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CONSTRUCTION OF AN EPITOPE-TAGGED CLONE OF GRA3 FOR ELECTROPORATION INTO *TOXOPLASMA GONDII*

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree Doctor of Medicine

by

Dina D. Strachan

class of 1994

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CONSTRUCTION OF AN EPITOPE-TAGGED CLONE OF GRA3 FOR ELECTROPORATION INTO *TOXOPLASMA GONDII*. Dina D. Strachan (Sponsored by Keith A. Joiner) Section of Infectious diseases, Department of Internal Medicine, Yale University, School of Medicine, New Haven, CT.

Abstract

GRA3 is a protein produced and secreted by the intracellular, apicocomplexan parasite *Toxoplasma gondii*. The parasite enters nucleated cells in a parasite-directed process which involves the formation of a parasitophorous vacuole (PV). Upon entry the membrane of this vacuole, the parasitophorous vacuole membrane (PVM) is devoid of intramembranous particles. Sometime after invasion, GRA3 appears associated with the PVM. The amount of GRA3 associated with the PVM increases as the membrane matures. It is thought that GRA3 may be involved in the intracellular survival of the parasite. Exploration into the topology of this protein with respect to the PVM may provide insight into the GRA3's role in intracellular survival. Construction of an epitope-tagged cDNA clone of GRA3 was attempted without success. Construction of an epitope-tagged genomic clone of GRA3 from a λ–DASH II phage library is proposed. Further experiments involving the electroporation of a plasmid carrying an epitope-tagged clone into *Toxoplasma gondii* are planned.

I) Introduction

A) Obligate intracellular parasites

Parasitism can be generally defined as the use of one organism by another as a source of food and reproductive energy. A parasite differs from a predator in that it does not require the immediate death of the exploited organism. Most commonly, parasites are much smaller than their hosts. This size difference opens up additional opportunities for parasites which are unavailable to predators. Intracellular parasites have evolved the capability to exploit the unique set of resources available by taking up residence inside of another cell. Adapting to intracellular life is not, however, without its challenges. Firstly, for a relationship of intracellular parasitism to be established, the parasite may have to acquire an adaptation which facilitates its entry into the host cell. Once inside the cell, the parasite is then faced with the task of avoiding destruction and the challenge of co-opting or redirecting the host cell's functions toward its own survival and reproduction. Unlike facultative intracellular parasites, which have maintained their ability to survive both inside and outside the host cell, obligate intracellular parasites, such as *Toxoplasma gondii*, have adapted so well to intracellular living that they can no longer survive and reproduce in the extracellular environment.

B) Conventional Mechanisms of Cell Entry

An intracellular parasite must, of course, enter a cell. Cell entry presents the challenge of crossing the plasma membrane. Small molecules may be transported directly into the cytoplasm of a target cell via a carrier protein located in that cell's plasma membrane. Instead of actually crossing the membrane, a number of enveloped viruses (e.g. HIV) gain direct access to the cytoplasm through the fusion of the viral envelope to the plasma membrane of the target cell (Kielian, 1990). By far, however, the most common mechanism of cell entry for macromolecules and other particles, is through processes involving the invagination of the plasma membrane and the formation of vesicles.

There are several known mechanisms by which cells take up foreign material into membrane-bound vesicles. These processes may operate for the internalization of inert substances, such as latex beads, as well as for complex organisms. They differ with respect to a number of features, including cell type involved, particle size, and sorting of the vesicular contents. Endocytosis is a term that was proposed in 1963 to include processes such as phagocytosis ("cell eating") and pinocytosis ("cell drinking") (Bo Van Deurs, 1989). Currently, however, endocytosis is often used synonymously with pinocytosis (although a distinction is sometimes made with respect to vesicle size); phagocytosis has been shown to be a distinct process.

Endocytosis

Endocytosis is a constitutive, or on-going, process which can be carried out by all eukaryotic cells. This mechanism of internalization can be divided into clathrin-dependent and clathrin-independent categories--both to be discussed. Clathrin-dependent endocytosis begins at a specialized area of plasma membrane, the coated pit, so named because it is an invagination to which clathrin, a structural protein, localizes (Mahaffey, 1989). Clathrin is probably linked to the plasma membrane, and to the cytoplasmic tails of membrane receptors, through proteins called adaptins (Pearse, 1988; Rodman, 1990). Assembly and attachment of the clathrin coat is followed by further invagination and the formation of the coated vesicle (Lin, 1991; Brodsky, 1988; Fine and Ockleford, 1984). Both fluid-phase and receptor-bound material may be internalized in the process. Soon after the formation of the vesicle, the clathrin coat is shed (Salzman, 1988). At this time, the vesicle is termed an endosome. The shed clathrin is recycled to the plasma membrane (reviewed in Pearse, 1987).

Receptor-mediated endocytosis (RME), like endocytosis in general, is a constitutive process. Uptake of ligand during receptor-mediated endocytosis, however, depends upon the localization of the receptor-ligand complex to the clathrin pit. A number of receptors,

such as the LDL and transferrin receptors, are clustered in the clathrin pit and are internalized regardless of whether they are bound to ligand (Brown and Goldstein, 1986). Other receptors, however, such as the epidermal growth factor (EGF) receptor, are distributed randomly on the cell surface and only localize to the clathrin pit to be endocytosed after binding with ligand (Brown, 1991). The signals for localization and internalization appear to be transmitted at the cytoplasmic tail of the receptor (reviewed in Pearse, 1987). The original evidence for this observation came from the finding that in a particular form of familial hypercholesterolemia the mutation resulting in the failure of internalization of the LDL receptor has been localized to the receptor's cytoplasmic tail (Davis, 1986b). Subsequently, a variety of coated pit localization signals most of which contain tyrosine, have been identified. Membrane proteins normally excluded from the pit (e.g. influenza virus hemagglutinin) have been induced to localize there after the addition of a tyrosine residue (Lazarovits, 1988).

The possible fates of internalized material are many. The various ligands contained in a single endosome may have distinct fates. The pathway that a receptor-ligand complex takes is influenced by a number of factors, including function, cell type and distribution of receptors (reviewed in Brown, 1991). During the vesicle's maturation process, ATP-driven proton pumps in its membrane acidify the vesicular contents (Yamashiro, 1984). This drop in vesicular pH has been shown to play a significant role in the sorting of the vesicular contents (reviewed in Mellman, 1986). Most commonly, the contents of endosomes are passed into the lysosomal system where they are degraded. Other ligands, however, are able to escape degradation through transport back to the plasma membrane and release from the cell. Pathways of receptor-mediated endocytosis have been classified based on the sorting of the endosome's contents (reviewed in Brown et al., 1983; Goldstein et al., 1985; Mikhailov, 1989; Shepherd, 1989).

The pathway of the LDL receptor, described by Brown and Goldstein in 1986, exemplifies a class of receptor-mediated endocytosis characterized by passage of the ligand

into the lysosomal pathway and recycling of the receptor (Brown and Goldstein, 1986). A drop in pH in the endosomal compartment results in the release of the ligand from the receptor. The receptor, which is membrane-bound, pinches off in a vesicle which returns to the plasma membrane. The released ligand, as well as the rest of the endosomal contents, enter the lysosomal pathway.

Another type of RME is represented exclusively by the transferrin receptor. Transferrin is a plasma protein which binds iron and transport it to cells. In its iron-associated state, transferrin binds its receptor, which is located in the clathrin pit, and is internalized (Dautry-Varsat, 1983; Thorstensen, 1990; Aisen, 1992) In the acidic environment of the endosome (pH 5.5), the iron is unable to remain bound to transferrin and the iron is released into the lumen of the compartment (Dautry-Varsat, 1983). The association between the iron-free protein, now called apotransferrin, and its receptor is stable at acid pH (Dautry-Varsat, 1983; Aisen, 1992). The two remain associated and escape degradation via a smaller vesicle which buds off of the endosomal membrane. This vesicle is recycled to the plasma membrane where the neutral pH results in the release of apotransferrin from its receptor (Dautry-Varsat, 1983).

Both receptor and ligand are degraded in the lysosomal pathway in a third type of receptor-mediated endocytosis. Ligands which follow this pathway (e.g. EGF) are usually involved in signal transduction (Brown, 1991).

Transcytosis is a process unique to polarized cells. The receptor-ligand complex is transported across the cell to the membrane opposite to the site of entry (Brandli, 1991; reviewed in Rodman, 1990). The ligand is released, whereas the receptor may be recycled or degraded. This class is commonly represented by immunoglobulin-receptor complexes which are transported across epithelial cells (Mostov, 1991; Abrahamson, 1981; Rodewald, 1980).

Clathrin-independent mechanisms of endocytosis have been identified (reviewed in van Deurs, 1989; Sandvig, 1991). Substances such as cholera toxin (Carpentier, 1989)

and ricin (Madshus, 1987) are known to be taken up in clathrin-independent processes. Vesicles involved in these alternative uptake processes may be naked or coated with a material other than clathrin as has been found in the collecting duct intercalated cells of the kidney (Brown, 1987). The most thoroughly studied structures involved in clathrin-independent processes are the caveolae, flask-shaped invaginations on the surface of many cells (Anderson, 1993; Anderson, 1992; Rohlich, 1976; Orci, 1978). These structures have been shown to have some endocytic function (Tran, 1987), however, as with the other clathrin-independent uptake pathways their role appears to be minor. Recent work by Hansen, et. al, however, has demonstrated that particles internalized in both types of endocytosis may have similar fates as Con A-gold taken up by a clathrin-independent mechanism was found to be delivered to endosomes containing transferrin (Hansen, 1993).

Phagocytosis

Phagocytosis, or "cell eating," is another major cellular uptake process. Generally speaking, phagocytosis is limited to "professional" phagocytes of the reticuloendothelial system. This group includes neutrophils, eosinophils, monocytes, and, most importantly, macrophages. The ability to engulf invading microorganisms allows these cells to play a critical role in the defense of the host against infection. Each cell type contains a distinct set of cytoplasmic granules which contain a variety of hydrolytic enzymes. These granules essentially function as lysosomes as fusion of the phagosome with the granules may result in the destruction of an engulfed organism.

Phagocytosis, unlike endocytosis, is a receptor-dependent process. Receptors on the surface of the phagocytes bind to particles opsonized by antibodies (exposing the Fc portion), the C3b or iC3b component of complement, or containing oligosaccharide residues recognized by the mannose-fucose receptor (Stossel, 1978; reviewed in Silverstein, 1993). Internalization of a particle in phagocytosis involves the circumferential binding of receptors to these ligands on the target particle in a process called

"zippering." (Griffin, 1975). A major criterion of phagocytosis is that the ligands be distributed evenly over the surface of the particle being engulfed as particles expressing ligands at only one pole can bind to the target cell, however, they cannot be internalized (Griffin, et al, J. Exp. Med, 1975).

Clathrin has been found to occasionally be present at the sites of initiation of phagocytosis (Aggeler, 1982), however, a number of findings, in addition to the "zippering" requirement, suggest that this process is distinct from endocytosis. Phagocytosis involves the mobilization of actin filaments, and unlike endocytosis, is inhibited by cytochalasin B (Aggeler, 1982; Stossel, 1978). In addition, the vesicles formed in phagocytosis, which are 1 μm or greater in diameter (Stossel, 1978), are consistently bigger than those formed in endocytosis which average about 150 nm. Endocytosis proceeds at 15-18°C, whereas phagocytosis does not occur at these temperatures.

The respiratory burst is another important feature of phagocytosis (Babior, 1992). Internalization of a particle via the Fc receptor, or the mannose/fucose (MFR) receptor, triggers the formation of reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI), as well as arachidonate and eicosinoid generation (Wright, 1983). Phagocytosis via he complement receptor, however, is not associated with oxidative activity (Wright, 1983).

C) Comparison of Conventional and Unconventional Routes of Cell Entry: Implications for Evasion of Death

Parasites may exploit "conventional" uptake processes in order to gain access to the intracellular environment. The entry of *Salmonella typhimurium* into epithelial cells, for example, may be linked to the phosphorylation of the EGF receptor (Galan, 1992). Transcytosis is exploited by some gonococci in crossing the columnar epithelium of the human fallopian tube mucosa (McGee, 1988). The drawback of this strategy, however, is that, should the host cell be a phagocyte, entry may trigger oxidative attack. Parasites may

defend themselves against oxidative attack through the production of enzymes which disable free radicals, such as catalase and superoxide dismutase (reviewed in James, 1991). Instead, however, many avoid this problem through the use of another phagocytic receptor, the complement receptor, which is not associated with a respiratory burst.

Another problem associated with the use of conventional uptake processes by intracellular parasites is that the organism may be passed into a degradative pathway. For some organisms, such as *S. typhimurium* and the *Leishmania* species which are internalized by phagocytosis, survival in the acidic phagolysosome is not problematic; it is, in fact, preferred. Most intracellular parasites, however, are apparently adapted to avoid contact with lysosomal contents.

The development of a parasite-driven mechanism of cell entry may confer an advantage on an intracellular parasites for a number of reasons. Firstly, it may simply be necessary for cell entry as some non-phagocytic cells lack the proper uptake apparatus which would allow the entry of the organisms. A parasite-driven strategy for cell entry may also provide a means by which the entering organism can avoid contact with the hostile environment of the lysosome.

The strategy used by *Trypanosoma cruzi* to enter cells is a special case. The infectious form of this parasite, the trypomastigote, enters cells in an active process. Unlike the epimastigote, which appears to be taken up by a cytochalasin B-inhibited process (i.e. phagocytosis) (Zingales, 1985), trypomastigotes can enter both phagocytic and non-phagocytic cells (Vickerman, 1985; Zingales, 1985). Trypomastigotes enter host cells in an active process which, ironically, involves the recruitment of lysosomes to the site of invasions. In this process, the parasite is exposed to the hydrolytic, lysosomal enzymes (de Titto, 1983). The lysosomes fuse to form the membrane which eventually envelops the incoming parasite. (Tardieux, 1992). Although *T. cruzi* is able to withstand exposure to the lysosomal environment for some time, it must, however, escape from this compartment into the cytoplasm. Trypomastigotes which are able to exit the lysosomal

compartment survive intracellularly, whereas epimastigotes, which remain behind, are killed (Vickerman, 1985).

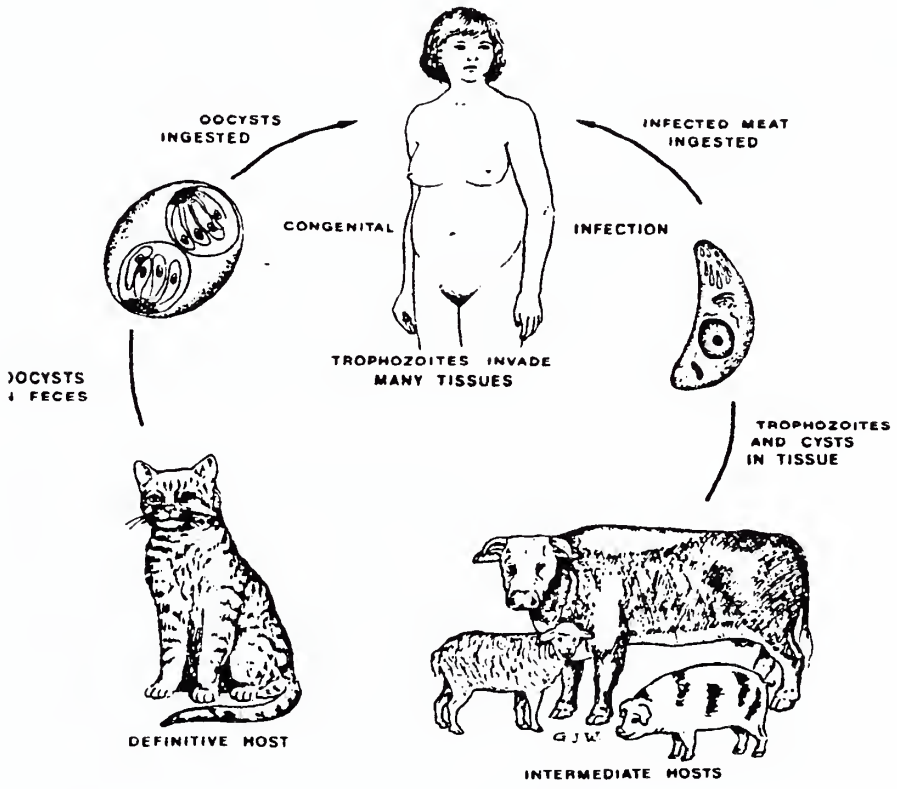
Coccidian parasites, such as those of the genus *Plasmodium*, and *T. gondii* , also enter cells in an active process involving mobilization of the parasites' own cytoskeletal apparatus. Additional support for the theory that invasion by these organisms is, at least in part, a parasite-driven process includes the great speed at which these organisms enter cells. Invasion is complete in 5-10 seconds, an order of magnitude greater than phagocytosis (Silverstein,1993). The compartments in which these organisms reside, the parasitophorous vacuoles (PV), have also been found to neither acidify nor fuse with lysosomes or other compartments in the endocytic cascade (Ward, 1993; Bannister, 1990; Moulder, 1985; Joiner, 1990; Jones, 1972a & b; Sibley, 1985). It is this fusion-incompetence of the parasitophorous vacuole, particularly in *T. gondii* , that has aroused interest in how the membranes of these vacuoles may differ from the membranes of vacuoles formed in other uptake processes and the role these differences might play in intracellular survival. Recent work has shown that the vacuolar membrane surrounding *T. gondii* is modified by parasite-derived proteins which may contribute to the function of this membrane. As one of the goals of the work described in this thesis is to understand how the vacuolar membrane surrounding *T. gondii* is modified by one of these proteins - GRA3- a more detailed description of *T. gondii* is appropriate:

D) *Toxoplasma gondii*: life cycle

Toxoplasma gondii, responsible for the disease toxoplasmosis, is an apicocomplexan parasite. As is characteristic of the organisms belonging to this phylum, *T. gondii* has a complex of secretory organelles at its apical end. These organelles, include the rhoptries, the micronemes, and the dense granules, which are thought to play a role in invasion and intracellular survival. *T. gondii* is capable of invading a variety of nucleated cell types in both mammals and birds. Although three forms of the parasite exist, the

oocysts and tissue cysts are usually responsible for infections in humans (see figure below)

The oocyst, containing the zygote form of the parasite, is shed only by felids. After passing through the animal's intestinal tract into the environment, the parasite sporulates, at which time it becomes infectious. Tissue cysts, which contain the slowly dividing bradyzoites, are usually encountered in raw or undercooked mutton, pork, or beef. Tachyzoites (trophozoites) are the actively replicating form of the parasite responsible for spread between cells and transplacental infection.



Life cycle of *Toxoplasma gondii* (Markell, 1992)

F) *Toxoplasma gondii*: clinical disease

Primary infection with *T. gondii* in the immunocompetent host is usually asymptomatic. Primary infection may rarely result in a flu-like syndrome. In

immunocompromised hosts and the congenitally infected infants, primary infection may lead to life-threatening disease. Through the actions of an intact immune system, however, the primary infection is self-limited as tachyzoites are forced to retreat into intracellular cysts. If the immune system is unable to control the primary infection, the tachyzoites will continue to infect other cells and result ultimately in serious sequelae such as chorioretinitis, myocarditis and fatal encephalitis. Once cysts have formed, the host is at life-long risk for opportunistic reactivation of the parasite should immunosurveillance fail.

The tissue cyst is the hallmark of latent infection. Until recently reactivation had not been a common medical problem outside the setting of Hodgkin's disease. With the development of more powerful immunosuppressive therapies and the growing problem of the acquired immunodeficiency syndrome (AIDS), however, disease resulting from recrudescence of *T. gondii* has become more commonly encountered. Current medical therapy is effective against the actively replicating tachyzoite form of the parasite only. Given the growing problem of reactivation toxoplasmosis, future investigations will look at ways of attacking the encysted bradyzoite.

G) Intracellular infection with *Toxoplasma gondii*

The tachyzoite can replicate or only inside a host cell. It enters in an active process (appendix A) involving the formation of a specialized, membrane-bound compartment, the parasitophorous vacuole (PV). Parasite uptake is thought to be induced by the rhoptries, a gland-like member of the set of apical organelles, whose activities are limited to the time of invasion (Kimata, 1987; Porchet-Hennere 1983; Schwartzman, 1986; and Perkins, 1992). The PV neither acidifies nor fuses with lysosomes at any time after invasion (Joiner, 1990; Jones, 1972a & b; Sibley, 1985). The membrane of this vacuole, the parasitophorous vacuole membrane (PVM), is devoid of host cell proteins upon entry (Joiner, 1990). It is believed that this absence of proteins in the PVM is responsible for the vacuole's fusion-incompetence (Joiner, 1990).

Once *T. gondii* has established residence in the host cell, the dense granules, another member of the set of apical organelles, release their contents (Charif, 1990; Leriche, 1991; Sibley, 1988). Secretion by the dense granules is followed by the formation of a tuboreticular network in the vacuolar space and the new appearance of intramembranous particles in the PVM (Porchet-Hennere, 1983). GRA3, one of five known dense granule proteins, is the major protein associated with the PVM. It is observed in increasing amounts as the vacuole matures (Dubremetz, 1993).

Transport across membranes requires the presence of membrane-associated proteins. In order to survive intracellularly for long periods of time, *T. gondii* must be able to interact with the host cell to obtain nutrients. Modification of the protein-deficient PVM is necessary to allow the parasite's long-term intracellular survival. GRA3 is thought to play a central role in the modification of the PVM and *T. gondii*'s long-term adaptation to intracellular life (Dubremetz, 1993; Ossorio, 1994; Joiner, 1994). I propose that the construction of a genomic clone of GRA3 provides the opportunity to further investigate the role of GRA3 in the intracellular survival of *T. gondii* as well as the protein's association with the PVM.

Experimental Methods and Design

Genomic library

A genomic library of *Toxoplasma gondii* inserted in the Eco RI site of λ–DASH II will be provided by David Roos, University of Pennsylvania.

Bacterial hosts

E. coli strain NM539 will be used as the host for the λ–DASH II vector. *E. coli* strain DH5α will be used as the host for the plasmid vector.

Plasmid vector

Bluescript (from Stratagene) will be used for transfection of DH5α and *T. gondii*.

Epitope tag

Oligonucleotide (c-myc) constructed at the Boyer center will be used (sequence = 5' - CGA GCA GAA GCT TAT CTC GGA GGA GGA TCT GAT - 3'; 5' - CGA TCA GAT CCT CCT CCG AGA TAA GCT TCT GCT - 3').

Parasites

T. gondii of the RH strain will be harvested from the peritoneum of Balb/C mice.

ATTEMPTED CONSTRUCTION OF A cDNA CLONE

I) PREPARATION OF THE TUBULIN PROMOTER FOR INSERTION INTO 18-3(4)

The tubulin promoter was obtained from the tub/CAT plasmid through digestion with NSI [5 µl plasmid, 1 µl NSI, 1 µl NSI buffer, 3 µl d H_2O] at 37°C for 2 hours. The promoter was gel purified and collected through 'freeze and squeeze.' DNA was precipitated according the the phenol/chloroform (P/C) protocol (described below). The promoter was then Klenowed [7.5 µl promoter DNA, 0.5 µl dNTPs (2.5 mM), 1 µl ligase buffer, 1 µl Klenow enzyme] at room temperature for 1/2 hour.

2) PREPARATION OF 18-3(4) FOR INSERTION OF THE TUBULIN PROMOTER

The native Cla I site was removed from 18-3(4), a Bluescript plasmid containing GRA3 at the Eco RI site, through digestion at the Xho I and Eco RV sites [1.5 µl plasmid,

1 µl Xho, 1 µl Eco RV, 2 µl buffer, 2 µl BSA, 12.5 µl dH_2O] at 37°C for 1 hour. 1µl Klenow enzyme and 1µl dNTPs was added to the tube and the reaction allows to run at room temperature for 1/2 hour. The linearized plasmid was gel purified and eluted using the GeneClean™ Kit.

3) INSERTION OF THE TUBULIN PROMOTER INTO 18-3(4) AND SCREENING

A) **Ligation of plasmid and insert**

Ligation reactions (16°C for 4 hours) were done using plasmid insert ratios of 1:1 and 1:3. DNA from each tube was isolated using the P/C protocol and resuspended in 10 µl of sterile water. For each reaction, 2 µl of DNA was added to an aliquot tube to which 40 µl of E. coli DH5α was added. Each sample was electroporated (2.5 volts, 25 amperes, 200 ohms). After electroporation, 800 µl of SOC media was added to each tube and the tubes were incubated at 37°C for 1 hour. Bacteria were innoculated in concentrations of 20 µl and 200 µl on LB + ampicillin (100 µg/ml) plates and incubated for 16 hours at 37°C.

A) **Screening for positive transformants**

Plates were screened for the presence of the promoter using the colony lift technique (described below). A plasmid prep (Sambrook, 1989) was performed on alleged positive transformants. DNA was cut with Cla I to determine the number of Cla I sites [1 µl plasmid, 1 µl Cla I, 1 µl buffer, 1 µl BSA, 6 µl dH_2O] at 37° C for 1 then run on a gel. A separate was then run to confirm the presence of the promoter [1 µl plasmid, 1 µl Kpn I, 1 µl Sac I, 2 µl buffer, 2 µl BSA, 13 µl dH_2O] at 37 °C for 1 hour and then run on a gel.

PROPOSED CONSTRUCTION OF A GENOMIC CLONE

I) ISOLATION OF A GENOMIC CLONE OF GRA3 FROM THE λ–DASH II LIBRARY

A) Screening of genomic libraries with a radioactive probe

i) Preparation of plating culture

50 ml of sterile LB medium supplemented with 0.2% maltose will be placed in a sterile flask and inoculated with a single bacterial colony of *E. coli* NM539. The culture will be grown overnight at 37°C with agitation of 250 cycles/minute. The cells will be centrifuged at 4000 g for 10 minutes at room temperature. The supernatant will be discarded and the pellet resuspended by vortexing in approximately 20 ml sterile 0.01M $MgSO_4$.

ii) Calculating the number of colonies required for screening

The number of colonies to be screened was determined to be 1.8×10^4 given a genome size of 80 megab (Sibley, 1993) and an insert size of 20 kb for λ–DASH. 10 90-mm plates will be innoculated and screened.

iii) Plating of the genomic library

Into one sterile tubes (13 mm x 100 mm; one tube per plate), a mixture containing 0.1 ml of the plating bacteria (*E. coli* NM539) with 0.1 ml of SM [5.8 g NaCl, 2 g MgSO4 7 H_2O, 50 ml 1M Tris Cl (pH 7.5), 5 ml 2% gelatin solution, H_2O to 1liter] containing 3×10^4 pfu (90-mm plates) of the bacteriophage λ–DASH library will be placed. The infected bacteria will be incubated for 20 minutes at 37°C. To each tube 2.5 ml of molten top agarose will be added and the mixture immediately poured onto a dry LB agar plate. The infected plates will be incubated for 3.5 hours at 42°C. (Sambrook, 1989)

iv) Transfer to filters

Nitrocellulose filters (NCF) will be overlaid on plates allowing 20 minutes for plaque absorption. Filters and agar will be marked with 18-gauge needle before removal of the filter. Special care will be taken to leave top agarose intact upon removal of the filter. Filters (plaque side up) will be dried at room temperature for 20 minutes (will do duplicate lift for control). Filters will be dipped sequentially for 30-60 seconds into 100 ml of the following three solutions: a) 0.2 M NaOH, 1.5 M NaCl, b) 2x SSC, 0.4 M Tris, pH 7.4, c) 2x SSC. Filters will be dried for 1 hour at room temperature on Whatman 3MM paper, then in a vacuum oven for 2 hours at 80°C.

v) Prehybridization

Filters will be placed in a heat-sealable plastic bag to which 10 ml of hybridization buffer S [3 ml 10% PVP (polyvinylpyrrolidone), 3 ml 10% BSA, 3 ml 10% Ficoll 400, 2.2 ml salmon sperm DNA stock (10 mg/ml), 15 ml 20 x SSC buffer stock, 250 mg yeast RNA, H_2O 10 150 ml] will be added. The bag will be incubated at room temperature for 2-3 hours.

vi) Labeling of synthetic cDNA probe

Boehringer Mannheim Biochemica labeling kit will be used. cDNA GRA3 probe (2 µl) will be added to 9 µl of dH_20 in a microfuge tube. Probe will be denatured by heating for 10 minutes at 100°C and then cooled on ice. The following will be added to the microfuge tube: 1 µl each of dATP, dGTP, and dTTP; 2 µl reaction mixture (from kit), 3 µl 50 µCi [$a^{32}P$] dCTP, and 1 µl Klenow enzyme. The contents of the tube will be incubated at 37°C for 1 hour. 2 µl of 0.2M EDTA will be added to the above to stop the reaction.

vii) Hybridization to filters and Primary Screen

^{32}P-labeled probe will be added to 10 ml of buffer and the mixture will be added to the bag. After the bag is sealed it will be incubated overnight at 65°C. The next day, the filters will be placed in plastic dishes for washing under the following conditions: i) 2x SSC/.1% SDS at room temperature for 20 minutes, ii) .2x SSC/.1% SDS at room temperature for 20 minutes, and iii) .2x SSC/.1% SDS at 65°C for 20 minutes. Filters will be incubated for an additional 30 minutes after changing the buffer and reducing the temperature to 55°C. Filters will be blotted between two pieces of of Whatman 3MM filter paper and air-dried for 30 minutes. Filters will be mounted on Whatman 3MM filter paper and covered with clear plastic wrap. Luminescent tape will be used for orientation and autoradiography will be performed overnight at -70°C with screen and X-ray film. The film will be developed the next day.

viii) Secondary Screen

200 µl of NM539 *E. coli* will be aliquoted into a 13 x 100 mm tube. 2.5 ml of LB top agar with 10 mM $MgSO_4$ at 48°C will be added and poured immediately into a 90-mm LB agar plate at room temperature and left to settle for 5 minutes. Each plate will be labeled with a grid with room for 50 secondary plaques. Film will be aligned with luminescent tape markers over filters marking the position of the filters' positioning holes on film. Plaques with a corresponding positive autoradiographic signal on the library plates will be transferred to individual squares on grid of bacterial lawn by gently puncturing lawn with toothpick. Plate will be incubated overnight at 37°C. Filters will be lifted and screened as described above.

ix) Tertiary screen

Phage from a single hybridizing secondary plaque generated by each primary signal will be transferred with a toothpick for each positive primary signal. Toothpicks will be

dropped into 10 ml of sterile TMG buffer [10 mM Tris, pH 7.4 0.5 ml of 2M stock, 10 mM $MgSO_4$ 1 ml of 1M stock, 0.01% gelatin 10 mg, add H_2O to 100 ml] in a sterile tube. The mixture will be mixed gently by hand to disperse phage. 0.25 and 2.5 µl TMG mixture will be placed into two sterile 13 x 100 mm tubes containing 200 µl of NM539 plating cells. 2.5 ml of LB top agar with 10 mM $MgSO_4$ at 48°C will be immediately poured over LB agar 90-mm plate at room temperature and allowed to harden for 15 minutes at room temperature. Plates will be incubated for 4 hours at 37°C. Tertiary grids will be hybridized and screened , as above. Positive signals are plaque purified clones. One positive from a primary positive will be transferred with a toothpick to 5 ml of TMG buffer, as above. 1 µl of TMG mix will be added to 200 µl of NM539 plating cells and plated as above. Plates will be incubate for 4 hours at 37°C.

B) **Preparation and purification of DNA from bacteriophage**

i) preparation of 10 ml Lysate

10 ml of LB medium supplemented with MgSO4 (10 mM final) will be added to a 50-ml capped, sterile tube. An agar plug containing a single plaque will be transferred to the tube with a sterile pipette. 50 µl of NM539 plating cells will be added to tubes which will then be put in a shaker (vigorous setting) with good aeration for 6-12 hours. Culture should become cloudy and then clear with lysis. After lysis, 100 µl of chloroform will be added to tube which will be shaken for an additional 2 minutes at 37°C. The tube will be spun for 10 minutes at 3,000 x g at room temperature to remove bacterial debris. Upper aqueous phase will be removed and placed in a new tube. 100 µl of sterile 1 M $MgSO_4$ solution will be added to each 10 ml of lysate.

ii) Tittering and plating of the 10 ml lysate of phage

A single colony of *E. coli* NM539 will be inoculated into 50 ml sterile LB medium with 20% maltose. Cells will be grown to an O.D. of 0.5 at 600 nm and collected by

centrifugation for 10 minutes at 3,000 x g. The pellet will be resuspended in 25 ml of sterile 10 mM MgSO4. A series of 10-fold dilutions of 1 µl of phage in TMG buffer will be made in microfuge tubes. 1 µl of diluted phage will be added to a sterile 12 x 75 mm culture tube. 200 µl of plating bacteria and 2.5 ml of top agar (cooled to 48-50°C) will be added to each culture tube. The mixture will then be immediately poured over bottom agar (37°C) on a 90-mm plate marked in a grid pattern. After top agar has hardened for 15 minutes, plates will be incubated at 37°C for 4 hours.

iii) DNA preparation of 10 ml Lysate solution: Isolation of DNA

10 ml of TM buffer and 320 µl of fresh DNAase I will be added to 10 ml of lysate and incubated for 15 minutes on ice after gentle mixing. 2 ml of 5 M NaCl and 2.2 g of solid PEG-6000 will be added (PEG must be completely dissolved). Mixture will be incubated on ice for 15 minutes and then spun for 10 minutes at 12,000 x g at 4°C. Supernatant will be discarded and pellet resuspended in 300 µl of TM buffer. After transfer of resuspended pellet to 1.5-ml microfuge tube, 300 µl of chloroform will be added. Phases will be separated through spinning for 5 minutes. Upper aqueous phase will be transferred to a new tube. Chloroform extraction will be repeated once more. 15µl of 0.5 M EDTA, pH 8.0, 30 µl of 5 M NaCl and 359 µl SS-phenol (salt -saturated phenol; Davis, 1986) will be added to the aqueous phase. After vortexing the tube will be microcentrifuged for 5 minutes. Extraction will be repeated with 350 µl of chloroform. DNA will be precipitated in 875 µl ethanol added to the aqueous phase. Tube will be placed on ice for 10 minutes then microcentrifuged for 5 minutes at 4°C. Supernatant will be discarded and DNA resuspended in 80% ethanol. Tube will be microcentrifuged for 2 minutes at 4°C. Ethanol will be discarded and DNA dried in vacuum. DNA will be resuspend in 50 µl of TE buffer (Davis, 1986).

C) **Detection and orientation of GRA3 gene and promoter**

The phage and vector will be digested with Bam HI, Eco RI, Eco RV, Hind I, Sac I, and Sal I. Fragments will be analyzed in a Southern blot analysis with hybridization as described in Davis for localization of GRA3 (Davis, 1986). GRA3 will be obtained through analysis of the Southern blot and gel purification.

To determine the presence of the promoter in the insert, the smaller fragment obtained after digestion of fragment containing GRA3 with Bam HI and gel purification will be inserted (upstream) into a plasmid containing the chloramphenicol acetyltransferase (CAT) gene. A functional analysis, as described in Soldati, will be performed (Soldati, 1993). Should the genomic promoter be absent, a tubulin promoter will be used in its place.

D) **Subcloning of plaque purified genomic GRA3 into plasmid vector**

GRA3 and genomic promoter isolated from the λ–DASH library will be precipitated according to the phenol/chloroform extraction (P/C) (Davis, 1986). A Cla I minus Bluescript plasmid will be digested with Eco RI for 1 hour. P/C will be repeated for both insert and plasmid. Linearized plasmid will be dephosphorylated with calf intestinal phosphatase (CIP) according to the Sambrook protocol 1.60 to prevent self ligation (Sambrook, 1989) . Two ligation reactions (16° C for 4 hours) will be done using a plasmid-to-insert ratios of 1:1 and 1:3. DNA will be isolated using P/C and suspended in 10 µl sterile water. 2 µl of the ligated plasmid for each reaction will be placed in separate aliquot tube to which 40 µl *E. coli* DH5α will be added. Each sample will be electroporated (2.5 volts, 25 amperes, 200 ohms). After electroporation , 800 µl of SOC media will be added to each tube and each will be incubated at 37°C for 1 hour. Bacteria will be plated on LB + ampicillin (100 µg/ml) plates , each batch in concentrations of 20 µl and 200 µl and incubated at 37°C for 16 hours. 16 colonies will be selected for plasmid prep (Sambrook, 1989).

To establish the uniqueness of the Cla I site, the constructed plasmid will be digested will Cla I for 1 hour and screened through gel electrophoresis.

II) Insertion of the epitope tag

Plasmid carrying genomic GRA3 will be cut will Cla I for 1 hour then precipitated according to the phenol/chloroform protocol. The linearized plasmid will be Klenowed and then dephosphorylated as described by Sambrook 1.60 (Sambrook, 1989). Two ligation reactions (16° C for 4 hours) using plasmid and the c-myc epitope to be inserted will be set up with a plasmid-to-insert ratio of 1:1 and 1:3. DNA will be isolated using P/C and suspended in 10 µl sterile water. 2 µl of the ligated plasmid will be placed in an aliquot tube (1 tube per batch) to which 40 µl E coli DH5α will be added. Each sample will be electroporated (2.5 volts, 25 amperes, 200 ohms) aiming for a time constant of 4.8. After electroporation, 800 µl of SOC media will be added to each tube and each will be incubated at 37°C for 1 hour. Bacteria will be plated on LB + ampicillin (100 µg/ml) plates, each batch in concentrations of 20 µl and 200 µl. and incubated at 37°C for 16 hours. Orientation of the epitope will be determined via sequencing.

III) Transfection of Toxoplasma by electroporation (Kim, 1993)

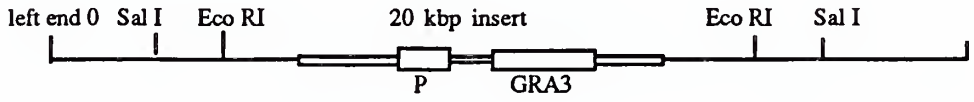
10-50 µg of plasmid linearized will be electroporated into 10^6 to 5 x 10^7 freshly lysed tachyzoites of the RH strain in Cytomix (van den Hoff,1992). The parasites will be used to infect human foreskin fibroblasts (HFF). Expression of the clone will be determined using a fluorochrome-labeled anti-c-myc IgG in a direct immunofluorescence analysis.

Outline of experimental design for construction of a genomic clone

I. Isolation of the genomic clone from the λ–DASH II (41, 900 bp) library

A. Screening of genomic libraries with a radioactive probe

λ–DASH II with genomic insert:



i) Preparation of plating culture
ii) Calculating the number of colonies required for screening
iii) Plating of the genomic library
iv) Transfer to filters
v) Prehybridization
vi) Labeling of synthetic cDNA probe
vii) Hybridization to filters and Primary Screen
viii) Secondary Screen
ix) Tertiary Screen

B. Preparation and purification DNA from bacteriophage

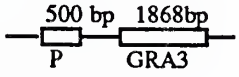
i) Preparation of 10 ml Lysate
ii) Tittering and plating of 10 ml Lysate
iii) DNA preparation of 10 ml Lysate solution: Isolation of DNA

C. Detection and orientation of GRA3 gene and promoter

digest with Eco RI, Eco RV, Sal I, Sac I, Bam HI, and Hind I
Southern blot analysis and hybridization
functional assay to detect promoter

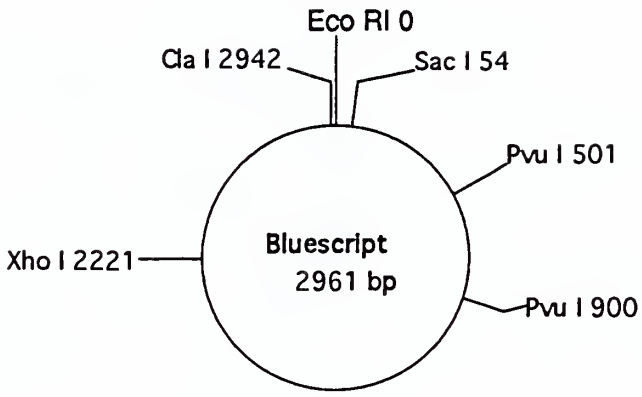
D. Subcloning of plaque purified genomic GRA3 into plasmid vector

Isolated fragment of GRA3 and promoter:

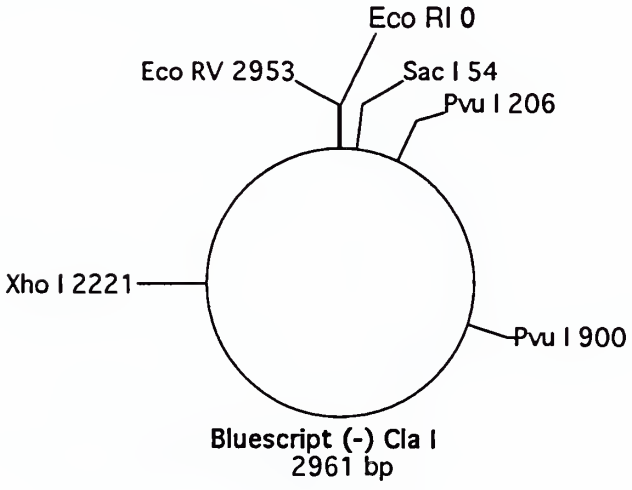


P/C; Klenow; P/C

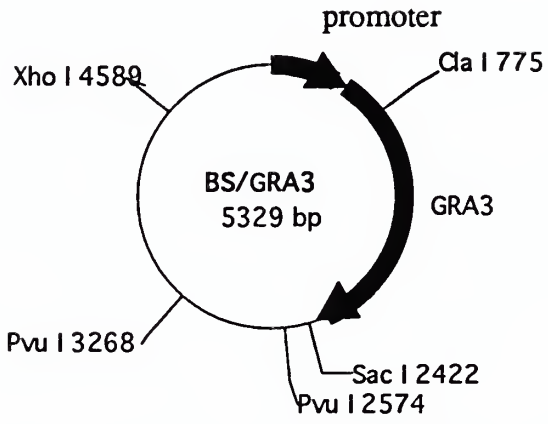
Bluescript:



Remove Cla I site: Klenow; ligate:



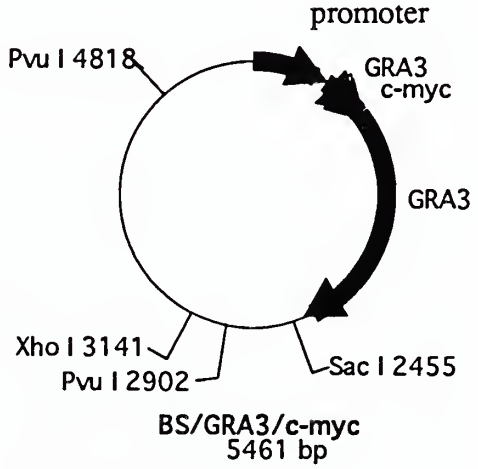
Ligate insert and plasmid:



Transfect bacteria; pick colonies; prepare DNA; digest with Cla I to detect added Cla I sites

II. insertion of the epitope tag

digest with Cla I; Klenow; dephosphorylate; ligate:



sequence

III. Transfection of Toxoplasma by electroporation

infect tissue culture; immunofluorescence

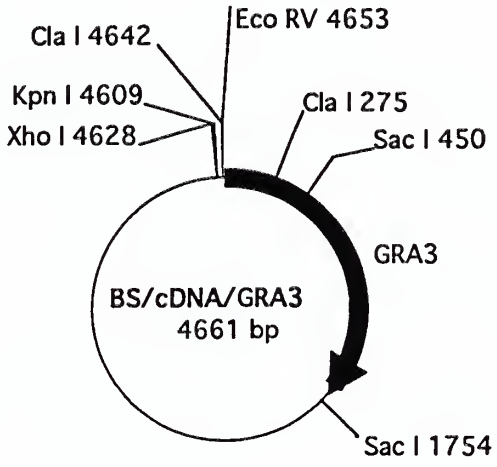
Results

No experiments to isolate a genomic clone have yet been conducted. Previous attempts to construct a plasmid containing an epitope-tagged version of GRA3 cDNA have were unsuccessful and are summarized here. Based on the lack of success of this system, and the desire to isolate genomic clones for subsequent knock-outs of GRA3, the decision was made to focus on isolation of GRA3 genomic clones. Construction of a plasmid (from Bluescript) carrying a copy of cDNA for GRA3 at the Eco RI site, and the tubulin promoter (500 bp) between the Xho I and Eco RV sites was attempted. Insertion of the tubulin promoter was not accomplished most probably secondary to technical problems.

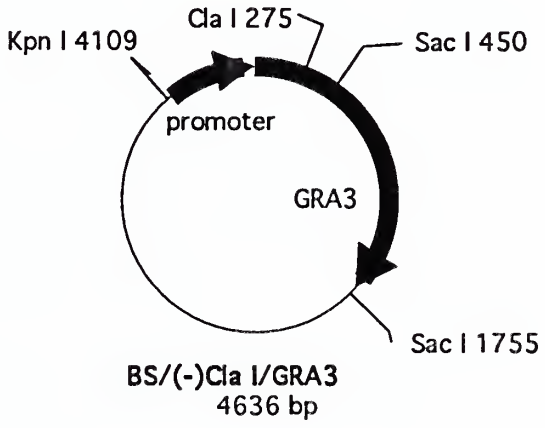
Ligation reactions were set up and, DH5α bacteria were transfected, as described above. Selection for transformants was performed plating electroporated bacteria on LB+ amp plates overnight at 37°C. Although the yield was never high, some colonies did appear on the plates. The plates were screened using the colony lift technique (described above) which, again, indicated that there were positive transformants. A plasmid prep was performed on these alleged transformants. The plasmids were cut with Cla I to and they linearized (see Appendix B: photo I 9/25/93) as would have been expected given the removal of the Cla I site in the Xho I to Eco RV segment. To screen for the presence of actual transformants v. reannealed plasmids with the Cla I site removed, cutting with Kpn I and Sac I was performed to determine the size of the segments and thus detect the presence of the promoter (see diagram). No 977 bp segment was seen on gel purification (see Appendix B: photo II 9/25/93) indicating that although the plates and the filters screened positive, there were no transformants carrying the promoter. These false positive results could be attributed to transformation of the bacteria by reannealed plasmid not containing the promoter. It is possible that the filters used on colony lift were either not washed sufficiently or that the plasmid contains a sequence with enough homology to the promoter that it can partially hybridize to it.

Outline of attempted construction of plasmid containing cDNA of GRA3

Bluescript plasmid containing indigenous Cla I site and GRA3:

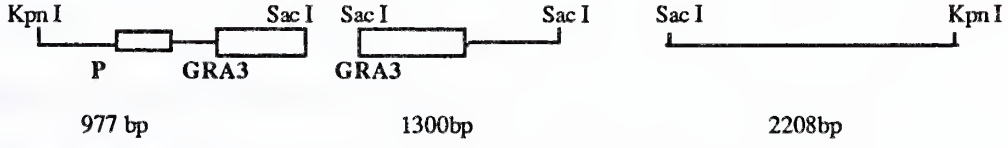


Excise Xho I-Eco RV segment to remove indigenous Cla I site.
Ligate tubulin promoter and plasmid:



Cut with Cla I: Does plasmid linearize?

Cut with Kpn I and Sac I: Do three fragments appear?



Discussion

A) Significance of GRA3: How proteins cross membranes

Construction of a genomic clone of GRA3 for electroporation into *T. gondii* creates the opportunity to do two things. First, it provides an opportunity to do knockout experiments. Second, it may also provide the opportunity to look at the topology of the protein in the membrane. GRA3's function with respect to the intracellular survival of *T. gondii* remains a mystery. It is thought, however, that given the appearance and association of the protein with the PVM sometime after invasion that perhaps this protein plays a role in the transport of nutrients from the host cell to the parasite.

The mechanisms used by small molecules to cross membranes are quite different from those used by particles and parasites to enter cells; vesicle formation is unnecessary. Pure lipid membranes are impermeable to water-soluble molecules. Thus the transport of small molecules into the cytosol takes place in processes, including passive diffusion, facilitated diffusion, and active transport, which all depend on the presence of specialized membrane-associated proteins. The function of a membrane-associated protein can, in part, be predicted by the topology of that protein with respect to the membrane. Understanding the nature of GRA3's relationship with the PVM could potentially uncover the role it may play in the parasite's survival.

B) Mechanisms of protein association with membranes

To understand how GRA3 may associate with the PVM, it is necessary to characterize the membrane-protein interaction. There are essentially two known classes of membrane-associated proteins. Integral membrane proteins (IMP) associate through interactions with hydrophobic domains in the lipid bilayer. In contrast, peripheral membrane proteins (PMP) associate with the membrane through ionic interactions with either integral membrane proteins or directly with the polar heads of the lipids in the

bilayer. These proteins can be distinguished from integral membrane proteins through extraction procedures which disrupt the ionic bonds.

Transmembrane proteins are a class of integral membrane proteins which completely cross the lipid bilayer. The Lenard & Singer model (Singer, 1990) predicts that all transmembrane proteins are amphipathic, having a required hydrophobic region able to span the membrane, as well as a hydrophilic region able to exist in both the extracellular and cytoplasmic environments. It is thought that the hydrophobic sequence crosses the membrane as an alpha helix containing at least 15 hydrophobic amino acid residues (Singer, 1990) . The photoreaction center in the membranes of photosynthetic bacteria is the only integral membrane protein for which this structure has been physically confirmed (Singer, 1990).

Singer describes four types of transmembrane proteins. Types I and II span the membrane only once, however, they differ as to whether the NH_2 terminus is oriented inside or outside of the cell. Type III, or polytopic proteins, have more than one hydrophobic region which crosses the membrane. The channel forming proteins form class IV. This class is divided into proteins that form polymers before or during insertion into the membrane and those which cross as a single folded protein.

As mentioned earlier, some proteins lack adequate hydrophobic residues to directly insert into a membrane. In such cases co- or post-translational modification can play an important role in the protein's association with the membrane. In their 1993 review of chemical modification of proteins in vivo, Reicker and McGee stated that peptide bond cleavage and formation, modification of the amino or carboxyl terminal amino acids, and modification of specific amino acid side-chain moieties as the three major types of protein modification. Of those mentioned, modification of a terminal amino acid or a side chain through the attachment of a lipid, a process known as acylation, could facilitate a protein's association with the hydrophobic portion of the membrane.

Prenylation, is a category of acylation in which a specific group of lipids, the isoprenoids, are added to proteins. This process is currently being studied with respect to its involvement in the membrane targeting and transformative properties of the Ras protein (Brown, 1993; Chow, 1992; Travis, 1993; Marshall, 1993) Present data suggest that one of two possible isoprenoids, a 15-carbon farnesyl or a 20-carbon geranylgeranyl, must be added to the Ras protein in order for it to be targeted to the membrane and cause transformation of the cell. The signal for farnesyl addition appears to be a CAAX box which is a terminal cysteine, with 2 aliphatic or linear amino acids, including either serine, methionine, or glutamine. Addition of the geranylgeranyl requires a CAAX ending in leucine or motifs CC or CXC. As will be explained below, GRA3 does not qualify for prenylation.

Glycosylphosphatidylinositol (GPI)-linkage is another mechanism by which proteins associate with membranes. GPI is added to the c-terminus of proteins in the endoplasmic reticulum at six possible sites, ser, glu, ala, sys, asp, or asn. These linkages can be disrupted by phospholipase C. GP-2, the major GPI-linked glycoprotein of pancreas zymogen granule membranes, and Tamm-Horsfall protein (THP), a GPI-linked protein associated with the apical vesicles in kidney thick ascending limb of loop of Henle cells (TALH) have been shown to compose a unique class of GPI-linked proteins which self-associate with membranes in a pH- and ion-dependent process (Fukuoka, 1992). Either mechanism of GPI-association is not likely to be involved in GRA3's association with the PVM as the protein is secreted in a soluble form which subsequently associates with the membrane.

C) GRA3 and the parasitophorous vacuole membrane

Given the above, what can we predict about the topology of GRA3 with respect to the PVM? Data exists which suggests that GRA3 is an integral membrane protein in the PVM (Ossorio, 1994). It is unclear, however, whether GRA3 is a transmembrane protein.

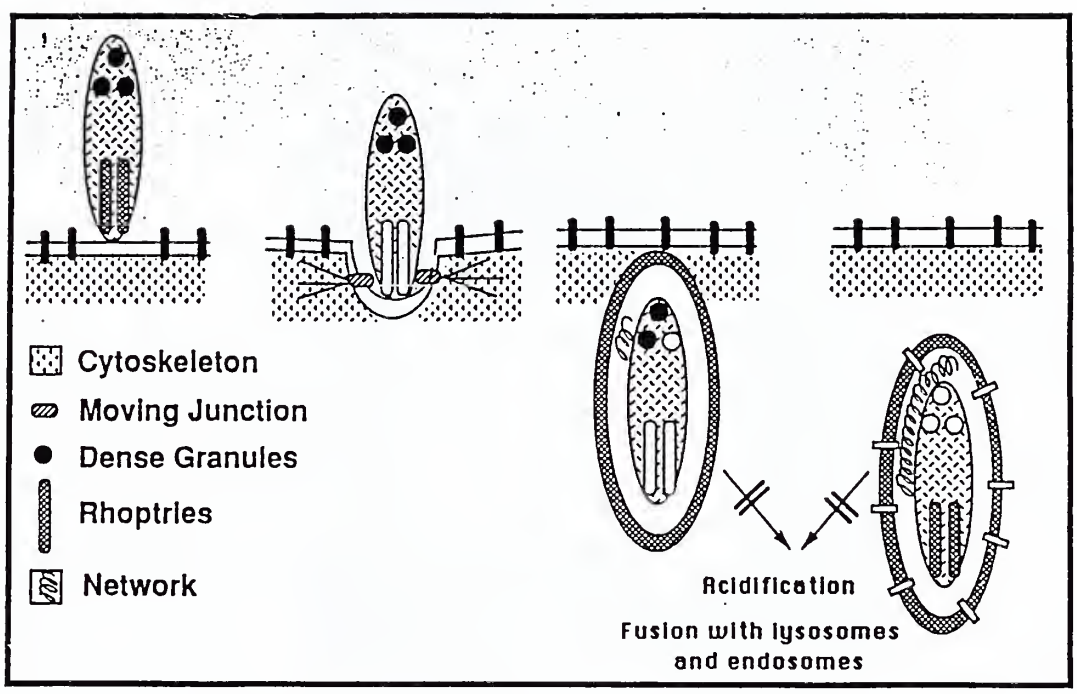
Although GRA3 does not have the 15-20 consecutive hydrophobic amino acids required to cross the plasma membrane, it has shorter hydrophobic regions that may allow it to interact with the membrane in some other way as well as several stretches of amphipathic helix (Bermudes, 1994). Another piece of data that suggests that GRA3 does not cross the plasma membrane was obtained in selective permeability studies. GRA3 was not detected on the cytoplasmic surface (Beckers, 1993 unpublished). This finding may reflect poor immunogenicity on the part of the epitope as the control also failed to label. In a study of parasite invasion, however, fluorescence to GRA3 was detected extending from the PVM into the host cell cytoplasm (Dubremetz, 1993) suggesting that the protein may in fact cross the membrane.

Exploration of the possibility that GRA3 is a peripheral membrane protein has suggested that the protein is not. GRA3 does not form ionic bonds to either the membrane or other membrane proteins (Ossorio, 1994). GRA3 is not released from the membrane in extremes of pH or $NaCO_3$ in extraction studies (Ossorio, 1994). In recent experiments, GRA3 also failed to crosslink any protein other than itself (Ossorio, 1993). Limited conclusions can be drawn from these finding, as in the case of the crosslinking study, however, rhoptry protein 2-3, a known transmembrane protein, also failed to label well.

GRA3's association with the PVM does not appear to be facilitated by the addition of a lipid moiety. Firstly, GRA3 does not label ^{14}C acetate studies (Ossorio, 1993). Nor does the protein does not meet the criteria (i.e. CAAX box, C-terminal hydrophobic stretch) for participation in a lipid anchoring process.

GRA3 may be a pore forming protein. The PVM of *T. gondii* has been found to have a pore-like channel which allows free diffusion of small charged and uncharged particles (Schwab, 1993). Unlike C9 and perforin, however, which are also soluble proteins that polymerize and insert into membranes, this pore does not result in cell lysis. GRA3 appears to be secreted from dense granules as a soluble molecule which forms

Appendix A



Active Cell Invasion by Coccidian Parasites

(printed with permission from Keith Joiner)

Appendix B

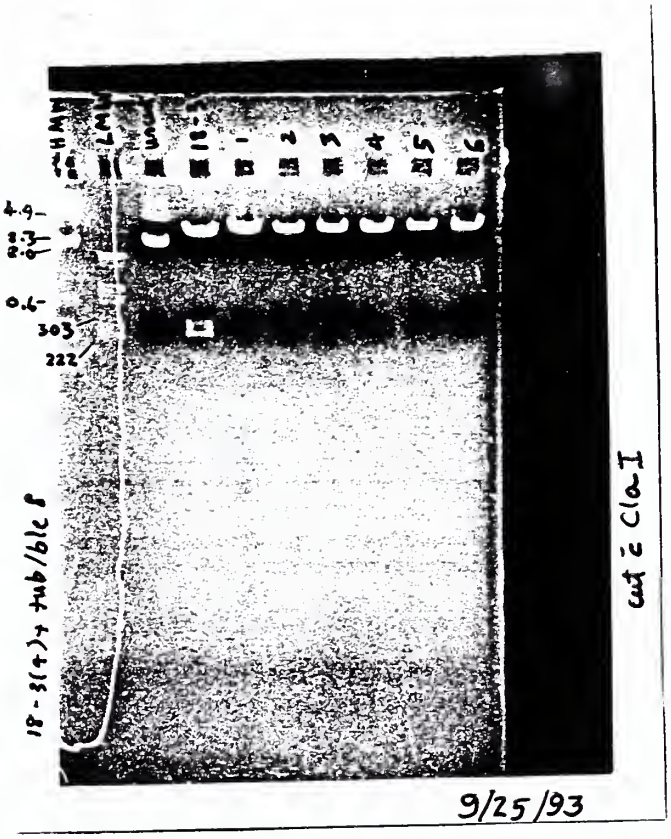


Photo I

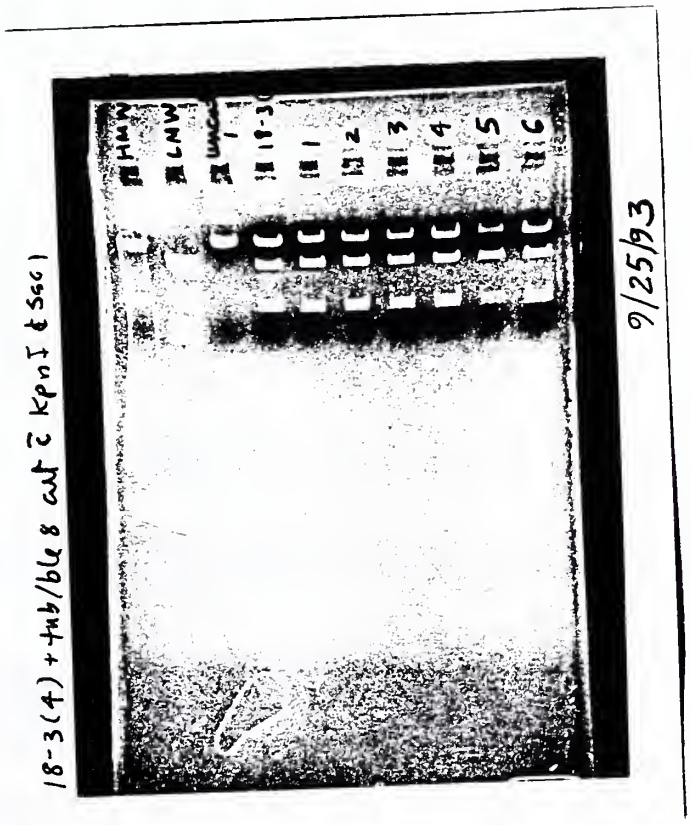


Photo II

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