James Rennie Bequest.

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Cell biology of plant and fungal tip growth

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Introduction

The meeting was based at the University of Siena, and sponsored by the NATO Science Program, the European Comission and other agencies. The research presented art the meeting included some of the latest cutting edge studies on plant and fungal cells. The focus on tip growth was directly relevant to my research as a Ph.D. student. As a mycologist, working primarily on fungi, it was good to meet many people in the mycological field as well as those working on plant cells. Comparing two different cell types greatly helped define the different mechanisms of tip growth.

Summary of the research program

Although it was a huge input of information, the subject of the meeting was so interesting that I attended over 50 different presentations over the one-week period. Questions could be addressed to the speakers following the talks resulting in extremely useful discussions. Talks that were particularly relevant have been summarised below:

Salamon Bartnicki-Garcia
Mathematical basis for the role of the Spitzenkörper and turgor in hyphal tip growth

Previously, a 2-D mathematical model of hyphal morphogenesis has been proposed. However this does not take into account the 3-dimensional nature of tip-growing cells. By means of computer-enhanced video microscopy and image analysis, it was possible to map with great accuracy the position of markers on the surface of elongating hyphae. These techniques have enabled the development of a new 3-D formulation of the hyphal morphogenesis model.

Harvey Hoch
Perception of external cues in fungal thigmotropism

The recognition of host surfaces is critical to pathogenesis and tissue invasion by fungal diseases. Recognition of the plant host surface begins when the fungal spore makes physical contact. Germination, growth and appressorium formation on the plant tissue surface involves perception of the topography by the fungus. This unique sensing was tested using artificial substrates to rule out the involvement of chemical

signals. Mechanisms by which the cells sense and mediate surface signals were discussed.

Rosamaria Lopez-Franco and Charles Bracker
Laser tweezers: Experimental tool for manipulating behaviour of apical vesicles and Spitzenkörper.
Laser microbeams are exceptionally effective at noninvasive probes for manipulating the posuition and behaviour of organelles in living hyphal tips of fungi growing in slide chambers on the microscope stage. Several techniques were employed to cause alterations in hyphal growth e.g. changes in direction, initiation of branching, variation in hyphal diameter, and initiation of localised bulges in hyphae. The presentation included some visually amazing sequences of computer-enhanced video microscopy and provided important clues about the mechanisms of hyphal growth in fungi.

Nicholas P. Money
Functions and evolutionary origin of hyphal turgor pressure.
The research includes measurement of turgor pressure in fungal hyphae using extremely sensitive apparatus. Interestingly, the cells of oomycete fungi actually grow faster when the turgor pressure is reduced. The precise measurement of the invasive forces exerted by single hyphae and experiments on fungal pathogens of plants and animals reveal their biomechanical behavior plays a more important role thin disease processes than recognised previously.

Patrick C. Hickey
Imaging organelle dynamics in growing fungal hyphae
My contribution to the scientific program consisted of an oral presentation, following a talk by Nick Read on the research. The presentation included a 15 minute video showing the unique scientific techniques developed at the University of Edinburgh. This included several time-lapse movies and 3-D movies generated during my Ph.D. research. In addition to the oral presentation at the meeting, I co-authored a chapter in the NATO Science Series book published from the symposium. A copy of the publication is included with this report.

Benefits of attending the meeting

The meeting allowed me to meet many old and new colleages, where we could discuss our research. Networking has resulted in several new collaborations to be forged, resulting in some excellent research and publications. The organisation was efficient and the quality of the hospitality was excellent. Besides the academic component, evening receptions and meals provided a good opportunity to make new friends. One particularly memorable experience was the final banquet, situated in the beautiful surroundings of a monastery owned by the University. Many thanks are due to Anja Geitmann and the other organisers of the Meeting.

Patrick C. Hickey

The vesicle trafficking network and tip growth in fungal hyphae

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Abstract. Experimental methods are described for analysing vesicle trafficking in living fungal hyphae involving confocal microscopy of fluorescent dyes. Results from the use of one of these dyes, FM4-64, are summarised. Based on this work, a speculative model of the vesicle trafficking network is presented. The likely multi-functional roles for endocytosis in hyphal tip growth are discussed. Finally, a new model (the *Exocytosis-Endocytosis Equilibrium [or 'Triple E'] model*) of how vesicle trafficking may regulate hyphal tip morphogenesis is proposed.

1. Introduction

Vesicle trafficking underpins hyphal tip growth. Although the significance of polarised secretion has been long appreciated as central to tip growth, only recently has it emerged that endocytosis may also play important roles in this process.

The growing tips of fungal hyphae characteristically possess a concentration of vesicles (the 'apical vesicle cluster'). In higher fungi (Ascomycota, Deuteromycota and Basidiomycota), these vesicles are the predominant components of a multi-component complex, which is usually called the Spitzenkörper (= 'apical body')[1-3]. The dynamic behaviour of this remarkable structure, and also smaller versions of it (so-called satellite Spitzenkörper [4-6]), have been shown to be intimately associated with the precise and often subtle growth pattern of the hyphal tip in ways which are consistent with Spitzenkörper delivering wall-building vesicles to local domains of the apical plasma membrane.

Increasing evidence for endocytosis in growing hyphal tips is coming from studies using the amphilic styryl dye, FM4-64 [6-8], which is commonly used as an endocytosis marker in budding yeast [9] and animal cells [10].

Vesicle trafficking is very dynamic and extensive within growing hyphal tips. A common rate of hyphal extension for *Neurospora crassa* growing in open culture in a Petri dish is ~ 36 μm sec^{-1}. In order for a 10 μm wide hypha of *Neurospora* to supply sufficient plasma membrane to the hyphal tip to maintain this growth rate it has been estimated that ~ 600 secretory vesicles per second would have to fuse with the apical plasma membrane [11]. However, even this magnitude of membrane transport must be a significant underestimate of the amount of vesicle trafficking being undergone within a growing hyphal tip because it does not take account of vesicles moving between organelles or the activity of endocytic vesicles.

The aims of this paper are to: (a) describe dye labelling approaches for the experimental analysis of vesicle trafficking in growing hyphal tips imaged with confocal microscopy; (b) summarise results that we have obtained in analysing vesicle trafficking using the dye FM4-64; (c) based on this data, provide a speculative model of the vesicle trafficking network in growing hyphal tips; (d) consider the likely multi-functional roles of endocytosis in hyphal tip growth; and finally (e) use our current knowledge of the vesicle trafficking network in growing hyphal tips to propose the *Endocytosis-Exocytosis Equilibrium* (or *'Triple E'*) *model* of hyphal morphogenesis.

2. Approaches for imaging vesicle trafficking in living fungal hyphae

Considerable emphasis is currently being placed on analysing the cell biology of individual living hyphae. Much of this has been due to important advances in imaging and dye technologies [12]. In this respect, we have developed techniques for monitoring vesicle trafficking by using confocal laser scanning microscopy of living hyphae loaded with the fluorescent dye FM4-64 [6].

Confocal microscopy has been used to image living hyphae at high spatial resolution without perturbing their growth. Dye-loaded hyphae of different species have been found to vary in their sensitivity to being scanned with the confocal microscope laser. However, repeated laser scanning at 3 sec intervals for periods greater than 10 min with a x60 (1.4 N.A.) objective are often possible without inhibiting the rate of hyphal extension, and using this approach animated time courses of growing hyphae are routinely obtained in our laboratory.

We have used three different styryl dyes (FM4-64, FM1-43 and TMA-DPH) commonly employed as indicators of endocytosis in other systems [e.g. 9, 10, 13], and assessed their suitability for monitoring vesicle trafficking in fungal hyphae. Comparison of the properties of these dyes (Table 1) showed that FM4-64 is best suited for this purpose [6].

Table 1. Comparison of the properties of FM4-64, FM1-43 and TMA-DPH for imaging vesicle trafficking in fungal hyphae using confocal microscopy. For further details see Fischer-Parton et al. (2000).

Property	FM4-64	FM1-43	TMA-DPH
Excitation wavelengths	488 or 514 nm	488 or 514 nm	351 and 364 nm
Emission wavelengths	> 550 nm	> 550 nm	> 400 nm
Staining of Spitzenkörper	Very good	Poor	Poor
Staining of mitochondria	Slowly (typically > 1 h)	Quickly (typically < 30 min)	None
Photobleaching	Reasonably resistant	Reasonably resistant except Spitzenkörper which photobleaches very rapidly	Poor
Phototoxicity	Not a major problem	Not a major problem	Very bad

Our published and unpublished findings support the endocytic uptake of dye shown in Fig. 1 as the predominant mechanism by which FM4-64 is internalised within hyphae [6].
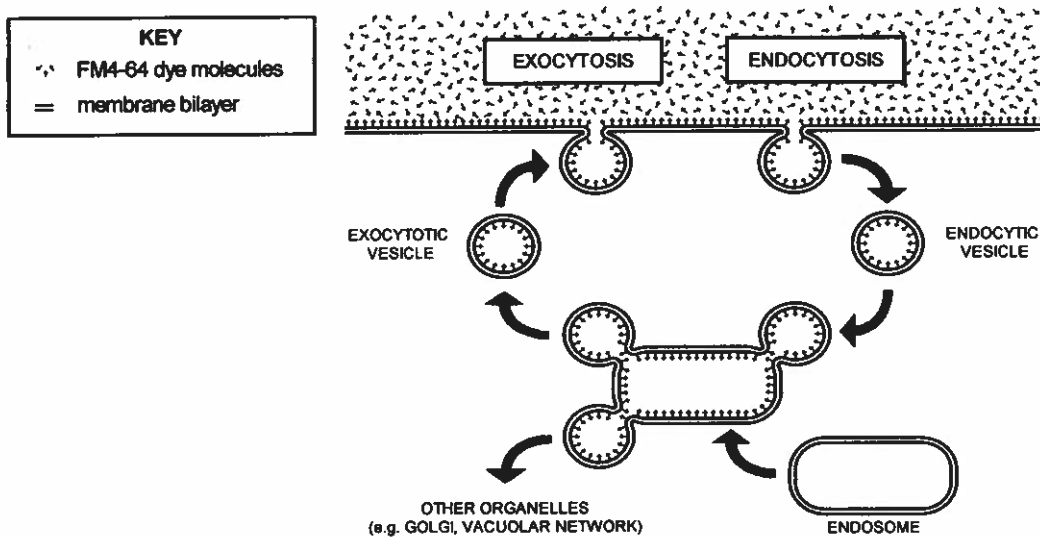
EXOCYTOSIS   ENDOCYTOSIS

EXOCYTOTIC VESICLE

ENDOCYTIC VESICLE

OTHER ORGANELLES
(e.g. GOLGI, VACUOLAR NETWORK)

ENDOSOME

Fig. 1. Main mechanism by which FM4-64 is probably taken up into fungal hyphae.

3. Experimental analysis of vesicle trafficking in fungal hyphae using FM4-64

Below is a summary of some of the main features of FM4-64 staining of hyphae:

(a) *The sequence of organelle staining is time-dependent.* The time course and pattern of hyphal staining with FM4-64 is broadly similar in the > 15 species representing the Zygomycota, Ascomycota, Basidiomycota and Deuteromycota [6, 14] we have examined. Initial staining of the plasma membrane is followed by staining in the cytoplasm (Fig. 2A), and then successive staining of putative endosomes (Fig. 2B), other membranous organelles, the apical vesicle cluster (Fig. 2C), vacuolar membranes and mitochondria. The time course of staining of hyphae of *N. crassa* are summarised in Table 2. This time-dependent staining of different organelles is consistent with dye internalisation by endocytosis and subsequent staining of different organelles by the distribution of dye via the vesicle trafficking network.

Fig. 2. Confocal images of hyphal tips of *N. crassa* showing the time course of FM4-64 staining. 25 μM FM4-64 was continuously present in the external medium. (A) Pronounced staining of the plasma membrane (30 sec after adding dye). (B) Staining of putative endosomes (1 min after adding dye). (C) Staining of the main Spitzenkörper (30 min after adding dye). Bar = 10 μm.

Time after adding FM4-64	Cell component stained
< 1 sec	Plasma membrane
< 10 sec	Putative endocytic vesicles
30 sec	Putative endosomes
1.5-3 min	Apical vesicle cluster
10 min	Subapical spherical vacuoles
15 min	Putative tubular vacuoles
~ 40 min	Mitochondria

Table 2. Summary of the time course of staining of a hypha of *N. crassa* continuously loaded with FM4-64.

(b) *Dye internalisation involves an active process which is energy dependent.* Loading hyphae with FM4-64 in the presence of the metabolic inhibitor sodium azide or at 4 °C allows staining of the plasma membrane whilst inhibiting uptake of the dye into the cytoplasm and staining of organelles [6, 7, 15]. This indicates that FM4-64 uptake into hyphae is an active, energy-dependent process (consistent with it being mediated by endocytosis) and does not involve unfacilitated diffusion.

(c) *Vesicles within Spitzenkörper become stained.* FM4-64 strongly stains the apical vesicle cluster within the main Spitzenkörper of growing hyphal tips (Figs. 2C, 3A and 3B; Table 2). Because it is assumed that the apical vesicle cluster is composed primarily of secretory vesicles, this observation indicates that the endocytic and secretory pathways within the vesicle trafficking network are integrated [6]. There is good agreement between the morphology and behaviour of stained Spitzenkörper [6] and unstained Spitzenkörper previously analysed by computer-enhanced, phase-contrast microscopy [3, 16]. Stained Spitzenkörper commonly possess a region exhibiting reduced staining [6]. This correlates with a differentiated 'core' region observed in unstained hyphal tips [3] and at the ultrastructural level [e.g. 1, 2, 17]. This region commonly possesses few vesicles although in some genera it contains a concentration of microvesicles [1]. Small satellite Spitzenkörper also arise in a location immediately below the plasma membrane several micrometers back from the main Spitzenkörper. Normally they migrate within 15 sec towards the main Spitzenkörper and fuse with it [5, 6]. If these satellite Spitzenkörper do not immediately migrate towards the main one then a bulge in the hypha typically appears adjacent to them indicating that they are delivering wall-building vesicles to the region of the plasma membrane which they are in close proximity with [5].

(d) *Fluorescence recovery after photobleaching (FRAP) indicate vectorial transport of secretory vesicles to the main Spitzenkörper.* In these experiments, Spitzenkörper of growing hyphae of *Rhizoctonia solani* which had been pulsed-stained with FM4-64 were photobleached with the laser of the confocal microscope without inhibiting tip growth. Fluorescence within the Spitzenkörper subsequently recovered within 10 min whilst the hyphae continued to grow. FRAP within the apical vesicle cluster is interpreted as involving the addition of new unbleached, stained wall-building secretory vesicles to the Spitzenkörper following their formation in a subapical hyphal region [18].

(e) *Internalised dye is recycled back to the plasma membrane.* FM4-64 only fluoresces within a hydrophobic environment (e.g. when inserted within a membrane [19]). Thus when

hyphae are immersed in dye, there is no background staining within the surrounding medium (Figs. 2 and 3). When hyphae are 'continuously loaded' with dye in this way the plasma membrane typically exhibits consistently pronounced staining However, if a hypha is 'pulse-loaded' with dye (i.e. the dye is washed out from the growth medium after a period of staining), the plasma membrane remains stained even for periods of 2 h or more (cf. Figs 3A and 3B). This indicates that dye internalised within hyphae by endocytosis is recycled back to be plasma membrane via exocytotic vesicles (Fig. 1). Furthermore, these results indicate that significant amounts of FM4-64 are not released into the lumen of exocytotic vesicles and lost during secretion as occurs dramatically in nerve cells loaded with the dye FM1-43 [20, 21].
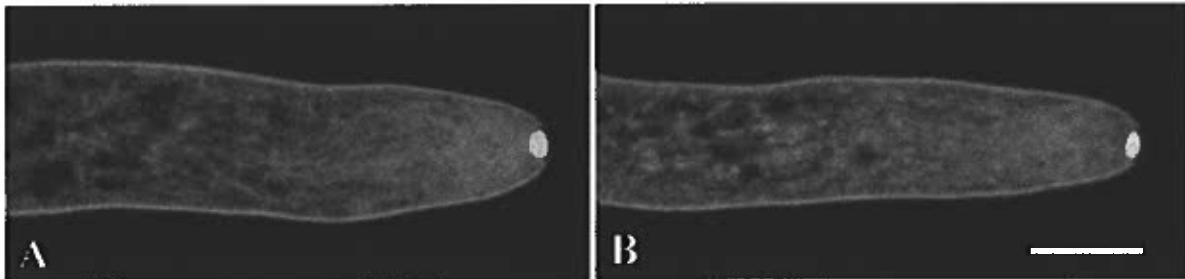

Fig. 3. Confocal image of a hyphal tip of *N. crassa* stained with 25 µM FM4-64 and imaged 2 h after initially applying dye. (A) Pulse-loaded hyphal tip (hyphae were incubated with FM4-64 for 30 sec before washing the dye out of the surrounding medium). Note that the plasma membrane has remained stained. (B) Continuously stained hyphal tip (the dye was left in the surrounding medium). Bar = 10 µm.

(f) FM4-64 may become distributed amongst membranes by a lipid sorting mechanism. Besides the time-dependent staining of organelle membranes by FM4-64 (which we interpret as primarily involving the transport of the dye between organelles via the vesicle trafficking network), we suggest that a process of 'lipid sorting' may also play a role in the differential staining of organelle membranes. It has recently been found that fluorescent lipid analogues undergo sorting to different organelles in animal cells solely on the basis of the chemistry of their hydrophobic tails. This is believed to be the mechanism by which lipids normally become distributed between different membranes of the endomembrane system [22]. With FM4-64 we find that some membranes (e.g. the plasma membrane) stain strongly, some membranes stain at a reasonable level (most organelles) whilst others (e.g. the nuclear membrane) do not appear to stain at all even though we would predict that the latter should stain up through connection with other components of the endomembrane system. With other styrl dyes which are internalised by endocytosis and which possess different hydrophobic tails (e.g. FM1-43) the pattern of membrane staining is different to that of FM4-64 (Table 1) [6].

4. Model of the vesicle trafficking network in hyphal tips

In Fig. 6 we provide a diagrammatic representation of our current working model of the vesicle trafficking network within growing hyphal tips. Below is a summary of the evidence for each of the component pathways which have been numbered in the diagram.
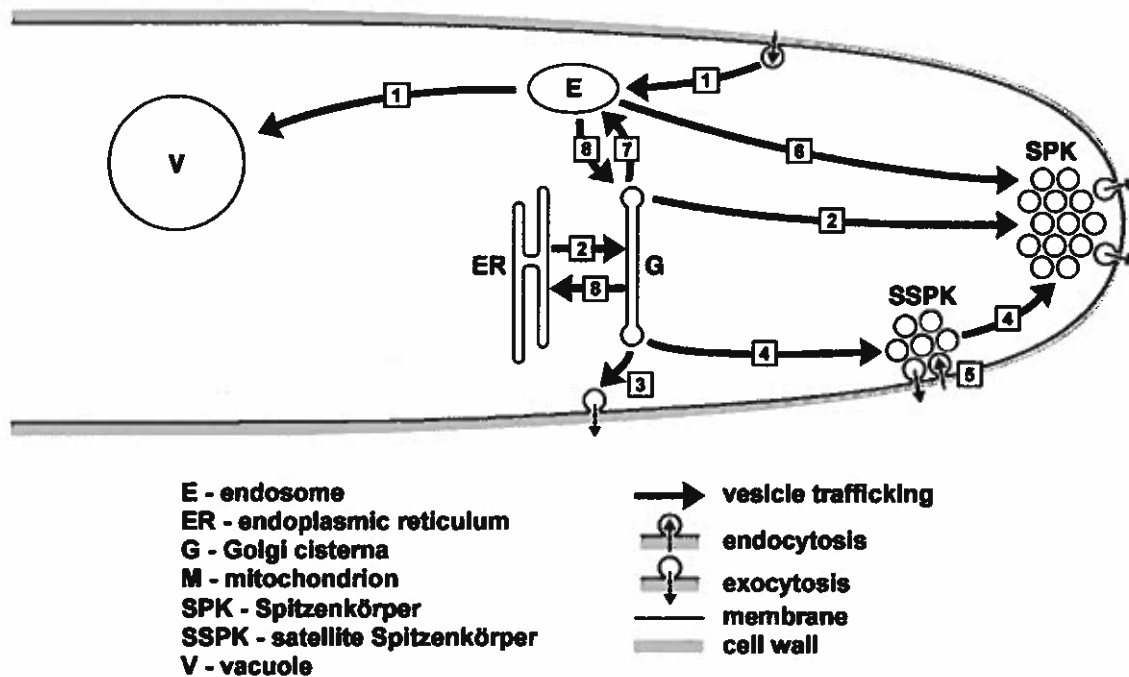
Fig. 4. Working model of the vesicle trafficking network within the growing hyphal tip (adapted from Fischer-Parton et al. 2000). Each of the numbered vesicle trafficking pathways are described in the accompanying text.

[1] *Plasma membrane → endocytic vesicles → endosomes → vacuole.* Dye fluorescence is initially detected within the cytoplasm within 10 sec of adding FM4-64 to hyphae and this is interpreted as representing stained endocytic vesicles which have been budded off from the plasma membrane. Although biochemical evidence for the presence of clathrin, the major coat protein of endocytic vesicles in animal and plant cells, has been found in *N. crassa* [23], convincing ultrastructural evidence for the existence of clathrin-coated vesicles or pits is lacking in filamentous fungi. The best candidates for endocytic vesicles in hyphae are filasomes [2, 6]. These are vesicles found throughout hyphae and which each possess a fibrillar coating rich in actin (Bourett and Howard, 1981; Roberson, 1992). The next small organelles to stain within the hyphal cytoplasm after endocytic vesicles (Fig. 2B) are believed to be endosomes. In animal and yeast cells endosomes act as the cell compartments responsible for molecular sorting, and are of two types 'early' and 'late' endosomes. Endosomes have not yet been identified at the ultrastructural level in fungal hyphae. However, one candidate for endosomes in hyphae are multivesicular bodies [e.g. see 2, 25] for which a function has not been established. Ten to fifteen minutes after adding FM4-64 to hyphae the spherical and tubular vacuolar elements become stained (Table 2).

[2] *Endoplasmic reticulum (ER) → Golgi → main Spitzenkörper → apical plasma membrane.* The traditional view of the secretory process involved in tip growth is that proteins synthesized on the ER are transported via vesicles to the Golgi within which they are processed and transported in secretory vesicles to the apical vesicle cluster within the main Spitzenkörper [2]. These secretory vesicles are then directed to the apical plasma membrane with which they fuse. An interesting feature of Golgi cisternae in filamentous fungi is that they do not typically form stacks as is characteristic of plant and animal cells [2].

[3] *ER → Golgi → subapical plasma membrane.* Secretion also occurs from subapical regions of fungal hyphae. This is particularly important during the delivery of wall-building vesicles to new sites of branch formation (Fig. 6) [26]. Other extracellular enzymes are possibly also secreted from subapical locations.

[4] *ER → Golgi → satellite Spitzenkörper → main Spitzenkörper → apical plasma membrane.* Satellite Spitzenkörper also appear to contain wall-building vesicles as is indicated by the observation that a bulge in a hypha often appears adjacent to these multicomponent structures [5]. However, it is not clear exactly where these vesicles are generated. One possibility is that at least some are derived from Golgi cisternae. The repeated delivery of wall-building vesicles by the fusion of satellite Spitzenkörper with the main Spitzenkörper is believed to result in the pulsed growth pattern of hyphae [4, 5].

[5] *Plasma membrane → endocytic vesicles → satellite Spitzenkörper → main Spitzenkörper → apical plasma membrane.* It is possible that satellite Spitzenkörper may also contain endocytic vesicles derived from the plasma membrane below which these Spitzenkörper characteristically arise. These endocytic vesicles could play a role in recycling proteins and lipids back to the hyphal tip (see Section 5).

[6] *Endocytic vesicles → endosomes → main Spitzenkörper → apical plasma membrane.* A second pathway which may be important for recycling membrane proteins and lipids back to the growing hyphal tip is via endosomes. This would have the added advantage that the endosomes could provide a 'molecular sorting' function to select those proteins and lipids to be returned to the hyphal tip for reuse (see Section 5). A third possible pathway for recycling membrane proteins and lipids may be from endosomes to the main Spitzenkörper via the Golgi.

[7] *ER → Golgi → endosomes → vacuoles.* Proteins (e.g. lytic enzymes) within vacuoles are ultimately derived from ER and then transported via the Golgi and endosomes in which they are respectively processed and sorted [27].

[8] *Retrograde vesicle trafficking.* Retrograde pathways of vesicle trafficking must occur to maintain the correct balance of membrane between different organelles and also to allow the recycling of specific molecules [27].

5. Importance of endocytosis for hyphal tip growth

We believe that endocytosis may play critical roles in hyphal tip growth. Important functions may include:

(a) *Removal of excess plasma membrane.* Calculations have indicated that in the tips of hyphae and pollen tubes, insertion of new membrane by secretory vesicle fusion results in an excess in apical plasma membane relative to the amount of cell wall components necessary to maintain tip extension [28-31]. Endocytosis could provide a mechanism to retrieve this membrane. Studies in which the time course of FM4-64 uptake into hyphae has been followed indicate that initial internalisation of the dye is concentrated in a region 2-20 μm back from the apical pole (Fig. 2A) [6, 7, 14]. Interestingly, it has been found that filasomes, which have been suggested as candidates for endocytic vesicles [6], are concentrated in this zone [24]. Furthermore, clathrin-coated endocytic vesicles have also been shown to be concentrated in a similar region in pollen tubes [31]. In chemically fixed hyphae, membranous aggregates associated with the plasma membrane and termed *plasmalemmasomes* or *lomasomes* [e.g. see 32] often develop. These structures which, in most cases are artefacts generated during the slow process of chemical fixation, may

represent the buildup of excess membrane which failed to be retrieved normally by endocytosis in the dying hyphae.

(b) *Generation of vacuolar system.* If the assertion is correct that an excess of membrane inserted by secretory vesicles into the apical plasma membrane has to be internalized, it may be that much of this membrane plays an important role in generating vacuolar membranes of the vacuolar system, especially in the apical hyphal compartment. When the vacuolar system in the apical compartments of *N. crassa* is stained using a vacuole-selective dye such as Oregon Green 488 carboxylic acid [33], we find that tubular and small, near spherical vacuolar compartments start to appear 10-30 μm back from the apical pole of the hyphal tip. Behind this region the tubular elements get progressively longer. The much larger spherical vacuolar compartments, characteristic of subapical hyphal compartments [6, 27], do not appear until the first septum back from the tip.
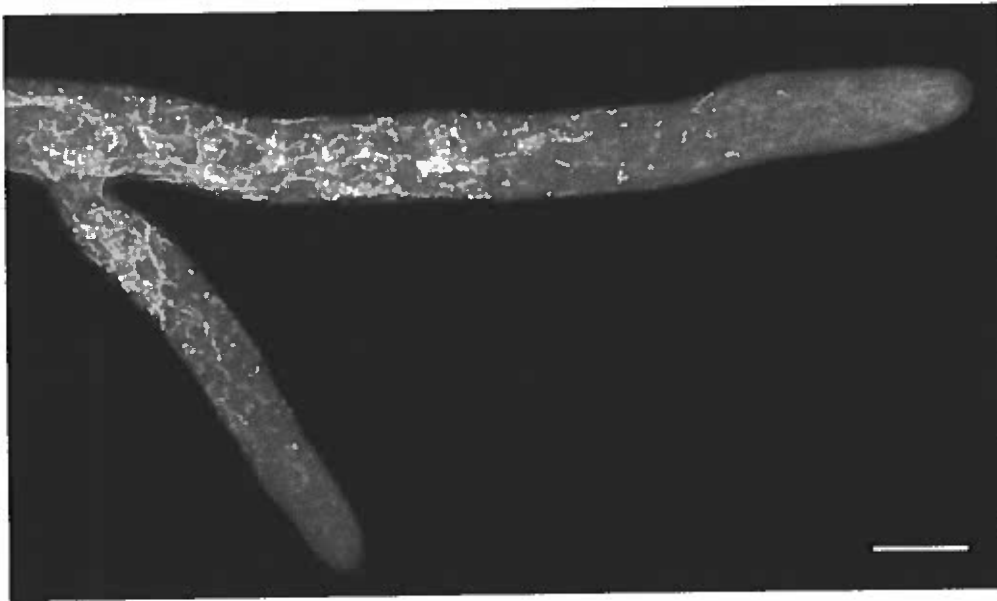

Fig. 6. Vacuolar network behind the growing hyphal tips of a main hypha and branch of *N. crassa* stained for 20 minutes with 50 μM of the vacuole-selective dye Oregon Green 488 carboxylic acid diacetate. Note the tubular and small, near spherical vacuolar compartments. Bar = 10 μm.

(c) *Recycling of membrane proteins.* Endocytosis could provide a mechanism for recycling enzymes and lipids involved in tip growth (e.g. cell wall synthases, ion transporters and possibly membrane lipids involved in signalling). This would maintain a concentration of these molecules in the tip region where they are active and where these molecules once recycled can be reused. Besides providing one of probably several mechanisms for polarising the distribution of important proteins and lipids in the tip region, recycling these molecules makes economic sense for a hypha rather than having to synthesise all of these molecules *de novo* which would require a much greater energy expenditure. In the budding yeast there are numerous examples of proteins with a polarised distribution concentrated at the site growth (i.e. buds). Furthermore, two of the three chitin synthases in budding yeast are recycled via endocytosis [34-37]. Endocytic recycling of membrane proteins has also been shown to occur in animal cells (e.g. in neurones [20]).

(d) *Transport of membrane proteins and lipids to the vacuole for degradation.* Besides endocytosis possibly supplying vacuolar membrane, many membrane proteins and lipids may also be transported to vacuoles for degradation. Fungal vacuoles are well established as the main lytic compartments in hyphae [27].

(e) *Uptake of molecules in fluid-phase of endocytic vesicles.* Small molecules may be taken up by endocytosis within the lumen of endocytic vesicles. This has been suggested as a possible mechanism for the uptake of certain small nutrients in budding yeast [38].

(f) *Uptake of signalling molecules involving receptor-mediated internalisation of ligands.* Pheromones and other signalling molecules may be internalised by receptor-mediated endocytosis in the hyphal tip region. In budding yeast, for example, this is the way in which α-factor is internalised [39] and results in yeast cells exhibiting polarized growth in the direction of the highest concentration of the pheromone [40]. There are a number of examples in different filamentous fungi where hyphae (e.g. trichogynes in *N. crassa*) exhibit positive chemotropisms towards pheromones. Endocytosis may also be important for the uptake of host signal molecules by hyphae in host-pathogen interactions.

6. The Endocytosis-Exocytosis Equilibrium model of hyphal morphogenesis

We have formulated what we term the *Endocytosis-Exocytosis Equilibrium* (or *'Triple E'*) *model of hyphal morphogenesis*. The background to this model is based on the following key attributes of fungal hyphae:

(a) They exhibit tip growth (i.e. hyphal extension is limited to a region occupying a few micrometers at the hyphal tip) and this involves polarized secretion [41].

(b) The major cell wall synthesizing enzymes (chitin and glucan synthases) involved in hyphal tip growth are integral membrane proteins [42].

(c) The cell wall in the tip region of a growing hypha is plastic but becomes progressively more rigid behind the tip; changes in hyphal morphology are only possible where the cell wall is plastic [26, 43].

In this paper and elsewhere [6, 7], it has been argued that extensive endocytosis occurs in the hyphal tip region.

Our Triple E model proposes that the amount of cell wall synthesizing plasma membrane encased by plastic cell wall in the hyphal tip region is regulated by the *dynamic equilibrium* between exocytosis and endocytosis. The predicted effects of the steady state of this equilibrium on hyphal morphogenesis are shown diagrammatically in Fig. 7. We further propose that the dynamic equilibrium in exocytosis and endocytosis will vary in different parts of the apical dome of a hypha because of gradients in each of these processes, and this may also influence the precise pattern of hyphal tip morphogenesis. Finally, we propose that the capacity for the endocytosis-exocytosis equilibrium to influence hyphal morphogenesis will be further modulated by the local plasticity of the encasing cell wall which again we would expect to vary in different regions of the hyphal dome.

In relation to possible gradients in exocytosis and endocytosis, it is well established that exocytosis is highly polarized in the growing hyphal tip [44] and various mechanisms have been proposed to explain this phenomenon (e.g. a mobile vesicle supply centre, calcium promoting localized vesicle fusion, and targeted transport along cytoskeletal elements to marked sites on the apical plasma membrane). Indeed, a range of physiological devices may be employed by hyphae to generate gradients in exocytosis [45]. Our preliminary evidence suggests that gradients in endocytosis also exist in the hyphal tip region with a concentration of endocytic activity just behind the main Spitzenkörper [14].
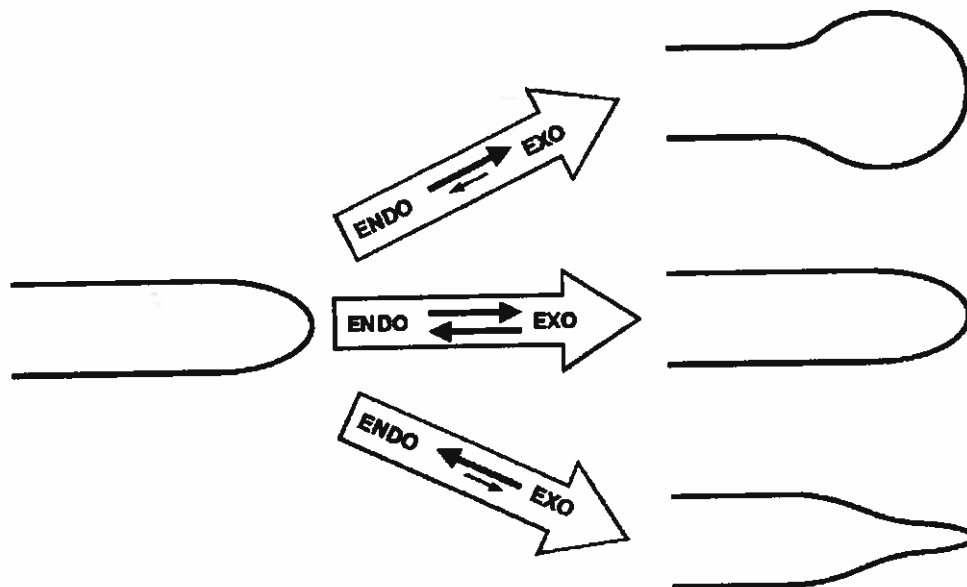
Fig. 7. A diagrammatic summary of the Endocytosis-Exocytosis Equilibrium ('Triple E') model of hyphal morphogenesis showing the effects of three different steady state equilibria between exocytosis and endocytosis in the hyphal tip region. This simplified diagram does not take account of likely gradients in the exocytosis-endocytosis equilibrium, or how gradients in cell wall plasticity, within the hyphal tip region might influence hyphal tip morphogenesis.

If the Triple E model proves to have credence in explaining important aspects of hyphal tip morphogenesis then it is clear that in order for a hyphal tip to maintain the normal hyphoid shape of an actively growing hypha [44] then exocytosis and endocytosis will have to be exquisitely regulated in a coordinated manner. A major challenge in the future will be to experimentally test the Triple E model by using pharmacological and genetic tools which interfere with these regulatory mechanisms, and to analyse the effects of these treatments in growing hyphae using methods similar to that described in this paper. It will also be interesting to determine whether a similar mechanism plays a role in the morphogenesis of tip-growing cells (e.g. pollen tubes and root hairs) of the plant kingdom where the mechanism of tip growth may have evolved independently of that in the fungal kingdom.

References
[1] S.N. Grove and C.E. Bracker, Protoplasmic Organization of Hyphal Tips Among Fungi: Vesicles and Spitzenkörper, *Journal of Bacteriology* **104** (1970), 989-1009.

[2] R.J. Howard, Ultrastructural Analysis of Hyphal Tip Cell Growth in Fungi: Spitzenkörper, Cytoskeleton and Endomembranes after Freeze-Substitution, Journal of Cell Science, 48 (1981), 89-103.

[3] R. López-Franco and C.E. Bracker, Diversity and Dynamics of the Spitzenkörper in Growing Hyphal Tips of Higher Fungi, Protoplasma 195 (1996), 90-111.

[4] R. López-Franco, S. Bartnicki-Garcia and C.E. Bracker, Pulsed Growth of Fungal Hyphal Tips, Proceedings of the National Academy of Sciences, USA 91 (1994), 12228-12232.

[5] R. López-Franco, R.J. Howard and C.E. Bracker, Satellite Spitzenkörper in Growing Hyphal Tips, Protoplasma 188 (1995), 85-103.

[6] S. Fischer-Parton, R.M. Parton, P.C. Hickey, J. Dijksterhuis, H.A. Atkinson and N.D. Read, Confocal Microscopy of FM4-64 as a Tool for Analysing Endocytosis and Vesicle Trafficking in Living Fungal Hyphae, Journal of Microscopy 198 (2000), 246-259.

[7] J. Hoffman and K. Mendgen, Endocytosis and Membrane Turnover in the Germ Tube of Uromyces fabae, Fungal Genetics and Biology 24 (1988), 77-85.

[8] N.D. Read, S. Fischer and R.M. Parton, Imaging Spitzenkörper, pH and Calcium Dynamics in Growing Fungal Hyphae, Pesticide Science 54 (1998), 179-181.

[9] T.A. Vida and S.D. Emr, A New Vital Stain for Visualizing Vacuolar Membrane Dynamics and Endocytosis in yeast, Journal of Cell Biology 128 (1995), 779-792.

[10] W.J. Betz, F. Mao and C.B. Smith, Imaging Exocytosis and Endocytosis. Current Opinion in Neurobiology 6 (1996), 365-371.

[11] A.J. Collinge and A.P.J. Trinci, Hyphal Tips of Wild-Type and Spreading Colonial Mutants of Neurospora crassa. Archives of Microbiology 99 (1974) 353-368.

[12] N.D. Read, Analysing the Cell Biology of Living Fungi, Mycologia, in preparation.

[13] D. Illinger and J.G. Kuhry, The Kinetic Aspects of Intracellular Fluorescence Labeling with TMA-DPH Supports the Maturation Model for Endocytosis in L929 Cells, Journal of Cell Biology 125 (1994), 783-794.

[14] P.C. Hickey and N.D. Read, unpublished.

[15] S. Fischer-Parton, Role of pH, Calcium and Vesicle Trafficking in Regulating Hyphal Tip Growth of Neurospora crassa. Ph.D. Thesis, University of Edinburgh (1999).

[16] N.D. Read, S. Fischer-Parton, R. López-Franco and C.E. Bracker, unpublished.

[17] T.M. Bourett and R.J. Howard, Ultrastructural Immunolocalization of Actin in a Fungus, Protoplasma 163 (1991), 199-202.

[18] J. Dijsterhuis and N.D. Read, unpublished.

[19] J.K. Angleson and W.J. Betz, Monitoring Secretion in Real Time: Capacitance, Amperometry and Fluorescence Compared. Trends in Neuroscience 20 (1997) 281-287.

[20] W.J. Betz and G.S. Bewick, Optical Analysis of Synaptic Vesicle Recycling at the Frog Neuromuscular Junction. Science 255 (1992) 200-203.

[21] J. Klingauf, E.T. Kavalali and R.W. Tsien, Kinetics and Regulation of Fast Endocytosis at Hippocampal Synapses. Nature 394 (1998) 581-585.

[22] S. Mukherjee, T.T., Soe and P.R. Maxfield, Endocytic Sorting of Lipid Analogues Differing Solely in the Chemistry of their Hydrophobic Tails, Journal of Cell Biology 144 (1999) 1271-1284.

[23] T.C. Caesar-Ton That, Isolation and Characterization of Coated Vesicles from Filamentous Fungi. European Journal of Cell Biology 43 (1987) 189-194.

[24] R.W. Roberson, The Actin Cytoskeleton in Hyphal Cells of Sclerotium rolfsii. Mycologia 84 (1992) 41-51.

[25] R.W. Roberson and M.S. Fuller, Ultastructural Aspects of the Hyphal Tip of Sclerotium rolfsii Preserved by Freeze-Substitution. Protoplasma 146 (1988) 143-149.

[26] G.W. Gooday, The Dynamics of Hyphal Growth. Mycological Research 99 (1995) 385-394.

[27] A.E. Ashford, Dynamic Pleiomorphic Vacuole Systems: are they Endosomes and Transport Compartments in Fungal Hyphae: Advances in Botanical Research 28 (1998) 119-159.

[28] J.M. Picton and M.W. Steer, Membrane Recycling and the Control of Secretory Activity in Pollen Tubes. Journal of Cell Science 63 (1983) 303-310.

[29] M.W. Steer, Plasma Membrane Turnover in Plant Cells. Journal of Experimental Botany 39 (1988) 987-996.

[30] C.E. Bracker, personal communication.

[31] J. Derksen, T. Rutten, I.K. Lichtscheidl, A.H.N. Dewin, E.S. Pierson and G. Rongen, Quantitative Analyses of the Distribution of Organelles in Tobacco Pollen Tubes – Implications for Exocytosis and Endocytosis. Protoplasma 188 (1995) 267-276.

[32] A. Beckett, I.B. Heath and D.J. McLaughlin, An Atlas of Fungal Ultrastructure. Longman, London (1974).

[33] L. Cole, G.J. Hyde and A.E. Ashford. Uptake and Compartmentalisation of Fluorescent Probes by *Pisolithus tinctorius* Hyphae: Evidence for an Anion Transport Mechanism at the Tonoplast but not for Fluid-Phase Endocytosis. *Protoplasma* **199** (1997) 18-29.

[34] J.S. Chuang and R.W. Schekman, Differential Trafficking and Timed Localization of Two Chitin Synthase Proteins, Chs2p and Chs3p. *Journal of Cell Biology* **135** (1996) 597-610.

[35] M. Ziman, J.S. Chuang and R.W. Schekman, Chs1p and Chs3p, Two Proteins Involved in Chitin Synthesis, Populate a Compartment of the *Saccharomyces cerevisiae* endocytic pathway, *Molecular Biology of the Cell* **7** (1996) 1909-1919.

[36] M. Ziman, J.S. Chuang, M. Tsung, S. Hamamoto and R.W. Schekman, Chs6p-dependent anterograde transport from the chitosome to the plasma membrane in *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* **9** (1998) 1565-1576.

[37] J.C.M. Holthuis, B.J. Nichols and H.R.B. Pelham, The Syntaxin Tlg1p Mediates Trafficking of Citin Synthase III to Polarized Growth Sites in Yeast. *Molecular Biology of the Cell* **9** (1998) 3383-3397.

[38] V. Dulic, M. Egerton, I. Elgundi, S. Raths, B. Singer and H. Riezman, Yeast Endocytosis Assays. *Methods in Enzymology* **194** (1991) 697-710.

[39] B. Wendland, S.D. and H. Riezman, Protein Traffic in the Yeast Endocytic and Vacuolar Protein Sorting Pathways. *Current Opinion in Cell Biolgy* **10** (1998) 513-522.

[40] W. Duntze, R. Betz and M. Nientiedt, Pheromones in Yeast. In: J.G.H. Wessels and F. Meinhardt (eds.), The Mycota Vol. 1. Springer-Verlag, Berlin, 1994, pp. 381-399.

[41] I.B. Heath (ed.), Tip Growth in Plant and Fungal Cells. Academic Press, San Diego, 1990'

[42] G.W. Gooday and N.A.R. Gow, Enzymology of Tip Growth in Fungi. In: I.B. Heath (ed.), Tip Growth in Plant and Fungal Cells. Academic Press, San Diego, 1990, pp. 211-232.

[43] J.G.H. Wessels, Cell Wall Architecture and Fungal Tip Growth. I.B. Heath (ed.), Tip Growth in Plant and Fungal Cells. Academic Press, San Diego, 1990, pp. 1-29.

[44] F.M. Harold, How Hyphae Grow: Morphogenesis Explained? *Protoplasma* **197** (1997) 137-147.

[45] S. Bartnicki-Garcia, Role of Vesicles in Apical Growth and a New Mathematical Model of Morphogenesis. I.B. Heath (ed.), Tip Growth in Plant and Fungal Cells. Academic Press, San Diego, 1990, pp. 211-232.