A novel motif in the yeast mitochondrial dynamin Dnm1 is essential for adaptor binding and membrane recruitment

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To initiate mitochondrial fission, dynamin-related proteins (DRPs) must bind specific adaptors on the outer mitochondrial membrane. The structural features underlying this interaction are poorly understood. Using yeast as a model, we show that the Insert B domain of the Dnm1 guanosine triphosphatase (a DRP) contains a novel motif required for association with the mitochondrial adaptor Mdv1. Mutation of this conserved motif specifically disrupted Dnm1–Mdv1 interactions, blocking Dnm1 recruitment and mitochondrial fission.

Introduction

Eukaryotic cells possess a family of dynamin-related proteins (DRPs), each of which is responsible for a specific cellular membrane-remodeling event. For example, the dynamin-related GTPase Drp1 (Dnm1 in yeast) mediates mitochondrial (Bleazard et al., 1999; Labrousse et al., 1999; Sesaki and Jensen, 1999) and peroxisomal fission (Koch et al., 2003; Li and Gould, 2003; Kuravi et al., 2006), Atlastin (Hu et al., 2009; Orso et al., 2009; Moss et al., 2011) and Mitofusins (Hales and Fuller, 1997; Hermann et al., 1998; Rapaport et al., 1998; Chen et al., 2003; Eura et al., 2003) play roles in ER and mitochondrial membrane fusion, respectively, and ARC5 (Gao et al., 2003) facilitates chloroplast membrane division. Like classical dynamin, DRPs self-assemble into highly ordered oligomers that use the energy of GTP hydrolysis to remodel lipid bilayers (Praefcke and McMahon, 2004).

Dynamin and DRPs have conserved GTPase, middle, and GTPase effector domains (see Fig. 1 A; van der Bliek, 1999). These domains mediate self-assembly and modulate GTPase activity. Dynamin and DRPs also contain nonconserved domains that, in some cases, have been shown to determine their cellular distribution and heterotypic interactions. For example, a proline-rich domain at the C terminus of dynamin facilitates its binding to a variety of actin-binding proteins. Dynamin also harbors a pleckstrin homology (PH) domain between its GTPase and GTPase effector domain, which is essential for interactions with the plasma membrane. In place of the PH domain, DRPs contain a region called Insert B (InsB) whose length and sequence varies. Whether or not there is a conserved function for InsB is not clear.

Although dynamin interacts directly with the lipid bilayer via its PH domain, DRPs do not initially interact with the lipid bilayers they remodel. Instead, they are recruited to specific cellular sites via interactions with membrane-associated adaptor proteins. In most cases, the DRP domains necessary for adaptor interactions have not been identified. However, structural studies of dynamin and several DRPs (Mears et al., 2007, 2011; Faelber et al., 2011; Ford et al., 2011) suggest that InsB is a likely candidate because it is predicted to reside at the base of the DRP oligomer, closest to the membrane.

Suppressor mutations in Mdv1 that restored Dnm1–Mdv1 interactions and fission identified potential protein-binding interfaces on the Mdv1 β-propeller domain. These results define the first known function for Insert B in DRP–adaptor interactions. Based on the variability of Insert B sequences and adaptor proteins, we propose that Insert B domains and mitochondrial adaptors have coevolved to meet the unique requirements for mitochondrial fission of different organisms.
InsB motif residues are important for Dnm1 function in mitochondrial fission

Yeast mitochondria form multiple tubular and branched structures that are continually remodeled by fission and fusion events. When fission is disrupted or blocked, mitochondria fuse to form netlike structures or a single interconnected tubule that often collapses to one side of the cell (Bleazard et al., 1999). To evaluate Dnm1 InsB motif function, we individually replaced the eight conserved residues with alanine and quantified the ability of each mutant protein to rescue fission defects in cells lacking the wild-type (WT) Dnm1 protein (dnm1Δ). As shown in Fig. 2 A, expression of WT Dnm1 rescued fission in up to 90% of dnm1Δ cells. In contrast, four Dnm1 mutant proteins (F606A, L607A, F610A, and F611A) failed to rescue mitochondrial morphology in this strain. Hereafter, these four loss-of-function proteins will be collectively referred to as Dnm1 InsBmut. Western blotting of whole cell extracts indicated that the Dnm1InsBmut proteins were expressed at levels similar to WT Dnm1 (Fig. S1 A). Thus, mutations in conserved residues of InsB block Dnm1 function in mitochondrial fission but do not affect protein stability.

We used the yeast mitochondrial fission machinery as a model to directly test the function of InsB in DRP–adaptor interactions and DRP1 membrane recruitment. In vivo, Dnm1 (the yeast DRP) is recruited to the mitochondrial outer membrane by Mdv1 (the adaptor; Tieu and Nunnari, 2000; Cerveny et al., 2001), which in turn associates with membrane-anchored Fis1 (Mozdy et al., 2000). Once on the membrane, Dnm1 co-assembles with Mdv1 into spirals that encircle and divide the mitochondrial tubule (Bhar et al., 2006; Nayelor et al., 2006). We identified a motif in the Dnm1 InsB domain that is required to recruit Dnm1 to mitochondria. The amino acid sequence of this motif is strictly conserved among fungi and is predicted to form a solvent-inaccessible helix (PSIPRED v2.6). Amino acid substitutions in this InsB helix inhibit the recruitment of Dnm1 to mitochondria and block fission. Importantly, these mutations do not impair Dnm1 self-assembly. Instead, they specifically disrupt the Dnm1–Mdv1 interaction. The disrupted interaction is rescued by suppressor mutations in the Mdv1 β-propeller domain to which Dnm1 binds. These findings identify a new functional motif in the Dnm1 InsB domain required for Mdv1 binding and recruitment of Dnm1 from the cytoplasm to its site of action on mitochondria.

Results

Dnm1 InsB contains a conserved sequence motif predicted to be solvent inaccessible

In a previous genetic screen (Bhar et al., 2006), we identified an InsB mutation (F610L) that interfered with Dnm1 function in mitochondrial fission. Alignment of Saccharomyces cerevisiae Dnm1 InsB with its fungal homologues revealed a motif of eight strictly conserved residues encompassing F610 (Fig. 1, A and B). These residues are predicted to form a short helix (PSIPRED v2.6) that is less accessible to solvent than surrounding residues (Fig. 1 C), suggesting that they are part of an interaction interface.

InsB motif residues are important for Dnm1 function in mitochondrial fission

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In the absence of WT Dnm1, the inability of Dnm1InsBmut proteins to support fission could be caused by their failure to self-assemble. To test this possibility, we coexpressed HA- and Myc-tagged versions of the same InsB mutant proteins in dnm1Δ cells and performed coimmunoprecipitation (coIP) assays. As shown in Fig. 2 B, each form of the HA-tagged Dnm1InsBmut protein was efficiently coprecipitated with the Myc-tagged version of itself. Thus, the F606, L607, F610, and F611 residues in InsB are not essential for Dnm1 self-assembly.

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proteins is indistinguishable from that observed for GFP-Dnm1 in cells lacking Fis1 or fission adaptor proteins (Mozdy et al., 2000; Griffin et al., 2005). This observation raised the possibility that mutations in Dnm1InsBmut proteins were disrupting interactions with the Mdv1 adaptor.

In vitro pull-down assays using purified GST-Mdv1-β-propeller and His-tagged Dnm1 showed that the Mdv1-β-propeller domain binds directly to Dnm1 (Fig. S1 B). To determine whether InsB mutations affected the Dnm1–Mdv1 interaction, we performed coIP experiments from dnm1Δ cells expressing Myc-tagged Dnm1 InsBmut proteins. Although full-length Mdv1 was efficiently coprecipitated by WT Dnm1-Myc, Mdv1 interaction with all of the Dnm1InsBmut-Myc proteins was dramatically reduced (Fig. 3 B). These combined results identify a novel motif in InsB essential for Dnm1–Mdv1 interaction and Dnm1 recruitment to mitochondria.

Suppressors of a dnm1InsBmut mutation cluster in the Mdv1-β-propeller domain

Using an integrated form of the dnm1F610A mutation, we performed a suppressor screen to identify residues in Mdv1 important for Dnm1–Mdv1 interaction (see Materials and methods and Table S1). Although the screen covered 84% of the MDV1 coding sequence, all but one suppressor mutation fell in the Mdv1-β-propeller domain (Fig. 4 A). Most of the affected residues localized to the top of the Mdv1-β-propeller model (Fig. 4 B, right),
In these two studies. It is also possible that mutation to glycine has a more significant effect on local protein structure and flexibility than mutation to glutamine.

Suppressor mutations in the Mdv1 β-propeller rescue mitochondrial fission defects caused by Dnm1F610A

In control studies, the Mdv1 suppressor proteins were all stably expressed in vivo (Fig. S1 C). To verify that the Mdv1 suppressor proteins were capable of rescuing fission, we expressed them from a plasmid in cells lacking WT Mdv1 and expressing Dnm1F610A protein from the genome. All of the Mdv1 suppressors rescued mitochondrial fission defects in this strain (up to 70% rescue). Representative results are shown in Fig. 5 A for the five best rescuing Mdv1 suppressors (Y418C, D539G, D539Y, L540P, and D576G). The same Mdv1 suppressors also rescued mitochondrial morphology defects caused by other Dnm1InsBmut proteins (F606A, L607A, and F611A; Fig. 5 B).

To test whether Mdv1 suppressors could rescue the mitochondrial recruitment of Dnm1F610A, Mdv1 suppressors and GFP-Dnm1F610A were coexpressed in an mdv1Δ strain. GFP mitochondrial puncta were visible in up to 70% of these cells (Fig. 5 C). The mitochondrial recruitment and puncta formation by GFP-Dnm1F610A suggested that Dnm1 interaction with the Mdv1 suppressor proteins had been restored. However, this interaction could not be detected in coIP assays, even after chemical cross-linking (unpublished data). Thus, the restored interaction between Dnm1F610A and Mdv1 suppressors is less robust than WT.

As an alternative, we evaluated Dnm1F610A–Mdv1 suppressor interactions using the yeast two-hybrid growth assay. Previous studies established that WT Dnm1 and WT Mdv1 interact in
Figure 4. Suppressors of a dnm1F610A mutation cluster in the Mdv1 β-propeller. (A) Domain structure of Mdv1 with indicated Dnm1F610A suppressor mutations [the number of mutations obtained for each allele is in parentheses]. NTE, N-terminal Extension; CC, coiled coil. (B) Top and side views of the Mdv1 β-propeller model (blue). Red indicates residues changed by suppressor mutations. The N terminus is in green. (C) Alignment of the portions of the Mdv1 (aa 408–643) and Caf4 (aa 329–566) β-propeller sequences. Residues affected by suppressor mutations are shown in red. Symbols below the sequence alignment indicate identity (*), strong similarity (:), and weak similarity (.) of amino acids.

Table 1. Characterization of Mdv1 suppressors of the dnm1F610A allele

<table>
<thead>
<tr>
<th>Mutations</th>
<th>WT mitochondrial morphologya</th>
<th>GFP mitochondrial punctab</th>
<th>Interaction with Dnm1c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNM1 mvlΔ</td>
<td>dnm1F610A mvlΔ</td>
<td>Dnm1</td>
</tr>
<tr>
<td>Mdv1</td>
<td>67 ± 7</td>
<td>0 ± 0</td>
<td>+</td>
</tr>
<tr>
<td>Y418C</td>
<td>6 ± 3</td>
<td>41 ± 16</td>
<td>+</td>
</tr>
<tr>
<td>K430R</td>
<td>9 ± 8</td>
<td>28 ± 4</td>
<td>+</td>
</tr>
<tr>
<td>E504G</td>
<td>19 ± 8</td>
<td>32 ± 16</td>
<td>+</td>
</tr>
<tr>
<td>D539G</td>
<td>9 ± 2</td>
<td>68 ± 11</td>
<td>+</td>
</tr>
<tr>
<td>D539Y</td>
<td>11 ± 1</td>
<td>61 ± 14</td>
<td>+</td>
</tr>
<tr>
<td>L540P</td>
<td>12 ± 5</td>
<td>58 ± 14</td>
<td>+</td>
</tr>
<tr>
<td>S541G</td>
<td>8 ± 4</td>
<td>38 ± 12</td>
<td>+</td>
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<tr>
<td>S557C</td>
<td>9 ± 8</td>
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<td>+</td>
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<tr>
<td>T558I</td>
<td>10 ± 8</td>
<td>34 ± 6</td>
<td>+</td>
</tr>
<tr>
<td>D576G</td>
<td>68 ± 17</td>
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<tr>
<td>D585G</td>
<td>45 ± 3</td>
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</tr>
<tr>
<td>D607G</td>
<td>15 ± 6</td>
<td>31 ± 12</td>
<td>+</td>
</tr>
</tbody>
</table>

aNumbers are the mean and standard deviation of at least three independent experiments, n = 300.
bGFP puncta localized to mitochondrial tubules.
cYeast two-hybrid assays. –, No growth on His or Ade minus medium; +, growth on His but not Ade minus medium; ++, growth on both His and Ade minus media. Growth on Ade minus medium is the more stringent indicator of protein–protein interaction.

this assay (Fig. 5 D; Tieu and Nunnari, 2000; Cerveny and Jensen, 2003; Karren et al., 2005). Although the interaction was severely disrupted when Dnm1F610A was paired with WT Mdv1, the interaction was partially (D539Y and L540P) or completely (Y418C and D539G) restored by substituting an Mdv1 suppressor protein for WT Mdv1 (Fig. 5 D). Surprisingly, the Mdv1D576G...
and cell-based assays confirm that the InsB–β-propeller interaction is critical for Dnm1–Mdv1 binding and Dnm1 membrane recruitment. The Dnm1 InsB motif and Mdv1 adaptor sequences required for this interaction are conserved in fungi but not in mammals or plants (Fig. 6). Thus, different InsB domains and adaptors may have coevolved in different organisms to mediate membrane targeting of mitochondrial dynamin-related GTPases.

Our mutational analysis demonstrates that four hydrophobic residues in the Dnm1 InsB motif are necessary for interaction with Mdv1. However, the purified Dnm1 InsB domain (aa 535–639) is not sufficient to bind the Mdv1 β-propeller in vitro (unpublished data). Because the conserved motif in InsB is hydrophobic, it may not adopt a functional conformation when InsB is expressed on its own. In full-length Dnm1, the InsB motif may normally be buried in the interior of the protein. In this case, a conformational change in Dnm1 would be required to expose critical residues in InsB for adaptor binding. A conformational change of this type could be stimulated by GTP binding to Dnm1. This scenario would be consistent with the previous finding that Mdv1 preferentially binds to the GTP-bound form of Dnm1 (Cerveny and Jensen, 2003; Naylor et al., 2006; Lackner et al., 2009).

Figure 5. Suppressor mutations in the Mdv1 β-propeller rescue mitochondrial fission defects caused by Dnm1F610A. (A) Quantification of mitochondrial morphology in mdv1Δ DNM1 and mdv1Δ dnm1:dnm1F610A strains expressing the indicated Mdv1 suppressor proteins. (B) Quantification of mitochondrial morphology in mdv1Δ dnm1Δ strains expressing indicated Mdv1 and Dnm1 proteins from plasmids. The mitochondria were visualized with MitoFluor Red 589 (Molecular Probes). (*) Plasmid-expressed WT Mdv1 rescued Dnm1F610A better than genome-expressed WT Mdv1 (Fig. 2 A). This is likely because of the higher steady-state abundance of the plasmid-expressed protein. (C) Quantification of GFP-Dnm1 localization in mdv1Δ dnm1Δ cells expressing the indicated Mdv1 and GFP-Dnm1 variants. [A and C] Black columns and error bars represent the mean and standard deviation of at least three independent experiments, n = 300.

Discussion

Although Dnm1 binding to Mdv1 and recruitment to the mitochondrial membrane is essential for fission, the Dnm1 domains required for this interaction were not known. Here we identify a novel motif in Dnm1 InsB that is specifically required for interaction with the Mdv1 β-propeller. Second-site suppression studies and cell-based assays confirm that the InsB–β-propeller interaction is critical for Dnm1–Mdv1 binding and Dnm1 membrane recruitment. The Dnm1 InsB motif and Mdv1 adaptor sequences required for this interaction are conserved in fungi but not in mammals or plants (Fig. 6). Thus, different InsB domains and adaptors may have coevolved in different organisms to mediate membrane targeting of mitochondrial dynamin-related GTPases.

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residues affected by the suppressor mutations likely delineate regions of the Mdv1 β-propeller in close proximity to Dnm1.

As new mitochondrial fission components were identified, it became clear that different organisms express unrelated adaptor proteins (Fig. 6 A; Tieu and Nunnari, 2000; Cerveny et al., 2001; Griffin et al., 2005; Nishida et al., 2007; Arimura et al., 2008; Gandre-Babbe and van der Bliek, 2008; Otera et al., 2010; Palmer et al., 2011; Zhao et al., 2011). We propose that the InsB domain provides some of the variability needed to mediate these diverse DRP–adaptor interactions. As shown in Fig. 6 (B and C), sequence alignments reveal little identity between InsB sequences of fungi, algae, plants, and mammals. Conversely, high identity is observed among InsB sequences of representative mammals (Fig. 6 C, 94%). The functional interaction we observe between Dnm1 InsB and the Mdv1 β-propeller in yeast may be recapitulated for DRP–adaptor interactions in other organisms. However, there are almost certainly additional DRP–adaptor interfaces that remain to be identified, especially in mammals, where a single DRP is able to interact with several structurally distinct adaptors (Fig. 6 A; Gandre-Babbe and van der Bliek, 2008; Otera et al., 2010; Palmer et al., 2011; Zhao et al., 2011). A recent study showed that mutation of a conserved residue in the Drp1 middle domain disrupts interaction with a
mitochondrial adaptor called Mff (Strack and Cribbs, 2012). The model that InsB domains mediate DRP interactions in other organisms can be directly tested in genetic and cellular studies, as well as structural studies of DRPs bound to their cognate adaptor proteins.

**Materials and methods**

**Yeast strains and plasmids**

For Dnm1–Dnm1 interaction experiments, functional HA- and Myc-tagged CoIP assays yeast cells were deconvolved and analyzed using Axiovision version 4.6 immersion objective was used to observe and image cells. For mitochondria promoter is leaky under these conditions and expresses approximately four-fold between the BamHI and SalI sites of the sequences encoding single substitutions F606A, L607A, N608A, Y609A, F610A, F611A, G612A, and K613A. Similar methods were used to generate pRS415-MET25-GFP-adenyGAD-Dnm1, pRS426-dnm1F610A-MYC, and pRS426-dnm1F610A-MycHA. To create pGAD-C1-DNM1 and pGAD-C1-dnm1F610A BamHI-DNM1-Sall and BamHI-dnm1F610A-Sall fragments were cloned between the BamHII and Sall sites of the pGAD-C1 vector. Similarly, BamHII-MDV1-Sall and BamHII-dnm1F610A-Sall fragments were cloned between the BamHII and Sall sites of the pGPD-C1 vector to generate pGPD-C1-MDV1 and pGPD-C1-dnm1F610A.

**Fluorescence microscopy**

Mitochondrial morphologies were quantified in WT, dnm1Δ, and dnm1Δmdv1Δ strains expressing the indicated proteins. The WT morphology category includes unbulbed or budded cells with more than two free tubule ends in the mother cell. The formation of GFP-Dnm1 mitochondrial puncta was quantified by analysis of deconvolved epifluorescence images of random fields of cells. Phenotypic quantification was reported as the mean and standard deviation of three independent experiments (total n ≥ 300 cells unless noted). Unless specified in the figure legend, the mitochondria were visualized by expressing mitochondrial-targeted fast-folding RFP (mRFP). Dnm1 Inb variants were expressed from the DNM1 promoter in the pRS415 vector. GFP-tagged Dnm1 variants were expressed from the pRS415-MET25 vector. mdv1Δ variants were expressed from the pRS416-MET25 plasmid. Yeast cells were grown at 30°C in selective synthetic dextrose medium containing 10 mg/ml methionine. Overnight cultures were diluted to 0.2 OD600 and grown for 3–5 h at OD600 0.5–1.0. The methionine-repressible MET25 promoter is leaky under these conditions and expresses approximately four-fold more protein at steady state than that expressed from the endogenous MET25 locus (Orr-Weaver et al., 1983). A microscope (Axioplan 2; Carl Zeiss) equipped with a 100× oil immersion objective was used to observe and image cells. For mitochondrial genome loss, and inability to grow on glycerol at 37°C. Mitochondrial morphologies were quantified in WT, mdv1Δ, and dnm1Δ strains grown on glycerol at 37°C. Candidate clones with verified phenotypes were sequenced to identify MDV1 mutations. In alleles with multiple amino acid changes, mutations were separated by site-directed mutagenesis. Mutations contributing to growth phenotypes were analyzed for mitochondrial morphology and GFP-Dnm1 localization.

**Mdv1 β-propeller modeling**

The β-propeller model shown in Fig. 4 was generated from the crystal structure of the Cdc4 WD40 repeat (pdb accession no. 1NEX) using the PHYRE Protein Fold Recognition server (Kelley and Sternberg, 2009). Residues 349–713 of Mdv1 are variably modeled as a seven- or eight-bladed β-propeller, depending on the structures most recently deposited in the PDB. The eight-bladed β-propeller model shown in Fig. 4 includes the majority of residues identified in the second-site suppressor analysis described here.

**Yeast two-hybrid analysis**

Yeast two-hybrid studies to analyze Dnm1–Mdv1 and Dnm1 self-interactions were performed in the Y187. 5 cerevisiae strain background (Takara Bio Inc.) via a growth assay as described previously (Guthrie and Fink, 2002). Interaction between His3- and GAD plasmids expressing the indicated fusion proteins was cotransformed into the Y187 reporter strain. Interaction between two fusion proteins leads to expression of one of several reporter genes in this strain, allowing the yeast cells to grow on S-dextrose minus histidine or minus adenine. WT Dnm1–Dnm1 interactions were performed in cells coexpressing GAD-Dnm1 WT and GBD-Dnm1 InsBmut. The Dnm1–Mdv1 interaction was tested in both directions. However, the interaction was only detected when Dnm1 and Mdv1 were fused with the GAD and GBD domains, respectively.

**Online supplemental material**

Table S1 shows a screen for mdv1 suppressors of dnm1F610A. Table S2 shows the plasmids used in this study. Fig. S1 shows expression, interaction, and assembly properties of Dnm1 and mdv1Δ variants. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201207079/DC1.
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