The Toxoplasma gondii Rhopty Protein ROP 2 Is Inserted into the Parasitophorous Vacuole Membrane, Surrounding the Intracellular Parasite, and Is Exposed to the Host Cell Cytoplasm

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Abstract. The origin of the vacuole membrane surrounding the intracellular protozoan parasite Toxoplasma gondii is not known. Although unique secretory organelles, the rhoptries, discharge during invasion of the host cell and may contribute to the formation of this parasitophorous vacuole membrane (PVM), no direct evidence for this hypothesis exists. Using a novel approach we have determined that parasite-encoded proteins are present in the PVM, exposed to the host cell cytoplasm. In infected cells incubated with streptolysin-O or low concentrations of digitonin, the host cell plasma membrane was selectively permeabilized without significantly affecting the integrity of the PVM. Antisera prepared against whole parasites or a parasite fraction enriched in rhoptries and dense granules reacted with the PVM in these permeabilized cells, indicating that parasite-encoded antigens were exposed on the cytoplasmic side of the PVM. Parasite antigens responsible for this staining of the PVM were identified by fractionating total parasite proteins by SDS-PAGE and velocity sedimentation, and then affinity purifying “fraction-specific” antibodies from the crude antiserum. Proteins responsible for the PVM-staining, identified with fraction-specific antibodies, cofractionated with known rhoptry proteins. The gene encoding one of the rhoptry proteins, ROP 2, was cloned and sequenced, predicting an integral membrane protein. Antibodies specific for ROP 2 reacted with the intact PVM. These results provide the first direct evidence that rhoptry contents participate in the formation of the PVM of T. gondii and suggest a possible role of ROP 2 in parasite-host cell interactions.

The protozoan Toxoplasma gondii, an obligate intracellular parasite, can invade and replicate within all nucleated mammalian and avian cells tested. During the invasion process a new membrane structure surrounding the entering parasite is formed in the host cell: the parasitophorous vacuole. Once inside the host cell the parasite remains within this vacuole where it multiplies rapidly (Pfefferkorn, 1990). The parasite extensively modifies the newly formed vacuolar space by the formation of an intravacuolar membranous network and the secretion of a number of proteins (Sibley and Krahenbuhl, 1988; Sibley et al., 1986; Achbarou et al., 1991; Leriche and Dubremetz, 1990).

The mechanisms underlying the formation of the parasitophorous vacuole and the origin of the material incorporated into its membrane, the parasitophorous vacuole membrane (PVM)↑, are not known. Because no endosomal or lysosomal markers are detectable within the vacuole at any stage during the infection (Joiner et al., 1990), this structure is unlikely to be formed by phagocytosis or a variant thereof. In addition, the PVM is, in early stages of infection, almost completely devoid of the intramembranous particles that are abundant in the host cell plasma membrane (Porchet-Henner and Torpier, 1983).

These observations have raised the possibility that the parasite itself may contribute to the formation of the PVM. The rhoptries, unique secretory organelles located in the apical part of the parasite at its site of attachment to the host cell, might play a role in the formation of the PVM (for review see Perkins, 1992). Evidence for this idea is indirect: (a) rhoptries discharge their contents during invasion, and (b) rhoptry proteins appear associated with the PVM immediately after invasion (Saffer et al., 1992). There are conflicting observations regarding the source of lipids in the...
been able to demonstrate, using a patch clamp method, a porelike structure with an exclusion limit of 1,300-1,900 daltons that give the growing parasite access to nutrients in the host cell cytoplasm. Throughout the life cycle of Plasmodium, this channel is open more than 98% of the time, is permeable to molecules of at least 200 daltons, and displays no detectable charge specificity.

The PVM functions described above are presumably mediated by specific PVM-associated proteins. We hypothesize that these proteins are derived from the parasite itself. In order for a PVM-associated parasite protein to be involved in any of the processes described above, it would have to be exposed on the host cell side of the PVM. We describe here a new approach for the identification of T. gondii proteins exposed on the host cell side of the PVM. Using this method we identified a Toxoplasma protein that is present in the PVM and faces the host cell cytoplasm. Throughout the infection this protein is present in the PVM, distributed in a distinct punctate or ribbon-like fashion. Since rhoptries discharge only at the time of host cell invasion, these data provide the first direct evidence with T. gondii that these organelles are intimately involved in the formation and/or modification of the PVM.

Materials and Methods

Buffers and Reagents

All chemicals were obtained from either Sigma Chem. Co. (St. Louis, MO) or J. T. Baker (Phillipsburg, NJ), unless indicated otherwise. Aprotinin, leupeptin, chymostatin, and pepstatin, were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Solutions were prepared using water prepared with the Nanopure system (Barnstead, Dubuque, IA). The pH values indicated were determined at room temperature.

The following monoclonal antibodies were used: Tg49 (Schwartzman, 1986) for ROP 1; T4 2F8 (Leriche and Dubremetz, 1991) for ROP 2 and 3; T3 4A7 (Sadak, et al., 1988) for ROP 2, 3 and 4; T5 1A11 (Leriche and Dubremetz, 1991) for ROP 7 (formerly po56); and T6 2H11 (Leriche and Dubremetz, 1991) for GRA3. Rabbit antisera to whole Toxoplasma (Ware and Kasper, 1987), a membrane fraction enriched in rhoptries and dense granules (Leriche and Dubremetz, 1991) were prepared as described.

Cell Propagation

Human foreskin fibroblasts (HFF) were grown in alpha-MEM containing 10% (vol/vol) FCS (JR Scientific, Woodland, CA) and 0.3 mg/ml glutamine. CHO cells were maintained in alpha-MEM containing 3.5% FBS.

Isolation of the ROP 2 Gene

A previously isolated fragment of the ROP 2 gene (Mercereau-Puijalon et al., 1993) was labeled using random priming (Boehringer Mannheim Biochemicals) and α-[32P]dCTP (Amersham Corp., Arlington Heights, IL). Using this as a probe, a cosmид library containing T. gondii genomic DNA (obtained from Dr. D. S. Roos, University of Pennsylvania) was screened by colony hybridization (Sambrook et al., 1989). Cosmid DNA was isolated from the positive colonies, digested with several restriction enzymes (New England Biolabs, Beverly, MA), separated by agarose electrophoresis and transferred by capillary transfer to Hybond-N membranes (Amersham Corp.). ROP 2-containing restriction fragments were identified by hybridizing the filter with the ROP-2 probe, subcloned in either pBluescript SK+ (Stratagene, La Jolla, CA) or pSP72 (Promega, Madison, WI) and sequenced using the Sequenase system (Amersham Corp.).

Preparation of an Antiserum to a β-Galactosidase-ROP 2 Fusion Protein

A fragment of genomic DNA, encoding the carboxy-terminal 365 amino acids of the predicted sequence of ROP 2, was inserted into the expression vector pMSgtll and transfected into the E. coli strain DH5a (Scherf et al., 1990). After induction with IPTG, the fusion protein was purified on p-aminobenzenyl-β-D-thiogalactoside agarose (Scherf et al., 1988). A rabbit was injected subcutaneously three times, at four week intervals, with 200 µg of the purified fusion protein mixed with either complete (for the first injection) or incomplete (for the remaining injections) Freund's adjuvant.

Parasite Propagation

The RH strain of Toxoplasma gondii was used throughout this study. Parasites were propagated in either BALB/C or Swiss-Webster mice (Charles River Labs., Wilmington, MA). Each mouse was injected intra-peritoneally with 0.5–1 × 10⁷ parasites in PBS using a 25-gauge needle. After 48-72 h the mouse was killed by asphyxiation in carbon dioxide, the abdomen was opened to expose the peritoneum and the parasites were recovered by peritoneal lavage using two washes with 2.5 ml PBS. Routinely, the parasite suspension was passed twice through a 27-gauge needle to disrupt any contaminating host cells, washed twice by centrifugation at 1,000 g in a 10 ml tube. The parasites were resuspended in PBS for use in experiments. Parasites were also grown continuously in the presence of aminoglycosides (streptomycin, 100 µg/ml and kanamycin, 50 µg/ml) to prevent contamination by adaptive variants and cells with low parasitoid load. Parasites were maintained in the presence of aminoglycosides.

Preparation of Digitonin-permeabilized Cells

For the preparation of digitonin-permeabilized cells, CHO or HFF cells were plated onto 12-mm circular coverslips (Fisher Scientific, Pittsburgh, PA) in 24-well tissue culture plates (Falcon 3047, Becton Dickinson Labware, Lincoln Park, NJ) at a density of 1–2 × 10⁵ cells per well. After 18-24 h, 10⁵ parasites, freshly isolated from infected mice, were added per well. After 16–18 h >95% of all cells on each coverslip contained at least one parasitophorous vacuole, with 1-16 parasites per vacuole. All incubations were performed in a 24-well plate placed on a metal block in an ice-water bath. The coverslips were washed three times with PBS and subsequently permeabilized by incubation in 0.002% digitonin (Calbiochem, La Jolla, CA) in PBS for 5 min. Digitonin concentrations in the range of 0.001-0.005 % usually gave good plasma membrane permeabilization without affecting the PMV. Concentrations below 0.001 % were ineffective in permeabilizing the plasma membrane, and concentrations higher than 0.01% resulted in permeabilization of the PVM. Excess detergent was removed by three washes with PBS. The solution (50-100 µl) containing the antibody preparation of interest in 3% BSA in PBS or in the neutralized elution buffer (see below) was placed onto the coverslip and allowed to incubate for 30 min. After the coverslips were washed three times with PBS to remove unbound antibodies, the cells were fixed for 15 min in 3% paraformaldehyde (wt/vol) in PBS. After one wash with PBS, the coverslips were incubated in 0.1 M lysine in PBS for 5 min. Cells were completely permeabilized for 5 min in 0.1% Triton X-100 in PBS, followed by three washes with PBS.

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labeled secondary antibodies, diluted 1:500 in 3% BSA in PBS, was placed from Parasites
on the coverslips and allowed to incubate for 30 min. Finally the coverslips were washed three times in PBS and mounted onto microscope slides using
Mowiol. Fluorescence microscopy was performed using a Nikon Microphot-
FXA, equipped for epifluorescence. Photographs were made using a Nikon
100x planapochromatic oil immersion objective at a final magnification of
312× on Kodak Tri-X film.

**Immunoelectron Microscopy**

Free parasites, suspended in ice-cold culture medium, were added to a flask
of confluent CHO cells and left on ice for 15 min to allow the parasites to
settle onto the cells. Subsequently the flask was incubated for 1 h at 37°C.
The infected CHO cells were then fixed for 90 min with 4% paraformalde-
hyde and 0.05% glutaraldehyde in 0.2 M Pipes pH 7.0. The monolayer was
washed with 10% FCS in PBS (PBSS) and scraped off the dish and pelleted.
The pellet was infiltrated with a solution containing 2.3 M sucrose and 20%
polyvinyl pyrrolidone (molecular mass 10 kD) in 0.1 M phosphate pH 7.4
for 4 h, and then frozen in liquid nitrogen. Sections were made at ~100°C
with a Leica Ultracut equipped with an FC4 attachment, and collected on
PBSS. Immunolocalization was performed by incubating sections with the
rabbit antiserum prepared against recombinant ROP2 protein (diluted 1:50
in PBSS), followed by 8 nm protein A-gold (diluted to an OD525 of 0.05
in PBSS). Preimmune rabbit antiserum was used as a control. The grids
were then stained with 0.4% uranyl acetate in 2% methyl cellulose and ex-
amined with a Hitachi H600 microscope.

**Preparation of a Rhoptry-enriched Fraction from Parasites**

A rhoptry fraction was prepared from tachyzoites, using a modification of
a published procedure (Leriche and Dubremetz, 1991). All manipulations
were performed on ice or at 4°C. Parasites that were isolated fresh from
infected mice or had been frozen in liquid nitrogen as described above, were
resuspended at a density of 10^6 parasites per ml in TES buffer (25 mM
Tris-HCl pH 8.0, 5 mM EDTA, 250 mM sucrose) containing leupeptin,
apro tin, pepstatin, and chymostatin at final concentrations of 5 μM, 1 μM, 5 μM,
and 100 μM, respectively. The suspension was transferred to a 15-ml conical centrifuge tube and washed glass beads (catalog number
H3640, Baxter Healthcare Corporation, Bedford, MA) were added until no free liquid was visible. The suspension was mixed at high speed, using a vortex mixer, until more than 95% of the parasites had been disrupted and
converted to parasite ghosts as judged by phase contrast microscopy. It was
usually sufficient to mix for 1 min to achieve this level of disruption. The beads were allowed to settle and the cell homogenate was retrieved from
the bottom of the tube using a Pasteur pipette. The beads were washed twice with a volume of TES equal to the original volume of the cell suspension.
The homogenate and the two washes were combined, and, after addition of
PBSF to a final concentration of 1 mM, centrifuged for 10 min at 1,000 g in
a Beckman GPR tabletop centrifuge to remove the few non-disrupted
parasites and the parasite ghosts. The supernatant was centrifuged for 90
min at 50,000 g in a Beckman 50Ti rotor. The supernatant was discarded
and the pellet resuspended in a volume of TES equal to 2.5 times the volume
of the original cell suspension. To this suspension 0.64 vol of 90% Percoll
(Pharmacia LKB Biotechnology, Piscataway, NJ) in TES was added to achieve a final concentration of 35% Percoll. After centrifugation for 35
min at 35,000 g in a Beckman 50Ti rotor, two distinct bands were visible
about 80% down the length of the gradient. Using colored Percoll density
marker beads (Pharmacia LKB Biotechnology), the density of both bands
was determined to be about 1.07 g/cm³. Both were collected from the gradient
using a Pasteur pipette and centrifuged for 90 min at 100,000 g in a
Beckman 50Ti rotor. The soft pellet on top of the Percoll pellet was recov-
ered and homogenized by 10 passes in a 1-ml Dounce (Wheaton, Millville,
NJ) homogenizer. The sucrose concentration was adjusted to 0.8 M by the
addition of 10% TES (5 ml per dish) and scraped off the dish into 1.5 ml of TES
using a rubber policeman. The CHO cells were disrupted by passing the
suspension twice through a 27-gauge needle. This treatment is sufficient to
completely disrupt the host cells and the PVM, yet leaves the parasites in-
tact. PMSF, leupeptin, aprotinin, pepstatin, and chymostatin were added
to final concentrations of 1 mM, 5 μM, 1 μM, 5 μM, and 100 μg/ml,
respectively. The parasites, essentially fragments of the host cells and intact host
cells were removed by centrifugation for 10 min at 1,000 g. The supernatant
(13 ml for ten 15-cm dishes) was overlaid onto a step gradient consisting of
15 ml 25% (wt/wt) sucrose in 10 mM Tris-HCl pH 8.0 and 10 ml 55%
(wt/wt) sucrose in 10 mM Tris-HCl pH 8.0. This was centrifuged for 2.5 h
at 100,000 g in an SW28 rotor. The membranous material at the 25%/55%
interface was collected using a Pasteur pipette, homogenized in a 1-ml
Dounce homogenizer and frozen in 100-μl aliquots in liquid nitrogen.

**Velocity Sedimentation of Detergent Lysates from Parasites**

All manipulations were performed on ice or at 4°C. Parasites were solubi-
ized in a buffer containing 1% Triton X-100, 100 mM KCl, 25 mM Tris-
HCl pH 8.0, 5 mM EDTA, 5 mM leupeptin, 1 μM aprotinin, 5 μM pepstatin, and 100 μg/ml chymostatin. In general, 100 μl of this
buffer was used to solubilize 10^7 parasites. For those gradients in which the
different fractions were used for the affinity purification of fraction-specific antibodies, 2-5 × 10^7 parasites were used per gradient.

After a 5-min incubation on ice, the lysate was centrifuged for 10 min at
13,000 g. The pellet was discarded and the supernatant was loaded onto a
5-ml linear 5-20% (wt/wt) sucrose gradient in 0.5% Triton X-100, 100
mM KCl, 50 mM Tris-HCl pH 8.0 and this gradient was centrifuged at
50,000 rpm in an SW50.1 rotor (Beckman Instrs.) until ω^2 = 4.5 × 10^11.
Twelve fractions (~420 μl each) were collected from the bottom of the gradients and the proteins were precipitated by the addition of 420 μl 20% TCA. After 30
min on ice the samples were centrifuged for 10 min at 13,000 g, the pellets
were washed once with acetone to remove the precipitated Triton X-100 and
the remaining protein pellets were dissolved in Laemmli sample buffer.
Identical results were obtained when CHAPS [3-(3-cholamidopropyl)-
dimethyl-ammonio-1-propane sulfonate] was used instead of Triton X-100.

To obtain an estimate of the S-value from the velocity sedimentation data,
BSA (4.6S, 67 kD), rabbit muscle lactate dehydrogenase (7.3S, 150 kD),
bovine liver catalase (11.4S, 240 kD), and rabbit muscle phosphorylase A
(15.4S, 370 kD) were used as standards and loaded onto a parallel gradient.

**Membrane Extractions**

Membrane preparations that were to be subjected to different extraction
procedures were, if necessary, diluted by the addition of 10 mM Tris-HCl
pH 8.0 to reduce the sucrose concentration to <0.25 M. Subcellular mem-
branes were collected by centrifugation for 1 h at 100,000 g in a 50Ti rotor.
The supernatant was discarded and the pellet resuspended in 250 mM
sucrose in 10 mM Tris-HCl pH 8.0 to a final protein concentration of 0.1-0.5
mg/ml.

The high salt and high pH extractions were performed by mixing 100 μl
of a membrane suspension with, respectively, 100 μl 2 M KCl in 10 mM
Tris-HCl pH 8.0 or 100 μl 0.2 M sodium carbonate pH 11. A mock extrac-
tion was always performed by incubation of 100 μl membrane suspension
with 100 μl of 250 mM sucrose in 10 mM Tris-HCl pH 8.0. The reactions
were incubated on ice for 15 min and subsequently pipetted on top of 100
μl 0.6 M sucrose in 10 mM Tris-HCl pH 8.0, containing phenol red, in a
0.35-ml airfuge tube. These were centrifuged for 20 min at 20 psi. The su-
pernatant fraction and a small amount of the cushion was recovered by
pipetting 220 μl from the top of the sample. The remainder of the cushion
was removed by aspiration and the pellet was dissolved in Laemmli gel-
loading buffer. The proteins that had been extracted from the membranes were recovered from the supernatant fraction by TCA precipitation and dis-
solved in Laemmli gel-loading buffer.

**Immunoprecipitations**

These were performed exactly as described previously (Leriche and
Dubremetz, 1991). For preparative immunoprecipitations of Toxoplasma proteins for the affinity purification of polyclonal antibodies, 1.2-4.5 × 10^7

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parasites, 5-μl ascites fluid and 50 μl of a 50% suspension of protein A-agarose (Boehringer-Mannheim) were used per reaction. For analytical immunoprecipitations 1-μl ascites fluid and 10-μl protein A-agarose were used.

**SDS-PAGE and Electrotransfer of Proteins to Nitrocellulose**

Protein gel electrophoresis was performed essentially as described by Laemmli, using the Mini-PROTEAN II electrophoresis cell (Biorad Labs., Richmond, CA). Usually, 10% gels were used in those experiments, where SDS-PAGE was to be followed by electrotransfer of proteins to nitrocellulose, pretaimed Mr markers were used (Biorad Labs.).

Transfer of proteins to nitrocellulose (Gelman Sciences, Ann Arbor, MI) after separation by SDS-PAGE was performed for 1 h at 100 V in 200 mM glycine, 25 mM Tris-base and 20% methanol, using a Mini-Trans-Blot Eletrophoretic Transfer cell (Biorad Labs.). After completion of transfer, the transfer of proteins was checked by briefly incubating the nitrocellulose in 0.2% Ponceau-S in 3% TCA followed by three rapid washes in distilled water. To block non-specific binding of antibodies, the nitrocellulose was incubated for 30 min at room temperature in BLOTTO (5% non-fat dried milk [Carnation, Los Angeles, CA] in Tris-buffered saline with 0.1% Tween 20 [TBST]). Incubation with the primary antibodies was performed in BLOTTO in heat-sealed plastic bags for 1-16 h at room temperature. Primary antibodies or antisera were used at dilutions of 1:1,000 for the detection of antigens. For the affinity purification of antibodies from antisera, dilutions of 1:25 or 1:100 were used (see below).

Non-bound antibodies were removed by three 10-min washes in TBST. The nitrocellulose was incubated for 1 h at room temperature with peroxidase-labeled goat-anti-mouse IgG or goat-anti-rabbit IgG (Biorad Labs.) at a 1:2,000 dilution in BLOTTO, followed by three 10-min washes in TBST to remove non-bound secondary antibodies. Bound antibodies were visualized using the ECL system (Amersham Corp.) or a solution of 0.6 mg/ml dianinobenzidine hydrochloride, 0.03% hydrogen peroxide, 0.3 mg/ml nickel chloride in 50 mM Tris-HCl pH 7.6.

**Affinity Purification of Antibodies on Nitrocellulose**

After separation by SDS-PAGE, proteins were transferred to nitrocellulose and incubated with BLOTTO as described above. The nitrocellulose was incubated for 4-16 h in a 1:25 dilution of the anti-Toxoplasma antiserum or a 1:100 dilution of the rabbit anti-rhoptry/dense granule antiserum in BLOTTO. Non-bound antibodies were removed by washing twice 15 min in TBST and once in PBS. The regions of interest were subsequently excised from the nitrocellulose, washed briefly in water, cut into small pieces and placed in a 1.5-ml centrifuge tube. To elute the bound antibodies, enough elution buffer (0.2 M glycine-HCl pH 2.8, 0.2% gelatin) was added to cover the nitrocellulose pieces.

For the affinity purification of antibodies from antisera, dilutions of 1:25 or 1:100 were used (see below).

Non-bound antibodies were removed by three 10-min washes in TBST. The nitrocellulose was incubated for 1 h at room temperature with peroxidase-labeled goat-anti-mouse IgG or goat-anti-rabbit IgG (Biorad Labs.) at a 1:2,000 dilution in BLOTTO, followed by three 10-min washes in TBST to remove non-bound secondary antibodies. Bound antibodies were visualized using the ECL system (Amersham Corp.) or a solution of 0.6 mg/ml dianinobenzidine hydrochloride, 0.03% hydrogen peroxide, 0.3 mg/ml nickel chloride in 50 mM Tris-HCl pH 7.6.

**Results**

**The PVM Contains Parasite-derived Proteins**

We reasoned that any parasite proteins involved in nutrient transport or interaction with host cell organelles would be exposed on the host cell side of the PVM. To gain access to this membrane, we explored different methods that might selectively permeabilize the plasma membrane of the host cell, yet leave the PVM intact. Permeability of the plasma membrane was assessed by failure to exclude the membrane impermeable dye trypan blue. The integrity of the PVM was determined by the inability of antibodies, directed against surface proteins of the parasite, to react with the parasites within the PVM. In infected cells treated with either streptolysin-O (data not shown) or low concentrations of digitonin, the host cell plasma membrane became permeable while the PVM remained intact (Fig. 1, A and B). Treatment with low concentrations of Triton X-100 or saponin resulted in either complete permeabilization or none at all (results not shown).

Assuming that PVM-associated parasite proteins were also present in the extracellular parasite, we reasoned that an antisemur prepared against extracellular parasites might contain antibodies directed against these PVM proteins. When permeabilized, infected cells were incubated with a polyclonal rabbit antisemur prepared against whole Toxoplasma, and bound antibodies were visualized using a fluorescent secondary antibody, staining of the PVM was observed (Fig. 1, C and D). In uninfected cells no structures reacted with the antisemur indicating that the molecules detected on the PVM were parasite-derived. Because the antisemur reacted well with extracellular parasites (Fig. 1 D), the absence of parasite staining within the PVM confirmed that this membrane was intact and impermeable to antibodies. The observed staining of the PVM is therefore testament to the presence in the antiserum of antibodies that recognize Toxoplasma proteins present on the host cell side of the PVM. Staining of the PVM with this antiserum was observed in both CHO cells and HFF cells after permeabilization with digitonin. Because the parasitophorous vacuole and its resident parasites can be seen more clearly in the large, flat HFF cells than in CHO cells, we present the immunofluorescence data only in the former, although the staining patterns were the same in both cell lines.

Using the same approach, both monoclonal antibodies and antisera directed against different molecules and organelles of Toxoplasma were tested for their ability to react with the host cell side of the PVM. An antisemur prepared against a partially purified preparation of rhoptries and dense granules (Leriche and Dubremetz, 1991) reacted strongly with the PVM in infected, digitonin-permeabilized cells (Fig. 1, E and F). The staining pattern observed with the anti-rhoptry/dense granule and anti-T. gondii antisera appeared qualitatively similar in that it was clearly punctate and evenly distributed over the PVM. The intensity of the labeling observed with the rabbit anti-rhoptry/dense granule serum was, however, much stronger.

None of the monoclonal antibodies or monospecific polyclonal antisera to specific Toxoplasma proteins already known to interact with the PVM, such as ROP 1 (Saffer et al., 1992) and the dense granule protein GRA3 (Achbarou et al., 1991), reacted under similar circumstances. These observations suggest that the antibodies that react with the PVM are directed against heretofore unknown proteins, or that the epitopes for the available monoclonal antibodies and monospecific antisera are simply not exposed on the host cell side of the PVM.

**The PVM Proteins Cofractionate with Members of a Family of Rhopty Proteins**

Both the anti-Toxoplasma and anti-rhoptry/dense granule antisera were found to react, by immunoblotting, with numerous Toxoplasma proteins in a whole parasite lysate fractionated by SDS-PAGE (Fig. 2).

The assumption was made that the protein(s) exposed on the host cell side of the PVM might be among those proteins detected by the anti-Toxoplasma and the anti-rhoptry/dense
Figure 1. T. gondii-encoded proteins are present on the host cell side of the PVM. Human foreskin fibroblasts growing on coverslips were infected with T. gondii at a ratio of about 10 parasites per host cell. After 16-18 h, the cells were washed in PBS and permeabilized with 0.002% digitonin in PBS as described in Materials and Methods. The permeabilized cells were incubated with (A and B) a monoclonal antibody (T4 IE5) directed against the major surface protein of the parasite, (C and D) a rabbit antiserum prepared against whole parasites, and (E and F) a rabbit antiserum prepared against a membrane fraction enriched in rhoptries and dense granules. Bound antibodies were visualized using a (A and B) rhodamine-conjugated goat-anti-mouse IgG or (C, D, E, and F) rhodamine-conjugated goat-anti-rabbit IgG antibodies. (A, C, E) Phase contrast; (B, D, and F) immunofluorescence. T. gondii encoded proteins were present in the PVM, accessible from the host cell side, and were distributed in a punctate fashion. Note the absence of staining of intracellular parasites by either the anti-surface monoclonal or the anti-whole parasite antiserum. Extracellular parasites are stained by the anti-whole parasite antiserum (arrow in C and D).

granule antisera on the immunoblots. If this were true, then one might be able to elute antibodies bound to specific proteins on the nitrocellulose after incubation with either antiserum and test them for their ability to react with the PVM in digitonin-permeabilized cells. Since the number of reactive protein bands was large, we eluted antibodies bound to proteins in five M, regions: larger than 116 kD, 80–116 kD, 50–80 kD, 30–50 kD, and smaller than 30 kD. As shown in

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Figure 2. Reaction of T. gondii proteins with the anti-whole parasite and the anti-rhoptry/dense granule antisera, as determined by immunoblotting. Whole parasites were lysed in SDS-PAGE sample buffer and three aliquots, each equivalent to about 5 × 10⁵ parasites, were loaded onto a 10% SDS polyacrylamide gel, electrophoresed, and transferred to nitrocellulose. Prestained molecular mass standards were included on the gels. The strips of nitrocellulose, corresponding to the three gel lanes, were excised. One strip was incubated with the anti-whole parasite antiserum (aRH), the other two strips were incubated with the anti-rhoptry/dense granule antiserum (~ROP/DG). Antibodies bound to the aRH strip and to one of the ~ROP/DG strips were visualized using a peroxidase-conjugated goat-anti-rabbit IgG antiserum and the ECL chemiluminescence detection system. The molecular masses of the pre-stained standards are indicated.

Fig. 3, only antibodies eluted from the 50–80-kD range reacted with the host cell side of the PVM. As the reaction was stronger using eluted antibodies from the anti-rhoptry/dense granule antiserum, this was used in subsequent experiments.

In the 50–80-kD Mₚ range, the anti-rhoptry/dense granule antiserum reacted with proteins of about 64–80 kD, 60 kD, 55 kD, and 50 kD (III A, B, C, and D in Fig. 2), suggesting that these might be the major proteins recognized by the anti-rhoptry/dense granule antiserum on the host cell side of the PVM. To determine with more precision which of these proteins was responsible for the staining pattern observed, tachyzoites were solubilized in non-ionic detergent and fractionated by velocity sedimentation on a linear sucrose gradient. Fractions were collected, subjected to SDS-PAGE, and transferred, in duplicate, to nitrocellulose membranes. These were incubated with the anti-rhoptry/dense granule antiserum. One of the blots was developed to reveal the fractionation behavior of the different proteins reactive with the antiserum (Fig. 4 A). For the companion blot the 50–80-kD range from each fraction was excised and the bound antibodies were eluted as described above. The eluted antibodies were tested for their ability to react with the host cell side of the PVM. As can be seen in Fig. 4 A, antibodies eluted from the fourth through the sixth fractions from the top reacted with the PVM in digitonin-permeabilized cells. Based on the fractionation behavior of standard proteins, the PVM-associated proteins had sedimentation coefficients of 7–11 S. Identical results were obtained if CHAPS was used as detergent instead of Triton X-100.

Other proteins with different Mₚ or sedimentation coefficients may also be present on the host cell side of the PVM, but may go undetected because the antisera tested may not contain antibodies directed against them. It is also possible that antibodies are present but do not bind to SDS-denatured proteins on the nitrocellulose. Lastly, antibodies may be inactivated during the elution procedure. We do not have any data to prove or disprove the first two possibilities. However, the latter is less likely since the antibodies eluted from all Mₚ ranges reacted on immunoblots with most of the proteins recognized by the anti-rhoptry/dense granule antiserum (data not shown) and also reacted with parasite-specific structures in infected cells that had been completely permeabilized with acetone (data not shown).

The Mₚ of the PVM proteins, taken with the fact that the PVM-staining antibodies were derived from an antiserum prepared against a partially purified rhoptry fraction, indicated that these proteins might be identical to a number of known rhoptry proteins with similar Mₚ: ROP 1 (60.5 kD), ROP 2 (55 kD), ROP 3 (59 kD), ROP 4 (60 kD), and ROP 7 (56 kD, formerly referred to as po56) (Leriche and Dubremetz, 1991; Sadak et al., 1988). To test this possibility, we determined the sedimentation behavior of the known rhoptry proteins in fractions from the same experiment used to determine the sedimentation behavior of the PVM proteins. ROP 1 was not included in these experiments as both monoclonal and polyclonal antibodies to this protein failed to react with the PVM in digitonin-permeabilized cells. As can be seen in Fig. 4 B, ROP 2, ROP 3, ROP 4, and ROP 7 have sedimentation coefficients that are indistinguishable from the PVM proteins, suggesting that one or more of these proteins may indeed be responsible for the PVM staining observed.

Given that on SDS-PAGE the Mₚ of the PVM proteins and ROP 2, 3, 4, and 7 is between 55 or 60 kD, but possess a sedimentation coefficient of 7–11 S, these proteins seem to be present in the parasite as oligomers, most likely trimers or tetramers. Because ROP 2 and 3, as well as ROP 7 can be immunoprecipitated separately from each other and ROP 4 (Sadak et al., 1988), it is likely that these proteins form homo-oligomers.

ROP 2 and ROP 4 or 7 Are Present on the Host Cell Side of the PVM

To determine whether antibodies to ROP 2, 3, 4, and 7 were
responsible for the staining pattern observed when digitonin-permeabilized cells were incubated with the rabbit anti-rhoptry/dense granule antiserum, we incubated the permeabilized cells instead with antibodies specific for these proteins. None of the monoclonal antibodies directed against ROP 2, 3, 4, or 7 that were tested in digitonin-permeabilized cells reacted with the PVM (data not shown), although they do react with the rhoptries in acetone-permeabilized preparations (Leriche and Dubremetz, 1991). The failure of these monoclonal antibodies to react with the PVM could indicate that either ROP 2, 3, 4, and 7 are not present on the host cell side of the PVM, or that the relevant epitopes are simply not accessible. To test the latter possibility, we prepared polyclonal antibodies to the four proteins. Rather than making specific polyclonal antisera to these proteins by immunizing animals with purified proteins, we affinity purified the desired antibodies from a crude antiserum. Monoclonal antibodies were first used to immunoprecipitate the proteins from a whole Toxoplasma lysate, followed by SDS-PAGE and transfer to nitrocellulose. The latter was used for affinity

Figure 3. Detection of PVM proteins using antibodies specific for T. gondii proteins found in specific size ranges on SDS-PAGE. A companion blot of those shown in Fig. 2 was incubated with αROP/DG and cut into five pieces corresponding to the molecular mass intervals indicated in Fig. 2. Antibodies bound to these pieces of nitrocellulose were eluted and used for immunofluorescence on digitonin-permeabilized, T. gondii-infected HFF cells as described in Materials and Methods. Antibodies were specific for proteins with an Mr on SDS-PAGE (A and B) larger than 106 kD, (C and D) of 80–106 kD, (E and F) 49.5–80 kD, (G and H) 29.5–49.5 kD, and (I and J) smaller than 29.5 kD. Bound antibodies were visualized using rhodamine-conjugated goat-anti-rabbit IgG antibodies. (A, C, E, G, and I) Phase contrast; (B, D, F, H, and J) immunofluorescence.
Figure 4. (A) Determination of the native molecular mass of PVM proteins using velocity sedimentation. A lysate of $5 \times 10^7$ parasites was prepared in buffer containing Triton X-100. After fractionation by velocity sedimentation on linear sucrose gradients, it was separated by SDS-PAGE and transferred (in duplicate) to nitrocellulose as described in Materials and Methods. The nitrocellulose pieces were incubated with the rabbit-antirhoptry/dense granule antiserum. One blot was used to visualize bound antibodies as described in the legend to Fig. 2. Antibodies bound to proteins in the 49.5–80-kD range were eluted from the nitrocellulose pieces corresponding to the different gradient fractions. These eluted antibodies were used immediately in immunofluorescence on digitonin-permeabilized, T. gondii-infected HFF cells. The relative intensity of PVM staining for the different antibody preparations is shown in the boxed region below the immunoblot in A. (B) ROP 2, 3, 4, and 7 have sedimentation coefficients similar to those found for the PVM proteins. Gradient fractions obtained from the gradient described above, were electrophoresed (in duplicate) on 10% SDS polyacrylamide gels and transferred to nitrocellulose. The nitrocellulose pieces were incubated with either a monoclonal specific for ROP 2, 3, 4 (T3 4A7) or ROP 7 (T5 1A11) and bound antibodies were visualized using peroxidase-conjugated goat-anti-mouse IgG antibodies and ECL detection system. The cross-reactive proteins with Mr less than those of ROP 2 and ROP 7 are not always seen and are presumably degradation products. The proteins used as sedimentation coefficient standards are described in Materials and Methods.

Figure 5. The specificity of antibodies reactive with the PVM in digitonin-permeabilized cells. Using ROP 2, 3, 4, and ROP 7 immunoprecipitated with monoclonal antibodies, antibodies to these proteins were purified from the rabbit anti-rhoptry/dense granule antiserum as described in Materials and Methods. After SDS-PAGE of a total parasite lysate on a 7.5% gel and immunoblotting, the nitrocellulose was incubated with the monoclonal antibodies to ROP 2, 3, 4 (T3 4A7, lane a) and ROP 7 (T5 1AI1, lane e), a rabbit antiserum prepared to a β-galactosidase-ROP 2 fusion protein (lane c) and with antibodies affinity purified on ROP 2, 3, 4 (lane b) and ROP 7 (lane d).

We conclude from these experiments that ROP 2 and ei-
Figure 6. ROP 2, 3, 4, and 7 are present in the PVM and exposed to the host cell cytosol. ROP 1 (Tg49), ROP 2, 3, 4 (T3 4A7), and ROP 7 (TS 1A1) were each isolated by preparative immunoprecipitation from 1.2-1.5 \times 10^7 parasites with specific monoclonal antibodies and used for the affinity purification of polyclonal antibodies specific for these proteins from the rabbit-anti-rhoptry/dense granule antiserum, as described in Materials and Methods. The affinity-purified antibodies were used in immunofluorescence on digitonin-permeabilized, \textit{T. gondii} infected HFF cells. (A, C, and E) Phase contrast; (B, D, and F) immunofluorescence. (A and B) ROP 1, (C and D) ROP 2, and (E and F) ROP 4 and 7.

Molecular Cloning of the ROP 2 Gene

A DNA fragment containing a part of the gene encoding ROP 2 was isolated by screening an expression library of \textit{T. gondii} genomic DNA in \lambda gt11, with the rabbit anti-rhoptry/dense granule antiserum (Mercereau-Puijalon et al., 1993). As shown in Fig. 5 (lane b), antibodies prepared to the protein product of a gene fusion between \beta-galactosidase and

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the phage insert react specifically with ROP 2 on immunoblots, indicating that the insert encodes all or part of ROP 2. The insert did not contain an open reading frame of sufficient size to encode a protein the size of the unprocessed form of ROP 2 (60 kD). To obtain the complete sequence of the coding region, the phage insert was used to screen a cosmid library of the *T. gondii* genome. Based on restriction analysis all positive cosmids contained the same fragment of *T. gondii* genomic DNA. Restriction fragments containing ROP 2-related sequences were identified by Southern hybridization analysis, subcloned, and their DNA sequence was determined as described in Materials and Methods. The complete sequence of the ROP 2 gene is shown in Fig. 7. The ATG present at position 439 was identified as the putative start codon in that it conforms to the consensus suggested by Kozak (1986) (a purine is present at the −3 position), and because stop codons in all three reading frames precede it. This start codon is followed by an open reading frame of 1686 base pairs, encoding a 561-amino acid protein with a predicted molecular mass of 64 kD and a predicted isoelectric point of 8.2. The gene fragment of ROP 2 isolated from the *λ*gt11 genomic library corresponds to base pairs 989 through 2234 of the sequence shown in Fig. 7. The conclusion that the identified open reading actually encodes ROP 2 is based on a number of observations. Most convincingly, antibodies prepared to a fusion of β-galactosidase and amino acids 184 through 561 of the open reading frame react specifically with the ROP 2 protein on Western blots (Fig. 5, lane c). In addition, the molecular mass and the basic isoelectric point of the protein predicted by this open reading frame, correspond well to the observed properties of the precursor form of ROP 2 (Leriche and Dubremetz, 1991; Sadak et al., 1988), a basic protein with an *M* of ~66 kD.

The DNA sequence of the ROP 2 gene is virtually identical to that reported for the partial cDNA clone Tg34 encoding a 54-kD protein, identified by Saavedra et al. (1991) as a major T cell antigen of *T. gondii*. In addition to including the initiation codon and the S′ non translated region, the sequence we obtained contains a G doublet at position 2022 and 2023, whereas the sequence reported for Tg34 contains a G triplet in the same position. Because of this difference, the predicted amino acid sequence of ROP 2 diverges at this point from that of Tg34 and is also 23 amino acids longer. The difference in sequence may be due to a single sequencing error, but the possibility that it reflects a strain difference cannot be discounted. The fact that the sequence of Tg34 (a cDNA clone) and the ROP 2 sequence (from a genomic clone) are virtually identical also confirms that the ROP 2 sequence does not contain introns. The presence of a 14 base pair palindrome immediately following the termination codon (Fig. 7, boxed region) was noted. The significance of this structure is unclear at this moment.

**Structure of the ROP 2 Protein**

The amino acid sequence predicted for ROP 2 has a number of interesting features. Two hydrophobic regions are predicted: the first one of these corresponds to a classical signal sequence, as can be expected for a secreted protein, and potential cleavage sites are located between residues 24 and 25, and between residues 26 and 27 in Fig. 7 (von Heijne, 1986). Cleavage of the signal sequence at one of these sites would result in a translocated protein of ~61 kD, close to the *M* found for the immature precursor form of ROP 2 detected in parasites early in pulse-chase experiments (Sadak et al., 1988). A second hydrophobic sequence is present between amino acids 466 and 486 and is largely composed of hydrophobic amino acids with the exception of an aspartic acid at position 472. This segment could form a membrane-spanning α-helix. The remainder of the ROP 2 sequence is characterized by a high abundance of charged amino acids and proline residues.

The ROP 2 protein is known to undergo posttranslational processing, in addition to cleavage of the signal sequence, resulting in a decrease in *M*, from 65 to 55 kD, consistent with the removal of 70–80 amino acid residues. It is not known whether this processing occurs at the amino or carboxy terminus or both, although a potential processing site is present as a cluster of basic amino acids at positions 111–116. It is important to note however, that even if processing were to occur at carboxy terminus, it would most likely not result in removal of the putative transmembrane domain.

When the amino acid sequence shown in Fig. 7 was compared to amino acid sequences contained within current databases no significant homology to known proteins was found, other than to the 54-kD Tg34 antigen, as reported above.

**ROP 2 Is Inserted into the PVM at the Time of Invasion and Is Exposed on the Host Cell Side**

To determine whether antibodies to ROP 2 were responsible for the staining pattern observed when digitonin-permeabilized cells were incubated with the rabbit anti-rhoptry/dense granule antisemur, we incubated the permeabilized cells instead with a ROP 2-specific antisemur, prepared by immunization of a rabbit with a fusion of β-galactosidase and residues 184 through 561 of ROP 2. The specificity of the antisemur is shown in Fig. 5 (lane c). In digitonin-permeabilized cells this antisemur clearly reacts with the PVM (Fig. 8) indicating that ROP 2 is indeed present on the host cell side of the PVM. In addition, ROP 2 appears to be present in the PVM of all intracellular parasites as soon as 10 min after addition of the parasites to host cells (Fig. 8, A and B), suggesting that rhoptry proteins are inserted into the PVM during invasion.

To determine more precisely whether ROP 2 is associated with the PVM itself, or with parasite or host cell structures present in close proximity to the PVM, we performed immunoelectron microscopy on *Toxoplasma*-infected cells using the ROP 2 antisemur. As would have been predicted from the results obtained at the light microscope level, gold label in the infected host cell was found almost exclusively (78 ± 10%) associated with the PVM (Fig. 9). No host cell structure appeared to react specifically with the ROP 2 antisemur. In the parasites, the antisemur reacted primarily (81 ± 10%) associated with the PVM (Fig. 9, inset). The weak labeling of the parasite surface observed in Fig. 9 is not seen consistently and is believed to be nonspecific.

No monospecific antisera directed against ROP 3, 4, or 7 are available at this moment, making it impossible to determine whether these proteins are present in the PVM as well.

**ROP 2 Is Intimately Associated with Membranes in the Rhoptries and in the PVM**

Determining the nature of the interaction of ROP 2 with the
Figure 7. Sequence of the ROP 2 gene. DNA and predicted amino acid sequence of the ROP 2 gene. The arrows indicate the potential signal sequence cleavage sites and the putative transmembrane domain is underlined. The asterisk indicates the position where an additional G is found in the sequence reported for Tg34, which is otherwise identical to the ROP 2 sequence. The boxed region indicates a 14 nucleotide palindrome of unknown significance. These sequence data are available from EMBL under accession number Z36906.
membranes in the rhoptries and the PVM has important implications for our understanding of how this protein becomes associated with the PVM and how the PVM is formed in general. If ROP 2 is soluble while in the rhoptries, but are more intimately associated with the PVM, one could conclude that they are capable of inserting into a preformed membrane. However, if this protein is an integral membrane protein in both the rhoptries and the PVM, one would have to conclude that protein-containing membranes from the rhoptries are inserted as a whole into the PVM. Analysis of the predicted amino acid sequence of ROP 2 reveals a protein with one potential transmembrane domain, suggesting that ROP 2 may be present in the rhoptry and/or the PVM as an integral membrane protein. Because no sequence information for ROP 3, ROP 4, and ROP 7 is available to date, no prediction can be made regarding their interaction with the membrane.

To determine the type of interaction between ROP 2 and membranes, we subjected rhoptry preparations from extracellular parasites and PVM-enriched preparations from infected cells to a number of treatments known to remove extrinsic proteins from membranes. After treatment of membranes with 1 M KCl or 0.1 M sodium carbonate at pH 11, ROP 2 still sedimented with membranes (Fig. 10). In the presence of detergent, ROP 2 does not sediment and instead remains in the supernatant (Fig. 10) indicating that the rapid sedimentation observed is not due to formation of large, insoluble protein aggregates. These results show that ROP 2 is tightly associated with membranes and is based on the presence of a potential transmembrane domain in its predicted amino acid sequence, most likely an integral membrane protein in both the rhoptries and in the PVM.

Discussion

The intracellular stages of the apicomplexan parasite Toxoplasma gondii are surrounded by the PVM, a membrane of uncertain origin and function. In an approach designed to identify parasite proteins that are exposed on the host cell side of the PVM and therefore likely to be important for the interaction of the parasite with the host cell, we identified ROP 2 and ROP 4 and/or ROP 7 as such proteins. These proteins are localized to the rhoptries in extracellular parasites, but are stably associated with the PVM in infected host cells. Using two ROP 2-specific antibody preparations this protein was clearly localized to the PVM by immunofluorescence and immunoelectron microscopy. ROP 4 and/or ROP 7 appear to be present in the PVM as well, but this conclusion is complicated by the observed cross-reaction of our polyclonal antibody preparations to ROP 7 with ROP 4.

The gene encoding ROP 2 was cloned and sequenced. The predicted amino acid sequence reveals a highly charged, proline-rich protein with a signal sequence and a potential transmembrane domain. The ROP 2 protein is localized to

Figure 8. ROP 2 is present in the PVM immediately after invasion and is exposed to the host cell cytosol. An antiserum to recombinant ROP 2 was used in immunofluorescence on digitonin-permeabilized, T. gondii-infected HFF cells (A and B) 10 min or (C and D) 16 h after the start of infection.
the rhoptries in extracellular parasites, but in the infected host cell it is detectable in the PVM immediately after parasite invasion and throughout the infection. The association of ROP 2 with these structures is very stable as it can only be extracted with detergent but not with either high salt concentrations or high pH. Taken together with the presence of a transmembrane domain in the predicted sequence, these observations suggest that ROP 2 is an integral membrane protein in both the rhoptries and the PVM.

The rhoptry proteins ROP 2, 3, 4, and 7 appear to be related based on several observations. First, several monoclonal antibodies react with both ROP 2 and ROP 3, with ROP 2, ROP 3, and ROP 4 (Leriche and Dubremetz, 1991), or with ROP 4 and ROP 7 (Dubremetz, J. F., unpublished observation). In addition, we show here that polyclonal antibodies affinity-purified on ROP 7 also cross-react with ROP 4. Second, these proteins have similar physical properties such as Mr, oligomerization state and basic pI (Leriche and Dubremetz, 1991). Finally, we present data suggesting that ROP 4 and/or ROP 7 is localized in the infected cell to the PVM as well. Although no sequence information is yet available for any of these proteins other than ROP 2, the observed cross-reactivities and similarities do suggest that ROP 2 may be a member of a family of proteins in the PVM that may perform distinct but related functions, such as the interaction with different types of host cell organelles. The test of this hypothesis awaits the generation of sequence information for ROP 3, ROP 4, and ROP 7 and antibodies that are specific for these proteins.

The observation that during invasion, or soon after, a membrane-associated protein is transferred from the rhoptries to the PVM, is the first direct evidence that the rhoptries may be the source of parasite proteins, and possibly also the lipids, in the PVM in cells infected with *T. gondii*. Although a similar experiment has not been performed in *T. gondii*, rhoptries in *Plasmodium* are known to contain lipid bilayer-like material as observed by electron microscopy. It is not clear how proteins such as ROP 2, and possibly lipids as well, would be transferred from the rhoptries to the PVM. Are the rhoptry contents secreted as membranous vesicles,
containing the PVM proteins, that spontaneously insert into the nascent PVM? Or is there a direct, physical connection between the rhoptries and the nascent PVM that allows the free lateral diffusion of protein and lipids from the rhoptry into the PVM? In the first model, supply of additional lipid and proteins to the growing PVM could be accomplished simply by the continued secretion from rhoptries by the multiplying parasites. In the second model, the continued supply of material from a rhoptry to the connected PVM would be complicated by the fact that the rhoptries in the mother cell disappear during the formation of the two daughter cells, implying that new PVM-rhoptry contacts must be formed throughout the infection. The first model is testable, in that it predicts that rhoptry contents, when isolated from purified, ruptured rhoptries, should spontaneously insert into artificial lipid bilayers.

Three other T. gondii proteins, ROP 1, GRA3, and GRA5 are also found associated with the PVM (Achbarou et al., 1991; Dubremetz et al., 1993; Lecordier et al., 1993; Saffer et al., 1992). As described above, the association of ROP 1 with the PVM is only seen at very early stages of the infection. In addition, ROP 1 cannot be detected on the host cell side of the PVM. GRA 3, on the other hand, is continuously secreted throughout intracellular development of the parasite as a soluble protein and becomes, in part, intimately associated with the PVM through hydrophobic interactions (Ossorio et al., 1994). The predicted amino acid sequence of GRA 3 lacks potential transmembrane domains (Bermudes, D., J. F. Dubremetz, and K. Joiner, manuscript submitted for publication), but does contain a short amphipathic helix. We have not detected GRA 3 on the host cell side of the PVM (data not shown). This indicates that GRA 3 is more likely to be involved in a process occurring within the lumen of the vacuole. The predicted amino acid sequence of GRA 5 reveals, in addition to an amino-terminal signal sequence, a potential transmembrane domain that could serve to anchor this protein in the PVM. Whether this protein is actually present in the PVM as a transmembrane protein remains to be determined. Both GRA 3 and GRA 5 have recently been shown to accumulate in the wall of T. gondii cysts (Dubremetz, J. F., unpublished observations; Lecordier et al., 1993), suggesting that these proteins may be involved in the formation of that structure.

Our understanding of nutrient acquisition by T. gondii has improved dramatically with the detection of pores in the PVM of sufficient size to allow free diffusion of small molecules into the vacuolar space (Schwab et al., 1994). One might speculate that the integral PVM proteins identified thus far might be involved in the formation of these pores. However, the amino acid sequence predicted for ROP 2 has only a single bona fide transmembrane domain, making it an unlikely but not impossible candidate for a pore-forming protein. Another function that the PVM proteins could be involved in is the interaction observed between the PVM and host cell organelles, most notably the mitochondria and the endoplasmic reticulum (Endo et al., 1981). The function of these interactions is not clear, but the parasite may derive a number of essential nutrients specifically from these organelles.

One of the most striking observations we made was that ROP 2 and ROP 4/7 are distributed in the PVM in either a punctate (Fig. 1 F, Fig. 3 F, Fig. 6, D and F) or a more ribbon-like fashion (Fig. 1 D). The significance of this organization is not clear, but it suggests that these proteins are organized in higher order structures in the PVM, possibly due to association with host cell structures. Initial experiments using immunoelectron microscopy (Fig. 9) and double label immunofluorescence experiments using mitochondrion-specific antibodies (data not shown) failed to demonstrate colocalization of ROP 2 with mitochondria. Further localization of the PVM proteins by immunoelectron microscopy and double-label immunofluorescence may give an un-
ambiguous answer regarding their involvement in the PVM-host organelle interaction.

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