PhLP3 Modulates CCT-mediated Actin and Tubulin Folding via Ternary Complexes with Substrates*

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Many ATP-dependent molecular chaperones, including Hsp70, Hsp90, and the chaperonins GroEL/Hsp60, require cofactor proteins to regulate their ATPase activities and thus folding functions in vivo. One conspicuous exception has been the eukaryotic chaperonin CCT, for which no regulator of its ATPase activity, other than non-native substrate proteins, is known. We identify the evolutionarily conserved PhLP3 (phosducin-like protein 3) as a modulator of CCT function in vitro and in vivo. PhLP3 binds CCT, spanning the cylindrical chaperonin cavity and contacting at least two subunits. When present in a ternary complex with CCT and an actin or tubulin substrate, PhLP3 significantly diminishes the chaperonin ATPase activity, and accordingly, excess PhLP3 perturbs actin or tubulin folding in vitro. Most interestingly, however, the Saccharomyces cerevisiae PhLP3 homologue is required for proper actin and tubulin function. This cellular role of PhLP3 is most apparent in a strain that also lacks prefoldin, a chaperone that facilitates CCT-mediated actin and tubulin folding. We propose that the antagonistic actions of PhLP3 and prefoldin serve to modulate CCT activity and play a key role in establishing a functional cytoskeleton in vivo.

A significant proportion of proteins requires the assistance of molecular chaperones to ensure proper biogenesis during and following translation on ribosomes (1, 2). Most chaperones that actively fold polypeptides depend on ATP hydrolysis, a process often regulated by cofactor proteins. For example, Hsp40 modulates the ATPase function of Hsp70, whereas factors such as GrpE or Bag1 control nucleotide exchange (3, 4). Hsp90 is also regulated by many cofactors, for example p23 and Cdc37 (5, 6). One class of toroid-shaped chaperones, termed chaperonins, are conserved across all domains of life and assist the folding of many cytosolic proteins (7, 8). In eukaryotes, the chaperonin containing TCP-1 (CCT, also termed TRiC or c-cpn) consists of two stacked rings each formed by eight related subunits (1, 2, 9). CCT binds substrate proteins and, through ATP-dependent conformational changes, encapsulates them in a central cavity. Upon release into the cytosol, the substrates may have folded to the native state or may require additional rounds of CCT binding and release (10, 11). CCT is required for folding nascent actin and tubulin and has been shown by immunoprecipitation studies to interact with a wide range (~10%) of polypeptides, many of which may be substrates (7). Unlike other ATP-dependent chaperones, there is no evidence that CCT cooperates with protein cofactors to modulate its ATP hydrolysis. Hop/p60, a cofactor of Hsp70 and Hsp90, promotes nucleotide exchange by CCT in vitro, but the significance of these findings is unknown (12).

CCT differs from its bacterial chaperonin counterpart, GroEL, in that it does not encapsulate its substrates with a GroES-like cofactor that fits over the chaperonin cavity (1). Instead, built-in protrusions within the apical regions of CCT close the central cavity during folding (9, 13). At least for actins and tubulins, another chaperone, prefoldin (PFD, also named GimC), participates in CCT-mediated folding. PFD is a heterohexameric complex that uses its octopus-like structure to clamp onto non-native proteins (14–16). In eukaryotes, PFD interacts with nascent chains and facilitates their transfer to CCT via direct interactions with the chaperonin (11, 15, 17–19). PFD also promotes the efficient release of native actin from CCT (11) by a mechanism that is not understood.

Recently, PhLP1 (phosducin-like protein 1) was shown to inhibit CCT-mediated folding, and a regulatory interaction was proposed (20). Phosducin, which does not interact with CCT, modulates retinal phototransduction by binding Gβγ subunits of transducin and preventing their reassociation with Gα following a signaling event (20, 21). The three known families of phosducin-like proteins likely participate in G-protein signaling, but they have also been implicated in other processes.

PhLP1, a close relative of phosducin, binds newly made Gβ and assists in the assembly of a Gβγ complex (22). A recent electron microscopy reconstruction of a mammalian CCT-PhLP1 complex shows that PhLP1 binds to the apical domains of multiple chaperonin subunits, simultaneously occluding the cavity of the cis ring and altering the conformation of the trans ring (23). However, the biological consequence of PhLP1 binding to the chaperonin is not completely understood. PhLP2 has an unknown but essential function in yeast and Dicyostelium, where it has been implicated in cell cycle progression and G-protein signaling (24, 25). PhLP3 (called APACD or TXNDC9 in mammals) has been linked to G-protein signaling in yeast but also influences tubulin function in both yeast and Caenorhabditis elegans (25–27). Deletion of the yeast PhLP3 gene orthologue, PLP1, rescues the benomyl supersensitivity of strains such as pac10Δ (a PFD subunit) or tub3Δ (an α-tubulin variant) that have an excess of undimerized β-tubulin (26). Folded but
undimerized β-tubulin is thought to interfere with normal microtubule assembly/function and is toxic when not associated with α-tubulin. Plp1p does not affect the levels of β-tubulin but rather its stability, so that in the absence of Plp1p some β-tubulin appears in nontoxic aggregates. Aside from the presence of aggregates, the deletion of Plp1p in yeast has not been shown to possess obvious defects, consistent with the wild-type (WT) phenotype of PhLP3 knockouts in Dictyostelium discoideum (24). Unlike yeast and Dictyostelium, however, C. elegans PhLP3 is essential as its disruption by RNA interference results in a failure of the first embryonic cell division. The arrested embryos possess short astral microtubules compared with control embryos, suggesting that PhLP3 plays a role in microtubule organization (27).

In this study, we identified human PhLP3 as a novel CCT-binding protein. We show that PhLP3 forms ternary complexes with CCT and either actin or tubulin and negatively impacts their folding. Functional assays suggest that this occurs by slowing the ATPase activity of the chaperonin and not through direct competition with substrates. In vivo, yeast PhLP3 appears to coordinate the proper biogenesis of actin and tubulin-β-cofactor A complex is present (25, 26). Actin and β-tubulin were expressed and purified as described (30).

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EXPERIMENTAL PROCEDURES

Purification of PhLP3 and CCT—Human PhLP3 (full-length; GenBank accession number NM_005783) and a truncated version (tPhLP3; residues 1–193), cloned into pRSETa6a, or GST-tagged variants (Fig. 1F) cloned into pGEX-6p, were produced in BL21[DE3]pLysS as described (16). Inclusion bodies were washed in 50 mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.75% Triton X-100, resuspended in 20 mM Tris-Cl, 8 M urea, pH 8.0, filtered, and passed over a Q-Phospho column (Amer sham Biosciences). Fractions containing pure PhLP3 or tPhLP3 were refolded by dialysis against 20 mM sodium phosphate, 100 mM NaCl, and 1 mM DTT, pH 8.0. GST-tagged proteins were purified with glutathione-Sepharose 4B as per the manufacturer's instructions (Amer sham Biosciences). CCT was purified from rabbit reticulocyte lysate as described (28) and was shown to be functional by its ability to refold denatured actin in vitro (data not shown and see Ref. 28).

Cell Culture—Human embryonic kidney (HEK) 293T cells were transfected with pCMV-Myc-PhLP3 or empty vector with PolyFect reagent (Qiagen). For immunoprecipitations, HEK cells were lysed mechanically in IP buffer (25 mM Tris-Cl, 100 mM KCl, 2 mM EDTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4), and cell debris was removed by centrifugation at 15,600 × g for 10 min. The supernatant was incubated with anti-Myc beads (Covance) at 4 °C for 60 min. Beads were washed three times in IP buffer and analyzed by SDS-PAGE and Western blot. TCP1 was detected with a rat anti-TCP1 antibody (StressGen Biotechnology). Cell lysate supernatants were fractionated on a Superose 6 column PC3.2/30 (Amer sham Biosciences). 52-μl fractions were collected, and 10 μl of each was loaded on an SDS-polyacrylamide gel and Western-blotted for Myc or TCP1. Separately run size standards were blue dextran (2 MDa), thyroglobulin (669 kDa), aldolase (150 kDa), serum albumin (69 kDa), and carbonic anhydrase (29 kDa).

In Vitro Translation, Folding Assays, and GST Pull-downs—Actin and tubulin were translated with the T7 quick-coupled translation kit (Promega) in the presence of 2 μl recombinant PhLP3 or tPhLP3 (29, 30). At time points, aliquots of the reaction were frozen in native gel running buffer and then thawed on ice and analyzed by native gel electrophoresis (30). GST pull-downs were done according to the manufactur-er’s instructions by using glutathione-Sepharose-4B (Amershams Biosciences). CCT (~100 nM) was incubated with ~10× m excesses of GST, GST-PhLP3, or GST-PhLP3 and 30× excess untagged PhLP3. CCT-fused truncations of PhLP3 were incubated in 10% reticulocyte lysate (Promega) for 30 min on ice.

Verification of Actin and Tubulin Folding Inhibition by PhLP3—We verified that PhLP3 inhibited native actin production by the addition of DNase I, which interacts only with native actin. In the absence of full-length PhLP3, a new band corresponding to a DNase I-actin complex appeared, but when PhLP3 was present, no such shift was observed because of the lack of native actin (see Ref. 30). To positively identify the lowest band in the tubulin-folding assay, we added taxol to the reactions to stabilize microtubules, thus removing αβ-heterodimers from the material loaded on the gel. The intensity of the lowest band was diminished in the presence of taxol, indicating that heterodimers are present (data not shown). However, the band did not disappear completely, leaving the possibility that some β-tubulin-β-cofactor A complex is present. 35S-Actin and β-tubulin were expressed and purified as described (30).

ATPase Activity Measurements—ATP hydrolysis at 30 °C was measured in folded buffer containing 2 mM [γ-32P]ATP and either 1 μM CCT alone, CCT with denatured client proteins (0.1 μg/ml actin or β-tubulin), CCT with PhLP3 (0.9 μg/ml) or CCT with denatured client proteins and either full-length or truncated PhLP3 (tPhLP3 at 1.1 μg/ml), by extraction of the [32P] phosphomolybdate complex formed in 1 N HCl as described (31).

Purification of Tubulin and Microtubule-associated Proteins—Dimeric αβ-tubulin was purified from pig brain by three polymerization cycles followed by phosphocellulose chromatography and stored at –80 °C in buffer D (0.05 M PIPES, pH 6.9, 0.5 mM EGTA, 0.25 mM MgCl2, 3.4 mM glycerol, and 200 μM GTP) (32). MAPs were isolated from microtubules by phosphocellulose and DEAE-Sephadex chromatography (33) and stored at –80 °C in buffer D.

Co-sedimentation Assay—Microtubules (50 μM tubulin) were assembled in buffer E (0.1 M K2PO4, pH 7.5, 0.5 mM EGTA, 0.5 mM MgCl2) supplemented with 3 mM MgCl2, 1 mM GTP, and a 2 M excess of taxol. After 15 min at 37 °C, the microtubule solution was supplemented with PhLP3 (to 50 μM) or MAPs (to 0.5 mg/ml) and incubated at 37 °C for 30 min before spinning at 200,000 × g at 37 °C for 10 min in a TL100-Tabletop ultracentrifuge (Beckman). Supernatant and pellet fractions were then analyzed on SDS-polyacrylamide gels.

Sedimentation Velocity Measurements—Sedimentation velocity experiments were carried out with a Beckman Optima XL-A analytical ultracentrifuge equipped with a 60 Ti four-hole rotor and cells with two-channel 12-mm path length centerpieces. Measurements were made at 45,000 rpm and 15 °C using tubulin and PhLP3 (0.3 and 0.44 mg/ml, respectively) in buffer E. The apparent distributions of sedimentation coefficients were obtained with the program DCDT (34).

Sample Preparation for Electron Microscopy—CCT was purified from bovine testis as described previously (15). The CCT-PhLP3 complexes were formed by incubating CCT and PhLP3 in a 1:10 molar ratio for 30 min at 25 °C. The CCT-tubulin complexes were formed by denaturing bovine brain tubulin (Cytoskeleton, Inc.) in 6 M guanidine hydrochloride and subsequent 100-fold dilution in buffer (20 mM HEPES, pH 7.4, 50 mM KCl, 5 mM MgCl2, 1 mM EDTA, 2 mM DTT) containing 0.9 μM purified CCT (chaperonin/tubulin molar ratio of 1:12). For the ternary complex between CCT, PhLP3, and unfolded tubulin, denatured tubulin was diluted 100 times in buffer containing PhLP3. After 5 min, CCT was added so that the CCT:PhLP3:tubulin molar ratio was 1:10:10.
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**TABLE 1**
List of yeast strains used

| Strain | Percentage | p value relative to wild-type | p value relative to pac10Δplp1Δ |
|--------|------------|-------------------------------|-------------------------------|
| WT     | 125        | 15.0                          | NA                           |
| plp1Δ  | 121        | 24.8                          | NA                           |
| pac10Δ | 145        | 37.2                          | p < 0.001                    |
| plp1Δ  | 134        | 53.0                          | p < 0.001                    |

**TABLE 2**
Statistics of cellular yeast phenotypes

| Phenotype                        | Strain | n   | Percentage | p value relative to wild-type | p value relative to pac10Δplp1Δ |
|----------------------------------|--------|-----|------------|-------------------------------|-------------------------------|
| Chromosome segregation defect in large budded cells | WT     | 126 | 15.0       | NA                           | NA                           |
|                                  | plp1Δ  | 121 | 24.8       | NA                           | NA                           |
|                                  | pac10Δ | 145 | 37.2       | p < 0.001                    | NA                           |
|                                  | plp1Δ  | 134 | 53.0       | p < 0.001                    | NA                           |
| Multinucleate large budded cells | WT     | 143 | 0.0        | NA                           | NA                           |
|                                  | plp1Δ  | 121 | 0.0        | NA                           | NA                           |
|                                  | pac10Δ | 309 | 4.5        | p < 0.001                    | NA                           |
|                                  | plp1Δ  | 228 | 14.5       | p < 0.001                    | NA                           |
| Anucleate unbudded cells         | WT     | 105 | 0.0        | NA                           | NA                           |
|                                  | plp1Δ  | 96  | 0.0        | NA                           | NA                           |
|                                  | pac10Δ | 100 | 6.0        | p < 0.025                    | p < 0.01                     |
|                                  | plp1Δ  | 156 | 19.2       | p < 0.001                    | NA                           |
| Proportion of total cells that are large budded | WT     | 451 | 35.3       | NA                           | NA                           |
|                                  | plp1Δ  | 384 | 35.9       | NA                           | NA                           |
|                                  | pac10Δ | 840 | 42.6       | p < 0.025                    | NA                           |
|                                  | plp1Δ  | 482 | 53.5       | p < 0.001                    | NA                           |
| Actin filaments in budded cells  | WT     | 141 | 85.2       | NA                           | NA                           |
|                                  | plp1Δ  | 165 | 88.5       | NA                           | NA                           |
|                                  | pac10Δ | 125 | 65.6       | p < 0.001                    | NA                           |
|                                  | plp1Δ  | 362 | 62.6       | p < 0.001                    | NA                           |
| Aberrant actin polarization, unbudded | WT     | 114 | 8.5        | NA                           | NA                           |
|                                  | plp1Δ  | 74  | 10.8       | NA                           | NA                           |
|                                  | pac10Δ | 84  | 39.7       | p < 0.001                    | NA                           |
|                                  | pac10Δ | 134 | 56.8       | p < 0.001                    | NA                           |
| Aberrant actin polarization, small-budded | WT     | 104 | 1.4        | NA                           | NA                           |
|                                  | plp1Δ  | 71  | 6.6        | NA                           | NA                           |
|                                  | pac10Δ | 86  | 3.0        | NA                           | p < 0.001                    |
|                                  | pac10Δ | 124 | 14.3       | p < 0.001                    | NA                           |
| Aberrant actin polarization, large-budded | WT     | 88  | 3.6        | NA                           | NA                           |
|                                  | plp1Δ  | 69  | 9.1        | NA                           | NA                           |
|                                  | pac10Δ | 61  | 20.0       | p < 0.01                    | p < 0.001                    |
|                                  | pac10Δ | 97  | 46.7       | p < 0.001                    | NA                           |
| Bud neck defect                  | WT     | 174 | 1.1        | NA                           | NA                           |
|                                  | plp1Δ  | 220 | 1.4        | NA                           | NA                           |
|                                  | pac10Δ | 190 | 0.5        | NA                           | NA                           |
|                                  | plp1Δ  | 303 | 6.2        | p < 0.01                    | NA                           |

*NA indicates not applicable.

**Electron Microscopy and Image Processing**—For electron microscopy of the various CCT complexes, 5-μl aliquots were applied to glow-discharged carbon grids for 1 min and then stained for 1 min with 2% uranyl acetate. Images were recorded at 0°-tilt in a JEOL 1200EX-II electron microscope operated at 100 kV and recorded at 60,000 nominal magnification. Micrographs were digitized in a Zeiss SCALM scanner with a sampling window corresponding to 3.5 Å/pixel. For two-dimensional classification and averaging, top views of CCT particles were selected, classified, and averaged using a maximum-likelihood multireference refinement algorithm (35) included in the XMIPP software package (36).

**Yeast Strains, Growth, and Microscopy**—For yeast strains, see Table 1. Yeasts were grown on YPD or YPD + 200 μg/ml genetechica sulfate (Invitrogen). Matings and tetrad dissections were performed as described (37). Latrunculin B (Sigma) sensitivity assays were performed according to Ref. 38. For microscopy, cells were grown to mid-log phase before live imaging or formaldehyde or methanol-acetic acid fixing and staining with TRITC-conjugated phalloidin (rho-phalloidin) or 4',6-diamidino-2-phenylindole (DAPI) (Sigma) according to manufacturer’s instructions (37). For cell sizing, cell diameters were measured perpendicular to the mother-bud axis at the largest point. Cellular defects were statistically validated using either an independent variable t test or χ² analysis as appropriate. The p values associated with particular statistical tests are shown for each phenotype scored in Table 2.

**RESULTS AND DISCUSSION**

Native PhLP3 Associates with CCT Likely as a Monomer Using Both N and C Termi—Based on literature showing that PhLP1 interacts with CCT, and that yeast and C. elegans PhLP3 are implicated in β-tubulin
folding and microtubule dynamics, respectively (20, 26, 27), we investigated whether mammalian PhLP3 also interacts with CCT. It could not be assumed that PhLP3 would bind CCT, as it has only 15% identity and 37% similarity to human PhLP1, whereas phosducin, which does not bind CCT, is 38% identical and 64% similar to PhLP1. However, translation of PhLP3 cDNA in rabbit reticulocyte lysate followed by native gel analysis showed a radiolabeled CCT co-migrating band similar to that seen for nascent actin (Fig. 1A). To observe the association of PhLP3 and CCT in vivo, we expressed Myc epitope-tagged PhLP3 in HEK cells and immunoprecipitated PhLP3 using a monoclonal antibody specific for the Myc epitope. CCT was efficiently co-immunoprecipitated from Myc-PhLP3-expressing cells but not from cells transfected with vector alone (Fig. 1B), suggesting that PhLP3 interacts physiologically with CCT. To assess whether the interaction is direct or indirect, we expressed and purified recombinant GST-tagged PhLP3 from *E. coli*, and we found that GST-PhLP3, but not GST alone, precipitated purified CCT complex in pull-down assays, indicating that CCT directly interacts with PhLP3 (Fig. 1C). GST-tagged PhLP3 also selectively bound CCT from either HEK cell or rabbit reticulocyte lysates (data not shown). The interaction of the purified components likely involves native PhLP3 because addition of excess folded (native) untagged PhLP3 (see below) competes with GST-PhLP3 for CCT binding in solution (Fig. 1D). These findings provide evidence that PhLP3 interacts with CCT in vivo in a folded, native form, rather than as a non-native substrate, similar to PhLP1 (20).

Untagged recombinant PhLP3 adopts a high degree of secondary structure and is thermally stable by circular dichroism, two indications that it is properly folded (data not shown). Moreover, PhLP3-CCT complexes resemble native PhLP1-CCT complexes by electron microscopy, and PhLP3 does not behave like a non-native substrate in functional assays with CCT (see Figs. 4–6 below). Sedimentation velocity analytical ultracentrifugation yielded measurements of 2.7S for PhLP3, consistent with its 26.5-kDa monomeric size (Fig. 2B). Additionally, when analyzed by size exclusion chromatography, Myc-PhLP3 from HEK cell extracts eluted not only at the position predicted for a PhLP3 monomer but also co-eluted with CCT (Fig. 1E). Our observations are therefore consistent with the existence of cytosolic monomeric and CCT-associated forms of PhLP3. Indeed, we also observed a cytosolic staining pattern for Myc- and green fluorescent protein-tagged PhLP3 by immunocytochemistry (data not shown and see Ref. 27).

Structurally, PhLP3 consists of a central domain with homology to thioredoxin, and flanking N- and C-terminal regions predicted to form a coiled coil and a potentially disordered region, respectively (39). We created several truncations of PhLP3 fused to GST and tested their ability to interact with CCT in 10% reticulocyte lysate (Fig. 1F). Removal of 27 N-terminal amino acids did not affect binding, but removal of the entire N-terminal region up to the thioredoxin domain (residues 65–226) abrogated CCT binding. Most interestingly, truncation of only 8 C-terminal amino acids strongly diminished CCT binding. Consistent with these findings, the thioredoxin domain alone (residues 65–191),...
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FIGURE 2. PhLP3 does not form binary complexes with unfolded actin, tubulin, or different forms of native tubulin. A denatured [35S-labeled actin or tubulin (D Actin or D β-tubulin, as shown) were diluted 1:100 into buffer containing no addition (−), PhLP3 (1.5 μM) (+), CCT (0.15 μM), or both and analyzed on a native gel. CCT-substrate complexes are indicated with an arrow. The position where native PhLP3 normally runs is also indicated. B, sedimentation coefficients for PhLP3, tubulin heterodimers, or a mixture of the two. C and D, microtubules assembled in vitro were pelleted at high speed with PhLP3 (C) or purified MAPs (D). The supernatant (S) and pellet (P) fractions were separated by SDS-gel electrophoresis and Coomassie-stained. C, buffer (S) corresponds to PhLP3 without microtubules added. The positions of α- and β-tubulin (Tubulin), PhLP3 or MAPs are indicated.

which is conserved in all phosducin and phosducin-like proteins, is not sufficient for CCT binding (Fig. 1F). Notably, both PhLP3 and PhLP1 seem to use similar regions for interaction with CCT, which suggests a similar binding mechanism (23).

PhLP3 Forms Ternary Complexes with CCT and Actin or Tubulin—In light of the role for yeast PhLP3 (PLP1) in β-tubulin biogenesis (26), we hypothesized that PhLP3 may act as a molecular chaperone that participates in CCT-mediated tubulin folding. We therefore investigated whether PhLP3 interacts directly with unfolded tubulin or actin, both of which are substrates of PFD and CCT (17, 18). Our results indicate that in vitro, PhLP3 does not appear to interact with non-native actin or tubulin in isolation in a native gel shift experiment (Fig. 2A), suggesting that it may not act as a chaperone. To assess whether PhLP3 may interact with other forms of tubulin, we tested for a potential interaction with α-and β-tubulin monomers exchanging in and out of heterodimers or with polymerized microtubules. PhLP3 was not found to interact with αβ-heterodimers undergoing exchange in solution (Fig. 2B). PhLP3 also did not co-precipitate with intact microtubules after pelleting by ultracentrifugation (Fig. 2C), although known MAPs did (Fig. 2D). Most interestingly, native PhLP3 did not interfere with the ability of CCT to bind the denatured substrates even at a 10:1 ratio (Fig. 2A). This is in contrast to the previous finding that the related protein PhLP1 seems to directly compete with substrates for CCT binding (20).

Because CCT interacts with both PhLP3 and tubulin, we tested if PhLP3 could affect tubulin biogenesis in a ternary complex with CCT and substrate. We translated α-tubulin, β-tubulin, or actin separately in [35S]methionine-supplemented rabbit reticulocyte lysate (which contains CCT) and could precipitate all three radiolabeled polypeptides with GST-PhLP3 (Fig. 3A). This result shows that PhLP3 may not be specific for β-tubulin, as suggested previously (26), and provides evidence for a ternary interaction. If the complex between PhLP3 and the nascent protein is binary instead of ternary, the interaction should take place in a heterologous E. coli in vitro translation system, which lacks CCT. Fig. 3B shows that E. coli lysate will not support the interaction between PhLP3 and translated β-tubulin unless exogenous CCT is added (Fig. 3B). These data corroborate the notion that PhLP3 does not interact directly with substrate proteins (Fig. 2) and instead provide evidence that PhLP3 binds to CCT-substrate complexes, forming CCT-PhLP3-substrate ternary complexes. Such a conclusion is supported by electron microscopy experiments (see below) and by functional assays with CCT, PhLP3, and either actin or tubulin (Fig. 6).

To confirm the presence of ternary complexes, we compared negative-stained electron microscopy images of CCT alone and CCT mixed with PhLP3, denatured tubulin, or both denatured tubulin and PhLP3. The average image of apoCCT (Fig. 4A) reveals an empty cavity, filled with stain and therefore darker, representing the unoccupied substrate interaction site of the CCT complex (40). The average image of the
PhLP3 inhibits actin and tubulin folding by CCT (20). Using Gt actinand tubulin folding assays. Previous data showed that mammalian stand how PhLP3 might affect CCT function, we carried out that the substrate and PhLP3 bind CCT in another inthecontextoftheternarycomplex. inFig.4, hardlynoticeable(comparethechaperonincavityinFig.4 CCT-tubulin complexes, in that the stain-penetrating regions are tubulin(Fig.4 average image of CCT in complex with both PhLP3 and denatured PhLP3 protein, the interaction seems to involve fewer CCT subunits. thechaperonincavity,verysimilartotheinteractionbetweenCCTand unfoldedsubstrates(Fig.5, (tPhLP3; residues 1–193) (Fig. 5, see under "Experimental Procedures"). In the presence of excess PhLP3, the addition of either denatured actin or tubulin alone, with PhLP3 alone, or with a substrate and PhLP3. In these assays, PhLP3 alone had no effect on the basal ATPase activity of CCT (Fig. 6A). As reported previously (28), the addition of either denatured actin or β-tubulin significantly increased ATP hydrolysis by CCT (Fig. 6, A and B). When either denatured actin or β-tubulin and PhLP3 were both added to CCT, ATP hydrolysis decreased well below the basal ATP hydrolysis levels of CCT alone (Fig. 6, A and B). As expected, truncated PhLP3 (tPhLP3), which does not interact with CCT, did not produce an inhibitory effect. The data are again consistent with PhLP3 acting in a ternary complex with CCT and either actin or tubulin rather than competing with them, because the ATPase activities of CCT-substrate or CCT-PhLP3 differ fromthatoftheCCT-PhLP3-substratecombination. HydrolysisdecreasedwellbelowthebasalATPhydrolysislevelsofCCT does not interact with CCT, did not produce an inhibitory effect. The data are again consistent with PhLP3 acting in a ternary complex with CCT and either actin or tubulin rather than competing with them, because the ATPase activities of CCT-substrate or CCT-PhLP3 differ from that of the CCT-PhLP3-substrate combination. HydrolysisdecreasedwellbelowthebasalATPhydrolysislevelsofCCT does not interact with CCT, did not produce an inhibitory effect. The data are again consistent with PhLP3 acting in a ternary complex with CCT and either actin or tubulin rather than competing with them, because the ATPase activities of CCT-substrate or CCT-PhLP3 differ from that of the CCT-PhLP3-substrate combination.

The substrate-dependent inhibition of chaperonin ATPase activity by PhLP3 provides a plausible explanation as to why actin or tubulin folding by CCT is inhibited by PhLP3 (Fig. 5, A and B) and hints at a complex allosteric relationship between the chaperonin, PhLP3, and substrate. In vivo, the concentration of PhLP3 is likely to be significantly less than that of CCT. However, the amount of PhLP3 may be sufficient to slow the reaction cycle of a significant proportion of newly formed CCT-actin and CCT-tubulin complexes. PhLP3 may recognize and act without folding productively or whether substrate turnover at CCT is reduced, leading to an occupied population of chaperonin complexes to which PFD cannot deliver substrates, is not known. Indeed, a possible mutual exclusivity between PFD and PhLP3 binding to CCT remains to be explored.

PhLP3 Inhibits the ATPase Activity of CCT Bound to a Substrate—Unlike the model proposed for PhLP1, the inhibition of actin or tubulin folding by excess PhLP3 is probably not caused by direct competition with substrate, because PhLP3 forms ternary complexes with CCT and substrate (Figs. 3 and 4). Another possible inhibitory mechanism would be if PhLP3 alters the ability of CCT to hydrolyze ATP. Fig. 6, A and B, shows the relative ATP hydrolysis activities of folding-competent rabbit CCT with either denatured actin or tubulin alone, with PhLP3 alone, or with a substrate and PhLP3. In these assays, PhLP3 alone had no effect on the basal ATPase activity of CCT (Fig. 6A). As reported previously (28), the addition of either denatured actin or β-tubulin significantly increased ATP hydrolysis by CCT (Fig. 6, A and B). When either denatured actin or β-tubulin and PhLP3 were both added to CCT, ATP hydrolysis decreased well below the basal ATP hydrolysis levels of CCT alone (Fig. 6, A and B). As expected, truncated PhLP3 (tPhLP3), which does not interact with CCT, did not produce an inhibitory effect. The data are again consistent with PhLP3 acting in a ternary complex with CCT and either actin or tubulin rather than competing with them, because the ATPase activities of CCT-substrate or CCT-PhLP3 differ from that of the CCT-PhLP3-substrate combination.

The substrate-dependent inhibition of chaperonin ATPase activity by PhLP3 provides a plausible explanation as to why actin or tubulin folding by CCT is inhibited by PhLP3 (Fig. 5, A and B) and hints at a complex allosteric relationship between the chaperonin, PhLP3, and substrate. In vivo, the concentration of PhLP3 is likely to be significantly less than that of CCT. However, the amount of PhLP3 may be sufficient to slow the reaction cycle of a significant proportion of newly formed CCT-actin and CCT-tubulin complexes. PhLP3 may recognize and act without folding productively or whether substrate turnover at CCT is reduced, leading to an occupied population of chaperonin complexes to which PFD cannot deliver substrates, is not known. Indeed, a possible mutual exclusivity between PFD and PhLP3 binding to CCT remains to be explored.

FIGURE 4. Electron microscopy of PhLP3-CCT and PhLP3-CCT-tubulin complexes. A–E, two-dimensional averages of negative-stained electron microscopy images of apoCCT (453 particles analyzed) (A), CCT-PhLP3 (847 particles) (B), CCT-PhLP1 (23) (C), CCT-tubulin (both α- and β-isofoms; 570 particles) (D), and CCT-PhLP3-tubulin complexes (530 particles) (E). Scale bar indicates 100 Å.

FIGURE 5. PhLP3 affects the folding of nascent actin and tubulin in vitro. Actin (A) or β-tubulin (B) was translated in the presence of excess PhLP3 (wt) or tPhLP3 (t; residues 1–193), and time points (as indicated) were analyzed on native gels (lower panel). The upper panel shows SDS-PAGE-analyzed translation products over time for the reactions. Tubulin* refers to either αβ-heterodimers or cofactor A-β-tubulin complexes (refer to "Experimental Procedures").
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on particular CCT-substrate complexes and modulate, rather than merely inhibit, CCT-mediated folding at physiologically relevant concentrations.

Given the prevalence of cofactors that modulate the ATPase of other chaperones, the absence of similar cofactors for CCT has been conspicuous. Many other nucleotide-dependent chaperones have at least one cofactor protein that influences ATP hydrolysis; Hsp70 has Hsp40 (4); Hsp90 has Aha1, p23, and Cdc37 (5, 6); and even the CCT homologue GroEL has GroES (42). For CCT, it will be interesting to establish if other phosducin-like proteins have similar or different effects on its ATPase activity, and to determine the significance of Hop/p60 as a nucleotide exchange factor for CCT (12). Direct effects of PFD on the CCT ATPase have not been tested, but because substrates stimulate the ATPase and PFD promotes delivery of substrates to CCT, PFD could have the indirect effect of stimulating the reaction cycle of CCT (11, 17, 18, 28). More importantly, this function of PFD would antagonize the inhibitory action of PhLP3.

Synthetic Interactions of PLP1 and Prefoldin Reveal Links to Tubulin and Actin Function in Vivo—To understand the relevance of our in vitro data to cytoskeletal function in vivo, we turned to S. cerevisiae. Yeasts lacking the PFD subunit PAC10 possess less folded tubulin overall but more β-tubulin relative to α-tubulin, an imbalance that is not tolerated by yeast and results in supersensitivity to the microtubule-depolymerizing drug benomyl (17, 43). In this context, the toxic levels of β-tubulin can be reduced if the folding of β-tubulin into its functional form is inhibited. The present model for yeast PhLP3 (Plp1p) function is that it promotes the folding of β-tubulin without influencing tubulin expression (26). Accordingly, PLP1 deletion suppresses pac10Δ benomyl sensitivity because less folded β-tubulin is produced (26) (Fig. 7A). We also found that at 20 °C, the plp1Δ mutant was itself more resistant to benomyl than WT (Fig. 7A), suggesting that even in WT cells a reduction in folded β-tubulin also counters benomyl-induced imbalances in αβ-tubulin levels (26).

On the surface, the apparently positive effects of PLP1 deletion on β-tubulin folding may seem to be in conflict with our in vitro data showing that excess PhLP3 inhibits tubulin folding. However, as mentioned previously, the in vivo ratio of PhLP3 to CCT is likely to be lower such that the modulatory effects of PhLP3 may actually be helpful. An increase in the time CCT and tubulin are associated could allow for more efficient folding and perhaps require fewer rounds of CCT binding and release. In the case of the bacterial chaperonin GroEL, substrate proteins that normally required several rounds of binding and release were shown to reach the native state while trapped in a mutant chaperonin unable to release polypeptides (44). Similarly, PhLP3 may increase substrate retention time, leading to a better folding yield within a particular CCT reaction cycle. It is also possible that a delay helps the quasi-native tubulin associate with downstream cofactors required for its assembly into heterodimers (41).

Six proteins (cofactors A–E and Cin4p/Ar12p) work downstream of CCT and PFD to promote the formation of αβ-tubulin heterodimers (41). Although PAC10 deletion in yeasts lacking any one cofactor leads to lethality or growth defects (45), in plp1Δ cells lacking either cofactor A (RBL2), C (CIN2), D (CIN1), or E (PAC2), no synthetic growth defects were observed (45). We surmise that because cofactor deletion strains have fewer polymerization-competent tubulin heterodimers, the deletion of PAC10, which leads to an excess of quasi-native β-tubulin, further interferes with microtubule assembly and therefore results in sickness or lethality. On the other hand, PLP1 deletion does not affect tubulin ratios, and this could be why it does not display synthetic interactions with cofactor deletions. Moreover, unlike the case for pac10Δ (Fig. 7A), there was no effect of PLP1 deletion on the benomyl supersensitivity of the cofactor deletions (Fig. 7B). This is consistent with the different effects of PFD and cofactor deletions on the rates of α- and β-tubulin. PLP1 deletion corrects the excess of quasi-native β-tubulin responsible for benomyl sensitivity in Δpac10 cells, although it has no observable effect on cofactor deleted cells, which have a normal ratio of α- to β-tubulin (46, 47).

The putative specificity of Plp1p for β-tubulin (26) implies that Plp1p acts on particular CCT-substrate complexes and/or may affect CCT-substrate complexes differentially, i.e. positively, negatively, or not at all. In addition to β-tubulin, in vitro data suggest that PhLP3 also impacts actin function (Figs. 3 and 5). Furthermore, it is known that PFD assists actin folding and that PFD subunit deletions are hypersensitive to the drug latrunculin (15), which specifically sequesters native actin monomers (38). Remarkably, we found that pac10Δ plp1Δ yeast were resistant to latrunculin relative to pac10Δ cells (Fig. 7C). As a control for specificity, we showed that latrunculin did not affect any of the tubulin cofactor deletions, consistent with literature showing no effect of latrunculin on microtubule function (data not shown and see Ref. 38). Latrunculin resistance implies that more folded actin is present in pac10Δ plp1Δ cells than in pac10Δ cells. In fact, rho-phalloidin staining of filamentous actin (F-actin) revealed lower staining intensity in pac10Δ plp1Δ cells than in WT or plp1Δ cells (Fig. 7D). More importantly, deletion of PLP1 in the same haploid pac10Δ strain restored F-actin staining intensity, suggesting that more F-actin is present in pac10Δ plp1Δ cells than in pac10Δ cells (Fig. 7D). These observations are consistent with published data showing that PFD deletions have an ~50% lower yield of actin folding (11) and with our data showing latrunculin resistance in pac10Δ plp1Δ cells relative to pac10Δ cells.

Aside from benomyl and latrunculin sensitivity, pac10Δ cells are sensitive to high osmolarity and low temperatures, likely representing actin and tubulin defects, respectively (Fig. 7, E and F) (17, 18). Although in

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each case plp1Δ cells were comparable with WT; we found that on high osmolarity media (1.5 M sorbitol (Fig. 7E) or 1 M NaCl (data not shown)) or on media grown at low temperatures (20 or 25 °C) pac10Δ plp1Δ cells grew slower than pac10Δ cells (Fig. 7F). Additional evidence that PLP1 and PFD work together to promote cellular viability was revealed in a large scale synthetic interaction study that showed that cells lacking PLP1 and any of five PFD subunits had a slow growth phenotype (45), a finding we have independently confirmed (data not shown).

Cellular Defects in pac10Δ Yeast Are Enhanced by PLP1 Deletion—To understand further the relationship between PFD and Plp1p, we examined the known pac10Δ cellular defects of increased cell size, aberrant chromosome segregation, and disorganization of cortical actin patches (17, 18) in plp1Δ and pac10Δ plp1Δ mutants. To determine the effects of PLP1 deletion on the pac10Δ phenotypes, we used differential interference contrast or fluorescence microscopy of yeast stained with DAPI (to stain nuclei) and rho-phalloidin (to stain F-actin). Although in each case the plp1Δ cells appeared WT, the deletion of PLP1 in a pac10Δ strain led to exacerbation of nearly all phenotypes examined.

As expected (17), when visualized by differential interference contrast, pac10Δ cells had a larger diameter perpendicular to the mother-daughter axis compared with WT cells (4.62 versus 4.40 μm WT, t = −4.467, p < 0.001). The pac10Δ plp1Δ cells were even larger on average than pac10Δ cells (5.05 μm, t = −5.914, p < 0.001). A significant proportion of pac10Δ plp1Δ cells also exhibited a thickening of the bud neck junction between mother and daughter cells, a possible indication of actin cytoskeleton defects (Table 2). DAPI staining revealed that, as published, pac10Δ cells often failed to segregate chromosomes properly to the large bud (37% defect; see Refs. 15 and 16) as compared with WT cells (15%). The pac10Δ plp1Δ cells exhibited even more penetrant defects (53%). Consistent with these observations, some pac10Δ cells were anucleate and unbudded (6%) or multinucleate and large budded (4.5%), two phenotypes not observed in WT cells but exacerbated in pac10Δ plp1Δ cells (19% anucleate; 14.5% multinucleate) (Fig. 8A; Table 2). A significant increase in the proportion of large budded cells was observed for pac10Δ yeast (43%) compared with WT yeast (35%), and a more severe defect was observed in pac10Δ plp1Δ yeast (53.5%). The increase in large budded cells suggests a G2/M cell cycle delay, potentially indicating a checkpoint response to the defects in DNA segregation (48). On the whole, these phenotypes are consistent with previously described microtubule and actin defects for PFD subunit deletions (17, 45).
Actin cables are denoted by an asterisk.

mid-log phase were fixed and stained with DAPI (budded pac10−/H9004 cells that were exacerbated by PLP1 deletion. In 20% of large budded pac10Δ cells, cortical actin was depolarized, whereas 47% of pac10Δ plp1Δ cells and only 3.6% of WT cells were depolarized. As published for another PFD subunit deletion (18), in pac10Δ yeast there was an abundance of unbudded cells with diffuse cortical actin patches (40%) compared with WT (8.5%) (Fig. 8B). Unbudded pac10Δ plp1Δ cells were slightly more affected (57%). Actin polarization in small budded pac10Δ cells was comparable with WT; however, the deletion of PLP1 in pac10Δ haploid cells led to significant depolarization (14 versus 3% of pac10Δ cells) (Fig. 8B; Table 2). Finally, rhodamine-phalloidin-stained actin filaments were visible in only 66% of pac10Δ cells with daughter buds compared with 85% of WT budded cells. This phenotype was not significantly altered in pac10Δ plp1Δ cells as 62% contained actin filaments, implying that actin cable formation is not further impaired by PLP1 deletion in pac10Δ cells (Table 2).

Although it has been suggested otherwise (26), in our hands the function of PLP1 appears to extend beyond tubulin to actin. Other genes whose mutations lead to latrunculin resistance promote actin instability in WT yeast (38); thus, Plp1p may regulate the levels of folded monomeric actin and in this way regulate filament stability. Plp1p also promotes microtubule instability, in this case by facilitating toxic quasienative β-tubulin production. Although PFD and Plp1p have opposing effects on microfilament and microtubule stability, as revealed by drug sensitivity, they clearly work together to promote cellular viability (Figs. 7 and 8 (45). Compared with PAC10 or PLP1 deletions, pac10Δ plp1Δ cells are slow growing and exhibit more significant tubulin defects (cold sensitivity and abnormal DNA segregation) and actin defects (osmosensitivity and incorrect actin-organization/polarization) (Fig. 8). plp1Δ cells are resistant to benomyl because a proportion of both tubulins aggregate (potentially more β-tubulin), rescuing strains that have an imbalance, such as pac10Δ, from the toxicity of excess β-tubulin (26). However, tubulin aggregation leads to fewer polymerization-competent heterodimers; the net result is that in strains where tubulin is already compromised, microtubule function is further reduced and gives rise to aggravated cold sensitivity and chromosome segregation defects (Figs. 7 and 8). The case for actin may be somewhat different; pac10Δ cells have ~50% the WT yield of folded actin (11), leading to latrunculin sensitivity and other phenotypes (17, 18). PLP1 deletion in a pac10Δ strain restores latrunculin resistance and the amount of F-actin visible by rhophalloidin staining to levels comparable with WT (Fig. 7, C and D). This suggests that Plp1p inhibits actin folding in a pac10Δ background, a notion consistent with our in vitro data (Fig. 5). However, pac10Δ plp1Δ cells are more sensitive to high osmolarity, are larger, and exhibit a greater number of cells with disorganized cortical actin than pac10Δ cells.

It is unclear why PLP1 deletion ameliorates certain pac10Δ actin phenotypes and exacerbates others. Although the in vitro data and the close genetic relationship with PFD suggest a direct effect on actin folding by Plp1p, there may also be indirect effects that could explain why PLP1 deletion gives rise to disparate actin phenotypes (Figs. 7 and 8). Plp1p and/or PFD may affect other CCT substrates independent of actin monomer production, and the effects on the substrate(s) could lead to enhanced defects in actin filament assembly or organization. The actin-related proteins represent candidates responsible for such indirect effects. Actin-related proteins regulate actin filament nucleation and organization, and some actin-related proteins are known CCT and prefoldin substrates (49). Alternatively, the speed and timing of actin production may be dysregulated in the absence of PFD and Plp1p, leading to defects in filament organization and function. Further experiments are required to understand precisely the effects of PFD and Plp1p acting on CCT downstream actin folding and organization. Moreover, the impact that the other phosducin homologues in yeast, Plp2, has on CCT, actin, and tubulin is unclear. A complete model of how PhLPs regulate CCT in yeast will require a better understanding of Plp2 function. Although Plp2p may function somewhat like Plp1p, it seems that the functions will differ to some degree because PLP2 is essential and PLP1 is not, and PLP1 overexpression cannot complement the loss of PLP2 (25).

Conclusions—In general, little is known about the biological functions of phosducin-like proteins outside of their role in G-protein signaling (21, 22, 24, 25). The following two studies have provided clues to a new cellular role for PhLP3: one in Saccharomyces cerevisiae, where Plp1p was implicated in β-tubulin folding (26); and the other in C. elegans, showing PhLP3 is important for correct microtubule architecture.
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(27). Based on our findings, we propose that PhLP3 acts as a novel cofactor of CCT that modulates its ability to fold substrate proteins. We show that PhLP3 binds to CCT, spanning the central cavity perhaps at the level of the apical domains above the substrate cavity, as does PhLP1 (23). We also show that PhLP3 can form ternary complexes with CCT and actin or tubulin and affect the folding of the two substrates in vitro. Finally, we show that PhLP3 slows the rate of ATP hydrolysis by CCT when in the presence of an unfolded substrate protein. The mechanism of PhLP3 function seems to be different from that proposed for PhLP1 (20). It is possible that the two proteins work differently at the level of CCT; alternatively, they could have different effects on different substrates. Indeed, the competition of PhLP1 for binding to CCT was shown with GtP

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