

## THE DEVELOPMENT AND BIOLOGY OF BRADYZOITES OF *TOXOPLASMA GONDII*

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### 1. ABSTRACT

*Toxoplasma gondii* is a protozoan parasite of mammals and birds that is an important human pathogen. Infection with this Apicomplexan parasite results in its dissemination throughout its host via the tachyzoite life-stage. After dissemination these tachyzoites differentiate into bradyzoites within cysts that remain latent. These bradyzoites can transform back into tachyzoites and in immunosuppressed individuals this often results in symptomatic disease. Both tachyzoites and bradyzoites develop in tissue culture and thus this crucial differentiation event can be studied. Recent advances in the genetic manipulation of *T. gondii* have expanded the molecular tools that can be applied to studies on bradyzoite differentiation. Evidence is accumulating that this differentiation event is stress mediated and may share common pathways with other stress-induced differentiation events in other eukaryotic organisms. Study of the stress response and signaling pathways are areas of active research in this organism. In addition, characterization of unique bradyzoite-specific structures, such as the cyst wall, should lead to a further understanding of *T. gondii* biology. This review focuses on the biology and development of bradyzoites and current approaches to the study of the tachyzoite to bradyzoite differentiation process.

### 2. INTRODUCTION

*Toxoplasma gondii* is a well-described ubiquitous Apicomplexan protozoan parasite of mammals and birds (1-3). The phylum Apicomplexa includes other important

pathogens such as *Plasmodium*, *Eimeria*, *Cyclospora*, *Babesia* and *Cryptosporidium*. The Apicomplexa are defined by the presence of a complex of apical secretory organelles (micronemes, rhoptries, and dense granules) (4). Infection rates with *T. gondii* vary throughout the world with some countries such as France having a greater than 90% prevalence rate (5). There are three infectious stages of this protozoan parasite: tachyzoites (asexual), bradyzoites (in tissue cysts, asexual) and sporozoites (in oocysts, sexual reproduction). Infection is typically acquired by ingestion of undercooked meat, such as rare lamb, harboring tissue cysts (which contain bradyzoites). Infection can also be acquired by ingestion of food contaminated with oocysts (which contain sporozoites) or by exposure to cat feces containing oocysts. Upon ingestion, sporozoites or bradyzoites will invade the intestinal epithelium, differentiate into the rapidly growing tachyzoite form, and disseminate throughout the body. In the intestine of definitive hosts (i.e. cats) oocysts develop after oral infection. In both definitive and intermediate hosts, tachyzoites, after dissemination, differentiate into bradyzoites that remain latent. Tachyzoites are also infectious, however, this life stage is not resistant to gastric secretions and is thus much less infectious via the oral route than either oocysts or bradyzoites.

In most individuals acute infection with *T. gondii* is asymptomatic or causes mild symptoms similar to a self-limited mononucleosis-like syndrome. If a seronegative (i.e. immunologically naïve) pregnant woman is infected,

transmission of this parasite to the fetus can occur with the development of a congenital infection that can result in a fetopathy (3,5). Transmission to the fetus is more frequent later in pregnancy, but disease in the fetus is usually more severe the earlier in pregnancy that infection and transmission occurs.

*Toxoplasma gondii* has long been recognized as an important opportunistic pathogen of immunocompromised hosts and has emerged recently as a major opportunistic pathogen of the AIDS epidemic (3,5,6). Although overwhelming disseminated toxoplasmosis has been reported, the predilection of this parasite for the central nervous system (CNS) causing behavioral and personality disorders and especially, fatal necrotizing encephalitis constitutes its major threat to patients with HIV infection (AIDS). CNS toxoplasmosis ranks among the 10 most commonly occurring opportunistic infections and malignancies in AIDS patients (2,3,6-8), and may well be a greater direct cause of morbidity and mortality than other more common opportunistic infections. Even in the era of highly active antiretroviral therapy (HAART) toxoplasmosis is still a problem for many patients. Greater than 90% of individuals with AIDS presenting with *T. gondii* encephalitis have serological evidence of prior *T. gondii* infection. The reported lifetime risk for development of CNS toxoplasmosis in a patient with AIDS and positive serology for *Toxoplasma* is from 6 to 12% with a mortality rate of over 50% (3). When all intracerebral infections in AIDS patients are considered, *Toxoplasma* has been found in about 20% of patients; often the diagnosis was unrecognized until found at autopsy (2,6-8).

In immunocompromised hosts, e.g. AIDS patients, the development of *Toxoplasma* encephalitis is believed to be due to the transition of the resting or bradyzoite stage to the active and rapidly replicating tachyzoite form. It is likely that in chronic toxoplasmosis bradyzoites in tissue cysts regularly transform to tachyzoites and that these active forms are removed or sequestered by the immune system. In mice new tissue cysts have been demonstrated to be formed during chronic infection (1,9,10). Such a dynamic equilibrium between encysted and replicating forms would lead to recurrent antigenic stimulation, possibly accounting for the life-long persistence of antibody titers found in chronically infected hosts (1,11). In addition to rodents (mice, hamsters and rats), tissue cyst rupture has been demonstrated to occur in a Panamanian night monkey (*Aotus lemurinus*) (11). Since AIDS patients with CNS toxoplasmosis usually have the same antibodies as immunocompetent patients with acute toxoplasmosis (12), it is likely that AIDS patients are unable to mount the cell mediated immune response needed to constrain unrestricted multiplication of tachyzoites which have formed from bradyzoites, thereby resulting in *Toxoplasma* encephalitis. Chorioretinitis, a late manifestation of congenital infection, is also ascribed to bradyzoite reactivation.

The clinical manifestations of *T. gondii* infections are influenced by a number of factors including the genetic background of the host and of the parasite. In mice susceptibility to acute infection has been mapped to several

loci (1,13-18). The number of tissue cysts formed in mouse brain appears to be regulated by the class I gene  $L^d$  (15). More tissue cysts are produced in mice that become mildly ill from infection than in those that become highly symptomatic. Tissue cyst persistence may vary with both the strain of *T. gondii* and the strain of murine host. Mice that are resistant to acute infection can be very susceptible to chronic infection with encephalitis suggesting that control of chronic infection is not linked to the loci that control susceptibility to acute infection (1,13-18). Interferon  $\gamma$  is clearly important in the control of infection. INF $\gamma$  knockout mice cannot control infection and anti-INF $\gamma$  can cause reactivation encephalitis in chronically infected mice (1,19,20).

*Toxoplasma gondii* strains have been divided into 3 groups based upon genotype and biochemical isoenzyme analysis of animal and human isolates (21-23). Most evidence suggests that the parasite has proliferated as clonal isolates. Group I isolates are virulent in the mouse model of toxoplasmosis with an LD<sub>100</sub> of less than 10 organisms. These strains are more frequently associated with congenital toxoplasmosis (25%) than would be expected by their distribution in chronically infected animals (<10%) and are associated with high parasitemia in animals (23-26). Type II strains comprise over 70% of isolates obtained from human toxoplasmosis and only 40% of isolates from animals (27). These strains differentiate from tachyzoites into bradyzoites at a relatively high frequency in the laboratory and are associated with high cyst burdens and chronic pathology in mice. Type III strains comprise approximately 45% of animal isolates, but are infrequently isolated from humans with clinical toxoplasmosis. The distribution of these 3 genotypes in subclinical human infection is unknown. Although circumstantial, these data are consistent with an influence of parasite genotype on whether infection will result in clinically apparent toxoplasmosis in humans.

### 3. THE MORPHOLOGY AND BIOLOGY OF BRADYZOITES AND CYSTS

*T. gondii* is an obligate intracellular parasite that replicates within a parasitophorous vacuole within host cells. Frenkel coined the term bradyzoite (brady = slow) to describe the stage present within tissue cysts; this stage is also called a cystozoite. Compared to tachyzoites (tachy = fast), this is a slowly replicating life stage. Like tachyzoites, bradyzoites remain intracellular and divide by a unique binary fission termed endodyogeny. The size of tissue cysts is variable, but on average a mature cyst is 50 to 70  $\mu$ m and contains from 1000-2000 crescent-shaped 7 by 1.5  $\mu$ m bradyzoites. Tissue cyst size is dependent on cyst age, the host cell parasitized, the strain of *T. gondii* and the cytological method used for measurement. Young and old cysts can be distinguished readily by their ultrastructural features (4,28-30). Degenerating cysts are often seen in the brains of mice with chronic toxoplasmosis (31).

In tissue young cysts as small as 5 $\mu$ m containing as few as two bradyzoites are well described. This is consistent with the hypothesis that the differentiation of a

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vacuole into one containing either tachyzoites or bradyzoites is an early event in the formation of the parasitophorous vacuole. Since bradyzoite specific markers, including those seen in the cyst wall, are present within one day in tissue culture (see next sections) it is possible the decision to become a bradyzoite or tachyzoite vacuole is made shortly after or at the time of invasion.

Bradyzoites develop in cysts within host cells in a variety of tissues, but they are more common in neural and muscular tissues (brain, heart, skeletal muscle and retina). In the CNS cysts have been reported in neurons, astrocytes and microglia (32-34). However, it is unclear which is the preferred or predominant cell in which cysts form and whether host cells influence cyst formation. In tissue culture both astrocytes and neurons have been demonstrated to support cyst formation (35-39). Intact tissue cysts are intracellular (i.e. the cyst wall is surrounded by the host cell membrane) and illicit a scant inflammatory response. It is now agreed that cysts are not static structures; they regularly break down or rupture host cells and reinvade others (40,41). When tissue cysts rupture, however, they elicit a strong inflammatory response resulting in the formation of glial nodules in the brains of chronically infected hosts.

Bradyzoites differ ultrastructurally from tachyzoites in that they have a posteriorly located nucleus, solid rhoptries, numerous micronemes and polysaccharide (amylopectin) granules (33,41). Lipid bodies are not seen in bradyzoites, but are numerous in sporozoites and occasionally seen in tachyzoites. Bradyzoites stain with periodic acid-Schiff (PAS) whereas tachyzoites do not. Bradyzoites are more resistant to acid pepsin (1 to 2 hour survival in pepsin-HCl) than tachyzoites (10 min survival) (42,43). The prepatent period (time to oocyst shedding) in cats following feeding of bradyzoites is shorter (3 to 7 days) than that following feeding of tachyzoites (over 14 days). This is the most sensitive biologic marker of mature functional tissue cysts (40).

On feeding cysts containing bradyzoites to mice, bradyzoites transform to tachyzoites and disseminate from the intestinal mucosa. Data suggests that penetration of the enterocytes occurs within 2 hours of oral feeding and transformation to tachyzoites is complete within 18 hours. Parasitemia is detectable within 24 hours and invasion of the lungs, brain and other organs is demonstrable by 4 days (40). Cyst formation is seen starting 6 days after infection (these cysts are functional by bioassay and express bradyzoite specific antigens by immunofluorescence).

The cyst wall is formed from the parasitophorous vacuole and has a ruffled appearance with precipitation of underlying material. It appears that the cyst wall is formed by the parasite and is enclosed in a host cell membrane (30,33). Outside the cyst wall the parasitophorous vacuole has been demonstrated to be wrapped in intermediate filaments. In astrocytes these glial acidic fibrillary proteins (GFAP) may be associated with maturation of the developing cyst (35). Nonetheless these filaments are not incorporated into the cyst wall as cysts purified from host cells do not stain

with anti-GFAP and no filaments are seen within the cyst wall by SEM or immuno-EM. The cyst wall is periodic acid-Schiff (PAS) positive, stains with some silver stains, and binds the lectins *Dolichos biflorus* (DBA) and succinylated-wheat germ agglutinin (S-WGA) suggesting that polysaccharides are present in the cyst wall (44). Data that the cyst wall binds S-WGA and is disrupted by chitinase are consistent with the presence of chitin (44).

## 4. BRADYZOITE SPECIFIC ANTIGENS AND GENES

In order to limit the confusion generated by using molecular weights alone to characterize gene products in *Toxoplasma*, the current classification for the proteins/genes of *T. gondii* is based upon the localization or function of the gene products. For example: SAG for surface antigens, GRA for dense granule proteins, ROP for rhoptry proteins, MIC for proteins in the micronemes, BAG for bradyzoite specific proteins and CST for cyst wall proteins. As proteins/genes in each class have been identified, they have been assigned numbers: thus the first surface antigen described is known as SAG1 (previously called p30). The gene is indicated by italics, i.e. *SAG1*, and the protein by capital letters, i.e. SAG1.

Lunde and Jacobs in 1983 demonstrated that tachyzoites (endozoites) and bradyzoites (cystozoites) were antigenically distinct using polyclonal antisera raised to each stage (45). Subsequent western blot and ELISA studies have confirmed that stage specific antigens exist and that tachyzoites, sporozoites and bradyzoites have unique as well as shared antigens (45-56). For example, tachyzoites and bradyzoites both contain SAG3 (p43) and GRA 1 to 7 (50,57). In addition, the ROP and MIC proteins are similar in both tachyzoites and bradyzoites. Numerous studies have contributed to our knowledge of the antigenic structure of the tachyzoite and many of the major tachyzoite genes have been cloned. Similar information is now being obtained about bradyzoites.

Intact cysts can be purified from brain tissue by several methods that take advantage of the specific gravity of cysts being about 1.056. The degree of purification is dependent on the host tissue and amount of blood contamination. Isopycnic centrifugation of 5ml of brain suspension added to 30 ml of a 45% Percoll solution is the most widely used method for purification of cysts from this homogenate, however, centrifugation on discontinuous gradients of 20-30% Percoll or centrifugation of homogenate in 20 or 30% dextran are also effective methods (58-61). Passing bradyzoites liberated from intact cysts by trypsin digestion (to disrupt cysts) over a Whatman CF11 column (to remove debris) will purify isolated bradyzoites. Alternatively magnetic beads which bind mAbs to bradyzoite specific surface proteins can be used to purify bradyzoites from solutions. Isolated cysts/bradyzoites have been used to create monoclonal antibodies (mAbs) (62).

Studies with monoclonal antibodies (mAbs) made to bradyzoites (see table 1) and tachyzoites have demonstrated that bradyzoites lack tachyzoite surface antigens SAG-1 (p30) and SAG-2 (p22) and have unique

**Table 1.** Bradyzoite-specific antigens

Name of Antigen	Monoclonal Antibody	Size on Immunoblot (kDa)	Location by immunofluorescence
BAG1/hsp30 (62) also called BAG5 (65)	7E5 (47)	28	Cytoplasm
BSR4 (p36) (64)	74.1.8 (48)		
SAG4 (p18) (63)	T84A12 (49)	36	Surface
None	T83B1 (49)	18	Surface
p21 (antigen appears late in development)	DC11(68)	not reactive	Surface
p34	T84G11 (49)	21	Surface
MAG1 (66)	T82C2 (49)	34	surface
None	None	65	matrix
None	E7B2 (67)	29	matrix
None	93.2 (Weiss unpublished)	not reactive	matrix
None	1.23.29 (Weiss unpublished)	19	matrix
CST1	73.18 (48)	116	cyst wall
None	CC2 (68)	115 (bradyzoite) 40 (tachyzoite)	cyst wall

surface antigens including SAG4/p18 (63) (reacts with mAb T83B1) (49) and BSR4/p36 (64), (mAb T84A12 (49)). The first bradyzoite genes cloned, BAG1/hsp30 (also known as BAG5) (62,65) which reacts with mAb 74.1.8 (48) and mAb 7E5 (47), and MAG1 (matrix antigen; (66)), were cloned using bradyzoite specific antibodies. Several other mouse monoclonal antibodies have been described that recognize other bradyzoite antigens: including other surface antigens of 21 kDa (mAb T84G10) (49) and 34 kDa (mAb T82C2) (49), and CST1 a 116 kDa cyst wall antigen (mAb 73.18) (48). In addition, 21 kDa (mAb 93.2 (Weiss LM unpublished data)), 29 kDa (mAb E7B2) (67); and 19 kDa (mAb 1.23.29 (Weiss unpublished)) matrix proteins have been described. A series of rat mAbs including mAb DC11 that recognizes a surface protein but is not reactive on immunoblot and mAb CC2 that recognizes a 115 kDa cyst wall antigen in bradyzoites and a 40 kDa antigen in tachyzoites (68) have also been developed. The relative contribution, if any, of these common and unique antigens to the development of the different types of parasitophorous vacuoles seen in bradyzoites (cysts) and tachyzoites remains to be determined.

The development *in vitro* of bradyzoites and the conversion of bradyzoites isolated from mouse brains into tachyzoites has been demonstrated employing bradyzoite specific mAbs (47-69-75) and this development can also be demonstrated by transmission electron microscopy (TEM) (74,76-80). Feeding experiments in cats have demonstrated that tissue culture derived cysts behave identically to cysts obtained from animal tissues (81) and are pepsin resistant (4,43,59).

## 5. THE DEVELOPMENT OF CYSTS AND BRADYZOITES *IN VITRO*

Although the development of tissue cysts *in vitro* in avirulent strains (type II/III) was reported over 40 years ago (82) interest in studying bradyzoite differentiation lay fallow until the recognition of stage specific antigens and the development of mAbs to these antigens. Nonetheless Matsubayashi suggested in 1963 that strains with a slower rate of replication were more likely to develop cysts *in vitro*

and that a slowing the replication of virulent strains could also allow cysts to develop *in vitro* (83). Given the morphologic similarity of bradyzoites and tachyzoites the use of light microscopy to study differentiation was difficult and subjective. The tissue cyst wall is phase lucent by phase-contrast microscopy and the vacuole often contains an odd number of organisms that are club shaped. Hoff, in 1977, demonstrated that *in vitro* produced cysts led to oocyte excretion in cats with a prepatent period consistent with that of tissue cysts (i.e. mature bradyzoites) (81). Using TEM it was demonstrated that while cyst-like structures were present within 3d of infecting host cells in tissue culture, by bioassay (in cats) mature cysts were not present until 6d post infection of host cells *in vitro* (40). Resistance to acid-pepsin can be used to demonstrate the formation of bradyzoites in tissue culture. TEM can demonstrate the formation of a cyst wall *in vitro*, however it is difficult to use TEM to obtain quantitative data on the percentage of vacuoles that contain bradyzoites versus tachyzoites. The matrix of cysts produced *in vitro* is often reduced (especially free floating cysts) compared to the matrix of cysts isolated from an *in vivo* infection.

Prolonged passage of *T. gondii* or other Apicomplexa *in vitro* may lead to the loss of an ability to differentiate into other stages. For example, prolonged passage of *Besnoitia jellisoni* *in vitro* leads to a loss of its ability to form tissue cysts in mice and many type II isolates (e.g. PLK) of *T. gondii* cannot form oocysts in cats (84). Additionally, we have observed that the efficiency of cyst production by avirulent *T. gondii* isolates decreases with prolonged *in vitro* passage (this correlates with the development of more rapid growth in tissue culture).

The bradyzoite specific mAbs developed in the 1990s facilitated monitoring bradyzoite development in response to manipulation *in vitro* and spurred interest in this important developmental transition. In tissue culture studies, it is evident that bradyzoites spontaneously convert to tachyzoites and that tachyzoites spontaneously convert to bradyzoites (43,47,59,69-80,85,86). The rate of conversion appears to be strain dependent. Thus, low virulence strains

i.e. high cyst forming strains in mice, such as ME49, have a higher spontaneous rate of cyst formation in culture than do virulent strains such as RH (72). The rate of replication of tachyzoites, which is greater than that of bradyzoites, enables tachyzoites to destroy the cell monolayer thereby preventing bradyzoite formation. Inhibiting the rapid growth of tachyzoites, either by drugs (pyrimethamine (70)), cytokines (interferons (70,72,74)), anti-toxoplasma serum or by frequent removal (77), allows the percentage of bradyzoites in culture to gradually increase, consistent with bradyzoites' slower replication rate.

Stress conditions are associated with the induction of bradyzoite development, i.e. there are more bradyzoites under these conditions than would be expected from the simple inhibition of tachyzoite replication. It was found that temperature stress (43°C (72)), pH stress (pH 6.6-6.8 or 8.0-8.2 (71,72,74)) or chemical stress (Na arsenite (72)) resulted in an increase in bradyzoite antigen expression by *T. gondii* in culture and for temperature and pH stress an increase in the observed number of cyst-like structures. In murine macrophage lines derived from bone marrow, IFN- $\gamma$  increased bradyzoite antigen expression, which appeared to be related to nitric oxide (NO) induction (70). When NO stimulation was inhibited by NMMA (N<sup>G</sup>-monomethyl-L-arginine), an inducible nitric oxide synthase inhibitor, bradyzoite induction was inhibited. Bradyzoite induction was enhanced by sodium nitroprusside (SNP), an exogenous NO donor (70).

Similarly, both oligomycin, an inhibitor of mitochondrial ATP synthetase function, and antimycin A, an inhibitor of the electron transport of the respiratory chain, increased bradyzoite antigen expression, although not to the same extent as NO (70,87). This was not due to an effect upon host cells because for these experiments parasites were grown in host cells with a non-functional mitochondrial respiratory chain. This suggests that parasite mitochondrial function may be important for stage interconversion. One hypothesis is that the bradyzoite is more dependent on the apicoplast (the plastid organelle of *T. gondii*) than is the tachyzoite. It is believed that the apicoplast is involved in lipid metabolism and thus the utilization of lipids may differ between bradyzoites and tachyzoites.

The contribution of the host cell to stage conversion remains to be elucidated. However, we (88) and others (89) have found that exposure of extracellular tachyzoites to stress conditions will result in an increase in bradyzoite differentiation, consistent with a direct effect of stress on the parasite. Clearly, defining the factors that influence stage interconversion may lead to new therapeutic modalities aimed at preventing reactivation.

When bradyzoite differentiation occurs in cell culture following infection with tachyzoites, all of the currently available markers for bradyzoite formation, with the exception of p21 (mAb T84G10), can be detected within 24 hours of infection (86,90). This includes markers of bradyzoite surface antigens as well as those related to cyst wall formation. By 3 days vacuoles are present in tissue culture that have the TEM characteristics of cysts. Reactivity

to mAb T84G10 (p21) does not appear until 5 days. As assessed by bioassay in cats mature/functional cysts are not formed, however, until at least 6 days in culture. Additional markers of mature functional cysts are needed to facilitate *in vitro* studies on cyst biology. When cells are infected by bradyzoites (from tissue cysts) differentiation to tachyzoites and the appearance of tachyzoite specific antigens (SAG1) occurs within 15 hours before cell division has occurred (71). Vacuoles containing organisms expressing only tachyzoite antigens are clearly evident within 48 hours of infection. Thus, conversion between these two stages is a rapid event and the commitment to differentiation may be occurring at the time of or shortly after invasion and formation of the parasitophorous vacuole.

## 6. THE IDENTIFICATION OF BRADYZOITE-SPECIFIC GENES

Several bradyzoite specific genes have been identified and cloned including: BAG1/hsp30(BAG5), MAG1, LDH2, p36 (BSR4), and p18(SAG4) (62-66, 91-93). Random sequencing of cDNA libraries (EST projects) from bradyzoites (P/ME49) *in vitro* and *in vivo* and tachyzoites (RH and P/ME49) has led to the identification of Apicomplexan specific genes and of genes which are increased or unique to the bradyzoite libraries (94-96). The tachyzoite and bradyzoite EST databases are accessible at [www/ParaDBs/Toxoplasma/ index.html](http://www/ParaDBs/Toxoplasma/index.html).

Several molecular biological methods have been used to identify genes that are induced during bradyzoite differentiation. Promoter trapping has been utilized to identify genes induced during bradyzoite differentiation. Transfection of a promoterless hypoxanthine-xanthine-guanine phosphoribosyltransferase (HGXPRT) gene can be used as a selectable marker in an HGXPRT knockout PLK strain *T. gondii* to identify genes that are unregulated during bradyzoite development *in vitro* by utilizing 6-thioxanthine (6-TX) or 8-azaguanine (8-AzaH) as negative selection and mycophenolic acid with xanthine (MPA-X) as a positive selection for the presence of active HGXPRT (64,93,97). By growing transfected parasites at pH 7.0 in the presence of 6-TX all organisms that have the HGXPRT gene on a constitutive or tachyzoite promoter will be killed. This population of organisms is then exposed to pH 8.0 and MPA-X. Under these conditions only parasites which express HGXPRT (i.e. those with a bradyzoite or constitutive promoter in front of the HGXPRT gene) will survive. This strategy may be "leaky", depending on the concentrations of 6-TX and MPA-X used. Nonetheless, the 6-TX and MPA-X selections can be repeated several times to enrich the population for organisms with HGXPRT under the control of a bradyzoite specific gene promoter. Using this approach 8 bradyzoite specific recombinant (BSR) strains were obtained. One of these, BSR4, was later found to be the bradyzoite surface antigen known as p36 (64).

Differential display also demonstrates differences in gene expression between bradyzoites and tachyzoites (Roos unpublished, (93)). Several genes have also been identified using a subtractive cDNA library approach (89).

Sixty-five cDNA clones were analyzed from a bradyzoite subtractive cDNA library of these many were identified that were exclusively or preferentially transcribed in bradyzoites. This included homologues of chaperones (mitochondria heat shock protein 60 and T complex protein 1), nitrogen fixation protein, DNA damage repair protein, KE2 protein, phosphatidylinositol synthase, glucose-6-phosphate isomerase and enolase.

From these studies several themes are emerging: 1) tachyzoites and bradyzoites express related genes encoding structural homologues in a mutually exclusive way, 2) metabolic genes that are stage specific glycolytic enzymes exist suggesting these stages are metabolically distinct and 3) stress related differentiation pathways and stress proteins are associated with these stage transitions.

### 7. CYST WALL AND MATRIX ANTIGENS

The formation of the cyst wall and parasitophorous vacuole matrix are early events accompanying bradyzoite differentiation. The cyst wall and matrix probably protect bradyzoites from harsh environmental conditions and also provide a physical barrier to host immune defenses. The cyst wall can bind both *Dolichos biflorus* (DBA) and succinylated wheat-germ agglutinin (S-WGA). This binding can be inhibited by competition with the sugar haptens N-acetylgalactosamine (GalNAc) for DBA and N-acetylglucosamine (GlcNAc) for S-WGA (44). Treatment with chitinase disrupts the cyst wall and eliminates S-WGA binding, consistent with the presence of chitin in this structure (44). The 116 kDa antigen identified by mAb 73.18 (CST1) binds to DBA (Weiss LM unpublished data). A similar sized antigen is recognized by the serum of animals with chronic infection (53,55,56) and by a rat monoclonal antibody (68). By 2D-electrophoresis this protein accounts for all of the DBA binding activity in the cyst wall and S-WGA binding is not seen on this protein. Both DBA and S-WGA have been used to demonstrate bradyzoite (cyst) formation in cell culture and are useful alternatives/adjuncts to immunofluorescence with bradyzoite specific mAbs.

MAG1 (U09029) is a 65 kDa protein expressed in the matrix of the cyst between bradyzoites and is also seen in the cyst wall (66). No significant expression of MAG1 is noted in tachyzoites. *MAG1* was isolated from a cDNA library prepared by a RACE technique from mRNA isolated from *in vivo* derived ME49 tissue cysts using a polyclonal anti-cyst rabbit antiserum absorbed against extracts from tachyzoites. Antibody to recombinant MAG1 reacts with extracellular material in the cyst matrix and to a lesser extent with the cyst wall, but not with the surface or cytoplasm of bradyzoites. There is no reaction with tachyzoites. Previous studies had demonstrated that animal and human sera recognize by immunoblot an immunodominant 67 kDa band in cyst lysate. Recombinant MAG1 is well recognized by sera from chronically infected animals and it is likely that it is the immunodominant 65 kDa band seen on immunoblot using whole cysts as the antigen (53,55). RT-PCR data indicates that mRNA for MAG1 is present in both tachyzoites and bradyzoites, but immunoblot confirms that protein is only

present in bradyzoites. The *MAG1* gene was 1356 bp predicting a protein of 452 amino acids with a 25 residue signal peptide at the amino terminus. The protein is predicted have multiple  $\alpha$ -helical domains and the high Glu content results in a predicted net charge of -27. The recombinant protein (AA<sub>26</sub>-AA<sub>452</sub>) has predicted size of 46 kDa but migrates at 65 kDa by SDS-PAGE. The transcript is 2 kb and the gene contains two introns, the first is 4bp upstream of initial coding ATG and the second 95 bp downstream of this ATG. None of the other bradyzoite genes cloned to date have an intron in the 5' untranslated region. The sequence of the splice junctions is the same as that seen in tachyzoites.

The dense granule protein GRA5 (L06091, (98)) is found in both tachyzoites and bradyzoites. In bradyzoites GRA5 has been demonstrated to be in the cyst wall and appears at the same time as other early bradyzoite markers (86).

### 8. SURFACE ANTIGENS

The EST sequencing project and cloning of *T. gondii* surface antigens has shown that many *T. gondii* surface antigens are members of a gene family with similar structure to SAG1 (99,100). Some of these are stage-specific (SAG1 and BRS4) while others (SAG3) are not. Family members are notable for several conserved motifs and conservation of cysteine arrangement. In addition, there are several smaller surface antigens that are not part of the SAG1 family. It is not clear what the function of the surface antigens is or why stage-specific ones exist. Roles for these surface antigens in evasion of host immune defenses and host cell invasion have been hypothesized.

SAG4/p18 (Z69373) is an 18 kDa protein surface protein of bradyzoites (63). A SAG4 homologue SAG4.2 (AF015715) has also been identified. No expression of SAG4 is seen in tachyzoites. SAG4 was cloned by affinity purification of SAG4 from bradyzoites using mAb T83B1 followed by protein microsequencing. Sense and antisense degenerate primers were then used to obtain a gene fragment and this PCR product was used to clone this gene from a genomic PLK strain *T. gondii* library. SAG4 is 172 amino acids with a predicted mass of 18.5 kDa. There is a signal peptide of 27 amino acids and it likely that this protein has a glycosylphosphatidylinositol (GPI) anchor. The *SAG4* transcription start site is about 250 bp upstream of the ATG initiation codon. The upstream region of this gene does not have the 9 bp sequence TGCTGTGTC seen in *BAG1* and *MAG1*. This gene, similar to *BAG1*, appears to be transcriptionally regulated during bradyzoite development.

BSR4/p36 (AF015290) is a surface protein specific to bradyzoites that reacts with mAb T84A12 (64). It was isolated from pH 8.0 treated *T. gondii* cultures in human fibroblasts using a promoter trap strategy. Transfection of a promoterless hypoxanthine-xanthine-guanine phosphoribosyltransferase (HGXPRT) gene was used as a selectable marker in an HGXPRT knockout PLK strain *T. gondii* to identify genes that were unregulated

during bradyzoite development *in vitro* employing 6-thioxanthine (6-TX) as a negative and mycophenolic acid with xanthine (MPA-X) as a positive selection for the presence of active HGXPRT. Using this approach 8 clones were obtained. One bradyzoite specific recombinant (BSR) strain, BSR4, lost the ability to bind mAb T84A12 but displayed other bradyzoite specific markers, suggesting that p36 was tagged by the HGXPRT insertion. BSR4 has homology to SAG1 and SAG3 and like other *T. gondii* surface proteins has a signal peptide and GPI anchor addition sequence. This gene demonstrates a restriction fragment length polymorphism between ME49 (PLK; Type II) and CEP (Type III) strains, which correlates with the lack of mAb T84A12 binding to CEP strain.

### 9. METABOLIC DIFFERENCES BETWEEN BRADYZOITES AND TACHYZOITES

Bradyzoite differentiation from tachyzoites is likely a programmed response related to a slowing of replication and lengthening of the cell cycle (101). Synchronization and evaluation of the cell cycle in *T. gondii* has been achieved by expression of the *Herpes simplex* thymidine kinase gene to allow the synchronization with thymidine and labeling of S phase nuclei with 5 bromo-2'-deoxyuridine (102). The tachyzoite cell is similar to those of higher eukaryotic cells and is characterized by major G1 and S phases and a relatively short G2+M. When *T. gondii* replication slows there is an increase in the G1 phase of the cell cycle. When VEG strain sporozoites are used to infect cells, they transform to rapidly growing tachyzoites with a half life of 6 hours. After 20 divisions these organisms shift to a slower growth rate with a half-life of 15 hours. Bradyzoite differentiation is not seen in the rapidly growing stages, but occurs spontaneously when the population shifts to a slower growth rate. This is analogous to the programmed expansion and differentiation seen in other coccidia. While spontaneous differentiation did not occur in the rapidly dividing organisms, stress conditions that slowed growth would induce differentiation in these organisms. Stress conditions would also induce a higher percentage of the organisms with a half-life of 15 hours to differentiate into bradyzoites.

Given the different rate of growth and location of bradyzoites in a thick walled vacuole it is likely that the energy metabolism of tachyzoites and bradyzoites is different. Tachyzoites utilize the glycolytic pathway with the production of lactate as their major source of energy, however, mitochondria with a functional TCA cycle exist and contribute to energy production. While both tachyzoites and bradyzoites utilize the glycolytic pathway for energy, data suggests that bradyzoites lack a functional TCA cycle and respiratory chain (61). Stage specific differences have been demonstrated in the activity of various glycolytic enzymes. Lactate dehydrogenase (LDH) and pyruvate kinase activity was higher in bradyzoites than tachyzoites while PP<sub>i</sub>-phosphofructokinase activity was higher in tachyzoites than bradyzoites (61). In addition, the bradyzoite enzymes were resistant to acidic pH. These data are consistent with bradyzoite energy metabolism being dependent on the catabolism of amylopectin to lactate and suggest that

bradyzoites are resistant to acidification resulting from the accumulation of these glycolytic products.

LDH2 (U23207) was isolated with MAG1 by screening the *in vivo* bradyzoite cDNA library with polyclonal anticyst serum (91,92). LDH2 is a 35 kDa cytoplasmic antigen that is expressed in bradyzoites but not in tachyzoites. Its expression appears to be transcriptionally regulated because *LDH2* mRNA is detectable by RT-PCR only in bradyzoites. Antiserum to recombinant LDH2 reacts strongly with a 35 kDa antigen with a PI of 7.0 in bradyzoites and also weakly with a 33 kDa antigen with a PI of 6.0 in tachyzoites. The tachyzoite antigen was cloned and was identified as LDH1 (U35118), which is a tachyzoite specific LDH. It had previously been noted that *T. gondii* grown *in vitro* (bradyzoites and tachyzoites) had two isoforms of LDH whereas those grown *in vivo* in mouse peritoneal fluid (only tachyzoites present) had only one isoform. In addition, the LDH activity of tachyzoite and bradyzoite extracts is different and it is believed that these two stage specific LDH isoforms account for this observation. LDH1 and LDH2 are 71.4% identical.

While a large percentage of bradyzoite-specific genes are of unknown function, some bradyzoite-specific genes appear to code for metabolic enzymes. For example, a stage specific enolase that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate is present (89,96). This is consistent with the hypothesis that utilization of the glycolytic pathway is different in tachyzoites compared to bradyzoites.

### 10. STRESS PROTEINS AND DIFFERENTIATION

In the *T. gondii* dBEST, BAG1 is the most abundant bradyzoite specific gene representing ≈3% of all clones that are bradyzoite specific. BAG1 has homology to small heat shock proteins (smHsps) with strongest homology to smHsps of plants. BAG1/hsp30 (X82213) (62), also known as BAG5 (U23944) (65), is a 28 kDa antigen expressed in the cytoplasm of bradyzoites. There is no expression of this antigen in tachyzoites or sporozoites. Antibodies to a 28 kDa antigen are seen in chronically infected animals and humans. The *BAG1* gene was isolated by two groups by screening cDNA libraries prepared from bradyzoites with monoclonal antibodies (mAb 74.1.8 (65) or mAb 7E5 (62)). As mAb 74.1.8 reacts with clones lacking the initial 28 amino acids and mAb 7E5 does not, it is likely that mAb 7E5 recognizes an N-terminal epitope. The *BAG1* mRNA is upregulated during bradyzoite formation as is the BAG1 protein, suggesting transcriptional regulation of its expression. Expression of BAG1 is seen early in differentiation *in vitro*, and cells expressing BAG1 are seen within 24 hours of exposure to pH 8.0 or other stress conditions. Polyclonal anti-BAG1 serum also reacts with the bradyzoites of *Neospora caninum* (103,104). *BAG1* is a single copy gene, but homologous genes are found in the dBEST. Immunization with recombinant BAG1 has been demonstrated to enhance protective immunity and reduce cyst formation during infection in mice with avirulent *T. gondii* isolates (105). The carboxyl-terminal region of BAG1 has a small heat

shock motif most similar to the small heat shock proteins of plants (carrot hsp 17.7). Near the N-terminus is a synapsin Ia like domain that may be involved in the association of this small heat shock protein with proteins during development. A traditional heat shock promoter region was not seen upstream of the transcription start site of *BAG1*, however, both *MAG1* and *BAG1* have an identical 9 bp region 5'TGCTGTGTC3' upstream of the transcription start site.

Further evidence for a role of stress-induced genes in bradyzoite development is the finding that *T. gondii* HSP70 homologues (AF045559, U85649, U85648) are induced during bradyzoite formation (88,106-108). Induction of HSP70 can be demonstrated at both the protein and RNA level. Quercetin, an inhibitor of HSP synthesis, was able to suppress HSP70 levels whereas indomethacin, an inducer of HSP transcription, was able to enhance the production HSP levels (88,109).

It is now accepted that bradyzoite differentiation is a stress-related response of *T. gondii* to environmental conditions such as the inflammatory response of the host to the tachyzoite stage. Bradyzoite differentiation probably shares features common to other stress induced differentiation systems such as glucose starvation and hyphae formation in fungi or spore formation in *Dicystelium* (110,111). These systems have demonstrated unique proteins related to specific differentiation structures in each organism as well as the utilization of phylogenetically conserved pathways. Many of these signaling pathways involve cyclic nucleotides and kinases as part of the regulatory system in differentiation. Heat shock proteins (hsps) are also involved in these pathways as chaperones for both regulatory and stage-specific proteins involved in differentiation.

Heat shock- or stress induced activation of a set of heat shock protein (hsp) genes, is a characteristic of eukaryotic and prokaryotic cells. The hsp genes have been implicated as chaperones for protein folding and transport (112-114). In eukaryotes, heat shock-induced transcription of hsp genes is under the control of heat shock transcription factor (HSF) which is activated post-translationally and binds specifically to the heat shock element (HSE) in the upstream promoter region of hsp genes (112-114). The hsps fall into several subfamilies, namely, the low molecular weight (16-35 kDa) or small hsps (smHsps), the hsp60 family, the hsp70 family (68-78 kDa), and the high molecular weight (89-110 kDa) hsp families (hsp90 and hsp100) (112-114). Of interest, in addition to affecting gene expression, heat shock can also change cellular metabolism in *Xenopus* with an interruption of oxidative phosphorylation leading to anaerobic glycolysis (115,116). In this regard it is interesting to note that a change in metabolic pathway utilization is believed to occur during bradyzoite differentiation.

In recent years, it has become clear that hsps are not just limited to stress responses but are developmentally regulated as well. The heat shock response of fungi such as *Saccharomyces cerevisiae* and *Neurospora crassa* has been extensively studied and the pattern of hsps induced varies

with fungal development (115-117)). Similar hsp associations with development and intracellular survival have been noted in protozoa. For example, in *Leishmania chagasi* hsps have been associated with the capacity to survive an oxidant stress and may play a role in promastigote to amastigote transition (118).

Temperature, pH, mitochondrial inhibitors, sodium arsenite and many of the other stressors associated with bradyzoite development *in vitro* are also associated with the induction of heat shock proteins. We examined extracellular *T. gondii* after a one-hour exposure to pH 8.1 versus pH 7.1 for the expression of inducible hsp70 using mAb C92F3A-5 (Stressgen). A 72kDa *T. gondii* protein was induced, demonstrating that a presumptive heat shock protein is induced by the pre-treatment protocol that also induced bradyzoite formation. *T. gondii* infected cultures treated with pH 8.1 showed 4 fold induction of the proteins to *T. gondii* grown in pH 7.1 treated cells (88,109). The 3 to 4 fold change in hsp70 levels demonstrated in *T. gondii* seen with stress as well as differentiation is consistent with the magnitude of the hsp70 response demonstrated in *Trypanosoma cruzi*, *Theileria annulata* and *Plasmodium falciparum* (119). Using immunofluorescence and immuno-EM reactivity with C92F3A-5 was demonstrated in *T. gondii* bradyzoites and not in tachyzoites. Similar results were recently obtained with *in vivo* cysts during reactivation in a murine model induced by anti- $\gamma$ -interferon (120), suggesting that hsp70 may be important in both tachyzoite to bradyzoite and bradyzoite to tachyzoite differentiation.

Quercetin, a bioflavonoid, has been reported to inhibit the synthesis of many heat shock proteins including hsp90, hsp70 and hsp27, while having no effect on the synthesis of other cellular proteins (121). Quercetin at 100  $\mu$ M inhibited the pH 8.1 associated induction of bradyzoite antigens and decreased the expression of hsp70 in pH 8.1 treated ME49 *T. gondii* as ascertained by immunoblotting with mAb C92F3A-5. Quercetin effects may be complex as it decreased growth or invasion of *T. gondii* and has been reported also to inhibit PI 3-kinase and phospholipase, mitochondrial ATPase, phosphodiesterases, and protein kinase C.

Nonsteroidal antiinflammatory drugs (NSAIDs) have been reported to induce heat shock proteins through effects on heat shock transcription factors (122). Treatment of cells with indomethacin has been associated with a decrease in the temperature required to obtain a heat shock response, i.e. a synergistic interaction resulting in a decrease in the heat shock threshold (122). Treatment with 100  $\mu$ g/ml indomethacin or media at pH 8.1 induces a 1.6 fold increase in bradyzoite antigen positive vacuoles. Combined indomethacin and pH 8.1 media lead to a 3.0 fold increase compared to controls (no indomethacin at pH 7.1). Immunoblotting with mAb 92F3A-5 demonstrated an increase in hsp70 in indomethacin treated *T. gondii*. Indomethacin appears to facilitate the development of bradyzoites and is likely to be synergistic with pH shock. Indomethacin's effect was consistent with its reported hsp effects.



It is likely that bradyzoite differentiation involves a number of heat shock proteins and other proteins classically associated with the stress response. BAG1 has homology to small heat shock proteins (smHsps) with strongest homology to smHsps of plants. Changes in the expression of smHSPs play an important role in cell regulation. Knock-out of *BAG1* affects the efficiency of cyst formation and knockout of smHsps in other organisms has been demonstrated to interfere with differentiation events (123-126). For example, the knockout of Acr (a 16 kDa smHsp) impairs the ability of *Mycobacterium tuberculosis* to grow in macrophages due to a decrease in transition to the stage associated with latency (127). Inhibition of hsp27 interferes with the granulocyte differentiation of human promyelocytic HL-60 cells and the differentiation of murine embryonic stem cells (125,128).

The mechanisms by which smHsps exert their effects during differentiation are avenues of active investigation by many laboratories. Small hsps have been associated with the determination of whether a cell undergoes apoptosis versus differentiation (125,129,130). This mechanism may be dependent on the redox-state of the cell (130). In this regard, smHsps have been postulated to act as specialized chaperones for enzymes such as glutathione reductase during differentiation. In plants smHsps are associated with metabolic enzymes related to chloroplast function. *T. gondii* also has a plastid organelle and it has been suggested that this organelle is involved in bradyzoite differentiation. Several laboratories have identified specific proteins that associate with smHsps during differentiation. For example, two novel hsp27 binding proteins that are developmentally regulated have been identified in Sertoli cells (125). The exact function of small hsps in *T. gondii* remains an area of active investigation.

## 11. GENETIC STUDIES ON BRADYZOITE BIOLOGY

It is possible to transfect *T. gondii* for transient or stable expression of genes (44,131-136). Successful selectable markers for transfection include *cat*, HGPRT, DHFR, *trp* and *ble* (44,131-140). Foreign DNA typically integrates at nonhomologous loci and this has allowed the development of insertional mutagenesis cloning strategies. As the size of the *T. gondii* genomic DNA insert increases, the frequency of homologous recombination increases, allowing gene replacements or knock-outs that permit the analysis of gene function (131). Since *T. gondii* is haploid knock-outs are feasible only if the gene is not essential. Recently, an episomal vector has been described which would allow complementation studies for essential genes (136).

Knockout of bradyzoite specific genes should not be essential as growth can occur in the tachyzoite stage. This strategy is being applied to bradyzoite specific genes. BAG1/hsp30 (BAG5) knockouts have been described by two groups (133,134). One knockout was done using HGXPRT as a selectable marker in an HGXPRT<sup>neg</sup> PLK strain of *T. gondii* and the other using CAT as a selectable marker in PLK strain. Cyst formation both *in vitro* and *in vivo* occurs in both knockouts, indicating that BAG1 is not essential for cyst formation. However, the number of cysts formed *in vivo*

in CD1 mice was reduced in one of the *bag1* knockouts and complementation of this knockout restored the production of similar numbers of cysts to that of the wild type PLK strain. When parasites were grown in sodium nitroprusside, a nitric oxide donor that induces bradyzoite formation, the *bag1* knockout grew faster than PLK. This may be a result of a difference in transition rate from the rapidly growing tachyzoite to the slowly growing bradyzoite stage in this *bag1* knockout.

Because the reduction of cyst burden observed with disruption of *bag1* was reversed by restoration of BAG1 expression, it appears that BAG1 influences the efficiency of cyst formation *in vivo*. That *bag1* knockout cannot block cyst formation in mouse brain implies that the BAG1 is not a trigger for bradyzoite differentiation. However, this does not mean BAG1 has no role in stage differentiation. The capacity to convert from tachyzoite to bradyzoite is key for *T. gondii* persistence in the host, and thus it is likely that multiple genes with redundant functions are involved in this process. It is of interest that BAG1 homologues that do not appear to be bradyzoite-specific were identified in the EST sequencing project (e.g. dbEST ID # 604948 and Ctoqual2\_349). It is possible that these homologues might be able to partially compensate for lack of BAG1

## 12. CHARACTERIZATION OF BRADYZOITE GENE EXPRESSION

Bradyzoite and tachyzoite specific promoter regions can be used to create reagents for the study of differentiation *in vitro* (10). Plasmids harboring the chloramphenicol acetyltransferase (CAT) gene contained within 5' and 3' flanking sequences from *T. gondii* have been used to monitor promoter activity and define minimal promoter sequences. These studies were performed with SAG1 (as a tachyzoite marker) and TUB1 (tubulin, a constitutively expressed housekeeping gene). An LDH2/CAT plasmid containing 1 kb of 5' flanking sequence (670 bp non transcribed and 30 bp 5'UTR) from the LDH2 gene as the promoter for CAT was used to evaluate pH 8.0 induced bradyzoite differentiation. At pH 7.0 no appreciable CAT activity was present, but at pH 8.0 there was a 45-fold increase in activity (91). Plasmids using a BAG1/CAT construct demonstrated similar activity with a 50-fold induction (141). These constructs have been used in both transient and stable transfection assays. Another reporter gene that is useful for these studies is Beta-galactosidase (beta-gal). Constructs containing BAG1/beta-gal and LDH2/beta-gal also demonstrate induction on exposure to stress conditions associated with bradyzoite formation.

*BAG1* reporter constructs have been used to map cis elements driving transcription (141). The BAG1 promoter has been mapped by the construction of deletions in a BAG1/CAT construct. A series of deletions in the BAG1 5'flanking sequences demonstrated that a 324 bp fragment, starting 60 bp upstream of the *BAG1* transcription start, is sufficient to confer stage specific regulation on the CAT reporter. This region does not include the 9 bp sequence TGCTGTCTC that is seen

upstream of both *MAG1* and *BAG1* transcription start sites. The majority of inducible activity is removed with a deletion of a critical 40 bp region between -284 and -324. This region contains a G/C rich palindrome CGCAGCG, however, no further studies have been done to map this region. There was no effect of the 3' flanking sequences of *BAG1* on the amount or stage-specificity of CAT expression. Whereas transient transfection containing the *BAG1*/CAT construct induced by pH 8.0 treatment had detectable activity at 24 hours, detectable activity was first detectable 3 days after induction of stable constructs.

A *BAG1*/HGXPRT construct was targeted into the uracil phosphoribosyl transferase locus (UPRT) of an HGXPRT<sup>neg</sup> PLK strain *T. gondii* (97). When integrated in the opposite direction of the UPRT gene, the *BAG1*/HGXPRT construct had a 50 to 100 fold inducibility, whereas integration in the same direction as the UPRT gene gave only a 6 fold inducibility. This transgenic parasite at pH 7.0 is resistant to 6TX or 8AzaH and sensitive to MPA-X (i.e. no expression of HGXPRT) and at pH 8.0 is sensitive to 6TX or 8AzaH and resistant to MPA-X (i.e. HGXPRT expressed). Insertional mutagenesis in this transgenic parasite can be used to identify positive and negative regulators of *bag1*. Knockout of a positive regulator will prevent expression of HGXPRT at pH 8.0 making these organisms resistant to 6TX and sensitive to MPA-X. Knockout of a negative regulator would make these organisms express HGXPRT at pH 7.0 and make them sensitive to 6TX and resistant to MPA-X at this pH.

A *T.gondii* hsp70 genomic PLK clone has been obtained (Weiss LM unpublished data). Beta-galactosidase expression constructs driven by *TgHSP70* as well as *SAG1*, *BAG1* and *GRA1* promoters were tested in transient transfection assays. Because many of the conditions that induce bradyzoite formation also reduce parasite number, constructs were cotransfected with a TUB1-CAT (chloramphenicol acetyl transferase) construct. Beta-galactosidase activity was normalized to CAT activity to ensure that differences in activity were not due to differences in parasite number. Sodium nitroprusside (SNP), a nitric oxide donor which induces bradyzoite differentiation, induced *BAG1* reporter expression and suppressed *SAG1* reporter expression. As expected, expression of the constitutive *GRA1* promoter construct was unaffected by SNP treatment. A 2 to 4 fold induction of *TgHSP70* promoter-driven beta-galactosidase activity was seen on exposure of the organisms to pH 8.1 compared to pH 7.1.

A cytosolic form of NTP has been generated that would deplete the parasite NTP stores on expression resulting in death of the organism (93). Using this cNTP gene an LDH2-cNTP2 construct has been made and demonstrated to kill bradyzoites during differentiation. LDH2-cNTP2 had is not expressed in tachyzoites and thus parasites that do not develop into bradyzoites are not killed. By insertional mutagenesis this construct could be used as a negative selectable marker to identify regulatory mutants that can not develop into bradyzoites.

In addition to transcriptional regulation of bradyzoite gene expression, post-transcriptional mechanisms are likely to be important for expression of some bradyzoite-specific proteins. In contrast to *LDH2*, *LDH1* transcripts are detectable by RT-PCR in both tachyzoites and bradyzoites. LDH1 protein is only detectable in tachyzoites. Similarly, semiquantitative RT-PCR indicates that *BSR4/p36* mRNA is present equally in both bradyzoites and tachyzoites, but BSR4/p36 protein is only expressed in bradyzoites. It has been postulated that the regulator of BSR4 is a translational control element present in the 5' UTR region of this gene. RT-PCR data indicates that mRNA for *MAG1* is present in both tachyzoites and bradyzoites, but immunoblot confirms that protein is only seen in bradyzoites. The mechanism of stage-specific expression of these bradyzoite proteins has not been elucidated.

### 13. SIGNALING PATHWAYS IN BRADYZOITE FORMATION

Although tachyzoites and bradyzoites are well defined morphologically, little is known about how interconversion from one to the other stage occurs or what signal(s) mediate this transformation. We are interested in defining the signaling pathways responsible for the tachyzoite-bradyzoite transition. *T. gondii*, like other organisms, must possess a way to sense and respond to its environment. Data from a number of model systems has implicated cyclic nucleotide signaling in stress-induced differentiation. The effect of cyclic nucleotides on bradyzoite differentiation was assessed by using non-metabolized analogues of cAMP and cGMP as well as forskolin (to stimulate a short pulse of cAMP). These experiments demonstrated that forskolin and cGMP could induce bradyzoite formation. Experiments with isolated extracellular *T. gondii* tachyzoites demonstrated that conditions inducing bradyzoites: pH8.1, Forskolin or SNP exposure, lead to a 3 to 4 fold elevation in cAMP levels but no consistent changes in cGMP were seen. Within 30 minutes, the cAMP levels are comparable to those seen in control parasites incubated in pH 7.1 media.

Most of the effects of cAMP within cells can be attributed to regulation of cAMP-dependent protein kinase A activity. We have identified and cloned several kinases from *T. gondii* that may serve as signaling molecules during the tachyzoite-bradyzoite transition. Among these are protein kinase A (Kim K. unpublished data) and a glycogen synthase kinase (GSK-3) homologue (142). Protein phosphorylation has proven to be a major mechanism of regulation of gene expression and integration and amplification of extracellular signals. The dbEST identified many *T.gondii* gene fragments predicted to encode signaling molecules such as protein kinases, phosphatases and G proteins. The presence of highly conserved signaling molecules suggests that many of the pathways identified in other eukaryotes are likely to be preserved in *T. gondii*.

### 14. OTHER FACTORS AFFECTING BRADYZOITE DEVELOPMENT

The cyst burden generated by *T. gondii* is undoubtedly influenced by multiple factors including the

genetic background of both the host (18) and parasite (25,143). In addition, it is likely that epigenetic factors also affect strain virulence and cyst formation. Anecdotal data suggest that both the acute virulence and efficiency of cyst formation are influenced by the number and frequency of passages in tissue culture or in animals (4). Sibley and coworkers have noted that the LD<sub>50</sub> of ME49/PLK for mice decreases from >10<sup>5</sup> to 10<sup>2</sup> with continued tissue culture passage after isolation from chronic infection (25). We have noted that with prolonged passage in tissue culture, the efficiency of spontaneous cyst production *in vivo* and *in vitro* declines but is restored after passage through mice. In addition, several antibiotics, including pyrimethamine (70), sulfadiazine (144) and atovaquone (87) have been reported to induce bradyzoite antigen expression. These are important considerations as illustrated by the report by Bohne *et al* (134) in which a *bag1* knockout did not show a decrease in cyst numbers. Several factors may have enhanced cyst production including sulfadiazine treatment and the use of C57/B6 mice that normally produce high cyst numbers and are very susceptible to *T. gondii* infection.

## 15. SUMMARY

Investigations into bradyzoite biology and the differentiation of tachyzoites into bradyzoites has been accelerated by the development of *in vitro* techniques to study and produce bradyzoites as well as by the genetic tools that exist for the manipulation of *T. gondii*. It is likely that unique structural antigens exist in bradyzoites as well as that developmental signaling pathways seen in other eukaryotes exist. The development of bradyzoite appears to be a stress mediated differentiation response and may share common mechanisms with similar starvation responses in yeast. The metabolic adaptations of the bradyzoite and the contribution of mitochondria, glycolysis and the apicoplast to these adaptations remain to be determined. The mechanism by which development is triggered and coordinated may eventually lead to novel therapeutic strategies to control this latent stage of Toxoplasmosis, perhaps allowing a radical cure of infections such as achieved with malarial chemotherapy. In addition, genetic strategies that prevent cyst formation may prove useful in the development of vaccine strains of this Apicomplexan protozoan pathogen.

## 16. ACKNOWLEDGEMENT

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