Mdv1 Interacts with Assembled Dnm1 to Promote Mitochondrial Division*§

Received for publication, July 21, 2005, and in revised form, October 5, 2005 Published, JBC Papers in Press, November 4, 2005, DOI 10.1074/jbc.M507943200

Kari Naylor†, Elena Ingerman‡, Voytek Okreglak‡, Michael Marino‡, Jenny E. Hinshaw§, and Jodi Nunnari‡,‡

From the †Section of Molecular and Cellular Biology, University of California, Davis, California 95616 and the ‡Laboratory of Cell Biochemistry and Biology, National Institutes of Health, NIDDK, Bethesda, Maryland 20892

The dynamin-related GTPase, Dnm1, self-assembles into punctate structures that are targeted to the outer mitochondrial membrane where they mediate mitochondrial division. Post-targeting, Dnm1-dependent division is controlled by the actions of the WD repeat protein, Mdv1, and the mitochondrial tetratricopeptide repeat-like outer membrane protein, Fis1. Our previous studies suggest a model where at this step Mdv1 functions as an adaptor linking Fis1 with Dnm1. To gain insight into the exact role of the Fis1-Mdv1-Dnm1 complex in mitochondrial division, we performed a structure-function analysis of the Mdv1 adaptor. Our analysis suggests that dynamic interactions between Mdv1 and Dnm1 play a key role in division by regulating Dnm1 self-assembly.

Mitochondria form a complex reticular structure that is maintained by a balance between ongoing mitochondrial division and fusion events (1). Disruption of mitochondrial function causes mitochondrial tubules to fragment (2). In contrast, disruption of mitochondrial division causes simple tubules to form highly interconnected net-like structures (3, 4). In yeast, three conserved dynamin-related GTPases control mitochondrial dynamics: the outer membrane protein, Fzo1, and the inner membrane protein, Mgm1, control mitochondrial fusion and the outer membrane protein, Dnm1, controls mitochondrial division (5–8). Numerous studies have established that the human orthologs of these components also function to regulate mitochondrial dynamics, indicating that the fundamental mechanisms underlying these events are conserved (9). Interestingly, there is increasing evidence suggesting that in human cells mitochondrial division and fusion components have been co-opted to regulate apoptosis (10).

Existing experimental evidence indicates that the yeast dynamin-related protein, Dnm1, is the master regulator of mitochondrial division. Dnm1 self-assembles and in vivo exists at steady state in punctate structures associated with the outer mitochondrial membrane, often at points of membrane constriction and fission (3, 4, 11, 12). Recent biochemical and structural analysis of pure Dnm1 indicates that Dnm1 self-assembly functions to drive the membrane constriction and fission events associated with mitochondrial division (13). Specifically, Dnm1 forms extended spirals, which possess diameters greater than dynamin-1 spirals, but which are, remarkably, equal to diameters of mitochondrial constriction sites in vivo. Dnm1 self-assembly proceeds through a rate-limiting nucleation step and nucleotide hydrolysis by assembled Dnm1 structures is highly cooperative with respect to GTP (13). These kinetic features of Dnm1 suggest that the key rate-limiting event in division is the regulation of Dnm1 self-assembly.

In yeast, two additional components, Fis1 and Mdv1, are required and function together with Dnm1 in mitochondrial division (5). Fis1 is a highly conserved C-tail anchored outer membrane protein comprised largely of a cytosolic superhelical tetratricopeptide repeat domain (14). An interaction of the human ortholog, hFis1, with the human Dnm1 ortholog, Drp1, has been reported, but the role of this interaction in division is unknown (15). To date, however, no interaction between Fis1 and Dnm1 has been detected, raising the possibility it is labile or not essential in yeast. In yeast, the most well defined role of Fis1 is as a mitochondrial receptor for Mdv1, where the Fis1·Mdv1 complex functions to trigger division (16, 17).

Mdv1 is a multidomain protein containing three major regions, an N-terminal extension of unknown structure (NTE), a central predicted coiled-coil (C-C), and a C-terminal WD repeat (WD), predicted to form a seven-bladed propeller. Previous two-hybrid and cytoplasmic observations indicate that the Mdv1 NTE mediates an interaction with Fis1, the Mdv1 WD mediates the interaction with Dnm1, and the central Mdv1 C-C mediates homo-oligomer formation (17). The WD is both necessary and sufficient for Mdv1 to interact with Dnm1 punctate structures (17, 18). Thus, Mdv1 functions as an adaptor linking Fis1 with Dnm1 during division.

Yeast cells possess a close Mdv1 structural homolog, CaF4, which also interacts and forms a complex with Fis1 (19). In the absence of Mdv1, a Fis1·CaF4 complex targets Dnm1 to the membrane; however, the Fis1·CaF4 complex only supports minimal division activity. Consistent with this, in cells lacking CaF4, mitochondrial morphology is reticular and indistinguishable from that of wild type. In cells lacking both Mdv1 and CaF4, Dnm1 is not stably targeted to mitochondria, an identical phenotype to fis1Δ cells. Thus, Fis1 targets Dnm1 to mitochondria only through its ability to interact with either Mdv1 or CaF4. Analysis of Mdv1 indicating that it functions as a linker between Fis1 and Dnm1 supports this conclusion (17). The role of the CaF4·Fis1 complex in mitochondrial division is unknown at present, but it is clearly dispensable and most likely regulatory.

In this report, we further examine the role that Mdv1 plays in mitochondrial division subsequent to Dnm1 targeting. Our analysis suggests that Mdv1 interacts with Dnm1 only when it is self-assembled into GTP-dependent ring-like structures and not when it exists as extended filaments, which form in the absence of GTP. We also show that the

† This work was supported by National Institutes of Health Grants 1R01EY015924 and 5R01GM062942 (to J. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Movie 1.

§ Present address: Section of Molecular and Cellular Biology, University of California, Berkeley, CA.

‡ To whom correspondence should be addressed. Tel.: 530-754-9774; Fax: 530-752-7522; E-mail: jmnunnari@ucdavis.edu.

3 The abbreviations used are: NTE, N-terminal extension; C-C, coiled-coil; WD, WD repeat β-propeller; SD, synthetic media with dextrose; S-Raf, synthetic media with raffinose; S-Gal, synthetic media with galactose; mito, mitochondrial targeting sequence; EM, electron microscopy; GMP-PCP, guanylyl 5′-β,γ-methyleneidiphosphonate; GFP, green fluorescent protein; CFP, cyan fluorescent protein; PIPE, 1,4-piperazinediethanesulfonic acid; dsRed, red fluorescent protein from Discoma sp.
Mdv1 WD domain plays both regulatory and structural roles in division via its interaction with Dnm1 and indicates that membrane division requires a dynamic Mdv1/Dnm1 interaction. We postulate that the critical activity of Mdv1 in division is to promote the assembly of Dnm1 into a division competent spiral-like structure.

EXPERIMENTAL PROCEDURES

Media and Yeast Genetic Techniques—Yeast strains used in this study are listed in Table 1.

Standard genetic techniques and yeast media, including yeast extract peptone glycerol (3% glycerol), SD, S-Raf (2% raffinose), and S-Gal (2% galactose), were prepared as previously described (20). Yeast transformations were performed as previously described (21).

Plasmids—A plasmid containing GAL-regulated MDV1 was constructed by inserting the KpnI-SacI piece of p416GALL (American Type Culture Collection) containing the GALL promoter and MCS into pRS314 to create p414GALL. GFP-EcoRI sites. EcoRI and BamHI sites were introduced by PCR amplification from pDH3 obtained from the Yeast Resource Center, resulting in (23) by SmaI digest and replacing it with CFP, which was PCR amplified inserted creating a mitochondrially targeted dsRedT1. A mitochondrial site in the vector backbone with Vent polymerase, dsRed.T1 (24) was constructed with 20–30 nucleotides on either side of the altered nucleotide(s) and were purified by polyacrylamide gel as described (25).

Random mutagenesis was performed using mutagenic PCR conditions to amplify nucleotides 1–687 of MDV1 and the resultant PCR products were used as megaprimer for whole plasmid amplification (26). Whole plasmid PCR products were transformed into DH5α cells to create a DNA library. Mdv1 mutants were screened and identified by their inability to rescue the glycerol growth defect in mdv1Δ cells harboring the temperature-sensitive fusion mutant fzo1-1 (16). Potential mutants were tested for Mdv1 expression by Western analysis and sequenced before further analysis.

Dnm1 Purification and Electron Microscopy—Both procedures were performed as previously described (13).

GTPase Assay—A GTPase assay in which substrate was continuously regenerated and GTPase activity measured continuously was performed as previously described (13). Reactions were carried out in 25 mM HEPES, 25 mM Pipes, pH 7.1, 110 mM imidazole, pH 7.4, 7.5 mM KCl, 160 mM NaCl, 5 mM MgCl2, 1 mM phospho(enol)pyruvate, 20 units/ml pyruvate kinase/lactate dehydrogenase, 600 μM NADH, 5.5% Me2SO, and 250 μM GTP. In each reaction, the concentration of wild type Dnm1 was 0.048 mg/ml (0.55 μM). To assess the effect of mutants Dnm1 K41A, Dnm1 S42N, and Dnm1 T62D/F on wild type Dnm1 GTPase activity, increasing amounts of each mutant were added to wild type Dnm1. Increasing concentrations of each of the mutant proteins, contained in a 40-μl volume of freezing buffer (20 mM HEPES, 20 mM Pipes, pH 7, 400 mM imidazole, pH 7.4, 400 mM NaCl, 20% Me2SO), were preincubated with 10 μg of wild type Dnm1. GTPase assay reactions were started by the addition of reaction buffer to the preincubated proteins.

All GTPase assay reactions were performed in 200-μl volumes, of which 150 μl was placed into wells of a 96-well plate. Depletion of NADH over time was measured for a duration of 40 min using a SpectraMax 250 96-well plate reader (Molecular Devices). Spectrophotometric data were transferred to Excel and the measured depletion of NADH over time was converted to a protein activity.

GTP Binding Assay—GTP binding assays were performed in 25 mM HEPES, 25 mM Pipes, pH 7, 90 mM NaCl, 60 mM imidazole, 0.5 μM [α-32P]GTP, 1 mM dithiothreitol, 5 mM MgCl2. Approximately 2.3 μg of protein was added to the GTP binding buffer in a 20-μl reaction volume. After incubating at room temperature for 5 min, the reactions were placed directly onto parafilm that was resting on top of the ice. The reactions were irradiated in a Spectrolinker XL-1500 (Spectronics Corp.) at 254 nm for 300 s, ~10 cm from the UV source. After cross-linking, 15 μl of each cross-linked reaction was placed into tubes containing 5 μl of 4X sample buffer, boiled for 5 min, and resolved on a 12% SDS-PAGE gel. The gel was stained with Coomassie, destained, dried onto Whatman paper, and wrapped in plastic. A phosphoscreen was exposed to the dried gel and scanned using a Storm system (Amersham Biosciences).

Two-hybrid Analysis—Full-length MDV1, 1–2142 nucleotides, or the WD region, 903–2142 nucleotides, were PCR amplified, introducing EcoRI and BamHI restriction sites, and ligated into pGAD and pGBDU to create pGAD-MDV1, pGAD-WD, pGBDU-MDV1, and pGBDU-WD (27). pGAD-FIS1Tr and pGBDU-FIS1Tr were created by ligation of PCR-amplified FIS11–381 nucleotides with introduced BamHI and Sall restriction sites into pGAD and pGBDU. pGADDNMI

| Strains | Genotype | Source | Source |
|---------|-----------|--------|--------|
| W303 | ade2–1; leu2–3,11,15; try1–1; ura3–1; can1–100 Mata | R. Rothstein | This study |
| JNY862 | W303, except mdv1Δ::his5+, Mata | This study | This study |
| JNY859 | W303, except dnm1Δ::his5+, dnm1Δ::his5+, Mata | This study | This study |
| JNY905 | W303, except dnm1Δ::his5+, Mata | This study | This study |
| JNY999 | W303, except fis1::his5+, dnm1Δ::his5+, Mata | This study | This study |
| JNY159 | Pj69–4A | James et al. (27) | |

**TABLE 1**

**Yeast strains**

| Strains | Genotype | Source |
|---------|-----------|--------|
| W303 | ade2–1; leu2–3,11,15; try1–1; ura3–1; can1–100 Mata | R. Rothstein |
| JNY862 | W303, except mdv1Δ::his5+, Mata | This study |
| JNY859 | W303, except dnm1Δ::his5+, dnm1Δ::his5+, Mata | This study |
| JNY905 | W303, except dnm1Δ::his5+, Mata | This study |
| JNY999 | W303, except fis1::his5+, dnm1Δ::his5+, Mata | This study |
| JNY159 | Pj69–4A | James et al. (27) |
and pGBDU-DNM1 were created by removing DNM1 from pRU23BD-DNM1 (28) and ligating it into pGAD and pGBDU using BamHI and Sall sites. For two-hybrid analysis, plasmids constructed as described above for pGBDU and pGAD were co-transformed into the yeast strain PJ69-4A and tested for interactions as previously described (27).

**Cytological Analysis**—Mitochondrial morphology was visualized using mito-dsRed or mito-CFP, created as described above. Dnm1-GFP was visualized by transforming cells with pHS20 as previously described (16, 17). Mdv1 was visualized by transforming cells with p414GALL-GFP-MDV1. To analyze Mdv1 and Dnm1 together, cells were transformed with pHS20 and p416GALL-dsRed/dimer2-MDV1. Transformants were grown in SD, subcultured into S-Raf media, further subcultured into S-Gal to induce expression of dsRed-tagged Mdv1 and mito-CFP, or supplemented with 1% dextrose to induce expression of GFP-tagged Mdv1, and imaged after 12 h of logarithmic growth. Cells were concentrated and mounted in 1.5% low melt agarose. All samples were imaged using a DeltaVision microscope with either a 100× 1.4 NA objective or 60× 1.4 NA objective. Step size of 0.2 μm was used for fixed time point analyses. For time-lapse analysis a single 0.2-μm section was imaged. Quantification and localization of Mdv1 puncta was performed using Velocity software to analyze movies of single yeast cell sections of mvd1-Δ strains expressing GFP-Mdv1 and GFP-Mdv1N544R. Mdv1 and Dnm1 mutants were determined to complement the respective null strains by the recovery of net-like mitochondria to reticular structures upon expression of Mdv1 and Dnm1 mutant alleles. Dominant negative phenotypes were determined by the interference of Mdv1 and Dnm1 mutants with wild type proteins as observed by the formation of nets upon the expression of the mutant alleles in an otherwise wild type background.

**RESULTS**

**Mdv1 Is Present in the Division Complex during Division**—Previously it has been shown by fixed time point analysis that Dnm1 is found in punctate structures associated with mitochondria and at mitochondrial constriction sites in cells (3, 4, 11). More recently, time-lapse analysis has shown that Dnm1 puncta are associated at sites of mitochondrial division in vivo (12). We have also characterized the behavior of Dnm1 and mitochondria in wild type cells using time-lapse microscopy analysis of Dnm1-GFP and mito-dsRed. Consistent with published observations, our analysis revealed that Dnm1-GFP was present in punctate structures of two distinct classes: those associated with mitochondria (Fig. 1, arrow), and those present in the cytosol (Fig. 1, arrowhead). Also consistent with previous observations (12), we observed that the mitochondrial associated Dnm1 puncta are restricted in movement. In contrast, we observed that cytosolic Dnm1 puncta were more dynamic and relatively less abundant than the mitochondrial associated Dnm1 puncta (Fig. 1, arrow). Cytosolic puncta exhibited velocities greater than 1 μm/s and also changed trajectory with greater frequency. The differences in behavior of the cytosolic and mitochondrial populations of puncta likely reflect a regulation of Dnm1 activity by mitochondrial localized Fis1 and Mdv1.

It has been shown that Mdv1 functions as an adaptor, interacting with both Fis1 and Dnm1 at a late step in the division pathway. Cytological analysis of the Mdv1-Dnm1 complex has shown that Mdv1 co-localizes with mitochondrial Dnm1 puncta (16, 30), but it is not known when during the division reaction Mdv1 interacts with Dnm1. Using time-lapse microscopy of GFP-Mdv1 and mito-dsRed, we found that Mdv1 puncta spend the majority of the time on the mitochondria and, unlike Dnm1 puncta, are rarely found in the cytosol (Fig. 2A). To determine when Mdv1 assembles with Dnm1 and the relationship of co-labeled structures with sites of fission, we conducted triple label experiments of GFP-Dnm1, dsRed-Mdv1, and mito-CFP. We observed from the analysis of fission events in triple labeled cells that Dnm1 puncta are first targeted to mitochondria, and then Mdv1 accumulates at sites of assembled Dnm1 structures (Fig. 2, B and C). This association was maintained until mitochondrial fission was completed (Fig. 2, B and C). This observation indicates that a structure(s) containing both Mdv1 and Dnm1 is required for the completion of mitochondrial fission.
involved in nucleotide binding and hydrolysis. It has been shown by yeast two-hybrid assays that Dnm1 self-interacts through its GED and middle domains (28). Thus, in addition to the GTPase mutants, we also analyzed Dnm1G385D, a previously reported middle domain mutant, which blocks self-assembly in vivo and in vitro (13, 31). As expected and consistent with previous observations all four Dnm1 mutants block division as observed by the inability to rescue dnm1Δ. Additionally they are dominant negative as indicated by net formation in wild type cells (data not shown) (18, 31).

We characterized this bank of Dnm1 mutants for GTPase activity, self-assembly, and in vivo behavior (Table 2). Recently we have characterized the kinetic and structural properties of Dnm1 (13). Our data indicate that, like dynamin, Dnm1 self-assembly stimulates its own GTPase activity ~16-fold (wild type activity, 50 min⁻¹) and that in the presence of GTP or non-hydrolyzable GTP analogs, Dnm1 self-assembles into spiral-like structures (13). In contrast, in the absence of nucleotides or the presence of GDP, Dnm1 forms extended slightly curved filaments. In addition, we have shown that Dnm1G385D cannot self-assemble and, as a consequence, exhibits lower GTPase activity (3 min⁻¹) (13). Our studies have demonstrated that Dnm1G385D exists as a nucleotide-independent stable dimer, suggesting that dimeric Dnm1 is the building block for the formation of higher order Dnm1 structures (13). Using identical experimental conditions, we have characterized the activity of the Dnm1 GTPase domain mutants and observe that, in contrast to wild type, and as predicted, no GTPase activity can be detected (up to 2 mM GTP) for any of these GTPase mutants. Interestingly, although Dnm1K41A possessed no activity on its own, it uniquely stimulated wild type Dnm1 GTPase activity, suggesting that Dnm1K41A can promote the self-assembly of wild type Dnm1 into structures that are activated for GTP hydrolysis (Fig. 3A).

To determine assembly activity of these Dnm1 mutants more directly, we assessed them using negative stain electron microscopy (EM), two-hybrid analysis, and in vivo cytology. Interestingly, EM analysis showed that Dnm1S42N and Dnm1T62D/F³ formed extended slightly curved filaments, even in the presence of the non-hydrolyzable GTP analog, GMP-PCP, similar to those formed by wild type Dnm1 in the absence of nucleotides or in the presence of GDP (Fig. 3B). This observation suggests that these mutants are unable to form the GTP-dependent ring and spiral-like Dnm1 structures, likely as a consequence of a nucleotide-binding defect. In contrast, EM analysis revealed that in the presence of GTP or GMP-PCP, Dnm1K41A self-assembled into ring-like structures. Interestingly, the diameter of Dnm1K41A rings is significantly larger than wild type Dnm1 rings (161 ± 13 versus 109 ± 16 nm). Thus, although our observation suggests that Dnm1K41A can bind GMP-PCP, its bound conformation is altered (Fig. 3B).

![FIGURE 3. Dnm1 GTPase mutants abolish GTPase activity and alter the assembly, properties, and localization of Dnm1. A, analysis of wild type Dnm1 GTPase activity in the presence of GTPase mutants K41A, S42N, and T62F as indicated. Wild type activity standardized to 100% is 6 min⁻¹. B, electron microscopy analysis of negatively stained Dnm1 GTPase assembly mutants in the presence of 1 mM GMP-PCP. Scale bar = 100 nm. C, GTP UV cross-linking binding assay of wild type Dnm1 and GTPase mutants. GTP binding was monitored by UV cross-linking as described. Top and bottom gels are from the same experiment: top is an autoradiogram of [γ-32P]GTP-labeled proteins; bottom is a Coomassie-stained protein. Lane 1, uncross-linked wild type Dnm1; lanes 2–6, UV cross-linked proteins: lane 2, wild type Dnm1; lane 3, Dnm1K41A; lane 4, Dnm1S42N; lane 5, Dnm1 T62D; and lane 6, bovine serum albumin. D, Dnm1-GFP GTPase mutant localization. Dnm1 mutants were visualized using C-terminal GFP fusions and mitochondria were visualized using mito-dsRed in dnm1Δ cells. A single 0.2-μm section is shown. Images are magnified ∼500 times.

The fact that Dnm1K41A can bind GMP-PCP suggests that this mutant may be involved in nucleotide-independent self-assembly. To test this observation we performed a UV cross-linking GTP binding assay to directly determine the capability of Dnm1 GTPase mutants to bind to GTP. As suggested from the EM analysis we observed that Dnm1K41A binds to GTP, whereas Dnm1S42N and Dnm1T62F have little or no binding capability (Fig. 3C).

Two-hybrid analysis supported the EM findings, specifically demonstrating that homotypic interactions occur between Dnm1S42N, Dnm1T62D/F, and Dnm1K41A binding and activation domain fusions (Table 2). In contrast, however, no interactions were observed between mutants in the presence of GTPase activity.}

### Table 2

**Summary of Dnm1 and Dnm1 mutant activities**

| Mutant   | Self-assembly | Localization of puncta in vivo | Interaction with Mdv1 |
|----------|---------------|-------------------------------|----------------------|
|          | EM            | Two-hybrid                    | Cytology⁴           | Two-hybrid⁴           |
| Dnm1     | +             | +                             | Mitochondrial and cytosolic |
| K41A     | +             | +                             | Mitochondrial       |
| S42N     | +             | +                             | Cytosolic           |
| T62D/F   | +             | +                             | Cytosolic           |
| G385D    | –             | –                             | Unassembled         |

* As defined by Dnm1 puncta co-localization with Mdv1.
* No growth on selective medium; + growth on selective medium. Five colonies for each construct pair were tested.
* Ring-like structures.
* Filamentous structures.
* Unassembled as defined in vivo is observed by the lack of puncta but presence of diffuse cytosolic localization.

---

⁴ Dnm1T62D and Dnm1T62F are phenotypically identical; all presented data are of Dnm1T62F unless noted.
the Dnm1G385D binding and activation domain fusions, consistent with in vivo and in vitro data demonstrating that Dnm1G385D possesses a defect in self-assembly (Table 2) (13, 31). Taken together, these observations also suggest that positive interactions with Dnm1 in the two-hybrid assay are specific for the formation of higher order structures. Thus, two-hybrid analysis of Dnm1 GTPase mutants supports EM data, indicating that they are capable of self-interaction. However, EM analysis demonstrated that among these mutants, only Dnm1K41A self-assembles into GTP-dependent ring-like structures, which is consistent with the ability of Dnm1K41A to stimulate wild type Dnm1 GTPase activity (Fig. 3, A and B).

Cytological analysis of these mutants also confirmed observations obtained from EM and two-hybrid analyses and uniquely placed them in the context of the division pathway in vivo. In vivo analysis of GFP-tagged Dnm1 GTPase mutants showed that in all cases mutant Dnm1 forms punctate-like structures consistent with self-assembly. However, more detailed time-lapse analysis revealed that Dnm1T62D/F and Dnm1S42N containing structures do not interact with mitochondria, are localized to the cytosol, and are structurally aberrant/larger as compared with wild type (Fig. 3D and Table 2). Previous analysis of Dnm1S42N and Dnm1T62F have also shown aberrant structures, however, these were reported to be associated with mitochondria (18). This difference in observations is possibly because of parental strain differences (18). Regardless, our observations, coupled with EM analysis of the Dnm1T62D/F and Dnm1S42N mutants showing that they cannot form GTP-dependent spirals, suggest that the observed aberrant in vivo structures are dead end aggregates. In contrast, in vivo analysis of the behavior of Dnm1K41A indicated that Dnm1K41A containing structures were exclusively localized to mitochondria and similar in morphology to wild type Dnm1 puncta (Fig. 3D and Table 2). These observations, together with EM analysis showing that Dnm1K41A can assemble into GTP-dependent rings suggest that the Dnm1K41A structures represent productive intermediates.

We examined the ability of Mdv1 to interact with this set of characterized Dnm1 mutants using cytological and two-hybrid analyses. Strikingly, based on both types of analysis, the ability of Mdv1 to interact with Dnm1 mutants was correlated with the ability of these Dnm1 mutants to self-assemble into ring and spiral-like structures. Specifically, as previously published, Mdv1 does not co-localize or interact in the two-hybrid assay with Dnm1T62F or Dnm1S42N (Table 2) (18). Additionally, Mdv1 does not co-localize or interact in the two-hybrid assay with Dnm1T62D or Dnm1G385D (Table 2). In contrast, Mdv1 does co-localize with mitochondrial associated Dnm1 and Dnm1K41A structures in vivo and interacts with Dnm1 and Dnm1K41A by two-hybrid analysis (Table 2). Taken together, our analysis suggests that Mdv1 interacts specifically with assembled GTP-dependent Dnm1 ring structures, which is consistent with a role for Mdv1 regulating Dnm1 assembly during division.

Structure Function Analysis of Mdv1 Confirms That the NTE Interacts with Fis1 and Indicates That the WD Region Functions as a Regulatory Domain via its Interaction with Dnm1—To further understand the role of Mdv1 in division, we undertook a detailed structure-function analysis approach. We made a variety of mutations in both the NTE and WD regions of Mdv1, which, from deletional analysis, are known to mediate the interactions with Fis1 and Dnm1, respectively (17). Using random PCR mutagenesis, we identified the mutation L148P within the WD domain for clarity (Fig. 4). The Mdv1 WD mutants are localized uniformly to the mitochondria. The arrow indicates a mitochondrial net-like structure. Class III Mdv1L148P expressed in mdv1-Δ, Mdv1S444R puncta localized to mitochondria and cytosol. The arrow indicates cytosolic Mdv1 puncta. Cells were grown to log phase in 5-Flu and subcultured into 5-Gal 1% dextrose for 12 h before imaging (% indicates mutation). Average % puncta on mitochondria was calculated using time-lapse images of mdv1-Δ expressing GFP-Mdv1 or GFP-Mdv1S444R using Volocity software as described. Images are a single 0.2-μm section and magnified ~500 times.

**TABLE 3**

Mdv1L148P disrupts the Fis1/Mdv1 complex and blocks division

| Two-hybrid interactions* | Mitochondrial morphology |
|--------------------------|--------------------------|
| Mdv1        | Dnm1        | Fis1        | Nets in mdv1-Δ (n = 50) |
| Mdv1        | +          | +          | +                        | 2% |
| L148P       | +          | +          | -                        | 75% |

*No growth on selective medium; + growth on selective medium. Five colonies for each construct pair were tested.
Behaved as loss of function mutants: they failed to complement the mitochondrial morphological defects of *mdv1*-Δ cells, had dominant negative effects, as defined by net formation in wild type cells, and colocalized with the mitochondria in a uniform pattern, indicating that they have lost their ability to interact with Dnm1 (Table 4 and Fig. 4B, panel 2). This conclusion is supported by two-hybrid analysis, which demonstrates that class II mutants lose their ability to interact with Dnm1, but maintain their interaction with Fis1 and Mdv1 (Table 5). An additional mutant, Mdv1R634E, was identified by its partial loss of function. Mdv1R634E only partially complements *mdv1*-Δ and is slightly dominant negative in wild type (Table 4) and interestingly it co-localizes with the mitochondria yet still forms a few puncta (data not shown). This suggests that Mdv1R634E has a partial loss of interaction with Dnm1 although no alteration of the interaction was detected by two-hybrid analysis (data not shown). As shown in Fig. 4A, these class II mutants are predicted to be on top of blades two and five, indicating that these regions mediate, either directly or indirectly, Mdv1/Dnm1 interactions.

Our class II loss of function mutants support the notion that WD domains form simple scaffolds that mediate protein-protein interactions (29, 32, 33). However, the phenotype of our Mdv1 class III mutant, Mdv1N544R, is not consistent with this model. Mdv1N544R failed to complement the mitochondrial morphological defect of *mdv1*-Δ and when expressed in wild type cells displayed a dominant negative phenotype; it blocked division and caused small mitochondrial net-like structures to form like the class II mutants (Table 4). However, in contrast to the class II mutants, GFP-Mdv1N544R localized to Dnm1-dependent punctate structures based on cytological analysis (data not shown). In fact, time-lapse analysis of Mdv1N544R behavior indicated that a significant fraction of Mdv1N544R puncta were present in the cytosol, unlike wild type Mdv1 puncta (Fig. 4B, panel 4, arrows, and supplemental movies 1, A and B). Quantification of the behavior of Mdv1 and Mdv1N544R puncta over time revealed that 65 ± 15% of Mdv1N544R puncta are on the mitochondria compared with 93 ± 4% of wild type Mdv1, showing a significant mislocalization of Mdv1N544R puncta into the cytosol (5 cells were analyzed for each strain for a total of 200 time points, approximately 10 Mdv1 punctate structures were present/0.2-μm section). Additionally, analysis of Dnm1 in the presence of Mdv1N544R showed no alteration of the localization of Dnm1 to both cytosolic and mitochondrial pools (data not shown). Further analysis of Mdv1N544R using the two-hybrid approach confirmed that it interacts with Dnm1 and significantly, demonstrated that the interaction is more stable, as seen by robust growth of positive cells in 3 days, rather than the 1 week growing time to detect the wild type Mdv1/Dnm1 interaction (Table 5). Together two-hybrid analysis and cytological data are consistent with the interpretation that Mdv1N544R more stably interacts with Dnm1, causing the mislocalization of Mdv1 to the cytosol.

To determine whether this more stable interaction caused by Mdv1N544R was capable of overcoming the inability of Dnm1S42N and Dnm1T62D/F to interact with Mdv1, we used both cytological and two-hybrid approaches to analyze the interaction between Mdv1N544R and Dnm1 GTPase mutants. The presence of the N544R mutation was not able to compensate for the loss of interaction of Mdv1 with Dnm1S42N and Dnm1T62D/F, although Mdv1N544R was capable of maintaining the interaction with Dnm1K41A as expected (data not shown). These findings emphasize the functional importance of the Mdv1/Dnm1 interaction and further suggest that during division this interaction is dynamic and that Mdv1 acts in a regulatory manner.

**DISCUSSION**

Current experimental findings support a model for mitochondrial division where Dnm1 exists in self-assembled structures at steady state...
in cells. These Dnm1 structures are localized in a dynamic manner to the cytosol and on the mitochondrial outer membrane. From cytological analysis, Dnm1 structures appear to have an intrinsic ability to interact with the outer mitochondrial membrane, perhaps via lipids, but are only stably retained on mitochondria through interactions with either of the adaptors, Mdv1 or Cafl, which are tethered to the mitochondrial membrane via Fis1. Data presented in this article shows that after Dnm1 is targeted to mitochondria, Mdv1 then accumulates at a subset of Fis1/Mdv1-Dnm1-containing structures to promote mitochondrial fission via Dnm1/Mdv1 WD domain interactions. Our experiments also provide insight into the nature of the post-targeting Mdv1-dependent fission via Dnm1. Mdv1 interacts efficiently with Dnm1 only when it is assembled into GTP-bound rings and spirals. Structural and biochemical characterization of Dnm1 indicates that GTP-dependent Dnm1 spirals possess diameters equal to those of mitochondrial constriction sites in vivo, suggesting the Dnm1 self-assembly functions to drive the membrane constriction event associated with mitochondrial division (13). Thus, the findings presented here raise the interesting possibility that Mdv1 functions in fission by stabilizing or promoting the formation of Dnm1 into spiral-like structures. Mdv1 may accomplish this by stabilizing the GTP bound form of Dnm1 or by acting as a nucleator, structurally constraining Dnm1 to specifically form rings and spirals on the surface of mitochondria. We found that a mutation in the Mdv1 WD domain that stabilizes the Mdv1/Dnm1 interaction blocks mitochondrial division. Thus, our data also indicate that the Mdv1/Dnm1 interaction within division complexes must be dynamic, perhaps reflecting the importance of Dnm1 assembly dynamics in division.

Acknowledgments—We thank David Wilson for advice on Mdv1 WD mutations. We also thank members of the Nunnari lab for discussion and review of the manuscript.

REFERENCES
1. Nunnari, J., Marshall, W., Straight, A., Murray, A., Sedat, J. W., and Walter, P. (1997) Mol. Biol. Cell 8, 1233–1242
2. Hermann, G. J., Thatcher, J. W., Mills, I. P., Hales, K. G., Fuller, M. T., Nunnari, J., and Shaw, J. M. (1998) J. Cell Biol. 143, 359–374
3. Bleazar, W., McCaffery, J. M., King, E. J., Bale, S., Mozyd, A., Tieu, Q., Nunnari, J., and Shaw, J. M. (1999) Nat. Cell Biol. 1, 298–304
4. Sesaki, H., and Jensen, R. E. (1999) J. Cell Biol. 147, 699–706
5. Shaw, J. M., and Nunnari, J. (2002) Trends Cell Biol. 12, 178–184
6. Osteryoung, K. W., and Nunnari, J. (2003) Science 302, 1698–1704
7. Meuser, S. L., and Nunnari, J. (2005) Curr. Opin. Cell Biol. 17, 389–394
8. Praefcke, G. J., and McMahon, H. T. (2004) Nat. Rev. Mol. Cell. Biol. 5, 133–147
9. Chen, H., and Chan, D. C. (2004) Curr. Top. Dev. Biol. 59, 119–144
10. Youle, R. J. (2005) Dev. Cell 8, 298–299
11. Otsuga, D., Keegan, B. R., Britsch, E., Thantcher, J. W., Hermann, G. J., Bleazar, W., and Shaw, J. (1998) J. Cell Biol. 143, 333–349
12. Legesse-Miller, A., Massol, R. H., and Kirchhausen, T. (2003) Mol. Biol. Cell 14, 1953–1963
13. Gierman, E., Perkins, E. M., Marino, M., Mears, J. A., McCaffery, J. M., Hinshaw, J. E., and Nunnari, J. (2005) J. Cell Biol. 170, 1021–1027
14. Suzuki, M., Neutzner, A., Tjandra, N., and Youle, R. J. (2005) J. Biol. Chem. 280, 21444–21452
15. Yoon, Y., Krueger, E. W., Oswald, B. J., and McNiven, M. A. (2003) Mol. Cell. Biol. 23, 5409–5420
16. Tieu, Q., and Nunnari, J. (2000) J. Cell Biol. 151, 353–365
17. Tieu, Q., Okreglak, V., Naylor, K., and Nunnari, J. (2002) J. Cell Biol. 158, 445–452
18. Cerveny, K. L., and Jensen, R. E. (2003) Mol. Biol. Cell 14, 4126–4139
19. Griffin, E. E., Graumann, J., and Chan, D. C. (2005) J. Cell Biol. 170, 237–248
20. Guthrie, C., and Fink, G. (1991) Methods Enzymol. 194, 1–933
21. Gietz, R. D., and Schiestl, R. H. (1994) Methods Mol. Cell. Biol. 5, 255–269
22. Campbell, R., Tour, O., Palmer, A., Steinbach, P., Baird, G., Zacharius, D., and Tsien, R. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7877–7882
23. Westermann, B., and Neupert, W. (2000) Yeast 16, 1421–1427
24. Bevis, B. J., and Glick, B. S. (2002) Nat. Biotechnol. 20, 83–87
25. Maniatis, T., Fritsch, E., and Sambrook, J. (1983) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Miyazaki, K., and Takenouchi, M. (2002) BioTechniques 33, 1033–1034, 1036–1038
27. James, P., Halladay, J., and Craig, E. E. (1996) Genetcs 144, 1425–1436
28. Fukushima, N. H., Britsch, E., Keegan, B. R., Bleazar, W., and Shaw, J. M. (2001) Mol. Biol. Cell 12, 2756–2766
29. Neer, E. J., Schmidt, C. J., Nambudripad, R., and Smith, T. F. (1994) Nature 371, 297–300
30. Cerveny, K. L., McCaffery, J. M., and Jensen, R. E. (2001) Mol. Biol. Cell 12, 309–321
31. Jensen, R. E., Aiken-Hobbs, A. E., Cerveny, K. L., and Sesaki, H. (2000) Microsc. Res. Tech. 51, 573–583
32. Komachi, K., and Johnson, A. D. (1997) Mol. Cell. Biol. 17, 6023–6028
33. Sprague, E. R., Redd, M. J., Johnson, A. D., and Wolfberger, C. (2000) EMBO J. 19, 3016–3027