Spatial and cellular localization of calcium-dependent protease (CDP II) in *Allomyces arbuscula*

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Summary

Immunogold labeling of calcium-dependent neutral protease II (CDPII) with specific antibodies in near median longitudinal ultrathin sections of *Allomyces arbuscula* showed that the enzyme is predominantly localized in the growing hyphal and rhizoidal apices. The tips in both cell type had more enzyme than the distal regions and showed a gradient distribution. Labeling of the ultrathin sections and western blot analysis of purified subcellular fractions showed that CDPII is mainly cytosolic. Catalytic activity of the enzyme measured with synthetic substrate (Bz-Arg-pNA) showed that 90% of its activity is present in the soluble fraction, although a small amount is associated with the nuclei (0.2%), plasma membranes (0.7%) and microsomes (3.9%). This association is discussed in the context of the functional role of the enzyme and its possible localized activation. Western blot analysis of the crude extract and indirect immunofluorescence of the fixed permeabilized hypahe after treatment with CDPII showed that the α-tubulin is a specific target of the enzyme.

Key words: *Allomyces arbuscula*, Calcium-dependent neutral protease, Immunogold labeling, Intracellular localization, Hyphal tips, α-tubulin a specific target

Introduction

Ca^{2+} as a second messenger is involved in cell signaling, initiation and regulation of a variety of inter- and intracellular reactions (Berridge, 1997; Clapham, 1995). Movement of Ca^{2+} either from outside or from stores inside, in response to a signal, is transient, as is accumulation of free intracellular Ca^{2+}, which rapidly combines with Ca^{2+}-binding proteins sequestered in storage organelles or pumped outside the cell. The free intracellular Ca^{2+} concentration, around 10^{-7} M, is lower than the calcium concentration in the internal stores, that is, mitochondria and endoplasmic reticulum/sarcoplasmic reticulum or the medium in which cell are growing (Carafoli, 1987). This difference provides sufficient gradient for Ca^{2+} influx into the cytosol and a transient rise in the free cytosolic Ca^{2+}, which appears in short pulses or waves of differing frequency and amplitude. These waves are interpreted differently by Ca^{2+}-binding proteins, which convert them into a variety of signals and biochemical reactions. Any change in the transient nature of free intracellular Ca^{2+} is detrimental and initiates an apoptotic process resulting in cell death (Trump and Berezesky, 1995).

The filamentous growth form, evolved and perfected by fungi, represents an extreme example of polarized growth because of its unrestricted nature in suitable environments in contrast to the restricted growth pattern of pollen tubes and nerve cells. In these polarized cell types, a small and restricted region, about the size of an average cell (approximately 10-20 μm diameter), the tip, is believed to be the center for growth-related activities. Numerous studies have emphasized the role of Ca^{2+} in polarized tip growth. Tip-high gradients of total and free cytoplasmic Ca^{2+} have been measured in the pollen tube and growing fungal hyphae (Brownlee and Wood, 1986; Nobiling and Reiss, 1987; Schmid and Harold, 1988; Jackson and Heath, 1993; Pierson et al., 1994; Torralba and Heath, 2001). In actively growing hyphae of *Saprolegnia ferax*, a steep tip-high gradient extends from the tip for about 5 μm, in contrast to *Neurospora crassa* where the gradient peaks about 3 μm from the tip (Levina et al., 1995; Hyde and Heath, 1997; Silverman-Gavrila and Lew, 2001). The precise role of the tip-based Ca^{2+} gradient is not clear but one possibility is that it may be involved in the regulation of secretion vesicles, which are abundant in this region, and the activities of Ca^{2+}-activated proteins.

We identified a Ca^{2+}-activated protease (CDP II) in the actively growing vegetative cultures of *Allomyces arbuscula* and found that it disappeared when the hyphae were induced to differentiate reproductive structures by transfer to dilute salts solution, thus interrupting the hyphal growth (Ojha and Turian, 1985). The enzyme was purified and shown to be a doublet of M_r 43-40 kDa in SDS-PAGE, irrespective of the method and speed of purification (Ojha and Wallace, 1988). Both peptides were active and contained serine residues that can be phosphorylated (Ojha and Favre, 1991; Ojha et al., 1994). The in vitro catalytic activity of the enzyme had an absolute requirement for Ca^{2+} and reduced cysteine SH group(s). Ca^{2+} seemed to induce conformational changes in the protein with two major consequences, at μM concentrations the enzyme bound to the plasma membranes, but at mM concentration of Ca^{2+} the catalytic activity was activated (Ojha, 1989). A second Ca^{2+}-dependent enzyme, CDP I, eluting at lower ionic concentrations than CDP II, has also been identified (Ojha,

1996b) and shown to be developmentally regulated (Ojha, 1996a).

Indirect immunofluorescence microscopic studies of the spatial distribution of CDP II showed it was predominantly localized in the growing hyphal tips of *A. arbuscula* and in fungi as divergent as ascomycete, *N. crassa* and basidiomycete, *Uromyces appendiculatus* (Huber and Ojha, 1994; Barja et al., 1999). Cytoskeletal proteins, particularly the actins and tubulins, have also been shown to be abundantly present in the growing hyphal tips (Raudaskoski et al., 1991; Barja et al., 1991; Huber and Ojha, 1994). The ultrastructural studies of hyphal tips had already shown an abundance of microtubules and microfilament in the tip region of the hyphae (Roos and Turian, 1977; Howard, 1981; Vargas et al., 1993), suggesting that these elements are probably involved in the maintenance of form and growth of the tips. Our finding that CDP II localized to the same region led us to assume that this colocalization must have some meaning. In this report we show the spatial and intracellular localization of CDP II at a higher resolution using immunogold labeling in the two growing cell types of *A. arbuscula*, namely hyphal and rhizoidal tips. The evidence obtained demonstrates the existence of an apico-basal gradient of the enzyme, which is mainly cytosolic, although a small amount is also associated with nuclei and the plasma membrane. We also show that α-tubulin is a selective target of CDP II – in response to a specific Ca^{2+} signal, proteolysis of α-tubulin can modify microtubule arrangements in the elongating hyphae.

Materials and Methods

Chemicals

Yeast extract, soluble starch and casamino acid were purchased from Difco. Other routine chemicals were from Fluka.

Strain, cultural conditions and light microscopy

Allomyces arbuscula strain Burma LD, our experimental strain, was maintained on solid YPSs medium (Emerson, 1941). For light microscopy, *A. arbuscula* were grown in liquid culture for 18 hours. Actively growing hyphal apex was prepared from a culture grown for 48 hours on solid YPSs medium. A thin plug of mycelium, a few mm from the colony front of the culture, was cut with a Pasteur pipette and mounted in 100 μl YPSs medium and grown for 3-4 hours in a sterile humidified chamber with further addition of the medium when necessary. Hyphal apices were observed with Nomarski differential interference contrast optics in a Zeiss axioplan microscope, the images were taken with Hamamatsu color chilled 3CCD camera and developed by Raster Ops video captor and treated using the Adobe PhotoShop 6 program.

Mycelia for the preparation of subcellular fractions or for electron microscopy and immunogold labeling of CDP II were obtained by inoculation of zoospores in liquid GCY medium (Turian, 1963). After 18-20 hours of growth at 30°C with forced aeration (Ojha and Turian, 1981), a small amount of the culture was saved for immunogold labeling and the rest was harvested by filtration. The mycelia were washed with de-ionized water, squeeze-dried and used for the subcellular fractions as described below.

Purification of CDP II, preparation of antibodies and immunoblotting

Details of the methods for enzyme purification, raising of anti-CDP II antibodies and their affinity purification have been described elsewhere (Ojha and Wallace, 1988; Huber and Ojha, 1994). Western blot experiments were done essentially according to the protocol of Towbin et al. (Towbin et al., 1979) with minor modifications. Proteins of cellular fractions were separated by SDS-PAGE (11%) and electroblotted onto nitrocellulose membrane, blocked overnight at 4°C with 5% BSA in TBS-Tween, incubated for 4 hours with 1:2000 diluted CDP II antibodies, washed and re-incubated for 2 hours with anti-rabbit IGg antibodies coupled to horseradish peroxidase, re-washed and developed with DAB-H_2O_2 as described earlier (Huber and Ojha, 1994).

Electron microscopy and immunogold labeling

The mycelia from an 18 hour culture were fixed for 2 hours at room temperature. In order to maintain the integrity of the culture and to keep any alteration owing to fixation at minimum, a concentrated solution of the fixative was added directly to the medium to obtain the final concentration of 0.5% glutaraldehyde, 4% paraformaldehyde and 100 mM phosphate buffer (NaH_2/K_2HPO_4, pH 7.4). The mycelia were collected by centrifugation and, pre-embedded in 1.5% agar at 45°C, dehydrated respectively in 70% and 100% ethanol (for 30 minutes each). The samples were then infiltrated sequentially in 2:1 (v/v) ethanol: LR White resin (Polysciences), 1:1 (v/v) ethanol: LR White for 30 minutes each, 1:2 (v/v) ethanol: LR White for 1 hour and finally 100% LR White for 24 hours at 50°C for polymerization.

Ultrathin sections were taken on nickel grids, incubated at room temperature (RT) for 2 hours in 50 mM phosphate buffer (K_2/KH_2PO_4, pH 7.0) containing 2% BSA, and then for another 2 hours (at RT) with anti-CDP II (polyclonal antibodies) diluted to 1:30 in phosphate buffer containing 2% BSA + 0.05% Tween-20. The sections were rinsed with the same buffer and incubated for 1 hour at RT with goat anti-rabbit antibodies conjugated to 20 nm gold particles diluted to 1:30 in the same buffer. After incubation, sections were washed with phosphate buffer, rinsed in distilled water, stained for 10 minutes in 2% uranyl acetate, 5 minutes in Reynold's lead citrate and examined at 60 kV in Philips M400 transmission electron microscope. The controls consisted of the use of pre-immune serum or CDP II antibody pre-incubated with purified enzyme at ratios of 1:0.8 and 1:1 for 30 minutes at room temperature and diluted to 1:30, as primary antibody.

Subcellular fractionation

Subcellular particles were prepared by a modification of the procedure described by Hahn and Covault (Hahn and Covault, 1990) for the isolation of the nuclei. Squeeze-dried mycelia from an 18 hour culture were extensively minced by a sharp onion cutter with frequent addition of small amounts of buffer A (0.3 M sucrose, 60 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 5 mM spermidine and 15 mM MOPS, pH 7.5). The pulp was then ground without abrasive to a very fine homogenate in pre-cooled mortar. Buffer A was added to complete the final ratio of 10 ml buffer per g of squeeze-dried mycelia. The homogenate was filtered through a four-layer cheese cloth and the filtrate was centrifuged for 15 minutes at 300 ***g***. The supernatant was recovered and re-centrifuged for 20 minutes at 2000 ***g*** in a Beckman JA20 rotor. The pellet contained the nuclei, and the supernatant the mitochondria and plasma membrane. The nuclear pellet was suspended in 3 ml buffer B (which is the same as buffer A except for the addition of EDTA and EGTA, both at 0.1 mM, and BSA, at 10 mg/ml). Percoll was then added to a final concentration of 27% and the mixture centrifuged for 20 minutes at 27,000 ***g***. The fluffy nuclear layer at the bottom of the tube was removed with a siliconized Pasteur pipette, diluted 10 times with buffer A, deposited carefully on a pad of 1 ml storage buffer (50% glycerol, 75 mM NaCl, 5 mM Mg-acetate, 0.85 mM DTT, 0.125 mM PMSF, 20 mM Tris-HCl, pH 7.9) in a siliconized corex tube and centrifuged at 2000 ***g*** for 20 minutes. The nuclear pellet thus obtained was suspended in storage

buffer and kept until use. The post-nuclear supernatant was centrifuged at 18,000 ***g*** for 20 minutes and the pellet containing mitochondria and contaminating nuclei was suspended in 3 ml buffer B, made up to 27% percoll and re-centrifuged at 27,000 ***g*** for 20 minutes. The fluffy mitochondrial layer from the top was recovered, diluted to 10 volumes with buffer A and re-centrifuged at 18,000 ***g*** for 20 minutes to pellet mitochondria, which were suspended in storage buffer.

Plasma membranes were sedimented from the post-mitochondrial supernatant by centrifugation at 41,300 ***g*** for 30 minutes and the pellet was washed by centrifugation and suspended in 200 µl buffer A. The supernatant from this centrifugation was subjected to ultracentrifugation at 100,000 ***g*** for 1 hour using a centricon TFT rotor. The pellet containing ribosomes, fragmented membranes and microbodies was suspended in 50 µl buffer A, and the clear supernatant was recovered and considered as soluble fraction. The enzyme activity in different fractions was measured using Bz-Arg-pNA as a substrate according to the procedure described earlier (Ojha et al., 1999).

In vitro proteolysis of tubulins in cell-free extract

The idea of using cell-free extract for testing the substrate specificity was based on the assumption that in crude extract there would be thousands of proteins, and given a choice the enzyme would cleave proteins only selectively, and these can be traced using specific antibodies. We chose tubulins because of their colocalization with CDP II in the tip region. Actively growing 18-hour culture was harvested, mycelia washed with cold distilled water, squeeze-dried, ground with quartz sand in a pre-cooled mortar and suspended in MOPS buffer containing protease inhibitors (Ojha et al., 1999). The homogenate was centrifuged in an Eppendorf microcentrifuge at 13,000 ***g***, the supernatant collected and concentration of proteins determined with Coomassie according to Bradford (Bradford, 1976). A pool of six reactions containing 75 µg of soluble protein was mixed with 3 µg CDP II, made up to a total volume of 120 µl with the enzyme reaction buffer containing 5 mM Ca^{2+} and 10 mM β-mercaptoethanol and incubated at 37°C. 20 µl samples (12.5 µg protein) were withdrawn at intervals, supplemented with 5 µl of 5× concentrated SDS-PAGE sample buffer, boiled for 3 minutes and processed on SDS-PAGE. As the control, two samples (containing 12.5 µg soluble proteins each) were incubated in the reaction mixture without CDP II or Ca^{2+}. The digested proteins in the samples were separated by SDS-PAGE and processed for western blotting as described elsewhere (Ojha et al., 1999; Barja et al., 1999).

In situ proteolysis and immunofluorescent localization of tubulins

Actively growing hyphae from the 18-hour culture were gently removed, fixed in 3% paraformaldehyde in phosphate buffer (unless stated otherwise, the phosphate buffer was 50 mM, pH 7.0), washed three times in the same buffer, the last wash being with the phosphate buffer, pH 6.5. The hyphae were then incubated at room temperature in a solution of Novozyme (5 mg/ml in phosphate buffer, pH 6.5) for 10 minutes, washed three times, for 5 minutes each, with phosphate buffer, pH 7.0. The plasmamembrane was permeabilised by treatment with 0.1% Triton-X100 for 10 minutes at room temperature, re-washed three times with phosphate buffer and then incubated for 1 hour at 37°C with CDP II (0.22 µg/µl, specific activity 1.176 mmol, pNA released L^{-1} min^{-1}) in the enzyme reaction buffer (20 mM Tris-HCl, pH 7.5, 3 mM EDTA, 4 mM $MgCl_2$, 5 mM Ca^{2+} and 10 mM β-mercaptoethanol). The hyphae were then washed five times, for 5 minutes each, with phosphate buffer and incubated for 2 hours at 37°C in phosphate buffer containing 3% BSA and further incubated with α- or β-tubulin-specific antibodies (Amersham N-356) at 1:10 dilution for 1 hour at the same temperature, washed five times, for 5 minutes each, with phosphate buffer and then incubated for another 2 hours with secondary antibody (goat anti-mouse IgG conjugated to FITC) diluted to 1:100 in phosphate buffer containing 3% BSA + 2% skimmed milk. The hyphae were then washed five times, for 5 minutes each, in phosphate buffer. The preparation was mounted on a slide in phenylenediamine and observed on a Leitz Orthoplan epi-illumination microscope equipped with fluotar optics with selective filter combination for viewing FITC conjugated antibodies. Photographs were taken on HP5 Ilford black and white films. The controls consisted of hyphae without CDP II treatment and α- or β-tubulin-specific antibody, respectively.

Results

Growing tips in *Allomyces*

Observation using the light microscope of actively growing hyphae under Nomarski optics showed two oppositely growing poles, the rhizoidal and hyphal (Fig. 1A). The rhizoids lacked

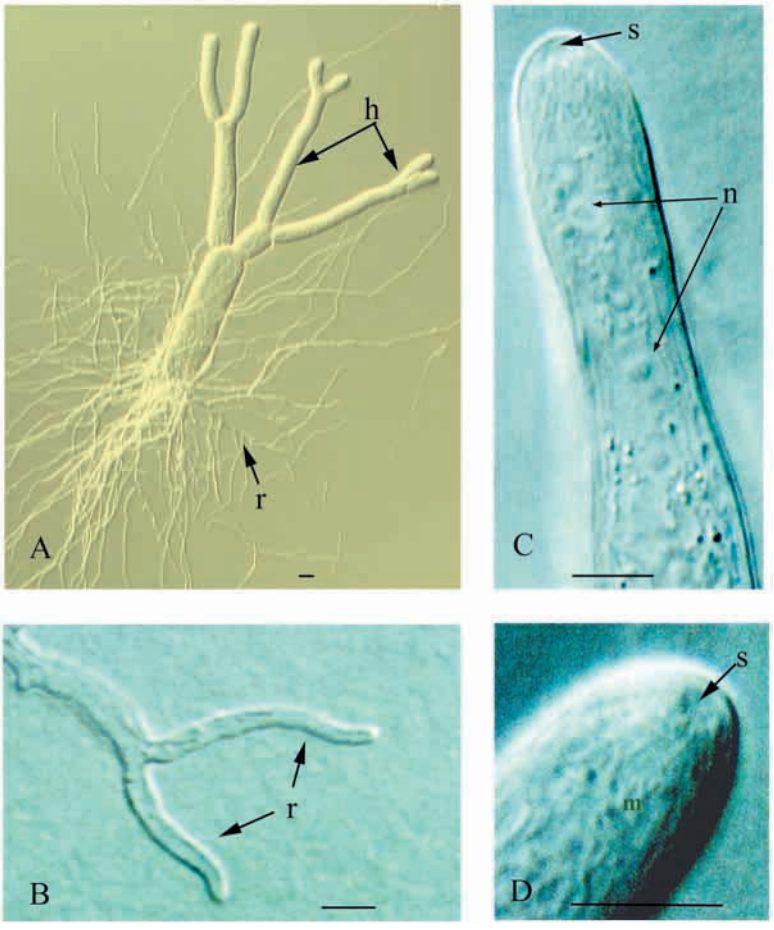

Fig. 1. Nomarski image of actively growing *Allomyces arbuscula* in liquid medium (YPSs) showing two oppositely growing poles. (A) General view of a young thallus representing two polarized cell types, hyphae (h) and rhizoids (r). (B) Rhizoidal pole. (C,D) Hyphal poles showing an apical exclusion zone containing a Spitzenkörper (s) and a sub-apical region containing nuclei (n) and mitochondria (m). Bar, 10 µm.

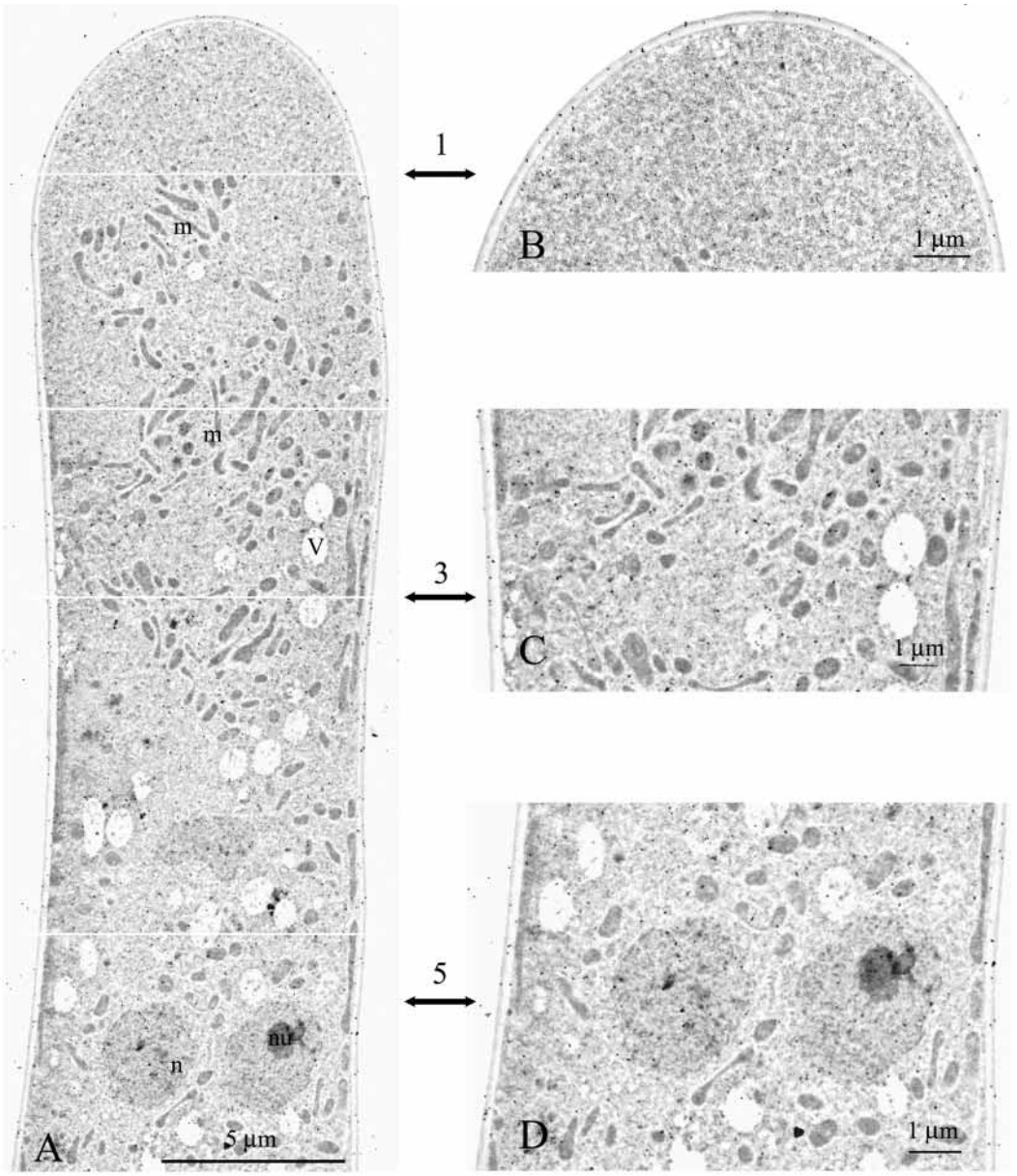

Fig. 2. Immunogold-labeled CDP II in a near median longitudinal section of hyphal apex of *Allomyces arbuscula* seen in an electron micrograph. (A) General view showing an apico-basal gradient of the enzyme. 1-5 correspond to the zones used for quantification of labels (see Table 2). (B) Higher magnification of the proximal tip region corresponding to zone 1. (C) Higher magnification of the distal region corresponding to zone 3 rich in mitochondria. (D) Higher magnification of zone 4 with nuclei. m, mitochondria; v, vacuole; n, nucleus.

nuclei but contained mitochondria, which were located in the sub-apical region leaving an apical exclusion zone. The details of cytoplasmic organization in the rhizoids were not clearly visible (Fig. 1B). By contrast, the tip and sub-apical regions were clearly discernible in the hyphal pole. The tip region was exempt from nuclei and mitochondria but contained a spherical pit-like structure of about 2.4-3 μm in diameter at the extreme apex representing the 'Spitzenkörper'. Arrays of filaments, most probably mitochondria, were seen radiating downwards from the Spitzenkörper zone (Fig. 1C,D). The sub-apical region contained centrally located nuclei, dispersed mitochondria, vesicles and microbodies (Fig. 1C).

Spatial distribution CDP II

Immunogold labeling of CDP II in ultrathin near median longitudinal sections of hyphal apices covering a length of approximately 32 μm from the tip showed an apico-basal gradient of the enzyme. Labeled grains were more abundant in the proximal than distal regions (Fig. 2A). This was further confirmed by actual counting of the labeled grains (Table 1) in three different regions of the section representing the apical exclusion zone (Fig. 2B), the proximal region rich in mitochondria but devoid of nuclei (Fig. 2C) and the zone further down where nuclei first appear (Fig. 2D). The 0-4.2 μm tip region (exclusion zone) contained approximately 1.8 times more enzyme than the sub-apical region, which was devoid of nuclei but rich in mitochondria, and about 2.5 times more than the region where nuclei start to appear, in the region about 26-32 μm further down (4 grains/μm^2 in the exclusion zone against 1.6 grains/μm^2 in the region between 26-32 μm from the tip). However, we think that this is an underestimate because the large number of grains counted in the mitochondria may not represent the reality, since in purified mitochondria very little labeling was observed (see Fig. 5A,B).

Table 1. Spatial distribution of CDP II (immunogold labels) in a near median longitudinal section of growing hyphal tip of *Allomyces arbuscula

Section	Distance from the tip (μm)	Surface area for the grain count in sections B, C and D (μm²)					Number of grains				Grains/μm² of the cytoplasm
		Total	Mitochondrial	Nuclear	Vacuolar	Cytoplasmic surface area	Cytoplasmic	Mitochondrial	Nuclear	Vacuolar	
1	0-4.2	41.56 [B]		–	–	41.56	164		–	–	3.95
2	4.2-9.8										
3	9.8-15.91	67.15 [C]	27 (56)	–	5.1 (6)	35.05	76	21	–	6	2.17
4	15.9-26.01										
5	26.01-32.72	84 [D]	16 (31)	16.25 (2)	10.09 (11)	41.66	66	6	29	7	1.6

*The counts correspond to the sections shown in Fig. 2.
Note that the numbers in parentheses are the actual number of organelles present.

This spatial distribution was more spectacular in the growing rhizoidal apex where a clear apico-basal gradient was visible. The labeled grains in the tip gave the image of projected spray from the tip and the number of grains in the 1 μm tip was twice that of the sub-apical region (Table 2; Fig. 3). The distribution of grains in the ultimate tip (0-1 μm) was cytoplasmic, but in the sub-apical regions, they were mainly localized along the plasma membrane and sometimes associated with the microtubules (Fig. 3B). The long filamentous mitochondrium present in the sub-apical region was devoid of labeled grains, which indicates the absence of the enzyme.

Controls using an incubation mixture containing a solution from which the CDP-II-specific antibody was stripped off by incubation with purified enzyme at ratios of 1:0.8 and 1:1 (w/w) showed only a few grains in the cytoplasm or organelles with the former ratio and none at all with a ratio of 1:1 (Fig. 4A,B). Similarly, very few labeled grains were observed when the hyphae were incubated with the pre-immune serum (Fig. 4C). These results indicated that the labeling reactions presented in Figs 2 and 3 were specific.

Table 2. Spatial distribution of CDP II (immunogold labels) in ultrathin longitudinal sections of growing rhizoidal tip of *Allomyces arbuscula*

Section	Distance from the tip (μm)	Number of grains	Numbers of grains normalized
1	0-1	118	100
2	1-2.5	46	39.0
3	2.5-4	49	41.5
4	4-5.5	43	36.4

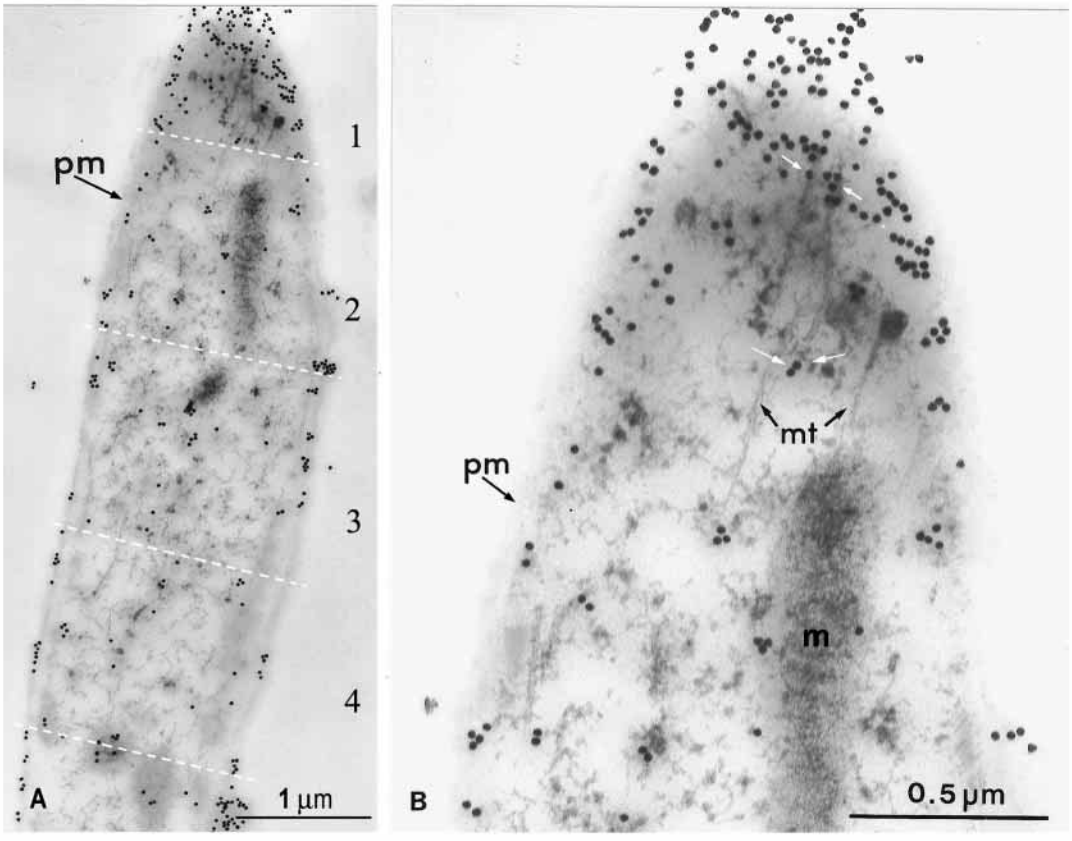

Fig. 3. Immunogold-labeled CDP II in a ultrathin longitudinal section of rhizoidal apex of *Allomyces arbuscula* seen in an electron micrograph. (A) General view showing abundance of the enzyme in the tip region. (B) Higher magnification of the 0-1.84 μm tip region. Plasma membrane (pm) (note the accumulation of the enzyme along the plasma membrane). m, mitochondria; mt, microtubule.

Intracellular distribution of CDP II

In the hyphal tip, labeled grains were predominantly located in the cytoplasm, but some were also present in the nuclei and attached to the plasma membrane (Fig. 2 and Fig. 5C-G). Occasionally, the enzyme was seen entrapped in the nuclear pore as if it were in the process of translocation from the cytosol to the nuclei (data not shown). The labeling in mitochondria was sparse, and grains were only seen in a few; by contrast, the enzyme was totally absent in the vacuoles (Fig. 2C,D; Fig. 5A). In the cytosol where the majority of the labeling was observed, single grains were mostly isolated, although sometimes aggregates of two to three grains were seen. As mentioned in the preceding section, in the rhizoid, grains were mainly cytoplasmic in the exclusion zone but predominantly associated with plasma membrane in the distal region (Fig. 3; Fig. 5G). Some aggregates of the enzyme were also found along the microtubules in the tip region (Fig. 3B). A comparison of the distribution of labeled grains in different zones showed that the major proportion of the enzyme is cytoplasmic. In the tip region, which was devoid of cell organelles, the labels were entirely cytoplasmic (Fig. 2A). Some labeling was found to be associated with mitochondria and nuclei in the sub-apical zones, but, the majority was cytoplasmic. The label counts in mitochondria could be an overestimate, as there was a total absence of labeling in the purified mitochondria and in the single large mitochondrium of the rhizoid (Fig. 3, Fig. 5A,B). Further, the purified mitochondrial fraction had very little enzyme activity (Table 3) and lacked enzyme antigens, as observed with western blot experiments (Fig. 6).

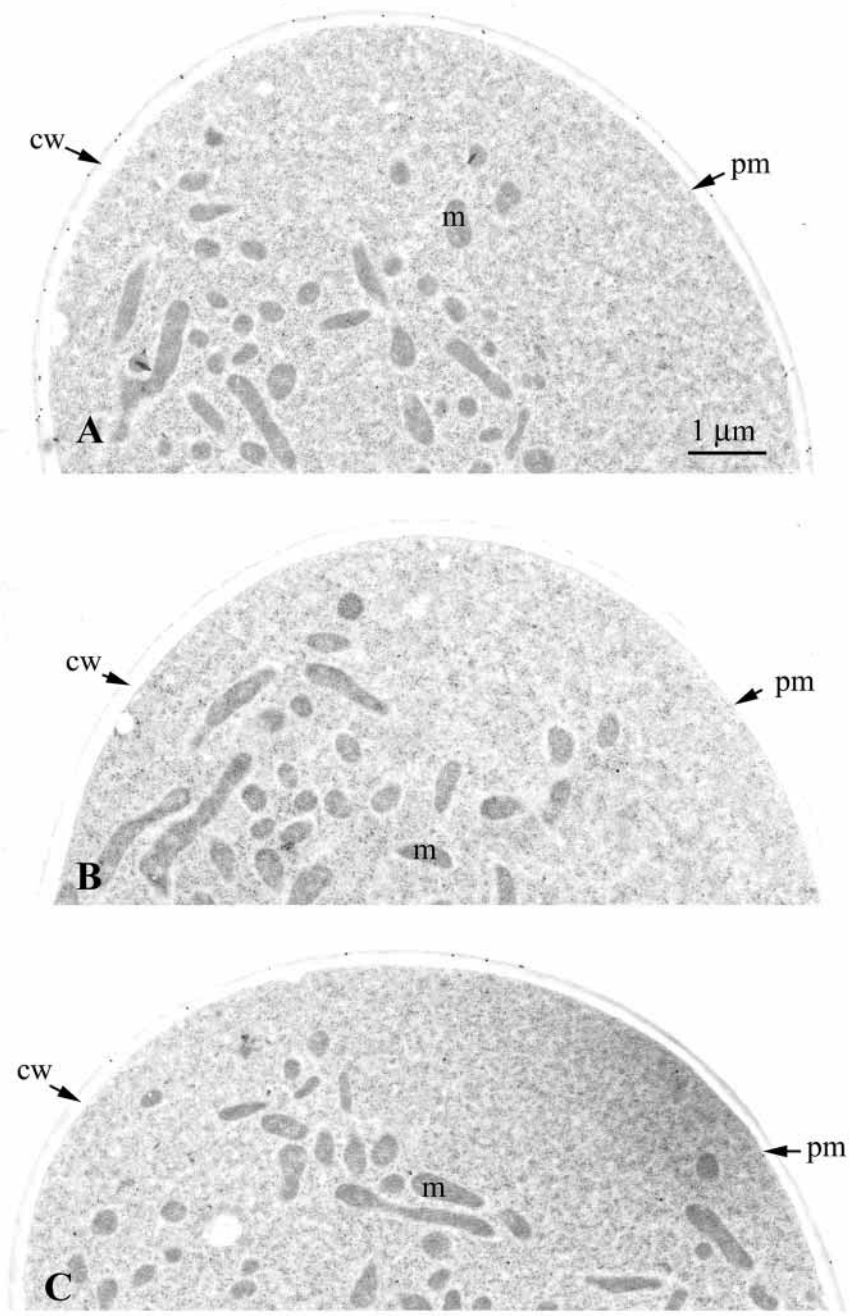

Fig. 4. Control experiments for immunogold labeling of CDP II in the ultrathin sections of hyphal apices of *Allomyces arbuscula*. (A,B) The incubation mixture was stripped-off for CDP-II-specific antibodies by pre-incubation with purified antigen at antibody-antigen ratio of 1:0.8 and 1:1 ratio respectively. (C) Pre-immune serum was used as primary antibody.

Immunogold labeling of CDP II in purified nuclei, mitochondria and plasma membranes

Localization of the enzyme in organelles was further studied by immunogold staining in purified organelles, and its distribution was compared to its in situ localization. As shown in Fig. 5B and D, the enzyme distribution in the isolated organelles corresponded well to that obtained using in situ labeling except for in mitochondria, which contained much fewer labeled grains in purified preparations than those in situ.

Cellular distribution of enzyme activity

Cellular distribution of the enzyme was further quantified by differential centrifugation of cell homogenate (prepared by mild cell disruption and the use of a buffer system known to protect the integrity of cell organelles) and measurement of the activity in different fractions. The results showed that 90% of the activity was associated with the soluble fraction and the remaining activity was distributed between the nuclei (0.2%), mitochondria (0.5%), plasma membrane (0.7%) and microsomal fractions (4%) (Table 3). The presence or absence of EGTA in homogenizing buffer did not affect the amount of activity recovered in the soluble fraction, indicating that the high activity in this fraction was not due to solubilization of the membrane bound enzyme by EGTA or EDTA present in the buffer (data not shown).

Table 3. Cellular distribution of CDP II activity*

Cell fraction	Total protein (mg)	Total units**	Specific activity (pNA mg/protein/minute)	% of total activity
Homogenate	896	42992	48	100
Nuclei	6.4	88	13	0.2
Mitochondria	9.7	218	22	0.5
Plasma membrane	5.5	290	52	0.7
Microbodies***	21	1685	8	3.9
Soluble	588	39030	66	90.8

*One of four independent experiments.
**1 unit of enzyme is defined as the amount of enzyme releasing 1 μM pNA.min^{-1}.
***100 K pellet.

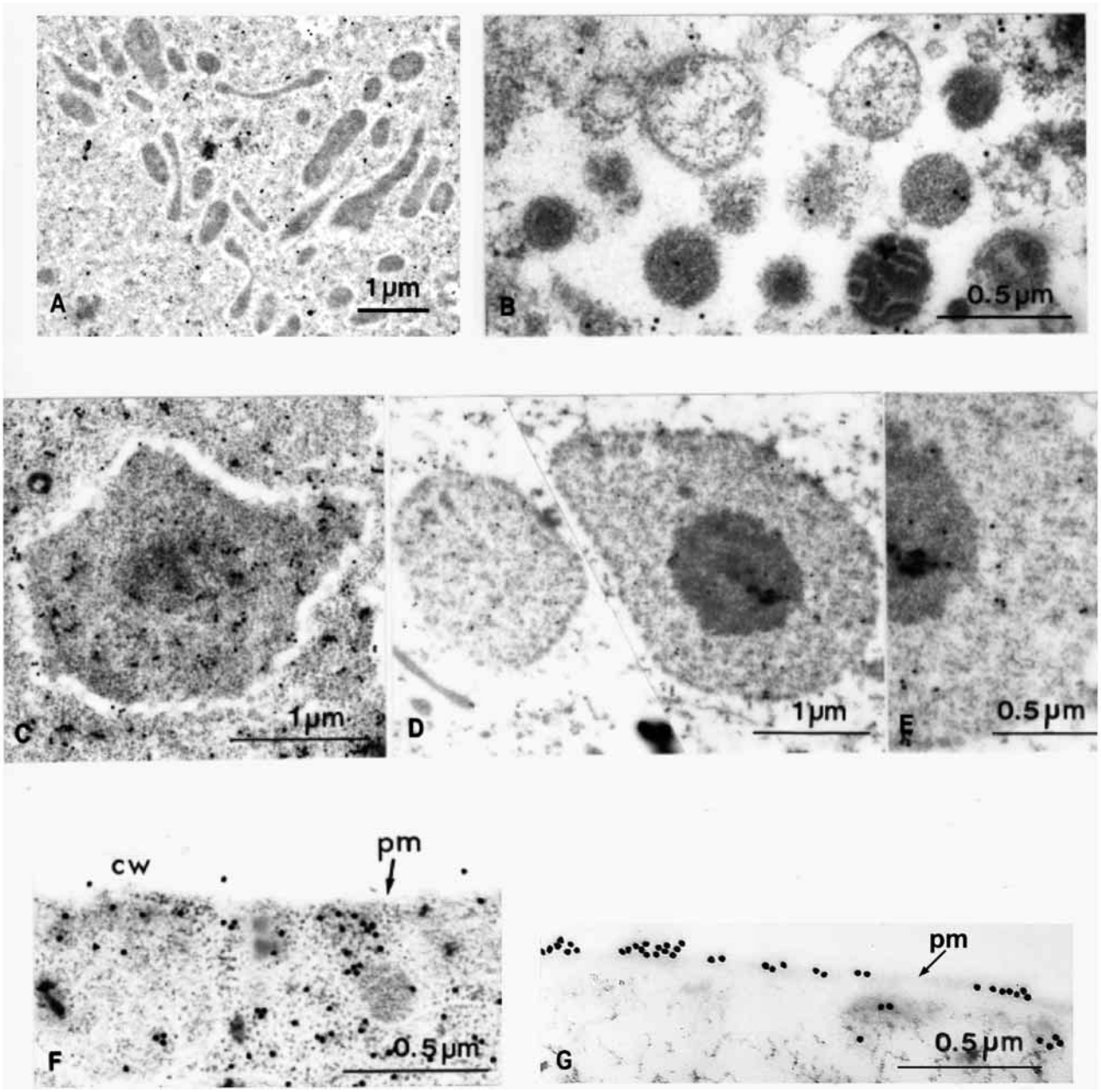

Fig. 5. Immunogold-labeled CDP II in thin sections of nuclei, mitochondria and membranes seen in an electron micrograph. (A,B) Labeling of in situ and purified mitochondria, respectively. (C,D) Labeling of in situ and purified nuclei, respectively. (E) Higher magnification of a portion of D. (F,G) Labeling along the plasma membrane (pm) in a section of hyphal tip and rhizoidal tip, respectively.

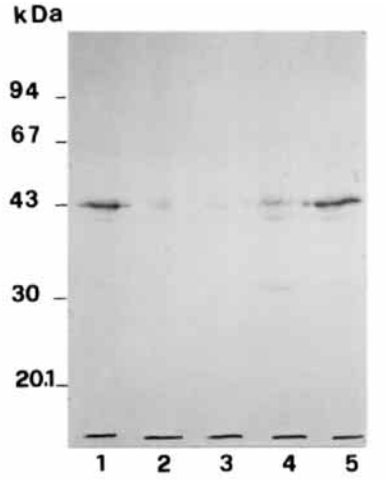

Fig. 6. Western blot analysis of CDP II in different subcellular fractions. Cell-free extract (300 ***g*** supernatant, 180 μg), nuclear (154 μg), mitochondrial (145 μg), plasma membrane (165 μg) and soluble (185 μg) protein fractions were electrophoresed in 11% SDS-PAGE and immunoblotted as described in Materials and Methods. Lanes: (1) cell-free extract; (2) nuclear; (3) mitochondrial; (4) plasma membrane; (5) soluble protein fraction.

Cellular distribution of the enzyme was further verified by western blot experiments with purified CDP II antibodies. As shown in Fig. 6, besides a small amount of reacting antigens present in the nuclear and plasma membrane (Fig. 6, lanes 2 and 4), the soluble fraction contained most of the antigens reacting with CDP II antibodies (Fig. 6, lane 5).

α-tubulin is a specific target of CDP II

A search for the integrity of tubulins in the cell-free extract after treatment with CDPII in the presence of Ca^{2+} by western blotting using specific antibodies showed a rapid proteolysis of α-tubulin. β-tubulin in the same extract was not affected, even after prolonged incubation. (Fig. 7C,D). An examination of the relative intensity of ponceau-stained bands after transfer from SDS-PAGE to nitrocellulose membrane did not show any massive general proteolysis (Fig. 7A,B). Similar analysis for the integrity of actins did not show any modification although purified protein was degraded rapidly (data not shown).

Incubation of fixed and permeabilised hyphae with CDP II in the enzyme reaction mixture and processing for immunofluorescence using α- or β-tubulin-specific antibodies showed a complete digestion of α-tubulin, with little effect on β-tubulin (Fig. 8A-D). These results confirmed those obtained with the crude extracts.

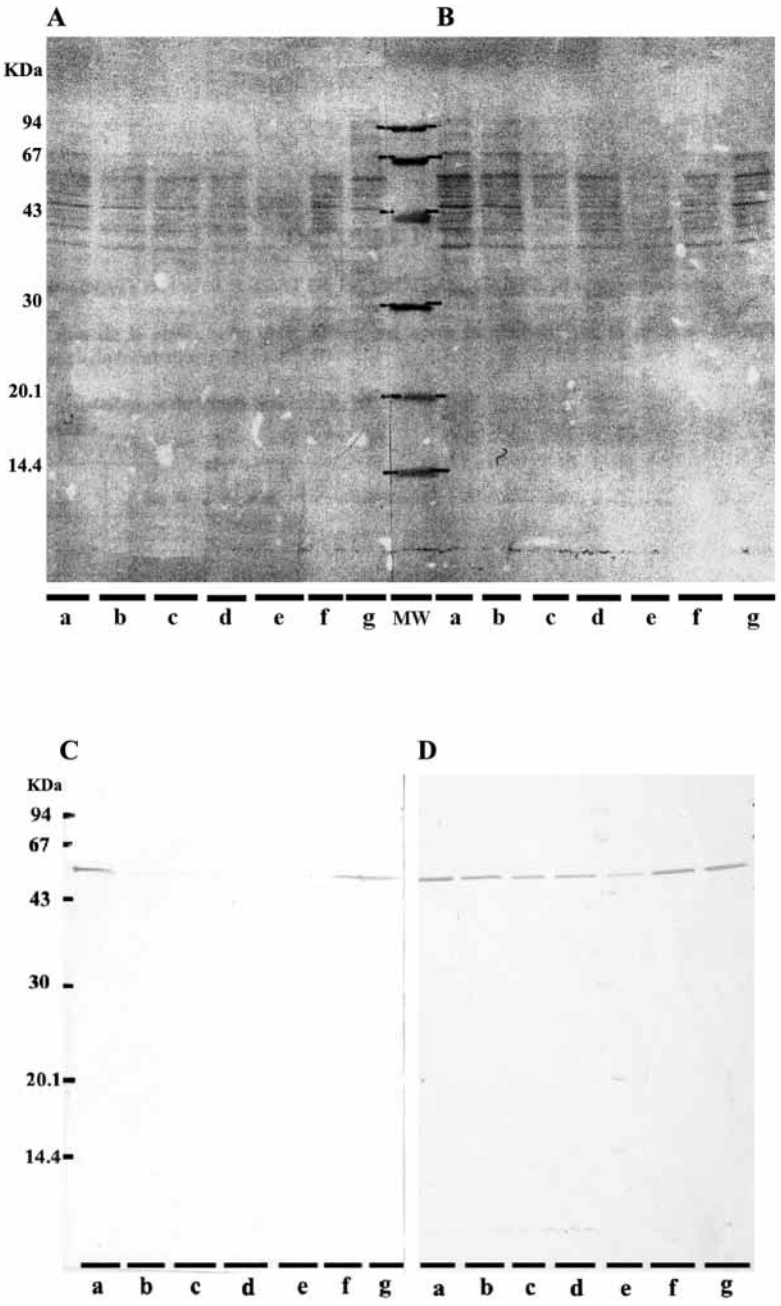

Fig. 7. Proteolysis of tubulins in the crude extract of *A. arbuscula* revealed by immunoblotting. 75 μg crude extract proteins were mixed with 3 μg CDP II, made up to a total volume of 120 μl with reaction buffer containing 5 mM Ca^{2+} and 10 mM β-mercaptoethanol and incubated at 37°C. 20 μl samples were withdrawn at intervals, added with 5 μl sample buffer and processed for western blotting as described in Materials and Methods. (A,B) Ponceau-stained proteins on nitrocellulose membrane after SDS-PAGE and electro-transfer. The lanes a-g correspond to the time course reaction with the CDP II as shown below (C,D). (C) Crude soluble protein digest immunoblotted with α-tubulin antibodies. (a) 0 minute incubation; (b) 10 minute incubation; (c) 20 minute incubation; (d) 30 minute incubation; (e) 60 minute incubation; (f) soluble proteins without enzyme; (g) soluble proteins + enzyme minus calcium. (D) Same as above but revealed with β-tubulin antibodies

Discussion

Allomyces arbuscula, like other filamentous chytridiomycetes, has two oppositely growing poles, the rhizoidal and hyphal poles. CDP II was predominantly localized in the tip region, forming an apico-basal gradient in both cell types. Much is known about the structural and physiological details of the growing hyphal tips in fungi. Growing hyphae have an apical

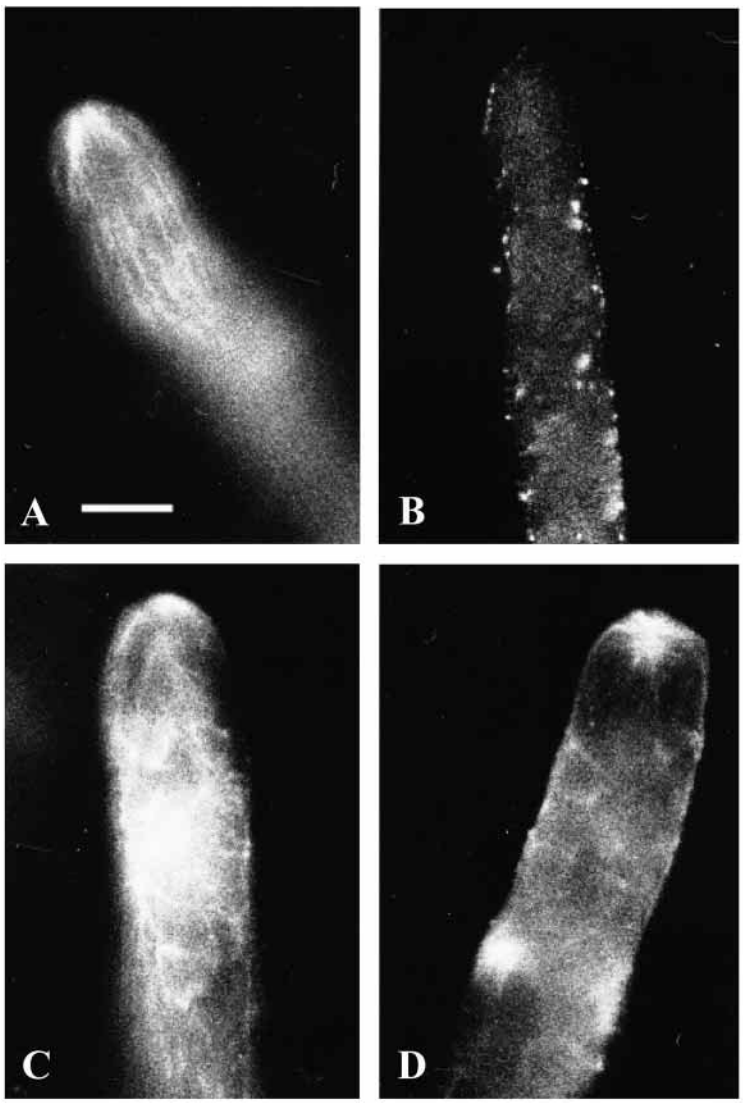

Fig. 8. Distribution and in situ digestion of tubulins in the growing hyphae of *A. arbuscula.* Samples were prepared as described in Materials and Methods. (A) Distribution of α-tubulin; (B) digestion of α-tubulin by CDP II; (C) distribution of β-tubulin; (D) digestion of β-tubulin by CDP II.

zone devoid of nuclei and mitochondria but are rich in spherical aggregates of vesicles and microbodies and a sub-apical zone containing centrally located nuclei, dispersed mitochondria and filamentous structures representing microfilaments and microtubules (Grove and Bracker, 1970; Grove et al., 1970; Roos and Turian, 1977; Howard, 1981). Likewise, physiological studies have demonstrated the existence of protons gradients, Ca^{2+} and an abundance of cytoskeletal proteins in the tip region (Turian, 1981; Turian, 1983; Schmid and Harold, 1988; Jackson and Heath, 1993; Barja et al., 1991; Barja et al., 1993; Gow, 1994; Robson et al., 1996; Srinivasan et al., 1996; Hyde and Heath, 1997). The presence of Spitzenköper in chytridiomycetes was not known until recently. In *Allomyces macrogynus*, Vargas et al. observed the presence of a spherical zone in the tip region of growing hyphae and showed it to be the Spitzenkörper equivalent in higher fungi (Vargas et al., 1993). We have observed a similar structure, a spherical pit of about 2.4-3 μm diameter, in the exclusion zone of the growing hyphal tips. Thus, the structural organization of the hyphal tip of *Allomyces* is no different from other fungi. No similar structure was discernible in the rhizoidal tips, and, as already known, nuclei were not present in the sub-apical region or further down the filament.

Immunogold labeling of the enzyme in ultrathin sections of the hyphal tip clearly demonstrated an apico-basal gradient, which confirmed our previous results obtained by indirect immunofluorescence microscopy (Huber and Ojha, 1994). The 0-4.2 μm tip region had 2.5 times more CDP II than the distal region (Table 1). An even more pronounced apico-basal gradient was observed in the rhizoidal tip (Fig. 3, Table 2) where labeling was absent in the filamentous mitochondria. The predominance of CDP II in the rhizoidal apex is interesting from a fundamental point of view. It raises the questions – how is sustained growth maintained at long distances in the absence of nuclei and nuclear gene related transcription and how is CDP II, coded by a nuclear gene, constantly transported to the tip?

In growing hyphal tips many investigators have documented the abundance of cytoskeletal proteins (Jackson and Heath, 1990; Kaminskyj and Heath, 1995; Barja et al., 1993; Huber and Ojha, 1994; Srinivasan et al., 1996), Ca^{2+} (Jackson and Heath, 1993; Hyde and Heath, 1997) and the presence of a pH gradient (Robson et al., 1996). The intracellular pH gradient (a pH of 8.2 in the tip and 6.8 in the distal region) in the growing hyphae of *N. crassa* showed a steep correlation between the magnitude and length of the pH gradient and the rate of hyphal extension (Robson et al., 1996). A decrease in the intracellular pH by weak acids resulted in the inhibition of growth in *S. ferax* (Bachewich and Heath, 1997). The optimal pH for the catalytic activity of the *Allomyces* Ca^{2+}-dependent proteases is in the range 7.4-8.0; thus the hyphal tip region provides an ideal environment for its catalytic activity. Although recent experiments by Parton et al. discount the role of pH in the regulation of tip growth (Parton et al., 1997), the tip is rich in various proteins with different pH requirements for their activity, and it is difficult to conceive that proteins that are optimally active at a given pH would function normally in a sub-optimal atmosphere. The pH, Ca^{2+} concentration and presence of colocalized proteins in the tip lead us to suggest a role for CDP II in the selective modification of cytoskeletal protein interactions between themselves and with the plasma membrane; thus, plasticity is maintained in this dynamic region. The experiments presented here have clearly demonstrated the predominant cytosolic localization and apico-basal gradient of the enzyme. A small amount of CDP II is also present in the nuclei and associated with the plasma membrane and microtubules. The localization of the enzyme along the plasma membrane is interesting as Hyde and Heath have shown an apico-basal Ca^{2+} gradient in the peripheral region along the plasma membrane in the growing hyphal tip of *S. ferax* (Hyde and Heath, 1997). If a similar gradient exists in the hyphal tip of *Allomyces*, the localized increase in Ca^{2+} along the plasma membrane and the translocation of the enzyme from the cytosol to the plasma membrane in response

to a rise in the free intracellular Ca^{2+} could activate its catalytic activity (Ojha, 1989).

The nuclear location could have a functional role in the regulation of the activity of transcriptional factors or other nuclear proteins; likewise, the enzyme aggregates along microtubules may be involved in the turnover of tubulin. Our results clearly identified α-tubulin as a specific target of the enzyme, although there might be many more proteins as well. The experimental conditions used to demonstrate this specificity are not necessarily physiological; more experiments are needed to understand the in vivo mechanism of CDP II activation, which must involve interactions with other proteins. The catalytic property of the enzyme has been studied using in vitro reactions, where its activation requires mM concentrations of Ca^{2+}, a concentration that is not available physiologically; this mystery of in vivo activation of the enzyme is unresolved. The free intracellular concentration of Ca^{2+} is considered to be around 10^{-6} to 10^{-7} M; however, a localized increase in Ca^{2+}, as along the plasma membrane (Hyde and Heath, 1997), can not be ruled out, although it can never attain concentrations in the mM range. Therefore, some other activator must exist that could lower the Ca^{2+} requirement of the enzyme activity. In this context it is interesting to note that the extensively investigated Ca^{2+}-dependent proteases (calpains) in the mammalian cells, the in vitro activation reactions, also require a non-physiological concentration of Ca^{2+}. But it has been shown recently that in vitro activation reactions can be activated by an endogenous protein activator (Melloni et al., 2000) at physiological Ca^{2+} concentration.

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