We have identified two novel proteins that colocalize with the subpellicular microtubules in the protozoan parasite *Toxoplasma gondii* and named these proteins SPM1 and SPM2. These proteins have basic isoelectric points and both have homologs in other apicomplexan parasites. SPM1 contains six tandem copies of a 32-amino-acid repeat, whereas SPM2 lacks defined protein signatures. Alignment of *Toxoplasma* SPM2 with apparent *Plasmodium* SPM2 homologs indicates that the greatest degree of conservation lies in the carboxy-terminal half of the protein. Analysis of *Plasmodium* homologs of SPM1 indicates that while the central 32-amino-acid repeats have expanded to different degrees (7, 8, 9, 12, or 13 repeats), the amino- and carboxy-terminal regions remain conserved. In contrast, although the *Cryptosporidium* SPM1 homolog has a conserved carboxy tail, the five repeats are considerably diverged, and it has a smaller amino-terminal domain. SPM1 is localized along the full length of the subpellicular microtubules but does not associate with the conoid or spindle microtubules. SPM2 has a restricted localization along the middle region of the subpellicular microtubules. Domain deletion analysis indicates that four or more copies of the SPM1 repeat are required for localization to microtubules, and the amino-terminal 63 residues of SPM2 are required for localization to the subpellicular microtubules. Gene deletion studies indicate that neither SPM1 nor SPM2 is essential for tachyzoite viability. However, loss of SPM1 decreases overall parasite fitness and eliminates the stability of subpellicular microtubules to detergent extraction.

**Toxoplasma gondii** is a human pathogen that causes serious opportunistic infections in immunocompromised individuals and can cause miscarriage or birth defects during primary infection of pregnant women (8). It is also a useful model system to study aspects of cell biology that are conserved with other, less experimentally tractable related apicomplexan parasites which cause medically and agriculturally significant diseases. *Toxoplasma* has been a particularly effective system to analyze aspects of the cytoskeleton including actin, actin-like proteins, myosin, inner membrane complex (IMC) proteins, and tubulin (3, 19, 20, 27, 30, 33, 34, 37, 38, 40–42, 51, 77). Since apicomplexan microtubules are selectively sensitive to disruption by dinitroaniline analogs, tubulin may represent an important target for therapeutic agents to treat infection with *Toxoplasma*, *Cryptosporidium*, and *Plasmodium* (4, 7, 11, 25, 52, 53, 59, 76). Therefore, we are particularly interested in understanding the organization and regulation of microtubules in *Toxoplasma* and other apicomplexans.

Apicomplexans have a complex life cycle that alternates between haploid asexual forms which replicate rapidly to cause the acute forms of disease and a transient diploid zygote which is formed when a macrogamete is fertilized by a motile microgamete (61). The asexual stages of apicomplexan parasites have two microtubule populations: spindle microtubules, which coordinate chromosome segregation, and subpellicular microtubules, which impose an elongated cell shape and cell polarity (36, 60–62, 64, 70). These microtubule populations are nucleated from distinct microtubule-organizing centers (MTOCs). During cell division, which occurs by a closed mitosis, the poles of intranuclear spindles are associated with a specialized region of the nuclear envelope termed the centrocone or spindle pole plaque, which is labeled by MORN1 (24, 30, 51, 75). The apical polar ring (APR), a circular MTOC found only in apicomplexan parasites, is located at the parasite apex and nucleates the subpellicular microtubules, which extend toward the parasite posterior in close association with the cytosolic face of the parasite pellicle (64, 72, 77). The coccidian subset of apicomplexan parasites (including *Toxoplasma*) builds an additional cytoskeletal element, termed the conoid, at the apex of the parasite (64, 68). This structure is thought to have evolved in the last common ancestor of apicomplexans and dinoflagellates and is retained by some apicomplexan lineages (48, 49). The conoid is composed of tubulin sheets (42) that spiral to form a cone-shaped organelle that can extend beyond the APR or retract within it to be surrounded by the basket of subpellicular microtubules (64). Conoid extension is thought to facilitate host cell invasion and is dependent upon calcium signaling (18, 57, 58, 66).

The apicomplexan pellicle is a composite structure built by the association of the plasma membrane with an inner membrane complex (IMC) formed from flattened vesicles (21, 67). Freeze fracture studies of the IMC bilayers reveal the presence of a striking array of intramembranous particles (IMPs) which likely reflect the transmembrane domains of integral membrane proteins in apicomplexan parasites (21, 60, 67). The IMP array is held in place by a lattice of intermediate-filament-like proteins associated with the IMC (3, 29, 55, 56). The subpellicular microtubules are closely associated with the cytosolic face of the IMC, and these microtubules demonstrate a periodicity which suggests that they directly associate with the IMC lattice (47, 60). The periodic association of unidentified proteins along subpellicular microtubules is evident both in thin sections and in negatively stained images or frozen-hydrated images of isolated microtubules (12, 60). Moreover, the heavy decoration of subpellicular microtubules may account for...
their unusual stability during isolation in the cold and in the presence of detergents and for many unpublished observations that diverse tubulin-specific antibodies do not label the full length of subpellicular microtubules, suggesting that tubulin epitopes are occluded by associated proteins. In this paper, we describe two proteins that specifically localize to the subpellicular microtubules in *Toxoplasma* tachyzoites. These proteins are conserved in many apicomplexan parasites and represent the first markers of the subpellicular microtubules in these parasites.

**MATERIALS AND METHODS**

Parasite maintenance in cell culture. *Toxoplasma* lines were grown in confluent monolayers of human foreskin fibroblast (HFF) cells as previously described (71).

Generation of endogenous fusion proteins. In-frame carboxy-terminal fusions of TGGT1_043740 (SPM1) and TGGT1_038020 (SPM2) genes to yellow fluorescent protein (YFP) or mCherry were created using established methods (43). A 1.0-kb fragment terminating before the stop codon of the SPM1 open reading frame was amplified from the *Toxoplasma* genome (RH strain) with primers SPM1 LIC 5’ and SPM1 LIC 3’ as listed in Table S1 in the supplemental material. A 1.0-kb fragment terminating before the stop codon of the SPM2 open reading frame was amplified from the *Toxoplasma* genome (RH strain) with primers SPM2 LIC 5’ and SPM2 LIC 3’ as listed in Table S1. The amplified genes were integrated into the pYFP.LIC.DHFR or pmCherry.LIC.DHFR vectors and transfected into *ku80*-null *Toxoplasma* parasites as previously described (77). Stable lines were isolated by selection in 1 μM pyrimethamine and single cell cloned.

Conceptual translation and protein alignment. We amplified the complete cDNA sequences for SPM1 and SPM2 with primers listed in Table S1 in the supplemental material (SPM1 cDNA 5’, SPM1 cDNA 3’, SPM2 cDNA 5’, and SPM2 cDNA 3’). These sequences validated the predicted protein sequences annotated in ToxoDB.org. Putative homologs of SPM1 and SPM2 were identified using the predicted amino acid sequence of SPM1 and SPM2 cDNAs to search the NR Protein database with BLASTP (2). We identified portions of homologous proteins in the genomes of *Eimeria* and *Sarcocystis* using servers at http://www.sanger.ac.uk/cgi-bin/blast/submitblast/e_tenella/omni and http://lims.ca.uky.edu/sarcblast/blast/blast.html. Repeats were identified manually and were aligned to each other both within individual proteins and between protein homologs using Clustal W (39).

Immunofluorescence assays and fluorescence microscopy. Immunofluorescence imaging was performed on *Toxoplasma*-infected 12-mm circular glass coverslips that were seeded with near-confluent HFF monolayers. In some cases, YFP- or mCherry-tagged SPM1 and SPM2 intracellular parasites were fixed with formaldehyde, permeabilized, and stained as previously described (62). In other cases, in order to expose SPM1 and SPM2 epitopes that are apparently masked, samples were fixed for 10 min in acetone at −20°C prior to blocking and staining. Antibodies for immunofluorescence include mouse monoclonal antibodies against green fluorescent protein (GFP) (Roche), anti-GRASP55 (22), anti-P30/SAG1 (DG52) (9), polyclonal RNG1, SPM1, and SPM2 mouse antisera, and rabbit *Toxoplasma*-specific tubulin antiserum (62). These antibodies were visualized with Alexa-594- and -488-conjugated antibodies (Invitrogen). DNA was visualized by Hoechst staining. Coverslips were mounted in a polyvinyl alcohol-based medium and imaged on a Zeiss Axiovert 200 M using the Axiovision camera and software, and images were processed in Photoshop 8.0.

Detergent extraction of *Toxoplasma* parasites. Extracellular parasites were passed through a 3-μm polycarbonate filter (GE Water & Process Technologies), centrifuged at 1,000 × g for 20 min at 4°C and then suspended in a small volume of media. For immunofluorescence, parasites were allowed to settle onto poly-l-lysine (Sigma)-coated coverslips for 15 min at room temperature. Coverslips were treated with 10 mg/ml deoxycholate (DOC) in phosphate-buffered saline (PBS) for 10 min and fixed in 3.7% formal saline (Sigma) at room temperature or −20°C acetone (Fisher Scientific) for 10 min. Samples were stained with antibodies as described above.

**Immunoblot analysis.** Pelleted tachyzoites (−5 × 10^6/well) were suspended in Tris-Tricine SDS sample buffer (NuSep) and resolved on a 10% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose (Whatman), blocked in 5% nonfat milk, and probed with polyclonal antisera that recognize SPM1 or SPM2 proteins or *Toxoplasma* β-tubulin, followed by Alexa-594 or 488 anti-mouse or anti-rabbit secondary antibodies (Invitrogen). The blots were visualized on a Typhoon Trio variable-mode imager (GE Healthcare).

Protein expression and purification. The coding sequences of SPM1 and SPM2 genes were resynthesized to optimize for expression in *Escherichia coli* (GenScript). Each codon-optimized gene was cloned into the IPTG (isopropyl-β-D-thiogalactopyranoside; Acros)-inducible pET-21a(+) vector (Novagen) by restriction enzyme cloning into BamHI and XhoI sites, creating an in-frame carboxy-terminal fusion to a His tag. Protein expression also required the use of BL21-CodonPlus bacterial cells. One-liter cultures were induced with 1 mM IPTG for 3 h, and cells were pelleted at 6,000 rpm for 15 min. The pellets were suspended in cold native purification buffer (50 mM NaH2PO4, 500 mM NaCl, pH 8.0) supplemented with 1× Halt protease inhibitor cocktail (Thermo Scientific), sonicated five times at 30-s intervals, and centrifuged at 2,912 × g for 10 min at 4°C. The supernatant was added to Ni-nitriolrotiactidic acid (NTA) agarose (Invitrogen) in native purification buffer and rocked for 1 h at 4°C. The resin was loaded on a gravity flow column and washed with 32 ml of cold wash buffer (native purification buffer supplemented with 20 mM imidazole), and proteins were eluted with 12 ml of cold elution buffer (native purification buffer containing 250 mM imidazole). Eluate was loaded into a 30,000-molecular-weight-cutoff Amicon Ultra centrifugal filter (Millipore) with an additional 45 ml of native purification buffer and centrifuged at 2,912 × g at 4°C to concentrate protein and remove imidazole. The protein concentration was determined by the bicinchoninic acid (BCA) method (Pierce) and samples were stored at −20°C. SPM1 and SPM2 antisera were generated in mice by Cocalico Biologicals, Incorporated (Reamstown, PA).

**SPM1 and SPM2 domain mapping.** We modified the established GRASP55-YFP construct (32) so that the GRASP55 gene was replaced by the SPM1 or SPM2 coding sequence. Full-length cDNAs for SPM1 and SPM2 were cloned in frame with YFP, and expression was driven by the α1-tubulin promoter. In order to generate amino- and carboxy-terminal truncations of SPM1 and SPM2, the full-length sequence was replaced by shortened cDNAs as amplified by primers listed in Table S1 in the supplemental material. For SPM1, we created mutations that truncated the 351-amino-acid protein at the carboxy terminus to make proteins that contained amino acids 1 to 310 (SPM1 with 5 repeats), 1 to 278 (SPM1 with 4 repeats), 1 to 246 (SPM1 with 3 repeats), 1 to 214 (SPM1 with 2 repeats), 1 to 182 (SPM1 with 1 repeat), and 1 to 150 (SPM1 with 0 repeats). We also created an amino-terminal truncation construct (SPM1 with 6 repeats and tail) to express the 201 terminal amino acids. For SPM2, we created carboxy-terminal truncations to make proteins of 1 to 255 (SPM2 ΔC1), 1 to 190 (SPM2 ΔC2), 1 to 114 (SPM2 ΔC3), and 1 to 59 (SPM2 ΔC4). We created amino-terminal truncation constructs to express the amino acids 64 to 315 (SPM2 ΔN1), 121 to 315 (SPM2 ΔN2), 147 to 315 (SPM2 ΔN3), and 222 to 315 (SPM2 ΔN4). Finally, we created SPM2 B1PM, which creates the point mutations P49A, G51A, and V52A in the Box 1 motif.

**SPM1 and SPM2 gene knockouts.** Gene knockout (KO) constructs were generated in order to introduce the dihydrofolate reductase (DHFR)-selectable marker into either the SPM1 or the SPM2 locus. The SPM1 knockout vector was created by amplifying 4.7- and 3.2-kb regions of the genome up- and downstream of the SPM1 locus with primers listed in Table S1 in the supplemental material (SPM1 KO US Flank 5’, SPM1 KO US Flank 3’, SPM1 KO DS Flank 5’, and SPM1 KO DS Flank 3’). The SPM2 knockout vector was created by amplifying 3.1- and 3.2-kb regions
of the genome up- and downstream of the SPM2 locus with primers listed in Table S1 (SPM2 KO US Flank 5', SPM2 KO US Flank 3', SPM2 KO DS Flank 5', and SPM2 KO DS Flank 3'). Both knockout vectors were based on the pMini-GFP.hh knockout vector with the DHFR-selectable marker replacing the HPT marker (26, 45). Vectors were electroporated into the knox0 knockout line and selected with 1 μM pyrimethamine. The SPM1 knockout was confirmed by DNA amplification using primers listed in Table S1 (SPM1 KON screening 5', SPM1 KON screening 3', SPM1 KOC screening 5', and SPM1 KOC screening 3') as well as immunoblots and immunofluorescence with SPM1 antisera. The SPM2 knockout was confirmed by DNA amplification using the primers listed in Table S1 (SPM2 KON screening 5', SPM2 KON screening 3', SPM2 KOC screening 5', and SPM2 KOC screening 3') as well as immunoblots and immunofluorescence with SPM2 antisera.

**Competition assay.** A competition assay, based on an established method, was performed to determine if the loss of SPM1 or SPM2 affects the fitness of the parasite line (54). An equal number of parasites from the spm1 or spm2 knockout lines and GFP-positive control parasite lines (containing a nonhomologous integration of the knockout vector) was inoculated into Tg2 rescue experiments were serially passaged every other day for 22 days. The remaining parasites from each flask were analyzed for relative numbers of nonfluorescent (knock-out) and GFP-fluorescent (control) parasites by flow cytometry (A-40 analyzer; Apogee Flow Systems).

**RESULTS**

**SPM1 and SPM2 colocalize with subpellicular microtubules.** In order to identify microtubule-associated proteins (MAPs) that interact with the subpellicular microtubules in apicomplexan parasites, we tagged a set of novel proteins that we hypothesized might be associated with the microtubule cytoskeleton. These proteins were selected from a larger group of previously identified mass spectroscopy hits in a Toxoplasma tachyzoite cytoskeleton fraction (41). We analyzed this previously identified proteome for possible interactions with subpellicular microtubules, and we identified two novel proteins that colocalize with the subpellicular microtubules, and these proteins were named SPM1 and SPM2 (Fig. 1).

**SPM1 has a 32-amino-acid repeat, while SPM2 lacks obvious motifs.** Both SPM1 and SPM2 are predicted to be soluble proteins with basic pl values, a characteristic biochemical trait for small proteins that interact with the acidic tubulin dimer (79). Toxoplasma SPM1 is predicted to be 38.8 kDa with a pl of 8.28, while Toxoplasma SPM2 is predicted to be a 34.7-kDa protein with a pl of 9.72. The Toxoplasma SPM1 protein has 6 copies of a 32-amino-acid repeat (Fig. 2A). SPM1 homologs are found in other apicomplexans, including various Plasmodium and Cryptosporidium species (Plasmodium berghei, P. chabaudi, P. knowlesi, P. yoelii, P. vivax, P. falciparum, Cryptosporidium muris, and C. parvum). Analysis of the SPM1 protein repeats in other apicomplexans indicates that individual Plasmodium species have expanded the number of central repeats, while keeping the amino-terminal head domain and the carboxy-terminal tail domain consistent sizes (Fig. 2B and E).

**The Cryptosporidium repeats have degenerated so that they are poorly conserved with each other and poorly conserved with the other SPM1 homologs (Fig. 2B). The Toxoplasma amino-terminal head domain is larger (137 residues) than that of Plasmodium species (82 or 83 amino acids), while the Cryptosporidium head domain is smaller (53 residues) (Fig. 2C and E). The tail domain of Toxoplasma SPM1 is of identical size (22 amino acids) and of nearly identical sequence to the Plasmodium tachyzoites, while the Cryptosporidium tail has two amino acid insertions (Fig. 2D).

**SPM1 and SPM2 localizes to distinct regions of the subpellicular microtubules.** SPM1 localizes to the full length of subpellicular microtubules in DOC-extracted membrane skeletons but is conspicuously absent from the conoid (Fig. 3A). Localization of the SPM1 protein is consistent between samples of parasites that express a YFP knock-in tag stained with anti-GFP antibody and distribution of the endogenous protein in wild-type (WT) tachyzoites labeled with anti-SPM1 antisera. Relative to SPM1, the SPM2 pattern is restricted to the middle region of the subpellicular microtubules (Fig. 3B). This localization is consistent in extracted samples of tachyzoites that express a YFP knock-in of SPM2 and when endogenous SPM2 protein is probed in wild-type parasites with anti-SPM2 antisera. SPM2 does not extend to the apical regions of the microtubules close to the APR or to the distal ends of the subpellicular microtubules, and SPM2 does not localize to the conoid.

In order to assess if SPM1 and SPM2 directly bind to microtubules, we engineered expression constructs to localize ectopically expressed protein in COS cells. In both cases, we observed diffuse YFP fluorescence rather than a microtubule-associated pattern (not shown). We also purified bacterially expressed SPM1 and

![FIG 1 SPM1 and SPM2 proteins colocalize with the subpellicular microtubules in Toxoplasma tachyzoites. Parasites were fixed ~8 h after infection of host HFF cells on coverslips and labeled with Toxoplasma-specific tubulin antisera and an anti-GFP monoclonal antibody. (A) Localization of SPM1-YFP (green) and Toxoplasma tubulin (red) in two intracellular parasites. Individual subpellicular microtubules are visible (arrows), and SPM1-YFP is found along the entire length of the subpellicular microtubules. The two individual parasites are numbered in the phase-contrast image. (B) Localization of SPM2-YFP (green) and tubulin (red) in four tachyzoites within a parasitophorous vacuole. SPM2-YFP localizes in a punctate fashion along the middle region of the individual subpellicular microtubules (arrows). The four individual parasites are numbered in the phase-contrast image.](ec.asm.org)
SPM1 for biochemical experiments. Since we were unable to express SPM1 or SPM2 protein in constructs that used the amplified cDNA sequences from *Toxoplasma*, the genes were resynthesized to optimize for *E. coli* codon usage. The SPM1-His6 fusion protein was abundantly expressed and easily purified on a nickel column. SPM2 purification was complicated by its incorporation into inclusion bodies and required denaturation for purification on a nickel column. The direct interaction of proteins with microtubules can be assessed by "pull-down" assays which use microtubules polymerized from purified tubulin to assess whether a protein...
tative MAP copellets with microtubules (it is a MAP) or remains in the supernatant with unassembled tubulin (it is not a MAP) (81). Although SPM1 copellets with microtubules polymerized from purified *Tetrahymena* tubulin, we suspect that this interaction reflects interaction of the basic SPM1 protein (pI 8.28) with tubulin, which carries an acidic charge, particularly on the carboxy-terminal tails (not shown). Since bacterially expressed SPM2 protein nonspecifically sticks to the nickel agarose column and has a basic pI (9.72), we did not test its interaction with microtubules in vitro.

Four repeats are required for correct localization of SPM1. We used domain deletion analysis to assess the redundancy of the six tandem copies of a 32-amino-acid repeat in *Toxoplasma* SPM1. We created a series of deletions to express amino- or carboxy-terminal truncations of SPM1 tagged with a carboxy-terminal YFP (Fig. 4A). Since expression of a full-length SPM1 cDNA construct driven by the *Toxoplasma* α1-tubulin promoter had apparently normal localization to the periphery of daughter parasite buds, we used this construct to express truncated proteins (Fig. 4B). These tagged and truncated proteins are overexpressed and must compete for binding with endogenous (untagged) SPM1 protein. Although the YFP signal for many of the truncated proteins localizes to the daughter bud periphery, it was impossible to see association with individual daughter microtubules. Moreover, as daughters complete endodyogeny, the signal decreases relative to that observed in young buds. We attribute this to lower affinity of the truncated proteins relative to untagged full-length SPM1. Although carboxy-terminal truncations that eliminate one or two of the repeats localize to *Toxoplasma* daughter buds (Fig. 4C), there is decreased association and increased cytosolic fluorescence correlated with increased deletion. Carboxy-terminal truncations that remove more than two of the repeats cause the protein to localize to the *Toxoplasma* cytoplasm (Fig. 4D). An amino-terminal deletion that contains the six 32-amino-acid repeats and the carboxy-terminal tail localizes to the APR region but does not extend the length of the subpellicular microtubules (Fig. 4E).

**The amino-terminal 63 amino acids are required to correctly target SPM2.** We created amino- and carboxy-terminal truncations of the SPM2 cDNA to assess the role of specific protein domains in targeting to the central region of the subpellicular microtubules (Fig. 5A). Since SPM2 lacks any defined motifs, we selected several methionines distributed throughout the length of the protein as sites to create amino-terminally truncated constructs. Carboxy-terminal truncation constructs were made to assess the importance of two conserved regions. The *Toxoplasma* α1-tubulin promoter was used to drive expression of YFP-tagged truncated proteins since the full-length SPM2 cDNA construct localizes to emerging daughter buds (Fig. 5B). Removing the amino-terminal 63 amino acids resulted in mislocalization to the parasite mitochondrion (not shown). The SPM2-ΔC1 and SPM2-ΔC2 (Fig. 5C) proteins localize to daughter buds, but further truncations lead to Golgi apparatus localization (SPM2-ΔC3) (Fig. 5D and E) or cytoplasmic distribution (SPM2-ΔC4; data not shown). Mutation of three amino acids (P49A, G51A, V52A) in the conserved 11-amino-acid box reduces but does not eliminate localization to daughter buds (SPM2 B1PM; data not shown). Deletion analysis indicates that 63 residues from the amino terminus of SPM2 are required for peripheral localization. Moreover, 190 amino-terminal amino acids are sufficient for localization to the daughter buds. This portion of the protein does not contain the larger carboxy-terminal conserved domain.

**Loss of SPM1 decreases parasite fitness.** In other systems, microtubule-associated proteins have been shown to influence microtubule organization and stability. We used standard gene

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**FIG 3** SPM1 and SPM2 localize to distinct regions of the subpellicular microtubules. (A) SPM1 (green) localizes to the full length of subpellicular microtubules (tubulin, red) in DOC-extracted membrane skeletons but is absent from the conoid (arrows). The pattern is consistent for both YFP-tagged SPM1 parasites labeled with anti-GFP antibody and wild-type tachyzoites labeled with anti-SPM1 antisera. (B) SPM2 (green) is located to a middle region of isolated subpellicular microtubules (tubulin, red) and is absent from the apical regions of the microtubules close to the APR. Labeling also ends prior to the plus ends of the subpellicular microtubules (arrows). Again, the pattern is consistent for YFP-tagged SPM2 parasites labeled with anti-GFP antibody and wild-type tachyzoites labeled with anti-SPM2 antisera.
inhibitory concentrations (IC50s) (not shown) (54). We also pre-
zalin, as assessed using a standard plaque assay to determine 50%
protein was not correlated with increased sensitivity to ory-
dinitroaniline-mediated disruption of microtubules. Loss of ei-
controls.
null parasite lines showed growth comparable to that of matched
in competition with matched controls, two independently derived
were quantified by flow cytometry, as previously described (54).
saged every other day to follow the relative performance of the
fresh monolayer of HFF host cells. This culture was serially pas-
out vector) and confirmed knockout lines were inoculated into a
control line (carrying a nonhomologous integration of the knock-
equivalent numbers of parasites from a GFP-expressing matched
FIG 4 Four carboxy-terminal SPM1 repeats are sufficient for subpellicular microtubule localization in Toxoplasma. (A) A schematic diagram drawn to scale that shows the fusion of GFP (green) to the full-length SPM1 cDNA (SPM1 FL) and truncations made for ectopic expression in Toxoplasma. Carboxy-terminal SPM1 truncations were made to remove the carboxy-terminal tail (orange) and sequentially remove the tandem repeats (teal). An amino-terminal truncation was made to remove the amino-terminal end (gray) and “header” region (yellow). (B to E) Immunofluorescence shows images of phase-contrast (left), merged (middle), and GFP fluorescence only (right). DG52 antibody (red) labels the SAG1 GPI-tagged surface protein which delineates the parasite body. The DNA is labeled with Hoechst (blue). (B) Expression of the full-length SPM1 cDNA by the strong α1-tubulin promoter shows localization to the parasite periphery in both maternal cells and daughter parasite buds. The SPM1 protein also shows discrete localization to an undefined structure that appears at the center of each daughter parasite (arrows). This spot is seen only when SPM1 is overexpressed and not in the YFP knock-in or with antibody staining. (C) SPM1 localization diminishes with each truncated repeat. SPM1 cDNA that contains four repeats continues to localize to all structures but with increased cytosolic GFP fluorescence. (D) Expression of a truncated SPM1 containing three repeats no longer localizes to cytoskeletal structures. (E) A SPM1 truncation that contains the six repeats and the carboxy-terminal tail region localizes to the apex of maternal and daughter parasites in a ring-like structure (arrows).

deletion techniques to create Toxoplasma tachyzoite lines with the
SPM1 gene or the SPM2 gene knocked out (Fig. 6A). Knockouts
were confirmed by amplification of the altered locus (Fig. 6B) and
by immunoblot analysis with anti-SPM1 or anti-SPM2 antisera
(Fig. 6C). Parasites with SPM1 or SPM2 genes deleted were viable.
To assess the overall fitness of spm1- and spm2-null parasite lines, equivalent numbers of parasites from a GFP-expressing matched control line (carrying a nonhomologous integration of the knock-
out vector) and confirmed knockout lines were inoculated into a fresh monolayer of HFF host cells. This culture was serially pas-
saged every other day to follow the relative performance of the
knockout and control cultures. The nonfluorescent (knockout
line) and fluorescent (matched control) population contributions were quantified by flow cytometry, as previously described (54).
In competition with matched controls, two independently derived
spm1-null parasite lines decreased over time, indicating a fitness
defect in the absence of SPM1 protein (Fig. 7A). In contrast, spm2-
null parasite lines showed growth comparable to that of matched
controls.

SPM1 stabilizes the subpellicular microtubules. We assessed the relative sensitivities of the spm1 and spm2 knockout lines to dinitroaniline-mediated disruption of microtubules. Loss of either protein was not correlated with increased sensitivity to oryzalin, as assessed using a standard plaque assay to determine 50% inhibitory concentrations (IC50) (not shown) (54). We also pre-
pared DOC-extracted parasite microtubule cytoskeleton samples to follow the localization of SPM2 in an spm1-null line and SPM1 in an spm2-null line. There was an absence of SPM2 labeling of
microtubules from spm2-null lines, but SPM1 localized in a fash-
similar to that of the wild type in the extracted subpellicular microtubules (Fig. 7B). A low level of apparent SPM2 labeling of
spm2-null parasites is due to the anti-mouse secondary antibody getting trapped in the area of the APR. Note that SPM2 antisem in combination with the same secondary antibody does not label this region in wild-type parasites (compare Fig. 7B with Fig. 4B).
We were unable to localize SPM2 in DOC-extracted samples from the
spm1-null line because the subpellicular microtubules from the
spm1-null parasites are completely disrupted by this treat-
ment, leaving only the conoid and associated APR in extracted preparations (Fig. 7C, middle). When the spm1-null line is com-
plemented by transfection with a vector that drives expression of full-length SPM1-YFP from the α1-tubulin promoter, the stable lines recover detergent-stable microtubules (Fig. 7C, right).

DISCUSSION
Proteins that associate with microtubules serve to influence microtubule stability and provide individual microtubule populations within the cell cytoplasm with specialized attributes. Microtubule-associated proteins (MAPs) have been studied for many years in vertebrate cells, particularly in neurons, which are enriched for specialized microtubule populations (10, 65, 69). These studies have led to the identification of a number of MAPs such as MAP1A, MAP1B, MAP2, tau, and the STOP proteins (5, 17, 44, 80). Other MAPs found in a wider array of organisms from yeast to vertebrates include microtubule motors and plus-end proteins such as EB1/Bim1/MAL3p, dynactin, APC, and CLIP-
170 (50, 78). MAPs can be categorized into sets that include microtubule motors and their regulators, microtubule-regulating proteins (plus-end proteins), and structural proteins. This latter group (MAP1, MAP2, tau, STOP proteins) includes a number of proteins that influence cold and drug stability. Many of these contain repeated motifs, such as the 46-amino-acid repeat found in STOP proteins.

Like neurons, protozoan organisms have abundant and elaborate microtubule cytoskeletons. Some protozoa, such as *Giardia lamblia*, *Trypanosoma brucei*, and *Toxoplasma gondii*, have unusual microtubule structures that have evolved in part to coordinate processes associated with the pathogenic lifestyles of these human parasites (14–16, 23, 46, 61, 74). The auger-like shape of trypanosomes is maintained by an array of corset microtubules which subtend the plasma membrane (46). These microtubules are densely packed, evenly spaced at 18 to 22 nm, and cross-linked to each other and to the plasma membrane by associated proteins. Previous work has identified a number of proteins that show detergent-insoluble association with the corset microtubules in *Trypanosoma brucei*, including MARP and WCB (46).

MARP (microtubule-associated repetitive protein) is perhaps the most extreme example of an associated protein with a repetitive structure (1, 35, 63, 73). Analysis of the conceptually translated full-length gene indicates that it has 76 copies of a highly conserved 38-amino-acid repeat and directly associates with in vitro-polymerized microtubules. The WCB protein is a 138-kDa protein.

**FIG 5** The 63 amino-terminal amino acids are necessary and 190 amino-terminal amino acids are sufficient for SPM2 localization. (A) A schematic diagram drawn to scale that shows the fusion of GFP (green) to the full-length SPM2 cDNA (light blue) and truncations made for ectopic expression in *Toxoplasma*. Carboxy-terminal and amino-terminal truncations of the SPM2 cDNA were made to probe the importance of conserved sequences (azure stripes) in the SPM2 gene. Region 1 consists of 11 conserved residues (alignment left), and region 2 contains 79 conserved residues (alignment right). The constructs are full-length SPM2-FP (SPM2 FL), 315 amino acids; SPM2 ΔC1, consisting of the amino-terminal 255 amino acids; SPM2 ΔC2, consisting of the amino-terminal 190 amino acids; SPM2 ΔC3, consisting of the amino-terminal 114 amino acids; SPM2 ΔC4, consisting of the amino-terminal 59 amino acids; SPM2 ΔN1, consisting of the carboxy-terminal 252 amino acids; SPM2 ΔN2, consisting of the carboxy-terminal 195 amino acids; SPM2 ΔN3, consisting of the carboxy-terminal 169 amino acids; SPM2 ΔN4, consisting of the carboxy-terminal 94 amino acids; and SPM2-B1PM, which has three mutations to the 11-amino-acid motif in box 1 (azure, 1). Clustal alignment of the two conserved regions is found in the following apicomplexan species: Py, *P. yoelii*; Pb, *P. berghei*; Pc, *P. chabaudi*; Pf, *P. falciparum*; Pk, *P. knowlesi*; Pv, *P. vivax*; Tg, *T. gondii*; Nc, *N. caninum*. In all of the Clustal alignments, individual amino acids are colored to reflect their chemical properties. Blue: acidic; green: hydroxyl/amine/basic/Q; magenta: basic; and red: small, hydrophobic (including aliphatic Y). (B to E) Immunofluorescence shows images of phase-contrast (left), merged (middle), and GFP fluorescence only (right). DG52 antibody labels the SAG1 GPI-tagged surface protein which delineates the parasite body (red). The DNA is labeled with Hoechst (blue). (B) Expression of full-length SPM2 shows localization to the apical periphery of both maternal and daughter (arrows) parasites. This pattern is less restricted than the localization in the YFP knock-in line. (C) Truncated SPM2 containing 255 amino-terminal amino acids (SPM2 ΔC1) localizes to daughter structures (arrows). (D) The 114 amino-terminal amino acids of SPM2 (SPM2 ΔC3) localize to the Golgi apparatus (arrows). (E) Golgi localization of SPM2 ΔC3 is validated by labeling with an antibody to GRASP55 that localizes to the *Toxoplasma* Golgi apparatus.
tein that contains five copies of a 32-amino-acid repeat with a central KSAED sequence (6). When WCB expression is silenced with RNA interference (RNAi), parasites exhibit slowed growth, aberrant morphology, and reduced organization of the corset microtubules. The observation of a 32-amino-acid repeat is reminiscent of the organization of SPM1 described here. However, the SPM1 repeat consensus is based on a distinct sequence.

Toxoplasma and other related apicomplexan parasites, such as Plasmodium and Cryptosporidium species, have a rigid, elongated cell shape that is imposed by the presence of an array of subpellicular microtubules that are distinct from the organization of corset microtubules in trypanosomes. Apicomplexan subpellicular microtubules originate at the APR and terminate posterior to the parasite nucleus. The close association of subpellicular microtubules with the cytosolic face of the parasite pellicle is clearly mediated by MAPs (21, 60, 67). Proteins that bridge the region between the microtubules and the overlying membrane are visible by electron microscopy (13, 47, 60) but have not been identified prior to this report. We describe two novel proteins that colocalize with the Toxoplasma subpellicular microtubules in tachyzoite-stage parasites.

Toxoplasma SPM1 is localized along the entire length of the subpellicular microtubules but does not localize to the conoid, the intraconoid microtubules, or the spindle. There are six copies of a 32-amino-acid repeat in the Toxoplasma SPM1 homolog and five copies (that are considerably more degenerate) in the Cryptosporidium SPM1 homolog. In Plasmodium spp., SPM1 homologs are of variable size because the repeat has expanded to different degrees. Interestingly, the Plasmodium repeats show the highest degree of conservation, which may be the consequence of successive gene recombination events which homogenized the repeat sequence while increasing the number of copies of this motif. Not surprisingly, the repeats are critical for correct localization of the SPM1 protein, and increased deletion of repeats is associated with reduced and ultimately eliminated localization to the Toxoplasma pellicle. We were able to create a spm1-null line which has reduced fitness relative to matched parasite controls. Moreover, we are not able to isolate DOC-extracted microtubule cytoskeleton samples

FIG 6 SPM1 and SPM2 are not essential genes. (A) We developed a knockout plasmid consisting of the DHFR-selectable marker flanked by upstream and downstream sequences from the SPM1 or SPM2 genes. This plasmid, which is based on the pMini-GFP.hh knockout vector, also contains a downstream GFP gene, with expression driven by the GRA1 promotor. In the case that the marker is integrated into the SPM1 or SPM2 locus by a double-crossover event, the GFP gene is lost (homologous recombination). In the case that the plasmid integrates elsewhere, the GFP gene is often retained (nonhomologous recombination), and these parasites serve as a matched control in competition assays and other experiments. Primers labeled A and B are specific for the termini of the SPM1 or SPM2 coding sequence. These specifically amplify a product from nonknockout lines but not from knockout lines. Primers labeled C and D are located upstream of the 5′ flanking sequence and within the DHFR gene. These specifically amplify a product from knockout lines. (B) Genomic DNA was isolated from individual lines in order to confirm homologous integration (KO) and nonhomologous integration events by gene amplification. The absence of an amplified DNA product from primer set A/B confirms the loss of the SPM1 (~2-kb) or SPM2 (~1-kb) gene locus. The presence of an amplified DNA product from primer set C/D (SPM1 KO primers, ~4.8-kb fragment; SPM2 KO primers, ~3.4-kb fragment) confirms a successful homologous amino-terminal integration event. (C) Immunoblots of lysate from control and spm1-null lines (left) or control and spm2-null lines (right) demonstrate that SPM1 and SPM2 proteins are missing from null (KO) lines but are present in parasites with a nonhomologous integration of the corresponding knockout vector (WT). The blots were simultaneously probed with rabbit anti-Toxoplasma tubulin and mouse anti-SPM1 (left) or -SPM2 (right) antibodies identified with distinct (594 and 488) Alexa-conjugated rabbit and mouse secondary antibodies, which are independently detected by a Typhoon Imager.
from spm1-null tachyzoites because the subpellicular microtubules in the knockout are disrupted by this treatment. An spm1-null line complemented with full-length SPM1-YFP recovers detergent-stable microtubules. These observations indicate that SPM1 is important to microtubule stability.

SPM2 has a more restricted localization pattern than SPM1: it is associated with the middle third of the subpellicular microtubules. SPM2 also lacks repeats and overt protein motifs, although there are apparent homologs of this protein in Neospora and Plasmodium but not Cryptosporidium. Protein alignments indicate that conservation of Plasmodium and Toxoplasma SPM2 proteins is restricted to residues 49 to 60 and residues 189 to 268 in the 315-amino-acid Toxoplasma protein. Surprisingly, although the majority of conservation between Toxoplasma and Plasmodium SPM2 proteins is in the carboxy terminus, the amino-terminal 63 residues are required for localization to the apical periphery of mother and daughter parasites. Up to 125 amino acids could be deleted from the carboxy-terminal end of SPM2 without affecting its localization. Since the significant conservation of SPM2 proteins begins at amino acid 189, localization of Toxoplasma SPM2 does not require the region of the protein that is most conserved with apparent Plasmodium homologs. This suggests that the small region of homology between amino acids 49 and 60 (AGxSSxRL xxL) is required for correct localization. We were able to create an spm2-null line, indicating that the protein is not essential for tachyzoite survival. Competition assays show that the spm2-null line grows comparably to control lines, indicating that the SPM2 protein is not required for overall tachyzoite fitness in vitro. However, it is entirely possible that in the more challenging environment of an animal infection, spm2-null parasites may exhibit a fitness defect.

The shared ancestry of apicomplexan, dinoflagellate, and ciliate lineages is reflected in a conserved organization of the pellicle, a structure formed by the association of a patchwork of flattened vesicles (alveoli) with the plasma membrane. A recent proteomic survey of the free-living ciliate Tetrahymena thermophila identi-
fied 529 novel proteins associated with a pellicle fraction (28). Nearly a quarter of these proteins have repetitive regions with an amino acid bias for K, E, Q, L, I, and V. Apicomplexans, including Toxoplasma, have similar proteins that localize to the pellicle in the region of the APR. It is interesting to note that the conserved regions of both SPM1 and SPM2 share aspects of the amino acid bias described for the repetitive pellicle proteins. Like the previously described Tetrahymena pellicle proteins, SPM1 contains a repetitive region; however, in addition to K, E, Q, L, I, and V, this 32-amino-acid motif also includes other conserved residues (P, F, S, Y). As described above, electron microscopy studies of Toxoplasma (60) and Plasmodium (47) subpellicular microtubules have detected an unidentified cytoskeletal element which binds to the subpellicular microtubules with a 32-nm periodicity. Similar studies of the subpellicular microtubules in spm1- and spm2-null parasite lines could clarify whether the 32-nm repeat is due to microtubule decoration with SPM1 or SPM2.

Diverse free-living and pathogenic protozoa have elaborate microtubule-based cytoskeletal structures that play critically important roles in polarity, motility, and cell shape as well as being essential for the segregation of chromosomes during cell division. Since proteins that selectively associate with distinct microtubule subsets imbue individual populations with specialized properties (such as membrane association), it is important to define the complement of associated proteins that provide these functions to individual microtubule arrays. Moreover, studies in other organisms have demonstrated that the expression of associated proteins can influence microtubule stability and therefore modulate sensitivity to microtubule-disrupting drugs. Previous studies defined a Toxoplasma protein (TgICMAP1) that specifically associates with the intracodonal microtubules (33). In this work, we describe the properties of two proteins that localize to the subpellicular microtubules in Toxoplasma tachyzoites. We anticipate that future studies will identify additional diverse and important proteins that associate with the subpellicular microtubules in order to build the complex cortical microtubule cytoskeleton which is essential to parasite survival.

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35. Hemphill A, Affolter M, Seebeck T. 1992. A novel microtubule-binding motif identified in a high molecular weight microtubule-associated protein from Trypanosoma brucei. J. Cell Biol. 117:95–103.

36. Hepler PK, Huff CG, Sprinz H. 1966. The fine structure of the exoerythrocytic stages of Plasmodium falciparum. J. Cell Biol. 30:333–358.

37. Herr-Gott A, et al. 2006. Functional and biophysical analyses of the class XIV Toxoplasma gondii myosin D. J. Muscle Res. Cell Motil. 27:139–151.

38. Hettemann C, et al. 2000. A dibasic motif in the tail of a class XIV apicomplexan myosin is an essential determinant of plasma membrane localization. Mol. Biol. Cell 11:1385–1400.

39. Higgins DG, Thompson JD, Gibson TJ. 1994. Using CLUSTAL for multiple sequence alignments. Methods Enzymol. 266:383–402.

40. Hu K. 2008. Organizational changes of the daughter basal complex during the parasite replication of Toxoplasma gondii. PLoS Pathog. 4:e10.

41. Hu K, et al. 2006. Cytoskeletal components of an invasion machine—the apical complex of Toxoplasma gondii. PLoS Pathog. 2:e13.

42. Hu K, Roos DS, Murray JM. 2002. A novel polymer of tubulin forms the conoid of Toxoplasma gondii. J. Cell Biol. 156:1039–1050.

43. Huynh MH, Carruthers VB. 2009. Tagging of endogenous genes in a Toxoplasma gondii strain lacking Ku80. Eukaryot. Cell 8:530–539.

44. Job D, Pabion M, Margolis RL. 1985. Generation of microtubule stability subclasses by microtubule-associated proteins: implications for the microtubule “dynamic instability” model. J. Cell Biol. 101:1680–1689.

45. Karasov AO, Boothroyd JC, Arrizabalaga G. 2005. Identification and disruption of a rhoptry-like homologue of sodium hydrogen exchangers in Toxoplasma gondii. Int. J. Parasitol. 35:285–291.

46. Kohl I, Gull K. 1998. Molecular architecture of the trypansomes cytoskeleton. Mol. Biochem. Parasitol. 93:1–9.

47. Kudryashov M, et al. 2010. Positioning of large organelles by a membrane-associated cytoskeleton in Plasmodium sporozoites. Cell. Microbiol. 12:362–371.

48. Leander BS, Keeling PJ. 2003. Morphostasis in alveate evolution. Trends Ecol. Evol. 18:395–402.

49. Leander BS, Kuvardina ON, Aleshin VV, Mylnikov AP, Keeling PJ. 2002. Proteolytic processing of TgIMC1 during maturation of the membrane skeleton of Toxoplasma gondii. Mol. Cell. 106:41240–41246.

50. Monteiro VG, de Melo EJ, Attias M, de Souza W. 2001. Morphological changes during conoid extrusion in Toxoplasma gondii tachyzoites treated with calcium ionophore. J. Struct. Biol. 136:181–189.

51. Morrissette NS, Mitra A, Sept D, Sibley LD. 2004. Dinitroanilines bind alpha-tubulin to disrupt microtubules. Mol. Biol. Cell 15:1960–1968.

52. Morrissette NS, Murray JM, Roos DS. 1997. Subpellicular microtubules associate with an intramembranous particle lattice in the protozoan parasite Toxoplasma gondii. J. Cell Sci. 110:35–42.

53. Morrissette NS, Sibley LD. 2002. Cytoskeleton of apicomplexan parasites. Microbiol. Mol. Biol. Rev. 66:21–38.

54. Muller N, et al. 1992. Identification and characterization of two repetitive non-variable antigens from African trypanosomes which are recognized early during infection. Parasitology 104:111–120.

55. Nichols BA, Chiappino ML. 1987. Cytoskeleton of Toxoplasma gondii. J. Protozool. 34:217–226.

56. Nunez J, Fischer I. 1997. Microtubule-associated proteins (MAPs) in the peripheral nervous system during development and regeneration. J. Mol. Neurosci. 8:207–222.

57. Pezzella N, et al. 1997. Involvement of calcium and calmodulin in Toxoplasma gondii tachyzoite invasion. Eur. J. Cell Biol. 74:92–101.

58. Porchet E, Torpier G. 1977. Freeze fracture study of Toxoplasma and Sarcocystis infective stages. Z. Parasitenkd. 54:101–124. (In French.)

59. Porchet-Hennere E. 1973. Quelques precision sur l’ultrastructure de Sarcocystis tenella. J. L’endozoite (Apres Coloration Negative). J. Eukaryot. Microbiol. 22:214–220.

60. Ramirez G, et al. 1999. Regulatory roles of microtubule-associated proteins in neuronal morphogenesis. Involvement of the extracellular matrix. Braz. J. Med. Biol. Res. 32:611–618.

61. Read M, Sherwin T, Holloway SP, Gull K, Hyde J. 1993. Microtubular organization visualized by immunofluorescence microscopy during erythrocytic schizogony in Plasmodium falciparum and investigation of post-translational modifications of parasite tubulin. Parasitology 106:223–232.

62. Roos DS, Donald RG, Morrissette NS, Moulton AL. 1994. Molecular tools for genetic dissection of the protozoan parasite Plasmodium gondii. Methods Cell Biol. 45:27–63.

63. Russell DG, Burns RG. 1984. The polar ring of coccidian sporozoites: a unique microtubule-organizing centre. J. Cell Sci. 65:193–207.

64. Schneider A, Hemphill A, Wyler T, Seebeck T. 1988. Large microtubule-associated protein of T. brucei has tandemly repeated, near-identical sequences. Science 241:459–462.

65. Seebeck T, Hemphill A, Lawson D. 1990. The cytosome of trypanosomes. Parasitol. Today. 6:49–52.

66. Sibert GJ, Speer CA. 1981. Fine structure of nuclear division and microgametogony of Eimeria nieschulzi Dieben, 1924. Z. Parasitenkd. 66:179–189.

67. Stokkermans TJ, et al. 1996. Inhibition of Toxoplasma gondii replication by dinitroaniline herbicides. Exp. Parasitol. 84:355–370.

68. Tran JQ, et al. 2010. RING1 is a late marker of the apical polar ring in Toxoplasma gondii. Cytoskeleton (Hoboken) 67:586–598.

69. Vaughan KT. 2005. Microtubule plus ends, motors, and traffic of Golgi membranes. Biochim. Biophys. Acta 1744:316–324.

70. Vedrene C, et al. 2002. Two related subpellicular cytoskeleton-associated proteins in Trypanosoma brucei stabilize microtubules. Mol. Biol. Cell 13:1058–1070.

71. Wallin M, Stromberg E. 1995. Cold-stable and cold-adapted microtubules. Int. Rev. Cytol. 157:1–31.

72. Ziegelbauer J, et al. 2001. Transcription factor MIZ-1 is regulated via microtubule association. Mol. Biol. Cell 8:339–349.