Forchlorfenuron, a phenylurea cytokinin, disturbs septin organization in *Saccharomyces cerevisiae*

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Septins, which are involved in cytokinesis, have been identified in a variety of fungi and animal cells. For analysis of the function of septin, drugs targeting septin would be useful; however, no such drugs have been available hitherto. By serendipity, we found that forchlorfenuron (FCF, N-(2-chloro-4-pyridyl)-N-phenylurea, 4PU300), a synthetic plant cytokinin, disturbed cytokinesis in *Saccharomyces cerevisiae*. Upon administration of FCF, septin structures at the bud neck became deformed and filament-like septin appeared outside of the neck. Under these conditions, the localization of actin was normal and Gin4, which is localized at the bud neck in a septin-dependent manner, was found to remain at the location of apparently normal septin at the bud neck, whereas it was not co-localized to the deformed septin at the bud neck or to septin seen outside the bud neck. FCF administration immediately induced production of sporadic septin structures outside the bud neck, and these structures disappeared promptly upon removal of the drug. Taken together, these findings indicate that FCF maybe a promising drug for investigating the structure and function of septin.

Key words: forchlorfenuron, septin, *Saccharomyces cerevisiae*

INTRODUCTION

During cell division, protein complexes with a particular function are assembled and disassembled in a spatiotemporally regulated manner. One well-known such structure is yeast septin. In *S. cerevisiae*, a filamentous structure called the neck filament was observed by electron microscopy at the bud neck, which is the cleavage site at mitosis (Byers and Goetsch, 1976a). This neck filament is about 10 nm in diameter and is localized just under the plasma membrane. The *S. cerevisiae* genes *CDC3*, *CDC10*, *CDC11*, and *CDC12* were identified by mutations that cause a defect in cytokinesis, and a mutation in any of these genes causes disappearance of the neck filament (Byers and Goetsch, 1976b). Later, *SHS1* (Mino et al., 1998) was added to this set of genes. The amino acid sequences deduced from the nucleotide sequences of these genes are similar to each other and these proteins are collectively designated septins (Haarer and Pringle, 1987; Ford and Pringle, 1991; Kim et al., 1991). Studies in which protein filaments were isolated from immunoprecipitates with anti-Cdc3 antibody and visualized by electron microscopy clearly indicated that septins are components of the neck filaments (Frazier et al., 1998).

Proteins structurally related to yeast septins have been identified in a variety of fungi and animals. For example, *Drosophila* septins, Pnut and Sep1, co-localize near the contractile ring in dividing cells. Loss of the Pnut function causes the formation of multinucleated cells (Neufeld and Rubin, 1994). Pnut, Sep1 and another *Drosophila* septin, Sep2, were purified and assembled into 7-nm filaments in vitro (Field et al., 1996). An important role of septins in mammalian cells was also shown by Kinoshita et al. (1997). They identified Nedd5, encoding a septin, as a highly expressed gene in mouse neural precursor cells and demonstrated that Nedd5 plays an essential role in cytokinesis. These data indicate that septins play a pivotal role in cytokinesis not only in yeast but also in *Drosophila* and mouse. However, mammalian septin has roles other than in cytokinesis, such as in membrane trafficking (Dent et al., 2002).

Septin is localized to the bud neck during cell division of yeast cells, disassembled after cytokinesis, and re-localized to an incipient bud site in the next cell cycle. How are the septin dynamics regulated? All of the septins except Cdc11 contain a predicted GTP-hydrolyzing site (P-loop). Also, septins except Cdc10 contain a coiled-coil structure at their C-terminal region that could serve in
protein-protein interactions. Cdc10 and Cdc12 were found to have GTPase activity and the GTP-bound form of Cdc12, but not its GTPase activity, was shown to be essential for the formation of septin filaments (Versele and Thorner, 2004).

Some mutations are known to cause changes in the morphology of septin filaments: ∆agin4 (Longtine et al., 1998), yck2Δ (Robinson et al., 1999), ∆cla4 (Cvrckowa et al., 1995; Weiss et al., 2000), ∆ste20 (Cvrckowa et al., 1995), ∆bni5 (Lee et al., 2002), and elm1 (Bouquin et al., 2000) are among such mutations and they may be in factors affecting septin dynamics. Versele and Thorner (2004) showed the involvement of phosphorylation of Cdc10 by Cla4 in septin filament formation at the bud neck. Since septin filaments contain multiple protein components, it is conceivable that a chaperone has some role in septin dynamics. In this context, it is very interesting that a mutant allele of the HSP104 gene caused structural alteration of septin filaments at the bud neck and that the growth defect of a special hsp104 mutant was suppressed by a mutation in the CDC12 gene (Schirmer et al., 2004). Thus, the number of factors known to affect septin dynamics is increasing.

To analyze the dynamics of cytoskeletal structures, specific inhibitors such as thiabendazole for tubulin (Friedman and Platzer, 1978; Davidse and Flach, 1978) and latrunculin A for actin (Coue et al., 1987) are useful. However, no specific drug that affects septin function or structure has been available until now. By chance, we found that an artificial cytokinin, forchlorfenuron (FCF), inhibited cytokinesis in S. cerevisiae, although the effective concentration for inhibition of yeast growth was several orders of magnitude higher than that effective as a plant hormone. This finding prompted us to examine the relationship between FCF and septin. FCF is a synthetic urea derivative that has potent cytokinin activity (Takahashi et al., 1978). An FCF-binding protein was found in mung bean extract and identified as a member of the major pollen allergen-related family (Fujimoto et al., 1978). However, this plant protein has no similarity to yeast septins.

In this study, we found that the administration of FCF caused sporadic appearance of septin filaments as well as deformation of septin filaments at the bud neck. The effect of FCF seen on septin filaments outside the bud neck was rapid and reversible.

### MATERIALS AND METHODS

#### Genetic manipulations

The yeast strains used in this study are listed in Table 1. Yeast cells were grown in rich medium (YPD) that consisted of 2% Polypepton (Nihon Seiyaku), 1% Bacto yeast extract (DIFCO), 2% glucose, 0.04% adenine sulfate and 0.005% uracil, or in synthetic complete medium (SC) that consisted of 0.67% Yeast Nitrogen Base without amino acids (DIFCO), 2% glucose, and appropriate supplements (Sherman et al., 1983). Sporulation medium consisted of 1% potassium acetate. Standard yeast genetic manipulations were performed as described previously (Sherman et al., 1983). Yeast transformations were performed by the lithium acetate method (Ito et al., 1983; Gietz and Schiestl, 1991). Escherichia coli DH5α (supE44 ΔlacU169 (80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1) was used for construction and propagation of plasmids. E. coli cells were grown in Luria-Bertani broth (LB), which consisted of 0.8% Bacto tryptone (DIFCO), 0.5% Bacto yeast extract (DIFCO), and 0.5% NaCl. The LB medium was supplemented with sodium ampicillin (40 mg/l) where appropriate. E. coli was grown at 37°C. E. coli transformations were carried out as described by Inoue et al. (1990). Agar was added to 2% to prepare solid media. Forchlorfenuron (Kyowa Hakko Kogyo Co., Ltd) was dissolved in 99.5% ethanol to 250 mM to prepare stock solution. Plasmids used in this study are listed in Table 2.

#### Morphological observation

Cells were fixed with 5% formaldehyde for 30 minutes at the temperature used for the cell culture as described previously (Iwase and Toh-e, 2004). After fixation, cells were washed with PBS (140 mM NaCl, 2.7 mM KCl, 3.8 mM NaHPO₄) three times and suspended in PBS. For actin staining, after fixation cells were mixed with 2% rhodamine-phalloidin (Molecular Probes, Inc.) for 2 hours, and washed three times with PBS. For indirect immuno-staining of Cdc3, after fixation cells were treated with 50 µg/ml Zymolyase and 2% β-mercaptoethanol in PBS containing 1.2 M sorbitol for 30 minutes, and then incubated in PBS containing 1% bovine serum albumin (BSA) for 20 minutes. Cells were treated with anti-Cdc3 rabbit antibody (Kim et al., 1991) at a1:200 dilution, followed by three washes with PBS, and then treated with Cy3-labeled sheep anti-rabbit IgG antibody (Chemicon International, Inc.) at a dilution of 1:200.
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After the samples were washed three times, they were mounted in p-phenylenediamine (1 mg/ml in 90% glycerol) and observed with a BX60 epifluorescence microscope (Olympus) and photographed with a PM-C35DX automatic camera.

**Time-lapse microscopy.** Cells were grown to exponential phase in SC liquid medium at 25°C and then 15 µl culture was put on a glass-bottomed dish coated with polylysine. Then a cover glass was placed on the culture. FCF was added into the space between the cover glass and the dish. The sample was observed with an Olympus IX70, an UplanApo100x/1.35 objective, and a SENSYSIII (Nippon Roper) cooled CCD camera using IP Lab software. Images were analyzed with IP Lab software, and the figures were created with Adobe Photoshop 5.0 software.

## RESULTS AND DISCUSSION

### FCF inhibits growth and cytokinesis of yeast.

By chance, we found that an artificial plant hormone, forchlorfenuron (FCF), inhibited yeast growth, as shown in Fig. 1. FCF at 1 mM inhibited the growth of the wild-type cells (Fig. 1Ae), whereas the cells grew when the concentration of FCF was 0.75 mM or lower (Fig. 1Aa–d). Among the strains tested, the ∆erg6 strain was found to be hyper-sensitive to FCF; its growth was inhibited by 0.5 mM FCF (Fig. 1A). This is probably explained by the fact that the permeability barrier toward various chemicals is reduced in the ∆erg6 mutant. The ∆erg6 cells treated with 0.5 mM FCF were found to be misshapen and to form chained cells (Fig. 1Bd), whereas wild-type cells grew in YPD medium containing 0.5 mM FCF and their morphology was normal (Fig. 1Bb). However, the wild-type cells treated with an inhibitory concentration of FCF showed a similar morphological change to that shown by the ∆erg6 cells treated with 0.5 mM FCF (Data not shown).

**FCF affects septin structure but not actin localization.** The morphology of the cells treated with a growth inhibitory concentration of FCF resembled that of cells with cytokinesis defects. Therefore, we examined whether FCF affects the localization of septin in wild-type cells in medium containing 1 mM FCF. Cells labeled with one of the septin-GFPs were cultured to mid-logarithmic phase in YPD medium and shifted to YPD medium containing 1 mM FCF. After 4 hours of incubation in the FCF medium, the cells became connected and the septin-GFP signals were seen as a string structure irrespective of which septin-GFP was examined (Fig. 2Aaa’–ee’), suggesting that the defect in cytokinesis caused by FCF seemed to be due to a change in septin. To examine whether the septin filaments sporadically produced upon FCF treatment are composed of septin subunits, the co-localization of Shs1-GFP and Cdc3 was examined. In Fig. 2B, we show that the Shs1-GFP signals (middle row) were superimposed onto the Cdc3 signals (bottom row) in the filamentous structures of septin, suggesting that the septin filaments that appeared after the administration of FCF contained a set of septin subunits. This result also indicates that the filamentous structures of septin-GFPs that emerged after administration of FCF were not an artifact due to the use of GFP-tagged septin. These results suggest that the filamentous structures were produced from genuine septin components.

To examine whether FCF disturbs actin distribution, Shs1-GFP cells treated with FCF for 4 hours were stained with rhodamine-phalloidin, and then actin and Shs1-GFP were observed (Fig. 3). Actin was localized normally in

### Table 2-i. List of vectors used in this study

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### Table 2-ii. List of plasmids used in this study

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<tr>
<td>pOS001</td>
<td>CEN URA3 GIN4-YFP (−600~+3426)</td>
<td>pTS913CU</td>
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cells treated with FCF, indicating that FCF did not disturb the actin orientation. This result demonstrated that Shs1-GFP was not co-localized with actin.

**Abnormal septin filaments do not contain Gin4.** All bud neck proteins so far tested except for casein kinase (Robinson et al., 1999) were localized at the bud neck in a septin-dependent manner (Gladfelter et al.,...
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To examine whether the localization of a bud neck protein whose localization is dependent on septin is affected by FCF, we examined Gin4, which is a Nim1-related kinase that is localized at the bud neck in a septin-dependent manner (Longtine et al., 1998). To this end, we constructed strain yOS001[pOS001], whose Shs1 was tagged with CFP and whose Gin4 was tagged with YFP. Four hours after the addition of FCF to the logarithmic phase culture of this strain, Shs1-CFP and Gin4-YFP were observed (Fig. 4). Shs1-CFP was seen at the bud neck as well as outside the bud neck as a filamentous structure. In some cells the Shs1-CFP signals at the bud neck were loosened. Gin4-YFP was co-localized only to the compact Shs1-CFP signals at the bud neck, and was

Fig. 2. The assembly of septin in liquid medium containing FCF. (A) The wild-type cells (W303a) containing CDC3-GFP (pM-1) (a, a'), CDC10-GFP (pM-2) (b, b'), CDC11-GFP (pM-3) (c, c'), or CDC12-GFP (pM-4) (d, d') plasmids and the cells whose chromosomal SHS1 gene was replaced with the SHS1-GFP gene (Masa6) (e, e') were grown at 25°C to mid-logarithmic phase in SC-URA (a,b,c,d, and e) and a portion of the culture was shifted to SC-URA containing 1.0 mM FCF (a', b', c', d', and e'). After 4 hours of incubation in FCF medium, the cells were harvested and fixed with 5% formaldehyde. GFP signals were observed. (B) Shs1-GFP and Cdc3 were co-localized in cells treated with FCF. Masa6 (Shs1-GFP cells) were grown at 25°C to mid-logarithmic phase in YPD and were shifted to YPD containing 1.0 mM FCF. After 4 hours of incubation in FCF medium, the cells were harvested, and fixed with 5% formaldehyde. Four different fields (a, b, c, and d) are shown. Top row: images obtained by phase contrast microscopy, middle row: Shs1-GFP signal, bottom row: Cdc3 observed by immunofluorescence using anti-Cdc3 antibody.
Fig. 3. FCF does not disturb actin filaments. Masa6 (Shs1-GFP) cells were grown at 25°C to mid-logarithmic phase in YPD and a portion of the culture was shifted to YPD or YPD containing 1.0 mM FCF. After 4 hours of incubation, the cells were harvested and fixed with 5% formaldehyde. Actin was observed by immunofluorescence using rhodamine-phalloidin and the Shs1-GFP signal was observed.

Fig. 4. Gin4-YFP was not co-localized with the septin filaments induced by FCF. yOS001 (Shs1-CFP) cells containing pOS001 (Gin4-YFP plasmid) were grown at 25°C to mid-logarithmic phase in SC-URA and a part of the culture was shifted to SC-URA containing 1.0 mM FCF. After 4 hours of incubation, the cells were harvested. The Shs1-CFP signal and Gin4-YFP signal were observed. Two different fields (upper row and lower row) are displayed. Shs1-CFP is shown by artificial blue color and Gin4-YFP is shown by artificial red color. Shs1-CFP signals not co-localized with Gin4-YFP are indicated by arrows.
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no longer co-localized to the loosened septin at the bud neck or to the septin filaments produced outside the bud neck. This result suggests that Gin4 is anchored to the normal septin ring at the bud neck and that it is released when the septin structure is loosened. It also shows that septin filaments outside the bud neck do not attract Gin4.

**Action of FCF is immediate and reversible.** To clarify how FCF immediately induces the appearance of septin filaments outside of the bud neck, we performed time-lapse analysis using Cdc11-GFP as septin. After adding FCF to a culture of Cdc11-GFP cells on the stage of a microscope, many GFP-dots newly emerged within a few minutes (Fig. 5A, arrows). The GFP signals became visible 1–1.5 minutes after the addition of FCF. Septin-GFP at the bud neck seemed to remain during this experimental period (Fig. 5A, upper panel). In addition, we examined whether the septin filaments produced in response to the treatment with FCF disappeared upon washing out the FCF. After septin filament production was induced by treatment with FCF for 4 hours, the cells were washed with liquid medium without FCF and the GFP signals were observed. The septin filaments outside of the bud neck vanished very quickly (Fig. 5B, arrows). These results suggest that septin filaments outside of the bud neck can be assembled in the presence of FCF and disassembled upon removing FCF.

**Induction of sporadic septin assembly by FCF.**
We demonstrated that FCF induced the production of abnormal septin structures that looked like filaments outside of the bud neck. Although septin filaments were produced sporadically, septin components were co-localized in the filamentous structures, suggesting that the structure of the septin complex was maintained in the presence of FCF. Thus, septin filaments are likely assembled sporadically in the presence of FCF, and FCF promotes the assembly of the septin components into the filamentous structure. On the other hand, cultures treated with FCF frequently contained cells with loosened septin at the bud neck (Fig’s. 1 and 2). How is this type of structure produced by the action of FCF if the action of FCF is assumed to be the promotion of septin assembly? Since septin at the bud neck did not undergo any quick change in the time-lapse observation, the loosening of the septin structure at the bud neck may be due to a different action of FCF on septins from its acute action. The observations described above indicate that FCF affords us a promising tool for investigating the role of septin in various phenomena.

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REFERENCES


