The organization of tip-growth-related organelles and microtubules revealed by quantitative analysis of freeze-substituted oomycete hyphae

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Summary

The distribution of organelles and microtubules in hyphal tips of the oomycete, *Saprolegnia ferax*, were quantitatively determined at high resolution from serial-section electron microscopy of freeze-substituted cells. All the organelles and the microtubules were non-uniformly distributed, each showing a characteristic longitudinal gradient starting at a different point behind the tip. In addition, when the cytoplasmic cross-sectional area was divided into radial regions, all organelles occurred preferentially in either the central (mitochondria and Golgi bodies) or the peripheral (microtubules, wall vesicles and spherical vesicles) region. The nuclei were so large as to span both regions but were always oriented with their centrioles facing the plasmalemma. Microtubules occurred in the extreme tips, became more abundant sub-apically, were predominantly short but increased in mean length with distance from the tip. The correlated patterns of organelle and cytoskeleton organization from this and previous work show that neither the microtubules nor the detected arrays of actin are sufficient to account for most organelle arrangements. However, on the basis of the distribution and orientation of the predominantly elongated wall vesicles, we suggest that the wall vesicles travel radially from their origin at the centrally located Golgi bodies to the cell periphery where they are transported longitudinally to the hyphal tip in conjunction with the plasmalemma-associated actin cables. Our data also suggest that the hyphae contain a cortical ectoplasm with which the nuclei interact, at least in part, *via* their centrioles and centriole-associated microtubules, and whose mechanical integrity is increased by both the peripheral actin cables and a high density of microtubules. We suggest that the endoplasm is less strong and has physiological properties that enhance the differentiation of endoplasmic reticulum and nuclear envelope into Golgi body production.

Key words: tip growth, microtubules, actin, organelles, organelle motility.

Introduction

Tip-growing cells such as hyphae, pollen tubes and root hairs are highly polarized. This polarization is at least partially determined by the requirements of localized cell wall synthesis. For example, vesicles carrying cell wall precursors reach their highest concentration at their site of exocytosis in the extreme tip of the cell (e.g. see Girbardt, 1969; Grove & Bracker, 1970; Grove *et al.* 1970; Heath *et al.* 1985; Hill & Mullins, 1980; Howard & Aist, 1980). However, other organelles also show a non-random distribution in the apical cytoplasm. For example, nuclei maintain well-regulated positions (e.g. see Heath, 1982; Herr & Heath, 1982; McKerracher & Heath, 1985) and mitochondria change in abundance along at least some hyphae (e.g. see Girbardt, 1979). These distributions provide clear evidence for a dynamic and selective system capable of generating and maintaining the patterns as the entire ensemble moves forward with the growing tip and presumably indicate largely unknown physiological requirements of the tip-growing systems. A comprehensive analysis of the organization of all organelles involved in the tip-growth process may help explain the physiology of the process and knowledge of the systems that generate the organization should indicate likely control mechanisms. Despite the morphogenic importance of the tip growth process to most plants, fungi and many algae, we currently have no clear analysis of the overall organization of all organelles in any organism and no comprehensive account of the elements responsible for generating that organization. Certainly there is evidence to show that microtubules play a role in tip organization (e.g. see Girbardt, 1979; Howard & Aist, 1980; Heath, 1982; Herr & Heath, 1982), but their precise involvement is unclear and it is even less clear how different organelles can be variously positioned in a

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common cytoplasm by a single cytoskeletal element. Furthermore, we now know that actin is concentrated in most growing tips but we lack clear indications of what, if any, its role is in the process. In principle, a high-resolution analysis of the patterns of organelle, microtubule and actin distributions correlated with organelle behaviour *in vivo* should clarify some of these uncertainties. Unfortunately such studies are technically difficult and have not been carried out in any tip-growing system. Descriptions of actin have generally not been correlated with organelle dispositions, except in one case where the behavior of a single type of organelle was found to be unrelated to actin organization (Heath, 1988). Most extensive descriptions of the overall organization of microtubules have employed immunocytochemistry, but they have not described the correlated organelle patterns and are of inferior resolution and of questionable reliability due to the rather harsh nature of the preparative techniques. Quantitative ultrastructural studies of microtubule patterns have not focussed on hyphal tips but have indicated an indirect relationship to nuclear positioning (e.g. see McKerracher & Heath, 1985, 1986; Meyer *et al.* 1988). Genetic analyses, again not focussed on hyphal tips, have suggested that whilst nuclear behavior is regulated in some way by microtubules (e.g. see Oakley & Morris, 1980) mitochondrial distribution is not (Oakley & Rinehart, 1985). Even the few studies of organelle distribution in hyphal tips have been mostly non-quantitative and focussed only on longitudinal distribution patterns (e.g. see Grove *et al.* 1970; Girbardt, 1969, 1979; Howard & Aist, 1980; Zalokar, 1959); thus non-random radial distributions would have been overlooked. In an attempt to clarify the total organization of organelles and cytoskeleton in a tip-growing system, we have undertaken a serial-section transmission electron microscopy analysis of freeze-substituted hyphal tips of the oomycete, *Saprolegnia ferax*. This technology provides the best currently attainable resolution and preservation, and these hyphae have been well suited because they are fast growing, large enough to reveal radial as well as longitudinal distribution patterns and have one of the most elaborate actin arrays known for any tip-growing system (Heath, 1987).

Materials and methods

Hyphae of *Saprolegnia ferax* (Gruith) Thuret (ATCC no. 36051) were grown on locust bean gum-coated dialysis membrane strips as described (Heath, 1987) for 22 h at approximately 23 °C. Portions of these strips bearing hyphae from the colony margin were cut and left on the agar surface to resume growth for approximately 1 h. These squares of tubing were rapidly (~2–3 s) transferred to a slam freezer, cryofixed and freeze-substituted as described (Heath, 1984). Hyphae were flat-embedded and tips showing normal morphology with no evidence of ice crystal formation were selected and mounted vertically for sectioning. Serial sections of these hyphal tips were collected on single slot grids and stained in uranyl acetate and lead citrate. A careful record was kept of all sections cut so that the precise number of each section recorded was known. Series encompassing the absolute hyphal tip (section number 0) were recorded by photographing approximately every third section and printing it at a final magnification of 37 600 times life size. Section thickness was assumed to be a uniform 60 nm (one tip) or 70 nm (two tips) throughout the series, on the basis of the number of sections needed to traverse approximately spherical structures in parts of the series. Two additional tips were analyzed from partial series of sections of hyphae grown in liquid medium and fixed by a conventional glutaraldehyde and osmium tetroxide protocol as described (e.g. see Heath, 1980).

In order to determine the radial distribution of organelles, the cytoplasmic areas of cross-sections were divided into a peripheral 44 % and a central 56 %. These values were selected because they coincided with the area values achieved on a reducing xerographic copier operated at 75 % linear reduction. The procedure used was to trace the outline of a photograph of a cross-section and reduce it on the copier to give an image of the outline encompassing only 56 % of the area of the original outline. The reduced outline was placed on a light box and the original photograph was superimposed in exact register and centered over the outline so that the outline showed through the photograph. Organelles were then scored in either the inner or outer region of the cytoplasm. Organelles spanning the boundary line were assigned to the zone containing the largest portion. In addition, the longest dimension of each wall vesicle profile (Heath *et al.* 1985) was also recorded. These analyses were performed on approximately every 20th section through the series to obtain representative samples through the length of the series.

The distributions of wall vesicles in the sub-apical zones of the three freeze-substituted hyphae were determined by constructing a radial grid with 36 lines at 10° intervals on a clear acetate sheet. This grid was centered over ×16 800 magnification prints of approximately every fifth section in the series and the position of each wall vesicle profile lying within 1 cm (= 0·6 μm) of the plasmalemma was recorded.

Data on the association between microtubules and organelles were obtained in one series of sections by analyzing all recorded sections and scoring each microtubule profile as 'associated' with an organelle if it lay within approximately one microtubule diameter of the membrane of the organelle. In a related analysis, mitochondria were tracked through the sections and each mitochondrion was thus identified, and its length and number of associated microtubules were recorded.

Microtubule lengths in one series of sections were recorded by determining their termination points in the series. However, since only approximately every third section was recorded, this procedure underestimates their true length.

Actin was fixed and stained with rhodamine-labelled phalloidin using Pipes-buffered formaldehyde as described (Heath, 1987).

Throughout the paper the term 'zone' will refer to a longitudinal portion of the hyphae whereas 'region' will be used to designate peripheral *versus* central cytoplasm.

Results

We have examined the distribution of organelles and microtubules for up to approximately 20 μm behind the hyphal tip. This zone includes the site of tip growth, the expanding region encompassing the transition to the mature hyphal diameter and the most apical populations of nuclei. It is far ahead of the zone of vacuolation so that the full hyphal volume is occupied by cytoplasm and is potentially available to all categories of organelles, i.e. there are no central vacuoles to exclude cytoplasm or

organelles as occurs in sub-apical regions. Representative views of freeze-substituted and conventionally fixed hyphae are shown in Fig. 1 to show the detectability of microtubules and the general quality of preservation of the cells used and to define visually the peripheral and central cytoplasm. The different appearances of the organelles have been reported in numerous studies of freeze-substituted *versus* conventionally fixed cells and are generally irrelevant to the present study. The differences in wall vesicle morphology were described by Heath *et al.* (1985).

Microtubule distribution

In all cytoplasmic areas almost all microtubule profiles are circular, which shows that they lie predominantly parallel to the long axis of the hypha. This conclusion is confirmed by tracking through serial sections. The rare exceptions to this are a few typically short microtubules emanating from the region of the centrioles.

Microtubules do occur in the most apical 1–2 μm but they are not abundant (Figs 2–4). They increase in number through the first 10 μm and become more numerous in the zone of the nuclei. In all zones the number of microtubules fluctuates substantially over short distances. These fluctuations are frequently evident as peaks and troughs defined by more than one point (i.e. section), thus showing that the variability is not a technical function of variable microtubule detectability in individual sections (Figs 2–4). This conclusion is supported by the fact that the cytoplasm predicted to contain a microtubule profile by the presence of one in that area in the preceding section was always examined especially carefully so that the termination of microtubules was very positively identified. The fluctuations in microtubule numbers along the length of the hypha suggest that many microtubules are short. This suggestion is confirmed in one hypha where the average length of microtubules was directly measured to be approximately constant at 0·12 μm over the first 15 μm zone with an increase to only 0·6 μm in the nucleus-containing region (Fig. 2A). In all regions combined, 92·5 % of the microtubules in this hypha were less than 1 μm long, 7·1 % were from 1 to 4 μm and the longest was 4·9 μm. These values are all slight underestimates because of the analysis of every third section only.

Microtubules do occur in both central and peripheral cytoplasmic regions but in all zones they were most abundant in the peripheral regions (Figs 2–4). This pattern is most pronounced in the zone below 8–10 μm (Fig. 4), with a more uniform distribution in the most apical zone where the total number is lower. In all but the most apical 1–2 μm, even those microtubules in the central region were mostly at its periphery (Figs 2, 3).

In all zones there was frequently an uneven circumferential distribution of microtubules but the higher density clusters did not consistently correlate with either one side of the hypha (e.g. the side against the substratum) or with the centriole-containing sectors when centrioles were present (Figs 2, 3).

Organelle distributions

No organelle shows either longitudinal or radial random distribution in the hyphal tip region. However, with the exception of Golgi bodies and nuclei, all organelles *can* occur in virtually all parts of the hyphae examined.

Wall vesicles reach their highest number in the most apical 5 μm zone but their highest density (not measured) is undoubtedly in the most apical ~1 μm zone where they occur almost exclusively of other organelles (Fig. 4). In the most apical zone they are approximately equally distributed between the peripheral and central regions but behind this zone they become much more restricted to the peripheral region (Fig. 4). Because many wall vesicles are tubular (Heath *et al.* 1985), the numbers of vesicles given in Fig. 4 are not strictly numbers of separate vesicles but numbers of cross-sectional profiles that do indicate abundance and relative volume fractions. Their tubular profiles permit analysis of their orientation in the hypha. If they were all perfectly aligned parallel to the long axis of the hypha, then their cross-sectional profiles would all be circular (assuming an even tubular shape) and a histogram of profile length would reveal a normal distribution with variation due only to diameter variation. Any deviation from this alignment would show up in the form of a 'tail' of longer profiles. The percentage of profiles in this tail would be proportional to the degree of deviation to the point where a perfect radial alignment would give all long profiles, the lengths of which would show the lengths of the vesicles. As predicted by the image in Fig. 1A, histograms of the maximum length profiles of wall vesicles typically revealed a fairly narrow peak at about 0·1 μm with a tail of longer lengths (not illustrated). The percentage of profiles greater than the mean is a convenient measure of the abundance of vesicles whose orientation is not parallel to the long axis of the hypha and is independent of possible differences in fixation or magnification fluctuation-induced changes in absolute values in different hyphae. The result of this analysis is shown in Fig. 5. There were, on average, 64 % more long profiles in the central regions relative to the peripheral cytoplasm. This difference was consistent along the lengths of the hyphae except in two regions. In some of the most sub-apical sections low numbers of vesicles produced some aberrant percentages (Figs 4, 5). In the extreme tip, the peripheral values approached those of the central values (Fig. 5). If the percentages of long profiles in the peripheral cytoplasm of the two most apical sections analyzed for each hypha are compared with the values for all other sections by a Student's t-test, the increased tip values are significantly different at the $P < 0{\cdot}001$ level. Thus, the predominantly parallel orientation of wall vesicles in the peripheral region becomes less organized in the extreme tip.

In the apical 20 μm zone, the peripheral wall vesicles tend to be at a sufficiently high concentration to form, typically, a nearly continuous layer adjacent to the plasmalemma. However, as their numbers diminish sub-apically (Fig. 4) they tend to form more discrete clusters in cross-sections. When plotted so that their longitudinal distribution can be seen, they frequently form chains stretching over several μm (Fig. 6). These

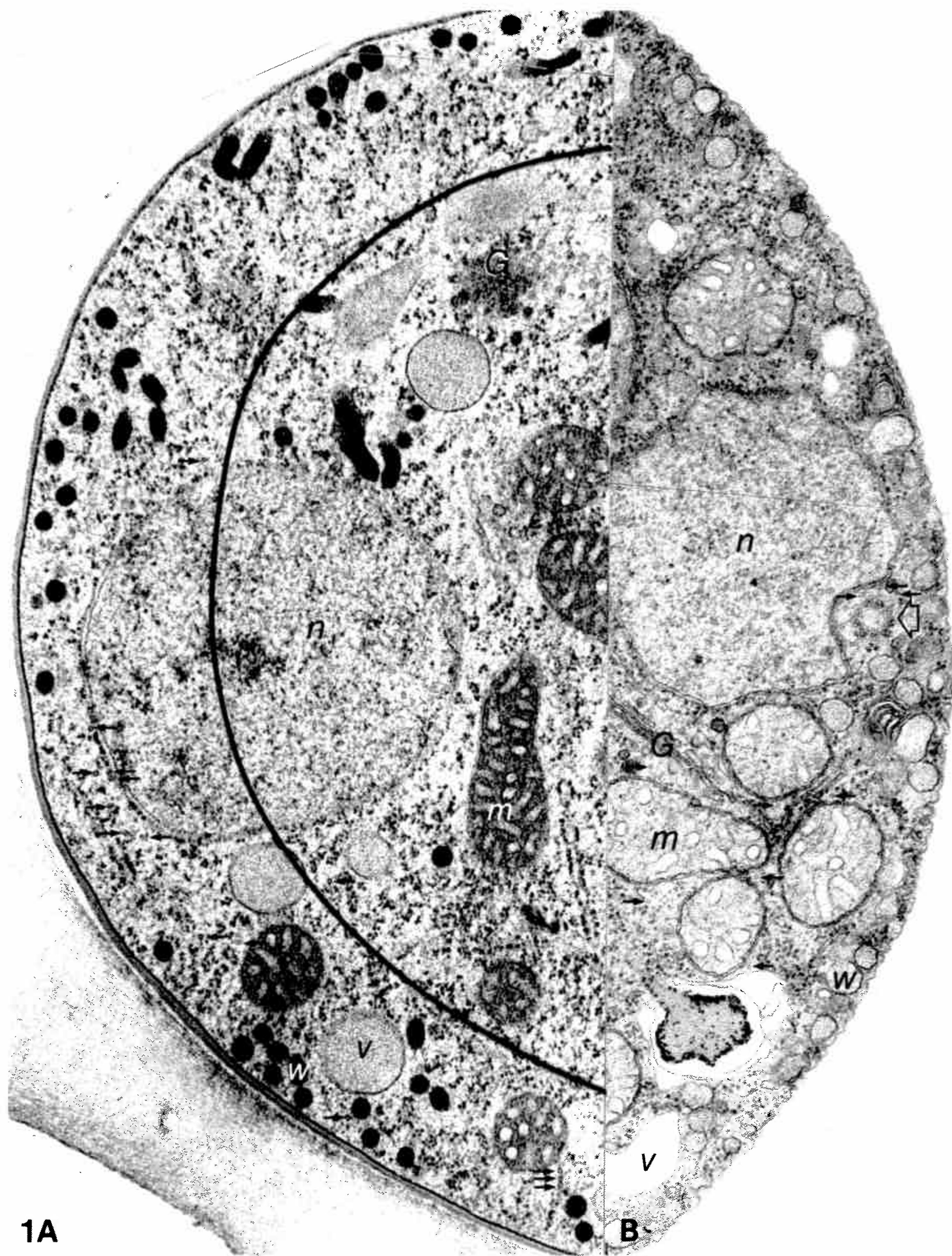

Fig. 1. Portions of sections at 14 μm in the freeze-substituted series shown in Fig. 2C (A), and at 7 μm in the conventionally fixed series shown in Fig. 3 (B), reproduced at the same magnification used for analysis of these series. Microtubules are easily detectable (arrows), as are wall vesicles (*w*), large spherical vesicles (*v*), mitochondria (*m*), Golgi bodies (*G*) and nuclei (*n*). A centriole (open arrow) associated with the nucleus in B is oriented towards the cell wall. The line on A shows the boundary between the central and peripheral cytoplasm as defined in Materials and methods. A,B, ×37 600.

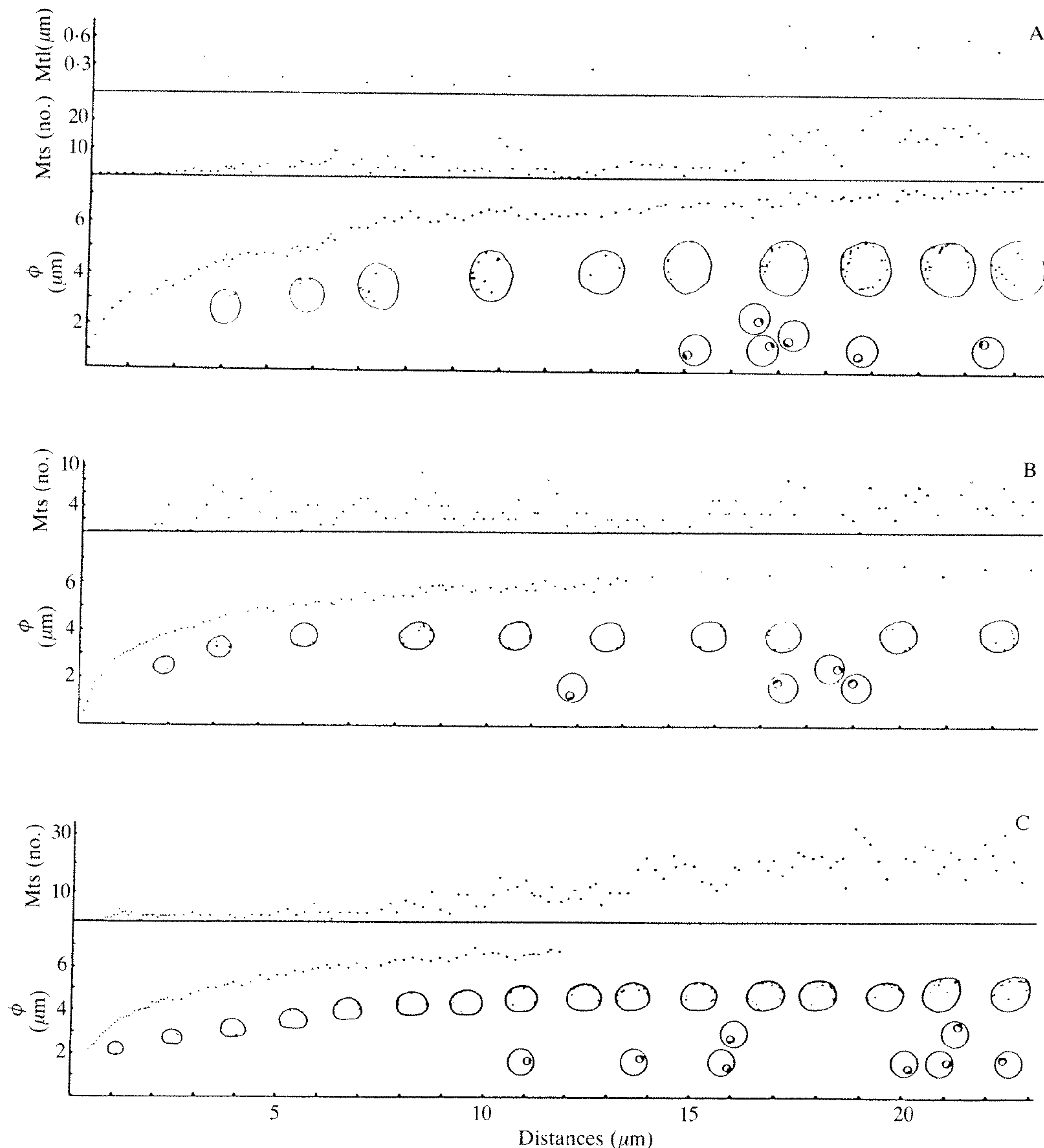

Fig. 2. Diagrammatic representation of key features of the three freeze-substituted hyphae analyzed. For each hypha: A–C, hypha diameter (ϕ, points on lower panels) and microtubule number per cross-section (Mts no.) are shown quantitatively. In the uppermost panel for hypha A only, the mean microtubule lengths in zones centered on the plotted points are shown (Mtl). Radial microtubule distributions are shown in the scale diagrams (produced by xerographic reduction of original tracings) in each lower panel. Nuclear positions (small inner circle) and centriole orientation (filled regions on nuclear circle) are shown diagrammatically in the regular circles in the lower panels. The longitudinal position of the nuclei is shown at the points where these centrioles are located but the nuclei extended considerably beyond these points. For each hypha, the abcissa shows distance from the hyphal tip (defined as the section containing the face view of the apical plasmalemma). Although given in μm, these distances are calculated from section numbers, as are the μm values for the upper panel in A.

chains are somewhat undulating and anastomosing in a pattern similar to the actin cables in the sub-apical regions (Fig. 6).

In contrast to the organization of the wall vesicles, the large spherical vesicles (Fig. 1) are rare in the apical 5 μm, become increasingly abundant sub-apically and show a marked preference for the peripheral cytoplasm throughout their range (Fig. 4).

Mitochondria are rare in the most apical 5 μm but attain their maximum abundance in the 5–10 μm zone and remain at that concentration through the rest of the analyzed zone. In all zones they occurred more frequently in the central cytoplasm but at the 20 μm zone they were more evenly distributed between regions (Fig. 4). Throughout the analyzed zone, in both regions, they are elongated and oriented predominantly with their long axes parallel to the long axis of the hypha. This latter point was demonstrated by tracking all mitochondria in

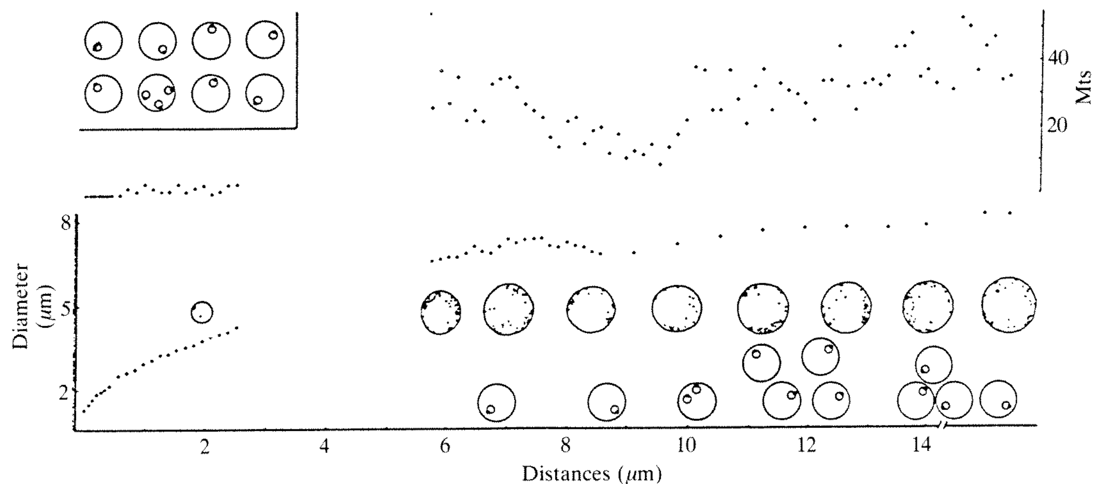

Fig. 3. Diagram of a conventionally fixed hypha constructed in the same way as for Fig. 2. There was a major gap in the series as shown, so the values above 5 μm are estimated and may be inaccurate. The diagrams on the upper left show the characteristics of nuclei in another conventionally fixed hypha partially sectioned through a region similar to the nucleus-containing region of the other hyphae. Mts, microtubules.

one hypha and subjectively ascertaining that most profiles were circular and thus transverse in the other two hyphae.

Golgi bodies are absent from the most apical 5 μm but occur at approximately equal frequencies throughout the rest of the examined zone. In all zones they were strongly concentrated in the central cytoplasm (Fig. 4).

Nuclei do not occur in the hyphal tips, they first appear in the 10–15 μm zone (Fig. 4). Because they are large, they typically span the boundary between the central and peripheral regions but the centrioles on all 40 nuclei examined were located on the peripheral side of the nucleus facing the cell wall (Figs 2–3). These centrioles lay with their centres at a mean distance of 0·7 μm (±0·4 μm, range 0·2 μm–1·6 μm) from the closest plasmalemma.

Microtubule–organelle associations

For reasons given in the Discussion, we have not undertaken an extensive analysis of inter-organelle and organelle–microtubule interactions but we did make two relevant observations. In the hypha shown in Fig. 3, of the 32 mitochondria that were completely within the series of sections analyzed, 78 % were associated with from 1–10 microtubules. Those mitochondria varied in length over a sevenfold range, with longer mitochondria being associated with more microtubules. However, the correlation coefficient of this association was only 0·5. In a reciprocal type of analysis on the hypha shown in Fig. 2A, each microtubule was examined along its length to determine what organelles were associated with it. Of the 268 microtubules, 8·6 % were associated with wall vesicles, 22·0 % with the large spherical vesicles, 18·7 % with mitochondria, 12·7 % with nuclei and 41·0 % had no associated organelles. These values total more than 103 % because some microtubules are associated with more than one organelle. When these associations were analyzed in each zone, as was done for microtubule length patterns, there were variations between zones but the variations did not correlate with any organelle distribution patterns or distance from the tip, except for the obvious absence of nuclear associations in the nuclear-free zone.

Fig. 7 summarizes, in semi-quantitative scale-diagrammatic form, a synthesis of all of the above results.

Discussion

The present results, combined with previous actin localizations in *Saprolegnia* hyphae (Heath, 1987), provide the only quantitative analysis of the organization of the cytoskeleton and organelles using the best currently available high-resolution three-dimensional techniques applied to a tip-growing cell. However, because even freeze substitution fails to reveal the actin system known from light microscopy (Heath, 1987) and there is evidence for less highly ordered actin permeating the entire cytoplasm (McKerracher & Heath, 1987), we cannot be confident that all structurally important components are yet revealed. Nevertheless, our data are the best possible with current technology and reveal many interesting features.

In general terms, our results show that *all* organelles occupy non-random distributions. These distributions cannot be maintained by any exclusionary mechanism because all organelles can be found in all regions and zones (with the exception of the exclusion of the nuclei and Golgi bodies from the extreme hyphal tips), nor does the simple presence or absence of either detected cytoskeletal system (i.e. microtubules or actin filaments) suffice. Thus we conclude that all organelles have the capacity to interact differentially with some unknown skeletal system or that there is extensive selectivity and non-obvious interactions with the known systems. Before discussing some of these possibilities further, it is important to emphasize the dynamic nature of the system because the tip itself is constantly growing forward at a

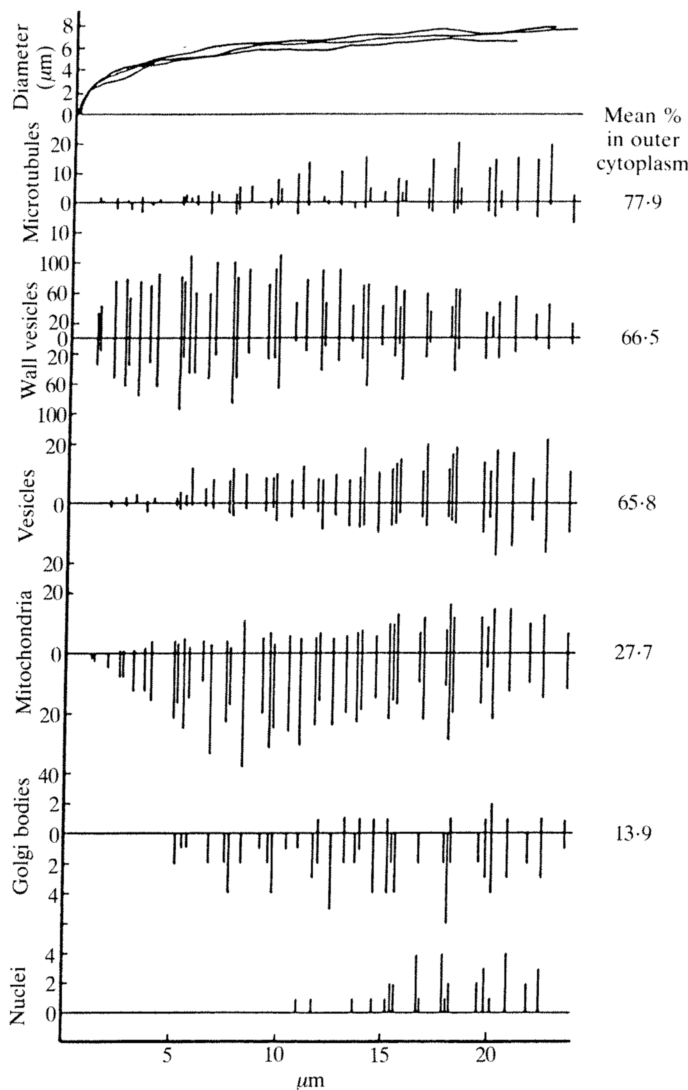

Fig. 4. Organelle distributions in the three freeze-substituted hyphae shown in Fig. 2. Hyphal diameters are shown in the upper panel. The lower panels show the number of organelle profiles in sections selected along the length of the hyphae. These numbers are shown as being in the central (below the zero line) or peripheral (above the line) cytoplasm, except for the nuclei, which typically traversed both regions and are therefore shown as total numbers. The mean percentage of profiles in the peripheral cytoplasm is shown to the right. Since the peripheral cytoplasm represents 46% of the total cytoplasm, a uniform distribution of organelles would give a value of 46% in this column. The separate values for each hypha are not identified, but for clarity different section numbers were selected for each hypha.

rate of about 12 $\mu m\ min^{-1}$ and the organelles are probably undergoing some synthesis and division, although the precise sites of organelle multiplication are totally unexplored.

We shall first discuss the contributions of our data to the understanding of organelle distribution mechanisms then deal with the characteristics of the oomycote microtubule system compared with those of other tip-growing cells.

Organelle-distribution mechanisms

One of the most enigmatic questions in tip-growth systems is the mechanism responsible for transporting the wall vesicles to the tip. We know that they are produced by the sub-apical Golgi bodies (Heath *et al.* 1971, 1985) and that they migrate to the tip prior to exocytosis. The analogy with microtubule-based vesicle transport in axons is obvious but, while disruption of microtubules can alter vesicle distribution patterns (Howard & Aist, 1980), it has long been recognized (Heath, 1975) that the frequency of wall vesicle–microtubule interactions is lower than expected if current axonal models (e.g see Sheetz *et al.* 1986) apply to tip-growing cells. Furthermore, if these models apply, it is difficult to explain the observations of normal tip growth in cells treated with anti-microtubule agents or with genetic lesions that render microtubules incompetent to move nuclei (e.g. see Oakley & Morris, 1980; Herr & Heath, 1982; Oakley & Rinehart, 1985; Meyer *et al.* 1988). Our data suggest an actin-based system as follows.

Because the wall vesicle-producing Golgi bodies are concentrated in the central cytoplasm, whereas the wall vesicles are relatively rare in the sub-apical central cytoplasm and most abundant in the peripheral regions, the majority of the wall vesicles must either be transported radially to the periphery then longitudinally, or directly longitudinally and very rapidly (therefore few would be detected in transit) in the central cytoplasm. We can test these alternative models in two ways with our data. First, because the wall vesicles are typically elongated (Heath *et al.* 1985), the mode of transport offering least resistance, and thus the most likely, would move them parallel to their long axes. Therefore, their orientation should indicate their direction of movement. Our data show that they are more radially oriented in the central cytoplasm and predominantly longitudinally oriented in the peripheral cytoplasm, except at the extreme tip where they are less oriented and presumably undergoing little transport having accumulated very close to their destination. These data are most consistent with a radial–longitudinal pathway. Second, if one assumes that each Golgi body in the studied zone contributes transported wall vesicles at an average uniform rate, then the number of vesicles at any point downstream (i.e. towards the tip) should be proportional to the number of upstream Golgi bodies. This prediction is shown by a high correlation coefficient when total Golgi bodies and wall vesicles are compared (Table 1, line A). The mean slope of this comparison (3·11) indicates that each Golgi body contributes three vesicles to the population. If transport is radial from the predominantly central Golgi bodies and then longitudinal in the peripheral cytoplasm, the correlation between central Golgi body numbers and peripheral wall vesicles should be high. Conversely, if transport is predominantly longitudinal through the central cytoplasm, the best correlation should be between central Golgi bodies and central wall vesicles. The higher mean and individual values for two of the three hyphae (line C *versus* line B, Table 1) support the radial–longitudinal model. Furthermore, if the radial–longitudinal model is not operating then the peripheral vesicles must

Table 1. *Correlations between Golgi body and wall vesicle distributions*

Comparison		Hypha A	Hypha B	Hypha C	Mean ± S.D.
A. Total Golgi bodies *versus* total wall vesicles					
	r	0·87	0·97	0·91	0·92 ± 0·05
	s	2·63	4·62	2·08	3·11 ± 1·34
B. Central Golgi bodies *versus* central wall vesicles					
	r	0·75	0·92	0·58	0·75 ± 0·17
	s	1·62	3·03	0·62	1·76 ± 1·21
C. Central Golgi bodies *versus* peripheral wall vesicles					
	r	0·90	0·79	0·90	0·86 ± 0·06
	s	1·75	2·41	1·61	1·92 ± 0·43
D. Peripheral Golgi bodies *versus* peripheral wall vesicles					
	r	0·90	0·83	0·77	0·81 ± 0·10
	s	8·76	12·36	17·72	12·95 ± 4·51

These values were obtained by comparing the number of wall vesicles in each region in selected sections (those used for Figs 4, 5) with the number of Golgi bodies in each region sub-apical to each section. For example, the most sub-apical Golgi body in the series was considered to be number 1 and therefore in the next more apical section there would be one Golgi body to produce the wall vesicles in that section, and in the section following the appearance of the next Golgi body there would be two and so on to the maximum number at the most apical Golgi body. This procedure is erroneous, in that there are more Golgi bodies below the lowest in the series, but this factor would only alter the position of the baseline number of vesicles, it should not influence the slope (s) or correlation coefficient (r) of the linear regression lines of the data through the zone of Golgi bodies examined.

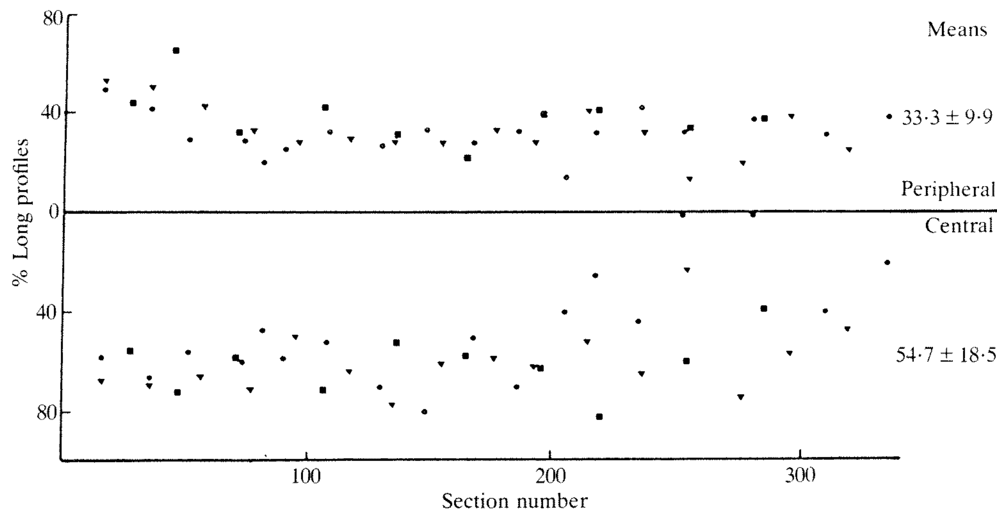

Fig. 5. Comparison of the length of profiles of wall vesicles in the peripheral and central cytoplasm in the same sections used for the data shown in Fig. 4. Percentage of profiles larger than the mean length for the total population of peripheral vesicles (ordinate) is shown in the two zones compared with distance from the hyphal tip (section number 0). A paired sample t-test of the arcsine-transformed percentages in the two regions in each section showed that they were indeed different at the $P < 0{\cdot}001$ level. Separate symbols are used for each hypha, circles for A, squares for B and triangles for C, with the letters as used in Fig. 2.

be produced predominantly by the peripheral Golgi bodies. While the correlation for this comparison is good (line D, Table 1) the mean slope (12·95) indicates that the peripheral Golgi bodies must be four times more active than the average population, which is unlikely.

Taken together, the above arguments and data are most consistent with the radial–longitudinal model, in which case a track-like role for microtubules is unlikely because they do not show a radial pattern in the central cytoplasm. Furthermore, the predominantly short lengths of the microtubules argue against a track-like involvement. The most obvious alternative is one involving actin, evidence for which is found in organelle translocations in other cell types, including pollen tubes (e.g. see Williamson, 1986; Adams & Pollard, 1986; Staiger & Schliwa, 1987; Herth *et al.* 1972; Franke *et al.* 1972). The detected actin arrays in the tip zone are exclusively peripheral and longitudinally oriented. This pattern, together with the observed close association between many wall vesicles profiles and the plasmalemma (e.g. see Fig. 1) supports the actin model for the longitudinal part of the transport pathway. Furthermore, given the elongated shape of the wall vesicles, even those profiles more remote from the peripheral actin arrays could be ends trailing from closely associated vesicle tips. The tendency for the wall vesicles in the sub-apical 40–50 μm zone to form undulating and anastomosing lines very similar in pattern to the actin cables in this zone (Fig. 6)

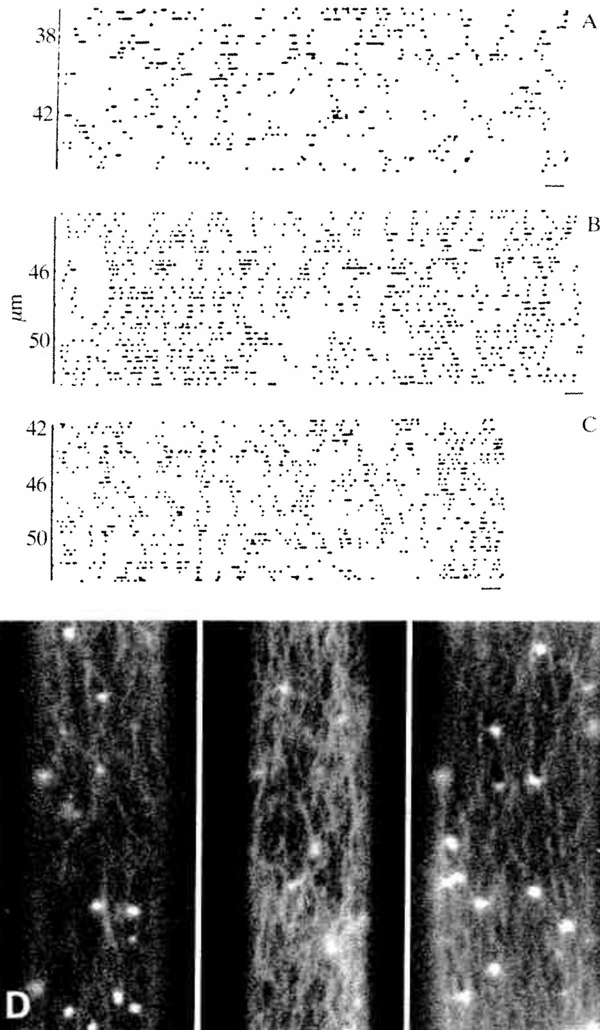

Fig. 6. A–C. The distribution of wall vesicles in the peripheral cytoplasm, adjacent to the plasmalemma, in the 37–53 μm zones of the three hyphae shown in Fig. 2. The distributions were determined as described in Materials and methods, then plotted with the abcissa as 360°. The absolute length of each degree is dependent on the diameter of the hypha in the analyzed zone and is indicated by a 1 μm scale at the bottom of each diagram. This method of plotting in effect shows the distribution as it would be seen if the hypha were slit longitudinally and opened out flat. In life the extreme left and right sides of each plot would be joined. The ordinate is given in μm as calculated from section numbers from the hyphal tip. The alignment of vesicle profiles is best seen by holding the page close to parallel to the line of sight and sighting along the page. The predominance of approximately longitudinal files (i.e. vertical) can be ascertained by similarly viewing the diagrams from other angles (e.g. the side) when comparable chains are absent. The size of the dots are approximately 50% above the life size of a transversely sectioned wall vesicle. In D are shown similarly magnified views of the peripheral actin array from a comparable region of hyphae to that shown in A–C. The similarity in pattern of the files of vesicles and actin cables is evident. ×2200.

is also consistent with them moving along the peripheral plasmalemma-associated actin cables. For the radial transport component, there is evidence for a low density, presumably more diffuse, actin array in the central cytoplasm (McKerracher & Heath, 1987), the orientation of which could be that required for this component. We conclude that wall vesicle transport is most likely *via* interactions between their membranes and actin filaments and that the few (8·6%) microtubules associated with them occur by chance alone.

Mitochondria are more likely than wall vesicles to be distributed *via* an interaction with microtubules. Morphological (e.g. see Heath & Greenwood, 1970; Heath & Heath, 1978) and inhibitor (Herr & Heath, 1982) studies in *S. ferax* and other fungi support this idea, as do our observations that 18·7% of microtubules are associated with mitochondria and 78% of mitochondria are so associated. However, the association data in this study are not convincing because we have not been able to devise a statistical test to determine whether the associations are due to chance, dictated by space constraints or to functionally significant causes. Some of our results show that other factors must be involved. For example, mitochondria are most abundant and maintained in longitudinal orientation in the central cytoplasm where microtubules are less frequent than in the periphery. Furthermore, if microtubules were dominant in determining mitochondrial positions, the plateau in abundance of mitochondria in the 5–10 μm zone should correlate with a similar plateau of microtubules, but this is not so. It is also difficult to see how a population of microtubules that are predominantly shorter than the mitochondria can be solely responsible for their positioning. However, our data are consistent with the concept that the microtubules may act as intermediates, possibly transiently, between the mitochondria and another positioning system. This concept would also be consistent with the data of Oakley & Rinehart (1985) for *Aspergillus nidulans*.

Nuclear positioning is justifiably frequently attributed to microtubules (reviewed by Heath *et al.* 1982; McKerracher & Heath, 1987) but the mechanisms are unclear (e.g. see Meyer *et al.* 1988). The unexpected consistent orientation of centrioles towards the cell surface in *S. ferax* is consistent with the idea that nuclear positioning is mediated by centriole-associated microtubules interacting with the plasmalemma as proposed for anaphase B movements by Aist & Berns (1981). The cortical actin arrays associated with the plasmalemma (Heath, 1987) may well be the plasmalemma-linked static component of this interaction. However, neither the presence and organization of such arrays, nor the abundance of cytoplasmic microtubules, are sufficient to determine the distribution of nuclei, because there is no transition in these parameters at the nucleus–nucleus-free boundary zone. Furthermore, the fact that the centrioles are typically lateral to the nuclei, as shown in Fig. 7 and deduced from the serial sections, shows that interphase nuclear positioning is not regulated solely by microtubules transmitting forces to the centrioles and thence to the nuclei, a point made previously with respect to anaphase B movements in this fungus (Heath & Greenwood, 1970). The present observations of many short microtubules in the vicinity of the nuclei are consistent with previous concepts of their role in nuclear positioning

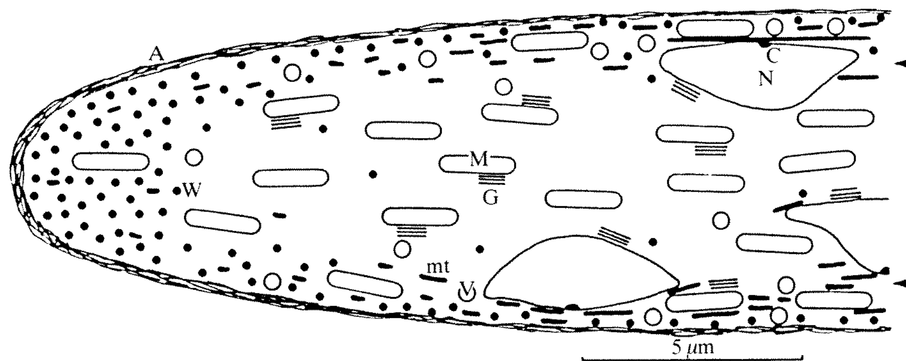

Fig. 7. Scale diagram of the region of hyphal tip analyzed. The salient features of wall vesicles (W), mitochondria (M), Golgi bodies (G), nuclei (N) with centrioles (C), large spherical vesicles (V) and microtubules (mt) are all accurately portrayed in simplified form from the data in Figs 2–5. The peripheral layer of actin (A) is depicted from the data of Heath (1987). Arrows on the right show the boundary between peripheral and central cytoplasm. The plasmalemma and cell wall are omitted for clarity but would be appressed to the exterior of the actin array. Wall vesicles are shown as uniformly spherical for differentiation from the short microtubule profiles but in fact many would be highly elongated.

(McKerracher & Heath, 1985) and thus extends the generality of these ideas to a very distantly related organism. The data shown in root hairs by Lloyd *et al.* (1987) are also consistent with these concepts, although these authors proposed alternative models.

The distribution of Golgi bodies is perhaps the most enigmatic of any of the patterns described here. Because most Golgi bodies are associated with either the nuclei (4 %) or endoplasmic reticulum and mitochondria (80 %) (Heath & Greenwood, 1971) one would predict their distribution to be similar to that of mitochondria, but such is not the case. They are largely constrained from association with the most apical and peripheral mitochondria and the peripheral portions of the nuclei. We have not analyzed the distribution of the endoplasmic reticulum in general because it is hard to recognize in some planes of section. Nevertheless, it is clearly present in both peripheral and central regions of cytoplasm and through all zones, so that Golgi body distribution is unlikely to be a simple function of endoplasmic reticulum distribution. Because Golgi bodies, unlike other organelles, are formed by membrane budding and fusion, it seems most likely that their distribution is determined by physiological regulation of the behavior of portions of the endoplasmic reticulum. Consequently, the predominantly central and sub-apical location of the Golgi bodies is probably indicative of differing physiological conditions in the central cytoplasm. This hypothesis may then explain the otherwise seemingly paradoxical situation of wall vesicle synthesis from central Golgi bodies when vesicle transport seems to occur in the peripheral cytoplasm.

Cytoskeletal functions

In general terms, the distribution and lengths of cytoplasmic microtubules do not correlate well with most types of organelle behavior and distribution, thus indicating either no, or an indirect, role in these processes. Furthermore, the predominantly short microtubules are unlikely to function directly in a track-forming role for the motility of the most prominent motile vesicles (unidentified in this work) because their track lengths are typically in excess of 5 μm (Heath, 1988). The salient features of the microtubule populations of these hyphae include their presence, albeit in low numbers, right to the hyphal tip, their concentrations in the cortical cytoplasm, their diverse but short lengths and the fact that 41 % show no association with any organelle. The hypothesis presented for the role of a similar population surrounding nuclei of another hyphal fungus, *Basidiobolus magnus* (McKerracher & Heath, 1986) fits the present data better than others. This hypothesis suggests that the microtubules function, at least in part, as reinforcing structures by interacting with undefined cytoskeletal elements (for example, the low concentration actin network referred to previously) to effect an increase in the tensile strength of the cytoplasm. This model predicts a higher-strength peripheral cytoplasm with a more fluid central 'endoplasm' and a weaker apical zone but does not preclude organelles interacting with the cytoplasm *via* microtubules. Indeed, there may be two or more populations of microtubules, those interacting with organelles and others reinforcing, as suggested in axons by Miller *et al.* (1987). Equally, there may be other linkages between organelles and the cytoplasm as discussed for the wall vesicles. Thus nuclei may be anchored in the peripheral cytoplasm by both centriole-associated microtubules and other membrane-associated linkages, but their position relative to the tip would be determined by the extensibility of the cytoplasm, which could be increased by anti-microtubule agents, thereby explaining the nocodazole data for these cells (Heath, 1982). A similar argument could explain the nuclear positioning data for root hairs obtained by Lloyd *et al.* (1987). If the peripheral cytoplasm were also linked to the peripheral actin arrays and thence to the cell wall in a manner analogous to focal contacts in mammalian cells, and as suggested to occur during tip growth initiation in *Fucus* zygotes (Kropf *et al.* 1988), one has a basis for an actin-based system to pull the cytoplasm, and associated organelles, forward as the tip extends. This concept equates the fungal protoplast with a tube-dwelling (the

cell wall) amoeba, an idea proposed by Reinhardt in 1892. In this model, the need for reinforcing microtubules in the extreme tip where the actin is most abundant is less, as observed, with greater reinforcement needed sub-apically to sustain the apex-generated tension through the sub-apical regions. Evidence for a peripheral layer of cytoplasm, with associated organelles, bound to the plasmalemma has been presented for *S. ferax* (McKerracher & Heath, 1987). However, we wish to make it clear that while this model fits the data for *S. ferax* and *B. magnus* we cannot extend it to other species until the details of their microtubule patterns have been elucidated with the same level of resolution and confidence in preservation as presented here. Aspects of this problem are addressed below.

Microtubule organization

A direct comparison of microtubule patterns between the present work and other reports of tip-growing systems is difficult because this is the only quantitative three-dimensional analysis using the resolution and preservation quality associated with electron microscopy of freeze-substituted tissue. Other electron microscopy studies have been neither quantitative nor three-dimensional, and immunocytochemical studies have been validly and adequately criticized by Lancelle *et al.* (1987). Nevertheless, a number of similarities between microtubule organization in *S. ferax* hyphae and other tip-growing cells can be detected. For example, a preponderance of peripheral longitudinally oriented microtubules occurs in root hairs (Seagull & Heath, 1980; Lloyd & Wells, 1985; Emons, 1987), pollen tubes (Raudaskoski *et al.* 1987; Lancelle *et al.* 1987), moss protonemata (Wacker *et al.* 1988; Doonan *et al.* 1988) and other hyphae (Hoch & Staples, 1985; Howard & Aist, 1979; Runeberg *et al.* 1986). However, fern protonemata also seem to possess circumferential microtubules (Wada *et al.* 1980; Murata *et al.* 1987) and some root hairs have variously helical arrays (Lloyd, 1983; Lloyd & Wells, 1985). Similarly, the preponderance of short microtubules in tips is seen in pollen tubes (Lancelle *et al.* 1987) and root hairs (Seagull & Heath, 1980; Emons, 1987). However, these latter reports have been questioned by immunocytochemical studies that highlight longer microtubules. This impression is partially illusory because lower magnifications produce images dominated by sub-apical regions where longer microtubules do dominate. Moreover, short microtubules are likely to be more labile to harsh fixation and permeabilization protocols and more likely to be overlooked in the images. Furthermore, preincubation of cells in microtubule-stabilizing buffers (Doonan *et al.* 1985) or calcium-chelating solutions (Derksen *et al.* 1985) to 'greatly improve preservation of microtubules' (Derksen *et al.* 1985) are quite likely to introduce significant artefacts, including microtubule polymerization. Finally, when working with isolated cells, such as severed root hairs, pollen or fungal germ tubes, or protonemata, if is difficult to know the precise growth behavior of the subsequently recorded cells at the time of fixation, thus the observed patterns may not reflect those of growing cells. Consequently, the prevalence of long microtubules in tip-growing cells is unclear. Nevertheless, if one concentrates solely on the most apical 20 μm considered in our work, which is the region of most relevance to the tip growth process itself, many immunofluorescent images support the existence of a sub-apically increasing number of microtubules, many of which are short. For example, these can be seen in *Uromyces* germ tubes (Hoch *et al.* 1987, fig. 4), radish root hairs (Lloyd & Wells, 1985, figs 1, 4), moss protonemata (Doonan *et al.* 1988, fig. 1B; Wacker *et al.* 1988, fig. 17), fern protonemata (Murata *et al.* 1987) and pollen tubes (Derksen *et al.* 1985; fig. 2C, D; Raudaskoski *et al.* 1987, figs 2, 3). Consequently, it seems likely that the patterns and subsequent interpretations reported here for *S. ferax* are likely to be widespread in tip-growing cells and thus of general significance. However, the differential distribution of microtubules in the peripheral *versus* central cytoplasm differs from the even distribution reported in *Trametes versicolor* by Girbardt (1979). The existence of radial differences in distribution is largely unexplored in other tip-growing cells, but these reported variations may indicate fundamental and widespread variability, which cannot be ignored in future studies aimed at determining possible universal models of tip-growth.

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