

# Regulators of GTP-Binding Proteins Cause Morphological Changes in the Vacuole System of the Filamentous Fungus, *Pisolithus tinctorius*

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Tubule formation is a widespread feature of the endomembrane system of eukaryotic cells, serving as an alternative to the better-known transport process of vesicular shuttling. In filamentous fungi, tubule formation by vacuoles is particularly pronounced, but little is known of its regulation. Using the hyphae of the basidiomycete *Pisolithus tinctorius* as our test system, we have investigated the effects of four drugs whose modulation, in animal cells, of the tubule/vesicle equilibrium is believed to be due to the altered activity of a GTP-binding protein (GTP $\gamma$ S, GDP $\beta$ S, aluminium fluoride, and Brefeldin A). In *Pisolithus tinctorius*, GTP $\gamma$ S, a non-hydrolysable form of GTP, strongly promoted vacuolar tubule formation in the tip cell and next four cells. The effects of GTP $\gamma$ S could be antagonised by pre-treatment of hyphae with GDP $\beta$ S, a non-phosphorylatable form of GDP. These results support the idea that a GTP-binding protein plays a regulatory role in vacuolar tubule formation. This could be a dynamin-like GTP-ase, since GTP $\gamma$ S-stimulated tubule formation has only been reported previously in cases where a dynamin is involved. Treatment with aluminium fluoride stimulated vacuolar tubule formation at a distance from the tip cell, but NaF controls indicated that this was not a GTP-binding-protein specific effect. Brefeldin A antagonised GTP $\gamma$ S, and inhibited tubule formation in the tip cell. Given that Brefeldin A also affects the ER and Golgi bodies of *Pisolithus tinctorius*, as shown previously, it is not clear yet whether the effects of Brefeldin A on the vacuole system are direct or indirect. *Cell Motil. Cytoskeleton* 51:133–146, 2002. © 2002 Wiley-Liss, Inc.

**Key words:** GTP $\gamma$ S; GDP $\beta$ S; Brefeldin A; tubulation; aluminium fluoride; cytoskeleton

## INTRODUCTION

Vacuoles of fungal hyphae exhibit an extraordinary range of dynamic behaviours. Membranous tubules may rapidly extend out from a parent spherical vacuole for distances of 60  $\mu$ m or more, and transiently connect it with a distant vacuolar compartment. Luminal content appears to pass through these interconnecting tubules, and the vacuole network may thus provide for more efficient bulk transfer of membrane and material than vesicular shuttling, a longer-recognised mode of endomembrane transport [Ashford, 1997; Ashford et al., 2001; Shepherd et al., 1993a,b]. The extent of vacuolar tubule formation varies within a colony and also with

external conditions [Hyde and Ashford, 1997]. Such dependency, and the spectrum of activities described for fungal vacuoles in general, have also been reported for endomembrane compartments of many other organisms,

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including plants and animals [see references in Ashford, 1997]. It now seems likely that all endomembrane compartments are capable of switching, at least under experimental conditions, between predominantly vesicular or tubular modes [Klausner et al., 1992; Mironov et al., 1997]. Probably due to their linear life-form, fungal vacuoles exhibit constitutive tubule formation as well developed as that of any endosomal-lysosomal system, and have proven fruitful systems for studies of this phenomenon.

We have recently shown that tubule extension from vacuoles in hyphae of *Pisolithus tinctorius* requires the presence of an intact microtubule network [Hyde et al., 1999]. Tubule formation in nearly all endomembrane compartments of other organisms studied so far also depends upon microtubules [e.g., Cooper et al., 1990; D'Arrigo et al., 1997; Donaldson et al., 1991; Swanson et al., 1987a,b; Terasaki et al., 1986]. For fungi it is not known what further cellular machinery, if any, is needed beyond microtubules for tubule extension. For animal and plant cells, several lines of evidence support the idea that various types of GTP-binding proteins (e.g., trimeric, monomeric, and dynamin-like proteins) are involved in regulating the vesiculation/tubulation equilibrium of endomembrane compartments [e.g., see Gilman, 1987; Klausner et al., 1992; Robinson and Kreis, 1992; Takei et al., 1995; Zhang et al., 2000]. In fungi, however, while GTP-binding proteins, and in particular monomeric (or small) GTP-ases are known to be involved in vesicle trafficking [e.g., see Takai et al., 2001], nothing is known of the role of GTP-binding proteins in endomembrane tubule formation.

In this study, we investigate the response of the vacuole system of *P. tinctorius* to GTP $\gamma$ S and GDP $\beta$ S, Brefeldin A (BFA), and aluminium fluoride. These compounds are well known for their ability to alter the vesiculation/tubulation equilibrium of animal cell endomembrane compartments [Gilman, 1987; Klausner et al., 1992; Robinson and Kreis, 1992; Takei et al., 1995]. By using these perturbing agents, and monitoring their effects on vacuolar behaviour in living hyphae by use of fluorescein-based dyes, we aim to determine if vacuolar tubule formation requires GTP-binding proteins, and if so, to gain some insight as to which class or classes are involved.

## MATERIALS AND METHODS

### Reagents and Antibodies

Oregon Green 488 carboxylic acid diacetate (carboxy-DFFDA, O-6151, 10 mg/ml DMSO at  $-20^{\circ}\text{C}$ ) and BODIPY-BFA (stock solution of 100 mM in DMSO at  $-20^{\circ}\text{C}$ ) were purchased from Molecular Probes (Eu-

gene, OR); mouse anti-actin monoclonal (clone: C4) antibody was obtained from ICN Biomedicals Inc. (Aurora, OH). Citifluor (PBS solution kept at  $4^{\circ}\text{C}$ ) was purchased from Alltech Associated Pty Ltd (Baulkham Hills, NSW, Australia). All other materials were purchased from Sigma (St. Louis, MO): anti- $\alpha$ -tubulin (stock kept at  $-20^{\circ}\text{C}$ , T-5168); BFA (0.5 mg/ml EtOH stock at  $-20^{\circ}\text{C}$ , B-7651); GTP $\gamma$ S (10 mg/ml H<sub>2</sub>O stock at  $-80^{\circ}\text{C}$ , G-8634); GDP $\beta$ S (50 mM H<sub>2</sub>O stock at  $-20^{\circ}\text{C}$ , G-7637); lysing enzymes (L-2265); aluminium chloride (A-3017), and sodium fluoride (S-1504).

### Fungal Cultures

*Pisolithus tinctorius* (Pers.) Coker and Couch, cultures, strain DI-15, isolated by Grenville et al. [1986] were grown on modified Melin-Norkrans (MMN) agar [Marx, 1969]. To provide material for experiments, a modification of the cellophane sandwich technique of Campbell [1983] was used. The inoculum was grown sandwiched between two discs of sterile cellophane placed on MMN agar according to Cole et al. [1997] for 8–14 days at  $23^{\circ}\text{C}$  in the dark.

### Fluorochrome Loading Drug Treatments

Wedges of actively growing hyphal tips were excised from a single colony and treated with carboxy-DFFDA (20  $\mu\text{g}/\text{ml}$ ), according to Cole et al. [1997]. After 30 min, the wedges were washed in reverse osmosis water with or without drugs for various times.

The effects of GTP $\gamma$ S, GDP $\beta$ S, Brefeldin A (BFA), and BODIPY-BFA were tested by observing hyphae after an incubation period of 15 (BFA and BODIPY-BFA) or 45 min (GTP $\gamma$ S and GDP $\beta$ S) in solutions containing various concentrations of the drugs (see Results), and then again 30 (BFA) or 45 min (GTP $\gamma$ S) after the drug was washed out with reverse osmosis water. Antagonism between GTP $\gamma$ S on the one hand and BFA and GDP $\beta$ S on the other were investigated by pre-treating the wedges with either 3.6  $\mu\text{M}$  BFA, 500  $\mu\text{M}$  GDP $\beta$ S or (as a control) reverse osmosis water for 15 min, and then treating with GTP $\gamma$ S (100  $\mu\text{M}$ ) in the presence of BFA (3.6  $\mu\text{M}$ ) or GDP $\beta$ S (500  $\mu\text{M}$ ) for 45 min. The effects of aluminium fluoride were examined after incubating hyphae in a fresh solution made from sodium fluoride and aluminium chloride in a ratio of 5 mM:30  $\mu\text{M}$  or 20 mM:10  $\mu\text{M}$ , for 60 min. This is the standard way of preparing aluminium fluoride solutions [e.g., Bigay et al., 1985; Kusner and Dubyak, 1994].

For some experiments, drugs were added to the fluorescent probe solution and the wash solutions. All observations were made with samples mounted in the last treatment solution. In all controls, the drug was excluded, but all other factors, including the time and ethanol

concentration (where necessary), were kept exactly the same. Other controls for the aluminium fluoride experiments were NaF only (5 and 20 mM),  $\text{AlCl}_3$  only (10 or 30  $\mu\text{M}$ ), and NaCl only (5 or 20 mM).

In all experiments, the results for each treatment are the mean of the results from three wedges from a colony, with a minimum of 100 hyphae scored in each wedge.

#### Freeze-Substitution and Immunocytochemistry

Hyphae, treated with  $\text{GTP}\gamma\text{S}$ , BFA, or BODIPY-BFA, as described above (but without carboxy-DFFDA fluorochrome loading), were freeze-substituted for immunocytochemistry. They were frozen in liquid propane at  $< -185^\circ\text{C}$ , and transferred to anhydrous ethanol at  $-90^\circ\text{C}$  for 3–4 days, then gradually warmed to room temperature, at  $5^\circ\text{C}/\text{hr}$ . Hyphae were then rehydrated in 50 mM potassium phosphate buffer (pH 6.8, kept at  $4^\circ\text{C}$ ); 95% for 30 min, 90% for 30 min, 80% for 30 min, 70% overnight, 60–0% by 10% steps at 15 min each. They were next incubated for 15 min in MES buffer (pH 5.5), and the walls digested with a lysing enzyme solution (10 mg/ml), containing 15  $\mu\text{l}$  of 20 mM PMSF protease inhibitor and 1% BSA in MES buffer, for 75 minutes at room temperature. Lysed hyphae were rinsed in MES buffer (2 $\times$ ) and then in phosphate buffered saline (PBS; 2 $\times$ ), and then permeabilized in 0.1% Triton X-100 in PBS for 15 min. Hyphae were rinsed again in PBS buffer (3 $\times$ ), transferred to embryo cups, and incubated in anti- $\alpha$ -tubulin (1:1,000 dilution in PBS + 1% BSA) or anti- $\alpha$ -actin antibody (1:400 dilution in PBS + 1% BSA) for 60 min at  $37^\circ\text{C}$ . They were then rinsed in PBS buffer as before, and incubated in sheep anti-mouse fluorescein isothiocyanate (SAM-FITC; 1:30 dilution in PBS + 1% BSA) for 60 min. Hyphae were then mounted in Citifluor and kept at  $4^\circ\text{C}$  overnight before observation.

#### Fluorescence Microscopy

Fluorescence micrographs of vacuole motility were taken with a Zeiss Axiophot microscope fitted with DIC and epifluorescence optics (filter combination used: BP-450-490, FT510, and LP515-585 for DFFDA; and LP515-585; BP546, FT580, LP590, for BODIPY-BFA), using a  $\times 40$ , 0.75NA objective. Individual images or sequences of images were captured with a real-time digital imaging set-up comprising an Image Point CCD camera (Photometrics, Tucson, AZ), a PCI-compatible LG3 framegrabber (Scion Corp, Frederick, MD), and Scion version of NIH image (public domain image analysis software), on a Macintosh 9500 computer.

## RESULTS

### Normal Vacuole Morphology in *P. tinctorius*

Hyphae of *P. tinctorius* consist of linear cells, which are on average 200  $\mu\text{m}$  long and separated from

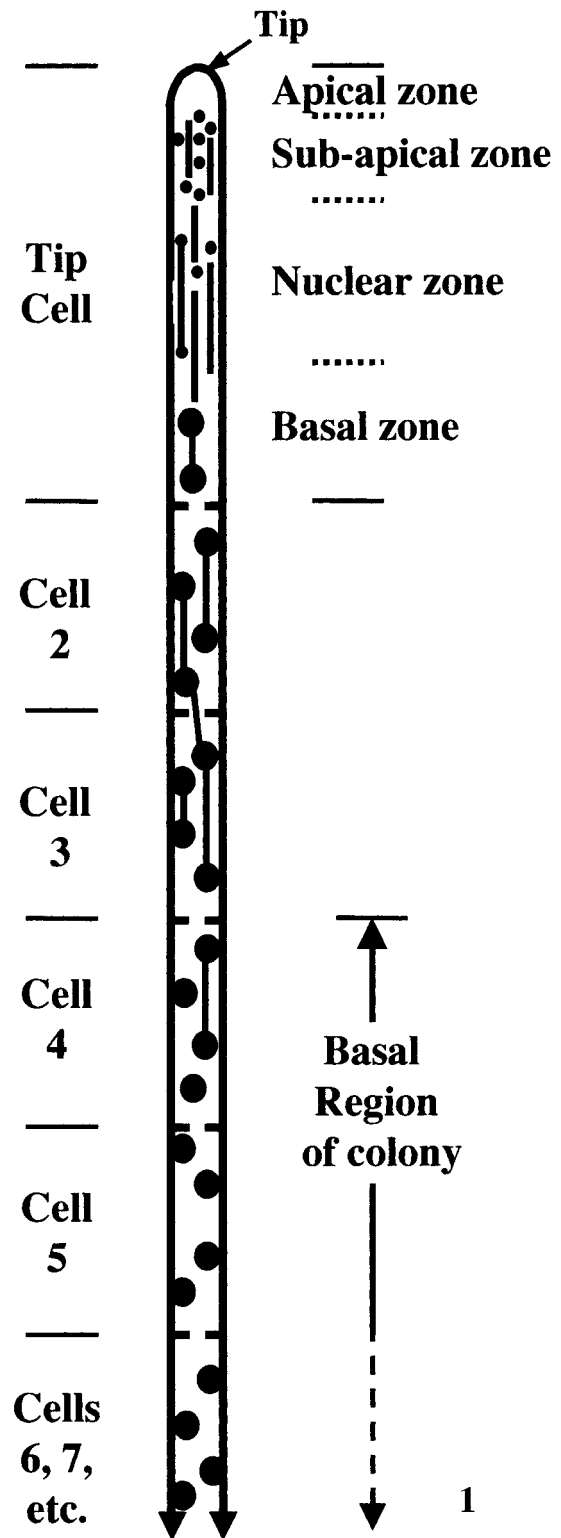
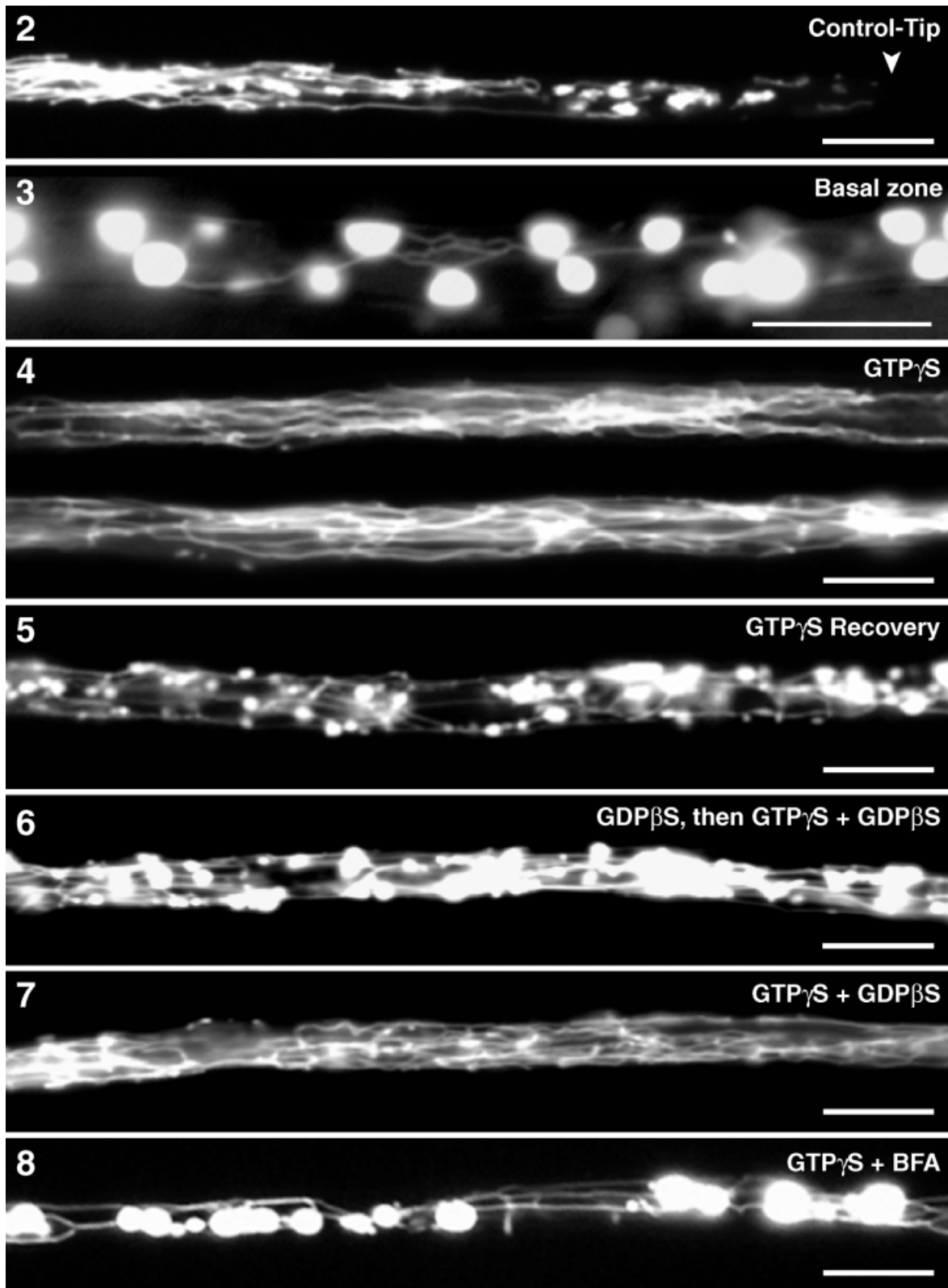


Fig. 1. A diagrammatic summary of a common pattern of vacuole morphologies in a hypha of *P. tinctorius* grown and examined under the standard conditions described in Materials and Methods. Note that the diagram is not to scale, e.g., the tip cell is not longer than other cells, but has been drawn such to allow for inclusion of more detail.



Figs. 2–8. Hyphal tip cells of *P. tinctorius* in which the vacuole system has been visualised by loading with the fluorescent dye Oregon Green. Bar = 10  $\mu\text{m}$ . Fig. 2. A typical control hypha, as shown here, contains both tubular and spherical vacuoles. *Arrowhead* marks the hyphal tip. Fig. 3. The vacuole system in cells of the basal zone of a tip cell. Vacuoles are mostly spherical, with some interconnecting tubules. Figs. 4–8. Hyphal tip cells showing the sub-apical and nuclear zones. The tip is to the right in all images. Fig. 4. The vacuole system in tip cells treated with 100  $\mu\text{M}$  GTP $\gamma$ S was predominantly composed of tubular vacuoles; this type of configuration is referred to in the text as a “complex tubule system.” Fig. 5. The effect of GTP $\gamma$ S on vacuole

morphology was reversed 45 min after flushing out the drug. Fig. 6. 500  $\mu\text{M}$  GDP $\beta$ S, if added before and then in conjunction with 100  $\mu\text{M}$  GTP $\gamma$ S, antagonised the response of the vacuole system to GTP $\gamma$ S. Vacuoles of a tip cell have both spherical and tubular vacuoles. Fig. 7. When 500  $\mu\text{M}$  GDP $\beta$ S and 100  $\mu\text{M}$  GTP $\gamma$ S were added simultaneously without GDP $\beta$ S pre-treatment, there was no antagonism of the GTP $\gamma$ S effect; e.g., the vacuole system of tip cells (one of which is shown here) was highly tubular. Fig. 8. 3.6  $\mu\text{M}$  Brefeldin A antagonised the response to 100  $\mu\text{M}$  GTP $\gamma$ S even if only added simultaneously, without any pre-treatment. In this tip cell, both tubular and spherical vacuoles are seen.

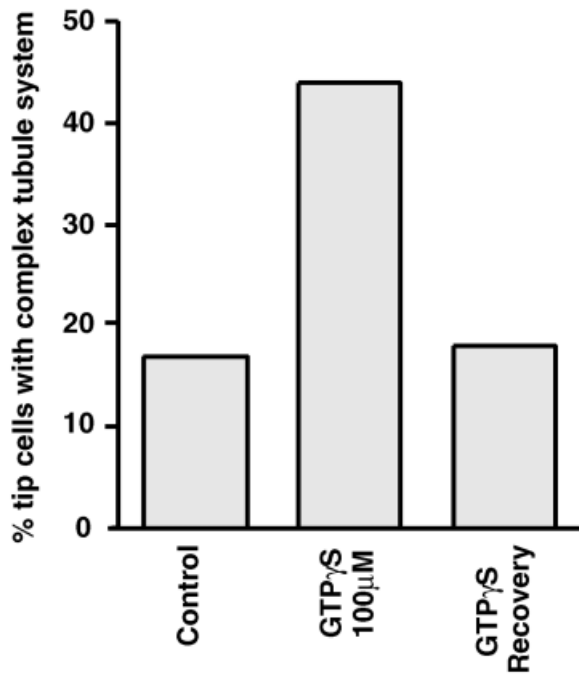


Fig. 9. Effect of 100  $\mu$ M GTP $\gamma$ S on vacuole morphology in tip cells. This drug increased the frequency of cells having a configuration similar to that shown in Figure 4. Percentages shown are the means of three experiments; at least 100 hyphae were counted in each experiment. Standard errors were too small to appear on graph.

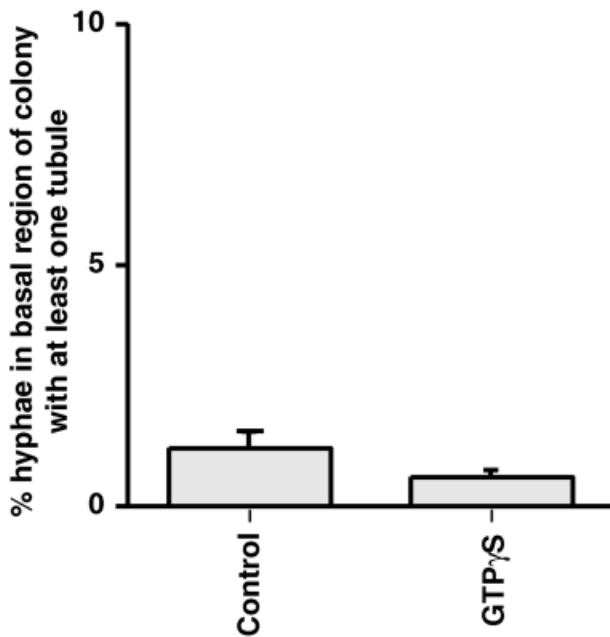


Fig. 10. Effect of 100  $\mu$ M GTP $\gamma$ S on vacuole morphology in cells from the basal region of a colony. There was little change in the frequency of tubules in this region. Percentages shown are the means of three experiments; at least 100 hyphae were counted in each experiment. Standard errors are shown.

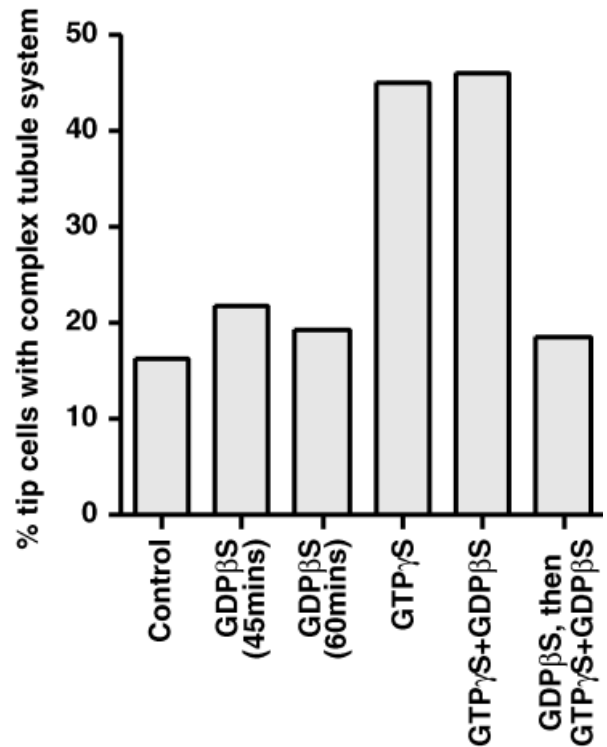


Fig. 11. Effects on the vacuole system of tip cells of 500  $\mu$ M GDP $\beta$ S, applied separately (GDP $\beta$ S columns) or added simultaneously with 100  $\mu$ M GTP $\gamma$ S (GTP $\gamma$ S + GDP $\beta$ S column) or added simultaneously with 100  $\mu$ M GTP $\gamma$ S after a 15-min pre-treatment with 500  $\mu$ M GDP $\beta$ S (GDP $\beta$ S, then GTP $\gamma$ S + GDP $\beta$ S column). Percentages shown are the means of three experiments; at least 100 hyphae were counted in each experiment. Standard errors were too small to appear on graph.

each other by perforated septa. For hyphae that have been grown and observed under our standard conditions, vacuoles show a roughly predictable progression of morphological forms along the length of the filaments (Fig. 1). In the apical zone, which extends for about one hyphal diameter or so behind the tip of the tip cell (about 5  $\mu$ m), vacuoles are typically rare or absent (Figs. 1 and 2). The remainder of the tip cell can be divided into a sub-apical zone (extending from 5  $\mu$ m to about 30–50  $\mu$ m behind the tip), a nuclear zone where two elongated nuclei are found (extending approximately a further 80–100  $\mu$ m behind the sub-apical zone) and the remaining basal zone (Fig.1). In the sub-apical zone, small ovoid-spherical vacuoles (henceforth called spherical vacuoles) are typically the predominant form, whilst tubular vacuoles are the more common vacuole type in the nuclear zone (Figs.1 and 2). Either in the basal zone of the tip cell, or at some point in the second cell, large spherical vacuoles eventually become the predominant form and remain so for all of the hypha behind the transition point (Figs. 1 and 3). Spherical vacuoles can, however, be interconnected by tubular vacuoles, with the frequency of inter-

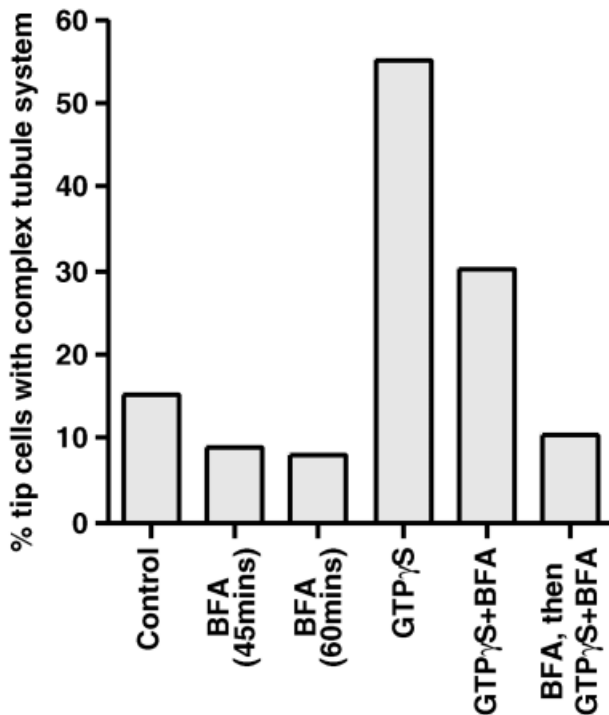
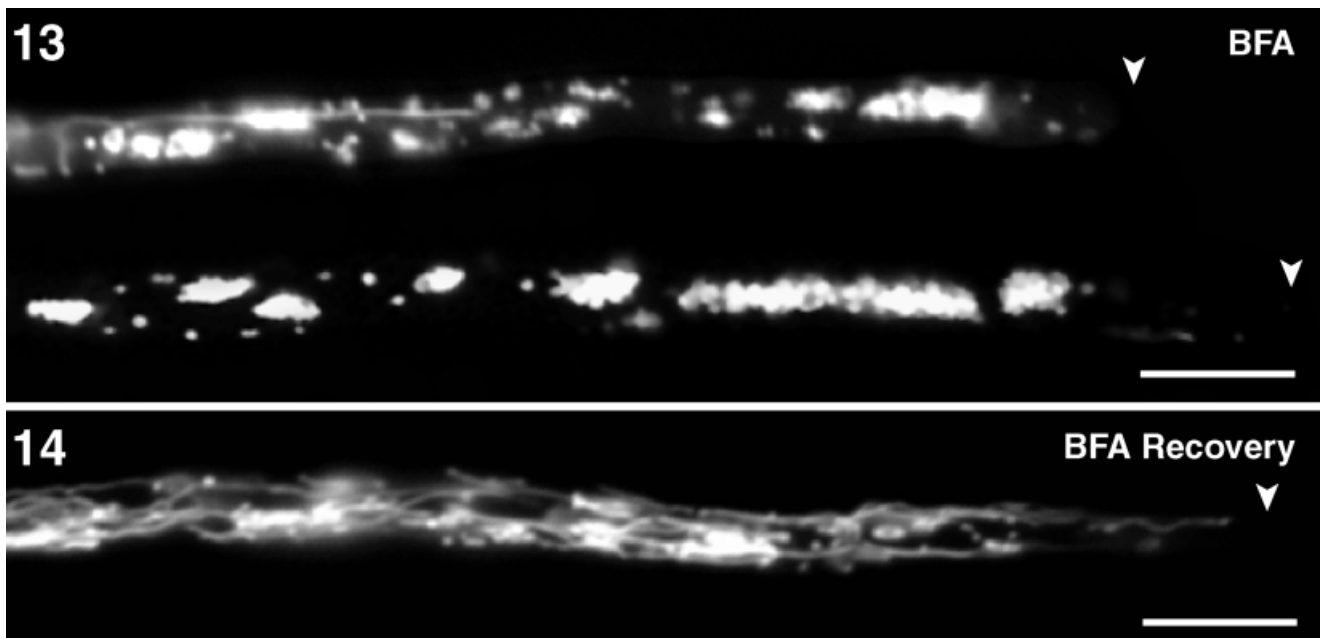


Fig. 12. Effects of 3.6  $\mu$ M Brefeldin A (BFA), applied separately or in conjunction with GTP $\gamma$ S, on vacuole morphology in tip cells. When applied by itself Brefeldin A reduced the frequency of vacuole systems with a complex configuration. Brefeldin A also antagonised GTP $\gamma$ S, especially when applied as a 15-min pre-treatment. Percentages shown are the means of three experiments; at least 100 hyphae were counted in each experiment. Standard errors were too small to appear on graph.

connection decreasing in cells further from the tip. In what we call the basal region of the colony (cell 4 and all cells beyond), interconnecting tubules are rare and only spherical vacuoles are usually seen (Fig. 1). Note that in the initial description of the nuclear zone by Shepherd et al. [1993b], the nuclear zone was described as containing few vacuoles; but in our recent work, in which we use hyphae that grow submerged rather than aerially, there is not any consistent decrease in vacuole frequency in this zone.

#### Effects of G-Protein Agonists and Antagonists on Vacuole Morphology in *P. tinctorius*

When hyphae were incubated in solutions containing GTP $\gamma$ S, there was, in brief, a significant increase in the predominance of tubular vacuoles in the first five cells (Fig. 4), which was recoverable (Fig. 5) and antagonised by GDP $\beta$ S (Fig. 6) and BFA (Fig. 8). To allow for a semi-quantitative analysis of these effects, we used the vacuolar configuration typical of GTP $\gamma$ S-treated cells (see Fig. 4) as an indicator of whether the vacuolar system was being affected by GTP $\gamma$ S or not. In cells with such a “complex tubule system,” tubules were not only more numerous than in typical controls (compare Figs. 2 and 4), but they were also more interconnected and formed an overall more reticulate network. As shown in Figure 9, 100  $\mu$ M GTP $\gamma$ S significantly increased the frequency of tip cells exhibiting a complex tubule sys-



Figs. 13, 14. The vacuole system in BFA treated tip cells of *P. tinctorius*. Arrowheads mark the hyphal tips; the sub-apical zone begins 5  $\mu$ m to the left of the tip. Bar = 10  $\mu$ m. Fig. 13. Two hyphae treated with 3.6  $\mu$ M BFA, showing a reduced frequency of tubular vacuoles, especially in the sub-apical zone (compare with Figs 2, 14). Fig. 14. The effect of BFA was reversed 30 min after flushing out the drug.

tem. In GTP $\gamma$ S-treated tip cells, tubular vacuoles were sometimes the only form seen. When the drug was washed out and hyphae left in GTP $\gamma$ S-free solution for 45 min before rescoring, tip cells recovered the typical range of control morphologies (Fig. 9). GTP $\gamma$ S also promoted tubule frequency in the second cell and as far back as the fifth cell (data not shown). For example, in the fifth cell some tubules were frequently seen after GTP $\gamma$ S treatment, whereas in most controls only spherical vacuoles were evident in this cell. In regions of the colony well behind the fifth cell, where tubular vacuoles are extremely rare, GTP $\gamma$ S had no effect on stimulating tubule frequency (Fig. 10).

The antagonism of these GTP $\gamma$ S effects by GDP $\beta$ S and BFA was also measured. When GDP $\beta$ S was added to the preparation for 15 min prior to the addition of GTP $\gamma$ S, it blocked the promotion of tubule formation normally caused by GTP $\gamma$ S treatment (Figs. 6, 11). Without a pre-treatment step, GDP $\beta$ S did not antagonise GTP $\gamma$ S (Figs 7, 11). GDP $\beta$ S itself, if added alone, had no effects on hyphae (Fig. 11).

BFA, at 3.6  $\mu$ M, also blocked the response of hyphal vacuoles to GTP $\gamma$ S, most strongly when exposure involved a pre-treatment phase (introduced 15 min before the combined BFA and GTP $\gamma$ S treatment; Fig. 12). This graph illustrates the antagonism by BFA of GTP $\gamma$ S effects in tip cells, but BFA also antagonised the effects of GTP $\gamma$ S as far back as the fifth cell (data not shown). Unlike GDP $\beta$ S, BFA also partly blocked the response to GTP $\gamma$ S when applied without a pre-treatment phase (Fig. 12). Even when applied alone, BFA also slightly reduced, in a recoverable fashion, the frequency of tip cells with a complex tubule system below that seen in controls (Figs. 12–14). Within the tip cell, the effects of BFA applied alone were most pronounced in the sub-apical zone. BFA reduced the number of hyphae with *at least one* tubular vacuole in this region from 86 to 19% (Fig. 15). The vacuole system was still present in the sub-apical zone, but typically only in the spherical form (Fig. 13). BFA applied alone had no effect on vacuoles in cells behind the tip cell (data not shown).

The response of the vacuole system to aluminium fluoride was also observed. Aluminium fluoride treatment involves addition of both NaF and AlCl<sub>3</sub> to the medium, and we used two combinations of these compounds: 5 mM NaF + 30  $\mu$ M AlCl<sub>3</sub> or 20 mM NaF + 10  $\mu$ M AlCl<sub>3</sub>. Both combinations (particularly the second) caused a significant increase in the frequency of tubular vacuoles (compared with controls in reverse osmosis water), but only in the basal region of the colony (Fig. 16). Tubular vacuoles were even seen in very old regions of the colony, where under our normal observation procedures tubules are typically completely absent (compare Figs. 17 and 18). However, treatment of hyphae with

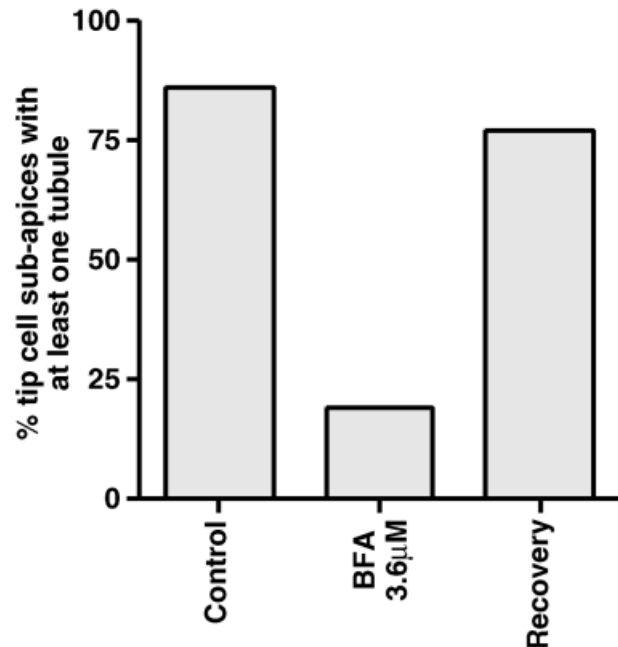


Fig. 15. Effects of 3.6  $\mu$ M BFA on vacuole morphology in the sub-apical region of tip cells. Frequency of tubules in this region was much reduced. Percentages shown are the means of three experiments; at least 100 hyphae were counted in each experiment. Standard errors were too small to appear on graph.

NaF, AlCl<sub>3</sub>, and NaCl also caused significant increases in tubule frequency in normally tubule-free regions of the colony (Fig. 16). As will be covered further in the Discussion, these results overall indicate that the responses seen are non-specific. Aluminium fluoride had no effect on the vacuole system in the first three cells. For example, when the frequency of tip cells with a “complex tubule system” was measured, treated preparations did not differ from controls (Fig. 19).

#### Effects of GTP $\gamma$ S and BFA on the Cytoskeleton

We also checked the response of the microtubule and actin cytoskeletons to GTP $\gamma$ S and BFA. Hyphal microtubules and actin patches at the tip of the tip cell were visualised by localisation of  $\alpha$ -tubulin and  $\alpha$ -actin after rapid freeze-fixation and freeze-substitution (our immunolocalisation procedure does not successfully label any longitudinal actin filaments that one would expect to be present in the hyphae) [e.g., see Heath, 1990]. BFA, and BODIPY-BFA, had a significant and recoverable (in the case of BFA) inhibition of actin cap formation at the hyphal tips (Figs. 20, 21, 23), but no effect on hyphal microtubules (not shown). The effects on actin caps are shown in Figure 23. GTP $\gamma$ S had no obvious effect on either microtubules or actin caps. For example, the  $\alpha$ -tubulin localisation patterns seen after GTP $\gamma$ S

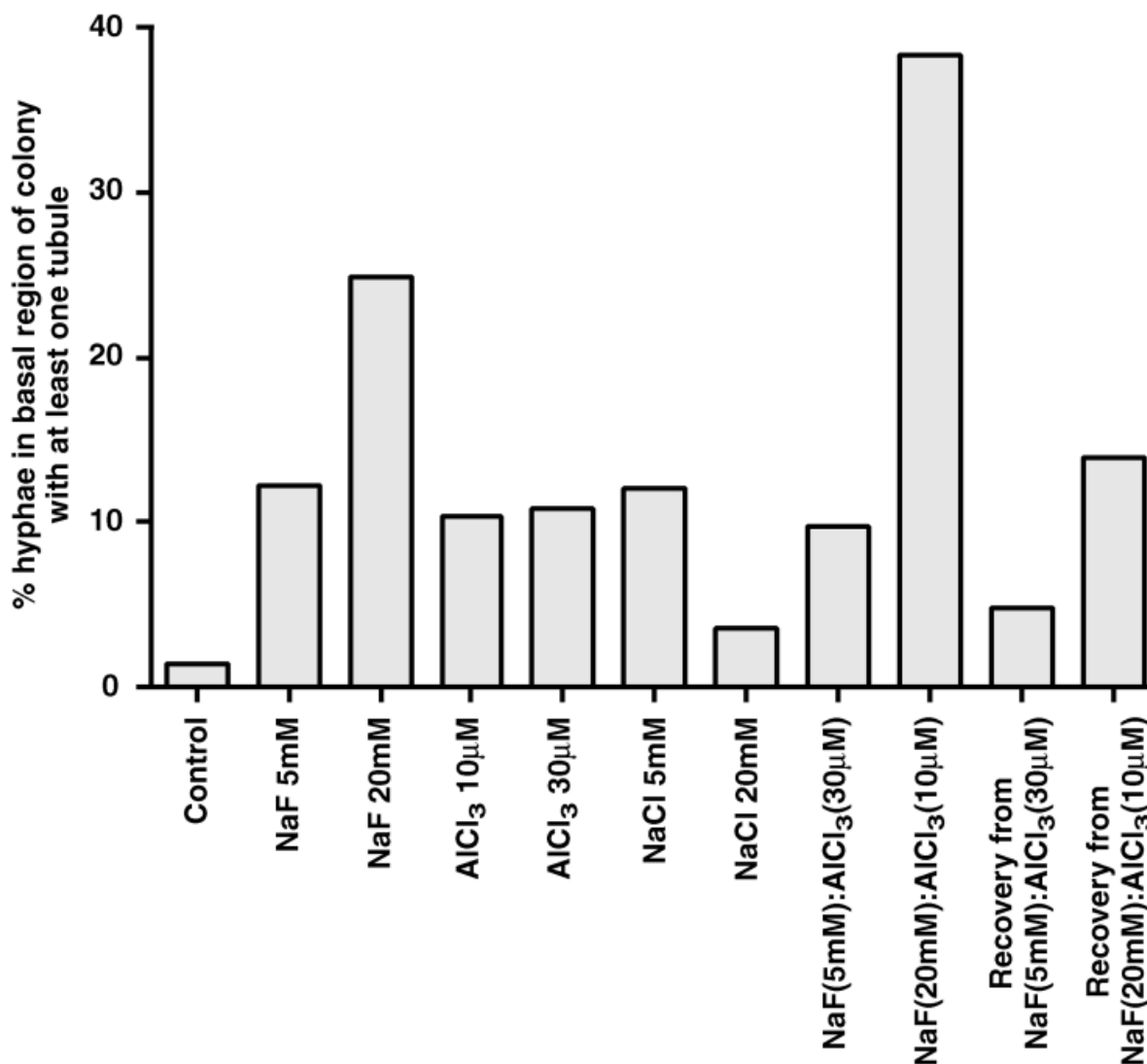


Fig. 16. Effects of aluminium fluoride and various controls on vacuole morphology in cells of the basal region of the colony. The frequency of tubules in these cells was increased. Percentages shown are the means of three experiments; at least 100 hyphae were counted in each experiment. Standard errors were too small to appear on graph.

treatment were similar to those that we have previously described for “control” cells (Fig. 22) [Hyde et al., 1999].

## DISCUSSION

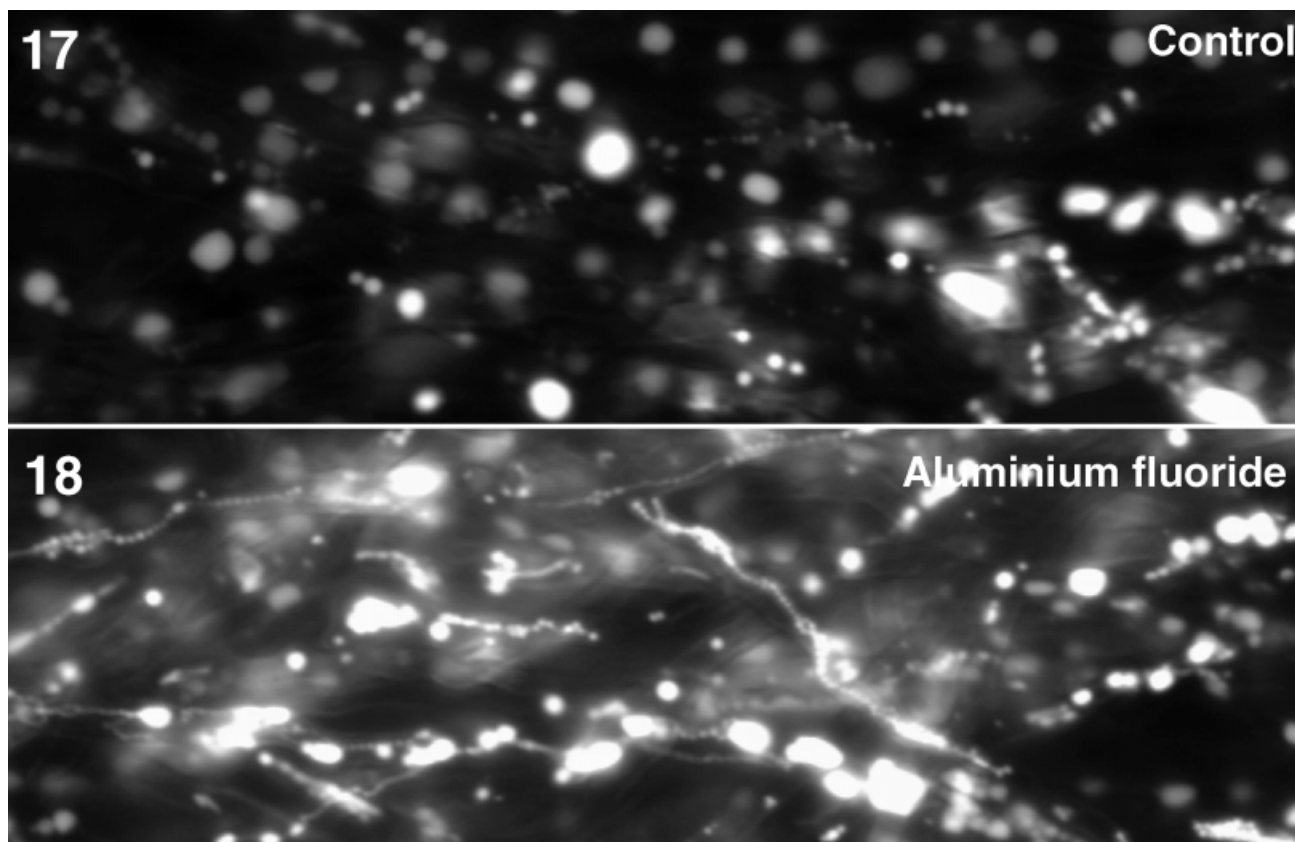
Three of the four drugs tested in this study (GTP $\gamma$ S, BFA, and aluminium fluoride) affected the predominant form of vacuoles in one or more regions of the hypha of *P. tinctorius* when applied independently (Fig. 24); the fourth drug GDP $\beta$ S functioned as an antagonist of GTP $\gamma$ S, if added before and during GTP $\gamma$ S treatment. While we believe that the responses to aluminium fluoride were unspecific, the results from the other drugs represent evidence consistent with the involvement of

GTP-binding proteins in the regulation of vacuolar motility in fungal hyphae and indicate possible differential regulation of the vacuole system in different regions of the hypha. We have previously reported on the effects of BFA (applied alone) on vacuolar morphology in *P. tinctorius* hyphae as part of a study of the effects of BFA on the ER and Golgi bodies [Cole et al., 2000].

### Localised Drug Effects Point to Functional Specialisation of Vacuole System

The three drugs that were effective when applied alone differed as to whether they promoted (GTP $\gamma$ S; aluminium fluoride) or inhibited the predominance of tubular vacuoles (BFA) and also with regard to their sites





Figs. 17, 18. The vacuole system in cells of the basal region of colony. Fig. 17. Control. Fig. 18. After treatment with aluminium fluoride. The image shows the presence of tubular vacuoles. Bar = 10  $\mu\text{m}$ .

of action (GTP $\gamma$ S: first five cells; BFA: tip cell, especially the sub-apical zone; aluminium fluoride, basal region of colony). While a partial explanation for these localised effects could be differential uptake of drugs, this cannot be the whole explanation. Although the effect of BFA, when applied alone, was localised to the tip cell, as an antagonist of GTP $\gamma$ S its influence extended through all of the first five cells.

Another explanation is that the form of the vacuole system is differentially regulated along the length of the hypha and thus sensitive to different perturbing influences. We have previously proposed that given differences in general cellular function along the length of the hypha (e.g., growth, uptake, storage, transport), one might expect functional specialisation within the vacuole system as well [Ashford, 1997]. This would be consistent with the observed morphological variations of the hyphal vacuole system along the length of the hypha [e.g., Shepherd et al., 1993b]. In plants, functional specialisation in different compartments of the vacuole system has been found even within a single cell [Rogers, 1998].

#### Which GTP-Binding Proteins Regulate Vacuole Morphology in *P. tinctorius*?

As well as exhibiting functional specialisation, it is also known that different pathways within the endomembrane systems (of non-hyphal systems at least) vary as to which native or introduced compounds and proteins they utilise or are regulated by. For example, three different vesicle coat proteins, COPII, COPI, and clathrin, have so far been identified at different points in vesicle trafficking between the ER, Golgi, and plasma membrane [Wieland and Harter, 1999]. In the formation of each class of vesicle, a unique ensemble of associated factors is involved, including one or more of a wide variety of GTP-binding proteins [Wieland and Harter, 1999] that fall into three major groupings (monomeric, trimeric, and dynamin-like proteins). Each of the four drugs utilised in our work have been found to affect different parts of the animal cell endomembrane network via their proposed perturbation of one or more of these classes of GTP-binding proteins [e.g., Gilman, 1987; Klausner et al., 1992; Padfield and Panesar, 1998; Robinson and Kreis, 1992; Stow and Heimann, 1998].

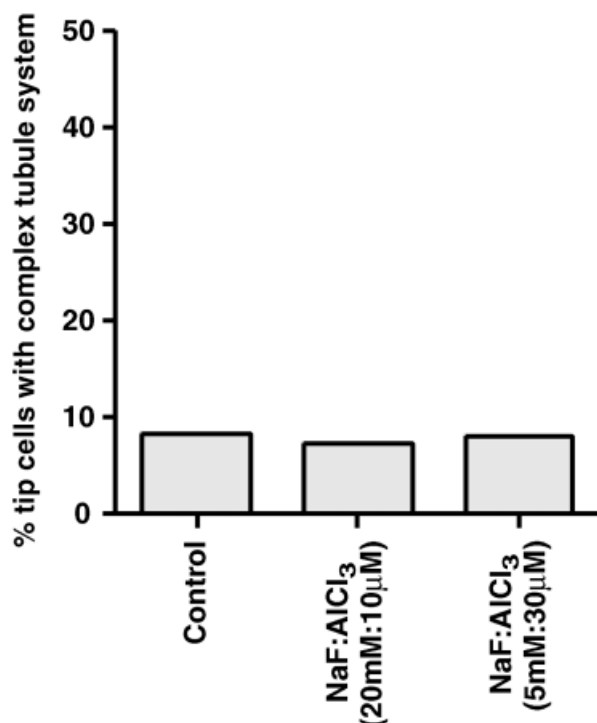


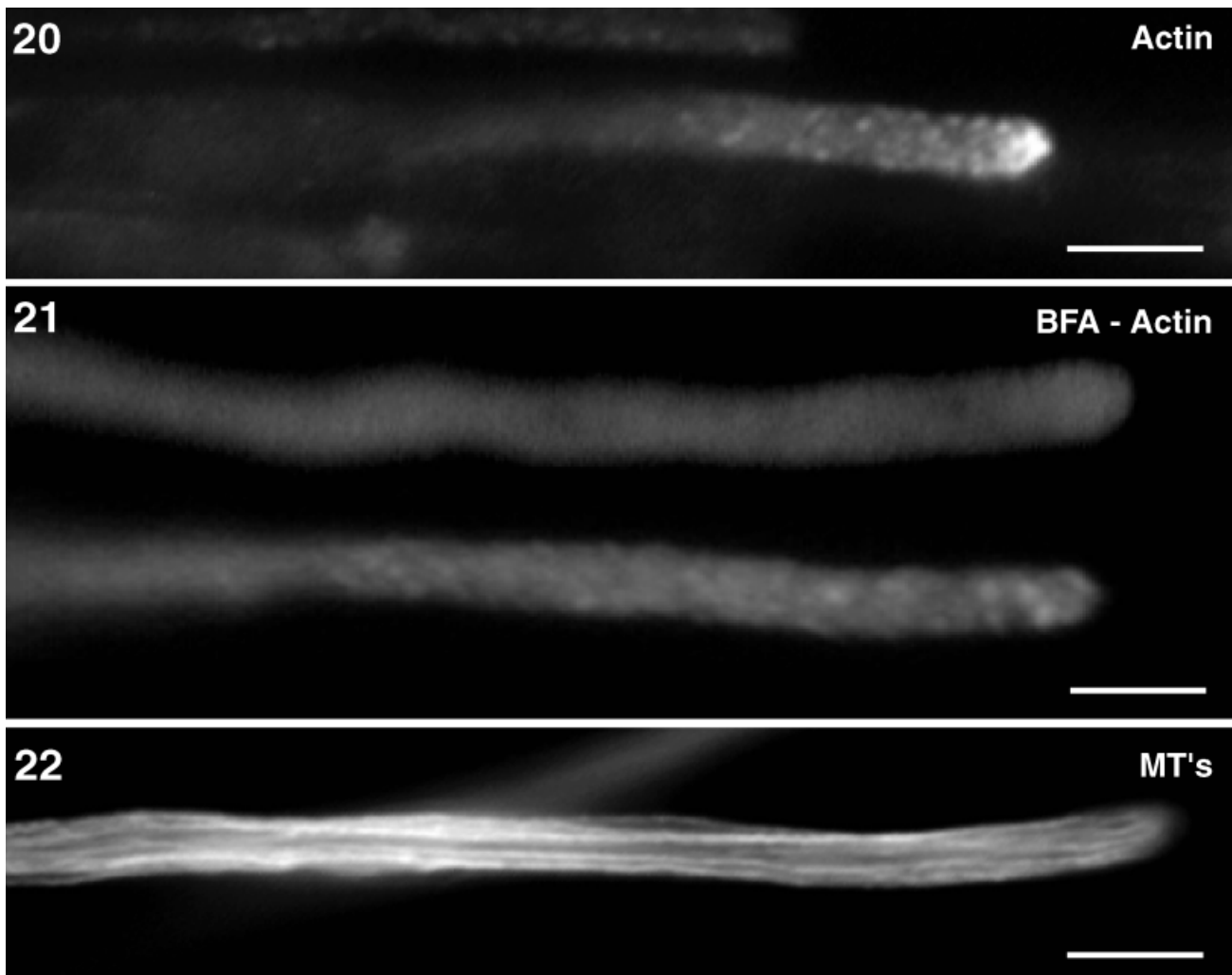
Fig. 19. Effects of aluminium fluoride on vacuole morphology in tip cells. No effects were observed. Percentages shown are the means of three experiments; at least 100 hyphae were counted in each experiment. Standard errors were too small to appear on graph.

It is fruitful to use the results from other organisms as a potential framework of reference for further clues as to the type of GTP-binding protein/s regulating vacuole morphology in *P. tinctorius*. The effects of GTP $\gamma$ S (and GDP $\beta$ S to a lesser extent) deserve the most attention for several reasons. Firstly, they are the least foreign of the molecules introduced, in that they are non-hydrolysable and non-phosphorylatable versions of naturally occurring regulators. In contrast, BFA and aluminum fluoride have more potential to trigger non-specific responses, for example BFA is a cellular toxin of fungal origin [e.g., see Satiat-Jeuemaitre et al., 1996] and aluminium fluoride is well known as a fungal toxin [e.g., Garciduenas and Cervantes, 1996]. Secondly, GTP $\gamma$ S caused the most significant response of any of the drugs: the tubulation promoted by GTP $\gamma$ S was dramatic in both its degree and in the length of hypha affected. Thirdly, the fact that the GTP $\gamma$ S response was antagonised by GDP $\beta$ S also supports the idea that this is truly a GTP-protein specific response and not an indirect effect. Interestingly, it was necessary to add GDP $\beta$ S as a pre-treatment to GTP $\gamma$ S to effect this antagonism. This is not entirely unexpected, since the antagonism due to GDP $\beta$ S does not involve competition for the sites of GTP action (which would cause a rapidly-occurring response); rather GDP $\beta$ S re-

places the normal cellular pool of GDP, and since it cannot be phosphorylated, less and less new GTP (the active state) will gradually be formed to replace that used metabolically. The fact that GDP $\beta$ S applied alone did not inhibit tubule formation also does not preclude classifying it as an antagonist, since in the two situations (control cell with few tubules; GTP $\gamma$ S-stimulated cell with many tubules) the equilibrium of GTP and GDP could vary dramatically and respond to applied GDP $\beta$ S in different ways.

Whilst trafficking by many compartments of the endomembrane system of animal cells is affected by GTP $\gamma$ S and GDP $\beta$ S, in only one process has GTP $\gamma$ S been reported to *stimulate* tubulation [Takei et al., 1995]. In other cases, GTP $\gamma$ S typically stimulates vesicle production, while GDP $\beta$ S promotes tubule formation [e.g., Robinson and Kreis, 1992]. For example, in its role of recruiting coat proteins to developing vesicle buds on the ER, the monomeric protein ARF requires exchange of its bound GDP with GTP, a reaction favoured by added GTP $\gamma$ S. The only reported case where GTP $\gamma$ S promotes tubule formation involves the GTP-binding protein dynamin in an in vitro axonal plasma membrane system, where vesicular pinching-off will *only* occur if dynamin-bound GTP is hydrolysed to GDP [Takei et al., 1995]. Thus, GTP $\gamma$ S, which is non-hydrolysable, promotes continued protrusion of nascent buds, which develop as tubules encased in rings of dynamin [Takei et al., 1995]. For mammalian Golgi bodies also, inhibition of dynamin action (by other means) also leads to membrane tubulation [Henley et al., 1999] and tubule formation is promoted by applied GTP $\gamma$ S [Fullerton et al., 1998]. In our study, GTP $\gamma$ S favours tubule formation, a result consistent with the dynamin model rather than the ER/ARF model. The result is also consistent with findings that show that dynamin-like proteins are found in *Saccharomyces cerevisiae*, and that their involvement in endomembrane trafficking is much wider than originally thought [for review see Schmid et al., 1998].

Although dynamin is, to our knowledge, the only GTP-binding protein of the endomembrane system that promotes tubulation when GTP $\gamma$ S is introduced, there may well be other as-yet-unidentified GTP-binding proteins that respond similarly. It is also possible that the tubulation stimulated by GTP $\gamma$ S in *P. tinctorius* results indirectly from activation of some GTP-binding protein outside the vacuole system proper. For example, GTP $\gamma$ S can promote microtubule assembly (tubulin is a GTP-binding protein) [Roychowdhury and Gaskin, 1986]; however although we know that vacuolar tubulation does depend upon microtubules [Hyde et al., 1999], in this study there was no obvious effects of GTP $\gamma$ S on hyphal microtubules.



Figs. 20–22. Hyphae of *P. tinctorius* that have been cryofixed and freeze substituted in ethanol before secondary labelling with antibodies. Bar = 10  $\mu$ m. Fig. 20. Control hypha secondarily labelled with anti- $\alpha$ -actin antibodies. Fig. 21. BFA-treated hypha secondarily labelled with anti- $\alpha$ -actin antibodies. Fig. 22. Control hypha secondarily labelled with anti- $\alpha$ -tubulin antibodies.

The responses of *P. tinctorius* hyphae to BFA also support the idea that vacuolar tubulation is regulated by some ensemble of coat proteins and factors other than the non-dynamain-involving ensembles characterised for trafficking by the ER and Golgi in animal cells. For these systems, there are no reports of inhibition of tubule formation by BFA, as was the case for *P. tinctorius*. In fact the opposite is true: BFA stimulates tubulation by the ER [e.g., Robinson and Kreis, 1992].

#### Responses to BFA and Aluminium Fluoride

Elsewhere we have reported that BFA alters the morphology of ER of *P. tinctorius* tip cells in similar fashion to that seen in animal cells, possibly due to blocking of transport out of the ER, or osmotic effects;

the morphology of the Golgi bodies is also affected [Cole et al., 2000]. Given the central roles of the ER and the Golgi bodies in the endomembrane system, it is difficult to know whether the effects of BFA on vacuoles are direct, or a consequence of perturbations of the ER and Golgi bodies. BFA also has an inhibitory effect on hyphal growth [Cole et al., 2000], which in itself can lead to changes in organelle distributions in tip cells of hyphae. While we did not monitor growth rates of *P. tinctorius* hyphae in this study, the disappearance of actin caps after BFA treatment is a sure indicator that growth has ceased [Heath, 1990]. However, the inhibition of vacuolar tubulation by BFA is unlikely to be a consequence merely of growth inhibition; in a previous study of *P. tinctorius* hyphae in which anti-actin drugs also caused disappearance of the actin caps, we found that

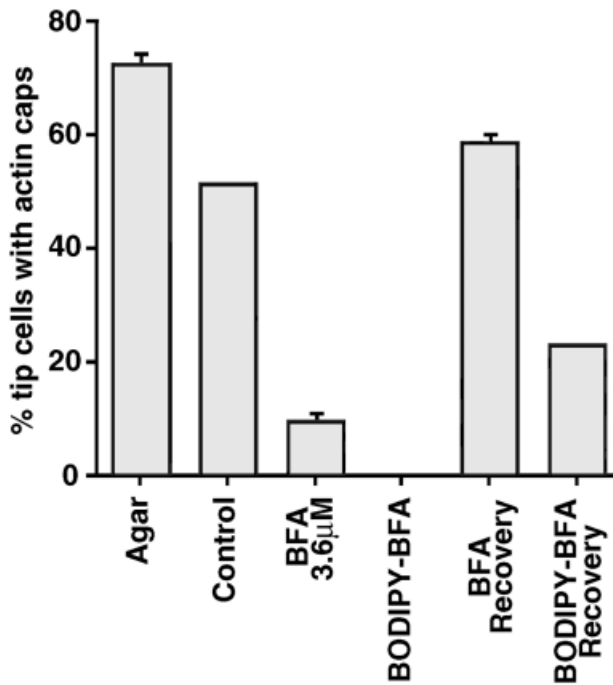


Fig. 23. Effects of BFA and BODIPY-BFA on actin caps. Percentages shown are the means of three experiments; at least 100 hyphae were counted in each experiment. Standard errors were too small to appear on graph.

there were actually more vacuolar tubules at the tip [Hyde et al., 1999].

Aluminium fluoride was the only drug to influence vacuoles in cells of the basal region of the colony, causing tubules to be seen in cells where they are usually infrequent or absent. In the literature, a response to aluminium fluoride is generally considered to be indicative of a process involving a heterotrimeric GTP-binding protein, but more recently it has also been found that aluminium fluoride can also influence some monomeric GTP-ases such as ARF and Ras proteins [Ahmadian et al., 1997; Finazzi et al., 1994]. For several reasons, we do not believe, however, that the effects of aluminium fluoride on *P. tinctorius* truly indicate a response involving a GTP-binding protein. Firstly, previous research indicates that of the two combinations of NaF and AlCl<sub>3</sub> that were used to form an aluminium fluoride solution (5 mM NaF + 30 μM AlCl<sub>3</sub> or 20 mM NaF + 10 μM AlCl<sub>3</sub>) in this study, the first combination should have had the greater effect on any process influenced by a GTP-binding protein [Bigay et al., 1985]. We found the opposite, suggesting a non-specific response to some other compound/s formed by the mixing of NaF and AlCl<sub>3</sub>. The results from our controls show that NaF itself can be a powerful stimulant of tubule formation, and the rise in tubule frequency when the concentration of NaF was raised from 5 to 20 mM mirrored that seen when com-

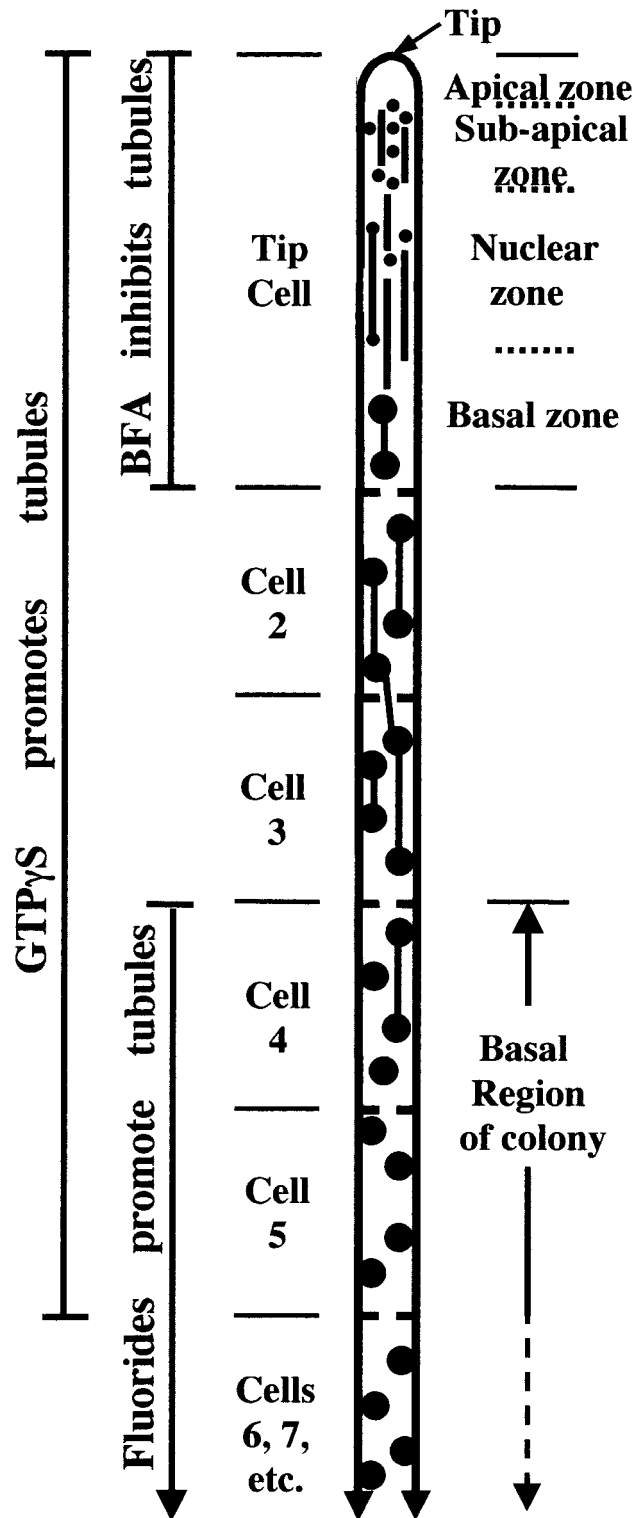


Fig. 24. Summary of the effects of BFA, GTP $\gamma$ S, and fluoride treatments, showing locations where the proportion of vacuoles that were tubular was either increased or reduced. For antagonistic effects, see text. Not drawn to scale.

paring the response to the 5 mM NaF/30  $\mu$ M AlCl<sub>3</sub> and 20 mM NaF/10  $\mu$ M AlCl<sub>3</sub> combinations. We do not know the reason for the response to NaF, but this compound is well known as an inhibitor of many phosphatases, kinases, and ATPases in fungi and other organisms [e.g., Arnold et al., 1987; Pinkse et al., 1999; Vargas et al., 1999], which could directly or indirectly influence tubule-forming activity. It should be noted here that some recent studies reporting the effects of aluminium fluoride on processes possibly regulated by GTP-binding proteins have not checked for non-specific effects of fluorides.

## CONCLUSIONS

In conclusion, our results indicate the involvement of GTP-binding proteins in vacuolar tubule production in both the hyphal apex and base. The results are consistent with the involvement of a dynamin-like protein. The plausibility of a role for dynamin in vacuolar motility is supported by the characterisation of dynamin-like proteins in *Saccharomyces cerevisiae*, in particular Dnm1p, which is proposed to play a role in endosomal transport [reviewed by Henley and McNiven, 1996]. Also, recently, it has been shown that disruption of a gene encoding a dynamin-related protein in *Aspergillus nidulans* results in highly fragmented vacuoles [Tarutani et al., 2001]. We think it probable that, given their extraordinary length, vacuolar tubules do require the support of an encasing protein, and thus are not likely candidates for "development by default," as has been suggested for some other tubule-forming systems [Klausner et al., 1992]. More work is now needed to determine if dynamin or some other protein does indeed play this role in fungal hyphae.

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