Published in final edited form as:
Mol Microbiol. 2012 July ; 85(2): 271–281. doi:10.1111/j.1365-2958.2012.08110.x.

Mechanism of the asymmetric activation of the MinD ATPase by MinE

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Summary

MinD is a component of the Min system involved in the spatial regulation of cell division. It is an ATPase in the MinD/ParA/Mrp deviant Walker A motif family which is within the P loop GTPase superfamily. Its ATPase activity is stimulated by MinE, however, the mechanism of this activation is unclear. MinD forms a symmetric dimer with two binding sites for MinE, however, a recent model suggested that MinE occupying one site was sufficient for ATP hydrolysis. By generating heterodimers with one binding site for MinE we show that one binding site is sufficient for stimulation of the MinD ATPase. Furthermore, comparison of structures of MinD and related proteins led us to examine the role of N45 in the switch I region. An asparagine at this position is conserved in four of the deviant Walker A motif subfamilies (MinD, chromosomal ParAs, Get3 and FleN) and we find that N45 in MinD is essential for MinE stimulated ATPase activity and suggest that it is a key residue affected by MinE binding.

Introduction

The Min system in *E. coli* contributes to the spatial regulation of division by preventing Z ring assembly away from midcell (Lutkenhaus, 2007). It consists of 3 proteins, MinC, MinD and MinE that undergo a coupled oscillation between the poles of the cell. MinD and MinE constitute the oscillator and MinC is a passenger in the oscillation through interaction with MinD (Hu and Lutkenhaus, 1999; Raskin and de Boer, 1999a, b). The MinCD complex is a potent inhibitor of Z ring assembly and the oscillation produces a time averaged concentration of this inhibitor on the membrane that is lowest at midcell where the Z ring forms (de Boer et al., 1990; Hu and Lutkenhaus, 2003; Meinhardt and de Boer, 2001). In the presence of ATP, MinD and MinE form traveling waves on a planar lipid layer that resemble the *in vivo* oscillation indicating that interactions between the MinD and MinE proteins and the membrane are sufficient to produce dynamic pattern formation (Ivanov and Mizuuchi, 2010; Loose et al., 2008).

The Min oscillation is fueled by ATP hydrolysis by MinD, which is stimulated by MinE (Hu and Lutkenhaus, 2001). MinD dimerizes in the presence of ATP and binds to the membrane through a C-terminal amphipathic helix (Hu and Lutkenhaus, 2003; Lackner et al., 2003; Szeto et al., 2002; Zhou and Lutkenhaus, 2003). MinC and MinE compete for overlapping sites located at the MinD dimer interface, however, MinE is able to displace MinC and stimulate ATP hydrolysis resulting in the release of MinD from the membrane (Hu et al., 2002; Hu et al., 2003; Lackner et al., 2003; Wu et al., 2011). To interact with MinD, MinE must switch from the latent (6 β-strands) to the active (4 β-strands) form, which releases the

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MinD binding regions sequestered at the MinE dimer interface (Park et al., 2011). This conformational rearrangement of MinE also releases cryptic N-terminal amphipathic helices that interact with the membrane (Park et al., 2011; Shih et al., 2011).

Although MinD is an ATPase, it is a member of the SIMIBI division of the large P loop GTPase superfamily (Leipe et al., 2002). It, along with ParA, Soj, FleN, ArsA, Get3, NifH and others, belong to the deviant Walker A motif family. These proteins undergo ATP dependent dimerization aided by the "signature lysine" (K11 in MinD) in this motif that spans the dimer interface and contacts ATP bound to the other subunit (Hester and Lutkenhaus, 2007; Leonard et al., 2005; Lutkenhaus and Sundaramoorthy, 2003; Wu et al., 2011; Zhou et al., 2005). Study of nucleotide hydrolysis by G proteins, members of the other division (TRAFAC) of the large P loop superfamily, indicates that efficient hydrolysis requires activation of a water molecule and neutralization of the negative charge developing during the transition state. These steps can be achieved by a variety of mechanisms involving various amino acids (Bos *et al.*, 2007). One well-characterized mechanism used by Ras and Rho employs an arginine finger to neutralize the developing negative charge and a glutamine to activate the water molecule.

Although the signature lysine promotes dimerization by coordinating the α- and γ-phosphate of ATP bound to the other subunit and functions like the arginine finger in G proteins, it is not sufficient to cause hydrolysis as MinE is still required. Also, the water molecule for nucleophilic attack of the γ-phosphate of ATP is activated by an aspartate residue (D40 in MinD) (Hayashi et al., 2001). An alanine for aspartic acid residue substitution of this key residue results in mutants in the case of Soj and MinD that lack ATPase activity and crystallize as ATP dependent dimers (Leonard et al., 2005; Wu et al., 2011).

Although ATP hydrolysis by MinD is stimulated by MinE the mechanism is not clear. Previous suggestions that MinE stimulates hydrolysis by causing release of the signature lysine (Ma et al., 2004) or acting as an arginine finger (Barilla et al., 2007) were ruled out by the structures of MinD and the MinD-MinE peptide complex (Park et al., 2011; Wu et al., 2011). Furthermore, a MinD mutant unable to bind the membrane is not stimulated by MinE suggesting that binding to phospholipids alters the structure of MinD making it competent for MinE induced ATP hydrolysis (Hu and Lutkenhaus, 2003).

MinD forms a symmetric dimer and in the crystal structure of the MinD-MinE complex MinE is observed on each side of the MinD dimer (Park et al., 2011). However, in the "Tarzan of the Jungle" model, which was proposed to account for the coupled behavior of MinD and MinE during oscillation, it was suggested that asymmetric binding of MinE to MinD (single occupancy of the symmetric MinD dimer by MinE) was sufficient to stimulate ATP hydrolysis and the release of MinD from the membrane. In this paper we test this asymmetric stimulation hypothesis through the generation of MinD heterodimers that only have one intact binding site for MinE. Furthermore, we compare the crystal structures of MinD and other related proteins to try and understand the mechanism of MinE-stimulated ATP hydrolysis. This analysis led us to MinD residue N45, which we find is critical for ATP hydrolysis and likely affected by MinE binding.

Results

Structural comparisons of MinD and Get3 implicate residue N45 in MinE stimulated MinD ATP hydrolysis

The structures of MinD and the MinD-MinE peptide complex, as well as various related proteins, were compared to get insight into the mechanism of MinE stimulated MinD ATP

hydrolysis. The structure of $MinD^{D40A}\Delta10$ (Wu et al., 2011) represents the prehydrolysis step (PDB 3Q9L) since MinD is a tight dimer and contains bound ATP and Mg^{+2} (referred to as free MinD). The signature lysine (K11) spans the dimer interface and contacts the α– and γ-phosphates of ATP bound to the other subunit. In the $MinD^{D40A}\Delta10$-MinE $peptide^{12–31}$ complex (Park et al., 2011) the ATP has been hydrolyzed and only ADP is visible (PDB 3R9I) (referred to as the MinD-MinE peptide complex). However, the MinD dimer in this complex is slightly more compact as the subunits have moved together by ~1.5 Å with the signature lysine contacting the α– and β-phosphates of ADP in the other subunit (Fig. 1S). Since crystallization was in the presence of ATP, hydrolysis must have occurred following crystallization but MinD is trapped in a tight dimer conformation.

Further comparison between free MinD and the MinD-MinE peptide complex revealed additional differences in the MinD structures, most of which are to accommodate MinE peptide binding, but some could also be due to the loss of the γ-phosphate (Fig. 1A). Several MinD residues move to accommodate R21 of MinE. R151 and R54 move to allow E53 to move into position so that its side chain can form hydrogen bonds with R21 of MinE. R21 also makes hydrogen bonds to the main chain carbonyls of MinD residues L48, S221 and N222. In free MinD residue E146 spans the dimer interface to contact the ribose ring, however, it no longer contacts the ribose ring in the MinD-MinE peptide complex and instead contacts S149 and S221 (Fig. 1B and data not shown). This latter contact may stabilize the interaction of R21 with S221.

The orientation of the highly conserved switch I residue N45 in the free MinD dimer is the same as in the MinD monomer (PDB:1G3R), although in the dimer N45 contacts S148 in the other subunit. In the structure of the MinD-MinE peptide complex N45 has moved away from S148 and has rotated upwards and is near D152 in the other subunit (Fig. 1B). Alignment of various members of the deviant Walker A family revealed an Asn in this position is conserved in MinD, FleN, chromosomal ParAs and Get3, whereas the equivalent position in other members varies but is often a serine or threonine (Fig. 1D). In the structure of the closely related Soj dimer (PDB:2BEK) the equivalent Asn is near the γ-phosphate of ATP and in the structure of Get3 (PDB:2WOJ) in the transition state (with ADP and AlF_4^-) the equivalent Asn (N61), in addition to the signature lysine, contacts AlF_4^- suggesting that it participates in the ATPase reaction, likely functioning to stabilize the transition state for hydrolysis (Mateja et al., 2009). Superimposing the Get3 structure with the two MinD structures revealed that the signature lysines overlap and residues N45/N61 are in similar positions (Fig. 1C). We therefore tested if N45 of MinD is important for ATP hydrolysis.

Previous examination of the MinD-N45A mutant revealed that it activated MinC as efficiently as WT MinD indicating it dimerized, bound ATP and the membrane (Zhou et al., 2005). ATP-dependent dimerization was confirmed by gel chromatography (data not shown). To determine the response of MinD-N45A to MinE, it was purified and tested in vitro. MinD-N45A bound to phospholipid vesicles in an ATP dependent manner and was able to recruit MinC (Fig. 2A). The addition of MinE displaced MinC while most of MinD-N45A remained on the membrane, suggesting MinE was unable to stimulate the ATPase activity of this mutant. Consistent with this MinE was recruited to the MinD-vesicle complex. Direct measurement of the ATPase activity confirmed that the ATPase activity of MinD-N45A, like that of MinD-D40A, is poorly stimulated by MinE (Fig. 2B). Thus, residue N45 of MinD affects MinEs' ability to stimulate MinD's ATPase activity.

Critical role of MinE residue R21

Residue R21 is absolutely conserved in MinE indicating it is likely to have a key role, although not as an arginine finger as suggested earlier (Barilla et al., 2007), since it is not near the γ-phosphate. In fact the signature lysine (K11) occupies the same position as the

arginine finger in the Ras-GAP complex (see discussion). In the MinDMinE peptide complex the R21 residue interacts with residues located on only one of the two MinD subunits (Fig. 1A). To more fully characterize the role of R21 we generated the MinE-R21A mutant. This mutant still bound to MinD in the bacterial 2-hydrid system, although the interaction was slightly reduced (Fig. 3A). The purified MinE-R21A mutant failed to stimulate the ATPase activity of MinD even at an elevated concentration that should overcome the binding deficiency (Fig. 4 and (Ghasriani et al., 2010)). Thus, R21 of MinE makes some contribution to MinD binding but has a critical role in stimulating the MinD ATPase. This result is consistent with the observation by Hsieh et al. (Hsieh et al., 2010) who found that the K19E/R21G double mutant failed to stimulate ATP hydrolysis by MinD although binding to MinD was only reduced by 50%.

MinE activation of MinD ATPase is asymmetric

To test whether MinE activation of the MinD ATPase requires MinE binding to both sides of the symmetrical MinD-ATP dimer we took advantage of MinD mutants that do not bind MinE. As previously shown with the bacterial 2-hybrid system, MinD mutants $MinD^{E53A}$ and $MinD^{N222A}$ do not interact with MinE although they both interact with MinC (Park et al., 2011). To ensure that MinD did not interact with MinE, we used the double mutant $MinD^{E53A/N222A}$. Bacterial 2-hydrid tests confirmed that this double mutant interacted with $MinD^{WT}$ (i.e. dimerizes [Fig. 3B]) but did not interact with MinE (Fig. 3A). It also binds the membrane and activates MinC (data not shown). Consistent with our expectation, the basal ATPase activity of the double mutant was minimally stimulated by MinE whereas the ATPase activity of the $MinD^{WT}$ was stimulated as shown previously (Hu and Lutkenhaus, 2001)(Fig. S2). MinD heterodimers that can only bind one MinE peptide were generated by incubating $MinD^{WT}$ with $MinD^{E53A/N222A}$ and adding ATP (Fig. 5A). In an initial experiment incubation of 4 μM $MinD^{WT}$ with 4 μM $MinD^{E53A/N222A}$ resulted in a rate of ATP hydrolysis equivalent to that observed with 8 μM $MinD^{WT}$ (Fig. S2). This result is consistent with $MinD^{WT}$ and $MinD^{E53A/N222A}$ forming a heterodimer and ATP bound to each subunit in the heterodimer undergoing hydrolysis.

To confirm this finding, we examined the effect of increasing the ratio of $MinD^{E53A/N222A}$ to $MinD^{WT}$. If our interpretation is correct, an excess of the mutant protein over the WT should not further stimulate the ATPase as the activity should be limited by the amount of $MinD^{WT}$ available to form heterodimers. Consistent with this prediction, a two or three fold excess of $MinD^{E53A/N222A}$ over WT MinD did not further stimulate the overall rate of ATP hydrolysis (Fig. 5B). This result indicates that the total enzyme activity is limited by the amount of $MinD^{WT}$ available in the reaction. This result is also consistent with all $MinD^{WT}$ subunits present as heterodimers and both subunits (mutant and WT) in heterodimers being stimulated by MinE to undergo ATP hydrolysis.

ATP hydrolysis by the subunit contacted by R21 is not required for the coupled MinD activation

The above results demonstrated that MinE binding to only one side of a MinD dimer was sufficient to stimulate ATP hydrolysis by both subunits. Although MinE binds at the dimeric interface of MinD, the interaction of MinE with the two subunits is not equivalent (Fig. 1A). As noted, the interaction of R21 of MinE is limited to residues located on one subunit of the MinD dimer. It is likely that the interaction of R21 with these residues (E53, L48, S221 and N222) in the $MinD^{WT}$ subunit in the heterodimer (Fig. 1A) initiates conformational changes leading to ATP hydrolysis by $MinD^{WT}$, which is coupled to the hydrolysis of ATP by the $MinD^{E53A/N222A}$ subunit.

To examine whether the activation of $MinD^{E53A/N222A}$ subunit within the heterodimer requires ATP hydrolysis by the WT subunit contacted by R21, we incubated an excess of the catalytic deficient mutant $MinD^{D40A}$ with $MinD^{E53A/N222A}$. $MinD^{D40A}$, like $MinD^{E53A/N222A}$, is poorly stimulated by MinE (Fig. 2B and 6A). In the heterodimer R21 contacts the D40A subunit (which can not hydrolyze the bound ATP) and any ATP hydrolysis must be due to the $MinD^{E53A/N222A}$ subunit. The ATPase activity of the reaction containing $MinD^{D40A}$ and $MinD^{E53A/N222A}$ is stimulated by MinE to a similar extent as $MinD^{WT}$ homodimer (Fig. 6A). The observation that ATPase was stimulated confirms that heterodimers form, since either mutant alone is not stimulated by MinE. This result suggests that the R21 residue of MinE interacts with the $MinD^{D40A}$ subunit ultimately leading to ATP hydrolysis by the $MinD^{E53A/N222A}$ subunit. This finding indicates that the $MinD^{D40A}$ subunit within the heterodimer undergoes the necessary conformational changes that occur upon MinE binding that is necessary for the activation of $MinD^{E53A/N222A}$ even though it is unable to hydrolyze ATP.

ATP hydrolysis by the MinD subunit contacted by R21 occurs in the absence of hydrolysis by the other subunit

To further explore the MinE activation of MinD we wanted to test if the ATPase activity of the subunit interacting with R21 of MinE requires ATP hydrolysis by the other subunit. For this purpose, $MinD^{WT}$ was incubated with excess amount of a catalytic deficient version of the $MinD^{E53A/N222A}$ mutant, $MinD^{D40A/E53A/N222A}$ (designated TM) to generate the $MinD^{WT}$-$MinD^{D40A/E53A/N222A}$ heterodimer. The ATPase activity of a reaction containing $MinD^{WT}$ and $MinD^{D40A/E53A/N222A}$ was similar to that of the $MinD^{WT}$ homodimer (Fig. 6B). This result indicates that MinE can stimulate hydrolysis of ATP bound to $MinD^{WT}$ in the absence of ATP hydrolysis by the $MinD^{D40A/E53A/N222A}$ subunit.

Discussion

MinD, like other P loop GTPase family members, uses a nucleotide switch to regulate its behavior (Wittinghofer and Vetter, 2011). In the ATP form it is a dimer that binds to the membrane, allowing it to take up residency in the cell, whereas in the ADP form it is a monomer that diffuses in the cytoplasm (Wu et al., 2011). MinE regulates this switch by binding to the membrane bound MinD dimer and stimulating ATP hydrolysis (Hu and Lutkenhaus, 2001).

For some P loop GTPases not all catalytic residues are present until an arginine finger is provided by a GAP (GTPase activating protein). However, trimeric G proteins such as transducin have all the catalytic residues and are activated by RGS proteins through stabilization of a key glutamine residue that coordinates a water molecule for nucleophilic attack and helps to stabilize the leaving group (Lambright et al., 1994; Sondek et al., 1994). This same principle applies to GTPases in the SIMIBI class of P loop GTPases, such as FlfH and SRP (Bange et al., 2011). In these latter cases all of the catalytic residues are brought together by dimerization (mechanistically referred to as GAD for GTPase activation by dimerization) with both subunits of the dimer contributing residues. However, GTPase activity is stimulated by an activator that stabilizes the catalytic center (Wittinghofer and Vetter, 2011). MinD and other deviant Walker A motif members resemble this latter group as dimerization brings together all the residues necessary for catalysis. How then does MinE stimulate hydrolysis?

In this study we found that the R21 residue of MinE is essential for stimulation of the MinD ATPase. Furthermore, the *minE-R21A* mutation separates MinE binding from stimulation, since this mutation has only a modest affect on the binding of MinE to MinD but markedly reduces the stimulation. This deficiency can not be overcome by increasing the

concentration of the MinE-R21A mutant, in contrast to another MinE mutant (MinE-I25R) that has reduced affinity for MinD but whose deficiency in stimulation can be overcome by increased levels of the mutant protein (Ghasriani et al., 2010).

A critical asparagine

Comparison of the structure of the MinD dimer to the structure of MinD in the MinD-MinE peptide complex indicated these structures represent the pre and post hydrolysis states respectively and revealed a change in the orientation of N45. This conformational change of N45, along with the equivalent residue (N61) in Get3 contacting AlF_4^- in the transition state complex (Mateja et al., 2009) suggested N45 could have a role in ATP hydrolysis. Our results show that N45 is essential for MinE stimulated ATP hydrolysis by MinD. The N45A mutant binds ATP, dimerizes and binds MinE but its ATPase is not stimulated. Together this suggests the following scenario (Fig. 7).

In the MinD monomer the signature lysine (K11) is restrained through a hydrogen bond with S148 and electrostatic interactions with D152 and E146 (Hayashi et al., 2001). Upon ATP binding K11 is released from these residues and promotes dimerization by contacting the α– and γ–phosphates of ATP in the other subunit (Wu et al., 2011), while S148 is occupied by N45 from the other subunit. Upon MinE binding R21 contacts the side chain of E53 and the backbone carbonyl of L48, S221 and N222 of MinD that leads to N45 of MinD reorienting to join the transition state by contacting the γ-phosphate as observed in the Get3-ADP-AlF_4^- structure. Following ATP hydrolysis N45 may contact D152, however, as the dimer dissociates K11 again makes a hydrogen bond to S148 and interacts electrostatically with D152 while E146 and N45 return to the positions they occupy in the monomer. It is not clear how the hydrolysis of ATP by both subunits is coupled even though MinE primarily influences only one subunit through R21.

Comparison of nucleotide hydrolysis by G proteins and the Deviant Walker A motif family proteins

During evolution of the deviant Walker A motif family from a P-loop GTPase nucleotide specificity switched from GTP to ATP. Comparison of the structure of MinD-E complex and Get3-AlF_4^- to Ras-GAP-AlF_3 (Scheffzek et al., 1997) is illustrative. Ras and heterotrimeric G proteins contain a catalytic glutamine residue that activates the attacking water molecule and with the aide of two positively charged residues, the Walker A motif lysine and the arginine finger, stabilizes the transition state (Fig. 8). In the “deviant Walker A motif” proteins the water molecule is activated by an aspartic acid residue (Hayashi et al., 2001; Leonard et al., 2005; Mateja et al., 2009) and our study suggests that an asparagine (N45/61) contributes to the stabilization of the transition state along with two positively charged residues, the signature lysine, which overlaps the arginine finger seen in Ras-GAP (Fig. 8), and the conserved lysine in the Walker A motif. Thus, the two roles of the glutamine in G proteins (activation of the water and stabilization of the transition state) appear split between two residues in MinD and Get3 (an aspartic acid and asparagine). We suggest that MinE binding to MinD allows the asparagine to move into position to participate in the transition state.

Asymmetric stimulation of MinD

Our impetus for this study was to test the assumption in the Tarzan of the Jungle model that MinE binding to one side of a MinD dimer is sufficient to stimulate ATP hydrolysis (Park et al., 2011). Our results clearly show that this is the case and, in part, explains the high Hill coefficient (2–4) observed in the stimulation of ATP hydrolysis by a monomeric MinE

activating peptide; one peptide is sufficient to stimulate ATP hydrolysis by both subunits (Ghasriani et al., 2010). Although MinE binds to MinD at the dimer interface, the interaction with the two subunits is very uneven with R21 of MinE interacting with E53, L48, N222 and S221 of one subunit (Park et al., 2011). We found that both subunits in the dimer are able to undergo ATP hydrolysis. In the first example the subunit unable to interact with R21 hydrolyzed ATP when the other subunit interacted with R21. In the second case we found if the catalytic activity of the subunit contacted by R21 was crippled with the D40A substitution the subunit unable to interact with R21 was still able to carry out ATP hydrolysis. This result suggests that MinE binding was able to induce the necessary conformational changes in MinD independent of ATP hydrolysis.

The concept of asymmetric stimulation of a dimeric ATPase is not new. Recently, it was shown that Hsp90 was asymmetrically stimulated by the Aha1 chaperone (Retzlaff et al., 2010). The investigative approach was similar to that used here. Heterodimers were constructed in which one of the subunits was mutated to disrupt Aha1 binding and the other was mutated so that it was catalytic deficient. The result demonstrated that Aha1 binding to one subunit could stimulate hydrolysis in trans. Stimulation in cis was also shown to occur. Although the ATPases are very different, the concept is very similar to what we observed with MinD and MinE.

The MinD/Mrp family has at least 8 subfamilies and includes proteins with diverse functions (Leipe et al., 2002). Among these are those that have an Asn residue conserved at the position of N45 of MinD including FleN associated with polar flagella (also called YlxH, MotR, FlhG or MinD2)(Bange et al., 2011), chromosomal ParAs, and Get3 required for insertion of tail anchored proteins in eukaryotes (Mateja et al., 2009). This conservation of the asparagine (Fig. 1D) indicates it is likely involved in ATP hydrolysis in these subfamilies as well. For at least ParA and MinD the ATP-dependent dimerization results in higher affinity for a surface and asymmetric activation of the ATPase allows rapid and efficient reversal of surface binding. Other members, ParC associated with polar flagellar (Ringgaard et al., 2011), MipZ involved in spatial regulation of division in α-proteobacteria (Thanbichler and Shapiro, 2006), the CpaE/TadZ subfamily associated with Type IV pili (Xu et al., 2011), MRP which is an Fe-S chaperone (Boyd et al., 2009; Hausmann et al., 2005), ArsA involved in arsenite resistance (Fu et al., 2010), Fe-protein (NifH) of the nitrogenase complex (Schindelin et al., 1997) and others do not have an asparagine residue at this position. Considering the remarkable variation in the mechanisms by which small G-proteins are activated by GAPs (Bos et al., 2007) it would not be surprising to see a multiplicity of activation mechanisms among members of the deviant Walker A motif subfamily.

Experimental procedures

Strain and plasmids

The *E. coli* K-12 strain JS964 (MC1061 *malP*::*lacI*^{q} Δ*min*::*kan*) was used in this study (Zhou et al., 2005). A *cya*-null strain BTH101Δ*min* (F^{-} *cya-99*, *araD139*, *galE15, galK16, rpsL1* [Str^{r}], *hsdR2, mcrA1, mcrB1* Δ*min::kan*) was used to assess interactions with the bacterial 2-hybrid system (Wu et al., 2011). The plasmids used in the bacterial 2-hybrid system were pCT25-MinD and pUT18-MinE and were described previously (Park et al., 2011). MinD proteins were expressed from derivatives of pZH115 (*minD*) and pZH115-10 (*minD*Δ*10*) (Wu et al., 2011). MinE-his has a C-terminal 6-histidine tag and was expressed from pJB216h (*minE-his*). This latter plasmid is similar to pJB216 (*minE*) (Park et al., 2011) except a 6X his tag was introduced in frame at the 3-end of the *minE* gene by PCR. Mutations were introduced into these plasmids by site directed mutagenesis to generate the

various alleles (*minD*^{N45A}, *minE*^{R21A}, *minD*$^{E53A\ N222A}$, *minD*^{D40A}, *minD*$^{D40A}\Delta 10$) used in this study. All plasmids were sequenced to verify the desired mutation had been introduced.

Bacterial 2-hybrid analysis

Plasmids carrying wild-type or mutant *minD* and *minE* alleles were transformed into BTH101Δ*min* and grown overnight at 37°C on LB plates containing 0.2% glucose, chloramphenicol (20 μg/ml) and ampicillin (100 μg/ml). Three colonies from each transformation were picked into 300 μl volume of LB broth and spotted onto fresh LB plates supplemented with chloramphenicol (20 μg/ml), ampicilin (100 μg/ml) and 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) at 40 μg/ml and 0.5 mM IPTG. The plates were photographed after 14–18 h of incubation at 30°C.

Structural comparisons

The crystal structure of the MinD dimer (PDB 3Q9L) was compared with structure of the MinD-MinE (12–31) peptide complex (PDB 3R9L). Superpositoin of the two structures was conducted using the program Superpose via the CCP4 interface (Krissinel and Henrick, 2004). For the superposition, chains A and B of the MinD dimer (residues 2-258) were fit to the corresponding residues in the MinD-MinE (12–31) peptide complex. The RMSD deviation between C-alpha atoms was found to be 0.924 Å with a maximum displacement observed at Ile 41 of chain B. An additional superposition was conducted in which all atoms of chain A and B (residues 2–258) were compared. An RMSD deviation of 1.223 Å was observed between all atoms. Additional structures were compared to the MinD dimer. These included Get3 (PDB 2WOJ), Soj (PDB 2BEK), MinD monomer (1G3R), and Ras-GAP (PDB 1WQI).

Multilamellar Vesicle (MLV) Preparation

E. coli phospholipids in chloroform were purchased from Avanti Polar Lipids (Alabaster, Ala). To prepare MLVs, the phospholipids were vigorously mixed and the chloroform was evaporated under constant flow of nitrogen gas. The dried *E. coli* phospholipid powder was thereafter suspended in Buffer A (25 mM Tris-HCl, pH 7.5, 50 mM KCl) at 10 μg/μL and incubated at 65°C for 2 hours with occasional vortexing. All aliquots of the MLV were stored frozen at −80°C.

MinD binding to MLVs

MinD^{N45A} (6 μM) was incubated with MLVs (400 μg/ml) in ATPase buffer (25 mM Tris-HCl [pH 7.5], 50 mM KCl, 5 mM $MgCl_2$). MinE (6 μM) and MalE-MinC (6 μM) was added along with 1 mM ATP or ADP and incubated at 24°C for 20 min. The vesicles were collected by centrifugation and the pellets analyzed by SDS-12% polyacrylamide gel electrophoresis.

MinD Purification

MinD$^{D40A}\Delta 10$ was purified from JS964 (Δ*min*) containing pZH115-40 Δ10. MinD^{WT}, MinD^{D40A} and MinD^{N45A} were purified from JS964 (Δ*min*) containing pZH115, pZH115-40 and pZH115-45, respectively (Zhou et al., 2005). JS964 (Δ*min*) carrying the expression plasmid was grown in 1 liter of LB containing ampicillin (100 μg/ml) and induced with 1 mM IPTG for 2–3 hours. Cell pellet was resuspended in buffer B (25 mM Tris-HCl [pH 7.5]), 20 mM NaCl, 1 mM EDTA, 2 mM DTT and 10 % glycerol) and lysed with a French press. For MinD proteins lacking the MTS (Δ10 versions) the clarified lysate was loaded on a DEAE column and MinD$^{D40A}\Delta 10$ eluted with a 60–120 mM NaCl gradient in buffer A. The peak fractions were pooled and run over a HiLoad Superdex 200 column in buffer B (10 mM HEPES-NaOH [pH7.0], 20 mM NaCl, 10% glycerol). The peak fractions

were collected and concentrated with a Vivaspin 20 (MW cutoff of 10 kDa) to ~10 mg/ml. The purification of full-length wild-type and mutant MinD proteins was carried out using the same method described above except a 20–120 mM NaCl gradient in Buffer A was used for the elution from a DEAE column. Also, the peak fractions from the Superdex column were not concentrated.

MinE Purification

For MinE-his purification, JS964 (Δ*min*) cells containing pJB216-h were grown at 37 °C in LB medium to an OD_{540} of approximately 0.4. IPTG (1mM) was then added and after 3 hours of incubation with IPTG, the cells were harvested by centrifugation and stored frozen at −80 °C. The cells were thawed on ice after suspension in buffer A (50 mM Tris-HCl, pH7.5, 100 mM NaCl, 10 mM imidazole). The cells were lysed by three passages through a French press at 16,000 psi and the supernatant was obtained after centrifugation at 12,000 × g for 30 min at 4°C. The supernatant was applied to a nickel affinity column (Qiagen) and proteins were eluted with buffer B (50 mM Tris-HCl [pH 7.5], 500 mM NaCl, 250 mM imidazole). The eluted protein samples were pooled and dialyzed against buffer C (25 mM HEPES-NaOH, pH7.0, 250 mM NaCl, 1 mM EDTA, 5 mM DTT) prior to final storage at −80 °C.

Measurement of MinD ATPase Activity

The hydrolysis of ATP was measured using the ATPase colorimetric assay kit that monitors the release of inorganic phosphate (P_i) (Innova Biosciences). The assay was performed according to the manufacturer's instructions with a minor modification to scale down reaction volumes. In a typical reaction MinD (2–4 μM) and MinE (4 μM) were mixed in reaction buffer (25 mM Tris-HCl, pH7.5, 50 mM KCl, 5 mM $MgCl_2$). After adding ATP (1 mM) and multilamellar vesicles (MLVs) (400 μg/ml), the reaction mixture was incubated at room temperature for 30 min. Once the reaction was quenched, the OD_{595} was recorded and the total enzymatic activity was quantified. To determine the ATPase activity of MinD heterodimers the two MinD proteins were mixed in the buffer before adding the MLVs. The ability of MinE-R21A mutant to stimulate The ATPase activity of MinD was assessed using this assay.

Supplementary Material

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Acknowledgments

We thank Todd Holyoak for reading the manuscript. This work was supported by Grant GM29764 from the National Institutes of Health.

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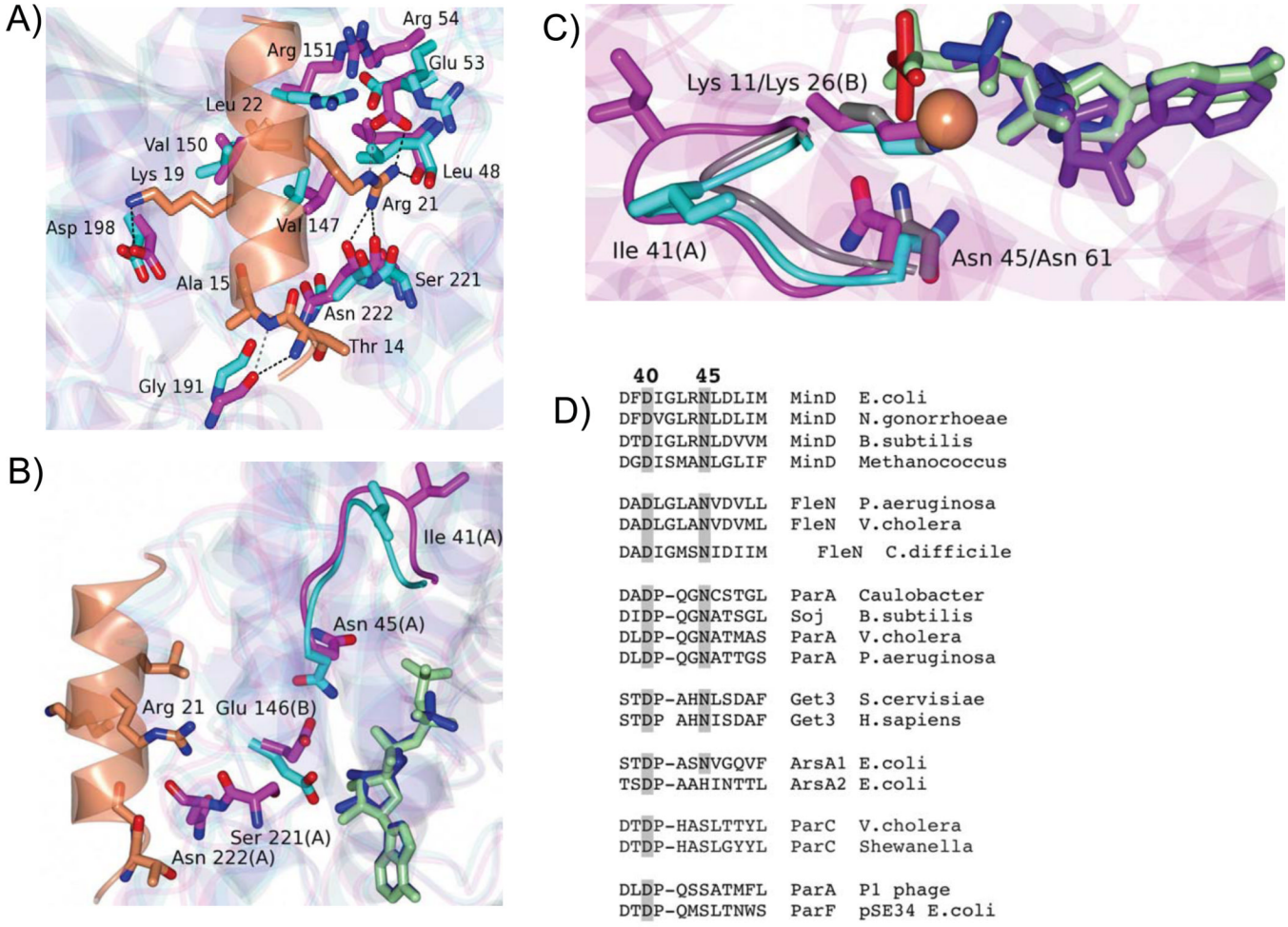

Fig. 1.

The impact of MinE binding on the structure of MinD and comparison to other deviant Walker A motif members. The structures analyzed were $MinD^{D40A}\Delta10$ (3Q9L), $MinD^{D40A}\Delta10$ with bound MinE peptide (12–31) (3R9I) and Get3 in the transition state containing ADP and AlF_4^- (2WOJ). (A) Residues in MinD move to accommodate R21 of MinE. Depicted are MinE (orange), free MinD (cyan) and MinD (magenta) from the complex with the MinE peptide. The dotted lines indicate interactions between residues. (B) Comparison of the ATP binding region from the MinD-MinE peptide (12–31) complex to the free MinD dimer (cyan) reveals that the orientations of N45 and E146 are altered in the MinD-E peptide complex. In the free MinD dimer, E146 forms hydrogen bonds with an ATP molecule (green). However, in the MinD-MinE (12–31) peptide complex, E146 rotates away from the ADP molecule (dark blue) and forms a hydrogen bond with S149 (not shown) and a close contact of 3.8Å with S221 which in turn forms a hydrogen bond with R21 of MinE. N45 in the free MinD dimer hydrogen bonds a water molecule that interacts with a Mg^{2+} ion and moves away from the ADP molecule in the MinD-MinE (12–31) peptide complex to form a hydrogen bond with Asp 152 (not shown). In this position it would be near the γ-phosphate if it was present. (C) Location of N45/N61 and K11/K26 of MinD and Get3, respectively, with respect to ATP. The ATP binding regions of MinD-MinE (12–31) peptide complex (magenta), the free MinD dimer (cyan) and GET3 (gray, PDB: 2WOJ) were superimposed. The ATP molecule in the free MinD dimer (green), ADP in the MinD-MinE (12–31) peptide complex (blue) and ADP in GET3 (purple) are shown. The

latter also contains AlF_4^- (red) and an Mg^{2+} ion (orange sphere). The signature lysine from MinD and Get3 (gray) overlap and the position of N61 of Get3 is between the positions of N45 observed in free MinD and the MinD-E peptide complex. (D) Alignment of residues in the Switch I region from MinD and several other deviant Walker A family members. Only a small sample of the sequences examined is shown. The positions of D40 and N45 in MinD are indicated. These residues are conserved in MinD, FleN, Get3 and chromosomal ParAs, however, Asn is not conserved in plasmid ParAs. It is also not conserved in ParC, which is closely related to ParA, but involved in location of chemotaxis proteins (Ringgaard et al., 2011).

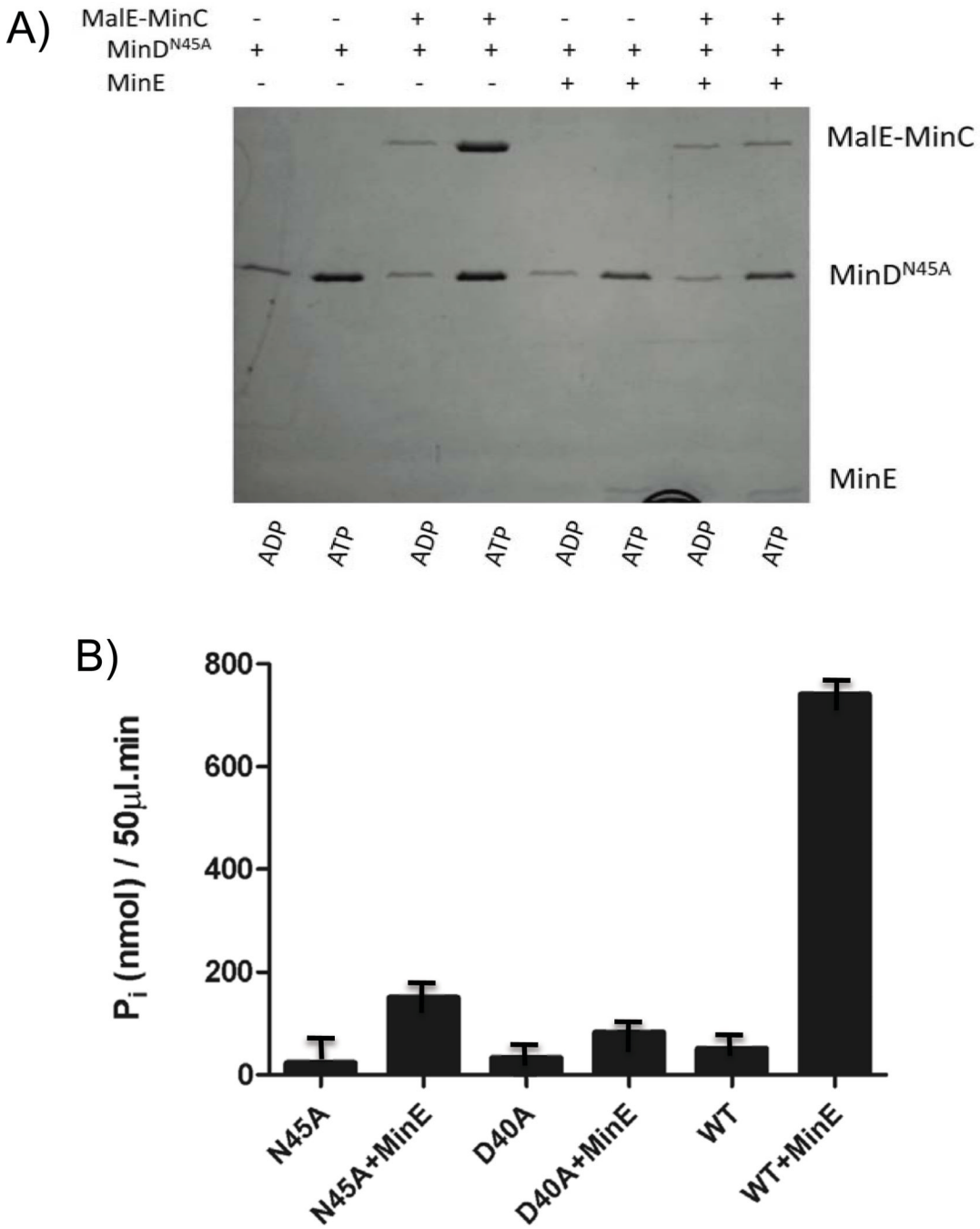

Fig. 2.

Analysis of the interaction of the MinD-N45A mutant with MinE. (A) The binding of MinD-N45A to the membrane, MinC and MinE was determined by phospholipid vesicle sedimentation assay *in vitro*. MinD-N45A (4 µµM) was incubated with phospholipid vesicles (400 µg/ml) in the presence of ADP or ATP (1 mM). MalE-MinC (4 µM) or MinE (4 µM) was added as indicated and the vesicles collected by centrifugation. Proteins bound to vesicles were assessed by SDS-PAGE. (B) MinD-N45A is deficient in MinE stimulated ATP hydrolysis. MinD-N45A, MinD or MinDD40A (4 µM) was incubated with phospholipid vesicles (400 µg/ml), ATP (1 mM) and MinE (4 µM) and the release of phosphate was assessed.

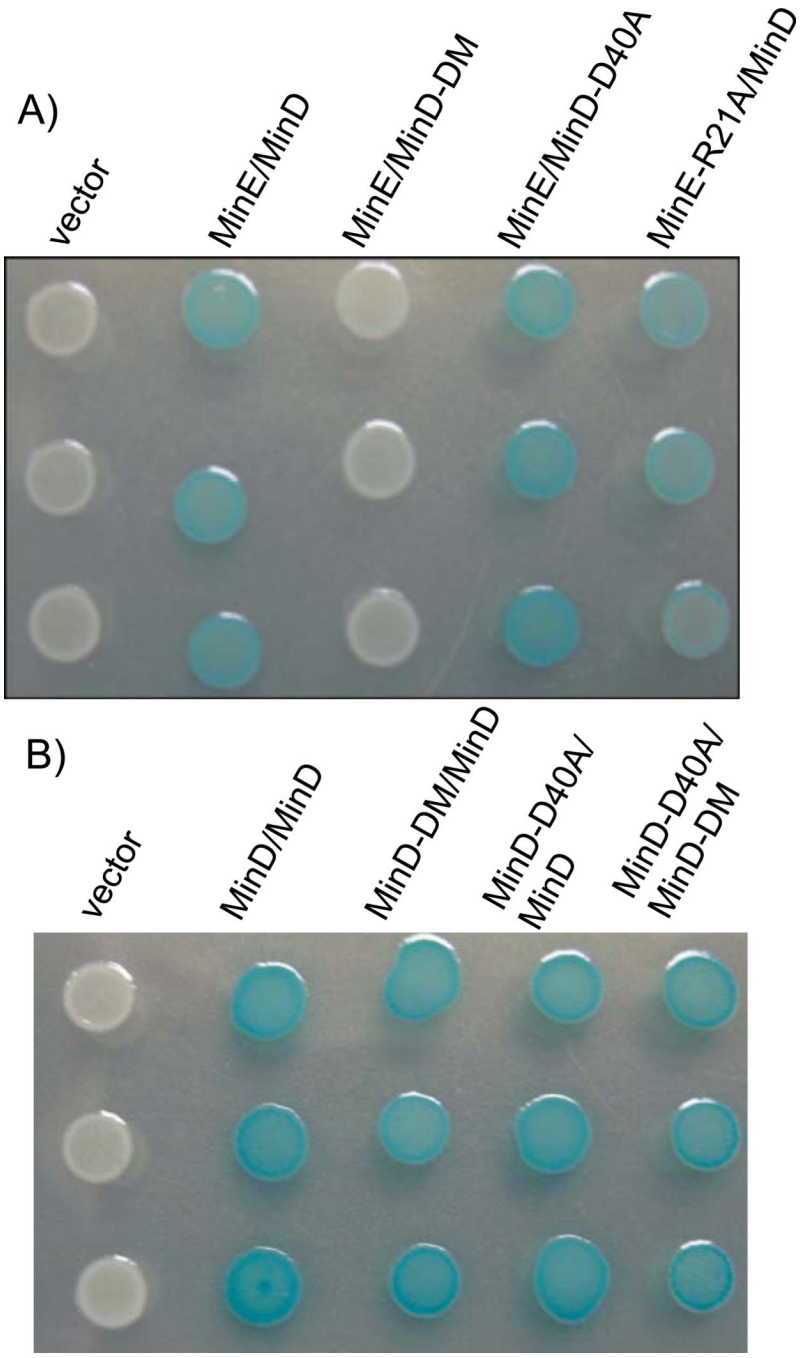

Fig. 3.

Assessment of MinD mutants for interaction with MinE and self interaction. (A) The interaction between MinE and MinD was assessed with the bacterial 2-hybrid system. Note the last column assesses the interaction between $MinE^{R21A}$ and MinD. For each sample three colonies were picked from the plasmid transformation into media and 100 μl of each spotted on indicator plates. (B) The interaction of various MinD mutants with $MinD^{WT}$ was assessed. Note the last column assesses the interaction between two MinD mutants: $MinD^{D40A}$ and the double mutant (DM), $MinD^{E53A\ N222A}$.

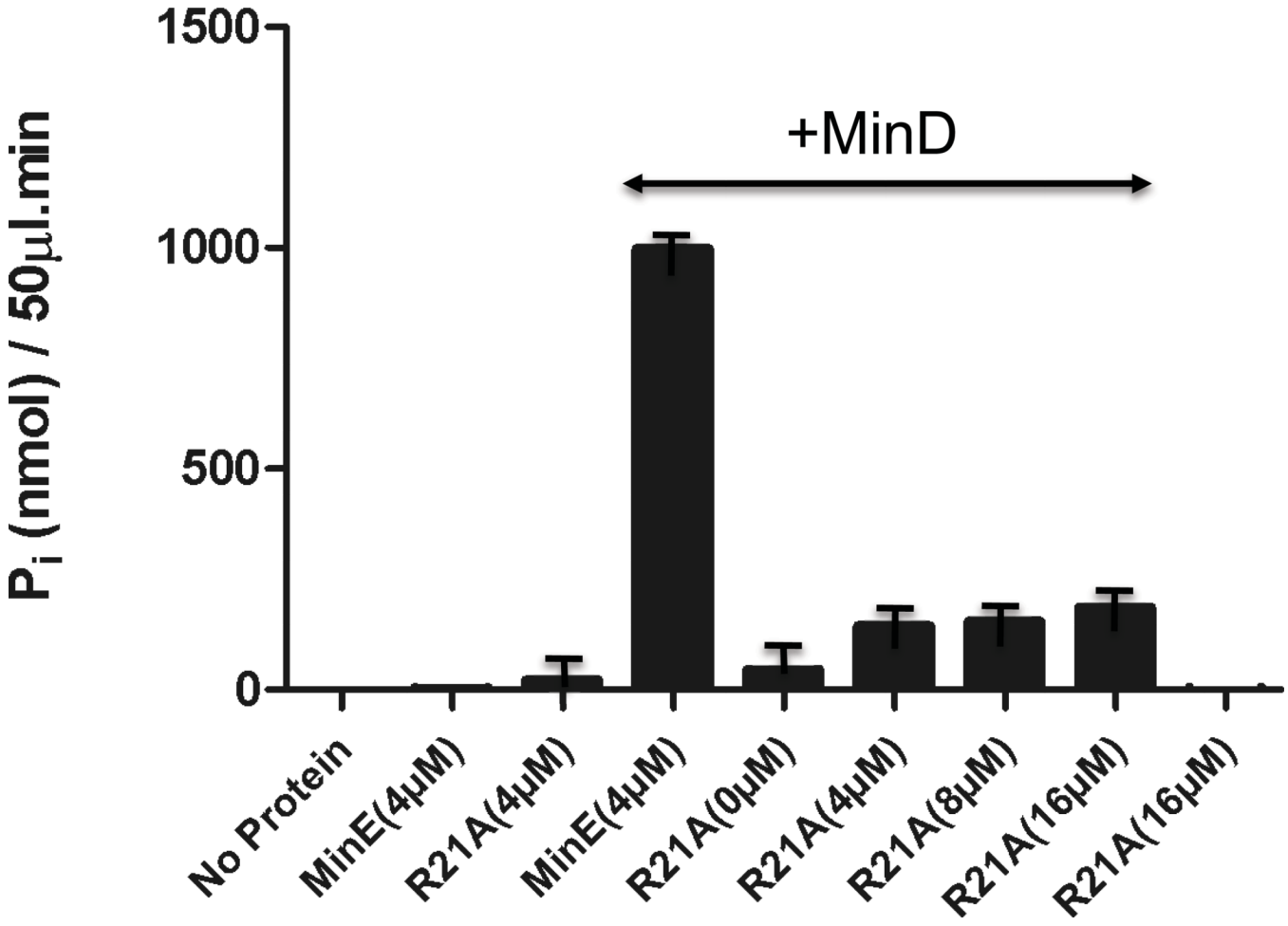

Fig. 4.

MinE-R21A fails to stimulate the ATPase activity of MinD. MinE-R21A was incubated with phospholipid vesicles (400 µg/ml), ATP (1 mM) and MinD (4 µM) and the release of phosphate was assessed. The MinE-R21A concentration ranged as indicated.

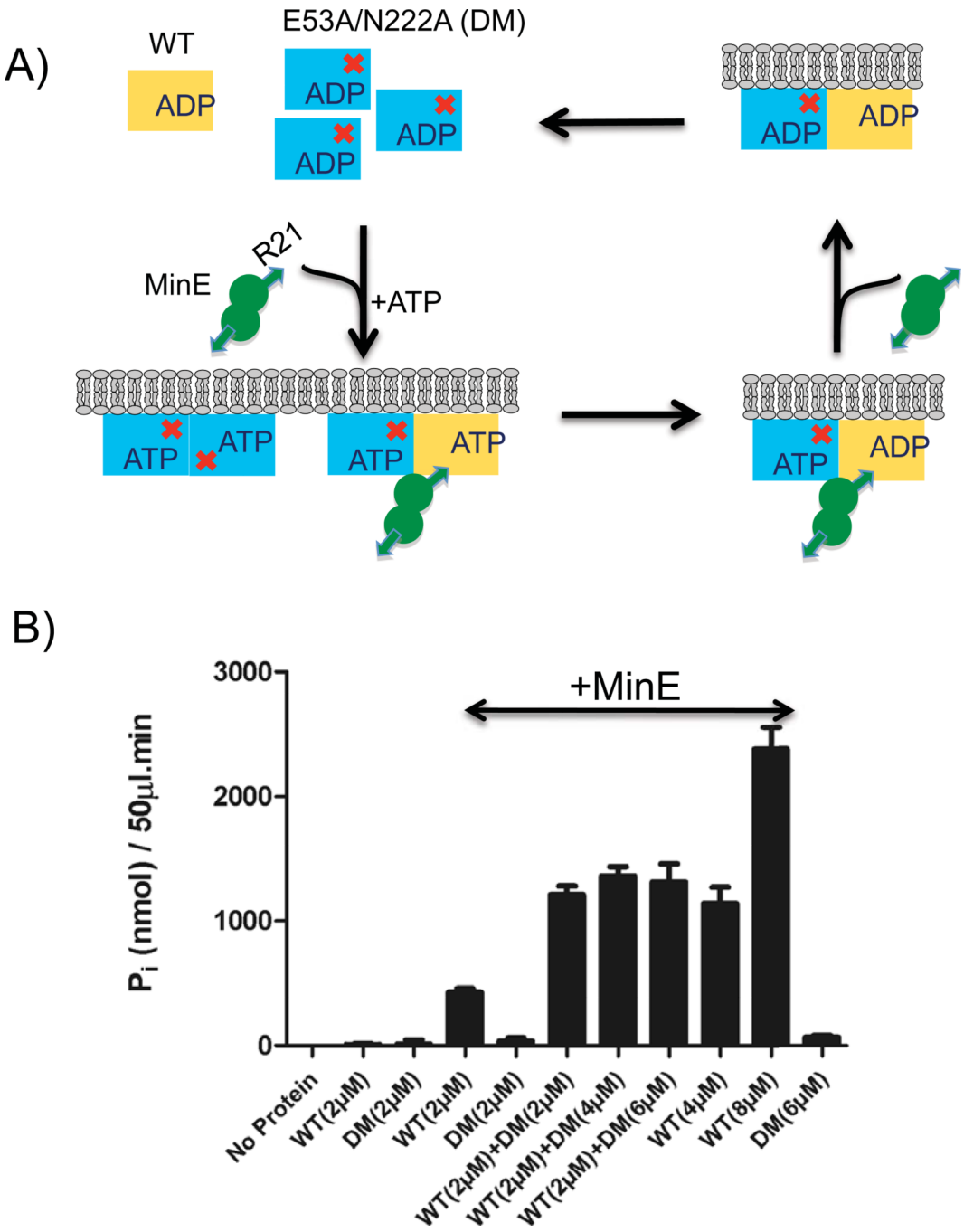

Fig. 5.

ATPase assay of MinD heterodimers. (A) Scheme depicting heterodimer formation and a sequential model for MinE activation of MinD ATPase. The red cross symbol indicates the double mutations (E53A/N222A). As MinE binds to the heterodimer, R21 contacts the $MinD^{WT}$ subunit resulting in the hydrolysis of ATP bound to that subunit, which in turn activates the $MinD^{E53A/N222A}$ subunit to hydrolyze ATP bound to it. If the WT subunit is replaced with the D40A mutant the ATP bound to $MinD^{E53A/N222A}$ is still hydrolyzed. (B) Heterodimer ATPase assay. The ATPase activity of the MinD heterodimer composed of $MinD^{WT}$ and $MinD^{E53A/N222A}$ (designated DM) is not affected by an excess of

MinD$^{E53A/N222A}$ mutant protein. The ATPase activity was determined after mixing the various proteins, phospholipid vesicles (400 μg/ml) and ATP (1 mM).

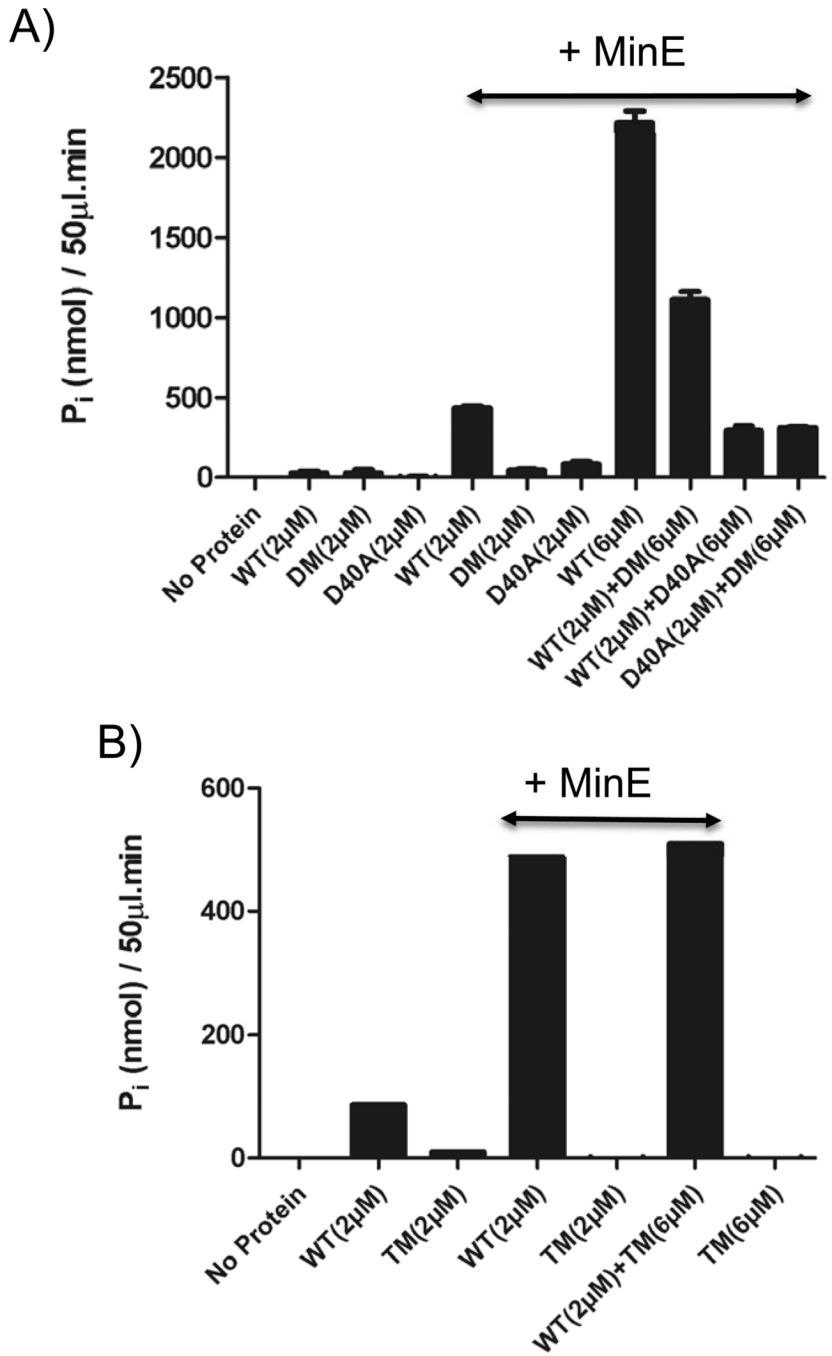

Fig. 6.

MinE stimulation of MinD heterodimers. (A) The $MinD^{D40A}$-$MinD^{E53A/N222A}$ heterodimer is stimulated by MinE. WT, D40A and DM refer to wild type ($MinD^{WT}$), catalytic-deficient mutant ($MinD^{D40A}$) and double mutant ($MinD^{E53A/N222A}$) protein, respectively. (B) Stimulation of the $MinD^{WT}$ -$MinD^{D40A/E53A/N222A}$ heterodimer by MinE. $MinD^{WT}$ and $MinD^{D40A/E53A/N222A}$, which is catalytic deficient and unable to bind MinE, were incubated together. The observation that hydrolysis occurs indicates the WT subunit within the heterodimer is able to hydrolyze ATP in response to MinE. TM refers to the triple mutant ($MinD^{D40A/E53A/N222A}$) protein.

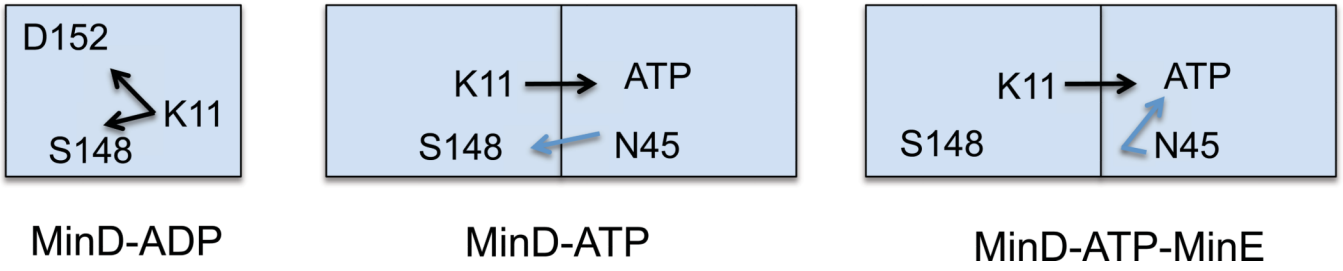

Fig. 7.

Effect of ATP and MinE on MinD residues involved in ATP hydrolysis. In the presence of ADP MinD is a monomer an K11 interacts with S148 and D152. Upon the addition of ATP this interaction is broken and K11 is free to interact with ATP bound to another subunit to promote dimerization, while S148 interacts with N45 across the dimer interface. Upon MinE binding the orientation of N45 changes and it interacts with the γ-phosphate of ATP in the same subunit to participate in ATP hydrolysis.

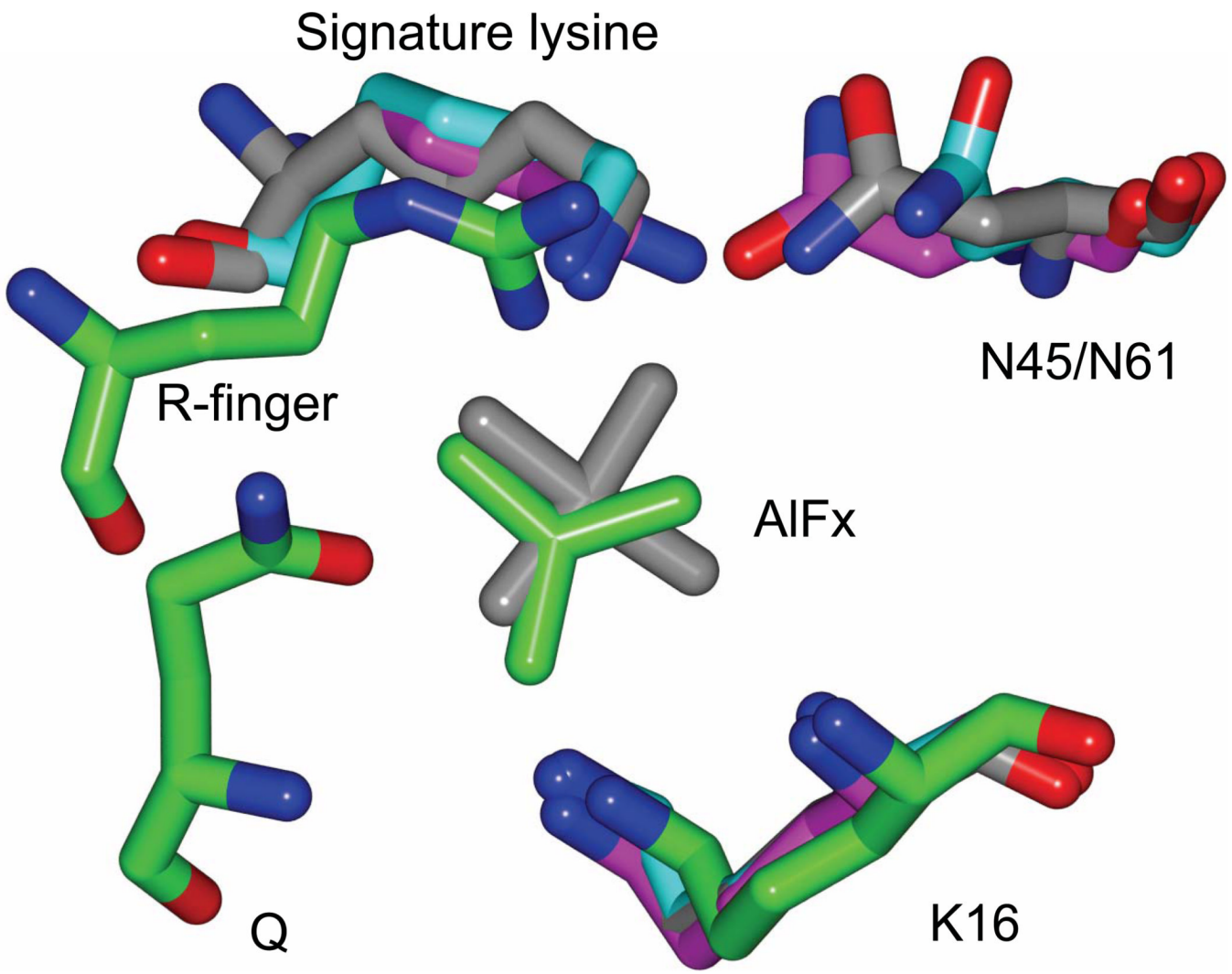

Fig. 8.

Comparison of the γ-phosphate binding regions of MinD, the MinD-MinE peptide complex, Get3-AlF_4^- and Ras-GAP-AlF_3 (PDB:1WQ1). Shown are MinD and Get3 residues that are known or proposed to stabilize the transition state. The AlF_4 is from Ras-GAP complex and the AlF_3 is from the Get3 structure. Free MinD residues are cyan, the MinD-E complex residues are magenta, Get3 residues are gray and residues from the Ras-GAP structure are green. The lysine on the lower right is the conserved lysine common to all Walker A motifs. On the upper left are the signature lysines from Get3 and MinD, which overlap the arginine finger from Ras-GAP. On the lower left is the catalytic glutamine from Ras. On the upper right are the asparagines from MinD and Get3. The glutamine in Ras has two roles, aligning the attacking water molecule and interacting with AlF_3. In MinD and Get3 an aspartic acid residue (not shown) aligns the attacking water molecule while in Get3 Asn 61 binds to the γ-phosphate in the transition state. We propose that N45 of MinD would do this in the transition state of MinD.