Journal of Microscopy, Vol. 198, Pt 3, June 2000, pp. 246–259.
Received 7 January 2000; accepted 3 March 2000

Confocal microscopy of FM4-64 as a tool for analysing endocytosis and vesicle trafficking in living fungal hyphae

S. FISCHER-PARTON, R. M. PARTON, P. C. HICKEY, J. DIJKSTERHUIS, H. A. ATKINSON & N. D. READ
Institute of Cell and Molecular Biology, University of Edinburgh, Rutherford Building, Edinburgh EH9 3JH, U.K.

Key words. Confocal microscopy, endocytosis, FM1-43, FM4-64, fungal hyphae, Spitzenkörper, tip growth, TMA-DPH, vesicle trafficking.

Summary

Confocal microscopy of amphiphilic styryl dyes has been used to investigate endocytosis and vesicle trafficking in living fungal hyphae. Hyphae were treated with FM4-64, FM1-43 or TMA-DPH, three of the most commonly used membrane-selective dyes reported as markers of endocytosis. All three dyes were rapidly internalized within hyphae. FM4-64 was found best for imaging the dynamic changes in size, morphology and position of the apical vesicle cluster within growing hyphal tips because of its staining pattern, greater photostability and low cytotoxicity. FM4-64 was taken up into both the apical and subapical compartments of living hyphae in a time-dependent manner. The pattern of stain distribution was broadly similar in a range of fungal species tested (*Aspergillus nidulans, Botrytis cinerea, Magnaporthe grisea, Neurospora crassa, Phycomyces blakesleeanus, Puccinia graminis, Rhizoctonia solani, Sclerotinia sclerotiorum* and *Trichoderma viride*). With time, FM4-64 was internalized from the plasma membrane appearing in structures corresponding to putative endosomes, the apical vesicle cluster, the vacuolar membrane and mitochondria. These observations are consistent with dye internalization by endocytosis. A speculative model of the vesicle trafficking network within growing hyphae is presented.

Introduction

Vesicle trafficking is fundamental to numerous activities in eukaryotic organisms, and underlies many of the basic processes involved in cell growth and differentiation. The vesicle trafficking network includes exocytosis and endocytosis (Gruenberg & Clague, 1992; Rothman, 1994). In filamentous fungi a reasonable amount is understood about exocytosis, whereas very little is known about endocytosis.

Correspondence to: Nick Read. Tel: +44 (0)131 650 5335; fax: +44 (0)131 650 5392; e-mail: nick.read@ed.ac.uk

Most of our current understanding of vesicle trafficking in filamentous fungi is concerned with tip growth and is based upon ultrastructural studies (e.g. Grove & Bracker, 1970; Howard, 1981), pharmacological treatments (e.g. Howard & Aist, 1980), analyses of mutants (Wu *et al.*, 1998; Seiler *et al.*, 1999) and mathematical modelling of vesicle trafficking in relation to tip growth (e.g. Bartnicki-Garcia *et al.*, 1989). During tip growth, extension of the hypha is confined to a region occupying only a few micrometres at the hyphal apex and involves highly polarized exocytosis. Secretory vesicles deliver membrane, cell wall precursors and wall-building enzymes to the hyphal tip, and many extracellular enzymes released into the surrounding medium are also believed to be secreted from this region (reviewed by Wessels, 1993).

In higher fungi (i.e. members of the Ascomycota, Basidiomycota and Deuteromycota) vesicle trafficking to the apex during tip growth is highly organized and involves the activity of a specific, multicomponent organelle complex which, in most cases, is called the Spitzenkörper (= 'apical body'). This structure is predominated by secretory vesicles which make up what is commonly described as an 'apical vesicle cluster' (Grove & Bracker, 1970; López-Franco & Bracker, 1996). The dynamic behaviour of the Spitzenkörper has indicated that it is intimately associated with the precise growth pattern of the hyphal apex (Girbardt, 1957; Bartnicki-Garcia *et al.*, 1995; López-Franco & Bracker, 1996).

From studies of tip-growing plant cells (Steer & Steer, 1989; Miller *et al.*, 1997) it has been suggested that membrane recycling via endocytosis is a critical part of the process of apical extension. However, reports of the existence of endocytosis in filamentous fungi have been conflicting. Work by Caesar-Ton That *et al.* (1987) identified a fraction from hyphae of *Neurospora crassa* that was rich in coated vesicles and possessed a major polypeptide with a molecular weight similar to that of the heavy chain of

clathrin, the major coat protein of endocytic vesicles in animal and plant cells (Hawes *et al.*, 1995; Mellman, 1996). However, convincing ultrastructural evidence for clathrin-coated vesicles or pits in fungal hyphae is lacking. Evidence against the occurrence of endocytosis in filamentous fungi was obtained by Cole *et al.* (1997), who were unable to observe uptake of membrane-impermeant fluorescent probes by fluid-phase endocytosis into hyphae of the basidiomycete *Pisolithus tinctorius.* Recently, uptake of the membrane-selective endocytosis marker FM4-64 by germ tubes of *Uromyces fabae* (Hoffmann & Mendgen, 1998) and hyphae of *N. crassa* and *Trichoderma viride* (Read *et al.*, 1998) has been taken as positive evidence for membrane internalization by endocytosis.

Amphiphilic styryl dyes, such as FM4-64, insert into the outer leaflet of the plasma membrane and are believed not to directly enter intact cells by unfacilitated diffusion (Illinger & Kuhry, 1994; Betz *et al.*, 1996). They have, therefore, been widely used as fluorescent reporters of endocytosis and other components of the vesicle trafficking network in animal cells (e.g. Betz *et al.*, 1996) and the budding yeast (Vida & Emr, 1995; Rieder *et al.*, 1996).

In this paper we have used confocal imaging of amphiphilic styryl dyes to study endocytosis and vesicle trafficking in living fungal hyphae. Although we tested three of these dyes (FM4-64, FM1-43 and TMA-DPH), most of our work has concentrated on FM4-64 because of its superior properties. Time courses of FM4-64 staining have been performed and considerable emphasis placed on imaging the stained apical vesicle cluster within growing hyphal tips. Possible pathways of dye internalization and distribution have been assessed. However, our analysis supports the view that endocytosis and vesicle trafficking are probably the predominant pathways by which FM4-64 is internalized and distributed between organelles within hyphae. Based on the interpretation of our results in the context of current knowledge in other cell types, we present a speculative model of the vesicle trafficking network within growing hyphae.

Materials and methods

Dyes and other chemicals

The dyes FM4-64, FM1-43 and TMA-DPH were obtained from Molecular Probes Inc. (Eugene, OR, U.S.A.). All other chemicals were supplied by Sigma (Poole, U.K.).

Fungal material

The following fungi were used: *Aspergillus nidulans* (R153 from Fungal Genetics Stock Center [FGSC], Arcata, CA, U.S.A.); *Botrytis cinerea* (from the Plant Disease Control Group, DuPont Co., Wilmington, DE, U.S.A.); *Magnaporthe grisea* (strain 0-42 from B. Valent, DuPont Co.); *Neurospora crassa* 74 A (strain 262 from FGSC); *Phycomyces blakesleeanus* (isolated from soil by P. Hickey, Edinburgh, U.K.); *Puccinia graminis* f. sp. *tritici* (isolate 84 from the National Institute for Agricultural Botany, Cambridge, U.K.); *Rhizoctonia solani* (No. 283 from E. E. Butler, University of California, Davis, U.S.A.); *Sclerotinia sclerotiorum* (from T. S. Abney, Purdue University, IN, U.S.A.); and *Trichoderma viride* (No. 2011 from J.F. Tuite, Purdue University).

Growth conditions and dye loading

Aspergillus nidulans, B. cinerea and *P. blakesleeanus* were grown on 2% w/v malt extract solidified with 2% w/v agar. Twenty-four-hour-old cultures of *P. blakesleeanus* and 48-h-old cultures of *A. nidulans* and *B. cinerea* were used for experiments. Agar bearing the leading edge of the colony was cut out and carefully placed, hypha side down, in liquid malt extract medium containing 25 μM FM4-64 on a glass coverslip.

Magnaporthe grisea was grown on oatmeal agar at 24 °C in continuous light (as described in Jelitto *et al.*, 1994). Conidia were harvested from 10-day-old cultures, inoculated onto glass coverslips in distilled water and incubated in darkened humid chambers. The resultant germlings were stained with 7.5 μM FM4-64.

Neurospora crassa, R. solani, S. sclerotiorum and *T. viride* were grown on Vogel's medium N (Vogel, 1956) plus 2% w/v sucrose (VMS medium) and prepared for microscopic observation on coverslips, as described by Parton *et al.* (1997). After 10 min of loading with 6.4 μM FM4-64, the medium containing dye was replaced with fresh medium lacking dye. *Neurospora* hyphae were similarly stained with 6.4 μM FM1-43 or 10 μM TMA-DPH. Additionally, *N. crassa* was grown on a thin layer of VMS medium solidified with 2% w/v agarose evenly spread on a glass coverslip (Parton *et al.*, 1997). Shortly before imaging, the mycelium was covered with 100 μL liquid VMS medium containing 6.4 μM FM4-64. For double staining of *N. crassa* hyphae with Rhodamine 123 and FM4-64, the stains were added sequentially. Firstly, 10 μM Rhodamine 123 was applied in 100 μL liquid VMS medium for at least 30 min then a further 100 μL medium containing 6.4 μm FM 4-64 was added.

Puccinia graminis urediospores (obtained from uredia on wheat as described by Read *et al.*, 1997) were inoculated onto glass coverslips and subsequently incubated in a humid chamber for 3–5 h to allow germ tubes to form. Germlings were loaded with dye by placing 20 mM HEPES-buffer (pH 7.2) containing 3.2 μM FM4-64 over them, followed by a coverslip supported by strips of lithographer's tape (No. 616, Scotch Brand).

For time course experiments, osmotic shock during dye

application was avoided by acclimatizing hyphae to standard liquid medium before experimentation.

Confocal microscopy

For routine confocal microscopy we employed a Bio-Rad MRC 600 confocal laser scanning microscope fitted with a 25 mW argon laser and connected to a Nikon Diaphot TMD inverted microscope with epifluorescence equipment (all supplied by Bio-Rad Microscience, Hemel Hempstead, U.K.). The laser power used was 1 or 3% of full intensity. Excitation was at 514 nm, and fluorescence was detected at >550 nm. Simultaneous confocal fluorescence images and corresponding brightfield images were collected. A $\times 40$ dry plan apo (NA 0.95) and a $\times 60$ oil immersion plan apo (NA 1.4) objective were used.

UV confocal microscopy of TMA-DPH was performed using a Leica TCS NT confocal microscope (Leica Microsystems Heidelberg GmbH, Germany) fitted with a 2 W UV argon laser. The Leica system was also equipped with a 100 mW argon ion laser, which allowed simultaneous imaging of Rhodamine 123 and FM4-64 without 'bleed through' of signal between the two channels used (488 nm excitation; Rhodamine 123 fluorescence detected at 530/30 nm, FM4-64 fluorescence detected at >640 nm). A $\times 63$ water immersion plan apo (NA 1.2) objective was used.

Results

Application and imaging styryl dyes in hyphae

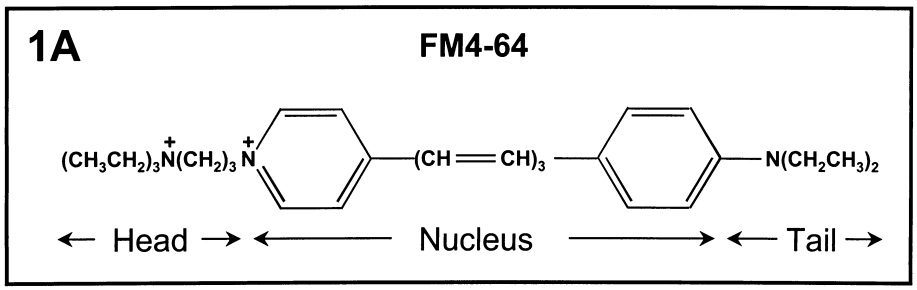
Amongst the most commonly used fluorescent dyes for imaging endocytosis in living cells are the styryl-based dyes FM4-64, FM1-43 and TMA-DPH (Figs 1A, 2A and 3A, Illinger & Kuhry, 1994; Betz *et al.*, 1996; Haugland, 1996). These compounds were tested in *N. crassa.* All three dyes were found to be taken up by both apical and subapical hyphal compartments in a time-dependent manner: immediate staining of the plasma membrane was followed by dye internalization and staining of organelles. All dyes stained the apical vesicle cluster within the Spitzenkörper (Figs 1B, 2B and 3B), although considerable differences were observed in the patterns of organelle staining and in the photosensitivity and phototoxicity of each dye.

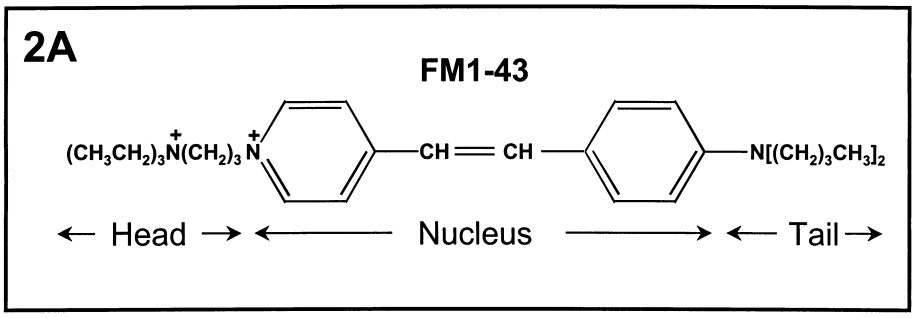
The staining pattern with each dye was examined over the apical 50 μm of hyphae, 40 min after dye application (Figs 1B, 2B and 3B). At that time FM4-64 had clearly stained the plasma membrane and Spitzenkörper region, with a more diffuse background staining of the cytoplasm. Staining of organelles was also evident but not marked. There was little staining of mitochondria (Fig. 1B). In addition, FM4-64 caused little disturbance to apical extension, even with repeated laser scanning at 15 s intervals over periods of 10 min when imaged with a $\times 40$ dry plan apo (NA 0.95) objective. The extension rate of dye-loaded hyphae imaged in this way was 16.7 ± 1.1 μm min^{-1} (SE) ($n = 11$), whilst the extension rate of control hyphae lacking dye was 17.2 ± 0.3 μm min^{-1} (SE) ($n = 10$). FM1-43 staining of the Spitzenkörper was similar except that it photobleached much more rapidly, making it more difficult to follow with time. In addition, the elongated mitochondria were more intensely stained with FM1-43 (cf. Figs 2B and 1B) unless the period of FM4-64 staining was significantly extended (Fig. 4). As with FM4-64, little significant effect on growth was observed (data not shown). TMA-DPH stained the plasma membrane and Spitzenkörper but the latter was less clearly stained than with FM4-64 (cf. Figs 3B and 1B). The cytoplasm was diffusely stained with TMA-DPH but pronounced staining of organelles was lacking (Fig. 3B). Repeated scanning with the UV laser led to drastic photobleaching of TMA-DPH and cessation of apical extension within three or four scans. These limitations render TMA-DPH useless for the type of prolonged examination required to image the dynamic behaviour of the Spitzenkörper or other aspects of vesicle trafficking.

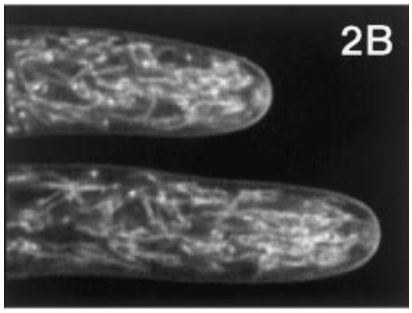
Characteristics of FM4-64 internalization

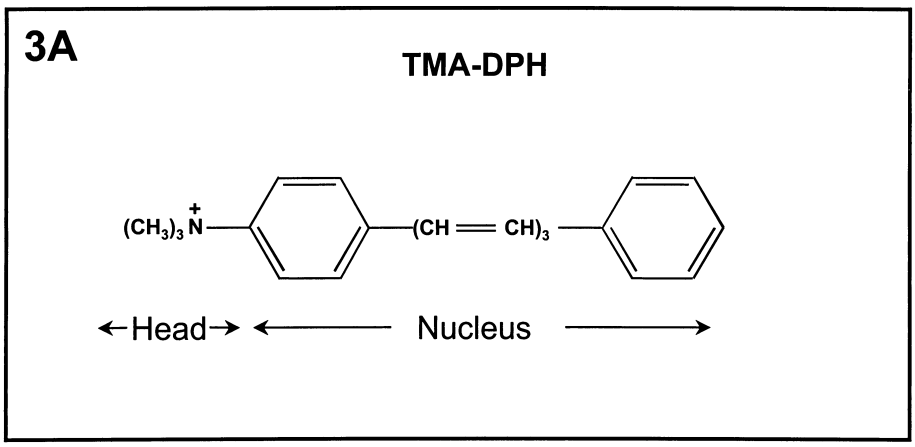
Examination of early dye uptake within hyphal tips revealed that after immediate plasma membrane staining, signs of internalized dye could first be discerned as early as 30 s following dye application, and more clearly after 60 s (Fig. 5). Initial dye internalization was observed by contrast adjusting images and could be seen as a slight staining of the apical cytoplasm, most obviously in a 10–15 μm long region commonly 8–10 μm from the apical pole. Discrete, roughly spherical fluorescent organelles, ~ 0.75 μm in diameter, which corresponded in size and in their time of appearance to the putative endosomes visualized by FM4-64 staining in budding yeast cells (Vida & Emr, 1995), were first discernible 110 s after dye application. These organelles tended to be more numerous or obvious in a particular region behind the extreme tip although the precise distance of this zone from the apical pole varied between hyphae (data not shown). Spitzenkörper staining was first evident after 180 s (Fig. 5). Subsequently, the Spitzenkörper region and the small, roughly spherical organelles became brighter whilst numerous other small organelles also became stained. In hyphae stained for longer than $\sim$ 15 min, numerous roughly circular regions of dye exclusion, $\sim$2–3 μm in diameter, could be seen up to within $\sim$20 μm of the tip (Figs 1B and 4). These regions of dye exclusion correspond to the size and location of nuclei (Zalokar, 1959). Staining of nuclear membranes was never observed.

The subapical compartments we investigated after staining with FM4-64 were within the peripheral growth zone of the mycelium (i.e. the mycelial region needed to maintain the maximum extension rate of the colony's leading hyphae) and possessed unplugged septa. These hyphal









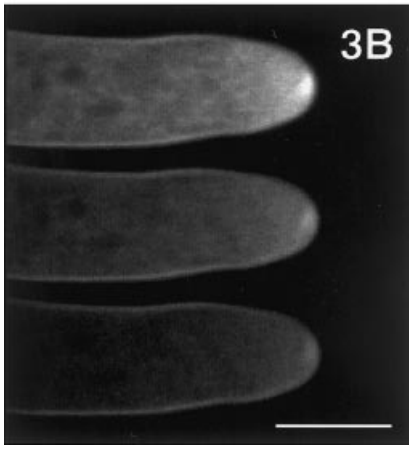


Fig. 1. (A) Molecular structure of endocytosis marker dye FM4-64. (B) Confocal image of growing hyphal tip of *Neurospora crassa* stained for 40 min with FM4-64; images 30 s apart; hyphal extension rate = 12.0 μm min^{-1}.
Fig. 2. (A) Molecular structure of endocytosis marker dye FM1-43. (B) Confocal image of growing hyphal tip of *Neurospora crassa* stained for 40 min with FM1-43; images 30 s apart; hyphal extension rate = 15.3 μm min^{-1}.
Fig. 3. (A) Molecular structure of endocytosis marker dye TMA-DPH. (B) Confocal image of growing hyphal tip of *Neurospora crassa* stained for 40 min with TMA-DPH; images 1 s apart; growth rate not determined.
Note the staining of elongated mitochondria in Fig. 2(B) but not in Figs 1(B) and 3(B). Bar = 10 μm.

compartments are metabolically very active and contribute to tip growth by the vectorial transport of cytoplasm and organelles towards growing hyphal apices (Trinci, 1971). In these compartments (Fig. 6) the immediate staining of the plasma membrane after dye application was followed within 1–2 min by the appearance of faintly stained roughly spherical organelles, ~0.75 μm in diameter, which were similar in appearance to the previously described organelles in the apical hyphal compartments. These organelles were distinct from the similarly sized, more-or-less spherical mitochondria found in subapical regions. This was shown by double staining with FM4-64 and the mitochondrion-selective stain, Rhodamine 123, which did not colocalize (Fig. 7). With time, FM4-64 staining increased in the small spherical organelles which were not mitochondria, and also in the surrounding cytoplasm and other organelles, until the small spherical organelles could no longer be clearly distinguished (3–9 min in Fig. 6). The large spherical vacuole, normally found lying adjacent to the septum of subapical hyphal compartments, was originally visible as a zone of dye exclusion. Initial staining of the vacuole membrane was observed after 9 min and the degree of staining increased thereafter (Fig. 6). Strong staining of the septum, lined on each

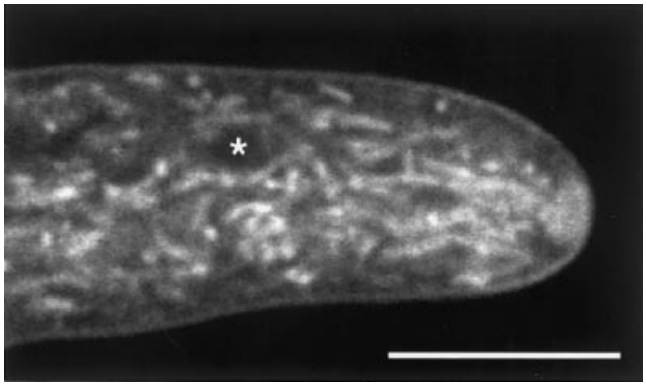


Fig. 4. Confocal image of a growing hyphal tip of *Neurospora crassa* stained for 105 min; hyphal extension rate = 11.5 μm min^{-1}. Note pronounced staining of elongated mitochondria (cf. Fig. 2B) and cytoplasmic region of dye exclusion (asterisk) which probably represents an unstained nucleus. Bar = 10 μm.

side by the plasma membrane, was also often observed. The intense dye fluorescence associated with the plasma membrane remained relatively constant during experiments in which dye was continuously present in the extracellular medium.

Application of FM4-64 in the presence of the metabolic inhibitor sodium azide inhibited dye internalization but still allowed staining of the plasma membrane of apical and subapical hyphal compartments (Fig. 8).

FM4-64 uptake into the apical hyphal region of different fungal species (Fig. 9) was broadly similar to that recorded for *N. crassa* (Fig. 5). However, there were clear differences between species in the rate and extent to which FM4-64 was taken up into organelles, most obviously the elongated mitochondria within hyphal tips. *Aspergillus nidulans*, in particular, accumulated FM4-64 more rapidly (i.e. within 30 min) within these mitochondria (Fig. 9D).

Staining of Spitzenkörper in different species

In higher fungi (Ascomycota, Deuteromycota and Basidiomycota) the Spitzenkörper is a highly dynamic and pleomorphic multicomponent structure believed to contain the secretory vesicles responsible for tip growth (Grove & Bracker, 1970; López-Franco & Bracker, 1996). López-Franco & Bracker (1996) investigated Spitzenkörper morphology in the unstained hyphal tips of 32 species using computer-enhanced phase-contrast microscopy. In the present study, we have compared FM4-64 staining of the apical vesicle cluster within the Spitzenkörper of seven higher fungal species: *N. crassa* (Fig. 9A), *S. sclerotiorum*

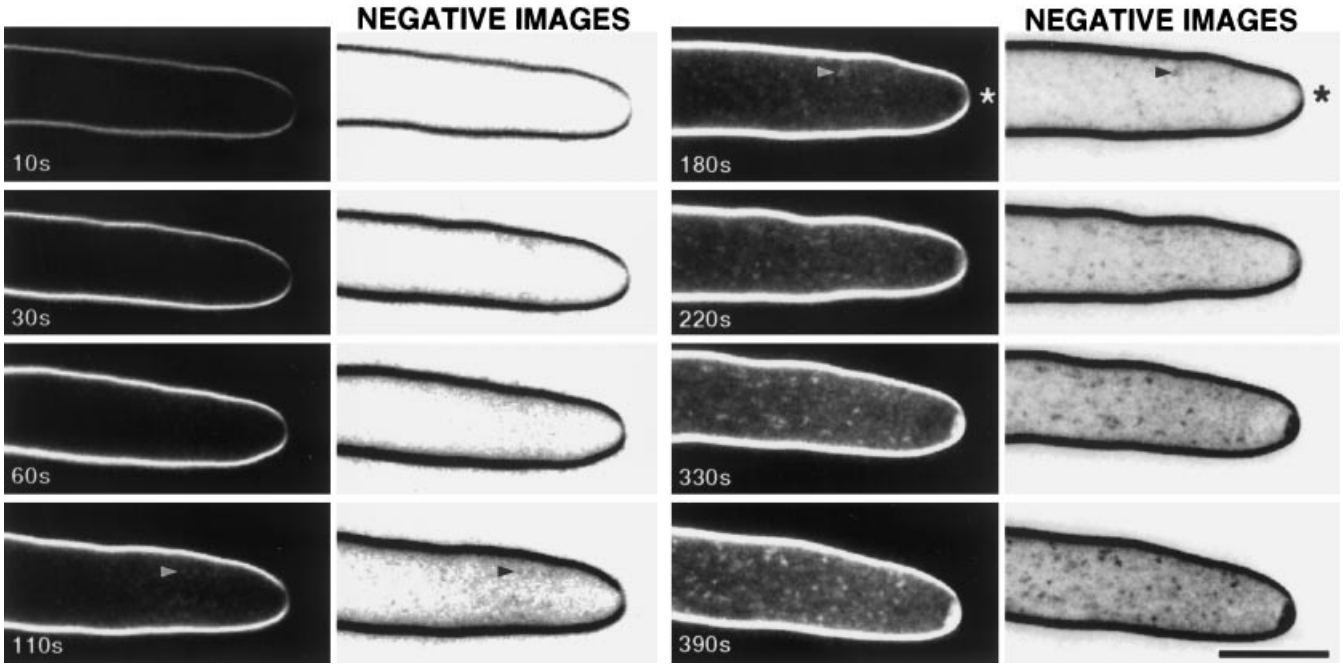


Fig. 5. Time course of FM4-64 internalization within the hyphal tip of *Neurospora crassa.* Numbers indicate time(s) after dye application (continuous loading). Confocal fluorescence images (median optical sections) are displayed alongside the same images contrast adjusted and shown in 'negative', in which dye-stained structures appear dark. Early uptake of dye is more easily seen in the contrast-adjusted negative images. The earliest signs of dye internalization can be seen after 30 s, most prominently within a (10–15 μm) region 8–10 μm from the apical pole. Small, roughly spherical organelles (arrowheads) are first evident between 110 s and 180 s. Staining of the Spitzenkörper region (asterisk) is first seen 180 s after dye application. Hyphal extension continued at an average rate of 21 μm min^{-1} throughout the time course. Bar = 10 μm.

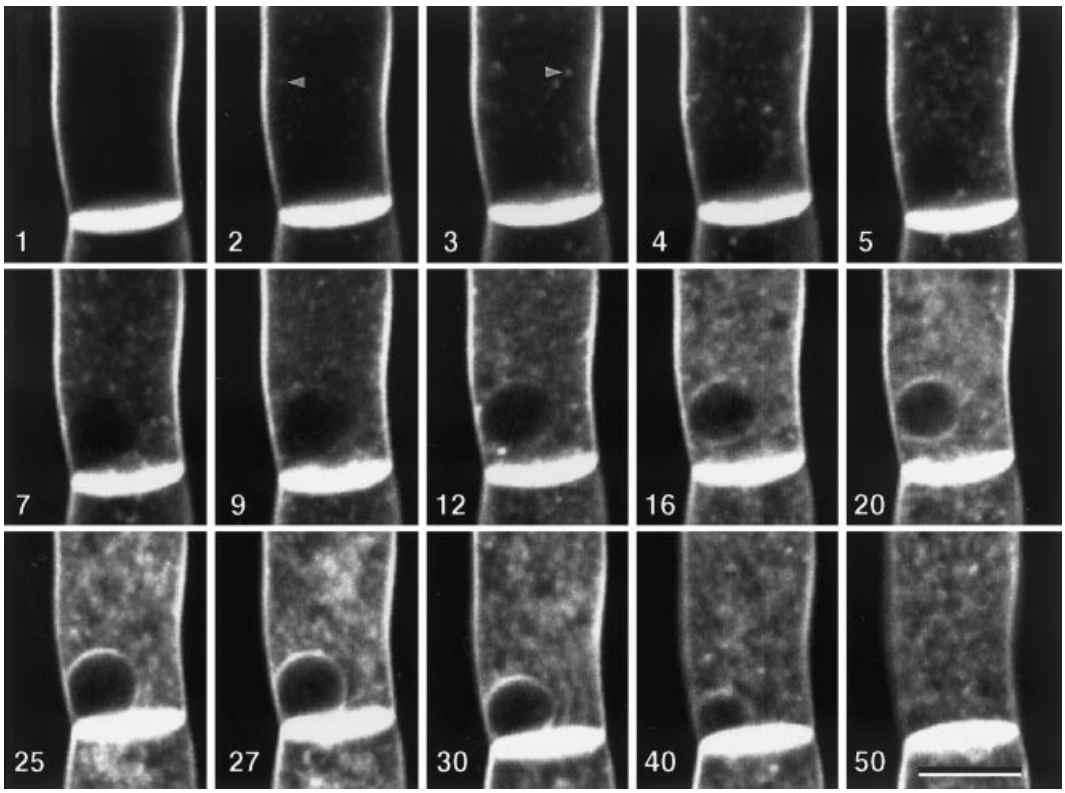


Fig. 6. Time course of FM4-64 internalization with continuous loading of a subapical hyphal region of *Neurospora crassa*. Numbers indicate time (min) after dye application. Small, roughly spherical structures (arrowheads) are clearly visible after 2 min. Bar = 10 μm.

(Fig. 9B), *M. grisea* (Fig. 9C), *A. nidulans* (Fig. 9D), *T. viride* (Fig. 9E), *R. solani* (Fig. 9F) and *B. cinerea* (Fig. 10). We also examined staining of the apical vesicle cluster within germ tubes of the basidiomycete rust fungus, *P. graminis* (Fig. 9G). It should be noted that the rust germ tube has been described previously as possessing an apical vesicle cluster but not a Spitzenkörper (Littlefield & Heath, 1979; Hoch & Staples, 1983; Kwon *et al.*, 1991). In general, there was good agreement between the morphologies of the brightly FM4-64 stained apical vesicle clusters with the unstained phase-dark Spitzenkörper of vegetative hyphae of *N. crassa, T. viride, S. sclerotiorum* and *R. solani* previously described by López-Franco & Bracker (1996). Our unpublished observations of the dynamic changes in position, morphology and size of the stained apical vesicle cluster of each species were also in agreement with previous descriptions of Spitzenkörper behaviour (Bartnicki-Garcia *et al.*, 1995; López-Franco *et al.*, 1995; Riquelme *et al.*, 1998).

After staining with FM4-64, the Spitzenkörper of *N. crassa, S. sclerotiorum, A. nidulans, T. viride, R. solani and B. cinerea* (Figs 9A, B, D–F and 10) were all visible as brightly fluorescent structures, which commonly had a distinct area of reduced fluorescence within them. In those species previously examined by López-Franco & Bracker (1996), the area of reduced fluorescence observed here correlates with their descriptions of the Spitzenkörper core. This core was often particularly large in *S. sclerotiorum* (Fig. 9B).

The appearance of the Spitzenkörper of *N. crassa* in optical sections (Fig. 9A) was roughly round but showed high variability in the shape and size of the core region, and varied from being circular to horseshoe-shaped. The Spitzenkörper of *A. nidulans* lacked an obvious negatively stained core (Fig. 9D) and was often surrounded by a diffusely fluorescent vesicle cloud (not shown). The Spitzenkörper of *S. sclerotiorum* (Fig. 9B), *T. viride* (Fig. 9E) and *B. cinerea* (Fig. 10) were roughly similar: more-or-less round with a central roughly circular core region which was most obvious in *S. sclerotiorum*. The Spitzenkörper of *R. solani* appeared as an irregularly shaped, bright horseshoe-like structure surrounding a darker core region. The germ tubes of *M. grisea* were very narrow, making it difficult to discern details of Spitzenkörper morphology. The Spitzenkörper appeared as a slightly flattened, sometimes crescent-shaped, band within the apical dome of the germ tube (Fig. 9C). The germ tubes of *P. graminis* possessed an FM4-64 stained vesicle cloud, which was most often rounded in shape yet highly pleiomorphic (Fig. 9G).

López-Franco *et al.* (1994, 1995) reported that the Spitzenkörper in many higher fungi was accompanied by one or more smaller migratory Spitzenkörper (called satellite

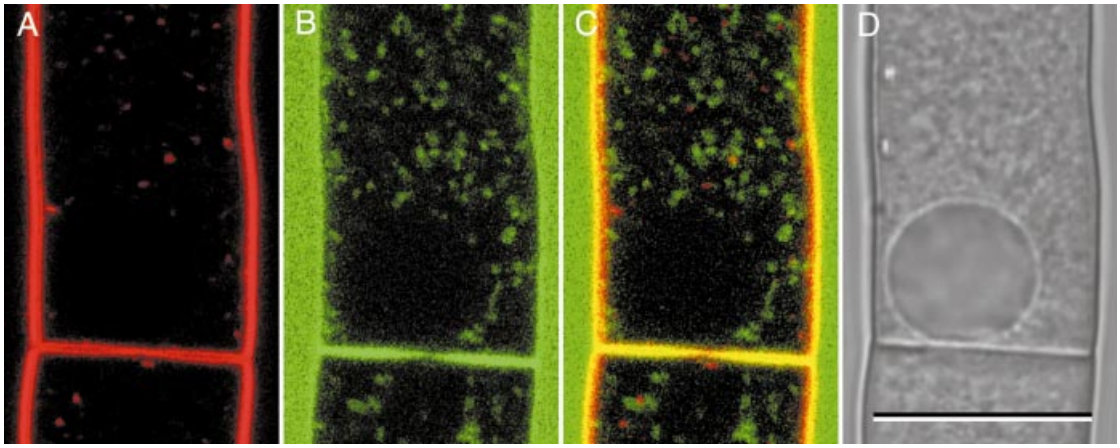


Fig. 7. Pseudocoloured confocal images of the subapical region of a *Neurospora crassa* hypha double stained with (A) FM4-64 loaded for ~4 min (red) and (B) Rhodamine 123 loaded continuously (green). (C) Overlay of (A) and (B) showing that putative endosomes labelled with FM4-64 are distinct from mitochondria labelled with Rhodamine 123 (colocalization = yellow). (D) Corresponding bright field image. Bar = 15 μm.

Spitzenkörper). FM4-64 was found to clearly stain satellite Spitzenkörper in *S. sclerotiorum, T. viride* (Fig. 9E) and *B. cinerea* (Fig. 10). Such satellites were most frequently observed in *B. cinerea*. Satellite Spitzenkörper generally emerged adjacent to the plasma membrane a few micrometres behind the main Spitzenkörper, migrated to the apex and finally merged with the main Spitzenkörper within 15 s (Fig. 10).

Finally, we also imaged the FM4-64 stained apical vesicle cluster of the lower fungus *P. blakesleeanus* (Fig. 9H). This fungus belongs to the Zygomycota, which have been reported to possess a crescent-shaped band of apical vesicles instead of a Spitzenkörper (Grove & Bracker, 1970). FM4-64 staining revealed a crescent-shaped zone of bright fluorescence in a zone corresponding to the expected location of these vesicles.

Discussion

In the present paper we have demonstrated FM4-64 staining of hyphae in a range of fungal species, spanning the Ascomycota, Deuteromycota, Basidiomycota and Zygomycota. We have shown that FM4-64 is clearly an excellent stain for tracking the apical vesicle cluster or main Spitzenkörper and satellite Spitzenkörper. FM4-64 also has significant potential for the study of endocytosis and other aspects of vesicle trafficking in living fungal hyphae. Below, we discuss how, with the aid of this dye, we can gain a better understanding of endocytosis and the vesicle trafficking network of hyphae.

Does endocytosis occur in filamentous fungi?

Little is known about endocytosis in filamentous fungi and neither physiological nor ultrastructural analyses have provided concrete evidence that it even occurs (Ashford, 1998). The best evidence for its occurrence so far available are reports of the uptake of FM4-64 by fungal hyphae (Hoffmann & Mendgen, 1998; Read *et al.*, 1998). Our current results further substantiate this with observations of FM4-64 uptake by nine different fungal species. In contrast to this, it is interesting to note that Cole *et al.* (1998) did not observe uptake of FM4-64 by healthy hyphae of *Pisolithus tinctorius* (Basidiomycota). The reason for this difference in results is unclear. Cole *et al.* (1997) also found that membrane-impermeant fluorescent probes, such as Lucifer Yellow carbohydrazide (LYCH), which is used as an indicator of fluid-phase endocytosis in budding yeast (Dulic *et al.*, 1991), were not taken up into hyphae of

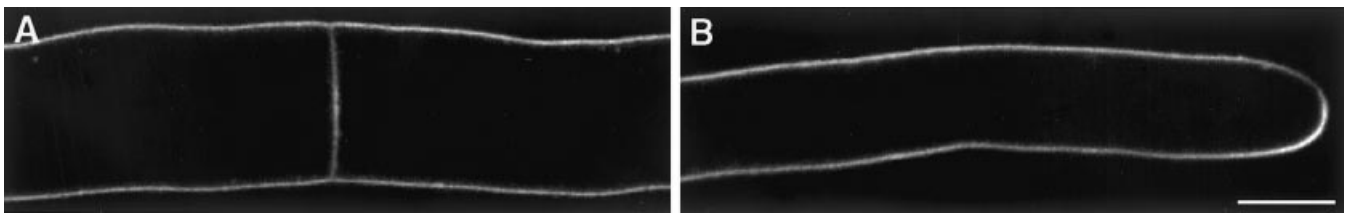


Fig. 8. Confocal images of (A) subapical and (B) apical hyphal compartments of *Neurospora crassa* loaded with FM4-64 for 45 min in the presence of 10 mM sodium azide (note that the sodium azide was added to hyphae 2 min before applying the dye). Bar = 10 μm.

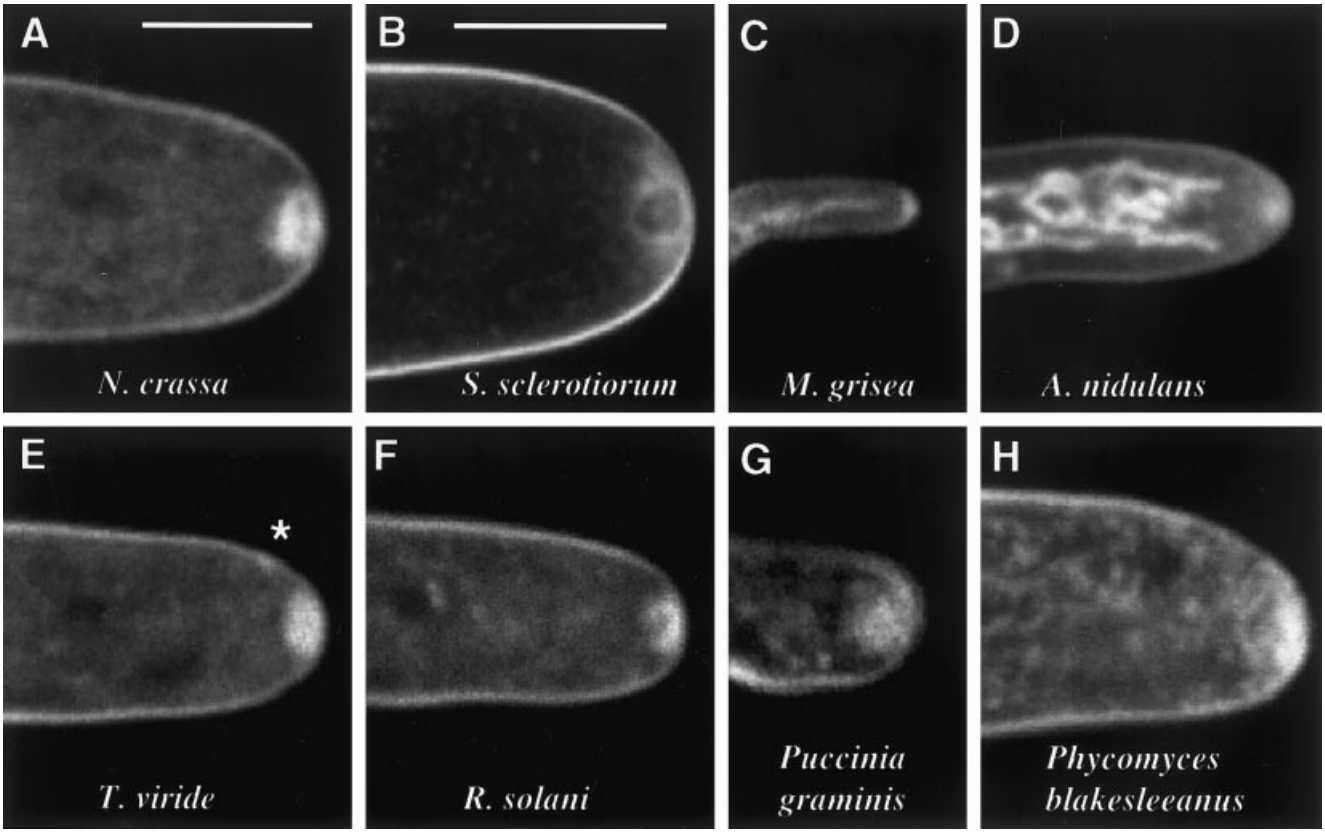


Fig. 9. Confocal images of growing hyphal tips of different species stained with FM4-64: (A) *Neurospora crassa*, (B) *Sclerotinia sclerotiorum*, (C) *Magnaporthe grisea*, (D) *Aspergillus nidulans*, (E) *Trichoderma viride* (asterisk indicates a satellite Spitzenkörper), (F) *Rhizoctonia solani*, (G) *Puccinia graminis*, (H) *Phycomyces blakesleeanus*. Bar in (A) for (A), (C)–(H), = 5 μm. Bar in (B) = 10 μm.

P. tinctorius (fluid-phase endocytosis involves the uptake of molecules in the lumen of endocytic vesicles). We found that although FM4-64 was rapidly internalized by *N. crassa*, LYCH and Oregon Green 488 10 kDa dextran did not appear to be taken up into hyphae of this fungus even after long periods of immersion in the dye (unpublished results).

In the light of such seemingly contradictory evidence, questions still need to be raised as to the occurrence of endocytosis in filamentous fungi and whether the styryl dyes do in fact reliably report endocytosis in filamentous fungi. In the absence of good evidence from other experimental techniques, the burden of proof presently lies heavily upon the FM4-64 data and is critically dependent upon the assumption that FM4-64 is internalized by endocytosis (Fig. 11A). This is certainly believed to be the case where it has been used with animal and yeast cells (Cochilla *et al.*, 1999).

Evidence supporting endocytic uptake of FM4-64

The FM4-64 molecule is composed of three elements (Fig. 1A): a hydrophobic tail (which promotes partitioning into membranes), a dicationic head (which prevents passage across membranes), and a body or nucleus (which determines the spectral properties of the dye). The structure of this dye therefore places a significant energetic barrier to direct passage across the plasma membrane (Betz *et al.*, 1996). Evidence against FM4-64 entering budding yeast cells by unfacilitated diffusion was obtained by inhibiting dye internalization in the presence of the metabolic inhibitor sodium azide, or by applying dye at low temperature (Vida & Emr, 1995). This was confirmed for fungal hyphae by our experimental observations with azide reported here. Similar inhibition was also obtained by Fischer-Parton (1999) when *Neurospora* hyphae were loaded with dye at 4 °C; when the temperature was subsequently increased to 25 °C normal dye uptake occurred.

Whilst unfacilitated diffusion of styryl dyes across membranes is unlikely, it is known that membrane phospholipids, which would similarly be expected to be resistant to passage from one side of membranes to the other, are able to cross over with the aid of flippases (Menon, 1995). Flippases are enzymes that facilitate the movement of specific phospholipids from one leaflet of the lipid bilayer of membranes to the other. Their activity is an essential requirement for membrane biosynthesis. In mammalian

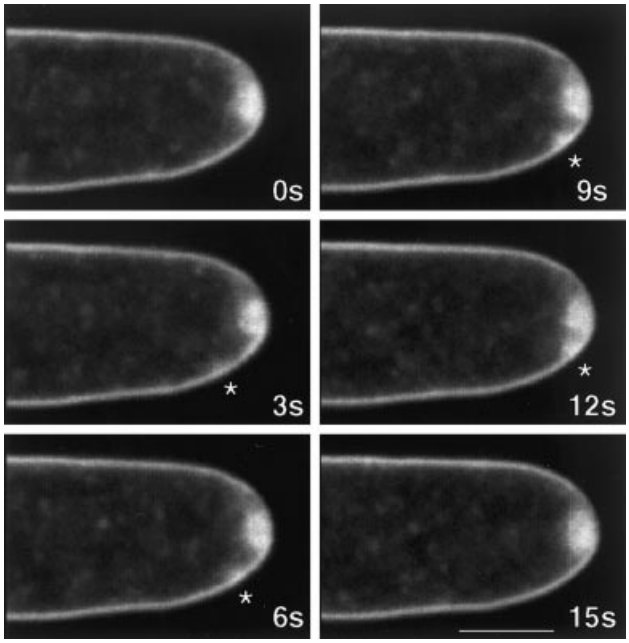


Fig. 10. Confocal images of an FM4-64-stained satellite Spitzenkörper (asterisk) of *Botrytis cinerea* showing a time course (in s) of different stages in its formation, migration and fusion with the main Spitzenkörper. Bar = 5 μm.

cells they have been identified in both the plasma membrane and endoplasmic reticulum (ER) (Menon, 1995) but they have not, to our knowledge, been described in fungi. It is conceivable that the action of flippases could provide an alternative mechanism for the internalization of FM4-64, FM1-43 and TMA-DPH (Fig. 11B). Once dye has been translocated to the inner leaflet of the plasma membrane by flippase activity, lipid transfer proteins may then transport dye molecules to the cytosolic face of the membranes of other organelles (Fig. 11B). Lipid transfer proteins have been identified in numerous eukaryotic cells, including filamentous fungi (Record *et al.*, 1998). Alternatively, owing to the water solubility of these dyes and reversible incorporation into many membranes, they could enter the cytosol from the cytoplasmic face of the plasma membrane and then label the external leaflet of organelle membranes (Illinger & Kuhry, 1994; Betz *et al.*, 1996). However, Illinger & Kuhry (1994), who used TMA-DPH to follow endocytosis in mammalian cells, recognized the possible significance of flippase activity for dye uptake but provided strong experimental evidence to discount it. Further evidence to support endocytic uptake comes from neuronal preparations, where it was shown that FM1-43 is confined to synaptic vesicles (Henkel *et al.*, 1996). Even stronger evidence that FM4-64 uptake follows an endocytic pathway comes from mutants in budding yeast defective in the vesicle trafficking network, in which styryl dye transport to the vacuole was inhibited (Vida & Emr, 1995). Indeed, in budding yeast FM4-64 has been successfully used in a fluorescence assisted cell sorter to screen for proteins involved in endocytosis (Gaynor *et al.*, 1998).

Our observations of the time-dependent sequence of FM4-64 internalization, with staining appearing in a defined sequence of organelles, is consistent with uptake by endocytosis as described for styryl dyes applied to animal and yeast cells (reviewed in Cochilla *et al.*, 1999). In these latter systems, the first obvious stained organelles have been identified as, or proposed to be, early endosomes (Vida & Emr, 1995; Cochilla *et al.*, 1999). The roughly spherical ~0.75 μm organelles observed here correspond well to this and thus we now refer to them as putative endosomes. However, it must be emphasized that endosomes have not yet been identified at the ultrastructural level in filamentous fungi.

The case for endocytosis in fungal hyphae

Although inconclusive, it is clear that uptake of FM4-64, FM1-43 and TMA-DPH provide the best evidence to date for endocytosis in filamentous fungi. The case for endocytosis in fungal hyphae is further supported by the wealth of knowledge available from research with the budding yeast fungus. Endocytosis and components of the endocytic pathway have been well characterized in this organism at the genetic, biochemical and ultrastructural levels (Pelham, 1997; Geli & Riezman, 1998; Prescianotto-Baschong & Riezman, 1998).

There is an array of physiological functions carried out in animal, plant and yeast cells which are mediated by endocytosis (Dulic *et al.*, 1991; Hawes *et al.*, 1995; Mellman, 1996; Geli & Riezman, 1998). Evidence that such activities are also carried out in filamentous fungi strengthens the case for endocytosis. Our data are consistent with the interpretation that endocytosis occurs in both apical and subapical hyphal compartments. At present we do not know exactly what roles endocytosis might serve in these different compartments. Nevertheless, based on what is known about the biology of hyphae and about endocytosis in other organisms we can speculate generally as to possible roles that endocytosis may serve in filamentous fungi.

Removal of excess plasma membrane. It has been suggested that, in pollen tubes, insertion of membrane by the fusion of vesicles delivering cell wall components to the apex exceeds the membrane necessary for tip extension (Picton & Steer, 1983). The most likely retrieval mechanism for superfluous membrane is endocytosis (Steer, 1988). This is supported by Derksen *et al.* (1995), who identified a zone 6–15 μm behind the tip with a concentration of clathrin-coated pits implicating it as a major site of endocytosis. In rust germ tubes, Hoffman & Mendgen (1998) observed initial FM4-64 uptake within a region 5–20 μm away from the apical pole, indicating active endocytosis in that zone. This broadly matches our observations of early uptake in *N. crassa*. Calculations of the amount

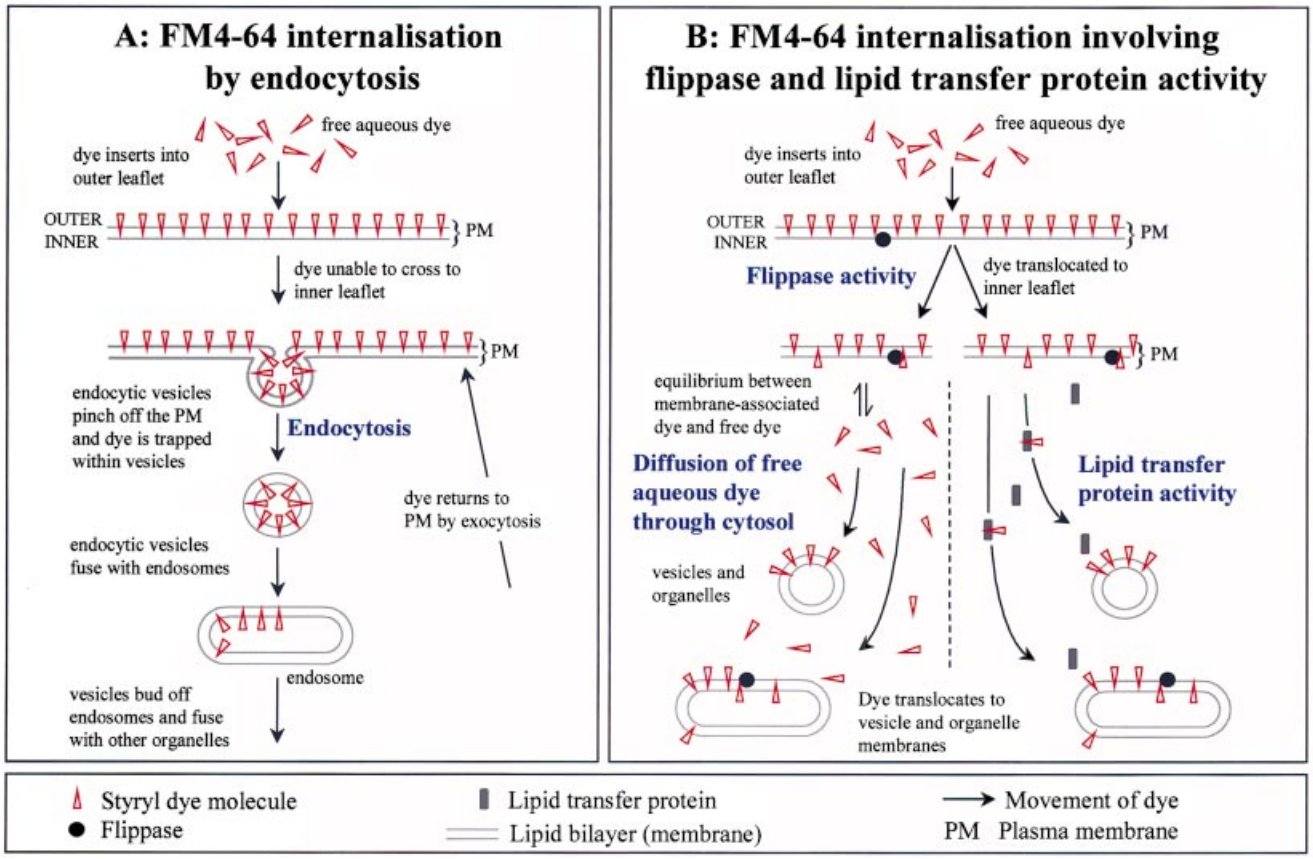


Fig. 11. Diagrams outlining possible alternative pathways of FM4-64 uptake by fungal hyphae. (A) Endocytic pathway of internalization. (B) Internalization involving the activity of flippases and lipid transfer proteins.

of apical vesicle membrane relative to the volume of these vesicles in fungal hyphae have indicated that a significant excess of membrane relative to wall material is probably added during hyphal tip growth (C. E. Bracker, personal communication, 1998). In subapical compartments endocytosis may be important for retrieving excess membrane delivered by secretory vesicles during septum formation.

Recycling of membrane proteins. Endocytosis in apical hyphal compartments may function in tip growth by providing a means for retrieving displaced membrane proteins (e.g. ion channels and cell wall-building enzymes) and returning them to the tip for re-use. In budding yeast, there is strong evidence that two of the three chitin synthases in the plasma membrane are recycled back to the plasma membrane via endocytosis (Chuang & Schekman, 1996; Ziman *et al.*, 1996, 1998; Holthuis *et al.*, 1998). It makes economic sense for a hypha to recycle and reuse some of the proteins involved in tip growth rather than to synthesize all of these proteins *de novo*. As discussed in the following section, rapid recycling of membrane proteins in hyphae may occur via endosomes and/or satellite Spitzenkörper.

Transport of membrane proteins and lipids to the vacuole for degradation. Our findings showed that spherical vacuoles in *N. crassa* became stained with FM4-64 in a manner similar to that reported for the vacuole of budding yeast, which functions as the site of degradation of plasma membrane proteins (Vida & Emr, 1995; Wendland *et al.*, 1998).

Uptake of molecules in the fluid phase of endocytic vesicles. This may be important for the uptake of certain nutrients (Dulic *et al.*, 1991).

Receptor-mediated uptake of ligands. It has been shown that, in budding yeast, the mating pheromone α-factor and its plasma membrane receptor are internalized by receptor-mediated endocytosis (Wendland *et al.*, 1998). Although filamentous fungi produce pheromones (Bölker & Kahmann, 1993), their mechanism of internalization has not been studied.

Tracking the vesicle trafficking network in fungal hyphae

FM4-64 was found to be of low toxicity and relatively resistant to photobleaching during repeated imaging. This makes it an excellent dye for following dynamic processes in living cells over time without perturbing those processes. Confocal imaging has allowed us to visualize putative endosomes and other organelles (e.g. mitochondria and vacuoles). However, it should be emphasized that visualization of individual vesicles is beyond

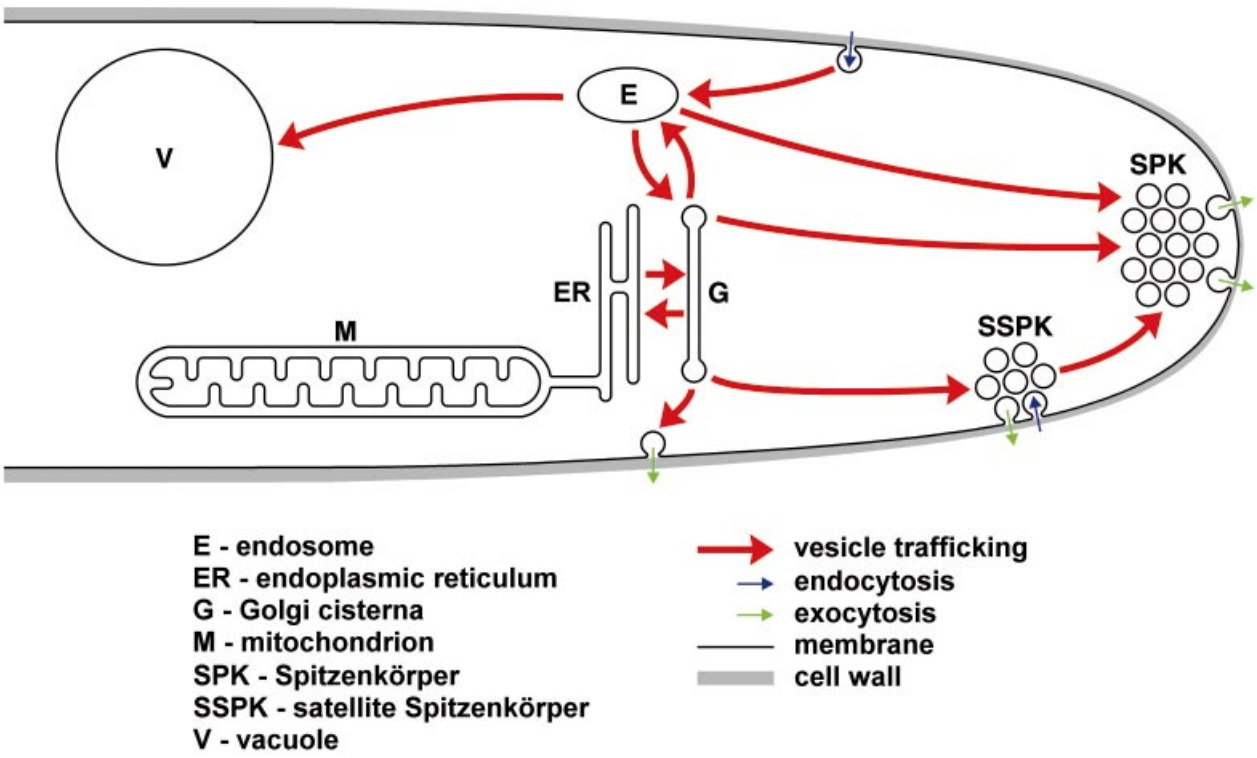


Fig. 12. A hypothetical model of the organization of the vesicle trafficking network in a growing hypha based upon the pattern of FM4-64 staining.

the limits of the imaging techniques employed here. This is because the vesicles are very small (typically < 100 nm in diameter) and are present at a high density within the hyphal cytoplasm. The diffraction limitation of fluorescence imaging means that such small, closely spaced fluorescing vesicles cannot be individually identified because they appear blurred together (Betz & Angleson, 1997).

On the basis of our observations with FM4-64 uptake we have proposed a speculative model (Fig. 12) which interprets our observations in the context of current knowledge of the vesicle trafficking pathways in yeast and animal cells (e.g. Mellman, 1996; Pelham, 1997; Geli & Riezman, 1998).

Our observation of diffuse fluorescence within the cytoplasm within 30 s of adding FM4-64 to hyphae may be interpreted as primarily representing a cloud of stained endocytic vesicles. Although endocytic vesicles have not been identified at the ultrastructural level, possible candidates are filasomes (Howard, 1981). These are vesicles possessing a fibrillar coating that contains actin (Bourett & Howard, 1991; Roberson, 1992). These coated vesicles were found to be concentrated principally in the first 12 μm of growing hyphal tips of the basidiomycete *Sclerotium rolfsii* (Roberson, 1992). This is consistent with our observations of initial dye internalization in a localized region behind the apical dome. The first FM4-64-stained organelles that we could visualize clearly in hyphae were small and roughly spherical and, as previously discussed, are interpreted as putative endosomes. In other cell types endosomes function as sorting compartments for proteins and lipids, and are classified into two functional types: 'early' and 'late' endosomes (Ashford, 1998; Prescianotto-Baschong & Riezman, 1998; Mukherjee *et al.*, 1999).

In subapical compartments, the next obviously stained organelle was the large spherical vacuole. This pattern of staining was similar to that in budding yeast, in which staining of yeast vacuolar membranes followed that of putative endosomes (Vida & Emr, 1995). In addition to the large spherical vacuoles, the vacuolar system in hyphae also consists of an extensive tubular network in both subapical and apical compartments (Ashford, 1998). We have also found that the membranes of these tubular vacuoles in apical hyphal compartments become clearly stained after prolonged immersion in FM4-64 (unpublished results).

In addition to vacuolar membranes we would also expect that both the Golgi and ER of fungal hyphae would become stained with FM4-64 via retrograde pathways which connect the endosomal system, Golgi and ER, as occurs in budding yeast (Pelham, 1997; Fig. 12). Staining by FM4-64 of these membranes in fungal hyphae would provide an explanation for the observed increase in staining of other organelles in the cytoplasm with time. However, it should also be stressed that the staining of the small, roughly spherical organelles proposed earlier to be endosomes could conceivably be Golgi instead.

The traditional view of hyphal tip growth has been that the wall-building, secretory vesicles which reside in the Spitzenkörper prior to exocytosis are generated exclusively by Golgi cisternae (e.g. Grove & Bracker, 1970; Howard, 1981). Recently, evidence has been obtained that indicates that satellite Spitzenkörper also supply wall-building vesicles to the growing hyphal tip (López-Franco *et al.*, 1994, 1995). Satellite Spitzenkörper arise immediately beneath the plasma membrane a few micrometres behind the apical pole, and then migrate towards and merge with the main Spitzenkörper. These fusion events were correlated with a transient increase in the hyphal extension rate and are believed to be responsible for the pulsed growth behaviour of fungal hyphae resulting from the pulsed delivery of wall-building secretory vesicles (López-Franco *et al.*, 1994, 1995). However, as yet, the source(s) of the vesicles within satellites is/are unknown. These vesicles may be derived from the Golgi and/or endocytic vesicles internalized from the plasma membrane.

We suggest that secretory vesicles may be additionally derived from endosomes. This is consistent with our observations that Spitzenkörper stained shortly after the putative endosomes. In the previous section we proposed that a likely function of endocytosis in hyphae is the recycling of proteins involved in tip growth back to the apical plasma membrane. Satellite Spitzenkörper may also have a role in this process. However, the longer route for recycling these proteins from endosomes to the Spitzenkörper via the Golgi cannot be discounted (Fig. 12).

Mitochondria were always stained by FM4-64 with longer incubation times than necessary to stain Spitzenkörper or putative endosomes, and these times varied between species. A possible explanation of this phenomenon, still consistent with FM4-64 internalization by endocytosis, is that FM4-64 stains mitochondria through direct contact with the ER (Fig. 12). Continuity between the ER and the outer mitochondrial membrane has been shown at the ultrastructural level in various cells including hyphae (Bracker & Grove, 1971; Franke & Kartenbeck, 1971). We found that FM4-64 and FM1-43 stained mitochondria at different rates in any one species and the rates of staining varied between species. An explanation for this may be found in the recent finding that fluorescent lipid analogues undergo endocytic sorting to different organelles in animal cells solely on the basis of differences in the chemistry of their hydrophobic tails (Mukherjee *et al.*, 1999). Variations between species (e.g. *N. crassa* and *A. nidulans*) in the composition of their organelle membranes may also explain the differential rates at which their mitochondria become stained by FM4-64.

The hypothetical model of vesicle trafficking presented in Fig. 12 now needs to be rigorously tested and, in particular, endocytic intermediates and components of the secretory pathways need to be identified and characterized. Towards this aim, we are currently performing double labelling experiments in living hyphae by following the time course of FM4-64 staining in which the green fluorescent protein has been targeted to specific organelles (e.g. Fernández-Ábalos *et al.*, 1998). This will need to be correlated with the localization of FM4-64 at the ultrastructural level as has been done for FM1-43 in nerve cells (Henkel *et al.*, 1996). The model in Fig. 12 is also being subjected to genetical analysis and in this respect our unpublished results have shown that FM4-64 is proving to be a very powerful tool for analysing growing hyphae of mutants compromised in vesicle trafficking.

Acknowledgements

We thank Charles Bracker, Rosamaría López-Franco and Bill Betz for extremely helpful advice and discussions. We are also grateful to all those providing cultures. The research was supported by an EC TMR Fellowship (to S.F.-P.), a BBSRC postdoctoral fellowship (to R.M.P.), a BBSRC studentship (to P.C.H.), a Leverhulme postdoctoral fellowship (to J.D.), and a BBSRC CASE studentship with AgrEvo UK Ltd (to H.A.A.).

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