

Ultrastructure of the *Aspergillus nidulans hypA1* restrictive phenotype shows defects in endomembrane arrays and polarized wall deposition

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Abstract: *Aspergillus nidulans* Eidam (G. Wint.) wild-type hyphal morphogenesis requires the *hypA* gene product. Like its homolog in *Saccharomyces cerevisiae* Meyen ex E.C. Hansen, *TRS120*, *hypA* encodes a cytoplasmic protein likely associated with endomembranes. *hypA* is not essential, but *hypA1* temperature-sensitive strains grow poorly at restrictive temperatures. In younger cells, endomembrane arrays were aberrant, only sometimes resembling wild type. In older cells, Golgi equivalents were swollen, impacted with electron-dense granules. In *hypA1* strains grown at 42 °C, the poorly polarized hyphae lack recognizable Spitzenkörper and have walls at least four-fold thicker than those of wild-type or *hypA1* strains grown at 28 °C. At restrictive temperatures, both hyphal width and wall thickness increase markedly in basal regions, suggesting wall deposition is impaired. Septa are thicker than in wild type, but have medial pores and Woronin bodies. Individual nuclei and mitochondria are smaller at 42 °C than at 28 °C, but each collectively occupies similar proportions of the cytoplasm. Mitochondrial cristae are reduced in number and width at 42 °C, possibly compromising metabolic efficiency; in older cells, cristae are widely spaced and randomly inserted. If *hypA1* cells grown at 42 °C are shifted to 28 °C, the thickened wall is precisely degraded for growth of wild-type branches, which form within 1 h, suggesting areas of nascent polarity formed at 42 °C require the *hypA* product for wild-type function.

Key words: endomembrane, filamentous fungus, electron microscopy, cell wall, secretion, *Saccharomyces TRS120*.

Résumé : Chez le type sauvage de l'*Aspergillus nidulans* Eidam (G. Wint.), la morphogénèse de l'hyphé nécessite le produit du gène *hypA*. Tout comme son homologue, le *TRS120* du *Saccharomyces cerevisiae* Meyen ex E.C. Hansen, le *hypA* code pour une protéine cytoplasmique vraisemblablement associée aux endomembranes. Le *hypA* n'est pas essentiel, mais les souches dont le *hypA* est sensible à la température poussent mal à des températures contraignantes. Chez les cellules plus jeunes, les ensembles d'endomembranes sont aberrants, ressemblant seulement à l'occasion à ceux du type sauvage. Chez les cellules plus âgées, les équivalents de l'appareil de Golgi sont gonflés, marqués par des granules denses aux électrons. Chez les souches possédant le *hypA* cultivées à 42 °C, les hyphes faiblement polarisées sont dépourvues d'un Spitzenkörper reconnaissable et ont des parois au moins quatre fois plus épaisses que le type sauvage, ou les souches de type *hypA* cultivées à 28 °C. À des températures contraignantes, la largeur des hyphes aussi bien que l'épaisseur des parois augmentent nettement dans les régions basales, ce qui suggère que la déposition des parois est affectée. Les septations sont plus épaisses que chez le type sauvage, mais possèdent des pores médians et des corps de Woronin. Les noyaux individuels et les mitochondries sont plus petits à 42 °C qu'à 28 °C, mais collectivement chacun occupe une proportion similaire par rapport au cytoplasme. Les crêtes des mitochondries sont réduites en nombre et en largeur à 42 °C, nuisant possiblement à l'efficacité métabolique; chez les cellules plus âgées, les crêtes sont largement espacées et insérées au hasard. Si des cellules de type *hypA* cultivées à 42 °C sont déplacées à 28 °C, les parois épaissies se dégradent précisément pour permettre la reprise de la croissance ramifiée du type sauvage en moins d'une heure, ce qui suggère que les zones de polarité naissantes formées à 42 °C nécessitent un produit du *hypA* pour fonctionner comme le type sauvage.

Mots clés : endomembrane, champignon filamenteux, microscopie électronique, paroi cellulaire, sécrétion, *Saccharomyces TRS120*.

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Introduction

Filamentous fungi are composed of tubular cells called hyphae. Wild-type *Aspergillus nidulans* Eidam (G. Wint.) hyphae are narrow cylindrical cells that extend only at their tips, a process called tip growth. Although the result is an elegantly simple polarized tubular cell containing cytoplasm and surrounded by a wall, hyphae can be morphologically distinctive for different species and (or) life-cycle stages. Even in the simplest situations, hyphal morphogenesis requires synthesis and directed deposition of wall materials, apex-directed migration of cytoplasm and organelles within the hypha, and support for the extending tip where the newly deposited wall is maturing (reviewed in Kaminskyj and Heath 1996; Shi et al. 2004). Defects in cell wall components can also cause abnormal hyphal morphogenesis (Shaw et al. 2002; Shaw and Momany 2002). In addition, mutagenesis screens in *A. nidulans* have identified gene products of unknown function that are essential for wild-type hyphal morphogenesis (e.g., Harris et al. 1999; Kaminskyj and Hamer 1998; Momany et al. 1999; Osherov et al. 2000).

Aspergillus nidulans hypA is needed for wild-type hyphal morphogenesis, although it is dispensable for growth and conidiation (Kaminskyj and Hamer 1998; Shi et al. 2004). *Aspergillus nidulans hypA* is represented by the temperature-sensitive alleles *hypA1* (Kaminskyj and Hamer 1998) and *hypA6* (*podA1*: Harris et al. 1999; *swoE1*: Momany et al. 1999). These alleles have the same genetic lesion and, consistent with this, have the same phenotype at 42 °C (Shi et al. 2004); they will be referred to collectively as *hypA1*. Strains with the *hypA1* temperature-sensitive allele grow slowly at 42 °C, producing wide hyphae with closely spaced septa (Kaminskyj and Hamer 1998). Both haploid *hypA* knockout (Shi et al. 2004) and *hypA1* strains grown at 42 °C (Kaminskyj and Hamer 1998) have slow, poorly polarized growth, and yet both conidiate to produce viable spores. When shifted from 42 to 28 °C, *hypA1* cells initiate wild-type branches at predictable sites (Sha 2003), suggesting that areas of nascent polarity exist in the cytoplasm.

Aspergillus nidulans hypA is homologous to *Saccharomyces cerevisiae* Meyen ex E.C. Hansen *TRS120* (Shi et al. 2004). This gene encodes a regulatory member of the transport protein particle (TRAPP) complex that mediates secretion via ER–Golgi trafficking (Guo et al. 2000; Sacher et al. 2000, 2001). At 42 °C, endomembrane arrays of *hypA1* strains visualized by the fluorescent probe FM4-64 differ from those of wild type by being patchy and poorly polarized (Shi et al. 2004), but they reform wild-type arrays after shifting to 28 °C (Sha 2003). At 42 °C, *hypA1* strains have reduced actin and cytoplasmic microtubule abundances, which retain normal distribution (Shi et al. 2004). Cytoplasmic microtubules also reform wild-type arrays after shifting to 28 °C (Sha 2003).

To extend the description of the *hypA1* phenotype (Shi et al. 2004) and to facilitate future analysis of *hypA*, we describe the ultrastructure of the *hypA1* temperature-sensitive phenotype at 42 °C and compare it with the wild-type *hypA1* phenotype produced at 28 °C (Kaminskyj and Hamer 1998).

Materials and methods

Aspergillus nidulans strains ASK30 (*hypA1*; *wA2*; *pyrA4*;

veA1; Kaminskyj and Hamer 1998) and ASH80 (*hypA6*, *biA1*, *pabaA6*; *veA1*; Harris et al. 1999) were grown at 42 °C for 14 h or 5 d, or at 28 °C for 14 h, on dialysis tubing overlying complete medium agar (Kaminskyj 2001). “Down-shifted” ASK30 cells were grown as mentioned for 14 h at 42 °C followed by 3 h at 28 °C. For transmission electron microscopy (TEM), cells were prepared as described in Kaminskyj (2000). Negatives were scanned with an Epson 1640SU flatbed scanner and EU33 film adaptor. Images were imported as TIFF files into MetaVue 4.6 (Universal Imaging Corporation, <http://www.image1.com>) and Adobe Photoshop 5.0. The epifluorescence protocol for wall staining is described in Kaminskyj and Hamer (1998).

Dimensions were measured on near-median sections, as assessed by membrane profiles. Linear and area measurements were taken using the tools in MetaVue. Hyphal wall thickness was measured in near-median sections of *hypA1* cells grown at 28 and 42 °C. Apical wall thickness was measured at the hyphal tip, and lateral wall thickness was measured at least 1 µm from septa for hyphae grown at 28 °C (the first septum is at least 50 µm behind the tip in *hypA1* cells grown at 28 °C) and about 10 µm from the tip for cells grown at 42 °C. Septum thickness was measured in near-median sections of septa grown at 28 and 42 °C that contained a pore or were within one section of doing so. Abnormal septa grown at 42 °C were excluded. Only one section was measured for each septum, but occasionally more than one septum was measured per hypha. Measurements were taken every 250 nm out from the pore and pooled.

Statistical analyses used Statview SE + Graphics v1.02, presented with Cricket Graph 1.3.1. Results are presented as mean ± SE.

Results

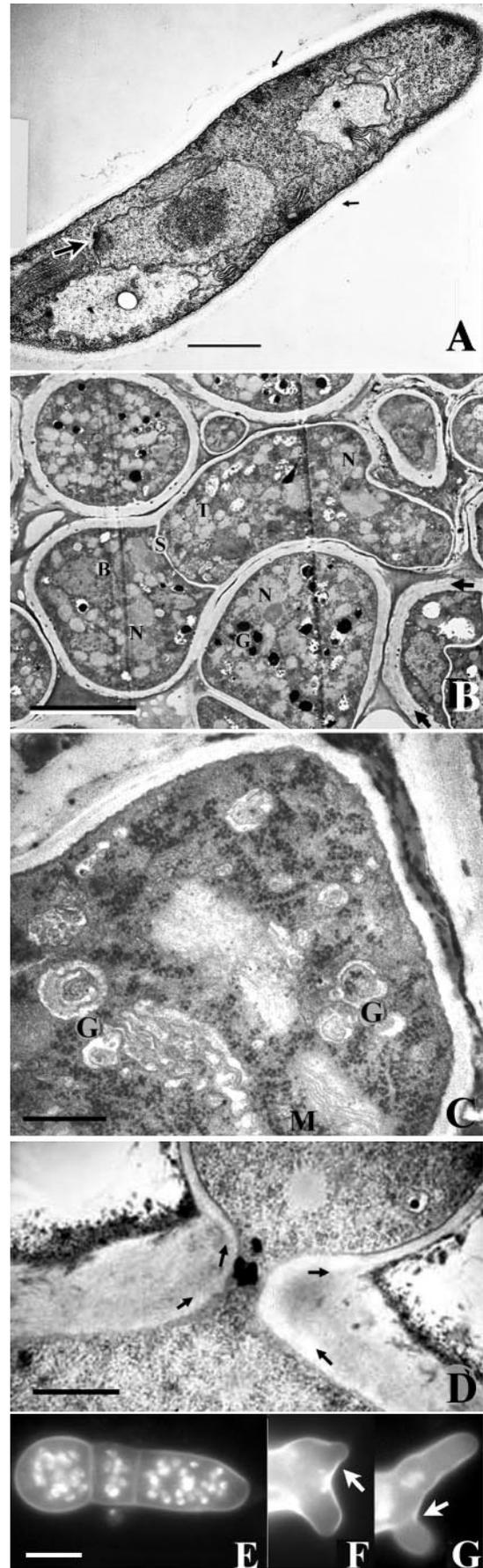
Walls and septa

A notable difference between *hypA1* cells grown at 28 and 42 °C, which prompted their initial isolation, is cell shape and septum deposition (Fig. 1). Strains with the *hypA1* allele germinate and grow slowly at 42 °C, producing wide cells with thick walls (cf. Figs. 1A and 1B). Grown at 28 °C, *hypA1* cells are tubular, and their walls are thin and electron translucent (Fig. 1A), whereas those grown at 42 °C are short and tapered, and their walls are thick and relatively electron dense (Figs. 1B, 3, 5B, 5C). The walls of cells grown at 42 °C often have layers or contain inclusions (Figs. 1B, 3, 5B, 5C), and wall thickness increases markedly with cell age. The increase in wall thickness was gradual, and *hypA1* cells grown at 42 °C do not have wall appositions.

In Fig. 1B, the hypha in the centre of the field is relatively young. There is a doubling in wall thickness from the apex of this tip cell (T) to the septum (S) at its base and a further increase in wall thickness in the basal cell (B). Other cells in Fig. 1B are older and have very thick walls, sometimes exceeding 1 µm (as discussed in the next paragraph, wild-type *A. nidulans* cell walls are <50 nm thick). Older cell walls of cells grown at 42 °C are relatively more electron dense than younger ones and can have layers and inclusions (Figs. 1B, 3, 5B, 5C).

Fig. 1. (A–D) Transmission electron micrograph of near-median sections of *hypA1* strains grown at (A) 28 °C; (B, C) 42 °C; and (D) 42 °C shifted to 28 °C for 3 h. (E–G) Epifluorescence micrographs of *hypA1* cells stained with Calcofluor White and Hoechst-33258 after growth at 42 °C for 24 h (E), or at 42 °C for 14 h then shifted to 28 °C for 60 min (F) or 90 min (G). (A) A cell grown at 28 °C has an electron-transparent wall (small arrows). The wild-type nucleus has a prominent nucleus-associated organelle (large arrow) and nucleolus. Vacuoles have a well-contrasted tonoplast. The true tip of this hypha is out of the plane of section. Scale bar = 1 μ m. (B) A 5-d-old *hypA1* colony grown at 42 °C shows that all cells grown at this temperature have much thicker walls than those grown at 28 °C. Cells are viable and contain abundant, organelle-rich cytoplasm; colonies of comparable age can conidiate. Older walls are relatively electron dense compared with walls of cells grown at 28 °C and may contain inclusions and (or) are layered (e.g., arrows). In the large cell in the centre of the field, the youngest wall, at the apex of the tip cell (T), is thinner than older wall near the septum (S), suggesting that wall deposition continues in subapical regions; the basal cell (B) wall is thicker yet. Nuclei (N) with prominent nucleoli are seen in all cells. Swollen vesicles with condensed, electron-dense contents, likely to be impacted Golgi (G), are common in older cells (recognizable by their thicker walls). Scale bar = 5 μ m. (C) Near-median section of an actively growing *hypA1* cell tip grown at 42 °C is characterized by a relatively thin, electron-lucent wall and abundant endomembranes, including putative Golgi equivalents (G) that are morphologically aberrant but do not contain impacted cores. Mitochondria (M) in young cells grown at 42 °C have parallel cristae, as in wild type, but they also have a pale matrix. Scale bar = 500 nm. (D) A cell grown at 42 °C was shifted to 28 °C for 3 h before fixation, showing the dramatic difference in wall thickness and appearance at the transition between these growth temperatures and the precise area of wall degradation for branching. Near the channel connecting the branch grown at 28 °C, the wall grown at 42 °C has a less electron-dense, inner layer (arrows) that is continuous with the wall grown at 28 °C. Scale bar = 1 μ m. (E–G) Epifluorescence micrographs typical of the 42 °C *hypA1* phenotype, showing thick walls, abundant nuclei, and closely spaced septa. Development of wild-type branches after downshift, with the arrows indicating the abrupt transition in wall thickness between growth at 42 and 28 °C. Scale bar = 5 μ m.

Apical and lateral wall thicknesses of *hypA1* germlings grown at 28 °C were similar at 44 ± 3 nm ($n = 36$ cells) and 48 ± 4 nm ($n = 48$ cells), respectively. Apical wall thickness was similar in 14-h- and 5-d-old *hypA1* cells grown at 42 °C (data not shown). Apical and lateral wall thickness of *hypA1* germlings grown at 42 °C was 168 ± 22 nm ($n = 36$ cells) and 274 ± 12 nm ($n = 76$ cells), respectively. Unlike cells grown at 28 °C, the lateral walls grown at 42 °C were significantly thicker than those at the apex ($P = 0.0001$, ANOVA). The difference in wall thickness between walls grown at 42 and 28 °C is particularly striking at the transition between growth temperatures in downshifted cells. A near-median section of a wild-type branch that formed after a downshift (Fig. 1D) shows that the thick wall of a cell grown at 42 °C was precisely degraded, forming a narrow channel subtending the wild-type branch grown at 28 °C. The change in wall thickness at the growth transition can also be seen in epi-



fluorescence images of downshifted *hypA1* cells stained with Calcofluor White (arrows in Figs. 1F, 1G). Using TEM, the inner layer of the wall grown at 42 °C at the degradation site was more electron lucent (Fig. 1D, arrows) and was continuous with the wall grown at 28 °C of the new branch. The wall grown at 42 °C around the branch site was also slightly thickened (Fig. 1D).

Septa of *hypA1* hyphae grown at 42 °C are considerably more closely spaced than those of *hypA1* hyphae grown at 28 °C and span wider cells (Fig. 1E; Kaminskyj and Hamer 1998). The septa grown at 42 °C had a trilamellar structure (Figs. 2A, 3, 5), near-medial pores up to 150 nm wide (Fig. 2), and associated Woronin bodies (Fig. 2). Thickness varied among *hypA1* septa grown at 42 °C (Fig. 3), presumably reflecting septa inserted at different times.

Septum thickness was measured in near-medial sections of septa grown at 28 and 42 °C ($n = 16$ and $n = 41$ septa, respectively) that contained a pore or were within one section of doing so. Clearly, some septa grown at 42 °C were abnormal (e.g., Fig. 5A), and these were excluded. Only one section was measured for each septum, but occasionally more than one septum was measured per hypha (Fig. 4). Close to the pore where there were measurements for both growth temperatures, septum thickness was significantly greater in cells grown at 42 °C than in those grown 28 °C ($P = 0.0001$, ANOVA).

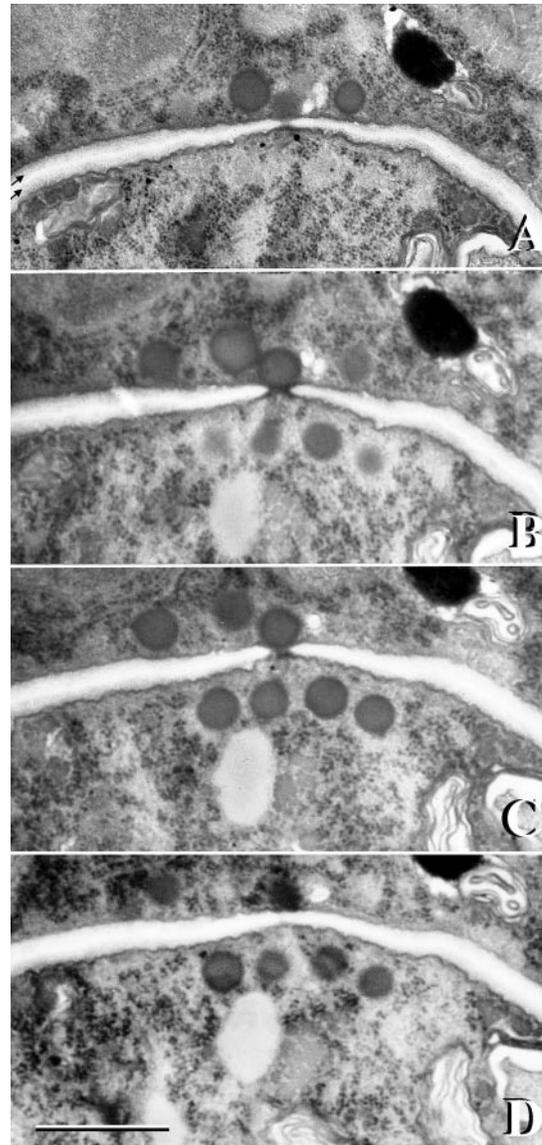
Woronin bodies associated with septal pores were similar in number to wild-type strains (Momany et al. 2002). At least eight Woronin bodies are associated with the septal pore shown in Fig. 2. As in wild type, each Woronin body was surrounded with a ribosome-free zone. At 42 °C, Woronin body diameter in *hypA1* cells was up to 300 nm, which is twice that of wild type (Momany et al. 2002). Woronin bodies were not unequivocally identified in *hypA1* cells grown at 42 °C except where associated with septal pores.

Endomembranes and Spitzenkörper

Cell wall deposition requires a functional endomembrane system for production of exocytic vesicles containing matrix material and wall fibril enzyme synthetic complexes. *Aspergillus nidulans hypA* is homologous to *Saccharomyces cerevisiae TRS120*, part of the TRAPP complex that mediates endomembrane traffic through the fungal Golgi equivalent (Shi et al. 2004). We examined endomembranes of *hypA1* cells grown at 42 °C to determine if the defects seen with FM4-64 and epifluorescence microscopy were apparent with TEM.

Both older and younger *hypA1* cells grown at 42 °C had abundant endomembranes (Figs. 1C, 5). Wild-type endomembrane arrays were seldom seen in cells grown at 42 °C, where instead the endomembranes formed convoluted whorls (Figs. 1C, 5) and multilamellate bodies (Fig. 5). Using the endomembrane probe FM4-64, *hypA1* cells have poorly polarized, patchy staining patterns, unlike wild-type strains and *hypA1* strains grown at 28 °C (Sha 2003; Shi et al. 2004). Also, there were areas of pale ribosome-free cytoplasm in *hypA1* cells grown at 42 °C, unlike in wild-type or *hypA1* cells grown at 28 °C, which sometimes appeared to contain membranous material (Fig. 5A). However, while these endomembranes are clearly unlike wild type, they

Fig. 2. Transmission electron micrograph of the septum shown in Fig. 1B grown at 42 °C. Serial sections spanning the near-medial septal pore, which has at least eight associated Woronin bodies. These are twice the diameter of wild-type Woronin bodies, but as in wild type, they are surrounded by a shell of ribosome-free cytoplasm. The trilamellar structure of this young septum is relatively faint (arrows in 2A). Scale bar = 1 µm.



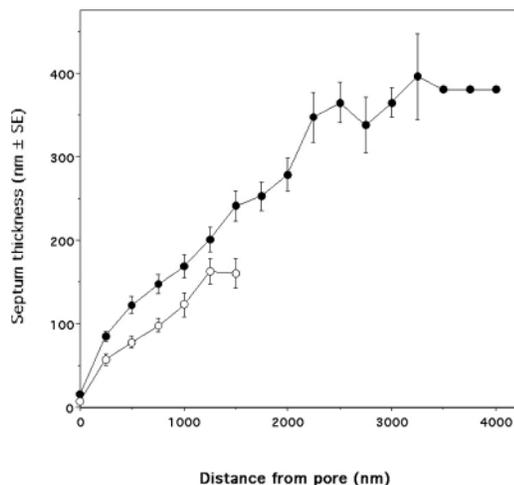
must be functional, since *hypA1* strains grown at 42 °C can complete their asexual life cycle.

At 28 °C, *hypA1* strain Golgi equivalents are single, multilobed cisterna, as in wild type (Beckett et al. 1974). At 42 °C in *hypA1* cells, recognizable Golgi equivalents were rare and found only in young cells. In older cells grown at 42 °C, dilated vesicles were surrounded by well-contrasted membranes that had electron-dark contents (Figs. 1B, 3, 5), which we interpret as being impacted Golgi equivalents. These were not seen in cells grown at 28 °C. The size of impacted Golgi equivalents increased in older cells grown at 42 °C, as judged by wall thickness (Fig. 1B). Impacted Golgi were reported for the restrictive phenotype of a

Fig. 3. Near-median sections through two *hypA1* septa grown at 42 °C likely to have been deposited at different times. Both septa have a trilamellar structure, the thicker one with additional darker layers. The hyphal wall is relatively electron dense and has layers (arrows), unlike walls of cells grown at 28 °C. The middle cell shows a future branch point (B, large arrow), which is associated with an impacted Golgi equivalent (G) but not with a vesicle cluster. Putative vacuole (V) lacking a well-contrasted tonoplast. Scale bar = 1 μ m.



Fig. 4. Septum thickness as related to distance from the medial pore in near-median sections of septa from *hypA1* hyphae grown 28 °C (open circles, $n = 16$) and 42 °C (closed circles, $n = 41$). Septum thickness was significantly greater for septa grown at 42 °C than for those grown at 28 °C, ranging between 0 and 1500 nm from the pore ($P = 0.0001$, ANOVA).



temperature-sensitive *TRS120* strain (Sacher et al. 2000). Multilamellate vesicles (Figs. 5A, 5C) may represent an early stage in impacted Golgi development. These were

common in *hypA1* cells grown at 42 °C but not in those grown at 28 °C.

The future growth direction of a fungal hypha is related to the position of the Spitzenkörper, an apical vesicle cluster (e.g., Grove et al. 1968, 1970; Lopez-Franco and Bracker 1996). Unlike growth at 28 °C, Spitzenkörper were not found in *hypA1* cells grown at 42 °C using electron microscopy, consistent with weakly polarized FM4-64 endomembrane staining patterns (Shi et al. 2004), with slow hyphal extension rate (Kaminskyj and Hamer 1998), and with aberrant cell growth and wall deposition patterns. Intriguingly, vesicles with dark-staining contents were occasionally seen underlying the cell membrane (Fig. 5B), where they might contribute to wall thickening.

Nuclei

Unlike walls, the nuclei of *hypA1* cells were similar at both growth temperatures except for their size. Nuclei in *hypA1* hyphae grown at 42 °C had prominent nucleoli and wild-type nucleus associated organelles (Fig. 6). Kaminskyj and Hamer (1998) suggested that the nuclei in cells grown at 42 °C were smaller than those in cells grown at 28 °C. Near-median profiles of *hypA1* nuclei grown at 28 °C were relatively elongate and averaged 1594 ± 142 nm \times 1027 ± 89 nm ($n = 22$ nuclei). The *hypA1* nuclei grown at 42 °C averaged 1273 ± 58 nm \times 1010 ± 46 nm ($n = 40$ nuclei), significantly different in length ($P = 0.017$, ANOVA) but similar in width. Nuclei in 14-h- and 5-d-old *hypA1* cells grown at 42 °C were similar in size (data not shown). On average, assuming nuclei at both growth temperatures approximated ellipsoids, *hypA1* nuclei grown at 42 °C had about 20% smaller volume than nuclei grown at 28 °C.

When expressed as number of nuclei per cell volume, and despite their apparent abundance, Kaminskyj and Hamer (1998) reported fewer nuclei per volume cytoplasm in *hypA1* cells grown at 42 °C than those grown at 28 °C. Areas occupied by nuclei compared with total cytoplasm were determined from electron micrographs of near-median sections of cells grown at 28 and 42 °C. The proportion of cytoplasm occupied by nuclei was higher in cells grown at 28 °C ($17.0\% \pm 2.9\%$, $n = 15$ sections) than in cells grown at 42 °C ($10.2\% \pm 1.9\%$, $n = 16$ sections), although this difference was not statistically significant ($P = 0.06$, ANOVA).

Mitochondria

Mitochondria of *hypA1* cells grown at 28 °C are vermiform (Fig. 7A), as in wild type (Beckett et al. 1974), whereas those of cells grown at 42 °C are ovoid (Fig. 7B). Near-median profiles of mitochondria in *hypA1* cells grown at 28 °C were 957 ± 102 nm \times 298 ± 33 nm ($n = 18$ mitochondria) compared with those of *hypA1* cells grown at 42 °C, which were 497 ± 25 nm \times 350 ± 13 nm ($n = 60$ mitochondria). Mitochondrial sizes were similar in 14-h- and 5-d-old *hypA1* cells grown at 42 °C. The mitochondrial lengths ($P = 0.0001$, ANOVA) but not widths ($P = 0.08$, ANOVA) were significantly different between the two growth temperatures. On average, mitochondria in cells grown at 42 °C had about 30% smaller volume than those in cells grown at 28 °C.

As well as differences in mitochondrial size and shape, there were internal differences in *hypA1* mitochondria at the

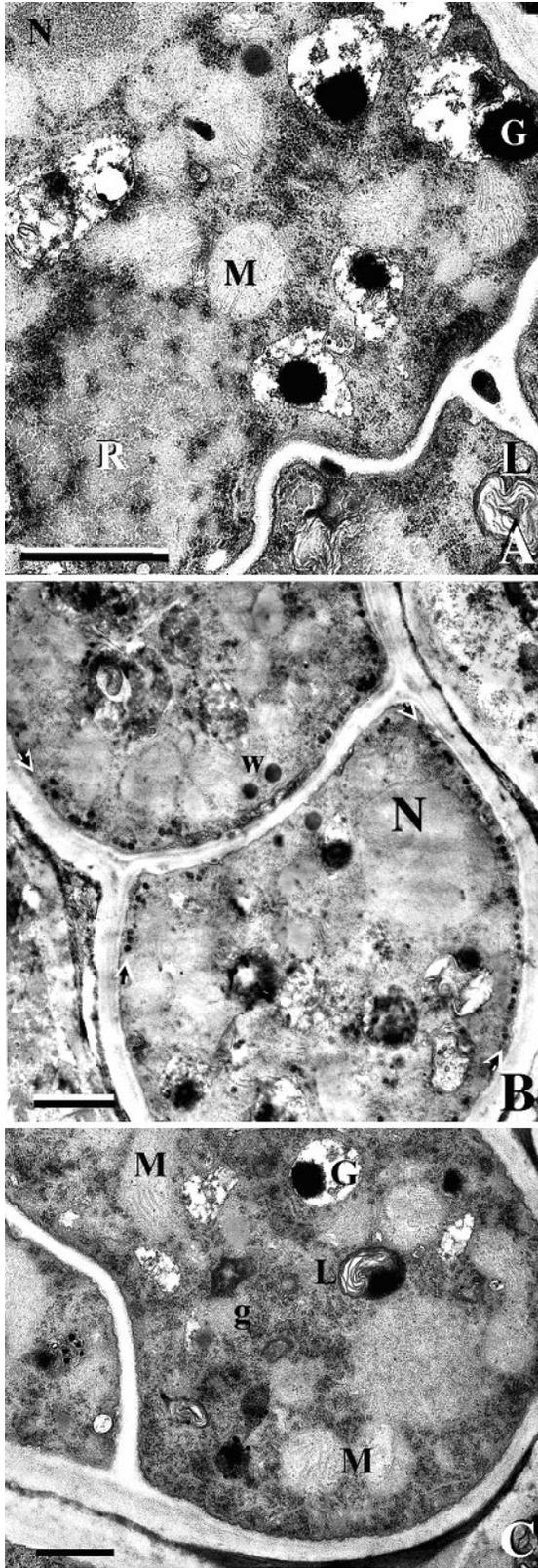
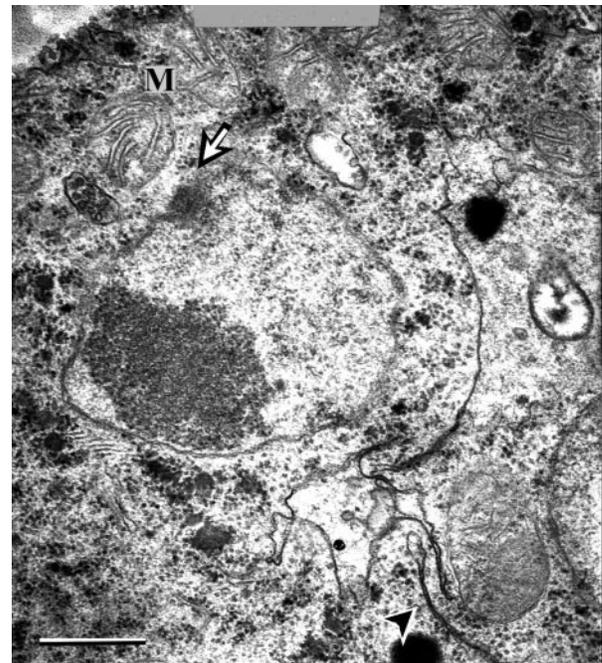


Fig. 5. Transmission electron micrograph of *hypAI* cells grown at 42 °C showing distinctive endomembranes and vesicles. Scale bars = 1 μ m. (A) Impacted Golgi equivalents (G). Reticulated cytoplasm (R) from which ribosomes are excluded, not seen in *hypAI* cells grown at 28 °C or in wild-type hyphae. Multilamellate bodies (L) are likely an early stage in impacted Golgi formation. N, nuclei; M, mitochondria. Note the abnormal septum. (B) Cells with dense vesicles (in line with the arrows) underlying the plasma membrane that might contribute to non-polarized wall thickening. The septum has a trilamellar structure. W, Woronin bodies. (C) Stages in formation of impacted Golgi equivalents in cells grown at 42 °C, from nearly wild-type (g), to multilamellate vesicles (L), to impacted (G). In this relatively young cell, mitochondria show both parallel and aberrantly inserted cristae, but in both cases the cristae are narrow and the matrix is less electron dense than in wild-type cells or in cells grown at 28 °C.

Fig. 6. Nucleus of a *hypAI* strain grown at 42 °C, with a prominent nucleolus and nucleus-associated organelle (arrow). Arrowhead points to a cisternum of endoplasmic reticulum. M, mitochondria. Scale bar = 1 μ m.

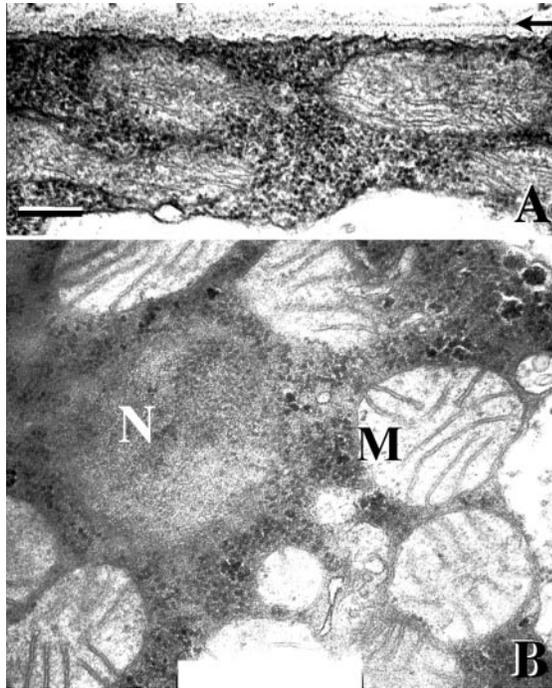


two growth temperatures. Like wild-type *A. nidulans* (Beckett et al. 1974), the cristae in *hypAI* mitochondria grown at 28 °C are parallel and platelike, inserted at the ends, and closely packed (Fig. 6A), whereas although cristae in mitochondria grown at 42 °C were platelike, they were in-

serted at the ends and sides and were relatively distant from each other (Figs. 5, 7). Insertion position was more aberrant in older cells grown at 42 °C. Cristae in *hypAI* mitochondria grown at 28 °C were 32 ± 3 nm ($n = 27$ cristae) across the lumen, significantly wider than those of mitochondria grown at 42 °C, which were 26 ± 1 nm ($n = 49$ cristae; $P = 0.007$, ANOVA). The matrix of mitochondria grown at 42 °C was less electron dense than that in cells grown at 28 °C, regardless of crista insertion position. Crista width and mitochondrial matrix density was similar in 14-h- and 5-d-old *hypAI* cells grown at 42 °C.

Cells of *hypAI* strains grow more slowly at 42 °C than at 28 °C (Kaminskyj and Hamer 1998), which might be

Fig. 7. Mitochondria of *hypA1* strains grown at (A) 28 °C and (B) 42 °C, showing typical morphologies, crista insertion and spacing, and matrix density. Arrow in A indicates the outer edge of the hyphal wall, which is less than 40 µm thick in this section. N, nucleus. Scale bars = 100 nm.



related to mitochondrial abundance as well as size and (or) efficiency. The average proportion of cytoplasm occupied by mitochondria was similar in *hypA1* cells grown at 28 and 42 °C, at $4.6\% \pm 0.7\%$ ($n = 15$ sections) and $5.9\% \pm 1.0\%$ ($n = 22$ sections) ($P = 0.33$, ANOVA), respectively.

Discussion

The ultrastructural defects in the *hypA1* restrictive phenotype are most notable in the endomembrane arrays and in cell wall deposition, which depends on properly targeted secretion coordinated with cell extension (Kaminskyj and Heath 1996). Clearly, part of the wild-type function of the *hypA* product is in the generation and maintenance of wild-type endomembrane arrays. Unlike the *hypA* homologs in *Schizosaccharomyces cerevisiae* (Sacher et al. 2001), *Schizosaccharomyces pombe* (Dai 2002), and *Ashbya gossypii* (T. Gaffney, personal communication), *A. nidulans hypA* is not essential (Shi et al. 2004). The homologous sequences from *S. pombe* and *S. cerevisiae* are able to rescue the *hypA1* defect (S.G.W. Kaminskyj, unpublished data). This is consistent with these genes having related but not identical function in their respective systems, as has been found for their cell cycle regulation (reviewed by Osmani and Ye 1997).

The *hypA1* restrictive phenotype is characterized by increased hyphal wall thickness compared with the permissive phenotype or with wild type. Although wall deposition in *hypA1* cells grown at 42 °C is weakly polarized (Fig. 1B; Kaminskyj and Hamer 1998), *hypA1* hyphal tip walls grown at 42 °C are at least four-fold thicker than those grown at 28 °C. Wall deposition must continue in subapical regions of

hypA1 cells grown at 42 °C to produce the tapered cell profiles and the smoothly increasing thickness of lateral compared with apical walls. Thus the *hypA1* walls grown at 42 °C must remain extensible, consistent with ongoing wall softening during hyphal tip growth (Bartnicki-Garcia 1973).

At 42 °C, *hypA1* germlings are easily ruptured despite their thick walls (Kaminskyj and Hamer 1998), with the burst points usually being adjacent to tips or septa, similar to preferential sites of branching after shifting to 28 °C (Sha 2003; data not shown). Burst points were not at the extreme end of hyphal tips, consistent with internal support from cytoplasmic actin arrays (Jackson and Heath 1990), which are slightly concentrated at *hypA1* cell tips grown at 42 °C (Shi et al. 2004). Wall thickness of *hypA1* cells grown at 42 °C does not prevent uptake of low molecular weight nutrients, but does reduce secretion of hydrolytic enzymes (Shi et al. 2004). Proteinaceous material trapped in *hypA1* walls grown at 42 °C is likely to contribute to their increased electron density. Wall-softening enzymes might be trapped in the thick *hypA1* walls grown at 42 °C, making them more fragile than walls grown at 28 °C.

When cells grown at 42 °C are shifted to 28 °C, the cell wall is precisely degraded to permit formation of a wild-type branch. Multiple branch sites occur predictably after downshift of *hypA1* cells, suggesting that some regions in *hypA1* cytoplasm in cells grown at 42 °C can rapidly initiate polarized secretion after a return to permissive temperatures, although like growing tips at 42 °C, these are not associated with recognizable Spitzenkörper. The thicker wall and electron-lucent inner layer at branch points triggered by downshift suggest that both wall-degrading enzymes and wall-building material were secreted. We are examining downshift-induced branching in *hypA1* strains as a model for Spitzenkörper ontogeny during wild-type mycelium development.

The increased thickness and relative lack of growth polarity in *hypA1* cells grown at 42 °C is consistent with *hypA* having a role in maintaining cell polarity and hyphal morphogenesis, presumably via regulation of function in the endomembrane system. During conidial swelling early in spore germination in *Aspergillus*, cell wall deposition is non-polar, and recognizable Spitzenkörper are not found (Dute et al. 1989). Peripheral vesicles were sometimes seen subjacent to the cell membrane in *hypA1* cells grown at 42 °C (Fig. 7B) and may contribute to nonpolarized wall thickening. A Spitzenkörper is not required for all types of cell wall deposition in filamentous fungi (Seiler et al. 1997; Wu et al. 1998), nor for the transient polar growth stages in yeasts, although it does correlate with polarized hyphal growth in many fungi (Lopez-Franco and Bracker 1996).

Septum thickness was significantly greater in *hypA1* cells grown at 42 °C than in those produced at 28 °C. Septa deposited by *hypA1* strains at both temperatures had a trilamellar structure, with the increased thickness at 42 °C being evident in all layers. Some *hypA1* septa grown at 42 °C were particularly electron dense, which cannot readily be explained by proteins being trapped during an inefficient secretory process; instead, this suggests mislocalized deposition of proteinaceous components. In *hypA1* cells grown at 28 °C, septum thickness doubled between the pore and the hyphal wall, whereas at 42 °C septum thickness quadrupled,

consistent with their spanning wider cells. This result suggests that ongoing deposition of central as well as peripheral layers in *hypA1* septa grown at 42 °C might be occurring across the septum, even after the septum was complete, as older septa were thicker than younger ones. Thus wild-type functions of the *hypA* product appear to include coordinating wall deposition with growth and restricting deposition to regions of cell and septum extension.

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