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The functionally important N-terminal half of fission yeast Mid1p anillin is intrinsically disordered and undergoes phase separation

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Abstract

Division of fungal and animal cells depends on scaffold proteins called anillins. Cytokinesis by the fission yeast *Schizosaccharomyces pombe* is compromised by the loss of anillin Mid1p (Mid1, UniProtKB P78953), because cytokinesis organizing centers, called nodes, are misplaced and fail to accumulate myosin-II, so they assemble slowly into abnormal contractile rings. The C-terminal half of Mid1p consists of lipid binding C2 and PH domains, but N-terminal half (Mid1p-N452) performs most of the functions of the full-length protein. Little is known about the structure of the N-terminal half of Mid1p, so we investigated its physical properties using structure prediction tools, spectroscopic techniques and hydrodynamic measurements. The data indicate that Mid1p-N452 is intrinsically disordered but moderately compact. Recombinant Mid1p-N452 purified from insect cells was phosphorylated, which reduces its tendency to aggregate. Purified Mid1p-N452 demixes into liquid droplets at concentrations far below its concentration in nodes. These physical properties are appropriate for scaffolding other proteins in nodes.

INTRODUCTION

Cytokinesis is the final step of the cell cycle, and the major events of the process are conserved from fungi to animal cells (1) where actin filaments, myosin-II (Myo2) and many other proteins form a cytokinetic contractile ring that invaginates the plasma membrane to

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Author Contributions

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Supporting Information.

Supplemental Figures. The functionally important N-terminal half of fission yeast Mid1p anillin is intrinsically disordered and undergoes phase separation. (File type, .pdf)

Accession Code of the proteins used in this study

P68139 (ACTS_CHICK)

P78953 (MID1_SCHPO)

Q9USI6 (MYO2_SCHPO)

form a furrow that cleaves the cell into two daughter cells. Defects in cytokinesis affect the survival of single-celled organisms and can favor tumor formation in multicellular organisms (2–4). Hence, regulation of the timing and position of cytokinesis is essential for cell division.

The fission yeast *Schizosaccharomyces pombe* prepares for cytokinesis during interphase with the accumulation of punctate structures called the nodes on the inside surface of the plasma membrane. These interphase nodes are of two distinct kinds (5–7). Type I nodes form around the equator during early interphase and contain kinases Cdr1p, Cdr2p and Wee1p. During G2 anillin Mid1p leaves the nucleus and joins Type I nodes already positioned around the nucleus on the plasma membrane. Type II nodes are composed of the scaffold protein Blt1p, kinesin Klp8p and Rho-GEF Gef2p. Type II nodes are components of the contractile ring. When the contractile ring disassembles at the end of constriction, the Type II nodes emerge (5–8) and diffuse on the inside of the plasma membrane until they are captured by stationary Type I nodes in the middle of the cell (5). During mitosis interphase nodes mature into cytokinetic nodes that form the contractile ring during the next cell cycle. Mid1p recruits in succession myosin-II (Myo2p heavy chain, Cdc4p light chain, Rlc1p1 regulatory light chain together called Myo2, UniProtKB Q9USI6), IQGAP Rng2p, F-BAR protein (FER/CDP4 homology domain-Bin-Amphiphysin-Rvs-like protein) Cdc15p and formin Cdc12p (9–12). Interactions of actin filaments (actin, UniProtKB P68139) nucleated by Cdc12p (13, 14) with Myo2 in neighboring nodes (15) produce forces that condense the nodes into a narrow contractile ring (16).

Fission yeast Mid1p, a protein of 920 amino acids with a molecular weight of 102 kDa, is a member of the anillin family of proteins, which participate in cytokinesis in fungi and animal cells. Crystal structures showed that C-terminal halves of both fission yeast and human anillins consist of lipid binding C2 and PH domains (17) that anchor the protein to the plasma membrane. The folds of the two C2 domains and of the two PH domains are similar in spite of limited sequence identity. Mid1p residues 583–787 form the C2 domain consisting of a β -sandwich, residues 787–807 form a central connector segment and residues 805–920 form a C-terminal PH domain (17). The C-terminal half of human anillin also has a coiled-coil domain that binds the Rho GTPase but is missing in Mid1p (17).

Anillin Mid1p is not essential for viability of fission yeast, but cytokinesis fails in one-third of cells with the *mid1* deletion mutation, while the cleavage furrow is misplaced or oblique in many of the surviving cells (15, 18, 19). Cells lacking Mid1p form interphase nodes that mature into cytokinetic nodes with Cdc12p but encounter problems assembling contractile rings for two reasons (20). First, these nodes do not locate normally in the middle of the cell, and second, they fail to accumulate Myo2. Formin Cdc12p in the nodes of *mid1* cells polymerizes actin filaments, which form strands containing Myo2 that slowly and unreliably attempt to form a contractile ring (20).

Phosphorylation during different stages of the cell cycle regulates several aspects of Mid1p function. Cdk1p (product of the *cdc2*⁺ gene) phosphorylates Mid1p T517 during the G2 phase of the cell cycle, forming a binding site for polo-like kinase (9, 21). Plo1p phosphorylates at least 6 sites among the first 100 residues of Mid1p during G2 (9), which

drives the export of Mid1p from nucleus to equatorial Type I nodes (21) and is required to recruit Myo2 to nodes (9). Plo1p localization to the contractile ring also depends on Mid1p (21).

The N-terminal halves of anillins have no sequence homology with known protein domains but have sequence motifs implicated in binding actin filaments (22), myosin-II (23), myosin light chain Cdc4p (10) and IQGAP (10). Co-sedimentation assays established that anillins from *D. melanogaster*, *X. laevis* and human bind filaments of purified actin (22, 24–26). *X. laevis* anillin purified from SF9 cells interacted with non-muscle myosin II from *X. laevis* (27), overexpressed *S. pombe* Mid1p immunoprecipitated with Myo2 from cell extracts (23), and Mid1p immunoprecipitated with Cdc4p and IQGAP from *S. pombe* lysates (10). In spite of lacking the C-terminal lipid-binding domains fission yeast Mid1p constructs consisting of residues 1–452 or 1–578 complemented the *mid1* null mutation remarkably well (28). During mitosis these N-terminal constructs concentrated in nodes around the equator that accumulated Myo2 and other contractile ring proteins. These cells formed contractile rings from strands of actin filaments and Myo2 slower than wild type cells, but placed the septum closer to its normal position and orientation than the *mid1* strain. Little is known about the structures of N-terminal halves of Mid1p and other anillins. Therefore, we characterized the physical properties of the functionally important N-terminal construct Mid1p-N452, consisting of residues 1–452. We discovered that this construct is intrinsically disordered, so we used hydrodynamic and spectroscopic methods appropriate to characterize this disorder.

MATERIALS AND METHODS

Sequence analysis.

Secondary structure prediction from the sequence of Mid1p-N452 was done using Jpred3 (29) and PSIPRED (30). Disorder prediction was carried out using PONDR-FIT (31), FoldIndex (32) and DisoPred2 (33). FoldIndex uses average hydrophobicity and net charge to predict disordered regions, while DisoPred employs artificial neural networks. PONDR-FIT uses combination of different methods to predict disordered regions. ExPASy-Protparam (34) was used to calculate the frequency of occurrence of different amino acids.

Cloning of Mid1p-N452.

Mid1p-N452 was amplified from *S. pombe* genomic DNA using 5' CTGTATTTTCAGGGATGCAAAGAGCAAGAGTTCTCATATAGAGAAGC 3' and 5' TTCCGGATCCATGGCTTATGATGGGTGACGTAAATCTTCAGAGCT 3' primers. Insert was cloned in frame into pFAST Bac HTA vector at the Sfo1 restriction site.

Purification of Mid1p-N452.

His-tagged Mid1p-N452 was expressed in the Hi5 insect cell line. Cells were infected with a second-generation baculovirus (made by transfecting SF9 cells with DNA and cellfectin (Sigma)) for 48 h at 27°C. Three hundred million cells were pelleted at 1000 rpm and lysed in 20 ml of cold lysis buffer (300 mM NaCl, 1 mM TCEP and 20 mM HEPES (pH 7.5)). Lysis buffer was supplemented with protease inhibitors (ROCHE – complete protease inhibitor cocktail; 0.5 tab/ 20 ml) and phosphatase inhibitors (Sigma – phosSTOP; 0.5 tab/

20 ml). The cells were broken by sonication (Branson Analog Sonifier 450A) at 10% amplitude for 15 cycles of 20 s on and 20 s off. Debris was removed by centrifugation at 48,000 g for 30 min at 4°C. About 20 ml of supernatant were loaded onto a 0.4 ml column of Ni-NTA agarose (QIAGEN) and shaken for 4–6 h at 4 °C. The unbound components were removed by washing the column with 30 ml of 20 mM HEPES (pH 7.5), 1 M NaCl, 1 mM TCEP and 30 mM imidazole (pH 7.5). Mid1p-N452 was eluted with 15 ml of 20 mM HEPES (pH 7.5), 300 mM NaCl, 1 mM TCEP and 300 mM imidazole (pH 7.5). The sample of ~15 ml was concentrated 3-fold to ~40 μ M by slow centrifugation (1000 rpm) in a centrifugal filter (Amicon ultra) and ~5 ml was loaded onto a size exclusion column, HiLoad 16/60 Superdex 75 pg column (GE Healthcare) equilibrated and eluted with 300 mM NaCl, 1 mM TCEP and 20 mM HEPES (pH 7.5). Fractions containing Mid1p-N452 were pooled and used immediately. The identity of Mid1p-N452 was verified with mass spectrometry. Protpram (34) calculated an extinction coefficient at 280 nm of 25,330 $M^{-1}cm^{-1}$ from the amino acid composition of Mid1p-N452.

Purification GST-Myo2-tail (1441–1526) and His-Mid1p-N309.

GST-tagged Myo2-tail (1441–1526) cloned in the pET 32a vector and His-Mid1p-N309 in the pQE80L vector were expressed in *E. coli* RIPL cells. Cell pellets were collected by centrifugation at 5000 rpm and suspended in ice cold lysis buffer containing 300 mM NaCl, 1 mM TCEP and 20 mM HEPES (pH 7.5). The cells were broken by 30 rounds of sonication (Branson Analog Sonifier 450A) at 40% amplitude for 30 s on and 20 s off. Debris was removed by centrifugation at 48,000 g for 30 min at 4°C. Samples of ~50 ml were incubated overnight at 4°C with 2 ml glutathione-agarose beads (GE Healthcare) for GST-Myo2-tail (1441–1526) and 2 ml NiNTA agarose beads (Qiagen) for His-Mid1p-N309. The beads were poured into columns and washed with 20 ml of 1 M NaCl, 1 mM TCEP and 20 mM HEPES (pH 7.5). GST-Myo2-tail (1441–1526) was eluted with 300 mM NaCl, 1 mM TCEP, 20 mM glutathione and 20 mM HEPES (pH 7.5), while Mid1p-N309 was eluted with 300 mM NaCl, 1 mM TCEP, 300 mM imidazole (pH 7.5) and 20 mM HEPES (pH 7.5). The proteins were further purified by size exclusion chromatography on a HiLoad 16/60 Superdex 75 pg column (GE Healthcare) equilibrated and eluted with 300 mM NaCl, 1 mM TCEP and 20 mM HEPES (pH 7.5). Fractions containing either GST-Myo2-tail (1441–1526) or Mid1p-N309 were pooled, concentrated by centrifugation at 1000 rpm in centrifugal filter (Amicon Ultra). We used the amino acid compositions and Protpram (34) to calculate extinction coefficients at 280 nm: GST-Myo2-tail (1441–1526) is 44,350 $M^{-1}cm^{-1}$; and Mid1p-N309 is 13,410 $M^{-1}cm^{-1}$.

Static light scattering.

Analytical size exclusion chromatography was performed for 300 μ g of Mid1p-N452 in 0.1 ml of 300 mM NaCl, 1 mM DTT and 20 mM HEPES (pH 7.5) on a Superdex 75p 10/300 GL column (GE Healthcare) on a Shimadzu liquid chromatography system coupled to a mini – DAWN TREOS multi angle static scattering detector (Wyatt) and a Shimadzu refractive index detector for absolute molecular weight determination. The molecular weight of Mid1p-N452 was determined based on light scattering and refractive index using ASTRA software (Wyatt).

Analytical ultracentrifugation.

Sedimentation coefficients of Mid1p-N452 in 300 mM NaCl, 1 mM TCEP and 20 mM HEPES (pH 7.5) were determined by sedimentation velocity at 40,000 rpm at 20°C using a Beckman XL-A analytical ultracentrifuge. Samples of 400 µL of Mid1p-N452 were loaded in a two-channel centerpiece fitted with quartz windows in a four-hole rotor. Radial scans were acquired at 280 nm for approximately 10 h. Data analysis was performed using the continuous $c(s)$ distribution model feature in SedFit v 10.3 (35). We report the mean of three independent measurements of the sedimentation coefficient. We used SEDNTREP to calculate the density and viscosity of buffers and their components and the partial specific volumes of the proteins from their amino acid composition.

Mass spectrometry.

Purified Mid1p-N452 was run on an SDS-PAGE and stained with Coomassie blue. Gel bands were cut into small pieces, washed on a tilt-table with 250 µL 50% acetonitrile for 5 min with rocking, and then washed twice with 50% acetonitrile with 50 mM NH_4HCO_3 for 30 min. The gel bands were dried by speed vacuum (Savant™ SPD131DDA Speed Vac™ concentrator) and resuspended in 30 µL of 10 mM NH_4HCO_3 containing 0.20 µg of digestion grade trypsin (Promega, V5111) and incubated at 37°C for 16 h. The soluble products of digestion were acidified using 5 µL 100% trifluoroacetic acid (TFA) in preparation for MS. Keck Center mass spectrometry facility at Yale University analyzed 5 µL samples of the peptides by LC-MS using a Waters nanoAcquity UPLC system (Acquity Peptide BEH C18 column) utilizing a binary solvent system (Buffer A: 100% water, 0.1% formic acid; Buffer B: 100% acetonitrile, 0.1% formic acid) coupled to a Thermo Scientific Q Exactive Plus mass spectrometer. Mass spectra were acquired in profile mode over the range of 300–1,700 m/z using 1 µscan, 70,000 resolution, AGC target of 3E6, and a maximum injection time of 45 ms. Spectra were analyzed with Scaffold software (36). Peptide coverage was calculated by dividing the total number of amino acids in peptides identified by MS by total the number total number of amino acids in the protein.

Circular dichroism spectroscopy.

CD spectra were measured with a Jasco J810 spectropolarimeter at intervals of 1 nm in 0.1 cm path length quartz cuvettes of 300 µL samples of 5 µM Mid1p-N452 in 300 mM NaF, 1 mM TCEP and 20 mM phosphate buffer (pH 7.4). The buffer spectra were subtracted from sample spectra and the units were converted from millidegrees to mean residual ellipticity (MRE) using calculated molecular weight and plotted using GraphPad prism. The spectrum was deconvoluted at the Dichroweb server (37) using CDSSTR algorithm (38).

Nuclear Magnetic Resonance spectroscopy.

Isotopically labeled Mid1p-N308 was expressed in M9 minimal media containing 1 g/L of ^{15}N -ammonium chloride and purified as described above. Nuclear magnetic resonance (NMR) spectra were collected for 25 µM of Mid1p-N452 in 20 mM HEPES (pH 7.5), 300 mM NaCl, 1 mM TCEP, 10 % D_2O . ^1H ^{15}N heteronuclear single quantum coherence (HSQC) experiments were done on V600b Varian VNMRs 600 MHz spectrometer and processed using Mnova.

Negative staining and electron microscopy.

Carbon-coated copper grids (Electron Microscopy Sciences) were glow discharged using a homemade glow discharge unit. A stock solution of 3 μ M Mid1p-N452 in 300 mM NaCl, 1 mM TCEP and 20 mM HEPES (pH 7.5) was clarified by centrifugation at 15,000 rpm and diluted 100-fold in the same buffer before a sample of 3.5 μ l was applied to the grid and incubated at room temperature for 30 s. Most of the sample was wicked from the grid, which was stained with freshly prepared 0.8% uranyl formate and air-dried. Specimens were observed with a JEOL-1230 electron microscope operating at 80 kV. Images were loaded on analyzed using ImageJ (39) and 61 particles were measured along two axes and mean is reported.

Actin co-sedimentation assay.

For co-sedimentation assays, 5 μ M, 10 μ M and 20 μ M ATP-actin were polymerized in 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole (pH 7.5) with 2 μ M of Mid1p-N452 at room temperature for 60 min. After ultracentrifugation at 100,000 g for 30 min at 20°C, equal amounts of the pellet and supernatant were analyzed by SDS-PAGE and Coomassie staining.

Myosin tail GST pull down assay.

For GST pull down assay purified GST- Myo2-tail (1441–1526) at a concentration of 17.5 μ M was incubated with GST beads (GE Healthcare) for 120 min at 4°C in rotisserie shaker. Next 10 to 200 μ l of GST beads bound to GST- Myo2-tail (1441–1526) was incubated with 10 μ M Mid1p-N452 for 120 min at 4°C in rotary shaker. The reaction volume was made to 250 μ l with 200 mM NaCl, 1 mM TCEP and 20 mM HEPES (pH 7.5) buffer. After pelleting the beads, Mid1p-N452 in supernatant was measure by SDS-PAGE, Coomassie staining and densitometry.

RESULTS

Sequence analysis of the N-terminal half of Mid1p.

Analysis of the sequence of Mid1p-N452 using the FoldIndex (32), PONDR-FIT (31), DisoPred (33) and DisEMBL (40) software predicted a few alpha-helices but mostly long disordered regions consisting of 73% (PONDR-FIT), 86% (DisoPred), 83% (DisEMBL) and 60% (FoldIndex) of the residues (Figure 1A, B). The longest disordered regions comprise 134, 129, 125 and 84 residues. Secondary structure prediction software PSIPRED (30) predicted only 4.7% alpha-helical residues (68–78, 81–87 and 436–440) plus 7.5% disordered helix (173–180, 311–326, 368–380). JPRED (29) predicted 4.9% alpha-helical residues (68–78, 81–87 and 370–376) (Figure 1A, B).

The amino acid composition of Mid1p-N452 is low in hydrophobic residues and enriched in residues found in disordered regions of other proteins (41, 42). Compared with the *S. pombe* proteome Mid1p-N452 has twice as much serine, is slightly enriched in aspartic acid, glutamic acid, proline, threonine and tyrosine, has less valine, leucine, isoleucine and phenylalanine and lacks cysteine and tryptophan (Figure 1D). Lysine, glutamic acid, aspartic acid, serine, asparagine and proline (43) add up to 47.5% of the total amino acids and the net

charge at pH 7 is -17.6 . In a plot of net charge vs. hydrophobicity Mid1p-N452 clusters with 54 disordered proteins rather than 105 ordered proteins (Figure 1C) (44). The absence of cysteine and small numbers of aromatic amino acids are also characteristic of disordered proteins.

The DISPHOS tool (41) predicted 48 of the 77 serines, 12 of 29 threonines and 13 of 17 tyrosines of Mid1p-N452 have a high probability of being phosphorylated (Figure 2A). Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry of Mid1p-N452 purified from Hi5 insect cells with phosphatase inhibitors (Figure 2B) confirmed that 22 of 48 predicted serines, 4 of 12 predicted threonines and 3 of 13 predicted tyrosines were phosphorylated. This included 6 of the 9 consensus Sid2p phosphorylation sites R-X-X-S (45), 3 of 4 minimal consensus Cdk1 phosphorylation sites S/T-P (46) and 3 of 8 consensus Plo1 phosphorylation sites (D/E)-X-(S/T)- Φ (where Φ is a hydrophobic residue) (47). Mid1p-N452 purified without phosphatase inhibitors was phosphorylated on 10 serines, 5 threonines and 1 tyrosine. This included 2 of 4 minimal consensus Cdk1 phosphorylation sites and 1 of 8 consensus Plo1 phosphorylation sites. The peptide coverage for the samples was 71.9% with phosphatase inhibitors and 77.7% without with phosphatase inhibitors.

Purification of Mid1p-N452.

Saha and Pollard (28) reported that Mid1p-N452 purified from *E. coli* was a hydrodynamically homogeneous particle with a sedimentation coefficient of 14.6 S and a molecular weight eight times larger than the polypeptide, whereas Sun et al. reported that their preparation of Mid1p-N452 from *E. coli* had a sedimentation coefficient of 1.2 S (17). The difference is explained by precipitation of our bacterial preparation of Mid1p-N452 when it was concentrated, leaving a bacterial contaminant in the supernatant, which we later identified as ArnA by mass spectrometry. The ArnA polypeptide has the same mobility as Mid1p-N452 by SDS-PAGE, and the ArnA protein consists of eight subunits (48) accounting for the analytical ultracentrifugation results.

Therefore, we developed a new method to purify His-tagged Mid1p-N452 from insect Hi5 cells infected with baculovirus carrying the expression construct. Chromatography on a Ni-NTA agarose affinity column and gel filtration yielded Mid1p-N452 free of contaminants, as analyzed by SDS-PAGE (Figure 3B) and mass spectrometry. The yield was ~ 0.5 mg from 150 million cells. We verified the identity of Mid1p-N452 by mass spectrometry and used the protein immediately after gel filtration for all the experiments.

Hydrodynamics of Mid1p-N452.

We studied the oligomeric state of Mid1p-N452 in buffer containing 300 mM NaCl, 1 mM TCEP and 20 mM HEPES (pH 7.4) by three methods, size exclusion chromatography combined with static light scattering (SLS) (Figure 3A) and sedimentation velocity ultracentrifugation (Figure 4 and Table 1). During gel filtration, Mid1p-N452 eluted as a single peak with a small partition coefficient compared with standard proteins (Figure 3B), corresponding to a Stokes' radius of 5 nm and a diffusion coefficient of 5.0×10^{-7} cm²/s. Static light scattering analysis of the peak of Mid1p-N452 gave a molecular weight of 60

kDa (Figure 3A), which is slightly larger than the 52.7 kDa molecular weight based on the sequence. Therefore, Mid1p-N452 is a monomer with a large hydrodynamic radius.

The sedimentation velocity profiles of Mid1p-N452 depended on whether the protein was prepared with or without phosphatase inhibitors (Figure 4A, B). Samples purified with phosphatase inhibitors had a main peak at 2.17 S and minor peaks at 4.0 S and 22 S. When corrected for standard condition, *i.e.* water at 20°C, $S(20,w)$ was 2.2 (mean) ($n = 3$; 2.5, 2.2 and 1.9). The diffusion coefficient (D) calculated from the spreading of boundary of the 2.17 S peak was calculated to be $4.5 \times 10^{-7} \text{ cm}^2/\text{s}$. SDS polyacrylamide gel electrophoresis after analytical ultracentrifugation showed no degradation. Given a sedimentation coefficient of 2.2 S, the two measurements of D gave molecular masses of ~43 kDa and ~43 kDa (Table 1), confirming that the main species is a monomer. The low sedimentation coefficient and large Stokes' radius are consistent with Mid1p-N452 purified with phosphatase inhibitors being disordered.

The sample purified without phosphatase inhibitors separated into multiple species with sedimentation coefficients ranging from 1 S to 28 S (Fig. 4B). Thus, Mid1p-N452 purified without phosphatase inhibitors has a strong tendency to self-associate into oligomers, as observed by Celton-Morizur et al. (2004) for Mid1p-N506 in crude extracts (49). All further experiments were done with Mid1p-N452 purified from insect cells with phosphatase inhibitor unless noted otherwise.

Electron microscopy.

Since the Stokes' radius and sedimentation coefficient are consistent with a range of shapes, we examined Mid1p-N452 by transmission electron microscopy. Micrographs of negatively stained dilute solutions of purified Mid1p-N452 purified with phosphatase inhibitors showed small particles with average dimensions of $4.8 \pm 1.7 \text{ nm}$ (mean $\pm$ SD, $n = 61$) along their short axes and $6.5 \pm 2.0 \text{ nm}$ (mean $\pm$ SD, $n = 61$) along their long axes (Figure 5A). Some of these particles were aggregated in small clusters and some particles were more elongated than the average particles.

Spectroscopy.

The hydrodynamic properties of Mid1p-N452 are consistent with a disordered protein, but this required conformation by independent spectroscopic methods, heteronuclear single quantum coherence spectroscopy (^1H - ^{15}N HSQC) and circular dichroism spectroscopy (50). The ^1H - ^{15}N HSQC NMR spectroscopy requires isotopically labeled protein. Since method for ^{15}N labeling are under development for insect cells, we expressed our labeled protein in bacteria. Since we were unable to separate the ArnA contaminant from Mid1p-N452, we used a shorter construct Mid1p-N308, which is not as functional at Mid1p-N452 (28) but includes two thirds of the longer construct predicted to be largely disordered by sequence analysis. Purified Mid1p-N308 is free of bacterial contaminants and has a sedimentation coefficient of 2 S (28). After uniform labeling of Mid1p-N308 with ^{15}N in bacteria and purification, we collected ^1H - ^{15}N HSQC NMR spectra. The peaks in the 2D NMR spectra overlapped and were characterized by limited ^1H chemical shift dispersion. These features are characteristic of intrinsically disordered proteins (IDPs) where conformational averaging

within the rapidly interconvertible ensembles results in poor dispersion (51). Flexibility allows significant chemical exchange of ^{15}N with bulk water resulting in reduction of ^1H - ^{15}N signal intensities and poor signal to noise ratio (52). Additionally, the ^1H - ^{15}N spectra of IDPs also have poor dispersion and broadening of peaks, which is due to lack of H-bonds and aromatic residues in the core of the protein.

The CD spectrum of Mid1p-N452 purified from insect cells with phosphatase inhibitors (Figure 5C) was typical of a disordered protein (53) with a minimum at 205 nm and a shoulder at 220 nm. Deconvolution of the spectrum with the CDSSTR algorithm (38) on Dichroweb server (37) predicted Mid1p-N452 to have 3.4% alpha-helix, 34.4% beta-strand, 15.3% turns and 46.1% coils, typical of intrinsically disordered proteins.

Solubility.

Mid1p-N452 purified from insect cells with phosphatase inhibitors aggregates over time. Differential interference contrast microscopy at room temperature (DIC) revealed that Mid1p-N452 at concentrations from 3.8 μM to 23 μM formed droplets, which increased in size over time (Figure 6A). The Discussion explains that these concentrations are far below those in nodes. Droplets formed earlier and grew faster at higher concentrations. As time passed numerous small droplets with diameters of $\sim 0.5 \mu\text{m}$ were replaced by fewer droplets of $\sim 10 \mu\text{m}$ in diameter.

We introduced a cysteine at the C-terminus of Mid1p-N452 for labeling with Alexa Fluor 488 C5 maleimide. Labeling increased the tendency of the protein to aggregate, so we used 10 μM for fluorescence microscopy. The labeled protein formed fluorescent droplets (Figure 6B), confirming that they are composed Mid1p-N452.

Binding of Mid1p-N452 to the tail of Myo2 and actin filaments.

In *S. pombe* Myo2 does not associate with nodes in cells lacking Mid1p (20). Mid1p immunoprecipitates with overexpressed Myo2, while the construct lacking 132 residues from C-terminus of Myo2 abolishes this interaction in *S. pombe* (23). Residues 142–254 of *X. leavis* anillin binds directly to nonmuscle myosin-II last 112 residues from C-terminus (27). Hence, we assessed the binding of purified Mid1p-N452 to GST-Myo2-tail, a fusion of GST to Myo2p residues 1441–1526 that we immobilized on glutathione beads. Mid1p-N452 bound to GST-Myo2 tail (1441–1526) with K_d of $\sim 2 \mu\text{M}$ ($n = 2$; 1.9 and 2.2) (Figure 7A). The data shown is representative of two experiments. Residues 127–371 of *Drosophila* anillin bind actin filaments (22), so we tested if Mid1p-N452 also interacts with actin filaments. We assessed binding of 2 μM Mid1p-N452 to a range of concentrations (5– 20 μM) of actin filaments by pelleting the filaments and SDS-PAGE of the pellets and supernatants (Figure 7B). All of the Mid1p-N452 remained in the supernatant indicating no direct binding between the two proteins.

DISCUSSION

This study focused on the structure of the functionally important N-terminal half of fission yeast anillin Mid1p. Except where noted otherwise, Mid1p-N452 was purified from insect cells with phosphatase inhibitors, which produced the most homogeneous samples. This

region of the protein can complement most of the functions lost when the *mid1*⁺ gene is deleted. Like the wild type protein, Mid1p constructs consisting of residues 1–506 (49) or 1–452 (20) exit the nucleus during interphase and accumulate in cortical nodes that mature to form cytokinetic nodes and assemble functional contractile rings during mitosis. These constructs lack the C-terminal; lipid-binding C2 and PH domains found in Mid1p and other anillins (17, 26, 54). The lipid-binding domains may not be essential for Mid1p function, because the Type I node scaffolding protein Cdr2p can bind independently to membrane lipids (55).

Mid1p-N452 is intrinsically disordered.

Sequence analysis (Figure 1A, 1B) predicted that Mid1p-N452 has little secondary structure (only 4.9% alpha-helix), and the CD spectrum (Figure 5B) is characteristic of an IDP (53, 56). The shoulder at 222 nm is not observed in fully expanded IDPs (56) and may indicate residual secondary structure.

The hydrodynamics, spectral properties and electron microscopy are consistent with Mid1p-N452 being a moderately compact, space-filling IDP. Mid1p-N452 has a low sedimentation coefficient (~2.2 S) and large Stokes' radius (5.0 nm). Other IDPs of ~450 amino acids have hydrodynamic radii ranging from 4 to 7 nm (57), quite different from a 50 kDa spherical protein, which has a maximum sedimentation coefficient ($S_{\max}$) of 4.9 S and a minimum Stokes' radius of 2.4 nm (58). The ratio $S_{\max}/S_{\text{obs}}$ is a measure of the departure of a particle from spherical; the very large value of 2.2 for Mid1p-N452 is consistent with a rod shaped, folded protein or a moderately compact, space-filling IDP. Electron microscopy ruled out a rod shape. A plot (53) of hydrodynamic radius vs. chain length (Supplementary Figure S1) shows that Mid1p-N452 falls between fully extended IDPs and compact proteins.

PONDR-FIT analysis of the N-terminal halves of *Drosophila*, human and *S. japonicus* anillins predicted large disordered regions (Supplementary Figure 3), similar to Mid1p-N452. Hence, large intrinsically disordered regions may contribute to common functions including myosin-II binding. Although the preparation for cytokinesis differs in some ways in *S. pombe* and *S. japonicus*, the absence of Mid1p compromises the recruitment of Myo2 to cytokinesis nodes in both species (5, 20).

Pairwise comparisons of the sequence of *S. pombe* Mid1p with the sequences of anillins from *D. melanogaster*, *H. sapiens* and *S. japonicus* show only ~20% identity and <40% similarity (Table 1; Supplemental Figure S2). The divergence of these sequences extends across the full lengths of these large proteins, including the C2 and PH domains, which retain common folds (17) in spite of their divergent sequences. Remarkably, the N-terminal halves of these proteins corresponding to Mid1p-N452 are their most conserved parts (Table 2), in spite of the tendency for IDPs to evolve rapidly (59). In addition, the two animal anillins have many inserted sequences: 253 residues in *Homo sapiens* anillin and 164 residues in *Drosophila melanogaster* anillin.

Mid1p-N452 undergoes phase separation.

Purified Mid1p-N452 monomers have a strong tendency to aggregate when concentrated. Some of the protein precipitates, but much of it forms droplets that grow in size and decrease

in number over time, as characteristic for phase separation of proteins (56). At a concentration of ~20 μM most of the protein partitioned into large droplets after 3 h.

Relevance of the physical properties of Mid1p-N452 to the functions of Mid1p.

The core of cytokinetic nodes consists of about ten copies of Mid1p, ten dimers of F-BAR protein Cdc15p and IQGAP Rng2p and 2 dimers of formin Cdc12p (60). Multiple pairwise interactions connect these node proteins (20). We confirm that Mid1p-N452 binds directly with micromolar affinity to a construct consisting of the last 85 residues from the end of the Myo2 tail (Figure 7A). Mid1p is also anchored indirectly to Myo2 by IQGAP Rng2p (9, 61, 62) and to formin Cdc12p by F-BAR Cdc15p (7, 9, 10, 13, 50, 61–66).

High-resolution fluorescence microscopy of live cells has provided data on the organization of the proteins in cytokinetic nodes. Both single molecule high-resolution co-localization (SHREC) (10) and FPALM super resolution microscopy (60) localized the C-terminus of Mid1p in cytokinetic nodes close to the plasma membrane as expected from its two lipid-binding domains. Three proteins that bind the N-terminal domain of Mid1p, the end of the Myo2 tail, F-BAR protein Cdc15p and IQGAP Rng2p are all located ~20–30 nm further from the plasma membrane than the C-terminus of Mid1p (60). Therefore, the N-terminal half of Mid1p extends only 20–30 nm further from the membrane than its C-terminal lipid binding domains. The intrinsic disorder and flexibility of Mid1p-N452 may facilitate each Mid1p molecule binding Cdc15p, Rng2p and the Myo2 tail simultaneously. For example, other IDPs with low hydrophobicity, high net charge and a lack of tertiary structure are capable of high affinity but rapidly-fluctuating, electrostatic interactions (67).

More than 2 mDa of protein are crowded together in nodes near the membrane. Crowding is often associated with intrinsically disordered proteins (68–71). We estimate the volume of the core of a node composed of Mid1p, Cdc15p and Rng2p to be $\sim 4 \times 10^{-20}$ L based on areas of single molecule localizations (60). Given 10 copies of Mid1p and 10 dimers of Cdc15p and Rng2p, their local concentrations are ~500 μM , far above the concentrations that favor phase separation by Mid1p-N452. Formation of a separate phase by Mid1p may facilitate the assembly of nodes. However, we note that the proteins are present in stoichiometric ratios (60), so intermolecular interactions seem to dominate over bulk phase separation.

Actin filament binding.

Drosophila anillin was discovered by chromatography on an actin affinity column (22) and constructs consisting of residues 127–371 bind ($K_d = 4.3 \mu\text{M}$) and bundle actin filaments. A central region of 82 residues (258 to 340) is essential for binding actin filaments, while the flanking residues were implicated in bundling actin filaments (22, 24). However, we did not detect Mid1p-N452 binding directly to muscle actin filaments, even at high concentrations (20 μM), so fission yeast Mid1p may be exceptional or bind actin filaments indirectly. The region of *S. pombe* Mid1p corresponding to residues 127–371 of *Drosophila* anillin is only 15.6 % identical, consistent with different function.

How does phosphorylation influence the physical properties of Mid1p?

Phosphorylation influences the self-association of Mid1p-N452 purified from insect cells. When protected by phosphatase inhibitors during purification, the protein is phosphorylated on multiple sites, including residues phosphorylated by Polo kinase (9). These phosphorylated preparations are much more homogeneous hydrodynamically (Figure 4A) than Mid1p-N452 purified without phosphatase inhibitors, which spontaneously forms oligomers (Figure 4B). Therefore, phosphorylation by Plo1p and other kinases may contribute to solubilizing Mid1p for export from the nucleus (9, 21, 24). The intrinsically disordered flexible nature of Mid1p might also facilitate its export from the nucleus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

Mid1p-N452 has large disordered regions. (A) POND-R-FIT (black) and DisoPred (magenta) and (B) FoldIndex (black) and DisEMBL (magenta) predict large disordered segments in the middle of Mid1p-N452. Bars show predictions of short alpha-helical regions by PSIPRED (red) and JPred3 (cyan). The colored area show alpha-helical regions predicted by both PSIPRED and JPred3 (cyan) or by PSIPRED only (green). (C) Plot of mean net charge vs. mean hydrophobicity for 105 ordered and 54 disordered proteins. Mid1p-N452 (blue) is included in the cluster of disordered proteins. (D) Comparison of the frequencies of each amino acid in Mid1p-N452 (grey) with full length Mid1p (black) and the rest of the *pombe* proteome (white). Serine is over-represented in Mid1p-N452.

Figure 2:

Phosphorylation of Mid1p-N452. (A) Comparison of predicted phosphorylation sites with mass spectrometry data. Residues predicted by disorder enhanced phosphorylation predictor DISPHOS to be phosphorylated: (○) serine, (□) threonine and (○) tyrosine. Symbols filled in magenta are residues identified to be phosphorylated by tryptic digestion and MALDI-TOF mass spectrometry in recombinant Mid1p-N452 purified from insect cells with phosphatase inhibitors. (B) Amino acid sequence of Mid1p-N452 with phosphorylated residues identified by LC-MS in boxes and consensus phosphorylation sites colored magenta for Sid2, grey for Plo1p and cyan for Cdk1 and yellow for Cdk1 and Plo1p. Note the presence of 6 Sid2p, 3 Plo1p and 3 Cdk1 kinase consensus sites.

Figure 3: Mid1p-N452 is monomeric and has a large Stokes' radius. (A) Size exclusion chromatography of Mid1p-N452 on Superdex 200 10/300 GL column in 20 mM HEPES (pH 7.4), 300 mM NaCl, 1 mM TCEP at 4°C. The red line shows the molecular weight of the protein in the peak calculated using static light scattering. (B) Calibration curve of Stokes' radius vs. V_e/V_o for proteins of known Stokes' radii: bovine serum albumin (3.48 nm), carbonic anhydrase (2.35 nm), cytochrome c (1.75 nm), aldolase (4.77 nm) and beta amylase (5.4 nm). The open symbol is Mid1p-N452, which has a Stokes' radius of 5.0 nm. Inset shows SDS-PAGE of the samples from the SEC peak stained with Coomassie blue and compared with standard proteins.

Figure 4: Sedimentation velocity analytical ultracentrifugation of Mid1p-N452 in 300 mM NaCl, 1 mM TCEP and 20 mM HEPES (pH 7.4) at 10°C. The graphs show the distributions of sedimentation coefficients $C(s)$. (A) A sample of 25 μ M Mid1p-N452 purified in presence of phosphatase inhibitors. The predominant peak is at 2 S. (B) A sample of 22 μ M Mid1p-N452 purified without phosphatase inhibitors. Multiple peaks in the sedimentation profile represent heterogeneous, self-associating species with larger sedimentation coefficients.

Figure 5:

Electron microscopy and spectroscopy of Mid1p-N452 confirm that it is intrinsically disordered. (A) Electron micrograph of 0.03 μM Mid1p-N452 purified from insect cells with phosphatase inhibitors applied to a carbon film in 300 mM NaCl, 1 mM TCEP and 20 mM HEPES (pH 7.4) followed by negative staining with 0.8% uranyl formate. Three particles are circled in black. (B) Two-dimensional ^1H - ^{15}N HSQC NMR spectrum collected at 25° C of 25 μM Mid1p-N308 isotopically labeled with ^{15}N and purified from *E. coli* in 300 mM NaCl, 1 mM TCEP and 20 mM HEPES (pH 7.4). The poor resolution of the individual peaks and narrow chemical shift dispersion of the peaks are characteristic of disordered proteins. (C) Far UV CD spectrum of 5 μM Mid1p-N452 purified from insect cells with phosphatase inhibitors in 300 mM NaF, 1 mM TCEP and 20 mM phosphate (pH 7.4), collected at 25°C. A negative peak at 203 nm confirms disorder.

Figure 6:

Phase separation of Mid1p-N452 purified from insect cells with phosphatase inhibitors observed by DIC and fluorescence microscopy. (A) Three different concentrations of Mid1p-N452 in 300 mM NaCl, 1 mM TCEP and 20 mM HEPES (pH 7.4) form droplets at room temperature that grow in size over time. (B) Spinning disk confocal fluorescence micrograph of 10 μM Mid1p-N452 labeled with Alexa 488 maleimide dye at C-terminal cysteine and incubated in the same buffer at room temperature for ~30 min.

Figure 7:

Equilibrium binding of Mid1p-N452 purified from insect cells with phosphatase inhibitors to a Myo2 tail construct and actin filaments. (A) GST pull down assay with 10 μM of Mid1p-N452 incubated for 120 min with a range of concentrations of GST-Myo2-tail (1441–1525) immobilized on glutathione beads in 250 μl of 300 mM NaCl, 1 mM TCEP and 20 mM HEPES (pH 7.4) at 4°C. After pelleting the beads, Mid1p-N452 in the supernatant was measured by SDS-PAGE, staining with Coomassie blue and densitometry (inset). The bound fraction was calculated by difference from the control in the first lane without beads. The graph shows bound fraction of Mid1p-N452 plotted against the GST-Myo2-tail (1441–1525) concentrations fit with a curve for $K_d = 2 \mu\text{M}$. (B) Actin filament binding assay with 2 μM of Mid1p-N452 and pelleting of 5 to 20 μM actin filaments in 50 mM KCl, 1 mM MgCl_2 , 1 mM EGTA, 10 mM imidazole (pH 7.5) buffer. After pelleting the

actin filaments, samples of the pellets and supernatants were analyzed by SDS-PAGE, staining with Coomassie blue and densitometry.

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Table 1:

Hydrodynamic properties of Mid1p-N452 calculated from light scattering, gel filtration and sedimentation velocity analytical ultracentrifugation. The primary measurements are bold. The molecular weight in the “Gel filtration” row was calculated with $S(20,w) = 2.2$ from the lower row. The molecular weight calculated from the amino acid sequence is 52.7 kDa.

Method	Stokes radius (Å)	D (cm ² s ⁻¹)	S (20,w)	Molecular weight (kDa)
Light scattering				60 (n = 2)
Gel filtration	49 (n = 1)	5×10^{-7} (n = 1)		43
Sedimentation velocity	****	4.5×10^{-7} (n = 3)	2.2 (n = 3)	42.7

Table 2:

Comparisons of the sequence identities of *S. pombe* Mid1p with the sequences of anillins from *S. japonicus*, *D. melanogaster* and *Homo sapiens* based on Clustal Omega multiple sequence alignment. Regions considered were the full-length proteins, the N-terminal 452 residues of Mid1p, and the C2 domain, the PH domain from x-ray crystal structure for C-termini of Mid1p and human anillin (17). The corresponding region for *S. japonicus*, *D. melanogaster* and *Homo sapiens* are in parentheses.

Sequence identities with Mid1p	Full Length	N-452 (residues)	C2 domain (residues)	PH domain (residues)
<i>S. japonicus</i>	29.8%	25.2% (1–463)	28.3% (591–822)	27.1% (839–934)
<i>D. melanogaster</i>	23%	21.6% (1–530)	18% (706–944)	23% (1072–1191)
<i>Homo sapiens</i>	17.2%	18.8% (1–604)	14.1% (801–986)	18.8% (987–1124)