. The highly conserved KKEK actin-binding region represents amino acids 22-25. *Chlamydomonas* produces a 50% homology with this region by positively matching amino acids 22 and 23. The anti-villin antibody sequence aligns with the same *Chlamydomonas* scaffold region as the villin headpiece conserved domain (figure 1.1).

The selected anti-Myosin-I antibody was produced using the human Myosin-I protein sequence. The antigenic region contains 98 amino acids spanning amino acids 530-628. The pairwise alignment between the antibody target sequence and the matching scaffold region in *Chlamydomonas* is shown in figure 4.2.

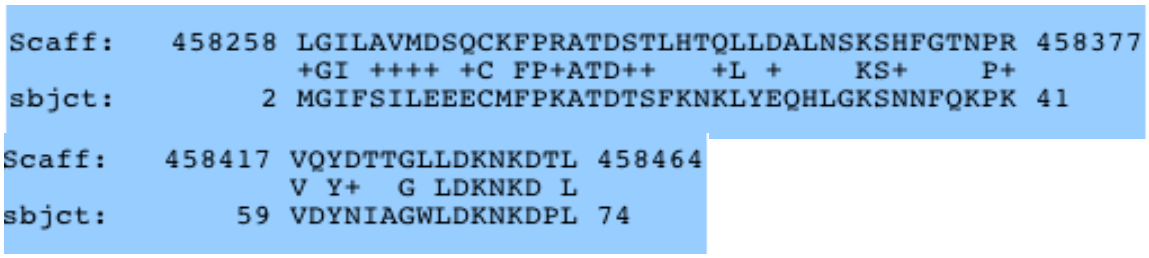


Figure 4.2: Anti-Myosin-I Pairwise Alignment in *Chlamydomonas*

Figure 4.2: Pairwise alignment from a TBLASTN search against the *Chlamydomonas* genome using the 98 antigenic amino acids from the commercially obtained anti-human Myosin-I antibody. The antigenic region of human Myosin-I is represented as the 98 amino acid subject sequence, while the region in *Chlamydomonas* producing a pairwise alignment is listed as the scaffold sequence. Scaffold numbers represent the location within the genome where the amino acid match occurs.

The pairwise alignment illustrated in figure 4.2 indicates an amino acid match occurs within two neighboring scaffold regions in *Chlamydomonas*. The alignment contains 22 positive amino acid matches out of 98, thus producing a 22% homology between the anti-Myosin-I sequence and *Chlamydomonas*. Although this alignment does not occur in the scaffold 6 region containing the Myosin-I conserved domain match (figure 3.2), it does indicate recognition of the antigenic antibody sequence with regions in *Chlamydomonas*.

Identification of hcVillin, hcFimbrin, and hcMyosin-I Genes in *Chlamydomonas*

To verify gene expression of hcVillin, hcFimbrin, and hcMyosin-I in *Chlamydomonas*, RT-PCR was performed using mRNA templates from the following

Chlamydomonas cell types: *mt*+ vegetative cells, *mt*– vegetative cells, *mt*+ gametes, and *mt*– gametes.

Using genomic DNA, a region spanning 200-300 bases at the beginning of the exon was selected for a PCR screen. Upstream and downstream primers were selected within this region and their amplification ability tested by performing a PCR reaction using purified plasmid DNA from the BACs as a template. PCR products from all five BACs aligned with the appropriate size KB marker, confirmed successful amplification using the selected primers (data not shown).

To determine the expression of hcVillin, hcFimbrin, and hcMyosin-I in *Chlamydomonas* cell types, a PCR reaction was performed using cDNA templates constructed by reverse transcribing mRNA from *mt*+ and *mt*– vegetative and gametic cells. Refer to Table 2 for the primer sequences used. Figure 5.1 graphically illustrates the PCR products of all three genes. Primer regions are labeled green with arrows indicating replication direction. Regions in red indicate exons containing cDNA while black regions represent introns.

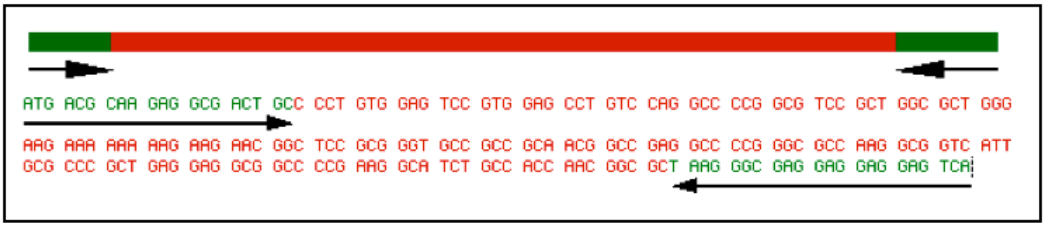


Figure 5.1: hcVillin PCR Product

Figure 5.1 illustrates the PCR product for hcVillin. Green regions indicate primer sequences and their replication directions, while the red codons represent hcVillin cDNA. The total PCR product size of hcVillin is 0.213 KB.

Figure 5.1 contains a 20 nucleotide upstream primer and a 22 nucleotide downstream primer within the hcVillin genomic sequence. Both primers, labeled in green with an arrow indicating replication direction, are located within a single exon and the entire PCR product is 213 base pairs in length.

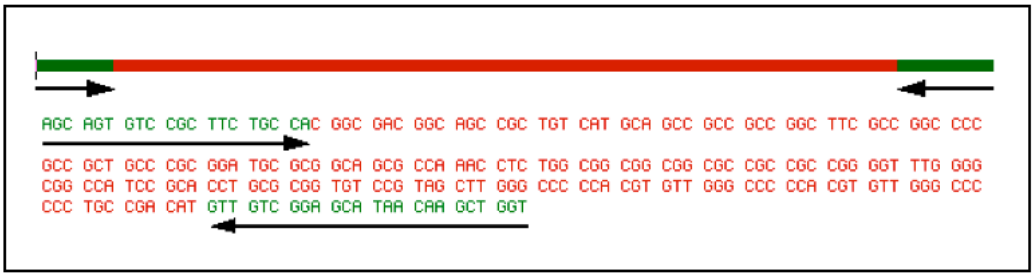


Figure 5.2: hcFimbrin PCR Product

Figure 5.2 illustrates the PCR product for hcFimbrin. Green regions indicate primer sequences and their replication directions, while the red codons represent hcFimbrin cDNA. The total PCR product size of hcFimbrin is 0.240 KB.

Figure 5.2 illustrates the PCR product for hcFimbrin. The primers, labeled in green, consist of a 20 nucleotide upstream primer and a 24 nucleotide downstream primer within the hcVillin genomic sequence. Both primers are located within a single exon and the entire PCR product is 240 base pairs in length or 0.240 KB.

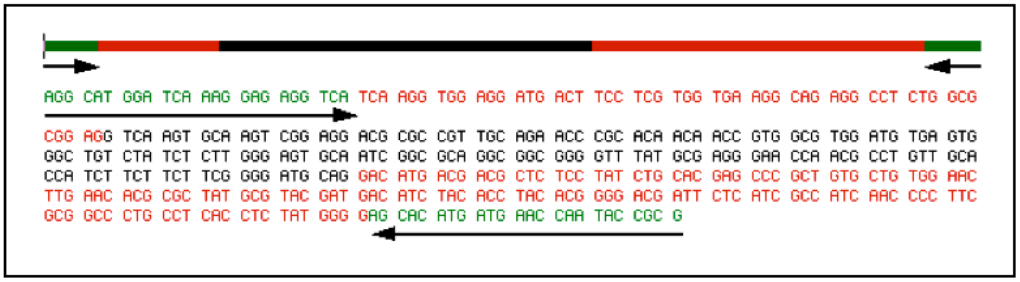


Figure 5.3: hcMyosin-I PCR Product

Figure 5.3 illustrates the PCR product for hcMyosin-I. Green regions indicate primer sequences and their replication directions. Red codons indicate exons containing cDNA while black regions represent introns within the genomic sequence of hcMyosin-I. The total PCR product size of hcMyosin-I is 0.244 KB.

Figure 5.3 displays the PCR product for hcMyosin-I. This product contains a 24 nucleotide upstream primer and a 24 nucleotide downstream primer within the hcMyosin-I genomic sequence. Both primers, labeled in green with an arrow indicating replication direction, are located within two exons. An intron, labeled in black, is located between these two coding regions. This entire PCR product is 244 base pairs in length.

The PCR products illustrated in figures 5.1, 5.2, and 5.3 were separated by size during gel electrophoresis and imaged under ultraviolet light using a Kodak Imager. Figure 6.1 illustrates DNA bands present in *Chlamydomonas* cell types using hcVillin specific primers.

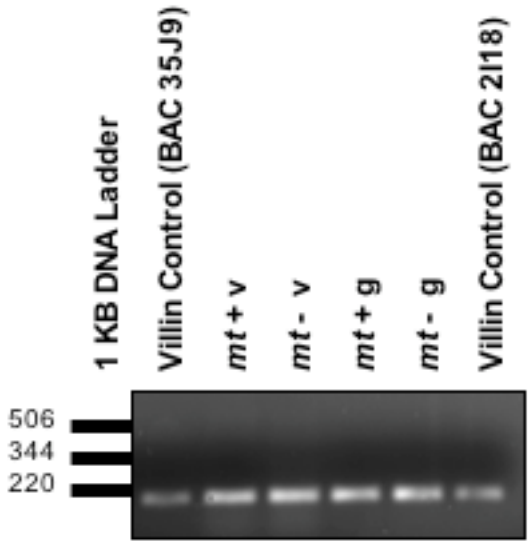


Figure 6.1: RT-PCR for hcVillin mRNA

Figure 6.1 is a DNA image of RT-PCR for hcVillin mRNA run on a 1.4% agarose gel. Molecular weight markers are labeled. Lanes: (1) Villin control BAC 35J9 (2) *mt*+ vegetative cells (3) *mt*- vegetative cells (4) *mt*+ gametes (5) *mt*- gametes (6) Villin control BAC 2I18. All cell samples contain 10µl cDNA template, 5µl distilled water, and 3µl 6x gel loading buffer.

Figure 6.1 contains the following cells in lanes 1-6: (1) Villin control containing BAC 35J9 cDNA template (2) *mt*+ vegetative cells (3) *mt*- vegetative cells (4) *mt*+ gametes (5) *mt*- gametes (6) Villin control containing BAC 2I18 cDNA template. All cell samples contain 10µl cDNA template, 5µl distilled water, and 3µl 6x gel loading buffer. DNA bands are present in all *Chlamydomonas* cell types and both controls near the predicted product size of 213 bases.

Figure 6.2 illustrates DNA synthesis in *Chlamydomonas* cell types when using hcFimbrin specific primers.

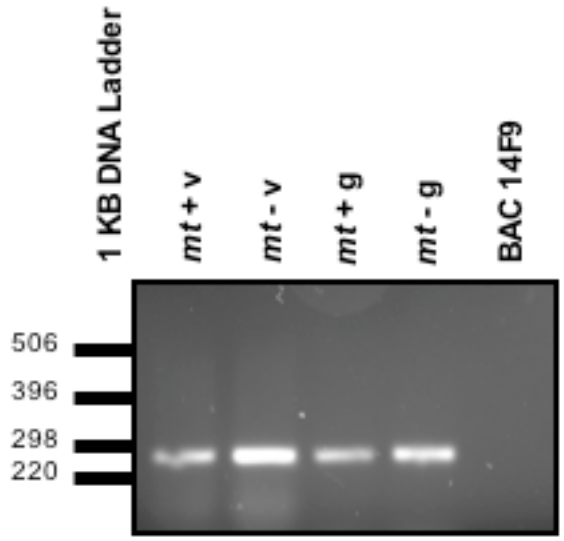


Figure 6.2: RT-PCR for hcFimbrin mRNA

Figure 6.2 is a DNA image of RT-PCR for hcFimbrin mRNA run on a 1.4% agarose gel. Molecular weight markers are labeled. Lanes: (1) *mt*+ vegetative cells (2) *mt*- vegetative cells (3) *mt*+ gametes (4) *mt*- gametes (5) BAC 14F9. All cell samples contain 10µl cDNA template, 5µl distilled water, and 3µl 6x gel loading buffer.

Figure 6.2 contains the following cells in lanes 1-5: (1) *mt*+ vegetative cells (2) *mt*- vegetative cells (3) *mt*+ gametes (4) *mt*- gametes (5) BAC 14F9 cDNA template. All cell samples contain 5µl cDNA template, 5µl distilled water, and 3µl 6x gel loading buffer. DNA bands are present in all four cell types near the predicted product size of 240 bases. DNA banding is not present in lane 5 containing BAC 14F9. Because hcFimbrin was not located within any BACs, the BAC nearest hcFimbrin was selected in hopes that it contained some part of the hcFimbrin gene.

Figure 6.3 illustrates DNA synthesis in *Chlamydomonas* cell types when using hcMyosin-I specific primers.

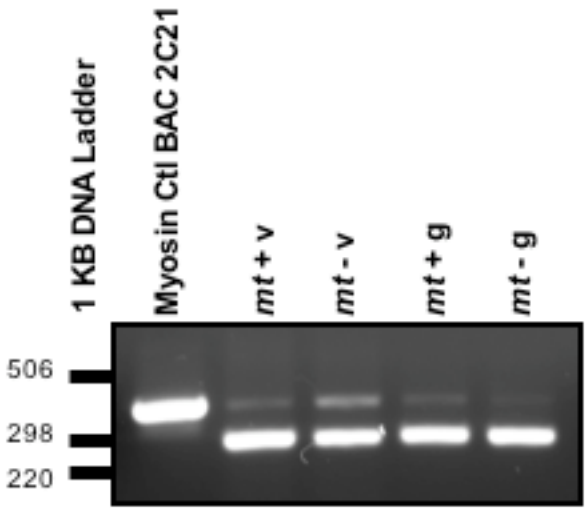


Figure 6.3: RT-PCR for hcMyosin-I mRNA

Figure 6.3 is a DNA image of RT-PCR for hcMyosin-I mRNA run on a 1.4% agarose gel. Molecular weight markers are labeled. Lanes: (1) Myosin Control BAC 2C21 (2) *mt*+ vegetative cells (3) *mt*- vegetative cells (4) *mt*+ gametes (5) *mt*- gametes. All cell samples contain 10µl cDNA template, 5µl distilled water, and 3µl 6x gel loading buffer.

Figure 6.3 contains the following cells in lanes 1-5: (1) myosin control BAC 2C21 cDNA template (2) *mt*+ vegetative cells (3) *mt*- vegetative cells (4) *mt*+ gametes (5) *mt*- gametes. All cell samples contain 10µl cDNA template, 5µl distilled water, and 3µl 6x gel loading buffer. Bright DNA bands present in all four cell types near the predicted product size of 244 bases represent cDNA PCR products. Faint bands of above the cDNA bands represent genomic DNA, as seen with the myosin control BAC.

Immunofluorescent Anti-Villin Labeling

The following *Chlamydomonas* cell types of both wild type and Ida5 actin mutant strains were labeled with primary α-villin and α -Myosin-I polyclonal antibodies: *mt*+ vegetative, *mt*- vegetative, *mt*+ unactivated, *mt*- unactivated, *mt*+ activated, and *mt*- activated. Primary antibody dilutions were 1:100 and secondary antibody dilutions were 1:2000. Similar results were observed in wild type *mt*- and Ida5 *mt*- cells (results not shown).

Immunofluorescent labeling of *mt*+ vegetative, *mt*+ unactivated, and *mt*+ activated cells of *Chlamydomonas* wild type and Ida5 strains with α-villin antibody is shown in figure 7. Figures 7A, 7C, 7E, represent wild type *Chlamydomonas* mating type plus cells while figures 7B, 7D, 7F, and 7H represent the conventional actin mutant, *Chlamydomonas* Ida5 strain.

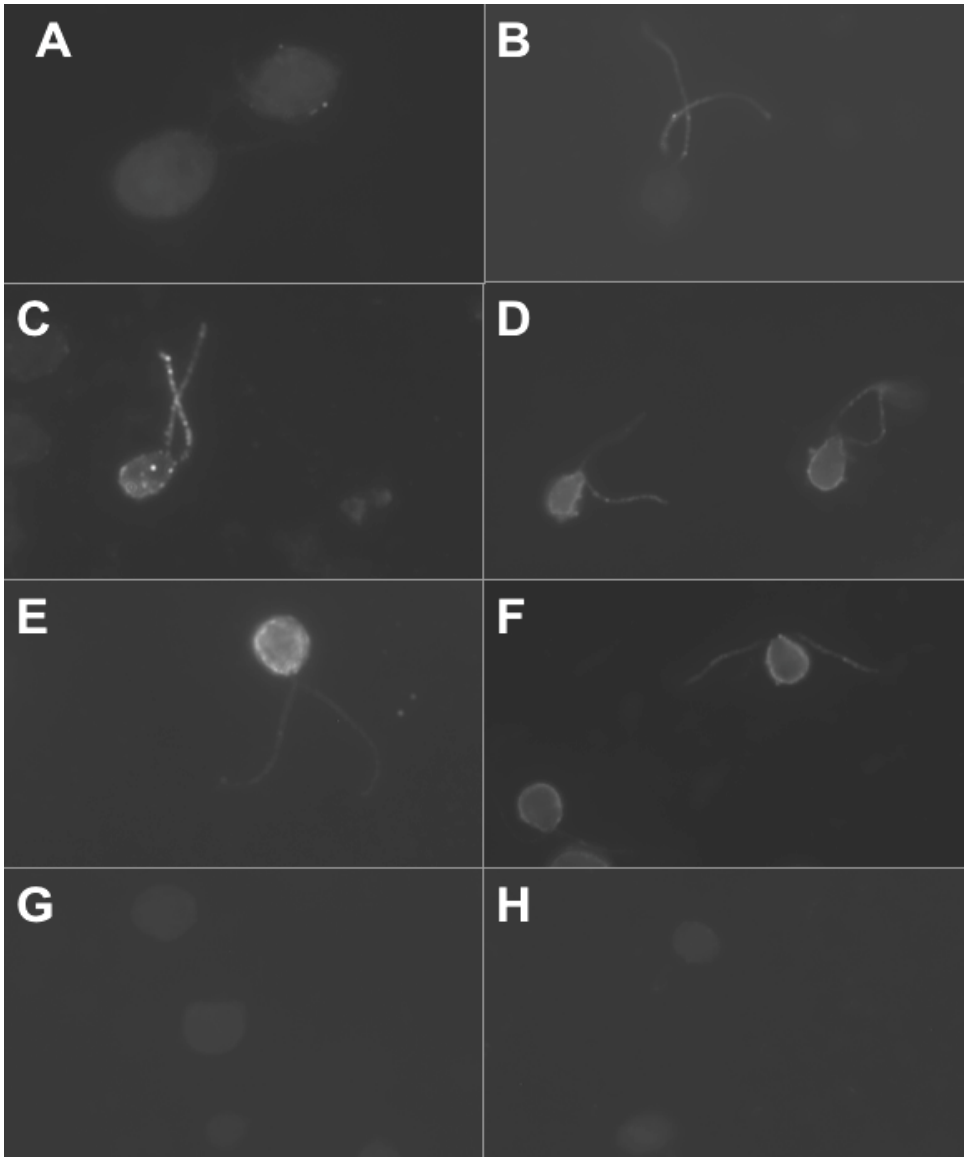


Figure 7: Anti-Villin Immunofluorescent Labeling of *Chlamydomonas* wild type and Ida5 *mt*+ Cells

Figure 7 illustrates the immunofluorescent labeling of *Chlamydomonas* wild type and Ida5 *mt*+ cells using a commercially obtained α-villin antibody. (A) wild type *mt*+ vegetative cells (B) Ida5 actin mutant *mt*+ vegetative cells (C) wild type *mt*+ unactivated cells (D) Ida5 actin mutant *mt*+ unactivated cells (E) wild type *mt*+ activated cells (F) Ida5 actin mutant *mt*+ activated cells (G) wild type *mt*+ activated cells with secondary antibody only (control) (H) wild type *mt*+ activated cells with α-ezrin antibody, a candidate protein that did not demonstrate significant alignments within the *Chlamydomonas* genome. All images were captured at the same exposure.

In the vegetative cells shown in figure 7, α-villin labeling is most apparent on flagella of Ida5 cells (Figure 7B), although patchy labeling is present on the outer cell body of wild type cells (Figure 7A). Labeling of the cell body and flagella is evident in the *mt*+ unactivated cells of both wild type (Figure 7C) and Ida5 (Figure 7D). Flagellar and cell body labeling is also apparent in *mt*+ activated wild type (Figure 7E) and Ida5 (Figure 7F) cells. Compared to wild type unactivated cells, labeling of the cell body is more intense in wild type activated cells. Wild type *mt*+ activated cells labeled with secondary antibody only served as the control to measure secondary antibody background fluorescence (Figure 7G). To compare labeling of a candidate protein that did not demonstrate significant alignments within the *Chlamydomonas* genome, wild type *mt*+ activated cells were labeled with α-ezrin (Figure 7H). Both the control and α-ezrin labeled cells did not exhibit the cell body and flagellar labeling evident in wild type and Ida5 vegetative, unactivated, and activated cells labeled with α-villin.

Immunofluorescent Anti-Myosin-I Labeling

Figure 8 illustrates the immunofluorescent labeling of α-Myosin-I using wild type *mt*+ and Ida5 mutant *mt*+ *Chlamydomonas* cells. Similar results were observed in wild type *mt*- and Ida5 *mt*- cells (results not shown).

Immunofluorescent labeling of *mt*+ vegetative, *mt*+ unactivated, and *mt*+ activated cells of *Chlamydomonas* wild type and Ida5 strains with α-Myosin-I antibody is shown in figure 8. Figures 8A, 8C, 8E, represent wild type *Chlamydomonas* mating type plus cells while figures 8B, 8D, 8F, and 8H represent the conventional actin mutant, *Chlamydomonas* Ida5 strain. Immunofluorescent labeling of hcMyosin-I using our commercially obtained α-Myosin-I antibody produced punctate labeling of the cell body

most evident in wild type *mt*+ unactivated cells (Figure 8C). Wild type *mt*+ activated cells labeled with secondary antibody only served as the control to measure secondary antibody background fluorescence (Figure 8E). To compare labeling of a candidate protein that did not demonstrate significant alignments within the *Chlamydomonas* genome, wild type *mt*+ activated cells were labeled with α-ezrin (Figure 8H). Both the control and α-ezrin labeled cells did not exhibit the punctuate labeling evident in wild type *mt*+ unactivated cells.

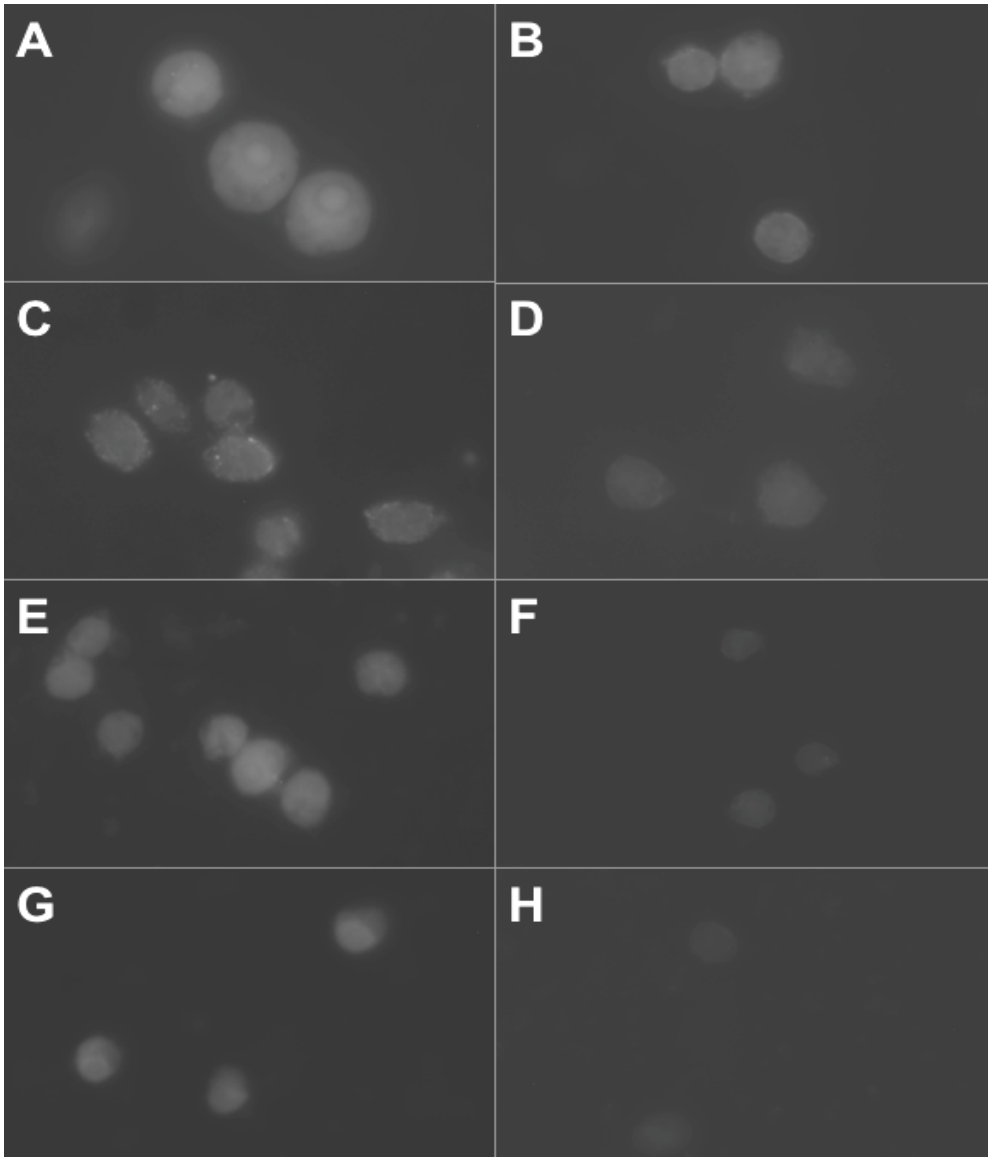


Figure 8: Anti-Myosin-I Immunofluorescent Labeling of *Chlamydomonas* wild type and Ida5 *mt*+ Cells

Figure 8 shows immunofluorescent labeling of *Chlamydomonas* wild type and Ida5 *mt*+ cells using a commercially obtained α-Myosin-I antibody. (A) wild type *mt*+ vegetative cells (B) Ida5 actin mutant *mt*+ vegetative cells (C) wild type *mt*+ unactivated cells (D) Ida5 actin mutant *mt*+ unactivated cells (E) wild type *mt*+ activated cells (F) Ida5 actin mutant *mt*+ activated cells (G) wild type *mt*+ activated cells with secondary antibody only to measure background fluorescence (H) wild type *mt*+ activated cells with α-ezrin antibody, a candidate protein that did not demonstrate significant alignments within the *Chlamydomonas* genome. All images were captured at the same exposure.

hcVillin Immunoblotting Results

Figure 9.1 shows an anti-hcVillin immunoblot. The following cells were present in lanes 1-5: (1) villin positive control (mouse kidney lysate) (2) *mt*+ unactivated cells (3) *mt*+ activated cells (4) isolated *Chlamydomonas* flagella (5) molecular marker. The arrow indicates anti-villin labeling of the positive control at the predicted weight of 92 kDa. There is no identified protein at the predicted molecular weight of hcVillin in any other lanes. There are distinct bands present in *mt*+ activated and *mt*+ unactivated cells at approximately 40 kDa.

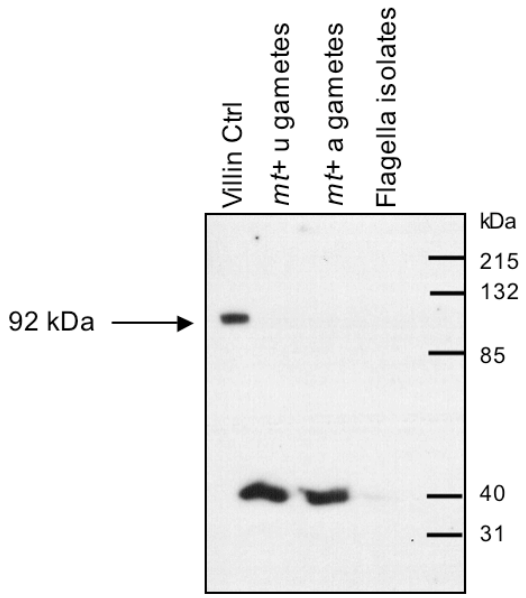


Figure 9.1: *Chlamydomonas* hcVillin Immunoblot

Figure 9.1 illustrates an immunoblot using anti-Myosin-I against the following cells in lanes 1-5: (1) villin positive control (mouse kidney lysate) (2) *Chlamydomonas mt*+ unactivated gametes (3) *Chlamydomonas mt*+ activated gametes (4) isolated *Chlamydomonas* flagella (5) molecular marker. The arrow indicates anti-villin labeling at the expected molecular weight of 92 kDa. No protein of the predicted size is apparent in any other lanes.

hcMyosin-I Immunoblotting Results

Figure 9.2 shows an anti-hcMyosin-I immunoblot. The following cells were present in lanes 1-5: (1) myosin positive control (mouse kidney lysate) (2) *mt+* unactivated gametes (3) *mt+* activated gametes (4) isolated *Chlamydomonas* flagella (5) molecular marker. The arrow indicates anti-Myosin-I labeling of the positive control at the predicted weight of 110 kDa. No protein of the predicted size is present in any of the other lanes.

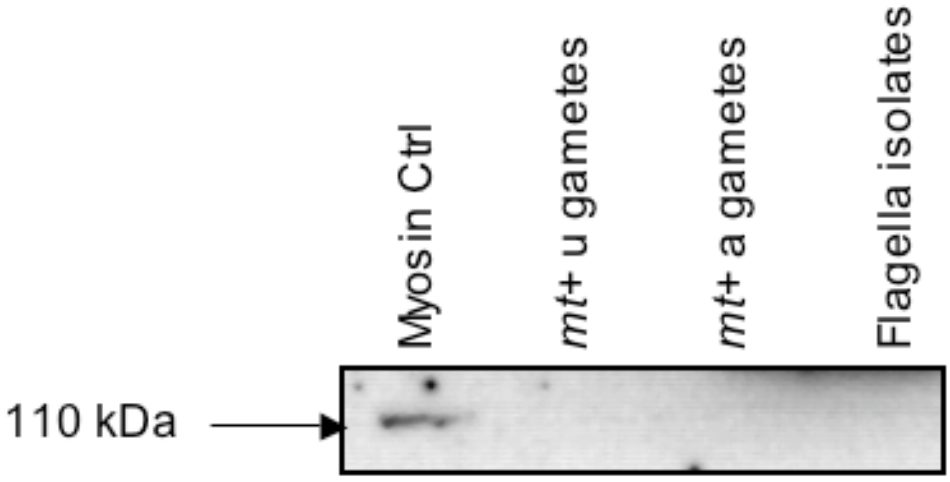


Figure 9.2: *Chlamydomonas* hcMyosin-I Immunoblot

Figure 9.2 illustrates an immunoblot using anti-Myosin-I against the following cells in lanes 1-5: (1) myosin positive control (mouse kidney lysate) (2) *Chlamydomonas* mt+ activated gametes (3) *Chlamydomonas mt+* unactivated gametes (4) isolated *Chlamydomonas* flagella (5) molecular marker. The arrow indicates anti-Myosin-I labeling at the expected molecular weight of 110 kDa. No protein of the predicted size is apparent in any other lanes. Approximately 3.5 x 10^8 cells were loaded into lanes 2-4.

DISCUSSION

The focus of this study was to identify actin-associated proteins in *Chlamydomonas reinhardtii* and to determine their possible role in the fertilization process. Because the fertilization tube of *mt*+ gametes resembles a microvillus, we hypothesized that actin-associated proteins present in microvilli may localize to this structure in *Chlamydomonas*. Through a multi-faceted process of database analysis, we identified three previously unknown hypothetical *Chlamydomonas* genes: hcVillin, hcFimbrin, and hcMyosin-I. Furthermore, RT-PCR verified the expression of mRNA from these three genes both in vegetative and gametic cells of *Chlamydomonas*.

Database Analysis

By performing a BLAST search of the nine actin-associated proteins known to be present in basic microvilli, three of these proteins produced significant matches within the *Chlamydomonas* genome that were previously unknown. The conserved domains of villin, fimbrin, and Myosin-I produced pairwise alignments containing 51%, 43%, and 63% homology respectively with *Chlamydomonas*. Literature review further confirmed the identity of profilin, one of the nine actin-associated proteins, in *Chlamydomonas*. A profilin-like protein that localized to the cell body and flagella of vegetative and gametic cells, as well as the base of the fertilization tube in *mt*+ gametes has been identified (Kovar *et al.,* 2001).

The remaining five candidate proteins did not produce significant matches within the *Chlamydomonas* genome given the search limit of an expect value of 10: ezrin, moeisin, radixin, fascin, and cofilin. However, future analysis may address cofilin using

broader search parameters. The small pairwise alignment of the cofilin conserved domain in *Chlamydomonas* along with the localization of profilin, suggests the presence of an actin-depolymerizing factor such as cofilin in *Chlamydomonas*.

Villin: A Multi-Faceted Actin-Associated Protein

We identified the presence of hcVillin, a villin-like gene, in *Chlamydomonas*. Villin, a 92kDa actin-associated protein, primarily functions as a filamentous actin binding protein but also possesses the dual ability to cap and sever actin filaments under calcium-dependent concentrations (Bretscher, 1986). The villin headpiece domain, located at the COOH terminus, uniquely identifies villin from its other gelsolin family members (Athman *et al.,* 2002). The villin headpiece contains a calcium independent actin-binding site located at amino acids 820-823. This KKEK amino acid motif is highly evolutionarily conserved (Athman *et al.,* 2002). Therefore, the villin headpiece domain served as the villin conserved domain sequence for this study.

Villin expression in adult vertebrates is tissue-specific and primarily occurs in the microvillus-rich region of the intestinal and kidney brush border (Athman *et al.,* 2002). During early embryogenesis, villin is expressed in intestinal epithelial cells of chick and mouse embryos and can serve as a developmental marker (Arpinet et al., 1988). However, villin expression is not restricted to vertebrates. The *Drosophila* gene QUAIL is a villin-like protein responsible for bundling actin filaments together, and four villin-like proteins have been identified in *Arabidopsis thaliana* (Klahre *et al.,* 2000). The discovery of *Arabidopsis* villin-like proteins marked the first instance in which a villin-like protein with high homology to animal villin was identified in plants. However, in contrast with *Drosophila* or mammalian villin, *Arabidopsis* villin-like proteins appear to

lack tissue-specific expression, thus suggesting plant villins are less specialized and serve a more general function such as cell division (Klahre *et al.,* 2000).

Chlamydomonas hcVillin Gene

We identified a hypothetical *Chlamydomonas* villin-like gene (hcVillin) that shows a 51% homology to the highly conserved villin headpiece domain. Although hcVillin does not contain the entire KKEK motif associated with the actin-binding domain in the villin headpiece, hcVillin does contain KK++, demonstrating 50% homology with these four highly conserved amino acids. BLASTX results of the hcVillin nucleotide sequence show similarity between hcVillin and other villin-like actin binding proteins across broad taxa.

Having identified the hcVillin gene, we were able to show that this gene is actively expressed in vegetative and gametic *Chlamydomonas* cells of both mating types (Figure 6.1). RT-PCR of mRNA from the various cell types was screened with hcVillin primers, demonstrating that the hcVillin gene is an actively transcribed gene. This finding is further supported by the presence of hcVillin mRNA in the Chlamydomonas EST database. Furthermore, the BLASTX results from the *Chlamydomonas* EST nucleotide sequence indicate that this expressed mRNA indeed resembles other villin-like proteins in other organisms.

hcVillin Protein Localization

Villin protein localization results utilizing immunofluorescent and immunoblotting techniques produced inconclusive results. Immunofluorescent labeling of hcVillin with our commercially obtained villin antibody was inconsistent. The antibody did label portions of the flagella and cell body in wild type and Ida5 actin-

mutant cells (Figure 7A-H). Control cells labeled with secondary antibody only (Figure 7G) and cells labeled with an irrelevant antibody did not demonstrate labeling of the cell body or flagella as seen with the villin antibody. However, this labeling was not consistent in every cell type and did not localize to the fertilization tubule. When compared to these controls, there appears to be an increased labeling associated with the villin antibody; however, further refinement is needed to fully interpret localization of the hcVillin protein.

Because Ida5 mutants lack the conventional actin gene, we hypothesized that these cells would not demonstrate any α-villin labeling. The fairly consistent outer cell body and flagellar labeling in Ida5 cells suggests a possible interaction between the novel actin protein (NAP) present in this mutant strain and the α-villin antibody. Since NAP has been shown to essentially takeover the function of actin in these mutants (Kato-Moura *et al.,* 1998), it is not unreasonable to anticipate an interaction between villin and NAP.

Similar to immunofluorescence, attempts to localize the endogenous hcVillin protein using immunoblotting were inconclusive. As indicated in figure 9.1, there is no identified protein at the predicted molecular weight of hcVillin. The positive villin control consisting of kidney lysate produced banding at the appropriate molecular weight of approximately 92 kDa. The presence of distinct bands at 40 kDa in the *Chlamydomonas* cells suggests a cross reactivity between α-villin and other *Chlamydomonas* proteins. One intriguing aspect is that 40 kDa is the molecular weight of actin.

Fimbrin: An Actin-Binding Protein

We also identified the presence of hcFimbrin, a fimbrin-like gene, in *Chlamydomonas*. Fimbrin, a member of the plastin family, is a 68 kDa actin-bundling protein first discovered in purified microvilli of the intestinal brush border (Bretscher and Weber, 1980). According to Hanein *et al.* (1997), fimbrin contains two actin binding domains located adjacent to each other within the 58 kDa COOH-terminal core. Each actin binding domain is a highly conserved 27 kDa sequence which contains two calponin homology (CH) domains. These CH domains cross link the actin filaments of the microvillus core bundle together, further enhancing the rigidity of the structure (Matsudaira, 1994; Hanein *et al.,* 1997). The conserved domain containing this CH domain served as the fimbrin conserved domain sequence for this study.

In humans, fimbrin crosslinks actin filaments in intestinal microvilli and the stereocilia of the inner ear (Volkmann *et al.,* 2001). Three human isoforms of fimbrin have been discovered and exhibit tissue-specific localization in cells, as reported with human villin (Hanein *et al.,* 1997). Like villin and Myosin-I, fimbrin is one of the key components of a basic microvillus structure (Bretscher, 1991).

***Chlamydomonas* hcFimbrin Gene**

A hypothetical *Chlamydomonas* gene for fimbrin was identified that shows a 43% homology to the highly conserved fimbrin CH actin-binding domain (Figure 2.2). Having identified the hcFimbrin gene, we were able to demonstrate the active expression of this gene in vegetative and gametic *Chlamydomonas* cells of both mating types (Figure 6.2). RT-PCR of mRNA from the various cell types using hcFimbrin primers was successful, demonstrating that the hcFimbrin gene is an actively transcribed gene. This

finding is further supported by the presence of hcFimbrin mRNA in the EST database, and that the hcFimbrin gene product resembles other fimbrin-like proteins in other organisms based on BLASTX results.

The absence of commercially available fimbrin antibodies limited our ability to identify the endogenous protein, as discussed in detail later. Generating a *Chlamydomonas* fimbrin antibody or a tagged form of the protein are the best routes for identifying the endogenous protein.

Myosin-I: An Actin-Binding Motor Protein

The third gene we identified is a myosin-like gene in *Chlamydomonas* - hcMyosin-I. Also termed brush border myosin-I, Myosin-I was first discovered in *Acanthameba castellanii* and is considered a major component of the cytoskeleton of intestinal microvilli (Collins and Borysenko, 1984). Along with actin, villin, and fimbrin, Myosin-I is the final protein necessary to create a microvillus-like structure *in vitro* (Coluccio and Bretscher, 1989). Myosin-I functions by laterally joining the actin filaments of the microvillus to the surrounding plasma membrane (Footer and Bretscher, 1994).

Classification of myosin proteins is complex and constantly changing. Currently, the eighteen identified myosin classes are grouped together based on similarity in structure, not function. All myosin proteins possess the highly conserved N-terminal motor head domain which contains ATP and actin binding sites (Tyska and Mooseker, 2002). The presence of this motor head domain in all myosin proteins makes it difficult to accurately identify a specific myosin class. Myosin-I is unique in that it contains only a single heavy chain in the N-terminal motor domain (McGoldrick *et al.,* 1995).

Chlamydomonas hcMyosin-I Gene

We identified a hypothetical *Chlamydomonas* myosin-like gene (hcMyosin-I) that shows a 63% homology to the conserved motor domain of Myosin-I. BLASTX results of the hcMyosin-I nucleotide sequence demonstrate similarity between hcMyosin-I and other myosin-like proteins across broad taxa (Figure 3.4).

Having identified the hcMyosin-I gene, we were able to show that this gene is actively transcribed in vegetative and gametic *Chlamydomonas* cells of both mating types (Figure 6.3) and is present in the *Chlamydomonas* EST database.

Because the conserved domain for Myosin-I consists of the highly conserved motor head domain present in all myosin proteins, we are unable to accurately classify hcMyosin-I as strictly a class I myosin protein. Future analysis may classify hcMyosin-I as a different class myosin protein.

hcMyosin-I Protein Localization

As seen with hcVillin, hcMyosin-I protein localization results were difficult to interpret. Immunofluorescent labeling of hcMyosin-I using our commercially obtained α-Myosin-I antibody produced a punctate labeling of the cell body most evident in wild type *mt*+ unactivated cells. This labeling did not localize to the fertilization tubule; however, this punctate distribution pattern is similar to Myosin-I distribution in other cell types (Conrad *et al.,* 1993). A more refined antibody should help to determine the significance of this punctate labeling. Furthermore, this antibody failed to identify a protein of appropriate size in immunoblotting (Figure 9.2). The antibody did recognize the positive control for which it was designed against, evident by the band present at approximately 110 kDa.

Identification of Endogenous Proteins

The inability of commercial antibodies to successfully recognize actin-associated proteins in *Chlamydomonas* suggests that our antibodies are not cross reactive with the *Chlamydomonas* version of these proteins. Determining the specific localization of these proteins in *Chlamydomonas* can be addressed with one of two strategies. (1) A tagged form of the gene can be constructed and transfected into *Chlamydomonas* for subsequent expression. (2) Antibodies against the *Chlamydomonas* protein can be generated instead of relying on commercially available antibodies typically against human isoforms of these genes.

The generation of *Chlamydomonas* specific antibodies has proven valuable in identifying other endogenous *Chlamydomonas* proteins (Tam and Lefebvre, 2002; Silflow *et al.,* 2001) including the fertilization tube associated FUS1 protein (Misamore *et al.,* 2003). Polyclonal antibodies can be generated against one of several forms of the protein. First, a specific antigenic region of the protein can be identified and a small (~ 15 amino acid) peptide can be used to generate the antibody. Second, a bacterially expressed form of the entire protein, or significant portion of the protein, can be used to generate polyclonal antibodies against the entire length of the protein. Third, the protein can be expressed in *Chlamydomonas*, purified using a small tag attached to the protein, and used to generate antibodies. Each of these strategies has had mixed success in *Chlamydomonas* in recent years.

More recently, generating tagged forms of the protein and looking at the localization in *Chlamydomonas* is proving to be a more reliable method for determining protein localization (W.J. Snell, personal communication). Epitope tagging involves

generating a recombinant form of the gene that contains an additional highly antigenic sequence added to the protein and typically under control of a high expression promoter. The human influenza virus hemagglutinin (HA) epitope tag has been used successfully in the past to identify *Chlamydomonas* proteins. This highly antigenic epitope is fused into the gene product and then expressed in *Chlamydomonas*. The FUS1 protein has recently been tagged with this epitope and this recombinant FUS1-HA protein has been shown to localize in the same region as the endogenous FUS1 protein (W.J. Snell, personal communication).

The critical step in designing the tagged form of hcVillin, hcFimbrin, and hcMyosin-I genes is placement of the HA tag in a region of the gene where it is least likely to impact protein function. Based on the relative position of the key functional domains in related proteins, I would propose that the HA tag be placed at the N-terminus of hcVillin, (away from the villin headpiece domain), the N-terminus of hcFimbrin (away from the two actin-binding/CH domains), and the COOH-terminus of hcMyosin-I (away from the highly conserved motor head domain).

***Chlamydomonas* Fertilization Tube Assembly**

Because the fertilization tube assembled upon activation of the *mt*+ gamete resembles a microvillus, it is generally considered to be a homologous structure (Wilson and Snell, 1998). Thus, we hypothesized that microvillus-associated proteins in other, primarily mammalian, systems would be present in the *Chlamydomonas* fertilization tubule. Our current inability to localize villin, fimbrin, and myosin to the fertilization tubule suggests one of two conclusions. Either the antibodies are not cross reactive with the *Chlamydomonas* version of these proteins or these proteins are not present in the

fertilization tubule. Having previously addressed the former explanation, I will now address the possibility that these proteins might not be found in the fertilization tubule.

The absence of these protein localizations suggests that perhaps the fertilization tubule of *Chlamydomonas* is not constructed like typical microvilli in other systems. Detailed observations of fertilization tube serial sections support this theory. Using electron microscopy, Detmers *et al.* (1983), examined the fertilization tube core by preparing serial sections of the tubule to determine its composition. Ultrastructure analysis revealed actin-filaments were not tightly arranged for maximum rigidity and support of the structure. The filaments appeared to be randomly arranged with very little crosslinks between them (Detmers *et al.,* 1983).

In most animal microvilli, such as those located in the brush border, the actin filaments are typically tightly bundled in discrete groups, and this bundling is attributed to greater rigidity of the structure. The low presence of crosslinks between actin filaments in the *Chlamydomonas* fertilization tubule may explain the absence of villin and Myosin-I, two actin-binding proteins, in the fertilization tube. Potentially, *Chlamydomonas* may not assemble their fertilization tubule like microvilli in other systems. Clearly, precise localization of the endogenous proteins from the three identified genes is needed to further test this hypothesis.

Summary

The purpose of this study was to identify actin-associated proteins in *Chlamydomonas* and determine their possible role in the fertilization process. We hypothesized that microvillus associated proteins in other systems would localize to the *Chlamydomonas* fertilization tubule. We identified three previously unknown

hypothetical *Chlamydomonas* genes: hcVillin, hcFimbrin, and hcMyosin-I. Extensive database analysis revealed sequence similarities between these *Chlamydomonas* genes and villin, fimbrin, and myosin-like proteins in other systems. Sequence homology for all three genes was high especially in the highly conserved regions of these protein families that are critical for the protein function. We documented that these *Chlamydomonas* genes are expressed in both vegetative and gametic cells of both mating types using RT-PCR of mRNA. Expression of these genes is further supported by their presence in Chlamydomonas EST libraries and that the expressed protein from these libraries is similar to like proteins from a broad range of taxa. We have yet to identify the endogenous proteins expressed by these genes. The absence of localization to the fertilization tubule suggests that either the commercially available antibodies do not cross react with the *Chlamydomonas* from the protein or that the *Chlamydomonas* fertilization tubule is not constructed like other microvilli. Generating *Chlamydomonas* specific antibodies or tagged forms of the proteins are the best routes for further identifying hcVillin, hcFimbrin, and hcMyosin-I proteins.

Appendix 1: Glossary of Database Analysis Terminology

Alignment: The resulting match between two sequences (query sequence and subject sequence) produced when a BLAST search is performed

Alignment hit report: Contains data about the selected sequences and provides a pairwise alignment of the two matching sequences

Alignment score color key: Located above the graphical alignment box, it displays different color ranges corresponding to varying significance of alignments based on the BLAST score

BLAST: Basic Local Alignment Search Tool searches for regions of similarity between nucleotide or protein sequences by comparing them to known sequence databases and determining the significance of the resulting matches

BLAST Score: Significance rating inversely proportional to expect value. Lower expect values produce higher BLAST scores which results in statistically significant sequence matches

BLASTX: compares a nucleotide query sequence translated in all reading frames against a protein sequence database

Conserved Domain: Areas of recurring units in polypeptide chains within proteins that have a distinct evolutionary origin and function

Conserved Domain Database: Located at NCBI website, it contains the sequence alignments with defining features that are conserved within each domain family.

Expect value: a factor that describes the number of sequence matches expected to occur simply by chance when searching a certain size database. The lower the E-value, or the closer it is to zero, the more significant the match

FASTA: Required format of most sequence alignment programs. The format begins with a single-line description of the sequence including the organism from which it is derived and the genbank identification number, followed by lines of amino acid sequence data

Graphical alignment box: Contains the specific location within the genome of significant alignments based on the selected expect value and illustrated by colored arrows generated by the alignment score color key

Pairwise Alignment: Aligns matching amino acids when two sequences are compared against each either by performing a BLAST analysis

Query Sequence: protein or nucleotide sequence of interest that is searched against a particular database.

Scaffold: specific locations of bases in the *Chlamydomonas* genome

Subject Sequence: protein or nucleotide sequence from a specific organism that is compared against a query sequence through a BLAST search

TBLASTN: compares a protein query sequence against a nucleotide sequence database translated in all six reading frames

TBLASTX: compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database

Appendix 2: Conserved Domain Families Used in Database Analysis

Ezrin, Radixin, and Moesin (ERM)

1: cd01220 Links
PH_CDEP: Chondrocyte-derived ezrin-like domain containing protein (CDEP) Pleckstrin homology (PH) domain. CDEP consists of a Ferm domain, a rhoGEF (DH) domain followed by two PH domains. PH domains share little sequence conservation, but all have a common fold, which is electrostatically polarized. PH domains also have diverse functions. They are often involved in targeting proteins to the plasma membrane, but few display strong specificity in lipid binding. Any specificity is usually determined by loop regions or insertions in the N-terminus of the domain, which are not conserved across all PH domains. PH domains are found in cellular signaling proteins such as serine/threonine kinase, tyrosine kinases, regulators of G-proteins, endocytotic GTPases, adaptors, as well as cytoskeletal associated molecules and in lipid associated enzymes. [cd01220|29869]

2: cd00836 Links
FERM_C: The FERM_C domain is the third structural domain within the FERM domain. The FERM domain is found in the cytoskeletal-associated proteins such as ezrin, moesin, radixin, 4.1R, and merlin. These proteins provide a link between the membrane and cytoskeleton and are involved in signal transduction pathways. The FERM_C domain is also found in protein tyrosine phosphatases (PTPs) , the tryosine kinases FAKand JAK, in addition to other proteins involved in signaling. This domain is structuraly similar to the PH and PTB domains and consequently is capable of binding to both peptides and phospholipids at different sites. [cd00836|29846]

3: pfam00769 Links
ERM: Ezrin/radixin/moesin family. This family of proteins contain a band 4.1 domain (pfam00373), at their amino terminus. This family represents the rest of these proteins. [pfam00769|24470]

4: smart00295 Links
B41: Band 4.1 homologues; Also known as ezrin/radixin/moesin (ERM) protein domains. Present in myosins, ezrin, radixin, moesin, protein tyrosine phosphatases. Plasma membrane-binding domain. These proteins play structural and regulatory roles in the assembly and stabilization of specialized plasmamembrane domains. Some PDZ domain containing proteins bind one or more of this family. Now includes JAKs. [smart00295|24244]

Villin

1: smart00262
GEL: Gelsolin homology domain; Gelsolin/severin/villin homology domain. Calcium-binding and actin-binding. Both intra- and extracellular domains. [smart00262|24237]

2: smart00153
VHP: Villin headpiece domain; [smart00153|3835]

Fimbrin

1: cd00014 Links
CH: Calponin homology domain; actin-binding domain which may be present as a single copy or in tandem repeats (which increases binding affinity). The CH domain is found in cytoskeletal and signal transduction proteins, including actin-binding proteins like spectrin, alpha-actinin, dystrophin, utrophin, and fimbrin, proteins essential for regulation of cell shape (cortexillins), and signaling proteins (Vav). [cd00014|28898]

2: COG5069 Links
SAC6: Ca2+-binding actin-bundling protein fimbrin/plastin (EF-Hand superfamily) [Cytoskeleton] [COG5069|14198]

Fascin

1: cd00257 Links
Fascin: Fascin-like domain; members include actin-bundling/crosslinking proteins facsin, histoactophilin and singed; identified in sea urchin, Drosophila, Xenopus, rodents, and humans; The fascin-like domain adopts a beta-trefoil topology and contains an internal threefold repeat; the fascin subgroup contains four copies of the domain; Structurally similar to fibroblast growth factor (FGF) [cd00257|29332]

2: pfam06268 Links
Fascin: Fascin protein. This family consists of several eukaryotic fascin or singed proteins. The fascins are a structurally unique and evolutionarily conserved group of actin cross-linking proteins. Fascins function in the organisation of two major forms of actin-based structures: dynamic, cortical cell protrusions and cytoplasmic microfilament bundles. The cortical structures, which include filopodia, spikes, lamellipodial ribs, oocyte microvilli and the dendrites of dendritic cells, have roles in cell-matrix adhesion, cell interactions and cell migration, whereas the cytoplasmic actin bundles appear to participate in cell architecture. [pfam06268|25141]

Profilin

1: cd00148 Links
PROF: Profilin binds actin monomers, membrane polyphosphoinositides such as PI(4,5)P2, and poly-L-proline. Profilin can inhibit actin polymerization into F-actin by binding to monomeric actin (G-actin) and terminal F-actin subunits, but - as a regulator of the cytoskeleton - it may also promote actin polymerization. It plays a role in the assembly of branched actin filament networks, by activating WASP via binding to WASP's proline rich domain. Profilin may link the cytoskeleton with major signalling pathways by interacting with components of the phosphatidylinositol cycle and Ras pathway. [cd00148|29067]

2: smart00392 Links
PROF: Profilin; Binds actin monomers, membrane polyphosphoinositides and poly-L-proline. [smart00392|24270]

3: smart00498 Links
FH2: Formin Homology 2 Domain; FH proteins control rearrangements of the actin cytoskeleton, especially in the context of cytokinesis and cell polarisation. Members of this family have been found to interact with Rho-GTPases, profilin and other actin-assoziated proteins. These interactions are mediated by the proline-rich FH1 domain, usually located in front of FH2 (but not listed in SMART). Despite this cytosolic function, vertebrate formins have been assigned functions within the nucleus. A set of Formin-Binding Proteins (FBPs) has been shown to bind FH1 with their WW domain. [smart00498|22818]

Cofilin

1: cd00013 Links
ADF: Actin depolymerisation factor/cofilin -like domains; present in a family of essential eukaryotic actin regulatory proteins; these proteins enhance the turnover rate of actin and interact with actin monomers as well as actin filaments. [cd00013|28897]

Myosin-I

10: cd01378 Links
MYSc_type_I: Myosin motor domain, type I myosins. Myosin I generates movement at the leading edge in cell motility, and class I myosins have been implicated in phagocytosis and vesicle transport. Myosin I, an unconventional myosin, does not form dimers. This catalytic (head) domain has ATPase activity and belongs to the larger group of P-loop NTPases. Myosins are actin-dependent molecular motors that play important roles in muscle contraction, cell motility, and organelle transport. The head domain is a molecular motor, which utilizes ATP hydrolysis to generate directed movement toward the plus end along actin filaments. A cyclical interaction between myosin and actin provides the driving force. Rates of ATP hydrolysis and consequently the speed of movement along actin filaments vary widely, from about 0.04 micrometer per second for myosin I to 4.5 micrometer per second for myosin II in skeletal muscle. Myosin II moves in discrete steps about 5-10 nm long and generates 1-5 piconewtons of force. Upon ATP binding, the myosin head dissociates from an actin filament. ATP hydrolysis causes the head to pivot and associate with a new actin subunit. The release of Pi causes the head to pivot and move the filament (power stroke). Release of ADP completes the cycle. [cd01378|30100]

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VITA

Jennifer Szopinski was born in Stevens Point, Wisconsin on July 27, 1981. She moved to Lewisville, Texas at the age of six and graduated with honors from Lewisville High School in 1999. She received her Bachelor of Science degree in Biology with a Communication Studies minor from Texas State University at San Marcos where she graduated Magna cum Laude in May, 2003. Jennifer entered the biology graduate program in August, 2003 at Texas Christian University, where she received her Masters of Science in Biology in May 2006. There she served as a teaching assistant for non-biology majors and received the 2005 & 2006 TCU Teaching Assistant of the Year Award. Jennifer is a member of *Beta Beta Beta* Biological Honor Society, *Alpha Chi* National Honor Society, and Golden Key International Honor Society.

Jennifer is the daughter of Jerry and Joann Szopinski, and the sister of Jessica Szopinski, future 2010 TCU graduate.

ABSTRACT

IDENTIFICATION OF THREE *CHLAMYDOMONAS REINHARDTII* GENES ENCODING ACTIN-ASSOCIATED PROTEINS

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The focus of this study was to identify actin-associated proteins in *Chlamydomonas reinhardtii* and to determine their possible role in the fertilization process. Because the fertilization tubule of activated *mt*+ gametes resembles a microvillus, we hypothesized that actin-associated proteins present in microvilli in other systems may localize to this structure in *Chlamydomonas*. We identified three previously unknown hypothetical *Chlamydomonas* genes: hcVillin, hcFimbrin, and hcMyosin-I. Extensive database analysis revealed sequence similarities between these *Chlamydomonas* genes and villin, fimbrin, and myosin-like proteins in other systems. Sequence homology for all three genes was especially high in the highly conserved regions of these protein families that are critical for the protein function. RT-PCR verified the expression of mRNA from hcVillin, hcFimbrin, and hcMyosin-I in *Chlamydomonas* vegetative and gametic cells. Immunofluorescent and immunoblotting experiments were performed using commercially available α-villin and α-Myosin-I antibodies against *mt*+ wild type and Ida5 actin mutant *Chlamydomonas* cells. When

compared to control cells, there appeared to be an increased labeling of the cell body and flagella associated with the villin antibody in both wild type and Ida5 cells. Immunofluorescent labeling of hcMyosin-I produced a punctate labeling of the cell body most evident in wild type *mt*+ unactivated cells. Neither α-villin nor α-Myosin-I localized to the fertilization tubule in *mt*+ activated gametes. The absence of commercially available fimbrin antibodies limited our ability to identify the endogenous hcFimbrin protein. Attempts to localize the endogenous hcVillin and hcMyosin-I proteins using immunoblotting were inconclusive. Our antibody failed to recognize the hcVillin and hcMyosin-I protein at the predicted sizes in *mt*+ unactivated cells, *mt*+ activated cells, and concentrated *Chlamydomonas* flagella. However, α-villin and α-Myosin-I antibodies did recognize villin and Myosin-I in the positive control for which they were designed against. Our current inability to localize villin, fimbrin, myosin-I to the fertilization tubule suggests one of two conclusions. (1) Commercially available antibodies do not cross react with the *Chlamydomonas* form of the protein. (2) The *Chlamydomonas* fertilization tubule is not constructed like other microvilli. Generating *Chlamydomonas* specific antibodies or tagged forms of the proteins are the best routes for further identifying hcVillin, hcFimbrin, and hcMyosin-I proteins.