Sfi1p has conserved centrin-binding sites and an essential function in budding yeast spindle pole body duplication

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Centrins are calmodulin-like proteins present in microtubule-organizing centers. The Saccharomyces cerevisiae centrin, Cdc31p, was functionally tagged with a single Z domain of protein A, and used in pull-down experiments to isolate Cdc31p-binding proteins. One of these, Sfi1p, localizes to the half-bridge of the spindle pole body (SPB), where Cdc31p is also localized. Temperature-sensitive mutants in SFI1 show a defect in SPB duplication and genetic interactions with cdc31-1. Sfi1p contains multiple internal repeats that are also present in a Schizosaccharomyces pombe protein, which also localizes to the SPB, and in several human proteins, one of which localizes close to the centriole region. Cdc31p binds directly to individual Sfi1 repeats in a 1:1 ratio, so a single molecule of Sfi1p binds multiple molecules of Cdc31p. The centrosomal human protein containing Sfi1 repeats also binds centrin in the repeat region, showing that this centrin-binding motif is conserved.

Introduction

Centrins are conserved calmodulin-like proteins and, like calmodulin, have roles in a number of different cellular processes (Schiebel and Bornens, 1995; Sullivan et al., 1998; Araki et al., 2001; Pulvermuller et al., 2002). In particular, centrins are ubiquitous components of microtubule-organizing centers (MTOCs), and are often located within different parts of the MTOC (Levy et al., 1996), again suggesting multiple roles. Two clear roles have been established. One is in the duplication of the MTOC (Byers, 1981; Middendorp et al., 2000; Salisbury et al., 2002), and the other is as constituents of contractile fibers within and attached to the MTOC, which can contract in response to changes in Ca\(^{2+}\) concentration (Salisbury et al., 1984). A related fibrous structure that also contracts in response to changes in Ca\(^{2+}\) concentration is the vorticellid spasmoneme (Weis-Fogh and Amos, 1972; Moriyama et al., 1999). Its principle constituent, spasmin (Amos et al., 1975), has homology to EF-hand domain proteins such as centrin (Maciejewski et al., 1999). In addition, the spasmoneme also has elastic properties that are not dependent on the Ca\(^{2+}\) concentration (Weis-Fogh and Amos, 1972). Thus, two properties, Ca\(^{2+}\)-independent elasticity, may be characteristic of centrin-containing fibrous structures.

Budding yeast has one centrin, Cdc31p, some of which is localized (Spang et al., 1993) to the half-bridge of the spindle pole body (SPB). The SPB is embedded in the nuclear envelope, and the half-bridge is a specialized rectangular area of the envelope attached to one side of the SPB (Byers and Goetsch, 1974; Adams and Kilmartin, 1999; O'Toole et al., 1999). The half-bridge plays an important role in SPB duplication. First, the new SPB starts to assemble on the distal cytoplasmic side of the half-bridge; and second, the half-bridge allows the transit of the partly assembled SPB across the two lipid bilayers of the nuclear envelope so that a nuclear spindle can be formed (Adams and Kilmartin, 1999). This transit process occurs by fusion of the cytoplasmic and nuclear lipid bilayers at the distal end of the half-bridge, followed by retraction of the half-bridge across the face of the newly assembled duplication plaque. This exposes the plaque to nucleoplasm, thereby allowing assembly of nuclear SPB components and completing SPB assembly (Adams and Kilmartin, 1999). The retraction process may rely on possible elastic properties of the half-bridge.

Cdc31p is essential for SPB duplication: temperature-sensitive mutants arrest with a single large SPB (Byers, 2003).
ever short NH2-termini of Cdc31p with proteins other than Kar1p are less Ca2+ sensitive (Geier et al., 1996). In addition, interactions of spasmmin with the spasmoneme (Amos, 1971; Amos et al., 1975), ciliary lattice (Klotz et al., 1997), are stable in the presence of EGTA. The proteins that copurified with ZCdc31p were of diverse function: Vps13p is involved membrane traffic (Brickner and Fuller, 1997), Thp1p is involved in mitotic recombination (Gallardo and Aguilar, 2001), and Hem15p is ferrochelatase (Labbe-Bois, 1990). One protein of particular interest, Sfi1p, was initially identified as a suppressor of the heat sensitivity associated with a particular mutation in adenyl cyclase (Ma et al., 1999). When Sfi1p was partly characterized, it was found not to be involved in the adenyl cyclase pathway, but it is an essential protein whose depletion causes a G1/M arrest with failure to form a mitotic spindle (Ma et al., 1999). This is similar to the phenotype of cdc31-1 (Byers, 1981), and indicates a possible function at the SPB.

To confirm that the interaction between Sfi1p and Cdc31p was reciprocal, Sfi1p-prA was used in a pull-down experiment under the same lysis conditions (Fig. 1 b). This pull-down was not as clean, but it did isolate Cdc31p together with another SPB component, Spc110p (Rout and Kilmartin, 1990; Kilmartin et al., 1993). It is not clear whether this reflects a direct interaction with Spc110p or with other SPB components such as Spc42p (Donaldson and Kilmartin, 1996), which are present in a complex with Spc110p (Elliott et al., 1999). At the low levels of protein present in Fig. 1 b, these other SPB components would be difficult to detect.

These experiments show a reciprocal interaction between Sfi1p and Cdc31p. In addition, inspection of the relative intensities of the Coomassie-stained bands for Sfi1p-prA and Cdc31p (the bands were too faint to scan accurately) suggests that Cdc31p is present in a molar ratio >1.

Localization of Sfi1p-GFP

Sfi1p was tagged with GFP and was found to localize to one or two spots that were coincident with nuclear DNA (Fig. 2, a and b). This suggests localization to the SPB region, which was confirmed by immunofluorescence (Fig. 2, c and d). Immuno-EM then defined the localization more precisely to the half-bridge (Fig. 2 e). The immuno-EM staining of Sfi1p in single SPBs appeared somewhat distal to the existing SPB, and in paired SPBs was more centrally located on the bridge compared with other bridge components such as Spc72p (Adams and Kilmartin, 1999). Whether this indicates a more specific localization of Sfi1p on the half-bridge or merely antigen accessibility is not clear. Cdc31p is also localized to the half-bridge (Spang et al., 1993), thus Sfi1p and Cdc31p are located in the same part of the SPB.

Phenotype of sfi1-3 and sfi1-7

The localization of Sfi1p to the half-bridge of the SPB suggests that it might have a function in SPB duplication. Temperature-sensitive mutants were prepared to check the arrest in more detail, in particular to determine whether SPB duplication had occurred and also to look for genetic interactions with CDC31. Two alleles, sfi1-3 and sfi1-7, were examined by immunofluorescent staining after arrest for 4 h at 36°C, and showed a very similar phenotype to the depletion (Ma et al., 1999), i.e., failure to form a mitotic spindle (unpublished data). However, cells synchronized in G1 with

Figure 1. SDS gel of proteins isolated from yeast cells containing ZCdc31p or Sfi1p-prA after binding to IgG-Sepharose. Bands were identified by matrix-assisted laser desorption/ionization mass spectrometry. Numbered bands contained heat shock proteins, proteosome components, ribosomal proteins, keratin, or nonspecific proteins (see Materials and methods).
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Factor (10 g/ml for 150 min at 23°C) and released at 36°C showed interesting differences between the two alleles. At 1.5 h after release, sfi1-7 cells passed through mitosis: 78% of the cells had spindles, and post-anaphase spindles appeared to show normal separating or separated DNA (Fig. 3 a). All of the cells then arrested with single microtubule asters from 2.5 to 5 h (Fig. 3 b). However, sfi1-3 cells (95% of the cells examined), despite budding at the same time as sfi1-7, did not pass through mitosis at 1.5 h, but remained arrested with single microtubule asters between 1.5 and 5 h (Fig. 3 c). These results show that sfi1-3 cells arrest during the first cell cycle, and sfi1-7 cells arrest during the second cell cycle. This behavior is very similar to that of different cdc31 alleles, where some arrest with single microtubule asters in the first cell cycle, and some arrest in the second (Byers, 1981; Vallen et al., 1994).

Both alleles were examined by EM to determine whether SPB duplication had occurred. The SPB-containing regions of asynchronous cells after 4 h at 36°C and synchronized cells between 2.5 and 5 h after release were examined by serial sectioning. In a total of 50 cells from both alleles, 49 had only single SPBs (Fig. 3 d–g). The one exception was an sfi1-7 cell with one normal SPB and one small partly assembled SPB still on the cytoplasmic side of the half-bridge, similar to the phenotype of mps2-1 (Winey et al., 1991).

About one third of the single SPBs had clear half-bridges (Fig. 3 e), but this is an underestimate, as the half-bridge is difficult to recognize unless it is almost perpendicular to the section. There was a clear increase in SPB size, the central plaque diameter increasing from 0.10 ± 0.02 μm (Fig. 3 d) to 0.16 ± 0.08 μm (Fig. 3 e–g). This is again similar to cdc31-1 (Byers, 1981), although the increase in SPB size is smaller.

In conclusion, the phenotypes of both sfi1-3 and sfi1-7 show striking similarities to cdc31 alleles (Byers, 1981; Vallen et al., 1994). All of these alleles arrest with a single large SPB in either the first or second cell cycle at the restrictive temperature.

Genetic interactions between sfi1-3, sfi1-7, and cdc31-1

The similarity between the phenotypes of sfi1-3, sfi1-7, and cdc31-1 suggest that there might be genetic interactions be-
between \textit{SFI1} and \textit{CDC31}. Overexpression of \textit{CDC31} on a 2-\mu m plasmid suppressed both \textit{sfi1-3} (Fig. 4 a) and \textit{sfi1-7} at 37\(^\circ\)C. There were also synthetic growth defects between both \textit{sfi1} alleles and \textit{cdc31-1}. Only one third of the expected \textit{sfi1-3 cdc31-1} double temperature-sensitive spores grew at 23\(^\circ\)C, and these all grew extremely slowly, produced a very variable colony size on streaking out, and were not studied further. All except one of the expected \textit{sfi1-3 cdc31-1} double temperature-sensitive spores grew at 23\(^\circ\)C, but slightly more slowly than the wild type, and failed to grow at all at 30\(^\circ\)C. Transformation of these cells with \textit{SFI1} restored growth at 30\(^\circ\)C, which was dependent on the presence of \textit{SFI1} (Fig. 4 b). These experiments show a synthetic growth interaction between the \textit{sfi1} alleles and \textit{cdc31-1}, and both sets of experiments show genetic interactions between \textit{SFI1} and \textit{CDC31}.

**Structural features and conservation of Sfi1 repeats**

\textit{Saccharomyces cerevisiae} Sfi1p is a very divergent protein. There is only 24.5\% sequence identity (Cliften et al., 2003) between the \textit{S. cerevisiae} and \textit{Saccharomyces castelli} proteins (these alignments are displayed at http://db.yeastgenome.org). This degree of divergence is similar or sometimes even worse for most SPB proteins; for example, Spc42p (Donaldson and Kilmartin, 1996) has only 16.5\% identity between the two species. However, \textit{S. cerevisiae} Sfi1p has a series of striking internal repeats (Fig. 5 a) containing a consensus sequence AX\_7LLX\_3F/LX\_2WK/R. This repeat structure is conserved in the other \textit{Saccharomyces} species, with the possible exception of repeats around W304 and F698, and sometimes the alanine is not as prominent. The gap between the clusters of repeats in \textit{S. cerevisiae} Sfi1p is between 23 and 35 amino acids.

Proteins with similar repeats to Sfi1p and with similar gaps between the repeats were identified by BLAST searching. These were an \textit{S. pombe} protein (T40750) called SpSfi1 here, and a human protein (KIAA0542) called hSfi1 here (Fig. 5, b and c). There are at least two versions of hSfi1 with almost identical sequence, but with stop codons after either 968 or 1242 amino acids (see Materials and methods); the version with 1242 amino acids is analyzed here. The re-
peats in hSfi1 are particularly uniform. Between residues 96 and 862 there are nine continuous 33mer repeats, one 35mer, a further nine 33mer repeats, one 37mer, and finally three 33mer repeats (the position of the conserved tryptophan, W, is shown diagrammatically in Fig. 5). The proline content within the repeat regions for all of the three proteins was low: one for Sfi1p, none for S. pombe Sfi1, and two for human Sfi1. There are no further consensus residues outside of the repeat region shown in Fig. 5, and no homology was detected between the NH2- and COOH-terminal domains of the three proteins.

Are there other proteins in these three organisms that also contain Sfi1 repeats? Because the repeat is quite heterogeneous in sequence, BLAST searching was not suitable, so ScanProsite (Gattiker et al., 2002) was used instead (see Materials and methods). This identified two more human proteins and the partial sequence of another containing multiple copies of this repeat (see Materials and methods). However, these sequences have many fewer repeats than hSfi1, and are thus less similar to the yeast proteins. No further S. cerevisiae proteins were identified. These results suggest that Sfi1 repeats are conserved between fungi and humans, and may be present in multiple proteins within one organism.

 Localization of S. pombe Sfi1 and human Sfi1
S. pombe Sfi1 (Fig. 5 b), a potential homologue of S. cerevisiae Sfi1p, was tagged with GFP to see whether it too localized to the SPB. Tagged S. pombe Sfi1 localized to one or two spots per cell coincident with the nuclear DNA (Fig. 6,

Figure 6. Localization of S. pombe Sfi1 (SpSfi1). (a and b) Fluorescence of unfixed cells containing SpSfi1p-GFP. GFP fluorescence (a) and DAPI fluorescence for DNA (b) show one or two GFP spots coincident with nuclei. (c–f) GFP fluorescence and immunofluorescence. GFP fluorescence (c and e) with anti-tubulin (d) to show localization of SpSfi1p to the ends of the mitotic spindle, and with the SPB marker anti-Sad1 (f) to show localization to the SPB region. Bars, 2 μm.

Figure 7. Transfection of HeLa cells with GFP-hSfi1. Cells stained with anti-GFP (a) and anti-γ-tubulin (b). An untransfected cell is shown at the bottom. Staining of the centrosome region with anti-GFP (c and f), anti-centrin 2 (d), GT335 (g), and corresponding merged images (e and h). Anti-GFP is green and anti-centrin 2 and GT335 are red. Bars, 5 μm.
Double labeling showed that these spots localized to the ends of the mitotic spindle (Fig. 6, a and b) and were coincident with Sad1 (Fig. 6, e and f), a marker for the SPB (Hagan and Yanagida, 1995). Thus, S. pombe Sfi1, like S. cerevisiae Sfi1p, localizes to the SPB region.

Of the four human proteins identified with potential Sfi1 repeats, hSfi1 seemed the most homologous to S. cerevisiae Sfi1p and S. pombe Sfi1. The other human proteins had between 4 and 10 repeats, whereas hSfi1 had 23 repeats, similar to the repeat number in the two yeasts (Fig. 5). In addition, as in the two yeasts, it had the repeats centrally positioned, similarly spaced, and also a low proline content in the repeat region.

hSfi1 was tagged at the NH2 terminus with GFP and was transfected into HeLa cells. A faint signal was detected with anti-GFP consisting of one or two dots within or close to the centrosomal γ-tubulin staining (Fig. 7, a and b). Similar results were found for both the 1242 and 968 amino acid forms of hSfi1, but the transfection efficiency was much higher for the 1242 amino acid form, so this was used for all the experiments. No staining was seen when GFP was placed at the COOH terminus of either form. The staining was almost completely coincident (Fig. 7, c–e) with an mAb, Cetn2 (Hart et al., 2001), against centrin 2, and almost coincident (Fig. 7, f–h) with an mAb, GT335 (Wolff et al., 1992), against glutamylated tubulin and a marker for centrioles (Bobinnec et al., 1998). These results indicate that hSfi1 localizes close to the centriole.

**Binding of Cdc31p to Sfi1p**

The prA pull-down experiments shown in Fig. 1 suggest the Cdc31p binds directly to Sfi1p. This was tested by GST pull-downs using proteins expressed in *Escherichia coli*. Because Cdc31p is conserved and the only conserved part of Sfi1p is the repeats, it seemed likely that Cdc31p would bind to this region. Accordingly, Sfi1p was initially divided into five domains (Fig. 8 a); the NH2-terminal domain (fusion 1), three domains containing the repeats (fusions 2–4), and the COOH-terminal domain (fusion 5). The SDS gel of the GST pull-downs (Fig. 8 b) shows the recombinant Cdc31p in the left lane, followed by the fusion protein (F), the supernatant from the pull-down (S), and the washed pellet from the pull-down (P) for each of the five constructs. It is clear that Cdc31p fails to bind to the NH2- and COOH-terminal domains, but does bind to the three fusions containing the repeats (Fig. 8 b). In addition, the relative intensities of the Coomassie-stained bands show that the molar ratio of Cdc31p to fusion is >1. Scanning of the gels showed between 3 and 4 mol of Cdc31p per mol of fusion protein (this number is approximate for fusion 3 because of proteolysis).
Increasing the Cdc31p concentration showed some evidence for saturation for fusion 2, where the molar ratio rose to ~5.5 compared with the six predicted repeats (Fig. 8 c).

Next, individual and smaller groups of repeats were examined. In designing the fusions for the individual repeats, it was assumed that the conserved tryptophan was the critical element, so it was placed centrally. All of these fusions were positive for Cdc31p binding (Fig. 8, d and e), although for 3b the affinity was low. The other fusions containing single repeats (2b, 3a, 4a, and 4b) showed molar ratios close to 1, which did not rise above 1.1 over a 10-fold range of Cdc31p concentration (unpublished data). For the fusions with 2 to 3 repeats, 2a unfortunately suffered from proteolysis, 2c gave a ratio of 1.1 in Fig. 8 d, which rose to 2.5 when the Cdc31p concentration was increased (unpublished data), compared with the predicted three repeats. The ratio for 4c containing two repeats never rose above 1.1 when the Cdc31p concentration was increased (unpublished data), suggesting that one of the repeats is inactive. As expected from the low free Ca²⁺ conditions of the yeast lysate pull-down (Fig. 1 a), the binding of Cdc31p to the GST fusions did not appear to be Ca²⁺-sensitive because it was unaffected by removal of Ca²⁺ with 5 mM EGTA (unpublished data). Together, these data suggest that most of the Sfi1 repeats in Sfi1p can bind Cdc31p.

Sfi1 repeats have been found in several human proteins (see Fig. 5 c and Materials and methods), so fusions were also prepared for hSfi1 (Fig. 8 f) and tested for binding to Cdc31p and human centrin 2 (Lee and Huang, 1993). The fusions containing Sfi1 repeats bound both Cdc31p (Fig. 8 g) and human centrin 2 (Fig. 8 h), whereas fusion 10 from the COOH terminus, containing no detectable Sfi1 repeats, did not bind either protein (Fig. 8, g and h). Human centrins 1 (Errabolu et al., 1994) and 3 (Middendorp et al., 1997) and mouse centrin 4 (Gavet et al., 2003) gave similar binding to fusions 6–9, but not fusion 10, with slight variations between the different centrins (unpublished data). Again, the binding of Cdc31p was unaffected by 5 mM EGTA, though the binding of centrin 2 was slightly decreased (unpublished data).

These results show that Sfi1 binds multiple molecules of Cdc31p directly through a series of conserved repeats. A human homologue of Sfi1p, hSfi1 containing similar repeats, also binds Cdc31p as well as mammalian centrins directly at these repeats.

Discussion

This paper describes a protein, Sfi1p, isolated from a pull-down using a functional tagged version of the budding yeast centrin, Cdc31p. Like Cdc31p (Byers, 1981; Spang et al., 1993), Sfi1p localizes to the half-bridge of the SPB and has an essential function in SPB duplication. SFI1 and CDC31 show genetic interactions, suggesting they interact in vivo. Cdc31p binds directly to multiple binding sites in Sfi1p that consist of a series of 17 novel repeats in the central region of the protein. This now brings the number of Cdc31p-binding proteins in the half-bridge to three; Kar1p (Biggins and Rose, 1994; Spang et al., 1995), Mps3p/Nep98p (Jaspersen et al., 2002; Nishikawa et al., 2003), and Sfi1p. This paper shows that the Sfi1 repeats are also present in two previously uncharacterized proteins, one from the evolutionarily distant yeast S. pombe, and another from humans. The human protein, hSfi1, binds centrin at the repeats, and all three proteins localize to the spindle pole.

Sfi1 repeats have some resemblance to WD repeats (Neer et al., 1994), but are clearly distinct because Sfi1 repeats lack the conserved aspartic acid (Garcia-Higuera et al., 1998) in WD repeats (that would be at position 16 in Fig. 5). Also, WD repeats lack the consensus leucine at position 14 (Fig. 5) of Sfi1 repeats. Both temperature-sensitive alleles of Sfi1p have mutations within the first and second repeats (see Materials and methods), and indeed, sfi1-3 has a mutation (F208C) in the consensus phenylalanine at position 19 in the first repeat. These results suggest that disabling one or two repeats may also affect the function of the rest of the repeats. Sfi1 repeats are apparently absent from the other proteins isolated in the Cdc31p pull-down (Fig. 1), and are not present in the three other proteins that directly bind Cdc31p, Kar1p (Biggins and Rose, 1994; Spang et al., 1995), Kic1p (Sullivan et al., 1998), and Mps3p/Nep98p (Jaspersen et al., 2002; Nishikawa et al., 2003). The Cdc31p-binding site in Kar1p has been identified (Biggins and Rose, 1994; Spang et al., 1995), but appears different from Sfi1 repeats. This heterogeneity in binding site motifs is very similar to the large variations between calmodulin-binding sites (Hoeflich and Ikura, 2002).

The most analogous calmodulin-binding domain to the centrin-binding Sfi1 repeats might be the IQ domains in the neck region of unconventional myosins (Chenery and Mooseker, 1992). Like the Sfi1 repeats, the myosin IQ domains show Ca²⁺-independent binding and are present in multiple continuous copies. They are spaced at a slightly shorter distance apart (23–26 residues), though other multiple IQ domains are spaced more widely, for example, 28–32 residues in the IQGAP-related protein Iqg1p (Terrak et al., 2003). Myosin IQ domains are thought to be present in a helical conformation between the NH₂- and COOH-terminal lobes of calmodulin (Houdusse et al., 1996); this model is based on the structure of scallop myosin with two calmodulin-like light chains bound (Xie et al., 1994; Houdusse and Cohen, 1996). The spacing of 23–26 residues between adjacent IQ domains is compatible with direct connections between the calmodulin molecules, which can explain some of the Ca²⁺-dependent regulation of the activity of unconventional myosins. Here, addition of Ca²⁺ could cause conformational changes in the already bound calmodulin. This may regulate the function of the motor domain because the first bound calmodulin may be directly in contact with that domain (Houdusse et al., 1996).

The Sfi1 repeat regions of the proteins described here generally lack prolines, and thus might adopt a helical conformation like the IQ repeats. The wider spacing between adjacent Sfi1 repeats (usually between 23 and 33 residues) may also be compatible with direct connections between the centrins, given the characteristic larger NH₂-terminal domains of centrins compared with calmodulin (Bhattacharya et al., 1993). This type of structure may have some relevance to other EF-hand domain or centrin-containing organelles such as the spasmomene (Amos et al., 1975) and striated flagellar roots (Salisbury et al., 1984), which rap-
idly contract on addition of Ca\textsuperscript{2+}. Spasmin or centrin are the main protein components of these organelles, so it was originally thought that they themselves might form the 2–7-nm filaments present in these organelles (Amos, 1975; Schiebel and Bornens, 1995). This now seems unlikely, given the homology of these proteins to EF-hand domain proteins or calmodulin. However, if a protein with multiple Sfi1 repeats were present in these organelles, then it could form a template for what would effectively be a short filament of spasmin or centrin. These filaments could then be made longer by end-to-end or staggered side-to-side interactions. Such a protein would be difficult to identify because it would be faintly stained in Coomassie-stained SDS gels of the organelle, due to the high molar ratio of spasmin or centrin to the Sfi1p-like protein. The association of at least spasmin with such a protein is likely to be Ca\textsuperscript{2+}-dependent because spasmomyosin are glycinated in 4 mM EGTA (Amos, 1971). Addition of Ca\textsuperscript{2+} to spasmin or centrin already bound to an Sfi1p-like protein could cause conformational changes, in the same way that addition of Ca\textsuperscript{2+} to calmodulin already bound to a K\textsuperscript{+} channel opens the gate (Schumacher et al., 2001). In the case of centrin bound to an Sfi1p-like protein, similar conformational changes could lead to a rapid shortening of a filament. A simple analogy might be the concertina part of a child’s flexible drinking straw. The extended form would be when the concertinas were stretched out, the conformational change would be equivalent to folding individual concertinas, thus leading to shortening.

What would be the role of Sfi1p be in SPB duplication? Clearly, if Sfi1p does form filaments, these might be important structural elements of the half-bridge. In addition, they may play a role in the proposed extension and retraction of the half-bridge during SPB insertion (Adams and Kilmartin, 1999). At present, there is no evidence for Ca\textsuperscript{2+} pulses at this point in the cell cycle, which might be necessary to expand and contract an Sfi1p-Cdc31p filament. However, an additional feature of centrin-binding filaments, at least in the case of the spasmomere (Weis-Fogh and Amos, 1972), is elasticity, which is not dependent on the Ca\textsuperscript{2+} concentration. Elastic proteins generally have multiple repeats (Tatham and Shewry, 2000), and in the well-studied example of titin, the stretching occurs by unfolding of tandem Ig-like repeats (Li et al., 2000). Possibly, Sfi1p-Cdc31p might be stretched during duplication plaque assembly by unfolding of Sfi1 repeats, with possible concomitant dissociation of some of the Cdc31p. After completion of assembly, tension would be released in some way and retraction would be driven by reassociation of Cdc31p.

Clearly, the multiple binding of centrin to the Sfi1 repeats is an interesting structural problem, and using some of the recombinant proteins described here, should be amenable to crystallographic investigation. In addition, using longer versions of these recombinant proteins, it should be possible to test any elastic properties or Ca\textsuperscript{2+}-dependent contraction.

Materials and methods

\textit{S. cerevisiae} strains

All \textit{S. cerevisiae} strains were prepared in K699 (W303a) background or the isogenic diploid K842 (Nasmyth et al., 1990), and yeast vectors used were the pRS series (Sikorski and Hieter, 1989). The \textit{cdc31-1} allele was backcrossed to K699 five times, and the sequence change in this allele, A48T (Vallen et al., 1994), was verified by PCR sequencing.

The construct to make ZCd31p was prepared by first placing a Ncol site by PCR at the initiator methionine of \textit{CDC31}; this would cause the change S2G, and did not appear to affect the function of the protein before the construct was used to rescue a deletion. The plasmid was amplified from pEZZ218 (GenBank/EMBL/DDB) accession no. M74186 between Ncol and Blp1I sites and was inserted in the correct orientation into the Ncol site place in \textit{CDC31}. This replaced the initiator methionine in Cd31p with the sequence: MGVSDFNKS...AQAPKFM, a total of 63 amino acids with the sequence between VDNK and AQAPK corresponding to the synthetic 8-amino acid Z domain (Nilsson et al., 1987). The HA-Cd31p construct was prepared by linker oligos again using Ncol and Blp1I sites and inserted into the sequence MGSSPYDVPDYAHH in place of the initiator methionine. Both constructs in the CEN vector pRS314 were able to complement a deletion of \textit{CDC31} with the cells showing an apparently normal growth rate.

Sfi1p was cloned by Vent PCR and by gap repair. Both constructs showed the same amino acid changes compared with the previously published sequence (Ma et al., 1999); these are probably due to strain differences and were: L96P, F278L, D541G, I623V, and C93E. Temperature-sensitive mutants were prepared by slight modifications of the gap repair procedure (Muhlrad et al., 1992). For \textit{sfi1-1}, the primers for the mutagenic PCR were 97–119 and 2820–2800, and the plasmid was gapped between Apg2420 and Sty1/2670 sites; for \textit{sfi1-7}, the primers were 340–361 and 2820–2800, and the plasmid was gapped between Mscl (748) and the same Sty1 site. All base pair numbers start from the A of the codon of the presumed initiator methionine. The PCR conditions were those for colony PCR (Akada et al., 2000) with 7.5 mM MgCl\textsubscript{2}, 0.2 mM dATP and dGTP, and 1 mM dCTP and dTTP. Transformants were replica plated onto fluoroorotic acid (FOA) medium (Boeke et al., 1984) to remove the wild-type URA3 plasmid, then onto warm (37°C) and ambient YEPD plates. Temperature-sensitive colonies were selected, and the plasmid was recovered. Plasmids conferring temperature-sensitive phenotype were transferred to the URA3 plasmid pRS306 and transformed into K699 to replace the wild-type gene with the temperature-sensitive allele by the popin-popout procedure described by Boeke et al. (1987). Ura\textsuperscript{-} transformants were patched onto FOA and...
Identification of other proteins with multiple Sfi1 repeats
A BLAST search with S. cerevisiae Sfi1p was not successful, as the Candida albicans homologues (listed at http://genolist.pasteur.fr/CandidaDB; gene name IPF5574) was used instead. A BLAST search with this Candida homologue identified S. cerevisiae Sfi1p (E value 2e−14), an S. pombe protein (GenBank accession no. T40750; E value 1e−6) called here Sfi1, and a human protein of 1212 amino acids KIAA0542 (BA2A5468; E value 0.096) called here hSfi1. All of these proteins have an unusual repeat structure (Fig. 5). The hSfi1 (KIAA0542) clone was sequenced and found actually to correspond to a splicing variant (T00322) containing 968 amino acids (apart from the substitution H32Z2Y). An Image EST clone (Image 5273112, B1463460) was sequenced and found to contain a full-length insert in the database sequence of hSfi1 with an additional insert of YFCFLAKDNVTHDHLQQRNLHQQFQGT between amino acids 385 and 386, and the partial sequences L0471 and P10165. This protein has 1242 amino acids. Apart from the above 31 amino acid insert and the substitution H438L, both versions of hSfi1 are identical, with the 968 amino acid form having a earlier stop codon. None of these ORFs could be followed continuously over a 120-kb region of human chromosome 22. Mice also have homologues (AAH46305 and AAH26390) that terminate at positions equivalent to 968 and 1242.

Other human proteins that might contain Sfi1 repeats were identified by ScanProsite (Gattiker et al., 2002) using the sequence AXVIFLXW/K/R/H and calling for at least three hits in the ORF. Out of >800,000 entries, this identified only Sfi1p, it’s S. pombe, Aspergillus, mouse, and human homologues, together with the complete sequence of one human protein, AAM34297 (five repeats between residues 757–879) and the partial protein, AAM4297 (five repeats between residues 757–879) and the partial homologue identified CAD38978 is quite tryptophan rich in parts of this region. Further by modulating the function of two TGN localization signals.

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