Dynamics of the actin cytoskeleton, hyphal tip growth and the movement of the two nuclei in the dikaryon of *Coprinus cinereus*

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We first examined the changes in distribution of F-actin during conjugate division in the apical cells of the dikaryon of *Coprinus cinereus* using indirect immunofluorescence microscopy, then followed hyphal tip growth and the movement of the two nuclei in the apical cells using differential interference contrast microscopy (DIC). In apical cells with interphase nuclei, F-actin occurred solely as peripheral plaques, which were distributed along the whole length of the cell and were more concentrated at the tips, where they formed caps. In the early prophase of conjugate division, F-actin was transiently concentrated, as diffused form and plaques, at hyphal regions where the two nuclei sit, and this was accompanied by transient disappearance of the actin cap at the hyphal tip in the majority of cells. The actin cap was also present at the tips of growing clamp cells from late prophase through metaphase and disintegrated during anaphase. In telophase, actin rings formed at the future septa. DIC revealed that, in early prophase, when the F-actin array occurs around the two nuclei and the actin cap is absent at hyphal tips, hyphae kept growing and the second nucleus accelerated its forward movement to catch up with the leading nucleus, which was still moving forward.

Key Words——actin; conjugate division; *Coprinus cinereus*; nuclear movement; tip growth.

Hyphal growth, which leads to the formation of a mycelium, is the most characteristic feature of fungi. It is a typical example of polarized growth which is based on mechanisms that ensure correct movements and positioning of organelles and polarized delivery of materials for localized cell extension. The actin cytoskeleton has been suggested to play a role in the mechanisms of hyphal tip growth as well as organelle movements (see Heath, 1990, 1994).

In many homobasidiomycetous fungi, the growth of dikaryotic hyphae is accompanied by conjugate division of the two nuclei with associated clamp connections, in which nuclear division, clamp cell formation and septation are under strict control in terms of time and space. It has been reported that F-actin transiently occurs in hyphal regions where the two nuclei sit in an early phase of conjugate division in *Coprinus cinereus* (Schaeff. ex Fr.) S. F. Gray sensu Konr. (Kamada and Tanabe, 1995), that an actin cap occurs in growing clamps in *Schizophyllum commune* Fr.: Fr. and *Paxillus involutus* (Batsch: Fr.) Fr. (Runeberg et al., 1986; Salo et al., 1989), and that actin rings occur at the future septa in the telophase of conjugate division in *C. cinereus* (Tanabe and Kamada, 1994; Tsukamoto et al., 1996). Thus the dikaryotic hyphae of homobasidiomycetous fungi exhibit marked

dynamics in F-actin distribution during conjugate division, and so should provide a unique opportunity for studying the roles of F-actin in nuclear movement as well as in hyphal growth.

In this study, we first examined the changes in F-actin distribution during conjugate division in the dikaryon of *C. cinereus* using indirect immunofluorescence microscopy. Then we followed hyphal tip growth and the movement of the two nuclei in living dikaryotic hyphae using differential interference contrast microscopy (DIC) in an attempt to examine the possible roles of F-actin in hyphal growth as well as in nuclear movements.

Materials and Methods

Strains and culture conditions A dikaryon constructed by mating two compatible wild-type homokaryons (5302 and 5401) of *C. cinereus* was used. CY-1 agar plates in Petri dishes 9 cm in diam (Kamada et al., 1982) were used for mycelial cultures. For DIC, hyphae were grown on CY-1 agar films (about 1 mm in thickness) on cover slips (24 × 60 mm). For fluorescence microscopy, hyphae were grown on cellophane, which had been soaked in CY-1 medium containing 0.5% (w/v) locust bean gum (Sigma, St. Louis, MO) (Heath, 1987), overlying CY-1 agar plates. Cultures were maintained at 28°C throughout this study.

Fluorescence microscopy Pieces of cellophane with

hyphae (5 × 5 mm) were cut out and treated according to Runeberg et al. (1986) with modifications. The hyphae on the cellophane were fixed with 4% (w/v) formaldehyde in 50 mM sodium phosphate buffer, pH 6.5, containing 5 mM $MgCl_2$, 5% (w/v) polyethylene glycol 6000, and 5 mM EGTA at 28°C for 2 h and washed in 50 mM sodium phosphate buffer, pH 6.5, containing 5 mM $MgCl_2$ with three changes for 30 min. Hyphal walls were then digested partially with 0.4% (w/v) lysing enzyme (Sigma) in 50 mM sodium phosphate buffer, pH 6.5, containing 5 mM $MgCl_2$ at 37°C for 10 min and washed in phosphate-buffered saline (PBS), pH 7.3, with two changes for 20 min. After washing, the hyphae were treated with 1% (w/v) NP-40 in PBS, pH 7.3, containing 5 mM phenylmethanesulfonyl fluoride (PMSF) and 5 mM EGTA for 20 min and washed in PBS, pH 7.3, with five changes for 50 min. The hyphae were then labeled with the mixture of a monoclonal actin antibody (Ab-1, Oncogene Science, Uniondale, NY) at a dilution of 1 : 100 and a monoclonal α-tubulin antibody (DM1A, Seikagaku, Tokyo) at a dilution of 1 : 500 for 12-15 h at 37°C. After labeling, the hyphae were washed in 1% (w/v) bovine serum albumin (BSA) in PBS, pH 7.3, with three changes for 30 min and labeled with the mixture of the secondary antibodies, rhodamine-conjugated anti-mouse IgM (Tago, Burlingame, CA) at a dilution of 1 : 100 for detection of actin and FITC-conjugated anti-mouse IgG (Cappel, West Chester, PA) at a dilution of 1 : 1000 for detection of
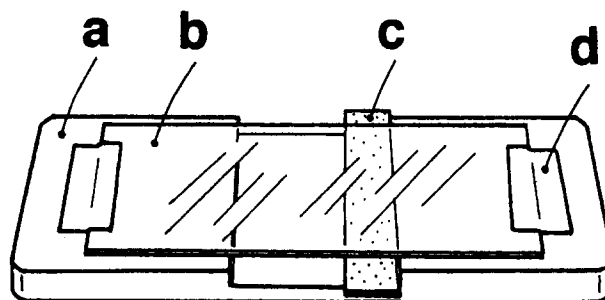

Fig. 1. A diagram of micro culture chamber used for observation of living hyphal tips. The glass slide (a), which is 2 mm in thickness, was hollowed 1 mm in depth at the middle part. The coverslip (b) on which hyphae were grown was set upside down on the slide and was fixed by two pieces of vinyl tape (d). To prevent the culture from drying, a piece of filter paper (c) placed at the edge of the chamber was kept wet by adding drops of water as needed.

tubulin for 8 h at 37°C. After labeling with the secondary antibodies, the hyphae were washed in PBS, pH 8.4, with three changes for 30 min and mounted in PBS, pH 8.4, containing 33% (w/v) glycerol, 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI), and 1 mg/ml p-phenylenediamine. The samples were observed with a Zeiss epifluorescence microscope.
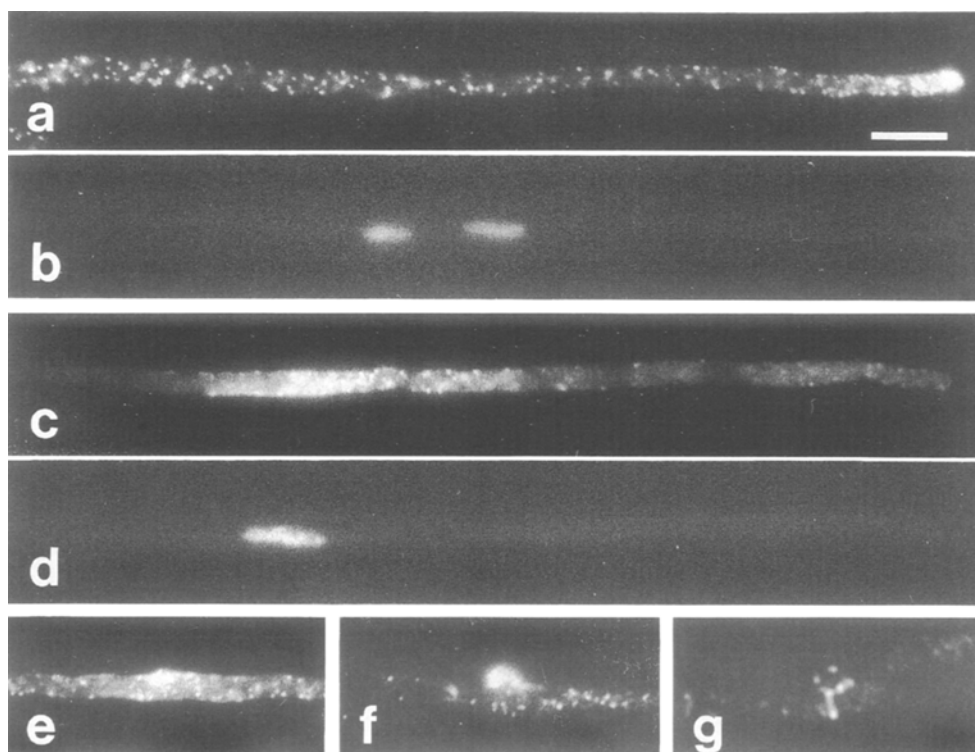
Measurement of nuclear movement and tip growth with

Fig. 2. Indirect immunofluorescence of apical cells of the dikaryon of C. cinereus with actin antibody (a, c, e, f, g) and DAPI staining of nuclei (b, d).
a, b, an apical cell in interphase; c, d, early prophase; e, f, late prophase; g, telophase. The hyphal apices are to the right of the figure. Bar, 10 μm.

DIC CY-1 agar film on the cover slip on which hyphae were grown was set upside down on a micro culture chamber after the film had been trimmed to fit in the chamber (Fig. 1). Growing apical cells of the main hyphae were observed from the reverse side of the cover slip under a Zeiss microscope equipped with DIC optics which was placed in a room maintained at 28°C. During measurement, the light of the microscope was kept off except when micrographs were taken. The images of apical cells were recorded on minicopy films (Fuji Photo Film, Tokyo) every few minutes, and the films were developed and printed out on black-and-white printing paper, Fujibro WP FM3 (Fuji Photo Film). The rates of tip growth and nuclear movements were worked out by direct measurement of these images. The middles of the nuclei were plotted for nuclear movements.

Results

Dynamics of F-actin in the apical cell of the dikaryon We examined the distribution of F-actin in a total of 482 apical cells of main hyphae of the dikaryon (Figs. 2, 3). The phases in the cycle of conjugate division (interphase,
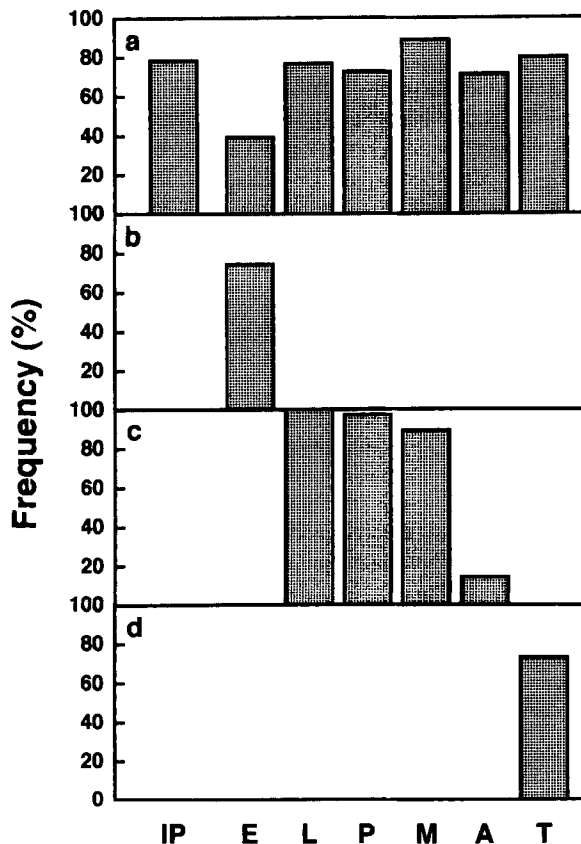

Fig. 3. Changes in frequency of actin caps at hyphal tips (a), actin arrays around nuclei (b), actin caps in the tips of clamp cells (c) and actin ring at the future septa (d) in various phases of conjugate division.
IP, interphase; E, early prophase; L, late prophase; P, prometaphase; M, metaphase; A, anaphase; T, telophase.

prophase, prometaphase, metaphase, anaphase, and telophase) were identified based on the configurations of the nuclei and cytoplasmic and mitotic microtubules as described by Tanabe and Kamada (1994). In apical cells with interphase nuclei, F-actin was distributed, solely as peripheral plaques, over the whole length of the apical cell, and was concentrated at hyphal tips to form actin caps in most apical cells (Figs. 2a, 2b, 3a). In the present study, we performed triple staining for observing microtubules, F-actin and nuclei simultaneously, which allowed us to subdivide the prophase into early prophase and late prophase.

In early prophase the two nuclei are getting closer together. F-actin is concentrated, as diffuse form and plaques, at hyphal regions where the nuclei sit in the majority of cells (Figs. 2c, 2d, 3b) and the actin cap is not recognizable at the region where a clamp cell forms. In late prophase the actin cap is recognizable in an incipient (Figs. 2e, 3c) or growing (Figs. 2f, 3c) clamp cell. In this phase, the actin arrays around the nuclei are absent (Fig. 3b). The actin cap in the clamp disappears during anaphase after clamp-cell formation was completed in metaphase (Fig. 3c). In telophase, F-actin is concentrated in rings at the sites of future septa (Figs. 2g, 3d). During actin ring formation, actin filaments as well as actin plaques are first concentrated around the future septa, then assemble into distinct actin rings (data not shown).

As shown in Fig. 3a, the frequency of actin caps in hyphal tips was clearly low in early prophase compared to those in the other phases: in all the phases except early prophase more than 70% of hyphal tips had actin caps, whereas in early prophase only 39% of hyphal tips had actin caps. Evidently, the disappearance of the actin cap in early prophase is relevant to the appearance of the actin arrays around the nuclei: 74% (55/74) of hyphal cells with an actin array around the nuclei lacked the actin cap, and 92% (55/60) of hyphal cells lacking the actin cap exhibited the actin array around the nuclei (Table 1). The reason for the lack of actin caps in 10-30% of cells in the phases other than early prophase is unknown at present.

Tip growth and the movements of the two nuclei in the apical cell of the dikaryon We followed tip growth and the movements of the two nuclei in the apical cells of the dikaryon over one to two cycles of conjugate division

Table 1. The relationship between the presence or absence of the actin array around the nuclei and that of the actin cap at the hyphal tip in apical cells of the dikaryon in early prophase.

Actin array around the nuclei[a]	Actin cap[a]	No. of cells observed
−	+	20
+	+	19
+	−	55
−	−	5

a) +, the actin array or the actin cap was present; −, the actin array or the actin cap was absent.
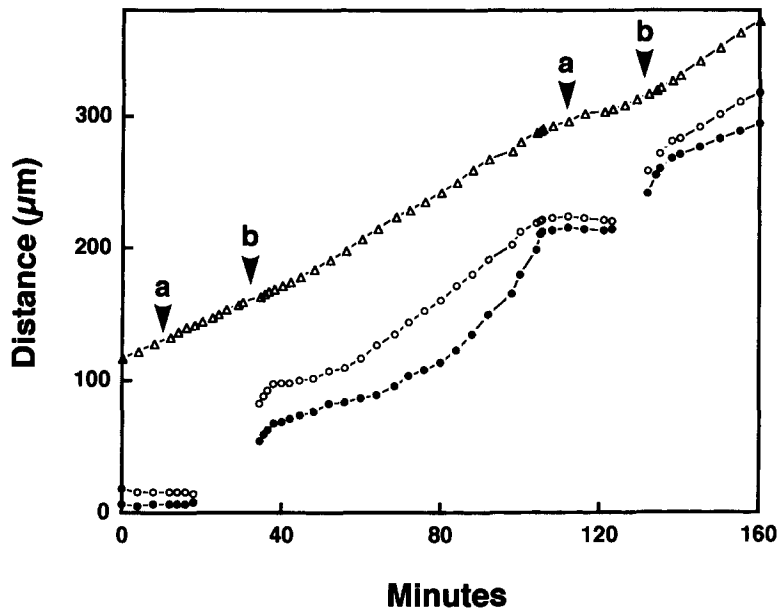
Fig. 4.   Growth of the hyphal tip and the movement of the two nuclei in an apical cell of the dikaryon.
Open triangles show tip growth.   Open circles and solid circles show the movements of the leading nucleus and the second nucleus, respectively.   Arrowheads a and b indicate the visible initiation of clamp cell formation and the reappearance of the two nuclei under DIC, respectively.

with DIC.   Figure 4 shows a typical result of the measurements.   As shown in the figure, the hypha kept growing throughout conjugate division.   The rate of growth fluctuated a little in the cycle of conjugate division.   However, the pattern of fluctuation was not consistent when the measurements were repeated, and the changes in the growth rate seemed not to be specific to any phases of conjugate division.   It is possible that some unknown internal and/or environmental factor(s) affect the rate of tip growth.   Average time required for completion of one cycle and the average rate of growth, as measured from visible initiation of clamp cell formation to the next initiation, were 65.7 ± 20.4 min (n = 31) and 2.23 ± 1.43 μm/min (n = 31), respectively.

The two nuclei were observable throughout by DIC except during the later half of conjugate division (Fig. 4). The leading nucleus moved forward to maintain a constant distance from the hyphal tip in interphase, but stopped moving a few minutes before clamp initiation was recognizable, and thus the distance between the hyphal tip and the leading nucleus began to increase. The second nucleus moved forward at almost the same rate as the leading nucleus to maintain a close spacing from the leading nucleus in interphase.   The rate of forward movement, however, started to increase $18.6 \pm$ 7.6 min (n = 32) before the initiation of clamp cell formation became recognizable, and the increased rate continued until the second nucleus made contact with the leading nucleus, which was still moving forward (Fig. 4). The forward movement of the second nucleus increased by 1.9 ± 0.7 (n = 32) times compared to that of the leading nucleus, and the increased rate continued for 8.1 ± 3.2 min (n = 32).   The two nuclei stopped moving forward after they made contact with each other, and ac-

cordingly the distance between the hyphal tip and the two nuclei became longer (Fig. 4).   After conjugate division, the two daughter nuclei exhibited rapid forward movements and resumed the original positions relative to the hyphal tip (Fig. 4).   The rapid forward movements of the nuclei after conjugate division have been reported in *S. commune* (Niederpruem, 1969) and *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (Kaminskyj et al., 1989).   In the dikaryon of *Polystictus versicolor* Fr., rapid forward movement of one of the daughter nuclei after conjugate division has been reported (Girbardt, 1968).

Discussion

In a previous study, we have reported that actin arrays appear around the two nuclei which are becoming closer together in the early phase of conjugate division in the dikaryon of *C. cinereus* (Kamada and Tanabe, 1995).   In this study, we showed that the actin arrays around the nuclei occur in the majority of cells in early prophase, and we found that the second nucleus accelerates its forward movement for the two nuclei to get closer and then make contact with each other.   These results imply that the actin arrays may be involved in some way in the acceleration of forward movement of the second nucleus.   F-actin around the nucleus has been reported in the chytridiomycetes *Neocallimastix* and *Orpinomyces* (Li and Heath, 1994), and the zygomycete *Neozygites* species (Butt and Heath, 1988).   In the former two organisms the actin arrays around nuclei have been suggested to be involved in nuclear motility and positioning, while in the latter F-actin around nuclei has been suggested to be actin storage for a new septum in the next phase in the cell cycle.   The actin arrays around nuclei in these organ-

isms, however, appear to be different from that found in *C. cinereus*: in the former, F-actin surrounds the nuclear envelope to form a nuclear shell or nuclear sheath; whereas in the latter, F-actin exists in the cytoplasm of hyphal regions where the nuclei sit and appears not to surround the nuclear envelope. Accordingly, the participation of F-actin in nuclear mobility may be different between these fungi. Further experimentation, for example, examination of the possible involvement of an actin-based motor protein which is now in progress, is needed to understand how F-actin mediates nuclear motility.

Actin caps occur at the tips of growing hyphae in filamentous fungi (Hoch and Staples, 1983; Runeberg et al., 1986; Heath, 1987, 1988, 1990; Salo et al., 1989; Jackson and Heath, 1990a), the growing end of fission yeast (Marks and Hyams, 1985), the growing buds in budding yeast (Adams and Pringle, 1984) and the tips of growing clamp cells in the dikaryons of basidiomycetous fungi (Runeberg et al., 1986; Salo et al., 1989). Thus the occurrence of the actin cap at the growing end of fungal cells appears universal. In addition to F-actin, myosin I is enriched at growing hyphal tips and plays a critical role in polarized growth (McGoldrick et al., 1995). Also, it has been reported that cytochalasins, a family of drugs that inhibit the function of actin filaments (Yahara et al., 1982), inhibit tip extension and cause apical swelling in several fungi (Allen et al., 1980; Grove and Sweigard, 1980; Tucker et al., 1986; Jackson and Heath, 1990b; Akashi et al., 1994). Jackson and Heath (1990b) have suggested that F-actin reinforces the extensible hyphal apex of the oomycete *Saprolegnia ferax* (Gruithuisen) Thuret, from the result that treatment of hyphae with cytochalasin E induces rapid changes in actin caps, and cap disruption was accompanied by transient growth rate increase. In addition, *Saprolegnia* hyphae have a spectrin homologue and an integrin homologue at the tip, which are likely to play a role in linking the cytoskeleton to the cell membrane and wall (Kaminskyj and Heath, 1995, 1996). In the present study, we found that the actin cap naturally disappears in early prophase of conjugate division; the time during which the actin cap is absent is estimated to be 8.2 min (65.7 min (the average time required for one division cycle to complete) × 60 (the number of hyphae which do not have the actin cap in early prophase)/482 (the total number of apical hyphae observed)). Also, we observed that the hyphae kept growing during conjugate division despite the absence of actin caps. Since dikaryotic hyphae are about 3 μm in diam (data not shown), hyphal volume at the tip would increase by 129.2 μm^3 ($\pi \times 2.25$ μm^2 × 2.23 μm/min × 8.2 min) during the period of 8.2 min when the actin cap is absent. So it would be expected that some changes in hyphal morphology would occur at the tips of dikaryotic hyphae during conjugate division if the actin cap were directly involved in hyphal morphogenesis. However, this was not the case. It may be that only a small amount of F-actin at the tip is directly involved in the mechanism of hyphal growth; it should be noted that a small amount of F-actin existed at the hyphal tip even when the actin cap was absent (Fig. 2c). The

bulk of F-actin at the hyphal tip may be maintaining polarity for polarized delivery or movement of materials, and/or constituting actin storage for continuous, localized cell extension rather than being involved directly in the mechanism of morphogenesis at the hyphal tip. The possibility also remains that the changes in the rate of tip growth and the shape of the tip were below the level of detection under the conditions used in this study.

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