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Association of host cell endoplasmic reticulum and mitochondria with the *Toxoplasma gondii* parasitophorous vacuole membrane: a high affinity interaction

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SUMMARY

The parasitophorous vacuole membrane (PVM) of the obligate intracellular protozoan parasite *Toxoplasma gondii* forms tight associations with host mitochondria and the endoplasmic reticulum (ER). We have used a combination of morphometric and biochemical approaches to characterize this unique phenomenon, which we term PVM-organelle association. The PVM is separated from associated mitochondria and ER by a mean distance of 12 and 18 nm, respectively. The establishment of PVM-organelle association is dependent on active parasite entry, but does not require parasite viability for its maintenance. Association is not a consequence of spatial constraints imposed on the growing vacuole. Morphometric analysis indicates that the extent of mitochondrial association with the PVM stays constant as the vacuole enlarges, whereas the extent of ER association decreases. Disruption of host cell microtubules partially blocks the establishment but not the maintenance of PVM-mitochondrial association, and has no significant effect on PVM-ER association. PVM-organelle association is maintained following disruption of infected host cells, as assessed by electron microscopy and by sub-cellular fractionation showing co-migration of fixed PVM and organelle markers. Taken together, the data suggest that a high affinity, potentially protein-protein interaction between parasite and organelle components is responsible for PVM-organelle association.

Key words: *Toxoplasma gondii*, Parasitophorous vacuole membrane, Mitochondria, Endoplasmic reticulum

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite that invades and replicates in nucleated cells of warm blooded animals (Joiner and Dubremetz, 1993). Parasite invasion is accompanied by the formation of a specialized non-fusogenic compartment termed the parasitophorous vacuole (PV), within which the parasite replicates (Joiner, 1994). The PV is delimited from the host cell by a specialized membrane termed the parasitophorous vacuole membrane (PVM) (Joiner, 1992). As the interface between the parasite and the host, the PVM must fulfill the requirements of providing both a safe haven from cellular defenses, while also ensuring access of the parasite to the nutrient rich host cytoplasm (Sinai and Joiner, 1997).

Formation of the PVM during cell invasion is an unusual process (Jones et al., 1972; Aikawa et al., 1977; Nichols and O'Connor, 1981). Invasion is associated temporally with discharge of anterior club-shaped organelles termed rhoptries (Sam-Yellowe, 1996), which likely participate in PVM formation (Beckers et al., 1994). Nonetheless, recent data suggests that the bulk of PVM lipids in the newly formed vacuole are derived from the host (Suss-Toby et al., 1996). Host cell plasma membrane proteins are excluded from the forming vacuolar membrane (Porchet-Hennere and Torpier, 1983; Carvahlo and Souza, 1989) by a transient structure termed the moving junction (Aikawa et al., 1978). By freeze fracture electron microscopy, the newly formed PVM is devoid of intramembranous particles (Porchet-Hennere and Torpier, 1983). Thus, the nascent PVM is an unusual membrane, which might be expected to have unique interactions with membrane-bound structures in the host cell. In fact, the failure of the PV to fuse with organelles of the endocytic cascade (Jones and Hirsch, 1972; Sibley et al., 1985; Joiner et al., 1990) is likely due to the exclusion of host proteins from the PVM which could serve as docking and fusion signals (Joiner et al., 1990).

Concomitant with and continuing after invasion, a striking morphological process is observed, namely intimate and extensive interactions between the PVM and host cell mitochondria and endoplasmic reticulum (ER) (Endo et al., 1981; De Melo et al., 1992). While mitochondrial profiles associated with the PVM are easily identified, the assignment of the ER is based on the reticulate nature and the presence of ribosomes on the PVM-associated membrane (Jones and Hirsch, 1972; Endo et al., 1981; De Melo et al., 1992). Thus, simultaneously with the failure of the PVM to interact with endosomes, lysosomes or other endocytic organelles, selective association is seen with two organelle systems. This process, which will

hereafter be referred to as PVM-organelle association, was noted in the earliest electron microscopic studies of *Toxoplasma*-infected cells (Jones and Hirsch, 1972). Surprisingly, despite the novel morphological nature of this interaction, little has been done to elucidate its function in parasite biology.

We hypothesize that the primary role of PVM-organelle association in the pathogenesis of *T. gondii* infections is in nutrient acquisition. Given the absence of a biological precedent for such an interaction, with this report we establish a morphological and biochemical foundation toward elucidating the molecular and physiological role of PVM-organelle association in the interactions between *T. gondii* and the host cell.

MATERIALS AND METHODS

Reagents

Phosphate-buffered saline (PBS) was made up to 137 mM NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4 and 1.8 mM KH_2PO_4, adjusted to pH 7.4. PBS^{2+} additionally contains 1 mM each of $CaCl_2$ and $MgCl_2$. Cell disruption buffer (CDB) contained 0.25 M sucrose, 50 mM Tris-Cl, pH 7.5 (American Bioanalytical), 25 mM KCl and 10 mM $MgCl_2$ with protease inhibitors (1 μM aprotinin, 5 μM antipain, 5 μM leupeptin (Boehringer Mannheim), 5 μM pepstatin A and 1 mM PMSF). Nitrocellulose filters used in immunoblot analysis were blocked using PET-M (PBS with 5 mM EDTA, 0.2% TX-100 and 5% dry milk powder). Immunoblot washes were performed using PET (PBS with 5 mM EDTA and 0.2% TX-100). Unless specifically indicated, reagents were purchased from Sigma (St Louis).

Parasite and cell culture

The RH strain of *Toxoplasma gondii* was maintained by serial intraperitoneal passage in female Swiss Webster mice as previously described (Beckers et al., 1994). *Leishmania amazonensis* amastigotes were obtained from the laboratory of Dr Dianne McMahon-Pratt (Yale University). *Coxiella burnetti* was grown in a BL3 facility (Rockefeller University, Dr Michel Rabinovitch) by serial passage in primary human foreskin fibroblasts.

Chinese hamster ovary (CHO) cells were obtained from ATCC (CCL 163) and were cultured in α-MEM supplemented with 3.5% fetal bovine serum (FBS), 2 mM L-glutamine and penicillin/streptomycin (100 units/ml per 100 μg/ml) at 37°C in 5% CO_2 atmosphere. Bone marrow macrophages were obtained from the aseptic aspiration of femoral bone marrow from Swiss Webster mice. Bone marrow aspirates were plated in RPMI with 10% FBS, 2 mM L-glutamine, penicillin/streptomycin (100 units/ml per 100 μg/ml) and stimulated with GM-CSF-containing supernatant from the overproducing LABNAC cell line at 20% concentration (Roberson and Walker, 1988). Human foreskin fibroblasts (HFF) were obtained from the Yale University Dermatology core facility at passage #3. All HFF cells used were passaged fewer than 10 times. HFF cells were maintained in Hepes-buffered α-MEM supplemented with 10% FBS, glutamine and penicillin/streptomycin (100 units/ml per 100 μg/ml). All tissue culture media were prepared at the Media Core Facility of the Department of Cell Biology, Yale University.

Antibodies

Antibodies used were rabbit anti-calnexin (diluted 1:1,500 in PET-M for immunoblot analysis) (antibody provided by Dr Ari Helenius, Yale University), rabbit polyclonal anti-GRA3 (Bermudes et al., 1994a), mouse monoclonal antibodies T6 2H11 against GRA3 (diluted 1:500 in PET-M for immunoblot analysis) (Leriche and Dubremetz, 1991) and T3 4A7 against ROP2,3,4 (diluted 1:500 in PET-M for immunoblot analysis) (Sadak et al., 1988).

Light and electron microscopy

Light and epifluorescence microscopy was performed on infected cells grown on sterile 12 mm coverslips in 24-well culture dishes (Falcon). All images were recorded on Kodak Elite 400 ASA slide film using a Nikon Microphot microscope. Cibachrome prints of images were scanned (Hewlett Packard, Scanjet IIcx) into Adobe Photoshop software and printed using a dye sublimation printer. PVM-associated mitochondria in infected cells were detected using the fixable membrane potential-sensitive dye MitoTracker (Molecular Probes). Infected cells were incubated in culture medium containing 0.5 μg/ml MitoTracker for 30 minutes, washed and chased in dye-free medium for an additional 15 minutes. Following three washes with PBS^{2+} monolayers were fixed with 3% paraformaldehyde in PBS^{2+}. MitoTracker accumulation was detected by fluorescence microscopy, by excitation using the rhodamine channel.

A standard protocol for the preparation of samples for electron microscopy was used unless specifically indicated. Infected cell monolayers were washed 3× with PBS and fixed for 3 minutes in 3% glutaradehyde (GA)/100 mM sodium cacodylate buffer, pH 7.2/3% sucrose. The monolayers were gently scraped with a bevel-edged teflon cell lifter and transferred to a microcentrifuge tube and pelleted in fixative. Fixation was continued for at least 1 hour on ice. Pellets were cut into approximately 1 mm^3 blocks and transferred into glass vials for further processing. Following three washes (5 minutes/wash) in 100 mM sodium cacodylate buffer, the blocks were osmicated on ice for 1 hour with 2% OsO_4 (EMS) in 100 mM cacodylate buffer. Following three washes in water, the blocks were treated in 50 mM sodium malate, pH 5.2 buffer (3×5 minutes/wash). Blocks were stained *en bloc* in 1% uranyl acetate (EMS) in 50 mM sodium malate buffer, pH 5.2, for 1 hour at room temperature. Following washes in water, the blocks were dehydrated in a series of graded ethanol (70%-100%) (Baker) and propylene oxide (EMS). The blocks were infiltrated in an Epon:propylene oxide 1:1 mix overnight, embedded in Epon and cured at 60°C overnight. The blocks were trimmed and sectioned using a ultramicrotome (Reichert/AO)with a diamond knife (Edgecraft). Ultrathin 60-80 nm sections were collected on grids contrasted with lead citrate and uranyl acetate and viewed on a JEOL C-100 electron microscope.

Morphometric analysis of PVM-organelle association

Thin sections were cut from epoxy resin-embedded, identically prepared pellets of *T. gondii* infected CHO cells, which had been treated as described below. These sections were randomly photographed and printed at known final magnifications. Estimates of the ratio of PVM surface area to the volume of PV (surface density) (S_v-PVM) was obtained using cross lattice overlays, as described (Weibel et al., 1969; Weibel, 1979) and reviewed by Gundersen et al. (1988). The surface densities of lengths of endoplasmic reticulum (S_v ER) and mitochondrial membranes (S_v M) associated with the PVM were similarly obtained. The distance between the PVM and associated organelle membranes was performed by direct measurement of images of known final magnification. All sampling, from the original material to the sectioning and photomicrography, followed sequential random sampling protocols (Meyhew and Gundersen, 1996).

Loading of macrophages with BSA-gold

15 nM BSA-gold was prepared as previously described (Slot and Geuze, 1985). BSA-gold was adjusted to an OD_{520} of 4 in bone marrow macrophage medium and added to macrophage monolayers for 30 minutes at 37°C in 5% CO_2. The monolayer was washed three times with PBS and the label chased in the presence of culture medium for 2 hours at 37°C in 5% CO_2. Under these conditions all the label is chased into lysosomes (Adams et al., 1982).

Parasites freshly isolated from a mouse peritoneum were used to infect the BSA-gold-loaded macrophages. A significant proportion of mouse-derived parasites are non-specifically opsonized, as determined by labeling with an FITC-conjugated anti-mouse secondary

antibody (data not shown). The cells were infected for 6 hours and processed for electron microscopy as described above.

Treatment of infected monolayers with pyrimethamine and nocodazole

Pyrimethamine

CHO cell monolayers were infected with parasites in the presence of microbicidal levels (10 μM) of pyrimethamine. Following infection for 20 hours, the monolayer was prepared for electron microscopy as described above.

Nocodazole

The role of host microtubules in the establishment and maintenance of PVM-organelle association was examined using nocodazole. This drug depolymerized host microtubules, as confirmed by immunofluorescence staining using a monoclonal anti-tubulin antibody (Amersham) (data not shown). Nocodazole treatment for 4 hours had no visible effect on the integrity of the CHO cell monolayer (data not shown).

To test the effects of nocodazole on establishment of PVM-organelle association, confluent CHO cell monolayers were infected in the presence or absence of nocodazole (10 μg/ml) for 4 hours prior to fixation and processing for electron microscopy. To test the role of microtubules in the maintenance of PVM-organelle association, monolayers infected for 16 hours were treated for an additional 4 hours with nocodazole (10 μg/ml). Control and treated samples were prepared for electron microscopy as described above. In all instances an identical volume of the carrier (ethanol for pyrimethamine, DMSO for nocodazole) was added to control infected monolayers.

Enrichment of PVM-organelle complexes by gradient centrifugation

A subcellular fractionation scheme was developed to enrich PVM-organelle complexes (Fig. 1). Six confluent CHO cell monolayers in 10 cm dishes were each infected for 20 hours with 2×10^7 parasites freshly isolated from a mouse peritoneum. An additional six dishes were left uninfected. Cells from both groups were washed thrice in PBS and collected by gentle scraping in 10 ml of PBS and pelleted at 1,000 ***g*** for 10 minutes at 4°C. Cell pellets were resuspended in 3 ml of CDB and the CHO cells were selectively disrupted in a ball-bearing homogenizer with a 0.0007 inch clearance. Under these conditions parasites are not disrupted (Ossorio et al., 1994). The lysate was centrifuged at 1,000 ***g*** for 10 minutes to remove parasites and cell debris (P1). The resultant supernatant, containing the organelle fraction (including PVM-organelle complexes in infected cells), was pelleted at 13,000 ***g*** (P13) using a SW41 Ti rotor, yielding an 'organelle pellet' containing both mitochondria and sheets of ER. Alternatively the supernatant was homogenized by a single passage in a French pressure cell (Amico) with a pressure of 10,000 psi. Under these conditions cellular organelles are converted into microsomal vesicles (data not shown). The French pressure homogenate was pelleted at 100,000 ***g*** (P100) to yield an organelle membrane pellet for use in sucrose equilibrium flotation gradients. This pellet in uninfected cells contains host organelle membranes and both host organelles membranes and the PVM in material from infected cells.

Material from these pellets was homogenized in the SW41 Ti centrifuge tube and incorporated into 750 μl of 60% sucrose in 10 mM Tris-Cl, pH 7.5 (w/w), using the pestle of a 1 ml Dounce homogenizer. Incorporated membranes were mixed in with an additional 750 μl of 60% sucrose. A continious gradient was established by overlaying 1.5 ml each of stock sucrose solutions in 10 mM Tris-Cl, pH 7.5 (w/w) of decreasing concentrations at 5% intervals from 55% to 25%. Sucrose equilibrium flotation gradients were centrifuged for 16-18 hours at 100,000 ***g*** in an SW41 Ti rotor (Beckman), establishing a linear gradient as determined by refractometry. Fractions were collected from the bottom-up by inserting a capillary to the bottom of the centrifuge tube and using a peristaltic pump (Pharmacia Frac 100) at 1 minute intervals calibrated with water to a flow rate of 1 ml per minute. 12 fractions of approximately 0.9 ml each and a 13th fraction of about 1.4 ml were routinely collected. 100 μl volumes of each fraction were precipitated using the chloroform-methanol precipitation technique of Wessel and Flugge (1984). Protein pellets were solubilized by boiling in SDS-PAGE sample buffer and subjected to electrophoresis and immunoblot analysis as described below.

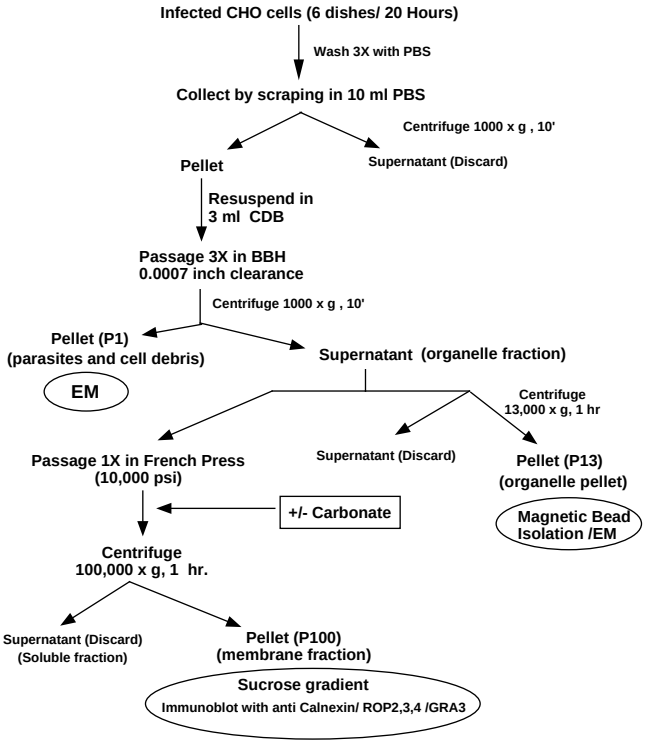

Fig. 1. Scheme for subcellular fractionation and enrichment of PVM-organelle complexes. Experimental approaches using particular fractions are circled. The identical fractionation scheme was used in parallel with uninfected cell controls. BBH, ball bearing homogenizer.

Effect of sodium carbonate on PVM-organelle complexes

The effect of 0.1 M sodium carbonate, pH 11, on the fractionation of organelle and PVM markers was assessed by sucrose equilibrium flotation gradients. Microsomal pellets (P100) from infected cells, prepared as described above, were resuspended in 3 ml of CDB and either an equal volume of CDB or 0.2 M Na_2CO_3. Following a 30 minute incubation on ice the membranes were re-pelleted at 100,000 ***g*** for 1 hour in a SW41 Ti rotor, and processed as decribed above for subcellular fractionation.

Magnetic bead isolation of PVM-organelle complexes

2×10^8 magnetic beads (Dynabead M-500 Subcellular) (Dynal) were crosslinked to affinity-purified goat anti-rabbit Fc antibodies (Pierce) according to the manufacturers instructions (Dynal). The beads were then coated with a rabbit anti-GRA3 antiserum at a final dilution of 1:15 for 2 hours on a rotating wheel at 4°C. Unbound antibody was removed by three washes in PBS with 5% BSA. The beads were collected with the magnet (Dynal) between washes.

PVM-organelle complexes pelleted as above at 13,000 ***g*** were resuspended in PBS with 5% FBS, 2 mM EDTA and protease inhibitors and following gentle douncing were incubated with GRA3-coated magnetic beads (2×10^7). The beads were incubated with slow rotation at 4°C for 16 hours. Following collection with the magnet, the beads were gently washed five times with PBS supplemented with

5% FBS, being collected with the magnet following each wash. Immuno-labeling of membranes captured by the beads was done prior to embedding as below. Bead/membrane complexes were fixed for 15 minutes with 4% formaldehyde in 50 mM phosphate buffer, pH 7.4. The beads were collected and washed five times in PBS + 5% FBS, before blocking with 15% glycine for 20 minutes. Following washes, the beads were resuspended in 400 µl PBS supplemented with 5% FBS to which 20 µl polyclonal rabbit anti GRA3 antiserum was added and allowed to incubate at room temperature with slow rotation for 45 minutes. Following bead collection with the magnet and five washes as above, the beads were incubated with Protein A-10 nM gold (Department of Cell Biology, University of Utrecht, The Netherlands) at a 1/30 dilution for 45 minutes. The samples were washed as above. Washed beads were resuspended in a 35 µl of 10% gelatin and incubated on ice for 5 minutes to gel. Gelatin-embedded beads were fixed in situ in 3% GA/0.1 M sodium cacodylate/3% sucrose for 15 minutes and prepared for electron microscopy as above, using the standard protocol with two modifications. Osmication and staining with uranyl acetate were performed for 15 minutes each.

SDS-PAGE and immunobot analysis

SDS-PAGE electrophoresis using 12% discontinuous gels was performed essentially as described by Laemmli (1970), using the Bio-Rad Mini-Protean gel electrophoresis systems. Immunoblot analysis was performed by electroblotting SDS-PAGE-resolved proteins onto nitrocellulose (Gelman) using the Bio-Rad Mini-Protean system. Nitrocellulose filters were blocked with PET-M and treated with primary antibodies diluted in PET-M, as described above. Following washes in PET, filters were incubated with species-specific HRP-conjugated secondary antisera as recommended by the manufacturer (Bio-Rad). The filters were washed with PET and the HRP signal detected using the ECL chemiluminescence system (Amersham) exposed on Kodak X-AR film. Sequential probing of immunoblots was conducted after inactivation of HRP with sodium azide (0.1% for 10 minutes). Densitometric analysis of immunoblots was achieved by scanning the gels using a Hewlett Packard flat-bed scanner into NIH-Image software run on a Power Macintosh computer.

RESULTS

PVM-organelle association, a basic description

A measuring system was developed for PVM-organelle association. This was done in order to permit a quantitative assessment of the kinetics and extent of the process in different host cell types and lines, and following different perturbations. Characteristics of the interaction in CHO cells are provided in detail.

The association of the PVM with organelle membranes was observed as an extremely intimate 'finger-glove' interaction (Fig. 2), with extensive membrane continuities between the PVM and organelle membranes (Fig. 2). Typically, contact between the outer mitochondrial membrane and the PVM was continuous over the entire length of the mitochondrial profile (Fig. 2A,C). The mean distance between the PVM and the mitochondrial outer membrane was 12.04 nm, with a standard deviation of 3.05 nm (n=41) (see Materials and Methods). Likewise, the entire membrane face of an ER profile was generally observed in association with the PVM (Fig. 2A,B). Unlike PVM-mitochondrial association, PVM-associated ER displayed stretches of membrane continuity, interspersed with stretches of discontinuity. This is reflected in the mean distance between the PVM and ER being 18.26 nm with a standard deviation of 9.24 nm (n=39) (Materials and Methods). As noted previously (Jones and Hirsh, 1972; De Melo et al., 1992), ribosomes were restricted to the membrane face of the ER, which was not involved in the association (Fig. 2A,B).

Morphometric quantitation of PVM-organelle association

PVM-organelle association was then examined in a variety of cell types and lines. Organelle association was observed in primary cells, including human foreskin fibroblasts, murine bone marrow derived macrophages (see below) and rat hepatocytes (data not shown), as well as in cultured cells, including CHO (Fig. 2), VERO and NIH 3T3-Balb/c fibroblasts (data not shown).

The extent of PVM-mitochondria and PVM-ER association was estimated for cells infected for 4 and 20 hours using standard morphometric techniques (see Materials and Methods). The extent of association is represented as the percentage of the PVM surface area associated with either mitochondria or ER, where the associated organelle is in direct contact with the PVM, using the criteria described above. The measurement of the surface to volume (Sv) ratio of PVM to the PV volume generates a parameter that is independent of the reference space (Gundersen et al., 1988; Weibel, 1979). This has the advantage of allowing direct comparison of the extent of organelle association, despite having PVs of different volumes, thereby permitting comparisons between both different time points and in response to treatments (see below).

For a 4 hour infection almost 75% of the PVM surface area was associated with organelles (Table 1), with mitochondria

Table 1. Morphometric analysis of PVM-organelle association

Sample treatment	Sample size (n)	SvPVM mean (s.e.)	SvM mean (s.e.)	SvER mean (s.e.)	Percentage PVM-mitochondrial association	Percentage PVM-ER association
Untreated, 4 hours	31	3.20 (0.16)	0.58 (0.07)	1.78 (0.22)	18	56
Nocodazole, 4 hours	31	3.78 (0.17)	0.33 (0.08)	1.79 (0.15)	9	47
Untreated, 20 hours	30	2.70 (0.19)	0.61 (0.06)	0.80 (0.13)	23	30
Nocodazole, 20 hours	30	3.22 (0.19)	0.81 (0.09)	1.21 (0.20)	25	38
Pyrimethamine, 20 hours	30	2.96 (0.14)	0.79 (0.09)	1.00 (0.14)	27	34

The extent of PVM-organelle association was measured at 4 hours and 20 hours post-infection.

Treatments included nocodazole (4 hours or 20 hours; see text and Fig. 6 for details) and pyrimethamine (20 hours). See text for details.

The specific parameters measured are the surface to volume ratios (Sv) of the PVM (SvPVM), PVM-associated mitochondria (SvM) and PVM-associated endoplasmic reticulum (SvER), all relative to the volume of the PV (see Materials and Methods). Mean surface densities and the standard error (s.e.) for the sample size measured are presented. The extent of PVM-mitochondrial and PVM-ER association is represented as a percentage of their surface densities (SvM and SvER) relative to Sv PVM. Values for mean surface densities and percentage association were rounded off to the level of significance indicated.

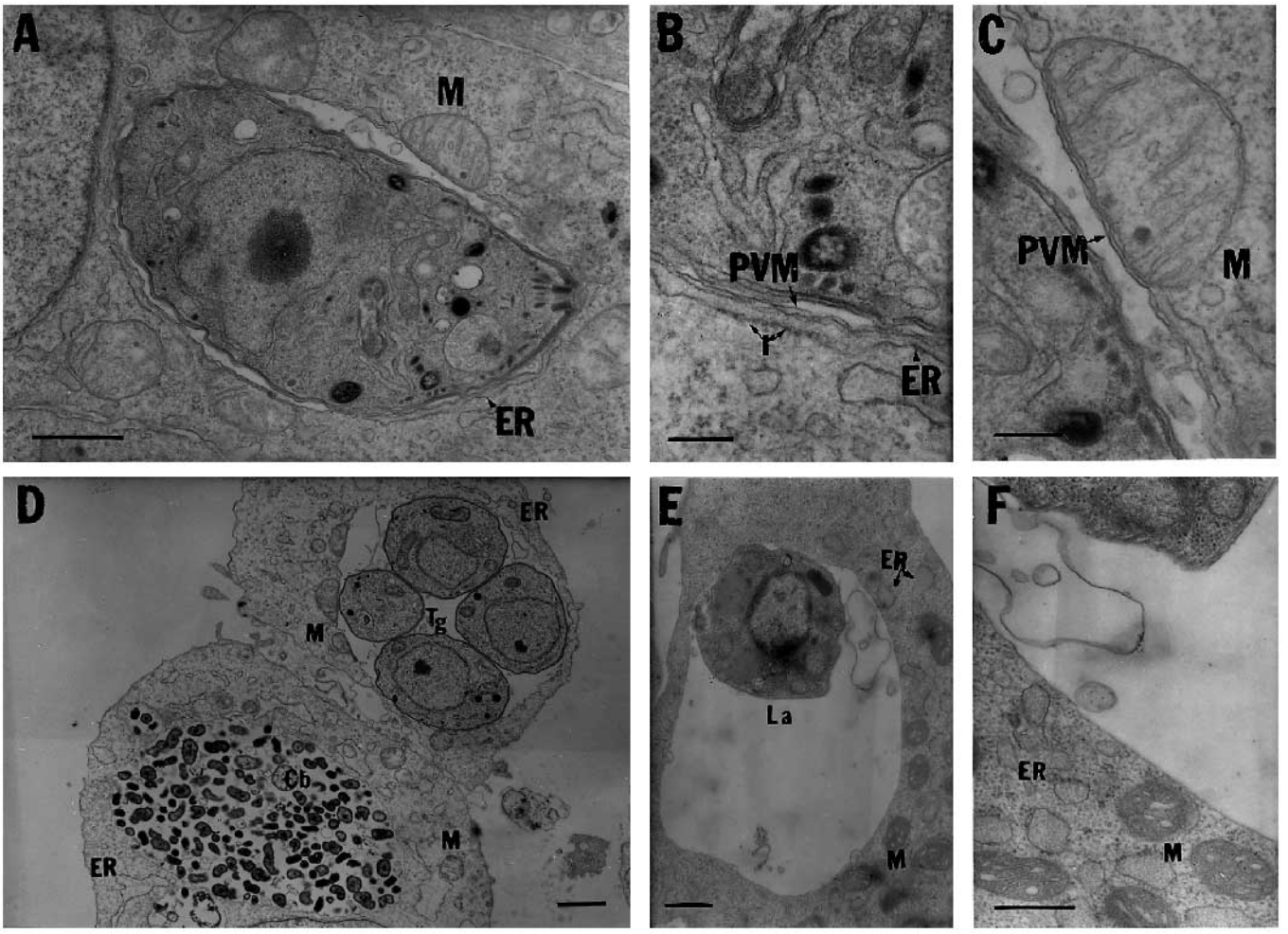

Fig. 2. Association of host endoplasmic reticulum and mitochondria with the *T. gondii* PVM is not a consequence of steric constraints within an infected cell. (A) CHO cell infected with *T. gondii* for 4 hours exhibiting association of host endoplasmic reticulum (ER) and mitochondria (M) with the PVM. (B) Higher magnification showing PVM-ER association. Ribosomes (r) are restricted to the face of the ER not involved in the association. (C) Higher magnification showing PVM-mitochondrial association. (D) Human foreskin fibroblasts infected with *C. burnetti* (Cb) for 48 hours prior to superinfection with *T. gondii* (Tg) for an additional 24 hours. Association of organelles is observed only with the *T. gondii* PVM. (E) The PVM *of L. amazonensis* (La) does not form tight associations with host mitochondria (M) and ER. (F) Higher magnification showing that membrane continuities between the *L. amazonensis* PVM and organelles are not observed despite their proximity. Bars: 2 μm (D); 1 μm (A and E); 0.5 μm (B,C,F).

and ER being associated with 18% and 56% of the PVM surface area, respectively (Table 1). At this time point, more than 85% of parasite vacuoles contained a single parasite (data not shown).

At 20 hours post infection, the majority of PVs contained 4 parasites (data not shown). The extent of PVM-mitochondrial association was unchanged or slightly more (Table 1), whereas the extent of PVM-ER association was roughly halved (Table 1). As a consequence, roughly half of the PVM surface was associated with organelles at this time point.

PVM-organelle association is not a consequence of steric constraints

Mitochondria and ER are the most abundant organelles in mammalian cells (Alberts et al., 1994), particularly in the perinuclear region where the *Toxoplasma* vacuoles develop (De Melo et al., 1992). To explore the possibility that organelle association was the result of the steric constraints imposed by the growing vacuole within the host cell, we examined whether vacuoles containing other pathogens exhibited this phenotype. Both *Leishmania amazonensis* and *Coxiella burnetti* grow within spacious vacuoles, which readily fuse with organelles of the endocytic cascade (Veras et al., 1994). Despite the presence of vacuoles considerably larger than those of *T. gondii*, host organelles were never observed to form closely apposed interactions with these pathogen vacuoles (Fig. 2D,E,F). While organelles were occasionally observed very close to the PVM, direct PVM-organelle continuities were never observed (Fig. 2F). Instead a minimum distance of approximately 30-50 nm, without any direct membrane contact between the PVM and organelles, was maintained. This is consistent with the organelle exclusion zone generated by repulsive interactions between negatively charged membranes and the associated hydration sphere. These observations are the first of several to suggest that specific components within the *T. gondii* PVM overcome these normally repulsive forces.

PVM-organelle association requires active parasite invasion

The route of *T. gondii* cell entry determines the fate of the parasite following internalization. As described above, active parasite entry results in the formation of a fusion-incompetent parasitophorous vacuole lacking membrane proteins from the host cell, and permissive for parasite replication (Joiner et al., 1990; Morisaki et al., 1995). If instead the parasite is internalized by phagocytosis, the vacuole (phagosome) membrane now contains phagocytic receptors and other plasma membrane proteins from the host cell, and readily fuses with lysosomes, resulting in parasite death (Jones and Hirsch, 1972; Joiner et al., 1990; Morisaki et al., 1995).

We tested whether PVM-organelle association was dependent on active invasion. Mouse bone marrow-derived macrophages loaded with BSA-gold as a marker for lysosomes (see Materials and Methods) were infected with parasites freshly isolated from the mouse peritoneal cavity. Under these conditions a significant proportion of the parasites are coated with murine IgG (data not shown), resulting in binding to and phagocytosis through Fc receptors in the bone marrow-derived macrophages (Joiner et al., 1990; Morisaki et al., 1995).

Often within the same macrophage, vacuoles fused and not fused with lysosomes were present concomitantly (data not shown). Only for those vacuoles not fused with lysosomes was organelle association observed (Fig. 3A). PVM-organelle association was not observed in vacuoles that fused with lysosomes, as assessed by delivery of BSA-gold into the vacuole lumen (Fig. 3B). In such vacuoles, parasites were killed (Jones and Hirsch, 1972; Sibley et al., 1985; Joiner et al., 1990). These results indicate that PVM-organelle association is dependent on active parasite invasion and the formation of a fusion-incompetent parasitophorous vacuole containing viable parasites. They do not distinguish, however, whether viable parasites are needed to maintain the association.

Killing the parasite after entry does not reverse organelle association

We therefore tested whether killing the parasite after active invasion reversed organelle association. Cells were infected and incubated in the presence of pyrimethamine at 10 μM for 20 hours. These conditions have been shown to be microbicidal (Silverman et al., 1997). Vacuoles with clearly dead and dying parasites continued to maintain associated organelles (Fig. 4A). This result indicates that neither a short-lived or diffusable secreted parasite component nor active parasite metabolism are necessary to maintain established organelle association. Furthermore, morphometric analysis revealed that pyrimethamine treatment has no effect on the extent of either PVM-mitochondrial or PVM-ER association (Fig. 4B, Table 1).

As has been previously reported, PVM-associated mitochondria retain their membrane potential and efficiently concentrate membrane potential-dependent fluorescent dyes including rhodamine 123 (Tanabe and Murakami, 1984) (data not shown) and MitoTracker (Fig. 5). Additionally, ablation of the mitochondrial membrane potential with NaN_3 or CCCP did not alter the extent of mitochondrial association (data not shown). The diffuse immunofluorescence staining pattern with the anti-calnexin antibody makes it imossible to assess PVM-ER staining at the light-microscopic level (data not shown).

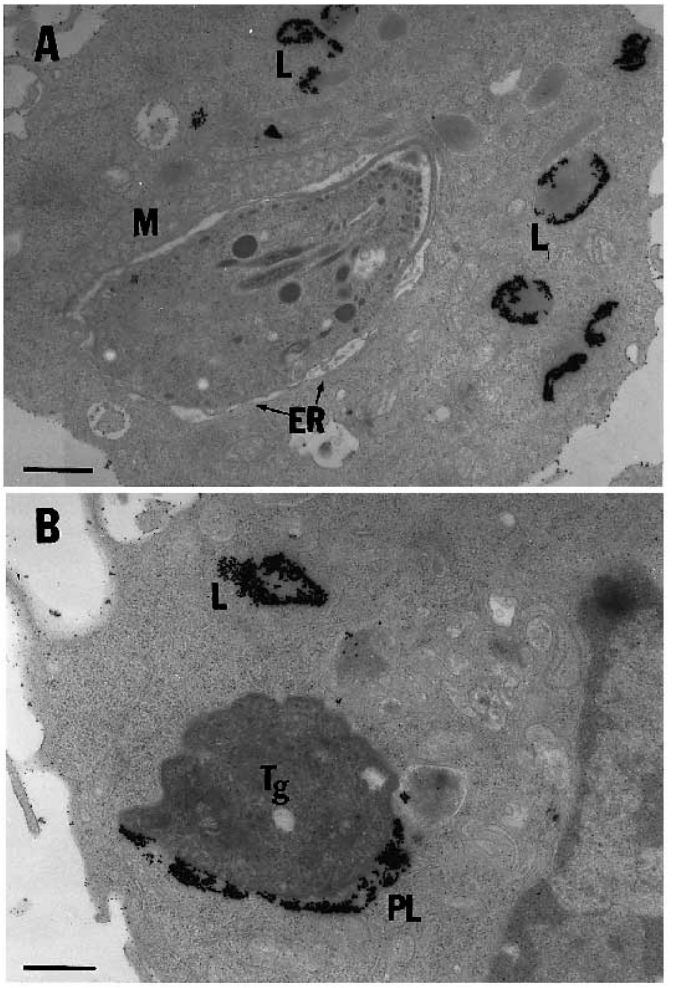

Fig. 3. Establishment of PVM-organelle association is dependent on active parasite invasion. (A) The establishment of a fusion-incompetent parasitophorous vacuole, preventing the delivery of the lysosomal (L) marker BSA-gold, is required for PVM-mitochondrial (M) and PVM-ER (ER) association. (B) Opsonized *T. gondii* (Tg) taken up by phagocytosis fuse with lysosomes (L) establishing a phagolysosome (PL). The phagolysosomal membrane does not associate with host organelles. Bars, 1 μm.

Role of host microtubules in PVM-organelle association

The distribution of both mitochondria and the endoplasmic reticulum is governed by the microtubule cytoskeleton (Kelly, 1990). We therefore examined the effect of nocodazole, a microtubule depolymerizing agent, on the establishment and maintenance of PVM-organelle association.

CHO cells pretreated with nocodazole were infected for 4 hours in the presence of the drug. Nocodazole treatment resulted in the dispersal of mitochondria from their normal perinuclear distribution (data not shown). This treatment did not affect the ability of vacuoles to establish in the perinuclear area (data not shown). The extent of PVM-ER association was essentially unaffected (Fig. 6A,B; Table 1), while roughly half as many mitochondria associated with the PVM (Fig. 6A,B; Table 1), suggesting a possible role for host microtubules in establishing PVM-mitochondrial association.

To determine if microtubules contribute to the maintenance

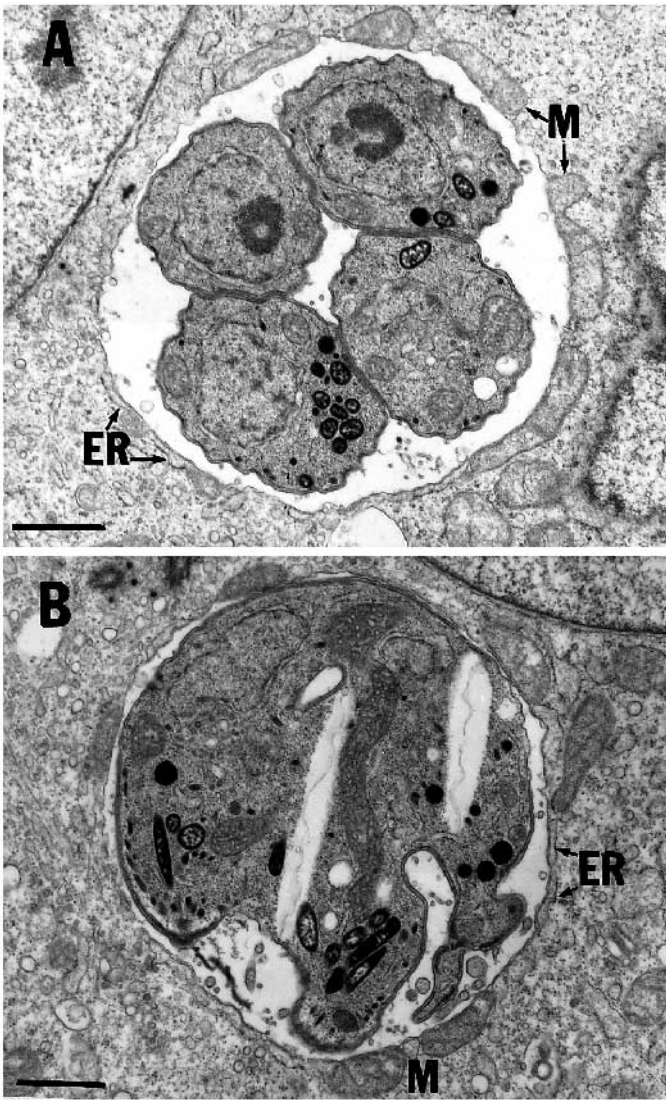

Fig. 4. Parasite viability is not required to maintain PVM-organelle association. Control (A) and pyrimethamine-treated (B) CHO cells infected with *T. gondii* for 20 hours. Pyrimethamine-treated *T. gondii* vacuoles continue to display PVM-organelle association despite the grossly deformed parasites they harbor. See Table 1 and text for quantitation. Bars, 1 μm.

of PVM-associated organelles, the extent of PVM-organelle association was quantitated in cells infected for 16 hours, followed by a 4 hour nocodazole treatment. There was no change in the extent of PVM-mitochondrial association, although a small increase in PVM-ER association was observed (Fig. 6C,D; Table 1).

PVM-organelle association is maintained following host cell lysis

The above results are consistent with the presence of a stable inter-molecular interaction between the PVM and associated organelles. To pursue this possibility, we examined whether the association was maintained following mechanical disruption of host cells using a ball-bearing homogenizer. Under these conditions selective host cell lysis is achieved (Ossorio et al., 1994), allowing collection in a low speed pellet (P1, see Fig. 1) of intact parasites with their partially associated PVM. Both mitochondria and ER were associated with PVM in these fractions (Fig. 7A), suggesting that a soluble host factor is not required to maintain organelle association.

The stability of the interaction was exploited to immuno-isolate PVM-organelle complexes. Magnetic beads were coated with an antibody to the luminal PVM marker GRA3 (Achbarou et al., 1991), and membranes from disrupted cells were affinity purified as described in Materials and Methods. Complexes containing both GRA3 and associated organelles were immuno-isolated from infected cells (Fig. 7B); co-precipitating mitochondria and ER are identifiable by the characteristic double membrane structure (Fig. 7B) or the presence of ribosomes (Fig. 7B). The presence of the PVM on magnetic bead-isolated organelle complexes was confirmed by immunogold labeling (gold beads) with an anti-GRA3 antibody (Fig. 7B) (see Materials and Methods). No membranes were isolated from organelle fractions of uninfected cells using the anti-GRA3 antibody(data not shown). This result further emphasizes the stable nature of the interaction, and indicates the lack of dependence on viable/metabolizing parasites or host cells to maintain the association.

PVM markers co-fractionate with the ER marker calnexin in sucrose density gradients

Given the stability of the interaction, experiments were initiated to purify PVM-organelle complexes by sucrose equlibrium flotation gradient centrifugation. The PVM markers

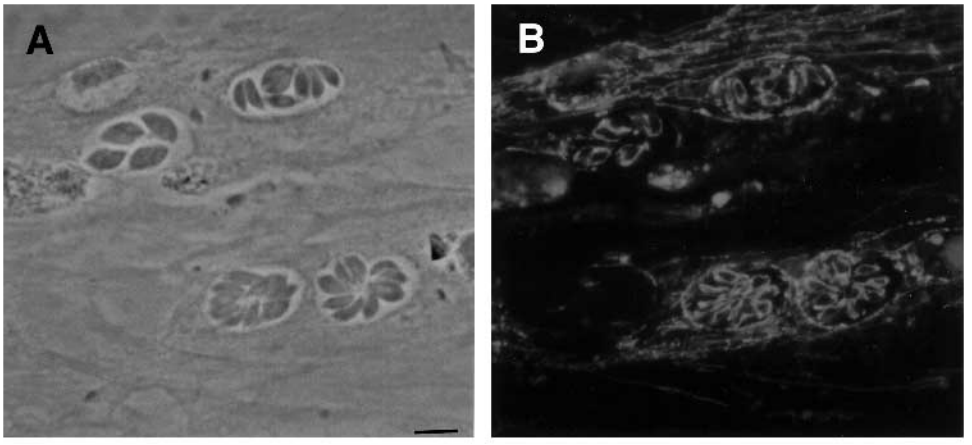

Fig. 5. PVM-associated mitochondria retain their membrane potential. Phase-contrast micrograph of HFF cells infected with *T. gondii* for 20 hours (A) and labeled with the potential sensitive dye MitoTracker (B). PVM-associated mitochondria accumulate the dye and follow the contour of the PV (A,B). MitoTracker accumulation is also observed in intracellular parasites (B). Bars, 5 μm.

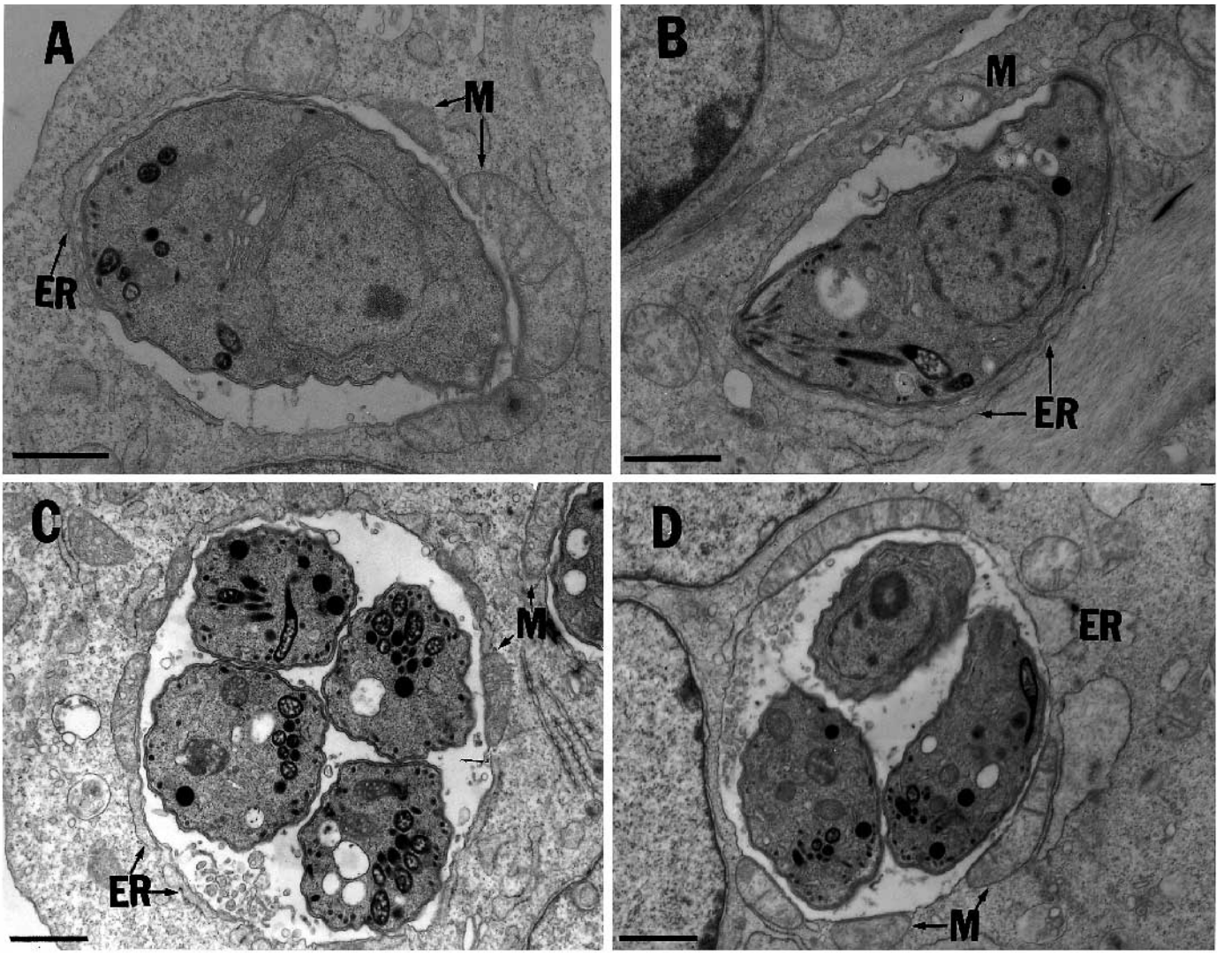

Fig. 6. Role of host microtubules in the establishment and maintenance of PVM-organelle association. CHO cells infected in the absence (A) or presence of nocodazole (B) for 4 hours. The PVM of nocodazole-treated parasites established both mitochondrial (M) and ER association (B). The frequency of mitochondrial association is reduced in nocodazole-treated cells (B) compared to the control (A). See Table 1 and text for quantitation. CHO cells infected for 20 hours (C) or for 16 hours followed by 4 hours in the presence of nocodazole (D). Microtubule disruption by nocodazole does not affect the maintenance of PVM-organelle association (D). See Table 1 and text for quantitation. Bars, 1 μm.

ROP2 (as well as ROP3 and ROP4) and GRA3, localizing to the cytoplasmic (Beckers et al., 1994) and luminal face (Achbarou et al., 1991) of the PVM respectively, co-fractionated with the host cell ER marker calnexin (Bergeron et al., 1994) (Fig. 8C,D). GRA3 is an ideal marker for the PVM in these experiments as it is a soluble protein within the parasite, associating with the PVM and network only following secretion into the PV (Ossorio et al., 1994). Only membrane-associated GRA3 floats up in these gradients. In addition, the distribution of calnexin in membranes from infected cells (Fig. 8C,D) was altered (peak fractions 7 and 8) compared to uninfected cells (Fig. 8A,B, peak fractions 8, 9). The change in distribution of calnexin in membranes from infected cells was consistently observed using both equilibrium flotation gradients (sample loaded at bottom of gradient) and equilibrium velocity gradients (sample loaded at top of gradient) (data not shown). Of importance, densitometric analysis reveals that only 4-8% of the calnexin signal in the peak is altered in its density (Fig. 8A,B,C,D) (data not shown), consistent with the notion that only a small subset of host ER is associated with the PVM.

GRA3 and ROP2,3,4 co-fractionate with the ER marker calnexin following treatment with sodium carbonate

Treatment of microsomes with 0.1 M Na_2CO_3, pH 11 causes the conversion of ER vesicles into membrane sheets, and the removal of peripheral membrane proteins (Fujuki et al., 1982). Treatment of the membrane fractions of infected cells with 0.1 M Na_2CO_3, pH 11, followed by sucrose equilibrium flotation gradient fractionation resulted in the shift of the calnexin signal to a lighter density (Fig. 8E,F, peak fractions 9-12) relative to untreated infected (Fig. 8C,D, peak fractions 7-8) and uninfected (Fig. 8A,B, peak fractions 8-9) cells. This change in the

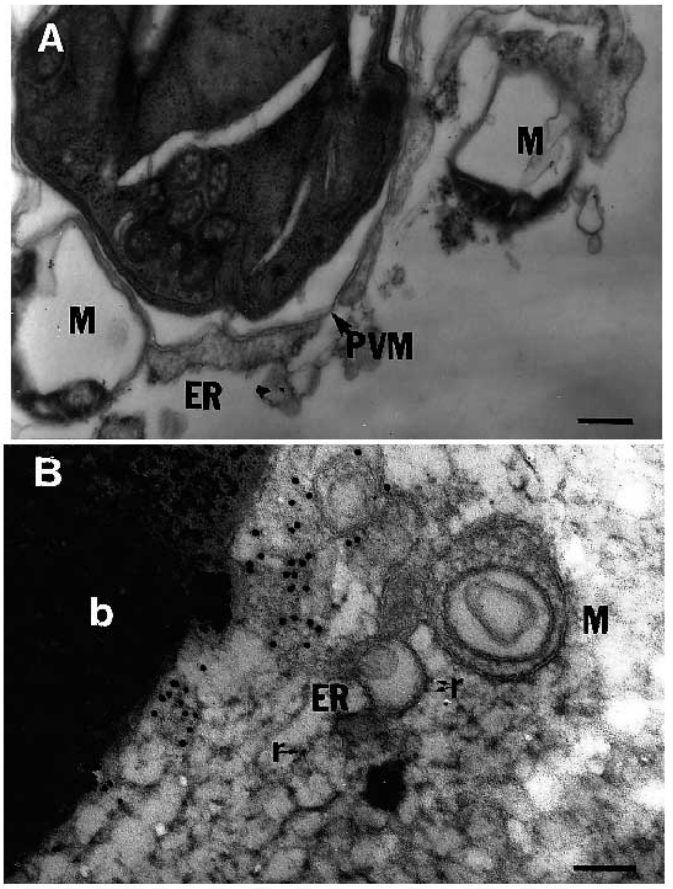

Fig. 7. PVM-organelle association is maintained following selective host cell disruption. (A) Association of mitochondria (M) and ER with the PVM following selective disruption of host cells. In this case the parasite pellet (P1, see Fig. 1) was examined, to allow unambiguous identification of the PVM surrounding intact parasites. (B) Immunoisolation of PVM-organelle complexes using magnetic beads (b) coated with anti-GRA3 antiserum. Isolated PVM, detected by immunogold staining with anti-GRA3 antiserum, forms a 'bridge' between the magnetic bead (b) and co-isolated organelles. The PVM is positively identified by immuno-gold labeling (10 nm gold beads) (see Materials and Methods). Mitochondria (M) are identified on the basis of their characteristic double membrane structure. ER is identified by the presence of ribosomes (r) on the membrane surface. Bars, 0.1 μm.

fractionation pattern was essentially mirrored by the PVM markers ROP2,3,4 and GRA3 (Fig. 8E,F), suggesting a stable interaction between these membranes. The cofractionation of the PVM markers with calnexin following carbonate treatment was also observed using sucrose equilibrium velocity gradients (data not shown).

DISCUSSION

Association of host cell organelles with the *T. gondii* PVM is an unprecedented biological process. As we show for the first time here, organelle association depends upon active entry of the parasite into host cells, and little else. Once established, viable parasites are no longer required to maintain the association. These features are highly reminiscent of the biogenesis and maintenance of a fusion-incompetent vacuole by the parasite, features ascribed to the unique characteristics of the PVM formed on invasion. Parenthetically, this process, which completely precludes PVM association with endocytic organelles, simultaneously promotes tight binding of mitochondria and ER. To achieve such an interaction, the PVM and organelle membranes must overcome both spatial and electrostatic constraints, suggesting the involvement of specific intermolecular interactions.

With nearly 75% of the PVM associated with organelles (Table 1) early after infection, these interactions may play a role in blocking PV fusion with endocytic organelles. We hypothesize, however, that the primary function for associated mitochondria and ER is to provide essential nutrients to the parasite. Although *T. gondii* viability is not dependent upon host-cell mitochondrial ATP production in tissue culture cells (Schwartzman and Pfefferkorn, 1982), the extent of ATP production by glycolysis in such cells precludes any conclusions regarding general parasite requirements for host cell ATP. It is likely that host cell mitochondria and endoplasmic reticulum provide lipids and products of intermediary metabolism to the intracellular parasite, but again no data exists in these areas. Defining the morphological and biochemical basis for organelle association is the first step in dissecting this fascinating and unprecedented process.

The percentage of the PVM surface occupied by mitochondria did not change between 4 and 20 hours (Table 1). At least two mechanisms can explain this observation. First, the rate at which mitochondria are added to the PVM matches the increase in the surface area of the PVM. Alternatively, attached mitochondria distend and elongate as the PVM expands, analogous to the increase in the size of an image drawn on the surface of an inflating balloon. Our own observations (see Fig. 4A, 5B, 7C, data not shown), and a report in the literature (Lindsay et al., 1993) indicate that PVM-associated mitochondria are deformed and stretched along the PVM. This scenario may be extended to suggest that PVM-mitochondrial association is established primarily in the nascent PV and that additional mitochondria do not bind as the vacuole matures.

The percentage of the PVM surface occupied by ER decreases markedly as the vacuole enlarges. At 20 hours post-infection, the extent of PVM-ER association is roughly half that observed for a 4 hour infection (Table 1). This suggests either that the ER is not as deformable as mitochondria, or that the net addition of ER to the PVM is less than for mitochondria, or that the ER association is less stable than for mitochondria. Distinguishing between these possibilities is complicated by the reticular nature of the ER, which precludes simple enumeration of ER structures.

Once established, association of the PVM with organelles does not require viable parasites. Microbicidal levels of pyrimethamine, resulting in dead and dying intravacuolar parasites (Fig. 4B), had no significant effect on the extent of PVM-organelle association (Fig. 4A,B, Table 1). This observation indicates that continual modification of the PVM is not required for the maintenance of associated organelles. Additionally, it suggests that the parasite 'receptors', if established in the PVM early after invasion, are stable and not subject to significant turnover.

Nocodazole inhibited by 50% establishment, but not the maintenance, of mitochondrial association with the PVM. This

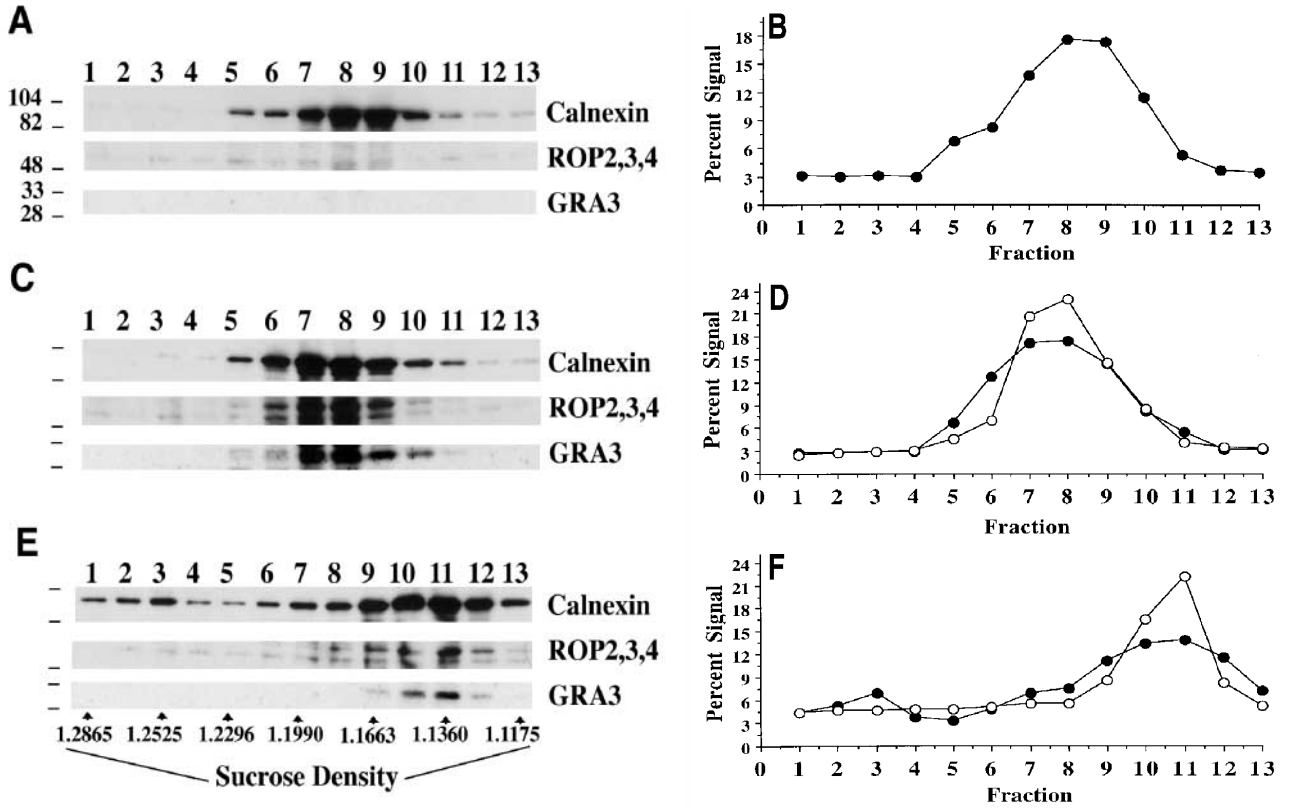

Fig. 8. Co-fractionation of PVM markers (ROP2,3,4 and GRA3) and calnexin in sucrose equlilibrium flotation gradients. Immunoblot detection of calnexin, ROP 2,3, 4 and GRA3 in fractions from sucrose equilibrium flotation gradients of membranes from P100 pellet (see Fig. 1). Membranes from uninfected cells (A,B), untreated infected cells (C,D) and carbonate-treated infected cells (E,F). Densitometric analysis of the distribution of calnexin (filled circles) and GRA3 (open circles) (B,D,F) for immunoblots (A,C,E).

result could reflect active participation of microtubules in 'delivering' mitochondria to the PVM. Alternatively, since nocodazole causes dispersion of mitochondria to the cell periphery (data not shown), with a reduced mitochondrial density in the perinuclear area occupied by the *T. gondii* vacuole, the effect of nocodazole could be more passive. In support of this latter possibility, at early time points after invasion, when the *T. gondii* vacuole is more peripheral in the cell, there is no apparent large-scale redistribution of mitochondria towards the vacuole (A. P. Sinai and K. A. Joiner, unpublished observations). This result indirectly suggests that mitochondrial association is not due to a chemotactic attraction of mitochondria towards the vacuole, but is more likely to occur as a consequence of the vacuole subsequently translocating to the perinuclear area of the cell (an event not inhibited by nocodazole).

The morphological data and subcellular fractionation experiments (Fig. 9) suggest a tight association between the PVM and organelles. PVM-associated organelles must overcome the repulsive forces inherent in the organelle exclusion zone, suggesting the involvement of specific inter-molecular interactions between the PVM and organelle membranes. Although these could be protein-protein, protein-lipid or lipid-lipid interactions, we favor the first of these mechanisms. The normal repulsive forces between organelles, contributed to by the hydration sphere around the membranes, generally maintain a distance of approximately 50 nm. The mean distance between the PVM and associated organelle membranes was 12 and 18 nm for mitochondria and ER, respectively, in *T. gondii*-infected cells. For purposes of comparison, the diameter of a ribosome is in the range of 30 nm, while most globular proteins range between 2 and 10 nm (Alberts et al., 1994). Hence, the proximity of organelles with the *T. gondii* PVM is compatible with a protein-protein interaction.

The prokaryotic intravacuolar pathogens *Legionella pneumophila* (Horwitz, 1983) and *Chlamydia psittaci* (Matsumoto et al., 1991) replicate in vacuoles that associate with host organelles (reviewed by Sinai and Joiner, 1997). Mitochondrial association with the *C. psittaci* vacuole morphologically resembles *T. gondii* PVM-organelle association (Paterson and de la Maza, 1981; Matsumoto et al., 1991), and is not dissociated following host cell disruption (Matsumoto, 1981). Matsumoto et al. found that the minimum distance separating the chlamydial vacuole and the mitochondrial outer membrane was 5.1 nm (Matsumoto, 1981; Matsumoto et al., 1991), although the average distance was not reported. The *C. psittaci* inclusion membrane initiates mitochondrial association coincident with the active replication of intravacuolar chalmydiae, suggesting a role in bacterial growth (Storz and Spears, 1978; Matsumoto et al., 1991). Studies with *L. pneumophila* mutants reveal a strong concordance between the extent of organelle association and the ability of intravacuaolar bacteria to grow (Berger and Isberg, 1993; Swanson and Isberg, 1995). The apparent link between organelle association and bacterial growth underscores the potential importance of these interactions (Sinai and Joiner, 1997).

The distribution of mitochondria within cells can be influenced by localized energy demands (Bereiter-Hahn, 1990). The *T. gondii* PV represents a potential ATP sink within the infected cell. The parasite secretes a potent nucleotide triphosphate hydrolase (NTPase) activity (Asai and O'Sullivan, 1983) into the lumen of the vacuolar space (Bermudes et al., 1994b; Sibley et al., 1994), which is capable of sequentially dephosphorylating nucleotide triphosphates (including ATP) to the monophosphate forms (Asai and O'Sullivan, 1983; Asai and Suzuki, 1990). Although the fraction of secreted enzyme which is enzymatically active is low (J. Silverman, A. Q. Qi, A. Riehl, C. Beckers, V. Nakkar and K. A. Joiner, manuscript in preparation), the vacuolar space may be a site of accelerated ATP degradation.

PVM-associated mitochondria are potentially physiologically distinct from other mitochondria within the cell. The studies of Tanabe and Murakami (1984), as well as our own observations (data not shown), indicate that PVM-associated mitochondria label more intensely with the membrane potential-sensitive dye rhodamine 123 than do other mitochondria in the cell. We have extended the studies of Tanabe and Murakami by the use of the fixable potential sensitive dye MitoTracker (Fig. 5). Interestingly, while rhodamine 123 fluorescence is not observed in intracellular parasites (Tanabe and Murakami, 1984) (data not shown), intense circumferential staining of intracellular parasites is observed using MitoTracker (Fig. 5). This staining pattern is distinct from that observed for extracellular parasites and suggests that the parasite plasma membrane or inner membrane complex may possess a charged membrane that could play a role in transport functions.

A common thread that ties the metabolic functions of mitochondria and ER in mammalian cells is their involvement in lipid metabolism (Trotter and Voelker, 1994). Significant membrane continuities between ER and mitochondria have been reported in several cell types from fungi to mammals, and may thus represent a normal cellular function (Franke and Kartenbeck, 1971; Moore et al., 1971; Montisano et al., 1982; Bereiter-Hahn, 1990). Interestingly, as observed for PVM-ER association, stretches of interaction between mitochondria and RER lack ribosomes at the interacting faces (Franke and Kartenbeck, 1971). Sites of membrane continuity between mitochondria and ER have been implicated in lipid trafficking (Trotter and Voelker, 1994; Vance and Shiao, 1996). This is most evident in the events surrounding the conversion of phosphatidylserine (PS) to phosphatidyethanolamine (PE) (reviewed by Trotter and Voelker, 1994; Vance and Shiao, 1996). Work by Vance and collegues has identified a unique membrane fraction that serves to directly link these organelles, facilitating the bulk transport of these lipids (Vance, 1990; Russinol et al., 1994). Such a pathway, linking two distinct cellular compartments by direct membrane contact (Trotter and Voelker, 1994; Vance and Shiao, 1996), may be used as a potential model for the proposed trafficking of lipids from the ER and mitochondria to the PVM at sites of associated organelles. Further lipid transport events, potentially along the the PV network, could deliver scavenged lipids to the growing parasite. The use of mutant cell lines deficient in these processes may provide insight into the functional basis of PVM-organelle association.

Preliminary experiments suggest that extracellular parasites, under conditions that permit protein synthesis, are deficient in their ability to synthesize de novo fatty acids from acetate (data not shown). Intracellular parasites, however, do accumulate de novo synthesized fatty acids, presumably synthesized from acetate in the host cell (data not shown). The proposed ability to transfer lipids directly from the host cell to the parasite at sites of PVM-organelle association would implicate appropriate transport activities in the PVM, in order to support ther needs of intravacuolar parasites.

The analysis of the functional contribution of PVM-organelle association to the biology of *T. gondii* will be greatly facilitated by the identification of the molecular basis for the interaction. In this report we establish the morphological and biochemical foundation of these highly unusual interactions, thereby defining an additional level of complexity in the interactions of a pathogen and host cell.

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