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Role of the parafusin orthologue, PRP1, in microneme exocytosis and cell invasion in *Toxoplasma gondii*

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Summary

The association of PRP1, a *Paramecium* parafusin orthologue, with *Toxoplasma gondii* micronemes, now confirmed by immunoelectron microscopy, has here been studied in relation to exocytosis and cell invasion. PRP1 becomes labelled *in vivo* by inorganic ^{32}P and is dephosphorylated when ethanol is used to stimulate Ca^{2+}-dependent exocytosis of the micronemes. The ethanol Ca^{2+}-stimulated exocytosis is accompanied by translocation of PRP1 and microneme content protein (MIC3) from the apical end of the parasite. Immunoblotting showed that PRP1 is redistributed inside the parasite, while microneme content is secreted. To study whether similar changes occur during cell invasion, quantitative microscopy was performed during secretion, invasion and exit (egress) from the host cell. Time-course experiments showed that fluorescence intensities of PRP1 and MIC3 immediately after invasion were reduced 10-fold compared to preinvasion levels, indicating that PRP1 translocation and microneme secretion accompanies invasion. MIC3 regained fluorescence intensity and apical distribution after 15 min, while PRP1 recovered after 1 h. Intensity of both proteins then increased throughout the parasite division period until host cell lysis, suggesting the need to secrete microneme proteins to egress. These studies suggest that PRP1 associated with the secretory vesicle scaffold serves an important role in Ca^{2+}-regulated exocytosis and cell invasion.

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Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan parasite capable of invading most vertebrate hosts. The parasite belongs to the phylum Apicomplexa that is responsible for a wide variety of illnesses such as toxoplasmosis (*Toxoplasma*), malaria (*Plasmodium*), crytosporidiosis (*Cryptosporidium*) and many veterinary diseases. *Toxoplasma gondii* is one of the major causes of opportunistic infection in AIDS patients and can result in severe damage of newborns during congenital infections.

Invasion of a host cell is crucial for the survival of *T. gondii* and is dependent on the mobilization of both intra- and extracellular Ca^{2+} (Pingret *et al*., 1996; Pezzella *et al*., 1997). The complete invasion process takes less than a minute (Morisaki *et al*., 1995). To invade host cells, *T. gondii* tachyzoites (the infective life stage of *T. gondii*) use sequential release from three types of secretory organelles. The microneme contents are released upon host cell contact leading to attachment of the parasite. Shortly thereafter, rhoptries release, and the parasite forms a parasitophorous vacuole while invading the target cell. The dense granules first begin their release when the parasite is in the parasitophorous vacuole within the host cell (Carruthers and Sibley, 1997).

Of the three secretory organelles, the micronemes are the only ones where secretion is Ca^{2+}-regulated (Carruthers and Sibley, 1999). Most of the micronemes are located at the apical end of the cell where they form an extended cap (Matthiesen *et al*. 2001). Secretion is accompanied by extrusion of the conoid, a basket-formed cytoskeletal structure at the extreme apical end through which the micronemes and rhoptries release their contents (Carruthers and Sibley, 1999). This region, besides the posterior end, is the only place where the tightly connected inner membrane complex of the cell cortex is discontinuous (Nichols and Chiappino, 1987).

Previous studies demonstrated the presence of a protein called *p*arafusin *r*elated *p*rotein (PRP1) in *T. gondii* (Matthiesen *et al*. 2001). PRP1 belongs to the phosphoglucomutase (PGM) superfamily. Phosphoglucomutase is an evolutionarily conserved cytosolic glycolytic enzyme that catalyses the interconversion of glucose-1-phosphate to glucose-6-phosphate. However, paralogues or isoforms of PGM exist in different eukaryotic cells, where they are implicated in Ca^{2+}-mediated signalling events that appar-

ently are not related to classical glucose metabolism (Kim *et al*., 1992; Lee *et al*., 1992; Subramanian and Satir, 1992; Subramanian *et al*., 1994; Kissmehl *et al*., 1998; Zhao and Satir, 1998; Fu *et al*., 2000). The deduced sequence of *T. gondii* PRP1 showed high homology to the superfamily member parafusin/PP63 (PFUS) that has been connected to Ca^{2+}-regulated exocytosis in *Paramecium* (Gilligan and Satir, 1982; Hohne-Zell *et al*., 1992; Subramanian and Satir, 1992). *In vivo* $^{32}P_i$ incorporation and association with the secretory vesicles are characteristic of *Paramecium* PFUS. Induction of exocytosis dephosphorylates the *Paramecium* labelled PFUS within milliseconds (Gilligan and Satir, 1982; Zieseniss and Plattner, 1985) and changes its localization (Zhao and Satir, 1998) suggesting that PFUS dissociates from the secretory vesicle. Dephosphorylation and dissociation of *Paramecium* PFUS are blocked in exo$^-$ mutants which suggests that the dephosphorylation and localization changes are linked to exocytosis.

The *T. gondii* PRP1 is a phosphoprotein that localizes to the apical third of the cell (Matthiesen *et al*., 2001). PRP1 co-localized with a rim/cap of apical micronemes by high-resolution fluorescence microscopy in *T. gondii* tachyzoites. Quantification of the co-localized fluorescence stain suggested that only mature micronemes ready for exocytosis (about 24%) have PRP1. PRP1 appeared as the major labelled protein when tachyzoites were incubated with inorganic ^{32}P. We concluded that PRP1 was the PFUS orthologue of *T. gondii* (Matthiesen *et al*., 2001).

Based on this conclusion, using studies on *Paramecium* PFUS as a paradigm, we hypothesized that PRP1 has a function in Ca^{2+}-regulated exocytosis of the microneme in *T. gondii* (Matthiesen *et al*., 2001). In the present study, to test this hypothesis, we have examined whether PRP1 phosphorylation state and/or cellular localization changed with exocytosis stimulated in isolated tachyzoites and in *in vivo* studies of host cell invasion by *T. gondii*.

Results

Recognition of a His-tag labelled PRP1 fusion protein

A full-length version of a PRP1 (Mr 68 kDa) recombinant fusion protein was generated with a N-terminal His-tag (3 kDa) for the investigation of PRP1 biochemical properties. Immunoblots of the expressed and purified His-PRP1 showed that H-15 (histidine tag specific antibody), rPRP1 (GST-PRP1-specific antiserum) and I-2 peptide (antibody specific to PRP1) antibodies all recognized the recombinant protein as a band at M_r 71×10^3 (data not shown). This confirms previous results with immunoprecipitated PRP1 from lysates of isolated tachyzoites (Matthiesen *et al*., 2001).

Localization of PRP1 by electron microscopy

PRP1 has been localized by immunofluorescence and deconvolution analysis to the micronemes in isolated tachyzoites (Matthiesen *et al*., 2001). To examine the localization of PRP1 to the apical end of the parasite in greater structural detail, both ultra-thin LR White- and cryo-sections were produced. Some sections were immunogold labelled with specific I-2 antibody. Both preparations gave identical results. Figure 1A shows a typical cryo-section of the apical conoid (C) ringed by microtubules (MT). The conoid is filled with micronemes identified by size and shape; additional micronemes line the micro-

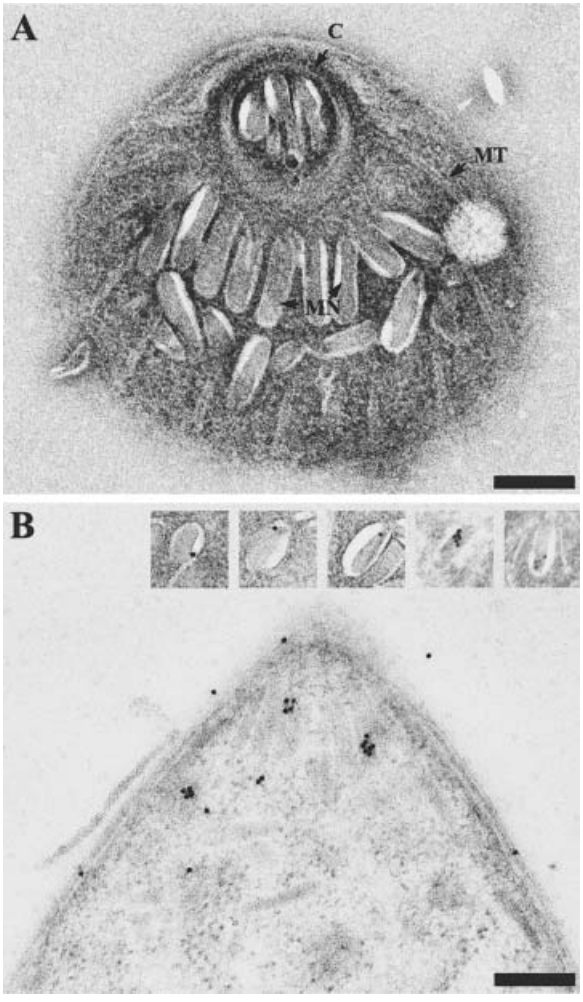

Fig. 1. Immunogold localization of PRP1 to micronemes of isolated *T. gondii* tachyzoites.
A. CryoEM image demonstrates that micronemes (MN) densely populate the region inside and below the apical located microtubule (MT) organizing conoid (C).
B. ImmunoEM images of the apical end of *T. gondii* labelled with the PRP1 specific I-2 antibody. In post-embedding gold particle labelling of LR white sections shows that PRP1 is localized to the micronemes. ImmunocryoEM (insert) confirms that the observed gold labelling is to the micronemes. The scale bar represents 200 nm.

tubules. Figure 1B shows clusters of gold localizing to organelles in the apical tip corresponding to the micronemes in LR White preparations. The insert shows a panel of gold-labelled micronemes from cryopreparations. Not all micronemes showed localization with PRP1, supporting previous data suggesting that only a fraction of mature organelles ready to release have PRP1 attached (Matthiesen *et al*., 2001).

PRP1 becomes phosphorylated in vitro *with UDP-glucose*

If PRP1 is a PFUS orthologue, it should have the ability to be phosphoglucosylated (Satir *et al*., 1990). To determine if PRP1 would serve as an acceptor for a glucose phosphotransferase (Fig. 2A), we prepared *T. gondii* lysates and incubated them with uridine (β-thio) diphosphate glucose [^{35}S] ([β-^{35}S]UDP-glucose). A band of the expected mobility for PRP1 (M_r 68 kDa) was labelled in lysates from isolated tachyzoites. Since the presence of the breakdown product [α-^{35}S]-glucose-1-phosphate from [β-^{35}S]-UDP-glucose could artifactually label PRP1 (Marchase *et al*., 1987; Satir *et al*., 1990), a chase was performed to determine if labelling was due to a potential labeling of an active PGM site of PRP1. Therefore, high excess levels of cold glucose-1-phosphate or UDP-glucose were used in order to chase the labelled species (Fig. 2A). No competition was observed with cold glucose-1-phosphate, but cold UDP-glucose reduced the labelling to 12%, indicating that a glucose phosphotransferase activity was the principal source of the labelling. Immunoblotting using the antiserum against PRP1 (rPRP1) showed that the lanes were loaded with the same amount of protein (Fig. 2B). Labelling was performed in the presence of 1 mM EGTA suggesting that the glucose phosphotransferase activity is not Ca^{2+}-dependent.

To confirm that PRP1 was the protein labelled by [β-^{35}S]-UDPglucose, we added recombinant His-PRP1 to *Paramecium* homogenates and labelled the mixture. As expected, label was incorporated into PFUS preserved in the homogenate, whether or not His-PRP1 was added. An additional labelled band was found on the autoradiogram at the expected size of the *T. gondii* His-PRP1 fusion protein, when His-PRP1 was present (Fig. 2C). We conclude that the glucose phosphotransferase in the *Paramecium* homogenate was also capable of adding labelled glucose-1-phosphate to His-PRP1.

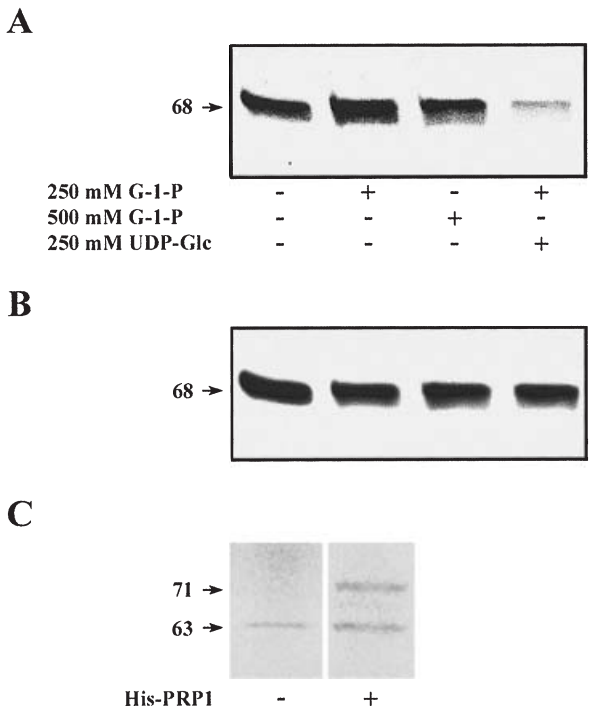

Fig. 2. *In vitro* labelling of PRP1 with [β-^{35}S]UDP-glucose.
A. The autoradiogram shows a predominant labelled protein at M_r 68 kDa in tachyzoite lysate. The labelling was chased with excess of glucose-1-phosphate (G-1-P, lane 2 and 3) or UDP-glucose (UDP-Glc, lane 4). Excess of unlabelled glucose-1-phosphate does not reduce the labelling by [β-^{35}S]UDP-glucose, but excess of unlabelled UDP-glucose does.
B. Immunoblot with rPRP1-specific antibody shows that the same amount of protein has been loaded in all lanes.
C. PFUS in *Paramecium* lysate gets labelled by [β-^{35}S]UDP-glucose. When *T. gondii* recombinant His-PRP1 is added to *Paramecium* lysate, it becomes labelled as well. The arrows and numbers ($\times 10^3$) indicate the M_r of the bands.

Stimulated exocytosis results in dephosphorylation of PRP1

We previously showed by immunoprecipitation that PRP1 becomes labelled *in vivo* when intact *Toxoplasma* tachyzoites were incubated with inorganic $^{32}P_i$ in MOPS-buffer (Matthiesen *et al*., 2001). It has earlier been demonstrated that these cells can be stimulated to exocytose their micronemes by ethanol treatment (Carruthers *et al*., 1999a). To determine if the Ca^{2+}-dependent exocytosis of micronemes was correlated with the phosphorylation state of PRP1, we stimulated secretion with Ca^{2+}-ethanol for 2 min (Fig. 3). When 200 mM ethanol was added to labelled cells, labelling of PRP1 was reduced significantly to 56 ± 15% (mean ± S.D)(*t*-test $P < 0.033$, $n = 3$). Ca^{2+}-dependence of the PRP1 dephosphorylation was examined by pretreatment of the tachyzoites with BAPTA/AM, a highly selective chelator of intracellular Ca^{2+}. The treatment with BAPTA/AM resulted in no dephosphorylation of PRP1, suggesting that dephosphorylation of PRP1 is dependent on intracellular Ca^{2+}.

PRP1 changes localization upon stimulated exocytosis

In Nomarski images an apical tip corresponding to the

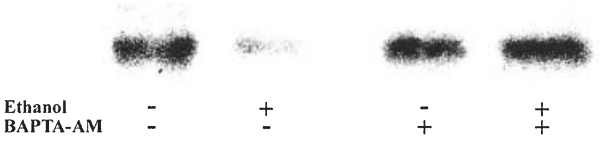

Fig. 3. Stimulated exocytosis effect on PRP1 phosphorylation state. Autoradiogram (ARG) of isolated *T. gondii* tachyzoites that have incorporated $^{32}P_i$ and were treated *in vivo* with or without 200 mM ethanol for 2 min. If the tachyzoites were pretreated with BAPTA/AM no dephosphorylation was observed. The level of dephosphorylation was determined with a phosphorimager from three independent experiments. Immunoblotting was done with rPRP1-specific antibody showing the same amount of PRP1 in the lanes (data not shown).

extruded conoid can be seen. In ethanol treated cells, the numbers of cells with extruded conoids increase, suggesting that these images indicate cells where microneme exocytosis has occurred (Fig. 4A, arrowheads). Tachyzoites were also examined for changes of microneme content (MIC3) and PRP1 localization by fluorescence microscopy when stimulated by ethanol for 2 min (Fig. 4A). Although there was some reduced staining, the strong apical staining of the non-stimulated tachyzoites for both markers disappeared when exocytosis was stimulated. Quantification of the fluorescence intensity showed a significant decrease in overall staining of PRP1 (*t*-test $P < 3.6 \times 10^{-5}$, Fig. 4B). The fluorescence intensity of MIC3 was reduced by 25% ($P < 0.043$, Fig. 4B).

Microneme content is exocytosed but PRP1 is redistributed upon stimulation

After ethanol treatment, the tachyzoites were spun down and the supernatant was examined to determine whether the observed decrease of PRP1 or microneme content fluorescence was a result of exocytosis. Whereas MIC3 antibodies were used for immunofluorescence studies, antibodies to microneme content protein MIC2 were used as a favourable marker for immunoblot studies of exocytosis because the cellular form of 115 kDa is processed to a 95 kDa upon secretion (Wan *et al.*, 1997; Carruthers *et al.*, 2000). Aliquots of supernatants before versus after ethanol treatment were measured. For quantification, the amount of MIC2 and PRP1 were determined in various concentrations of whole cell lysates. There was some minor spontaneous release of MIC2 in the presence of Ca^{2+}-containing buffer alone, but about 28% of total MIC2 was released and processed within 2 min of ethanol addition (Fig. 5). No PRP1 was detected in the tachyzoite supernatant, suggesting that parasite lysis is minimal in our procedure and that PRP1 is not secreted, but redistributed within the tachyzoites upon exocytosis.

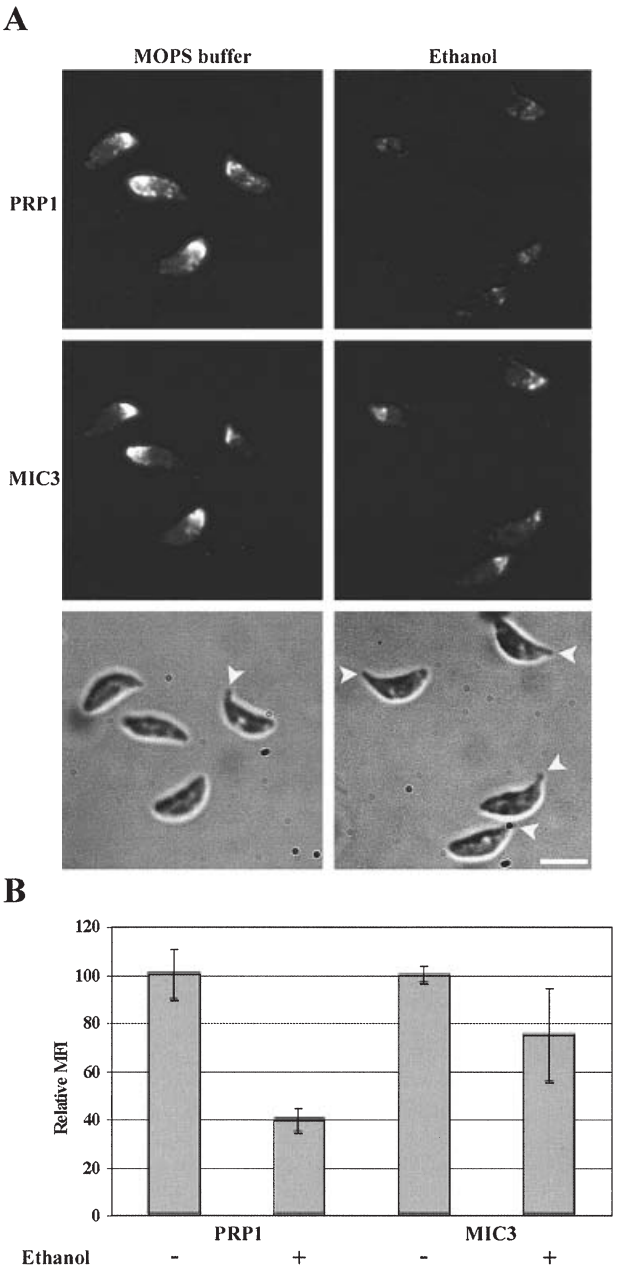

Fig. 4. Stimulated exocytosis effect on PRP1 localization.
A. Immunolocalization of PRP1 (I-2 antibody) and microneme secretory organelle protein MIC3 (MIC3 antibody) in isolated tachyzoites before and after ethanol-stimulated exocytosis. The apical staining of the tachyzoites is reduced after exocytosis. The arrowheads show extruded conoids. The digital images before and after stimulated exocytosis were taken with identical exposure time. The scale bar represents 5 μm.
B. Quantification of the difference in the relative mean fluorescence intensity (MFI) of PRP1 and MIC3 after the ethanol-stimulated exocytosis in tachyzoites. The bars represent the standard deviation.

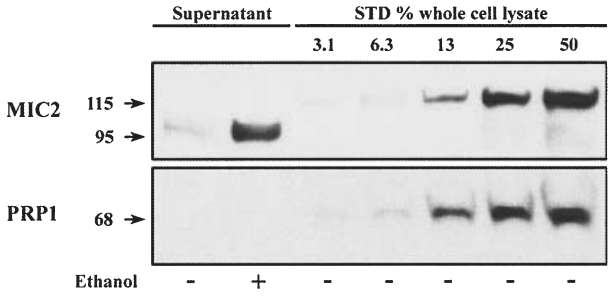

Fig. 5. PRP1 is not released as a secretory product. No recognition of PRP1 in extracellular fluid was observed when exocytosis was stimulated with MOPS-buffer or ethanol in immunoblots with antiserum to PRP1 (rPRP1). The content protein MIC2 was secreted upon stimulation. A serial dilution of tachyzoite lysate (STD whole cell lysate), from non-ethanol stimulated parasites, was used to estimate the amount of ethanol-secreted MIC2 proteins. The arrows and numbers ($\times 10^3$) indicate the M_r of the bands.

In vivo *cell invasion*

A time-course experiment was performed to examine the localization of PRP1 and MIC3 when the parasite invades a human foreskin fibroblast (HFF). Isolated tachyzoites were allowed to invade host cells for 5 min, then free tachyzoites were removed by washing. The parasites in the HFF cells were followed through several replication cycles to host cell lysis after about 40 h.

Consistent with the results for Ca^{2+}-ethanol induced exocytosis in isolated tachyzoites, there was significant reduction in fluorescence intensity of PRP1 and MIC3 within the parasitophorous vacuole 2 min after contact of tachyzoites with HFF cells (Fig. 6). MIC3 fluorescence then began to increase quickly and was at preinvasion levels by 15 min after initial contact. PRP1 fluorescence took longer to recover and full recovery of localization was not seen until 1 h after invasion. As the parasite replicated, apical co-localization of PRP1 and MIC3 was maintained until host cell lysis. The results were quantified by measurements of relative mean fluorescence intensity (MFI) (Fig. 7), with the MFI of PRP1 and MIC3 both standardized to 1.0 in the preinvasion isolated tachyzoites. At 2 min after invasion, both MFIs dropped to 9% of preinvasion levels. MIC3 recovered to preinvasion levels by 15 min and then continued to increase, rising to about 4× the preinvasion levels before parasite replication began 8 h after contact. PRP1 did not recover to preinvasion levels until 1 h after invasion and then increased as well to about 4× the preinvasion level. PRP1 and MIC3 levels both remained balanced at about 4× the preinvasion throughout the replication period. At host cell lysis MIC3 dropped drastically, returning to levels found in preinvasion cells. PRP1 fluorescence levels persisted to variable extents in different parasites, but also dropped on average to preinvasion levels.

Discussion

We previously hypothesized that *T. gondii* PRP1 has a function in Ca^{2+}-regulated exocytosis of the microneme based upon its structural similarity to *Paramecium* PFUS (Matthiesen *et al*., 2001). PRP1 was localized to micronemes by high-resolution fluorescence microscopy (Matthiesen *et al*., 2001). We have confirmed the localization to micronemes of the conoid region by immunoelectron microscopy. Likewise, PFUS has been identified as a 'coat' protein of the dense core secretory vesicles (trichocysts) of *Paramecium*. These vesicles undergo exocytosis in a Ca^{2+}-dependent manner, accompanied by changes in localization and phosphorylation of PFUS (Zhao and Satir, 1998). It is the first time that a PGM superfamily member other than *Paramecium* PFUS has been localized to a secretory organelle. We have now examined the localization and phosphorylation characteristics of PRP1 during exocytosis and host cell invasion by *T. gondii*. With the results presented here it seems probable that PRP1 is the PFUS orthologue of *T. gondii*.

PRP1 is labelled by [β-^{35}S]UDP-glucose and shares this characteristic phosphoglucosylation with other PGM superfamily members, but not PGM itself, in organisms ranging from yeast to mammalian cells (Srisomsap *et al*., 1988; Satir *et al*., 1990; Dey *et al*., 1994; Veyna *et al*., 1994; Chilcoat and Turkewitz, 1997). The glucose transferase that phosphoglucosylates PFUS apparently also phosphoglucosylates PRP1. The dominant protein labelled with $^{32}P_i$ in *T. gondii in vivo* is PRP1 (Matthiesen *et al*., 2001). We show that PRP1 loses $^{32}P_i$ label when Ca^{2+}-dependent exocytosis of the micronemes is stimulated with ethanol. In PFUS, this loss of label is probably due to a dephosphoglucosylation, removing labelled Glc-1-P from the protein via a Ca^{2+}-activated phosphodiesterase (Satir *et al*., 1990; Subramanian and Satir, 1992). If this is so for PRP1, which we suggest, it would imply that the carbohydrate cycle in signal transduction, suggested for *Paramecium* exocytosis (Subramanian and Satir, 1992) is found in other alveolate protozoa, and elsewhere as well.

Ethanol has been shown to stimulate microneme exocytosis by elevating the intracellular levels of Ca^{2+} in the cytoplasm of the parasite (Carruthers *et al*., 1999a; Lovett *et al*., 2002). We have confirmed that microneme content protein is released into the supernatant and processed after ethanol-Ca^{2+} stimulation. The reduced fluorescence of microneme content protein after ethanol stimulation indicates as well that exocytosis of micronemes has taken place. Neither rhoptries nor dense granules are stimulated to exocytose by ethanol and the treatment results in low parasite lysis (Carruthers *et al*., 1999a), as we have confirmed. Ethanol stimulation results in a drop in apical PRP1 fluorescence staining. PRP1 is not secreted, but

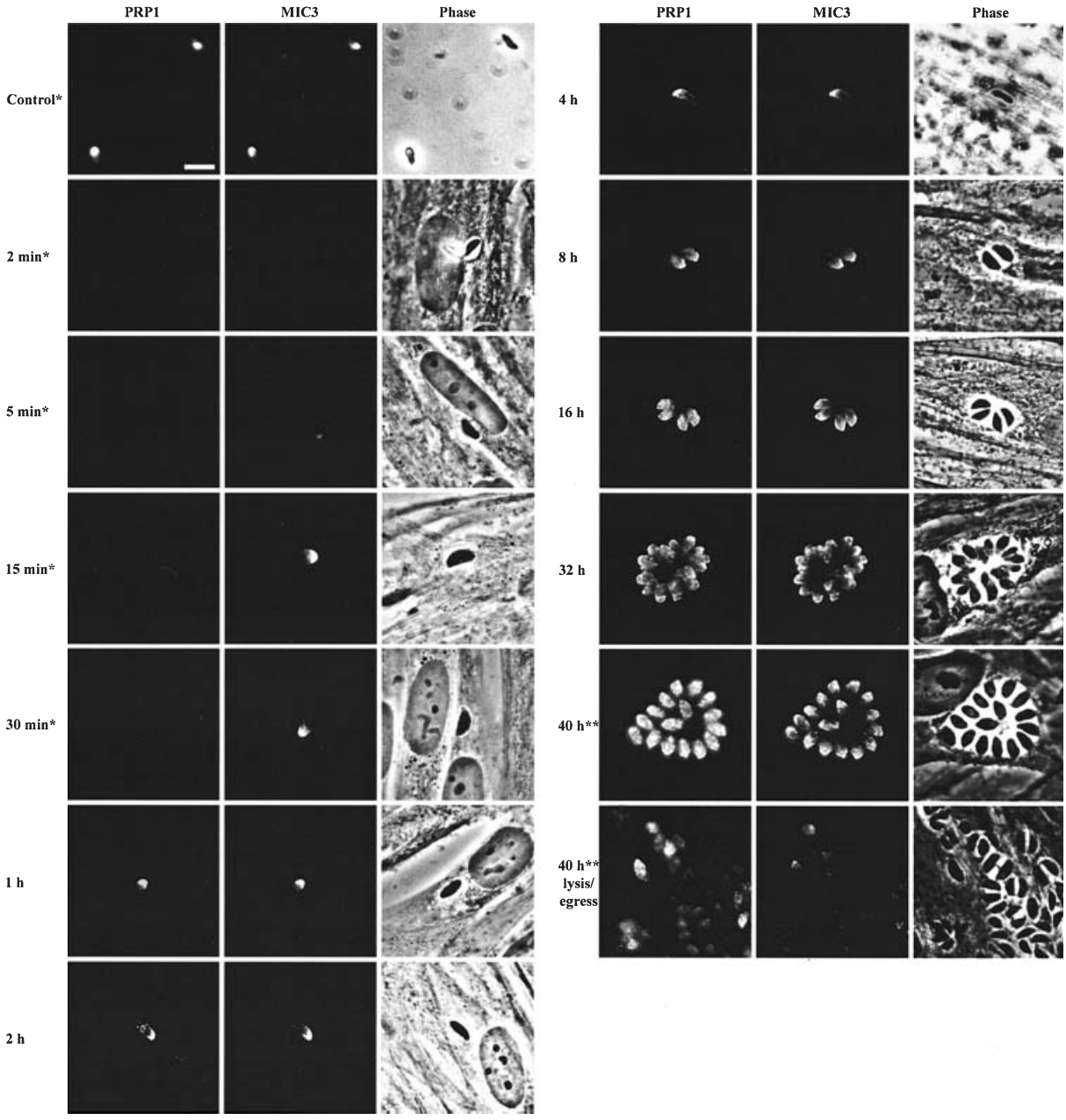

Fig. 6. The effect on PRP1 localization in *T. gondii* by host cell invasion. A time-chase experiment was started at 0 min by inoculating tachyzoites onto HFF cells. Parasites that did not invade were washed away at 5 min. The fluorescence micrographs show that staining of the apical end is reduced after invasion (2 min) for both PRP1 and the microneme content protein MIC3. Replication starts at about 8 h and 'rosettes' with the apical ends of the tachyzoites directed outward have been formed at 32 h. Control is the tachyzoites used for the inoculation (time 0). The scale bar represents 10 μm. The parasites were all, except for the lysed out parasites at 40 h, chosen blindfolded and were all (including the lysed out parasites at 40 h) acquired with the same exposure time. Intensity has been scaled individually for the images from 1 h to 32 h to aid visualization. The images labelled (*) and (**) have the same scaling respectively. For quantification of intensity levels, see Fig. 7.

remains in the cytoplasm in a soluble form not able to bind significant amounts of fluorescent antibody. The lack of recognition of the soluble form is probably due to post-translational modification and/or conformation changes of PRP1. A fraction of 24% on average of microneme proteins has been reported to be released in the supernate upon invasion (Carruthers *et al.*, 1999b). The quantification of micronemal fluorescence in tachyzoites here stimulated with ethanol showed here a 25% reduction. This reduction in fluorescence correlates to the approximately 28% of microneme content released in the supernate by ethanol stimulation shown here and by others by immu-

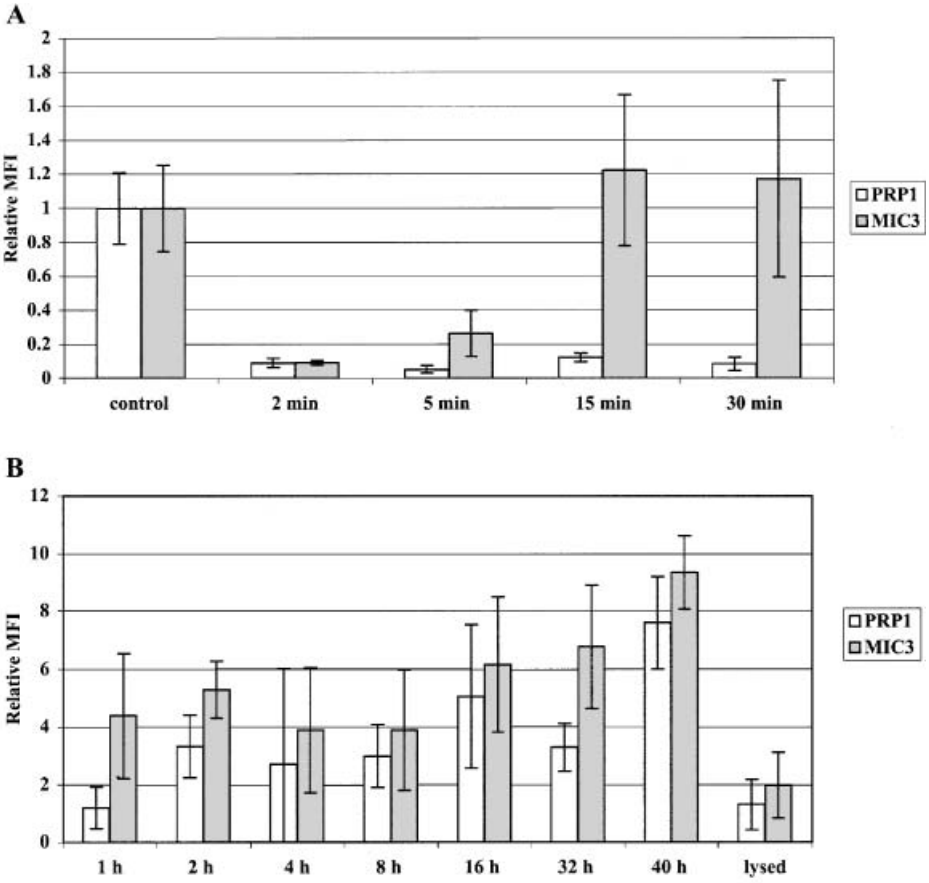

Fig. 7. The quantitative analysis of the mean fluorescence intensity (MFI) in *T. gondii* before and after invasion of HFF cells.
A. The column chart shows MFI of PRP1 (white) and MIC3 (grey) the first 30 min after inoculation of tachyzoites. The analysis confirms the qualitative observations, made in Fig. 6, that both PRP1 and MIC3 localization are reduced in the apical end after invasion.
B. The chart continues from (A) showing what happens with the localization in the parasitophorous vacuole from 1 h until the beginning lysis of the HFF cells at 40 h after invasion. The level of MIC3 and PRP1 MFI increases several fold above preinvasion levels 1 (MIC3) or 2 h (PRP1) after invasion. Notice that the scale of the units on the *y*-axis in (B) is different than in (A). Standard deviations are indicated by the bars.

noblot (Carruthers *et al*., 1999a). We have earlier reported that on average 24% of microneme contents co-localized with PRP1 as an apical rim (Matthiesen *et al*., 2001). The ethanol-stimulation data further support the suggestion that PRP1 is assembled, probably as a coat-associated protein, on a subpopulation of micronemes competent for exocytosis.

The change of phosphorylation of PRP1 was similar to the change in fluorescence staining upon ethanol-stimulated exocytosis. The dephosphorylation of PRP1 was prevented by BAPTA/AM, which efficiently blocks microneme secretion as well (Carruthers *et al*., 1999a). Because dephosphorylation and changes in localization of PRP1 take place at the same time, the two are likely to be related. When PRP1 is $^{32}P_i$ labelled in tachyzoites before Ca^{2+}-ethanol treatment, PRP1 and microneme content protein co-localize. The dephosphorylation of PRP1 at the time of exocytotic stimulation may lead to its dissociation from micronemes.

PRP1 may play an indirect role in exocytosis by regulating intracellular Ca^{2+} levels. This has been suggested by a knockout of the major isoform of PGM in *Saccharomyces cerevisiae* which led to an increase in Ca^{2+} uptake rate resulting in a remarkable increase in total cellular Ca^{2+} (Fu *et al*., 2000). The authors hypothesized that the accumulation of Glc-1-P was coupled to the elevated Ca^{2+} levels by some mechanism. The same group recently confirmed their hypothesis and showed that the cellular Ca^{2+} homeostasis in yeast is responsive to the relative levels of Glc-1-P and Glc-6-P (Aiello *et al*., 2002). *Saccharomyces cerevisiae* is, however, not capable of regulated exocytosis, but the results may suggest a general regulatory role for PGM superfamily members that are orthologues of PRP1.

The presence of an orthologue in *Tetrahymena thermophila*, a ciliate closely related to *Paramecium* (Sogin and Silberman, 1998), was suggested by the presence of a 62 kDa protein which showed dephosphorylation in a Ca^{2+}-dependent manner when exocytosis was induced (Satir and Murtaugh, 1988). One *Tetrahymena* PGM-like gene was cloned and the macronucleus knockout of the gene did not cause any obvious defect in exocytosis (Chilcoat and Turkewitz, 1997). The conclusion of this knockout study was that it was unlikely that the PGM-like molecule would have a potential role preceding membrane fusion. Neither the localization of the PGM-like protein nor the ultrastructure of the mutant was examined. It is not clear if the *Tetrahymena* protein has a function in Ca^{2+} homeostasis as observed with the major isoform of PGM in *S. cerevisiae*. Additionally, that no phenotypic effect was observed could be due to redundancy or substitute pathways (Matthiesen *et al*., 2001).

Although ethanol stimulation is useful in testing what happens when elevated Ca^{2+} levels stimulate microneme exocytosis, it does not reproduce all signalling events that occur during parasite attachment, invasion and formation of the parasitophorous vacuole. The majority of the tachyzoites used in the ethanol experiments are not ready or capable of invading host cells. The invasion protocol has the advantage of effectively synchronizing the tachyzoite population being examined, as parasites that do not invade fibroblasts within 5 min are washed away; in addition the full repertoire of signalling in a system close to the natural course of tissue invasion is utilized. These are particular advantages for studying the role of PRP1 protein in microneme exocytosis.

After invasion the staining intensity of both the PRP1 and the microneme content protein MIC3 was greatly reduced. These data are similar to the results obtained with ethanol-stimulated exocytosis, but not surprisingly, the effect was more dramatic, probably because only *T.* gondii tachyzoites capable of successful invading host cells *in vivo* were quantified, whereas all tachyzoites were quantified when ethanol stimulated including not competent of invasion. The results support the hypothesis that changes in PRP1 localization accompany microneme exocytosis *in vivo*.

The fluorescence intensity level of MIC3 returns to preinvasion levels sooner than does that of PRP1, which suggests that after invasion, micronemes are resynthesized, but only later are coated with PRP1. The co-localization of fluorescence is re-established by 1 h after invasion and thereafter persists with highest intensity at the apical ends of the parasites. Interestingly, the fluorescent intensities of PRP1 and MIC3 increased to about 4× the preinvasion levels and remained high throughout parasite divisions. One possible explanation is that inside the parasitophorous vacuole, fluorescent intensity is increased artificially, but a second explanation, which we think more likely, is that both molecules continue to be synthesized as the cell cycle progresses. At host cell lysis, when *T. gondii* egress and new cell invasion occurs, the fluorescence intensity returns to about preinvasion levels. It may be that microneme proteins are released from tachyzoites into the parasitophorous vacuole immediately before egress of the host cell.

Although fluorescence microscopy as used here is quantitative (Femino *et al*., 1998), without additional knowledge of how antigen binding changes with protein concentration, conformation and post-translational modifications and further controls such as quantitative immunoblots, it is not possible to convert the quantitative fluorescence directly into changes in protein amount. A further difficulty is posed by background fluorescence when the antigen (e.g. PRP1) is at low concentration. Nevertheless, the fluorescent changes seen on PRP1 and MIC3 during invasion and egress sufficiently pronounced and reproducible, so that they are meaningful and, even if not fully quantified, strongly indicative of real changes in protein concentration and localization.

The mechanism of host cell egress by *T. gondii* and other parasite Apicomplexa is not well understood. Studies of egress strongly suggest that the same Ca^{2+}-dependent signal transduction components regulate both *T. gondii* invasion and egress. The characteristics for invasion and egress are very similar, but no reports have described if the parasite micronemes are secreted during egress (Hoff and Carruthers, 2002), which our data suggest.

In summary, the data presented here show that profound effects on the characteristics of *T. gondii* PRP1 accompany host cell invasion. PRP1 becomes dephosphorylated (dephosphoglucosylated) in a Ca^{2+}-dependent manner and its localization to the apical end of the parasite is reduced, presumably as a consequence of the signal transduction events leading to Ca^{2+}-dependent microneme exocytosis. Exocytosis of micronemes is essential in the initiation of invasion of host cells by *T. gondii* and other Apicomplexan parasites and is possibly needed in egress too. The staining intensity pattern and localization of PRP1 during invasion and replication of *T. gondii* tachyzoites follows the same staining intensity patterns as microneme content protein. However, whereas micronemal protein is released from the cell upon invasion and then resynthesized as the parasite duplicates, PRP1 is initially re-distributed within the cytoplasm at invasion, and only later relocalizes to the newly synthesized micronemes. The returning co-localization supports the conclusion that PRP1 sits as a component of a scaffold on the cytoplasmic side of the apical micronemes capable of Ca^{2+}-dependent exocytosis and that PRP1 may play an important role in that process during parasitic invasion.

Experimental procedures

Antibodies

The rPRP1-specific antibody is a polyclonal mouse antibody against purified recombinant glutathione S-transferase (GST) - PRP1 (rPRP1) (Matthiesen *et al*., 2001). I-2 is an affinity-purified rabbit antipeptide antibody prepared against the *Paramecium* PFUS I-2 peptide (Subramanian *et al*., 1994; Zhao and Satir, 1998) that specifically recognizes PRP1 in *T. gondii* (Matthiesen *et al*., 2001). The mouse monoclonal antibodies 6D10 against MIC2 (Wan *et al*., 1997) and T42F3 against MIC3 (Garcia-Reguet *et al*., 2000) *Toxoplasma gondii* microneme protein were used to labelled microneme contents. H-15 is an affinity-purified rabbit peptide antibody against the polyhistidine domain of His-tagged proteins (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell lines, cell culture and harvesting

Toxoplasma gondii strain RH tachyzoites were maintained by serial growth in human foreskin fibroblasts (HFF) in Dulbeccos's modified Eagle medium (DMEM) (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Life Technologies) and 2 mM glutamine (Complete DMEM). Cultures were maintained at 37°C in 5% CO_2. The tachyzoites were collected from lysed cultures by centrifugation (400 *g*, 10 min). After washing, either in phosphate-buffered saline (PBS), MOPS-buffer (20 mM MOPS, 37 mM NaCl, 2.7 mM KCl, pH 7.4) or complete DMEM, parasites were passed through 20, 23 and 25-gauge needles, then separated from host cell material by passage through a 3.0 μm-pore Nucleopore filter. The tachyzoites were then resuspended after centrifugation or further washed twice in buffer or medium before use.

Construction, expression and purification of His-PRP1

Polymerase chain reaction was performed on the C20 *PRP1* cDNA clone (Matthiesen *et al*., 2001), containing the entire open reading frame, to obtain the C-terminal 0.33 kb product from the internal *Nco* I restriction site to the stop codon. The antisense primer BHS09 (5′-CCC AAG CTT CCT ACG TAA TGA CAG TTG GC-3′) was used to add a C-terminal *Hin*d III restriction site upstream of the stop codon. The sense primer BHS10 (5′-GTC GAC GAG AAT CTC TTG-3′) started 20 bp upstream from the internal *Nco* I restriction site. The PCR product was purified (QIAquick protocol for PCR, Qiagen, Valencia, CA/USA), cut with *Nco* I and *Hind* III, gel purified (QIAquick gel extraction kit, Qiagen), and directionally cloned into pRSETB (Invitrogen, San Diego, CA/USA). The clone was verified by DNA sequencing. A 1.6 kb *Nco* I digested gel purified fragment that begins at the start codon of *PRP1* and ends at the internal *Nco* I site (Matthiesen *et al*., 2001) was then cloned into the *Nco* I site of pRSETB upstream of the C-terminal 0.33 kb insert. The construct containing the full-length cDNA of His-PRP1 was transformed into *E. coli* BL21DE3 cells by electroporation. A colony was picked from LB-plates (with 35 μg ml^{-1} chloramphenicol and 50 μg ml^{-1} ampicillin) and grown in 2 ml SOB medium with antibiotics at 37°C overnight. One ml of the culture was used to inoculate 100 ml SOB medium at 37°C. When the culture reached an $OD_{600} = 0.5$ expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C. Cultures were grown for 200 min and harvested by centrifugation. His-PRP1 fusion protein was purified on a Probond™ Ni^{2+}-column (Invitrogen) using an imidazole gradient in pH 6.0 under native conditions following the Xpress™ System Invitrogen User's Guide (Invitrogen). The eluted His-PRP1 was first dialysed against 0.1 mM PIPES, 1 mM EDTA, 0.015% NaN_3, pH 7.5 at 4°C overnight, then against protein storage buffer (0.1 mM PIPES, 2 mM $MgCl_2$, 0.5 mM EDTA, 0.01% NaN_3, pH 7.0). The protein concentration was determined by BCA protein assay using albumin as standard (Pierce Chemical, Rockford, IL, USA).

Immunoblotting

SDS-PAGE was performed on 10% polyacrylamide gels (Laemmli, 1970). The proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA) using semidry electroblotting. After blocking in Tris-buffered saline/5% non-fat milk for 1 h, blots were incubated with primary antibody (rPRP1 1 : 600, I-2 1 : 500, MIC2 1 : 5000 or H-14 1 : 800) for 1 h at room temperature and washed. The blots were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or -anti-mouse IgG antibody (Boehringer Mannheim, Germany) for 30 min and washed again. Labelled proteins were visualized by chemiluminescence detection of HRP activity (NEN™ Life Science Products).

Electron microscopy (EM)

The parasites were harvested using PBS. For cryoimmunoEM, cells were fixed in double strength fixative (4% paraformaldehyde, 0.05% glutaraldehyde, 0.1 M cacodylate buffer) for 20 min. The pellet was washed in PBS with 0.05 M glycine, embedded in gelatin, cryoprotected in 2.3 M sucrose, and frozen in liquid nitrogen. Thin sections were done at −110° to −130°C using a Leica UCT cryoultramicrotome (Leica, Bannockburn, IL/USA), transferred to 100 mesh nickel grids (Formvar- and carbon-coated, Polysciences, Warrington, PA, USA), and placed in PBS. The grids were then transferred to PBS, 0.1 M glycine for 15 min, blocked with goat serum block solution (Aurion, Electron Microscope Sciences, Fort Washington, PA/USA) for 1 h, washed five times, incubated with I-2 antibody (diluted 1 : 10) overnight at 4°C, washed seven times, incubated with goat anti-rabbit conjugated to 10 nm gold (Aurion, diluted 1 : 100) for 2 h at 4°C and washed eight times. The above washes and dilutions were done in PBS, 0.1% acetylated bovine serum albumin (BSA-c, Aurion), pH 7.4. Then the grids were washed three times in PBS, fixed 5 min in 2% glutaraldehyde in PBS, washed one time in PBS, and three times in H_2O. Staining was done in 2% uranyl acetate for 15 min and embedding in 0.75% methylcellulose at 0°C for 30 s. For immunoEM, cells were fixed in double strength fixative (4% paraformaldehyde, 0.05% glutaraldehyde, 0.1 M cacodylate buffer), dehydrated through a graded series of ethanol, and embedded in LR White resin (London Resin Company, Surrey, England). Ultrathin sections were cut on a Reichert Ultracut E and transferred to 200 mesh nickel grids (Polysciences) and put in PBS. The grids were transferred to PBS, 0.05 M glycine for 15 min and treated as above until the staining step. The cells were then stained with 4% uranyl acetate for 15 min, washed five times with double distilled H_2O, stained with lead citrate for 2 min and washed five times with H_2O. JEOL 100CXII or JEOL 1200EX

transmission electron microscopes were used to examine the cells on the grids at 80 kV.

In vitro *[β-^{35}S]-UDP-glucose labelling*

The tachyzoites (5×10^8 cells) were harvested, washed with PBS, resuspended in 300 µl lysis buffer [50 mM Tris-HCl, pH 7.3, 2 µM leupeptin, 1.5 µM antipain, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.012 TIU (trypsin inhibitor unit) ml^{-1} aprotinin], and lysed by sonication (3×10 s, setting 4.5; Sonifier cell disrupter, Model W1850, Branson Ultrasonic, CO, USA). The protein concentration was determined using the method of Bradford (1976). One hundred µg of the lysate was incubated in lysis buffer with 0.3 µCi [β-^{35}S]UDP-Glucose (50 µCi ml^{-1}, 300 Ci mmol^{-1}, American Radiolabeled Chemicals, St Louis, MO, USA) and 1 mM EGTA and 0 or 200 or 400 mM glucose-1-phosphate and 0 or 200 mM UDP-glucose in to a total volume of 50 µl for 30 min at 22°C. The samples were boiled for 8 min in 50 µl 2 × SDS sample buffer and subjected to SDS-PAGE, immunoblotting and autoradiography. Analysis of the data was carried out using a GS-700 Imaging Densitometer and Multi-Analyst PC software (Image Analysis Systems, version 1.1, Bio-Rad, Hercules, CA, USA).

In vivo *labelling of PRP1*

Tachyzoites were harvested and resuspended in 0.4 ml MOPS-buffer (see above) after filtration, and 3.5×10^8 cells were incubated with 50 µCi [$^{32}P_i$] inorganic orthophosphoric acid (NEN™ Life Science Products, Boston, MA/USA) at 37°C for 20 min. As a control for the requirements of internal Ca^{2+} for dephosphorylation of PRP1, tachyzoites were pretreated with 10 µM BAPTA/AM [bis-(o-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid/acetoxymethyl ester] (Molecular Probes, Eugene, OR, USA).

Twenty-five µl MOPS-buffer containing 2 mM Ca^{2+} and 400 mM (or 0 mM) ethanol was pre-equilibrated to 37°C in a water bath. Twenty-five µl labelled cells were added and incubated for 2 min before being cooled on ice (Carruthers *et al*., 1999a). The samples were boiled for 5 min in 50 µl 2× SDS sample buffer, separated by SDS-PAGE and exposed to PhosphorImager screens (Molecular Dynamics, La Jolla, CA, USA) and processed for autoradiography. Analysis of the data was carried out using the IMAGEQUANT version 3.3 software (Molecular Dynamics).

Stimulated exocytosis

Freshly harvested tachyzoites, using MOPS-buffer, were syringed through a 23-gauge needle and washed once. The secretion assay was performed in 96-well round-bottom microtitre plates (Carruthers and Sibley, 1999). Fifty µl MOPS-buffer containing 2 mM Ca^{2+} and 400 mM (or 0 mM) ethanol was pre-equilibrated to 37°C in a water bath for 30 s. Fifty µl tachyzoites were added and incubated for 2 min before being cooled on 0°C ice slurry. Fifty µl cells were then transferred to 50 µl 6% paraformaldehyde in PBS on ice and fixed for 20 min. The tachyzoites were permeabilized for 2 min in PBS, 0.1% Triton X-100 and processed for immunofluorescence.

For examination of the presence of secreted PRP1 in extra cellular fluid (MOPS-buffer), tachyzoites were syringed, filtered and washed. The secretion assay was performed as above but with 70 µl cells/buffer instead of 50 µl. A sample was taken of the parasites on the ice slurry for serial loading of whole cell tachyzoite lysate. The rest of the parasites were then centrifuged (1200 *g*, 5 min) at 0°C. The supernatants and the samples for tachyzoite lysate were boiled with 2× SDS-PAGE sample buffer for 5 min. For PRP1 70 µl of each sample (in SDS-PAGE sample buffer) was concentrated with Microcon YM-3 (Millipore, Bedford, MA, USA) at 14000 *g* for 25 min. The samples used for MIC2 were not concentrated. See the further procedure under 'Immunoblotting' above.

In vivo *invasion*

The HFF were grown in 38 mm Petri dishes with glass coverslip bottoms (MatTek Corporation, MA, USA) in complete DMEM. 5×10^6 freshly lysed parasites were added to the HFF cells and the Petri dishes were incubated at 37°C. Two minutes after inoculation, one dish was washed twice with PBS and fixed with 3% paraformaldehyde in PBS. At 5 min, another Petri dish was fixed as above. The other Petri dishes were washed twice in 37°C PBS to remove non-invading parasites and incubated with complete DMEM at 37°C. The lysed tachyzoites used for inoculation were fixed and used as control for time 0 min. The remaining Petri dishes were fixed after incubation times of 15, 30 min, 1, 2, 4, 8, 16, 32, 40 and 47 h. The cells were permeabilized for 10 min (the control for 5 min) in PBS with 0.1% Triton X-100.

Immunofluorescence

The cells were blocked in washing buffer (PBS, 3% BSA, 0.03% Tween 20). The preparations were incubated with primary antibodies I-2 (1 : 800) and MIC3 (1 : 1000) for 15 min (30 min for invasion experiments), washed, and incubated with secondary FITC-labelled anti-mouse IgG (1 : 75) or CY3-labelled anti-rabbit IgG (1 : 800) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 15 min (60 min for invasion experiments) and washed again. Then the preparations were mounted in PBS, 70% glycerol, 2% *N*-propyl-gallate.

Fluorescence microscopy, digital image acquisition and image analysis

Fluorescence imaging of the stimulated secretion experiment was performed using a Provis AX70 microscope with a PlanApo 60×, 1.4 NA objective and 100 W mercury lamp for epi-illumination (Olympus, Melville, NY, USA). Images were acquired using a Photometrics CoolSNAP HQ digital CCD camera (Roper Scientific, Tuscon, AZ, USA) using IPLab Windows version 3 acquisition software (Scanalytics, Fairfax, VA, USA). Imaging of the invasion experiment was done on an inverted IX70 microscope with a 40× Ph2 LWD objective (Olympus). Images were taken with a Photometrics digital CCD camera (SCSI PXL KAF1400-G2**,** Roper Scientific) using IPLab Macintosh version acquisition software (Scanalytics).

Using IPLab, a region of interest (ROI) was drawn around the phase image of each cell in the image. Each ROI was transferred from the phase image to an aligned fluorescence image of the same field. In each field of cells, an additional ROI was drawn in an area without cells. IPLab measured the total fluorescence

intensity (TFI) and area for each ROI in the fluorescence channel. The measurements were imported to Microsoft Excel where the mean fluorescence intensity (MFI) of each ROI was calculated. For each field of cells, the MFI of the ROI drawn in areas without cells was subtracted from the MFI of the ROIs from each cell measured in the field.

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