Identification of Obscure yet Conserved Actin-Associated Proteins in 

*Giardia lamblia*

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Consistent with its proposed status as an early branching eukaryote, *Giardia* has the most divergent actin of any eukaryote and lacks core actin regulators. Although conserved actin-binding proteins are missing from *Giardia*, its actin is utilized similarly to that of other eukaryotes and functions in core cellular processes such as cellular organization, endocytosis, and cytokinesis. We set out to identify actin-binding proteins in *Giardia* using affinity purification coupled with mass spectroscopy (multidimensional protein identification technology [MudPIT]) and have identified >80 putative actin-binding proteins. Several of these have homology to conserved proteins known to complex with actin for functions in the nucleus and flagella. We validated localization and interaction for seven of these proteins, including 14-3-3, a known cytoskeletal regulator with a controversial relationship to actin. Our results indicate that although *Giardia* lacks canonical actin-binding proteins, there is a conserved set of actin-interacting proteins that are evolutionarily indispensable and perhaps represent some of the earliest functions of the actin cytoskeleton.

In addition to being a major parasite infecting more than 280 million people each year, *Giardia lamblia* (synonymous with *Giardia intestinalis* and *Giardia duodenalis*) belongs to one of the earliest diverging groups of eukaryotes (1–4). Therefore, investigation of *Giardia* biology has the potential to uncover evolutionarily deep cellular mechanisms. However, the placement of *Giardia* near the root of the eukaryotic tree, in addition to the placement of the root itself, is contentious (5). Nevertheless, *Giardia* is the most divergent eukaryote that can be manipulated in the laboratory with molecular and genetic tools (4, 6–10). In addition, many pathways in *Giardia* have fewer components than in the well-studied model eukaryotes (4). Thus, the combination of *Giardia*’s highly divergent and minimalistic genome provides a unique perspective through which cellular processes may be examined. This perspective may potentially define both the minimal requirements for function and the portions of cellular mechanisms constrained throughout evolution.

A major point of divergence between *Giardia* and other eukaryotes is the cytoskeleton (11). *Giardia* lacks the canonical actin-binding proteins, once thought common to all eukaryotes, which perform critical functions in other eukaryotes (12). Their absence may indicate a split from the last eukaryotic common ancestor before the canonical set of actin-binding proteins was established. Alternatively, *Giardia* may have evolved a novel set of actin-interacting proteins that allowed for the gradual loss of the canonical set of actin-binding proteins (11, 13). Our previous work has shown that despite the lack of canonical actin-binding proteins, *Giardia* actin (giActin) is required for conserved cellular functions, including membrane trafficking, cytokinesis, polarity, and control of cellular morphology (13). The *Giardia* cytoskeleton is also quite elaborate, suggesting the presence of cytoskeletal regulators (Fig. 1). That giActin performs similar functions to actin in other eukaryotes suggests these processes were already associated with actin at the time *Giardia* split from the other eukaryotes (13). We have also shown that giRac, the sole Rho family GTPase in *Giardia*, regulates actin despite the absence of all actin-binding proteins known to link G-protein signaling to the actin cytoskeleton (Arp2/3, formin, wave, myosin, and cofillin) (13). Therefore, *Giardia* must contain a novel set of actin-interacting proteins comprised of ancient yet undiscovered and/or *Giardia*-specific actin regulators. We sought here to identify actin-binding proteins using actin affinity chromatography coupled with multidimensional protein identification technology (MudPIT) (14). The discovery of *Giardia*-specific actin-binding proteins with essential functions would open an avenue to potential therapeutic targets, while the discovery of conserved proteins would highlight an ancient relationship between actin and the identified protein, worthy of further exploration.

**MATERIALS AND METHODS**

**Strain and culture conditions.** *Giardia lamblia*, strain WBC6 was cultured as described previously (15). Morpholino knockdown experiments and quantitative Western blotting were performed as described previously (9, 13). Large volume high-yield cultures required a method to increase the surface area. We filled standard wide-mouth media bottles with cut-to-length “jumbo drinking straws” and autoclaved them before filling with media (see Fig. S1 in the supplemental material). *Giardia* cell counts increased by ∼30% in straw-filled 500-ml bottles versus those without. After 3 days of growth, we did not observe unattached cells at the bottom of straw-filled culture vessels that are typical of overgrown cultures, while bottles without straws had a layer of cell sediment. After 72 h of growth 1-liter cultures regularly reach 2.5 × 10⁶ cells/ml, exceeding maximum trophozoite concentrations obtained with Farthing’s roller bottles, without needing specialized equipment (16).

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 Constructs. The TS-Actin vector was constructed by modifying pGFPapac (17). A BamHI site was first introduced between BsrGI and NotI of enhanced green fluorescent protein (eGFP) using an oligonucleotide adapter; all primer sequences can be found in Table S1 in the supplemental material. The glutamate dehydrogenase (GDH) promoter was exchanged for the actin promoter by excising GDH with HindIII and exchanging for the actin promoter by excising GDH with HindIII and subsequently ligated into the same position. Finally, the vector was digested with BamHI and NotI so that the actin gene could be ligated into the vector. All PCR amplification steps were performed with iProof high-fidelity polymerase (Bio-Rad), and the resulting vectors were verified by sequencing. The putative actin-interacting proteins were PCR amplified from genomic DNA and inserted into the pKS 3HA.NEO vector (10) using the restriction sites indicated in Table S1 in the supplemental material. All resulting constructs except for TS-Actin, GL50803_6744, and GL50803_13273 were linearized and integrated into the genome by homologous recombination to generate endogenously tagged proteins.

**Actin affinity chromatography.** One-liter straw-packed and sterilized bottles were filled with medium and inoculated with two 13-ml confluent cultures containing wild-type (WT) or TwinStrep-giActin cell lines. After 3 days the cultures were incubated in an ice water bath for 1 h to detach cells. The media and unattached cells were transferred to centrifuge bottles and pelleted at 750 × g for 15 min. The resulting cell pellet was washed in 10 ml of cold HEPES-buffered saline, transferred to 15-ml conical tubes, and pelleted again. The cell pellet was resuspended in an equal volume (1–2 ml) of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 7.5% glycerol, 0.25 mM CaCl₂, 0.25 mM MgCl₂, 0.25 mM ATP, 0.05 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 2 × Halt protease inhibitors [Pierce]). The pellet was stored overnight at −80°C and, after thawing, the cells were sonicated, and the extract cleared at 10,000 × g for 10 min. The lysate was added to 200 µl of Streptactin-Sepharose beads (IBA) previously equilibrated with lysis buffer. Binding was performed for 1.5 h with end-over-end mixing at 4°C. The beads were washed once in batch (100 mM Tris, pH 8.0, 150 mM NaCl, 7.5% glycerol, 0.25 mM CaCl₂, 0.25 mM ATP, 0.5 mM DTT) and then moved into a chromatography column (Bio-Rad) and washed four additional times with one column bed volume of wash buffer. Protein was eluted with 6 half-column bed volumes with elution buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 7.5% glycerol, 0.25 mM CaCl₂, 0.25 mM ATP, 0.5 mM DTT, 2 mM 1-biotin).

**Actin pelleting assay.** TwinStrep-Actin was purified as described above and then dialyzed for 2 h in G buffer (5 mM Tris, pH 8.0, 0.2 mM CaCl₂, 0.5 mM DTT). After a buffer exchange, the actin was dialyzed overnight. The dialyzed actin was cleared by centrifugation at 100,000 × g for 30 min to remove aggregates. A 1/10 volume of 10× KMEI80 (800 mM KCl, 10 mM MgCl₂, 10 mM EGTA, 100 mM imidazole [pH 7.0]) was added to the cleared actin, followed by incubation for 30 min at room temperature. The KMEI80-actin mixture was then centrifuged at 100,000 × g for 30 min.

**Mass spectrometry.** Mass spectrometry was performed by the Vincent J. Coates Proteomics/Mass Spectrometry Laboratory at UC Berkeley. The protein solution was adjusted to 8 M urea, subjected to carboxyamidomethylation of cysteines, and digested with trypsin. The sample was then desalted using a c18 spec tip (Varian). A nano-LC column was packed in a 0.5×20 cm packing material (Varian), followed by a pressure bomb and washed extensively with buffer A (see below). The sample was then directly coupled to an electrospray ionization source mounted on a Thermo-Fisher LTQ XL linear ion trap mass spectrometer. An Agilent 1200 high-pressure liquid chromatograph equipped with an emitter tip. The column consisted of 10 cm of Polaris c18 5-µm packing material (Varian), followed by 4 cm of Partispher 5 SCX (Whatman). The column was loaded by using a pressure bomb and washed extensively with buffer A (see below). The column was then directly coupled to an electrospray ionization source mounted on a Thermo-Fisher LTQ XL linear ion trap mass spectrometer. An Agilent 1200 high-pressure liquid chromatograph equipped with a split line so as to deliver a flow rate of 300 nl/min was used for chromatography. Peptides were eluted using an eight-step MudPIT procedure (14). Buffer A was 5% acetonitrile–0.02% heptafluorobutyric acid (HFBA); buffer B was 80% acetonitrile–0.02% HFBA. Buffer C was 250 mM ammonium acetate–5% acetonitrile–0.02% HFBA; buffer D was the same as buffer C, but with 500 mM ammonium acetate. The programs SEQUEST and DTASelect were used to identify peptides and proteins from the *Giardia* database (18, 19).

**Immunoprecipitation and Western blotting.** Immunoprecipitation began with a single confluent 13-ml tube per cell line. After detachment, cells were pelleted at 900 × g and washed once in HBS. The cells were resuspended in 300 µl of lysis buffer (50 mM Tris 7.5, 150 mM NaCl, 7.5% glycerol, 0.25 mM CaCl₂, 0.25 mM ATP, 0.5 mM DTT, 0.5 mM PMSF,
0.1% Triton X-100, 2× Halt protease inhibitors (Pierce) and sonicated. The lysate was cleared by centrifugation at 10,000 × g for 10 min at 4°C and then added to 30 μl of anti-HA beads (Sigma). After 1.5 h of binding, the beads were washed four times with wash buffer (25 mM Tris 7.5, 150 mM NaCl, 0.25 mM CaCl₂, 0.25 mM ATP, 5% glycerol, 0.05% Tween 20) and then boiled in 50 μl of sample buffer. Western blotting was performed as described previously (13). Rabbit anti-giActin polyclonal (13) and anti-HA mouse monoclonal HA7 antibody (Sigma-Aldrich) were both used at 1:3,000. Fluorescent secondary antibodies (Li-Cor) were used at 1:15,000, horseradish peroxidase-linked anti-rabbit antibodies (Bio-Rad) were used at 1:7,000.

**Microscopy.** Fixations were performed as described previously (13). Anti-HA mouse monoclonal HA7 antibody (Sigma-Aldrich) was used at 1:125, and anti-mouse and anti-rabbit secondary antibodies were used at 1:200 (Molecular Probes). Images were acquired on a DeltaVision Elite microscope using a 100× 1.4 NA objective and a CoolSnap HQ2 camera. Deconvolution was performed with SoftWorx (API, Issaquah, WA). Maximal projections were made with ImageJ (20), and figures were assembled using the Adobe Creative Suite (Mountain View, CA).

**RESULTS**

We set out to identify giActin interactors via an affinity chromatography approach utilizing the TwinStrep Tag (21–23). Actin is notoriously sensitive to chimeric fusions, because epitope or fluorescent protein fusions may cause steric interference or otherwise affect filament formation and dynamics (24). Thus, we devised a strategy to test whether our TwinStrep-Ag fusion (TS-Actin) was functional *in vivo*. Previous work demonstrated that actin can be effectively depleted with translation-blocking morpholinos (13). These antisense morpholinos bind to the start of the transcript and block translation initiation machinery from recognizing the start codon (9, 13). Therefore, by fusing TwinStrep to the N terminus of giActin, we generated a morpholino-insensitive version of giActin. In this case, morpholino treatment is expected to block translation of endogenous actin, whereas it should have no effect on the transgenic version. We also sought to maintain actin levels near endogenous levels by driving expression of our TS-Actin fusion with the native actin promoter.

Quantitative Western blotting with an anti-giActin polyclonal antibody (13) indicated that although we used the native promoter, there was roughly a 4-fold increase in total actin levels compared to nontransgenic controls; ca. 75% of this was TS-Actin (Fig. 2A). The higher levels of transgenic actin are presumably due to the copy number of our episomal maintained construct exceeding the number of endogenous actin genes. Morpholino treatment of the TS-Actin-expressing cell line behaved as predicted; the N-terminal epitope tag protected TS-Actin from being depleted by anti-actin morpholinos, while the endogenous actin was depleted to ~20% of control levels (Fig. 2A). Further, we examined the morpholino-treated cells for morphological defects associated with actin depletion such as abnormal cell shape, out-of-position flagella, and multiple or out-of-position nuclei (13). In the control-treated cell line we observed a slight increase in the number of abnormal cells: 6.6% for TS-Actin (n = 600) versus 1.9% for wild-type (n = 400), indicating that the increased actin levels and/or the epitope tag mildly interfered with normal actin function (Fig. 2B). The transgenic line was, however, resistant to morpholino depletion since the proportion of abnormal cells remained at 6.8% (n = 600) after morpholino treatment. In contrast, 35.2% of the WT cells (n = 500) treated with the anti-actin morpholinos had abnormal morphology. Therefore, we conclude that TS-Actin can partially rescue endogenous actin depletion, indicating that TS-Actin is functional *in vivo*.

A particular challenge of producing large-scale *Giardia* cultures, sufficient for biochemical analysis, is the need to provide surface area for adherent growth. *Giardia* is an extracellular parasite that colonizes the host intestine by attaching via its “suction cup” organelle, the ventral disc (25, 26). Likewise, in the laboratory *Giardia* trophozoites grow attached to the sides of the culture tubes. Cultures cease to proliferate after the culture tubes are confluent with cells. Free-floating cells are often observed to have an aberrant morphology, indicating the importance of surface attachment, possibly because *Giardia* divides by an adhesion-dependent mechanism (27, 28). Custom “inside-out” roller bottles have been used by others to grow high-yield *Giardia* cultures, but these are not commercially available (16). We developed a low-cost high-yield method of growing *Giardia* by inserting common polypropylene drinking straws into wide mouth bottles (see Fig. S1 in the supplemental material and see Materials and Methods). Using our high-surface-area culture system, 1-liter cultures of WT and the TS-Actin transgenic cell lines produced ~2–ml cell pellets. Extracts from these cell pellets were affinity purified in parallel. The elutions from a pilot experiment were concentrated before sodium dodecyl sulfate (SDS) analysis so that ~50% of the eluted protein could be analyzed by SDS-PAGE. Many unique bands are apparent in the TS-Actin sample (Fig. 3A). The purification was repeated for mass spectroscopy analysis; Fig. 3B represents 5% of the elutions that were analyzed by mass spectroscopy. Table 1 lists 57 proteins that were unique to the TS-Actin sample and had a minimum of five detected peptides. The complete list, including low-abundance hits and proteins also identified in our mock control, is given in Table S2 in the supplemental material. Bioinfor-
matics analysis was utilized to place these hits into six categories (Table 1; see Table S2 in the supplemental material).

We identified several hits that support the quality and relevance of this data set. For example, we identified all eight subunits of the TCP-1 chaperonin complex, which has an important role in folding actin (29). In addition, two proteins, p28 dynein light chain (p28 DLC) and centrin, were found in the giActin interactome, which we had previously hypothesized to be conserved actin-interacting proteins (13). Genetic and biochemical analysis of flagellar components has demonstrated that actin has an important role in flagella, where it functions in the inner dynein arm complexes (30–32). Within the inner dynein arms, p28 DLC and centrin, have been demonstrated to directly interact with actin (32). In Giardia, actin is readily detectable within all eight flagella, and both p28 DLC and centrin are conserved (13). In terms of peptides per molecular weight, p28 DLC was the most abundant interactor identified in our analysis. In addition to these examples, homologs of several other proteins that have been reported to interact with actin, we colocalized actin and the tagged interactors (Fig. 4B). Each protein displayed a localization pattern consistent with its proposed function. p28 DLC localized to flagella. Centrin localized to the basal bodies and around a portion of the internal axonemes of the posterior flagella. ARP7, TIP49, and ERK2 localized to the nuclei with various amounts of non-nuclear localization. HSP70 and 14-3-3 were found throughout the cell with slight enrichment at the cell anterior. None of these conserved proteins colocalized with prominent filamentous actin structures (see Fig. 1), which is consistent with the idea that they complex with G-actin (discussed below). It should be noted that standard tools such as fluorescent phalloidin and DNase I typically used to distinguish between monomeric and filamentous actin do not work in Giardia (13).

DISCUSSION

In this study, we undertook a biochemical approach to identify actin interactors in Giardia. Our easily adopted method for growing large-scale cultures and the use of the TwinStrep tag have the potential to make the process of defining interactomes routine in Giardia. During the course of our study, Svard and coworkers published a TAP-tagging approach for proteomics in Giardia (37). Similar to our approach, these researchers used two tandem Strep II tags but also included a Flag tag, the entirety of which is known as the SF-TAP tag. They overcame the surface area issues associated with SF-TAP tags, although these attempts to validate dynamin (Fig. 4A) and myeloid leukemia factor (MLF; data not shown) were unsuccessful. Both dynamin and MLF have been shown to interact with actin and alter filament organization in other eukaryotes (35, 36). Although these hits may be false positives, it is also possible that the C-terminal tag disrupted interaction or that the lower concentration of cell extracts in our immunoprecipitation experiments versus large-scale affinity chromatography failed to maintain integrity of the complex.

To better understand the relationship between these conserved interactors and actin, we colocalized actin and the tagged interactors (Fig. 4B). Each protein displayed a localization pattern consistent with its proposed function. p28 DLC localized to flagella. Centrin localized to the basal bodies and around a portion of the internal axonemes of the posterior flagella. ARP7, TIP49, and ERK2 localized to the nuclei with various amounts of non-nuclear localization. HSP70 and 14-3-3 were found throughout the cell with slight enrichment at the cell anterior. None of these conserved proteins colocalized with prominent filamentous actin structures (see Fig. 1), which is consistent with the idea that they complex with G-actin (discussed below). It should be noted that standard tools such as fluorescent phalloidin and DNase I typically used to distinguish between monomeric and filamentous actin do not work in Giardia (13).

The genome of Spiromonocus salmonicida, another diplomonad and close relative of Giardia, was recently released (33). As part of our analysis, we compared our list of putative actin interactors to the S. salmonicida genome (Table 1) (33). Although most of the identified proteins are present in S. salmonicida, several appear to be specific to Giardia. We also searched the S. salmonicida genome for the presence of canonical actin-binding proteins. Intriguingly, we found that S. salmonicida contains several actin-binding proteins not found in Giardia; these include formin, coflin, and co-ronin (see Table S3 in the supplemental material). S. salmonicida, however, lacks many canonical actin-binding proteins, including the Arp2/3 complex, nucleation-promoting factors, dynactin, capping protein, and myosin. Nevertheless, the subset of canonical actin-binding proteins in S. salmonicida suggests the loss of such proteins from Giardia. Without additional genomes, we can only speculate whether the diplomonads ever had the full complement of actin-binding proteins; however, the absence of myosin in
like kinase (S. Gourguechon and W. Z. Cande, unpublished data); because we did not find Polo in the actin data set, we believe the low-abundance hits are likely false positives. The identity of these low-abundance hits may be useful for others using our same approach; therefore, we have identified the overlapping hits in Table S2 in the supplemental material.

Although once controversial, it is now clear that actin is part of the nucleoskeleton responsible for many nuclear processes, including transcriptional regulation, chromatin remodeling, and...
general maintenance of genome organization and integrity (reviewed in reference 38). In contrast to localization studies performed in model eukaryotes, where it is difficult to detect actin in the nucleus, gIACTIN is readily detectable in the nuclei, suggesting that it has an important role in nuclear function (see Fig. 3B). Although we validated complex formation with actin for ARP7 and TIP49 (the second most abundant hit in terms of peptides/molecular weight), we identified six other proteins containing do-

FIG 4 Validation of identified interacting proteins. (A) Immunoprecipitation from Giardia extracts of C-terminally HA-tagged interactors, followed by anti-actin Western blotting demonstrates that these proteins interact with actin. (B) Colocalization of actin and HA-tagged interacting proteins in Giardia trophozoites. Actin is green, HA tagged proteins are red, DNA is blue. The first three columns are maximal projections, and the last column is a single slice through the middle of the cell. Arrowhead marks centrin localization associated with the posterolateral flagella. Scale bar, 5 μm.
 mains that are consistent with a role in actin-based chromatin remodeling. It has been put forth that several proteins known to function in the cytoskeleton have roles in the nucleus; thus, they may have originally evolved to serve the genome (38). Our identification of conserved nuclear proteins and the lack of core cytoskeletal regulators are consistent with this notion.

Actin’s role in the flagella is well established but largely ignored. Biochemical fractionation of flagella has shown that six of the seven inner dynein arm complexes are associated with actin (39). A conventional actin mutant of Chlamydomonas, ida5, lacks four of the inner-arm dynein complexes and, in this mutant, an actin-like protein, NAP, is upregulated to substitute for actin in the remaining two inner-arm dynein complexes, thus demonstrating the importance of actin to axonemal structure and function (39). Within the inner-arm dynein complexes, actin is thought to exist as a monomer in a complex with either a dimer of p28 or a monomer of centrin (32). Using super-resolution microscopy, we observed a regular repeating pattern for actin within cells corroborate an actin–14-3-3 interaction (49). We define the 14-3-3 interactome in both conserved and Giardia proteins (see above), both because of the evolutionary implications.

Our identification of an actin-ERK2 complex in Giardia corroborate the seven inner dynein arm complexes are associated with actin (41). A conventional actin mutant of Chlamydomonas, ida5, lacks four of the inner-arm dynein complexes and, in this mutant, an actin-like protein, NAP, is upregulated to substitute for actin in the remaining two inner-arm dynein complexes, thus demonstrating the importance of actin to axonemal structure and function (39). Within the inner-arm dynein complexes, actin is thought to exist as a monomer in a complex with either a dimer of p28 or a monomer of centrin (32). Using super-resolution microscopy, we observed a regular repeating pattern for actin within cells corroborate an actin–14-3-3 interaction (49). We define the 14-3-3 interactome in both conserved and Giardia proteins (see above), both because of the evolutionary implications.

Of the conserved proteins identified, 14-3-3 and ERK2 may be the most intriguing since they are likely regulators of actin dynamics or actin-related processes. 14-3-3 is known to play an important role in cytoskeletal regulation in other eukaryotes (40); however, the relationship between 14-3-3 and actin is complicated by multiple isoforms of 14-3-3 and conflicting results about 14-3-3 interactions with actin (reviewed in reference 41). In addition to our identification of 14-3-3 as an actin interactors, several efforts to define the 14-3-3–actin 39-3 interaction in both Giardia and mammalian cells corroborate an actin–14-3-3 interaction (42–44). However, the current view is that 14-3-3 regulates actin through phospho-dependent interaction with the actin-depolymerizing protein cofilin and does not bind to actin directly (45, 46). Notably, Giardia lacks both cofilin and its regulatory kinase LIM. Perhaps a more direct regulation of actin by 14-3-3 underlies the well-characterized cofilin–14-3-3 interaction. Our analysis of 14-3-3’s role in actin interrogation of their function will be a challenge. Simultaneously, if functional experiments demonstrate these proteins to be essential, they will become potential therapeutic targets to treat giardiasis.

In this first exploration of the giActin interactome, we found both conserved and Giardia-specific interactors. The subset of canonical actin-binding proteins in S. salmonicida suggests loss of actin-binding proteins from Giardia. Therefore, the retention of actin-interacting proteins in the nucleus and flagella suggest these processes are the most constrained of any actin processes. In any case, the role of actin in the nucleoskeleton and flagella are likely some of actin’s most ancient functions and remain relatively unexplored compared to the role of actin in the cytoskeleton. The set of novel/Giardia-specific proteins remain intriguing. Many of these proteins have no recognizable domains; therefore, elucidation of their function will be a challenge. Simultaneously, if functional experiments demonstrate these proteins to be essential, they will become potential therapeutic targets to treat giardiasis.

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