Structural Insights into the Intrinsic Self-Assembly of Par-3 N-Terminal Domain

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SUMMARY

Par-3, the central organizer of the Par-3/Par-6/atypical protein kinase C complex, is a multimodular scaffold protein that is essential for cell polarity establishment and maintenance. The N-terminal domain (NTD) of Par-3 is capable of self-association to form filament-like structures, although the underlying mechanism is poorly understood. Here, we determined the crystal structure of Par-3 NTD and solved the filament structure by cryoelectron microscopy. We found that an intrinsic “front-to-back” interaction mode is important for Par-3 NTD self-association and that both the lateral and longitudinal packing within the filament are mediated by electrostatic interactions. Disruptions of the lateral or longitudinal packing significantly impaired Par-3 NTD self-association and thereby impacted the Par-3-mediated epithelial polarization. We finally demonstrated that a Par-3 NTD-like domain from histidine ammonia-lyase also harbors a similar self-association capacity. This work unequivocally provides the structural basis for Par-3 NTD self-association and characterizes one type of protein domain that can self-assemble via electrostatic interactions.

INTRODUCTION

The polarization of cells is fundamental for a variety of biological processes, including cell differentiation, proliferation, and morphogenesis (Arimura and Kaibuchi, 2007; Drubin and Nelson, 1996; Martin-Belmonte and Mostov, 2008; Nelson, 2003; Wodarz, 2002). Many proteins/protein complexes, as hallmarks of cellular polarization, are restrictedly distributed at the cell cortex and thus define specific membrane microdomains to establish and maintain cell polarity (Knoblich, 2010; Macara, 2004; Pellettieri and Seydoux, 2002; Shin et al., 2006; St Johnston and Ahringer, 2010). Genetic screenings have identified a set of Par (partitioning defective) proteins (i.e., Par-1–Par-6) as essential factors for cytoplasmic partitioning in the early C. elegans embryo (Kemphues, 2000; Kemphues et al., 1988; Schneider and Bowerman, 2003). Among them, Par-3 and Par-6, together with atypical protein kinase C (aPKC), form a tripartite Par-3/Par-6/aPKC complex that is located at the anterior cell cortex of worm embryos (Goldstein and Macara, 2007; Hung and Kemphues, 1999; Tabuse et al., 1998). The Par-3/Par-6/aPKC complex is evolutionarily conserved and plays essential roles in regulating a diverse range of polarization processes in addition to zygote development in C. elegans (e.g., oocyte morphogenesis and neuroblast asymmetric division in Drosophila and epithelial polarization, neuronal differentiation, and directional cell migration in mammals) (Etienne-Manneville and Hall, 2003; Goldstein and Macara, 2007; Jan and Jan, 2001; Ohno, 2001; Suzuki and Ohno, 2006).

In the Par-3/Par-6/aPKC complex, Par-3 is a multimodular scaffold protein with an N-terminal domain (NTD), three PDZ domains (PDZ1–PDZ3), one aPKC-binding domain (aPKC-BD), and a C-terminal region (CTR) (Figure S1A available online). In addition to interacting with Par-6 (Joberty et al., 2000; Lin et al., 2000), the PDZ1 domain can also bind to several membrane receptors to localize Par-3 onto the membranes or junctions (Chan et al., 2006; Ebnet et al., 2001; Itoh et al., 2001). Given that the PDZ2 domain is capable of recognizing phosphoinositide phosphates (PIPs) (Wu et al., 2007) and the PDZ3 domain can associate with PTEN (a PI(3,4,5)P3 phosphatase) (Feng et al., 2008; von Stein et al., 2005), the presence of both PDZ2 and PDZ3 domains enables Par-3 to integrate PIP signaling events (Martin-Belmonte and Mostov, 2008; Wu et al., 2007). The conserved aPKC-BD can bind to and be phosphorylated by aPKC, which is essential for the apical exclusion of Par-3 in epithelial cells (Morais-de-Sá et al., 2010; Nagai-Tamai et al., 2002). Although the CTR is predominantly unstructured, it can also directly interact with several essential cell polarity regulators (Chen and Macara, 2005; Nishimura and Kaibuchi, 2007; Nishimura et al., 2004, 2005). Consequently, Par-3 functions as a signaling hub by interacting with multiple proteins to coordinate various cellular events during polarization.
As a signaling hub, Par-3 can further self-associate to induce the high order organization of the Par-3/Par-6/aPKC complex, which is mediated by its conserved NTD (Figure S1A). The NTD-mediated self-association is essential for the apical membrane localization of Par-3 and the development of epithelial tight junctions, likely via generating multiple binding sites for apical anchoring and then increasing the interaction avidity of Par-3 with the apical membrane (Benton and St Johnston, 2003; Feng et al., 2007; Mizuno et al., 2003). Moreover, the NTD-mediated oligomerization of Par-3 is also required to maintain distinct Par protein microdomains that are critical for cell polarity maintenance in the early C. elegans embryo (Dawes and Munro, 2011; Li et al., 2010). Recently, the solution structure of a monomeric mutant of the Par-3 NTD has been determined, which revealed that the domain adopts a PB1 domain-like fold containing two oppositely charged surfaces, indicating that the Par-3 NTD potentially self-associates into an elongated homo-oligomer with a “front-to-back” interaction mode (Feng et al., 2007). As a result, the preliminary electron microscopic study of Par-3 NTD demonstrated that it tends to form a filament-like structure (Feng et al., 2007). However, the molecular basis underlying the self-association of the Par-3 NTD into the filament is not well understood.

In this study, we determined the crystal structure of the wild-type Par-3 NTD at 2.9 Å resolution, which revealed details of the “front-to-back” self-association interaction interface. From cryoelectron microscopy (cryo-EM) and atomic force microscopy (AFM), we further observed the helical symmetry of the Par-3 NTD filament and solved its structure at the nominal resolution of 6.1 Å. Based on both the cryo-EM map and crystal structure, we were able to build a pseudomonomeric model of the filament using molecular dynamics flexible fitting (MDFF). The Par-3 NTD self-assembles into a “left-handed” helical filament. The “front-to-back” self-association electrostatic interface observed in the crystal structure is responsible for the lateral packing of the filament. The longitudinal packing of the filament is also mediated by electrostatic interactions. Mutations of essential residues at either the lateral or the longitudinal packing interfaces significantly disrupted the Par-3 NTD self-association and thereby impacted the Par-3-mediated polarization of epithelial cells. Finally, we investigated another protein, histidine ammonia lyase (HAL), and found that its Par-3 NTD-like domain also possesses a similar self-association capacity. Hence, this work not only provides a high-resolution structure of the Par-3 NTD filament but also characterizes one type of protein domain that can self-assemble via electrostatic interactions.

RESULTS

Crystal Structure of the Wild-Type Par-3 NTD

Given that the structure of the Par-3 NTD monomeric mutant (Feng et al., 2007) cannot provide intermolecular contacts for self-association, we aimed to structurally characterize the wild-type Par-3 NTD (residues 2–83) was determined to 2.9 Å resolution by molecular replacement (Figure 1A; Table 1). Two molecules were found in the asymmetric unit. Except for the C-terminal residue (D83 for one molecule and Q82 and D83 for the other), all of the other residues were resolved according to well-defined electron density. The root-mean-square deviation (rmsd) for the Cα atoms of the wild-type Par-3 NTD and the monomeric mutant (Feng et al., 2007) is only 0.9 Å. As expected, the Par-3 NTD adopts a ubiquitin/PB1 domain-like fold containing five β strands (β1–β5) and two α helices (α1 and α2). The five-stranded β sheet wraps around the long amphipathic α1 helix and together forms the core of the structure, of which one open site is capped by the short α2 helix (Figure 1A). Two Par-3 NTD molecules are present in the asymmetric unit and extensively pack with one another in a “front-to-back” manner (Figure 1A). The “front” site of the Par-3 NTD is positively charged and composed by residues from β1, β2, β5, and α1, which can be divided into two basic patches, B1 and B2 (Figures 1B and S1B). In contrast, the “back” site of the molecule is negatively charged and contributed by residues from β4, β5, and α2, which can also be separated into two acidic patches, A1 and A2 (Figures 1B and S1B). The interactions between the two Par-3 NTD molecules are chiefly mediated by these two charged surfaces with an A1/B1 and A2/B2 interaction pattern (Figures 1A and 1B), which confirms our previous suggestions (Feng et al., 2007). In the A1/B1 interaction interface, the residues K2 from β1 and K72 from the α2/β5-loop form electrostatic interactions with the residues D70’ and D73’ from the α2/β5-loop at the top, whereas the residues R11 from β2 and R74 from β5 form electrostatic interactions with the residue D55’ from the β3/’β4’-loop at the bottom (Figure 1C). Additionally, the residues T4 from β1 and V13 from β2 form hydrophobic interactions with the residues H53’ from the β3/’β4’-loop, L59’ from β4’, and V68’ from α2’ in the center (Figure 1C), thus stabilizing the A1/B1 interface. In the A2/B2 interaction interface, the residues R33 and K36 from the α1 helix form extensive electrostatic interactions with the residues D66’, D62’, and D63’ from the β4/’α2’-loop (Figure 1C).

Helical Packing in the Crystal of the Par-3 NTD

The crystal of the Par-3 NTD belongs to the space group P4_12_2 (Table 1). The self-rotation function from the diffraction data indicated the existence of a noncrystallographic 8-fold axis within the crystal, which is parallel to the c axis (Figures S2A and S2B). Indeed, by investigating the crystal packing, we found a helical stacking of the Par-3 NTD along the c axis (43 screw axis) (Figures S2C and S2D). One asymmetric dimer of the Par-3 NTD is related to the adjacent dimer by imposing a 43 screw symmetry. Surprisingly, the packing interface between these two asymmetric units is identical to the “front-to-back” interface observed within the dimer of the asymmetric unit (Figure 1A). This universal interface within the dimer and between the dimers thus yields the additional 81 screw symmetry along the c axis, which is consistent with the self-rotation crystallographic analysis (Figures S2A and S2B). Consequently, the Par-3 NTD packs into a left-handed helical structure in the crystal (Figures S2E and S2F). Each turn of the helix is composed of eight Par-3 NTD monomers with the helical pitch and inner and outer diameters of approximately 45 Å, 30 Å, and 90 Å, respectively. More interestingly, from either the top or the bottom view of the helical structure, the positive and negative charges are alternately distributed (Figure S3), suggesting that the two adjacent layers of the helical structure may further stack with each other via...
the charge complementation. Taken together, the universal “front-to-back” Par-3 NTD packing interface within the crystal may also suggest the intrinsic oligomerization property of this domain.

**Helical Structure of the Par-3 NTD Filament by cryo-EM and AFM**

Our previous electron microscopic (EM) studies of the negatively stained protein sample suggested that most of the Par-3 NTDs assemble into a filament-like structure in vitro (Feng et al., 2007). Whether the self-assembled Par-3 NTD filament adopts a similar helical stacking structure that was observed in the crystal (Figure S2) is not clear. To further uncover the molecular basis governing Par-3 NTD self-association, we next utilized cryo-EM to determine the molecular structure of the Par-3 NTD filament. Consistent with the negative stain EM studies, cryo-EM micrographs indicated that the Par-3 NTD exhibits a filament-like structure (Figure 2A). We collected 6,460 cryo-EM micrographs, selected approximately 84,000 segments of Par-3 NTD filaments, and found that most (83%) possess a diameter of 10 nm, some filaments (16%) possess a diameter of 11 nm, and a small fraction (1%) possess a diameter of 15 nm. The Fourier transform of the selected 10 nm filament revealed the layer line diffraction pattern (Figure 2B), indicating that the Par-3 NTD filament possesses a helical assembling symmetry. Although the diffraction of the thin filament is limited to only 10 Å (Figure 2B), the power spectra of most raw cryo-EM micrographs demonstrate a contrast transfer function modulation that is higher than 5 Å (Figure 2C).

The Par-3 NTD filament structure was successfully reconstructed using the iterative helical real space reconstruction (IHRSR) method (Egelman, 2000) and EMAN1 package (Ludtke et al., 1999). The projection of the 3D structure, the class-averaged image at the equivalent orientation, and the matched class-averaged image from a reference-free two-dimensional classification analysis demonstrated a good consistency (Figure S4A). Either a featureless cylinder or a filament structure that was imposed with an incorrect helical symmetry was used as an initial model during the helical symmetry refinement, and both converged to an identical model with the correct helical
rmsd for the C atoms in the original cross-correlation coefficient from 0.87 to 0.92. The approach (Chan et al., 2011) was then utilized to refine the initial symmetry-restrained molecular dynamics flexible fitting (MDFF) cryo-EM map and obtained an initial structural model. The crystal structures of the Par-3 NTD monomers into the map (Figures 2E and S4D). We then fit the structure with each turn of the helix containing 8.2 Par-3 NTD monomers (Figures 2E and 3A). Compared with the helical packing in the crystal (Figure S2), the significant difference to the Par-3 NTD filament is its extensive longitudinal interlayer packing, which likely produces a shorter helical pitch (~29 Å) but a wider inner (~39 Å) and outer diameter (~100 Å) (Figures 2E and 3).

The Par-3 NTD filament was also observed using atomic force microscopy (AFM) under ambient conditions (Figure S4E). The length of the filaments can reach more than 400 nm, and the diameter (D_{AFM} = ~18.1 nm) of the filaments measured by AFM is approximately twice the diameter (D_{TEM} = ~10 nm) observed in cryo-EM, which is due to both the tip convolution and the dehydration effect (Dong et al., 2006; Markiewicz and Goh, 1995). The helix chirality of the Par-3 NTD filament can be directly determined as left-handed by inspecting the high-resolution topography of a single filament (Figure 2F), which is consistent with the left-handed helix chirality determined from the cryo-EM reconstruction (Figure 2E). The height (h_{AFM} = ~5.3 nm) of the filament in AFM topography (Figure 2G) is smaller than the diameter (D_{TEM} = ~10 nm) measured in cryo-EM, which is due to the compression from the tip. However, the Fourier transform of the line profile along the filament direction revealed the significant 0.06 nm^{-1} (~16 nm repeating space) frequency of the filament (Figure 2H), which corresponds to the primary translation-repeating unit of the filament. Considering that each turn of the helix contains 8.2 Par-3 NTD monomers (Figures 2E and 3A), we could deduce that the filament possesses a translational symmetry for every five turns of the helix and the translation-repeating space is approximately 15 nm (five helix pitches). Therefore, the spacing observed in AFM is consistent with that of the filament. The good agreement between the cryo-EM and AFM studies strongly confirmed the reliability of our structural model of the Par-3 NTD filament.

### Table 1. Data Collection and Structural Refinement Summary for Par-3 NTD

<table>
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<th>Data Collection</th>
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<tr>
<td>Space group</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Resolution range (Å)</td>
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<td>No. of unique reflections</td>
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<tr>
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<tr>
<td>Rmerge (%)</td>
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<tr>
<td>Structure Refinement</td>
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<td>Water</td>
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</table>

### Ramachandran Plot

<table>
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</thead>
<tbody>
<tr>
<td>Most favored regions (%)</td>
</tr>
<tr>
<td>Additionally allowed (%)</td>
</tr>
<tr>
<td>Generously allowed (%)</td>
</tr>
</tbody>
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### Lateral and Longitudinal Packing Interfaces of the Par-3 NTD Filament

The packing interface of the two lateral adjacent molecules within the Par-3 NTD filament possesses an identical "front-to-back" mode observed in the crystal packing (Figures 3B and 3C). When we compared the crystallographic dimer with the two lateral adjacent molecules in the filament (which is named as the filament dimer hereafter) by superposing one of their monomers, we found that the relative position of the other monomer in the filament dimer is slightly different from that in the crystallographic dimer: the nonsuperposed monomer in the filament dimer is slightly different from that in the cryo-EM reconstruction (Figure 2D). When we compared the crystallographic dimer with the two lateral adjacent molecules in the filament (which is named as the filament dimer hereafter) by superposing one of their monomers, we found that the relative position of the other monomer in the filament dimer is slightly different from that in the crystallographic dimer (Figure 2D). When we compared the crystallographic dimer with the two lateral adjacent molecules in the filament (which is named as the filament dimer hereafter) by superposing one of their monomers, we found that the relative position of the other monomer in the filament dimer is slightly different from that in the crystallographic dimer (Figure 2D). When we compared the crystallographic dimer with the two lateral adjacent molecules in the filament (which is named as the filament dimer hereafter) by superposing one of their monomers, we found that the relative position of the other monomer in the filament dimer is slightly different from that in the crystallographic dimer (Figure 2D). When we compared the crystallographic dimer with the two lateral adjacent molecules in the filament (which is named as the filament dimer hereafter) by superposing one of their monomers, we found that the relative position of the other monomer in the filament dimer is slightly different from that in the crystallographic dimer (Figure 2D). When we compared the crystallographic dimer with the two lateral adjacent molecules in the filament (which is named as the filament dimer hereafter) by superposing one of their monomers, we found that the relative position of the other monomer in the filament dimer is slightly different from that in the crystallographic dimer (Figure 2D). When we compared the crystallographic dimer with the two lateral adjacent molecules in the filament (which is named as the filament dimer hereafter) by superposing one of their monomers, we found that the relative position of the other monomer in the filament dimer is slightly different from that in the crystallographic dimer (Figure 2D). When we compared the crystallographic dimer with the two lateral adjacent molecules in the filament (which is named as the filament dimer hereafter) by superposing one of their monomers, we found that the relative position of the other monomer in the filament dimer is slightly different from that in the crystallographic dimer (Figure 2D).
distributed either from the top or from the bottom view of the fila-
mment, indicating that the interlayer stacking is likely to also be
mediated by electrostatic interactions (Figures 3F and 3G). In the
longitudinal packing interface formed by four Par-3 NTD
molecules (molecules a, b, c, and d in Figures 3D and 3E), the
residues R20 and K22 from molecule c at the bottom layer
form electrostatic interactions with the residues E52, D55, and
D80 from molecule a at the top layer. Conversely, the residues
R9 and R11 from molecule b at the top layer form electrostatic
interactions with the residues D67 from molecule c and D18
from molecule d at the bottom layer (Figure 3E). As a result,
the charge complementation between the two layers is respon-
sible for the longitudinal interlayer stacking.

Evaluation of the Key Residues for Par-3 NTD Self-
Association

A series of point mutations were generated in previous studies
on either the positively charged “front” or the negatively charged
“back” site of the Par-3 NTD, such as V13D and D70K (Feng
et al., 2007). All of these mutations have been demonstrated to
dramatically dissociate the Par-3 NTD homo-oligomer (Figures 4A and 4B; Feng et al., 2007), confirming that the residues involved in the lateral intermolecular interactions are essential for Par-3 NTD self-association. To further probe the essential role of the residues involved in hydrophobic interactions in the middle of the lateral packing interface, we generated a T4E mutation on the A1/B1 interaction site (Figure 1C). As expected, this mutation significantly disrupted Par-3 NTD oligomerization based on both size exclusion analysis and a chemical crosslinking assay (Figures 4A and 4B). Next, we wondered whether the charged residues responsible for the longitudinal interlayer packing are also essential for Par-3 NTD self-association. We further generated an R9A mutation (which disrupts the longitudinal stacking but did not affect the lateral packing; see Figure 3E) and examined the oligomerization behavior of this mutant. The R9A mutation could largely impair the high order oligomerization of the Par-3 NTD (Figures 4A and 4B), indicating that the longitudinal packing is also essential for its self-association. However, based on the size exclusion profile, the elution volume of the R9A mutant is smaller than that of other Par-3 NTD mutants (Figures 4A and 4B), suggesting that the R9A mutant retains the lateral intermolecular interactions and can form small oligomers that were observed in the negative stain electron microscopy (Figure S6). Taken together, all of the above biochemical data demonstrated that both the lateral packing and the longitudinal stacking are essential for Par-3 NTD self-association.

Par-3 NTD Self-Association Is Essential for Epithelial Polarization

We have demonstrated that the NTD-mediated oligomerization is critical for the membrane localization of Par-3 in Madin-Darby canine kidney (MDCK) cells (Feng et al., 2007). To further evaluate the role of Par-3 NTD self-association for epithelial polarization, we used the “calcium-switch” assay to determine the effects of the point mutations, which dissociate the Par-3 NTD homo-oligomer, on the repolarization of MDCK cells. Consistent with previous studies (Wu et al., 2007), compared with the wild-type control, knockdown of the endogenous Par-3 severely delayed the repolarization of MDCK cells after the calcium switch, and transfection of the wild-type (WT) rat Par-3 could largely rescue this repolarization defect (Figures 4C and 4D). In contrast, transfection of the Par-3 NTD self-association defective mutants containing the point mutations either in the lateral packing (V13D/D70K and T4E) or in the longitudinal stacking (R9A) led to significant delays in the repolarization of MDCK cells (Figures 4C and 4D). Thus, Par-3 NTD self-association is also essential for Par-3-mediated epithelial polarization.

The N-Terminal Domain of Histidine Ammonia-Lyase Is Also Capable of Self-Association

The N-terminal domain (NTD) of histidine ammonia-lyase (HAL) shares similar primary sequence homology with the Par-3 NTD (Figure S1). We predicted that the HAL NTD may possess a self-association capacity similar to the Par-3 NTD. To test this hypothesis, we generated a structural model of the HAL NTD (with a confidence value of 99%) utilizing the protein structure prediction server Phyre (Kelley and Sternberg, 2009). The predicted model shows a similar Par-3 NTD structural fold and also contains a positively charged “front” site and a negatively charged “back” site (Figure S7A), which strongly suggests that it is also capable of self-association, likely via a “front-to-back” mode. More significantly, this assumption was further confirmed by the observation of HAL NTD oligomers from negative stain electron microscopy (Figure S7B). However, the
Structure

Structure Basis for Par-3 NTD Self-Association

HAL NTD does not tend to form a helical filament but rather self-assembles into circular oligomeric particles, likely due to the absence of equivalent charged residues that are essential for the longitudinal packing of the Par-3 NTD filament (Figure S1B). Taken together, the HAL NTD is indeed capable of self-association, and thus, the Par-3 NTD-like domains in other proteins are able to form homo-oligomers.

DISCUSSION

The NTD-mediated self-association has been demonstrated to be essential for the specific cellular localization of Par-3 (Benton and St Johnston, 2003; Mizuno et al., 2003). We have determined the solution structure of a monomeric mutant of the Par-3 NTD and have found that the domain is capable of forming filament-like structures (Feng et al., 2007). However, both the intermolecular interactions for self-association and the molecular basis governing the formation of the filament-like structure are poorly understood. Here, we determined the crystal structure of the wild-type Par-3 NTD, which reveals the intermolecular interactions in Par-3 NTD self-association at the atomic level. Consistent with previous suggestions (Feng et al., 2007), the two Par-3 NTD molecules in the asymmetric unit pack with each other in a “front-to-back” manner through extensive electrostatic interactions (Figure 1). Based on both the reconstructed cryo-EM map and the crystal structure, we further built the atomic model of the Par-3 NTD filament (Figure 2), which demonstrated that it adopts a compact helical structure with extensive “front-to-back” lateral packing and “layer-to-layer” longitudinal stacking. Thus, the work presented in this study provides the pseudoatomic model of the Par-3 NTD filament-like homooligomer.

In addition to the NTD, Par-3 contains three central PDZ domains and a C-terminal region that are capable of interacting with many membrane receptors and cell polarity regulators (Figure S1A). Thus, the synergistic coordination of the NTD-mediated self-association would further enhance the avidity of Par-3 for binding to these target proteins. Moreover, Par-3 is the central scaffold of the Par-3/Par-6/aPKC complex and interacts with both Par-6 and aPKC (Figure S1A). The NTD-mediated self-association of Par-3 would also likely be able to further organize a large dynamic Par-3/Par-6/aPKC signalosome (based on the Par-3 NTD helical filament-like structure shown in Figure 3) for enhancing the local activity of this complex during cell polarity regulation. In addition to the essential role for the membrane localization of Par-3 (Feng et al., 2007), we further demonstrated that the NTD-mediated self-association is also critical for epithelial polarization (Figure 4), thus firmly establishing the biological significance of the NTD-mediated self-association.

Although the lateral and longitudinal packing in the Par-3 NTD filament are both essential for its self-association, disruption of these two types of intermolecular packing led to slightly different effects (Figures 4A and 4B). Mutations of the residues involved in the lateral packing (V13D/D17K and T4E) nearly abolished Par-3 NTD self-association, whereas the mutation (R9A) in the longitudinal packing retained a certain capacity for self-association (Figures 4A, 4B, and S6). Hence, it is possible that the lateral packing might provide the primary interactions for the initial self-assembly, whereas the longitudinal packing could be responsible for the further elongated stacking of the helical filament.

The Par-3 NTD contains a conserved signature “GILD” sequence motif (Figure S1B). In the Par-3 NTD structure, the “GILD” motif forms a short β strand located at the bottom of the negatively charged surface, which is directly involved in the intermolecular “front-to-back” interactions through both hydrophobic and electrostatic interactions (Figures 1 and S1B). Thus, the conserved “GILD” sequence motif plays a critical role in Par-3 NTD self-association. HAL possesses a Par-3 NTD-like domain at its N terminus, which contains most of the essential charged residues that are equivalent to the residues of the Par-3 NTD at the “front-to-back” interface in addition to the “GILD” motif (Figure S1B). Both the structural prediction and the EM-based study further confirmed that the N-terminal Par-3 NTD-like domain of HAL is also capable of self-association, likely via the “front-to-back” interaction mode (Figure S7B). Because the Par-3 NTD-like domain of HAL does not possess the equivalent essential residues for the longitudinal stacking of the Par-3 NTD filament (Figure S1B), it does not form a filament-like structure. Nevertheless, the “front-to-back” intramolecular interactions do induce the formation of the circular oligomeric particles and the “front-to-back” interaction interface could be partially adapted to form different sizes of particles (Figure S7B). Interestingly, this manner of assembly could also be observed from the negative stain electron microscopy of the Par-3 NTD (Figure S7C). In addition to the formation of a filament, the Par-3 NTD can also form a circular particle that possesses an identical diameter (~10 nm) as the filament. In the circular particle, instead of a helical assembly, the Par-3 NTD partially adapts its “front-to-back” interaction interface to form a closed ring structure (Figure S7C). Taking all of the above together, we may deduce that the Par-3 NTD/Par-3 NTD-like domain is likely one type of protein domain that can self-assemble into a circular ring structure or sometimes a helical filament by its “front-to-back” electrostatic interaction mode.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification

The DNA of the Par-3 NTD (residues 2–83) was amplified by PCR from the full-length rat Par-3. The sequence was then inserted into a modified PET-32a vector. The final expressed Par-3 NTD contains a thioredoxin (Trx)-His_{6} tag at its N terminus. Point mutations of the Par-3 NTD were generated using the standard PCR-based mutagenesis method and confirmed by DNA sequencing. Recombinant proteins were expressed in Escherichia coli BL21-CodonPlus host cells at 16°C. The Trx-His_{6}-tagged proteins were purified using Ni^{2+}-NTA affinity chromatography (GE Healthcare) followed by size exclusion chromatography (Superdex 200, GE Healthcare). After cleavage of the tag, the resulting proteins were further purified by an additional size exclusion chromatographic step.

Crystallization, Data Collection, and Structure Determination

The purified Par-3 NTD was concentrated to 8 mg/ml in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol (DTT), and 1 mM EDTA. Crystals were obtained at 16°C in 1.6 M ammonium sulfate and 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, using the vapor diffusion method (sitting drop). Crystals were flash-frozen in liquid nitrogen for X-ray diffraction experiments. Diffraction data were collected at the beamline BL17U at the Shanghai Synchrotron Radiation Facility (SSRF) using an MX225 charge-coupled device (CCD) detector. For details concerning the determination of the crystal structure, see the Supplemental Information. The statistics for data collection and structural refinement are summarized in Table 1.
**Cryoelectron Microscopy and Image Processing**

The purified Par-3 NTD was concentrated to 2 mg/ml in 50 mM Tris-HCl, 100 mM NaCl, 1 mM DTT, and 1 mM EDTA. A drop (3.5 μl) of the Par-3 NTD sample was applied to a 300 mesh Gilholo carbon grid (Jiangsu LifeTrust, China) that was pretreated in a plasma cleaner (PDC-32G, Harrick Plasma). The grid was then blotted 3.0 s with a blot force of three at 100% humidity using an FEI Vitrobot (Mark IV) prior to flash-freezing in liquid ethane that was cooled by liquid nitrogen. The Par-3 NTD filament was imaged with an FEI Titan Krios cryoelectron microscope that was operated at 300 kV and equipped with a Gatan UltraScan 4000 CCD camera. Low dose images (20 e^-/Å^2) were automatically collected with the automatic collection package LegoIn (Carragher et al., 2000). The nominal magnification was set to 96,000, which corresponds to a pixel size of 0.933 Å. The defocus range was set to 2 to 3 μm. A total of 6,460 cryo-EM micrographs were collected.

The defocus value of each micrograph was determined by CTFFIND3 (Mendell and Grigorieff, 2003). The images were further processed using the IHRSR method (Egelman, 2000) and EMAAn1 package (Ludkte et al., 1999). For details concerning the image processing, see the Supplemental Information. During the refinement, a SPIDER script was embedded for correspondence analysis of each image class (Frank et al., 1996; Shaikh et al., 2008), which was wrapped in the Appion package (Lander et al., 2009). The final cryo-EM map was sharpened using EMBFACTOR (Fernández et al., 2008). Cryo-EM maps were segmented, displayed, and fit with atomic models using UCSF Chimera (Pettersen et al., 2004). All structural figures were generated by UCSF Chimera and PyMOL (Delano, 2002).

Other detailed experimental procedures can be found in the Supplemental Information.

**ACCESSION NUMBERS**

The coordinate of the crystal structure of Par-3 NTD is deposited in the Protein Data Bank (PDB) with the accession number 46P. The cryo-EM map of the Par-3 NTD filament is deposited in the Electron Microscopy Data Bank with the accession number EMD-2237, and its corresponding pseudoatomic model is deposited in the PDB with the accession number 3ZEE.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.str.2013.04.004.

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**Figure 4. Evaluation of Key Residues for Par-3 NTD Self-Assembly**

(A) Analytical size exclusion analysis of the WT Par-3 NTD and its various mutants. The elution volumes for the molecular weight protein markers are indicated at the top of the elution profile. The WT Par-3 NTD self-associated and thereby eluted at the void volume. The point mutations in the lateral packing interface, such as R9A, also severely impaired the assembly, the mutant retained a certain capacity for self-association.

(B) Chemical crosslinking assay of the WT Par-3 NTD and its various mutants.

(C) Disruption of Par-3 NTD self-association severely compromised the repolarization of MDCK cells during a calcium switch assay. The repolarization of MDCK cells after the calcium switch was assayed by staining the tight junction marker ZO-1. MDCK cells were transfected with the pSUPER vector (control) or pSUPER-Par-3 (Par-3 small hairpin RNA [shRNA]), and GFP was coexpressed as a marker for the transfection efficiency. The constructs encoding the C-terminal GFP-tagged WT rat Par-3 and its various mutants (V13D/D70K, R9A, and T4E) were cotransfected with pSUPER-Par-3 to rescue the repolarization defect induced by the Par-3 knockdown. Cells were subjected to the “calcium switch” assay and fixed at the indicated time points after the calcium switch (0, 0.5, 1, and 2 hr). Scale bar: 10 μm.

(D) Quantification of the average intensity of ZO-1 staining per cell. The average intensity of ZO-1 staining per cell at the indicated time points was normalized to that of each ZO-1 staining at the high calcium medium condition. Each bar represents the mean ± SD of three randomly selected fields from imaging slides. Statistical analysis was performed using two-tailed Student’s t test by comparing various experiments with the control experiment, with *p < 0.05 and **p < 0.01. See also Figures S6 and S7.


