Dimeric Dnm1-G385D Interacts with Mdv1 on Mitochondria and Can Be Stimulated to Assemble into Fission Complexes Containing Mdv1 and Fis1*

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Interactions between yeast Dnm1p, Mdv1p, and Fis1p are required to form fission complexes that catalyze division of the mitochondrial compartment. During the formation of mitochondrial fission complexes, the Dnm1p GTPase self-assembles into large multimeric complexes on the outer mitochondrial membrane that are visualized as punctate structures by fluorescent labeling. Although it is clear that Fis1p/Mdv1p complexes on mitochondria are required for the initial recruitment of Dnm1p, it is not clear whether Dnm1p puncta assemble before or after this recruitment step. Here we show that the minimum oligomeric form of cytoplasmic Dnm1p is a dimer. The middle domain mutant protein Dnm1G385Dp forms dimers in vivo but fails to assemble into punctate structures. However, this dimeric mutant stably interacts with Mdv1p on the outer mitochondrial membrane, demonstrating that assembly of stable Dnm1p multimers is not required for Dnm1p-Mdv1p association or for mitochondrial recruitment of Dnm1p. Dnm1G385Dp is reported to be a terminal dimer in vitro. We describe conditions that allow assembly of Dnm1G385Dp into functional fission complexes on mitochondria in vivo. Using these conditions, we demonstrate that multimerization of Dnm1p is required to promote reorganization of Mdv1p from a uniform mitochondrial localization into punctate fission complexes. Our studies also reveal that Fis1p is present in these assembled fission complexes. Based on our results, we propose that Dnm1p dimers are initially recruited to the membrane via interaction with Mdv1p/Fis1p complexes. These dimers then assemble into multimers that subsequently promote the reorganization of Mdv1p into punctate fission complexes.

Mitochondrial fission controls organelle shape, copy number, and function in most eukaryotic cells. Recent studies in mammalian cells highlight the importance of mitochondrial fission in programmed cell death (1–5), calcium homeostasis (6, 7), and neuronal development and function (8). Although the molecular machinery regulating mitochondrial fission is conserved from fungi to mammals, a mechanistic understanding of distinct steps in mitochondrial fission complex assembly has come mainly from studies in the budding yeast, Saccharomyces cerevisiae.

Three proteins are known to be essential for mitochondrial fission in yeast. Dnm1p is a conserved GTPase related to dynamin and contains an N-terminal GTPase domain, a middle domain, and an α-helical GTPase effector domain (9–12). Fis1p is a conserved tail-anchored outer mitochondrial membrane protein with a single tetratricopeptide repeat-like fold facing the cytoplasm (13, 14). Mdv1p contains an N-terminal extension, a domain predicted to form coiled-coils, and seven WD repeats predicted to form a β-propeller (15–17). A paralog of Mdv1p called Caf4p also binds both Dnm1p and Fis1p but is not essential for fission (18). Thus, Caf4p either plays a regulatory role in fission or is only required under specific, as yet unidentified physiological conditions.

Interactions between these three proteins are required to form fission complexes that catalyze mitochondrial division. Fis1p initially recruits Mdv1p to the membrane, and this Fis1p-Mdv1p complex mediates recruitment of Dnm1p (13, 15, 17–19). By fluorescence microscopy, Dnm1-GFP can be visualized as punctate complexes at discrete sites along mitochondria (9, 11, 20). In this study we refer to these punctate structures as Dnm1p “multimers,” although we do not exclude the possibility that cells may contain additional multimeric Dnm1p structures that are too small to visualize. In addition, large Dnm1-GFP punctate structures can be observed moving rapidly in the cytoplasm (21–23). Sometime after Dnm1p multimers appear on mitochondria, Mdv1p accumulates at these sites followed by mitochondrial fission (15, 17, 23). We refer to punctate structures on mitochondria containing both Mdv1p and Dnm1p as “mitochondrial fission complexes.” Interestingly, Fis1p has not been visualized in punctate fission complexes along with Dnm1p and Mdv1p, although it is thought to mediate post-assembly steps in mitochondrial fission (13, 19, 24). This finding raises the possibility that Fis1p physically interacts with the fission complex components before but not after their assembly into punctate fission complexes.

Dnm1p molecules are proposed to cycle between small oligomeric subunits in the cytoplasm and the large, multimeric fission complexes that are found at mitochondrial constriction sites during fission (11). In support of this idea, purified Dnm1p forms a dimer that can further assemble into large, multimeric rings and spirals (25). The minimum oligomeric state of cytoplasmic Dnm1p in vivo is not known. However, the predominant cytoplasmic form of both dynamin (26) and Drp1 (27), the mammalian Dnm1p homolog, is reported to be tetramer.

Although significant progress has been made, several key steps in fission complex assembly are not understood. First, it is not clear whether Dnm1p recruitment to mitochondria occurs before or after formation of large Dnm1p multimers. Second, the specific event required to stimulate Mdv1p reorganization from a uniform to a punctate appearance in fission complexes has not yet been defined. Third, the physical presence of Fis1p in the assembled fission complex has not been demonstrated.

In this study we show that the minimum oligomeric form of cytoplasmic Dnm1p is a dimer. Using the dimeric middle domain mutant

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Dnm1\textsuperscript{G385D}p, we demonstrate that Dnm1p dimers are effectively recruited to mitochondria and stably interact with Mdv1p. Although Dnm1\textsuperscript{G385D}p is reported to be a terminal dimer \textit{in vitro} (25), we describe conditions that promote assembly of Dnm1\textsuperscript{G385D}p into complexes containing Mdv1p and Fis1p, some of which catalyze fission. Thus, we have defined a role for the middle domain in stabilization of Dnm1p self-interactions that lead to the formation of Dnm1p multimers. By controlling the assembly state of Dnm1\textsuperscript{G385D}p, we provide the first direct demonstration that Mdv1p reorganization into punctate fission complexes is stimulated by Dnm1p multimerization on the mitochondrial membrane. Our results support a model of fission complex assembly in which Dnm1p dimers are initially recruited to Fis1p-Mdv1p complexes on mitochondria, where they self-assemble further into multimeric structures. These structures then promote the reorganization of Mdv1p into punctate fission complexes that also contain Fis1p.

**EXPERIMENTAL PROCEDURES**

**Strain and Plasmid Construction**—Yeast strains used in this study were derived from the FY genetic background (28). Standard methods were used for growth, transformation, and genetic manipulation of \textit{S. cerevisiae} (29, 30) and \textit{Escherichia coli} (31). To construct pRS425 + 3MYC-DNM1, an XhoI/Sall fragment of pRS316 + 3MYC-DNM1 (provided by R. Jensen and H. Sasaki) was ligated into pRS425 digested with XhoI/Sall. pRS426 + DNM1–3HA was constructed as described (10). pRS415 + DNM1\textsuperscript{G385D}, pRS425 + 3MYC-DNM1\textsuperscript{G385D}, and pRS426 + DNM1\textsuperscript{G385D}–3HA were created by site-directed mutagenesis (QuickChange site-directed mutagenesis kit, Stratagene) using plasmids pRS415 + DNM1, pRS425 + 3MYC-DNM1, and pRS426 + DNM1–3HA as template. pRS416 + DNM1\textsuperscript{G385D}–GFP was created by site-directed mutagenesis (Stratagene) using pRS416 + DNM1–GFP as template. Integration of DNM1\textsuperscript{G385D} at the DNM1 locus was carried out using a two-step replacement technique. First, the DNM1 open reading frame was replaced with \textit{URA3} using standard homologous recombination techniques (32). Second, \textit{URA3} was replaced with DNM1\textsuperscript{G385D} by homologous recombination, and integrants were selected by growth on 5-fluoroorotic acid. Mutations, disruptions, and replacements were confirmed by PCR, DNA sequencing, and Western blotting (where appropriate).

**Fluorescence Microscopy and Quantification of Mitochondrial Pheno-types**—Mitochondrial morphology was analyzed and quantified in cells expressing a fast folding mitochondrial matrix-targeted form of red fluorescent protein (mt-RFP)\textsuperscript{2} from p414GPD-mtRFP\textsuperscript{f} (19) or a mitochondrial targeted form of green fluorescent protein (mt-GFP) from pVT100U-GFP\textsuperscript{2} plasmid (provided by B. Westermann). For \textit{in vivo} localization of Dnm1p, Dnm1p-GFP was expressed from pRS416 (13). For \textit{in vivo} localization of Mdv1p, GFP-Mdv1p was expressed from pRS415–MET25 (19, 33). Expression of GFP-Mdv1p from the un-induced MET25 promoter produced sufficient GFP-Mdv1p (2–5-fold increase over endogenous Mdv1p levels) for visualization with no adverse effects on mitochondrial morphology or fission protein localization. Before quantification, strains were grown overnight at 30 °C, diluted to 0.5 \textit{A}\textsubscript{600} and grown for an additional 2–3 h in log phase. For experiments using sodium azide (Na\textsubscript{3}N) and sodium fluoride (NaF), cells were grown in the appropriate synthetic media to log phase and incubated with 10 mM Na\textsubscript{3}N and 10 mM NaF at 30 °C for 15 min before observation. Mitochondrial morphology and fission protein localization were visualized using a Zeiss Axioplan 2 Imaging microscope (Carl Zeiss) (34). Images were captured, processed by deconvolution, and assembled as described previously (34). For each strain, 3 independent experiments of 100 cells each were performed, the average and S.D. of which are reported.

**Co-immunoprecipitation Analysis**—Co-immunoprecipitation (co-IP) assays were performed on the indicated yeast strains grown in the appropriate synthetic media to a density of 1–1.5 \textit{A}\textsubscript{600}. For Dnm1p-Dnm1p co-IPs, all strains were transformed with the following plasmids: pRS426 + DNM1–3HA (10), pRS425 + 3MYC-DNM1, pRS426 + DNM1\textsuperscript{G385D}–3HA, and pRS425 + 3MYC-DNM1\textsuperscript{G385D}. Co-IPs were performed from whole cell lysates as described previously (10) using anti-HA-agarose-conjugated beads (Sigma). For Dnm1p-Fis1p co-IPs with cross-linking, yeast strains expressing Dnm1p (DNM1) or Dnm1\textsuperscript{G385D}p (DNM1\textsuperscript{G385D}) from the genomic locus were transformed with either pRS415MET25 + 9MYC-FIS1 (13) or pRS415MET25 plasmids. For Dnm1p-Mdv1p co-IPs with cross-linking, dnm1\textsubscript{Δ} cells expressing endogenous Mdv1p were transformed with either pRS425 + 3MYC-DNM1 or pRS425 + 3MYC-DNM1\textsuperscript{G385D} plasmids. Dnm1p-Fis1p co-IPs and Dnm1p-Mdv1p co-IPs were performed as described previously (19). Briefly, log phase cells growing in synthetic dextrose media were harvested at 4200 x g for 5 min and incubated in buffer TD (100 mM Tris-SO\textsubscript{4}, pH 9.4, 10 mM dithiothreitol) for 15 min at 30 °C. After washing twice with buffer SP (1.2 mM sorbitol, 20 mM potassium phosphate, pH 7.4), cells were incubated with zymolase (6 mg/ml) for 40 min at 30 °C. Spheroplasts were treated with 2.5 mM dithiobis(succinimidyl propionate) (DSP) (Pierce) for 30 min, incubated with 50 mM glycine for 15 min to quench the cross-linking reaction, and homogenized in buffer SH (1.2 mM sorbitol, 40 mM HEPES-KOH, pH 7.4). 50 A\textsubscript{600} units of homogenized cells were centrifuged at 12,500 x g for 10 min. The pellet was solubilized with IP buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.07% Triton X-100) containing 1:50 protein inhibitor mixture (Calbiochem) for 15 min, and the supernatant was incubated with anti-c-Myc conjugated agarose beads for 1 h. Where indicated, spheroplasts were treated with 10 mM NaN\textsubscript{3}/NaF for 15 min at 30 °C before dithiobis(succinimidyl propionate) cross-linking. Co-IP samples were analyzed by 10% SDS-PAGE and Western blotting using anti-HA (University of Utah), anti-Myc (Santa Cruz Biotechnology), anti-porin (Molecular Probes, Inc.), anti-Mdv1p (J. Nunnari), and affinity-purified anti-Dnm1p (11) antibodies as indicated.

**Gel Filtration Analysis**—100 A\textsubscript{600} of each indicated yeast strain in log phase was solubilized and lysed in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na\textsubscript{2}HPO\textsubscript{4}, 1.4 mM KH\textsubscript{2}PO\textsubscript{4}, pH 7.4) containing 1:100 protease inhibitor mixture (Sigma) as described previously (35). Cleared whole cell lysates were centrifuged at 100,000 x g for 30 min, and the resulting supernatant (~3 mg/ml) was loaded on a Sephacryl-S300 column (16/60; Amersham Biosciences) and separated in the presence of phosphate-buffered saline (35). Proteins in collected fractions were precipitated in 10% trichloroacetic acid (TCA), washed with cold acetone to remove residual TCA, resuspended in sample buffer, separated by 10% SDS-PAGE, and analyzed by Western blotting using affinity-purified anti-Dnm1p antibody. The molecular mass of Dnm1p or Dnm1\textsuperscript{G385D}p was calculated based on the elution profile of high molecular weight gel filtration standards (Amersham Biosciences) run separately.

**RESULTS**

**Mutation of a Conserved Glycine in the Dnm1p Middle Domain Blocks Protein Function and Causes Dominant Fission Defects**—The middle domain of Dnm1p contains a glycine residue (Gly-385) that is conserved among members of the dynamin family (Fig. 1A). In genetic

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\textsuperscript{2} The abbreviations used are: mt-RFP, mitochondrial matrix-targeted red fluorescent protein; GFP, green fluorescent protein; IP, immunoprecipitation; HA, hemagglutinin; DSP, dithiobis(succinimidyl propionate).
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A. Dnn1p

| Species | S.c. | S.c. | S.p. | A.t. | C.e. | H.s. | H.s. | H.s. | MGRS-10His-HA-Dnm1p |
|---------|------|------|------|------|------|------|------|------|---------------------|
| Dnm1p   | SLVQLMNKFSSTFAISDFTSSDINTKELCG | SVUSSMTDFSNYAGILDEAKLESLQELG | ILLQNNMNFAFSTFDGNSNIPITKELCS | ALLNFLKCYAESYSSLKGESEMSTSLSL | RTLLQ11TRFAVYCTIGTARIKFTTEELCG | ATLLQ11TFTCNYCTIETKAYTIELC | KALLQMVQFAVDPEKRISSGQIDYTELCS | KALLQMVQFGVDEKRISSGQIDYTELCS |

B. Co-IP of 3Myc-Dnm1G385Dp

| Strain | p-empty | pDNN1 | pDNM1| pDNM1G385Dp |
|--------|---------|-------|------|-------------|
| dnm1Δ  | 0 0 0 0 | 0 0 0 0 | 0 0 0 0 | 0 0 0 0 |
| DNN1+  | 0 0 0 0 | 0 0 0 0 | 0 0 0 0 | 0 0 0 0 |

C. Western Blot Analysis

| Strain  | DIC | GFP | % cells with punctate GFP |
|---------|-----|-----|--------------------------|
| dnn1Δ+  | 99.3 ± 1.2 | 3.3 ± 5.8 |
| pDNN1-GFP | 99.3 ± 1.2 | 99.3 ± 1.2 |
| pDNM1G385Dp-GFP | 3.3 ± 5.8 | 3.3 ± 5.8 |

FIGURE 2. Dnm1G385Dp forms a complex with itself in a co-immunoprecipitation assay. Myc-tagged and HA-tagged forms of Dnm1p or Dnm1G385Dp were co-expressed in dnm1Δ cells. HA-tagged Dnm1p proteins were immunoprecipitated using anti-HA agarose beads. Co-IP of Myc-tagged Dnm1 proteins was detected by immunoblot using anti-Myc antibodies (top panel). Immunoprecipitation of HA-tagged Dnm1p or Dnm1G385Dp was detected with anti-HA antibodies (middle panel). Western blot shows total loaded (low), flow-through (FT), and eluted (E) proteins. The mitochondrial outer membrane protein porin is provided as a loading control (bottom panel). The asterisk denotes nonspecific binding of 3Myc-Dnn1p to beads.

expressing Dnm1G385Dp in the presence of wild-type Dnm1p had fission competent mitochondria, indicating that Dnm1G385Dp causes severe dominant fission defects. In a dnm1Δ background, Dnm1G385Dp displayed very little fission activity, suggesting that this protein likely blocks activity of the native fission machinery. The inability of Dnm1G385Dp to support fission was not due to reduced protein levels, as Western blot analyses of whole cell extracts from these strains confirmed that similar levels of Dnm1p and Dnm1G385Dp were expressed at steady state (data not shown).

Unlike wild-type Dnm1-GFP, which assembled into multimeric punctate structures on the outer mitochondrial membrane (Fig. 1C), the Dnm1G385D-GFP protein localized diffusely in the cytoplasm. Thus, residue Gly-385 in the middle domain is critical for stable assembly of Dnm1p on mitochondria. However, the G385D mutation does not prevent Dnm1G385Dp from interacting with wild-type Dnm1p. In cells expressing both wild-type Dnm1-GFP and mutant Dnm1G385Dp, the Dnm1-GFP protein was restricted to the cytoplasm in 60% of the cells (data not shown), suggesting that Dnm1G385Dp mediates dominant effects by binding to and sequestering the wild-type protein in non-functional complexes.

Dnm1p and Dnm1G385Dp Self-Interact and Form Cytoplasmic Dimers at Steady State—In a previous study, we demonstrated that Dnm1p forms complexes with itself in vivo (10). In this study, co-IP studies were used to determine whether the G385D mutation affected Dnm1p complex formation with itself. Fig. 2 shows that co-IP of 3Myc-Dnm1G385Dp with Dnm1G385D-3HAp (Fig. 2a, lane 6, α-Myc) was only slightly less efficient than co-IP of 3Myc-Dnm1p with Dnm1-3HAp (Fig. 2a, lane 3, α-Myc), indicating that Dnm1G385Dp proteins are able to form complexes in vivo. These interactions were specific, as we did not observe significant co-IP of porin (an outer membrane protein) under these conditions (Fig. 2, lanes 3, 6, 9, 12, α-porin) or of 3Myc-tagged Dnm1p when an HA-tagged protein was absent (Fig. 2, lane 12, α-Myc). In separate experiments, mutant 3Myc-Dnm1G385Dp co-immunoprecipitated efficiently with wild-type Dnm1-3HAp (data not shown), consistent with the idea that Dnm1G385Dp is a dominant negative because it binds to and interferes with the function of the wild-type Dnm1p protein.

Although Dnm1G385Dp formed complexes in the co-immunoprecipitation assay, we were unable to detect Dnm1G385Dp-GFP assembled into punctate structures. A recent in vitro study reported that MGRS-10His-HA-Dnm1p purified from insect cells formed dimers that further assembled into rings and spirals in low salt buffer (25). These ring-like structures are presumed to represent the punctate
and eluted in the 157-kDa fraction even in the absence of Mdv1p and Fis1p (Fig. 3B). Relative to the Dnm1p profile, a larger portion of Dnm1G385Dp eluted in early fractions corresponding to a slightly larger molecular weight in the dnm1Δ strain background (Fig. 3B, top panel, fractions 5 and 6). Although the physiological relevance of this observation is currently unclear, these results demonstrate that the minimal dimeric form of Dnm1p is not disrupted by the G385D middle domain substitution.

It is important to note that the S300 column used in this gel filtration experiment only resolves small Dnm1p cytoplasmic oligomers and not large complexes. Therefore, this experiment does not address the oligomeric state of the large, rapidly moving punctate structures visualized by Dnm1p-GFP in live cells.

Sodium Azide/Sodium Fluoride Treatment Promotes Assembly of Dnm1G385D-GFP into Mitochondrial Fission Complexes—Although Dnm1G385Dp proteins interact to form cytosolic dimers, these dimers do not form visible punctate complexes. It is possible that Dnm1G385Dp dimers never interact further to form multimers. Alternatively, Dnm1G385Dp may form multimers that are unstable and rapidly dissociate, shifting the equilibrium to the dimeric form. In vitro, dynamin family members including Dnm1p assemble into stable rings and spirals in the absence of nucleotide or the presence of GDP and disassemble upon GTP hydrolysis (25, 44, 45). We reasoned that if Dnm1G385Dp forms unstable complexes, then depleting cells of GTP might stabilize these complexes, allowing their visualization. To test this idea, cells were treated with sodium azide and sodium fluoride (NaN3/NaF), which inhibits the cytochrome oxidase complex and enzymes of the tricarboxylic acid cycle, respectively. As a consequence, ATP production is rapidly abolished, and ATP pools required for GTP production are depleted (46–50).

We examined the organization and distribution of Dnm1-GFP or Dnm1G385D-GFP in cells depleted of GTP by a brief 10 mM NaN3/NaF treatment. As shown in Fig. 4, Dnm1-GFP localized to punctate fission complexes on mt-RFP-labeled mitochondria in 100% of the cells in the population (Fig. 4, top row), and NaN3/NaF treatment did not alter the punctate appearance or mitochondrial localization of wild-type Dnm1-GFP (Fig. 4, second row). By contrast, the Dnm1G385D-GFP staining
experiments in fzo1Δ dnm1Δ cells, which have tubular mitochondria. The lack of fusion, which prevents net formation, enhances detection of fission events in these cells. As shown in Fig. 5, mitochondria fragmented in the majority of fzo1Δ dnm1Δ cells expressing wild-type Dnm1p both in the presence and absence of NaN3/NaF (Fig. 5, first and second row). Significantly, when fzo1Δ dnm1Δ cells expressing Dnm1G385Dp were treated with 10 mM NaN3/NaF, mitochondria fragmented in 41.3% of cells. By contrast, only 4.6% of untreated cells contained fragmented mitochondria (Fig. 5, fifth and sixth row). Cells carrying the empty vector did not show any increase in mitochondrial fragmentation when treated with NaN3/NaF, indicating that stimulation of mitochondrial fission under these conditions is Dnm1p-dependent (Fig. 5, third and fourth row) (16). These results indicate that the Dnm1G385Dp punctate structures formed in the presence of NaN3/NaF mediate fission.

Assembly of Dnm1G385Dp into Stable Multimers Is Not Required for Interaction with Mitochondrial Mdv1p—Both co-IP (21) and yeast two-hybrid (17, 18, 21) studies suggest that Dnm1p interacts with Mdv1p. We performed co-IP assays from membrane-enriched fractions to examine the possibility that defects in these Dnm1p-Mdv1p interactions were responsible for the inability of Dnm1G385Dp to form stable mitochondrial fission complexes in vivo. As shown in Fig. 6, a fraction of Mdv1p from chemically cross-linked membrane fractions reproducibly co-immunoprecipitated with 3Myc-Dnm1p (Fig. 6, lane 2, α-Mdv1p). Importantly, membrane-bound Mdv1p also co-immunoprecipitated with 3Myc-Dnm1G385Dp (Fig. 6, lane 4, α-Mdv1p) at levels similar to wild type. It is important to note that the fractions used for these experiments were generated from cells containing dimeric Dnm1G385Dp but lacking Dnm1G385Dp stable multimers. The ability of mitochondrial Mdv1p to interact with Dnm1G385Dp suggests that 1) stable assembly of Dnm1G385Dp into multimeric complexes is not a prerequisite for Mdv1p interaction, 2) dimeric Dnm1G385Dp is efficiently recruited to mitochondria, and 3) the instability of Dnm1G385Dp multimers is not due to a Dnm1G385Dp-Mdv1p interaction defect.

Assembly of Dnm1G385Dp into Stable Multimers Is Essential for Reorganization of GFP-Mdv1p into Punctate Structures—In wild-type cells, Fis1p-Mdv1p complexes are uniformly distributed on the cytoplasmic face of the mitochondrial outer membrane (15, 17, 19). GFP-Mdv1p is also found in punctate structures that co-localize with Dnm1p (15, 17). However, GFP-Mdv1p fails to form visible punctate structures in dnm1Δ cells, suggesting that Dnm1p plays an essential role in their formation. However, the specific step that promotes Mdv1p reorganization from a uniform mitochondrial distribution into punctate structures is unknown. We hypothesized that Dnm1p multimerization drives pattern was converted from diffuse cytoplasmic (Fig. 4, third row) to discrete puncta after NaN3/NaF treatment (Fig. 4, fourth row). The formation of Dnm1G385Dp-GFP puncta was very rapid, occurring within 5 min of NaN3/NaF treatment, and was rapidly reversed upon NaN3/NaF removal (data not shown). Quantification revealed that greater than 80% of these Dnm1G385Dp-GFP-containing puncta stably co-localized with mt-RFP-labeled mitochondria in individual cells, suggesting that they are bona fide fission complexes. These findings are consistent with the idea that Dnm1G385Dp dimers form unstable multimeric complexes in vivo that rapidly disassemble unless they are stabilized by GTP depletion.

In wild-type cells, NaN3 treatment stimulates Dnm1p-dependent fission, leading to mitochondrial fragmentation (Fig. 4, second row, mt-RFP, and Fekkes et al. 16). If Dnm1G385Dp multimers that form after treatment with NaN3/NaF are physiologically relevant, they should also stimulate fission. However, in dnm1Δ cells expressing Dnm1G385Dp, we did not observe significant mitochondrial fragmentation after treatment with NaN3/NaF (Fig. 4, fourth row, mt-RFP). We reasoned that fission events might be difficult to detect in cells already containing mitochondrial nets. To test this possibility, we performed the same
Mdv1p reorganization into punctate structures. To test this idea, we examined the localization pattern of GFP-Mdv1p in Dnm1G385Dp-expressing cells incubated in the absence or presence of NaN3/NaF. In both the absence and presence of NaN3/NaF treatment, GFP-Mdv1p formed mitochondrial puncta in cells expressing wild-type Dnm1p (Fig. 7, first and second rows). The NaN3/NaF treatment had no detectable effect on the size or shape of these puncta. By contrast, a uniform mitochondrial GFP-Mdv1p localization was observed in cells lacking Dnm1p whether or not NaN3/NaF was added (Fig. 7, third and fourth rows). Importantly, GFP-Mdv1p puncta were not observed in the majority of untreated cells expressing Dnm1G385Dp (Fig. 7, fifth row). Because Dnm1G385Dp forms dimers under these conditions that are able to bind and co-IP with Mdv1p, we conclude that interaction of dimeric Dnm1G385Dp with Mdv1p is not sufficient to promote reorganization of Mdv1p into puncta. Significantly, GFP-Mdv1p puncta were restored in 63% of cells expressing Dnm1G385Dp after treatment with NaN3/NaF (Fig. 7, sixth row). As described above, dimeric Dnm1G385Dp assembles into multimeric complexes after NaN3/NaF treatment. Thus, stabilizing Dnm1G385Dp multimeric complex formation stimulates the production of GFP-Mdv1p punctate structures. This GFP-Mdv1p puncta formation depends on assembly of Dnm1p or Dnm1G385Dp, as similar structures are not present in dnm1Δ cells or in untreated DNM1G385Dp cells. Together, our co-IP and localization results provide a direct demonstration that stable assembly of Dnm1p into multimeric complexes is required to reorganize Mdv1p into punctate structures.

Fis1p Is Present in Assembled Fission Complexes on Mitochondria—Fis1p is essential for Dnm1p recruitment to mitochondria (13–17) and is also thought to play an important role after assembly of the fission complex (13, 19, 24). However, unlike Mdv1p and Dnm1p, fluorescently tagged Fis1p does not form visible puncta associated with fission complexes. This observation raises the possibility that Fis1p is required to initially recruit fission complex components but does not associate with these components after they form punctate structures. Alternatively, Fis1p may be an integral component of assembled complexes but is present in low stoichiometric amounts compared with Mdv1p and Dnm1p, making visualization by fluorescence microscopy difficult. We described conditions in which mitochondrial Mdv1p and Dnm1G385Dp are reorganized into punctate structures after NaN3/NaF treatment. To test whether Fis1p is present in these complexes, we performed co-IP experiments between Fis1p and Dnm1G385Dp from DSP cross-linked membrane fractions in the presence or absence of NaN3/NaF. A fraction of Dnm1p co-immunoprecipitated with 9Myc-Fis1p both in the absence and presence of NaN3/NaF (Fig. 8, lanes 2 and 4, α-Dnm1p). This interaction was specific as Dnm1p failed to precipitate from cells lacking 9Myc-Fis1p (Fig. 8, lane 10, α-Dnm1p). We did not detect Dnm1G385Dp in the majority of untreated cells expressing Dnm1G385Dp (Fig. 7, fifth row). Because Dnm1G385Dp forms dimers under these conditions that are able to bind and co-IP with Mdv1p, we conclude that interaction of dimeric Dnm1G385Dp with Mdv1p is not sufficient to promote reorganization of Mdv1p into puncta. Significantly, GFP-Mdv1p puncta were restored in 63% of cells expressing Dnm1G385Dp after treatment with NaN3/NaF (Fig. 7, sixth row). As described above, dimeric Dnm1G385Dp assembles into multimeric complexes after NaN3/NaF treatment. Thus, stabilizing Dnm1G385Dp multimeric complex formation stimulates the production of GFP-Mdv1p punctate structures. This GFP-Mdv1p puncta formation depends on assembly of Dnm1p or Dnm1G385Dp, as similar structures are not present in dnm1Δ cells or in untreated DNM1G385Dp cells. Together, our co-IP and localization results provide a direct demonstration that stable assembly of Dnm1p into multimeric complexes is required to reorganize Mdv1p into punctate structures.
**Dynamics of Mitochondrial Fission Complex Assembly**

**FIGURE 8.** Dnm1(G385D)-Fis1p complexes are only detected in a co-IP assay after treatment with NaN₃/NaF. Western blot showing Dnm1p and Dnm1(G385D)p co-immunoprecipitation with Fis1p before (indicated by −) and after (indicated by +) treatment of intact cells with 10 mM NaN₃/NaF. Myc-tagged Fis1p was immunoprecipitated using anti-Myc-agarose-conjugated beads. Co-IP of Dnm1 proteins was detected by immunoblot using native Dnm1p antibodies (top panel). Western blot shows total loaded (L) and eluted (E) proteins. Immunoprecipitation of Myc-tagged Fis1p was detected with anti-Myc antibodies (middle panel). The mitochondrial protein porin is provided as a loading control (bottom panel). Genes indicated are genomic unless preceded by p, which denotes plasmid-borne.

Co-immunoprecipitation with 9Myc-Fis1p (Fig. 8, lane 6, α-Dnm1p), suggesting that any amount of Dnm1p associated with Fis1p is below the level of detection under these conditions. However, Dnm1(G385D)p co-immunoprecipitated efficiently with Fis1p in the presence of NaN₃/NaF, indicating that Fis1p is a stable component of assembled fission complexes on mitochondria.

**DISCUSSION**

The results described here advance our understanding of fission complex assembly in several important ways. First, we demonstrate that the minimum cytoplasmic form of Dnm1p is a dimer. Second, our experiments support a role for the Dnm1p middle domain in the stabilization of Dnm1p multimers during or after assembly. Third, our data suggest that assembly of Dnm1p into stable multimers is not required for Dnm1p-Mdv1p interaction or mitochondrial recruitment of Dnm1p. Fourth, we demonstrate that reorganization of Mdv1p into punctate structures requires assembly of Dnm1p into multimers on mitochondria. Finally, we show that Fis1p is physically present in assembled fission complexes with Mdv1p and Dnm1p (25). Each of these points is discussed in detail below.

**Cytosolic Dnm1p Is a Dimer—**Our gel filtration assays demonstrate that the minimum soluble form of yeast Dnm1p under physiological conditions is a dimer. Moreover, we show that Dnm1p dimerization is not dependent upon the presence of Mdv1p or Fis1p. These results are consistent with an in vitro study reporting that yeast Dnm1p purified from insect cells forms a dimer in solution (25).

However, the mammalian homolog of Dnm1p, Drp1, migrates as a tetramer in gel filtration studies (27). We suggest that the differences in oligomeric state may reflect variations in the assembly pathway of mitochondrial fission complexes between species. This view is supported by the fact that whereas Mdv1p is an important component of mitochondrial fission in yeast, its mammalian homolog has not yet been identified.

**The Middle Domain Is Required to Stabilize Multimeric Dnm1p Complexes—**We demonstrated previously that the Dnm1p middle domain interacts with itself and the GTPase effector domain/assembly domain (10). Although these findings suggest that the middle domain is important for Dnm1p self-assembly, this idea has never been rigorously tested in vivo. In this study we demonstrate that Dnm1(G385D)p dimerizes to the same extent as the wild-type Dnm1 protein. However, this amino acid substitution compromises the ability of Dnm1p to form stable multimers. Dnm1(G385D)p might fail to form multimeric complexes because the G385D substitution blocks dimer-dimer interactions required for multimer assembly. Alternatively, Dnm1(G385D)p may form multimers that disassemble more rapidly, preventing visualization of stable fission complexes. By treating cells with NaN₃/NaF, we demonstrated that dimeric Dnm1(G385D)p is capable of assembling into functional fission complexes containing both Mdv1p and Fis1p. We suggest that the primary effect of the G385D middle domain mutation is to enhance disassembly of Dnm1p complexes, shifting the equilibrium to the dimeric state. The molecular basis for this enhanced disassembly is currently unclear. One possibility is that the G385D substitution alters the Dnm1p GTPase cycle in vivo by disrupting Dnm1p intramolecular interactions, resulting in premature GTP hydrolysis and rapid disassembly of Dnm1p(G385D)p-containing multimers.

Dynamin and Dnm1p multimers assemble in the absence of nucleotide or the presence of GDP in vitro (25, 44, 45). In this study we show that Dnm1(G385D)p can form multimeric complexes on mitochondria after treatment to deplete cellular GTP. Furthermore, mitochondrial fission is partially restored under these conditions. The simplest interpretation of this result is that Dnm1(G385D)p self-interactions are stabilized when Dnm1(G385D)p is in the GDP-bound or empty state, resulting in the formation of visible multimeric structures that can catalyze fission. A recent study reported that Dnm1(G385D)p is a terminal dimer in vitro (25). It is possible that Dnm1(G385D)p multimers also assemble in vitro under conditions not yet tested. Alternatively, the ability of Dnm1(G385D)p multimers to assemble upon NaN₃/NaF treatment may require the presence of additional proteins or factors that promote assembly in vivo but are absent in vitro.

**Assembly of Dnm1p into Multimers Is Not Essential for Dnm1p-Mdv1p Interactions or Dnm1p Recruitment to Mitochondria—**Mdv1p interacts with Dnm1p in mitochondrial fission complexes (17, 19, 21). It has been proposed that Mdv1p only interacts with multimeric Dnm1p (23). This model was based on the observations that Mdv1p fails to interact with Dnm1(G385D)p by the yeast two-hybrid assay and that Mdv1p fails to form punctate structures on mitochondria in cells expressing Dnm1(G385D)p. We show here that dimeric Dnm1p(G385D)p forms a complex with mitochondrial Mdv1p in a co-IP assay. Thus, assembly of Dnm1p into stable multimers is not required for Dnm1p-Mdv1p interaction. Moreover, although Mdv1p can interact with dimeric Dnm1(G385D)p, this interaction does not stabilize Dnm1(G385D)p multimer formation. It is important to note that we failed to detect interactions between Dnm1p(G385D)p and Mdv1p using a sensitive yeast two-hybrid protocol (data not shown), consistent with published results (23). However, co-IP assays reproducibly detected this interaction, indicating that the two-hybrid assay may not always be a reliable means of assessing physiologically relevant interactions between these proteins.

Membrane-bound Mdv1p is required to recruit Dnm1p to mitochondrial fission complexes (18, 19). We and others (21–23) have observed Dnm1p multimeric structures moving rapidly in the cytoplasm in live cells, leading to the hypothesis that Dnm1p multimers first assemble in the cytoplasm and are subsequently recruited to Mdv1p on mitochondria. However, these rapidly moving puncta have never been shown to stably bind mitochondria and subsequently become functional fission complexes. An alternative interpretation is that these cytoplasmic Dnm1p multimers are the products of completed fission reactions or simply Dnm1p storage units. Here we show that dimeric Dnm1p(G385D)p is efficiently recruited to mitochondrial Mdv1p, consistent with the idea that multimerization occurs after recruitment of dimeric Dnm1p to the membrane.

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Mdv1p Reorganization into Punctate Structures on Mitochondria Requires Stable Assembly of Dnm1p Multimeric Complexes—Previous studies established that Mdv1p-Fis1p complexes are distributed uniformly on mitochondrial tubules (15, 17–19). In addition, Mdv1p forms punctate structures that co-localize with Dnm1p-containing fission complexes (15, 17). When Dnm1p is absent, Mdv1p remains uniformly distributed on mitochondria and fails to form punctate structures (15, 17), suggesting that Dnm1p is required for the reorganization of Mdv1p into visible puncta. Here we demonstrate that Mdv1p fails to form mitochondrial puncta even when it is associated with dimeric Dnm1p. However, when Dnm1p multimers are stabilized by NaN3/NaF treatment, Mdv1p forms visible mitochondrial puncta, providing a direct demonstration that assembly of Dnm1p into multimeric complexes promotes the reorganization of Mdv1p from a uniform to a punctate mitochondrial localization. Consistent with these observations, in time-lapse studies, GFP-Mdv1p puncta appear after Dnm1p-GFP puncta become visible on mitochondria (23). Exactly how Mdv1p is reorganized during this process is not yet clear. Mdv1p puncta formation may reflect a change in the stoichiometry of Dnm1p and Mdv1p in the fission complex due to Mdv1p self-assembly. In support of this model, Mdv1p has been shown to form a complex with itself in co-IP experiments (18). Further studies are required to determine the nature of Mdv1p reorganization into these punctate fission complexes.

Fis1p Is Present in Assembled Fission Complexes—Fis1p is essential for Dnm1p recruitment to mitochondria (13, 15–17) and also plays a role after assembly of the fission complex (13, 19, 24). Despite the apparent stability of the fission complexes visualized in intact cells, the presence of Fis1p in these structures has been difficult to demonstrate. In the co-IP studies described here we did not detect Fis1p in a complex with dimeric Dnm1p even after chemical cross-linking. However, this interaction was detected after NaN3/NaF treatment, which stabilizes Dnm1p multimers. These results indicate that Fis1p remains associated with fission complexes after assembly. Given our results, it is surprising that GFP-Fis1p does not form visible puncta similar to those observed for Dnm1p-GFP and GFP-Mdv1p in fission complexes. It is possible that there are fewer Fis1p molecules compared with Dnm1p and Mdv1p in an assembled fission complex.

Stepwise Assembly of Mitochondrial Fission Complexes—This study provides new insights regarding the spatial and temporal dynamics of mitochondrial fission complex assembly (Fig. 9). We propose that fission complex assembly begins when Dnm1p dimers are recruited to pre-existing Mdv1p-Fis1p complexes on mitochondria (Fig. 9, step 1). These mitochondrial dimers, which are in a dynamic equilibrium with the cytoplasmic pool of Dnm1p dimers, further assemble to form multimeric complexes (Fig. 9, step 2). Dnm1p multimerization then drives the reorganization of Mdv1p from a uniform mitochondrial distribution into punctate fission complexes that also contain Fis1p (Fig. 9, step 3). In later steps, these fission complexes are activated, leading to division of the mitochondrial compartment. How these three proteins work together in the assembled complex to achieve fission is the focus of our ongoing studies.

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