Host Cell-Directed Interactions with *Toxoplasma* Influence Pathogenesis

The host molecular environment can influence parasite growth and cyst development

Jay R. Radke, C. Amy Eibs, and Philip D. Fox

Toxoplasma gondii infects an estimated 1.5 million humans annually in the United States and likely many more around the globe. Although in healthy individuals infection produces few symptoms, bradyzoites form parasite cysts that remain permanently in host tissues. Recrudescence from these cysts in the immunocompromised host can give rise to the lytic tachyzoite stage responsible for overt clinical toxoplasmosis. Thus, transmission to human populations can be dangerous not only to the developing fetus and in AIDS, but also to those undergoing chemotherapy or treatment with immunosuppressive drugs following organ transplant.

Toxoplasma is a single-cell protozoan belonging to the phylum *Apicomplexa* and an obligate intracellular parasite of humans and animals alike. It traverses distinct stages in its life cycle, moving between a definitive feline host, where gametes and oocysts form, and a multitude of intermediate animal hosts, where tachyzoite and bradyzoite life-stages proliferate. Oocysts shed into the environment are likely sources of human infections, and in extreme cases, contaminated soils or municipal water supplies have led to human epidemics. Oocysts are also the primary source of infections of livestock destined for slaughter and human consumption. In mammalian hosts, tachyzoite stages replicate rapidly, but growth is ultimately self-limiting and leads to the bradyzoite or tissue cyst form responsible for permanent infection.

Clinical toxoplasmosis causes public health problems in two principal settings. First, primary intrauterine infections in the first trimester are a leading cause of infection-related birth defects, affecting as many as 1 in 10,000 live births in the United States. It is estimated 85% of women of childbearing age are susceptible to primary *Toxoplasma* infection, and the incidence of new infections in this population can exceed 80% in some underdeveloped countries. Clinical manifestations include low birth weight, ocular lesions, and hydrocephalus. Since prenatal screening early in pregnancy is unable to delineate the time of any natural infection, and no studies are available to indicate the efficacy of antiparasite treatment during pregnancy, strategies for preventing and controlling congenital toxoplasmosis remain controversial. Clinical toxoplasmosis is even more common in AIDS patients where latent infection can reactivate, leading to uncontrolled

Summary

- Bradyzoite differentiation is critical to chronic *Toxoplasma* infection, and tissue cysts containing growth-arrested bradyzoites are commonly found in tissues of the central nervous system and muscle, but this relationship is not well understood.
- Animal cells induced to express increased levels of cell-division autoantigen-1 (CDA1) can slow parasite replication and induce bradyzoite development in Type II and Type III strain parasites.
- Acutely virulent Type I strains are able to ignore the CDA1-induced host signal and proliferate without differentiation. This difference provides the basis for genetic studies to isolate resistance factors.

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A Straightforward Approach to Getting at How Things Work

For Jay Radke, growing up on a farm and ranch has helped to prepare him for a career in research. "Problem-solving on the farm was a pragmatic process, and one that demanded a straightforward definition of the problem and an expedient, cost-effective, solution," he says. "Little value was placed on extended discussion once we decided how to proceed, since these challenges, large and small, could arise almost daily during calving season or harvest. I think these experiences have suited me well in the lab."

Radke was born in Iowa, but raised on a farm and ranch in south-central Montana, where his family also owned a commercial dairy herd. He learned to ride horses and rope livestock at an early age. "As kids, we all developed a certain proficiency at unique, diverse skills that are mostly foreign to those who grow up in other lifestyles," he says. "Driving trucks and operating heavy machinery were common at an early age, and, yes, roping calves was done from the back of a horse—still among the most special things I have done, personally.

"The lifestyle also required some ingenuity and creative thinking in a work life that often required building and repairing everything from fences and flat tires to radiators and transmissions," he adds. "Much of this hands-on training came from my father, who, I'm convinced, can fix most anything with twine string and his Leatherman."

Today Radke, 44, is an associate research professor in Veterinary Molecular Biology at Montana State University in Bozeman, where he studies communication between host cells and infectious pathogens. "Identifying and understanding these key molecular interactions will identify potential targets for the development of therapeutics and vaccines," he says.

Although he spends more time these days at the bench than in the saddle, his farm background, where high expectations were natural, continues to have an impact on his scientific approach. "High expectations for myself and those around me have definitely carried over into my adult life and career—often exasperating my own children as well as those who work in the laboratory with me, but all in a good way I hope," he says.

"It is in the context of farm life that my interest and appreciation for how things work first arose," he adds. "I am rarely satisfied without all the details when I am learning new things. I have a difficult time taking someone's word for the how-and-why without all the evidence in front of me."

He cannot point to a single teacher as having influenced his career path, but has definitely benefited from the efforts of many. He says, "Teaching is a noble profession. On their best days, teachers can perform miracles that last a lifetime."

Radke received a B.S. in biology in 1986 and a Master's degree in Computer Science in 1990, both from Eastern Montana College (now the Billings campus of Montana State University) in Billings. He earned his doctorate in biochemistry in 1996 from Montana State University in Bozeman.

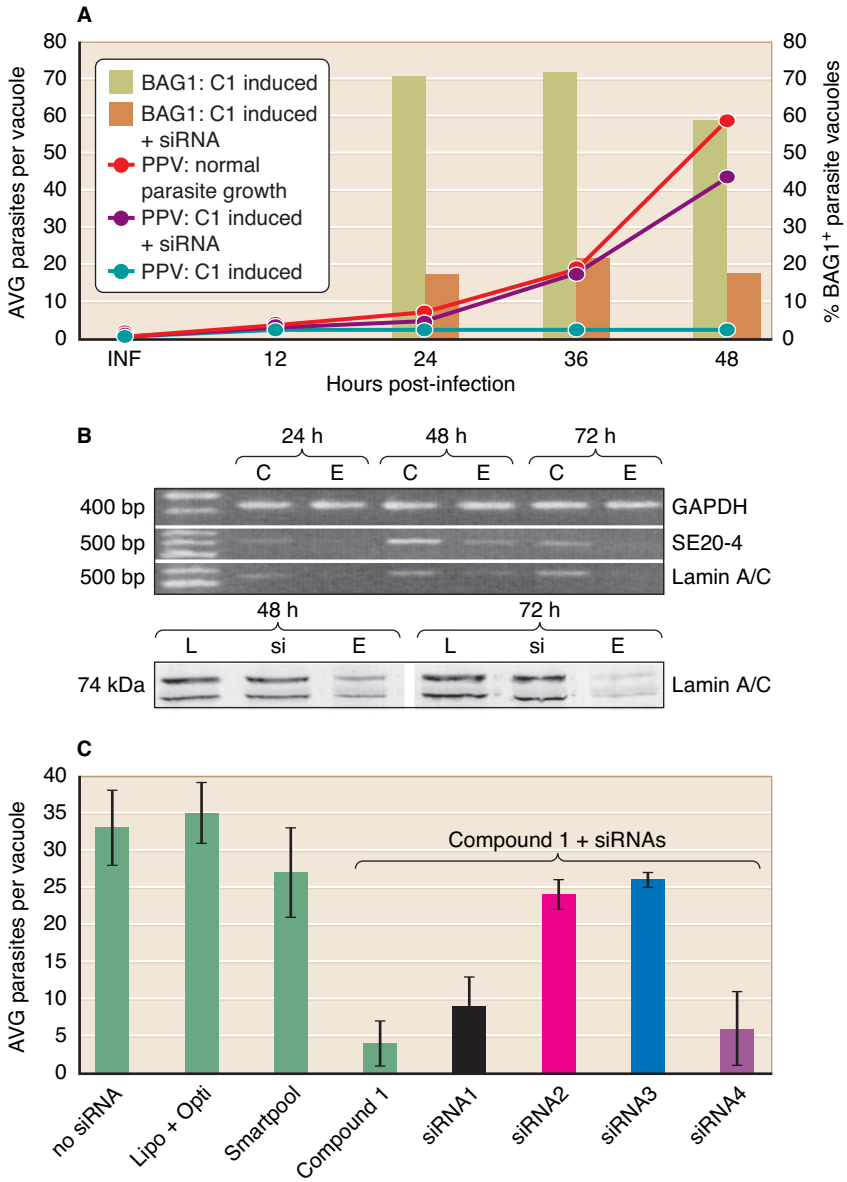
Radke credits his doctoral advisor, Kenneth Hapner, with teaching him to examine and evaluate research results. "I've yet to meet anyone who is able to scrutinize experimental results like him—and with such excitement—even for the little things," he says. His postdoctoral mentor, Michael White, is largely responsible for his interest in infectious diseases. "It may yet be a dubious honor to have influenced my career, but he has taught me to see a bigger picture and to frame questions in the context of rational cell biology." He adds, "in Mike's lab, it was natural for us to ask hard questions and expect to find the answers in our results. Sometimes that meant we built and tested new model systems, or adapted and optimized novel experimental strategies to get what we needed. Mike's approach was always confident and fearless and that has definitely influenced the way I want to approach science in my own laboratory."

Radke has been married for 25 years; his wife is an educator and social worker who currently works for the Human Resources Development Council in Bozeman. They have two boys and two girls, ranging in age from 26 to 19. "All have worked for me in some capacity while growing up, although sometimes not willingly," he says. "Three have used science to pursue diverse careers, so the tissue culture and media skills—and dishwashing—they learned early have served them well in the short term. They have all grown into smart and dedicated young adults."

Radke published papers and presented research at meetings with his two eldest sons, and one daughter is preparing to present her first poster at a scientific meeting this spring. However, their career paths diverge from his: "One is now headed off to law school, one will be a teacher, the other a chef, and the last is still thinking," he says.

Marlene Cimons
Marlene Cimons is a freelance writer in Bethesda, Md.

FIGURE 1



Multivariate microarray analyses of Compound 1-treated host cell gene expression identified cell division auto-antigen 1 (CDA1) among 79 other mRNAs with potential roles in the induction of bradyzoite development. (A) Human foreskin fibroblasts (HFF) cells transfected with siRNAs against CDA1 and co-treated with Compound 1 prior to parasite infection allow normal parasite growth (purple line) and BAG1 expression (orange bars) when compared with growth in untreated cells (red line), and with growth (green) and BAG1 expression (green bars) in cells pre-treated with Compound 1. (B) The loss of CDA1 mRNA when cells were treated with CDA1-specific siRNAs was measured by RT-PCR of total RNA at 24, 48, and 72 h following transfection. Lamin A/C controls demonstrate siRNA-mediated knockdown of individual genes. Transfection with siRNAs against Lamin A/C, with non-specific siRNAs or with Lipofectamine alone, was unable to antagonize Compound 1 inhibition of parasite growth or induction of BAG1 expression (results not shown). RT-PCR primers specific to glyceraldehyde-3-phosphate dehydrogenase were used as a control for RNA quality. RT-PCR experiment: 'C' = Compound-1 pre-treatment (3 h/3 mM) only; 'E' = HFF cells pretreated with both Compound-1 and siRNA(s) against either CDA1 or Lamin A/C. Protein blot: 'L' = HFF cells pre-treated with Lipofectamine, but without siRNAs; 'si' = HFF cells pre-treated with siRNAs against either CDA1 or Lamin A/C, but without transfection reagent (Lipofectamine); E = same as above. (C) Cotransfection of HFF cells with individual siRNAs separately, and then treatment with Compound 1 prior to parasite infection demonstrate that siRNAs 2 and 3 were responsible for antagonizing the Compound 1-induced growth inhibition. Compare bars for host cells treated with siRNAs 2 and 3 with untreated cells (no siRNA), and also with Compound 1-treated cells (Compound 1). siRNAs 1 and 4 do not significantly antagonize the Compound 1-induced inhibition of parasite replication.

tachyzoite replication and severe or fatal encephalitis.

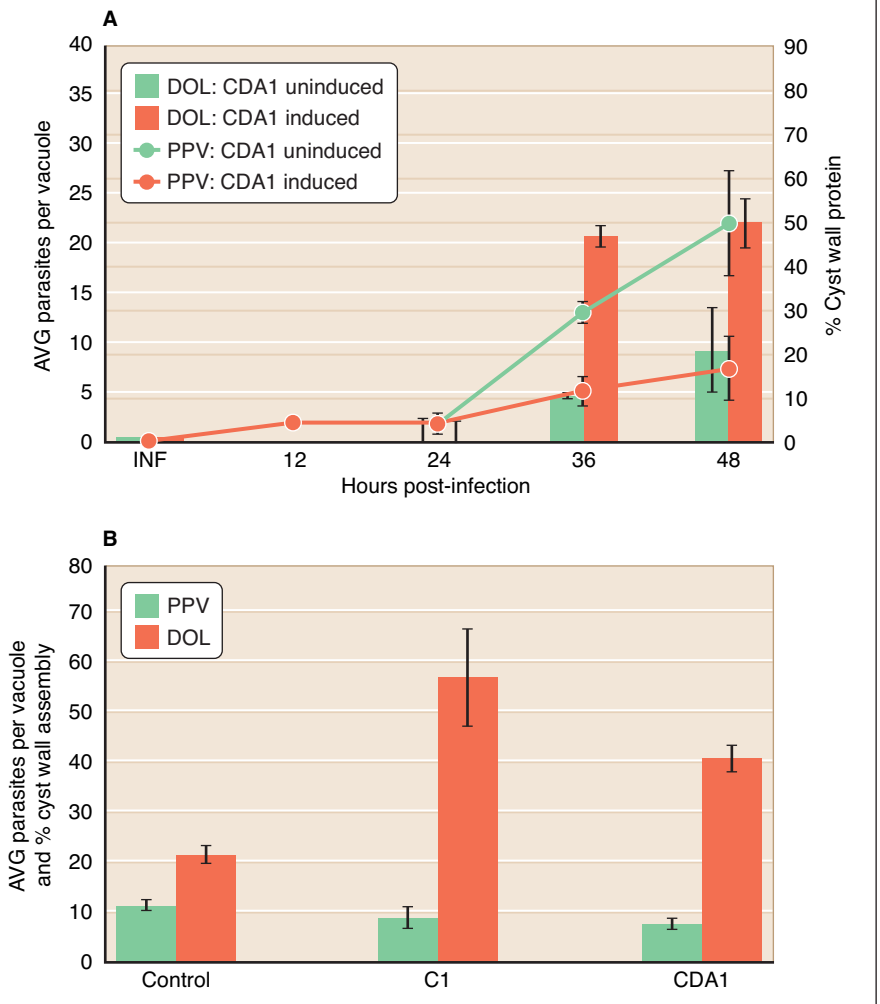
Pyrimethamine and sulfonamides are standard treatments for infection, but can be mildly toxic, which prevents continued use in long-term prophylaxis required in AIDS. And because no drugs are available to treat the encysted bradyzoite form, significant research efforts are directed at identification and development of new strategies to control acute virulence and cyst development. Recent findings suggest that infection and pathogenesis can be dictated by both host- and parasite-specific factors, although the details of specific host-pathogen interactions are not well understood.

Parasite-Directed Interactions Influence Growth and Development

Toxoplasma can alter the molecular environment of the host cell after infection, but whether bradyzoite development results from ongoing modifications to the host or is a manifestation of the cell types where bradyzoites are commonly found is not known. While acute infections can be characterized by rapid tachyzoite replication, bradyzoite development and tissue cyst formation are marked by slowed tachyzoite replication and the onset of a state of extended dormancy as differentiation progresses. During replication the parasite recruits host mitochondria and the endoplasmic reticulum (ER) to the parasitophorous vacuole to acquire host lipoate cofactors, according to Frank Seeber of Philipps Universität, Marburg, Germany, and also acquires host cell cholesterol via the low-density lipoprotein (LDL) pathway, according to Isabella Coppens of the Johns Hopkins Bloomberg School of Public Health in Baltimore, Md.

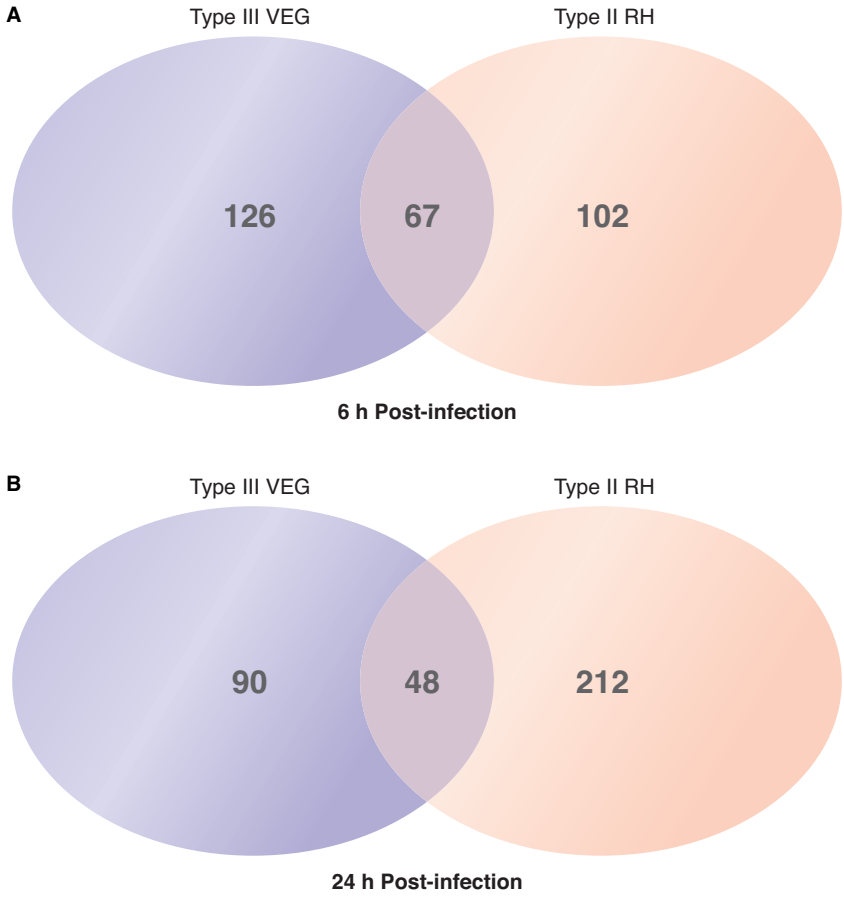
Interference with acquisition of either lipoate or cholesterol from the host cell reduces parasite replication. In contrast, bradyzoites in tissues cysts are mostly growth arrested. Thus, it is not surprising that parasite cysts are found mostly in long-lived or terminally differentiated cells of the central nervous system and mature muscle. Anthony Sinai in the College of Medicine at the University of Kentucky has demonstrated that a parasite-specific kinase(s) is able to mediate interference with host cell apoptosis, and as such,

FIGURE 2



Overexpression of host cell CDA1 in infected cells slows parasite growth and induces bradyzoite development. (A) HeLa cells expressing the tetracycline repressor protein transfected with the pcDNA4 TO-CDA1 plasmid inhibit parasite growth and induce cyst wall protein when grown in media containing tetracycline to induce expression of the CDA1 transgene. Compare parasite growth in transfected HeLa cells cultured without tetracycline (growth measured as average parasites per vacuole, PPV, green line) with that of parasite growth in media containing tetracycline to induce CDA1 expression (orange line). Concurrent with the observed inhibition of parasite replication, cyst wall protein is also expressed. Compare background levels of cyst wall protein (green bars) with levels induced by over-expression of CDA1 (orange bars) at 36 and 48 h. (B) Stable cell line expressing the CDA1 transgene under control of the tetracycline inducible promoter. Compare growth (PPV, green bars) and cyst wall protein (DOL, orange bars) between cells treated with 3 mM Compound 1 (C1) and HeLa cells stably expressing the CDA1 transgene (CDA1).

FIGURE 3



Venn diagrams quantify changes in host cell mRNA levels that differ when comparing infection with Type I RH and Type III VEG strain parasites. (A) Hybridization results identify 126 and 102 unique transcripts altered ≥5-fold when comparing VEG with RH infected cells at 6 h postinfection, respectively. (B) At 24 h postinfection, there are 90 unique mRNAs in cells infected with VEG, but in cells infected with RH, the number of unique transcripts nearly doubles to 212. Comparison of mRNA pools at 6 and 24 h demonstrates that 165 of the 212 mRNAs observed in cells infected with RH were unique at 24 h postinfection. Similar analyses in cells infected with VEG parasites identify 80 transcripts unique among the 90 altered at 24 h postinfection (results not shown). These observations further suggest significant regulation of distinct mRNA populations based on strain type and time postinvasion. Parasite infections were established at ≈ 1:1 to 1:3 multiplicity of infection (MOI) in confluent human foreskin fibroblast monolayers. DNA microarray hybridizations were completed using Agilent Technologies Whole Human Genome Oligo Array according to standard methods (Agilent Technologies Inc., Santa Clara, Calif.).

the parasite may have evolved overt strategies for making its host cell into a long-term home.

Recently, analyses of quantitative trait loci (QTL) from genetic crosses between avirulent strain types have also identified the parasite-specific rhoptry protein, designated ROP16. This protein is secreted into the host cytosol during the initial cell invasion and ultimately traffics to the host cell nucleus where it activates the STAT signaling pathways and the expression of IL-12, according to John Boothroyd of Stanford University School of Medicine, Palo Alto, Calif. Also discovered via QTL analyses, expression of the virulent Type I ROP18 allele in typically nonvirulent Type II or III strain parasites enhances virulence in mice, according to L. David Sibley, Department of Microbiology, Washington University School of Medicine, St. Louis, Mo. Both of these parasite genes encode kinase domains, and thus encode proteins that could directly alter specific host targets during infection. Peter Bradley, Department of Microbiology, Immunology and Molecular Genetics, University of California at Los Angeles has demonstrated that parasite-specific protein phosphatase 2C (TgPP2C) can also be found in the host nucleus soon after invasion and further carries the potential to directly alter host cell proteins. However, little is yet known of specific target molecules in the host.

The Host Cell Can Influence Growth and Bradyzoite Development

A distinct role for the host cell in bradyzoite development is less well defined, but recent studies demonstrate the host can significantly influence pathogenesis. Analysis of host cell gene expression in a cell infected with *Toxoplasma* provides some basis for understanding host cell effects on growth and development. For instance, host cell hypoxia-inducible factor 1 (HIF1) is required for tachyzoite growth in hypoxic cells, according to Ira Blader of the University of Oklahoma Health Sciences Center in Oklahoma City. The laboratory of John Boothroyd at Stanford University School of Medicine reports differences in host cell gene expression when comparing tachyzoite with bradyzoite infections. These early studies suggest involvement of a diverse cross-section of host biochemical pathways, but it remains difficult to identify changes in transcript levels that

are biologically relevant without correlation to definable, altered parasite phenotypes.

We recently demonstrated that a trisubstituted pyrrole, designated compound 1, alters host cells to slow parasite replication and induce early bradyzoite development in Type II and III parasite strains. Cysts induced by the Compound 1-altered host cell are morphologically indistinguishable from those induced by growth in alkaline media, a standard strategy for the study of differentiation in vitro, and the kinetics of induced parasite cyst wall and BAG1 proteins further suggest the altered host cell likely acts on the parasite via its natural bradyzoite development pathway.

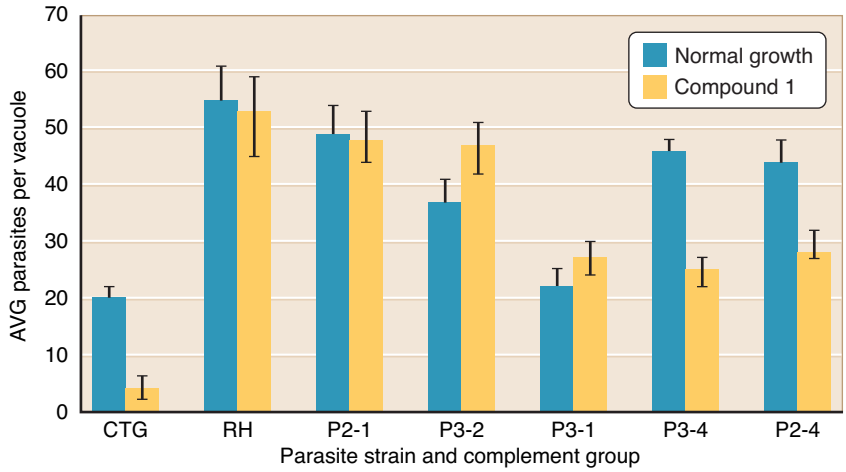
Using a multivariate DNA microarray approach, we have identified host cell mRNAs that are modulated by compound 1 in concert with parasite growth inhibition and induction of bradyzoite-specific antigens. From a subset of these host mRNAs, the mRNA encoding cell division autoantigen 1 (CDA1) was upregulated 50-fold in the Compound 1-altered cell. CDA1 is a nucleosome assembly related protein (NAP) involved in arrest of the host cell replication cycle. The protein can be phosphorylated in vitro by cyclin D1/CDK4, cyclin A/CDK2, and cyclin B/CDK1. Changes in the CDK phosphorylation sites abrogate the negative growth effects of this protein on the host cell.

To verify the role of CDA1 in the induction of bradyzoite differentiation, we used siRNAs directed against distinct regions of the CDA1 coding sequence to knockdown CDA1 mRNA in a Compound 1-altered cell. When CDA1 is missing in such cells, parasite growth is restored and the bradyzoite-specific BAG1 protein is no longer expressed (Fig. 1A and B). Conversely, we expressed CDA1 alone in the cell and then infected with the parasite, and found that increased CDA1 inhibits parasite replication and induces expression and assembly of bradyzoite-specific cyst wall protein (Fig. 2A). Thus, increased levels of CDA1 in host cells enable bradyzoite differentiation and early cyst formation in Type II and Type III parasite strains.

It is significant that acutely virulent Type I strains are unresponsive to the Compound 1-altered environment and to the overexpression of CDA1 alone. These parasites do not slow growth or express bradyzoite-specific antigens in the Compound 1-altered host environment. Compound 1 targets parasite cGMP-dependent protein kinase (PKG), and direct treatment with compound 1 reduces extracellular gliding motility and invasion. However, because these phenotypes are abrogated in transgenic parasites with a mutant PKG allele, we believe that the effects on Type I parasites are independent of those associated with Type II and Type III strains. Consistent with this observation, accepted alternate methods to induce parasite development in vitro are also less able to inhibit replication or induce bradyzoite-specific protein expression in Type I strains. This is congruent with differences in host cell gene expression when comparing infection with Type I RH and Type III VEG parasites (Fig. 3).

We considered that Type I strains may harbor a mutation that alters how these parasites initiate the bradyzoite developmental program. To test this possibility, we used genetic complemen-

FIGURE 4



Complementation suggests a genetic basis for the observed resistance of Type I strains to the Compound 1- or CDA1-altered host cell environment. Complementation of the avirulent Type III CTG strain with a virulent Type I resistance factor and selection for pyrimethamine and Compound 1 resistance reveals complementation groups with definable growth resistance to the Compound-1 treated cell. The growth of the Type III CTG and Type I RH strains, in untreated cells (green bars) and in cells treated with Compound-1 (yellow bars), are shown to aid comparison with the complement groups designated P2–1, P3–2, P3–1, P3–4 and P2–4. Note that resistance to Compound 1 in groups P2–1 and P3–2 is similar to that seen in RH. The growth and resistance of P3–1 is similar to that observed for CTG. Resistance in the Compound-1-treated host cell appears intermediate when comparing complement groups P3–4 and P2–4 with P2–1, P3–2; and P3–1.

tation to transfer a gene from the Type I RH ToxoSuperCos cosmid library into Type III strain CTG parasites (cosmid library obtained from Boris Striepen of the University of Georgia, Athens) and identified parasite populations resistant to the Compound 1-altered host cell (Fig. 4). These findings point to a genetic basis for Type I resistance to a host cell environment that readily induces Type II and Type III parasite strains to initiate bradyzoite differentiation.

As with parasite-specific molecules that affect the host cell environment, little is known of how host cell CDA1 and others influence parasite development. Whether additional factors present in the Compound 1 or CDA1-altered host cell act on the parasite, or are directly altered by infection, is unknown, but host cell influences on bradyzoite development may be manifest in cell and tissue-specific pathologies. It is also unclear whether Type I resistance is related to known differences in development among strain types, but it is possible that Type I strains respond to stimuli other than that induced in the CDA1-altered cell, or harbor a mutation that subverts the response observed in Type II and Type III strains. Traits like inherent growth rate, virulence, and tissue migration appear to be inherited, such that the transfer of resistance to Type III parasites suggests a genetic mechanism may underlie developmental differences in Type I strains. Further investigation of host biochemical pathways with respect to tissue tropism and variation in strain type response will increase our understanding of the potential links between the host cell molecular environment and the pathogenesis of *Toxoplasma* infection.

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