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Growth and guidance of the fungal hypha

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Hyphal growth – hallmark of the fungi

Fungi probably evolved in terrestrial ecosystems as scavengers of organic detritus. While the excursions of motile unicellular micro-organisms are limited ultimately by the water films in which they are trapped, the invention of the hypha brought new advantages in facilitating the crossing of barren, dry or inhospitable zones between isolated pockets of vegetation or other sources of nutrients. The exploratory tip-growing hypha is perfectly adapted for survival in a world of feast or famine since it can sequester the biosynthetic resources of many tens, thousands or, in some cases, hundreds of thousands of microns of hyphal tubes to drive the forward progression of the apex. It can therefore forge across or through solid substrates, spreading into and assimilating nutrient-rich zones as a branching mycelium. The hyphal growth habit is a most adaptive lifestyle and accounts in no small measure for the success of the fungi, which, as a group, out-number the types of vascular plants by at least fivefold and, in terms of biodiversity, are second only to the insects.

The process of hyphal growth has been studied for over a century but its understanding today is still superficial. What is clear is that cell-wall biosynthesis and cell extension are confined largely to the tapering apical region (Gooday, 1971; Gooday & Trinci, 1980; Gow 1994a) and that the membrane and enzymes required to expand the apex are packaged in microvesicles (Bracker *et al.*, 1976; Bartnicki-Garcia *et al.*, 1978; Bartnicki-Garcia & Bracker, 1984) that are then translocated through the hypha to the cell surface at the apex via actin microfilaments or possibly microtubules (Howard & Aist, 1977; Heath, 1990). These vesicles accumulate in the hyphal tip, where the high density of cell membrane renders the cell refractile in the phase-contrast microscope, to create an apical body or Spitzenkörper (Girbardt, 1969; Grove & Bracker, 1970; Howard, 1981; see work by Franco & Bracker described in Gow, 1994a). The vesicles are then exocytosed, unloading the wall biosynthetic enzymes which synthesize chitin and glucan in a matrix of mannoprotein. The structural poly-

saccharides are initially produced as plastic microfibrils which can be deformed under the global force of turgor pressure, but these are thickened gradually, cross-linked and rigidified to create the rigid lateral fungal cell wall as they are displaced laterally by the insertion of new material (Wessels, 1986, 1993).

Therefore, by necessity, my interest in the process and consequences of the tip growth of hyphae has encompassed many aspects of cellular physiology – wall biosynthesis, cytoskeleton function, secretion, cell polarity, vectorial metabolism as well as fungal ecology.

Cell biology and physiology of hyphal growth

In bacteria and in yeasts, such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, some of the most incisive insights into the control of cell growth have come through the analysis of mutants (*fts*, *cdc*, etc.). The same cannot be said for filamentous fungi, where many of the most illuminating studies of hyphal development have come through careful observation of the growth of wild-type mycelia. Robertson observed the growth of hyphae and their swelling and bursting responses to mechanical and osmotic perturbations and deduced that the apex of a hypha must be plastic and that the cell wall is rigidified sub-apically (Robertson, 1959). More recently, Wessels and his co-workers provided an elegant biochemical explanation of this model. They demonstrated that nascent chitin is synthesized in relatively amorphous strands which become thicker as chitin chains accrete by hydrogen bonding to form microfibrils. These are then cross-linked gradually to glucan chains in sub-apical regions of the hyphae. In non-growing tips the rigid glucan–chitin microfibril complexes are found at the extreme apex, preventing cell expansion (Wessels, 1986, 1993). I draw attention to these studies because this is one of very few examples where hyphal phenomenology has been unravelled at the molecular level. Other important insights into the process of hyphal growth have come from the work of Bartnicki-Garcia, Bracker, Girbardt, Gooday, Harold, Heath, Hoch, Trinci, Wessels and others. Most of these workers approached the problem of

Fig. 1. Two germ tubes of *C. albicans* showing vacuolated intercalary compartments (V) with centrally positioned nuclei (N), and septa (S) with phase-dark, non-vacuolated, growing apical compartments. Specimens were mounted in a concentrated protein solution of refractive index 1.369 to increase contrast between the vacuolated and non-vacuolated regions. From Gow & Gooday (1982b), with permission. Scale bar 20 μ m.

hyphal growth from the holistic, cellular perspective, working inwards towards the details of the underlying mechanisms. As yet we lack the complementary information at the molecular level, but we can take advantage of some of the many insights offered to us from genetic studies of the growth of yeasts. My own work on hyphal growth has involved cellular, physiological and molecular experiments at various stages. There have been some rewards from bridging these various perspectives although it has also highlighted the gulfs that exist between them.

Filamentous growth of *Candida albicans*

The germ tube of the dimorphic human pathogen *C. albicans* has been the focus of my interests for many years. We have, over the years, worked with this fungus at the cellular, physiological and molecular levels in order to understand the mechanism that regulates the yeast to hyphal transition and the role of germ-tube growth in the obligate association of *C. albicans* with animal hosts.

Budding of *C. albicans* is essentially the same as the budding of diploid cells of *S. cerevisiae*. However, yeast cells of *C. albicans* can form parallel-sided true hyphae in media containing whole serum or *N*-acetylglucosamine, or when stationary-phase cells are exposed to an increase in temperature and medium pH. Time-lapse analysis of mycelial development on serum-containing agar showed that germ tubes all grew at a linear rate but that branches emerged eventually at an exponential rate so that the overall increase in mycelium length was a logarithmic function (Gow & Gooday, 1982a). Linear growth kinetics are unusual for filamentous fungi since the increasing volume of cytoplasm in a lengthening germ tube provides an increasing biosynthetic resource for the apex. Growth is therefore autocatalytic and exponential (Gow, 1989a; Prosser, 1994). However, detailed examination of the germ tubes of *C. albicans* showed that the cytoplasmic

volume supporting growth remained relatively constant since the protoplasm in the parent yeast cell migrated forward with the tip, leaving behind an extensively vacuolated cell to the rear (Gow & Gooday, 1982b, 1984; Gow *et al.*, 1986). Only the apical cell remains metabolically active and a series of extensively vacuolated, uninucleate cells are left in its wake (Fig. 1). These intercalary compartments can remain inactive for protracted periods and are presumed to be arrested in the G1 phase of the cycle (see issue cover picture). This cell cycle arrest of sub-apical cells contrasts with the growth of pseudohyphal cells of *S. cerevisiae*, where each daughter bud continues to grow in synchrony with the mother cell from which it was born (Gimeno *et al.*, 1992; G. R. Fink, personal communication). However, the vacuolated hyphal compartments of *C. albicans* do eventually start to regenerate and synthesize cytoplasm at the expense of the vacuolar space. This occurs prior to the formation of secondary germ tubes and branches (Gow & Gooday, 1982b, 1987a; Gow *et al.*, 1986). Thus the hyphae of *C. albicans* consist of a series of discrete compartments with autonomous cell cycles, despite the fact that hyphal septa each contain a small micropore sufficient for ionic or molecular communication, but too small to allow organelle migration (Gow *et al.*, 1980; Gooday & Gow, 1983). This mode of growth would seem to be well adapted for exploration of the environment, particularly under conditions of nitrogen stress, since migration occurs with minimal expense to protein synthesis. Here we can draw a parallel with the foraging pseudohyphae of *S. cerevisiae*, which are also formed under conditions of nitrogen stress (Gimeno *et al.*, 1992).

The role of hyphal growth of *C. albicans* in pathogenesis is a hotly debated topic (Odds, 1988, 1994; Matthews, 1994; Gow, 1994b). While some histological sections of diseased tissues are virtually free from hyphal cells (Odds, 1994) other studies suggest that hyphae are better able to gain a foothold in the primary invasion of a host tissue (Martin *et al.*, 1984; Sobel *et al.*, 1984). Experiments using animal models and strains that are debilitated in hyphal formation have shown that the yeast cells are undoubtedly pathogenic in their own right (Simonetti & Strippoli, 1973; Shepherd, 1985; Ryley & Ryley, 1990) but equivalent hyphal-competent strains penetrate more deeply into the host tissues (Ryley & Ryley, 1990). From a teleological point of view tip-growing hyphae would seem well adapted for penetration of surfaces such as epithelia and it is with that in mind that we have looked for behavioural adaptations of the germ tube when grown in contact with a surface.

Despite the fact that *Candida* normally exists as an epiphyte on the superficial layers of human tissues, most studies of growth have cultivated the hyphae in liquid cultures. When we grew the filamentous form on a variety of inert surfaces we observed that hyphal growth was influenced strongly by contact or touch (Sherwood *et al.*, 1992; Sherwood-Higham *et al.*, 1994; Gow *et al.*, 1994a). Helical hyphae were formed when filamentous growth was induced on certain firm surfaces including Cellophane and other membranes, agars or gels (Fig. 2a; Sherwood-

Fig. 2. Diagram of the thigmotropic response of hyphae of *C. albicans*: (a) helical growth induced by growth on firm surfaces; (b) penetration of pores of Nuclepore membranes; (c) contact guidance by scratches in a membrane or (d) ridges on a polystyrene replica. From Gow *et al.* (1994a) with permission.

'The Electric Fungus'

Fig. 3. Investigations of the electrical currents of fungi lead to a shocking conclusion! Courtesy of G. M. Gadd.

Higham *et al.*, 1994; Gow *et al.*, 1994a). Hyphae were straight when growing through agar or when sandwiched between two membranes, suggesting that asymmetric contact with one side of the hyphal tip is necessary to induce the helical growth response (Sherwood-Higham *et al.*, 1994). We have attributed helical growth to the rotation of the apex during cell extension. A second class of behavioural response was observed when hyphae were grown on various contoured surfaces (Fig. 2b–d). Hyphae penetrated the pores of Nuclepore membranes that were overlaid on serum-containing agar, then followed the upper or lower face of the membrane until they encountered a second pore, which they then re-entered (Fig. 2b). Because hyphae grew away from the underlying agar as readily as they grew towards it, the reorientation of the apex at the pore lips was not due to chemotropism, but rather to the avidity for the surface which mediated

thigmotropism or contact guidance (Sherwood *et al.*, 1992; Gow *et al.*, 1994a). Similarly, hyphae that encountered grooves or ridges were reoriented by them and ran parallel to them (Fig. 2c, d). Thus *C. albicans* hyphae make use of topographical cues to guide the forward progression of the tip. Such thigmotropic behaviour is well recognized for hyphae of plant pathogens such as the rusts (Wynn, 1981; Hoch *et al.*, 1987; Read *et al.*, 1992) and we have speculated that thigmotropism may confer similar advantages for medically important fungi by facilitating tissue penetration through microscopic wounds, membrane evaginations and sites where the integrity of the epithelium is weakened. No direct clinical evidence exists for this as yet, although some sections of *Candida* infected tissues apparently show alignments of the infiltrating hyphae (see Odds, 1994).

Regulation of dimorphism

The biochemical and physiological basis of dimorphic regulation has proved one of the most elusive topics in mycology. The situation is at its most frustrating for *C. albicans*, where the literature is both vast and contradictory. A huge range of environmental factors have been shown to be capable of influencing the balance between yeast and hyphal growth, including various nutrient regimes, the presence of various chemical inducers (serum, *N*-acetylglucosamine, calcium ions, etc.) and physical perturbations such as external pH and temperature (summarized in Odds, 1985, 1988). Few general principles emerge but it is evident that there is no single environmental trigger for dimorphism and that there must be several independent signal transduction pathways that can bring about a change from yeast to hyphae (see Gow, 1988).

We showed that changes in external pH that lead to the yeast to hyphal transition also induce changes in internal pH (pH_i) which precede and predict morphogenesis (Stewart *et al.*, 1988, 1989). Similar changes in pH_i have also been reported elsewhere (Kaur *et al.*, 1988), although the data differ quantitatively in some respects. We showed that cells destined to form germ tubes exhibited a greater rise in pH_i than cells that would form buds and that appropriate concentrations of inhibitors of the plasma membrane ATPase, such as diethylstilboestrol, could attenuate rises in pH_i and prevent morphogenesis without reducing cell viability (Stewart *et al.*, 1988). Strains of *C. albicans* that had been selected on the basis of their impaired ability to form germ tubes did not exhibit the rapid cytoplasmic alkalinization observed under culture conditions that normally induced dimorphism (Stewart *et al.*, 1989). Thus pH_i is one of several candidates that have been proposed to act during signal transduction or as second messengers of dimorphic regulation (for reviews see Gadd, 1994 and Gow, 1994b).

A second putative second messenger for *Candida* dimorphism is calcium ions, which may act via interactions with calmodulin and/or by affecting inositol phosphate metabolism (Sabie & Gadd, 1989; Gadd, 1994). We

showed that the calmodulin inhibitors such as trifluoperazine, chlorpromazine and R24571 could interfere specifically with the dimorphic transition without affecting growth by budding, and we hoped this would allow us to test the perennial hypothesis that the switch to hyphal development promoted pathogenesis. Unfortunately, while we were able to demonstrate the efficacy of these compounds in suppressing germ-tube formation *in vivo*, the doses of drug that had to be applied in animal models to prevent or ameliorate infection were too high to contemplate use of calmodulin inhibitors therapeutically (Buchan *et al.*, 1993). However, we have continued to maintain an interest in Ca^{2+} transport in *Candida* and other fungi in terms of their role in hyphal growth and guidance mechanisms (discussed below). Signal transduction in the newly characterized dimorphic response of *S. cerevisiae* is already helping to clarify some of the possible regulatory networks that may operate in *C. albicans*, but despite the intensity of effort there is much that remains to be understood in this important aspect of hyphal development.

Ionic currents

It has already been mentioned that the apical growth of filamentous fungi can be considered as a question of vesicle translocation and localized exocytosis. An important question concerning the regulation of polarized hyphal growth is what marks the site at which growth-supporting vesicles are allowed to fuse with the cell membrane. In the fungi we know that vesicle exocytosis coincides with an actin-rich part of the cell (e.g. Anderson & Soll, 1986; Marks & Hyams, 1985; Heath, 1990) and that proteins such as Spa2 and Myo2 may serve as markers for membrane patches where vesicles can fuse (Snyder, 1989; Johnston *et al.*, 1991; Lille & Brown, 1992; reviewed in Gow, 1994a). However, the regulation of cell polarity is akin to taking apart a Russian doll – there are layers within layers of organizational controls. Thus, although we can infer that the polarized distribution of actin is likely to be significant in the polarized growth process it is still not clear what enables actin to become polymerized at one end of a cell or how specific cytoskeletal-binding proteins become inserted in the membrane at a distinct location. To understand what is important and special about the apex, it is reasonable to study those aspects of cellular physiology that are particular to it. In this respect I became interested in the ionic/electric currents that are generated by fungi and other tip-growing eukaryotes (Gow, 1989b).

An electrical current, in a biological context, is a circulation of charge-carrying ions through and around a cell or tissue. Electrical currents, which are very commonly generated by eukaryotic cells, are caused by asymmetries in the distribution of ion transport processes in the cell membrane so that patches of membrane are enriched for ion pumps or ion porters. Current flows between the sites of net current efflux and net influx in both the cytoplasmic and extracellular spaces. In a wide range of eukaryotes the shape of the current map is correlated with the structural organization of the cell or

tissue (Gow, 1984, 1989b; Nuccitelli, 1990). This observation led to the hypothesis that ion currents may play an active role in polarity and morphogenesis.

In many cases, in particular with narrow hyphal cells of fungi, these currents and associated electric fields are often small (Fig. 3) and cannot be detected with conventional voltage-sensitive microelectrodes. They can, however, be measured with an instrument called the vibrating probe (Jaffe & Nuccitelli, 1974), which has a vibrating voltage-sensing element and enables signals to be detected extracellularly at a single frequency against a background noise that would otherwise overwhelm them.

The first fungi to be examined with vibrating microelectrodes were the chytridiomycete *Blastocladiella emersonii* and the oomycete *Achlya bisexualis* in Frank Harold's laboratory (Stump *et al.*, 1980; Kropf *et al.*, 1983). Both fungi drive currents that are carried mostly by protons, which turn out to be the main current-carrying ions for all fungi that have been examined to date (Kropf *et al.*, 1984; McGillivray & Gow, 1987). Oomycetes such as *Ach. bisexualis* are not true fungi (Gunderson *et al.*, 1987), but they have hyphae that are functionally identical to those of true fungi and have additional advantages for electrophysiology in being relatively large, robust and fast growing. Careful characterization of the *Achlya* current suggested that the current represented a spatially extended chemiosmotic circulation with the apex being rich in amino acid proton symporters and the sub-apical hyphal membrane containing a concentration of proton pumping ATPases (Kropf *et al.*, 1984; Gow *et al.*, 1984). Protons normally enter the growing hyphal apex. The scheme illustrated in Fig. 4 for *Ach. bisexualis* was also consistent with studies of gradients of external pH (Gow *et al.*, 1984) and membrane potential that were obtained with ion-selective or static voltage-sensing electrodes (Kropf, 1986). Initial experiments showed a tight correlation between the ability of the fungus to drive an inward apical current and the ability of the tip to extend. Moreover, the site of formation of vegetative (Kropf *et al.*, 1983) or sexual branches (Gow & Gooday, 1987b) was predicted by the establishment of a new current. Further attempts to reproduce the conditions that allowed the measurement of the new currents of sexual antheridial branches were unsuccessful (Cho *et al.*, 1991). However, the fact that new currents predicted new sites of growth in a variety of cell systems (Gow, 1989b; Nuccitelli, 1990) again suggested that ion currents may have some function in cell polarity and the regulation of cell extension.

However, several studies argue strongly that currents are not involved directly with the control of apical extension of fungal hyphae. Firstly, the direction of the current of *Ach. bisexualis* or *Neurospora crassa* could be shown to be attenuated or even reversed transiently without any consequence to tip extension rate (McGillivray & Gow, 1987; Takeuchi *et al.*, 1988; Schreurs & Harold, 1988; Cho *et al.*, 1991). In the chytridiomycete *Allomyces macrogynus* the current was found to be constitutively outward at the apex even when the fungus exhibited 'reversed development' by widening the tip progressively towards the rear of the hypha (Youatt *et al.*, 1988; De

Fig. 4. Circulating ion currents in the rhizoids and sporangium of *B. emersonii*, hypha of *Ach. bisexualis* and hypha and rhizoids of *All. macrogynus* as described in the text. The arrows indicate movements of ions; amino acid substrates for nutrient symporters are indicated by 'a'.

Silva *et al.*, 1992). Thus the direction of growth did not affect the direction of the current.

Outward current of *All. macrogynus*, as in the other chytrid *B. emersonii*, was found at the region of the tapering rhizoids that function in anchoring the fungus to leaves and other substrates in the fresh water environments in which they proliferate. Thus for three aquatic species inward protonic current was found at the rhizoids of *B. emersonii* and *All. macrogynus* and at the hyphal apex of *Ach. bisexualis* (Fig. 4). Interestingly, these regions are all chemotropic (Kropf & Harold, 1982; Schreurs *et al.*, 1989; Youatt *et al.*, 1988), suggesting that the true reason for the inward current was that proton-driven nutrient transporters were concentrated in those regions. Inwardly directed ion current therefore indicates sites of local nutrition rather than local extension (De Silva *et al.*, 1992). Although this study did not illuminate how the process of tip extension is regulated, it served as a valuable cautionary tale for those interested in this branch of electrophysiology in non-microbial systems. It is also worth noting that currents are one example of a physiological process that can only be studied in the intact system. A current cannot be cloned, purified or subjected to the type of molecular analyses that constitute the present preferred route towards the solution of a developmental problem.

Galvanotropism

In the course of our work on the ionic currents of hyphae we learnt a trick that has enabled us to guide and steer the hyphal tip experimentally. When a fungus is exposed to an exogenous electrical field, growth is redirected towards the anode or cathode (galvanotropism). We have made use of this phenomenon to examine the growth and guidance of the hyphal apex.

Germ-tube formation, germ tube and hyphal growth, branch formation, budding and rhizoid growth are all polarized by electrical fields (McGillivray & Gow, 1986;

Gow, 1987; Youatt *et al.*, 1988; Crombie *et al.*, 1990; Lever *et al.*, 1994). All fungi that have been examined respond to electrical fields; some reorient towards the anode, while others do so towards the cathode. Germ tubes and buds of *C. albicans* grow towards the cathode of an electrical field and germ tubes retain a memory of having once been in an electrical field, even when it was switched off prior to cell evagination (Crombie *et al.*, 1990). These observations suggest that the electrical field may redistribute proteins that are important for cell extension by electrophoresis or electroosmosis and that the polarization of these proteins decays only gradually with time. Studies with animal cells have demonstrated that membrane-bound proteins are indeed redistributed in electrical fields (Jaffe, 1977; Orida & Poo, 1978; Poo, 1981; Stollberg & Fraser, 1988). One consequence of an imposed electric field is therefore to influence the localization of membrane proteins. Autoradiographic studies on protoplasts of *Schizophyllum commune* that were regenerated in an electrical field suggest that chitin synthase is not one of the target proteins (De Vries & Wessels, 1982). Electrophoresis is, however, not enough to explain the various galvanotropic responses that have been documented.

If hyphae or rhizoids are grown for extended periods in an electrical field, growth in the plane of the field is inhibited increasingly. For *N. crassa*, the hyphae take on a new orientation perpendicular to the field (McGillivray & Gow, 1986). The rhizoids on *All. macrogynus* stop abruptly so that the length of the in-field vector is the same (De Silva *et al.*, 1992). This results in a moustache-like fringe of rhizoids (Fig. 5). As a cell grows in the plane of the field the cathodal end of the cell will become depolarized increasingly while the anodal end will become hyperpolarized. Therefore, a possible explanation of these various growth inhibitory effects is that they are due to the increasingly large perturbations of the membrane potential at the anodal and cathodal ends of the cells.

Fig. 5. Galvanotropism of rhizoids of *All. macrogynus*. The rhizoids grow initially towards the anode but extension is ultimately arrested so that the length of the rhizoids in the plane of the electric field (arrow) is the same. Scale bar represents 100 μm . From De Silva *et al.* (1992), with permission.

Two other observations are also worth noting. Firstly, we observed recently that the galvanotropic responses of fungal hyphae are influenced markedly by external pH (Van Laere, 1988) and have an isoelectric point at which the fungi are unresponsive to the field (Lever *et al.*, 1994). This result again suggests an electrophoretic model for protein sorting. However, hyphae of *N. crassa* were more anodotropic at alkaline pH while the reverse was true for hyphae of *Aspergillus fumigatus* and *Coprinus cinereus* (Lever *et al.*, 1994). On a theoretical basis, increasing pH would be expected to make all proteins more negative and so one might expect all fungi to be affected in a qualitatively similar way if the mechanism involved electrophoretic or electroosmotic displacement only. This finding suggests therefore that effects of external pH on galvanotropism are multifactorial.

The second additional observation that must be accounted for is that galvanotropism of fungi is apparently inhibited by the removal of external Ca^{2+} from the growth medium or by calcium ion blockade (Lever *et al.*, 1994; Wittekindt *et al.*, 1989; A. D. B. Buchan, G. W. Gooday & N. A. R. Gow, unpublished). This has led us to propose that calcium channels may be one target protein that electrical fields act on. The plausibility of this hypothesis is

supported by recent observations of a tip-high gradient of calcium and calcium channels in hyphae of *Saprolegnia ferax* (Garrill *et al.*, 1992, 1993; Jackson & Heath, 1993b; Levina *et al.*, 1994) and in view of the many reports of calcium ions influencing tip extension rates, actin morphology and branching frequencies (Schmid & Harold, 1988; Jackson & Heath, 1989; Dicker & Turian, 1990; Robson *et al.*, 1991; Gow *et al.*, 1992; reviewed in Gow, 1994a and Gooday & Gow, 1994). There are several classes of calcium channels, including those that are opened by membrane depolarization (voltage-gated channels) and those which are not. The conductance of voltage-gated channels would be increased by a reduction in membrane potential, while the conductance of non-voltage-gated channels would be decreased (Robinson, 1985; Bedlack *et al.*, 1992; Davenport & Kater, 1992). These properties seem to fit the requirements for a transducer of the electrical field effects we have observed.

Our most recent model for the mechanism by which electrical fields induce polarized growth incorporates both protein electrophoresis and induced membrane potential effects. This model proposes that anodotropic fungi may have an apex enriched for non-voltage-gated channels and that the main influence of the electrical field is to bring about anodal electrophoresis and increased conductance of these channels (Fig. 6). For cathodotropic fungi it is proposed that the electric field effect is due mainly to the opening of calcium channels at the depolarized, cathodal end of the cell. In both cases it might be envisaged that the immediate consequence of the induced asymmetries in calcium-ion transport is to create local hot spots in cytoplasmic calcium concentration and associated alterations in calcium-stimulated processes such as actin polymerization, vesicle exocytosis and chitin synthesis that may be critical for tropic growth and cell extension (Brawley & Robinson, 1985; Onuma & Hui, 1988; Martinez-Cadena & Ruiz-Herrera, 1987; Jackson & Heath, 1993b; Levina *et al.*, 1994). It should be noted that not all electrical field responses can be mediated by actin since we have shown that bacteria also exhibit marked galvanotropic responses (Rajnicek *et al.*, 1994). However, electrical fields are potentially useful tools for studying hyphal growth since they provide a means for creating active alignments of hyphae and studying what processes are necessary for such orientation responses.

Molecular biology of hyphal growth

The minimal extent to which molecular biology has impinged on the study of tip growth of filamentous fungi contrasts starkly with the extensive genetic analyses of the growth cycle of budding and fission yeasts (e.g. Drubin, 1991; Madden *et al.*, 1992). Our work on the molecular biology of hyphal development has again been focused on *C. albicans*. Because this fungus is asexual and constitutively diploid it is recalcitrant to genetic analysis. Nonetheless, methods are now in place to isolate, analyse and disrupt *Candida* genes and thereby gain a clearer view of the regulation of the yeast to hypha dimorphic transition and virulence of this pathogenic organism.

Fig. 6. Model accounting for the anodotropic and cathodotropic growth of different filamentous fungi. In (a) for an anodotropic fungus it is proposed that the electric field displaces non-voltage-gated calcium channels to the positive pole and that the field increases conductance through the channels by hyperpolarizing the apex. In (b) for a cathodotropic fungus it is proposed that the electric field opens voltage-gated channels at the depolarized cathodal end of the cell. In both cases increased calcium ion conductance is proposed to stimulate local growth, perhaps by stimulating actin assembly. From De Silva *et al.* (1992), with permission.

Molecular approaches

In collaboration with Al Brown and Graham Gooday at Aberdeen we have isolated and analysed a range of genes whose expression is regulated by the yeast to hyphal transition or is implicated in pathogenicity. To assess the importance of a given gene in dimorphism and pathogenicity we have first performed detailed Northern analyses to determine the temporal pattern of gene expression during growth and dimorphism and then created null mutant strains to assess the phenotypic consequence of gene disruption. Gene disruption in diploids such as *C. albicans* is not trivial because disruptions must normally be made in each of the two homologous alleles at each locus to observe a phenotype. In addition, there is a paucity of available selectable genetic markers in this organism, which constrains the number of sequential gene replacement experiments that can be performed. A further bugbear is that *C. albicans* has a non-universal codon usage so that CUG codons are decoded by a novel seryl tRNA rather than with leucine (Santos *et al.*, 1993). This non-standard translational event prevents the use of standard heterologous reporter genes (*lacZ*, *gus*, *luc*, etc.) or heterologous selectable markers in *Candida*.

Kleckner's group devised a method, called 'ura-blasting', that enables multiple disruptions of *Saccharomyces* genes to be made using *URA3* as a single selectable marker (Alani *et al.*, 1987). This method employs a ura-blaster cassette in which the selectable marker, *URA3*, is flanked by a directly repeated sequence of a bacterial origin, *hisG*. This *hisG::URA3::hisG* cassette is cloned into the open reading frame of the isolated gene target to create a disruption cassette *in vitro*. This is used to inactivate the target gene by integrative transformation in a *ura3*⁻ background, selecting for uridine prototrophs (Alani *et al.*, 1987). After the first allele has been disrupted, the selectable marker can be recovered by treatment of *ura3*⁺

transformants with 5-fluoroorotic acid (FOA), which selects for *ura3*⁻ segregants. FOA is a substrate for the wild-type *URA3* gene product (orotidine-5'-phosphate decarboxylase), which converts it into the toxic derivative 5-fluorouracil (Boeke *et al.*, 1984). As a result, all cells are killed except those segregants that have undergone a *cis*-recombination event between the two *hisG* copies with the consequential loss of the *URA3* gene. The result is that the *URA3* selectable marker can be recycled many times to disrupt second or further alleles (Alani *et al.*, 1987). This method has now been adapted for use in *Candida* by Fonzi, who made constructs incorporating the *C. albicans URA3* gene into the ura-blaster cassette and generated isogenic auxotrophic strains of *C. albicans* which have not been exposed to random mutagenesis programmes (Fonzi & Irwin, 1993; Gow & Fonzi, 1994). The availability of these tools is having a major impact on our ability to perform precise reverse genetic experiments in *C. albicans*.

Genetics of dimorphism

One possible approach to understanding regulation of fungal dimorphism is to isolate and analyse genes that are expressed preferentially in either the budding or hyphal growth forms. We used polyclonal antisera from candidosis patients, many of whom had AIDS, to screen a λ ZAP cDNA expression library which had been prepared from mRNA isolated from hyphal cells of *C. albicans* (Swoboda *et al.*, 1993). Despite the fact that the antisera we used were first depleted for antibodies that reacted with yeast cell extracts, the cDNA clones that were isolated and identified by sequencing did not encode hyphal-specific functions. Instead, amongst the ten genes analysed in detail, three encoded glycolytic enzymes and one encoded a ribosomal binding protein (Swoboda *et al.*, 1993; R. K. Swoboda and others, unpublished). The expression of all of these genes was regulated during

dimorphism, but there was no tight correlation between the temporal pattern of expression and the kinetics of germ-tube emergence (Swoboda *et al.*, 1993, 1994a; Gow *et al.*, 1995). We have now examined the expression of nearly 30 mRNAs during the yeast to hyphal transition of *C. albicans* (Gow *et al.*, 1995). Of these, only two showed no significant change in mRNA level during the induction of hyphal growth. Most mRNAs showed a transitory or sustained increase, but some showed a transitory or sustained decrease in expression. Since many of these mRNAs encoded proteins with housekeeping functions (glycolytic enzymes, heat-shock proteins, translation factors, etc.), we conclude that many or most *C. albicans* genes are regulated during morphogenesis, but few of these genes play an active regulatory role in the control of dimorphism (Gow *et al.*, 1993, 1995; Swoboda *et al.*, 1993, 1994a, b). A few genes have been found whose expression is increased specifically during germ-tube formation, i.e. whose expression was not strain dependent or related to the method of germ-tube induction or to the physical and chemical changes in environment that are imposed on cells during germ-tube growth. These few genes include *HYR1* (for hyphal regulation), which was isolated as a false positive in an immunological screen for a signal transduction pathway gene (A. P. J. Brown, D. A. Bailey, P. Feldmann, M. Bovey & N. A. R. Gow, unpublished; Gow *et al.*, 1995), and three secretory aspartyl proteinase genes (Hube *et al.*, 1994), discussed below. We are now performing the disruption of the *HYR1* gene in *C. albicans* to establish its role in hyphal growth. One aim of these studies is to obtain a genetically clean dimorphic mutant that could be used to establish the significance of the yeast to hyphal transition in *Candida* infections.

Chitin synthesis

Since chitin is a major structural component of the fungal cell wall, it is likely to be important for the control of cell-shape changes associated with dimorphic transitions in *C. albicans*. Three chitin synthase genes have been isolated and sequenced from *C. albicans* (Au-Young & Robbins, 1990; Chen-Wu *et al.*, 1992; Sudoh *et al.*, 1993). Each gene has an equivalent homologue in *S. cerevisiae*, where the function of each has been analysed carefully by gene disruption (Bulawa, 1993). Despite the sequence homologies between the *Candida* and *Saccharomyces* genes, gene disruption analysis has shown that corresponding homologues may not be functionally equivalent. While on sabbatical in Phil Robbins' laboratory, I disrupted the *C. albicans* *CHS2* gene using the ura-blaster protocol outlined above (Fig. 7; Gow *et al.*, 1994b). Chen-Wu *et al.* (1992) had shown that this gene was expressed preferentially in the hyphal form. We later confirmed this and showed that *CHS3* (*CSD2*) expression also increased in hyphae in a variety of media. We also showed that *CHS1* was expressed in both yeast and hyphae, but not in hyphae grown using *N*-acetylglucosamine as a chemical inducer (Schofield, 1994; Gow *et al.* 1995). In this light it was of interest to determine whether germ-tube formation would be affected by disruption of the *C. albicans* *CHS* genes. The

Fig. 7. Sequential disruption of the *C. albicans* *CHS2* gene using the ura-blaster protocol. The structure of the wild-type and disrupted Δ *hisG::chs2* alleles and the probe used to screen transformants is shown in (a). The open reading frame is indicated by the open box. Restriction sites relevant to the Southern analysis (b) are X (*XhoI*), P (*PstI*), H (*HindIII*) and H (destroyed *HindIII* site). Lane 1 is DNA from the parental strain and lanes 2, 3 and 4 are from strains following one, two or three rounds of transformation, strain purification and treatment with 5-FOA. For *PstI* digests, the disruption results in the loss of a band at 4.7 kb and a new band at 4.0 kb. For *XhoI* digests, wild-type alleles generate two bands at 3.6 and 5.6 kb and disrupted alleles generate a single band at 8.5 kb. Lane 4 therefore is a homozygous null mutant. The relative band intensities are reversed in lanes 2 and 3, suggesting that there were three *CHS2* alleles in this strain. In lane 2 there are two wild-type alleles and one null allele, while in lane 3 there are two null alleles and one wild-type allele. From Gow *et al.* (1994b), with permission.

phenotype of the *chs2* homozygous null mutant was such that it produced germ tubes as efficiently as the parental strain but at a slightly slower rate. The germ tubes had a 40% reduction in their chitin content, but the chitin content of yeast cells was not affected (Gow *et al.*, 1994b). The virulence of prototrophic *chs2* null mutants was attenuated slightly, but the change was barely significant. By comparison of nucleotide sequences, the *Candida* *CHS2* gene is more similar to the *Saccharomyces* *CHS1* gene and the *Candida* *CHS1* gene is more similar to the *Saccharomyces* *CHS2* (Bowen *et al.*, 1992). However, the characteristics of the *Candida* *chs2* null mutants contrast with the phenotype of either the *S. cerevisiae* *chs1* mutant, which has a defect in the repair of the post-partition septum, or *chs2*

mutants, which have no primary septum and a normal or elevated chitin content (see Bulawa, 1993, for a review). Recently, we have shown that the *Candida chs1* homozygous null mutant is probably lethal (C. Munro, A. Buchan, A. Brown & N. Gow, unpublished). This again contrasts with the phenotype of the equivalent null mutant in brewers' yeast, where *CHS2* (and *CHS1*) have been shown to be non-essential (Bulawa *et al.*, 1986; Bulawa & Osmond, 1990). This is particularly interesting in view of the fact that the *Candida CHS1* gene was isolated by heterologous expression in a *Saccharomyces chs1* mutant. These findings make the point that mutations in equivalent genes in *Candida* and *Saccharomyces* may not generate the same phenotype.

Putative virulence genes

C. albicans is normally a commensal of humans and it has been argued that the term 'virulence factor' is misleading in this context. However, *C. albicans* is the most common fungal infection in humans and several aspects of its general physiology, including germ-tube formation, do seem to correlate with the invasiveness of various clinical isolates. In particular, the relative ability to adhere to surfaces and the level of protease production of a strain seem to be most important for virulence (Cutler, 1991; Matthews, 1994).

We have therefore examined the expression of the ever-increasing family of *C. albicans* secretory aspartyl proteinase genes (Hube *et al.*, 1991; Wright *et al.*, 1992; White *et al.*, 1993; Miyasaki *et al.*, 1994; Monod *et al.*, 1994). Of the seven genes studied, six were found to be regulated differentially and one was silent under all conditions examined (Hube *et al.*, 1994). *SAP1* and *SAP3* were regulated in response to the phenomenon of phenotypic switching (for a review see Soll, 1992) and *SAP2* was found to be the dominant transcript in budding cells. Indeed, alignments of the N-terminal amino acid sequences indicated that essentially all biochemical analyses of *C. albicans* proteases that have been reported to date may relate solely to the activity of the *SAP2* gene product (Hube *et al.*, 1994). An interesting finding was that *SAP4*, *5* & *6*, which are closely homologous sequences, were expressed only in germ tubes in media containing serum as a source of nitrogen. It is possible that these gene products may have a non-enzymic role, possibly in adherence. The argument for this is that aspartyl proteinases have an acidic pH optimum and are not active at neutral pH, which favours filamentous growth of *C. albicans*. Also, the aspartyl protease inhibitor pepstatin A has been shown to inhibit adherence of *C. albicans* to tissue surfaces, suggesting that proteases might play a role in adherence (Borg & Rchel, 1988). In order to clarify the roles of these various genes and their importance as putative virulence factors we are now undertaking a collaborative project with Michel Monod's laboratory to create the requisite strains with single and multiple gene disruptions at *SAP* loci.

Mannoproteins are the dominant antigens, adhesins and immunomodulators in *C. albicans* (Cutler, 1991; Douglas, 1992; Calderone, 1993). We have also begun an analysis of

genes encoding the mannosyltransferase gene family (Gow *et al.*, 1995; C. Westwater, E. Buurman & N. A. R. Gow, unpublished). Isolation and disruption of specific mannosyltransferase genes should enable us to make specific alterations in the O- and N-linked glycosyl oligosaccharides and thereby assess the contribution of particular mannan moieties to the host-fungus interactions.

Future directions and anastomosis

Hyphal growth is of interest in terms of the basic process by which cell polarity is established and maintained and because hyphal growth is linked intimately with many important biotechnological, ecological, and pathological processes. Although the filamentous fungi are a diverse and critically important group of organisms our detailed understanding of their growth remains sketchy. The molecular biological analysis of hyphal growth is as yet in its infancy and it has been argued that molecular biology may not provide the appropriate means to deduce answers to some of the most difficult questions, such as how hyphal morphogenesis is controlled (Harold, 1990). However, perhaps our best hope for improving our understanding of the fungal lifestyle is to arrange the proper marriage of cellular, physiological and molecular approaches to piece together the essential molecular, sub-cellular and cellular aspects of hyphal growth. Alexander Flemings' legacy to mankind is but one example of the rewards of studying filamentous fungi and their properties. It is likely that the study of hyphal growth will surely continue to inspire and reward fundamental and applied mycological research in the years to come.

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