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

Geoffrey J. Hyde,* Danielle Davies, Louise Cole, and Anne E. Ashford

School of Biological Earth and Environmental Science, University of New South Wales, Sydney, Australia

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 (GTPγS, GDPβS, aluminium fluoride, and Brefeldin A). In *Pisolithus tinctorius*, GTPγS, a non-hydrolysable form of GTP, strongly promoted vacuolar tubule formation in the tip cell and next four cells. The effects of GTPγS could be antagonised by pre-treatment of hyphae with GDPβS, 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 GTPase, since GTPγS-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 GTPγS, 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: GTPγS; GDPβS; 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 μm 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.

Contract grant sponsor: Australian Research Council.

*Correspondence to: Geoffrey J. Hyde, School of Biological Science, University of New South Wales, Kensington, NSW, 2052, Australia. E-mail: G.Hyde@UNSW.edu.au

Received 20 February 2001; Accepted 14 November 2001

Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/cm.10015

© 2002 Wiley-Liss, Inc.
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; Takei et al., 1995; Zhang et al., 2000]. In fungi, however, 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, 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]. 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°C) and BODIPY-BFA (stock solution of 100 mM in DMSO at −20°C) were purchased from Molecular Probes ( Eugene, OR); mouse anti-actin monoclonal (clone: C4) antibody was obtained from ICN Biomedicals Inc. ( Aurora, OH). Citifluor (PBS solution kept at 4°C) was purchased from Alltech Associated Pty Ltd (Baulkham Hills, NSW, Australia). All other materials were purchased from Sigma (St. Louis, MO): anti-α-tubulin (stock kept at −20°C, T-5168); BFA (0.5 mg/ml EtOH stock at −20°C, B-7651); GTPyS (10 mg/ml H2O stock at −80°C, G-8634); GDPβS (50 mM H2O stock at −20°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°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 μg/ml), according to Cole et al. [1997]. After 30 min, the wedges were washed in reverse osmosis water. Antagonism between GTPyS and GDPβS on the one hand and BFA and GDPβS on the other were investigated by pre-treating the wedges with either 3.6 μM BFA, 500 μM GDPβS or (as a control) reverse osmosis water. Antagonism between GTPyS on the one hand and BFA and GDPβS on the other were investigated by pre-treating the wedges with either 3.6 μM BFA, 500 μM GDPβS or (as a control) reverse osmosis water for 15 min, and then treating with GTPyS (100 μM) in the presence of BFA (3.6 μM) or GDPβS (500 μM) 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 μM or 20 mM:10 μM, 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), AlCl₃ only (10 or 30 µM), 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 GTPγS, 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°C, and transferred to anhydrous ethanol at −90°C for 3–4 days, then gradually warmed to room temperature, at 5°C/hr. Hyphae were then rehydrated in 50 mM potassium phosphate buffer (pH 6.8, kept at 4°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 µl 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×) and then in phosphate buffered saline (PBS; 2×), and then permeabilized in 0.1% Triton X-100 in PBS for 15 min. Hyphae were rinsed again in PBS buffer (3×), transferred to embryo cups, and incubated in anti-α-tubulin (1:1,000 dilution in PBS + 1% BSA) or anti-α-actin antibody (1:400 dilution in PBS + 1% BSA) for 60 min at 37°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°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 ×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 µm long and separated from...
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 μm. 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 μM GTPγS 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 GTPγS on vacuole morphology was reversed 45 min after flushing out the drug. Fig. 6. 500 μM GDPβS, if added before and then in conjunction with 100 μM GTPγS, antagonised the response of the vacuole system to GTPγS. Vacuoles of a tip cell have both spherical and tubular vacuoles. Fig. 7. When 500 μM GDPβS and 100 μM GTPγS were added simultaneously without GDPβS pre-treatment, there was no antagonism of the GTPγS effect; e.g., the vacuole system of tip cells (one of which is shown here) was highly tubular. Fig. 8. 3.6 μM Brefeldin A antagonised the response to 100 μM GTPγS even if only added simultaneously, without any pre-treatment. In this tip cell, both tubular and spherical vacuoles are seen.
each other by perforated septa. For hyphae that have been
grown and observed under our standard conditions, vacu-
oles show a roughly predictable progression of morpho-
logical 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
μm), 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 5–30–50 μm behind
the tip), a nuclear zone where two elongated nuclei are
found (extending approximately a further 80–100 μm
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 typ-
ically 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 intercon-
ected by tubular vacuoles, with the frequency of inter-

Fig. 9. Effect of 100 μM GTPγS 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 experi-
ment. Standard errors were too small to appear on graph.

Fig. 10. Effect of 100 μM GTPγS 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 experi-
ment. Standard errors are shown.

Fig. 11. Effects on the vacuole system of tip cells of 500 μM
GDPβS, applied separately (GDPβS columns) or added simulta-
neously with 100 μM GTPγS (GTPγS + GDPβS column) or added
simultaneously with 100 μM GTPγS after a 15-min pre-treatment with
500 μM GDPβS (GDPβS, then GTPγS + GDPβS column). Percent-
ages 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 GTPγS, 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 GDPβS (Fig. 6) and BFA (Fig. 8). To allow for a semi-quantitative analysis of these effects, we used the vacuolar configuration typical of GTPγS-treated cells (see Fig. 4) as an indicator of whether the vacuolar system was being affected by GTPγS 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 μM GTPγS significantly increased the frequency of tip cells exhibiting a complex tubule sys-

![Graph](image1.png)

**Fig. 12.** Effects of 3.6 μM Brefeldin A (BFA), applied separately or in conjunction with GTPγS, 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 GTPγS, 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.

![Images](image2.png)

**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 μm to the left of the tip. Bar = 10 μm. Fig. 13. Two hyphae treated with 3.6 μM 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 GTPγS-treated tip cells, tubular vacuoles were sometimes the only form seen. When the drug was washed out and hyphae left in GTPγS-free solution for 45 min before rescoring, tip cells recovered the typical range of control morphologies (Fig. 9). GTPγS also promoted tube 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 GTPγS 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, GTPγS had no effect on stimulating tubule frequency (Fig. 10).

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

BFA, at 3.6 μM, also blocked the response of hyphal vacuoles to GTPγS, most strongly when exposure involved a pre-treatment phase (introduced 15 min before the combined BFA and GTPγS treatment; Fig. 12). This graph illustrates the antagonism by BFA of GTPγS effects in tip cells, but BFA also antagonised the effects of GTPγS as far back as the fifth cell (data not shown). Unlike GDPβS, BFA also partly blocked the response to GTPγS 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 AlCl3 to the medium, and we used two combinations of these compounds: 5 mM NaF + 30 μM AlCl3 or 20 mM NaF + 10 μM AlCl3. 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 NaF, AlCl3, 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 GTPγS and BFA on the Cytoskeleton**

We also checked the response of the microtubule and actin cytoskeletons to GTPγS and BFA. Hyphal microtubules and actin patches at the tip of the tip cell were visualised by localisation of α-tubulin and α-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. GTPγS had no obvious effect on either microtubules or actin caps. For example, the α-tubulin localisation patterns seen after GTPγS...
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 (GTPγS, 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 GDPβS functioned as an antagonist of GTPγS, if added before and during GTPγS 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 (GTPγS; aluminium fluoride) or inhibited the predominance of tubular vacuoles (BFA) and also with regard to their sites

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.
of action (GTPγS: 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 GTPγS 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].

**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 μm.
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 GTPγS (and GDPβS 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 aluminium fluoride have more potential to trigger non-specific responses, for example BFA is a cellular toxin of fungal origin [e.g., see Satiat-Jeunemaitre et al., 1996] and aluminium fluoride is well known as a fungal toxin [e.g., Garciduenas and Cervantes, 1996]. Secondly, GTPγS caused the most significant response of any of the drugs: the tubulation promoted by GTPγS was dramatic in both its degree and in the length of hypha affected. Thirdly, the fact that the GTPγS response was antagonised by GDPβS also supports the idea that this is truly a GTP-protein specific response and not an indirect effect. Interestingly, it was necessary to add GDPβS as a pre-treatment to GTPγS to effect this antagonism. This is not entirely unexpected, since the antagonism due to GDPβS does not involve competition for the sites of GTP action (which would cause a rapidly-occurring response); rather GDPβS replaces 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 GDPβS 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; GTPγS-stimulated cell with many tubules) the equilibrium of GTP and GDP could vary dramatically and respond to applied GDPβS in different ways.

Whilst trafficking by many compartments of the endomembrane system of animal cells is affected by GTPγS and GDPβS, in only one process has GTPγS been reported to stimulate tubulation [Takei et al., 1995]. In other cases, GTPγS typically stimulates vesicle production, while GDPβS 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 reactionfavoured by added GTPγS. The only reported case where GTPγS 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, GTPγS, 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 GTPγS [Fullerton et al., 1998]. In our study, GTPγS 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 GTPγS 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 GTPγS in P. tinctorius results indirectly from activation of some GTP-binding protein outside the vacuole system proper. For example, GTPγS 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 GTPγS on hyphal microtubules.
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-dynamin-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...
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 AlCl₃ that were used to form an aluminium fluoride solution (5 mM NaF + 30 μM AlCl₃ or 20 mM NaF + 10 μM AlCl₃) 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 AlCl₃. 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. 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.

---

**Fig. 24.** Summary of the effects of BFA, GTPγS, 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 µM AlCl₃ and 20 mM NaF/10 µM AlCl₃ 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.

ACKNOWLEDGMENTS

We thank Jim Pearse and Xiao Mei Niu for their excellent technical assistance during the course of this work, and Rita Verma for comments on typical vacuolar morphology. Australian Research Council grants to G.J.H. and A.E.A. are also acknowledged.

REFERENCES


