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Mining the *Giardia* genome and proteome for conserved and unique basal body proteins

Tineke Lauwaet^{a,*}, Alias J. Smith^a, David S. Reiner^{a,c}, Edwin P. Romijn^{b,d}, Catherine C. L. Wong^b, Barbara J. Davids^a, Sheila A. Shah^{a,e}, John R. Yates 3rd^b, and Frances D. Gillin^a

^a Department of Pathology, University of California San Diego, San Diego, CA, 92103, USA

^b The Scripps Research Institute, La Jolla, CA, USA

Abstract

Giardia lamblia is a flagellated protozoan parasite and a major cause of diarrhea in humans. Its microtubular cytoskeleton mediates trophozoite motility, attachment and cytokinesis, and is characterized by an attachment disk and eight flagella that are each nucleated in a basal body. To date, only 10 giardial basal body proteins have been identified, including universal signaling proteins that are important for regulating mitosis or differentiation. In this study, we have exploited bioinformatics and proteomic approaches to identify new *Giardia* basal body proteins and confocal microscopy to confirm their localization in interphase trophozoites. This approach identified 75 homologs of conserved basal body proteins in the genome including 65 not previously known to be associated with *Giardia* basal bodies. Thirteen proteins were confirmed to co-localize with centrin to the *Giardia* basal bodies. We also demonstrate that most basal body proteins localize to additional cytoskeletal structures in interphase trophozoites. This might help to explain the roles of the four pairs of flagella and *Giardia*-specific organelles in motility and differentiation. A deeper understanding of the composition of the *Giardia* basal bodies will contribute insights into the complex signaling pathways that regulate its unique cytoskeleton and the biological divergence of these conserved organelles.

Abstract

*Corresponding author. Tineke Lauwaet, Department of Pathology, 214 Dickinson Street, CTF-C, University of California San Diego, San Diego, CA, 92103-8416, USA Tel.: +1 619 543 7825; fax: +1 619 543 6614. tlauwaet@ucsd.edu.

^cCurrent address: Sanford-Burnham Medical Research Institute, La Jolla, CA, USA

^dCurrent address: Philips Research Labs, 5656 AE Eindhoven, The Netherlands

^eCurrent address: Life Technologies Corporation, Carlsbad, CA, USA

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Supplementary data associated with this article.

Keywords

Giardia; Basal body; Flagella; Centrin; Cytoskeleton; Proteomics; Immunolocalization

1. Introduction

Basal bodies are eukaryotic microtubule organizing centers that nucleate flagella/cilia and function as spindle poles during cell division. Although basal bodies have been studied for over 100 years, their composition, function and biological origins remain incompletely understood (Debec et al., 2010). Recent proteomic analyses of isolated human centrosomes and basal bodies in the non-flagellated protist *Dictyostelium discoideum*, the flagellated alga *Chlamydomonas reinhardtii* and the ciliate *Tetrahymena thermophila* are excellent resources for comparative and functional studies (Andersen et al., 2003; Keller et al., 2005; Reinders et al., 2006; Kilburn et al., 2007). Nonetheless, additional models are needed.

The protozoan *Giardia lamblia* offers a valuable model as it is a binucleate pathogen with a complex cytoskeleton that mediates swimming, attachment and differentiation into and from dormancy. *Giardia* is a major cause of water-borne diarrheal disease worldwide (Adam, 2001) and exists in two life stages: the motile trophozoite and the dormant infectious cyst. Ingested cysts are activated by gastric acid but trophozoites only emerge in the small intestine. During this excystation, the cytoskeleton rapidly reassembles, allowing trophozoites to swim and to attach to small intestinal enterocytes where they divide and can cause disease. Trophozoites that are swept downstream must differentiate into cysts before fecal excretion to survive outside the host. Both the rapid giardial excystation and the more gradual encystation require global remodeling of the parasite's cytoskeleton (Adam, 2001).

The complex giardial cytoskeleton is largely microtubular and is composed of a ventral attachment disk, a median body, eight basal bodies and four pairs of flagella: anterior, posterior-lateral, ventral and caudal. Each of the eight flagellar axonemes is templated in a

basal body that is located between and slightly anterior to the two nuclei. Each flagellar pair runs through different parts of the cell body and exits at distinct sites. Intracellular parts of the flagella are associated with diverse electron dense structures of unknown function: paraflagellar dense rods (PFR) along the posterior-lateral and anterior axonemes, marginal plates along the anterior axoneme and the funis along the caudal axonemes (Elmendorf et al., 2003; Nohynkova et al., 2006). The disk and paraflagellar structures are unique to *Giardia*. The functions of the different flagellar pairs have not been resolved but each pair is suggested to play a distinct role in swimming, steering and attachment (Elmendorf et al., 2003). The median body is a stack of unorganized microtubules that is located posterior to the nuclei and whose function is unknown.

The morphology of centrioles and basal bodies is well conserved from protists to mammals and is characterized by nine radially arranged blades of triplet microtubules forming a small cylinder with a diameter of 250 nm (Fig. 1A) (Chapman et al., 2000; Beisson and Wright, 2003; Dutcher, 2003a; Benchimol, 2005; Nohynkova et al., 2006; Sagolla et al., 2006). Certain *Giardia* basal bodies are anchored to the anterior portion of the nuclei by centrin-rich fibers, and to the ventral disk microtubules and the funis by two microtubular roots (Mariante et al., 2005; Nohynkova et al., 2006). Similar to the centrioles in metazoan cells, *Giardia* basal bodies are located at the spindle poles during mitosis (Sagolla et al., 2006; Davids et al., 2008). However, *Giardia* has two nuclei and undergoes semi-open mitosis involving two extranuclear spindles. Two basal bodies or centrioles, perpendicular to one another and surrounded by a pericentriolar matrix, form a centrosome. In contrast to most eukaryotes, each *Giardia* interphase cell has the eight basal bodies needed to form four centrosomes of the two spindles. It is not yet known when the eight basal bodies duplicate and which of the basal bodies form the spindle poles. *Giardia* basal body assembly and duplication molecular machinery is only poorly understood, largely because so few giardial basal body proteins have been identified: the universal basal body proteins α - and γ -tubulins, centrins 1 and 2, and the signaling proteins calmodulin, PKAc, PKAr, PP2A-C, ERK1 and aurora kinase (Table 1) (Meng et al., 1996; Nohynkova et al., 2000; Ellis et al., 2003; Reiner et al., 2003; Correa et al., 2004; Davids et al., 2008). To date, aurora kinase is the only mitotic kinase that has been studied. *Giardia* aurora kinase is phosphorylated during mitosis and is important in microtubule nucleation (Davids et al., 2008).

These studies also showed that the universal signaling proteins PP2Ac, PKAc, PKAr and ERK1 that dock to the giardial basal bodies have crucial roles in encystation and/or excystation (Abel et al., 2001; Ellis et al., 2003; Gibson et al., 2006; Lauwaet et al., 2007). Remarkably, the localization of these signaling proteins to the basal bodies is constitutive over the life cycle. However, their localization to the anterior PFR changes dramatically in response to the external signals of growth or encystation (Abel et al., 2001; Ellis et al., 2003; Gibson et al., 2006; Lauwaet et al., 2007). This stimulus-responsive localization suggests that these signaling proteins might dictate the distinct behavior of *Giardia*-specific organelles during growth and differentiation.

In this study, our goal was to identify conserved and novel *Giardia* basal body proteins and to analyze their localization in interphase trophozoites. We test the hypothesis that other basal body proteins also localize to *Giardia*-specific organelles in distinct patterns. A more

complete understanding of basal body proteins and their localizations to different cytoskeletal organelles will aid studies on the functional dynamics between basal bodies and other cytoskeletal structures in both life cycle and cell cycle events. It will also augment our understanding of signaling protein functions.

In contrast to the better studied ciliates, *C. reinhardtii* and *T. thermophila*, the basal bodies of *Giardia* are deeply embedded in the cell body and are attached to intracellular parts of the flagellar axonemes. Due to this and the inability to deflagellate *Giardia*, isolation of basal bodies free of axonemal contaminants is challenging. To overcome these technical limitations, we mined the *Giardia* genome for conserved basal body proteins and used proteomic approaches to identify new *Giardia* basal body proteins and confocal microscopy to confirm their localization in interphase trophozoites. This study enhances the understanding of the composition of the *Giardia* basal bodies and extends the understanding of the biological divergence of these conserved organelles.

2. Materials and methods

2.1. HMMER search of genome

A dataset containing the predicted protein sequences of *G. lamblia* was downloaded from <http://giardiadb.org>. The single sequence query JackHMMER program is part of the HMMER3 software package (Johnson et al., 2010) and was used to query the *G. lamblia* protein dataset for the conserved basal body proteins listed in Table 1 and Supplementary Tables S1 and S2. The inclusion threshold was set at 0.001.

2.2. Cell culture

Giardia trophozoites (strain WB, clone C6, American Type Culture Collection GL50803) were cultured in modified TYI-S-33 medium with bovine bile (Diamond et al., 1978; Keister, 1983). Trophozoites were grown to late log phase, at ~80% confluence and, where specified, were treated for 14 h with 8 μ M nocodazole (Sigma, USA) to facilitate subsequent depletion of the α - and β -tubulin rich flagellar axonemes from the basal body fraction. Viability of cells was determined by propidium iodide exclusion and remained >95% after nocodazole exposure.

2.3. Isolation of Giardia basal bodies

Giardia basal bodies were isolated by a modification of a protocol used for isolating basal bodies from the flagellated green alga *Spermatozopsis similes* (Geimer et al., 1997). Non-synchronized log phase cultures of *Giardia* trophozoites (6.5×10^8 trophozoites), mostly in interphase (<5% in mitosis), were chilled on ice and harvested by centrifugation. Trophozoites were washed three times with PBS and extracted by sequential 10 min incubations in 0.5% and 1.5% Triton-X-100 in PBS at room temperature to deplete membranes and cytoplasm. The resulting cytoskeletons were pelleted (800 g, 5 min), resuspended in 10% sucrose in TE buffer (10 mM Tris-HCl, pH 7.4, 2 mM EDTA), sonicated on ice (4×15 s, 30% pulse intensity, with 30 s intervals) and incubated overnight with Benzonase® nuclease (Novagen, USA) at 4°C to reduce viscosity of the lysate and to facilitate fractionation of the cytoskeleton components. The lysate was layered onto an

overnight-linearized sucrose density gradient (20–65%) and centrifuged at 12,500 g for 1 h at 4°C in an ultracentrifuge with the SW41 swinging bucket rotor (Beckman, USA). One ml fractions were collected from the side of the tube with a 20-gauge needle. Each fraction was diluted 1:1 with MT buffer (30 mM HEPES, 5 mM Na₂-EGTA, 15 mM KCl, pH 7.0) and centrifuged at 38,000 g for 30 min. Pellets were washed three times, resuspended in MT buffer and kept at 4°C. Protein concentrations of fractions were determined by the Coomassie Plus protein assay (Pierce, USA). Centrin and alpha-tubulin immunolabeling of intact cells, cytoskeletons and fractionated lysates is shown in Supplementary Fig. S1.

2.4. ELISA

Bottoms of 96 well plates (Nunc-Immuno Plate MaxiSorp Surface, Nalge Nunc International, USA) were coated with 0.5 µg protein from each fraction for 2 h at room temperature or at 4 °C overnight. Wells were blocked with 3% BSA in PBS + 0.05% Tween-20 for 1 h and subsequently probed with one of the following antibodies: monoclonal antibodies (mAbs) against: human centrin (20H5) (Salisbury et al., 1988), α-tubulin (T-9026, Sigma, USA), γ-tubulin (Nohynkova et al., 2000), then with anti-mouse horseradish peroxidase (HRP) or anti-rabbit HRP conjugates (Pierce, USA). All antibodies were diluted in 3% BSA in PBS + 0.05% Tween-20. After rinsing the plate, signals were revealed by adding 100 µl of SureBlue™ (KPL, USA) and reactions were stopped with 100 µl of 0.1 M HCl. O.D. was measured in an ELISA plate reader (Molecular Devices, USA) at 450 nm.

2.5. Proteomic analyses

The fractions to be analyzed were washed three times in MT buffer, pelleted at 20,800 g and trichloroacetic acid precipitated (6%) overnight at 4 °C. Trichloroacetic acid precipitates from basal bodies purified from nocodazole-treated or control vegetative cells were resuspended in 200 mM Na₂CO₃, pH 11, then adjusted to 8 M urea, reduced and alkylated as previously described (Wolters et al., 2001). Proteinase K (5 µg) was added to the sample and incubated at 37°C for 5 h in a Thermomixer (Brinkmann, USA) (Wu et al., 2003). The digestion was stopped by the addition of formic acid to 5% and microcentrifuged at 18,000 g at 4°C for 15 min to remove particulates. The protein digest was pressure-loaded onto a fused silica capillary desalting column containing 5 cm of 5 µm Polaris C18-A material (Metachem, USA) packed into a 250 µm inner diameter (i.d.) capillary with a 2 µm filtered union (UpChurch Scientific, USA). The desalting column was washed with buffer containing 95% water, 5% acetonitrile and 0.1% formic acid. After desalting, a 100 µm i.d. capillary with a 5 µm pulled tip packed with 10 cm of 5 µm Aqua C18 material (Phenomenex, USA) followed by 3 cm of 5 µm Partisphere strong cation exchanger (Whatman, USA), was attached to the filter union. The entire split-column (desalting column–filter union–analytical column) was placed inline with an Agilent 1100 quaternary HPLC (Agilent, USA) and analyzed using a modified 10-step separation method described previously (Wolters et al., 2001). The buffer solutions used were 5% acetonitrile/0.1% formic acid (buffer A), 80% acetonitrile/0.1% formic acid (buffer B), and 500 mM ammonium acetate/5% acetonitrile/0.1% formic acid (buffer C). Step 1 consisted of a 70 min gradient from 0–100% buffer B. Steps 2–9 had the following profile: 3 min of 100% buffer A, 2 min each of 10, 20, 30, 40, 50, 60, 90 and 100%, respectively, buffer C, a 10 min

gradient from 0-15% buffer B, and a 97 min gradient from 15-45% buffer B. In the final step, the gradient contained: 3 min of 100% buffer A, 20 min of 90% buffer C and 10% buffer B, a 10 min gradient from 0-15% buffer B, and a 107 min gradient from 15-70% buffer B. As peptides eluted from the microcapillary column, they were electrosprayed directly into a LTQ mass spectrometer (ThermoFinnigan, USA) with the application of a distal 2.4 kV spray voltage. A cycle of one full-scan mass spectrum (400-1800 m/z) followed by five data-dependent MS/MS spectra at a 35% normalized collision energy was repeated continuously throughout each step of the multidimensional separation. Application of mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcaliber datasystem. Poor quality spectra were removed from the dataset using an automated spectral quality assessment algorithm (Bern et al., 2004).

MS/MS spectra remaining after filtering were searched with the SEQUEST™ algorithm (Eng et al., 1994) against the *G. lamblia* ('giardia14') database, concatenated to a decoy database in which the sequence for each entry in the original database was reversed (Peng et al., 2003). No enzyme specificity was considered for any search. SEQUEST results were assembled and filtered using the DTASelect (version 2.0) program (Cociorva et al., 2006). DTASelect 2.0 uses a linear discriminant analysis to dynamically set XCorr and DeltaCN thresholds for the entire dataset to achieve a user-specified false positive rate (5% in this analysis). The false positive rates are estimated by the program from the number and quality of spectral matches to the decoy database. *Giardia* proteins were identified by two or more different peptides in three independent analyses. Proteins were queried using BLASTP searches (Altschul et al., 1990) against the Swissprot database.

2.6. Epitope tagging of proteins

The region containing the presumed promoter (>100 bp upstream of the start codon) and coding sequences for Gene IDs GL50803_104685, GL50803_6744, GL50803_16973, GL50803_4689, GL50803_4692, GL50803_11867, GL50803_13352, GL50803_15218, GL50803_16279, GL50803_92498, GL50803_104150, GL50803_12057, GL50803_41512, GL50803_16202, GL50803_14373, GL50803_134441, GL50803_11992, and GL50803_15455 were amplified from *G. lamblia* strain WB clone C6 genomic DNA with appropriate primers (Supplementary Table S2) (see www.giardiadb.org for gene details). The PCR products and the vector encoding the C-terminal AU1 tag (Weiland et al., 2003) or hemagglutinin (HA) tag (Touz et al., 2003) were digested with the respective restriction enzymes (Supplementary Table S2). Digested inserts and vectors were gel extracted using a QIAquick Gel Extraction Kit (Qiagen, USA), and ligated overnight at 14°C. Plasmids were transformed into *Escherichia coli* JM109 (Promega, USA). Bacteria were grown overnight in Luria broth and plasmid DNA was purified using a Maxiprep kit (Qiagen, USA) and sequenced (Etonbio, USA). Trophozoites were electroporated with 50 µg of plasmid DNA and transfectants were maintained through puromycin selection (Knodler et al., 1999).

2.7. Calmodulin antibody

The coding sequence of giardial calmodulin was PCR-amplified from *G. lamblia* genomic DNA using primers 5'aggacgggatccatggaggagacatgcc3' and 5'ccctcgagttacctctgcagatattcttac3'. The resulting product was restriction digested and

ligated into the expression vector pET 28a (Novagen, USA) containing a carboxy-terminal polyhistidine tag. Plasmids containing the calmodulin insert were transformed into BL21DE3 bacteria (Novagen, USA) and induced at 37°C with 0.5 mM of IPTG for 2 h to express recombinant his-tagged calmodulin, which was then purified over Ni-NTA agarose (Qiagen, USA). Antibody to this protein was developed in chickens by a standard protocol (QED Bioscience Inc., USA).

2.8. Immunofluorescence analysis

Trophozoites were harvested by chilling and allowed to adhere to coverslips at 37°C for 10 min. Whole trophozoites were fixed in situ with methanol (−20°C), permeabilized for 10 min with 0.5% Triton X-100 in PBS (Abel et al., 2001) and blocked for 1 h in 5% goat serum, 1% glycerol, 0.1% BSA, 0.1% fish gelatin and 0.04% sodium azide. Coverslips were subsequently incubated for 1 h with the FITC labeled rat mAb against HA (Roche, USA) and the mouse mAb against centrin (20H5) or with the mouse mAb against AU1 (raw ascites, Covance, USA) and the rabbit pAb against centrin (J. Salisbury Mayo Clinic, USA) or with the chicken anti-calmodulin antibody and the mouse mAb against centrin. Finally, the coverslips were incubated with a goat anti-mouse, anti-rabbit or anti-chicken Alexa 568 conjugate, washed, postfixed with 4% paraformaldehyde, rinsed with PBS and mounted with Prolong Gold with DAPI (Molecular Probes, USA). Staining was monitored and photographed on a Nikon Eclipse E800 microscope with an X-Cite™ 120 fluorescence lamp and 1000× magnification (Nikon Instruments Inc., USA). Confocal images were taken with the Leica TCS SP5 system attached to a DMI 6000 inverted microscope (Leica, USA).

3. Results and Discussion

3.1. HMMER search for conserved basal body proteins in the *Giardia* genome

To identify *Giardia* basal body proteins, we used a list of proteins that have been immunolocalized to basal bodies/centrosomes in human, *Chlamydomonas* and *Tetrahymena*. Homologs of conserved basal body proteins that were detected in assemblage A of the *Giardia* genome by JackHMMER searches (Johnson et al., 2010) are listed in Table 1, while the proteins of which no homologs were found in the *Giardia* genome are listed in Supplementary Table S1. Searches of the *Giardia* assemblage B and E genomes revealed that with the possible exception of Cep 76 (GL50803_5672), which was not detected in assemblage B, all genes are syntenic (data not shown). Since several of the identified *Giardia* proteins have no functional annotation (Table 1) (www.giardiadb.org/giardiadb), we refer to these proteins by their Gene ID numbers and use the annotation of their conserved basal body homolog in the classification and discussion below.

3.1.1. Proteins involved in centrosomal assembly/duplication—Previous genomic analyses showed that giardial pathways and multicomponent structures have fewer identifiable components than in yeast or other eukaryotes (Morrison et al., 2007). Our mining of the *Giardia* genome identified few proteins known to be crucial for centrosomal maturation and duplication cycle in metazoan cells (Azimzadeh and Bornens, 2007; Kleylein-Sohn et al., 2007; Loncarek and Khodjakov, 2009). For example, in mammalian cells, centriolar duplication is initiated by complexing of the cell cycle dependent kinase 2

(cdk2) with cyclins A and E whereas in *Giardia*, cyclin E is missing (Supplementary Table S1). Based on previous suggestions that cyclin A and cyclin E have redundant functions in certain eukaryotic cell types, we hypothesize that in *Giardia*, the role of cyclin E in the G1-S phase transition is taken over by cyclin A (Kalaszczynska et al., 2009; Ferguson et al., 2010).

The centriolar components SAS-4/CPAP, SAS-6 and BLD10/Cep135 belong to a universal module of functionally conserved proteins involved in the early steps of centrosome/basal body assembly and establishment of their nine-fold symmetry (Salisbury, 2003; Matsuura et al., 2004; Nakazawa et al., 2007; Culver et al., 2009; Carvalho-Santos et al., 2010). We confirmed the presence of a *Giardia* SAS-4 homolog (Hodges et al., 2010) and identified a SAS-6 but not a BLD-10 homolog. In *Chlamydomonas*, SAS-6 and BLD-10, and in *Tetrahymena*, SAS-6a, are coiled coil proteins that localize to the cartwheel (Fig. 1A), the earliest nine-fold symmetrical structure to appear during eukaryotic centriolar assembly. It is possible that in *Giardia*, the function of BLD-10 is lost or taken over by other cartwheel proteins. For example, homologs of the *Tetrahymena* cartwheel proteins Bbc29 and Bbc82 (Kilburn et al., 2007) are suggested to have functions in early basal body assembly and are present in the *Giardia* genome (Pearson and Winey, 2009). Alternatively, since the structures of the BLD10 homologs are quite divergent (Carvalho-Santos et al., 2010), it is possible that the HMMER search did not detect the *Giardia* homolog.

Other proteins that are indispensable for centrosomal duplication in humans such as Polo kinase 4 (Plk4/ZYG-1), Synpolydactyly 2 (SPD2/Cep192) and centrosomal protein 110 (CP110) are taxon-specific and are not found in protists (Carvalho-Santos et al., 2010; Hodges et al., 2010) including *Giardia* (Supplementary Table S1). SPD2 and CP110 are only found in metazoans and Plk4/SAK/ZYG-1 plays an important role upstream of SAS-6 and SAS-4 (Delattre et al., 2006) but is only found in opisthokonts, a group of eukaryotes that includes animals, fungi, choanoflagellates and organisms with a single posterior flagellum (Carvalho-Santos et al., 2010).

3.1.2. Basal body scaffolding proteins—Similar to *Chlamydomonas* and *Tetrahymena*, the *Giardia* genome lacks genes encoding the centrosomal large coiled-coil proteins, pericentrin/kendrin, centrosome and Golgi localized protein kinase N-associated protein (CG-NAP)/A kinase anchoring protein (AKAP450), the microtubule anchoring proteins (FGFR1 Oncogene Partner) FOP, centrosome associated protein 350 kDa (CAP350) (Yan et al., 2006) and members of the dynactin family (Supplementary Table S1). CG-NAP/AKAP450 and pericentrin are scaffolding proteins that provide a platform for signaling proteins such as PP2Ac and PKAc (Takahashi et al., 1999; Diviani et al., 2000). Since both PP2Ac and PKAc localize to the *Giardia* basal bodies (Abel et al., 2001; Lauwaet et al., 2007), we propose that the function of the scaffolding CG-NAP/AKAP450 and pericentrin are replaced by numerous other coiled-coil proteins in the giardial genome (Morrison et al., 2007).

3.1.3. Conserved *Chlamydomonas* and *Tetrahymena* basal body proteins—The structure and composition of basal bodies has been studied in depth in *Chlamydomonas* and *Tetrahymena*. Our JackHMMER analysis of the *Giardia* genome detected numerous

homologs of the immunolocalized *Chlamydomonas* and *Tetrahymena* basal body proteins. We found 10 homologs of the 16 immunolocalized *Tetrahymena* basal body component (Bbc) proteins, 10 of the 19 immunolocalized *Chlamydomonas* proteome of centriole (POC) proteins and five homologs (Table 1) of the eight immunolocalized *Chlamydomonas* basal body proteins with upregulated genes (BUG) (Keller et al., 2005, 2009). The genes encoding BUG proteins are upregulated in *Chlamydomonas* flagellar regeneration and are suggested to have important functions during flagellar assembly (Keller et al., 2005). We did not identify *Giardia* homologs of *Chlamydomonas* OFD1/BUG11 and NPHP-4/POC10 which were found to be associated with ciliary diseases in humans (Keller et al., 2005) (Supplementary Table. S1).

3.2. Proteomic analysis of basal body enriched fractions

To identify basal body proteins unique to *Giardia* and confirm the expression of *Giardia* basal body homologs in trophozoites, we prepared a basal body enriched fraction from vegetative trophozoites (see Section 2.3, Supplementary Fig. S1) and analyzed its composition by Multidimensional Protein Identification Technology (MudPIT) analysis (Delahunty and Yates, 2007). Since immunofluorescence analysis and ELISA studies revealed large amounts of α -tubulin-containing structures (mainly flagella) in the fractions of the initial experiments, cells were treated with nocodazole as was previously reported for isolation of centrosomes from T-lymphoblastic cells (Bornens et al., 1987). The nocodazole concentration used had no detectable effect on cell viability (>99%) or motility and did not deflagellate cells or greatly affect cellular morphology at the light microscopy level (Supplementary Fig. S1). Nocodazole appeared to facilitate the detachment of flagella during subsequent sonication of the isolated cytoskeletons and greatly reduced flagellar contamination in the final basal body enriched sucrose gradient fractions (Supplementary Fig. S1). Sucrose gradient fractions were tested for enrichment of the known basal body marker proteins, centrin and γ -tubulin, relative to α -tubulin by ELISA (Fig. 1B). Centrin and γ -tubulin were enriched by ~71% and ~40% in the 20% sucrose fraction (fraction 8), respectively, compared with the untreated control (Fig. 1B). We analyzed the protein composition of this basal body enriched fraction (BBEF) by MudPIT (Wolters et al., 2001; Keller et al., 2005). Individual peptides were matched to proteins in the *Giardia* genome (GiardiaDB release 1.1) (Morrison et al., 2007). Three independent analyses detected a total of 21,266 peptides corresponding to 363 proteins identified by at least two different peptides (Supplementary Table S2). Of these, only 38 are homologs of proteins that have been localized to either *Chlamydomonas* or *Tetrahymena* basal bodies or to human centrosomes (Table 1). The remaining 325 proteins include homologs of proteins that have been identified in published basal body proteomes but whose localization to basal bodies have not been confirmed, as well as *Giardia*-specific basal body proteins and contaminants. Immunolocalization studies are needed to distinguish between true basal body and contaminating proteins.

3.3. Immunolocalization of selected candidate basal body proteins

We epitope tagged and immunolocalized 16 conserved basal body proteins present in both the *Giardia* genome (Tables 1 and Supplementary Table S1) and proteome analyses (Supplementary Table S2). Cells expressing epitope tagged proteins were double labeled

using antibodies against the HA or AU1-tag and centrin. Proteins were considered localized to *Giardia* basal bodies if confocal microscopy confirmed centrin co-localization with the tagged protein. Localized basal body proteins are discussed below within the context of their functions and localization patterns.

3.3.1. Calcium binding EF-hand proteins—Centrins are calcium-binding EF-hand proteins that belong to the calmodulin family, concentrate in the lumen of the centriole, and are considered universal basal body markers (Bornens and Azimzadeh, 2007). Nonetheless, the function of centrins in centriolar duplication is species-specific. Centrins are required for *Tetrahymena* and *Chlamydomonas* centriolar assembly, while there is no evidence for a role for centrin in *Drosophila* and *Caenorhabditis elegans* centriolar duplication (Koblenz et al., 2003; Stemm-Wolf et al., 2005; Azimzadeh and Bornens, 2007). In humans, the function of centrin is controversial since initial studies using small interfering RNA (siRNA) in HeLa cells demonstrated that centrin 2 is required for centriole duplication (Salisbury et al., 2002), while more recent studies in which two centrins were depleted had no effect on centriole formation (Kleylein-Sohn et al., 2007).

Giardia has two centrins whose functions have not been studied. Heterologous human centrin antibodies immunolocalized *Giardia* centrin to the basal bodies, posterior-lateral PFR (Fig. 2A) and in some studies to the median body (Meng et al., 1996; Correa et al., 2004). Since heterologous antibodies recognized both centrins (Meng et al., 1996; Correa et al., 2004), we individually epitope tagged both centrins and found that centrins 1 (Gene ID GL50803_6744) and 2 (Gene ID GL50803_104685) have identical localization patterns and localize to the basal bodies and posterior-lateral PFR (Fig. 2A). Centrin is universally found in basal bodies and centrosomes but its localization to additional structures is not unusual. For example, in *T. thermophila*, centrin localizes to the oral crescent that lines the cystosome, in *Trypanosoma brucei* centrin 2 localizes to an additional bi-lobed structure near the Golgi apparatus, and *Paramecium* centrin localizes to the infraciliary lattice (Madeddu et al., 1996; Moudjou et al., 1996; McLaughlin and Buhse, 2004; He et al., 2005), suggesting that centrin may have species-specific functions.

Calmodulin, a well-studied mediator of calcium signaling pathways (Reiner et al., 2003), has not been found to localize to basal bodies/centrosomes in other organisms. However, *Giardia* calmodulin (Gene ID GL50803_5333) has high sequence similarity with the *Chlamydomonas* and *Tetrahymena* basal body proteins POC6 and Bbc82, respectively, and was found to localize to basal bodies and cytoplasmic puncta (Reiner et al., 2003). We now demonstrate, using detailed confocal microscopic analyses, that calmodulin immunolocalizes between and anterior to the nuclei and overlaps with centrin in several but not in all basal bodies (Fig. 2A). Thus calmodulin, which is essential for excystation, can be considered a valid giardial basal body protein, further expanding insights into its potential functions. Future studies are needed to evaluate interactions between calmodulin and other basal body proteins.

We also identified another EF-hand protein (Gene ID GL50803_41512) in the BBEF proteome, currently annotated as ‘flagella associated protein’, that is homologous to the protofilament ribbon protein Rib72/POC9 in *Chlamydomonas*, and basal body component

73 (Bbc73) in *Tetrahymena*. Rib72/POC9 localizes to human centrosomes (Keller et al., 2009) and to the microtubule scaffold of the *Tetrahymena* basal bodies (Kilburn et al., 2007), while the epitope-tagged *Giardia* homolog (Gene ID GL50803_41512) localizes to the basal bodies and the cytoplasm (Fig. 2A). In *Chlamydomonas*, Rib72 localizes to the flagella, regulating axonemal assembly and flagellar motility (Ikeda et al., 2003). Since the *Giardia* homolog does not localize to flagella, we suggest that the *Giardia* homolog (Gene ID GL50803_41512) has a distinct function.

3.3.2. Tubulins and ring complex components— α - and β -tubulins are major conserved structural components of basal bodies and flagellar axonemes, which are abundant in *Giardia* basal bodies and other cytoskeletal structures such as the median bodies and disk (Supplementary Fig. S1). γ -tubulin is present in centrosomes/basal bodies and cytoplasm in most eukaryotes and spindle poles in yeast. In *Giardia*, γ -tubulin only immunolocalizes to the basal bodies of interphase and late mitotic cells, and does not localize to the spindle poles during mitosis (Nohynkova et al., 2000). Although we did not detect γ -tubulin peptides by MudPIT, we readily quantitated γ -tubulin in the BBEF using the more sensitive ELISA (Fig. 1B). γ -tubulin functions in a ring complex that nucleates microtubule assembly. Most metazoan cells have two γ -tubulin ring complexes: a γ -tubulin small complex (γ TuSC) and a large γ -tubulin ring complex (γ TuRC). γ TuSC is composed of two γ -tubulin chains, a γ -tubulin complex component (GCP) 2 and a GCP3. The larger γ TuRC is composed of several γ TuSC in addition to GCP-4, -5 and -6. Similar to *Saccharomyces cerevisiae*, *Chlamydomonas* and *Tetrahymena*, *Giardia* only has homologs of GCP-2 and -3 (Table 1), suggesting that only the smaller γ TuSCs are formed (Cuschieri et al., 2007; Raynaud-Messina and Merdes, 2007) and are likely sufficient for centrosomal microtubule nucleation for spindle formation, and may be crucial in mitosis and cell cycle progression (Raynaud-Messina and Merdes, 2007). We identified the *Giardia* homolog of GCP-3 (Gene ID GL50803_12057) in the BBEF and the HA-tagged protein immunolocalizes to the *Giardia* basal bodies (Fig. 2B; Table 1).

In *C. elegans*, γ -tubulin is also required for the recruitment of SAS-4 (see Section 3.1.1.) (Dammermann et al., 2008). The *Giardia* SAS-4 homolog (Gene ID GL50803_13352) is present in the BBEF, colocalizes with centrin to the basal bodies and localizes to the median body and the posterior-lateral PFR (Fig. 2B).

δ - and ϵ -tubulins are required for basal body assembly in metazoan cells and in *Chlamydomonas* (Dutcher, 2003b), but are not found in *S. cerevisiae*, *Drosophila melanogaster* or *C. elegans* genomes. Surprisingly, both δ - and ϵ -tubulins are present in the *Giardia* genome (Morrison et al., 2007). Although the associations of δ - and ϵ -tubulins to the *Chlamydomonas* basal bodies are microtubule-independent (Dutcher, 2003) we only found *Giardia* ϵ -tubulin in the basal body enriched fraction from non-nocodazole treated trophozoites, suggesting *Giardia* ϵ -tubulin is an integral basal body component linked by tubulins. We did not find δ - tubulin in the BBEF.

3.3.3. Mitotic kinases and phosphatases—The four major families of mitotic kinases that control chromosomal segregation during the cell cycle are Never in Mitosis Gene A-related (NEK), Polo, Aurora and Cdk, which are serine/threonine kinases with members that

localize to the centrosomes/basal bodies in mammalian cells (Malumbres and Barbacid, 2007). To date, aurora kinase is the only cell cycle kinase that has been shown to be crucial for giardial cell division and localizes to basal bodies and spindle poles in mitotic trophozoites (Davids et al., 2008).

NEK are important regulators of eukaryote cell cycle and axonemal microtubule dynamics (Quarmby and Mahjoub, 2005; O'Regan et al., 2007). We identified 11 NEK homologs in the BBEF and immunolocalized two (Fig. 2C). In addition to their basal body localization, one NEK (Gene ID GL50803_16279) localizes to the ventral disk as well as to the intracellular portions of the posterior-lateral and caudal flagellar axonemes and/or their associated structures. The other NEK (Gene ID GL50803_92498) additionally localizes to the anterior PFR and axonemes, ventral disk, median body and intracellular portions of the caudal and posterior-lateral axonemes (Fig. 2C). NEK also localizes to the flagella and basal bodies in *Chlamydomonas* and *Tetrahymena*, where they have roles in flagellar disassembly and length control (Mahjoub et al., 2002; Bradley and Quarmby, 2005; Wloga et al., 2006). Humans and most non-ciliated organisms have 10 or fewer NEKs, but the number of NEKs tends to be higher in ciliated/flagellated organisms (Parker et al., 2007). This group is dramatically expanded in the *Giardia* genome which contains 199 NEKs, none of which has previously been localized or functionally characterized (Morrison et al., 2007) (unpublished data). Recently, six NEKs have been identified in a proteomic analysis of the *Giardia* mitosome but their localization has not been confirmed (Jedelsky et al., 2011). The localization of our immunolocalized NEKs to the basal bodies and flagellar associated structures supports earlier speculation (Parker et al., 2007) that the high number of *Giardia* NEKs might reflect the need to regulate the complex process of flagellar maturation and segregation during *Giardia* cytokinesis (Nohynkova et al., 2006; Parker et al., 2007).

Giardia has only one Polo-like kinase that is homologous to Plk1 (Gene ID GL50803_104150) (Morrison et al., 2007). Plk1 plays a role in human centrosomal duplication (Liu and Erikson, 2002; Tsou et al., 2009), spindle assembly, cytokinesis, DNA damage response (Dai, 2005) and the recruitment of γ -tubulin to the centrosome (Haren et al., 2009). HA-tagged *Giardia* Plk1 localizes to puncta in the disk and co-localizes with centrin to the basal bodies (Fig. 2C). This suggests that the basal body and cell cycle related functions of Plk1 are conserved in *Giardia*.

3.3.4. Homologs of basal body proteins with flagellar functions—The BBEF contains homologs of basal body proteins that have flagellar functions in other organisms. For example, human ciliary protein SPAG6 (sperm associated antigen 6) localizes to the transition zone of the basal body in *Tetrahymena* and to the basal bodies and the central pair microtubules of the flagellar axoneme in *Chlamydomonas*. Mutation in the *Chlamydomonas* SPAG6 homolog PF16 results in flagellar paralysis (Smith and Lefebvre, 1996; Sapiro et al., 2000), supporting its role in flagellar motility. Immunolocalization of the *Giardia* homolog of SPAG6 (Gene ID GL50803_16202) shows that this protein mainly localizes to the basal bodies, and weakly to the flagella associated anterior and posterior-lateral PFR and median body (Fig. 2D). Another example is the coiled-coil protein Rib43a which, together with tektins and alpha- and beta-tubulins, constitutes the main structural component of the electron dense ciliary and flagellar protofilaments. Recent studies have shown that ribbon

proteins also localize to basal bodies in *Chlamydomonas* (Norlander et al., 2000). The *Giardia* Rib43a homolog (Gene ID GL50803_11867) localizes to the basal bodies, posterior-lateral PFR and axonemes, caudal axonemes and median body (Fig. 2D). Although the functions of both the PFR and median body are currently unknown, the presence of both SPAG6 and Rib43 homologs in the flagellar associated PFR and median body might indicate a function for these structures in flagellar assembly and/or motility.

3.3.5. Homologs of conserved basal body proteins of unknown function—We epitope tagged and immunolocalized (Fig. 2E) the *Giardia* homologs of *Chlamydomonas*' BUG14/POC16 (Gene ID GL50803_15218) and *Tetrahymena* Bbc52 (Gene ID GL50803_13467) which localize to the human centrosomes and *Tetrahymena* basal bodies, respectively (Keller et al., 2005; Kilburn et al., 2007), and whose functions are not yet known. The *Giardia* BUG14/POC16 homolog Gene ID GL50803_15218 co-localizes with centrin to the basal bodies and localizes to the posterior-lateral, and faintly to the anterior and caudal PFR, and the median body (Fig. 2E). According to a recent phylogenetic centriole study, GL50803_15218 is homologous to WDR16, a protein of unknown function that localizes to basal bodies in trypanosomes and is expected to have centriolar functions based on its presence in a variety of ciliated eukaryotic cells (Hodges et al., 2010).

The Bbc52 homolog Gene ID GL50803_13467 localizes to the basal bodies and all flagellar pairs except for the ventral pair (Fig. 2E). Our finding that the basal body localization of these functionally uncharacterized proteins is conserved highlights them as valuable candidates for future functional studies in *Giardia* and other organisms.

3.3.6. Dynamin—We detected a large number of dynamin peptides in the BBEF. Dynamin is a GTPase that has been well characterized in *Giardia* (Gaechter et al., 2008). In vegetative trophozoites, dynamin (Gene ID GL50803_14373) localizes to peripheral vesicles and the bare area in the center of the ventral disk (Adam, 2001) and has a major function in endocytosis (Gaechter et al., 2008). Previous studies in rat fibroblasts and in HeLa cells have shown that dynamin 2 co-localizes with γ -tubulin to centrosomes and is responsible for centrosomal cohesion and cell cycle progression (Thompson et al., 2004; Ishida et al., 2011). Our confocal analysis of epitope tagged *Giardia* dynamin in trophozoites confirms the localization to the peripheral endosomal-lysosomal vesicles and an unknown structure central and dorsal to the disk (Gaechter et al., 2008). In addition, we find partial overlap of the central dynamin staining with centrin in the basal bodies (Fig. 3), explaining the presence of dynamin in the BBEF. Further studies are needed to define the interaction of basal body-associated dynamin with other organelles in vegetative and encysting cells.

3.3.7. Homologs of conserved basal body proteins that do not localize to the giardial basal bodies—We found that some of the proteins in the BBEF do not immunolocalize to the basal bodies in vegetative *Giardia* trophozoites despite their presence in human, *Chlamydomonas* and/or *Tetrahymena* basal bodies/centrosomes. Examples are: Enolase (Johnstone et al., 1992), 14-3-3 protein (Pietromonaco et al., 1996), Bbc20 (Kilburn et al., 2007; Sedjai et al., 2010), BUG21/PACRG (Keller et al., 2005), and Ran (Chen et al., 1994). Instead, ours and others' studies show that protein 14-3-3 (Lalle et al., 2006), enolase (Ringqvist et al., 2008), TCP-1 (Gene ID GL50803_11992) (Fig. 4) and BUG21/PACGR

(Gene ID GL50803_15455) (Fig. 4) localize to the cytoplasm of vegetative trophozoites. Bbc20 (Gene ID GL50803_134441) localizes to the anterior, posterior-lateral and caudal PFR (Fig. 4). Ran (Gene ID GL50803_15869) localizes to *Giardia* nuclei (Chen et al., 1994). The centrosomal localization of Ran in Hela cells is mediated by the matrix A anchoring protein AKAP450 (Keryer et al., 1993) and the absence of AKAP450 in the *Giardia* genome might help to explain the localization of *Giardia* Ran.

It is likely that these proteins appear in our BBEF as contaminants due to their abundance in the cell cytoplasm (TCP-1, PACGR) or their localization to structures that are associated with the basal bodies (e.g. nuclei (Ran), PFR (Bbc20)). Due to their proximity to the basal bodies, additional co-affinity purification and functional studies are necessary to determine whether these proteins do indeed interact with basal body proteins. Alternatively, it is possible that some of these proteins localize to the basal bodies but that the C-terminal epitope disrupted the normal pattern of localization. This is not likely because previous C-terminal-tagged proteins that are expressed under their own promoters localize accurately (Reiner et al., 2003; Sun et al., 2003), as we have confirmed with centrin (Fig. 2).

3.3.8. Unique basal body proteins in *Giardia*—Proteomic analysis can systematically identify novel *Giardia*-specific basal body proteins that have no detectable homology to proteins in other species. We epitope tagged three such proteins that are present in the BBEF. The proteins with Gene ID GL50803_4689 and 4692 both localized to the basal bodies and not to other recognizable cytoskeletal structures or to scattered puncta in the cytoplasm (Fig. 5). Similar to the homologs of known basal body proteins (Figs. 3, 6) and previously characterized giardial basal body proteins (Abel et al., 2001; Ellis et al., 2003; Gibson et al., 2006; Lauwaet et al., 2007), the protein with Gene ID GL50803_16973 localizes to the basal bodies, the median body and to the posterior-lateral PFR (Fig. 5). Despite their distribution to puncta in the cytoplasm, these proteins appeared to be concentrated in the basal bodies with centrin.

Future studies are necessary to determine whether these and the other proteins in our BBEF which are unique to *Giardia* have a role in *Giardia* basal body regulation.

Our genomic and proteomic approach identified 75 *Giardia* homologs of conserved basal body proteins in the *Giardia* genome, 65 of which had not previously been associated with *Giardia* basal bodies. Our confocal studies confirmed the basal body localization of 13 of these newly identified proteins in interphase trophozoites. In addition, we identified three unique proteins in the BBEF with no homology to conserved basal body proteins and immunolocalized those to the basal bodies. While the functional analysis of each protein is beyond the scope of this study, our findings greatly enhance the current understanding of these proteins which were previously only known as ‘hypothetical proteins’ or were named after a structural repeat or domain in their amino acid sequence.

We demonstrate that 13 of the 16 homologs of conserved basal body proteins that we immunolocalized, indeed localize to the basal bodies, suggesting a basal body-specific role for these proteins in *Giardia*. However, in addition, most of these conserved basal body proteins also localize to cytoskeletal structures unique to *Giardia* (PFR, ventral disk, median

body) (Fig. 6). This was previously observed for the universal signaling proteins, PP2Ac, PKAc, PKAr, and ERK1 (Abel et al., 2001; Ellis et al., 2003; Gibson et al., 2006; Lauwaet et al., 2007). The functions and protein compositions of the median body and PFR are largely unknown. Therefore, the differential localization of conserved basal body proteins to these structures might yield valuable insights into their roles in motility, cell division and attachment. They hold new clues to the signaling pathways that regulate these functions. For example, the anterior flagella are suggested to be involved in forward motion, swimming and steering, and the caudal flagella are suggested to function as a rudder and in cell detachment (Elmendorf et al., 2003). While PP2Ac, PKAc and PKAr localize to both flagellar pairs, NEK (GL50803_92498) and Rib43A localize only to the anterior flagellar pair while NEK (GL50803_16279) (Fig. 2C) and ERK1 localize to the caudal pair (Abel et al., 2001; Ellis et al., 2003; Gibson et al., 2006; Lauwaet et al., 2007). Phosphorylated aurora kinase localizes to the anterior PFR in mitotic cells (see below), suggesting that it helps to regulate this flagellar pair.

Mitotic kinases such as NEK (GL50803_16279 and GL50803_92498), Plk1 and ERK additionally localize to the ventral attachment disk in interphase cells. In contrast, we did not detect Aurora kinase (AurK), a well-known human basal body protein in the BBEF. We found previously that *Giardia* AurK localizes to the nuclei during interphase, while during mitosis it is activated by phosphorylation and targets to the basal bodies/spindle poles and fibers, the anterior paraflagellar dense rods, the median body, as well as the parental attachment disk (Davids et al., 2008). AurK inhibitors disrupt both the cytoskeleton and mitosis. Taken together, these observations strongly suggest distinct roles for these kinases in the disassembly of the disk during *Giardia* cytokinesis (Tumova et al., 2007).

Moreover, it is remarkable that few basal body proteins localized to the extracellular parts of the flagella and none localized to the ventral flagella. This suggests that the ventral flagellar motility is likely regulated by distinct signaling pathways. The ventral flagellar pair is the most motile and extracellular and has a characteristic sine wave beating that is proposed to regulate the suction of the attachment disk (Holberton, 1973; Elmendorf et al., 2003).

Our confocal microscopy studies showed that despite the overlapping localization of centrin with basal body proteins, there is heterogeneity in the basal body staining patterns. Even centrin immunolocalization patterns can vary in some cells (Fig. 2), possibly reflecting different stages of the cell cycle. While some proteins clearly localize to distinct basal body puncta, others (e.g. calmodulin, dynamin, unique proteins) localize to a less defined area that overlaps with centrin labeled basal bodies. These different patterns might be due to the localization of proteins to distinct parts of the basal bodies (e.g. cartwheel, lumen, transition zone, PCM) (Fig. 1A). Previous studies suggested that not all *Giardia* basal bodies have the same protein composition. For example, γ -tubulin localizes to the basal bodies of the ventral and posterior-lateral but not the caudal and anterior flagella (Nohynkova et al., 2000). High-resolution electron microscopy studies are needed to determine the exact basal body location of each protein.

Our study demonstrates that a combined genomic and proteomic approach is a valuable tool for identifying conserved and unique candidate basal body proteins and confirms that

immunolocalization is important for authentication. Some conserved basal body proteins did not localize to the *Giardia* basal bodies, reflecting divergent localizations and possible distinct functions of these proteins in different organisms. Future studies are likely to reveal additional basal body proteins. Nonetheless, our identification and localization of previously unknown *Giardia* proteins contributes to the annotation of the genome. Moreover, it greatly enhances the understanding of the composition of the basal bodies and unique cytoskeleton of *Giardia*, and suggests roles in cell cycle and flagellar regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Enrichment of *Giardia* basal body proteins in the basal body enriched fraction (BBEF) (fraction 8). (A) Cartoon of a basal body attached to a flagellar axoneme. (B) Nocodazole treatment (open symbols) reduces the amount of α -tubulin and increases the amount of centrin and γ -tubulin in the 20% sucrose fraction (fraction 8), compared with the untreated control (closed symbols). The relative amounts of α -tubulin, γ -tubulin and centrin in each fraction were determined by ELISA (O.D. at 450 nm). The scales of the Y-axes are different in each graph, reflecting the abundance of each protein.

Fig. 2.

Immunolocalization of conserved basal body proteins to the *Giardia* basal bodies. (A) (a) *Giardia* trophozoites immunolabeled with a monoclonal antibody (mAb) against human centrin (20H5) and trophozoites expressing AU1-tagged centrin 1 (Gene ID GLGL50803_104685), or centrin 2 (Gene ID GL50803_6744) immunolabeled with a mAb against the AU1 epitope. (b) Confocal images show a *Giardia* trophozoite immunolabeled with antibodies against calmodulin (GL50803_5333) and centrin. (c) *Giardia* trophozoites expressing epitope tagged homologs of the EF hand protein Rib72 (GL50803_41512). (B) γ -

tubulin ring complex component-3 (GCP-3, GL50803_12057) and SAS-4 (GL50803_13352). (C) Mitotic kinases: NEK (Gene ID GL50803_16279; Gene ID GL50803_92498) and Polo kinase 1 (Plk1) (Gene ID GL50803_104150). (D) Proteins with flagellar functions in other organisms, SPAG6 (GL50803_16202) and Rib43a (GL50803_11867). (E) Conserved basal body proteins of unknown function, Bbc52 (GL50803_13467) and BUG14/POC16 (GL50803_15218). All were detected in the basal body enriched fraction (BBEF) (Supplementary Table S2), and were double immunostained with antibodies against the hemagglutinin (HA) or AUI-tag (Supplementary Table S3) and mAb 20H5 against centrin (see Section 2.8.). Co-localization of centrin and the proteins was analyzed by confocal microscopy. Images show centrin and HA or AUI labeling of a 0.1 μm optical section at the level of centrin labeled basal bodies. Colocalization of centrin and the HA or AUI tagged proteins are shown in the merged image. The insert shows a 1.5 $\times$ magnification of the basal body area. Total HA/AUI immunolabeling (projection) and a differential interference contrast image (DIC) through the entire cell are shown. Scale bar, 5 μm .

Fig. 3.

Immunolocalization of dynamin and centrin. *Giardia* trophozoites expressing hemagglutinin (HA)-tagged dynamin (Gene ID GL50803_14373) were double immunostained with an antibody against centrin and co-localization of both proteins was confirmed by confocal microscopy. Images show centrin and dynamin labeling of a 0.1 μm optical section at the level of centrin labeled basal bodies. The image labelled 'merge' shows co-localization between centrin and dynamin and is flanked by XZ and YZ confocal cross sections. Total cellular dynamin immunolabeling (projection) and a differential interference contrast (DIC) image of the entire cell are shown. Scale bar, 5 μm .

Fig. 4.

Immunolocalization of conserved basal body proteins that do not specifically localize to the basal bodies in vegetative *Giardia* trophozoites. Images show the immunolocalization of the AUI or hemagglutinin (HA)-tagged *Giardia* homologs of the conserved basal body proteins Bbc20 (Gene ID GL50803_134441), TCP-1 (Gene ID GL50803_11992) and PACGR (Gene ID GL50803_15455) that are present in the basal body enriched fraction (BBEF) (see Supplementary Table S2). Scale bar, 5 μ m.

Fig. 5.

Immunolocalization of *Giardia*-specific basal body proteins. *Giardia* trophozoites expressing AU1-tagged GL50803_4689/4692/16973 proteins that are present in the basal body enriched fraction (BBEF) were double immunostained with an antibody against centrin. Total cellular tagged protein (projection) for each protein is shown and a 0.1 μm optical section at the level of centrin labeled basal bodies is shown in the subsequent two panels. Co-localization of centrin and the epitope-tagged unique protein is shown in the image labelled 'merge'. A differential interference contrast (DIC) image is shown for each cell. Scale bar, 5 μm .

Fig. 6.

Cartoon summarizing the localization of all *Giardia* basal body proteins identified to date in interphase trophozoites. Proteins labeled as anterior, posterior-lateral and caudal flagella localize to either the intracellular axoneme, paraflagellar dense rods (PFR) or entire flagella (see Figs. 2, 3, 4 and 5). Numbers are the last five digits of the protein's Gene ID 50803_. Aurora kinase (Gene ID: 50803_5358) was not included in this cartoon because it only localizes to basal bodies in dividing cells. Proteins with * were localized in previous studies (Abel et al., 2001; Ellis et al., 2003; Reiner et al., 2003; Gibson et al., 2006; Lauwaet et al., 2007).

Table 1

Conserved basal body proteins in the *Giardia* genome. Proteins shown to immunolocalize to human centrosomes, *Chlamydomonas* and/or *Tetrahymena* basal bodies (column 1) were used as queries in JackHMMER searches against the current release of the *Giardia lamblia* protein dataset. These conserved centrosomal/basal body proteins are listed with their NCBI accession number (column 2), species (column 3, H = human, T=*Tetrahymena*, C=*Chlamydomonas*), and reference for basal body localization (column 4). *Giardia* homologs of these proteins are listed with their Gene ID (<http://www.giardiadb.org/giardiadb/>) and JackHMMER score (column 5) and current annotation in the *Giardia* database (column 6). Immunolocalization of *Giardia* homologs is shown in column 7 (see also Fig. 6) and referenced in column 8. Gene ID of proteins detected in the basal body enriched fraction (BBEF) are listed in column 9. Proteins that were only found in the BBEF of non-nocodazole treated cells are labeled (-N). BB, basal body; C, cytoplasm; D, ventral disk; F, flagella; MB, median body; N, nuclei; PFR, paraflagellar dense rods; a, anterior; c, caudal; p, posterior-lateral; v, ventral; PM, plasma membrane; PV, peripheral vesicles.

Basal body protein	Accession ID	Organism	Reference (other organism)	Gene ID (GL50803_) and score of <i>Giardia</i> homolog	<i>Giardia</i> Annotation	Localization in <i>Giardia</i>	Reference <i>Giardia</i>	In Proteome (GL50803_)
Proteins involved in centrosomal assembly/duplication								
cyclin A	CAA02087	H	1	14488(1.8e-86)	Cyclin A			-
Cdk2	NP_001789	H	1	8037(1.4e-105), 16802(7.6e-104)	Kinase, CMGC CDK			8037, 16802
SAS-4/CPAP	NP_060921	H	2,3	13352(5.1e-117)	T-complex protein 10	BB, pPFR, MB	Fig. 2B	13352
SAS-6	XP_001019742.1	T	2	14874(1.3e-51)	Hypothetical protein			-
POC1	TTTHERM_01308010	T	4, 5, 6	33762(2.4e-60)	WD-40 repeat protein			-
POC1A	NP_056241.2	H	5, 6	33762(1.2e-82)	WD-40 repeat protein			-
Centrins / Calcium binding proteins / EF hand proteins								
centrin 1	NP_004057.1	H	7	104685(5.8e-67), 6744(3.3e-65)	Caltractin, Centrin	BB, pPFR, MB	28, 36, Fig. 2A	104685
centrin 2	NP_004335.1	H	7	104685(9.6e-65), 6744(8.7e-64)	Caltractin, Centrin	BB, pPFR, MB	28, 36, Fig. 2A	104685
centrin 3/POC6	NP_004356.2	H	5	104685(1.1e-62), 6744(7.7e-57)	Caltractin	BB, pPFR, MB	28, 36, Fig. 2A	104685
Bbc37	TTTHERM_00267880	T	4	104685(9.5e-49)	Centrin; Caltractin	BB, pPFR, MB	28, 36, Fig. 2A	104685
BBc82	TTTHERM_00703920	T	4	104685(1.3e-24) / 5333(4.9e-24)	Centrin; Caltractin / Calmodulin	BB, pPFR, MB / BB, C	28, 36, Fig. 2A	104685, 5333
Bbc73	TTTHERM_00143690	T	4	41512(3.2e-270)	Flagella associated protein	BB, C	Fig. 2A	41512
POC9/Rib72	NP_060570.2	H	5	41512(9.2e-263)	Flagella associated protein	BB, C	Fig. 2A	41512
Tubulins and ring complex components								
alpha tubulin	AAA91576	H	7	103676(8.8e-239), 112079(8.8e-239)	Alpha tubulin	BB, F, D, MB	29	103676, 112079
beta tubulin	AAB59507.1	H	7	101291(2.2e-241), 136020(2.2e-241), 136021(2.2e-241)	Beta tubulin	BB, F, D, MB		101291, 136020
delta tubulin	NP_057345	H	7	5462(1.1e-99)	Delta tubulin			-
epsilon tubulin	XP_001703303.1	C	7	6336(1.3e-84)	Epsilon tubulin			6336(-N)
gamma tubulin	NP_001061	H	8	114218(4.4e-201)	Gamma tubulin	BB	8	-

Basal body protein	Accession ID	Organism	Reference (other organism)	Gene ID (GL50803_) and score of <i>Giardia</i> homolog	<i>Giardia</i> Annotation	Localization in <i>Giardia</i>	Reference Giardia	In Proteome (GL50803_)
GCP 2	Q9BSJ2.2	H	7	17429(2e-218)	Tubulin, small gamma tubulin complex gcp2			-
GCP 3	Q96CW5.2	H	7	12057(9e-196)	Gamma tubulin ring complex	BB	Fig. 2B	12057
GCP 4	Q9UGJ1.1	H	7	17429(1.2e-63)	Small gamma tubulin comple gcp2			-
Mitotic kinases and phosphatases								
Plk1/SAK	NP_005021.2	H	7	104150(2.5e-185)	NEK kinase / Polo kinase (PLK)	BB, D, pPFR	Fig.2C	-
Aurora A kinase	NP_003591.2	H	9	5358(2.2e-79)	Aurora kinase	BB, D, aPFR (in mitotits)	9	-
Nek 2	NP_002488.1	H	10	87945(1e-305), 137696(4.5e-234)	NEK kinase			87945(-N)
Homologs of basal body proteins with flagellar functions								
SPAG6	TTHERM_00157940	T	4	16202(3.6e-303)	Axoneme central apparatus protein	BB, aPFR, pPFR, MB	Fig. 2D	16202
Rib43a	NP_056468.1	H	5	11867(5.1e-176)	Coiled-coil protein	BB, aPFR, pPFR, MB	Fig. 2D	11867
POC1B	NP_758440.1	H	5, 6	15026(3.5e-84)	Src-associated protein-like protein			-
Signaling proteins described to localize to <i>Giardia</i> basal bodies								
PKA catalytic subunit	NP_002721.1	H	11	11214(3.2e-89), 86444(5.4e-82)	Kinase, AGC PKA; Kinase, AGC PKA	BB, aPFR, cF	11	-
ERK1	AAH13992.1	H	12	17563(4.7e-96), 22850(7.6e-90)	Kinase, CMGC MAPK; Kinase, CMGC MAPK	BB, cF, D	12	-
PP2A catalytic subunit	NP_002706.1	H	13	5010 (1.7e-140)	Protein phosphatase 2A, catalytic subunit	BB, aPFR, pPFR, D	30	-
Conserved basal body proteins with no described function								