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Actin-Like Protein 1 (ALP1) is a component of dynamic, high molecular weight complexes in *Toxoplasma gondii*

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Abstract

Apicomplexan parasites, such as *Toxoplasma gondii*, rely on actin-based motility for cell invasion, yet conventional actin does not appear to be required for cell division in these parasites. Apicomplexans also contain a variety of actin-related proteins (Arps); however, most of these not directly orthologous to Arps in well-studied systems. We recently identified an apicomplexan-specific member of this family called Actin-Like Protein 1, (ALP1), which plays a role in the assembly of vesicular components recruited to the inner membrane complex (IMC) of daughter cells during cell division. In addition to its enrichment at daughter cell membranes, ALP1 is localized throughout the cytoplasm both diffusely distributed and concentrated in clusters that are detected by fluorescence microscopy, suggesting it forms complexes. Using quantitative optical imaging methods, including fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP), we demonstrated that ALP1 is a component of a large complex, and that it readily exchanges between diffusible and complex-bound forms. Sedimentation and density gradient analyses revealed that ALP1 is found in a freely soluble state as well as high molecular weight complexes. During cell division, ALP1 was dynamically associated with the IMC, suggesting it rapidly cycles between freely diffusible and complex forms during daughter cell assembly.

Keywords

Actin-like protein; FRAP; FLIP; protein complex; protozoan parasite

INTRODUCTION

The phylum Apicomplexa consists of many parasitic protozoa including *Toxoplasma gondii*, the causative agent of toxoplasmosis and *Plasmodium spp.*, which are responsible for malaria. Apicomplexans are most closely related to ciliates and dinoflagellates (Baldauf 2003), hence their biology is highly divergent from common model organisms. Apicomplexans are polarized cells with a unique microtubule organizing center and specialized secretory organelles concentrated at their apical ends. The parasite cytoskeleton consists of subpellicular microtubules bound to the inner surface of the inner membrane complex (IMC), a structure made up of flattened cisternae tethered to an array of protein filaments called the subpellicular network (Morrisette and Sibley 2002). Gliding motility relies on the small myosin TgMyoA, embedded within the inner membrane complex (Meissner et al. 2002; Gaskins et al. 2004), and the formation of actin filaments located between the IMC and the plasma membrane (Wetzel et al. 2003). Actin polymerization is essential for motility and cell invasion in apicomplexans (Dobrowolski and Sibley 1996), yet

parasite actin is highly dynamic. Within the parasite, actin exists primarily in an unpolymerized state that is capable of rapid polymerization into short, unstable filaments (Sahoo et al. 2006). Gliding motility in apicomplexans also relies on the apical secretion of adhesins followed by the active translocation of adhesin-receptor complexes from the anterior to posterior end of the cell (Sibley 2004).

The repertoire of actin binding proteins is very limited in these parasites (Gordon et al. 2007). For example, while they contain orthologues of profilin, actin-depolymerizing factor, capping protein, and coronin; other actin sequestering and cross-linking or bundling proteins are absent (Baum et al. 2006). They also completely lack common regulators of actin nucleation such as the Arp2/3 complex, a protein complex ubiquitous among other eukaryotes (Gordon and Sibley 2005). Instead, apicomplexans contain a greatly expanded repertoire of actin-like proteins, several of which are unique to this phylum (Gordon and Sibley 2005).

Actin-related proteins (Arps) are members of the actin superfamily that includes proteins that share an actin fold, yet have diverse functions (Frankel and Mooseker 1996). Arps share a high sequence homology to actin (~20–60% amino acid identity) (Schafer and Schroer 1999), and their nomenclature is based on their level of divergence from actin (Schroer et al. 1994). Arps carry out diverse roles in the cell as components of very large macromolecular complexes (Schafer and Schroer 1999; Olave et al. 2002). For example, Arp1 and Arp11 are components of dynactin, a protein complex that transports vesicular cargo through its interactions with the microtubule-motor protein dynein. The dynactin complex has 11 different protein components, some found in multiple copies, and a molecular mass of 1.2 MDa (Schroer et al. 1996; Schroer 2004). Arp2 and Arp3 are involved in actin nucleation of branched actin filaments from mother filaments (Machesky and Gould 1999). In addition to Arp2 and Arp3, the Arp 2/3 complex is made up of 5 other protein subunits that are all critical to its function (Gournier et al. 2001). Finally, Arps 4–10 function in the nucleus as components of chromatin-modifying and chromatin-remodeling protein complexes (Olave et al. 2002). The nuclear Arp4 alone is present in several complexes including the NuA4 histone acetyltransferase and several members of the ATP-dependent SWI2-SNF2 family (Olave et al. 2002). Apicomplexans contain orthologues of Arp1, Arp4, and Arp6 (Gordon and Sibley 2005), although their roles have not been studied in these parasites.

While apicomplexans rely on actin-based motility for migration and cell invasion, pharmacological studies suggest that conventional actin is not necessary for cell division (Shaw et al. 2001). We recently described a novel actin-related protein in apicomplexans (ALP1) (Gordon et al. 2008), which is involved in endodyogeny, a mechanism of cell division that is unique to these parasites. During endodyogeny, daughter parasites bud internally within the maternal cell and must synthesize an entirely new cytoskeleton, including a new IMC, each division cycle (Morrisette and Sibley 2002). Our previous studies indicate that ALP1 may participate in the initial construction of the IMC by recruitment of vesicles that are derived from the secretory system (Gordon et al. 2008). In dividing and non-dividing cells, ALP1 is found both diffusely distributed and clustered in discrete foci throughout the cytoplasm (Gordon et al. 2008). Since there is a strong precedence for actin-related proteins to function in the context of larger protein complexes, we hypothesized the fluorescent ALP1 foci represent multimeric protein structures. Using both cellular and biochemical approaches, we determined that ALP1 is transiently associated with high molecular weight protein complexes in the parasite.

MATERIALS AND METHODS

Cell Culture

Parasite strains used in this study include the RH strain (ATCC # 50838), RH GFP 5-Ser⁶⁵Thr (Kim et al. 2001) and RH GFP(2)-ALP1 (Gordon et al. 2008). Parasites were serially passed into human foreskin fibroblasts (HFF) monolayers every 48 hr, as previously described (Gordon et al. 2008). Host cell monolayers were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 20 mM HEPES, pH7.5, and 10 µg/ml gentamicin.

Live cell imaging

Plastic 35 mm microwell dishes containing coverslip inserts (MatTek Cultureware, Ashland, MA) that had been previously seeded with HFF cell monolayers were inoculated with parasites expressing either GFP(2)-ALP1 and or GFP alone and grown for ~ 18–24 hr. Ringer's medium (155 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 3 mM NaH₂PO₄, 10 mM HEPES, pH 7.4, 10mM glucose) supplemented with 10% FBS was added to culture dishes immediately before imaging. Live-cell imaging was done on a heated stage using a LSM 510 Meta laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY) equipped with a 63×, 1.4 numerical aperture Zeiss Plan Aplanachromat oil objective.

Fluorescence Recovery After Photobleaching (FRAP)

FRAP was performed using the LSM 510 Meta laser scanning confocal microscope (see above) using an Argon laser set at 6.0 Amp tube current. Each FRAP experiment consisted of 50 total sampling scans with bleaching after the 5th scan. Sampling line scans were done at 488 nm, 3% laser output to prevent unwanted bleaching (pixel dwell time 2.56 µs, scan times 393.21 ms). Photobleaching was performed by line scanning for 1 or 10 iterations (where stated) at 488 nm, 100% laser output. Bleaching times were held constant for each condition at .029 ms and .291 ms respectively. The size of the bleach spot was held constant (0.9025 µm²) for all experiments. Fluorescence recovery was measured as a function of time using the LSM 510 software (Carl Zeiss, Thornwood, NY). Data tables were imported for analysis into the spreadsheet program Microsoft Excel OSX (Microsoft Corporation, Redmond, WA). All experiments were reported as a percentage of the initial fluorescence intensity and normalized to an internal region of the cell not subject to bleaching to correct for loss of fluorescence due to diffusion during bleaching. Separate experiments were averaged to create a representative curve for each condition (i.e. 1 bleach iteration versus 10 iterations). These curves were imported into the graphing program Prism (GraphPad Software, San Diego, CA) and the half-time to recovery ($t_{1/2}$) was calculated by nonlinear regression using a one-phase exponential equation.

Relative mobility fractions were calculated using the equation $M\% = ((F_{\max} - F_{\text{bleach}}) / (F_i - F_{\text{bleach}})) * 100$, where $M\%$ = mobile fraction, $F_{\max}$ = the maximum % fluorescence recovered, F_{bleach} = % fluorescence immediately following the bleach, and F_i = % initial fluorescence.

Fluorescence loss in photobleaching (FLIP)

FLIP experiments were performed using the same parameters as FRAP except that scan times were increased (3.12 µs pixel dwell time, scan time = 1.93 s) and cells were bleached for 10 iterations (100% 488 nm laser output) every 5th scan for 50 scans. The total time for each individual bleach was 0.830 ms. Before and after images were taken of each sample by line scan (averaged over 8 scans for higher resolution). The loss of fluorescence intensity was measured as a function of time and quantitative measurements of the bleaching at all regions of interest (ROI) were recorded using the LSM 510 software. Data was imported

into the spreadsheet program Excel and normalized as a percentage of the initial fluorescent intensities for each ROI and plotted vs. time.

Sedimentation Assays

Freshly egressed parasites were harvested as described previously (Gordon et al. 2008), and resuspended in PBS and Triton X-100 was added to a 2% final concentration, followed by protease inhibitors (10 μ g/ml E64, 100 μ g/ml 4-(2-aminoethyl)-benzene sulfonyl fluoride hydrochloride (AEBSF), 100 μ g/ml N α p-tosyl-Lysine-chloromethyl ketone hydrochloride (TLCK), 10 μ g/ml leupeptin) and DNase I. Cells were lysed at room temperature for 2 hr, and lysates were pre-cleared by low-speed centrifugation at 16,000g for 15 min. Soluble fractions were then divided into two equal volumes and spun by ultracentrifugation at 100,000g for 1 hr at 4°C in a Beckman Optima TL Ultracentrifuge (Beckman Coulter Inc., Fullerton, CA) using the TLA 100.3 rotor. Pellets were washed twice by gentle resuspension in PBS and then centrifuged at the same speed for 10 min before being resuspended in sample buffer. Soluble fractions were precipitated overnight at 4°C using 10% Trichloroacetic acid (TCA). The pellet and soluble fractions were resuspended in sample buffer and aliquotes were run on 10% SDS-PAGE gels followed by western blotting to detect ALP1. Signals were quantified using a *Fuji FLA5000 phosphorimager and ImageGauge V4.22* (Fuji Film Medical Systems U.S.A., Stamford, CT) and the proportion of ALP1 in each fraction determined as a % of the total.

Sucrose Gradients

Freshly egressed parasites ($\sim 5 \times 10^9$ cells) were harvested and lysed in detergent as described above. The soluble fraction from the low-speed spin was layered onto linear 5–40% sucrose gradients prepared in F-Buffer (50 mM KCl, 2 mM MgCl₂) using an AutoDensi Flow (Labconco, Kansas City, MO). Gradients were spun at 35,000 rpm ($\sim 150,000$ g) in a Beckman L8-80M ultracentrifuge using a SW41 swinging bucket rotor (Beckman Coulter, Inc., Fullerton, CA) for 20 hr at 4°C. Fractions were removed from the gradients in 500 μ l aliquots by reversing the pump flow of the AutoDensi Flow. Gradients were also run with a series of standards (Bovine serum albumin (BSA, 4.4S), Lactate dehydrogenase (LDH, 6.95S), Catalase (CAT, 11.4S), Ferritin (FER, 17.6S) and Thyroglobulin (THY, 19.4S)) and their migration was used to calibrate the gradients based on the known S units of these proteins. Fractions were precipitated by addition of 10% TCA, stored overnight at 4°C, pelleted at 16,000g for 30 min at 4°C, washed twice in 100% methanol, and allowed to dry. Samples were resuspended in equal volumes of sample buffer and analyzed by western blotting (see below).

Western blotting

Gradient fractions were electrophoresed on 10% SDS-PAGE followed by transfer to nitrocellulose using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's recommendations. Following incubation with primary antibodies (rabbit anti-ALP1 at 1:10,000), secondary antibodies conjugated to horseradish-peroxidase (1:20,000) (Jackson ImmunoResearch Laboratories, West Grove, PA) and detected with ECL plus (Amersham Biosciences, Piscataway, NJ). Signals were detected by phosphorimager analysis with correction for local background and data was analyzed using Microsoft Excel OSX.

The pellet fraction from the sucrose gradient was resuspended in 500 μ L of 50 mM Tris buffer, pH7.5 and 10 μ L of the sample was loaded per well onto a 10% SDS-PAGE gel. A portion of the gel was stained with Biosafe™ Colloidal Coomassie Stain (BioRad, Hercules, CA) and another portion was transferred to nitrocellulose for Western blot analysis as described above. Signals were detected with Super Signal West Pico ECL reagent (Thermo

Fisher Scientific, Pittsburgh, PA) with a subsequent exposure to autoradiography BX film (MidSci, St. Louis, MO).

RESULTS

Time-lapse analysis of GFP(2)-ALP1 versus GFP in live cells

Previous studies using immunofluorescence localization revealed that ALP1 is localized both diffusely and clustered in discrete foci throughout the cytoplasm of the parasite (Gordon et al. 2008). We used time-lapse confocal microscopy of transgenic parasites expressing either ALP1 tagged at the N-terminus with a tandem GFP tag (GFP(2)-ALP1) and compared this to free GFP to better characterize the nature of these ALP1 foci. In previous studies we have shown that GFP(2)-ALP1 colocalizes and co-fractionates with native ALP1, hence this fluorescent fusion protein serves as a surrogate marker for the behavior of ALP1 (Gordon et al. 2008). GFP(2)-ALP1 was excluded from the nucleus and exhibited a pattern of diffuse staining with dispersed foci, while GFP alone was more uniformly distributed and was not excluded from the nucleus (Fig. 1). In this regard, GFP(2)-ALP1 differs somewhat from native ALP1, which is not excluded from the nucleus (Gordon et al. 2008). To compare the patterns of GFP to GFP(2)-ALP1 quantitatively, the change in fluorescence intensity along a linear transect was plotted over time as shown for a representative ~ 3.5 s time interval (Fig. 1, red arrows depict the transect plotted). Intensity profiles for GFP(2)-ALP1 showed a wide range in pixel intensities that fluctuated frame-to-frame, suggesting that the foci were dynamic (Fig. 1A, top traces). In contrast, GFP fluorescence appeared uniform in intensity with only slight changes detected throughout the time series (Fig. 1B, top traces). Fluctuations in pixel intensity were compared using a 2-dimensional fluorescence intensity heat map, where the level of fluorescence was displayed as a spectrum (red being the most intense to blue being the least) (Fig. 1A and B, lower panels). An enlargement of small regions of each cell revealed changes in the intensity pattern, reflecting the movement of fluorescent foci (Fig. 1A and B, black boxes). In cells expressing GFP(2)-ALP1, regions of high fluorescence intensity shifted positions at each time-point (Fig. 1A, lower plots), whereas GFP remained highly constant (Fig. 1B, lower plots). Whether the movement of GFP(2)-ALP1 foci reflects rapid assembly and disassembly, or rapid migration of stable clusters in and out of the plane of focus cannot be discerned from these experiments. Regardless, these results demonstrated that foci of GFP(2)-ALP1 were highly dynamic within the cytosol.

Fluorescence recovery after photobleaching (FRAP)

Time-lapse studies suggested that the GFP(2)-ALP1 fluorescent foci represent dynamic complexes. To further assess their stability, we used FRAP analysis to compare the diffusion rates of GFP(2)-ALP1 to free GFP. Monomers of ALP1 (~ 50 kDa) and the fusion protein GFP(2)-ALP1 (~110 kDa) would not be expected to differ substantially in diffusion through the cytosol from free GFP (dimer ~ 60 kDa), based on FRAP analysis, unless ALP1 existed in higher order complexes (Sprague and McNally 2005). Intracellular parasites expressing GFP(2)-ALP1 were used to determine experimental parameters for photobleaching using 0.9025 μm^2 circular regions-of-interest (ROI) for 1 to 10 iterations with 100% laser output. *Toxoplasma* parasites are crescent shaped with a length ~5–7 microns and a width of ~2 microns. The very small cell volume of the parasite, estimated at 1.6×10^{-14} L (Rodrigues et al. 2002), combined with the rapid diffusion of GFP, presents some unusual technical challenges for performing FRAP in parasites. For example, because GFP diffuses rapidly (estimated diffusion rate of $20 \mu\text{m}^2 / \text{s}$ (Sprague and McNally 2005)), the total cellular fluorescence drops rapidly, even when bleaching a single ROI. Consequently, the resulting image after bleaching typically failed to show a discretely resolved dark spot, and recovery invariably led to less than 100% of the initial intensity, despite rapid recovery kinetics (data

not shown). To control for this, we monitored a second ROI that was not bleached and adjusted the recovery of the bleached region for this initial loss in total cellular intensity.

Typical photobleaching images for GFP(2)-ALP1 or GFP alone following 10 iterations are shown in Fig. 2A. Following photobleaching of GFP(2)-ALP1, loss of fluorescence was regional and slow recovery was observed within the cell (Fig. 2A, top). Photobleaching of GFP under these same conditions resulted in much greater and widespread loss of fluorescence, reflecting faster diffusion (Fig. 2A bottom). In the example shown in Fig. 2A, the remaining signal was confined to the nuclei (two daughter cell nuclei are shown in a dividing cell), which gradually recovered over time. As discussed below, when GFP was bleached less using a single iteration, there was less overall loss and recovery was also rapid.

To examine the rates of recovery, measurements of fluorescence recovery were taken every ~0.4 s over a 20 s time interval, and the data from several experiments were normalized and averaged to generate a representative curve (Fig. 2B). This rate of sampling was optimized to prevent further bleaching from the laser due to sampling and was also limited by the need to optimize pixel resolution from the relatively weak signal of GFP(2)-ALP1. Comparison of the recovery curves after 10 iterations of bleaching (0.291 ms, 100% laser output) showed GFP(2)-ALP1 recovered much slower than free GFP (Fig. 2B). However, there was a difference in the overall effect of bleaching on GFP versus GFP(2)-ALP1. Since conditions had been optimized for GFP(2)-ALP1, and free GFP was more mobile, the later cells underwent greater loss of signal intensity during the bleach period. When adjusted for loss of total cell fluorescence, there was much less photobleaching of GFP (20%) compared to GFP(2)-ALP1 (60%) (Fig. 2B). Therefore, it was possible that GFP recovered faster because, under these conditions, it was more resistant to photodamage by the laser or conversely, that GFP(2)-ALP1 was more sensitive. To correct for this, we minimized the time of bleaching by using a single iteration (0.029 ms, 100% laser output). Under these new bleaching conditions both proteins showed reduced bleaching (GFP ~15%, and GFP(2)-ALP1 ~30%), and yet GFP(2)-ALP1 still demonstrated slower recovery (Fig. 2B, 1 iterations). In all cases, both proteins were 100% mobile as reflected by nearly 100% recovery over time (after adjustment for total cell loss), suggesting that neither protein was immobilized within the cell (Fig. 2C).

To compare the relative rates of diffusion, the $t_{1/2}$ of fluorescence recovery was calculated from the time-lapse curves. When compared to itself, the $t_{1/2}$ for recovery of either protein did not vary between bleaching conditions (1 versus 10 iterations). However, the $t_{1/2}$ of fluorescence for GFP(2)-ALP1 was ~4× slower than GFP alone calculated using either 1 or 10 iterations (Fig. 2D). The reported $t_{1/2}$ for GFP and proteins of similar molecular mass is actually much faster than this, and is on the order of 0.014 s (Sprague and McNally 2005). Since we observed a significant amount of GFP within the nucleus of the parasites, the discrepancy in the recovery rate may reflect delays due to compartmentalization of the protein. An alternative explanation is that our measurements were limited by the relatively long sampling time required to image GFP(2)-ALP1, due to its relatively weak fluorescence. Nonetheless, under our experimental conditions, the $t_{1/2}$ of GFP(2)-ALP1 was considerably slower than GFP alone, suggesting that ALP1 is found in large complexes that retard diffusion.

Cell fractionation and sucrose density gradient analysis of ALP1 in parasite lysates

While photobleaching experiments suggested a tendency of ALP1 to form complexes, these approaches lacked sufficient resolution to define the complex. To examine the biochemical properties of ALP1 complexes, we generated detergent extracts of parasites and analyzed them by sedimentation. Ultracentrifugation of detergent extracts of parasites at 100,000g for 1 hr revealed that ~45% of ALP1 was in the insoluble fraction, demonstrating that it

partitions into two discrete subpopulations (Fig. 3A). Sucrose density gradients were used to further characterize the size distribution of ALP1 in detergent extracts. Lysates of parasites were separated on a 5–40% sucrose density gradient by ultracentrifugation at 100,000g for 20 hrs. Western blotting of gradient fractions showed ALP1 was primarily found in fractions that migrated with sedimentation (S) values of 5–7, suggesting it was monomeric or in small complexes (Fig. 3 B, C). ALP1 was also detected in higher density fractions toward the bottom of the gradient and in the pellet, suggesting the formation of large complexes (Fig. 3B,C). Separation of this pellet fraction by SDS PAGE and staining with Coomassie Blue revealed a complex pattern of proteins, with ALP1 being a relatively minor component (Fig. 3D). Attempts to immunoprecipitate ALP1 did not yield sufficient mass amounts of proteins to identify the nature of the complexes, despite the fact that we could confirm immunoprecipitation of ALP1 by western blot (Fig. 3D). Hence, the nature of the complexes and whether they involve homopolymeric interactions or other binding partners is unresolved.

Interaction of GFP(2)-ALP1 with the IMC during daughter cell formation

ALP1 was previously shown to play a role in cell division during which it associates with the forming IMC of daughter cells (Gordon et al. 2008). Previous time-lapse recordings have indicated that GFP(2)-ALP1 associates with forming daughter cells at a very early stage, and then continues to accumulate on the IMC as it elongates; hence the pattern of fluorescent GFP(2)-ALP1 can be used to stage cell division (Gordon et al. 2008). Fluorescence loss in photobleaching (FLIP) of parasites expressing GFP(2)-ALP1 was used to examine this association in dividing parasites within host cells. We were specifically interested in addressing whether ALP1 was stabilized when bound to daughter cells; this association might be predicted to result in a slower loss of fluorescence intensity compared to cytoplasmic ALP1. Parasites were subjected to photobleaching by choosing a region of interest that was distant from the nascent daughter cells to ensure photobleaching only affected cytoplasmic populations of ALP1 (Fig. 4A, ROI 1, bleach panels). Loss of fluorescence was recorded at non-bleached regions that included the growing IMC in daughter cells and randomly chosen areas in the cytoplasm (Fig. 4A, ROI 2–4, bleach panels). Photobleaching (high laser intensity, see methods) was repeated in ten pulses that were spaced every 5th scan for 50 scans, with the intermittent scans being used to acquire images of the cell. By the 50th scan, most cells had lost the majority of their fluorescence throughout the cell, indicating that GFP(2)-ALP1 was highly mobile and readily diffused in and out of the ROI (Fig. 4A, 'after' panels). Quantitative measurements of fluorescence levels were plotted as a function of time to determine the rate of fluorescence loss (Fig. 4B). There was no significant difference in the rate of fluorescence loss between cytoplasmic GFP(2)-ALP1 and GFP(2)-ALP1 localized to daughter cells, regardless of whether the cells had just begun to divide (Early in Fig. 4) or were at very late stages in cell division (Late in Fig. 4). Collectively, these data indicate that ALP1 protein complexes were not stabilized through interactions with the IMC on forming daughter cells.

DISCUSSION

Actin-related proteins are members of a protein superfamily that is characterized by a common protein domain, the actin fold (Holmes et al. 1990). Structurally, these proteins are extremely similar, yet their incorporation into various different multimeric protein complexes has led to considerable diversification of their cellular functions (Schafer and Schroer 1999.). ALP1 is an actin-related protein that is unique to apicomplexans and plays a role in parasite cytokinesis during formation of the inner membrane complexes of daughter cells (Gordon et al. 2008). In addition to its localization on daughter cell membranes during cell division, ALP1 is found in bright, fluorescent foci and diffusely dispersed throughout

the cytoplasm. Here we demonstrate that ALP1 foci are dynamic within live cells, apparently undergoing turnover between freely diffusible and complex forms. This dynamic nature was also apparent when ALP1 interacted with the forming IMC of daughter cells, suggesting it undergoes rapid association and dissociation.

Although ALP1 has previously been detected on the IMC membranes of forming daughter cells (Gordon et al. 2008), FLIP analysis revealed that ALP1 was not stabilized through this interaction. Instead, GFP(2)-ALP1 was bleached from the IMC at the same rate as the rest of the cytosol, suggesting it freely exchanges between these two compartments. Previous studies have shown the IMC proteins in daughter cells are also dynamic (Hu et al. 2002). FRAP analysis of YFP-tagged IMC1 revealed that recovery was complete within 20 min on daughter cells, while the mother IMC did not recover during this time frame (Hu et al. 2002). This result demonstrates that during construction of the daughter cells, IMC proteins are added on a continual basis. The IMC consists of two components that must be built in parallel. Previous studies have shown that ALP1 is found in association with secretory vesicles that are shuttled to the daughter cell cytoskeleton, where they are assembled (Gordon et al. 2008). The proteins that comprise the subpellicular filaments underneath these vesicles (i.e. IMC1) must also traffic to the forming daughter cell cytoskeleton, where they assemble into a network of woven filaments. Finally, these two components are linked to form the complete structure of the IMC (Striepen et al. 2007). The association of GFP(2)-ALP1 with these IMC membranes is highly transient as FLIP analysis indicated complete loss within 1 min. These data are consistent with the previously proposed role of ALP1 in mediating trafficking of vesicles derived from the ER and targeted to the forming IMC (Gordon et al. 2008).

Time-lapse confocal microscopy demonstrated that GFP(2)-ALP1 was distributed diffusely within the cytosol of the parasite and also formed discrete foci that were not observed with free GFP. The rapid changes in intensity and location of fluorescent foci of GFP(2)-ALP1 (hot spots visible in the heat maps) that were observed in time-lapse recordings could result from movement of stable ALP1 complexes or rapid association and dissociation within the cytoplasm. FRAP analyses showed delayed recovery of GFP(2)-ALP1 vs. free GFP yet did not provide evidence of a biphasic recovery, which would have indicated the presence of two distinct populations (Reits and Neefjes 2001). Uncomplexed GFP(2)-ALP1 would be expected to have a diffusion rate similar to free GFP (as imaged here), hence the delayed recovery is consistent with exchange between freely diffusible and complexed forms of ALP1. Moreover, FLIP analysis did not detect a stable population of discrete foci that were resistant to bleaching. Collectively, these data support a model in which there is free and rapid exchange between monomeric forms of ALP and those found in higher order complexes. More rapid techniques to measure diffusion rates such as fluorescence correlation spectroscopy could provide more accurate estimates of the diffusion of ALP1 complexes (Lippincott-Schwartz et al. 2001).

Cell fractionation studies indicated that ALP1 can form protein complex that are relatively large. Sedimentation assays showed that ~45% of ALP1 fractionated to the insoluble pellet when lysates were spun either at 100,000g. Sucrose density gradient analyses indicated that while a portion of ALP1 was monomeric, a large fraction also existed in stable complexes that pelleted in 40% sucrose. Due to the fact that the ALP complexes sedimented under the gradient conditions used here, we were unable to provide precise estimates of their size; however, further analysis with differential sedimentation or gel filtration should enable more precise estimates. The ALP1 complexes appeared to be much more stable in cell extracts compared to the inherently rapid turnover of ALP1 complexes that was observed in live cells. Hence, it is possible that factors necessary for exchange are diluted when cell lysates were separated over gradients, leading to a stable complex. The low abundance of ALP1 in

the cell (approximately 1/100th of conventional actin) has thus far hampered efforts to define the composition of these complexes. Previously studies have shown that over-expression of ALP1 is highly detrimental to cell division suggesting it interacts with a sensitive step in daughter cell formation (Gordon et al. 2008). As such, formation of complexes by ALP1 may be a mechanism to control the level of freely available ALP1 and hence regulate its activity.

Collectively, our data indicates that ALP1 transiently associates with a larger protein complex of high molecular mass. While ALP1 complexes appear to be highly dynamic within live cells, other actin-related proteins form stable complexes in cells such as Arp1 and Arp11 in the dynactin complex (Gill et al. 1991; Lees-Miller et al. 1992; Eckley and Schroer 2003) or Arp 2 and Arp3 in the Arp2/3 complex (Machesky and Gould 1999; Pollard and Beltzner 2002). The complex is highly dynamic as shown by time-lapse microscopy, FRAP and FLIP analysis of photobleaching in live cells. Further characterization of ALP1 and the interactions it makes with other proteins in the cell will help elucidate the cellular role of this protein and offer valuable insight into the strategies used by these parasites to regulate cytoskeletal structures.

List of Abbreviations

ALP	Actin-Like Protein 1
Arp	actin-related protein
GFP	green fluorescent protein
FRAP	fluorescence recovery after photobleaching
FLIP	fluorescence loss in photobleaching

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Fig. 1. Time-lapse analysis of changes in GFP(2)-ALP1 vs free GFP

Time-lapse imaging of parasites expressing either A) GFP(2)-ALP1 or free B) GFP as examined by confocal microscopy. Transect lines (red arrows) were drawn for each image and changes in fluorescence intensities were tracked over time. * indicate the position of the nuclei in the images. Quantitative measurements of pixel intensities are plotted at the top; Y axis is relative intensity and X axis is distance along the transect. Below the intensity plots, two-dimensional fluorescence intensity maps display pixel intensities where red is the most intense and blue is the least. Black boxes represent a region in the cell that has been zoomed to visually track pixel intensity patterns over time. Scale bar = 1 micron.

Fig. 2. Fluorescence recovery after photobleaching (FRAP)

A) Representative images of both GFP(2)-ALP1 and free GFP before bleaching, immediately after bleaching, and after cells have fully recovered. Cells shown were bleached for 10 iterations over a $0.9025 \mu\text{m}^2$ circular region. Red circles illustrate the region targeted by the laser but are not drawn to scale. Scale bar = 5 microns. B) Recovery curves showed GFP(2)-ALP1 recovered more slowly than GFP under conditions optimized for GFP(2)-ALP1 (upper panel, 10 bleach iterations, 0.291 ms total bleach time) or set for a minimal bleach time (lower panel, 1 bleach iteration, 0.029 ms total bleach time). Bleach recovery was normalized as a % of initial fluorescence. An internal ROI (not shown) was measured to correct curves for nonspecific bleaching. Each curve represents an average of three or more experiments for a given condition. C) GFP(2)-ALP1 recovered completely despite delayed response. The mobile fractions of GFP(2)-ALP1 and GFP were calculated for all experiments using both bleaching conditions. D) Comparison of the half time of recovery showed that ALP1 recovered ~4X slower than GFP. Since half times were similar under both conditions of bleaching they were averaged and plotted as mean $\pm$ S.D. from three or more experiments.

Fig. 3. Separation of ALP1 complexes in cell lysates by centrifugation

A) ALP1 fractionated into both the pellet and supernatant following centrifugation at 100,000g for 1 hour, 4°C. Resolved on 10% SDS-PAGE gel and Western blotted using rabbit anti-ALP1 antiserum and analyzed using a phosphorimager. B) Sucrose gradient separation detected ALP1 fractions of 6–8 S units that likely represent monomer or dimer forms, as well as associated with higher molecular weight complexes near the bottom of the gradient and in the pellet (P). L is parasite lysate for comparison. Lysates were separated on a 5–40% sucrose gradient by ultracentrifugation for 20 hours, 4°C. Fractions were precipitated, run on an SDS-PAGE gel, and detected by Western blotting using rabbit anti-ALP1. The higher band at ~ 70 kDa is a nonspecific reaction product. C) Plot of abundance of ALP1 in each fraction based on phosphorimager analysis of western blotting from gel run in B. Standards run in separate gradient are shown in S units for comparison. D) Detection of ALP1 in the pellet of the sucrose gradient by western blotting. Samples were resolved by SDS PAGE, stained with Coomassie Blue, or western blotted with rabbit anti-ALP1 (α -ALP1).

Fig. 4. Stability of GFP(2)-ALP1 in dividing cells as determined by fluorescence loss in photobleaching (FLIP)

A) Cytoplasmic ROIs were analyzed using FLIP of early dividing cells (top) or late in cell division (bottom), as defined by the structure of forming daughter cells. Early: ALP1 staining was enhanced at the poles of the dividing nucleus, seen in a characteristic horseshoe shape. ROI 1 represents the bleached region in both panels (red circles). In the early panel (top), ROI 2 and ROI 4 represent daughter cell IMC while ROI 3 is the posterior end of the maternal cell. Late: ALP1 staining was apparent on daughter cells that have fully formed, but have not begun to bud. In the Late panel (bottom), ROI 2 and ROI 3 are on the daughter cell IMC and ROI 4 is on the maternal cytoplasm. Before and After images represent the average of 8 scans. Scale bars = 1 micron. B) Quantitative measurements of the decrease in fluorescence intensity for all ROIs are represented as a function of time. Oscillations in the bleach curves are due to multiple rounds of bleaching followed by a fluorescence recovery phase. Curves have been normalized as a percent of initial intensities.