**Background:** Drp1 oligomerization and activity is critical for mitochondrial fission.

**Results:** GTP hydrolysis is required for Drp1 constriction of lipid bilayers. The variable domain of Drp1 regulates self-assembly and is not required for constriction of lipid bilayers.

**Conclusion:** The core machinery of Drp1 is sufficient to mediate lipid assembly, constriction, and disassembly.

**Significance:** Characterization of the mechanoenzymatic properties of Drp1 advances our understanding of mitochondrial fission.

Mitochondria are dynamic organelles that continually undergo cycles of fission and fusion. Dynamin-related protein 1 (Drp1), a large GTPase of the dynamin superfamily, is the main mediator of mitochondrial fission. Like prototypical dynamin, Drp1 is composed of a mechanochemical core consisting of the GTPase domain, middle domain, variable domain (VD),3 and the GTPase effector domain (GED) (see Fig. 2A). GTPase activity is essential for Drp1 function, and an alanine mutation at a conserved lysine in the GTPase domain (K38A) inhibits Drp1 activity and mitochondrial division (17, 18). The middle and GED domains promote self-assembly through oligomerization interaction interfaces (19). The role of the VD is currently debated, but recent studies have identified a role in lipid interactions (20, 21). Collectively, these domains work in concert to promote cycles of protein assembly and disassembly at sites of membrane remodeling. The enzymatic activity of Drp1 is proposed to mediate conformational changes within these oligomeric protein complexes that promote membrane scission.

In solution, Drp1 exists as a mixture of predominantly dimers and tetramers, which correspond to the cytosolic forms of the protein (22). In vitro interactions with GTP and lipids lead to the assembly of larger oligomers that represent the macromolecular fission machinery (23). Specifically, Drp1 forms large oligomers in the presence of nonhydrolyzable GTP analogs (18, 22–24). The addition of Drp1 to negatively charged liposomes also leads to oligomerization, forming protein-lipid tubules (18, 19).

It is unclear how Drp1 associates with membrane bilayers, because bioinformatics analyses find no apparent lipid-binding domain in the Drp1 sequence when compared with the other

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3 The abbreviations used are: VD, variable domain; GED, GTPase effector domain; DOPS, 1,2-diol-eoyl-sn-glycero-3-[phospho-L-serine]; CL-mix, cardiolipin-containing lipid mixture; PH, pleckstrin homology; CBP, calmodulin-binding protein; BME, β-mercaptoethanol; GMPPCP, βγ-methyleneguanosine 5′-triphosphate.
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dynamin family members. For dynamin, a pleckstrin homology (PH) domain interacts with phosphatidylinositol 4,5-bisphosphate at the plasma membrane. An analogous lipid-interacting role has been proposed for the Drp1 VD (25), but no structural information is available for this unique sequence. The VD has also been proposed to function as an autoinhibitory domain of oligomerization based on cellular studies (26). Therefore, Drp1 assembly and subsequent fission events are likely regulated by interactions between the VD and the mitochondrial outer membrane. Still, it is not clear whether the VD is a vital component of the fission machinery.

As stated, the GTPase activity of Drp1 is essential to mediate mitochondrial fission. Several dynamin family members, including Drp1, have the ability to constrict lipid bilayers upon the addition of GTP (24, 27, 28). For dynamin, it has been shown that GTP hydrolysis is required for lipid constriction (28, 29). However, it has also been shown that a dynamin mutant deficient in GTPase activity can achieve a superconstricted state when GTP is added (30). For the yeast homolog of Drp1, Dnm1, it has been shown that GTP binding stabilizes the protein oligomer on lipid bilayers, and GTP hydrolysis leads to membrane constriction (27). Similarly, Drp1 has been shown to constrict lipid bilayers upon the addition of GTP (19, 24). However, “constriction” was also noted in conditions where either GDP or a nonhydrolyzable GTP analog was added (19). Therefore, the separate roles of nucleotide binding and hydrolysis in mediating Drp1 constriction remain undefined.

In this study, we examine the cycle of Drp1 assembly, constriction, and release from lipid bilayers. We show that Drp1 interactions with lipid templates yield tubular structures with a broad distribution of diameters that are stabilized upon GTP binding. To address the independent roles of GTP binding and subsequent hydrolysis in Drp1 constriction, we use time-resolved EM to examine changes to Drp1-lipid tube morphologies. Although GTP binding stabilizes the protein oligomer, GTP hydrolysis is required for maximal constriction of the underlying lipid bilayer. These studies also suggest that Drp1 undergoes cycles of disassembly and reassembly on the lipid template. We also found that removal of the VD does not impair membrane-dependent Drp1 self-assembly nor membrane constriction, which suggests that the mechanoenzymatic core of Drp1 is sufficient for membrane remodeling in vitro. Moreover, we show that the VD acts as a negative regulator of Drp1 self-assembly by preventing the formation of large, functionally diminished oligomers in solution. The VD also regulates the curvature of Drp1 oligomers formed on lipid templates, which affects subsequent constriction. Collectively, these results highlight distinct conformational states of Drp1 oligomers that drive cycles of protein assembly and constriction to promote mitochondrial membrane fission.

EXPERIMENTAL PROCEDURES

Drp1 Expression and Purification—Human Drp1 isoform 1 (UniProtID: 000429-1, residues 1–736) was cloned into pCAln-EK vector (Agilent) using BamHI and SalI as restriction sites, resulting in a construct with an N-terminal calmodulin-binding protein (CBP) tag. The Drp1 ΔVD and K38A construct were made using the QuikChange mutagenesis kit (Agilent Technologies). The VD (residues 517–639) was deleted at sites described previously (19).

Drp1 was expressed and purified as described previously (31, 32) with some modifications. Briefly, CBP-Drp1 was transformed into Escherichia coli BL21 DE3 cells. The cells were grown in LB broth and induced with 1.0 mM isopropyl β-D-thiogalactopyranoside for ~19 h at 18 °C. The overnight culture was harvested, and cell pellets were resuspended in a cell lysis/binding buffer (500 mM arginine, pH 8, 300 mM NaCl, 10 mM β-mercaptoethanol (BME), 5.0 mM magnesium chloride, 1.0 mM imidazole, and 2 mM CaCl2) with 100 ng/ml lysosome (Sigma), 4 units/ml DNase (Sigma), and complete EDTA-free protease inhibitor mixture tablets (Roche). The resuspended cells were then passed through a micro fluidizer (M-110 Y; Microfluidics, Newton, MA), and the lysate was centrifuged in a Beckman Coulter Optima L-look Ultracentrifuge (50.2 Ti rotor) at 40,000 rpm (184,048 × g) for 30 min at 4 °C. The supernatant containing CBP-Drp1 was mixed with pre-equilibrated calmodulin affinity resin (Agilent Stratagene), allowed to batch bind for 1 h at 4 °C, and then loaded onto a column fitted into the AKTA Purifier 10 (GE Pharmacia). The resin was washed with lysis/binding buffer until the absorbance at 280 nm returned to baseline, and the target protein was eluted with elution buffer (500 mM arginine, pH 8, 300 mM NaCl, 10 mM BME, 5 mM MgCl2, and 2.5 mM EGTA). Fractions were collected, pooled, and concentrated using centrifugal 50-kDa molecular mass cutoff filter units (Millipore). The concentrated protein was then loaded onto a Superdex 200 gel filtration column (GE LifeSciences) equilibrated in HEPES column buffer (HCB300: 50 mM HEPES, pH 8, 300 mM NaCl, 10 mM BME). CBP-Drp1 fractions were collected, aliquoted, and flash frozen in liquid nitrogen with 5% glycerol. Samples were stored at −60 °C.

Liposome Preparation—All lipids were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Liposomes were prepared by drying 100 mol% 1,2-dioleoyl-sn-glycero-3-[phospho-1-serine] (DOPS) or a cardiolipin-containing lipid mixture (CL-mix) in chloroform under a nitrogen stream. CL-mix contained 40 mol% 1,2-dioleoyl-sn-glycero-3-phosphocholine, 35 mol% 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine, and 25 mol% bovine heart cardiolipin (CL). The dried lipid was stored in vacuum overnight and rehydrated in resuspension buffer (HCB100: 50 mM HEPES, pH 8.0, 100 mM NaCl, 0.5 mM MgCl2, 10 mM BME). The lipid was then extruded using a polycarbonate membrane of a defined size (Whatman) to generate uniform vesicles.

Dynamic Light Scattering—Dynamic light scattering measurements of liposome size were performed in a DynaPro Nanostar (Wyatt Technologies) instrument according to previously published methods (33). Samples (50 μl) of liposomes (200 μM) prepared by extrusion were analyzed in an Eppendorf UVette at room temperature. Autocorrelation curves from a set of 10 acquisitions (10-s integration time each) were analyzed using Dynamics version 7.1.3 software (Wyatt Technologies) to resolve the relative size distribution of liposomes within each sample.

EM Analysis and Time Course Experiments—Negative stain EM was used to visualize Drp1 structures. Sample mixtures...
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were prepared using WT or mutant Drp1 incubated with DOPS at room temperature for at least 30 min. To study the effect of nucleotides on the Drp1-lipid tubes, Drp1 samples were preincubated with DOPS to allow lipid-induced oligomerization. Subsequently, 1 mM nucleotide (GTP, GMPPCP, or GDP) was added, and aliquots of the sample reactions were taken at specified time points. For all samples, five microliters of reaction mixture were applied to carbon-coated grids, followed by 2% uranyl acetate stain (Electron Microscopy Sciences), and excess liquid was blotted in between applications. Images were acquired on a Tecnai T12 electron microscope (FEI, Co.) using a 4000 × 4000 CCD camera (Gatan).

Drp1 Lipid Oligomer Measurements—Tube outer diameters were measured using the ImageJ software (53). Diameters were measured to the outer edge of the protein layer on each side of the protein-lipid tubule. Extended or branched tubules were measured multiple times at increments no less than 300 nm apart. To account for heterogeneity within individual tubes, maximum and minimum widths were measured. Tubes that were not uniformly decorated with protein were omitted.

Sedimentation Assay—To quantify Drp1 oligomerization, a sedimentation assay was conducted similar to what has been described previously (34, 35). Large oligomers formed by Drp1 samples, in the presence of ligands, were found in the pellet after a medium speed centrifugation. Specifically, protein was diluted in HCB100 to 0.2 mg/ml, and specified WT and mutant samples were incubated at room temperature with DOPS liposomes for at least 30 min. The mixtures were then spun at 13,200 rpm (16,100 × g) for 30 min in a tabletop centrifuge (Eppendorf). The supernatant and pellet fractions were not uniformly decorated with protein were omitted.

GTPase Assay—Drp1 function was assessed using a continuous GTPase assay (36). A master mix was prepared to achieve the following final concentration in the assay solution (50 mM HEPES-KOH, pH 8, 150 mM NaCl, 0.5 mM MgCl2, 10 mM BME, 4 mM phosphoenolpyruvate, 0.3 mM NADH, 10 units of pyruvate kinase/lactate dehydrogenase, and 1 mM GTP). Extruded DOPS liposomes were prepared as above (1-μm filter; Whatman). Drp1 was added to a final concentration of 0.2 μM, whereas DOPS was added to specified reactions at a final concentration of 198 μM (1:1, mass:mass). A 96-well quartz plate (Molecular Devices) was preheated and maintained at 37 °C during the experiment. The decrease in the NADH absorbance at 340 nm was monitored (VersaMax; Molecular Devices), and the rate of NADH oxidation was measured and used to calculate the GTPase activity (kₚₒ) of Drp1 in each condition. The NADH oxidation was also monitored in the absence of protein using buffer alone with and without DOPS as controls. The experimental values were normalized to correct for background.

90° Light Scattering—WT Drp1 and ΔVD (0.1–0.2 mg/ml) were preassembled on pure DOPS liposomes (198 μM; extruded 1-μm diameter) in HCB100 buffer. Scattered light from these samples was measured continuously at 350 nm (0.5-nm bandwidth; 0.5-s integration time) using a Fluorolog 3–22 photon-counting spectrophotometer (Horiba, Edison, NJ), before and after addition of nucleotide (1 mM final concentration). Samples were contained in a 4-mm × 4-mm quartz cuvette (Starna cells, Atascadero, CA) and temperature-equilibrated at 25 °C throughout the measurement. For comparison, the initial scatter obtained prior to addition of nucleotide under each condition was normalized.

RESULTS

Drp1-Lipid Oligomers Constrict within Minutes of GTP Addition—Full-length human Drp1 was expressed with a CBP tag and purified for our studies. Arginine was included in the purification buffer to suppress aggregation (37). This modification of the purification protocol improved the amount of protein in the soluble fraction and also aided in resolving a homogenous Drp1 population from larger aggregates during gel filtration. The protocol was critical in isolating a soluble and active pool of protein for all Drp1 constructs in this study. WT Drp1 was examined using negative stain EM (Fig. 1A), and electron densities corresponding to protein complexes were found uniformly distributed on the EM grid.

Consistent with previous studies (20, 22), we found that Drp1 favored oligomerization in the presence of negatively charged lipid preparations. In our studies, DOPS was found to induce the formation of more extended and abundant Drp1 oligomers when compared with other preparations with negatively charged lipids. In fact, the CBP-tagged Drp1 exhibited a high propensity to oligomerize on DOPS liposomes (Fig. 1B) and also displayed a higher basal and lipid-stimulated activity (Fig. 1C) compared with untagged Drp1 (19, 20). This was advantageous because it afforded us the opportunity to examine a large population of polymers and perform rigorous statistical analyses to evaluate changes in polymer morphology. Therefore, DOPS was a suitable template for our time-resolved EM measurements.

Although Drp1 has previously been demonstrated to construct synthetic membrane bilayers in a nucleotide-dependent manner in vitro (19, 24), a rigorous statistical examination of the membrane curvatures generated under various nucleotide-bound states has not been performed. Interactions with GTP have been proposed to promote conformational changes in Drp1 assemblies on lipid templates (18, 19, 24, 27). To examine the specific role of nucleotide interactions in Drp1 constriction and disassembly, a series of experiments were performed using various guanine nucleotides.

To begin, EM images were acquired, and the diameters of protein-lipid tubules were measured at discrete time points before and after nucleotide addition. Before GTP was added (0 min), lipid-bound Drp1 oligomers displayed a broad distribution of diameters that averaged 60 ± 12 nm (Fig. 1D and Table 1). Upon further examination, WT Drp1 is capable of sampling larger diameters (Fig. 1D). This trend was calculated as a measure of skewness (Table 1), which defines the asymmetry of a distribution of variables about its mean. For skewed distributions, the median can be a more accurate measurement of central tendency. Therefore, we have also included median values in Table 1. For WT Drp1-lipid tubes, the distribution is left
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Upon the addition of GTP, immediate constriction of Drp1-lipid oligomers was observed (a 11-nm decrease on average after 1 min; Fig. 1G). Five minutes after addition of GTP, the diameter of the Drp1-lipid oligomers decreased considerably to an average diameter of 39 ± 9 nm (a 22-nm decrease; Fig. 1, E and G). It should be noted that roughly two-thirds of the measured diameters were less than 40 nm (dashed lines in Fig. 1), a diameter sufficiently narrow to place juxtaposed lipid bilayers under considerable strain (30, 38). Therefore, the addition of GTP to Drp1 oligomers promotes significant constriction of the underlying lipid template.

Overall, these measurements represent a distribution of the total population of oligomers observed during the experiment. Qualitatively, there appeared to be fewer tubes to measure after GTP addition, which is consistent with Drp1 dissociation after GTP hydrolysis. Therefore, Drp1 oligomers constrict upon GTP hydrolysis to narrow the underlying lipid tubule diameter to promote membrane fission. Subsequent conformational rearrangements lead to Drp1 release, initiating additional dynamic cycles of Drp1 assembly and disassembly, which has been observed previously with other dynamin family members (27, 29). This trend is evident as Drp1-lipid oligomers decrease in diameter initially and then recover over time (Fig. 1G). After 60 min, Drp1-lipid oligomers recovered to a larger average diameter of 53 ± 11 nm (Fig. 1, F and G).

GTP Hydrolysis Is Required for Drp1-induced Constriction of Lipid Bilayers—To date, it is unclear whether GTP binding, GTP hydrolysis or GDP binding are required for Drp1 constriction of lipid bilayers. Therefore, we tested each distinct condition and examined Drp1-lipid tube morphologies.

The posthydrolysis product, GDP, was added to preformed Drp1-lipid tubules. GDP interactions appeared to have little effect on WT Drp1-lipid tube morphology (average diameter of 59 ± 7 nm 15 min after addition; Fig. 2E). Fewer large Drp1-lipid tubes were observed compared with WT Drp1 + DOPS alone, which results in a more symmetric distribution (decreased skewness value; Table 1).

To mimic the effect of GTP binding, GMPPCP, a nonhydrolyzable analog of GTP, was added to preformed lipid tubes. Fifteen minutes after GMPPCP addition, the protein-lipid oligomers averaged 51 ± 7 nm in diameter (Fig. 2, D and F, and Table 1). This decrease in average diameter was in part due to the lack of larger protein-lipid tubes and also due to a conformational stabilization at ~50 nm that may prime the polymer for constriction. This observed stabilization is further supported by the measured decrease in standard deviation (Table 1).

Although this average size is significantly less than the 60-nm starting diameter, it did not approach the narrow diameters observed when GTP hydrolysis was permitted (Fig. 2G). Indeed, none of the measured diameters were less than 40 nm (dashed line in Fig. 2F). Comparatively, 15 min after GTP addition, preformed WT Drp1 lipid tubes achieved maximal constriction that was 13 nm narrower than when GMPPCP was added (38 ± 7-nm average diameter; Fig. 2, D and G). Therefore, GMPPCP stabilized the conformation of the Drp1-lipid oligomers over time, but a sizable constriction of the lipid template was not observed (Fig. 2D).

tail-limited because the lipid template resists curvature generated by the protein. In fact, diameters under 40 nm were not observed (highlighted by dashed gray lines in Fig. 1).
To better understand the mechanism of Drp1 constriction, Drp1 K38A, a GTPase-defective mutant, was utilized. The well-characterized alanine mutation at lysine 38 (K38A) results in a GTP hydrolysis-deficient mutant because of an inability to coordinate the γ-phosphate of the nucleotide (18). Analogous mutations in other dynamin family members have a similar defect (39), and hydrolysis is prevented even though GTP binding occurs under saturating conditions (39, 40). In this study, Drp1 K38A was found to interact with GTP-agarose beads similar to WT, which is consistent with these previous findings that Drp1 K38A can bind but not hydrolyze GTP (data not shown).

When examined by EM, K38A in solution forms small oligomers that are distributed throughout the EM grid similar to WT (Fig. 2B). In the presence of DOPS, K38A readily oligomerized and tubulated the membrane template as well (Fig. 2C). The protein-lipid tubes were nearly identical in size when compared with WT (60 nm average for WT versus 61 nm average for K38A lipid tubes; Table 1).

To test the hypothesis that GTP hydrolysis is required for constriction, Drp1 K38A-lipid tube diameter was monitored after addition of GTP. Drp1 K38A-lipid tubes did not constrict in the presence of GTP (62 nm average diameter at 15 min; Fig. 2D and J, and Table 1). GMPPCP was also added to K38A, and the conformational stabilization effect was not observed, because K38A lipid tubes maintained an average diameter of 59 nm (Fig. 2I and Table 1).

Deletion of the Variable Domain (VD) Leads to Drp1 Hyperoligomerization—To study the role of the VD in mediating membrane constriction, a ΔVD Drp1 mutant was isolated (Fig. 3A). Again, the arginine purification protocol was critical in promoting the solubility of the mutant protein. We found that, at high concentrations, ΔVD began to precipitate in solution. Therefore, ΔVD was purified and analyzed at lower concentrations (1 μM) than had been used in previous studies (19). A sedimentation assay was used to measure the oligomerization of WT, K38A, and ΔVD Drp1 in solution and in the pres-
ence of lipids. Interestingly, ΔVD pelleted much more (76%) than WT Drp1 (16%) and K38A (12%) in solution (Fig. 3B), which suggested that the mutant forms larger species. Negative stain EM confirmed this hypothesis because ΔVD was found to form ordered filamentous structures in solution (Fig. 3C). Therefore, removal of the VD promotes premature Drp1 oligomerization in solution.

ΔVD Mutant Tubulates and Constricts Lipid Bilayers—Sedimentation of WT Drp1 (70%) and K38A (68%) was higher in the presence of DOPS than alone in solution, which is consistent with the formation of large protein-lipid tubes observed by EM (Figs. 1B and 2C). Interestingly, ΔVD sedimentation was also elevated (96 ± 3%) when DOPS was added. To determine the cause of this increase, EM was used to visualize the sample, and ΔVD-lipid tubes were observed (Fig. 3D). Interestingly, ΔVD Drp1 formed protein lipid tubules that were larger on average compared with WT (82 ± 25 nm; Table 1) and displayed an even broader distribution of diameters (Fig. 3E), which is evident from an increased standard deviation and skewness value (Table 1).

First, the effect of GTP binding on the larger ΔVD lipid tubes was tested with GMPPCP incubation. After 15 min, the protein lipid tube diameters decreased ~19 nm (63 ± 15 nm; Table 1). Similar to WT Drp1, we believe this change is due to a conformational stabilization, which is further supported by a large decrease in the measured standard deviation. Therefore, GTP binding stabilizes the ΔVD Drp1 oligomer on the lipid template.

Separately, GTP was added to determine whether this mutant contains the basic functional components needed to constrict a lipid template upon hydrolysis. Five minutes after GTP addition, the ΔVD-lipid tubes constricted to an average diameter of 47 ± 6 nm (Fig. 3F). The magnitude of this constriction was larger than WT (a 30-nm difference versus a 22-nm change, respectively), but a large majority of oligomers did not constrict below 40 nm (94% greater than 40 nm; dashed line in Fig. 3F), unlike WT Drp1 (30% greater than 40 nm). Therefore, the constriction machinery remained intact, but the final dimensions of ΔVD constriction were larger because of the increased starting diameter.

WT and ΔVD Drp1 Self-assemble on Liposomes Containing Cardiolipin—To ensure that Drp1 interactions with lipid are not exclusive to DOPS liposomes, WT and ΔVD Drp1 were added to CL-containing lipid templates (CL-mix). Both DOPS and CL-mix liposomes were found to be largely spherical, heterogeneous, and featureless in solution (Fig. 4A and B). WT Drp1 tubulated the CL-mix liposomes to diameters comparable with those formed on DOPS (57 ± 6 nm; Fig. 4C and D, and Table 2). In contrast, the oligomers formed by ΔVD in CL-mix were much larger (266 ± 49 nm; Fig. 4E and F).

To further test the ability of the ΔVD mutant to induce curvature on lipid bilayers, the starting diameters of DOPS and CL-mix liposomes were measured using dynamic light scatter-
FIGURE 4. WT and ΔVD Drp1 associate with DOPS and CL-mix liposomes to form ordered protein-lipid tubes. A and B, DOPS and CL-mix liposomes were visualized using negative stain EM. Scale bar, 1 μm. C–F, WT and ΔVD Drp1 form protein-lipid tubes on DOPS and CL-mix liposomes. WT Drp1-lipid tube morphology is largely unaffected by lipid composition (C and D), whereas ΔVD-lipid tubes exhibit larger diameters with DOPS and CL-mix liposomes (E and F). Scale bar, 100 nm. G and H, shaded gray areas represent the starting distribution of liposome diameters measured using dynamic light scattering. The diameters for WT and ΔVD Drp1 oligomers were measured by EM using DOPS (G, n = 197, n = 146) and CL-mix liposomes (H, n = 152, n = 86) as indicated.
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**TABLE 2**
The VD modulates Drp1 curvature on lipid templates

| Sample conditions | WT Drp1 | ΔVD Drp1 |
|-------------------|---------|---------|
|                   | Mean    | Median  | S.D. | Skew* | Mean    | Median  | S.D. | Skew* |
| CL mix (0.2 μm)   | 63.1    | 65.5    | 8.3  | 1.1   | 190.3   | 173.5   | 86.5 | 0.5   |
| CL mix (1 μm)     | 56.6    | 61.9    | 5.5  | 0.9   | 266.1   | 269.1   | 49.2 | −0.9  |
| DOPS (0.2 μm)     | 61.9    | 61.3    | 5.6  | 0.6   | 75.3    | 61.0    | 35.5 | 2.5   |
| DOPS (1 μm)       | 60.0    | 58.0    | 11.7 | 1.5   | 81.8    | 75.7    | 24.8 | 1.9   |

*This value refers to the skewness (or asymmetry) of the distribution. The values closer to 0 are more symmetric (i.e. Gaussian), whereas those approaching +1 or −1 have more of a right-tailed or left-tailed distribution, respectively.

ing (Fig. 4, G and H, gray shaded areas). Lipid extrusion through a 1-μm filter resulted in a broad distribution of liposome diameters, mostly between 300 and 450 nm. When WT and ΔVD Drp1 were added to the DOPS liposomes, both were able to tubulate the lipid template (Fig. 4G), and the diameters were measured by EM (Table 2). When added to CL-mix liposomes, WT Drp1 was able to impose curvature, whereas ΔVD Drp1 was not. The diameters of ΔVD-CL-mix tubules were measured by EM, and these complexes were a similar size when compared with the starting diameters of the CL-mix liposomes measured by dynamic light scattering (Table 2 and green line versus gray-shaded areas in Fig. 4H). When the liposomes were filtered with a 0.2-μm filter, a similar trend was observed (Table 2). Therefore, WT and ΔVD Drp1 can associate with both lipid preparations in vitro, but the ΔVD Drp1 oligomer is deficient in generating membrane curvature, especially on CL-containing membranes.

Drp1 ΔVD Exhibits Lipid-stimulated GTPase Activity—To assess protein function, a continuous GTPase assay (36) was used to measure the GTP hydrolysis activities of WT, K38A, and ΔVD Drp1 (Fig. 5A). As shown previously, WT Drp1 exhibited robust basal GTPase activity at 13 ± 1 min⁻¹. In the presence of DOPS liposomes, Drp1 activity was stimulated ~10-fold (123 ± 11 min⁻¹). This lipid-induced stimulation was similar in magnitude to what has been reported previously (19).

As expected, K38A Drp1 exhibited no detectable GTPase activity in solution or in the presence of a lipid template (Fig. 5A). At concentrations lower than had been studied previously (19), ΔVD Drp1 exhibited GTPase activity in solution that was diminished compared with WT (a 2.6-fold reduction). Consistent with the ability of ΔV to form ordered oligomers on a lipid template, a ~10-fold stimulated activity was observed when DOPS was added (30 ± 5 min⁻¹), although the total enzyme activity was less than WT Drp1 in the presence of DOPS liposomes (Fig. 5A). Therefore, ΔVD Drp1 has the ability to form lipid-induced oligomers that stimulate GTPase activity, and this activity promotes constriction of the underlying membrane.

GTP Hydrolysis Mediates Drp1 Disassembly—After GTP-induced constriction, WT Drp1-lipid tube diameters appeared to recover to their starting value (53 ± 6 nm). This suggests that Drp1 disassembles after GTP hydrolysis and subsequently rebinds lipid templates. An alternative interpretation is that the recovery of Drp1-lipid diameters was due to the relaxation of the Drp1 polymers on the membrane rather than recycling through disassembly and rebinding. However, this model is inconsistent with the observed decrease in the number of Drp1-lipid tubes observed by EM after GTP was added.

To monitor Drp1 polymerization dynamics on a lipid template, 90° light scattering experiments were conducted. Previous studies have established that the scattering intensity of a network of protein-decorated membrane tubules decreases as a function of both membrane constriction and protein disassembly. When GTP was added to preformed WT Drp1-DOPS lipid tubules, an immediate decrease in scattering intensity was...
observed (Fig. 5B, red trace). A similar experiment with ΔVD Drp1 showed protein disassembly in much the same manner as WT (Fig. 5B, green trace). Conversely, when GMPPCP was added to either preformed WT or ΔVD Drp1-lipid tubes, no such decrease was observed (Fig. 5B, blue and orange traces, respectively). Therefore, Drp1 disassembly is dependent on GTP hydrolysis.

The hyperoligomeric properties of the ΔVD mutant also provided the opportunity to use EM to observe disassembly of Drp1 polymers after the addition of GDP or GTP. As stated earlier, ΔVD alone in solution forms filamentous structures (Fig. 5C). The addition of GDP did not induce any change in ΔVD oligomerization (Fig. 5D). However, after the addition of GTP for 30 min, almost no ΔVD filaments were observed on the grid (Fig. 5E).

**DISCUSSION**

In this study, we examined the combined roles of guanine nucleotide and lipid interactions on the self-assembly properties of Drp1. In addition, the K38A and ΔVD mutants provided valuable insight into the roles of GTP hydrolysis and the largely uncharacterized VD in promoting membrane constriction.

Moreover, the ΔVD mutant offered valuable information regarding Drp1 self-assembly. Previous studies have characterized the formation of large protein aggregates in vitro, and in cell culture when the VD was removed (19, 26). We have shown that removal of the VD yields a hyperoligomeric form of Drp1 that assembles into well ordered filaments (Fig. 3C) rather than disordered aggregates. Electron microscopy was used to identify the unique structural properties of the ΔVD mutant. The hyperoligomeric properties of this mutant are consistent with the hypothesis that the variable domain occludes a Drp1 self-assembly motif (Fig. 6A). In this way, the variable domain serves to negatively regulate Drp1 oligomerization in solution and retain Drp1 in a smaller, active state (Fig. 6B). This may explain why removal of the VD leads to the formation of large filaments, which do not retain WT Drp1 activity (Fig. 5A).

It should be noted that these results do not exclude the possibility of an interaction occurring between the VD and lipid. Rather, we propose that the variable domain of WT Drp1 likely conceals a self-assembly motif to limit the formation of larger assemblies in solution (Fig. 6A). When the VD is removed, the negative regulation is alleviated, and unopposed assembly commences. WT Drp1 interactions with lipid may expose the self-assembly motif to promote oligomerization. Therefore, the ΔVD mutant bypasses this regulatory mechanism because ΔVD oligomers in solution readily assemble on lipid bilayers.

A deficiency in protein oligomer curvature was apparent when ΔVD Drp1 was added to lipid templates as well. This
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result supports a model wherein the VD plays a role in promoting membrane curvature. ΔVD-lipid oligomers exhibit a larger average diameter compared with WT on DOPS liposomes (Fig. 4G), and the diameter difference is even more profound on CL-mix liposomes (Fig. 4H). In fact, ΔVD Drp1 was unable to significantly reshape the CL-mix liposomes. This difference could partially be due to the lipid template itself, because CL-mix liposomes are less pliable than DOPS liposomes (41). This is in part due to the conical structure of cardiolipin, which favors negative membrane curvature and likely resists the positive curvature imposed by Drp1. In addition, the head groups of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine and 1,2-dioleoyl-sn-glycero-3-phosphocholine do not exhibit charge repulsion found in DOPS liposomes, so fewer membrane defects would be available for protein interactions. Very large protein-lipid tubes (>200 nm) were still observed when ΔVD was added to the DOPS template, which demonstrates a clear deficit in generating membrane curvature. Therefore, VD interactions with the lipid bilayer and/or within the multimeric protein assembly influence curvature directly.

The VD has been proposed to interact directly with negatively charged lipid (20, 21). Nevertheless, the ΔVD mutant was found to assemble on both negatively charged liposomes used in these studies. This suggests that Drp1 can associate with lipid independently of the VD, and weak electrostatic interactions near the base of the middle/GED stalk may be sufficient to promote protein-lipid tubule formation in vitro. As such, the multimerization of the ΔVD protein likely provides an accumulation of charge that drives lipid association and bypasses specific regulatory interactions between the VD and lipid. This domain is also the site of several post-translational modifications that regulate Drp1 localization to mitochondrial membranes in cells (42–45). It is possible that the VD of Drp1 is analogous to the PH domain in dynamin and likely participates in membrane interactions. This interaction may target the protein to the mitochondrial surface, but our results suggest that membrane interactions mitigate the negative regulation of the VD and promote Drp1 self-assembly.

Interestingly, the removal of the PH domain in dynamin also leads to a hyperoligomeric form of the protein that readily sediments in solution (46) and retains GTPase activity (47, 48). Therefore, it appears that the variable and PH domains both function to negatively regulate the premature, nonproductive protein assembly influence curvature directly.

Following assembly of Drp1 on lipid bilayers, we have identified three additional steps in the Drp1 constriction process (Fig. 6B). Before constriction, Drp1 undergoes a nucleotide-induced stabilization on lipid bilayers. GTP binding caused a slight but significant reduction in oligomer diameter because Drp1 assumes a more ordered conformation. This stabilization was disrupted by the K38A mutation, because adding GTP to preformed K38A-lipid tubes has no effect on membrane morphology. This result is surprisingly different from dynamin, because the homologous mutant, K44A, forms a superconstricted polymer in the presence of GTP (30). Drp1 K38A does not constrict to this intermediate state in the presence of GTP, which highlights the importance of lysine 38 in mediating conformational changes that promote this stabilization.

After GTP binding, GTP hydrolysis promotes Drp1 constriction of the lipid templates. Within seconds of GTP addition, highly constricted WT Drp1-lipid oligomers were observed with some diameters measuring less than 30 nm, a distance that would seem sufficient to mediate lipid mixing between juxtaposed membrane leaflets (38, 49). Moreover, because many of our diameter distributions were skewed (Table 1), the mean values are likely conservative representations of Drp1-induced membrane constriction.

Nonhydrolyzable GTP analogs and GDP are incapable of mediating constriction to a significant degree. However, GDP appears to reduce the formation of much larger oligomers, which is evident in the lower skewness value (0.1; Table 1). This may explain the perceived constriction reported previously in the presence of GDP (19). Nevertheless, these data clearly demonstrate that GTP hydrolysis is essential for maximal constriction and eventual fission of the mitochondrial membrane.

Our results also showed that ΔVD Drp1 oligomers could undergo a conformational stabilization and constrict lipid bilayers upon nucleotide addition (Fig. 6B). Therefore, the mechanoenzymatic core of Drp1 (GTPase, Middle, and GED) is sufficient for GTP-induced constriction of lipid bilayers. This minimal protein machinery is regulated by the VD sequence, which controls the oligomeric properties of Drp1 in solution and on membranes. These differences limit lipid tube constriction, because the ΔVD oligomers were not able to constrict liposomes to the smaller diameters observed with WT Drp1 assemblies. Therefore, the VD ensures that Drp1 oligomers attain a geometry that promotes full constriction of lipid tubules.

Lastly, we examined whether Drp1 GTPase activity mediates oligomer disassembly, which would then lead to successive membrane binding and constriction events. In agreement with the EM results, we found that WT Drp1 depolymerizes when GTP hydrolysis occurs, whereas GTP binding stabilizes the oligomer (Fig. 6B). ΔVD Drp1 oligomers, both in solution and on lipid templates, disassemble after GTP addition. ΔVD-lipid oligomers are also stabilized by GTP binding, and a 19-nm decrease in average diameter was observed when GMPPCP was added. This may explain the slight decrease and subsequent plateau in the light scattering value (Fig. 5B). Although removal of the VD enhances self-assembly, it does not prevent protein disassembly.

The EM time course experiments allowed for visualization of changes in Drp1-lipid morphology over time. However, a significant population of lipid fragments or vesicles was not observed in our studies. As with other dynamin family members, Drp1 likely requires several cellular factors to efficiently complete the fission process. These cellular factors would include partner proteins, which are responsible for the recruitment of Drp1 to the outer mitochondrial membrane. The endoplasmic reticulum has also been implicated in inducing fission sites through extensive mitochondrial contacts and interactions with associated cytoskeletal structures (50–52).

Taken together, our data demonstrate the dynamic ability of Drp1 to accommodate different oligomeric conformations and sizes depending on ligand interactions. Our data support a model where the VD serves to limit Drp1 self-assembly in the
cytosol, and membrane interactions relieve this inhibition to promote Drp1 oligomerization at mitochondrial fission sites (Fig. 6A). GTP binding causes a conformational stabilization of these polymers, and hydrolysis is required to promote maximal constriction of the underlying membranes. This process leads to disassembly of Drp1 to allow for rebinding and successive cycles of membrane constriction in a processive manner (Fig. 6B). Overall, this work identifies fundamental biochemical and structural characteristics of Drp1 and the core components that form the contractile apparatus to drive membrane fission.

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