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Biochemical characterization of human dynamin-like protein 1

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Human dynamin-like protein 1 (DLP-1) is involved in the fission of mitochondrial outer membranes, a process that helps to maintain mitochondrial morphology and to reduce the accumulation of functional and structural defects in mitochondria. DLP-1 is a ~80 kDa membrane-interacting protein and contains a GTPase domain, a middle domain, a putative PH-like domain and a GTPase effector domain (GED). While the GED has been suggested to be important on protein oligomerization and GTPase activation, functional relationships between the other domains especially the roles of the middle domain in protein activity remains less clear. In this study, we have investigated the biochemical properties of recombinant DLP-1 wild-type and selected mutants, all expressed in *Escherichia coli*. The middle domain mutants G350D, R365S and ΔPH (lacking the putative PH-like domain) severely impair the GTPase activity, but have no obvious effects on protein tetramerization and liposome-binding properties, suggesting these mutants probably affect protein intra-molecular interactions. Our study also suggested that proper domain–domain interactions are important for DLP-1 GTPase activity.

Keywords: DLP-1/dynamin/GTPase activity/middle domain/mitochondrial fission.

Abbreviations: DLP-1, human dynamin-like protein 1; Dnm1, DLP-1 homolog in yeasts; Fis1, mitochondrial fission 1 protein; GED, GTPase effector domain; hGBP1, human guanylate-binding protein 1; PH domain, pleckstrin homology domain; PIs, phosphoinositides; ΔPH, DLP-1 lacking the putative PH-like domain.

The dynamics of continuous fission and fusion events are essential for maintaining normal mitochondrial morphology and reducing the number of functional defects that could lead to a variety of diseases. Human mitochondrial fission is regulated by dynamin-like protein 1 (DLP-1) (*1*–*4*), a highly conserved GTPase in dynamin superfamily (*5*). Although many detailed mechanisms remain unclear about mitochondrial fission, it is believed that during the fission event, DLP-1 is recruited by mitochondrial surface proteins and then forms a ring-like structure surrounding the mitochondrial outer membranes (*6*). Via hydrolysis of GTP, DLP-1 interacts with a number of protein molecules and forms a protein complex to facilitate mitochondrial fission on a GTPase activity-dependant manner (*7*, *8*).

DLP-1 is ~80 kDa protein and consists of an N-terminal GTPase domain (approximately 330 residues in length) with conserved GTP-binding motifs; a approximately 170 residue middle domain; a approximately 105 residue insert of unknown function; and a approximately 100 residue GTPase effector domain (GED) with putative roles in both self-assembly and stimulation of GTPase activity (*8*). The insert of unknown function is topologically analogous to the PH domains in dynamins, and we therefore will use the term 'PH-like' domain for convenience only, and not to infer structural or functional similarity. In contrast, the middle domain has been proposed to interact with the GED and have potential roles in protein self-assembly and tetramerization. The hypothesis was mostly based on structural and biochemical studies on the other dynamin superfamily proteins: the crystal structure of human guanylate-binding protein 1 (hGBP1), a dynamin-related protein, shows that the GED folds back to interact with the middle domain and extends to the GTPase domain probably regulates the GTPase activity (*9*); the low resolution three-dimensional structure of dynamin, derived from cryoelectron microscopy, also suggested a similar structural arrangement of the middle and GED domains (*10*); finally, yeast two-hybrid assays of dynamin and related proteins showed direct interactions between the GED and the middle domains (*11*–*13*). Meanwhile, point mutations in the middle domain of dynamin (*14*) and Dnm1 (DLP-1 homolog in yeasts) (*15*, *16*) affect the assembly of tetramer or higher ordered oligomers, as well as protein GTPase activity. To study the roles of the middle domain and the PH-like domain in DLP-1, we generated the middle domain mutations G350D and R365S, and the deletion of the PH-like domain.

DLP-1 is a membrane-interacting mammalian protein, therefore is likely to be difficult for heterogeneous expression and purification in bacteria. In the present study, to obtain sufficient amounts of DLP-1 for functional and structural studies, we have successfully purified recombinant DLP-1 and its mutants with high

yield by optimizing expression and purification conditions. We have also analysed the biochemical and physical properties of wild-type DLP-1 and selected mutants to investigate the impact of key amino-acid residues or specific domains on the protein functions.

Materials and Methods

DNA cloning, protein expression and purification

The DNA encoding for full-length DLP-1 isoform 2, the GTPase domain (residues 1–307) and the PH-like domain (residues 497–607) were amplified from cDNA IMAGE (clone ID 3882922) by PCR and cloned into pLW01 expression vector. Mutagenesis was performed using GeneEditor *in vitro* mutagenesis system (Promega). For the ΔPH, a flexible linker of approximately 20 amino acids was engineered between the middle and the GED domain. For protein expression, constructed plasmids were transformed into *Escherichia coli* host C41 (DE3) competent cells. Fresh single colonies were inoculated into LB media containing 100 μg/ml ampicillin and shaked at 37°C overnight at 200 r.p.m. Twenty milli litres of this culture was transferred to 1 l LB media and grown at 37°C until the OD_{600} reached to 0.8–1.0. The protein expression was then induced by adding 0.02 mM IPTG and the cells were incubated with shaking at 12°C or room temperature for 17 h. For protein purification, cell pellets were re-suspended in buffer A (50 mM sodium phosphate, 300 mM NaCl, 5% glycerol, 10 mM β-mercaptoethanol, 0.1 mM EDTA, pH 8.0). After sonication, the crude cell extract was centrifuged at 4°C for 20 min at 12,000*g*. The supernatant was loaded onto a pre-equilibrated Ni-NTA column (Qiagen). The column was washed and eluted with buffer A containing 25 and 200 mM imidazole, respectively.

Ion exchange and size exclusion chromatography

The pooled and concentrated eluates from Ni-NTA were loaded onto a 1 ml HiTrap Q ion exchanger (GE Life Sciences) pre-equilibrated with buffer B (20 mM Tris–HCl, pH 8.5). Protein was eluted off the column with a linear concentration gradient of NaCl (0–1 M) at a flow rate of 1 ml/min. The peak fractions containing purified target protein were pooled and concentrated. For size exclusion chromatography, a Superdex 200 or 75 column (GE Life Sciences) was pre-equilibrated with buffer C (20 mM HEPES, 150 mM NaCl, pH 7.2). The protein solution was eluted at a rate of 0.5 ml/min. The size exclusion column was calibrated by monitoring the elution of molecular weight standards from GE Life Sciences.

Measurement of GTPase activity

The GTPase activity was measured using a Malachite Green phosphate assay kit (Bioassay systems) at room temperature. The reaction included 1 μM purified target protein with 0.05% bovine serum albumin, 16 μM GTP and 2 mM $MgCl_2$ in buffer C at an 800 μl volume. At different time points, 200 μl malachite green reagent was added to stop the reaction and the mixture was incubated at room temperature for 10 min to allow colour to develop. Absorbance at 650 nm was measured spectrophotometrically, and the amount of released inorganic phosphate was determined by using a standard curve of known phosphate concentrations. All data points represent an average of at least three independent measurements.

Liposome-binding assay

Interactions between protein and lipids were analysed as described (*17*, *18*). Briefly, the lipids, consisting phosphoinositides (Sigma), were incubated in TBS buffer (50 mM Tris–HCl, 100 mM NaCl, pH 7.0) at 37°C for 1 h. After vortexing for 5 min, the solution was centrifuged at 20,000*g* at 4°C. The pellet containing 200 μg liposomes was resuspended in 100 μl TBS, followed by the addition of 5 μg protein sample. The binding reaction was performed by incubating the mixture at 30°C for 30 min. Then, the solution was centrifuged at 20,000*g* at 4°C and the distribution of target proteins in the pellet and supernatant fractions was investigated by SDS–PAGE.

Results and discussion

Protein expression and purification

Several mutant forms of DLP-1 were constructed, which allowed us to investigate the impact of specific domains or key amino-acid residues on the biochemical properties of DLP-1. The alignment of the DLP-1 protein sequence with its homologues from other organisms and an analysis of putative secondary structure made it possible to determine essential residues and putative domain boundaries. With the exception of the PH-like domain, other regions of the protein are highly conserved and contain regular secondary structures with combination of helices and strands (data not shown). We created a construct of DLP-1 ΔPH (deletion of the PH-like domain from residues 497–607; Fig. 1A) to investigate whether the PH-like domain has a role in the structure and/or function of DLP-1. The sequence alignment of a part of the DLP-1 middle domain with those of its homologues from other organisms identified several highly conserved residues (Fig. 1B). We chose to study residues G350 and R365 since the equivalent residues in human dynamin and yeast Dnm1 were shown to be involved in the assembly of tetramers and higher ordered oligomers, as well as in maintaining high GTPase activity (*14–16*). We were interested in determining whether the G350 and R365 are important in DLP-1 GTPase activity and protein tetramerization. We also made constructs to isolate stable and soluble GTPase domain (residues 1–307; Fig. 1A) and PH-like domain (residues 497–607; Fig. 1A) to explore whether they are critical for protein oligomerization.

In an effort to produce DLP-1 and mutant proteins for functional and structural studies, several conditions regarding recombinant expression of membrane-interacting protein in *E. coli* were optimized. First, pLW01 vector was chosen for DLP-1 expression, as it was successfully used to express the membrane bound cytochrome P450 (*19*) and a number of membrane-related proteins in our laboratory (R. M. Garavito, unpublished data) in *E. coli*. Second, *E. coli* C41 (DE3), a strain often allows high-yield expression of membrane-related proteins perhaps due to the formation of internal membranes (*20*, *21*), was used as the expression host. Last, the growth conditions including temperature and concentration of the inducer (IPTG) were optimized to reduce the rate of protein synthesis and minimize formation of inclusion bodies. With all these optimizations, we were able to successfully express and purify DLP-1 full-length protein, the GTPase domain, the middle domain mutants G350D and R365S, the PH-like domain, and the ΔPH. At least 10–20 mg/l culture of purified recombinant DLP-1 species were obtained after purification by Ni-NTA column and ion exchange chromatography (Fig. 1).

GTPase activity

The DLP-1 WT showed high GTPase activity (Fig. 2A), as opposed to the GTPase null mutant K38A, which is consistent with the results from previous studies (*12*, *22*). While the isolated GTPase domain

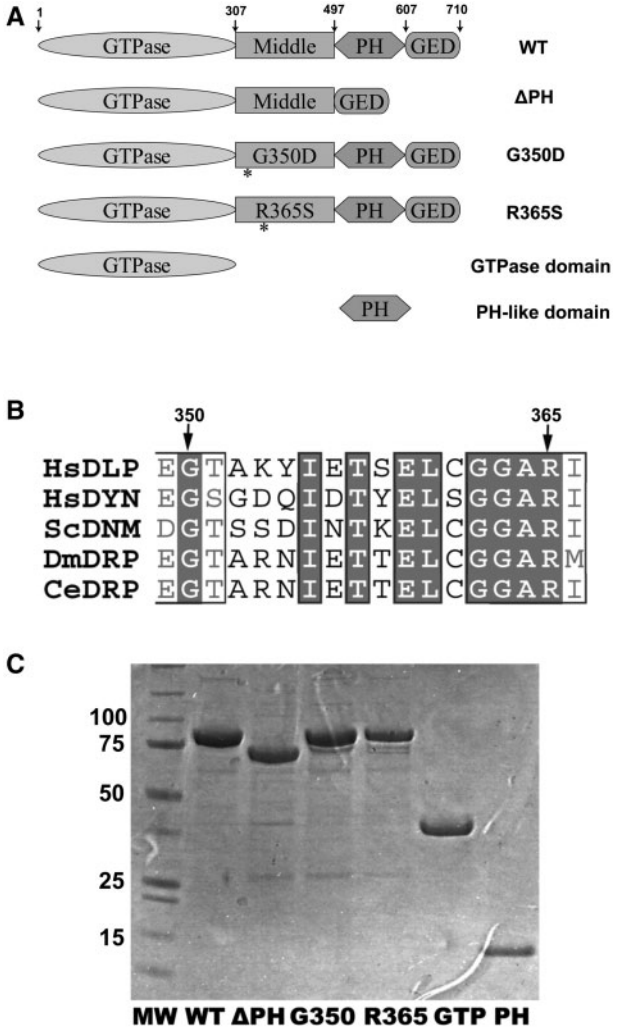

Fig. 1 Expression and purification of DLP-1 WT and mutants. (A) Schematic illustrations of the DLP-1 constructs that were successfully expressed and purified. G350D and R365S are point mutations in the middle domain; and ΔPH lacks the PH-like domain (residues 497–607). The isolated GTPase (residues 1–307) and the PH-like domain (residues 497–607) were also expressed and purified. (B) Sequence alignment of a portion of the middle domain of *Homo sapiens* DLP-1 (HsDLP) with *H. sapiens* dynamin (HsDYN), *Saccharomyces cerevisiae* Dnm1 (ScDNM), *Drosophila melanogaster* dynamin-related protein (DmDRP), *Caenorhabditis elegans* dynamin-related protein (CeDRP). The G350, and R365 of DLP-1 are indicated by arrows. (C) The SDS–PAGE of purified recombinant DLP-1 WT and mutants. From left to right: molecular weight markers; DLP-1 WT; ΔPH; G350D; R365S; GTPase domain; the PH-like domain.

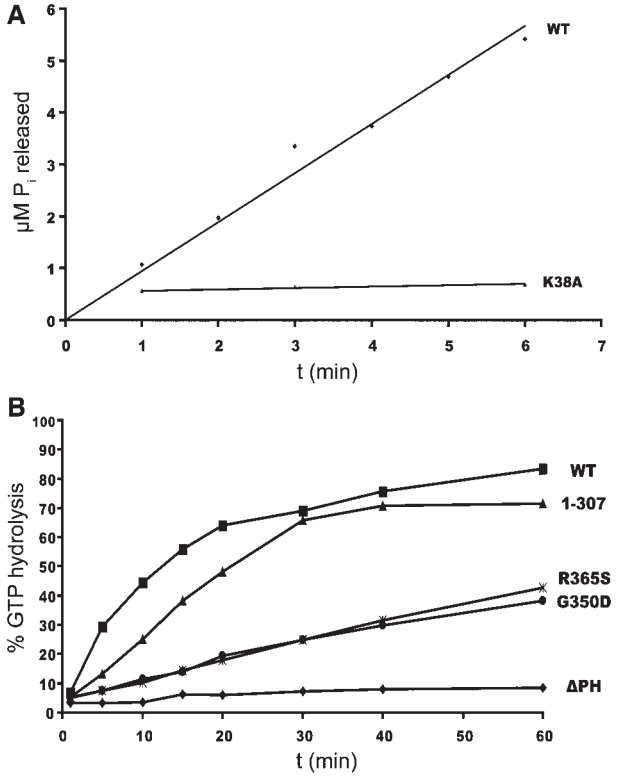

Fig. 2 GTPase activity of DLP-1 WT and mutants. (A) GTP hydrolysis by DLP-1 WT was plotted as time (min) versus released inorganic phosphate (μM). DLP-1 WT activity is shown as squares, while the K38A negative control is shown as triangles. (B) GTP hydrolysis by DLP-1 WT and mutants. The data were plotted as time (min) versus hydrolyzed GTP/total GTP (%). Squares, WT; triangles, GTPase domain (residues 1–307); circles, G350D; stars, R365S; rhomboids, ΔPH. All data points represent an average of three independent measurements.

demonstrated an activity lower but comparable to wild-type DLP-1, the G350D and the R365S mutations showed much lower GTPase activities (Fig. 2B). Surprisingly, the ΔPH totally abolished the GTPase activity (Fig. 2B). These results indicate that the middle domain and the PH-like domain are important for DLP-1 GTPase activity.

Protein tetrameric state

DLP-1 forms higher order structures upon binding to membranes, but exists primarily as tetramers in the cytosol (*12*, *13*). Since the G350D, the R365S, and the ΔPH exhibited reduced GTPase activities, we were interested in studying the oligomeric states of these species to analyse whether the decreased activities were related to protein tetramerization. Analytical size exclusion chromatography showed that the DLP-1 WT can be isolated as a ~330 kDa species, which corresponds to the tetrameric state (Fig. 3A and Table I), consistent with previous reports (*12*, *13*). Similarly, the G350D and the R365S were eluted also at ~330 kDa (Fig. 3B and C and Table I), displaying stable tetrameric forms that are almost indistinguishable from that of the wild-type protein. These results suggest that the DLP-1 G350D and the R365S mutations do not markedly impact DLP-1 tetramer, nor are the reduced GTPase activities a consequence of the loss of the protein's quaternary structure. Although the middle domain may be important in maintaining GTPase activity and protein tetramerization, the structural impact of these two mutations are clearly not causing a major disruption of the tetrameric interactions. A recent report coincidently investigated the effects of DLP-1 G350D on protein tetramerization and observed that G350D do not significantly inhibit DLP-1 tetramer formation (*23*), which is consistent with our results. The report also investigated the

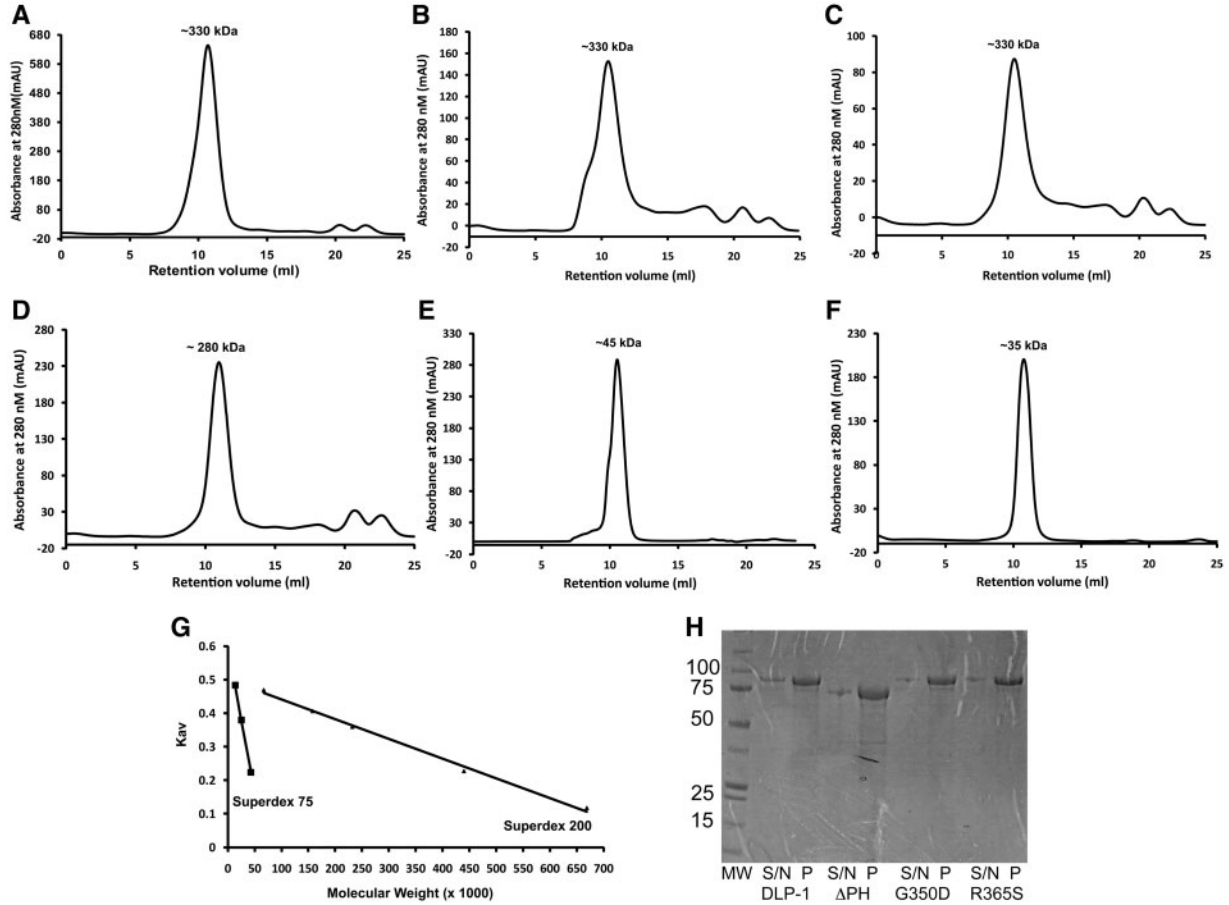

Fig. 3 Size exclusion chromatography and liposome-binding assay of DLP-1 WT and mutants. Panels A–D show chromatography on a Superdex 200 10/30 column of DLP-1 WT, G350D, R365S and ΔPH, respectively; panels E and F show chromatography on a Superdex 75 10/30 column of the PH-like domain and GTPase domain, respectively. Panel G shows the Kav versus molecular weight plot of protein standards. The standards were plotted as Kav versus molecular weight. Kav was obtained by the formula of $\text{Kav} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of each molecular weight markers, V_0 is the void volume, and V_t is total volume of the column. Panel H shows the SDS–PAGE of liposome-binding assay. MW, protein molecular weight standard (in kDa); SN, supernatant fraction showing non-bound protein; P, pellet fraction showing bound protein.

Table I. Estimated molecular weight of DLP-1 WT and mutants by size exclusion chromatography.

Species	Calculated MW	Oligomeric state
DLP1 WT	~330,000	Tetramer
G350D	~330,000	Tetramer
R365S	~330,000	Tetramer
ΔPH	~280,000	Tetramer
GTPase domain	~35,000	Monomer
PH-like domain	~45,000	Tetramer

higher order states of the DLP-1 mutants, which is out of the scope of our study.

The loss of GTPase activity in the DLP-1 ΔPH deletion mutant raises questions: Does the loss of the PH-like domain disrupt the tetrameric state of the DLP-1 ΔPH? Is the PH-like domain essential for tetramer formation? The analytical size exclusion chromatography revealed that the DLP-1 ΔPH exists as a ~280 kDa species, suggesting that stable tetramers were being formed (Fig. 3D and Table I). Hence, the loss of the PH-like domain does not obviously impact tetramerization. Interestingly, size exclusion chromatography showed that the PH-like domain could be isolated as a ~45 kDa tetramer (Fig. 3E and Table I). Although we could not exclude the possibility that the PH-like domain plays an active role in protein tetramerization, the fact that the ΔPH forms a stable tetramer in solution suggests that the PH-like domain may not be the major determinant of DLP-1 tetramerization.

Size exclusion chromatography showed that the GTPase domain (residues 1–307) is a ~35 kDa monomer as an isolated species (Fig. 3F and Table I), indicating that this domain may not be involved in DLP-1 tetramerization. In addition, during our search for possible domain boundaries for the GTPase domain, secondary structure prediction suggested a long helix ending at residue 323, and sequence alignments favoured residue 340 as the domain boundary (data not shown). Although also we isolated and purified residues 1–323 and 1–340 fragments of the GTPase domain, these species were quite aggregated as judged by size exclusion chromatography (data not shown). This may suggest that the segment from residues 308 to 340 may be a hydrophobic transition

region between the GTPase domain and middle domain helix bundle.

Studies have described the GED of dynamin or related proteins as a key player in protein oligomerization and higher order structure formation (*5*, *24*). Also, the middle domain was considered to be critical for the formation of these structures (*10*, *14*, *16*). We have attempted to express DLP-1 fragments containing the middle domain or the GED deleted in order to study directly the effects of these two domains on protein tetramerization. Unfortunately, all of the cloned and expressed species were found in insoluble inclusion bodies, despite all attempts to optimize the expression conditions. This may suggest that loss of either the middle or GED domain exposes substantial amount of hydrophobic surface to solvent, which then results in protein aggregation. Since the isolated GTPase domain itself is a monomer, and the PH-like domain does not seem to be essential for the protein tetramerization, our results may suggest quite important roles for the middle and GED domains in DLP-1 tetramerization.

Liposome-binding properties

Wild-type DLP-1 has been reported to bind lipid membranes *in vitro*, and can eventually cause them to form tubules (*22*). We investigated whether the mutant DLP-1 species impaired the liposome-binding properties. In a liposome-binding assay, DLP-1 or mutants were incubated with liposomes, and after centrifugation, distribution of bound (in the pellet fraction) and non-bound (in the supernatant fraction) protein was analysed by SDS–PAGE. The control experiment using blank buffer without liposomes showed that DLP-1 or mutants were distributed exclusively in the centrifugal supernatant fraction (data not shown). However, when incubated with liposomes, the wild-type DLP-1, as well as the G350D, R365S and ΔPH mutations, were dominantly fractionated in the pellet fraction (Fig. 3H), indicating that the wild-type DLP-1 binds membranes and that those mutant forms of DLP-1 did not display significant changes in the liposome-binding properties of the protein. Since these mutants also retained proper tetrameric states (Fig. 3 and Table I), it is likely that the quaternary protein structure is one of the factors required for the effective membrane binding.

Putative models for the molecular interactions disrupted by DLP-1 mutants

Protein sequence analysis of DLP-1 showed that the consensus motifs required for GTP/GDP binding and hydrolysis are located in the N-terminal 300 amino acids (the isolated GTPase domain, data not shown). Moreover, the crystal structure of hGBP1 (*9*), a protein in the same family with DLP-1, does not reveal any directly interactions between the GTPase and middle domains. However, the hGBP1 structure clearly demonstrated that the GED folds back to strongly interact with the middle domain and reaches to the GTPase domain, probably to coordinate and stimulate GTPase activity. Furthermore, the structure of human dynamin from electron microscope supported the similar protein organization (*10*) and yeast two-hybrid results also suggested the interactions between the GED and middle domains (*11–13*). Therefore, the overall domain organization of DLP-1 has been commonly considered as that described in Fig. 4A. The G350D and R365S mutations possibly cause conformational changes within the middle domain of DLP-1, which interfere with the intra-molecular interactions, such that the GTPase and GED domains no longer interact appropriately, and subsequently affect protein GTPase activity (Fig. 4B). Given the fact that the two residues are relatively close together, the similar low GTPase activities exhibited by both point mutations may indicate that they have elicit the same functional or structural effect.

The unaltered tetrameric state in the middle domain mutants support the hypothesis that the reduction of GTPase activation in the G350D and R365S mutants arises from the loss of intra-molecular interactions mediated by the middle domain. How exactly the G350D and R365S mutations disrupt the middle domain interactions with the GED and GTPase leading to reduced GTPase activity is unclear. The reduction of GTPase activity caused by G350D and R365S mutations in the middle domain without the loss of the tetrameric state is similar to the phenotypes reported for the K679A mutation in the GED domain of DLP-1 (*12*). Because of complementary intra-molecular

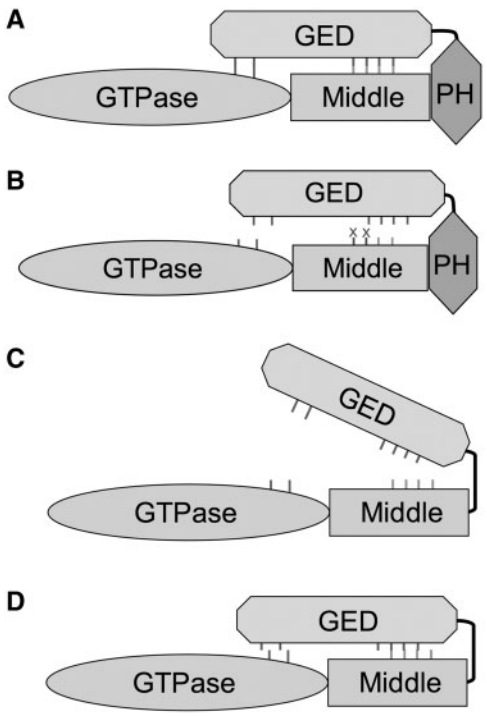

Fig. 4 Schematic models of putative DLP-1 intra-molecular interactions. In wild-type DLP-1 (A), the GTPase effector domain (GED) forms close and specific interactions with the middle and GTPase domains to enhance GTPase activity. However, G350D and R365S mutations, shown as red X's in (B), disrupt these interactions, resulting in significantly reduced GTPase activity. For ΔPH, the missing PH-like domain (PH) could result in a substantial loss of inter-domain interactions (C) or in the formation of a more compact, but inactive domain arrangement (D).

interactions between the middle domain and the GED, it would be expected that mutations on either side would disrupt the similar interactions and cause similar defective 'open' state (Fig. 4B). These parallel phenotypes of mutations in adjacent domains provide further evidence that the middle domain interacts with the GED. Taking the activities and protein tetramerization results of the G350D and R365S, our findings support the idea that proper intra-molecular interactions with the middle domain are important for normal GED functions on DLP-1 activity.

It is hard to interpret the result that the deletion of the PH-like domain abolished all significant GTPase activity as the GTPase domain is intact in the DLP-1 ΔPH. A possible interpretation is that deletion of the PH-like domain also disrupts the GED interactions with the middle and GTPase domains. This structural change may cause the GED domain to lose all interactions with the GTPase domain (Fig. 4C) or to interact tightly, but non-specifically with the GTPase domain, thus blocking the GTPase active site (Fig. 4D). To test these two hypotheses, a limited proteolysis experiment was performed to compare the proteolytic patterns of the full-length protein and the DLP-1 ΔPH deletion mutant. The full-length DLP-1 was digested by proteases trypsin, chymotrypsin and proteinase K leaving a protease-resisting 49 kDa fragment (Supplementary Figs S1A and S2). Mass spectrometry analysis combined with N-terminal sequencing identified this fragment to be from residue 82 to 516. The residue 82 is located to the GTPase active site, and residue 516 is in the PH-like domain. Since the PH-like domain was deleted in the DLP-1 ΔPH, the residue 516 is no longer a site of proteolytic cleavage. If the GTPase active site is blocked by inappropriate interactions with the GED domain, then the residue 82 might no longer be accessible to proteases. The limited proteolysis results showed that the DLP-1 ΔPH was indeed more resistant to proteolysis than the full-length protein (Supplementary Fig. S1B), which is consistent with our hypothesis that blocking of the active site abolished GTPase activity in DLP-1 ΔPH (Fig. 4D), rather than the loss of all interactions (Fig. 4C).

Summary and perspective

Recombinant DLP-1 wild-type protein and mutant species were expressed and purified from *E. coli* to study the roles of key residues and specific domains in protein biochemical and biophysical properties. The middle domain mutations G350D and R365S, and ΔPH affect GTPase activity, but have no significant impact on protein tetrameric state and liposome-binding properties. These mutants are likely to affect protein activity by disrupting proper intra-molecular interactions. Our study provides insights into the roles of the middle and PH-like domains in DLP-1 activation towards better understanding of mitochondrial dynamics. DLP-1 is believed to be recruited by integral membrane protein Fis1 to the mitochondrial outer membranes and form a complex for mitochondrial division in a GTPase activity-dependant manner (*7*, *8*). Since the G350, R365, and the integrity of the PH-like domain are important for the DLP-1 GTPase activity, it is likely that mutations on these residues or domain would affect normal function of the mitochondrial fission complex. Although the mutations had no much impact on protein tetramerization, it is possible that subtle structural differences caused by the mutations affect interactions of DLP-1 with Fis1 or other components of the fission complex. High resolution crystal structure of DLP-1 would help determine detail protein organization and provide critical information on its function in mitochondrial fission.

SUPPLEMENTARY DATA

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

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