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Toxoplasma gondii Sporozoites Form a Transient Parasitophorous Vacuole That Is Impermeable and Contains Only a Subset of Dense-Granule Proteins†

MICHAEL TILLEY,[1] MARIA E. FICHERA,[2] MARIA E. JEROME,[1] DAVID S. ROOS,[2] AND MICHAEL W. WHITE[1]*

Department of Veterinary Molecular Biology, Montana State University, Bozeman, Montana 59717,[1] and Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6018[2]

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Toxoplasma gondii **sporozoites form two parasitophorous vacuoles during development within host cells, the first (PV1) during host cell invasion and the second (PV2) 18 to 24 h postinoculation. PV1 is structurally distinctive due to its large size, yet it lacks a tubulovesicular network (C. A. Speer, M. Tilley, M. Temple, J. A. Blixt, J. P. Dubey, and M. W. White, Mol. Biochem. Parasitol. 75:75–86, 1995). Confirming the finding that sporozoites have a different electron-dense-granule composition, we have now found that sporozoites within oocysts lack the mRNAs encoding the 5′ nucleoside triphosphate hydrolases (NTPase). NTPase first appears 12 h postinfection. Other tachyzoite dense-granule proteins, GRA1, GRA2, GRA4, GRA5, and GRA6, were detected in oocyst extracts, and antibodies against these proteins stained granules in the sporozoite cytoplasm. In contrast to tachyzoite invasion of host cells, however, sporozoites did not exocytose the dense-granule proteins GRA1, GRA2, or GRA4 during PV1 formation. Even after NTPase induction, these proteins were retained within cytoplasmic granules rather than being secreted into PV1. Only GRA5 was secreted by the sporozoite during host cell invasion, becoming associated with the membrane surrounding PV1. Microinjection of sporozoite-infected cells with fluorescent dyes showed that PV1 is impermeable to fluorescent dyes with molecular masses as small as 330 Da, indicating that PV1 lacks channels through which molecules can pass from the host cytoplasm into the vacuole. By contrast, lucifer yellow rapidly diffused into PV2, demonstrating the presence of molecular channels. These studies indicate that PV1 and PV2 are morphologically, immunologically, and functionally distinct, and that PV2 appears to be identical to the tachyzoite vacuole. The inaccessibility of PV1 to host cell nutrients may explain why parasite replication does not occur in this vacuole.**

Apicomplexan parasites, such as *Toxoplasma gondii* tachyzoites, safely enter host cells and reside within a specialized vacuole that is produced during the process of penetration. Extracellular tachyzoites attach to host cells and become oriented with their anterior end adjacent to the host cell plasmalemma, whereupon they actively invade the host cell by forming the parasitophorous vacuole (10, 14–16, 25, 30, 32, 33, 40). Although the outlines of penetration into host cells are now well established, the relative roles and timing of host and parasite contributions to the parasitophorous vacuole remain unclear.

Once inside the host cell, tachyzoites modify the parasitophorous vacuole via exocytosis of parasite constituents, including the contents of the electron-dense granules (7). As a result of this activity, a network of membranous tubules is assembled in the intravacuolar space (36) and parasite proteins can be detected in the parasitophorous vacuolar membrane (PVM) (3, 4, 7, 27). The parasitophorous vacuole has been shown to contain channels through which molecules with masses of <1,300 Da can freely diffuse, equilibrating between the intravacuolar space and the host cytoplasm (31). At least seven distinct proteins are secreted from tachyzoite dense granules, including GRA3 and GRA5 (associated with the PVM) and GRA1, GRA2, GRA4, GRA6, and 5′ nucleoside triphosphate hydrolase (NTPase), all of which are found within the intravacuolar space and are associated with the tubulovesicular network (7, 35). The functions of these proteins are unknown, although NTPase is suspected to play a role in nucleoside salvage from the host cell (5, 35). Thus, the parasitophorous vacuole is thought to serve two essential functions: ensuring safe entry into the host cell and facilitating access to host cell nutrients.

In contrast to the parasitophorous vacuole formed by tachyzoites, sporozoite entry is characterized by a large vacuole which lacks an intravacuolar network (39). By 24 h postinfection the large parasitophorous vacuoles disappear and parasites are observed in small vacuoles (39), indicating that sporozoites form a primary and then a secondary vacuole (PV1 and PV2, respectively)—a sequence confirmed by time-lapse video microscopy (37). In this report, we have used immunological and microinjection experiments to probe the nature of the first and second sporozoite vacuoles and have found distinct differences between these structures.

MATERIALS AND METHODS

Cell culture and parasite purification. Human foreskin fibroblasts (HFF) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) newborn calf serum. Bovine pulmonary artery endothelial cells (CPA; ATCC CCL 207) were grown in RPMI 1640 with 10% fetal bovine serum.

Sporulated oocysts from an avirulent strain (VEG) of *T. gondii* originally isolated from an AIDS patient (28) were purified from cat feces by centrifugal elutriation on a Beckman JE-6B elutriation system with a 4.8-ml Sanderson elutriation chamber as described previously (39). Purified oocysts were incubated in 10% (vol/vol) Clorox (in phosphate-buffered saline [PBS]) at room temperature for 30 min, collected by centrifugation, and washed three or four times in Hanks' balanced salt solution (GIBCO, Gaithersburg, Md.) to remove residual Clorox. Sporozoites were excysted according to published methods (38), washed

* Corresponding author. Phone: (406) 994-4705. Fax: (406) 994-4303. E-mail: uvsmw@gemini.oscs.montana.edu.

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in PBS, and, in some cases, filtered through 3.0-μm-pore-size polycarbonate filters (Nucleopore Corp., Pleasanton, Calif.) to remove sporocysts, unbroken oocysts, and oocyst debris. Sporozoites were suspended in culture medium containing 1% fetal bovine serum or newborn calf serum, 50 μg of dihydrostreptomycin/ml, and 50 U of penicillin G/ml and inoculated into cultured cells. VEG strain tachyzoites were obtained from cultures of HFF cells initially inoculated with sporozoites and serially propagated in HFF cells according to standard protocols (29).

SDS-PAGE and immunoblotting. Extracts of sporozoites (5×10^8/ml) and tachyzoites (2×10^8/ml) were prepared for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as described previously (39). Briefly, purified oocysts suspended in lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 5 mM EDTA) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine HCl, 10 mM 1,10-phenanthroline, and 10 μg each of antipain, chymostatin, leupeptin, and pepstatin A/ml) were broken with glass beads on a vortex mixer. After removal of the glass beads, Nonidet P-40 was added to a final concentration of 2% (vol/vol), and the lysate was then incubated on ice for 30 min followed by centrifugation for 10 min at $12,000 \times g$. Tachyzoites grown in HFF cells were passed through a 3.0-μm-pore-size Nucleopore filter, washed two times in PBS, and extracted as above, with the Nonidet P-40 added to the lysis buffer during disruption of the cell pellet. Samples were boiled for 3 min in Laemmli reducing buffer, electrophoresed, and transferred to nitrocellulose. The nitrocellulose strips were treated for 1 h in blocking buffer (5% [wt/vol] nonfat dry milk in 50 mM Tris [pH 8.0]–150 mM NaCl) except for the blot, which was probed for anti-GRA6 that was blocked in undiluted horse serum. All blots were probed for 2 h with the primary antibody diluted 1:250 (1:500 in the case of NTPase) and washed six times for 5 min each time in blocking buffer. Primary antibodies were as follows: anti-GRA1, monoclonal antibody (MAb) TG17-43 (8); anti-GRA2, MAb TG17-179 (8); anti-GRA3, MAb T6 2H11 (23); anti-GRA4, polyclonal rabbit antiserum (kindly provided by David Sibley); anti-GRA5, MAb TG17-113 (21); anti-GRA6, mouse polyclonal antiserum (22); and anti-NTPase, polyclonal rabbit antiserum (5). Secondary reagents were as follows: alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin G (IgG) (Promega, Madison, Wis.) diluted 1:5,000 or goat anti-rabbit IgG (Sigma, St. Louis, Mo.) diluted 1:2,500 in blocking buffer. Antigen detection was accomplished by incubating the blots in the appropriate alkaline phosphatase-conjugated antibody for 1 h, washing as described above, and developing in nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate.

Immunofluorescence assays. CPA cells were grown in 8-well chamber slides and inoculated with 10^5 sporozoites or tachyzoites. At various intervals, cells on slides were washed three times in PBS, fixed with 3% paraformaldehyde in PBS, treated in acetone for 10 min at 4°C, and air dried. The slides were incubated with the various anti-GRA protein antibodies for 1 h in a humid chamber and then washed three times in PBS followed by a single wash in PBS–0.3% rabbit serum. The slides were treated with the secondary antibody fluorescein-conjugated anti-rabbit or anti-mouse IgG (Sigma) diluted 1:64 in Dulbecco's modified Eagle medium containing 2.5% goat serum for 1 h in the dark, washed four times in PBS, mounted with Gel/Mount (Fisher Scientific, Pittsburgh, Pa.) solution containing 2.5% (wt/vol) diazabicyclo[2.2.2.7]octane, and observed on a Nikon epifluorescence microscope. Matched irrelevant antibody controls were included for each anti-GRA antibody.

RT-PCR assay. Total RNA was prepared from 4×10^7 tachyzoites purified as described above and from an excystation mix containing sporozoites, sporocysts, and intact oocysts derived from 7×10^6 oocysts (~5.6×10^7 sporozoites). Samples were washed three times in PBS, lysed in a 4 M guanidine isothiocyanate solution containing 0.125% (wt/vol) Sarkosyl, 24 mM sodium citrate, 0.8% β-mercaptoethanol, and 40 μg of Baker's yeast (Sigma Chemical Co., St. Louis, Mo.) tRNA/ml as a carrier, and processed as described previously (2, 9). The total RNA preparation was then extensively treated with RNase-free DNase (Ambion, Austin, Tex.) to remove potentially contaminating genomic DNA. Reverse transcription (RT) was performed as described previously (1) with minor modifications. Reactions contained 5 μg of total RNA and 1 μM of the various antisense oligonucleotides. Control reactions were performed by omitting the reverse transcriptase and/or RNA. A 2-μl sample from each (RT) reaction mixture was then used for PCR with a GeneAmp 2400 thermal cycler (Perkin-Elmer, Foster City, Calif.) programmed for denaturation at 94°C, followed by 25 cycles of 94°C for 30 s, 65°C for 30 s (52.5°C for NTPase), and 72°C for 30 s. A final extension step was performed at 72°C for 5 min. The following oligonucleotide primers were obtained from Ransom Hill Bioscience Inc. (Pomona, Calif.): (i) spanning nucleotides (nt) 839 to 1319 of the SAG1 gene (6), sense (5′-CAAGCCAGAGCCTCATCGGTCGTC-3′) and antisense (5′-CGCG ACACAAGCTGCGATAGAGCC-3′); (ii) regions of the small subunit rRNA spanning nt 1097 to 1595 of the *T. gondii* rRNA sequence (13) (these regions exhibit specificity for coccidia), sense (5′-GGAAAACGTCATGCTTGACTTC TCCTG-3′) and antisense (5′-CTCACAAGATTACCTAGACCTATCGGCC-3′); and (iii) oligonucleotides differentiating NTPase1 from NTPase2 or -3 (provided by Keith Joiner, Yale University [5]) producing cDNA-derived fragments of 1,036 nt (NTPase1) or 1,039 nt (NTPase2 or -3), sense (for both NTPase1 and NTPase2 or -3) (5′-GGCGAAGAAACTCATGCAACTC-3′), antisense (NTPase1-specific) (5′-TCTCTCGTCCCGTTATAAAC-3′), and antisense (NTPase2 or -3-specific) (5′-GCATACCTCCGCCCGTGACG-3′).

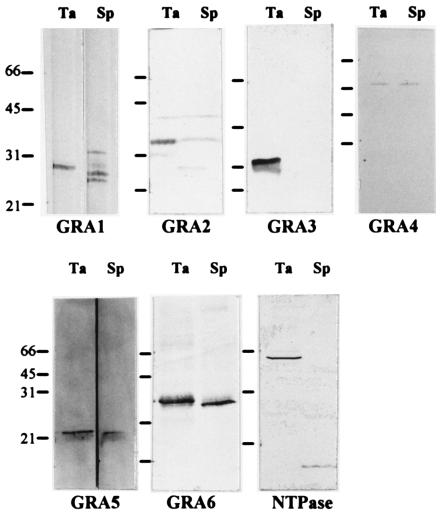

FIG. 1. Western blots of *T. gondii* dense-granule proteins. Equal amounts of protein from VEG strain tachyzoite or sporozoite (oocyst) extracts were electrophoresed under reducing conditions on SDS–12% PAGE gels. Nitrocellulose blots were probed with anti-GRA antibodies as described in Materials and Methods. Molecular mass markers (in kilodaltons) are indicated to the left of each panel; antigen mobility in VEG tachyzoite extracts is identical to that observed for RH strain parasites (not shown). Note that GRA2 is less abundant in the oocyst extracts than in those of tachyzoites, while GRA3 and NTPase proteins are completely lacking. Additionally, note the three major and one minor species of GRA1 in sporozoites. Ta, tachyzoites; Sp, sporozoites.

Microinjection. Microinjection of sporozoite-infected CPA cells was performed as previously reported (31). CPA cells were grown on 22-mm-diameter glass coverslips attached to 60- by 15-mm dishes with silicon sealant (732 RTV; Dow Corning), and were inoculated with sporozoites. At various times after inoculation, the standard growth medium was changed to minimal essential medium buffered with 20 mM HEPES (pH 7.2) and containing Hank salts, L-glutamine, and 3% (vol/vol) dialyzed fetal bovine serum. Microinjection was performed at ambient temperature; needles were prepared from 1.0-mm-diameter glass capillary tubing (model TW100F-4; World Precision Instruments, Sarasota, Fla.) on a P-87 Flaming/Brown micropipette puller (Sutter Instrument Co., Novata, Calif.). Individual infected cells were injected with lucifer yellow (1% aqueous solution of the dilithium salt) or fluorescein (0.15 mM in 5% ethanol) with a Narishige micromanipulator and automated injector and observed with a Zeiss Axiovert 35 fluorescence photomicroscope. At least eight parasite-infected host cells were microinjected and observed at each time point.

RESULTS

Expression of dense-granule proteins in oocyst extracts. Our previous studies (39) demonstrated that *T. gondii* sporozoites do not express the tachyzoite protein GRA3. We therefore examined sporozoites for expression of other dense-granule proteins which are known to be secreted into the tachyzoite parasitophorous vacuole. VEG strain oocyst (sporozoite) extracts were electrophoresed, transferred to nitrocellulose, and incubated with a series of antibodies that recognize dense-granule proteins. Equal protein loading and transfer was confirmed by staining the blots with Ponceau S (data not shown). Consistent with our previous report (39), dense-granule proteins were differentially expressed in tachyzoites and sporozoites (Fig. 1). In addition to GRA3, oocyst extracts lacked detectable levels of the NTPases (5), while apparently normal GRA4, GRA5, and GRA6 were present at similar levels in both preparations (Fig. 1). GRA2 was detected in sporozoites at lower levels than in the tachyzoite extracts. The anti-GRA1 antibody revealed a complex pattern of sporozoite antigens, which were absent in the tachyzoite extracts.

Because the anti-NTPase antiserum used in these studies does not distinguish between the different NTPase isoforms,

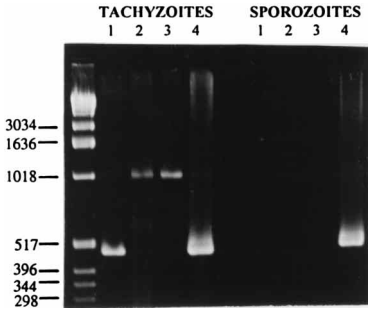

FIG. 2. NTPase mRNA expression in tachyzoites and sporozoites. RT-PCR amplification of NTPase1, NTPase3, and SAG1 mRNAs was performed as described in Materials and Methods, and the products were separated on a 1.2% agarose gel. Lane 1, SAG1 mRNA; lane 2, NTPase1 mRNA; lane 3, NTPase3 mRNA; lane 4, *T. gondii* small-subunit rRNA. The numbers on the left correspond to molecular size markers in nucleotides. All amplified bands are of the expected size.

we examined NTPase mRNA in sporozoites by RT-PCR as shown in Fig. 2. Total RNA from tachyzoites and sporozoites was purified, and RT-PCR was performed with primers specific for NTPase1 and NTPase2 or -3 sequences (5). Amplified products of the predicted size were identified in tachyzoite RNA with both NTPase primer sets but not in sporozoite total RNA (Fig. 2, lanes 2 and 3), confirming the absence of this important protein in sporozoites. These results suggest that the developmental regulation of protein expression in sporozoites is due to changes in transcript levels rather than to an inhibition of translation. In support of this conclusion, SAG1 primers specific for the major tachyzoite-specific surface antigen P30 (6) amplified a cDNA product from tachyzoite total RNA but not from sporozoite RNA (Fig. 2, lane 1). The presence of RNA in both preparations was confirmed by using primers specific for the *T. gondii* small-subunit rRNA (Fig. 2, lane 4), although it is recognized that the presence of rRNA control may not accurately reflect mRNA levels. No amplification was seen in reactions from which RNA or reverse transcriptase (data not shown) was omitted, indicating that the PCR products were not due to genomic DNA contamination.

Dense-granule proteins are differentially expressed during the development of sporozoites into tachyzoites. Our earlier studies established the time frame for induction of GRA3 and SAG1 during sporozoite development, with the first detectable expression occurring 12 to 15 h after sporozoite inoculation (39). The kinetics of expression for other tachyzoite dense-granule proteins are summarized in Table 1. In situ localization of GRA5, GRA1, and NTPase by immunofluorescence is shown in Fig. 3 to 5, respectively. As noted previously, tachyzoite dense-granule proteins can be categorized based on their localization within the tachyzoite parasitophorous vacuole: GRA3 and GRA5 are PVM associated, while GRA1, GRA2, GRA4, GRA6, and NTPases are localized to the tubulovesicular network. Once expressed (>12 h postinfection), GRA3 is secreted into PV1 and associates with the PV1 membrane (39). A similar localization was obtained with the anti-GRA5 antibody, and because GRA5 is expressed in sporozoites within oocysts, GRA5 was detected in the PV1 membrane at all the times it was examined after infection (Fig. 3).

The network-associated dense-granule proteins revealed a pattern of expression and in situ localization different from those of GRA3 and GRA5. While sporozoites inhabited the distinctive PV1, the staining of anti-GRA1, -GRA2, and -GRA4 antibodies was confined to dense granules within the sporozoite cytoplasm and no staining was observed outside of the parasite itself (Table 1). Figure 4B and C show typical images of early (2 h postinoculation) and late (18 h postinoculation) PV1s stained with anti-GRA1. By contrast, in tachyzoite-infected cells or in host cells where sporozoites had moved into a PV2 (>18 h postinfection) most of the GRA1, GRA2, and GRA4 was localized to the vacuolar space (Table 1). The diffuse anti-GRA1 staining that rims the tachyzoite in Fig. 4A* indicates that most of the GRA1 has been exocytosed into the lumen of the parasitophorous vacuole, consistent with previous reports (36). Vacuolar GRA1 also can be seen in PV2s containing parasites that have started to replicate (Fig. 4D).

NTPase antigen was detected in VEG tachyzoites but not in extracellular sporozoites, indicating that the induction of the NTPases must occur during sporozoite development, analogous to that of GRA3 and SAG1 (39). Like that of these other induced proteins, NTPase expression is first detected ~12 h after inoculation with sporozoites (Table 1 and Fig. 5), but unlike GRA3 expression, NTPase localization within PV1 is distinct from the localization seen in tachyzoite vacuoles. At 12 h postinfection, NTPase expression is visible but confined to discrete granules within the sporozoite cytoplasm (Fig. 5C), comparable to GRA1, GRA2, and GRA4 staining. Only within the second vacuole (PV2) is vacuolar staining evident. Figure 5D shows NTPase staining of a newly formed PV2 (in addition to granular staining of the parasite itself). This particular vacuole appears to have arisen from a parasite that had recently left the adjacent, unstained PV1.

TABLE 1. Antigen localization following infection with *T. gondii* sporozoites

Parasite stage	Time post-infection (h)	Vacuole	Localization of antigen to[a]:					
			GRA1	GRA2	GRA3	GRA4	GRA5	NTPase
Sporozoite	0		P	P	–	P	P	–
	2	PV1	P	P	–	P	V	–
	6	PV1	P	P	–	P	V	–
	12	PV1	P	P	V (30)	P	V	P (25)
	18	PV1	P	P	V (50)	P	V	P (60)
	24	PV2	N	N	V + N	N	V	N
Tachyzoite	0		P	P	P	P	P	P
	12	TV	N	N	V + N	N	V	N

[a] Immunofluorescence assays were performed at various times after sporozoite infection of CPA cells. TV, tachyzoite vacuole. Data reflect predominant staining pattern: P, parasite dense granules; V, vacuolar membrane; N, intravacuolar network; –, no staining. Numbers in brackets indicate percentage of cells with predominant staining pattern in samples where only some vacuoles were stained (100% where not indicated).

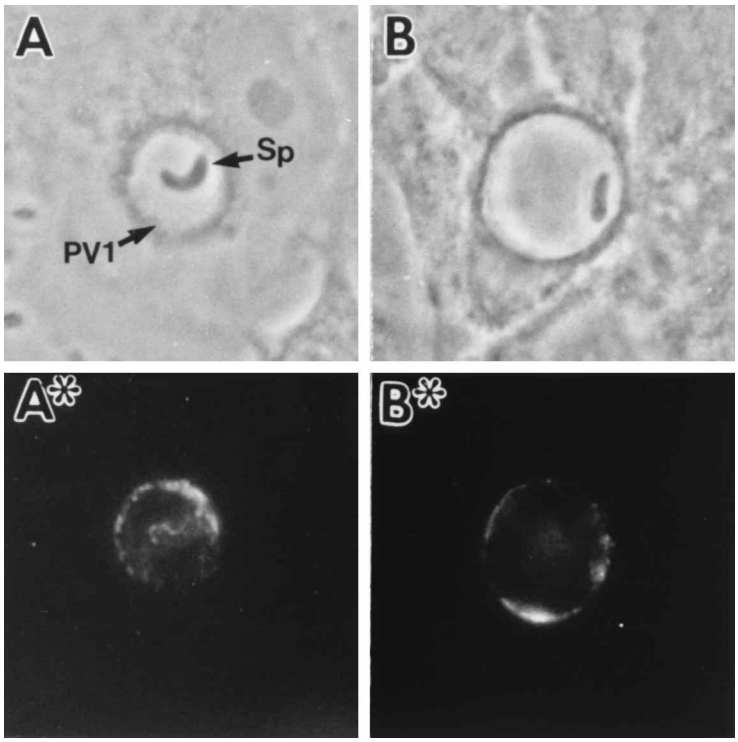

FIG. 3. Indirect immunofluorescence of GRA5 in sporozoite-infected CPA cells. Cells were fixed 6 (A) and 24 (B) h postinfection and stained with an anti-GRA5 antibody. Immunofluorescence images (bottom) correspond to the phase-contrast micrographs shown at top. Note the extensive staining of the PV1 membrane (the patchiness of membrane staining is typical of membrane-associated dense-granule proteins [12, 21]). Sp, sporozoite. Magnification, ×1,600.

PV1 lacks molecular pores. The ultrastructural differences between PV1 and PV2, combined with the apparent lack of parasite replication in PV1, led us to ask whether PV1 contains the molecular channels recently defined by microinjection of fluorescent dyes into tachyzoite-infected host cells (31). These channels permit molecules of <1,300 Da to freely diffuse between the vacuolar space and the host cell cytoplasm (31) and are likely critical for nutrient acquisition by the parasites. Rapid diffusion of the dye was observed throughout the host cell following microinjection of lucifer yellow (457 Da) into the cytoplasm of CPA cells harboring a sporozoite in the PV1, but the dye was excluded from the vacuole (Fig. 6). Fluorescein, which has a slightly smaller molecular mass (332 Da) than lucifer yellow, was also excluded from PV1 (not shown). Similar results were obtained up to 24 h postinoculation. These results suggest that PV1 is surrounded by an intact membrane, and that previous ultrastructural observations of fragmented membranes (39) may have been artifacts.

In several cases, the microinjection process appeared to induce sporozoites to leave PV1. Interestingly, sporozoite penetration of the PV1 membrane did not cause the vacuole to collapse and no dye was observed to enter the vacuole. The residual, empty PV1 remained impermeable to lucifer yellow throughout the period of observation (5 to 10 min). Attempts to directly microinject PV1 indicate it is a relatively sturdy structure which can be readily moved by the microinjection needle. Microinjecting PV1 led to a partial collapse of the vacuole, suggesting there is a pressure difference between the intact vacuole and the host cytoplasm.

By 48 h postinoculation all sporozoites have left PV1-type vacuoles and are seen within PV2s, where replication commences. Microinjected lucifer yellow rapidly equilibrated across these vacuoles, although the dye was excluded from the cytoplasm of individual parasites within the vacuole (Fig. 6B), supporting the view that PV2 is identical to the tachyzoite vacuole (31).

DISCUSSION

Dormant within oocysts, *T. gondii* sporozoites are poised to invade the intestinal mucosa once they are ingested by a suitable animal host. After passage through the stomach, infectious parasites are released from the oocyst within the small intestine (11). In contrast, the proliferative tachyzoite form of the parasite is not found naturally outside the animal and is unable to survive passage through the gut. It is likely that sporozoites have evolved stage-specific mechanisms facilitating their survival, and radiolabeling of sporozoite surface proteins has revealed two major (and several minor) stage-specific protein species (17, 19). The sporozoite surface appears to have very few proteins in common with tachyzoites: the major tachyzoite surface antigens, SAG1 and SAG2, are not detected on sporozoites.

Differences between sporozoites and tachyzoites are not limited to surface antigens. Probing *T. gondii* sporozoite extracts with a tachyzoite anti-rhoptry antiserum reveals stage-specific differences in the apex-associated organelles (39). Stage-specific rhoptries have also been documented in the asexual stages of the chicken coccidian *Eimeria tenella*, although the developmental functions of these differences are unknown (20, 41). *Toxoplasma* sporozoites lack the dense-granule proteins GRA3 (39) and NTPase (Fig. 1). Regulation of the NTPase (and SAG1) appears most likely to be at the transcript level (Fig. 2) (transcripts for other GRA proteins have not been examined). Both the NTPase and GRA3 proteins are first observed ~12 h postinfection, suggesting that they may share developmental regulatory elements, although preliminary

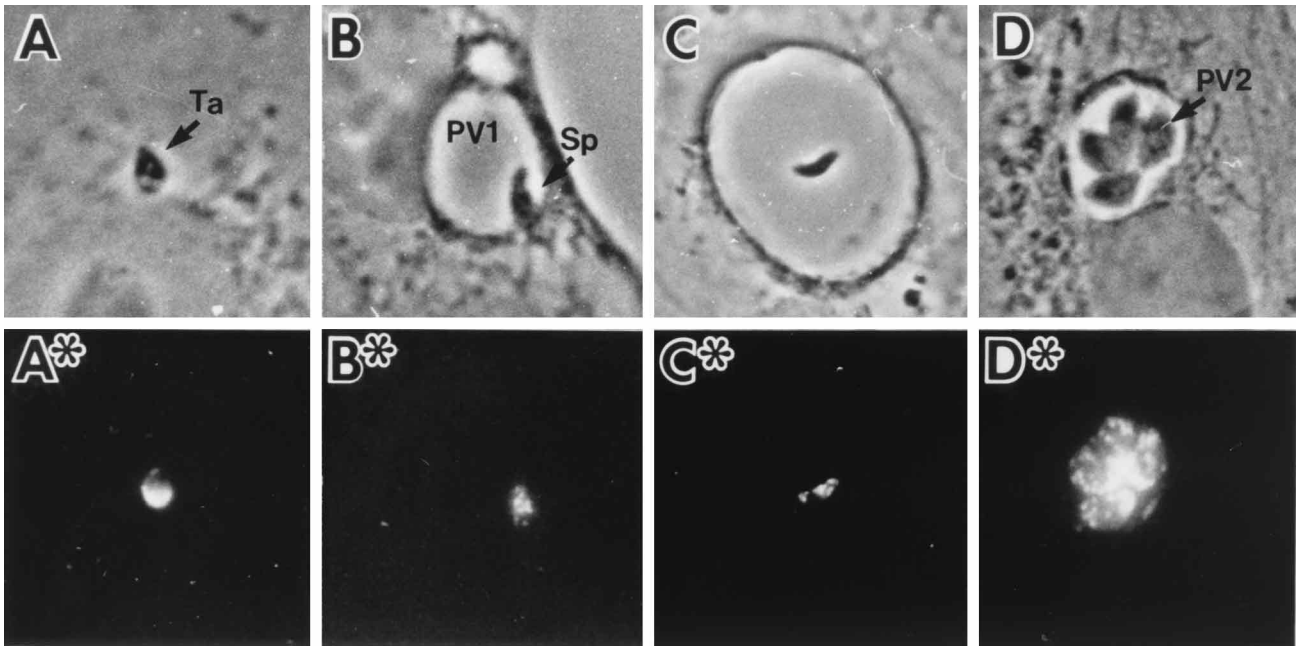

FIG. 4. Indirect immunofluorescence of GRA1 in sporozoite-infected CPA cells. CPA cells were fixed 2 (A and B), 18 (C), or 40 (D) h after infection with VEG strain tachyzoites (A) or sporozoites (B to D) and stained with anti-GRA1 antibody. Immunofluorescence images (bottom) correspond to the phase-contrast micrographs above. Compare the diffuse extracellular staining of GRA1 that surrounds the parasites in panels A* and D* to the intracellular, granular staining of the sporozoites in panels B* and C*. Magnification, ×2,400 (A and B), ×1,800 (C), and ×2,000 (D).

comparison of the putative promoter regions failed to reveal any obvious shared features which might be responsible.

Other dense-granule proteins are present in both forms of the parasite, although the GRA2 levels are somewhat reduced, and multiple antigens cross-react with the GRA1 antibody (whether these are related antigens from different gene[s] or alternative or processed products of the GRA1 gene is not known). Dense-granule proteins are known to be glycosylated

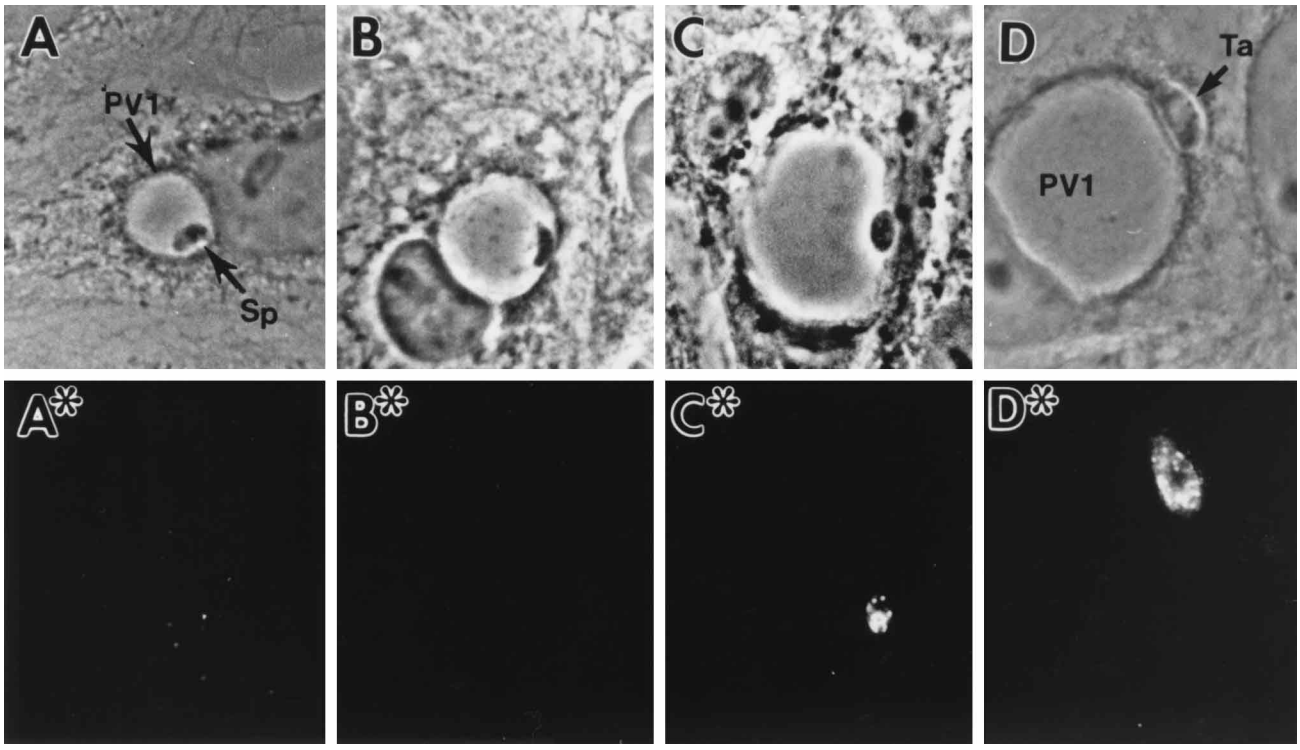

FIG. 5. NTPase expression during sporozoite development in CPA cells. NTPase was detected in infected CPA cells by indirect immunofluorescence 2, 6, 12, or 18 h after sporozoite inoculation (A to D, respectively). Immunofluorescence images (bottom) correspond to the phase-contrast micrographs above. NTPase is first detected as intracellular granular staining ~12 h postinfection and is only secreted in PV2, not PV1. Sp, sporozoite; Ta, tachyzoite. Magnification, ×1,500.

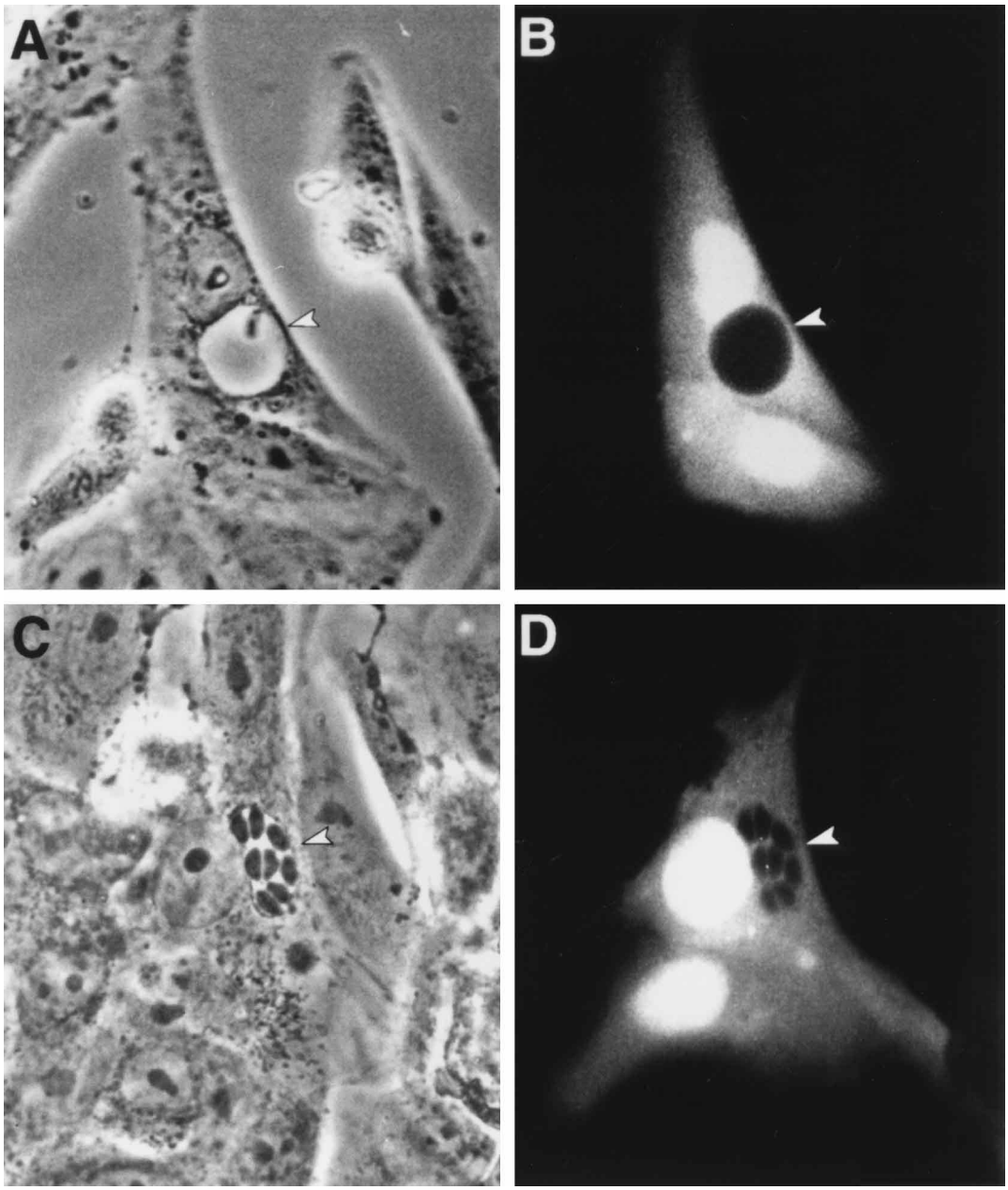

FIG. 6. Microinjection of sporozoite-infected CPA cells with lucifer yellow. (A and B) Note the exclusion of dye from PV1s 12 h after infection with sporozoites. (C and D) Dye freely diffuses into a PV2 48 h postinfection but is excluded by the parasite plasma membrane; eight parasites are visible within this vacuole, indicating three cell division cycles. Fluorescence images at right correspond to the phase-contrast micrographs at left. Bright-staining regions result from entrapment of lucifer yellow within the host cell nucleus.

(3, 8), suggesting that different posttranslational processing could account for the sporozoite GRA1 species. Collectively, these findings are consistent with previous observations of stage-specific antigen expression in *Toxoplasma* (17, 19). All of the known dense-granule proteins are coordinately expressed in tachyzoites, but the sporozoite observations indicate that this is not essential.

In contrast to host cell invasion by *T. gondii* tachyzoites, which enter relatively tight-fitting parasitophorous vacuoles and proceed to replicate intracellularly, sporozoites form unusually large parasitophorous vacuoles containing a single parasite, which fails to replicate (39). Replication commences only after the parasite leaves this first vacuole (PV1) and enters a new one (PV2), which more closely resembles the tachyzoite vacuole (Table 1).

Apart from its large size, one of the most striking characteristics of PV1 is the absence of the intravacuolar tubulovesicular network found in PV2 and the tachyzoite vacuoles (36, 37, 39). These structural observations are consistent with the failure of intracellular sporozoites resident within PV1 to secrete the dense-granule proteins GRA1, GRA2, GRA4, and NTPase (Table 1 and Fig. 4 and 5). It is interesting to note that there is a close (but not perfect) correlation between dense-granule proteins which are not secreted and network associa-

tion in tachyzoite vacuoles. Why sporozoites should fail to secrete these proteins is puzzling, as they are certainly capable of secreting GRA3 and GRA5 into PV1 (Table 1 and Fig. 3) (39). It is also not possible to completely explain the block in secretion of certain dense-granule proteins into PV1 by some kind of sequestration event occurring within the oocyst: NTPase is only synthesized after establishment of the invading parasite within PV1 (Table 1), but the enzyme is not secreted until entry into PV2 (Fig. 5). In contrast, GRA3, which is synthesized at the same time, is exocytosed immediately (Table 1) (39). These data support previous hypotheses that dense granules are not homogeneous (GRA2) (36), although we have not tested this directly. Signals responsible for induced protein secretion in *Toxoplasma* have not been elucidated, but host and parasite factors are both implicated (34, 36). Intravacuolar Ca^{2+} concentration appears to play an important role, as extracellular tachyzoites undergo dense-granule exocytosis in buffers containing cytosolic concentrations of Ca^{2+} ($<$10 mM) and are inhibited by extracellular Ca^{2+} concentrations greater than 1 mM (34).

Another intriguing observation is the absence of pores which permit small molecules from the host cytoplasm (e.g., ATP) to enter PV2 (Fig. 6) or the tachyzoite vacuoles (31). The inability to obtain critical nutrients via PV1 might explain why sporozoites fail to replicate intracellularly and stay in this vacuole for a relatively short time before reinvading the host cytoplasm to establish PV2. The combination of PV1 impermeability, lack of a tubulovesicular network, and absence of network-associated dense-granule proteins may be more than coincidence. Ultrastructural studies on the intravacuolar network in tachyzoite vacuoles suggest that network tubules may be continuous with the PVM and exposed to the host cytoplasm (26, 36). In this context GRA2, with its predicted amphipathic alpha-helix motif, might be a component of the pore (24, 36). The failure of sporozoites to secrete GRA2 until their entry into PV2 is consistent with the appearance of pores at that stage. Conversely, association of GRA3 and GRA5 with the PV1 membrane is not sufficient for pore formation.

Coccidian sporozoites (including *T. gondii*) are notable for their ability to survive within oocysts in the environment for years, apparently utilizing stored energy sources, such as amylopectin. In this arrested growth state, sporozoites must be very efficient at economizing on metabolic demands. It may be that enzymes such as NTPase, which are presumed to play an important role in parasite metabolism (5), are downregulated in the dormant sporozoite. If this model is accurate, PV1 could provide the sporozoite with a protected environment within the host cell where gene expression is induced—albeit one in which access to host cell nutrients is restricted.

During sporozoite development within PV1, the parasite surface changes so that organisms emerging from the sporozoite-infected cell are capable of interacting with new host cells to produce a vacuole comparable to the tachyzoite vacuole. These parasites express the tachyzoite surface protein SAG1 (39) and all known tachyzoite dense-granule proteins (Table 1). We have also followed expression of a 30-kDa sporozoite surface protein, which declines almost immediately after sporozoite entry into PV1 and is completely absent from parasites replicating within PV2 (13a). Why the sporozoite and tachyzoite surfaces are different is not known, but it is intriguing to speculate that stage-specific surface proteins mediate distinct interactions with different host tissues. SAG1 has been shown to influence parasite binding to host cells (reviewed in reference 18), suggesting that sporozoite attachment to host cells may be affected by stage-specific surface proteins. Further studies are needed to characterize the parasite proteins associated with PV1, which can be stained with sporozoite-specific antiserum that fails to recognize PV2 or tachyzoite vacuoles (13a).

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