GPI-ANCHORED PROTEINS IN CILIATED PROTOZOA:
FROM CA++ SIGNALING TO MITOCHONDRIAL EXTRUSION

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by
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Ciliates are an ancient eukaryotic lineage comprised of roughly 7,000 extant species. These include historically important models for the study of cell biology and genetics, most notably, *Tetrahymena* and *Paramecium*. One of the characteristic features of ciliates is their extensive cell surface comprised of plasma and ciliary membranes enriched in GPI-anchored proteins known as immobilization antigens or i-antigens. Antibodies against these proteins trigger a number of behavioral changes in ciliates including discharge of cortical secretory granules and arrest of cell movement (hence the term immobilization antigens). Although ciliate Immobilization antigens were discovered more than a hundred years ago, the signaling mechanisms underlying these effects are still unknown. To investigate transmembrane signaling events in response to i-antigen clustering we used *Ichthyophthirius multifiliis*, a parasitic ciliate, and *Tetrahymena thermophila*, a well-studied free-living ciliate as model systems. Using a variety of molecular and cell biological techniques we show that antigen clustering is accompanied by mobilization of intracellular Ca$^{++}$ and the formation of membrane aggregates (blebs) at the cell surface that migrate to the tips of cilia where they are shed. Remarkably, cross-linking of i-antigens also leads to mitochondrial extrusion both in
Tetrahymena and Ichthyophthirius. Release of mitochondria from intact cells was shown directly by negative stain and thin-section transmission electron microscopy. Using confocal imaging in conjunction with antibodies against HSP60 and ATP synthase, extruded mitochondria were shown to co-localize with plasma membrane blebs. Mitochondrial extrusion appears to be Ca++ dependent and can be induced in response to heat shock. Cells survive the response and regain their normal architecture and swimming behavior following the extrusion. Several recent reports have suggested that mitochondria can be jettisoned from mammalian cells under conditions of oxidative stress. While the precise mechanisms responsible this phenomenon are unclear, the fact that protozoa and mammalian cells are both capable of ejecting their mitochondria would strongly suggest the process is evolutionarily conserved, and raises interesting questions regarding the advantages this phenomenon may have for cells.
The author of this work, Yelena Bisharyan, the oldest of three children, was born in Yerevan, Armenia on July 2, 1976 to Ashot Bisharyan and Emma Papeyan. She grew up in the city of Yerevan where she attended Missak Manoushian high school. During her high school years she was also admitted to School of Arts in Yerevan from where she graduated with a diploma in acting. After graduating from high school with honors she successfully passed the tests and was admitted to Yerevan State University’s department of Cell Biology and Genetics. During her first year in university she met her college sweetheart, Ashot Papoyan. The couple got married shortly after graduating in 1999 and moved to Ithaca, New York. Yelena started working as a technician in Dr. Peter Bruns’s laboratory, in the department of Genetics and Development at Cornell University. In 2001 she transferred to Ted Clark’s laboratory in the department of Microbiology and Immunology. In 2003 she applied and got accepted to graduate school in the field of Immunology, through Cornell’s Employee Degree Program. She greatly enjoyed years spent in Clark lab, where she divided her time between work and graduate study.
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Chapter one

Introduction and Literature Review
A. Ciliate i-antigens: involvement in signal transduction

A.1 Ciliated protozoa as biological species

Protozoa are a diverse group of single-celled organisms representing the oldest eukaryotic phylum. They include free-living, commensal and obligate parasitic species including some of the most important human and veterinary pathogens. Among the most well studied protozoa are the ciliates, with roughly 7000 extant species. Ciliates have a rich history in biological research that dates to van Leeuwenhoek in the early 1700s. They are predominantly free-living organisms, but include several parasitic species such as *Ichthyophthirius multifiliis*, a major pathogen of freshwater fish. As the agent of “white spot” disease, or Ich, *I. multifiliis* is responsible for high fish mortality and heavy economic losses in commercial aquaculture (129). Confined to the skin and gill epithelia, *Ichthyophthirius* can be readily visualized in host tissues, and has served as a useful model for the study of host-parasite interactions in lower vertebrates (32). The ciliates include two other important models that have been widely used in cell biology and genetics, namely, *Paramecium* and *Tetrahymena*. The first animal-like cell to be grown in pure culture, *Tetrahymena* has been particularly effective as a model system, and has been responsible for a large number of fundamental discoveries ranging from RNA self-splicing to the mechanics of ciliary beat (3). Its importance as a research tool prompted an ambitious effort to sequence the large (>10^8 bp) *Tetrahymena* macronuclear genome. That effort was successfully completed in 2004 (35). Analysis of the genome revealed major expansion of certain gene families including one for GPI-anchored surface proteins.
A.2 GPI anchored proteins in protozoa

GPI anchored surface proteins are typically abundantly expressed on the plasma membranes of parasitic protozoa. It has been suggested that these proteins form a protective coat at cell surface. Their linkage to the membrane via a glycolipid anchor permits extremely close packing at the cell surface without disruption of the lipid bilayer, a feature that would not be possible in the case of transmembrane proteins (84).

GPI-anchored proteins are thought to be important players in host parasite interactions, and may function in avoidance of the host immune system. For example, in the case of *Trypanosoma*, it is widely believed that the dense packing of GPI-linked variable surface glycoproteins (VSGs) may protect the parasite by shielding critically important surface receptors from the antibody binding (16, 45). Nevertheless, free-living protozoa that do not interact with the immune systems of vertebrate hosts also express abundant GPI-anchored surface proteins. The case of the common pond-water ciliates, *Tetrahymena* and *Paramecium* express abundant GPI-linked coat proteins known as immobilization antigens, or i-antigens that are similar in structure to the VSGs of trypanosomes. While the functions of these proteins are not known, it is believed they may play a protective role in general, for example, in response to environmental stress in which case they could be involved in membrane adaptation, or as a shield against natural predators (87).

Although GPI-anchored proteins are typically linked to the plasma membrane, spontaneous as well as stimulus-dependent shedding of these proteins has been described in a number of different protozoan species (16, 45) Organisms such as *Toxoplasma* and *Plasmodium*, shed surface proteins during invasion of host cells. This is thought to involve the activation of
membrane-associated proteases that can shave surface antigens, as well as trim adhesive proteins released from secretory organelles at the anterior end of the cell. During invasion, a complex of cell surface and adhesive proteins is capped at the posterior end of the cell by a mechanism that involves linkage of actomyosin-dependent motors to the cell surface via transmembrane and GPI-linked proteins. During the final steps of shedding, these proteins are cleaved by membrane-associated proteases, and the cap is released (16). In ciliates the shedding of GPI-anchored proteins from the cell surface has been documented, however the mechanisms responsible for their release are not well understood (87).

A.3 Immobilization antigens

The most abundant GPI-anchored proteins in ciliates are known as immobilization antigens, or i-antigens. I-antigens were discovered in Paramecium over 100 years ago when cells exposed to antisera against whole cell preparations became immobilized and killed (hence the name immobilization antigens). The i-antigens can form up to several percent of total cell protein in various hymenostome species, and are specifically enriched in ciliary membranes. While their precise function is still unknown, immobilization antigens have been extensively studied in Paramecium, Tetrahymena, and Ichthyophthirius (23, 46, 119). The genes for several i-antigens have been cloned and characterized. Based on their deduced sequences, the proteins vary in size from 30-310 kDa, and are characterized by cysteine rich repeats that span their length. They have hydrophobic signal peptides at their N and C termini that control ER localization and GPI anchor addition, respectively (21, 22, 24). Paramecium and Tetrahymena contain multiple i-antigen genes that
are expressed in mutually exclusive fashion such that only one i-antigen is expressed at the cell surface at any given time. In response to environmental stimuli (temperature, pH) cells undergo antigenic shift, changing expression from one i-antigen to another (23).

A.4 Behavioral changes in ciliates in response to i-antigen crosslinking.

The idea that GPI-anchored proteins are involved in signaling in ciliates comes from a number of observations on *Ichthyophthirius*, *Paramecium* and *Tetrahymena*. Antibodies directed against the i-antigens of these cells cause reversal of ciliary beat and immobilization suggesting that these proteins initiate intracellular signaling events leading to increase in intracellular [Ca$^{++}$] (23, 46). This is supported by the further observation that i-antigen clustering can result in the discharge of cortical secretory granules (mucocysts in *Ichthyophthirius* and *Tetrahymena*, and trichocysts in *Paramecium*) (20, 53, 63, 119). In general, regulated secretion in all eucaryotic cells involves calcium signaling (53), and previous studies in mammalian cells have demonstrated that GPI anchored proteins can initiate signaling cascades leading to increase in intracellular Ca$^{++}$ (50, 80, 94).

A hallmark of signal transduction via membrane receptors is their clustering at the cell surface. This appears to be the case with the i-antigens as well. Monovalent antibody subfragments (Fab) directed against the i-antigens do not immobilize cells, nor do they induce secretion of mucocysts or trichocysts. However, the addition of secondary antibody (that is, bivalent antibody against Fab) results in rapid immobilization and mucocyst secretion, which clearly shows that these reactions require clustering of i-antigens on the cell surface. This is also nicely demonstrated by passive immunization studies on
Ichthyophthirius multifiliis. As mentioned above, Ich i-antigens are targets of the host’s immune system and elicit the production of specific antibodies in response to infection (80). Furthermore, mouse monoclonal antibodies against these proteins confer strong protection against parasite infection when administered passively to naïve fish. The mechanism of protection involves a novel behavioral effect in which parasites exit the host skin prematurely in response to antibody binding (21, 22). I-antigen-specific mAb Fab fragments fail to elicit protection against parasite infection when administered passively. However, when secondary (bivalent) anti-mouse IgG is administered to fish subsequent to Fab injection, parasite rapidly exit the host, indicating that protection requires cross-linking of i-antigens at the parasite surface (rather than blocking receptor ligand interactions within the host skin) (21, 22).

A.5 Signaling through GPI anchored proteins and lipid rafts

Ironically, while GPI-anchored proteins are thought to have important functions in protozoa, few studies have addressed their potential role in transmembrane signaling. Since there was abundant evidence that this can occur in ciliates (23), the original goal of this dissertation was to elucidate the mechanisms involved, and address the subsequent reactions leading to shedding of GPI-anchored proteins from the cell surface. On a practical level, this may be an important step towards the development of therapeutic protocols against more distantly related parasitic protozoa such as the apicomplexans, which form a sister group to the ciliates. In more basic terms, how proteins restricted to the outer leaflet of the plasma membrane can initiate intracellular signaling events in protozoa is an interesting question by itself. One of the simplest ways for GPI anchored proteins to participate in
transmembrane signaling is through their physical association with transmembrane proteins that have intrinsic kinase activity, or whose cytoplasmic tails can associate with signaling molecules that can trigger downstream signaling cascades upon ligand binding. In mammals, a number of GPI-linked proteins have been shown to participate in transmembrane signaling via such mechanisms (72). Nevertheless, in a majority of cases, signaling through GPI-anchored proteins in mammals appears to involve non-receptor, Src-family tyrosine kinases (120). GPI-anchored proteins, and doubly acylated Src-family kinases, partition into membrane microdomains known as lipid rafts (56, 83). Lipid rafts are transient microdomains enriched in sphingolipids and sterols. The saturated hydrocarbon chains of sphingolipids allow sterols to be tightly intercalated forming a liquid ordered state. These microdomains are present in almost all eukaryotic cell types examined to date, and appear to participate in diverse cellular functions such as cell signaling, lipid trafficking, endocytosis and exocytosis (52, 81, 85, 89, 103). Due to their physical state, proteins that contain lipid modifications (such as glycosylphosphatidylinositol; palmytol, etc.) preferentially associate with rafts. As a result, the clustering of GPI anchored proteins on the outer leaflet of the membrane are thought to aggregate acylated Src family tyrosine kinases on the inner leaflet resulting in autophosphorylation and downstream signaling events including Ca$^{2+}$ mobilization, actin reorganization, MAP kinase activation and cytokine secretion (56, 85).

The existence of lipid rafts, and the association of GPI anchored proteins with these membrane domains has now been shown in ciliates and apicomplexans (30, 31). However, the idea that signaling in these organisms can occur through an association between lipid rafts and GPI anchored
proteins has yet to be explored. While current models could be applied to protozoans, *bona fide* (i.e. single-specificity) tyrosine kinases are thought to have evolved with the metazoans. However, the recent isolation of these proteins from the choanoflagellate, *Monosiga brevicollus* (a unicellular relative of metazoans), suggests that tyrosine kinases may have arisen before the emergence of multicellular organisms (69, 118). Just how far back these proteins date and whether ciliates have precursors to the single-specificity tyrosine kinases found in metazoa is not yet known. A search of the *Tetrahymena* genome database has revealed possible candidates; however, it is difficult to ascribe enzyme activities on the basis of predicted sequence alone.

A.6 Ca++ in ciliated protozoa

As in other organisms, a variety of essential processes are regulated by changes in intracellular Ca++ in the ciliates. These include exocytosis (10, 71, 100, 101), endocytosis (99), direction of ciliary beat (58, 59), and cell contraction (68) to name a few. Potential sources of Ca++ in ciliates are the plasma membrane, alveolar sacs, endoplasmic reticulum (ER) and mitochondria (Figure 1.1). The plasma membrane, which is continuous with ciliary membranes, is rich in Ca++ pumps, Na+/Ca++ exchange proteins, Ca++ channels, and other ion channels that are themselves regulated by Ca++ and/or Ca++/Cam (calmodulin). Regulation of Ca++ influx and efflux in these cells is likely to be complex. Voltage dependent Ca++ channels are thought to be restricted to ciliary membranes, while mechanosensitive and hyperpolarization-sensitive Ca++ channels are presumed to be localized on the plasma membrane (100). Alterations of the membrane due to mechanical
stress, changes in membrane fluidity, or hyperpolarization/depolarization, are likely to cause activation or inactivation of specific combinations of channels leading to particular physiological outcomes. One level of control on this system could be the strategic localization of voltage-gated ion channels on ciliary membranes, and mechanosensitive and hyperpolarization-sensitive channels on the plasma membrane. The presence of excitable and non-exitable membranes in different regions of the same cell, suggests that the site of membrane perturbation could significantly alter downstream physiological events (100). By the same token, alterations in Ca$$^{++}$$ flux in one region of the cell could change the properties of the membrane in a different region.

An interesting morphological feature of ciliates (shared with dinoflagellates and apicomplexa) are the alveolar sacs located just beneath the plasma membrane (Figure 1.1). Alveolar sacs are believed to act as Ca$$^{++}$$ storage compartments. In general, the sacs are separated from the plasma membrane by several nanometers although visible membrane-to-membrane links between the two have been described (100, 111). The nature of these links have yet to be determined but are thought to represent structures analogous to those which occur between SR cisternae and the plasmalemma in muscle (100, 111). In Paramecium, Ca$$^{++}$$ within the aveloar sacs associates with a high-capacity binding protein (CaBP) similar to calsequestrin. The alveolar sacs are thought to be important players in shaping and regulating cytosolic Ca$$^{++}$$ in ciliates. Excess Ca$$^{++}$$ taken in through the plasma membrane is presumably stored in the sacs and then released upon activation of Ca$$^{++}$$ release channels triggering exocytosis of dense core secretory granules and other calcium-dependent physiological events. Based on this model, Ca$$^{++}$$
channels that participate in Ca\(^{++}\) influx into the alveolar sacs are located on the side facing the plasma membrane, while the channels involved in Ca\(^{++}\) release are located on the side facing the cytoplasm\(^{(100)}\).

The endoplasmic reticulum can also act as a Ca\(^{++}\) storage compartment in ciliates\(^{(47)}\). Calreticulin-like proteins are thought to be the main types of CaBP in the ER. The dual distribution of Ca\(^{++}\) in the ER and alveolar sacs, suggests that these compartments regulate different Ca\(^{++}\)-dependent functions. For example, while the alveolar sacs may regulate Ca\(^{++}\) homeostasis at the cell cortex and act as a readily source of Ca\(^{++}\) for dense core granule exocytosis, the ER may provide Ca\(^{++}\) for regulating intracellular particle transport, or the maintenance of Ca\(^{++}\) homeostasis in deeper regions of the cell such as the nucleus \(^{(100)}\).

In addition to the ER and alveolar sacs, mitochondria are also involved in Ca\(^{++}\) regulation and establishment of cellular Ca\(^{++}\) homeostasis in ciliates. In vitro experiments with *Tetrahymena* have shown that these cells contain a specific Ca\(^{++}\) transporting system similar to mammals, suggesting their role in Ca\(^{++}\) storage in these cells \(^{(67, 68)}\). Morphological studies in *Tetrahymena* strongly suggest that mitochondria are associated with the ER as is the case for other systems. Indeed, the interplay between mitochondria and the ER as Ca\(^{++}\) buffering organelles is well established in mammalian cells \(^{(42, 93)}\). The presence of alveolar sacs makes Ca\(^{++}\) regulation in the ciliates even more complicated. One could imagine that ciliates require a more a complex Ca\(^{++}\) buffering system due to their unicellular nature, and the fact that they must be nimble in balancing intracellular [Ca\(^{++}\)] within a constantly changing environment.
Figure 1.1 Arrangement of Ca\(^{++}\) buffering organelles in *Tetrahymena thermophila* VOC (Voltage Gated Ca\(^{++}\) Channels), PM (Plasma Membrane), AS (Alveolar Sacs), DCG (Dense Core Granules), ER (Endoplasmic Reticulum), M (Mitochondria)
**B. Mitochondria as moonlighting organelles**

**B.1. Mitochondria are the cell's powerhouses**

Mitochondria generate much of the cell’s energy currency in the form of ATP using Oxidative Phosphorylation and are consequently among the most essential organelles in eukaryotic cells. Depending on the cell’s energy needs they can occupy a major fraction of its total volume (1, 37). In *Tetrahymena*, for example, mitochondria occupy 15% of the cytoplasm (37). Mitochondria are membrane delimited organelles and one of their most striking features is the large amount of internal membrane they contain (1, 95). Each mitochondrion has two highly specialized membranes that play an important role in its function. Together these two membranes create two separate mitochondrial compartments, namely, the internal matrix space, and intermembrane space (1, 95). The outer mitochondrial membrane (OMM) is thought to be similar to the cell’s plasma membrane (1). One of the components of OMM is a transport protein called porin, which creates large (2-3 nm) aqueous channels through the lipid bilayer allowing diffusion of molecules 5000 Daltons or less to the intermembrane space (1, 114). OMM also contains ion channels, transporters and enzymes that are essential in lipid metabolism. Mitochondrial outer membrane is also involved in highly selective protein import into the organelle which is essential for mitochondrial function and biogenesis. The sorting and assembly pathway of outer membrane proteins involves three molecular machines: the translocase of the outer membrane (TOM complex), the sorting and assembly machinery (SAM complex), and the MDM complex (mitochondrial distribution and morphology) (1, 7, 114).

The region between the inner and outer mitochondrial membranes
known as the **intermembrane space** contains channel proteins (porins) that allow free diffusion of small molecules from the cytosol, and makes the mitochondrial intermembrane space chemically equivalent to the cytosol with respect to small molecules (1, 114).

The inner mitochondrial membrane (IMM) is highly specialized. It has a very complex structure, including all of the complexes of the electron transport system, the ATP synthase complex, and transport proteins (1, 114). The IMM is folded into cristae, which greatly expand its surface area and allows many more electron transport complexes than would otherwise be possible if the inner membrane were shaped like the outer membrane. IMM also contains a high proportion of cardiolipin, a double phospholipid with four fatty acids. Because of its structure, cardiolipin helps make the IMM impermeable to ions and is essential for maintaining the electrochemical gradient across the membrane. Thus the inner membrane is freely permeable only to oxygen, carbon dioxide, and water (1, 114). The electron transport chain is located on the inner membrane of the mitochondria. It consists of five extremely complex transmembrane structures called complexes I-V (discussed bellow) (1, 79, 114).

The space surrounded by IMM is the mitochondrial **matrix**. It contains highly concentrated mixture of enzymes involved in TCA cycle. Compared to the cytoplasm, this space is more viscous due to its low water and high protein content. The IMM also contains several copies of mitochondrial DNA, ribosomes and tRNAs, along with enzymes required for mitochondrial gene expression (1, 114).

**B.2 Respiratory Chain Complexes**

Cells generate ATP via two pathways namely, glycolysis, in which glucose is
the primary energy source, and oxidative phosphorylation, in which fatty acids and pyruvate serve this purpose. During oxidative phosphorylation, electrons are transferred from a donor molecule (such as NADH) to an acceptor molecule (such as O₂) through a set of mediating biochemical reactions in an electron transport chain resulting in the transfer of H⁺ ions across a membrane. In mitochondria the electron transport chain is very complex and includes 5 complexes located in inner mitochondrial membrane (79, 127). Electron transfer between these complexes is accomplished by the mobile coenzymes ubiquinone (in the lipid membrane, from Complexes I and II to Complex III) and cytochrome c (in the intermembrane space, from Complex III to Complex IV). **Complex I** is the NADH dehydrogenase, the latter removes two electrons from NADH and transfers them to ubiquinone (coenzyme Q) which as a result is reduced to ubiquinol (CoQH₂) (figure 1.2). This is a free lipid soluble carrier and is free to diffuse into the membrane (79, 127). At a same time Complex I transfers four protons across the membrane and produces a proton gradient (membrane potential) with the inside negative and outside positive. **Complex II** is a succinate dehydrogenase that removes electrons from the succinate and transfers them to Quinon pull via FAD (79, 127), (Figure1.2). **Complex III or Cytochrome bc₁ Complex** removes two electrons from QH₂ and sequentially transfers them to two molecules of cytochrome c. Cytochrome c is a highly soluble heme-containing protein capable of undergoing oxidation and reduction leading to transfer of electrons between complexes III and IV. When electron transfer is hindered (for example, by high membrane potential, point mutations, or respiratory inhibitors such as antimycin A), Complex III may leak electrons to oxygen resulting in the
formation of superoxide, a highly-toxic species, which is thought to contribute to the pathology of a number of diseases, including aging (79, 127). **Complex IV** is cytochrome C oxidase. It removes four electrons from four molecules of cytochrome c and transfers them to molecular oxygen (O₂), producing two molecules of water (H₂O). At the same time, it moves four protons across the membrane, producing a proton gradient. The energy that is released as the electrons flow down the electrochemical gradient is used to pump protons out across the mitochondrial inner membrane though complexes I, III and IV. The potential energy stored in electrochemical gradient is used to import Ca++ and various proteins into the mitochondrion, and generate ATP via **complex V** ATP synthetase. Matrix ATP is then exchanged for cytosolic ADP by the inner membrane adenine nucleotide translocators (ANT)(79, 127).
Figure 1.2 Respiratory Chain Complexes in the Inner Mitochondrial Membrane (image adapted from www.biocenter.helsinki.fi/bi/biophys/images/)

Ilya Belevich ©
Early electron microscope studies of mitochondria in *Tetrahymena* and *Paramecium* describe elongate or oval structures composed of two membranes (56). The inner mitochondrial membrane in ciliates forms tubular crista (Figure 1.3). Thin sections of *Tetrahymena* cells show very characteristic distribution of these organelles just beneath the plasma membrane and alveolar sacs (Figure 1.3), although numerous mitochondria can also be found distributed randomly within the cytoplasm. When labeled with mitotrackers or immunofluorescently for confocal microscopy, mitochondria in actively growing cells appear as punctuate dots distributed close to the cell surface along the ciliary rows (Figure 1.3). As *Tetrahymena* cells age (stationary phase) some of the mitochondria move towards the center and become more spherical. This can be visualized in immunofluorescently labeled cells when working with stationary cultures (Bisharyan and Clark unpublished) and by electron microscopy(37).

*Tetrahymena* generally contain about 600 mitochondria per cell (58) although the number can increase to roughly 1200 just before division. This is correlated with the rate of cell growth and energy needs (37). *Tetrahymena* has a linear mitochondrial DNA (44) with 55 putative genes: three ribosomal RNA genes, eight transfer RNA genes, 22 protein-coding genes with putatively assigned functions, and 22 additional open reading frames of unknown function (44, 88). This compares with higher eukaryotes in which most mtDNA is circular and contains only 13 genes that are involved in oxidative phosphorylation (43).

Mitochondrial DNA in the parasitic ciliate *Ichthyophthirius multifiliis* has
not been extensively studied. However, based on morphological studies mitochondria in *Ichthyophthirius* have a characteristic elongated tubular appearance (Figure 1.3). In the infective theront stage of the parasite, mitochondria are concentrated at the anterior end of the cell just under the plasma membrane.

Electron microscopic analyses combined with direct calcium measurements have shown that dense Ca$^{++}$ granules are present in mitochondria of *Tetrahymena*. These granules show age dependant changes in Ca$^{++}$ concentration. For example compared to mitochondria from exponentially growing cells, mitochondria from stationary phase cells contain a large endogenous Ca$^{++}$ pool and dramatically loose their stability to increased concentrations of Ca$^{++}$ (36, 37).
Figure 1.3. Intracellular Distribution of Mitochondria in Ciliates. A. B. Confocal images of *Ichthyophthirius multifiliis* theronts fixed and labeled with antibodies against mitochondrial ATP synthase and HSP60 respectively C. Confocal *Tetrahymena thermophila* labeled with antibodies against ATP synthase D. TEM micrograph of a thin section of *Tetrahymena* cell showing mitochondrial distribution in the cell cortex along the basal bodies (m-mitochondria, bb-basal bodies).
B.4 Involvement of mitochondria cell Ca\(^{++}\) homeostasis

Ca\(^{++}\) plays an important role in regulating mitochondrial function and bioenergetics. At the same time, mitochondria act as Ca\(^{++}\) stores and are thus involved in cell Ca\(^{++}\) homeostasis by regulating and shaping Ca\(^{++}\) signals (38, 42). Because of their involvement in cell Ca\(^{++}\) homeostasis, mitochondria are often strategically located near the Ca\(^{++}\) release channels on the ER as well as beneath the plasma membrane (41, 123). Current models propose that physical interaction between mitochondria and the ER occurs via a Mitochondria-ER linker (75), which is supported by electron microscopy (28) studies as well as reconstruction of a synthetic linker (75). This linker is thought to allow rapid and very efficient Ca\(^{++}\) transfer between the two (75).

The proportion of mitochondria that associates with ER has been shown to be relatively constant (40), which is somewhat at odds the fact that mitochondria are also dynamic, motile organelles. In the context of the work presented in this thesis, interactions between mitochondria and the plasma membrane are especially interesting. In a majority of cells, a subset of mitochondria is located beneath the plasma membrane (41) and participates in subplasmalemmal Ca\(^{++}\) buffering and maintenance of the activity of Ca\(^{++}\) entry into the cytoplasm (54). A physical junction between mitochondria and plasma membrane Ca\(^{++}\) channels has recently been hypothesized (54, 55). Indeed, this junction is thought to be established in response to Ca\(^{++}\). The connection of mitochondria with the ER on the one hand, and with the plasma membrane on the other (both of which can supply Ca\(^{++}\)), along with their ability to accumulate substantial amounts of Ca\(^{++}\) makes mitochondria one of the central players in establishing and maintaining Ca\(^{++}\) homeostasis within the cell. Once Ca\(^{++}\) is elevated in the cytosol it is immediately taken up by pumps and exchangers to

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counteract the Ca\(^{++}\) rise (48). There are two main sources of Ca\(^{++}\) entry to the
cytosol, internal stores and the extracellular space. The internal stores
contribute to Ca\(^{++}\) release once IP3 receptors and ryanodine receptors (RyR)
are activated following cell stimulation (42). This, in turn, leads to store
operated Ca\(^{++}\) entry from the extracellular space. In cells with excitable
membranes, Ca\(^{++}\) flows into the cytosol via voltage gated Ca\(^{++}\) channels first,
which can then contribute to Ca\(^{++}\)-induced Ca\(^{++}\) release from the internal
stores (12). Mitochondria are known to be involved in both processes, and in
the case of excitable cells, their role is more clear and consistent (4). For
example, mitochondria rapidly accumulate Ca\(^{++}\) in the initial phase of Ca\(^{++}\)
entry, then slowly release it back to the cytosol prolonging the plateau phase
of Ca\(^{++}\) entry (4, 12). In the case of Ca\(^{++}\) release from internal stores (the main
source of which is the ER), the role of mitochondria is less clear. On the one
hand, mitochondria have been shown to accelerate and slow down Ca\(^{++}\) wave
propagation when Ca\(^{++}\) is released from the internal stores (12). On the other
hand, it has also been shown that oscillations in cytosolic Ca\(^{++}\) are
accompanied by Ca\(^{++}\) concentration oscillations in mitochondria and the ER
(57). This is counterintuitive for two reasons, first mitochondria themselves act
as Ca\(^{++}\) stores, and second the ER and mitochondria are connected and both
contribute to cytosolic Ca\(^{++}\) buffering (40, 57).

Another aspect of influence on cytosolic Ca\(^{++}\) signaling by mitochondria
is their ability to exhibit spatial Ca\(^{++}\) buffering in distinct areas of the cytosol
(96). A great example of this is how mitochondria are located around the
granular pole in pancreatic acinar cells to form a belt that retains Ca\(^{++}\) in the
granular pole (61, 96, 97).
Mitochondria contribute to cellular Ca\(^{++}\) homeostasis by taking up and releasing Ca\(^{++}\) therefore shaping Ca\(^{++}\) signals within the cells. Free cytosolic Ca\(^{++}\) is transported into the mitochondrial matrix where it can activate mitochondrial dehydrogenases and boost ATP production (42, 110).

Mitochondria have a high capacity for Ca\(^{++}\). This allows them to act as significant Ca\(^{++}\) stores in the cell, even more so than the ER (132). The ability of mitochondria to buffer and store Ca\(^{++}\) depends on phosphate and physiological concentrations of adenine nucleotides. The level of free Ca\(^{++}\) in the matrix depends on formation of an instantly reversible Ca\(^{++}\) phosphate complex (38, 132). The pathways of Ca\(^{++}\) entry into mitochondrial matrix are known as mitochondrial calcium uniporter (MCU) (70), the rapid mode (RaM) and the mitochondrial ryanodine receptor (42). Uptake via the mitochondrial calcium uniporter is considered the primary route of Ca\(^{++}\) influx into the mitochondria (62). MCU represents a gated, highly selective ion channel that can be activated by Ca\(^{++}\) itself and inhibited by a number of chemical reagents including lanthanides, ruthenium red, and others (70). The second pathway of entry, RaM, facilitates very rapid Ca\(^{++}\) influx into the mitochondria and is assumed to play a role in providing mitochondria with an immediate Ca\(^{++}\) burst when needed as a way of bypassing the slower pathway of MCU-mediated Ca\(^{++}\) uptake (42). The ryanodine receptor (RyR) represents the third mechanism of mitochondrial Ca\(^{++}\) import. Mitochondrial RyR is activated at low concentrations of Ca\(^{++}\) and is very similar to the RyR of skeletal muscle (42, 62). While Ca\(^{++}\) is essential for mitochondrial function and ATP synthesis, excess Ca\(^{++}\) can damage mitochondria and result in cell death in the absence of an effective Ca\(^{++}\) export mechanism (38). The main mechanism of Ca\(^{++}\)
export is the mitochondrial Na\(^+\)/Ca\(^{++}\) exchanger referred as NCX mito. Therefore the NCX mito is considered to be the physiological counterpart of MCU (33). Although the existence of both of the channels has been identified based on pharmacological criteria, the actual proteins that account for their activities are still unknown.

There are two sides of the effect of Ca\(^{++}\) on mitochondrial function. On the beneficial side Ca\(^{++}\) is a hallmark stimulatory signal for activation of numerous mitochondrial enzymes acting as positive effectors of oxidative phosphorylation. However, when overloaded, Ca\(^{++}\) becomes a pathological signal leading to opening of mitochondrial permeability transition pore (PTP) and subsequent initiation of apoptosis if not controlled (17, 27). The PTP is composed of different mitochondrial proteins located in both the inner mitochondrial membrane and outer mitochondrial membrane (OMM) that associate with each other to form a large channel. Upon opening of the PTP channel, mitochondrial membrane potential is lost and matrix solutes less than 1.5 kDa in size, along with Ca\(^{++}\), are released from mitochondria (17, 27, 124). These include pro-apoptotic molecules cytochrome c, AIF (apoptosis inducing factor), some pro-caspases, as well as pyridine nucleotide and ADP/ATP (77, 124).

The primary constituents of PTP are the outer mitochondrial membrane protein, VDAC, the inner mitochondrial membrane protein, ANT, and the Matrix protein cyclophilin D (5, 6). The voltage-dependent anion channel (VDAC) and adenine nucleotide translocase (ANT) are mitochondrial proteins that perform well-established roles in the cell. VDAC, a mitochondrial porin, is a large H\(_2\)O-filled pore that allows low M\(_r\) solutes to permeate freely across the outer membrane and gain access to the solute-specific transport systems of
the inner membrane (5, 6, 106). ANT mediates ADP–ATP exchange across the inner membrane, essential for the basic bioenergetic function of the organelle in supplying ATP to the cytosol (106). Cyclophilin-D (CyP-D) is an essential constituent of the permeability transition pore as well being a member of the cyclophilin protein family. It is thought to facilitate conformational change of the complexed protein by catalyzing the rotation of surface-exposed peptidyl prolyl bonds (106). In addition, cyclophilins (at least, CyP-A in *E. coli*) preferentially bind cis-Pro isomers. Thus, CyPs may catalyze the formation of, and stabilize, a particular conformation of the target protein (5). Other proteins such as members of Bcl 2 family also associate with PTP and play major roles in PTP opening, and apoptosis initiation as well as inhibition (5).

PTP opening does not always lead to apoptosis. In fact, transient PTP opening is thought to play a role in Ca$$^{2+}$$ release from overloaded mitochondria thus serving as a safety valve to prevent excess Ca$$^{2+}$$ from causing mitochondrial failure and cell death (13).

### B.6 Crosstalk between mitochondrial Ca$$^{2+}$$ and ROS

Reactive oxygen species are molecules formed by one electron reduction of oxygen. They include free radicals such as superoxide, hydroxyl radical and singlet oxygen as well as non-radical species such as hydrogen peroxide. These are highly reactive and can damage proteins, lipids and nucleic acids. In order to survive, cells have to maintain fine balance between ROS generation and ROS detoxification. Mitochondria are considered the main source of physiological production of ROS (13). Roughly 1 to 2% of the total electrons transported through the respiratory chain leak to produce...
superoxide anions (O$_2^-$), which can rapidly be converted into the more reactive H$_2$O$_2$ and subsequently to hydroxyl radicals (OH$^-$). More specific sites of ROS production in mitochondria are considered Complexes I and III (13, 127). Recently matrix enzymes such as alphaketoglutarate dehydrogenase, and other components of dehydrogenase complexes of the Krebs cycle have been also considered as important sources of ROS within mitochondria (103). Mitochondrial oxidative stress is caused by an imbalance between ROS generation and ROS detoxification (38). To balance the production of ROS, mitochondria house a sophisticated defense network of enzymatic and nonenzymatic antioxidants against ROS. Some of these include GSH (glutathione redox couple), NADH/NAD+, thioredoxin as well as the enzymes superoxide dismutase (SOD), GSH reductase etc (38).

The impact of mitochondrial Ca$^{++}$ uptake on mitochondrial ROS homeostasis is somewhat controversial. According to dogma, Ca$^{++}$ uptake by mitochondria induces membrane depolarization, which should result in uncoupling of the electron transport chain and a decrease in ROS production. However, a fundamentally different model has been proposed in which uncoupling leads to increased ROS generation because increased electron flux is needed to maintain membrane potential, thereby resulting in a higher probability of electron slippage and increased rate of superoxide generation at Complex III (38, 106, 125).

It has also been shown that Ca$^{++}$ can activate nitric oxide synthase (NOS) and generate NO (49, 109), which can have an inhibitory effect on Complex IV. Complex IV inhibition, in turn, can lead to increased production of ROS at the site of Complex III. According to this model, while Ca$^{++}$ can stimulate oxidative phosphorylation electron flux, it can also lead to partial
inhibition of the electron transport chain therefore increasing the probability of electron slippage to O$_2$ (79, 127).

Just as Ca$^{++}$ can influence ROS generation, cellular redox state can significantly modulate Ca$^{++}$ signaling (113, 131). This is the underlying concept of Ca$^{++}$ and ROS crosstalk. Many cellular processes are regulated by the redox state of the proteins, which can change the activity of enzymes and ion transporters (113, 131). In the context of Ca$^{++}$ signaling, many Ca$^{++}$ channels/pumps/exchangers are activated or inhibited by their redox state. These include ryanodine receptors, IP3 receptors, SERCA and PMCA pumps as well as plasma membrane Na/Ca exchanger (38, 113, 131). Since both Ca$^{++}$ and ROS play such important roles in regulating mitochondrial homeostasis, it is essential that mitochondria have the ability to manage both. In doing so, mitochondria utilize the ROS produced via oxidative phosphorylation as a signaling molecules for regulating Ca$^{++}$ transport. In summary, stimulation of mitochondrial Ca$^{++}$ signals by ROS and increased ROS generation results in positive feedback loop (38, 113, 131). While on the one hand physiological increases of Ca$^{++}$ are beneficial and necessary for mitochondrial metabolism and function, Ca$^{++}$ overload can be damaging and become pathological. The same is true for ROS - as a signaling molecule it is important, but at high levels ROS can lead to oxidative stress and mitochondrial dysfunction with more global effects at the cellular level (38, 42).

**B.7 Heat shock response and reactive oxygen stress**

Heat shock response is a ubiquitous adaptation mechanism of organisms ranging from bacteria to mammals that helps them to survive and adapt to a wide range of environmental challenges. Heat shock, like many
other stressors induces specific and highly regulated signaling cascades that function to restore cellular homeostasis. The most notable heat shock induced signaling pathways are the MAP-kinase (mitogen activated protein kinases), ERK (extracellular signal regulated kinase), SAPK1 (stress activated protein kinase 1), c-Jun N terminal kinase, and SAPK2-p38 with protein kinase B (PKB) (8, 92). While the mechanisms responsible for activation of these pathways are still unclear, protein denaturation, chromatin structure perturbation, and changes in cellular redox state are all possible consequences of heat shock that could trigger the above mentioned signaling pathways (15). More likely than not, the potential initiating factor for heat shock response is the membrane alteration (126). Different reports suggest that heat shock signals originate at the plasma membrane and could induce changes in membrane fluidity, which in turn would lead to a variety of cell stress responses varying from activation of voltage gated ion channels in excitable cells (65, 108) to clustering of certain growth factor receptors resulting in autophosphorylation and activation of downstream signaling cascades. These can lead to elevated intracellular Ca++ along with reactive oxygen stress. An important consequence of heat shock triggered signaling is the coordinated activation of a number of stress response proteins called heat shock proteins (64, 76). Heat shock proteins are a highly conserved and functionally interactive network of chaperone proteins that are either constitutively expressed or rapidly induced in response to a variety of chemical, environmental and physiological stresses. Heat shock proteins are classified into subfamilies according to their molecular weight (e.g. small Hsps [16-30 kDa]; Hsp40; Hsp60 [which is a mitochondrial protein]; Hsp70; Hsp90; and the large Hsp110). One of the primary functions of the Hsps are to act as
molecular chaperones that can disaggregate, refold and renature misfolded proteins (64, 76). Consequently, Hsps play a role in housekeeping and regulation of protein quality control under normal conditions, but are also essential for cell survival and death decisions under conditions of stress (64, 76). Due to their versatile function, Hsps can intervene following oxidative stress at several levels. First, they play a crucial role in clearance of damaged proteins by selecting and directing aberrant proteins to the proteasome or lysosomes for degradation (78). Second, in cases when misfolded proteins can be rescued, Hsps can refold damaged proteins (64). Heat shock proteins have also been shown to associate with membranes, particularly the GroEL chaperonins of bacteria, and their eukaryotic analogs, the HSP60s. Membrane bound Hsps can prevent aggregation of membrane-localized enzymes. This idea is supported by evidence that GroEL can bind to model membranes, which, in turn, is dependent on the physical state of the membrane (9, 128). In yeast, it has been shown that the integral plasma membrane protein Hsp30 can reduce membrane fluidity at higher temperatures (98, 116). Thus heat-induced membrane perturbation causes induction of Hsps, which then associate with membrane and reduce the induction signal in a feedback loop. Hsps can also negatively regulate apoptosis by binding and inhibiting members of apoptotic cascade. Additionally, some Hsps have anti-inflammatory actions (78, 92).

**B.8 The role of mitochondria in apoptosis**

Apoptosis (programmed cell death) is a highly-regulated mechanism of cellular self-destruction that plays critical roles in a wide variety of physiological processes in fetal and adult tissues resulting in removal of
unwanted cells and maintenance of tissue homeostasis (60). Defects in the control of apoptosis can lead to many diseases such as cancer, stroke, heart failure, neurodegeneration, etc (26, 34, 66). There are multiple pathways that can lead to apoptosis; the most well characterized pathways are the extrinsic and intrinsic pathways. The extrinsic pathway consists of cell surface receptors of TNF-related family (death receptors CD95/FAS and TRAIL) and their cytoplasmic counterparts and inhibitory molecules (FADD or FLIP)(105). The intrinsic pathway, in which mitochondria are central players, is also well characterized (107, 121). Mitochondria play a crucial role in regulating apoptosis by providing many important pro-apoptotic factors. Both intrinsic and extrinsic apoptotic signaling pathways converge and lead to caspase activation and nuclear fragmentation (105, 121). The intrinsic (mitochondrial) pathway can be initiated by a variety of stress stimuli including UV radiation, heat, DNA damage and elevated cytosolic Ca++ (121) These diverse forms of stress are sensed by the cell and are translated to signaling cascades directed to mitochondria. Alterations in outer mitochondrial membrane that result from these signals leads to opening of mitochondrial permeability pore and release of pro-apoptotic mitochondrial proteins (60, 122). The most important of these is Cytochrome C, which plays a crucial role in activating the cascade of cell death. In addition to Cytrochrome C, a number of other pro-apoptotic factors, including Omi/Htr2A , Smac/Diablo, AIF and endoG, are also released from mitochondria (60, 121, 122). The last two are only released in the case of severe damage to both the outer and inner membranes. Once the pro-apoptotic proteins are released from mitochondria, Cytochrome C activates Apoptosis Protease Activating Facor-1 (APAF-1) thereby initiating the formation the apoptosome. The apoptosome acts as a large platform for
recruiting and activating the caspase cascade. The whole process is regulated by additional factors like Smac/Diablo, which are capable of interacting with caspase inhibitors and interfering with their inhibitory action. AIF (apoptosis inducing factor) and OMI (mitochondrial protease) are thought to contribute to cell death by penetrating into the nucleus and contributing to nuclear condensation and DNA degradation. However, the role of these proteins in apoptosis has been debated since they are only released in later stages of the process, and genetic defects of these proteins induce a phenotype resembling mitochondrial diseases rather than reduction of cell death (as would be expected in the case of an Apaf-1 knockout for example). This raises the possibility that AIFI and Omi are normally required for mitochondrial homeostasis, and once mitochondrial membranes are compromised they leak into the cytoplasm and redistribute to other organelles (60, 121, 122).

The release mitochondrial pro-apoptotic factors is controlled by Bcl-2 family proteins. Bcl-2 proteins include the pro-apoptotic members, Bax and Bak, and the anti-apoptotic members, Bcl-2 and Bcl-xL, along with a series of BH3 domain pro-apoptotic proteins, a representative of which is, Bid, that functions upstream of Bax and Bak. The relative ratios of anti- and pro-apoptotic Bcl-2 members dictate the sensitivity or resistance of cells to various apoptotic stimuli, including hypoxia, oxidants, heat and Ca^{++} overload (27, 60, 121).

Besides anti-apoptotic Bcl-2 proteins, apoptosis is also regulated by heat shock proteins. Hsps have been shown to block apoptosis by interfering with caspase activation. In many different cellular models, over expression of Hsp27, Hsp60, Hsp70 and Hsp90 has been shown to inhibit apoptosis and prevent caspase activation. By contrast, depletion of the same Hsps makes
cells more sensitive to apoptotic signals. Mechanism of apoptosis inhibition occur at three different levels, namely, 1) upstream of mitochondria, by modulating signaling pathways, 2) at the level of the mitochondria themselves, by controlling the release of apoptogenic molecules and 3) at the post-mitochondrial level, by blocking apoptosis at a later phase than any known survival enhancing drug or protein (78, 92).

While the role of apoptosis or apoptosis-like mechanisms in unicellular eukaryotes is still being explored, various forms of apoptotic cell death have been described in protozoa, clearly challenging the view that apoptosis emerged with evolution of multicellular organisms (14, 74). To date, apoptosis-like processes have been described in Trypanosomes, Leishmania, Dictyostelium and Tetrahymena. In Tetrahymena, the most well characterized of these is referred to as “programmed nuclear death” (PND) which occurs during conjugation and leads to fragmentation of macronuclear DNA. While the precise mechanisms that lead to this phenotype have yet to be determined, there is some evidence that mitochondria play a role through the release of AIF into the parental macronucleus which then initiates DNA degradation (14, 74).

C. Objectives and organization of dissertation

The original goal of this project was to identify signaling mechanisms triggered by cross-linking of GPI-anchored proteins (immobilization antigens) in ciliated protozoa. The work was prompted by observations made over 100 years ago in Paramecium, which showed that rabbit antisera prepared against whole cells cause rapid loss of cell motility. While the target antigens involved in this response are well known, the mechanisms responsible for
immobilization are still unclear. To conduct our studies we used *Ichthyophthirius multifiliis* a parasitic ciliate that infects freshwater fish, and *Tetrahymena thermophila*, a well-studied free-living ciliate as model systems. We hypothesized that clustering of i-antigens with specific antibodies leads to Ca$^{++}$ mobilization either by directly activating Ca$^{++}$ channels on the plasma and ciliary membranes, or through additional downstream pathways leading to Ca$^{++}$ release from internal stores. Our underlying hypothesis was that elevated cytosolic Ca$^{++}$ leads to a number of behavioral changes including immobilization of the cells. To test this, we constructed and expressed a Ca$^{++}$ sensitive GFP reporter construct in *Tetrahymena* that helped to prove the hypothesis and show that immobilization is, in fact, a Ca$^{++}$-dependent process (Chapters 2 and 3). To further clarify the mechanisms leading to Ca$^{++}$ elevation, the association of i-antigens with detergent-resistant membranes, or “lipid rafts” was investigated with the idea that raft-mediated signaling might lead to Ca$^{++}$ mobilization. These studies revealed major changes in lipid raft composition in response to i-antigen clustering. Surprisingly, the i-antigens themselves were lost from raft fractions following antibody binding and were shed into the medium. The entire process was accompanied by the formation of 50-100 nm vesicles at the cell surface, aggregation of the vesicles on the plasma membrane, and shedding from the ciliary tips where vesicles typically coalesce prior to being released from cells (chapter 2).

In the process of carrying out these studies we made a surprising discovery that mitochondria are extruded from both *Tetrahymena* and *Ichthyophthirius* in response to i-antigen cross-linking (Chapter 3). This was a diversion from our original goal but provided us with a unique opportunity to study an unusual and novel phenomenon. Mitochondrial extrusion could have
major implications in fundamental cell biology (chapter 3) and became the centerpiece of the dissertation. To understand the mechanisms of mitochondrial extrusion we expanded the work beyond i-antigens and demonstrated that mitochondrial extrusion can be induced by another form of cell stress, namely, heat shock.

To summarize the work, I provide a model and future directions, which are discussed in detail in Chapter 4.
REFERENCES


20. **Clark T. G., Dickerson, H. W.** 1997. Antibody-mediated effects on


100. Plattner, H., and N. Klauke. 2001. Calcium in ciliated protozoa:


120. **Stefanova, I. e. a.** 1991. GPI-anchored cell surface molecules complexed to protein tyrosine kinases. **254**:1016-1019.


Chapter two

Ciliate Immobilization antigens trigger Ca$^{++}$ mobilization in response to antibody binding accompanied by major changes in membrane composition
Abstract

Ciliate GPI-anchored proteins were discovered in *Paramecium* at the beginning of last century. Antibodies against these proteins trigger a number of behavioral changes in ciliates, among which are DCG (dense core granule) secretion and arrest in cell movement (hence the name immobilization antigens). Despite the fact that immobilization antigens have been subjects of extensive studies in their connection with antigenic variation and host parasite interactions, signaling mechanisms induced by clustering of these proteins on the cell surface are still unknown. Using a chimeric GFP reporter we show that antibody binding to i-antigens induces sustained Ca^{++} response, which can be blocked by Ca^{++} chelators and L-type channel inhibitors Verapamil and Nifedipine. Lack of extracellular Ca^{++} partially restored cell movement, indicating that immobilization is a Ca^{++} dependant process. Interestingly changes in intracellular Ca^{++} were accompanied by major rearrangements in the lipid raft proteome and loss of i-antigens, as well as other raft associated proteins, from the cell surface. TEM analysis revealed that antigens were being shed from the cell surface in membrane vesicles that seemed to originate from plasma membrane and coalesce at the ciliary tips prior to being released into the surrounding space.
**Introduction**

Immobilization of ciliated protozoa by antibodies against GPI-anchored surface proteins (i-antigens) was described more than 100 years ago. Despite this, and despite the fact that regulation of ciliary beat is a fundamental problem in cell biology, the precise mechanisms responsible for immobilization are still unknown. I-antigens are abundant proteins distributed on the plasma and ciliary membranes (figure 3.1A), and physical cross-linking of cilia in the presence of bivalent antibodies could potentially inhibit cell movement. However, a number of observations suggest that lateral clustering of GPI-anchored proteins in the plasma membrane results in a transmembrane signaling event that alters normal ciliary beat. First, at lower antibody concentrations the cells undergo ciliary reversal, followed by backward swimming, a classical avoidance reaction in ciliates (9, 27). Ca\(^{++}\) controls ciliary beat frequency, and ciliary reversal has been shown to be a result of series of complex interactions between voltage gated ion channels, Ca\(^{++}\) and ciliary ATP levels (1, 25). Second, antibodies against i-antigens trigger discharge of secretory granules in a variety of ciliates (9, 18). In ciliates, as well as in all other eukaryotic cells, regulated secretion has been shown to occur as a result of elevated Ca\(^{++}\) near the docking sites of secretory granules, where it initiates Ca\(^{++}\) dependant membrane fusion and subsequent release of granule content (2, 17, 27). Third, immobilization antigens are GPI-anchored proteins (10), which allows them to associate with lipid rafts, which are considered to be platforms for organizing signaling molecules, ion channels and ordered lipids on the plasma membrane (16, 21, 29). Crosslinking of GPI-anchored proteins in higher eukaryotes has been shown to initiate
transmembrane signaling events leading to Ca\(^{++}\) mobilization in the cells. This is nicely demonstrated in mammalian cells using a GFP tagged GPI-anchor (14, 26).

With the idea that calcium could be involved as a second messenger in the phenomenon of immobilization, we transformed the free-living ciliate *Tetrahymena thermophila* with a circularly permutated GFP reporter gene construct that allowed continuous monitoring of intracellular Ca\(^{++}\) in live cells. Cross-linking of GPI-linked surface proteins with specific monoclonal antibodies triggered rapid and sustained increases in intracellular Ca\(^{++}\) in these cells. Such increases were blocked by the addition of either calcium chelators, or L-type calcium channel inhibitors to the culture medium, suggesting the involvement of plasma membrane ion channels in the process. Notably, the same drugs partially restored normal motility in a majority of antibody-treated cells in culture. By contrast, cells treated with the calcium ionophore, A23187, in the absence of antibody underwent rapid cessation of forward movement followed by backward swimming. Taken together, these observations argue strongly that elevation in intracellular Ca\(^{++}\) is responsible for immobilization of ciliated protozoa in response to antibody binding.

Clustering of raft associated proteins in higher eukaryotes has been shown to result in enrichment in lipid rafts (15, 23). In contrast, i-antigen clustering resulted in progressive loss of these proteins from raft fractions. This was consistent with previous reports showing release of antigens into the surrounding space in *Paramecium* (18) followed by antibody crosslinking. Transmission electron microscopy has revealed that i-antigens undergo massive reorganization in response to antibody binding, entering membrane vesicles and forming larger aggregates that migrate to the tips of the cilia.
before being shed into the culture medium. We posit that elevation of
intracellular Ca\(^{++}\) and subsequent immobilization are directly attributable to the
activation of excitatory L-type channels on ciliary membranes following whole-
scale reorganization of GPI-anchors in the lipid bilayer. Involvement of i-
antigens in membrane dynamics and adaptation in unicellular ciliates and their
effect on ciliary function are discussed.

Results

I-antigens are abundant proteins on the surface of ciliated protozoans:
Our lab has a long standing interest in immobilization antigens as vaccine
candidates against the parasitic ciliate *Ichthyophthirius*, the causative agent of
white spot disease in fish (35). *Ichthyophthirius* i-antigens are primary targets
of the host immune response and good vaccine candidates (6-8). For the
purpose of vaccine development, *Tetrahymena thermophila* is being used as a
recombinant protein expression system for *Ichthyophthirius* i-antigens (3).
Crosslinking of parasite i-antigens on the surface of recombinant *Tetrahymena*
immobilizes the cells in culture, indicating that parasite antigens expressed in
a free living ciliate are capable of inducing similar downstream effects as in the
parasite itself (Figure 2.1B,C supplemental movie) (3, 6). This prompted us to
use *Tetrahymena* as a model system to study the physiological function of
parasite antigens, and downstream signaling events associated with
crosslinking of these proteins on the cell surface.

I-antigen clustering induces sustained Ca\(^{++}\) mobilization in the cytosol.
Based on observations that i-antigen cross-linking can induce reversal of
ciliary beat, immobilization, and regulated secretion in a variety of ciliate
species (4,13) we hypothesized that clustering of i-antigens triggers an
Figure 2.1 Immobilization antigens. A Confocal image of *Ichthyophthirius* theronts labeled with monoclonal antibodies against immobilization antigens, showing uniform distribution of antigens on ciliary and plasma membranes. B and C Confocal images of representative *Tetrahymena* cell cultures expressing *Ichthyophthirius* i-antigen G52B prior (B) and after (C) treatment with Ichthyophthirius i-antigen specific antibodies. Size bar is 50µm
A

B -ab

C +ab

10µm
increase in intracellular Ca\textsuperscript{++} which in turn leads to the observed responses. To determine whether intracellular Ca\textsuperscript{++} is elevated in response to i-antigen cross-linking, a calcium-sensitive reporter construct, GCamP2 (24), was introduced into *Tetrahymena* cell lines expressing *Ich* i-antigens.

As previously described (24), GCamP2 consists of a fusion protein with an N-terminal calmodulin binding peptide and a C-terminal calmodulin domain that are separated by a circularly permuted, green fluorescent protein (GFP) (24). In the absence of calcium, the fusion protein (GCamP2) is only weakly fluorescent (Figure 2.2A). However, as calcium concentrations increase above the Kd for the reporter, calmodulin binds to its ligand, resulting in a conformational change in the modified GFP component and a large increase in fluorescence that can be readily measured (24). The chimeric reporter gene (a gift from Dr. Michael Kotlikoff’s laboratory at Cornell University) was engineered into a *T. thermophila* expression vector that has a neo selection cassette, and cadmium-inducible metallothionin gene promoter for robust expression (Figure S2.1) of the reporter in *T. thermophila* following the addition of cadmium to the growth medium (34).

Shortly after crosslinking i-antigens on the surface of double transgenic *Tetrahymena* cells expressing GCamP2 reporter and parasite antigens, several fold increase in GFP fluorescence within the cytosol was easily detectable under the confocal microscope (Figure 2.2B). Spectrofluorimetric measurements showed a 2-4 fold increase in GFP fluorescence which was equivalent to increased cytosolic Ca\textsuperscript{++} in response to treatment (Figure 2.2C). To test if GFP fluorescence increases can be prevented in the absence of Ca\textsuperscript{++}, common Ca\textsuperscript{++} chelators EGTA and Bapta AM were used. As expected both of the chemicals inhibited increase of GFP fluorescence in response to
antibody treatment (Figure 2.3A). To test the hypothesis and to demonstrate that depletion of Ca^{++} has an effect on cell behavior following antibody treatment, cells were induced to undergo immobilization in buffer with no Ca^{++}. As expected treatment of the cells with i-antigen specific antibodies in the absence of Ca^{++} resulted in a marked decrease in the number of cells that were immobilized (Figure 2.3C and supplemental movie), and in a reduction of the overall duration of the response. This was manifested by cells starting to swim within 15-30 min following immobilization in no Ca^{++} buffer, compared to the controls in buffer with Ca^{++} in which immobilization typically lasts between 1 to several hours. If elevated cytosolic Ca^{++} was the underlying cause of immobilization, we reasoned that cells would immobilize in response to treatment with Ca^{++} ionophore, since we showed earlier that treatment with A23187 resulted in several fold increase in GFP fluorescence with the Ca^{++} reporter (Figure 2.2D). To test this, cells were treated with different concentrations of ionophore A23187. At lower concentrations (1-2 µmolar) the ionophore caused backward swimming followed by clustering of 50% of the cells consistent with previous reports (25). At higher concentrations (5-10 µmolar) the cells stopped swimming shortly after treatment and slowly settled on the bottom of the plate, indicating that the ionophore was inducing behavioral changes in the cells similar to those observed following the addition of antibodies against i-antigens (data not shown). Next we tried to further dissect the signals leading to Ca^{++} elevation within the cytoplasm. Given the excitable nature of ciliary membranes, we hypothesized that antibody induced clustering of i-antigens triggers activation of voltage gated ion channels on ciliary membranes, leading to increase in intracellular Ca^{++}. To test this, L-type channel inhibitors
Figure 2.2 Ca\(^{++}\) mobilization in response to i-Ag clustering. A and B. Convocal micrographs of live double transgenic Tetrahymena expressing Ichthyophthirius antigens and a GCamP2 reporter prior to (A) and after (B) antigen crosslinking induced by the addition of antibodies against immobilization antigens. C. Spectrofluorimetric readings of the same cultures in the presence and absence of the same antibodies. D. Spectrofluorimetric readings of GCamP2 expressing Tetrahymena in the presence of ionophore A23187.
Figure 2.3 Immobilization of the cells is Ca\textsuperscript{++} dependent. A. Spectrofluorimetric readings of double transgenic *Tetrahymena* expressing Ichthyophthirius antigens and a GCamP2 reporter following treatment with antibodies against i-antigens incubated with of Bapta AM and EGTA. B. Double transgenic *Tetrahymena* cells in the absence of immobilizing antibodies. C. Double transgenic *Tetrahymena* following treatment with i-antigen specific antibodies. Note that cells are still immobilized 2hr after treatment, and remain clustered together in multicellular aggregates. D. Same culture as C, only in the absence of Ca\textsuperscript{++}. Note the cell aggregates are much smaller and there are a large number of swimming cells. E and F. Spectrofluorimetric readings of double transgenic *Tetrahymena* incubated with L type channel inhibitors Nifedipine and Verapamil prior to antibody treatment.
Figure A: Graph showing relative fluorescence intensity over time for different conditions: -ab –Ca++ (black line), Bapta +ab (magenta line), EGTA+ab (green line).

Figures B, C, and D: Microscopic images of cell cultures under different conditions. B shows -ab –Ca++, C shows +ab +Ca++, and D shows +ab –Ca++.

Figure E: Graph showing time course of relative fluorescence intensity for -ab –Ca++ (black line) and Nifedipine+ab (purple line).

Figure F: Graph showing time course of relative fluorescence intensity for +ab (black line) and +ab +verapamil (purple line).
Nifedipine and Verapamil were used to inhibit Ca$$^{++}$$ fluxes in response to antigen crosslinking. Treatment with both of the inhibitors resulted in complete abrogation of GFP florescence increase in the presence of anti-i-antigen antibodies, indicating possible involvement of voltage gated calcium channels in this process. The fact that L-type channel inhibitors resulted in complete inhibition of Ca$$^{++}$$ signals after treatment lead to the hypothesis that i-antigen clustering on the cell surface directly affects channel activity, resulting to channel opening and Ca$$^{++}$$ influx into the cells. Given their abundance on the membranes, we hypothesized that aggregation of i-antigens leads to changes in membrane composition, which in turn affect channel activity. As GPI anchored proteins, immobilization antigens have been shown to associate with detergent resistant membranes known as lipid rafts in *Tetrahymena* and *Paramecium* (19, 36). Given this, any specific changes were considered more likely to occur in lipid rafts.

*Ichthyophthirius* i-antigens associate with DRMs (detergent resistant membranes) in *Tetrahymena*: To test the hypothesis that i-antigen clustering induces changes in lipid raft composition we first verified that recombinantly expressed *Ichthyophthirius* i-antigens associate with *Tetrahymena* DRMs. Cells were treated for 1 hr with nonionic detergent Triton X-100 either at 4°C or 37°C, and the resulting material centrifuged 16,000 x g. Quantitative densitometric analysis of Western blots of material from cell pellets and supernatant fractions showed that a substantial fraction of i-antigens (at least 50% based on this method) were associated with detergent insoluble material that pelleted at 4°C (Figure 2.4). The corresponding absence of GPI-anchored proteins in pellets isolated at 37°C clearly argues
that Ich i-antigens associate with *Tetrahymena* DRMs (Figure 2.4). These results are consistent with those of Ko and Thompson who showed that endogenous GPI-anchored proteins are enriched in detergent resistant membrane fractions of *Tetrahymena* (19).

**Clustering of Immobilization antigens induces major rearrangements in lipid raft proteome.** To test if clustering of i-antigens was inducing changes in lipid raft composition, detergent resistant membranes (equivalent to lipid rafts) from transgenic *Tetrahymena* were isolated prior to and after treatment with antibody for varying time periods. Analysis of lipid raft fractions by 2D gel electrophoresis revealed a number of dramatic changes in raft proteome that occurred as a result of antibody treatment (Figure 2.5). These involved a loss of numerous proteins as well as recruitment of new proteins to detergent resistant raft fractions (Figure 2.5 B, C, D). Most of the changes occurred within 5 min to 1hr after i-antigen clustering. The loss of different raft associated proteins in response to treatment raised the question of whether i-antigens themselves were being lost from lipid rafts.

**I-antigens disappear from lipid rafts in response to antibody treatment:** To examine the fate of i-antigens in rafts after treatment with antibodies, lipid rafts were isolated based on flotation on sucrose gradient prior to and 1hr following antibody crosslinking. Consistent with previous data, roughly 50% of total i-antigens associated with lower density detergent resistant fractions in resting cells (Figure 2.6 A), and antibody crosslinking resulted in progressive loss of i-antigens from lipid raft fractions by 1hr after treatment (Figure 2.6 A).

**I-antigens are shed from the cell surface into the extracellular space:** In the case of both *Tetrahymena* and *Paramecium*, i-antigens have been shown
to be released from the cell surface following antibody crosslinking, as well as in response to environmental changes such as temperature and pH (9). If *Ichthyophthirius* antigens were being shed as well, one would predict that they could be recovered from the medium following low-speed centrifugation of cells. To test this, *Tetrahymena* cells expressing Ich i-antigens were incubated for 1 hr in the presence or absence of specific antibodies, and then separated from media by centrifugation at 300 X g. To avoid cell contamination, the media was spun a second time and examined under the microscope to ensure that no cells were present. As predicted, a substantial fraction of the total i-antigen present in cells (>55%) was recovered from the media subsequent to antibody-mediated cross-linking (Figure 2.6B). In contrast, little if any protein was present in the medium surrounding cells not treated with antibody (Figure 2.6B). Taken together, these data clearly show that i-antigens are being shed from lipid rafts as well as ciliary and plasma membranes as a result of clustering in the membrane.

I-antigens are shed as hydrophobic proteins, possibly with the GPI-anchor intact. To gain insights into the mechanism of shedding we examined the state of the shed antigens. If the GPI-anchor were being cleaved prior to shedding, i-antigens would be expected to partition into the hydrophilic phase when treated with nonionic detergent Triton-X114. This has previously been demonstrated for Ich i-antigens following treatment of the protein with exogenous GPI-PLC (10), which resulted in a shift of the protein from hydrophobic to a hydrophilic state. To test this idea, the media containing the shed material was collected and treated with TritonX-114 detergent, followed by incubation at 30ºC to form detergent micelles which trap any hydrophobic proteins and partition them into the detergent phase when spun at low speed.
Western blotting of the detergent and aqueous phases showed that i-antigens partition to detergent phase, strongly suggesting that they were still hydrophobic, either due to retention of their GPI anchor, or due to their association with other hydrophobic proteins in the membrane (Figure 2.5 C).

When crosslinked with antibodies i-antigens formed aggregates at the ciliary tips, which were subsequently released into the media. When crosslinked with specific antibodies, *Tetrahymena* and *Paramecium* i-antigens have been shown to aggregate at the ciliary tips, where they are subsequently released into the surrounding space (18). To test whether this is also true for *Ichthyophthirius* i-antigens, a series of confocal experiments were conducted with the parasite as well as *Tetrahymena* expressing parasite i-antigens. Results of time course experiments are shown in Figure 2.7. In both types of cells, prior to antibody addition, i-antigens were uniformly distributed on ciliary as well as plasma membranes (Figure 2.7A, D). Shortly after cross-linking, however, i-antigens began to redistribute towards the ciliary tips (Figure 2.7B, E), and in most cases, were no longer visible by 30 min to 1 hr (Figure 2.7 C, F) indicating that the antigens were being lost from the cell surface.

**Immobilization antigens are shed in membrane vesicles.** In order to better understand whether i-antigens were being released as individual proteins or in association with membrane vesicles, we conducted TEM analysis of the cells prior to and following treatment with antibody for varying time periods. Examination of thin sections revealed 50-100nm membrane vesicles originating from plasma membranes 5 min after antibody treatment (Figure 2.7G), which then aggregated at later time points and were visible at the ciliary tips by 30 min (Figure 2.7 H, G, K). Examination by TEM of shed material collected from the media of the cells treated with antibody revealed large
amounts of vesicles, indicating that vesicles originating from plasma membrane were being shed from the cells (Figure 2.8A). Overall data obtained from TEM analysis of antibody treated cells was very consistent with previously obtained confocal results showing aggregation and coalescence at the ciliary tips (Figure 2.7 A-F). To our surprise there were numerous mitochondria, either intact or broken down, in the pellet along with shed vesicles (for further information the reader is referred to chapter 3 of the thesis). To confirm that the vesicles seen with TEM actually represented the aggregates seen with the confocal microscope, thin sections showing cells with membrane vesicles were labeled with anti-i-antigen antibodies followed by gold labeled secondary antibodies. The vesicles stained very heavily with anti-i-antigen antibodies (Figure 2.8B), in sharp contrast to the controls that showed uniform distribution of gold along the ciliary and plasma membranes (Figure 2.8C).

**Shed vesicles are a complex mixture of proteins.** Next we asked if vesicles contained other proteins in addition to i-antigens. This was based on the observation that a number of other raft associated proteins were being lost upon treatment with antibodies along with i-antigens. To answer the question, shed material from treated cells was collected and analyzed on a 2-dimensional gel (Figure 2.8 D). Analysis of the shed material revealed a complex mixture of proteins, and a Western blot of the same gel showed that the i-antigens were not the predominant proteins in the mixture (Figure 2.8D inset). This of course does not exclude the possibility of contamination by the proteins released from the mucocysts and mitochondria due to the cells undergoing regulated secretion and mitochondrial extrusion (for details see chapter 3 and chapter 2 Introduction).
Figure 2.4 *Ichthyophthirius* i-antigens associate with *Tetrahymena* DRMs. Recombinant *Tetrahymena* cells expressing *Ichthyophthirius* i-antigens were treated with Triton-X100 at 37°C and 4°C as described in results section. The resulting cell lysates were centrifuged to separate detergent soluble and insoluble fractions and pellets and supernatants subjected to Western blotting. Blots were probed with primary antibody against i-antigens.
Figure 2.5 Time dependant rearrangement of the lipid raft proteome following i-Ag crosslinking. 2D gel analysis of pooled lower density DRM fractions from a sucrose gradient of recombinant *Tetrahymena* cell lysates prior to (A) and 5min (B), 20min (C) and 1hr (D) following treatment with antibodies. Blue circles indicate raft proteins that are being lost following i-antigen clustering with specific antibodies, red circles indicate proteins that are being enriched or recruited to lipid rafts in response to the same treatment.
Figure 2.6 I-antigens are shed from lipid rafts into the extracellular space as hydrophobic proteins. A. Western blot of sucrose gradient fractions collected from cell lysates of *Tetrahymena* expressing Ich i-antigens. Cells were collected immediately before and 1 hr after treatment with i-antigen specific antibody. Fractions 1-6 represent lower density raft fractions. Blot is probed with antibody against i-antigens. B. Western blot of cells and supernatants from the same *Tetrahymena* strain as in A, harvested prior to and 1hr after antibody treatment. Shows the release of antigens into the media in antibody treated samples. C. Western blot of the supernatant from the same *Tetrahymena* strain as in A, harvested from cells prior to and 1hr after antibody treatment. Supernatants were treated with TritonX114 and the detergent phase was separated from aqueous phase following incubation at 30°C. Shed antigens partition into the detergent phase.
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Figure 2.7 I-antigens are shed from the cell surface in membrane vesicles: **A, B, C**, Confocal images of recombinant *Tetrahymena* expressing Ich i-antigens labeled with antibodies against i-antigens before, 15min (B) and 1hr (C) after crosslinking with antibodies. **D, E, F** Confocal images of *Ichthyophthirius* labeled with same antibodies before (D), 15min (E) and 40min (F) after treatment. **C, F** insets show merged fluorescence and bright field images of the same cells. **G, H, I, K** Transmission electron micrographs of cells treated with antibody 5min (G) and 30min (H, J, K) after antibody treatment. (c-cilia, mv-membrane vesicles, mc-mucocytes) The arrows show membrane vesicles estimated to be 50-100nm.
Figure 2.8 I-antigens are in shed in vesicles that are a mixture of proteins: A, Transmission electron micrograph of vesicles harvested from culture supernatant treated with antibodies. Shows aggregated vesicles. The inset shows higher magnification of the same image. B, C, Immunogold labeled cryosections of cells undergoing vesicle shedding (B) show heavy labeling of vesicles with antibodies against i-antigens in contrast with the controls that show uniform distribution of gold on plasma and ciliary membranes (C). D, 2-Dimentional analysis of harvested vesicles from the media of antibody treated cells shows numerous other proteins in addition to i-antigens.
Using *Tetrahymena thermophila* expressing a GCamp2 GFP reporter gene, we show that crosslinking of i-antigens at the cell surface induces a sustained increase in cytosolic Ca++ that we believe contribute to number of behavioral changes, most notably immobilization of the cells. Based on spectrofluorimetric readings, elevated Ca++ signals could be detected within seconds of antibody addition and peaked between 3-5 min after treatment. Within the same time frame, 90-100% of cells became immobilized suggesting that increased levels of intracellular Ca++ and immobilization are linked. Indeed incubating cells with immobilizing mAbs in the absence of Ca++ decreased the rate and duration of immobilization and restored normal swimming behavior to ~ 50% of the cells in culture indicating that extracellular Ca++ is required for complete immobilization to occur. To determine whether elevated intracellular Ca++ was required to immobilize cells in response to i-antigen clustering, we investigated the effects of the calcium chelator BAPTA-AM at concentrations that allow forward swimming (it should be noted that some level of extracellular Ca++ is required to maintain ciliary beat and its complete removal with high concentrations of calcium chelators will completely immobilize cells even in the absence of i-antigen crosslinking). At moderate concentrations of BAPTA-AM that allowed forward swimming (*albeit* at a slower rate), we found that elevation of intracellular Ca++ in response to antibody was blocked, and at the same time, cells were no longer immobilized. This would clearly suggest that increased intracellular Ca++ is a prerequisite for immobilization in response to i-antigen crosslinking.

To determine the mechanisms underlying Ca++ mobilization, we investigated the effects of the L-type calcium channel blockers Verapamil and
Nifedipine on intracellular Ca++ in transgenic cells expressing the GFP reporter construct. Both drugs blocked elevation of Ca++ in response to antibody suggesting the involvement of voltage gated ion channels in the process. While the concentrations of Verapamil and Nifedipine used in these experiments were in the 50 μM range, we cannot not entirely rule out the involvement of other types of Ca++ channels in the response since both inhibitors can act nonspecifically on a range of channels, particularly in ciliates (28). In this regard, it would be of interest to study the entire process in pawn mutants in Paramecium which are known to lack ciliary voltage-gated Ca++ channels, and which would be predicted to swim following i-antigen cross-linking (4, 5, 32, 33).

The possible involvement of L-type calcium channels in this case is highly suggestive of a model in which opening of the channels triggers the release of Ca++ from internal stores and sustained elevation of intracellular Ca++ lasting hours. The most likely source of calcium from internal stores would be the alveolar sacs, a set of membranes common to alveolates (namely, apicomplexans, ciliates and dinoflagellates) that surround the cell just beneath the plasma membrane. Alveolar sacs are operationally equivalent to the sarcoplasmic reticulum of muscle (31), and due to their localization just beneath the plasma membrane can respond quickly to stimuli by releasing of Ca++ into the cytosol. Elevated Ca++ itself can act as a negative feedback and reduce inward Ca++ currents as the cells try to reestablish the altered Ca++ homeostasis. Consistent with this, Kung et al. have shown that inward Ca++ current in Paramecium decreases within 30 min of treatment with i-antigen-specific antibodies (30). Nevertheless, how calcium impinges on ciliary beat and cell motility in these cells is a complex issue. Ciliary beat frequency is
modulated by the interplay between internal Ca\(^{++}\) levels and other second messengers such as cGMP and cAMP, as well as ATP as an energy source. It has been shown that Ca\(^{++}\) regulates ciliary beat by modifying ATP levels (1, 25) via mechanisms that are not well understood. In Chapter 3, we demonstrate that mitochondria are jettisoned from cells in a Ca\(^{++}\)-dependent process following i-antigen crosslinking (see Chapter 3). If elevated intracellular Ca\(^{++}\) levels cause mitochondrial damage and/or extrusion, then immobilization of cells could be attributed to Ca\(^{++}\) induced depletion of intracellular ATP. To determine whether mitochondrial ATP generation is directly responsible for cessation of cell movement, one could test whether microinjection of ATP into immobilized cells would restore normal swimming behavior.

Regulation of calcium channel activity has been shown to depend on membrane environment (1, 12), and a variety of stimuli that alter membranes can act as triggers to activate them. These stimuli can affect properties of the lipid bilayer itself, or can activate specific kinases or phosphatases that alter channel behavior directly through phosphorylation/dephosphorylation (12). Here we show for the first time that clustering i-antigens on the cell surface induces major changes in lipid raft composition manifested by the loss of selected proteins (including i-antigens) and recruitment of others into rafts. In the course of my thesis, I was able to show that those changes are accompanied by serine threonine as well as tyrosine phosphorylation of specific lipid raft associated proteins (data not sown). Whether or not these changes are responsible for activation of Ca\(^{++}\) channels located on the ciliary and plasma membrane is not clear and would have to be directly demonstrated.
Despite this, changes in lipid raft composition were accompanied by major changes in the membrane itself visible by transmission electron microscopy. In particular, i-antigen crosslinking resulted in the formation of 50-100 nm membrane vesicles that aggregated over time and accumulated at the tips of cilia before being shed into the extracellular space. Based on immunolabeling with specific antibodies, these vesicles originate at the plasma membrane and are enriched in i-antigens as expected. Nevertheless, when analyzed by 2D gel electrophoresis, membrane aggregates where found to contain a complex mixture of other proteins in addition to the i-antigens themselves. Since i-antigens preferentially associate with lipid rafts, one would expect that some of the proteins lost from detergent-resistant raft fractions (shown in Figure 2.3) are associated with shed membrane vesicles. Indeed, given that rafts are considered platforms for the assembly of signaling complexes, a more thorough analysis of detergent-resistant membrane fractions and shed vesicles is warranted using LC/MS/MS.

Shedding of membrane microvesicles has been described in number of other cell types (if not all). Recent reports indicate that shedding vesicles are involved in surface membrane traffic, horizontal transfer of proteins and mRNAs among neighboring cells, and intercellular signaling and communication (11, 13, 20). Immobilization antigens have been shown to be released from the surfaces of free-living and parasitic ciliates under variety of conditions including environmental stress (22). If vesicle formation and shedding is a generalized mechanism of surface antigen release in ciliates it would be tempting to speculate that the process is required for rapid phenotypic adjustments to constantly changing conditions encountered by ciliates in their environment. I-antigens are abundant GPI anchored proteins.
and would be expected to play a role in membrane fluidity maintenance and adaptation. I propose that the release and uptake these proteins in response to changes in the environment, allows ciliates to vary their lipid and membrane protein repertoire in order to increase membrane fitness and enhance cell survival.

**Materials and Methods**

* Tetrahymena cultures: Recombinant *Tetrahymena* strains expressing *Ichthyophthirious* i-Antigens IAG52B (G5) and IAG48 (G1) were made as previously described (ref). Cell lines were grown in NEFF (0.25% proteose peptone, 0.25% yeast extract, 0.55% glucose, and 0.033 mM FeCl3) at 30ºC with constant shaking at 90 rpm and allowed to reach late log to early stationary phase. At this stage cultures were induced with 2µg/ml CdCl2 and harvested 12-16 hours after induction. Cells were harvested by centrifugation 300xg for 3min.

* Ichthyophthirius growth and maintenance: *Ichthyophthirius multifiliis* strain G5 was maintained on channel catfish as previously described. Trophonts were collected by gently rubbing the infected fish after which the parasites were incubated overnight at room temperature in carbon filtered water. After trophonts hatched and formed theronts the culture was filtered with fine wire mash filters to get rid of throphont debris. Theronts were harvested by low speed centrifugation at 300xg for 3-4min and resuspended in 10mM Hepes buffer pH 7.3.

* Lipid raft isolation: Cell lines were grown and harvested as described above. For lipid raft isolation 25ml culture at the density of 1x10^6 cells/ml was spun down and cell pellet was immediately resuspended in 500µl of buffer
supplemented with protease inhibitors E64 and PMSF (Sigma). Equal volume of cold Isolation buffer (25mM Tris pH 7.3, 150mM NaCl, 5mM EDTA) containing 2% Triton X-100 was added to the pellet and transferred to pre-chilled Dounce homogenizer. Cells were lysed by 10-15 strokes and left on ice for 1hr. Subsequently, the cell lysate was mixed with 2ml 2M Sucrose and layered with a sucrose gradient starting from 0.9-0.2M from the bottom of the tube to the top respectively. The tubes containing the gradient preparations were ultra-centrifuged at 247 000xg using Backman SW41Ti rotor for 15-17hr. 1ml fractions were collected starting from the top and analyzed.

**I-antigen crosslinking and sample preparation:** Tetrahymena cells expressing the recombinant Ich i-antigens were harvested by centrifugation 300xg and resuspended in pre-warmed (30°C) buffer (10mM Tris-Cl 1mM CaCl$_2$ pH 7.3). For crosslinking, I-Ag monoclonal antibody G361 was added to the samples at the final dilution 1:100 after which cells were incubated at 30°C non-shaking for varying periods of time depending on the experiment. Controls for each experiment were treated the same way only the primary antibody was not added to the samples. For western blotting 10ml (1x10$^6$cells/ml) of controls and treated cultures were harvested by centrifugation. 50µl of resulting 500µl pellet was processed with protease inhibitors and 2X SDS Sample buffer as described in Maniatis. Supernatants were centrifuged once more at 300xg for 5min to remove remaining cells and 100µl aliquote of resulting supernatant was checked under a dissecting microscope with 20x objective to ensure that there were no cells left in it. Supernatant samples were TCA precipitated as follows: samples were placed on ice and 1/10 volume of 0.15% DOC was added followed by 15min incubation. After incubation 1/10 volume of 70% TCA was added and samples were left on ice
for additional 30min then centrifuged at 13Kxg for 20min in cold. Resulting pellets were washed in 100% Acetone once for 5min, centrifuged again at 13,000xg for 5min and air dried at room temperature. Protein pellets were resuspended in 1X Alkaline SDS buffer containing protease inhibitors and boiled for 3min.

**Western blotting:** Whole cell lysates of parasite theronts and *Tetrahymena* cells were separated on 12% SDS-page gel in mini protean II Cell (BIO-RAD) and transferred to Trans-Blot pure nitrocellulose membrane (0.45µm, Bio-Rad) in mini trans blot transfer cell (BIO-RAD) using a transfer buffer containing 39mM Glycine, 48mM Tris (pH 8.3), 0.037% SDS and 20% methanol. After the transfer membranes were incubated in PBS buffered 5% milk at 4 ºC overnight. The next day the primary rabbit anti-G5 polyclonal antibody (1:2000 dilution in blocking buffer) was added and membranes were incubated at room temperature with agitation for 1hr. Membranes were washed 3 times for 5 min each in PBS, and incubated with HRP labeled secondary goat anti-rabbit IgG (Bio-Rad) for additional 1hr (1:10000 dilution in blocking buffer). Membranes were washed again with PBS 3 times for 5min each and incubated for 5 min in 10 ml equal volume mixture of Supersignal West Pico Luminol/Enhancer Solution and Stable Peroxide Solution (Pierce Biotechnologies). After the incubation membranes were drained and exposed to Kodak BioMAX MS film for 10 min. In some cases pictures were taken using a CCD camera (Chemiginius)

**2D electrophoresis:** Lipid raft fractions (fractions from the top 1-5) were pulled and subjected to TCA acetone precipitation as described above. Precipitated protein pellet was resuspended in 200µl rehydration buffer (2M Thiourea, 7M Urea, 4% Chaps). For rehydration, the IPG strips (Bio-Rad)
were incubated with samples in a focusing tray under a low voltage (50V) overnight (active rehydration). The next day the strips were focused as follows: step1- 250V, for 15min, step2- 8000V 35,000 – 50,000 V-hr total, Rapid Ramp at 20C. After focusing was finished the strips were washed in reducing buffer 3x for 15min each. The second dimension was run by securing the IPG strips on 12% SDS page gel and sealing with melted 1% agarose LE, the gels were run under 70V constant for 12hr. Staining was done with SyproRuby according to manufacturer’s recomendations. Pictures were taken using a UV source and CCD camera (Chemigenious Bio-Rad).

Microscopy

**TEM Fixation and Embedding:** Samples were fixed in 4% glutaraldehyde in 0.2M Sodium Cacodylate pH7.4 for 40min at room temperature. After fixation cells were washed with 0.1M Sodium Cacodylate pH 7.4 3x for 10min each at 4 C, after which cells were fixed again in 2% osmium tetroxide at room temperature for 1hr. Cells were washed again 3x for 10 min each followed by dehydration series in ethanol from 10% to 100% and acetone from 50% to 100%. Samples were infiltrated with eponaraldehyde overnight then added with accelerator and cured overnight in 60ºC incubator. Thin sections were prepared using a Reichert uiltramicrotome. The thickness of the sections was checked using the interference -color method. The 90-110nm  (silver-gold) sections were ironed by waving a small heating plate over them and immediately collected by touching with a formvar coated grids from above the surface of the water. The grids were dried and kept for staining

**Staining the sections:** One drop of uranyl acetate for each grid was placed on paraphilm in a covered Petri dish. Grids were floated, sections
down, on the drops for 5-7 min and the Petry dish was covered with a dark box. A second parafilm lined Petry dish was used for lead citrate staining. A few NaOH pellets were put into the dish to absorb CO₂ 2min prior putting drops of lead citrate onto the parafilm. The grids were removed from the uranyl acetate stain and rinsed for 10 sec in each of three boiled and cooled water. All liquid (with the exception of a film of water) was removed from the grids with a filter paper wedge. After which the grids were floated on top of lead citrate drops for 5-7 min. Grids were rinsed for 10 second in each of three containers of boiled and cooled water and were put on a filter paper to dry before examination with the electron microscope.

**Imaging:** Stained, mounted samples were examined using a Technai12 transmission electron microscope. An accelerating voltage of 80-100 KV, a condenser aperture of 100µm and an objective aperture 20µm was used for all operations. Emission was set at 2 or 4 and magnifications ranged from 3,000X- 100,000X.

**Immunofluorescent labeling and confocal microscopy:** After inducing with 2µg/ml CdCl₂ for 16-24 hr, cells were harvested by low speed centrifugation. Cells were washed with PHEM buffer pH 6.9 (60mM PIPES, 25mM HEPES 10mM EDTA and 2mM MgCl₂) and fixed in 3% Paraformaldehyde in PHEM buffer pH 6.9 overnight. After fixation cells were washed twice with 0.1% BSA-PBS for 5min each followed by incubation with monoclonal antibodies G361 and 10H3diluted 1:100 in 1% BSA-PBS for 1hr. After 1 hr cells were washed again twice for 5min each with 0.1% BSA -PBS and incubated with secondary anti mouse IgG (Invitrogen) labeled either with FITC or TRITC diluted 1:500 in 1% BSA-PBS for 1hr. Then cells were washed with 0.1% BSA-PBS three times 5 min each and put on coverslips with
mounting media. Images were taken by using an OLYMPUS laser scanning confocal microscope using 63X objective.

**Construction of GCamp2 vector for expression in **Tetrahymena thermophila: **GFP-calmodulin-M13 fusion construct was amplified using primers 5’gcataGGATCCgtcgactcatcacgtcgtaaagtgaataaacaggg 3’-Fwd and 5’ccataggcgcgcctcacttcgctgtcatcattgtaactttcg 3’-Rev in order to engineer a *Tetrahymena* stop codon and restriction sites BamH I and Asc I were added at the 3’ and 5’ ends of the construct. Resulting PCR product was cloned into Topo vector (Invitrogen) according to manufacturers directions followed by transformation into bacteria. Resistant colonies were screened by colony PCR (using above mentioned primers) and plasmids containing the insert was isolated from positive colonies. Isolated plasmid was cut using restriction enzymes Asc I and BamH I. The restriction reaction was run on a 1% Agarose and a band corresponding to the size of the insert was cut and gel purified. Insert was cloned into pXS76 *Tetrahymena* expression vector containing neo cassette for Paromomycine selection. *Tetrahymena* expressing *Ichthyophthirius* i-antigens were transformed biolistically as described previously (Figure S-3.1).

**Calcium measurements & Inhibition experiments:** Double transgenic *Tetrahymena* were washed into Tris-HCl, 2mM CaCl₂ pH7.3 buffer and fluorescence of the cell suspension was monitored using a PTI spectrofluorimeter equipped with Felix 32 software. Fluorescence changes in GFP were monitored in live cells over time as they were treated with either antibodies or ionophores. For inhibition experiments, 10ml aliquots of cell cultures were incubated with appropriate inhibitors 1hr at 30ºC in growth medium. After incubation cells were washed into Tris-HCl pH7.3 and...
fluorescence changes in response to various treatments were monitored as above. To block Ca\textsuperscript{++} currents Verapamil was used at 50µM, Nifedipine 30µM, Bapta AM 10-20µM and EGTA at 15mM concentrations.
REFERENCES


Supplemental Figures
Figure S-2.1 Expression of GCamp2 in Tetrahymena: A. Chimeric construct Gcamp2 was cloned into expression vector suitable for recombinant protein expression in Tetrahymena. As shown in the figure the vector contains neo cassette for Paromomycin resistance, Tetrahymena metallothionin promoter and flanking regions for homologous recombination. B. Western blots of double transgenic Tetrahymena verifying the expression of parasite antigens and chimeric reporter GCamp2. The blots were probed with antibodies against i-antigens and GFP for GCamp2.
A

Calmodulin binding sites

Calm binding peptide

Promoter

ATG

Stop

5' Flank (MTT1) Neo

3' Flank (MTT1)

Homologous Recombination

MTT 1

B

Clone A

Clone B

Anti-i-Ag

Anti-GFP
Chapter three

Ca++ Dependent mitochondrial extrusion
in unicellular eukaryotes
Abstract.

Mitochondria are highly dynamic structures capable of interacting with each other and with other intracellular organelles. Indeed, recent studies in mammals suggest that mitochondria can also communicate with the extracellular environment through the release of both DNA and protein. While intriguing, mechanisms that could account for the release of mitochondrial constituents from live cells remain unclear. Here we demonstrate that ciliated protozoa can jettison mitochondria as intact organelles before they unload their contents to the extracellular space either in a soluble form, or in association with “exosome”-like membrane vesicles on plasma and ciliary membranes. The process is rapid and can be triggered by various stimuli including lateral clustering of GPI-anchored surface proteins, as well as heat shock. Mitochondrial extrusion is accompanied by sustained increases in intracellular Ca$^{++}$ and is inhibited by Verapamil and BAPTA-AM arguing strongly for the involvement of calcium in initiation of the response. These results could account for recent observations in mammalian cells and suggest that mitochondrial extrusion is an evolutionarily conserved process with important implications for cellular physiology as a whole.
Introduction.

Mitochondria generate much of the cell’s energy currency in the form of ATP. At the same time, they play critically important roles in a variety of other intracellular processes including calcium homeostasis, maintenance of the cell’s redox state, carbohydrate and lipid metabolism, cell growth, and apoptosis (14, 17, 42, 50, 54). They are capable of fusion and fission and associate with other intracellular organelles, most notably the endoplasmic reticulum (ER) (3, 11, 30). Indeed, mitochondrial constituents have been demonstrated to be present outside the cell suggesting that these organelles are far more dynamic than previously thought (33, 40, 55, 56).

Numerous studies over the past decade have identified mitochondrial proteins in association with the plasma membrane, membrane vesicles (so-called exosomes), and tissue fluids of mammals. These include subunit proteins of ATP synthase (28, 36, 38), superoxide dismutase (34), pyruvate dehydrogenase (41), subunit 2 of NADH dehydrogenase (18), HSP60 (24, 29, 49) and others (4, 13, 35, 46). In some instances, these extracellular mitochondrial proteins have been shown to perform unexpected functions. For example, F1ATP synthase associated with the plasma membrane of endothelial cells is capable of generating ATP outside the cell, and can act as a receptor for angiostatin (28). In the same vein, the β-catalytic chain of ATP synthase, and the mitochondrial chaperone HSP60 have been identified as cell surface receptors for high-density lipoproteins such as apolipoprotein A-I in various cell types (5, 36).

While other roles have been ascribed to ectopically localized mitochondrial proteins, the mechanisms responsible for their release from living cells are central unanswered questions. The majority of these proteins
normally associate with the inner mitochondrial membrane and lack canonical
signal peptides that could potentially direct them to the plasma membrane or
constitutive secretory pathway. Although it is now recognized that some
proteins can reach the exterior of the cell without transiting the ER and golgi
(22, 52), how this occurs is still unknown.

Perhaps even more puzzling are recent reports describing catapult-like
release of mitochondrial DNA (mtDNA) from mammalian granulocytes (55,
56). Eosinophils and neutrophils appear capable of jettisoning mitochondrial
DNA in response to reactive oxygen stress associated with bacterial infection.
While dying neutrophils release chromatin to the surrounding tissue space (6),
mitochondrial DNA appears to be extruded from live cells and is thought to
play an essential role in trapping microbial pathogens and limiting their spread
(55, 56). Mitochondrial DNA has also been shown to be associated with
exosomes released from cultured astrocytes and glioblastoma cells (20).
Exosomes are believed to play a general role in intercellular information
exchange, and could potentially carry mtDNA from cell to cell (10, 15, 25, 32).
Transfer of mtDNA from stem cells to cells with nonfunctional mitochondria as
a means of rescuing aerobic respiration has, in fact, been described (47). As
in the case of mtDNA extrusion from granulocytes, the mechanisms
responsible for the transfer of the mitochondrial genome from cell to cell
remain unclear.

Here we show that mitochondrial DNA and protein are released from
ciliated protozoa following rapid expulsion of whole mitochondria from viable
cells. Ciliates, most notably Paramecium and Tetrahymena, have served as
important models for basic studies in eukaryotic cell biology and genetics
since the 1920s. Shedding of GPI-linked surface proteins in response to
antibody binding in these cells led us to examine the nature of material
released in two ciliate species, *Tetrahymena thermophila*, and the common
fish parasite, *Ichthyophthirius multifilis*. Along with aggregated membrane
vesicles, we were surprised to find large numbers of mitochondria within the
extracellular space. Morphological analyses suggest that mtDNA and protein
associate with shed membrane vesicles following extrusion of intact organelles
from cells. Possible molecular mechanisms responsible for mitochondrial
extrusion and the potential benefits of this process for overall cellular
physiology are discussed.

**Results**

**I-antigen shedding from plasma and ciliary membranes.**

Heterotrichous ciliates have abundant GPI anchored surface proteins known
as i-antigens that vary their expression in response to environmental stimuli
(9). Consistent with previous reports (26), we found that i-antigens were shed
from the cell surface following cross-linking with specific antibodies. As shown
in Figure 3.1, prior to antibody treatment, the proteins were uniformly
distributed on ciliary and plasma membranes of *Ichthyophthirius multifilis*
(Ich), and *Tetrahymena thermophila* made transgenic for the expression of i-
antigens from Ich. In both cases, addition of mAb G3-61 caused rapid
cessation of cell movement, along with aggregation and coalescence of i-
antigens at the tips of the cilia before their release into the culture medium
(Figure 3.1). I-antigen shedding was verified by Western blotting analysis of
cell-free supernatant fractions and was complete within 30-60 min of antibody
addition (Chapter 2, Bisharyan and Clark). As shown by transmission electron
microscopy (TEM), lateral clustering of i-antigens in *Tetrahymena* induced the
formation of 50-100 nm exosome-like vesicles at the cell surface that assembled into larger aggregates, first on the plasma membrane, and then on cilia (Figure S3.1). These aggregates were eventually released into the medium and could be visualized in thin sections of high-speed pellets derived from cell-free culture supernatant fractions (Figure S3.2).

Mitochondrial extrusion in response to antibody treatment. Because of potential artifacts associated with conventional TEM, negative stain was applied to gain further insight into the nature of the material released from cells. *T. thermophila* were placed on Formvar coated grids, incubated with antibody for 60 min, and then gently removed before staining the grids with uranyl acetate (UA). As expected, large membrane aggregates resembling those seen by transmission EM were visible in these samples (Figure S3.2). Surprisingly, however, numerous electron dense structures ranging from 1.2-1.7μm in diameter were also present on the grids (Figure 3.2). These structures contained an outer membrane enclosing a tubular endomembrane system. In many instances the outer membranes were broken open splaying inner tubular membranes onto the grids. At high magnification, the walls of the tubules were studded with characteristic lollypop-shaped structures that bore all the hallmarks of mitochondrial ATP synthase. Aside from membrane vesicles and presumptive mitochondria, no other identifiable structures were present on the grids. With the idea that mitochondria were being jettisoned from cells in response to i-antigen cross-linking, *Tetrahymena* were fixed at different times following mAb addition and examined by TEM. Structures that were readily identified as mitochondria were visible outside the cells within 30 min of antibody treatment (Figure 3.3). With increasing time, the number of extracellular mitochondria increased and reached a peak at ~2 hr.
Figure 3.1 Confocal analysis of Tetrahymena and Ichthyophthirius membrane aggregates prior to and after treatment with antibodies against i-antigens. **A-C.** Ichthyophthirius theronts were fixed with 4% paraformaldehyde and labeled with monoclonal antibodies specific to i-antigens (G361) followed with secondary anti-mouse IgG (Rhodamin). Arrows show the uniform distribution of i-antigens on both plasma and ciliary membranes. **D-E.** Live theronts were treated with G361 antibodies for 30min prior to fixation and labeled with the same secondary antibody. Arrows indicate membrane aggregates which are evident at the ciliary tips in both fluorescent and phase contrast images. **F-H.** Recombinant Tetrahymena thermophila cells expressing Ichthyophthirius i-antigens labeled with G361 followed by anti-mouse IgG (FITC). **I-K.** Recombinant Tetrahymena were incubated with G361 for 30min fixed and stained as described above showing similar response to antibody against recombinant antigens (scale bar is 10µm).
following antibody treatment when at least 1-2 could be visualized outside each cell. Mitochondria in the extracellular space were sometimes positioned near a pocket in the plasma membrane with veils of membrane extending from the organelle towards the cell surface. However, in all cases the plasma membrane was intact and there were no instances in which mitochondria could be seen in transit across the lipid bilayer suggesting that extrusion itself is rapid. Based on the size of mitochondria and the relative dimensions of the cells, rough estimates based on TEM suggest that 5-20% of the total intracellular mitochondrial pool was being released.

**Mitochondrial DNA and protein in the culture supernatant.** To determine whether mitochondrial extrusion resulted from a transient disruption of the plasma membrane that was not discernible by TEM, a membrane impermeable dye, Sytox orange, was added to cells just prior to antibody treatment. Although Sytox orange readily stained mitochondria and nuclei of dead and dying *Tetrahymena* (data not shown), the vast majority of cells in culture showed no labeling of intracellular organelles before or after antibody treatment (Figure 3.4). Nevertheless, within 5 min of antibody addition, bright punctuate staining could be visualized outside the cells. Staining of extracellular material intensified with time, and, as with i-antigen labeling, appeared to accumulate in refractile structures at the ciliary tips (Figure 3.1 and 3.4).

Extracellular material labeled with Sytox orange was presumed to be DNA either within or released from extruded mitochondria. To verify this, PCR was performed on cell-free culture supernatant fractions using oligonucleotide primers specific for mitochondrial or nuclear genes. As shown in Figure 3.4E, primers that span a 496 bp region of the gene encoding the α-subunit of
Figure 3.2 Analysis of shed material by Negative Staining. Recombinant *Tetrahymena* expressing i-antigens were left on an EM grid in the presence of antibodies specific to i-antigens for 1hr. Cells were gently removed and deposited material was negatively stained with Uranyl Acetate. **A.** Extruded intact mitochondrion showing the inner membrane tubular network. **B, C** and **D.** Arrows indicate broken down mitochondria with released inner membrane crystae. The inset shows characteristic lollipop structures of F1 subunit of ATP-synthase (scale bar for A, B, and C is 0.5µm; for D 100nm).
Figure 3.3 TEM analysis of thin sections of *Tetrahymena* cells undergoing mitochondrial extrusion after treatment with antibodies against i-antigens: A, B, C, D TEM micrographs show 1 to several mitochondria extruded from the cells but still attached to them via what looks like a veil of membrane extending from the organelle (A, C) (scale bar is 0.5µm). Note the plasma membrane in these cells is still intact as well as other intracellular organelles and cilia (scale bar is 0.5µm) The black arrows show the extruded mitochondria and the gray arrows show the veil of membranes extending from them. (m-mitochondria, c-cilia, mc-mucocytes, bb-basal bodies, mv-membrane vesicles)
*Tetrahymena* mitochondrial ATP synthase produced an amplicon of the correct size, while no detectable band was seen with primers for a macronuclear-specific gene sequence from *T. thermophila* (Figure 3.4E). The absence of contaminating nuclei in cell-free culture supernatant fractions was confirmed by Western blotting with antibodies against *T. thermophila* histone H2A (Figure 3.4E).

To further examine whether transient disruption of the plasma membrane accompanies mitochondrial extrusion, cell-free culture supernatant fractions from *T. thermophila* at different time points after antibody treatment were screened for the presence of tubulin, one of the most abundant proteins in the cell. As shown in Figure 3.4F, Western blots of culture supernatant fractions had no detectable signals for either α-tubulin, as a probe for the cytosolic protein, or polyglycylated-tubulin, as a marker for tubulin in cilia. By contrast, the same fractions showed strong signals for the mitochondrial chaperone, HSP60, within 30 min of i-antigen cross-linking, reaffirming the presence of mitochondria outside the cell. Interestingly, the relative signals from both HSP60 and tubulin increased with time after antibody addition relative to histone H2A.

**Cells survive mitochondrial extrusion.** While the preponderance of evidence indicated that the plasma membrane was intact following antibody treatment, the consequences of i-antigen shedding and mitochondrial extrusion on overall cell viability were not known. To test this, single cells were manually isolated from control and antibody-treated cultures using fine-bore micropipettes and their ability to divide over a 24 hr period was measured. In both cases, >75% of cells exhibited robust (logarithmic) growth following transfer to fresh media with no statistical differences between control
and antibody-treated cells in terms of their ability to divide (Figure 3.4G).

Mitochondrial constituents localize to membrane aggregates. When cross-linked at the cell surface, i-antigens coalesce at the tips of cilia prior to their being shed within aggregated membrane vesicles (see above). The accumulation of Sytox orange stain near the tips of cilia raised the interesting possibility that mitochondrial constituents associated with these membrane vesicles as well. To test this directly, confocal immunofluorescence microscopy, as well as immunogold EM were carried out on control and antibody-treated cells that were double-labeled with antibodies against mitochondrial ATP synthase on the one hand, and the i-antigens (to track aggregated membrane vesicles) on the other. Confocal imaging showed a normal distribution of both proteins in control cells, with ATP synthase being localized to orderly rows of mitochondria at the cell cortex, and the i-antigens being uniformly distributed on plasma and ciliary membranes (Figure 3.5A-D). This pattern was drastically altered in response to i-antigen cross-linking. Following antibody treatment, mitochondrial rows disappeared, and ATP synthase showed characteristic punctate labeling of the plasma membrane and ciliary tips reminiscent of Sytox orange staining (Figure 3.5E-H). I-antigens were similarly redistributed into distinct domains around the cell periphery and ciliary tips. When staining with the two probes was compared, clear instances of co-localization were found (Figure 3.5E-F).

At the level of the EM, thick sections of antibody-treated cells showed electron dense mitochondria either in close proximity to, or embedded within membrane aggregates on the cell surface (Figure 3.5I). In many instances, these mitochondria were small and appeared to lack outer membranes suggesting that they had undergone fragmentation. Immunogold labeling of
Figure 3.4 Mitochondrial DNA and proteins are extruded from the cells. A, B, C, D. Confocal images of live *Tetrahymena* cells stained with Sytox orange before and 30-40 min after antibody treatment show extracellular structures that brightly label with Sytox orange resembling membrane aggregates. Note the cells don’t show any nuclear labeling as an indication that the cell membranes are still intact. E. PCR amplification of mitochondrial (top panel) and nuclear (middle panel) DNA from the media harvested from the cells prior to and after treatment with antibodies and Western blot (lower panel) of the same samples with anti-histone H2A. (Scale bars are 0.5µm and 100nm). F. Western blot of cells and media collected from the cells not treated and treated with antibody for varying times, probed with anti- I-ag, HSP60, Histone H2A, PGP-tubulin and alpha-tubulin from top to bottom respectively. G. Cell proliferation analysis of single cell isolation from *Tetrahymena* before, 30min and 1hr after inducing the cells to undergo mitochondrial extrusion. The experiment is a representation of three independent experiments. In each experiment 250 single cells were isolated for each time points and the controls.
thin-sectioned material yielded similar conclusions (Figure 3.5J-K). Antibodies specific for ATP synthase showed strong labeling of intact and fragmented mitochondria intermixed with membrane vesicles that were also labeled, but to a lesser extent. Equivalent sections stained with i-antigen-specific secondary antibodies showed intense labeling of membrane vesicle aggregates interspersed with mitochondrial fragments that were, by and large, unlabeled.

**Mitochondrial extrusion in Ichthyophthirius.** While it was clear that mitochondria were being jettisoned from transgenic *Tetrahymena* expressing heterologous i-antigens, it was important to show that the same phenomenon occurred in cells expressing the native proteins. To test this, mitochondrial distribution was examined by confocal immunofluorescence microscopy in the parasitic ciliate, *Ichthyophthirius multifiliis*, before and after treatment with i-antigen specific antibodies. As shown in Figure 3.5, both the shape and organization of mitochondria were clearly different in *Ichthyophthirius* when compared with *Tetrahymena*. Using anti-HSP60 antibodies as a probe, mitochondria in *Ichthyophthirius* were tubular in appearance (rather than round) and were arranged at the anterior of the cell rather than in cortical rows (Figure S3.4). Nevertheless, following i-antigen cross-linking, massive redistribution of mitochondria was seen, along with clear evidence for extracellular localization of the probe (Figure 3.5L-S and Figure S3.4). Indeed, co-localization of i-antigens and mitochondrial proteins at the cell surface and in ciliary tips was more apparent in *I. multifiliis* than in *Tetrahymena*, offering additional evidence that mitochondrial constituents and elements of the plasma membrane can intermix.

**Mitochondrial extrusion in response to heat shock.** As indicated above, i-antigen cross-linking in *Tetrahymena* resulted in a >2 fold increase in
the expression of HSP60 (Figure 3.4F, and Bisharyan, unpublished).

Upregulation of HSP60 expression in response to heat shock and other forms of stress has been well documented in ciliates and other species (7, 21, 37). Moreover, heat shock has been shown to cause a redistribution of mitochondria in *Tetrahymena* from the cell cortex to the interior, along with a precipitous decline in intracellular ATP (16). With the idea that heat shock might itself induce mitochondrial extrusion, wild-type and transgenic *T. thermophila* were incubated at 40°C for 30 min and the release of mitochondrial DNA and protein to the extracellular space was examined. In the case of wild-type *Tetrahymena*, heat shock caused rapid appearance of Sytox orange staining outside the cell as determined by fluorescence microscopy (Figure 3.6A), as well as the appearance of HSP60 in cell-free culture supernatant fractions as measured by Western blotting (Figure 3.6B). Indeed, the signals generated for HSP60 appeared even more robust than those seen following i-antigen cross-linking in the same transgenic cells (Figure 3.6C). Lastly, heat shock induced i-antigen shedding (data not shown).

Mitochondrial extrusion is dependent on elevated intracellular Ca++. Previous studies have suggested that calcium plays an important role as a second messenger in the downstream events surrounding i-antigen cross-linking (9). To examine this more directly, relative levels of intracellular Ca++
Figure 3.5 Mitochondrial proteins colocalize with plasma membrane aggregates: A-H. *Tetrahymena thermophila* double stained with antibodies against i-antigen and ATP synthase. Top row cells not crosslinked show characteristic spacing of mitochondria around the cell periphery along the ciliary rows and i-Ag labeling of ciliary and plasma membranes, lower crosslinked for 2hr show dramatic redistribution of mitochondria to the plasma membrane blebs and subsequent colocalization with plasma membrane proteins i-antigens. I. A thick section transmission electron micrograph of treated cells shows membrane vesicles intermixed with extruded mitochondria and immunogold labeled thin sections with anti-i-antigen and anti-ATP-synthase from left to right respectively. Both of the antibodies showed labeling of membranes aggregates. L-S. *Ichthyophthirius multifiliis* theronts double stained with antibodies against i-antigen and HSP-60 show very tubular mitochondrial labeling in theronts in resting cells which changes to extracellular labeling upon antibody treatment. Red (ATP synthase) Alexa fluor 633, green (i-antigens) FITC (fluorescin iso-tio cianate), blue (nucleous) DAPI (4,6-diamino-2-phenylindole).
were determined in double-transgenic *Tetrahymena* co-expressing recombinant i-antigens from *Ichthyophthirius*, and the GFP-based calcium reporter construct, GCamP2(39), following treatment with the i-antigen specific mAb, G3-61. As shown in Figure 3.7, cells undergo a rapid and sustained increase in intracellular [Ca^{++}] within seconds of antibody addition. Calcium mobilization in this case was found to be sensitive to the L-type calcium channel inhibitor Verapamil, as well as the calcium chelator BaptaAM (Figure 3.7C).

Since mitochondria are both affected by calcium and play a role in shaping calcium signals by acting as calcium stores, the role of Ca^{++} mobilization in mitochondrial extrusion was examined. First, HSP60 levels in cell-free culture supernatant fractions were measured following i-antigen cross-linking in the presence or absence of either Verapamil or BaptaAM. Consistent with a role for Ca^{++} mobilization in mitochondrial extrusion, the release of HSP60 into culture supernatant fractions in response to antibody was completely inhibited by both drugs (Figure 3.7D). To determine whether elevated intracellular Ca^{++} was sufficient to induce the response, the calcium ionophore, A23187, was added directly to the medium and the culture supernatant fractions tested for mitochondrial DNA and protein. The results of these studies were, nevertheless, inconclusive since levels of A23187 that induced comparable increases in intracellular [Ca^{++}] compared with antibody treatment were toxic to *T. thermophila* causing damage to the plasma membrane (as measured by the entry of Sytox orange dye) and cell death.
Figure 3.6 Heat shock induces mitochondrial extrusion: A. Confocal images of live *Tetrahymena* cells subjected to heat shock and stained with Sytox Orange. B. Western blot of media collected from recombinant *Tetrahymena* cells expressing i-antigens. The samples are as follows from left to right heat shocked, antibody treated and untreated controls. C. Western blot of media collected from wild type *Tetrahymena* subjected to heat shock.
Figure 3.7 Mitochondrial extrusion is Ca^{++} dependent: A. Spectrofluorimetric readings obtained from double transgenic *Tetrahymena* expressing GcamP2 and i-antigen. Cells treated with antibody show several fold increase in GFP fluorescence as a result of elevated intracellular Ca^{++} in contrast to the controls, which are either non treated or treated with irrelevant antibody. B. Confocal analysis of double transgenic *Tetrahymena* treated (left) and not treated (right) showing increase in fluorescence in antibody treated cells. C. Increase in Ca^{++} can be blocked by Ca^{++} chelator Bapta AM or L-type channel inhibitor Verapamil. D. Western blot of the media collected from the cells untreated, treated with antibodies alone or combined with Bapta AM and Verapamil.
A

![Graph showing fluorescence intensity over time with different conditions: +Irrelevant Ab, No Ab, +Ab, Verapamil +Ab, +Bapta +Ab, +Ab, -Ab, Vrp Bapta +Ab, and control Vrp Bapta.](image)

B

![Images showing cells labeled with antibodies: +ab and -ab.](image)

D

![Western blots showing HSP60 expression under different conditions: control, Vrp, Bapta, +Ab, and -Ab.](image)
Discussion

The work described here provides direct evidence for rapid extrusion of mitochondria from ciliated protozoa in response to either heat shock, or the lateral clustering of GPI-anchored proteins on plasma and ciliary membranes. The association of mitochondrial DNA and protein with membrane vesicles on the surfaces of these cells is highly reminiscent of recent reports of catapult-like discharge of mitochondrial DNA from vertebrate eosinophils and neutrophils (55, 56), and of mitochondrial proteins on the exterior of other mammalian cell types, often in association with exosomes (20, 48). While the precise mechanisms underlying these phenomena are still unknown, the ability of cells from such widely diverged taxa to release mitochondrial constituents to the extracellular space would clearly suggest that the phenomenon is deeply rooted in evolution, and has fundamental importance in eukaryotic cell biology as a whole.

Based on TEM, estimates of the number of mitochondria released from *T. thermophila* in response to antibody treatment range between 30-160/cell, or 5-25% of the total intracellular pool in log phase cells (1, 12). Because cells were washed extensively prior to fixation and significant numbers of mitochondria released into the medium may have been lost during processing, this should be considered a minimal estimate. Nevertheless, Western blots comparing the relative signal strength of HSP60 in cell pellets versus cell-free culture supernatant fractions from antibody-treated cells suggested similar numbers, that is, 10-20% of mitochondrial HSP60 being present outside the cell. When levels of HSP60 in culture supernatant fractions from heat-shocked and antibody treated cells were compared (Figure 2.6C), signals from heat shock samples were considerably higher suggesting that temperature shock
could be a stronger stimulus for mitochondrial extrusion than i-antigen cross-linking. In this regard it should be noted that previous studies measuring intracellular ATP pools in *T. thermophila* using $^{31}$P NMR spectroscopy showed a precipitous decline of 50% immediately following heat shock (16). While the drop in ATP levels that accompanies heat shock may not reflect mitochondrial extrusion per se, the number of mitochondria jettisoned from cells is likely to depend on the nature and strength of the stimulus involved, and appears to be substantial in the case of both heat shock and antibody treatment.

In considering how mitochondrial extrusion is triggered in these cells, both heat shock and i-antigen clustering would be expected to alter a basic property of plasma and ciliary membranes, namely, fluidity of the lipid bilayer. I-antigens comprise over 10% of total membrane protein in ciliates (31), and are strongly associated with detergent-resistant membranes or “lipid rafts” that are believed to form liquid-ordered domains within the plasma membrane. Based on work in other systems, coalescence of GPI-anchored proteins in response to antibody binding would be expected to increase membrane fluidity at least locally, and in the case of whole-scale shedding, even globally. Similarly, heat shock is known to increase membrane fluidity in a wide variety of cell types (2, 23, 45, 51, 53). An important consequence of that effect may be the rapid and sustained increase in intracellular Ca$^{++}$ seen here.

Because ciliates are difficult to load with calcium sensitive dyes, we used a circularly permuted GFP reporter construct to measure changes in intracellular Ca$^{++}$ in response antibody binding. The substantial increases in intracellular Ca$^{++}$ that were observed following antibody treatment were not entirely surprising given the known physiological responses of ciliates to i-antigen cross-linking, namely, regulated secretion of dense core granules.
(mucocysts) and reversal of ciliary beat, both of which are calcium-dependent phenomena. Moreover, the ability of Verapamil to inhibit the sustained increases in intracellular Ca++ shown here was not entirely surprising given that reversal of ciliary beat in response to antibody binding is thought to involve voltage-gated ion channels. Again, based on reports in other systems activation of excitatory calcium channels is consistent with the increase in membrane fluidity predicted above (27, 44) and may explain how calcium mobilization is initiated.

While it remains to be demonstrated that changes in membrane fluidity leading to opening of L-type calcium channels play a role in these events, the fact that Verapamil and BAPTA-AM blocked mitochondrial extrusion clearly demonstrated that release of these organelles to the extracellular space is a calcium dependent phenomenon. To demonstrate that Ca++ mobilization was sufficient to induce the response, cells were treated with the calcium ionophore, A23187, but rather than inducing mitochondrial extrusion, plasma membranes became leaky and cells died.

The fact that mitochondria exit cells as intact organelles was clearly demonstrated by negative stain and transmission electron microscopy. However there were no instances in which mitochondria were caught transiting the plasma membrane suggesting that the process itself is relatively rapid. An exhaustive survey of many fields yielded a number of examples in which extracellular mitochondria were positioned close to an invagination in the plasma membrane, and their were numerous examples of mitochondria containing veiled membranes at one end extending towards the plasma membrane (Figure 3.3). Given these observations, there are a limited number of models that could account for how mitochondria exit the cell. Perhaps the
least likely would involve fusion of the outer mitochondrial membrane with the plasma membrane itself. The presence of intact mitochondria outside cells would rule against such model, unless a second set of membranes were capable of surrounding the extruded inner tubules. A more reasonable model might involve transient opening of the plasma membrane allowing mitochondria to move through. A more extreme version of the same basic model would involve mitochondria being forced through the plasma membrane in the absence of a fusigenic event emerging on the outside with their membranes intact. Both scenarios would require motive force to drive organelles across the membrane itself. Lastly, one could imagine that mitochondria being taken up into cytosolic vacuoles that subsequently fuse with the plasma membrane spitting intact organelles into the culture medium where they subsequently break down. While there is no direct evidence that this in fact occurs, such a model is suggestive of autophagy and raises the interesting possibility that mitochondrial extrusion is an alternative pathway for the removal of damaged mitochondria triggered by a specific set of conditions related to stress. That such a pathway exists has been suggested in two recent reports, one describing extrusion of fragmented mitochondria in murine embryonic fibroblasts undergoing TNF-α induced cell death (40), and the other describing the elimination of damaged mitochondria from HeLA cells treated with a respiratory chain inhibitor and an uncoupler of oxidative phosphorylation (33). In the first instance, fragmented mitochondria were taken up into vacuoles and then released from cells as “naked” mitochondria prior to apoptotic cell death. In the second (termed “mitoptosis”), damaged mitochondria clustered around the nucleus and then gathered into a vacuole in which they decomposed. The vacuole, or “mitoptotic body” eventually
protruded from cells and was presumed to release material to the extracellular space (33).

As with rapid extrusion of mitochondrial DNA from eosinophils and granulocytes (55, 56), elimination of decomposing mitochondria from poisoned HeLa cells was dependent on the formation of reactive oxygen species (ROS). Indeed, Skulachev and co-workers have argued ROS can serve as the trigger for the elimination of damaged mitochondria from cells as a means of surviving oxidative stress. While vacuolar membranes surrounding mitochondria were not visible in *Tetrahymena*, upregulation of HSP60 and tubulin expression in these cells is a classic indication of stress (21, 43), and it is not unreasonable to think that the stimuli underlying mitochondrial extrusion in this case could damage these organelles. In this regard, mitochondria can store calcium, but are also injured when calcium levels exceed a certain threshold (19). I-antigen cross-linking leads to sustained increases in intracellular Ca++ in these cells. Moreover, the peripheral location of mitochondria just beneath the plasma membranes of ciliates would put them at a site of high Ca++ in the event of calcium influx across the membrane. Although not shown here, TEM studies conducted in our laboratory suggest the presence many more electron-dense crystalline arrays (believed to be calcium deposits) in the mitochondria of antibody-treated cells than in those of controls. Finally, antibody-treated cells often failed to survive additional treatment with microtubule destabilizing agents suggesting again that were under considerable stress (data not shown).

While these observations are consistent with a model in which cells extrude damaged mitochondria to survive oxidative stress, the results described here invoke other recent studies demonstrating the presence of
ectopic mitochondrial DNA in mammalian astrocytes and glioblastoma cells (20), as well as mitochondrial protein in human breast carcinoma and melanoma cells in association with exosomes released from the plasma membrane (48). Exosomes are believed to be important in membrane adaptation and information exchange between cells, and may play a critical role in transfer of mitochondrial DNA from cell-to-cell (15, 25, 32). The association of mitochondrial constituents with exosomes in mammalian cells is strikingly similar to what we describe here in terms of membrane vesicle formation, and intermixing of these vesicles with mtDNA and proteins following mitochondrial extrusion in ciliates. Even more remarkable is the fact that despite their unicellular life styles, Tetrahymena, as well as Ichthyophthirius, cluster into tight multicellular aggregates in the process of mitochondrial extrusion. Whether they exchange information under these conditions is not known but is currently being tested.

Although many questions remain regarding the biological significance and underlying mechanisms responsible for this phenomenon, the work described here provides unequivocal evidence that mitochondria can be jettisoned from ciliated protozoa in a process that is dependent on sustained increases in intracellular Ca\(^{++}\). The relationship between this process and the release of mitochondrial constituents from mammalian cells is clearly interesting, and may shed new light on conserved organellar functions.

**Materials and Methods**

*Tetrahymena cultures*: Recombinant *Tetrahymena* strains expressing Ichthyophtirious i-Antigens were made as previously described. Cell lines were grown in NEFF (0.25% proteose peptone, 0.25% yeast extract, 0.55%
glucose, and 0.033 mM FeCl₃) at 30°C with constant shaking at 90 rpm and allowed to reach late log to early stationary phase. At this stage cultures were induced with 2µg/ml CdCl₂ to express the recombinant proteins and harvested 12-16 hours after induction. Cells were harvested by centrifugation 300xg for 3min.

**Ichthyophthirius growth and maintenance:** *Ichthyophthirius multifiliis* strain G5 was maintained on channel catfish as previously described. Trophonts were collected by gently rubbing the infected fish after which the parasites were incubated overnight at room temperature in carbon filtered water. After trophonts hatched and formed theronts the culture was filtered with fine wire mash filters to get rid of throphont debris. Theronts were harvested by low speed centrifugation at 300xg for 3-4min and resuspended in 10mM Hepes buffer pH 7.3.

**I-antigen crosslinking and sample preparation:** Transgenic *Tetrahymena* cells expressing Ich i-antigens were pelleted by centrifugation at 300xg and resuspended in pre-warmed (30°C) buffer containing 10mM Tris-Cl, 1mM CaCl₂ at pH 7.3. For i-Ag crosslinking: monoclonal antibody G361 was added to the samples at the final dilution of 1:100, after which, cells were incubated at 30°C non-shaking for varying periods of time depending on the experiment. Negative controls for each experiment were treated identically, with the exception of primary antibody which was withheld. For Western blotting: 10ml cultures (1x10⁶ cells/ml) of control and treated cells were harvested by centrifugation and cell pellets and supernatant samples were kept separately. 50µl of the resulting 500µl pellet was processed with protease inhibitors and 2X SDS sample buffer as previously described (Maniatis). Supernatants were centrifuged once more at 300xg for 5min to remove
remaining cells and 100µl aliquote of resulting supernatant was checked under the microscope using a 20x objective to ensure that there were no cells left in it. Supernatant samples were TCA precipitated as follows: samples were placed on ice and 1/10 volume of 0.15% DOC was added followed by 15min incubation. After incubation 1/10 volume of 70% TCA was added and samples were left on ice for additional 30min then centrifuged at 13.000xg for 20min in cold. Resulting pellets were washed in 100% Acetone once for 5min, centrifuged again at 13.000xg for 5min and air dried at room temperature. Protein pellets were resuspended in 1X Alkaline SDS buffer containing protease inhibitors and boiled for 3min.

**Western blotting:** Samples were separated on 12% SDS-page gel in mini protean II Cell (BIO-RAD) and transferred to Trans-Blot pure nitrocellulose membrane (0.45µm, Bio-Rad) in mini trans blot transfer cell (BIO-RAD) using a transfer buffer containing 39mM Glycine, 48mM Tris (pH 8.3), 0.037% SDS and 20% methanol. After the transfer membranes were incubated in PBS buffered 10% milk at 4 ºC overnight. The next day the primary antibody was added and membranes were incubated at room temperature with agitation for 1hr. Membranes were washed 3 times for 5 min each in PBS, and incubated with HRP labeled secondary antibody. Membranes were washed again with PBS 3 times for 15min each and incubated for 5 min in 10 ml equal volume mixture of Supersignal West Pico Luminol/Enhancer Solution and Stable Peroxide Solution (Pierce Biotechnologies). After the incubation membranes were drained and exposed to Kodak BioMAX MS film for 10 min. In some cases pictures were taken using a CCD camera (Chemiginius).

**Confocal Microscopy:** For fluorescence microscopy experiments cells
were fixed in ice cold 50mM Hepes pH7.4 buffer containing 4% Paraformaldehyde for 1hr on ice. Subsequently, the samples were washed in 50mM Hepes pH 7.3 and blocked with 1% BSA-PBS at room temperature for 15min. After that, samples were incubated with primary antibodies (anti-ATP-synthase and anti-HSP60) for 1hr at room temperature. After incubation in primary antibody cells were washed and incubated for 1hr with FITC labeled secondary antibodies (anti-rabbit IgG and Alexa 633 labeled anti-mouse IgG (Invitrogen)), after which cells were mixed with mounting medium. Images were acquired with a Leica SP5 confocal microscope using 63x water objective.

**Negative staining:** 10 µl of sample containing cells was placed on formvar-coated grids, treated for 1 hr with mAb G361, and then drained to remove the cells. Grids were processed as previously described (ref).

**TEM Fixation and Embedding:** Samples were fixed in 4% glutaraldehyde in 0.2M Sodium Cacodylate pH 7.4 for 40min at room temperature. Following fixation cells were washed 3 times (10 min each) with 0.1M Sodium Cacodylate pH 7.4 at 4ºC, after which cells were fixed again in 2% osmium tetroxide at room temperature for 1hr. Cells were washed again 3x for 10 min followed by dehydration series in ethanol from 10% to 100% and acetone from 50% to 100%. Samples were infiltrated with eponaraldehyde overnight then added with accelerator and cured overnight in 60ºC incubator.

**For Immuno-EM fixation:** Samples were fixed in 0.15% glutaraldehyde and 4% paraformaldehyde in 40mM Hepes pH 7.4 for 1hr on ice, then washed 2x in 40mM Hepes pH 7.4. Samples were dehydrated by washing 2x in 50% ethanol followed by 2 washes in 70% ethanol on ice. After which cells were resuspended in 2:1 mixture of LR White and 70% ethanol respectively and
incubated on ice for 30 min. Cells were transferred to 100% LR White for 1hr at 4C then transferred to fresh LR White for overnight infiltration at room temperature. Next day the infiltrated cells were transferred to beam capsules with fresh LR White and cured for 24 hr at 50ºC.

**PCR:** 10mls of *Tetrahymena* culture was washed in 10mM Tris-HCl, 2mM CaCl$_2$ pH 7.4 and treated with antibodies to undergo mitochondrial extrusion. Cells were removed by centrifugation and media was collected and checked to ensure there were no cells left in it. DNA was precipitated using 1/10 volume isopropyl alcohol in the presence of 1µg/ml Glycogen. Resulting pellet was diluted in 100 µl 1x PCR buffer and 5µl of it was used for PCR. For positive control, cell pellet was collected from 1ml of culture and diluted in 500 µl PCR buffer with detergent Tween 20 and Proteinase K, followed by 1 hr incubation at 55C. Proteinase K was inactivated by incubating cell lysate at 95C for 10 min. 2.5µl of sample was used for PCR using primers `atp9-nad1_b FWD CTCTACCACTGAGCTACTTA` and `atp9-nad1_b REV AAAAAATCTGATAAAAACGTGTT` for mitochondrial DNA.

**Ca++ measurements:** Double transgenic *Tetrahymena* cells expressing i-antigens and GCamP2 were transferred into 10mM Tris, 1mM Ca$^{++}$ buffer pH7.3 and changes in GFP fluorescence were monitored using a Felix 32 spectrofluorimeter. 2µM final concentration of ionophore A23187 (Sigma Aldrich) and mab-G361 1:100 final dilution were added to cultures while monitoring live cells for fluorescence changes. For inhibition 50µM Verapamil and 2µM Bapta AM were added to cultures and incubated for 1hr. Cells were washed out of the treatment into 10mM Tris pH 7.4 and fluorescent readings were taken immediately in the presence and absence of mab-G361.

**Inhibition experiments:** Recombinant *Tetrahymena* cells expressing i-
antigens were incubated with Verapamil and BaptaAM as described above for 1hr in growth media. After incubation 10ml aliquots of cell cultures were taken and washed once in 10mM Tris pH 7.5 followed by 1hr antibody treatment to induce mitochondrial extrusion. After 1hr of treatment supernatants were harvested and analyzed by western blotting for the presence of HSP60 as described above.
REFERENCES


Supplemental Figures
Figure S-3.1 TEM analysis of shed material collected from antibody treated cells: A. Lower magnification transmission electron micrograph of a field with shed membrane vesicles and mitochondria harvested from Tetrahymena cultures treated with antibodies against i-antigens (scale bar is 2µm). The insets (B, D) show higher magnification of highlighted sections. C, E, F. Shed mitochondria collected from the media of treated cells by high speed centrifugation intermixed with membrane vesicle (E, D).
Figure S-3.2 Negatively stained shed material harvested from media of antibody treated cells: *Tetrahymena* cells were treated with antibodies and left on the grid for 1hr. The images represent the shed material that was deposited on the grid after the cells were removed. A. Membrane vesicles intermixed with inside out mitochondrial inner membranes. B, C Aggregated membrane vesicles D, E, F. Inside out inner mitochondrial membranes released as a result of mitochondrial break down after extrusion.
Figure S-3.3 TEM micrographs of cells undergoing mitochondrial extrusion and membrane vesicle shedding: A. Longitudinal section of cilia associated with membrane vesicles. B, C. Thin sections of *Tetrahymena* with one to several mitochondria extruded however still attached to the cell surface. Inset shows a higher magnification image of mitochondria surrounded with what appears to be an extra layer of membrane. D. Thick section with a cell showing mitochondria and membrane vesicles intermixed on a cell surface. (c-cilia, mv-membrane vesicles, m-mitochondria)
Figure S-3.4 Confocal micrographs of Ichthyophthirius prior to and after treatment with antibodies against i-antigens. A. Z sections through Ichthyophthirius cells that haven’t been treated with antibodies fixed and labeled with anti-mitochondrial HSP60 and anti-i-antigen antibodies showing intracellular localization of mitochondria and uniform distribution of i-antigens on the ciliary and plasma membranes. B. Same as above only the cells were treated with antibody before fixation. Note the pattern changes dramatically after antibody treatment, mitochondrial HSP60 now colocalizes with i-antigen aggregates. Red (ATP synthase) Alexa fluor 633, green (i-antigens) FITC (fluorescin iso-tio cianate), blue (nucleous) DAPI (4,6-diamino-2-phenylindole).
Chapter four

Summary and Future Directions
Summary

The work described in this dissertation identifies signaling mechanisms associated with the clustering of GPI-anchored proteins on ciliated protozoa, along with their downstream consequences. Collectively the data presented in this dissertation support a model (Figure 4.1) in which i-antigen cross-linking induces the formation of membrane vesicles, the aggregation of these vesicles on plasma and ciliary membranes, and their movement to ciliary tips from where they are eventually shed into the extracellular space. Based on electron microscopy, immunogold labeling and 2-D gel analysis, vesicles originate from plasma membrane and contain many proteins in addition to the i-antigens. Changes in membrane composition associated with vesicle shedding most likely contribute to opening of plasma membrane Ca$$^{++}$$ channels and sustained elevation of Ca$$^{++}$$ within the cytosol. In our studies we found that elevation of intracellular Ca$$^{++}$$ could be blocked by L-type channel inhibitors, Verapamil and Nifedipine, supporting the hypothesis that Ca$$^{++}$$ influx originates at the plasma membrane and subsequently triggers Ca$$^{++}$$ release from internal stores. Elevated intracellular Ca$$^{++}$$ in turn contributes to a number of changes at the behavioral and cell biological levels manifested by dense core granule secretion, immobilization, and, unexpectedly, mitochondrial extrusion. Once extruded, mitochondria appear to break down and release their contents to the extracellular space where they intermix with plasma membrane vesicles that are subsequently shed into the surrounding medium. With the idea that antibody-treatment induces a generalized stress response, we subjected cells to heat shock and showed that it induced the exact same response (that is, i-antigen shedding and mitochondrial extrusion) suggesting that both stimuli act via similar mechanisms.
Figure 4.1 A model summarizing downstream effects triggered by lateral clustering of i-antigens on the cell surface. (For detailed explanation refer to Summary in Chapter 4)
Future directions

The work presented in this dissertation provides foundation for future investigations into two fundamental areas of cell biology. The first would attempt to elucidate the mechanisms and biological significance of mitochondrial extrusion, and the second would investigate membrane vesicle shedding and its role in membrane adaptation and intercellular communication.

Mitochondrial Extrusion: Here we provide direct evidence that intact mitochondria are extruded from two species of protozoa in response to major alterations in plasma membranes resulting from antibody-induced clustering of GPI-anchored proteins on the cell surface or to heat shock. Changes in membrane composition are likely to result in activation of voltage-gated calcium channels either by directly affecting the lipid environment surrounding the channels or through phosphorylation/dephosphorylation of specific proteins involved in regulation of channel activity (8). As a result, we hypothesize that calcium influx triggers a release from internal stores leading to sustained elevation in intracellular Ca++. Mitochondria are involved in establishing calcium homeostasis by shaping and regulating calcium signals, however, they can also be damaged by Ca++ above a threshold level (10). Although we hypothesize that mitochondria jettisoned from cells could be damaged and functionally impaired due to Ca++ uptake, we have no direct evidence that this is in fact the case. In order to assess the functional state of mitochondria either prior to, or in the process of extrusion, there are three relatively simple tests that we could perform to lay the ground work for further investigations. First, it would be informative to monitor changes in intracellular ATP levels in response to antibody treatment using a commercially available
ATP measurement kit (Invitrogen). Alternatively, it would be useful to monitor oxygen consumption rates by cells as an indication of respiring mitochondria prior to, and at varying times after treatment. The drop in ATP levels and impaired respiration would be indicative of compromised mitochondrial function. Mitochondrial damage has been shown to result in loss of outer mitochondrial membrane potential and subsequent release of reactive oxygen species in the cytoplasm causing oxidative stress (7, 20). To test if any of the treatments leading to mitochondrial extrusion induce changes in mitochondrial membrane potential, dyes that are sensitive to membrane potential such as GC1 could be used. Labeled mitochondria could be monitored for changes in emission spectra over time following treatment with antibodies (or heat shock) using either confocal microscopy or spectrofluorimetry. Finally it would be helpful to understand the role of reactive oxygen stress in mitochondrial extrusion. The availability of dyes that are sensitive to reactive oxygen species would make this a relatively fast and simple assay involving incubation of cells in dye followed by treatment with antibody or heat shock. If elevated ROS is seen prior to extrusion, it would be interesting to treat cells with inhibitors of ROS to determine whether mitochondrial extrusion is also blocked, and therefore ROS dependent. This would provide additional insights into the mechanism of extrusion.

To directly test whether Ca^{++} is taken up by mitochondria as a prelude to extrusion, the Ca^{++} sensitive GFP reporter construct used in these studies could be targeted to mitochondria by engineering a mitochondrial targeting sequence into the fusion protein. This would allow direct measurements of mitochondrial Ca^{++} in response to various treatments that induce extrusion in real time.
To better understand the mechanisms underlying mitochondrial extrusion, it is important to further characterize the pool of extruded mitochondria. Mitochondria released from cells could be harvested from the culture supernatant in isoosmotic buffer and their functional state assessed by determining ATP production and oxygen consumption over time as described above.

If we conclude that mitochondria destined for extrusion are damaged or functionally impaired it would of great interest to determine whether this process can serve as an alternative pathway to mitochondrial autophagy (mitophagy) as a mechanism of recycling the damaged organelles. Extrusion of fragmented mitochondrial material has been shown to occur in mammalian cells by two independent groups(14, 15). If mitochondrial extrusion is an alternative pathway to autophagy it would interesting to see if blocking autophagy would have an effect on mitochondrial extrusion. There are several approaches that could be used to address this. In the first case, cells could be starved to induce autophagy (6) and then treated with 3-methyladenine, a common inhibitor of autophagosome formation (16). In the second case cells would be induced to undergo mitochondrial extrusion in the presence of 3-methyladenine. Depending on whether these two pathways merge at a certain stage, or whether they are two independent pathways alternating each other, different outcomes would be expected. If one sees an increase in mitochondrial extrusion it could mean that mitochondrial extrusion and autophagy are independent alternative pathways. If blocking autophagy inhibits mitochondrial extrusion this could mean that the two pathways merge at a certain point and blocking the stage before they diverge would inhibit both.
To better understand how mitochondria exit the cell, *Tetrahymena* were fixed for TEM within seconds of antibody treatment and at varying times thereafter hoping to capture organelles in transit across the plasma membrane. Unfortunately at no time did we visualize mitochondria in the process of extrusion. This could be explained in a number of ways including limited sample size, rapid movement, or a mechanism that is intrinsically difficult to visualize. To determine whether mitochondria might be budding off the plasma membrane in enclosed vesicles, we performed section tilts on thin-sections and looked for additional layers of membrane surrounding extruded mitochondria. In some cases mitochondria appeared to have a third layer in addition to the inner and outer mitochondrial membranes (Figure 4.2) although, in general, this was extremely hard to discern. Alternatively, mitochondria may be taken up into vacuoles prior to extrusion and then released when vacuole membranes fuse with the plasma membrane. While there is no direct evidence that this occurs, one could imagine 3 likely sources of vacuolar membranes, namely, the alveolar sacs (which are specific to Alveolates), the plasma membrane, and phagosomal/endosomal membranes. This could be examined using antibodies specific for all four membrane systems and determining whether any of the markers co-localize with extruded mitochondria, or the outer mitochondrial membrane at the light and electron microscopic levels. How mitochondria exit cells remains a puzzle that is ripe for future investigation.
Figure 4.2 Transmission electron micrograph of extruded mitochondria. The arrows show what appears to be an additional layer of membrane surrounding the organelles. Scale bar is 0.5µm.
Release of mitochondrial DNA has recently been described in mammalian eosinophils and granulocytes where it appears to have a role in trapping bacteria and limiting the spread of microbial pathogens within the host (21, 22). Furthermore, transfer of mitochondrial DNA between the cells has been reported to occur in stem cells (17), and mitochondrial DNA release in association with exosomes has been documented in astrocytes and glioblastoma cells (11, 13), and is believed to be a mechanism by which cells exchange information. To test whether mitochondrial extrusion in ciliates is indeed followed by an exchange or transfer of information we propose two experiments.

First, in order to test whether cells can transfer mitochondrial DNA to other cells we would incubate a *T. thermophila* cell line that carries resistance to chloramphenicol (due to a mutation in mitochondrial DNA) with a cell line containing a paromomycin resistance cassette (neo) in the nucleus, and then induce mitochondrial extrusion with heat shock. After the extrusion is complete (~ 1-2 hr), single cells would be isolated and allowed to undergo several rounds of fission, after which individual clones would be tested for double resistance to paromomycin and chloramphenicol.

Second, to determine whether cells can exchange whole mitochondria (rather than DNA alone) one could generate cell lines expressing either GFP- or RFP-fusion proteins targeted to mitochondria. Differentially labeled cells would then be mixed and induced to undergo mitochondrial extrusion. After 1-2 hr, exchange of mitochondria between cells could be visualized using confocal microscopy.

Finally we would like to expand this work beyond the ciliates and look at mitochondrial extrusion in other cell types.
Vesicle shedding and immobilization: One of the novel findings presented in this dissertation is the phenomenon of vesicle shedding in response to i-antigen clustering. Small (50-100 nm) vesicles have been shown to be released from the surfaces of many cell types (5) including other protozoa (19) and participate in important biological processes such as rapid phenotypic adjustments to new conditions (5, 9, 12), surface membrane traffic, and horizontal transfer of protein, RNA (9, 13) and now mitochondrial DNA (11) among the neighboring cells. The exact nature or function of vesicles shed by ciliates is not entirely clear. However, based on the fact that i-antigens are shed under conditions of stress (heat shock, pH etc) (4) and that shedding is accompanied by remarkable changes in membranes, it would be tempting to speculate that they are involved in rapid phenotypic adjustments, specifically membrane adaptation to changes in the extracellular environment. In unicellular organisms this could serve as a rapid mechanism of increasing membrane fitness in response to environmental stress.

To understand the motive force responsible for vesicle shedding we conducted preliminary experiments in which i-antigen cross-linking was induced in the presence of microtubule or actomyosin-based inhibitors. Results shown in figure 4.3 indicate that these inhibitors block i-antigen shedding, and at the same time alter cell viability. This would clearly suggest a role for microtubule and/or actomyosin-based motors in driving the process, and argues that shedding itself is essential for survival. In the context of membrane adaptation, it would be informative to see if inhibition of i-antigen shedding also inhibited membrane rearrangements described earlier in the thesis.

Since one hypothesis that could account for opening of calcium
channels involves a change in membrane composition (Figure 4.1) it would be interesting to monitor Ca\textsuperscript{++} changes in response to i-antigen cross-linking in cells that were treated with actomyosin and microtubule inhibitors. If Ca\textsuperscript{++} mobilization, in addition to shedding, were blocked, it would support a model in which changes in membrane composition lead to channel activation.

As argued above, shed vesicles may serve as vehicles for intercellular communication and information exchange. Despite the fact that ciliates are unicellular organisms, the idea that membrane shedding could be a mechanism of cell to cell communication is especially appealing given the cells themselves cluster together and form multicellular aggregates during the process (Figure 4.4). TEM of these cells demonstrates that they intimately connected with vesicles often trapped in between them (Figure 4.4 D, F).

Since exosome vesicles in metazoan cells seem to originate from lipid rafts (1, 18) it is likely that they are enriched in various signaling molecules and lipids. Intercellular communication via shed vesicles in ciliates would be an attractive idea, given the fact that these are single celled organisms, and vesicles enriched in non-diffusible signaling substances would greatly facilitate cell-to-cell communication in a pond setting.

To test this hypothesis and to better understand the nature as well as the function of membrane vesicles in ciliates it would be of interest to identify the proteins and the lipids associated with them. Shed vesicles can be harvested by high speed centrifugation and analyzed for their protein and lipid content using mass spectrometry and TLC respectively. To test if vesicles play a role in intercellular communication it would be interesting to incubate the cells that are undergoing vesicle shedding with wild type cells and look for behavioral changes in the latter. Alternatively wild type cells could be
Figure 4.3 I-antigen shedding and cell survival: Recombinant *Tetrahymena* expressing Ich i-antigens were incubated in the presence of microtubule and actin inhibitors for 1hr prior to treating with antibodies. For cell survival assays 150 single cells were hand isolated to individual hanging drops and cell proliferation was monitored over 24hr period. I-antigen shedding was assed based on western blotting of material collected from antibody treated cells in the presence or absence of inhibitors. (Oryz-Orizaline, Noc-Nocadosole, CytB-CytochalazineB)
Figure 4.3 Intercellular communication in *Tetrahymena*. A. A phase contrast image of recombinant *Tetrahymena* cells expressing *Ichthyophthirius* i-antigens immobilized in the presence of antibodies against i-antigens. Upon addition of antibody, the cells stop swimming and cluster on a bottom of the plate forming large aggregates. Scale bar is 50µm. B. Confocal micrograph of immobilized *Tetrahymena* fixed and labeled with secondary anti-mouse IgG conjugated with Rhodamine. Shows a cluster of cells with the i-antigen containing vesicle aggregates trapped between them. C, E. Transmission electron micrographs of immobilized *Tetrahymena*, show pairs of cells with cell-to-cell contact areas with membrane vesicles trapped between them. D and F are higher magnification images of C and E respectively. Scale bars for C is 2µm, E 10µm, D and F 1µm.
incubated with harvested vesicles to look for changes in behavior, or the transfer of vesicle proteins to the cells.

Finally we show that elevation of intracellular Ca\(^{++}\) in response to antibody binding responsible for immobilization of cells. This raises the intriguing possibility that immobilization could be a result from ATP depletion due either to mitochondrial extrusion, calcium-induced mitochondrial damage, or both. To test this hypothesis one could microinject immobilized cells with ATP. If cell motility were restored it would prove that ATP depletion is responsible for immobilization and answer a question that has lingered for more than 100 years (4).

The overall goal of this dissertation was to elucidate signaling mechanisms triggered by antibody induced lateral clustering of GPI anchored proteins in protozoan cells. The main focus of our study was ciliate i-antigens as an example of abundant GPI anchored proteins in protozoa. I-antigens have been extensively studied for their role in antigenic variation in free-living ciliates (4) and as targets of the host immune response in the case of *Ichthyophthirius* (2-4), however, prior to these studies, little or no work had been done on understanding their role in transmembrane signaling. The data presented here demonstrates that ciliate i-antigens are associated with detergent-resistant membrane fractions and that their clustering on the cell surface leads to sustained mobilization of intracellular Ca\(^{++}\), loss of cell motility, and mitochondrial extrusion. We believe these studies have important implications for membrane biology in unicellular eukaryotes, and raise important questions regarding membrane dynamics and adaptation to environmental stress in general.

As a result of this work we discovered mitochondrial extrusion from the
cells in response to Ca^{++} induced stress, which became the main focus of my dissertation research. Mitochondrial extrusion is a novel finding that expands our knowledge of mitochondrial dynamics and has broad implications for cell biology in general.


