

## Subcellular localization of 14-3-3 proteins in *Toxoplasma gondii* tachyzoites and evidence for a lipid raft-associated form

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### Abstract

A polyclonal antibody was raised against a *Toxoplasma gondii* 14-3-3–glutathione *S*-transferase fusion protein obtained by cloning a 14-3-3 cDNA sequence determined from the *T. gondii* database. This antibody specifically recognized *T. gondii* 14-3-3 without any cross-reaction with mammalian proteins. Immunofluorescence microscopy studies of the tachyzoites or the *T. gondii*-infected cells suggested cytosolic and membranous localizations of 14-3-3 protein. Different subcellular fractions were prepared for electrophoresis analysis and immunodetection. 14-3-3 proteins were found in the cytosol, the membrane fraction and Triton X-100-resistant membranes. Two 14-3-3 isoforms were detected. The major one was mainly cytoplasmic and to a lesser extent membrane-associated, whereas the minor isoform was associated with the detergent-resistant lipid rafts.

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### 1. Introduction

14-3-3 proteins constitute a family of highly conserved proteins, present in all eukaryotic organisms [1]. Mammalian cells have been shown to contain five to eight different 14-3-3 genes encoding different isoforms designated by Greek letters [2,3]. In the case of higher plants, 10 isoforms have been identified in *Arabidopsis thaliana* [4], while in the case of yeasts, two isoforms have been described for *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* [5–7]. One isoform was identified in *Candida albicans* [8]. Most 14-3-3 isoforms are located in the cytoplasm, although some are associated with membranes, as

described for the green alga *Chlamydomonas reinhardtii* and for rat brain [9–11]. Although the exact function of 14-3-3 proteins has not been completely elucidated, they are known to stimulate protein–protein interactions by acting as molecular scaffolds or chaperones, and are involved in the regulation of the subcellular localization of proteins, and in the activation or inhibition of enzymes [12]. In mammals, 14-3-3 proteins have been shown to be involved in the regulation of mitosis [13,14] and in signal transduction via the mitogen-activated protein kinase cascade [15,16].

Concerning parasites, the number of 14-3-3 isoforms, their subcellular distribution and their functions are not so well established. We only know that their mRNA is expressed in the protozoon *Plasmodium falciparum* [17] and 14-3-3 proteins are present in helminths such as *Echinococcus multilocularis* and *Echinococcus granulosus* [18] or *Schistosoma mansoni*. In these latter parasites, they have

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been reported to be involved in parasite growth and survival [19].

*Toxoplasma gondii*, another protozoon, is an intracellular parasite known to cause severe congenital infection in humans and animals [20]. As a sporozoite, its sexual reproduction occurs in the intestine of its definitive hosts, the cat, while its asexual multiplication involves nucleated cells of various hosts, in particular humans [20]. 14-3-3 protein has been shown to be present in *T. gondii* during the feline enteroepithelial stage and has not been detected in the tachyzoite, the asexual parasite form [21]. Tachyzoites are able to invade cells and multiply at a high rate in a particular intracellular organite, the parasitophorous vacuole [22,23]. Since previous studies in mammals have involved 14-3-3 proteins in cell multiplication, we chose to reinvestigate the presence of 14-3-3 protein during the asexual stage of the parasite.

The present study reports the presence of a 14-3-3 protein homolog in a *T. gondii* RH strain, a virulent strain with a high tachyzoite multiplication rate. The generation of fusion proteins from the tachyzoite mRNA enabled the production of a rabbit polyclonal anti-*T. gondii* 14-3-3 antibody. This antibody was used to localize 14-3-3 protein in the tachyzoite. This study is the first report of the presence of two different 14-3-3 isoforms in tachyzoites. One is mainly cytosolic and membrane-associated, while the other is only present in the lipid rafts which are Triton X-100-resistant membrane microdomains.

## 2. Materials and methods

### 2.1. Parasite and cell lines

The virulent RH strain of *T. gondii* is maintained by intraperitoneal passages in OF1 female mice. The tachyzoites were collected by peritoneal washing with physiological serum [24]. HeLa cells were grown in Dulbecco's modified Eagle's medium. The THP1 (human monocyte) cell line and THP1 cells infected by the RH strain were grown in RPMI Glutamax medium (Invitrogen, France). Both media were supplemented with 5000 U ml<sup>-1</sup> penicillin, 5000 µg ml<sup>-1</sup> streptomycin and 10% fetal calf serum (Invitrogen, France). Cultures were performed in a humidified 5% CO<sub>2</sub> incubator at 37°C.

### 2.2. Reagents

The protease inhibitor cocktail (leupeptin, aprotinin and pepstatin A) was purchased from Sigma (France). SAG1 (IgG2A) monoclonal antibody (clone PIE1E7) was obtained from Bio-Mérieux (France) and diluted to 1:1000 before use. GRA1 monoclonal antibody was kindly provided by C. Mercier (CNRS, Grenoble, France) and diluted to 1:1000 and 1:20 000 for immunofluorescence and Western blot, respectively. The secondary antibodies were

anti-rabbit Alexa-conjugated antibody used at 1:400 dilution (Interchim, France), and goat anti-mouse phycoerythrin-conjugated antibody diluted to 1:200 (Zymed, USA). All antibodies were diluted in phosphate-buffered saline (PBS) and 1% bovine serum albumin (BSA) for immunofluorescence staining.

### 2.3. Production of *T. gondii* 14-3-3 recombinant protein

Koyama's *T. gondii* Express Sequence Tag database was searched to obtain the open reading frame sequence of 14-3-3 protein that was used to design the following primer pair: (i) sense 5'-ACGGAATTCACGATGGCGGAG-GAAATC-3', creating an *EcoRI* site near the start codon, and (ii) antisense 5'-ACGTCGACTTACTGATCAGCT-TGTTCTG-3', including a *SalI* site near the stop codon [25]. These primers were used to amplify the tachyzoite 14-3-3 cDNA from RNA purified with Trizol reagents (Invitrogen, France). The 14-3-3 cDNA was cloned into the *EcoRI* and *SalI* sites of the pBluescript KS<sup>+</sup> vector (Stratagene, USA). The 14-3-3 pBluescript clone was digested and subcloned into the pGEX-4T-2 expression vector (Amersham Pharmacia Biotech, France). The resulting glutathione *S*-transferase (GST) fusion protein was expressed in *Escherichia coli* DH5α (Invitrogen, France) after induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 4–6 h at 37°C. The protein was then solubilized and purified with glutathione *S*-Sepharose 4B beads (Amersham Pharmacia Biotech, France).

### 2.4. Production and purification of anti-recombinant *T. gondii* 14-3-3 antibody

Antiserum production was carried out by intradermal injections of GST fusion *T. gondii* 14-3-3 protein to rabbits (Covalab, France). Anti-*T. gondii* polyclonal 14-3-3 antibodies were purified on immobilized protein G column (Pierce, USA). After anti-GST antibody depletion by incubation with GST protein and glutathione *S*-Sepharose 4B beads, anti-*T. gondii* polyclonal 14-3-3 antibodies were diluted to 1:250 for immunofluorescence staining and to 1:2000 for Western blot analysis. For antibody characterization, 1.5 × 10<sup>6</sup> HeLa cells were transfected using the lipofectamine method (Invitrogen, France) with 5 µg of *T. gondii* 14-3-3 homologous cDNA subcloned into mammalian vectors PciNeo (Promega, France) and PcDNA (Invitrogen, France).

### 2.5. SDS-PAGE and immunoblot analysis

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis using a 15% gel, then transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore) [26]. Immunodetection was performed using the specific polyclonal or monoclonal antibodies (see Sections

2.2 and 2.4). Detection was performed by peroxidase conjugation using the anti-species secondary antibody (Dako, France) diluted to 1:2000 for anti-rabbit antibody and 1:1000 for anti-mouse antibody. The antigens were detected using enhanced chemiluminescence detection reagents (Amersham, France). The densitometric analysis of the antigens was performed by densitometric scanning with Kodak Digital science 1D Image Analysis software.

### 2.6. Immunofluorescence microscopy

Tachyzoites obtained from ascites were washed three times in PBS, fixed with PBS/2% paraformaldehyde for 10 min, then washed extensively with PBS. The purified polyclonal anti-*T. gondii* 14-3-3 antibody and monoclonal antibodies against SAG1 or GRA1 were applied for 1 h at 37°C. After washing three times with PBS, the parasites were incubated for 45 min at 37°C with mixed secondary antibodies. *T. gondii*-infected THP1 cells were fixed like parasites and permeabilized for 30 min in PBS containing 1% BSA and 0.25% Triton X-100. Then antibodies were applied as described above. The free parasites and *T. gondii*-infected THP1 cells were both deposited on slides, then counter-stained with bisbenzimidazole 3358 used at 0.1 µg ml<sup>-1</sup>. After drying, the slides were mounted in Vectashield (Vector Laboratories, Germany) and were observed under a fluorescence microscope (Axioplan Zeiss, France).

### 2.7. Subcellular fractionation

10<sup>9</sup> tachyzoites were extracted in buffer I (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, protease inhibitors and 1 mM phenylmethylsulfonyl fluoride) by serial cycles of freezing (–180°C) and thawing (37°C). After centrifugation at 600 × *g* for 5 min, the supernatants (LSS) were centrifuged at 150 000 × *g* for 30 min to separate the membranous pellet (HSP), further suspended in buffer I, from supernatant (HSS).

### 2.8. Preparation of Triton X-100-resistant membrane complexes by gradient centrifugation

2 × 10<sup>9</sup> tachyzoites were incubated at 4°C in 2 ml of buffer II (10 mM Tris-HCl, pH 7.5, 1 mM MgCl<sub>2</sub>, 150 mM NaCl) containing protease inhibitors, 60% sucrose and 1% Triton X-100. After 1 h incubation, the extract was overlaid with 4 ml of 50%, 40%, 35%, 30%, 25% sucrose and 3.5 ml of 20%, 15%, 10%, 5% sucrose in buffer II. After ultracentrifugation at 121 000 × *g* for 24 h at 4°C, 1-ml aliquots were collected from the bottom to the top of the gradient tube. Proteins were titrated with the D<sub>c</sub> Protein assay system (Bio-Rad, France). The sucrose concentration was determined with a RRA refractometer (PZO, Poland).

## 3. Results

### 3.1. Characterization of recombinant *T. gondii* 14-3-3 antibodies

Anti-*T. gondii* 14-3-3 homologous antibodies depleted of anti-GST antibodies showed, after SDS electrophoresis and Western blot, no reaction with GST (Fig. 1A, lane 1) and a positive reaction with GST-*T. gondii* 14-3-3 homologous fusion protein which migrated at 61 kDa (Fig. 1A, lane 2). These antibodies recognized only one band migrating at 36 kDa in tachyzoite cytosol extracts (Fig. 1A, lane 3), indicating the presence of a 14-3-3 protein homolog in *T. gondii* tachyzoites. The differences in apparent molecular mass observed between the protein detected in Fig. 1A, lane 2 and that in lane 3 are due to the presence of the GST sequence. When lysates from HeLa cells transfected with different vectors were used, only cells containing the 14-3-3 homologous cDNA vector reacted positively at 36 kDa for PciNeo and 40 kDa for PcDNA (Fig. 1B). The difference in molecular mass is related to the presence of a histidine tag in the PcDNA vector.

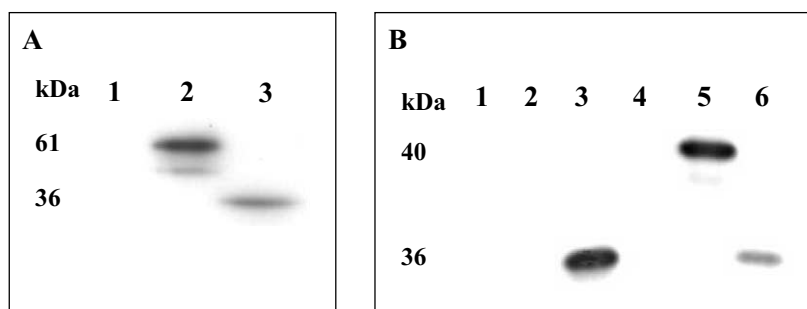


Fig. 1. Characterization of rabbit polyclonal anti-*T. gondii* 14-3-3 antibody after electrophoresis and immunoblotting. A: 1 µg of GST protein (lane 1), 14-3-3 homologous fusion protein (lane 2), cytosol of RH strain tachyzoites (lane 3). B: 10 µg of proteins obtained from untransfected HeLa cells (lane 1); PciNeo vector (lane 2); PciNeo/14-3-3 insert (lane 3); PcDNA vector (lane 4); PcDNA/14-3-3 insert (lane 5); cytosol of RH strain tachyzoites (lane 6).

### 3.2. Localization of *T. gondii* 14-3-3 protein homolog by immunofluorescence analysis

The 14-3-3 protein homolog was localized in free *T. gondii* tachyzoites by immunofluorescence analysis using the polyclonal anti-*T. gondii* 14-3-3 homologous antibody and antibodies specific for either cytosolic (GRA1) [27] or membrane glycosylphosphatidylinositol (GPI)-anchored (SAG1) proteins [28]. When both anti-*T. gondii* 14-3-3 and SAG1 antibodies were used simultaneously on unpermeabilized tachyzoites, the SAG1 labeling was effectively found in the pellicle of the tachyzoites whereas 14-3-3 antibody produced a more diffuse labeling with spots in the cytosol. Moreover, the presence of yellow spots in the overlay view (Fig. 2A) suggests a partial co-

localization of 14-3-3 protein with SAG1 in the *T. gondii* membrane.

When both anti-*T. gondii* 14-3-3 and GRA1 antibodies were used simultaneously on unpermeabilized tachyzoites (Fig. 2B), the 14-3-3 labeling was similar to that observed in Fig. 2A, as expected. The very low labeling obtained with GRA1 antibody (Fig. 2B) suggests a weak diffusion of the antibody into the parasites. This absence of labeling confirms the cytosolic localization of the protein. The overlay view of GRA1 and 14-3-3 (Fig. 2B) showed no well-defined yellow spots, indicating the absence of protein overlapping.

Tachyzoites were then treated with Triton X-100 before antibody labeling. Under these conditions, the binding of SAG1 disappeared, suggesting either a membrane solubi-

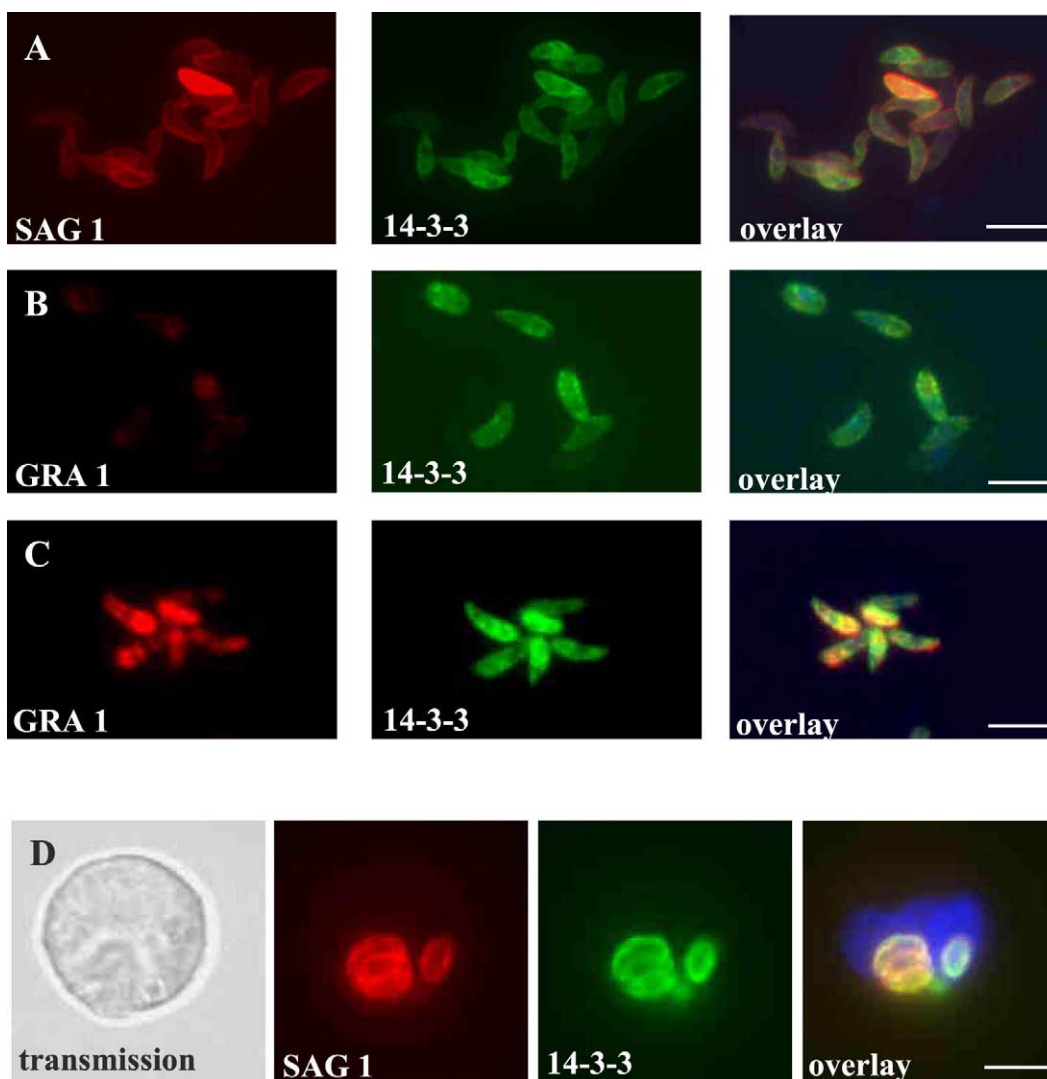


Fig. 2. Immunofluorescence of paraformaldehyde-fixed, double-stained *T. gondii* parasites or THP1 cells infected with *T. gondii*. Antibodies used for double staining were: line A, anti-14-3-3 antibody and monoclonal SAG1 antibody; line B, anti-GRA1 monoclonal antibody and anti-14-3-3 antibody; line C, parasites permeabilized with Triton X-100 were stained with monoclonal anti-GRA1 antibody and anti-14-3-3 antibody; line D, infected cells were fixed and permeabilized before staining with mixed anti-SAG1 and anti-14-3-3 antibodies. The stains were revealed with anti-species secondary antibodies (see Section 2.2). Counter-staining was performed with bisbenzamide 3358. The images were acquired with an Axioplan microscope (Zeiss, France) and the overlapping analysis was performed with Soft imaging system software (Eloise, France) (bar = 5  $\mu$ m).

lization or the absence of epitope recognition by the specific antibody, while the 14-3-3 labeling persisted (data not shown).

When both anti-*T. gondii* 14-3-3 and GRA1 antibodies were used simultaneously on permeabilized tachyzoites (Fig. 2C), both views showed intense labeling with red or green spots, reflecting effective antibody penetration. The presence of intense yellow labeling in the overlay view indicates a colocalization of both proteins in the cytosol of free tachyzoites.

To see if intracellular tachyzoites as well as free tachyzoites can induce 14-3-3 production, RH strain-infected THP1 cells were studied. Without permeabilization, proteins could not be detected in tachyzoites with either 14-3-3 or SAG1 antibodies. Cells were then permeabilized to allow the penetration of the antibodies. This enabled tachyzoite membrane labeling by both 14-3-3 and SAG1 antibodies (Fig. 2D), showing the integrity of the parasite membrane. Furthermore, colocalization of the two proteins in the tachyzoite membrane was observed in the overlay view (Fig. 2D).

### 3.3. Subcellular distribution of 14-3-3 proteins

The different subcellular fractions obtained from freshly prepared tachyzoites were analyzed by SDS-PAGE and Western blot analysis using the anti-14-3-3 antibody. 14-3-3 protein was detected in the post-nuclear fraction (Fig. 3, lane LSS). After high-speed centrifugation of the LSS, the protein was concentrated in the soluble cytosolic

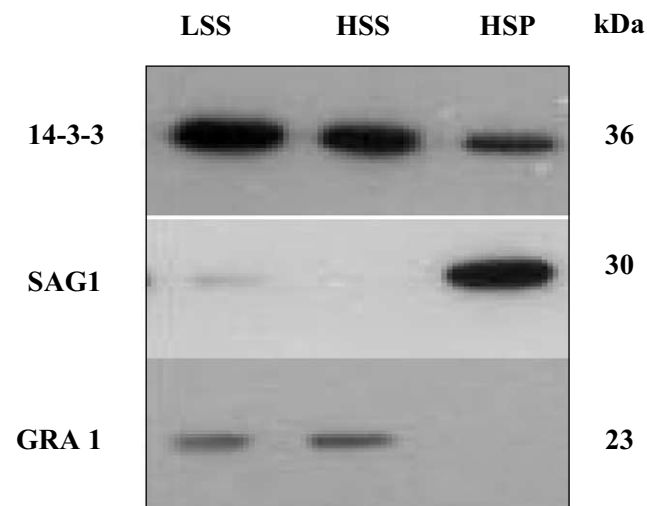


Fig. 3. Western blot analysis of subcellular fractions of *T. gondii*. Tachyzoites were lysed by serial freezing and thawing in lysis buffer. The homogenates were centrifuged at low speed ( $600\times g$ ). The post-nuclear fraction (LSS) was subjected to high-speed centrifugation to separate the supernatant (HSS) and the pellet (HSP). Aliquots of the different subcellular fractions (1  $\mu$ g protein per lane) were separated by 15% SDS-PAGE, then transferred to PVDF membrane before immunostaining with either polyclonal anti-14-3-3 antibody raised against the GST/14-3-3 fusion protein depleted of anti-GST antibody, or monoclonal anti-SAG1 and anti-GRA1 antibodies.

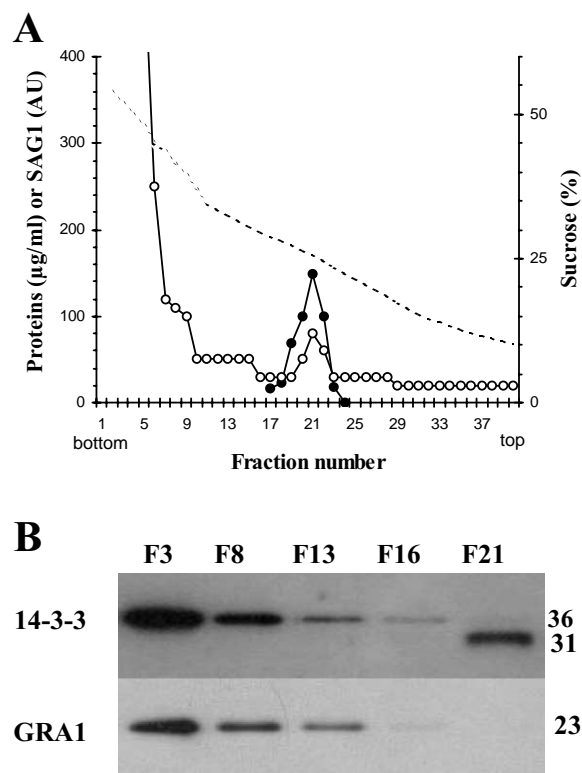


Fig. 4. Density gradient detection of *T. gondii* DRM and 14-3-3 protein association. A: The amounts of total proteins (open circles) and the percentage of sucrose (dotted line) were measured for each fraction. 1  $\mu$ g of the fractions which formed the DRM peak were analyzed by Western blotting with monoclonal SAG1 antibody and densitometric scanning (dark circles). The amount of SAG1 protein is expressed in arbitrary units. B: Different gradient fractions were analyzed with the polyclonal anti-14-3-3 antibody depleted of anti-GST antibody or with monoclonal GRA1 antibody.

fraction (Fig. 3, lane HSS) but was also present at a lower level in the membrane fraction (Fig. 3, lane HSP). In agreement with microscopic data, these results show the presence of 14-3-3 protein in the membrane and in the cytosol of *T. gondii*.

14-3-3 subcellular distribution was confirmed by using the above-mentioned reference proteins as controls. (i) SAG1, detected in trace amounts in the post-nuclear fraction (Fig. 3, lane LSS), was absent from the soluble cytosolic fraction (lane HSS), while it was concentrated in the membrane fraction (lane HSP). (ii) GRA1 was detected at the same level in LSS and HSS, while it was absent from HSP (Fig. 3). Results obtained with reference proteins indicate a good fractionation of cytosol and membranes of *T. gondii* tachyzoites in our conditions.

### 3.4. Detection of 14-3-3 protein in *T. gondii* detergent-resistant membranes

The plasma membranes of many cell types contain specialized membrane microdomains, the lipid rafts, which are resistant to non-ionic detergents (such as Triton X-



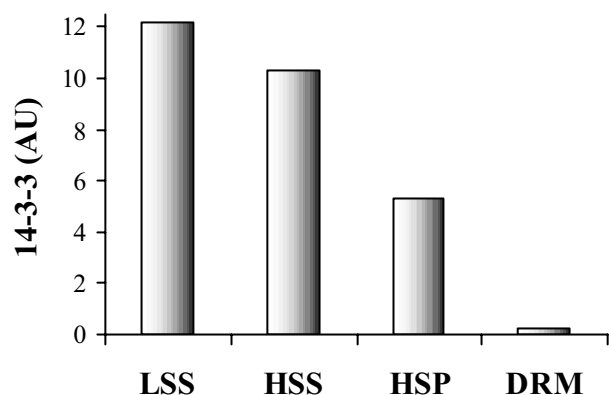


Fig. 5. Densitometric analysis of the subcellular 14-3-3 protein distribution. The post-nuclear (LSS), membrane supernatant (HSS), total membrane (HSP) and DRM fractions were analyzed by SDS-PAGE and transferred to PVDF membrane before Western blotting with anti-14-3-3 antibody. The intensities of each 14-3-3 spot are expressed in arbitrary units/ $10^9$  tachyzoites.

100) used at 4°C. These detergent-resistant membranes (DRM) are enriched in GPI-anchored proteins [29,30]. To get more information about the localization of 14-3-3 protein in the *T. gondii* membrane, Triton X-100-resistant membranes were prepared from the tachyzoites. After ultracentrifugation, a weak peak of proteins (Fig. 4, open circles) was observed in the gradient between 26 and 24% sucrose. To check whether this peak corresponded to DRM, the presence of SAG1, a GPI-anchored protein of *T. gondii*, was tested by immunodetection, revealing that SAG1 (Fig. 4A, dark circles) was actually associated with the peak of proteins. 14-3-3 distribution along the gradient was compared to that of the soluble protein GRA1 of *T. gondii* (Fig. 4B). High levels of both proteins were detected at the bottom of the gradient tube, then decreased progressively until fraction 13, while only 14-3-3 was present as an intense band in the DRM peak (fraction 21). The molecular mass of the DRM-associated 14-3-3 protein (14-3-3<sub>DRM</sub>) was found to be slightly lower than that of the non-DRM 14-3-3 protein, suggesting the presence of two different forms in the tachyzoites.

The amount of 14-3-3 protein present in the different subcellular fractions was estimated by measuring spot intensities after immunoblotting (Fig. 5). The 14-3-3<sub>DRM</sub> isoform represented less than 4% of the total membrane-associated 14-3-3 protein.

#### 4. Discussion

In this work, we have shown that 14-3-3 protein is present in the asexual form of *T. gondii*, corresponding to the tachyzoite stages, the form of the parasite that is virulent in humans. Moreover, 14-3-3 was detected when tachyzoites were free and when they were infecting human THP1 cells. 14-3-3 protein was previously described as absent in RH strain tachyzoites [21]. This apparent contra-

dition between our results and those of Koyama et al. [21] might be due to differences in the immunoaffinities of the antibodies used in each study.

Detection by immunofluorescence microscopy showed that 14-3-3 protein is cytosolic since it could be detected in permeabilized parasites, along with the cytosolic GRA1 protein. 14-3-3 protein is also membrane-associated, as evidenced by its colocalization with SAG1 protein in non-permeabilized parasites. These two localizations were confirmed by immunodetection of 14-3-3 in the subcellular fractions. Under these conditions, the amount of 14-3-3 protein present in the cytosol and in the membranes could be roughly estimated. 14-3-3 protein is mainly cytosolic, but it is also found with the membrane-associated protein fraction. Both cytosolic and membrane-associated 14-3-3 proteins have the same apparent molecular mass. This is the first report of a membranous form of 14-3-3 protein in the tachyzoites of *T. gondii*. In the cases of rat brain and *C. reinhardtii* subcellular localization of 14-3-3 proteins, most 14-3-3 isoforms were cytosolic, and only a small proportion was selectively membrane-associated [9,10], which is in agreement with the results presented here.

The localization of 14-3-3 protein in the parasite membrane was assessed using DRM prepared from *T. gondii*. A large proportion of the 14-3-3 protein was solubilized by Triton X-100. However, a new minor isoform (14-3-3<sub>DRM</sub>) with a lower apparent molecular mass was Triton-resistant at 4°C and was found to be specifically bound to DRM. This apparent low level of the 14-3-3<sub>DRM</sub> isoform could be due to its lower affinity for the antibodies. This DRM selectivity was not previously described in studies on 14-3-3 subcellular localization [20,32].

The 14-3-3<sub>DRM</sub> isoform could be produced either (i) by other genes encoding different protein isoforms, as described for another parasite, *Schistosoma mansoni* [19], or (ii) by post-translational modifications of the protein as is the case for the  $\beta/\alpha$  and  $\zeta/\delta$  isoforms in mammals [31]. Analyzing the differences between the two 14-3-3 isoforms of *T. gondii* would be interesting, although it would require a significant quantity of isoform. Until now, the low amount of 14-3-3<sub>DRM</sub> associated with DRM has not made this structural analysis possible. However, different 14-3-3 isoforms were found in all eukaryotes. These forms may be associated with different functions, as shown for mammals; for example, 14-3-3 $\zeta$  has been found to interact with the weel kinase, which plays a key role in cell cycle progression by inactivating cyclin-dependent kinases [32], whereas 14-3-3 $\sigma$  is induced by p53 in response to  $\gamma$ -irradiation and other DNA-damaging agents [13].

In the case of parasites, 14-3-3 proteins have been reported to play a role for the helminth, as they are implicated in the tumor-like growth process of *E. multilocularis* metacystode [18]. In *S. mansoni*, 14-3-3 proteins have a role in host immunity, parasite development and survival [34]. A physical association with Raf protein, a signal

transduction protein localized in rafts [33–35], was also reported for *S. mansoni* [19]. Rafts are postulated to function as platforms supporting numerous cellular events [36,37]. Their roles are presently unknown for protozoa. The presence of a DRM-specific 14-3-3 isoform in *T. gondii* could be correlated with the involvement of the lipid rafts in signal transduction. So, studying the different 14-3-3 proteins of *T. gondii* tachyzoites could be important to unravel their roles in parasite growth and its survival in the host.

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