The WD repeat protein, Mdv1p, functions as a molecular adaptor by interacting with Dnm1p and Fis1p during mitochondrial fission

Quinton Tieu, Voytek Okreglak, Kari Naylor, and Jodi Nunnari

Section of Molecular and Cellular Biology, University of California, Davis, Davis, CA 95616

Yeast mitochondrial fission is a multistep process during which the dynamin-related GTPase, Dnm1p, assembles into punctate structures that associate with the outer mitochondrial membrane and mediate mitochondrial division. Steps in the Dnm1p-dependent process of fission are regulated by the actions of the WD repeat protein, Mdv1p, and the mitochondrial outer membrane protein, Fis1p. Our previous studies suggested a model where Mdv1p functions to regulate fission at a post-Dnm1p assembly step and Fis1p functions at two distinct steps, at an early point, to regulate Dnm1p assembly, and later, together with Mdv1p, to facilitate Dnm1p-dependent mitochondrial fission. To test this model, we have examined the physical and functional relationship between Mdv1p and Fis1p and present genetic, biochemical, and two-hybrid data indicating that a Fis1p–Mdv1p complex is required to regulate mitochondrial fission. To further define the role of Mdv1p in fission, we examined the structural features of Mdv1p required for its interactions with Dnm1p and Fis1p. Data from two-hybrid analyses and GFP-tagged domains of Mdv1p indicate that it contains two functionally distinct domains that enable it to function as a molecular adaptor to regulate sequential interactions between Dnm1p and Fis1p and catalyze a rate-limiting step in mitochondrial fission.

Introduction

In the budding yeast Saccharomyces cerevisiae, mitochondria in logarithmically growing cells form a branched and continuous tubular structure distributed at the cell cortex. The formation and maintenance of this branched reticulum requires a balanced frequency of fusion and fission events (Nunnari et al., 1997; Shaw and Nunnari, 2002). Mitochondrial fusion is controlled by the evolutionarily conserved mitochondrial outer membrane GTPase, Fzo1p (Hales and Fuller, 1997; Hermann et al., 1998; Rapaport et al., 1998; Santel and Fuller, 2001). Mitochondrial fission is a multistep process regulated by the conserved dynamin-related GTPase, Dnm1p, which localizes to the outer mitochondrial membrane and assembles into punctate structures associated with sites of mitochondrial constriction (Otsuga et al., 1998; Smirnova et al., 1998; Bleazard et al., 1999; Labrousse et al., 1999). Members of the dynamin-related GTPase family are required during cellular membrane remodeling events, such as the formation and scission of clathrin-coated vesicles from the plasma membrane during endocytosis. Mutations in DNM1 cause mitochondria to form net-like structures of interconnected mitochondrial tubules that are the result of unopposed mitochondrial fusion (Bleazard et al., 1999; Sesaki and Jensen, 1999).

We and others have identified and characterized two additional proteins, Mdv1p and Fis1p, that act together with Dnm1p to facilitate fission (Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001). Mdv1p is a predicted soluble protein containing at least three regions: an NH$_2$-terminal region of indeterminate structure, a predicted coiled-coil (C-C)* central domain, and a COOH-terminal seven-WD repeat domain. Cytology, genetics, and two-hybrid analyses indicate that Mdv1p interacts with Dnm1p in punctate structures to mediate mitochondrial fission (Fekkes et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001). Although Mdv1p is not required for the assembly of Dnm1p into punctate structures, Dnm1p-containing structures lacking Mdv1p are not able to complete division.

*Abbreviations used in this paper: AD, activating domain; BD, binding domain; C-C, coiled coil; DSP, dithiobis(succinimidylpropionate); MBP, maltose-binding protein; NTE, NH$_2$-terminal extension; SB, sorbitol buffer.
Results and discussion

Dnm1p puncta assemble in fis1-L80P cells, but fission is impaired

Our previous studies suggested a model where Fis1p functions at two distinct steps in the mitochondrial fission pathway (Tieu and Nunnari, 2000). Specifically, we proposed that Fis1p functions early in the fission pathway to regulate the assembly of Dnm1p into punctate structures and target Dnm1p to mitochondrial membranes (Mozdy et al., 2000; Tieu and Nunnari, 2000). A second function of Fis1p was inferred from the observation that in the absence of Dnm1p, Mdv1p remains associated with mitochondria in a Fis1p-dependent manner (Tieu and Nunnari, 2000). This observation suggests that Fis1p also functions together with Mdv1p later in the fission pathway. Based on these results, we proposed a model of mitochondrial fission where Dnm1p puncta associate and assemble on mitochondria in a Fis1p-dependent manner. Within these structures, Mdv1p interacts with Fis1p or a Fis1p-dependent component, resulting in the catalysis of mitochondrial division (Tieu and Nunnari, 2000).

We have tested this model for mitochondrial fission by examining the functional and physical relationship between Mdv1p and Fis1p and by determining the structural features of Mdv1p required for its activities. Here we present genetic, cytological, and biochemical evidence that Fis1p interacts with Mdv1p to regulate a rate-limiting, Dnm1p-dependent event during mitochondrial fission and report that Mdv1p performs the role of a molecular adaptor by interacting with both Dnm1p and Fis1p.

Table 1: Quantification of mitochondrial morphology and Dnm1p-associated puncta

| Strain       | No. cells scored | Branched reticular | Nets | Dnm1p localization |
|--------------|------------------|--------------------|------|--------------------|
|              |                  | %                  | %    | No. cells scored   | No. Dnm1-GFPp puncta/cell |
| WT           | 107              | 97                 | 3    | 40                 | 26 ± 7                    |
| fis1-Δ       | 103              | 1                  | 99   | 30                 | 6 ± 3                     |
| mdv1-Δ       | 99               | 0                  | 100  | 30                 | 23 ± 6                    |
| fis1-L80P    | 92               | 16                 | 84   | 40                 | 21 ± 4                    |

Figure 1. The fis1-L80P mutation disrupts mitochondrial fission, but Dnm1p-containing puncta have wild-type characteristics.

Mito-GFP was used to visualize mitochondrial morphology. Dnm1p was visualized using a Dnm1p–GFP fusion protein. (A) Mitochondrial morphology of representative wild-type, gene deletion, and fis1-L80P cells. (B) Dnm1p–GFPp localization pattern in fis1-L80P cells. Bars, 2 µm.
These observations suggest that the fission defect observed in fis1-L80P cells is not the result of a defect in the assembly of Dnm1p-containing puncta, but rather occurs as a result of a defect at a later step in the division pathway. Significantly, the phenotypic characteristics of fis1-L80P cells are similar to those observed in mdv1-A cells (Fig. 1, A and B; Tieu and Nunnari, 2000). Together with our previous observation that in dnm1-A cells, Mdv1p remains associated with mitochondria in a Fis1p-dependent manner, these results support our hypothesis that Fis1p functions with Mdv1p to regulate fission at a post-Dnm1p assembly step.

Overexpression of Mdv1p suppresses the fission defect in fis1-L80P cells

We asked whether overexpression of Mdv1p could suppress the observed fission defect in fis1-L80P cells to further test our hypothesis that a Mdv1p-dependent, post-Dnm1p assembly step is specifically blocked in fis1-L80P cells. To overexpress Mdv1p in cells, we used the GAL1 promoter. When cells containing the GAL1-MDV1 plasmid were grown under inducing conditions, using the carbon source galactose, Mdv1p was overexpressed ~20-fold as assessed by SDS-PAGE and Western blotting with anti-Mdv1p antibodies (Fig. 2 A).

Examination of mitochondrial morphology in wild-type cells with mito-GFP indicated that overexpression of Mdv1p had no effect on mitochondrial structure (Fig. 2, B and C). This observation is in contrast to what has been observed in studies of Dnm1p, where overexpression results in mitochondrial fragmentation and indicates that, unlike Dnm1p, the concentration of Mdv1p in wild-type cells is not rate limiting for fission (Sesaki and Jensen, 1999; Fukushima et al., 2001). As expected, net-like mitochondrial structures observed in mdv1-A cells were transformed into tubular branched structures upon Mdv1p overexpression, indicating that fission is restored and that Mdv1p expressed from the GAL1 promoter is functional (Tieu and Nunnari, 2000; Fig. 2, B and C). Significantly, mainly branched reticular mitochondrial structures, characteristic of wild-type cells, were observed when Mdv1p was overexpressed in fis1-L80P, indicating that overexpression of Mdv1p suppresses the fission defect in fis1-L80P cells (Fig. 2, B and C). Mitochondrial nets, however, persisted in fis1-A cells when Mdv1p was overexpressed, demonstrating that suppression of the fission defect in fis1-L80P cells is allele specific (Fig. 2, B and C). Taken together, these observations are consistent with our hypothesis that a Mdv1p-dependent post-Dnm1p assembly step is specifically blocked in fis1-L80P and suggest that Mdv1p interacts with Fis1p during fission.

Mdv1p and Fis1p are in a complex

We previously used the two-hybrid assay to demonstrate that Dnm1p and Mdv1p interact (Tieu and Nunnari, 2000). In this study, we also used the two-hybrid assay to determine whether Mdv1p and Fis1p interact. Interactions between activating domain (AD) fusion proteins and binding domain (BD) fusion proteins were assessed by monitoring the expression of the stringent GAL2-ADE2 reporter gene. We tested both the full-length AD--FIS1 protein fusion and a construct lacking the transmembrane domain, AD--FIS1-A128--155, with BD--MDV1 to assess protein–protein interactions. Cells expressing a combination of AD--FIS1 and BD--MDV1 constructs displayed growth on media lacking adenine, indicating that Fis1p and Mdv1p specifically interact (Fig. 3 A; unpublished data). Cells harboring both the AD--FIS1-A128--155 and BD--MDV1 plasmids displayed more robust growth on media lacking adenine. Thus, not surprisingly, the Fis1p transmembrane domain interfered with the Fis1p--Mdv1p interaction in the assay.

To test whether a Mdv1p--Fis1p interaction occurs within the context of the mitochondrial membrane, we performed
immunoprecipitations with anti-Mdv1p from isolated detergent-solubilized mitochondria and whole cell extracts. We were unable to detect a Mdv1p–Fis1p complex in cell extracts, suggesting that a Mdv1p–Fis1p interaction might be labile in vitro. To overcome the possible instability associated with the Mdv1p–Fis1p interaction, proteins were cross-linked in vivo with the bifunctional, reversible cross-linker dithiobis(succinimidylpropionate) (DSP), and extracted under denaturing conditions before immunoprecipitation with antibodies. After immunoprecipitation, cross-links were reversed with a reducing agent and precipitates were analyzed by SDS-PAGE and Western blotting with anti-Mdv1p and Fis1p antibodies.

Western blot analysis of fractions from the anti-Mdv1p immunoprecipitation revealed that a significant fraction of Mdv1p from wild-type cells was present in the precipitate (Fig. 3 B, lanes 1–3). Significantly, we observed that a fraction of Fis1p from cross-linked extracts was reproducibly coimmunoprecipitated with anti-Mdv1p antibodies (Fig. 3 B, lanes 1–3). As a control for specificity, anti-Mdv1p antibodies were used to perform immunoprecipitations from DSP–cross-linked extracts from wild-type and mdv1−/H9004 cells were immunoprecipitated with anti-Mdv1p antibodies and fractions were analyzed by SDS-PAGE and Western blotting as described in the Materials and methods.

Figure 3. Fis1p interacts with Mdv1p by two-hybrid and coimmunoprecipitation analyses. (A) Two-hybrid analysis of MDV1 and FIS1. Interaction between AD fis1 Δ128–155 and BD MDV1 two-hybrid vectors was assessed by growth of indicated transformants on SD media as described. (B) Immunoprecipitation with anti-Mdv1p antibodies from DSP–cross-linked cells. In vivo, DSP–cross-linked extracts from wild-type and mdv1−/H9004 cells were immunoprecipitated with anti-Mdv1p antibodies and fractions were analyzed by SDS-PAGE and Western blotting as described in the Materials and methods.

The Mdv1p–Fis1p interaction is abolished in fis1-L80P cells
Taken together, our genetic, biochemical, and two-hybrid analyses suggest that Fis1p interacts with Mdv1p to regulate a rate-limiting, post-Dnm1p assembly step in the fission pathway. In this context, these observations further suggest that an alteration in the Fis1p–Mdv1p interaction in fis1-L80P cells is specifically responsible for the observed defect in mitochondrial fission. To test this idea, we biochemically analyzed the Fis1p–Mdv1p interaction in fis1-L80P cells.

We first examined the stability and intracellular localization of Fis1p and Mdv1p in fis1-L80P cells. Wild-type and fis1-L80P cell extracts were fractionated by differential centrifugation, and analyzed by SDS-PAGE and Western blotting. Consistent with a mitochondrial localization, in extracts from both wild-type and fis1-L80P cells, the majority of Mdv1p and Fis1p cofractionated with porin, the mitochondrial marker, in the mitochondrial-enriched pellet fraction (Fig. 4 A, compare lanes 1–3 with 4–6). In addition, levels of Mdv1p and Fis1p were similar in fis1-L80P and wild-type cell extracts (Fig. 4 A, compare lanes 1–3 with 4–6). These results indicate that both Fis1p and Mdv1p are expressed stably and localized correctly to mitochondria in fis1-L80P cells. Thus, the fission defect ob-

Figure 4. The Fis1p–Mdv1p interaction is disrupted in fis1-L80P cells and restored upon overexpression of Mdv1p. (A) Fractionation of cell extracts from wild-type and fis1-L80P cells by differential centrifugation. Fractions were analyzed by SDS-PAGE and Western blotting as described. (B) In vivo, DSP–cross-linked extracts from cells harboring either pGAL1 or pGAL1-MDV1, grown in SGal media, were immunoprecipitated with anti-Mdv1p antibodies and analyzed by SDS-PAGE and Western blotting.
served in fis1-L80P cells is not simply the result of Fis1p and/or Mdv1p instability.

To test whether the Mdv1p–Fis1p interaction is altered in fis1-L80P cells, we determined whether Fis1p coimmunoprecipitated with Mdv1p under conditions where a complex was detected in wild-type cells (Fig. 3). Interestingly, when Mdv1p was immunoprecipitated from DSP–cross-linked extract from fis1-L80P cells with anti-Mdv1p antibodies, we failed to detect Fis1p in the precipitates, in contrast to wild-type cells (Fig. 4 B, pGAL1). These results suggest that a complex containing Mdv1p and Fis1-L80Pp fails to form, or that interactions within the complex are weakened and thus harder to detect. Consistent with the latter possibility is our observation that overexpression of Mdv1p in fis1-L80P cells suppresses the mitochondrial fission defect. Thus, we tested whether increasing the amount of Mdv1p in the fis1-L80P cells could, by mass action, restore the Mdv1p–Fis1p interaction observed by coimmunoprecipitation with anti-Mdv1p antibodies.

Wild-type, mde1-Δ, and fis1-L80Pp cells harboring pGAL1-MDV1 were grown in galactose to induce overexpression of Mdv1p, DSP cross-linked, and immunoprecipitated with anti-Mdv1p antibodies. Western blot analysis of fractions from wild-type cells indicated that Fis1p coimmunoprecipitated with Mdv1p either with or without overexpression of Mdv1p (Fig. 4 B, pGAL1 and pGAL-MDV1, lanes 1 and 4). Interestingly, overexpression of Mdv1p did not increase the fraction of Fis1p that coimmunoprecipitated with Mdv1p, suggesting that Mdv1p is not limiting for this interaction. As expected, when Mdv1p is overexpressed in mde1-Δ cells, Fis1p can be observed in the immunoprecipitates, in contrast to immunoprecipitates from mde1-Δ cells harboring the pGAL1 vector without the MDV1 gene (Fig. 4 B). Significantly, overexpression of Mdv1p in fis1-L80P cells restored the Mdv1p–Fis1p interaction as detected by coimmunoprecipitation (Fig. 4 B, pGAL1 and pGAL-MDV1, lanes 1 and 4). These observations indicate that the Mdv1p–Fis1p interaction is defective in fis1-L80P cells and that overexpression of Mdv1p can restore this interaction, as detected by coimmunoprecipitation. These data suggest that the mitochondrial fission defect observed in fis1-L80P cells is the result of a defective Fis1p–Mdv1p interaction, suggesting a role for this interaction at a late post-Dnm1p assembly step in mitochondrial fission.

Mdv1p functions as a molecular adaptor in fission

Data presented in this manuscript demonstrate that Mdv1p interacts with Fis1p during fission to catalyze a rate-limiting step. We had previously shown that Mdv1p also interacts with Dnm1p in punctate structures within cells in a Fis1p-independent manner (Tieu and Nunnari, 2000). Thus, to gain further insight into the molecular mechanism of mitochondrial fission, we examined the regions of Mdv1p responsible for its interactions with Fis1p and Dnm1p.

Mdv1p contains at least three distinct regions: a novel NH₂-terminal extension region (NTE), a middle region predicted to form a C-C structure, and a COOH-terminal region that contains seven WD repeats predicted to form a seven-bladed propeller structure (WD) (Fig. 5 A; Tieu and Nunnari, 2000). To analyze the structural features of Mdv1p required for its interactions with Dnm1p and Fis1p, we constructed GAL1-regulated GFP fusions to each of these putative domains and examined their localization patterns after expression in wild-type, mde1-Δ, fis1-Δ, dnm1-Δ, and fis1-Δ dnm1-Δ cells. We also examined mitochondrial morphology, particularly in wild-type and mde1-Δ cells, to determine whether expression of Mdv1p domains produced dominant negative phenotypes or could complement the loss of MDV1 function, respectively.

The localization of GFP–tagged Mdv1p domains and mitochondrial morphology in representative cells are presented and summarized in schematic form in Fig. 5 B. As expected, GFP–tagged Mdv1p was localized to punctate structures primarily associated with mitochondria in wild-type, mde1-Δ, and fis1-Δ cells, but not in dnm1-Δ, consistent with our previous observations showing that GFP–Mdv1p interacts and colocalizes with Dnm1p in these structures in a Fis1p-independent manner (Fig. 5 B, panels 1–4). In addition, in mde1-Δ cells, mitochondrial tubular structures characteristic of wild-type cells were observed, indicating that, as previously published, GFP–Mdv1p is functional (Fig. 5 B, panel 2; Tieu and Nunnari, 2000). Also as previously shown, GFP–Mdv1p was observed uniformly localized to mitochondria in dnm1-Δ cells (Fig. 5 B, panel 4; Tieu and Nunnari, 2000). The functional and biochemical data presented in this study indicate that Mdv1p’s mitochondrial localization pattern in dnm1-Δ cells reflects a Dnm1p-independent interaction between Mdv1p and Fis1p. Consistent with this interpretation, in cells lacking both Dnm1p and Fis1p, Mdv1p was observed in a diffuse pattern, associated with the cytosolic fraction (unpublished data; Tieu and Nunnari, 2000).

Mitochondrial net-like structures were observed in mde1-Δ strains expressing each Mdv1p region alone, indicating that no single region was sufficient for wild-type levels of mitochondrial fission (Fig. 5 B, panels 2, 6, 10, and 14; 100%, n = 50 in all strains). However, in wild-type, mde1-Δ, and fis1-Δ cells expressing a GFP-tagged version of WD region of Mdv1p, GFP fluorescence was observed in punctate structures, primarily associated with mitochondria (Fig. 5 B, panels 9–12). The localization of GFP–WD to punctate structures was not observed in dnm1-Δ cells, indicating that Dnm1p is required for their formation (Fig. 5 B, panel 12). In addition, punctate structures labeled by GFP–WD also contained Dnm1p, as assessed by colocalization of Dnm1–dsRed in wild-type cells (Fig. 5 C). Taken together, these observations suggest that the WD region is sufficient to interact with Dnm1p.

GFP–NTE labeled both mitochondria and punctate structures in wild-type cells (Fig. 5 B, panels 5–8). Unlike GFP–WD, however, the punctate structures labeled by GFP–NTE probably do not reflect an interaction of NTE with Dnm1p and may be the result of GFP–NTE self-aggregation because they also were observed in dnm1-Δ and dnm1-Δ fis1-Δ cells (Fig. 5 B, panel 12; unpublished data). In addition, GFP–NTE-labeled punctate structures were observed localized at the cell cortex, not associated with mitochondria. The dispersive mitochondrial labeling pattern observed for GFP–NTE in wild-type cells was not observed in fis1-Δ, suggesting that the NTE interacts specifically with Fis1p in cells (Fig. 5 B,
Consistent with this interpretation, GFP–NTE also was observed to be associated with mitochondria in \( \text{dnm}1-\Delta \) cells (Fig. 5 B, panel 8). In contrast, GFP–WD was observed in a diffuse, cytosolic pattern in the majority of \( \text{dnm}1-\Delta \) cells (Fig. 5 B, panel 12). Thus, the cytological analysis of \( \text{Mdv1p} \) domains suggests that the WD and NTE regions are each sufficient to interact with \( \text{Dnm1p} \) and \( \text{Fis1p} \), respectively.

Interestingly, in all cell types examined, the GFP-tagged C-C region of \( \text{Mdv1p} \) was observed in a diffuse pattern, consistent with a cytosolic localization, suggesting that this region is not involved in mediating interactions with either \( \text{Dnm1p} \) or \( \text{Fis1p} \) (Fig. 5 B, panels 9–16). Interestingly, in contrast to the NTE region, expression of GFP-tagged C-C and WD in wild-type cells caused mitochondrial net-like structures to form (Fig. 5 B, panels 9 and 13; 80% net-like structures, \( n = 58 \), and 19% net-like structures, \( n = 59 \), respectively), indicating that these regions interfere with mitochondrial fission in a dominant negative manner.

To independently test our cytological observations, we also examined the structural features of \( \text{Mdv1p} \) required for its interactions with \( \text{Dnm1p} \) and \( \text{Fis1p} \) using the two-hybrid assay. We tested both the full-length \( \text{Mdv1p} \) and \( \text{Dnm1p} \) protein fusions and \( \text{Fis1p} \) with regions of \( \text{Mdv1p} \) to determine protein–protein interactions. An interaction was indicated by cells growing on media lacking adenine (indicated by + in Table II).

As previously shown, full-length \( \text{Mdv1p} \) was observed to interact with both \( \text{Fis1p} \) and \( \text{Dnm1p} \) in this assay. However, the expression of GFP-tagged C-C and WD regions in wild-type cells caused mitochondrial net-like structures to form (Fig. 5 B, panels 9 and 13; 80% net-like structures, \( n = 58 \), and 19% net-like structures, \( n = 59 \), respectively), indicating that these regions interfere with mitochondrial fission in a dominant negative manner.

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**Figure 5.** \( \text{Mdv1p} \) functions as a molecular adaptor during mitochondrial fission. (A) Predicted structural domains of \( \text{Mdv1p} \) (NTE, C-C, and WD [seven-WD repeat domain]). (B) Dual localization of mitochondria labeled with MitoTracker (in red, left) and GFP-tagged \( \text{Mdv1p} \) regions (in green, middle) in cells. Overlay (right image) was obtained by merging the signals from MitoTracker and GFP-tagged \( \text{Mdv1p} \) regions. Indicated strains were grown in SRaf, subcultured in SGal, supplemented with 1% dextrose to partially repress galactose-induced overexpression of \( \text{Mdv1p} \) domains for optimal visualization, and imaged by deconvolution microscopy. Dextrose was omitted for the cytological analysis of \( \text{Mdv1p} \) regions in \( \text{fis}1-\Delta \) cells because higher expression levels were required to detect interactions with \( \text{Dnm1p} \)-containing structures. In cells, schematically represented, GFP-tagged \( \text{Mdv1p} \) regions and mitochondria are depicted in green and red, respectively. (C) GFP–WD and \( \text{Dnm1} \)–dsRed colocalize in punctate structures in wild-type cells. Cells were grown and analyzed as described in B. Bars, 2 \( \mu \text{m} \).
say (Fig. 3 A; Tieu and Nunnari, 2000). In addition, we observed that full-length Mdv1p interacted with full-length Mdv1p in this assay, suggesting that Mdv1p is able to oligomerize. The WD region was observed to interact with Dnm1p exclusively, consistent with our cytological observations using GFP-tagged WD. The NTE region was expressed as a BD or AD fusion only when the C-C region was included (NTE/C-C; unpublished data) and specifically displayed an interaction with Fis1Δ128–155p, consistent with our cytological analysis of the NTE region alone.

We also observed an interaction of NTE/C-C with full-length Mdv1p by two-hybrid analysis (Table II). Indeed, two-hybrid analysis indicates that the C-C region alone was sufficient to specifically interact with Mdv1p, suggesting that Mdv1p forms a higher order homo-oligomeric structure via the predicted C-C region (Table II). Analysis of the primary structure of Mdv1p by the MultiCoil algorithm indicates that Mdv1p has a high probability of forming a parallel dimeric C-C structure (Wolf et al., 1997). It is possible, therefore, that the C-C region exerts its dominant negative effect on mitochondrial fission in wild-type cells by preventing the formation of an Mdv1p dimer (Fig. 5 B, panels 13–16). The localization of the GFP-tagged C-C region to the cytosol in cells further suggests that full-length Mdv1p interacts with both Dnm1p and Fis1p in a homodimeric or higher order complex.

A molecular model for mitochondrial fission

Data presented in this paper support a model, similar to the one recently proposed where Fis1p plays two distinct and separable roles during mitochondrial fission (Shaw and Nunnari, 2002). Early in the fission pathway, Fis1p targets Dnm1p to mitochondrial membranes and regulates its assembly probably via a direct interaction. Dimeric Mdv1p coassembles with Dnm1p in Dnm1p-containing punctate structures where it functions, specifically at a rate-limiting step where fission is triggered. Here we have provided genetic and biochemical evidence that Fis1p also functions at this rate-limiting step, by interacting with Mdv1p. Our observations also indicate that Mdv1p plays the role of a molecular adaptor, whose two functional domains are separated by a C-C region. Our analysis supports a model where the WD domain of Mdv1p mediates an interaction with assembled Dnm1p. Given that expression of the WD region interferes with mitochondrial fission in a dominant negative manner, we infer that the localization of Mdv1p to assembled Dnm1p structures is important for its ability to stimulate fission. Our data also support a role for the NTE region of Mdv1p as a molecular switch that interacts in a regulated manner with Fis1p, triggering conformational changes within an assembled Dnm1p structure that bring about the division of mitochondrial membranes. Elucidation of the stoichiometry of Dnm1p, Fis1p, Mdv1p, and any other factors within the complex(es) we have identified will help answer the question of how these components function in generating the force required to coordinately divide the outer and inner mitochondrial membranes.

Materials and methods

Media and yeast genetic techniques

Yeast strains used in this study are listed in Table III. Standard genetic techniques and yeast media, including YPG (3% glycerol), YPGal (2% galactose), SD, SRaf (2% raffinose), and SGal (2% galactose), were prepared as previously described (Guthrie and Fink, 1991). Yeast transformations were performed as previously described (Gietz and Schiestl, 1991).

Strains, plasmid construction, and yeast two-hybrid analysis

The fis1-L80P allele was isolated as an extragenic suppressor of fzo1-1 cells as described and identified by analyzing linkage from a cross to an fzo1-1 fis1-3 strain (Tieu and Nunnari, 2000). The mutation was identified by amplifying the FIS1 locus in fis1-L80P cells by PCR using Vent polymerase (New England Biolabs, Inc.) and sequencing the products directly (Davis Sequencing, University of California, Davis).

A plasmid containing GAL1-regulated MDV1 was constructed by introducing EcoRI and BamHI sites by PCR amplification of the MDV1 ORF, followed by subcloning into p416GAL (American Type Culture Collection [ATCC]), generating the pGAL1-MDV1 plasmid. The GFP-tagged Mdv1p domains were constructed by PCR amplifying S65T GFP lacking the 3’ terminator codon using oligonucleotides engineered with 5’- and 3’-NcoI sites. The amplified products were ligated with an XbaI site and SpeI. Two-hybrid constructs were made by introducing regions of the MDV1 ORF and introducing EcoRI and BamHI restriction sites by PCR. Full-length MDV1 was amplified from the 5’ ATG initiator to nucleotide 2142. The WD region was amplified from nucleotide 903 to 2142 including a 5’ ATG initiator, the NTE/C-C region was amplified from the 5’ ATG initiator to nucleotide 903, and the C-C region was amplified from nucleotide 723 to

![Table II. Two-hybrid interactions between Mdv1p and Mdv1p domains, Fis1Δ128–155p and Dnm1p](image)

| Strains | Genotype | Source |
|---------|----------|--------|
| JSY1826 | leuΔ1, his3Δ200, trp1Δ63, ura3-52, Mata | Mozdy et al., 2000 |
| JSY2977 | leuΔ1, his3Δ200, trp1Δ63, ura3-52, fzo1-1, Mata | Mozdy et al., 2000 |
| JSY1371 | leuΔ1, his3Δ200, ura3-52, dnm1Δ::HIS3, Mata | Otsuga et al., 1998 |
| JNY854 | JSY1826, except mdv1Δ::his5+, Mata | Tieu and Nunnari, 2000 |
| JNY855 | JSY1826, except fis1Δ::his5+, Mata | This study |
| JNY866 | JSY1826, except fis1-L80P, Mata | This study |
| JNY159 | PJ69-4A | James et al., 1996 |
903 with a 5' ATG initiator. DNA encoding all four regions of Mdv1p were subcloned into both pGAD and pGBDl plasmids. All 3' primers contained TGA stop codons. pGAD-Fis1Δ128–155 and pGAD-DNM1 were gifts from J. Shaw and A. Mozdy, University of Utah, Salt Lake City, UT. For two-hybrid analysis, plasmids constructed as described above from pGBDl and pGAD were cotransformed into the yeast strain Py64-4A and tested for interactions as previously described (James et al., 1996).

For the production of anti-Fis1p antibodies, the FIS1 PCR product was amplified from the 5' ATG initiator codon to nucleotide 384 (FIS1 lacking the transmembrane and intermembrane space regions) and subcloned in frame with maltose-binding protein (MBP) coding sequence into pMAL-C2 (New England Biolabs, Inc.: using a 5' BamHI site and a 3' HindIII site. All plasmid constructs were analyzed by sequencing and contained no additional mutations.

Cytological analysis
Mitochondrial morphology was analyzed and quantified using Mitotracker CMXR (Molecular Probes) or mito-GFP expressed from the plasmid pYX232 (provided by B. Westermann, Ludwig Maximilians Universität, Muenchen, Germany) as previously described (Tieu and Nunnari, 2000). Dnm1p was visualized and quantified by transfecting cells with plasmid pHS20 (DNM1–GFP) (gift from R. Jensen, John Hopkins University, Baltimore, Maryland) or pECJN233 (DNM1–dsRED) as previously described (Tieu and Nunnari, 2000). pGAL1 plasmids containing GFP-tagged Mdv1p regions were transformed into JSY1826, JNY854, JNY855, and JY1371. Transformants were grown in SD, subcultured into SRA media, further subcultured into SDG, supplemented with 1% dextrose to induce expression of GFP-tagged Mdv1p domains, and imaged after 12 h of logarithmic growth. All samples were imaged using either a Leica confocal or DeltaVision microscope with either a 100× 1.4NA or 60× 1.4NA objective.

Biochemical analyses
Soluble MBP-Fis1Δ128–155 was expressed in Escherichia coli (BL21 [DE3]) at 37°C and purified using amylase affinity chromatography (New England Biolabs). Anti-Fis1p polyclonal antibodies were produced in rabbits by injection of the purified MBP–Fis1Δ128–155 fusion protein (Co- Vance Research, Inc.).

Cell extracts were fractionated by differential centrifugation and analyzed by SDS-PAGE and Western blotting as previously described (Tieu and Nunnari, 2000). Immunoprecipitation of Mdv1p or Fis1p was performed from yeast cells grown in rich or SD media to a density of 1 OD600. After washing in water, 10 OD600 equivalents of cells were resuspended in 0.1 ml of sorbitol buffer (SB; 1.2 M sorbitol, 10 mM Hepes, 1 mM MgCl2) digested with 10 μl of 10 mg/ml lytic yeast enzyme (80,000 U/g; ICN Biomedicals) for 15 min at 30°C, and washed twice with 10 volumes of cold SB. After treatment with lytic enzyme, cells were resuspended in 0.92 ml of cold SB, and 0.08 ml of 18 mM DSP (Pierce Chemical Co.) in DMSO was added and cells were incubated for 2 h on ice to cross-link proteins. Cross-linking was terminated by quenching with 0.15 ml of 1 M glycine, pH 8.0, and cells were washed twice with two volumes of cold SB. Cross-linked cells were resuspended in 0.6 ml of 20 mM Tris, pH 8.0, 50 mM ammonium acetate, 2 mM EDTA, and 2 ml of solution containing 0.2% SDS, 20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 0.02% NaN3 for 5 min. Insoluble material was pelleted by centrifugation at 65,000 rpm for 30 min at 4°C. The renatured sample was incubated with 50 μl protein A agarose beads equilibrated with buffer containing 0.2% SDS, 20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 0.02% NaN3 (IPS) at 4°C with gentle shaking for 15 min. The cleared supernatant was removed to a new eppendorf containing 20 μl of IP5-equilibrated anti-Mdv1p-protein A cross-linked beads, and incubated at 4°C with gentle shaking for 45 min. The cross-linked anti-Mdv1p-protein A agarose beads were prepared as previously described (Harlow and Lane, 1998). Specifically, a total of 6 mg of crude anti-Mdv1p antibodies were cross-linked to 2.0 ml of protein A agarose beads (Santa Cruz Biotechnology, Inc.) with 20 ml of the water-soluble cross-linker dimethylpimelimidate. 25 μl of anti-Mdv1p–protein A cross-linked beads (antibody beads) was used per immunoprecipitation with 10 OD600 equivalents of cells prepared as described above. The protein-bound resin was washed five times with 1 ml of cold PBS, resuspended with 30 μl SDS-PAGE loading buffer, boiled for 5 min, and subjected to SDS-PAGE and Western blotting.

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