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In Vitro Studies on the Interactions Between Toxoplasma Gondii and Its Host Cell.

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In vitro studies on the interactions between Toxoplasma gondii and its host cell

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IN VITRO STUDIES ON THE INTERACTIONS
BETWEEN TOXOPLASMA GONDII AND ITS HOST CELL

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by

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LIST OF ABBREVIATIONS

DMEM - Dulbecco’s Modified Eagle’s Medium
FBS - Fetal bovine serum
BSA - Bovine serum albumin
DAPI - 4'-6' diaminodino-2-phenyl-dihydrochloride
PBS - Phosphate buffered saline
Toxoplasma gondii is an obligate intracellular protozoan parasite of the Phylum Apicomplexa. The parasite resides in the cytoplasm of its host cells in a membrane bound compartment called the parasitophorous vacuole. All growth and development of the parasite occur within this compartment. In this thesis, the interactions between the Vero cells, a fibroblast-like cell line, and the parasite which occur during the intracellular growth of the parasite were addressed. The impact of infection on the host cell intermediate filaments and microtubular cytoskeletal elements was examined by immunofluorescence microscopy. Host cell intermediate filaments were found to overcoat the parasite vacuole beginning shortly after invasion and persisting throughout the course of infection. Evidence indicates the host cell vimentin binds to the parasitophorous vacuole serving to dock the vacuole to the host cell nuclear surface. Host cell microtubules were also found to overcoat the parasitophorous vacuole and were increasing accumulated around the parasite compartment throughout the infection. Unlike the intermediate filaments however, the microtubules were not involved in juxtanuclear positioning of the vacuoles. Neither were the microtubules found to be involved in the mitochondrial or vimentin overcoating of the vacuoles.
However, the infection had a dramatic impact on the organization of the host cell endoplasmic reticulum. This data suggests the microtubular associations with membranous organelles might be disrupted in infected cells. A membranous network occurs within the parasitophorous vacuole which is thought to transport membrane and proteins from the parasite to the parasitophorous vacuolar membrane. The presence of GTP-binding proteins on the networks was investigated. A 25 kDa GTP-binding protein was found to be present on the networks. Additionally, the parasite was found to contain a 41 kDa GTP-binding protein. The 41 kDa protein was not found on the networks but rather localized to the inner membrane complex, a parasite organelle involved in secretion of the networks. These results indicate GTP-binding proteins are important in the secretion of the membranous networks into the parasitophorous vacuole and may function in directing membrane traffic within the parasitophorous vacuole.
INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite belonging to the Phylum Apicomplexa. The definite host of T. gondii is the cat, or any member of the cat family, while a wide variety of birds and mammals, including man, can serve as the intermediate host (Frenkel 1973). In man, T. gondii causes both an acute and chronic infection. The acute phase of infection is characterized by the rapidly replicating form of the parasite called the tachyzoite. Tachyzoites can invade a variety of host cell types and the infection quickly disseminates throughout the body (Frenkel 1988). The acute phase of infection is self-limiting because of the host's effective immune response (Frenkel 1988). However, some parasites survive the immune response and persist in the host in tissue cysts, preferentially located in the central nervous system and striated muscle. The cysts contain a slowly replicating form of the parasite called the bradyzoite form. The cysts, containing viable parasites, can persist throughout the lifetime of the host (Frenkel 1988).

In the immunocompetent host these cysts are quiescent and cause no illness in the host. In immunosuppressed hosts
however, cysts rupture, releasing viable parasites and resulting in an active infection. Toxoplasmosis is one of the most prevalent opportunistic infections afflicting AIDS patients (McCabe & Remington 1988). Reactivation of toxoplasma infections is a serious problem in AIDS patients causing serious disease and often resulting in mortality. A better understanding of the biology of \textit{T. gondii} is of obvious importance.

\textit{T. gondii} resides in the cytoplasm of its host cells within a unique endocytic compartment that is formed at invasion. The membrane of this compartment is derived from the host cell plasma membrane but soon after invasion parasite proteins are found within the membrane of this compartment (Kimata & Tanabe 1988; Sibley et al., 1986). Modification of this membrane is due to the parasites secretion of a membranous network into the parasitophorous vacuole (Sibley et al., 1986). Elaboration of this network occurs soon after invasion and is thought to be involved in the inhibition of phagolysosomal fusion (Sibley et al., 1986). The development of this modified endosomal compartment is thought to be crucial to the establishment of intracellular infection.

The initial interactions between the host cell and parasite that occur during invasion, have been well
studied (Jones et al., 1972, Nichols et al., 1983). However, little is understood however about the subsequent intracellular events associated with T. gondii infection. The present series of reports address the interactions that occur between T. gondii and its host cells during the intracellular growth of the parasite. Studies were done in an in vitro system to facilitate experimental manipulation. Vero cells, a monkey fibroblast-like cell line isolated from the kidney, was selected as the host cell for all the studies reported here. A fibroblast, rather than a phagocytic cell, was selected for study since in the host animal, the majority of T. gondii replication occurs in nonphagocytic cells.

The initial studies addressed the impact of the parasite on the host cell domain throughout the course of infection. The cytoskeleton is a network of filamentous fibers that traverse the cytoplasm of all eukaryotic cells. The cytoskeleton, composed of microfilaments, microtubules and intermediate filaments, are involved in a variety of cell functions. Several viruses and intracellular bacterial parasites have been found to interact with the cytoskeleton of their host cells, either affecting host cell function by disrupting these elements (Bonneau 1985) or in some instances utilizing the host cytoskeleton for their own needs (Finlay et al., 1991).
Interactions of protozoan parasites with the cytoskeletal elements of their host cells has not been well studied. The first report examines the interaction between the parasitophorous vacuole of *T. gondii* and the intermediate filaments of Vero cells during the course of infection.

The organization of the intermediate cytoskeletal network is dependent upon the microtubular cytoskeleton (Klymkowsky et al., 1989). Together the intermediate filaments and microtubules form a scaffold around which other cytoplastic components are spatially organized. Therefore, in the second chapter the impact the parasite on the host cell microtubular cytoskeletal elements was examined. Additionally, since many organelles of cells are attached to the cytoskeletal elements and previous studies (Jones et al., 1972) have reported the association of the vacuole with other host cell organelles, the relationship of the microtubular cytoskeletal and association of the vacuole with other host cell organelles was also examined.

The parasitophorous vacuole is a complex highly specialized organelle. The vacuole is characterized by the intraphagosomal network which completely fills the lumen of the vacuole, forms connection with the vacuolar membrane and constitutes a considerable surface area. All traffic between the host cell and the parasite must cross
the vacuolar membrane and the intraphagosomal network has been suggested to be specialized for this task (Schwartzman et al., 1992). Evidence indicates the network transports membrane and proteins between the parasite and the vacuolar membrane (Leriche et al., 1991; Torpier et al., 1993). GTP-binding proteins are proteins that have been implicated to be involved in membrane traffic events from a variety of organisms (Bourne et al., 1990; Hall 1990). In the third chapter, the presence of GTP binding proteins on the networks was investigated.
CHAPTER 1

OVERCOATING OF TOXOPLASMA GONDII PARASITOPOPHOROUS VACUOLE WITH VIMENTIN TYPE INTERMEDIATE FILAMENTS
INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite that is ubiquitous in nature and capable of infecting most species of mammals, including man. Infection with T. gondii is often asymptomatic and induces a lifelong protective immunity against reinfection (Frenkel 1988). However, T. gondii if acquired congenitally can cause serious disease and in immunocompromised patients can be fatal (McCabe & Remington 1988). The onset of the AIDS epidemic and the resultant increase in prevalence of toxoplasmosis has fostered a renewed interest in understanding the biology of this parasite.

T. gondii resides within a unique membrane compartment within the cytosol of host cells that is resistant to phagolysosomal fusion. This compartment is formed at invasion by invagination of the host cell plasma membrane. Within minutes after invasion however, the parasite elaborates a membranous network into the vacuole which incorporates parasite proteins into the parasitophorous vacuole membrane (Leriche et al., 1991, Kimata & Tanabe 1987; Sibley et al., 1986). As the primary interface between the host cell and the parasite, modification of
parasitophorous vacuole membrane may account for the resistance to phagolysosomal fusion (Sibley et al., 1986). The establishment of this unique intracellular compartment is thus thought to be crucial to intracellular survival of the parasite and many studies have focused on further characterization of this compartment.

The intermediate filaments are cytoskeletal elements, 9-11 nm in diameter, that form a filamentous network throughout the cytosol, extending from the nuclear surface to the plasma membrane (Steinert & Roop 1988). Intermediate filaments are a dominant component of the cytosol to which many subcellular organelles and macromolecules are attached. Several viruses have been found to interact with the intermediate filaments of their host cells (Murti & Goorha 1989; Bonneau et al., 1990). Interaction with host cell cytoskeletal elements has not been studied in many protozoan parasites.

In this study the interaction between host cell vimentin type intermediate filaments in T. gondii infected Vero cells was examined by immunofluorescence microscopy. An association of the host cell intermediate filaments with the T. gondii parasitophorous vacuole was observed. The association of vimentin with the T. gondii parasitophorous vacuole begins shortly after invasion and persists
throughout the course of infection. Data presented here suggest the host cell vimentin binds to the \textit{T. gondii} parasitophorous vacuole and furthermore that binding to the host cytoskeletal elements may serve to dock the parasite compartment to the host cell nuclear surface.

**MATERIALS AND METHODS**

**Media, chemicals and antibodies**

The medium used in all experiments and to maintain tissue cells was Dulbecco's Modified Eagle's Medium (DMEM) (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml of penicillin and streptomycin. The antibodies used were mouse anti-vimentin (clone VIM 3B4; Boehringer Mannheim Chemicals) and anti-mouse IgG conjugated to fluorescein (Sigma Chemical Co.). Okadaic acid and calyculin A was obtained from Calbiochem.

**Tissue culture and infection**

Vero cells were grown in DMEM containing 10% FBS and 100 U/ml penicillin and streptomycin and maintained in a 37°C incubator in the presence of 5% CO$_2$. For experimental purposes, Vero cells were cultured onto poly-L-lysine coated coverslips. \textit{T. gondii}, RH strain, were propagated in Vero cells, an African green monkey cell line. To
infect cultures, parasites were liberated from Vero cells by forcing a suspension of infected cells through a 27-gauge needle. Extracellular T. gondii cells were purified from remaining host cells by filtration through 3.0 \( \mu \text{m} \) polycarbonate filters and washed three times by centrifugation at 250 g for 10 minutes. Cultures were infected by addition of parasites in fresh medium to Vero cells. After 15 minutes at 37°C, the inoculum was removed, the monolayer rinsed once and incubated with fresh medium.

**Indirect immunofluorescence staining**

Cells grown on coverslips were fixed in 1.75% formaldehyde for 10 minutes and permeabilized with 0.1% Triton X-100 for 15 minutes. All coverslips were blocked with FBS for 15 at 37°C and subsequently washed in phosphate buffered saline (PBS) for 5 minutes. After blocking, coverslips were incubated in anti-vimentin antibody for 60 minutes at 37°C, subsequently washed 3 times in PBS + 10% BSA, and then incubated in rhodamine conjugated anti-mouse IgG. Control staining was done by omitting the first antibody from the above protocol. After antibody labelling, coverslips were counterstained with 0.1% (wt/vol) solution of 4′-6′ diamino-2-phenyl-dihydrochloride (DAPI) in PBS for 1 minute. Coverslips were mounted in PBS + glycerol (3:1). Light microscopic observations were made with a
Nikon photomicroscope equipped with an epifluorescence system. Micrographs were taken with Kodak TMAX 400 film.

Drug treatments

A 1 mM stock solution of okadaic acid was dissolved in dimethysulphoxide and stored at -20°C. Infected cells were treated with either 0.1 µM or 1 µM okadaic acid. Infected cells were incubated with okadaic acid for 2 hours at 37°C and then fixed and processed for indirect immunofluorescence (see above). A 100 µM stock solution of calyculin A was dissolved in ethanol and stored at -20°C. Infected cells were treated with 10 nM Calyculin A for 2 hours at 37°C.

RESULTS

Growth of *T. gondii* in Vero Cells

*T. gondii* in Vero cells are found within a parasitophorous vacuole which is characteristically located next to the host cell nucleus (Fig.1). *T. gondii* invade at the cell periphery, but within 10-90 minutes arrive at the host cell nucleus. In this juxtanuclear position, the parasite replicates every 4-6 hours. As the parasite replicates the vacuole greatly increases in size and after 24 hours can
Figure 1 - Growth of *T. gondii* infected Vero cells as viewed by phase contrast(b) and the nuclear stain, DAPI(a) showing an uninfected cell (on the left) and an infected cell with multiple parasitophorous vacuoles(pv). Note the corresponding DAPI stain clearly shows the presence of individual parasites (arrow) as well as the location of the host cell nucleus (n). x 1,560.
contain up to 32 individual parasites. Host cells often are infected by more than one parasite and therefore may contain numerous parasitophorous vacuoles, as illustrated in Fig. 1.

Organization of vimentin type intermediate filaments in \textit{T. gondii} infected Vero cells

\textit{T. gondii} infected Vero cells exhibited a reorganization of vimentin around the parasitophorous vacuoles (Fig. 2). In the host cells containing young vacuoles, a layer of vimentin surrounds each of the parasitophorous vacuoles, but otherwise the organization of vimentin appears largely unperturbed (Fig. 2a and b). In the more mature vacuoles, the network of vimentin type intermediate filaments are rearranged around the parasitophorous vacuole and an accumulation of vimentin is present at the perimeter of the parasitophorous vacuoles (Fig. 2c and d, arrow). Again, even at the late stage of infection, the impact to the vimentin organization was largely confined to the area surrounding the parasitophorous vacuole (Fig. 2a and c).

In heavily infected cells, however, the vimentin reorganization was more extensive. Nearly all the vimentin in the host cell was in association with the parasitophorous vacuole. Furthermore, the host cell vimentin has now been clearly reorganized into unique cagelike formations around the parasitophorous vacuoles.
Figure 2 - Immunofluorescence staining for vimentin (a,c,e) and the corresponding DAPI stain (b,d,f) of T. gondii infected Vero cells, showing the rearrangement of host cell vimentin around the parasitophorous vacuoles.

2a & b. Infected cells containing numerous young vacuoles, each with 1-2 parasites; note each vacuole is clearly enveloped with vimentin. 2c & d. Infected Vero cell with a mature vacuole, showing the accumulation of vimentin at the periphery of the vacuole (arrow) and the network of filaments surrounding the vacuole. 2e & f. A heavily infected cell, showing the intermediate filament cagelike formations around each parasitophorous vacuole (arrows).
Kinetics of the vimentin reorganization

The vimentin intermediate filament organization of Vero cells was analyzed at 30 minutes, 12 hours and 18 hours after infection with *T. gondii* (Fig. 3). Reorganization of the intermediate filaments around the parasitophorous vacuole began within the first 30 minutes following invasion. Evidence of a vimentin association with the parasitophorous vacuole first began to appear coincident with the arrival of the parasite at the host cell nuclear surface (Fig. 3a and b). The parasitophorous vacuole is faintly rimmed with vimentin. Additionally, areas of vimentin accumulation are evident around the vacuoles (Fig. 3a, arrow). Similar vimentin overcoating was not present around parasitophorous vacuole prior to their arrival at the host cell nucleus. At 12 hours post invasion, the vimentin overcoating of the parasitophorous vacuole was more pronounced (Fig. 3c and d; arrow). By 18 hours post invasion, a layer of vimentin was still present on the cytosolic surface of the parasitophorous vacuole, but there is now evidence of a network of intermediate filaments which encages the parasite compartments (Fig. 3e and f). Note as the parasites multiplied, there was a progressive involvement of intermediate filaments from the host cell nuclear surface, appearing to restrain the enlarging parasitophorous vacuole to the host nuclear surface.
Figure 3 - Kinetics of vimentin rearrangement in *T. gondii* infected Vero cells. Immunofluorescence against vimentin (a,c,e) and the corresponding DAPI stain (b,d,f) of Vero cells at 30 minutes (a & b), 12 hours (c & d) and 18 hours after infection (e & f). Note the beginning of vimentin accumulation around the newly formed parasitophorous vacuole at 30 minutes (small arrows); 3c & d. Vimentin overcoating of the parasitophorous vacuole is more pronounced; 3e & f. Note the presence of vimentin at the cytosolic surface of the parasitophorous vacuole (arrow) and the intermediate filaments surrounding the parasitophorous vacuole. x 3570.
Effect of disruption of the intermediate filament network on the parasitophorous vacuole

The phosphatase inhibitors, okadaic acid and calyculin A were used to depolymerize the intermediate filament networks. Both agents cause hyperphosphorylation of the intermediate filaments and hence promote the depolymerization of the intermediate filament networks (Ishihara et al., 1989; Yatsunami et al., 1991). Infected cells were incubated in either 0.1 μM or 1 μM okadaic acid for 2 hours at 37°C (Fig. 4). The lower concentration (0.1 μM) resulted in partial disassembly of the vimentin with most of the filaments in the cytoplasm disrupted, but still present around the nucleus. At the lower concentration, despite the absence of intermediate filament networks in the cytosol, the vimentin overcoating the parasitophorous vacuole was retained (Fig. 4a and b). The higher concentration (1 μM) of okadaic acid however caused a near total disassembly of the filaments, in which almost all of intermediate filaments, including those at the nuclear surface, were depolymerized. At the higher concentration of okadaic acid, the intermediate filament assemblage around the parasitophorous vacuoles was lost. Additionally, the parasitophorous vacuoles were displaced from the nuclear surface into the peripheral cytoplasm (Fig. 4a and b). The loss of the intermediate filament
Figure 4 - Effect of okadaic acid on vimentin arrangement around *T. gondii* parasitophorous vacuoles; immunofluorescence of the vimentin networks (a,c) and the corresponding DAPI stain (b,d). 4a & b. Infected cells treated at the low concentration of okadaic acid (0.1 μM), showing the retention of vimentin overcoating around the parasitophorous vacuoles. 4c & d. Infected cells treated with the higher dose of okadaic acid (1 μM) showing the near total disassembly of intermediate filament networks. Note the absence of vimentin at the nuclear surface and around the parasitophorous vacuoles and that the parasitophorous vacuoles are dispersed throughout the cytosol. The faint background staining of the cytoplasm of the cell is probably due to the presence of vimentin monomers now in the cytosol. x 1800.
assemblage around the vacuoles was observed to coincide with the loss of intermediate filaments from the host cell nuclear surface. Similarly, displacement of the parasitophorous vacuoles from the host cell nuclear surface was also observed when the intermediate filament networks were disrupted with calyculin A (data not shown).

DISCUSSION

In this study we report the presence of host cell intermediate filaments overcoating the parasitophorous vacuoles of *T. gondii*. The vimentin overcoating begins within an hour after invasion and is initiated at the host cell nuclear surface. The parasitophorous vacuole undergoes a maturation process during the first hour after invasion that is crucial for establishment of infection. The maturation process involves 1) incorporation of the parasite proteins into the parasitophorous vacuolar membrane, 2) accumulation of host cell endoplasmic reticulum and mitochondria around the parasitophorous vacuole and finally 3) arrival of the parasitophorous vacuole at the host cell nuclear membrane, accompanied by an enlargement in size (Sibley 1988). Results from this study indicate vimentin overcoating is an additional feature of the maturation of the parasitophorous vacuole, occurring in the same time frame as parasite modification.
of the vacuole and acquisition of host cell mitochondria and endoplasmic reticulum.

The immunofluorescence data presented here suggest a binding affinity of vimentin for the membrane of the parasitophorous vacuole. Immunofluorescence and electron microscopic studies have shown a physical connection between vimentin and various intracellular membranes, including the plasma and nuclear membranes, mitochondria and various small intracellular vesicular membranes (Murphy & Grasser 1984; Traub 1985) and in vitro studies have demonstrated a high affinity of vimentin for cellular lipids (Perides et al., 1986a; 1986b). Vimentin binding to membranes is mediated either through interactions with integral membrane proteins or alternatively via direct lipid interactions. Vimentin binding to the plasma membrane and nuclear membrane for instance has been demonstrated to be mediated through binding to the proteins, ankyrin and lamin respectively (Georgatos et al., 1985; Georgatos & Blobel 1987a, 1987b). Direct binding of vimentin to lipid bilayers has been demonstrated to occur via the binding of the amino terminal end of vimentin to the hydrophobic domains of lipid bilayers (Perides et al., 1987).
Since the membrane of the parasitophorous vacuole is derived in part from the host cell plasma membrane at invasion, it is possible that host cell ankyrin is present in the parasitophorous vacuolar membrane and mediates the binding of vimentin to the parasitophorous vacuole. However, the timing of parasitophorous vacuole vimentin overcoating argues against this as vimentin overcoating does not appear on the newly invaded parasitophorous vacuole, as would be expected if ankyrin derived from the host cell plasma membrane at invasion, was present. Additionally, preliminary evidence from our laboratory from immunolocalization studies indicates ankyrin is not present in the parasitophorous vacuolar membrane. The fact that the parasitophorous vacuole acquires an affinity for vimentin only after extensive parasite modification of the membrane of the parasitophorous vacuole has occurred, suggests parasite products, rather than the host cell, are responsible for the vimentin affinity.

Parasite modification of the vacuolar membrane involves incorporation of parasite proteins and lipids, into the parasitophorous vacuolar membrane either of which could mediate vimentin binding. The rhoptries, parasite organelles whose contents have been suggested to contribute lipids to the parasitophorous vacuolar membrane (Nichols et al., 1983), have been found to contain high
cholesterol to phospholipid content and high amounts of phosphatidic acid (Foussard et al., 1991). The presence of these lipids in the parasitophorous vacuole membrane is interesting in light of the fact that acidic phospholipids have been shown to be necessary for vimentin binding to lipid vesicles and furthermore that cholesterol plays a synergistic role in this reaction (Perides et al., 1986a; 1987). Additionally, vimentin binding to lipids was shown to stabilize vimentin filaments. Thus a binding of vimentin to the parasitophorous vacuole could explain, in part, the apparent enhanced stability of the intermediate filaments in the presence of okadaic acid.

In addition to an apparent binding affinity of vimentin for the parasitophorous vacuole, the progressive restructuring of intermediate filaments around the parasitophorous vacuole indicates additional interactions are occurring between the parasite compartment and the intermediate filaments. Intermediate filament proteins are highly susceptible to modification by Ca$^{2+}$ activated proteases, which cleave the amino terminal end (Inagaki et al., 1989). Since intermediate filaments are often in association with Ca$^{2+}$ sequestering membranes, it has been suggested that activation of intermediate filament proteins in response to signals that induce an influx of Ca$^{2+}$ into the cytosol, is a common mechanism by which
intermediate filaments are regulated (Traub 1985). For instance, during receptor mediated endocytosis, vimentin type intermediate filaments bind to the newly formed endosomes, (Dellagi & Brouet 1982; Herman & Albertini 1982). Endocytosis is accompanied by a transient increase in intracellular Ca\(^{2+}\) and the concentration of Ca\(^{2+}\) around the endocytic compartments is high enough to activate these Ca\(^{2+}\) dependent proteases. The intermediate filament proteins are thus cleaved in the vicinity of the endosome and the newly processed vimentin filaments then have a high probability of binding to the nearby membrane. Given the parasitophorous vacuole is surrounded by host cell mitochondria and endoplasmic reticulum, it is possible the intermediate filament assembly around the parasitophorous vacuole is regulated by calcium fluxes from these organelles. In light of this mechanism, it would be very interesting to determine if calcium fluxes in the vicinity of the parasitophorous vacuole occurs throughout the course of infection.

The unusual assemblies of vimentin intermediate filaments in the vicinity of the parasitophorous vacuole could also be a result of metabolic activities occurring in the parasitophorous vacuole. Different kinds of ions and ionic strengths have been shown in vitro to influence the assembly and structure of vimentin filaments (Hofmann et
al., 1991). Calcium in particular was found to slow the rate of vimentin assembly and result in thicker filaments. Indeed it is likely, that the parasitophorous vacuole, a metabolically active compartment effects the ionic composition and strength in the immediate cytosol. Thus metabolic activity in the parasite compartment may create localized conditions which facilitate the unusual intermediate filament assemblies observed around the parasitophorous vacuole. This explanation is consistent with the absence of impact on the intermediate filament in the cytosol distal to the parasitophorous vacuole.

In addition to the possible binding of vimentin to the parasitophorous vacuole, data indicate a functional role for the vimentin in docking of the parasitophorous vacuole to the host nuclear surface. First of all, the immunofluorescence data suggests the vimentin cytoskeleton attaches to the parasitophorous vacuole early after invasion and as the parasite grows remain associated with the enlarging parasitophorous vacuole, appearing to restrain it to the host cell nuclear surface. Secondly, the ability of the vimentin depolymerizing agents to displace the parasitophorous vacuole from the host cell nuclear surface also supports this hypothesis. Given the parasite is incapable of de novo purine synthesis (Schwartzman & Pfefferkorn 1982), attachment of the
parasite compartment to the host nucleus may enable the parasite to intercept the molecular traffic of purines passing into and out of the nucleus.

Overcoating of the parasitophorous vacuole with host cell intermediate filaments may also serve other functions for the parasite. Given that many cellular organelles and macromolecules, such as heat shock proteins (Leicht et al., 1986), protein kinase C (Murty et al., 1992) and creatine phosphokinase (Eckert et al., 1980) are attached to the intermediate filaments, envelopment of the parasitophorous compartment with intermediate filaments may serve to bring nutrients and metabolites within the vicinity of the parasite. Interestingly, during adipogenesis, an arrangement of vimentin-endoplasmic reticulum-mitochondria occurs around the developing lipid globules (Franke et al., 1987). The authors suggested this arrangement could provide the developing lipid globule with the metabolites and enzymes necessary for lipidogenesis. Recent data indicate vimentin does play a functional role in supplying the metabolites to the developing lipid (Sarria et al., 1992). Likewise, the trilaminar envelopment of *T. gondii* vacuoles with host cell vimentin-endoplasmic reticulum-mitochondria may also serve to redesign the cellular milieu in the vicinity of
the parasite to provide the nutrients and metabolites needed by the developing parasites.

Utilization of the host cell cytoskeletal elements has been reported to occur in a variety of other intracellular pathogens. Viruses, for instance, utilize the cytoskeleton of their host cells to support some metabolic needs (Bonneau et al., 1985; Murti & Goorha 1989) and several intracellular bacterial pathogens utilize host cell cytoskeletal elements during invasion of host cells (Finlay et al., 1991; Tilney et al., 1992; Young et al., 1992). Cytoskeletal interactions are known for a few other intracellular protozoan parasites. Eimeria vermiformis (Adams & Bushnell 1989) for instance, induces cytoskeletal arrangements very similar to those reported here to occur with T. gondii and the protozoal parasite, Trypanosoma cruzi, has been shown to impact the cytoskeletal elements of its host cell (Low et al., 1992; Paulin et al., 1988).
CHAPTER 2

FLUORESCENT STUDIES ON ALTERATIONS IN THE HOST CELL DOMAIN IN TOXOPLASMA GONDII INFECTED VERO CELLS
Toxoplasma gondii enter host cells by an active invasion process which results in the enclosure in a membrane compartment. The first hour after invasion is a dynamic period during which modifications in the parasitophorous vacuole, crucial for the establishment of the intracellular infection, occur. Additionally during this time, the vacuole acquires associations with the following host cell components: 1) the parasitophorous vacuole moves towards and docks at the host cell nucleus; 2) the parasitophorous vacuole accumulates host cell mitochondria and 3) the host cell endoplasmic reticulum envelopes the parasitophorous vacuole. This overcoating of the parasitophorous vacuole with host cell organelles persists throughout the course of infection. As reported in Chapter 1, the parasitophorous vacuole acquires host cell intermediate filaments during the initial hour post invasion. Vimentin overcoating of the parasitophorous vacuole also persists throughout the course of T. gondii infection.

The intermediate filaments codistribute with the microtubules within a cell and the extended organization of the intermediate filaments is dependent upon interactions with microtubules (Gyoeva and Gelfand 1991; Klymkowsky et al., 1989). Together the intermediate
filaments and microtubules provide an interactive scaffold around which other cytoplasmic components are spatially organized. For example, a number of important enzymes are associated with the intermediate filaments, and interactions between vimentin type intermediate filaments and mitochondria are well documented (Klymkowsky et al., 1989; Summerhaynes et al., 1983). The microtubules are important for maintaining membranous organelle distribution of the endoplasmic reticulum and the Golgi network in cells. In addition, the microtubules serve as tracks along which a variety of intracellular organelles, including mitochondria, lysosomes and endocytic vesicles, move (Cooper et al., 1990; Pfister et al., 1989; Terasaki et al., 1986). This cytoskeletal scaffolding system is not rigid but rather is a dynamic complex with the capacity to reorganize in response to different developmental and environmental cues.

Therefore, we questioned whether there might be an interdependence between the movement of cytoplasmic organelles to the parasitophorous vacuole and the cytoskeletal system. This is indicated by the overcoating of the parasitophorous vacuole with both host cell cytoskeletal elements and organelles. Interactions of host cell microtubules with the parasitophorous vacuole were studied using fluorescent probes and the relationships
between the microtubules and vacuole associations with the intermediate filaments, host cell nucleus, mitochondria and host cell endoplasmic reticulum were examined. Microtubules were observed to overcoat the parasitophorous vacuoles early in infection and were increasingly accumulated at the vacuolar surface over the course of infection. Evidence in this report show that microtubules do not appear to affect mitochondria positioning, vimentin overcoating or the juxtanuclear position of the vacuoles. However, the parasitophorous vacuole development had a dramatic impact on the organization of the host cell endoplasmic reticulum. The implications of the cytoplasmic changes in the host cell that occur throughout the course of infection are discussed.

MATERIALS AND METHODS

Cell and parasite culture
Vero cells were grown in DMEM containing 10% FBS and 100 U/ml penicillin and streptomycin and maintained in a 37°C incubator in the presence of 5% CO₂. T. gondii, Rh strain, were propagated in Vero cells. Infection of cultures and liberation of parasites were done as described in Chapter 1. For immunofluorescent labelling or staining of the mitochondria with Rhodamine 123 or the endoplasmic reticulum with DiOC6(3) (3,3′-dihexyloxycyanine iodide)
carbocyanine iodide), cells were plated on coverslips 24 hours prior to use.

**R123 labelling**

Rhodamine 123 was dissolved in dimethyl sulphoxide at a concentration of 1 mg/ml as a stock solution. Mitochondria labelling was done as described by Chen (1989). Briefly, cells grown on coverslips were incubated with 10 µg/ml R123 in culture media for 10 minutes at 37°C, rinsed in medium and mounted on a microscope slide with media and sealed with nail polish. Stained cells were viewed with epifluorescent illumination using a 40X Fluor lens.

**DiOC6(3) labelling**

DiOC6(3) (3,3′-dihexyloxacarbocyanine iodide) was dissolved in ethanol at 0.5 mg/ml as a stock solution. Labelling of cells with DiOC6(3) was done as described by Terasaki (1989). Briefly, live cells grown on 12 mm glass coverslips were stained in culture medium containing 0.5 µg/ml of fluorescent dye for 10 min. Alternatively cells on coverslips were fixed in 0.25% glutaraldehyde in a Hank’s balanced salt solution (pH=7.4) containing 0.1 M sucrose for 2-3 minutes at room temperature. Cells were stained with 2.5 µg/ml of fluorescent dye in buffer for 30 sec at room temperature. The coverslips, after brief rinsing, were mounted on a microscope slide with a drop of
dye free buffer and viewed with a Nikon microscope equipped with epifluorescence using a 60X (NA 1.4) Plan Apo lens.

**Drug treatments**

Host cells were incubated in 10μg/ml nocadazole for 2 hours at 37°C to depolymerize the microtubules. Alternatively cells were incubated with 10 μg/ml colchicine for 12 hours at 37°C. These drug treatments were found to result in complete depolymerization of the microtubules in 95-100% of the cells, as determined by indirect immunofluorescence staining for tubulin.

**Indirect immunofluorescence**

Cells were fixed and processed directly on the glass coverslips. Cells to be stained with anti-tubulin were fixed in methanol:acetone (1:1) for 10 min at 4°C. Cells to be stained with anti-vimentin were fixed in 1.75% formaldehyde for 10 minutes and subsequently permeabilized with 0.1% Triton X100 for 15 minutes. All coverslips were incubated in FBS for 15 min at 37°C to block nonspecific binding. Coverslips were subsequently washed in PBS for 5 min and then incubated in the primary antibody for 60 min at 37°C, washed 3x in PBS, and then incubated in the secondary antibody for 45 min at 37°C. Anti-tubulin (Boehringer-Mannheim) was diluted 1:2 and anti-vimentin
(Boehringer-Mannheim) was diluted 1:3. The secondary antibody used was anti-mouse IgG conjugated to fluorescein.

RESULTS

Organization of host cell microtubules in *T. gondii* infected Vero cells

Microtubule organization in infected Vero cells was assessed via indirect immunofluorescence antibody labelling using a monoclonal antibody to tubulin (Fig. 5). Host cell microtubules encircled the parasitophorous vacuole, appearing as a thick band at the perimeter of the vacuoles (arrow, Fig 5a). Each parasitophorous vacuole was distinctly overcoated with microtubules as illustrated in the infected Vero cell in Figure 5 which has 3 mature vacuoles (i.e., each vacuole has 8-16 parasites). In all infected Vero cells examined, the parasitophorous vacuoles were located where the microtubules were the most dense, indicating vacuoles were located near the microtubule organizing center. The impact of the parasites on the microtubules was confined to the vicinity of the vacuoles. The microtubules in the cytoplasm distal to the parasites, appeared normal and showed no evidence of disruption.
Figure 5 - Microtubular arrangement around the parasitophorous vacuoles (a) and the corresponding DAPI stain (b) illustrating the location of the parasites. Note the thick band of microtubules overcoating the vacuoles (arrow). The microtubules in the cytosol distal to the parasites are intact and show no evidence of depolymerization. Note the parasites, which also contain tubulin, stain faintly with anti-tubulin. x1950
The impact of the parasite on the host cell microtubules at different stages of the infection are illustrated in Figure 6. Association of host cell microtubules with the parasitophorous vacuole was first evident when vacuoles are located at the host cell nucleus (Fig. 6a and b). As *T. gondii* infection progressed, the microtubules remained associated with the vacuoles and accumulated around the perimeter of the vacuoles (Fig. 6a and b; small arrow). In the terminal stages of *T. gondii* infection (Fig. 6a and 6b; long arrow), the microtubular envelopment of vacuoles was absent. A faint fluorescence around the vacuole persisted due to the labelling of the tubulin present in the parasites. However, the microtubules in the vicinity of the vacuole at this stage of infection, appeared to be depolymerized. Additionally, at this stage of infection, the organization of microtubules in the cytoplasm was disrupted. The microtubular organizing center was no longer apparent and the microtubules were condensed into large cables.

The terminally infected cell illustrated in Fig. 6c and d (small arrow) also displays microtubular depolymerization in the vicinity of the vacuole. The absence of microtubules over the surface of the vacuole was most obvious at the dark spot which corresponds to the site where parasites were absent (Fig. 6c, small arrow). In
Figure 6 - Immunofluorescence staining with anti-tubulin (a,c) and the corresponding DAPI stain (b,d), illustrating the microtubular rearrangement around the parasite vacuoles at different stages of infection. Microtubular accumulation around the parasite vacuole is evident early in the infection (small arrow, Fig 6a & b). Note however that in very heavily infected cell (long arrow, Fig. 6a & b), microtubules are now largely depolymerized over the vacuoles and the microtubules around the perimeter are still present but are now condensed into a thick band. Note the cell in Fig. 6c, which contains an "empty" (long arrow, Fig. 6c & d) illustrating the microtubules are still intact over mature vacuoles. Note the very heavily infected to the right (small arrow, Fig. 6d & e) shows evidence of microtubular depolymerization. X1600
rare instances "empty" vacuoles (i.e., T. gondii free) were observed (Fig. 6c, long arrow). This was considered a fixation artifact since empty vacuoles were never observed in live cells. Examination of the empty parasitophorous vacuole showed the microtubular filaments over these large vacuoles were intact around the vacuole (Fig 6c, large arrow). The presence of intact microtubules over mature vacuoles (i.e. vacuoles containing 24-32 parasites) indicated depolymerization of microtubules around the vacuoles occured only in the terminal phases of infection.

Affect of microtubular depolymerization on juxtanuclear positioning and vimentin overcoating

Microtubules were induced to depolymerize by incubations in nocadazole for 2 hours at 37°C. As illustrated in Figure 7a, after 2 hours in nocadazole the microtubules were completely disassembled. Despite microtubule disassembly, the parasitophorous vacuoles retained a juxtanuclear position (Fig. 7b). Similar results were observed when microtubules were depolymerized with colchicine (not shown).

The effect of microtubular depolymerization on intermediate filament cages around the vacuoles was assessed. As shown in Figure 8a and b, a two hour incubation in nocadazole caused a retraction of the
Figure 7 - Effects of nocadazole on the microtubules. Immunofluorescence against tubulin, illustrating the total depolymerization of the microtubules (a) and the corresponding DAPI stain (b) demonstrating the parasites. Note the parasites, depicted at the arrows, are not displaced from the host cell nuclear surface. x1900
intermediate filament network from the cell periphery onto
the nucleus in the uninfected cells. In *T. gondii*
infected cells however, while the juxtanuclear collapse
was still seen, vimentin filaments in the vicinity of the
vacuole were still apparent (Fig. 8a and b, arrow). Longer
incubations in nocadazole (4 hours) caused a complete
collapse of the vimentin in the infected cells (Fig. 8c
and d). However, even when vimentin networks were totally
collapsed, the vimentin overcoating of the parasitophorous
vacuole was still evident.

**Mitochondrial distribution in infected Vero cells**
The mitochondrial dye, R123 was used to visualize
mitochondrial overcoating of parasitophorous vacuole in
Vero cells (Fig. 9). This dye has been shown to stain the
mitochondria of extracellular but not intracellular
parasites (Tanabe 1984). The mitochondria in uninfected
cells were long, filamentous and extend throughout the
cell. In the infected cells, the mitochondria were clearly
localized around the parasitophorous vacuole (arrows, Fig.
9a and b). Mitochondrial overcoating was most obvious in
the mature vacuoles (arrow, upper left, Fig. 9a and b).
Here the mitochondria conformed to the perimeter of the
parasitophorous vacuole. Mitochondria began to associate
around parasitophorous vacuole within 30 minutes after
Figure 8 - Effects of nocadazole on the vimentin overcoating of the vacuole at 2 hours (a,b) and 4 hours (c,d) incubation in the drug. Immunofluorescence against vimentin (b,d) and the corresponding DAPI stain (a,c). Note in the uninfected cells, nocodazole causes the intermediate filaments to collapse to one side of the nucleus. In the infected cells a juxtanuclear collapse was still seen, but vimentin filaments in the vicinity of the vacuole were still apparent (Fig. 8a and b, arrow). Longer incubations in nocadazole (4 hrs) caused a complete collapse of the vimentin in the infected cells (Fig. 8c and d); note however that even when vimentin networks were totally collapsed, the vimentin overcoating of the parasitophorous vacuole was still evident (arrows). x1800
infection. Note for example, mitochondrial overcoating was evident on the parasitophorous vacuole adjoining the host cell nucleus (arrow, middle right). Shortly after invasion there was R123 fluorescence associated with intracellular *T. gondii* (arrow, lower left). It was not clear whether the fluorescence was due to *T. gondii* parasite or the vacuole. The capacity for the parasitophorous vacuole or parasite to incorporate the dye apparently occurred only during the initial minutes post-invasion as judged by the fact that this was only observed for *T. gondii* parasites in the very distal positions in the host cell cytoplasm and never on parasites docked at the host cell nucleus.

The influence of microtubules on mitochondrial overcoating of the parasitophorous vacuole was assessed by treating infected cells with nocadazole for 2 hours to induce disassembly of the microtubules. Microtubule depolymerization had no effect on the association of the mitochondria with the vacuoles as determined by R123. Examination of the nocadazole treated cells with electron microscopy showed no significant differences in morphology in those mitochondria docked at the vacuolar surface.
Figure 9 - Mitochondrial overcoating of the parasite vacuoles as viewed by phase contrast (a) and staining with R123 (b), which stains the host cell mitochondria. Arrows indicate the presence of the parasite vacuoles. Note the presence of the host cell mitochondria around the parasite vacuoles. Mitochondria were most obvious around the mature vacuole (arrow, far left) but also evident around the young vacuole at the host cell nuclear surface (arrow, to the right). Additionally, note the vacuole in the peripheral cytoplasm (arrow, foreground) stains intensely with R123, whereas the more mature intracellular stages do not stain with the dye.
Organization of the endoplasmic reticulum in Vero cells during *T. gondii* infection

The organization of the endoplasmic reticulum was examined with the fluorescent probe, DiOC6(3). Although DiOC6 stains the endoplasmic reticulum, mitochondria and other intracellular membranes, the endoplasmic reticulum was easily identified by its distinctive morphology (Terasaki et al., 1984, 1989). Stained endoplasmic reticulum appeared as a reticulate network while mitochondria were larger vesicular structures. As shown by Terasaki et al. (1989), lightly fixing the cells in glutaraldehyde preserves the endoplasmic reticulum staining and eliminates the toxic side effects of the dye and the possible photodamage that occurs when viewing unfixed cells. Both living and lightly fixed cells were examined. Both had comparable stainings and therefore, fixed cells were used in this study.

Staining of infected cells with DiOC6(3) showed a significant reorganization of the host cell endoplasmic reticulum during the course of *T. gondii* growth and reproduction. The endoplasmic reticulum of Vero cells in the early stages of infection remained unchanged. The endoplasmic reticulum displayed the typical reticulate type network and extended from the cell center out to the cell periphery (Fig. 10a and c). In heavily infected Vero
Figure 10 - Organization of the endoplasmic reticulum in early infected cells (a,c) and a late stage of infection (b,d). Note the reticulate nature of the endoplasmic reticulum present in the early stage of infection vs the swollen endoplasmic reticulum present in the late stage of infection (small arrows, a and b). A enlargement of the endoplasmic reticulum illustrating the reticulate vs. swollen cisternae of the endoplasmic reticulum is shown in 10c and d respectively. Note, the presence of a young parasite vacuole containing 2 parasites depicted at the large arrow in 10a vs. the large number of parasites (p) in 10b.
cells however, the reticulate like nature of the endoplasmic reticulum was totally disrupted (Fig. 10b and d). The endoplasmic reticulum appeared "swollen" and was retracted from the cell edge. The central region of the cell contains the Golgi apparatus and the microtubule organizing center and the overlapping membranes make observation of the endoplasmic reticulum in this region of the cell difficult. However, it appears the endoplasmic reticulum membranes in this area of the cell are also swollen, indicating the endoplasmic reticulum was disrupted throughout the entire cell. In more heavily infected cells the endoplasmic reticulum was even further fragmented with the peripheral cytosol totally devoid of endoplasmic reticulum membranes.

The parasites and the vacuolar membrane also stained intensely with DiOC6(3) (Fig. 10). The staining of the vacuolar membrane was probably due in part to the host cell endoplasmic reticulum membranes which envelopes the parasitophorous vacuole. However the dye also stained the parasite interior, probably due to staining of the parasite endoplasmic reticulum and other internal membranes.
DISCUSSION

In this study the impact of the *T. gondii* domain on various components of the host cell cytoplasm was examined. Host cell microtubules associate with the parasitophorous vacuole beginning when the vacuoles first appeared at the host cell nucleus and persisting throughout the course of infection. The microtubules accumulated at the perimeter of the vacuoles as a band appearing to partition the parasitophorous vacuoles from the surrounding host cell cytoplasm. Host cell mitochondria were also observed to overcoat the vacuoles at about the same time. Despite the association of microtubules with the vacuoles, depolymerization of microtubules did not remove intermediate filaments or mitochondrial overcoating of the parasite vacuoles or cause displacement of the parasite vacuoles from the host cell nucleus. These results indicate the association of the vacuole with these host cell organelles is independent of the microtubules. Infection was found to cause a disruption in the organization of the host cell endoplasmic reticulum, an organelle whose distribution is highly dependent upon microtubules.

The interactions of the parasitophorous vacuoles with host cell microtubules are similar to those reported to occur
with the intermediate filaments (Chapter 1). First, the initiation of microtubular interactions occurs when the vacuole first appears at the host cell nucleus and microtubules increasingly accumulate around the enlarging vacuole. Secondly, as with the intermediate filaments, infection had little impact on the microtubules in the areas of the cytoplasm peripheral to the vacuoles. Since cytoskeletal structures present in the same cell interact with one another forming a integrated cytoskeleton, any alterations or disruptions of one system may lead to structural reorganizations of other systems. The similar effect of infection on the organization of the microtubules and intermediate filaments, may in part reflect the interrelationship between these two cytoskeletal systems.

Several observations indicate that the interactions of the intermediate filaments in the vicinity of the parasitophorous vacuole are distinct from those of the microtubules. First, while the microtubules were associated with the vacuole there was no evidence of a specificity of the microtubules for the vacuolar surface as there appeared to be with the intermediate filaments. Secondly, the retention of vimentin overcoating in nocadazole treated cells also indicated the vacuoles have an affinity for vimentin independent of the microtubules.
When nocadazole treated cells were stained with anti-tubulin on the other hand, there was no evidence of tubulin binding to the vacuolar surface. Finally, the fact that depolymerization of the microtubules did not displace the parasitophorous vacuoles from their juxtanuclear position, indicates that vacuole docking to the nucleus does not involve microtubules. These observations, together with those reported in Chapter 1, indicate that the intermediate filaments are interacting directly with the vacuolar surface, perhaps serving to localize the vacuole to a discrete location within the host cell. The overcoating of vacuoles with microtubules on the other hand does not appear to be involved in maintaining the position of the vacuoles in the host cell cytoplasm.

Since the endoplasmic reticulum and mitochondria are attached to the microtubular filaments in most cells (Summerhayes et al., 1983), the arrangement of these organelles around the vacuoles might also be expected to be dependent upon cytoskeletal elements. However, depolymerization of the microtubules had no effect of the overcoating of the vacuole with mitochondria. This in part could be due to a retention of vimentin overcoating around the vacuoles, as in many cells, mitochondria are also attached to intermediate filaments (Mose-Larsen et al., 1982; Summerhaynes et al., 1983). However, the
mitochondrial overcoating of the vacuole is reported to occur earlier (Jones et al., 1972) than vimentin overcoating, indicating the mitochondrial association with the vacuole is independent of cytoskeletal elements examined to date.

An accumulation of host cell microtubules around the parasitophorous vacuoles is nonetheless quite interesting, especially given the dynamic events that occur between the parasite compartment and the host cell membrane compartments. During the first hour of infection, fusion of the host cell lysosomes with the newly formed vacuole is inhibited (Sibley et al., 1986). During the same time exocytosis of membrane out of the vacuole is also occurring (Sibley 1992). Thereafter the host endoplasmic reticulum membranes envelope the parasite vacuole and increasingly accumulate around this compartment throughout the rest of the infection. Microtubules are known to be involved in the movement of membranous organelles in eukaryotic cells (Parton et al., 1991). Thus, it may be that the microtubular arrangement around the vacuoles is involved with the control of host cell membrane organelles in the vicinity of the vacuole.

It was surprising to find the endoplasmic reticulum membranes disrupted in the periphery of heavily infected
cells given that the microtubules in infected cells remain polymerized throughout this region during infection. In normal cells the distribution of the endoplasmic reticulum into the peripheral cytosol is dependent upon intact microtubules (Terasaki et al., 1986). When the microtubules are depolymerized, the endoplasmic reticulum network is retracted from the cell periphery and large cisternae begin to form (Terasaki et al., 1986). The appearance of these membranes is similar to that seen in the endoplasmic reticulum of moderately infected cells. The endoplasmic reticulum is an unstable configuration of membranes that requires a continued interaction with microtubules to remain extended (Schultze & Kirschner, 1987). Thus it could be that the swollen endoplasmic reticulum seen in *T. gondii* infected cells is due to a disruption of the interaction between the endoplasmic reticulum and microtubules.

In the related parasite, *Eimeria*, the cytoplasm of host cells exhibit a distension of the endoplasmic reticulum and the ability of host cells to endocytose is diminished (White et al., 1992). In a similar fashion the swollen endoplasmic reticulum observed in this study may also reflect an impairment in the host cells endocytic pathways. Coccidean parasites in general (e.g., *T. gondii*, *Sarcocystis* and *Eimeria* sp.) interfere with the fusion of
host cell lysosomes with the parasitophorous vacuole membrane. Perhaps the capacity to interfere with lysosomal fusion is a reflection of a more general process whereby these parasites can disrupt the endosomal system of the host cell. Indeed the apparent disruption of the endoplasmic reticulum-microtubular connection observed in this study suggests other membranous organelles attached to the microtubules, such as lysosomes (Collot et al., 1984), may also be disrupted.

In summary, a number of changes in the host cell cytoplasm over the course of infection have been described in this study. Through the course of infection the parasite dramatically restructures the cytoplasm of the host cell. The host cell cytoskeletal elements, endoplasmic reticulum and mitochondria are all reorganized around the parasite compartment. While the importance of these interactions are not fully understood at this time, they represent striking capacity of the parasite to modulate host cell structure.
CHAPTER 3

ASSOCIATION OF GTP-BINDING PROTEINS WITH THE TUBULAR NETWORK IN THE PARASITOPHOROUS VACUOLE OF TOXOPLASMA GONDII
Intracellular survival of *Toxoplasma gondii* is dependent upon establishment of the modified endosomal compartment. This compartment is derived from the host cell plasma membrane upon invasion, but within minutes parasite proteins are found in the parasitophorous vacuolar membrane (Kimata & Tanabe 1987; Nichols et al., 1983). Modification of the vacuolar membrane is thought to be due to the rapid secretion of a membranous network into the vacuole upon invasion (Sibley et al., 1986, 1989). The membranous network is thought to be involved in the resistance to phagolysosomal fusion and acquisition of nutrients from the host cell. The secretion of the intraphagosomal network is a crucial event in the establishment of infection and a further understanding of this network is of obvious importance to understanding the host parasite interactions.

The intraphagosomal network is a highly ordered structure comprised of membranous tubular elements. The tubular elements of the network are secreted from the dense granules (parasitic organelles) shortly after invasion. Throughout infection the network is increasingly accumulated in the vacuolar space and numerous connections with the vacuolar membrane are formed. Dense granule
proteins are found on the networks and on the vacuolar membrane (Jones et al., 1972; Sibley et al., 1986; Nichols et al., 1983; Sheffield & Melton 1968). These observations have lead to the suggestion that the networks are involved in contributing membrane and transporting parasite proteins to the vacuolar membrane. The mechanisms by which the networks may accomplish these processes are not understood.

GTP-binding proteins are ubiquitous membrane-associated molecules which have recently been found to be involved in the regulation of membrane traffic in a variety of eukaryotic cells (Bourne et al., 1990; Hall 1990). Two distinct families of GTP-binding proteins are known; the heterotrimeric (signal-transducing) G proteins and the monomeric GTP-binding proteins. Both the heterotrimeric and monomeric GTP-binding proteins function is a similar manner (Fig. 11a and b). The binding of guanine nucleotides is common to all such proteins, with the GTP-binding protein cycling between an inactive GDP-bound and active GTP-bound forms. Activation of GTP-binding proteins is catalyzed by agonist-liganded receptors and inactivation is due to the intrinsic GTPase activity of these proteins.
Figure 11 - Schematic of mechanism by which the heterotrimeric (a) and the monomeric (b) GTP-binding proteins are thought to function. Both heterotrimeric and monomeric GTP-binding proteins are inactive in the GDP bound state and active in the GTP bound state. Note the heterotrimeric G proteins are composed of 3 subunits (α, β and γ) which dissociate when the α subunit is in the GTP bound state. Trimeric GTP-binding proteins are stimulated by agonist(A)-liganded receptors (R), which catalyze a GDP/GTP exchange, and inactivated by their intrinsic GTPase activity. The monomeric GTP-binding proteins are composed of only one subunit of small molecular weight (20–30 kDa) which contains the GTP-binding site; the subunit in the diagram is designated p21. Like the trimeric GTP-binding proteins, the monomeric proteins are activated by an interaction with a receptor, which catalyses the GDP/GTP exchange and likewise inactivated by their intrinsic GTPase activity which cleaves the terminal phosphate and returns the protein to the GDP-bound state.
The larger heterotrimeric G proteins are composed of 3 subunits (α, β, and γ). The α-subunit contains the GTP binding site and effects a cellular response by modulating the activity of membrane bound enzymes which generate second messengers (Gilman 1987). Recently, the β and γ subunits have also been recognized to play a role in transmembrane signalling (Clapman & Neer 1993). The α subunits are diverse, ranging in weight from 39-52 kDa and are thought to confer specificity to the G-protein-receptor and effector interactions. The monomeric G proteins are composed of a single subunit of 20-30 kDa, which contains the GTP-binding site. The small molecular weight G proteins have been implicated in the targeting of membrane vesicles to and fusion with appropriate acceptor compartments in the secretory pathway (Hall 1990). The heterotrimeric GTP-binding proteins have recently also been implicated in the secretory pathway in eukaryotic cells and it is now thought that both monomeric and heterotrimeric GTP-binding proteins are involved in the regulation of membrane traffic (Bombsel & Mostov 1992).

In this study the presence of GTP-binding proteins on the intraphagosomes network in T. gondii was investigated. The small molecular weight G proteins were identified by a specific GTP blot assay and the trimeric G proteins were identified using an antibody to the α subunit. T. gondii
was found to possess small molecular weight G protein(s), at least one of which is present on the intraphagosomal networks. Additionally, the parasite was found to contain a 41 kDa protein indicative of an α subunit of a trimeric G protein. This G protein was not found on the networks but rather localized to an intracellular compartment which undergoes a dramatic reorganization during invasion. By immunoelectron microscopy this compartment was identified as the inner membrane complex, a compartment involved in secretion of the networks. Results indicate a trimeric G protein in conjunction with inner membrane complex is involved in regulation of secretion of the intraphagosomal network. Furthermore the presence of the monomeric G proteins on the intraphagosomal network suggests mechanisms may exist for the intraphagosomal network to regulate membrane traffic in the parasitophorous vacuole.

MATERIALS AND METHODS

Cell and parasite culture
Vero cells were grown in DMEM containing 10% FBS and 100 U/ml penicillin and streptomycin and maintained in a 37°C incubator in the presence of 5% CO₂. *T. gondii*, Rh strain, were propagated in Vero cells. To infect cultures, parasites were liberated from host cells by forcing a
suspension of infected cells through a 27-gauge needle. Extracellular T. gondii cells were purified from remaining host cells by filtration through 3.0 μm polycarbonate membrane filters and washed three times by centrifugation at 250xg for 10 minutes. Cultures were infected by the addition of parasites in fresh medium to Vero cells. After 15 minutes at 37°C, the inoculum was removed, the monolayer rinsed once and incubated with fresh medium.

Isolation of the intraphagosomal network
Tachyzoites were harvested from infected Vero cells in Hank’s balanced salt solution supplemented with 1 mM calcium and 10 mM Hepes. Freshly isolated, washed T. gondii cells were incubated for 30 min at 4°C in dissociation buffer consisting of calcium-magnesium-free phosphate-buffered saline, pH 7.2 containing 0.1mM EDTA, 0.1 mM dithiotreitol, 1 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml tosyl lysine chloromethyl ketone. The protocol used to isolate the intraphagosomal network of T. gondii is illustrated in Figure 14. After incubation in dissociation buffer, intact T. gondii cells were pelleted and the supernatant clarified by five spins at 1,000xg for 10 min each. The clarified supernatant was fractionated by centrifugation, first at 10,000xg for 60 min then at 100,000xg for 120 min. The 100,000xg pellet was washed
twice in dissociation buffer and centrifuged at 100,000xg for 60 min.

**SDS polyacrylamide gel electrophoresis**

*T. gondii* cells and fractions isolated as described above were boiled 2 min in 1% SDS, 2% 2-mercaptoethanol, 1% Triton X-100, 10 mM Tris-HCl (pH=6.8), containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 1 μg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml tosyl lysine chloromethyl ketone. Proteins were separated by electrophoresis in 5-20% or 5-15% gradient polyacrylamide gels using a Laemmli discontinuous system (Laemmli 1970). Gels were stained with 0.25% Coomassie Blue dissolved in 40% methanol + 10% acetic acid and destained in 40% methanol + 10% acetic acid.

**Immunoblotting**

Proteins were transferred from SDS polyacrylamide gels to nitrocellulose paper by electrophoresis at 30 V for 8 hours at 4°C, using the glycine-methanol buffer system. Blots to be probed with antibody were blocked in PBS + 5% milk and subsequently incubated with primary antiserum diluted 1:100 in PBS + 5% milk for 4 hours at 4°C. Blots were washed three times in PBS + 5% milk followed by a final wash in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5 + 5% milk. Blots were then incubated with alkaline phosphatase
conjugated anti-rabbit antibody, diluted 1:500, incubated for 2 hours at room temperature. After extensive washing, blots were reacted with chromogenic substrate BCIP/NBT (5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium).

GTP blot assay
The GTP blot assay was done as described by Lapetina (1987) to detect small molecular weight GTP-binding proteins. This method has been shown to allow renaturation of the monomeric GTP-binding proteins and thus nucleotide binding capacity is retained. Nitrocellulose filters were incubated in 20-mM Tris, pH 7.7, 0.25% Tween 20, 2 mM DTT, 50 μM MgCl₂, and 1 nM carrier-free [α-³²P]GTP. Blots were incubated with the radiolabelled GTP for 60 min at room temperature, then washed twice for 10 minutes in buffer lacking radiolabelled GTP. In some experiments, 10 μM-unlabeled GTP or ATP was included in the incubation. Blots were air-dried, and apposed to Kodak X-OMAT AR film for 24-48 hours and bound [α-³²P]GTP was detected by autoradiography. In some instances blots were subsequently used for immunoblotting.

Indirect immunofluorescence
Indirect immunofluorescence was done as described in Chapter 1.
Immunoperoxidase localization

Infected Vero cells were fixed in cold 2% formaldehyde, 0.1% glutaraldehyde in 0.1 M cacodylate buffer for 30 minutes at 4°C. Cells were successively incubated in antisera at 37°C and washed with PBS at 4°C in primary antibody (anti-Ga) diluted 1:100, followed by peroxidase-conjugated anti-rabbit antibody diluted 1:20 in PBS. Cells were refixed in 2.5% glutaraldehyde for 1 hr at 4°C and subsequently rinsed in Tris-HCl, pH=7.4, developed for 3 minutes with diaminobenzidine and fixed in 1% osmium tetroxide. Cells were dehydrated in acetone, embedded in Epon and thin sections were examined. Infected cells were also prepared for immunogold labelling by fixation in 1% formaldehyde, 0.1% glutaraldehyde for 10 minutes and subsequently dehydrated in ethanol and embedded in LR white or alternatively dehydrated in acetone and embedded in Epon.

RESULTS

Development of the intraphagosomal network in T. gondii infected Vero cells

The intraphagosomal network in T. gondii infected Vero cells is shown in Figure 12. In the mature parasitophorous vacuole, the intraphagosomal network is comprised largely
Figure 12 - Views of *T. gondii* in Vero cells illustrating the intraphagosomal network in the vacuole. (a) Electron micrograph of *T. gondii* (P) in Vero cells (HC) illustrating the intraphagosomal networks (N). Note the networks occupy most of the intravacuolar space; x 12,000. (b) Detail of the networks (N) illustrating tubular elements comprising the networks; x 18,000x. The small arrow denotes immature dense granule and the large arrow a mature dense granule.
of tubular elements of 40-60 nm in diameter (Figure 12a). The tubules occupy considerable space within the vacuole, largely filling the lumen. The tubules are formed in the dense granules of the parasite and secreted into the intraphagosomal lumen at the lateral surface of the parasite. As shown in Figure 12b, parasites contain 2 types of dense granules, an immature form which is electron dense and located toward the cell interior (small arrow) and a mature form which contains tubulovesicular membranes and forms near the outer membrane of the parasite (large arrow).

The intraphagosomal network was found to be responsive to the phosphatase inhibitor, okadaic acid. When cells were grown in the presence of this drug, the amount of network in the vacuole was greatly enhanced. The enhanced expression of the tubules allowed for a more detailed examination of tubule structure. As shown in Fig. 13a, membranous tubules are found inside the dense granule prior to their release into the lumen. Release of the tubular vesicles appears to occur through only a small opening as the tubular extension (arrow, Fig. 13a) suggests. The tubules are clearly membranous in nature as evidenced by the trilaminar appearance of the tubules (Figure 13b). Furthermore, in cross section, it is also evident material is present in the interior (arrow, Fig.
Figure 13 - Views of okadaic acid induced expressions of the intraphagosomal network. (a) Note the dense granule(g) just below the surface of the parasite with vesicular tubes inside; note the long tube projecting from the parasite surface (arrow). (b) details of tubes of networks illustrating the trilaminar appearance (arrows) indicative of the membranous nature of the tubular network. (c) tubes in cross section showing central dense core indicating presence of material in the interior of the tube. Also note amorphous "fuzzy" material to the right which is commonly seen around the tubes. x 100,000.
Additionally, a "fuzzy" amorphous material is often present around the tubules (Fig. 13c).

The intraphagosomal network exhibits a calcium responsiveness, adhering to *T. gondii* cells in the presence of calcium and dispersing in the absence of calcium. This calcium responsiveness was used to isolate the intraphagosomal network from *T. gondii* cells as described by Sibley et al. (1986). A schematic of the isolation procedure is illustrated in Fig. 14c. The isolation of intraphagosomal networks by this method was verified by transmission electron microscopy of the 100,000xg pellet. The 100,000xg pellet was found to consist of membrane-delineated vesicles of similar size and appearance as those found in the parasitophorous vacuole (Figure 14a and b), indicating the material purified was comparable to that of intact networks.

**Identification of a small molecular weight GTP-binding protein on the intraphagosomal network**

Low molecular weight GTP-binding proteins were detected by probing Western blots with \( \alpha^{32}P \) GTP, followed by autoradiography. \( \alpha^{32}P \)GTP labelled a band with molecular mass of approximately 25 kDa (Figure 15b) in both the tachyzoites and the isolated intraphagosomal network fraction. The retention of GTP binding activity in the
Figure 14 - Electron micrograph of isolated intraphagosomal network fraction. (a) low magnification (x 20,000) and (b) high magnification (x40,000) illustrating the membranous profiles. (c) Schematic of isolation protocol.
Figure 15 - Coomassie blue stained (a) lane 1 - MW marker, lane 2 - T. gondii cells; lane 3 intraphagosomal network; GTP blot (b), lane 1 & 2 - T. gondii, lane 3 & 4 - T. gondii + dissociation buffer; lane 5 - isolated intraphagosomal network fraction.
tachyzoites after the removal of the intraphagosomal network indicates this GTP-binding protein is present within the parasite as well as on the networks.

Comparison of the profile of GTP-binding proteins from Vero host cells and the parasite indicated the GTP-binding protein was unlikely to be a contaminant from the host cell (Figure 16b). Vero cells had 2 labelled bands corresponding to proteins, of 23 and 25 kDa, indicating at least 2 GTP-binding proteins, as would be expected of mammalian cells. The intact parasites (lane 3 and 4) and the intraphagosomal network fraction (lane 2) contained a single band at approximately 25 kDa, consistent with the results of the first blot. This same membrane preparation was probed with a polyclonal antibody to T. gondii (Figure 16a). The antibody reacted with parasite proteins in the area corresponding to the area demonstrating GTP-binding activity. The immunoreactive material, which was not present in the host cells, supports the interpretation that these were T. gondii proteins. In the western blot, it appeared 2 proteins may be present in the intact parasite while the intraphagosomal network fraction contained only 1 protein in this range.
Figure 16 - Western blot (16a) and corresponding GTP blot (16b); lane 1 - Vero cells; lane 2 - intraphagosomal network fraction, lane 3 - *T. gondii* cells; lane 4 - *T. gondii* cells
Nucleotide binding was specific for GTP because excess cold GTP could prevent \( [\alpha^{32}\text{P}] \) binding, but excess ATP did not (Figure 17). A shorter incubation time was used to attain better resolution of the bands; note there is a faint indication of 2 separate bands around 25 kDa.

**Identification of trimeric G proteins**

The heterotrimeric G proteins were identified via western blotting using the antibody, GA/1. This antibody is directed against a conserved peptide within the GTP-binding site and is specific for the alpha subunits of the trimeric G proteins (Simonds, 1989). The antibody reacts to G\(_{s}\), G\(_{i}\), G\(_{o}\) and G\(_{q}\). The antibody recognized a single protein of approximately 41 kDa in the *T. gondii* cells (lanes 3-5) and 2 proteins of 42 and 36 kDa respectively, in Vero cells, the host cells. The different banding pattern in the tachyzoite preparation and the Vero cells is indicative that the parasite preparation is free of host cell contamination. Infected cells were probed with GA/1 to verify the protein in the parasite was distinct from either of those found in the host cells (Vero cells). The antibody recognized 4 bands, with 2 bands of approximately 36 and 45 kDa, corresponding to the host cell G proteins and 2 bands of approximately 41 kDa, corresponding to parasite proteins. The presence of G proteins in the intraphagosomesomal network fraction was also
Figure 17 - GTP blot demonstrating specificity of the GTP subunit. Lane 1 & 2 - tachyzoites incubated with [α-32P]-GTP; lane 3 & 4 - tachyzoites incubated with [α-32P] and cold 10 mM GTP; lane 5 & 6 - tachyzoites incubated with [α-32P] and 10 mM cold ATP.
assayed by western blotting with the GA/1 antibody. No proteins were detected in the intraphagosomal network fraction (lane 2). No bands were detected in the 20-30 kDa range in either the parasite preparations or the host cells, indicating this antibody does not react with the monomeric G proteins.

**Immunofluorescence of the heterotrimeric G proteins in infected cells**

The GA/1 antibody was used to localize the trimeric G proteins in the parasitophorous vacuole via indirect immunofluorescence. A tubular staining pattern was observed in the parasitophorous vacuole (Figure 19). The close correspondence of the tubular staining pattern with the presence of the individual parasites, as evident by counter staining with DAPI, indicates the G protein α subunit is confined to the individual parasites. There appeared to be no staining in the lumen of the vacuole or parasitophorous vacuole membrane. This supports the result of the Western blot which showed no reactivity on the networks. The tubular staining pattern assumed a "wishbone" configuration. This is most evident in the parasite in the lower right (arrow, Figure 19a). The close correspondence of the staining with the contour of the parasite shape indicates the G protein is located on or
Figure 18 - Western blot of parasite and host cells probed with GA/1 antibody, which reacts with the consensus peptide from the GTP binding site of GTP binding proteins. Lane 1,3-5; T. gondii tachyzoites; Lane 2 - isolated intraphagosomal network fraction; Lane 6 - infected Vero cells; Lane 7 - Vero cells. The position of the molecular weight standards are indicated on the left.
just beneath the surface of the parasite. Interestingly in extracellular tachyzoites this tubular staining pattern was not evident (large arrow, Figure 19a). Additionally, in some vacuoles, there was evidence of an intermediate staining pattern with more prominent staining near one end of the parasite and the tubular staining present on only one side (Figure 19c and d).

**Reorganization of G protein compartment upon invasion**

Because of the different staining pattern observed in the extracellular and intracellular tachyzoites, the organization of the G proteins was analyzed in tachyzoites prior to invasion and at 15, 30 and 60 minutes after invasion using immunofluorescence and the GA/1 antibody (Figure 20a-d). The G proteins in the extracellular parasites displays a bipolar staining with the majority of fluorescence in one end of the parasite as shown in Figure 20a. In this figure, the parasites are near the host cell but have not yet invaded (as determined by phase microscopy). Based on the orientation of the parasite toward the host cell upon invasion (the anterior end penetrates host cell), the predominant staining appears in the posterior of the parasite. Staining of the parasites was not observed in cells which were not permeabilized indicating that the G protein(s) are an intracellular antigen.
Figure 19 - Immunofluorescence staining with the GA/1 antibody of *T. gondii* infected Vero cells (a & c) and the corresponding DAPI stain (b & d). Arrows designate the prominently staining tubular extensions found along the lateral surface of the parasite. x3600
Within the first hour after invasion, the G protein(s) undergoes a dramatic change in localization as indicated in figures 20b-d. The beginnings of the tubular pattern is evident within 15 minutes after invasion as shown in figure 20b. By 30 minutes post invasion, the posterior portion of the parasite still exhibits prominent staining, but most parasites now show evidence of a tubular extension on one side (arrows). By 1 hour after invasion, the tubular pattern observed in mature vacuoles is attained. Note the staining appears to conform to the contour of the parasite indicating it is located just below the cell surface.

The reorganization of the G protein(s) appears to begin almost immediately after invasion, moving primarily from a concentration in the posterior part of the parasite, to a tubular configuration, over the first hour after invasion. A composite view of the reorganization of the G protein(s), representing the various stages, is presented in Figure 20e. In the extracellular parasite, the protein(s) are concentrated in the anterior and posterior ends of the parasite. Immediately upon invasion however, the movement of the protein from this posterior position to the lateral side is evident. Subsequently, fluorescence is seen on both sides of the parasite and finally, the mature "wishbone" configuration is attained at one hour.
Figure 20 - Immunofluorescence staining using the GA/1 antibody at 0 (a), 15 (b), 30 (c) and 60 min (d) after invasion. Arrows denote the movement of the G protein from a posterior location to the lateral side of the parasite; (e) a composite of the sequential stages identified in the movement of the G protein over the course of the first hour after invasion (1-4) and the mature configuration observed at 4 hours post invasion (5). x2800
after invasion. The staining does not extend the entire length of the parasite, but appears to be discontinuous at the posterior end of the parasite. The mature wishbone staining pattern persists throughout the infection.

**Effect of Brefeldin A on the G protein compartment**

To further define the nature of the compartment containing the G protein(s), the effects of brefeldin A on extracellular tachyzoites and the infected host cells was assessed. Brefeldin A has been shown to cause the Golgi compartment to mix with the endoplasmic reticulum in a wide variety of eukaryotic cells (Klausner et al., 1992). Mixture of the Golgi with the endoplasmic reticulum is evident within 15 min at 37°C and is evidenced by a punctate staining pattern. As shown in Figure 21, incubation in Brefeldin A resulted in a diffuse punctate staining pattern of the G protein in the tachyzoites. This effect was evident within 15 minutes. The G protein compartment in intracellular parasites however, was largely unaffected at this time. Incubation in the drug was continued for up to 2 hours to ensure the drug had penetrated the host cell. In the majority of cells, the tubular staining pattern remained intact indicating the proteins had moved to a resistant compartment. Some disruption of the localization of the G protein(s) was evident at this time as shown in Figure 21. However,
Figure 21 - Immunofluorescence staining using the GA/1 antibody, demonstrating the effects of brefeldin A on the distribution of the G protein in extracellular parasites (a and b) and intracellular parasites (c and d). In the extracellular parasites brefeldin A induces a vesiculation of the G protein (arrows a vs. b). In the intracellular parasites the tubular pattern is still evident (arrow) at 30 minutes in the drug; at 2 hours in the drug the tubular pattern is somewhat condensed but is vesiculation of the G protein compartment, as observed in the tachyzoites did not occur (compare b to d). x3700
rather than a vesiculation of the fluorescence, a condensation of fluorescence occurred, indicating this was not due to mixing of the golgi with the endoplasmic reticulum. Cells were also observed to exit the host cells at this time indicating the drug had a toxic effect on the intracellular parasites or the host cells.

**Immunoelectron microscopy of the G protein compartment**

To more precisely determine the location of the G protein compartment, electron microscopy immunolocalization techniques were used. Infected cells were reacted with the GA/1 antibody, followed by incubation with a horseradish peroxidase conjugated secondary antibody. Labelling was done with lightly fixed cells prior to embedding. This labelling procedure gave a slight reaction product in a few intracellular tachyzoites (Figure 22). For example, a mild reaction product is present at the anterior end on the parasite on the right (arrows, Fig. 22a-d). The structure evidencing a reaction product at the anterior end of the parasite, corresponds to the inner membrane complex. While the reaction was modest, comparable labelling was not observed in controls. Additionally, labelling when present, was consistently found on the inner membrane complex.
Figure 22 - Immunoperoxidase reaction, using the GA/1 antibody, of *T. gondii* infected Vero cells. Note the reaction product in the anterior region of the parasite (arrows), labelling the interior membranes corresponding to the inner membrane complex. x18,000 (a & b); x 36,000 (c & d).
In order to facilitate access of the label to the parasites, tachyzoites were liberated from the host cells and incubated in the presence of the GA/1 antibody and labelled with horseradish peroxidase conjugated secondary antibody. This procedure also labelled the inner membrane complex confirming the results observed in the permeabilized intact cells. Labelling of the inner membrane complex was enhanced in tachyzoites where the pellicle was slightly disrupted exposing the inner membrane complex to the GA/1 antibody (Figure 23). This indicates the mild reaction was due to inaccessibility of the antigen to the antibody. Labelling of sections with the GA/1 antibody and a gold conjugated secondary antibody was attempted but were unsuccessful due to nonspecific binding of the antibody to the LR white resin, and a lack of reactivity using Epon.

DISCUSSION

In this study the presence of GTP-binding proteins on the intraphagosomal network in the parasitophorous vacuole of T. gondii was investigated. A small molecular weight GTP-binding protein of approximately 25 kDa was found to be present on the networks. An approximately 41 kDa protein was found within the parasite. This corresponds to the
Figure 23 - Immunoperoxidase labelling, using the GA/1 antibody, of tachyzoites. (a); arrows denote the uniformity of the label around the perimeter of the cell (x 24,000); (b) detail of an area where the membranes are disrupted showing labelling of the inner membranes (x 75,000); (c) detail of another region of the cell showing label on the inner membrane complex where membranes are not disrupted (x 75,000).
molecular mass of the α subunit of the heterotrimeric G proteins. This protein was found to undergo a dramatic relocalization upon invasion, moving from a posterior location to the lateral sides of the parasite. Preliminary localization studies identified this protein to be localized to the inner membrane complex. These data suggest the heterotrimeric G protein, in conjunction with the inner membrane complex, may be involved in secretion of the networks into the vacuole.

The monomeric G proteins are a large family (about 80 members) and are classified into the ras, rab and ARF subfamilies. Western blotting using an antibody to ras-like proteins (data not shown), indicated this protein was not a member of the ras type subfamily, indicating it may be related to one of the other subfamilies. Rab and ARF, are associated with membranes and both are thought to function in membrane trafficking (Balch 1990, Pryer et al., 1992). The rab type GTP-binding proteins are thought to directionally target vesicles between specific membrane compartments in the secretory pathway in a role analogous to the GTP-binding protein ET-TU in protein synthesis (Bourne 1988). The ARF type GTP-binding proteins on the other hand regulate membrane traffic by promoting vesiculation or tubulation of membrane vesicles by controlling the "coating" on vesicles; the presence of a
membrane coat causes vesiculation and the absence results in tubulation of the vesicle (Klausner et al., 1992). Both of these proteins are highly conserved and have been identified to function in membrane traffic in cells as diverse as mammalian cells to yeast. Additionally, ARF type proteins have been identified in the protozoan, Giardia, indicating these proteins are also found in protozoans (Murtagh et al., 1992).

Further studies are necessary to determine the subfamily to which the 25 kDa G protein is related. However, the presence of a rab or ARF like GTP-binding protein on the networks suggests intriguing possibilities as to how they may function on the networks. For example, ARF like GTP-binding protein cause dramatic changes in the morphology of vesicular structures simply by regulating coating or uncoating of the membranes (Klausner et al., 1992). In an analogous fashion, an ARF type protein on the networks could regulate the assembly of the network from a vesicular to tubular configuration providing an effective way for the intraphagosomal network to connect with the parasitophorous vacuolar membrane. That is, linearization of the network tubules would facilitate elongation of the tubules and thus eventual contact with the parasitophorous vacuolar membrane. A mechanism to facilitate rapid contact with the parasitophorous vacuolar membrane is of obvious
importance because incorporation of proteins into this membrane is thought to prevent phagolysosomal fusion. Thereafter mechanisms to promote elongation of tubules would contribute membrane and proteins to the parasitophorous vacuolar membrane, essential for vacuolar growth and parasite survival. It is not known if T. gondii has "coating" proteins, but a "fuzzy" material was often observed in association with the tubules in the vacuole. Interestingly this fuzzy material is most prominent near recently secreted tubules (Fig.13a) as would be expected if uncoating proteins were involved in the linearization of the tubulovesicular vesicles upon secretion.

Likewise, if the 25 kDa GTP-binding protein were a member of the rab subfamily, this would indicate that the networks have the capability to specifically target membrane vesicles within the vacuole. There is evidence to support the idea that the networks have mechanisms to direct membrane flow to discrete sites within the vacuole. For example, proteins on the intraphagosomal network undergo differential targeting in the parasitophorous vacuole with some proteins found on the vacuolar membrane and intraphagosomal network while others are found on the networks only (Achbarou et al., 1991). Similar reports of differential targeting of parasite proteins in the bradyzoite stage of development have also been reported
(Torpier et al., 1993). The ability to direct membrane traffic offers the possibility of distinct domains and therefore that functional specialization on the networks and on the vacuolar membrane may exist.

The presence of a monomeric G protein on the networks, which are secreted and therefore external to the cell, is unusual given most GTP-binding proteins are found on internal membranes. However, association of low molecular weight GTP-binding proteins with some secretory products has been reported. For example, low molecular weight GTP-binding proteins are secreted from mammary epithelial cells in association with lipid globules (Ghosal et al., 1993); in association with released exosomes during reticulocyte maturation (Vidal and Stahl, 1993) and with rat lamellar bodies in alveoli cells (Rubins et al., 1992). In these situations the GTP-binding proteins are found on membranes which are secreted as vesicles, analogous to the secretion of membranous vesicles of the intraphagosomal network. This suggests association of small molecular weight GTP-binding protein with complex secretory products may be a general phenomenon. In the developing lipid globule and the lamellar body in the rat, the GTP-binding proteins were found to associate with the developing lipid and lamellar body respectively and in both instances it was thought these proteins may serve to
direct membrane materials into these developing organelles. Likewise the role of the 25 kDa G protein in *T. gondii* may function similarly in the complex events accompanying the formation of the tubulovesicular membranes inside the dense granule. Indeed the development of the lamellar body in the rat alveoli, bears a striking morphological similarity to the lamellae observed in the developing dense granule (Gil 1985).

In this study a heterotrimeric G protein with an alpha subunit of approximately 41 kDa was found in the parasite. Immunolocalization of the 41 kDa G protein with electron microscopy and light microscopy, indicates this protein is found predominantly within the parasite on the inner membrane complex. Although the immunoperoxidase labelling was modest, the tubular staining pattern observed on the light level corresponds precisely with known morphology of the inner membrane complex. This organelle is found just beneath the parasite outer membrane and consists of a series of flattened cisternae which are continuous around the parasite except for an opening at the anterior and posterior end.

The localization of the G protein to the inner membrane complex is interesting given the inner membrane complex has been identified as an compartment involved with
secretion in *T. gondii* (Charif et al., 1990). Notably, at an early stage in dense granule maturation, a transient contact of the dense granule to the inner membrane complex was observed. Thereafter, the membrane formation occurred and membrane whorls were subsequently formed inside the dense granule. Secretion of these membranous vesicles from the dense granule occurs with the dense granule in close opposition to the inner membrane complex and involves a small localized disruption in the inner membrane complex and the outer membranes of the parasite. These observations indicate the inner membrane complex may be involved in formation of membranes in the dense granule as well as the secretion of the networks. The localization of 41 kDa G protein α subunit, to the inner membrane complex suggests it may be part of a complex required later for signal transduction leading to exocytosis of the dense granule membrane as well as formation of the tubules within the dense granule.

The heterotrimeric G protein identified in *T. gondii* was approximately 41 kDa. The molecular weight indicates it may be a Go,-like subunit (Gilman 1987). Go, are 40-41 kDa subunits which can be ADP-ribosylated by pertussis toxin and are known to inhibition adenylate cyclase or stimulation of K⁺ channel in some cell types. Interestingly Go, are known to be involved in exocytotic
processes from a variety of cells. For example, G\textsubscript{i} type proteins have been identified on the secretory granules of mast cells (Aridor & Sagi-Eisenberg 1990), chromaffin cells (Vitale et al., 1991) and the acrosome of sperm (Glassner et al., 1991; Karnik et al., 1992). In these systems a G\textsubscript{\alpha} type proteins has been implicated to operate downstream of the generation of second messengers, functioning in the late stages of exocytosis mediating fusion with the plasma membrane (Gomperts 1990). The 41 kDa G\textsubscript{\alpha} in T. gondii may operate in a similar fashion in the secretion of the dense granules.

The immunofluorescence data indicate that the 41 kDa G protein \textalpha subunit is translocated to the inner membrane complex upon invasion. The recompartmentalization of the G protein upon invasion may be related to the involvement of this protein in the regulation of network secretion. The positioning of a 41 kDa protein on the inner membrane complex may make these membranes secretion competent, for example, or alternatively impose an inhibitory effect so as to ensure secretion proceeds at a regulated rate. It is notable that the secretion that occurs immediately after invasion is distinct from that which occurs throughout the infection cycle. That is, the exocytosis from the dense granules immediately after invasion occurs primarily from posteriorly located dense granules and
involves release of large amounts of the network and large openings through the plasma membranes. Secretion of the networks after the first hour postinvasion however, occurs primarily at the lateral sides and involves release of small amounts of the network with only small disruptions through the outer membrane and the inner membrane complex. The regulatory mechanisms involved in the secretion of the networks immediately after invasion may be distinct from those controlling secretion throughout the rest of the infection cycle.

Precisely how the G protein(s) are redistributed to the inner membrane complex at invasion, is not clear. Translocation of the Ga subunit upon stimulation has been observed in mouse mastocytoma cells (Negishi et al., 1992). A Ga, for example has been found to translocate from the cytosol to the plasma membrane upon stimulation. In this system it was found Ga, is not myristilated (as most other Ga are) and hence stimulation released it from the membrane. Translocation of the Ga subunit to the plasma membrane was found to require complexing with other proteins. In the present study, the western blots of infected cells revealed two bands compared to one detected in extracellular parasites. This suggests that a modification of the 41 kDa protein may be occurring which could be involved in the distribution of this protein.
within the organelle. Additionally, the movement of the protein was often observed from discrete areas in the posterior and anterior ends, areas where the inner membrane complex is discontinuous. The anterior and posterior ends of the inner membrane complex are "thickened" suggesting they could have different properties from other areas of the inner membrane complex and may in fact serve as specialized sites for translocating this protein within the inner membrane complex.

In summary this study has identified GTP-binding proteins of approximately 25 and 41 kDa in T. gondii. Localization data indicate that both of the GTP-binding proteins may be important in the intraphagosomal network. Further studies to define the subtype of the G proteins involved may yield information on the precise cellular functions of the networks.
SUMMARY

The preceding reports examine a number of features of the interactions that occur between Toxoplasma gondii and Vero cells during the intracellular stage of the infection. T. gondii vacuoles were found to associate with the host cell vimentin type intermediate filaments. Association of the intermediate filaments occurred when the vacuoles arrived at the host cell nuclear surface and appeared to involve filaments emanating from this surface. Depolymerization of the vimentin filaments resulted in a displacement of the vacuole from the host cell nuclear surface indicating the intermediate filaments may serve to "dock" the vacuole next to the host cell nucleus.

Interactions of the parasite vacuole with other host cell components were also studied. Namely the host cell microtubules were observed to accumulate at the periphery of the vacuoles throughout the course of infection while the microtubules in the distal cytosol were unaffected. The association of the vacuole with the host cell intermediate filaments, mitochondria and host cell nucleus was found to be independent of the microtubules. The host cell endoplasmic reticulum however was found to be disrupted during the course of infection, indicating the normal
disrupted in *T. gondii* infected cells. The overcoating of the vacuoles with microtubules therefore might be involved in controlling the host cell membrane traffic around the parasitophorous vacuole. These observations underscore the importance of the interactions with host cell components that occur at the vacuolar surface and implicate this host parasite interface to be a very dynamic surface.

The intraphagosomal network itself may contribute to this interface though transport of parasite proteins and lipids to the parasitophorous vacuole membrane. The presence of GTP-binding proteins on the network indicates this structure is highly organized and has mechanism to regulate the flow and distribution of parasite proteins on the parasitophorous vacuolar membrane. Additionally the findings of a GTP-binding protein involved in the secretion of the network also indicates the intraphagosomal network is a highly regulated structure. Further studies on the cellular functions of this network should provide more insights into the functions of the intraphagosomal network.


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Candidate: Sandra Karen Halonen

Major Field: Zoology

Title of Dissertation: In vitro Studies on the Interactions Between Toxoplasma gondii and Its Host Cell

Approved:

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Major Professor and Chairman
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

October 29, 1993