Allosteric Modulation of Drp1 Mechanoenzyme Assembly and Mitochondrial Fission by the Variable Domain*1

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Background: Mechanisms of Drp1-mediated mitochondrial fission are poorly understood. Results: Substitution of the Drp1 variable domain causes a spectrum of assembly and activity phenotypes but does not compromise the stalk domain-mediated recruitment of Drp1 to the mitochondrial anchoring protein Mff. Conclusion: The variable domain modulates Drp1 activity through oligomeric assembly. Significance: Insight into Drp1 regulatory mechanisms is essential for understanding mitochondrial biology.

The mechanoenzyme dynamin-related protein 1 (Drp1) hydrolyzes GTP to power mitochondrial fission, a process required for organelle biogenesis, quality control, transport, and apoptosis. The pleckstrin homology domain of dynamin is essential for targeting to and severing of lipid tubules, but the function of the corresponding variable domain (VD, or insert B) of Drp1 is unknown. We replaced the VD of Drp1 with a panel of linker sequences of varying length and secondary structure composition and found that the VD is dispensable for mitochondrial recruitment, association with the Drp1-anchoring protein Mff (mitochondrial fission factor), and basal and protonophore-induced mitochondrial fragmentation. Indeed, several ΔVD mutants constitutively localized to the outer mitochondrial membrane (OMM) and fragmented mitochondria more efficiently than wild-type Drp1. Consistent with an autoinhibitory role of the VD, we identified Arg-376 in the Drp1 stalk domain as necessary for Mff interaction, assembly into spirals, and mitochondrial fission. Switching the length of N- and C-terminal α-helical segments in the VD-replacing linker converted Drp1 from constitutively active and OMM-localized to inactive and cytosolic. Other hypoactive ΔVD mutants formed stable and characteristically shaped aggregates, including extended filaments. Phosphorylation of a PKA site bordering the VD disassembled the filamentous ΔVD mutant and accelerated cytosolic diffusion of full-length Drp1. We propose a model for regulation of Drp1-dependent mitochondrial fission, in which posttranslational modifications in or near the VD alter the conformation of a membrane-proximal oligomerization interface to influence Drp1 assembly rate and/or geometry. This in turn modulates Arg-376-dependent OMM targeting of Drp1 via multivalent interactions with Mff.

Fission and fusion reactions determine mitochondrial shape and interconnectivity and are critical for biogenesis as well as recycling of the organelle by mitophagy. In addition, the mitochondrial fission/fusion equilibrium controls ATP synthesis, calcium buffering, as well as production and sequestration of reactive oxygen species by mitochondria (1, 2). A group of large GTPases of the dynamin family of mechanoenzymes are responsible for mitochondrial fission and fusion. Fusion of the outer mitochondrial membrane (OMM)3 is catalyzed by the transmembrane GTPases Mfn1/2 (mitofusin-1 and -2), whereas inner membrane fusion involves the intermembrane space-localized GTPase Opal (optic atrophy 1) (3). Mutations in Mfn2 and Opal cause common hereditary neurological disorders (4, 5). In opposition to Mfn1/2 and Opal, fission of both outer and inner mitochondrial membranes is carried out by a single enzyme, dynamin-related protein 1 (Drp1). Drp1 knockout in mice results in mid-gestational lethality (6, 7) and a spontaneous, hypomorphic Drp1 mutation was shown to cause severe birth defects and early infant death (8). Current models of mitochondrial fission borrow from those proposed for dynamin-mediated scission of endocytic vesicles from the plasma membrane (9, 10), although alternative models also have been proposed (11). Briefly, Drp1 is thought to function as a “pinchase,” polymerizing into spirals around mitochondria. Cross-bridging of GTPase domains from adjacent spiral rungs induces a GTP hydrolysis-dependent conformational change, which leads to spiral constriction and severing of the organelle. Dynamin and Drp1 have in common GTPase, middle (MID), and GTPase effector domains (GED, see Fig. 1). According to the recently solved crystal structure of dynamin 1, the MID and GED form a three-helix bundle termed the stalk (12, 13). Dynamin has an additional C-terminal proline-rich domain and a pleckstrin-homology (PH) domain that separates MID and GED and mediates binding to phosphoinositol lipids (9, 10). In place of the PH domain, Drp1 features an 80–130-residue long sequence referred to as insert B or variable domain (VD) because it shows the least evolutionary sequence conser-

1 This work was supported, in whole or in part, by National Institutes of Health Grants NS043254, NS056244, and NS057714 (to S. S.)
2 □ The abbreviations used are: OMM, outer mitochondrial membrane; GED, GTPase effector domains; PH, pleckstrin homology; EGFp, enhanced GFP; HBBS, Hank’s balanced salts; FRAP, fluorescence recovery after photobleaching; BREr, bioluminescence resonance energy transfer; PC, Pearson’s coefficient; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; Drp1, dynamin-related protein 1; Mff, mitochondrial fission factor; VD, variable domain.

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vation and because it is subject to insertion of two alternative exons in vertebrates (see Fig. 1). The VD contains all eight identified sumoylation sites (14), as well as a cyclin-dependent kinase 1/cyclin B phosphorylation site that promotes mitochondrial fission during mitosis (15). At the border of the VD and GED is a PKA phosphorylation site that inhibits mitochondrial fission (16, 17). This highly conserved serine is at positions 656 and 637 in the longest Drp1 splice forms of rat and human, respectively but is referred to here as Ser_{PKA} for simplicity. 7 residues downstream of Ser_{PKA} is a cysteine, S-nitrosylation of which promotes mitochondrial fragmentation, although mechanisms are controversial (18, 19).

Mitochondrial fission factor (Mff), a ~30-kDa OMM-anchored protein, is required for Drp1 recruitment to mitochondria and subsequent mitochondrial fission (20, 21). Mff oligomerizes via a coiled-coil domain and binds via its N terminus directly to Drp1. The Mff interaction site on Drp1 has, however, not been mapped.

Here, we use linker substitution mutagenesis to investigate the function of the Drp1 VD. Our results indicate that the VD is an autoinhibitory domain that modulates mitochondrial fission through oligomeric assembly of Drp1. We also provide evidence that Ser_{PKA} phosphorylation inhibits mitochondrial fission by disrupting Drp1 assembly and identify a residue in the Drp1 stalk domain necessary for Mff-dependent recruitment to mitochondria.

**EXPERIMENTAL PROCEDURES**

Vectors—Drp1 mutants were based on the rat splice variant lacking all three alternative exons. ΔVD mutants were generated using a PCR-based method, amplifying N-terminal GTPase-MID and C-terminal GED sequences using nested primers encoding α-helical and random coil linker sequences. The two PCR fragments were joined by ligation using a silent NotI site included in one α-helical segment. Point mutations in Drp1 and Mff mutants were made by full plasmid synthesis according to the QuikChange protocol (Stratagene). H1 promoter-shRNA cassettes to silence endogenous Drp1 or Mff were inserted into the PciI sites of pEGFP-C1-based vectors according to the QuikChange protocol (Stratagene). H1 promoter-shRNA cassettes to silence endogenous Drp1 or Mff were generated starting with pEGFP-C1-based vectors by coexpression of GFP-Drp1 with mitochondria was quantified as the maximum value of 1 for perfectly circular mitochondria. Colocalization of GFP-Drp1 with mitochondria was quantified as the Pearson’s or Manders’ coefficient using the JaCoP plug-in for ImageJ software (24).

**Bioluminescence Resonance Energy Transfer (BRET)—** HeLa cells transfected with GFP-Drp1 and Renilla luciferase Mff at 1:1 ratios were detached from the substrate with 0.25% trypsin/2 mM EDTA and suspended to 10^6 cells/ml in HHBS. Mitochondrial Morphometry and Colocalization—Mitochondria channel images were analyzed using a custom ImageJ macro described previously (22, 23). Shape metrics reported here are aspect ratio (long axis divided by short axis of a best fit ellipse) and form factor (perimeter^2/(4π × area)), both of which have a minimum value of 1 for perfectly circular mitochondria. Colocalization of GFP-Drp1 with mitochondria was quantified as the Pearson’s or Manders’ coefficient using the JaCoP plug-in for ImageJ software (24).

**Fluorescence Recovery after Photobleaching (FRAP)—** FRAP of GFP-Drp1 in HeLa cells was carried out as described (22). Diffusion coefficients of cytosolic GFP-Drp1 were approximated using the equation 0.224 × r^2 / τ_{BP}, where r is the radius of the photobleached area and τ_{BP} is the time of half-recovery obtained by curve fitting (25, 26). To quantify mitochondrial interconnectivity, a square area next to the nucleus containing mitochondria was bleached with the 633 nm laser line of a Zeiss LSM 510 confocal microscope and recovery of MitoTracker Deep Red fluorescence was tracked by capturing images every 5 s for 3–4 min. Recovery curves were normalized to prebleach intensity and corrected for acquisition bleach (26) using a custom-written ImageJ macro.

**Bioluminescence Resonance Energy Transfer (BRET)—** HeLa cells transfected with GFP2-Drp1 and Renilla luciferase Mff at 1:1 ratios were detached from the substrate with 0.25% trypsin/2 mM EDTA and suspended to ~2 × 10^6 cells/ml in HHBS. Cell suspensions (40 μl) were dispensed into white 96-well plates, and luminescence reactions were started by automated injection of DeepBlueC coelenterazine substrate (10 μl of 25-
Autoinhibition by the Drp1 Variable Domain

![Diagram](image)

**FIGURE 1. Sequence and phenotypes of Drp1 VD deletion mutants.** The Drp1 VD (green) and adjacent GED (blue) contain sites for phosphorylation, sumoylation, and S-nitrosylation (underlined S, K, and C), and two alternatively spliced exons contribute to the VD (insertion point marked by triangle). At two N- and C-terminal borders (i, inside; o, outside), the VD and portions of the MID were replaced with artificial linkers composed of α-helical (h, H) and random coil (c) segments.

μM in HHBS). Emission at 410 nm (Renilla luciferase) and 513 nm (GFP2) was recorded using a Synergy 4 plate reader (BioTek) and BRET signals were calculated as Em513/Em410 nm (GFP2) was recorded using a Synergy 4 plate reader.

**RESULTS**

**Variable Domain Substitution Affects Morphology of Cellular Drp1 Assemblies**—To characterize the role of the Drp1 variable domain, we selected deletion boundaries based on crystal structures of dynamin 1 and sequence alignments of dynamin 1 and Drp1 (12, 13). Outer (o) N- and C-terminal borders of VD deletions corresponded to transitions of α-helices of the stalk domain to disordered sequences that link to the PH domain in dynamin 1. More conservative, inner (i) deletion boundaries preserved additional 17 residues at the N terminus and 5 residues at the C terminus, the latter including the SerPKA phosphorylation site (Fig. 1). The VD and flanking sequences were replaced by 10 to 28-residue-long artificial linkers composed of different permutations of modules forming 1.5 α-helical turns (h = EAAAR) and flexible, hydrophilic coils (c = GGSGGGSGG). Inadvertent internal duplication during PCR-based mutagenesis resulted in a third linker module containing five consecutive alanines (H = EAAAAAR) (Fig. 1).

Drp1ΔVD mutants were expressed in human HeLa or HEK293 cells as N-terminally tagged GFP fusion proteins from plasmids that also silence endogenous Drp1 via shRNAs to mitigate artifacts due to overexpression or coassembly of wild-type and mutant Drp1 (16). All 16 ΔVD mutants analyzed efficiently coimmunoprecipitated with wild-type HA-tagged Drp1 (Fig. 2A), indicating that the VD is not required for dimerization and that these large scale deletions do not globally perturb protein folding. We also examined Drp1ΔVD association with coexpressed, V5-tagged Mff by coimmunoprecipitation. As reported previously (20), this interaction assay requires chemical cross-linking of intact cells, presumably to stabilize Drp1 and/or Mff oligomers. The two representative ΔVD mutants (6 and 13) selected for these experiments both interacted with Mff, although ΔVD13 did so with reduced efficiency (Fig. 2B). In contrast, a Drp1 point mutant (R376E, discussed further below) abrogated Mff coimmunoprecipitation.

Wild-type GFP-Drp1 assumed a mixed diffuse cytosolic and punctate mitochondrial localization in live HeLa cells, which was also observed with the majority of ΔVD mutants albeit to varying degrees (Fig. 3, first row). The remaining six Drp1ΔVD mutants formed characteristically shaped intracellular aggregates, ranging from large (3 = ohhchhi) to small globular (5 = ohho), to drop-shaped (2b = icHho), and filamentous (1 = ichhi, Fig. 3, middle and bottom rows; summarized in Fig. 1). Drp1ΔVD1 formed extended and uniformly fine filaments that associated into bundles of variable caliber. These filaments did not colocalize with microtubules, actin, or vimentin (data not shown) and may therefore correspond to linear Drp1 homopolymers.

**Drp1 VD Is Autoinhibitory Domain**—To assess the mitochondria-fragmenting activity of the Drp1ΔVDs, transfected HeLa cells were immunofluorescently labeled for COX2 to stain mitochondria, and epifluorescence micrographs were subjected to digital morphometry (22, 23). Mitochondrial shape changes were quantified as aspect ratio and form factor, both of which have a minimum value of 1 for perfectly circular objects. Replacement of endogenous with wild-type GFP-tagged and RNAi-resistant Drp1 did not appreciably change mitochondrial morphology as compared with...
untransfected, GFP- and shRNA-negative cells (Fig. 4A). Expression of the aggregate-forming Drp1ΔVD mutants uniformly resulted in mitochondrial elongation presumably as a consequence of unopposed fusion. The cytosolic/mitochondria-localized ΔVD mutants, in contrast, promoted significant mitochondrial fission compared with wild-type Drp1, with mitochondria in ΔVD6/ohchto expressing cells displaying the smallest aspect ratios and form factors. A notable

FIGURE 2. Complex formation of Drp1 mutants. HEK293 cells were cotransfected with either triple HA-tagged Drp1 WT (A) or V5-Mff (B) and the indicated GFP-tagged Drp1 mutants, and lysates were analyzed for coimmunoprecipitation (coIP) with HA- or V5-directed antibodies, respectively. For the Mff::Drp1 coimmunoprecipitations in B, cells were cross-linked with dithiobis(succinimidyl) propionate (30 min, 0.75 mM) prior to lysis.

FIGURE 3. Localization and morphology of cellular Drp1ΔVD aggregates. HeLa cells were transfected with the indicated GFP-Drp1 expression plasmids, which also silence endogenous Drp1, and MitoTracker Deep Red-stained mitochondria (red) and Drp1 (green) were visualized by live epifluorescence microscopy 16–24 h later.
exception was ΔVD13/ohhcho, which was largely cytosolic but caused mitochondrial elongation similar to the aggregating Drp1ΔVD mutants (Fig. 4A).

To confirm that variable domain deletion can activate Drp1, we assessed mitochondrial interconnectivity in live cells by FRAP. Recovery of MitoTracker Deep Red fluorescence into a laser-bleached 5 × 5 μm area near the nucleus was monitored in HeLa cells expressing wild-type Drp1 as well as two hyperactive ΔVD constructs (3b/ohchhi, 6/ohchho). Both mutants slowed the extent and rate of mitochondrial dye refilling as quantified as fluorescence recovery integrated over the first 3 min (area under the curve, AUC, Fig. 4C), indicating that visually smaller mitochondria are indeed physically separated.

For a subset of Drp1ΔVD mutants with mixed cytosolic/mitochondrial distribution, we also evaluated colocalization with mitochondria (Pearson’s coefficient, PC) as a function of mitochondrial morphology (form factor) in fixed and immunofluorescently labeled HeLa cells. A negative, linear correlation was observed, with Drp1ΔVD6/ohchho expressing cells displaying the smallest mitochondria and highest colocalization, and the linker-reversing Drp1ΔVD13/ohhcho placing at the opposite end of the spectrum (Fig. 4D).
Dissipation of the proton gradient causes mitochondrial swelling and rapid, Drp1-dependent mitochondrial fragmentation (28). Because all previously characterized regulatory post-translational modifications of Drp1 occur within or near the mitochondrial border (4), we asked whether the VD is necessary for stimulation of mitochondrial fission by the protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). We examined Drp1ΔVD4, a mixed cytosolic/mitochondrial mutant with moderately impaired activity that lacks all sumoylation and phosphorylation sites, but retains the nitrosylation site in the GED. Another mutant carried a Cys→Val substitution to also block nitrosylation (∆VD4 CV). FCCP treatment (5 μM, 20 min prior to fixation) resulted in mitochondrial translocation of Drp1 as well as fragmentation of the organelle, regardless of whether HeLa cells expressed GFP-Drp1 wild-type, ∆VD4, or ∆VD4 CV (Fig. 4E). Thus, FCCP-mediated mitochondrial fragmentation does not require any of the known posttranslational modification sites in Drp1. Drp1ΔVD6 did not respond to uncoupler treatment, likely because this mutant is already maximally active and mitochondria-associated.

Because mitochondria in cells expressing Drp1ΔVD6 were highly punctiform, on average smaller than those in FCCP-treated, wild-type Drp1 expressing cells (Fig. 4E), we examined them ultrastructurally for signs of dysfunction. To identify GFP-Drp1ΔVD6-positive cells at the electron microscope level, we subjected them to GFP-catalyzed diaminobenzidine photo-oxidation prior to embedding (29). Mitochondria in Drp1ΔVD6-expressing HeLa cells were ultrastructurally indistinguishable from those in untransfected cells, displaying close apposition of inner and outer membranes and abundant cristae (Fig. 4F).

Collectively, these results reveal that the VD is dispensable for basal and uncoupler-stimulated mitochondrial targeting and severing by Drp1. ∆VD mutants with decreased fission activity feature either the more conservative N-terminal deletion border (i...i), a linker beginning with a three-turn α-helix (hh), or both. Therefore, the autoinhibitory function of the Drp1 VD appears to be mediated by an α-helical extension of the stalk-forming MID domain.

Phosphorylation Modulates Drp1 Assembly—Drp1 cycles between the cytosol and the OMM, and Drp1 turnover at the OMM correlates with mitochondrial fission rates (22, 30). Because some hypoactive Drp1ΔVD mutants, such as ∆VD5, form mitochondria-localized puncta, we reasoned that slow turnover may contribute to their functional impairment. To test this hypothesis, we measured GFP-Drp1 dynamics in HeLa cells by FRAP. Indeed, the hypoactive Drp1 mutants ∆VD1, ∆VD2, ∆VD5, and ∆VD13 displayed decreased fluorescence recovery, whereas hyperactive ∆VD6 exchanged at a similar rate as wild-type Drp1 (Fig. 5, A and B).

Assembly of Drp1ΔVD1 into prominent linear filaments afforded us with the opportunity to examine the effect of SerPKA phosphorylation on Drp1 oligomerization. Either phosphoinositide substitution of SerPKA with Asp or coexpression of Drp1ΔVD1 with the PKA catalytic subunit resulted in apparent disassembly of Drp1 filaments, as quantified by an increase in colocalization of Drp1 with mitochondria (PC, Fig. 5, C and D). Drp1ΔVD1 with a SerPKA to Ala substitution cannot be phosphorylated and displayed decreased colocalization with mitochondria when expressed by itself, indicative of enhanced filamentous assembly; the Ala mutation also attenuated the Drp1-dispersing effect of PKA expression (Fig. 5D).

To investigate whether SerPKA phosphorylation also modulates assembly of full-length Drp1, we measured diffusion rates of cytosolic GFP-Drp1 by FRAP. Whereas mitochondria-associated Drp1 turns over with a half-time (t1/2) of >10 s (22, 30, 31), obeying reaction/binding-limited kinetics (26), photobleaching GFP-Drp1 in an area devoid of mitochondria resulted in faster recovery (t1/2 = 2–5 s) that varied with the size of the photo-bleached area, was well fitted by a single exponential curve and therefore followed diffusion-dominated kinetics. Drp1 G363D, a well characterized mutant that dimerizes (Fig. 2A) but does not form higher-order oligomers (32, 33), diffused faster than wild-type Drp1 (Fig. 5F). These results show that FRAP can be used to estimate Drp1 oligomer stoichiometry and that cytosolic Drp1 is tetrameric or larger. Pseudophosphorylated (SerPKA→Asp mutant) Drp1 recovered faster, whereas nonphosphorylatable (SerPKA→Ala) Drp1 recovered more slowly than the wild-type fission enzyme (Fig. 5, E and F). Together with experiments involving the filamentous Drp1ΔVD1 mutant, these findings demonstrate that SerPKA phosphorylation inhibits oligomeric assembly of Drp1 in the cytosol.

The Variable Domain Is Not Required for Drp1 Recruitment by Mff—Mff coimmunoprecipitates with hyperactive Drp1ΔVD6 as well as with wild-type Drp1, but association with hypoactive Drp1ΔVD13 is decreased (Fig. 2B). To examine whether Drp1 recruitment to the OMM by Mff requires the variable domain, we either overexpressed or inhibited Mff alongside replacing endogenous with GFP-tagged, wild-type, or mutant Drp1. Mff inhibition was accomplished by combined shRNA-mediated knockdown of endogenous Mff and expression of an RNAi-resistant, dominant-negative mutant that cannot bind Drp1 (Mff two-repeat mutant VPEK23AAA,VPER53AAA; see Fig. 7E). Mff overexpression enhanced association of Drp1 with mitochondria (increase in PC) and mitochondrial fission (decrease in form factor), whereas Mff inhibition resulted in increased cytosolic localization of Drp1 and mitochondrial elongation. Wild-type and hyper- (∆VD6) and hypoactive (∆VD1, ∆VD13) Drp1 responded similarly to Mff manipulation (Fig. 6, A and B), indicating that the VD is not required for Mff-dependent recruitment of Drp1 to the OMM and mitochondrial fission.

Arg-376 Is Necessary for Drp1 Association with Mff—The finding that reversal of the linker secondary structure (∆VD6/ohcho→∆VD13/ohcho) converts Drp1 from hyper- to hypoactive indicates that the conformation of the variable domain is a key determinant for Drp1 assembly and fission activity, likely by allosterically modulating multivalent interactions with Mff (Fig. 6, C and D, see “Discussion”). We sought to identify the Mff interaction domain in Drp1 to gain further support for this hypothesis. A threading model of Drp1 was generated based on the dynamin 1 structure (12, 13). Using this structure model (Fig. 7A) and sequence alignments of dynamin family proteins, candidate residues constituting the Mff binding interface were identified according to two criteria: 1) surface exposure facing the OMM and 2) sequence conservation...
Hypoactive Drp1ΔVD mutants display slow turnover and SerPKA phosphorylation disassembles ΔVD1 and full-length Drp1 complexes. HeLa cells expressing GFP-Drp1 wild-type or indicated ΔVD mutants were analyzed for mobility of Drp1 complexes by FRAP (A, B, E, and F) and for Drp1 localization to mitochondria by quantitative epifluorescence microscopy (C and D). A, frames of representative confocal time series (5-s interval). B, Drp1 fluorescence recovery over time and total recovery quantified as area under the curve (AUC, means ± S.E. of 10–18 cells/condition). C, representative images of cells co-expressing the indicated Drp1 mutants ± PKA catalytic subunit; D, Drp1::mitochondria colocalization (Manders' coefficient) was quantified as a measure of filamentous Drp1ΔVD1 disassembly (means ± S.E. of 120 cells/condition). E and F, diffusion coefficients (F, means ± S.E. of 24–43 cells/condition) of the indicated full-length GFP-Drp1 proteins were estimated by photobleaching an area of cytosol devoid of mitochondria and capturing images (E, representatives) at a 2-s interval. Student’s t test comparison with Drp1 WT; *, p < 0.05; **, p < 0.005; ***, p < 10⁻⁵.
FIGURE 6. **Mff recruits Drp1 and induces mitochondrial fission independent of the VD.** A and B, HeLa cells expressing plasmids to replace endogenous Drp1 with the indicated GFP-tagged mutants were also transfected with plasmids to either to overexpress (↑) or inhibit (↓) Mff, the latter by combined Mff knockdown and expression of dominant-negative Mff (two-repeat mutant, 2RM=VPEK23AAAVPERS3AAA). After 24 h, cells were fixed, immunofluorescently labeled for Mff (V5 tag) and mitochondria (COX2) and analyzed for mitochondrial shape and Drp1::mitochondria colocalization by epifluorescence microscopy (representative images in A, plot of means ± S.E. of 70–140 cells/condition in B). C, surface-filling representation of dynamin 1 dimer (Protein Data Bank code 3SNH, PH domain omitted for clarity) viewed along the long axis of a lipid tubule and highlighting residues that connect to the unstructured L2 oligomerization loop (red). Residues corresponding to outside (o) borders of VD deletion in Drp1 and connecting to unstructured linkers leading to the PH domain are colored green. P denotes the SerPKA phosphorylation site, while the residue colored yellow (Phe-372) corresponds to the Mff-binding (bdg.) Arg-376 in Drp1. D, model. Posttranslational modifications in or adjacent to the VD modulate Drp1 assembly geometry or rate as mimicked by different linker substitutions (see “Discussion”). aa, amino acids.
among metazoan Drp1 orthologs, but divergence in other dynamin family proteins, including Dnm1p from yeast, which does not have a Mff ortholog. A short H9251-helical stretch in the Drp1 MID domain was identified, in which three amino acids spaced three residues apart and therefore on the same H9251-helical face meet both criteria (Fig. 7, A and B). The second of these three residues, Arg-376, was mutated to glutamate. Drp1 R376E displayed completely cytosolic localization and replacement of endogenous Drp1 resulted in mitochondrial elongation exceeding that of the least active Drp1 VD mutant, VD13. Although Mff overexpression recruited Drp1 wild-type and ΔVD13 into Mff complexes at the OMM to enhance mitochondrial fission, it had no effect in HeLa cells expressing Drp1 R376E (Fig. 7, C and D). In addition, Drp1 R376E failed to coimmunoprecipitate with Mff (Fig. 2B). However, the mutant efficiently coimmunoprecipitated with wild-type Drp1 (Fig. 2A), indicating that the dimerization interface is not compromised. To investigate oligomeric assembly, detergent lysates of transfected HEK293 cells were subjected to analytical size exclusion chromatography on a Superdex 200 column. Both wild-type and R376E mutant GFP-Drp1 fractionated into a broad profile larger than 160 kDa, with roughly half of the proteins eluting in

**FIGURE 7.** Drp1 Arg-376 is necessary for Mff-mediated recruitment of Drp1 to mitochondria and mitochondrial fission. A, schematic representation of the Drp1 dimer modeled after dynamin 1 (Protein Data Bank code 3SNH, one-to-one threading structure prediction by PHYRE2 (36)) and viewed from the mitochondrial surface. The VD was modeled with low confidence and is therefore not shown. The middle portion of the first stalk helix (α1) is expanded, highlighting Arg-376 and adjacent residues predicted to contribute to the Mff interface. B, alignment of dynamin-related protein sequences centered on Arg-376, with residues conserved in metazoan Drp1 orthologs only colored red. C and D, HeLa cells cotransfected with plasmids replacing endogenous with wild-type or mutant GFP-Drp1 and either empty vector (□) or Mff overexpression plasmids (○) were analyzed for mitochondrial shape and Drp1 localization (representative images in C, plot of means ± S.E. of 70–140 cells/condition in D). The R376E mutation blocks basal and Mff-stimulated association of Drp1 with mitochondria and mitochondrial fission. E, the indicated wild-type and mutant Mff and Drp1 cDNAs were coexpressed in HeLa cells as fusions to Renilla luciferase and GFP2, respectively, and association was detected as BRET. Either Drp1 R376E or Mff VPEK23AAAA/VPER53AAAA (2RM) mutation block in vivo complex formation (plotted are means ± S.D. of duplicates from a representative assay).
the void volume (>1 MDa, supplemental Fig. S1). Thus, Arg-376 is not required for Drp1 assembly into higher-order complexes.

We measured BRET between Mff fused to Renilla luciferase and Drp1 fused to blue light-excitable GFP utilizing the blue light emitting substrate DeepBlueC. BRET was employed to establish that Drp1 and Mff are within ~100 nm of each other in intact cells and that this interaction requires Arg-376 of Drp1. Coexpression of wild-type Renilla luciferase Mff and GFP2-Drp1 in HeLa cells resulted in robust and specific BRET signals that were abolished by mutations in the two N-terminal repeats of Mff (Mff 2RM VPEK23AAAA/VPER53AAAA). Although GFP2-Drp1 R376E was expressed at wild-type levels, it was not excited by Renilla luciferase Mff beyond background levels (Fig. 7E), further substantiating the critical role of Drp1 Arg-376 in Mff interaction and mitochondrial fission.

**DISCUSSION**

The PH domain of dynamin has an essential role in targeting the endocytosis motor to the phosphoinositide-rich plasma membrane; in addition, insertion of the PH domain has been postulated to perturb the outer leaflet of the lipid bilayer to complete membrane scission (9, 10). The PH domain of dynamin 2 is a hot spot for mutations that cause dominant intermediate Charcot-Marie-Tooth disease (34), underscoring the importance of this part of the protein. The present report demonstrates that the analogous structure in Drp1, the variable domain or insert B, has a distinct, non-essential function, modulating Drp1 assembly and activity through posttranslational modification and perhaps alternative splicing. In fact, the VD of Drp1 may be classified as an autoinhibitory domain because the most extensive VD deletions cause constitutive OMM localization and activation of Drp1, resulting in highly punctiform, but ultrastructurally normal mitochondria. Assuming the role of the dynamin PH domain, OMM-anchored proteins, such as Fis1 in yeast and Mff in metazoans have evolved to localize Drp1 to mitochondria.

**Drp1 Forms Spirals around Mitochondria**—The availability of hyperactive Drp1 mutants with enhanced OMM localization permitted visualization of GFP-Drp1 fission complexes with minimal interference by cytosolic fluorescence. GFP-Drp1ΔVD6 was coexpressed with Mff in HeLa cells to further reduce cytosolic Drp1 signals. Notably, endogenous Drp1 was similarly enriched in mitochondria associated foci, with little extramitochondrial Drp1 detectable by indirect immunofluorescence (Fig. 8A). Extended Drp1ΔVD6 helices were observed frequently in live HeLa cells stained with MitoTracker Deep Red (Fig. 8B). Time lapse microscopy at 37 °C of HeLa cells coexpressing Mff revealed association of loosely wound, spiral-shaped GFP-Drp1 complexes (both wild-type, Fig. 8C and ΔVD6, data not shown) with mitochondrial fission sites. These extended spirals generally compacted to bands presumably composed of stacked helical rungs before scission of the underlying membrane occurred, leaving roughly equal-sized Drp1 complexes attached to the new mitochondrial poles. Pole-associated Drp1 puncta diminished in size over time but could often be tracked for several minutes following a fission event (Fig. 8C). Endogenous Drp1 formed similar structures on mitochondria (Fig. 8A).

**FIGURE 8. Drp1 forms spirals in cells.** A, endogenous Drp1 (green) in HeLa cells assumes punctate, band-shaped, and more complicated morphologies on mitochondria (red) according to immunofluorescence labeling. B, when coexpressed with Mff, GFP-Drp1ΔVD6 forms extended spirals around MitoTracker-stained mitochondria in live HeLa cells. C, time lapse microscopy shows wild-type GFP-Drp1 forming spirals prior to fission and Drp1 complexes remaining associated with the ends of divided mitochondria for several minutes.
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indicative of considerable flexibility. In contrast, secondary structure prediction suggests that the Drp1 VD is largely unstructured, with short α-helices connecting to the GED and MID domain. Deletion and linker substitution mutagenesis demonstrates that in particular, the length of the N-terminal VD α-helix is a critical determinant for Drp1 assembly and activity, with longer α-helices resulting in inhibition and shorter α-helices promoting hyperactivation of the protein. Linker flexibility is also important, as Drp1VΔVD5/ohho, a mutant lacking the random coil-forming poly-Gly-Ser (c) segment between MID and GED is inactive. The dynamin 1 crystal structure (12, 13) offers a plausible explanation for how linker composition could influence Drp1 function. The disordered L2 loop that mediates higher-order dynamin assembly and the structurally resolved MID C terminus and GED N terminus are in close proximity at the membrane proximal end of the dynamin stalk (Fig. 6C). The predicted α-helical N- and C-terminal extensions of the Drp1 VD, the latter containing SerPKA, could therefore constrain the flexibility of L2 loops of adjacent Drp1 dimers to influence the diameter and/or pitch of the assembled Drp1 helix. In the case of hyperactive Drp1 mutants such as ΔVD6, this may confer Drp1 oligomers with a geometry or flexibility that maximizes multivalent interactions with Mff at the OMM (Fig. 6D). At the other extreme, the ichhli linker of ΔVD1 may force Drp1 to polymerize into relatively rigid linear filaments that cannot efficiently engage with Mff along the steep curvature of the OMM (that is, unless Mff is overexpressed, Fig. 6A). The ΔVD13/ohhcho linker confers diffuse cytosolic localization, but does not affect diffusion rate by FRAP or assembly of Drp1 into megadalton complexes by gel filtration (data not shown), suggesting that Drp1ΔVD13 self-assembles into perhaps highly curved geometries incompatible with mitochondrial recruitment by Mff. Finally, puncta-promoting linkers such as ΔVD5/ohho could result in Drp1 assembly geometries that facilitate unconstrained interstrand interactions in the cytosol.

Structure model-based mutagenesis uncovered a Mff interface on Drp1, with the highly conserved Arg-376 identified as necessary for Mff binding based on BRET, coimmunoprecipitation, and functional assays. The two Arg-376 residues in the Drp1 dimer model are situated midway down the stalk domain and opposite of the dimer interface, thus facing laterally, but also toward the OMM (Fig. 7A). Drp1 Arg-376 aligns with Phe-372 in dynamin, which forms the center of a stalk::PH domain interface in one of the two crystal structures (12). The middle segment of the first stalk domain helix (α1) therefore has a conserved role in dynamin and Drp1, binding to intra- and intermolecular membrane adaptors, respectively. Given that Mff oligomerizes to at least a dimer (20), the placement of Arg-376 is compatible with a role for Mff in not only the initial recruitment of Drp1 to the OMM but also in cross-linking adjacent rungs of the Drp1 spiral. In doing so, Mff could contribute to spiral compaction, a step we inferred from time lapse microscopy as necessary for inter-rung dimerization of GTPase domains, assembly-stimulated GTP hydrolysis, and ultimately mitochondrial fission.

Our results also shed light on the mechanism by which phosphorylation of the highly conserved SerPKA residue at the border between the VD and GED inhibits mitochondrial fission (16, 17, 22). PKA phosphorylation is shown to inhibit assembly of Drp1ΔVD1 into conspicuous linear filaments and to increase diffusional mobility of full-length Drp1 in the cytosol. According to the dynamin crystal structure and dynamin-to-Drp1 sequence alignments, SerPKA is just N-terminal of the structurally resolved GED and thus in close proximity to the assembly interface formed by MID domain loop L2 (Fig. 6C) (12,13). SerPKA phosphorylation could thus inhibit Drp1 higher-order assembly by electrostatic repulsion between two phosphates or by altering conformation/accessibility of L2. Our model (Fig. 6D) predicts that if phosphorylation occurs in the cytosol or is constitutive (Ser→Asp substitution), Drp1 is retained in the cytosol because it only inefficiently forms short oligomers that expose multiple, low affinity binding sites (involving Arg-376) for avidity-based mitochondrial recruitment via Mff (20). Once Drp1 is at the OMM, phosphorylation mediated for instance via the mitochondrial PKA/AKAP1 complex (22) may preferentially target the exposed ends of partially assembled oligomers, thereby preventing completion of fission-competent spirals and causing the buildup of large, but inactive Drp1 complexes that are stabilized by lateral interactions via GTPase domains. This model explains why Drp1 SerPKA→Asp is mostly cytosolic, whereas Drp1 phosphorylated by mitochondrial PKA/AKAP1 accumulates at the OMM (22, 35). Inhibition of oligomeric assembly and hence assembly-stimulated GTP hydrolysis may also rationalize the controversial effects of SerPKA phosphorylation on in vitro GTPase activity of Drp1 (16, 17).

A Drp1 mutant that lacks all known posttranslational modifications sites (Drp1ΔVD4 CV) is rapidly recruited to FCCP-depolarized mitochondria to carry out membrane scission. Although we cannot rule out unidentified sites on Drp1, these results imply that posttranslational modifications of Drp1 have a modulatory, rather than a “trigger” function. Perhaps modification of Mff or simply negative membrane curvature due to localized swelling (“beading”) promotes Drp1 accumulation on uncoupled mitochondria.

Lastly, the hyperactive Drp1 mutants described in this study should provide useful tools to examine the physiological and pathophysiological consequences of enhanced mitochondrial fission and to experimentally validate the models proposed here using biophysical and structural approaches.

Acknowledgments—We thank Chantal Allamargot at the Central Microscopy Research Facility for expert electron microscopy services, Ernesto Fuentes and Ann Murray for providing access to and help with chromatography equipment, and Songhai Chen for help with BRET.

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