Fungal Genetics and Biology **30,** 63–70 (2000)
doi:10.1006/fgbi.2000.1198, available online at http://www.idealibrary.com on IDEAL®

Cellular Distribution of COT1 Kinase in *Neurospora crassa*

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Accepted for publication April 26, 2000

Gorovits, R., Sjollema, K. A., Sietsma, J. H., and Yarden, O. 2000. Cellular distribution of COT1 kinase in *Neurospora crassa. Fungal Genetics and Biology* 30, 63–70. The *Neurospora crassa cot-1* gene encodes a Ser/Thr protein kinase, which is involved in hyphal elongation. Many vacuoles, abnormally shaped mitochondria, and nuclei, along with differences in the structure of the cell wall and hyphal septa, were observed in hyphae of the *cot-1* mutant shortly after a shift to the restrictive temperature. Immunolocalization experiments indicated that COT1 was associated with the cytoplasmic membrane; COT1 was also detected in the cytoplasm. The membrane-associated COT1 was absent from the *cot-1* mutant when shifted to the restrictive temperature, as was a lower molecular weight isoform of COT1. We propose that COT1 may be involved in several cellular processes, and the spatial and temporal regulation of COT1 activity involves trafficking of the kinase within the fungal cell and its possible interaction with additional proteins.

Index Descriptors: COT1 kinase; fungal growth and development; *Neurospora crassa.*

Establishment and maintenance of cell polarity are key processes in developmental biology. Continuous cell polarity is observed throughout most phases of fungal growth and development. In filamentous fungi this results in continuous cell elongation at hyphal tips and in budding yeast in reiterated production of daughter cells at specific positions. Therefore, fungi are outstanding models for studying cell polarization.

One of the approaches to study hyphal elongation is the analysis of mutants that are defective in hyphal proliferation or structure. Under standard growth conditions *Neurospora crassa* grows by continuous hyphal elongation and branching which results in the formation of spreading radial colonies. The large number of factors involved in hyphal growth is reflected by the great number of mutants, defective in hyphal elongation/branching, which have been isolated (Perkins *et al.,* 1982). Some of these mutants, designated *cot,* display a compact colonial temperature-sensitive phenotype under conditions favoring spreading growth in the wild type. The *cot-1* strain exhibits normal spreading radial growth at or below 25°C, but mutant colonies grow slowly with extensively branched hyphae at or above 32°C (Collinge *et al.,* 1978; Mitchell and Mitchell, 1954).

The *cot-1* gene has been isolated, and, on the basis of the deduced COT1 amino acid sequence, the gene has been predicted to encode a Ser/Thr-specific protein kinase which shares significant similarity with members of the Rho-kinase subfamily (Yarden *et al.,* 1992; Justice *et al.,* 1995; Wissmann *et al.,* 1997; Lauter *et al.,* 1998; Verde *et al.,* 1998). Other members of this Ser/Thr-kinase family are encoded by the *Drosophila warts*/*lats* gene and the human *DM* gene. The *warts*/*lats* gene is required for the control of cell proliferation as well as for normal morphogenesis (Justice *et al.,* 1995; Xu *et al.,* 1995). Mutations in

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1087-1845/00 $35.00

DM result in myotonic dystrophy (Mahadevan *et al.*, 1993). Verde *et al.* (1998) have shown that the fission yeast *orb6* gene, another member of this kinase family, is required for cell polarity maintenance during interphase. Thus, functional expression of these genes is required for normal cell differentiation in different organisms.

The proper spatial and temporal cellular distribution of the proteins involved in hyphal elongation is most likely a prerequisite for the success of cell growth. The proper localization of such proteins may well be an integral part of the regulatory mechanisms involved in the activation of such proteins/enzymes. This may well be the case for regulators of hyphal elongation, such as the COT1 kinase. Several reports on the cellular distribution of the structurally related *DM* gene product suggest that this kinase is located in different mammalian tissues as well as different subcellular locations which include both cytosolic and membrane-associated forms of the enzyme (Kameda *et al.*, 1998; Pham *et al.*, 1998).

Antibodies, raised against COT1, detected and immunoprecipitated a predominant 73-kDa polypeptide in *N. crassa* extracts, whose abundance was constant under all growth conditions tested. An additional, lower molecular weight COT1 isoform (67 kDa) present in the wild type was not detected in *cot-1*, grown at the restrictive temperature. The cellular distribution of COT1 is unknown. However, the presence of more than one COT1 isoform has been suggested to be indicative of the occurrence of posttranslational events (such as subcellular distribution) which may be involved in COT1 function (Gorovits *et al.*, 1999).

In this study, we have analyzed the ultrastructural changes that occur in the fungal cell following the impairment of COT1 function. We have detected COT1 in different subcellular fractions of *N. crassa* and have determined that the abundance of the membrane-associated form of COT1 kinase is significantly reduced in the *cot-1* mutant when grown at restrictive temperatures. Based on the results obtained by Western analyses and indirect immunolocalization procedures, we suggest that the two COT1 isoforms are differentially localized and are likely to have different roles within the fungal cell.

MATERIALS AND METHODS

N. crassa Strains, Media, Growth, and Transformation Conditions

N. crassa wild-type (74-OR23-1A; FGSC987) and *cot-1* (FGSC 4065) strains were used throughout this study. Procedures used for fungal growth and other manipulations are described in Davis and de Serres (1970). Strains were grown in either liquid or solid (supplemented with 1.5% agar) Vogel's medium with 1.5% (w/v) sucrose.

For electron microscopy studies the organisms were grown in a thin layer of solid medium (0.5 mm) between two cellophane membranes placed on top of solid medium. Both wild type and *cot-1* were grown overnight at 25°C, transferred to 37°C (for 4 h) in order to impose the temperature-induced change in COT1 activity, and subsequently fixed.

Electron Microscopy

Mycelium was fixed for 2 h on ice by removing the top membrane and placing fixative (2.5% formaldehyde, 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) directly on the mycelium. For ultrastructural studies the fixative was removed by washing with water and replaced with 1.5% $KMnO_4$. After 15 min at room temperature, the mycelium was washed with water and small blocks were cut from the edge of the colony and stained overnight for higher contrast in 1% uranyl acetate. After dehydration in a series of 50–100% ethanol washes, the material was embedded in Epon 812 (Serva). For immunolabeling, the permanganate staining was omitted and the material dehydrated immediately after fixation and embedded in Unicryl (Biocell). For better visualization of septa, mycelia were treated after fixation for 2 h with a mixture of two parts 1% OsO_4 and one part 5% $K_2Cr_2O_7$, then washed with water, stained overnight with 1% uranyl acetate, washed, dehydrated, and embedded in Epon 812. Ultrathin sections were cut in an LKB microtome and placed on formvar/carbon-coated nickel, single-hole grids. For immunolabeling, each grid was incubated for 5 min in a drop of 0.5% BSA in PBS and subsequently with a 10^{-3} dilution of anti-COT1 antibodies (αCOT17 or αCOT60; Gorovits *et al.*, 1999) in PBS amended with 0.5% BSA, washed with phosphate-buffered saline (PBS), and treated with 15-nm gold-labeled goat anti-rabbit IgG (Amersham). All samples were visualized with a Philips CM 10 electron microscope.

Immunoblotting

N. crassa mycelial samples were frozen in liquid nitrogen, pulverized, and suspended in extraction buffer [50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 10 mM

EGTA, 0.5% Triton, 0.3% β-mercaptoethanol, and Complete (Boehringer Mannheim) protease inhibitor mixture]. The samples were homogenized by 10 strokes in a Dounce homogenizer in order to produce total extract samples. Samples were frozen in liquid nitrogen and stored at −70°C. The cytosolic fraction and nuclei were initially separated from the crude extract by centrifugation at 4000*g* (20 min at 4°C). The pellet (containing the nuclei) was extracted by incubation (30 min at 4°C) in the presence of extraction buffer amended with 110 mM KCl and a protease inhibitor mixture. The cytoplasmic fraction and nuclear extracts were clarified by ultracentrifugation at 100,000*g* (60 min at 4°C) (Wang *et al.*, 1994). After ultracentrifugation, the cytoplasmic pellet (membrane fraction) was washed with lysis buffer and resuspended in the same buffer with 110 mM KCl. Glycerol (10% v/v) was added to the membrane (designated P100), cytoplasmic (S100), and nuclear (NS100) fractions and the samples were maintained at −70°C prior to use.

Proteins were separated by 7.5 or 10% gradient SDS–PAGE. Western blotting was performed according to standard procedures (Sambrook *et al.*, 1989). Anti-COT1 antibodies (αCOT17 and αCOT60; Gorovits *et al.*, 1999) were used (at a 1:20,000 dilution) for immunoblotting throughout this study. For Western blot analysis membranes were incubated at room temperature for 2–4 h or at 4°C overnight with the primary antibody. Goat peroxidase-coupled secondary antibodies (Amersham) were used at a 1:20,000 dilution, followed by signal detection with ECL reagents (Sigma).

Protein Kinase Assays

The kinase reaction was carried out in 50 μl kinase buffer (100 mM Mes, pH 6.8, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 0.3% β-mercaptoethanol, 5 mM $MgCl_2$, 5 mM ATP) containing 2 μM [γ-^{32}P]ATP (600–800 GBq/mmol) and partially purified cytoplasmic membrane protein extracts (10–20 μg) with or without 10 μg per sample of the exogenous substrate myelin basic protein (MBP, Sigma). Following a 30-min incubation (30°C), the reaction mixtures were spotted onto Whatman p81 paper and washed three times with ice-cold 75 mM H_3PO_4. Incorporation of ^{32}P into the substrate was assessed by scintillation counting, and specific activity (nmol/min/mg protein) was calculated.

RESULTS

Ultrastructure of cot-1

When grown at a permissive temperature (25°C), wild type and the *cot*-1 mutant of *N. crassa* were phenotypically almost indistinguishable; no features detectable by electron microscopy differed from the wild type (Fig. 1A). The well-structured cytoplasm with many mitochondria in the multinucleate compartment between two septa was clearly visible. However, extensive branching of the hyphae occurred in the *cot-1* mutant at 37°C, leading to what has been called a barbed wire appearance, clearly visible by light microscopy.

Sections prepared from such mycelia exhibited an extremely disorganized cytoplasm (Fig. 1B). Many vacuoles were visible, some with vesicle-like inclusions; mitochondria and nuclei appeared to be abnormally shaped, hyphal compartments became much shorter, and many extrusions or branch initiations were visible. In addition, the wall of the hypha appeared to be more loosely structured and less compact than the wild type. Noteworthy was the difference in appearance of the septa. When grown at 25°C, the septa were uniformly structured and compact (Fig. 1C), as they were in the wild type. However, in the mutant grown at 37°C, about half of the septa showed that a multilayered structure had formed between two adjacent hyphal compartments, and extrusion of polysaccharide material at the contact site was clearly visible (Fig. 1D). This phenomenon was most pronounced in the younger part of the growing colony. This was expected, as the older hyphae were formed at the permissive temperature when hyphal morphology was apparently normal and no posttemperature-shift modifications of septal structures were expected.

Immunolocalization of cot-1

Treatment of thin sections of both wild type and the *cot-1* mutant with antibodies raised against recombinant COT1 (Gorovits *et al.*, 1999) followed by treatment with secondary antibodies labeled with 15-nm gold particles was used to visualize the localization of COT1. Antibodies prepared against a full-length recombinant COT1 polypeptide (αCOT60) detected a significant membrane-associated protein and a dispersed signal distributed throughout the cytoplasm in preparations of wild type and *cot-1* grown at the permissive temperature (Fig. 2, wild type not shown). However, in *cot-1*, grown at the restric-

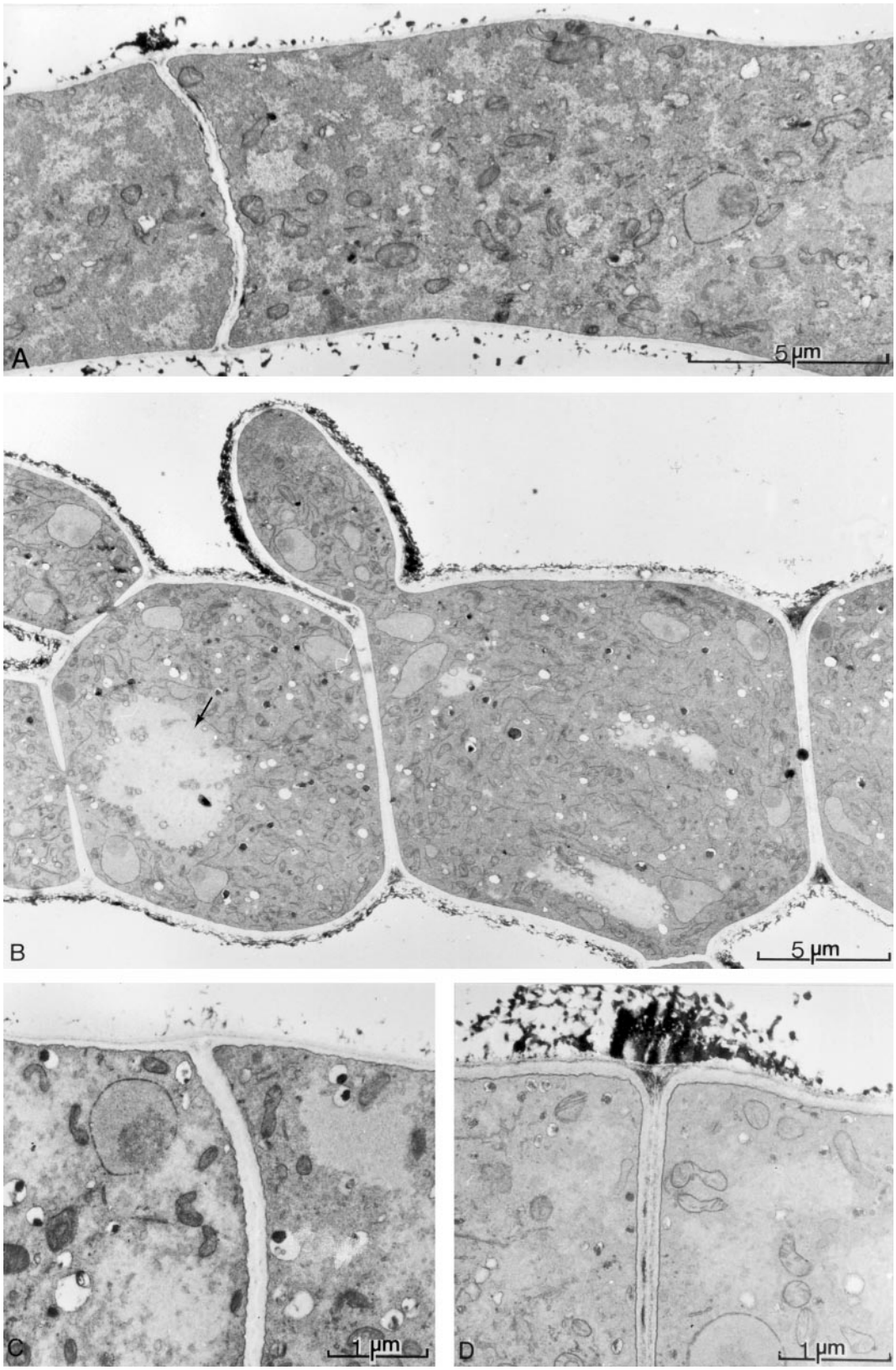

FIG. 1. Ultrastructure of *cot-1* before and after a shift to the restrictive temperature. (A) *cot-1* grown for 20 h at 25°C; (B) *cot-1* grown for 16 h at 25°C and for 4 h at 37°C. (The arrow points to vesicle-like inclusions inside a vacuole.) (C) Structure of a septum of *cot-1* grown for 20 h at 25°C; (D) structure of a septum of *cot-1* mutant grown for 16 h at 25°C and for 4 h at 37°C.

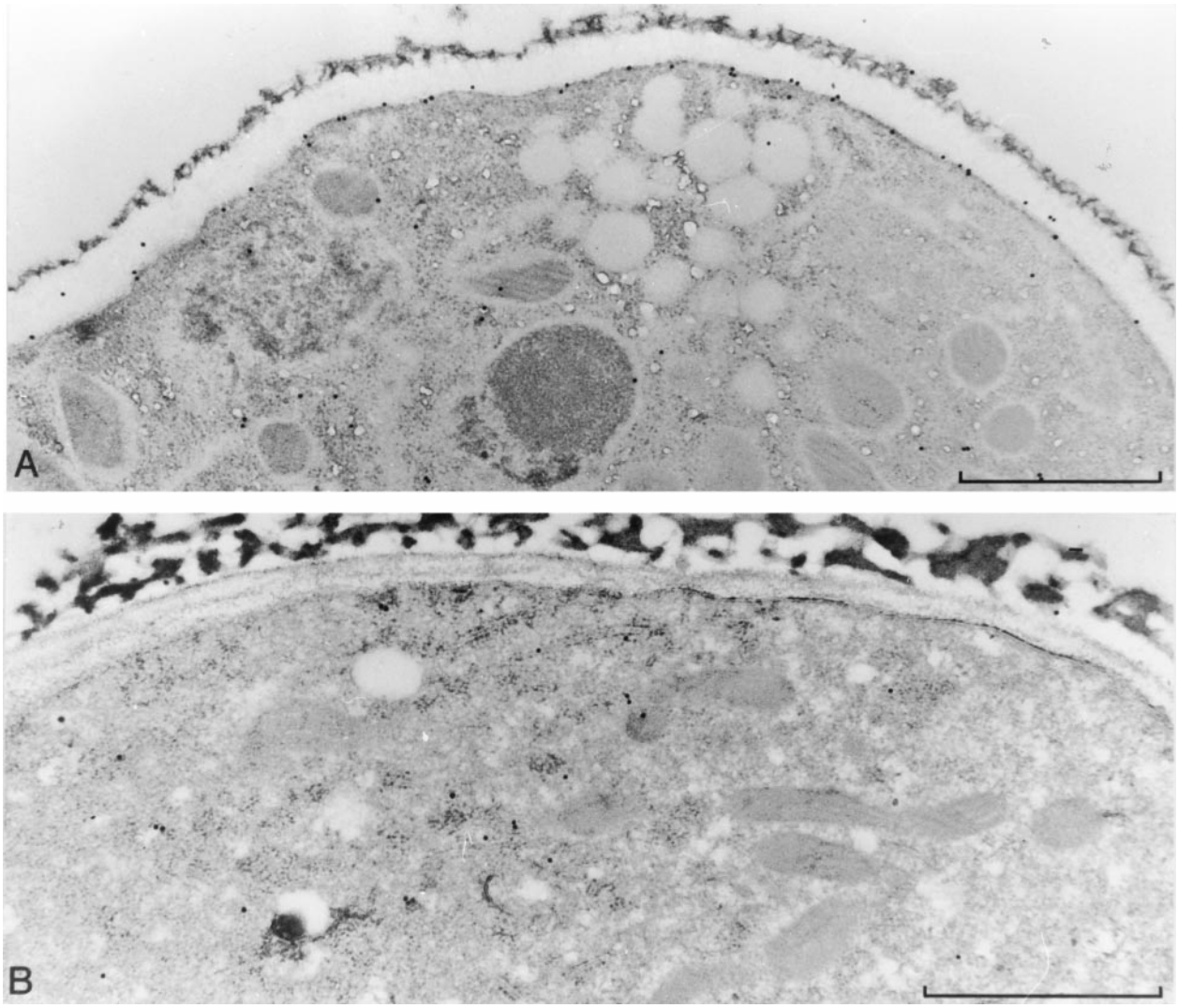

FIG. 2. Immunolocalization of COT1 using αCOT60 in *N. crassa.* (A) *cot-1* grown at 25°C for 20 h; (B) *cot-1* grown for 16 at 25°C h and for 4 h at 37°C.

tive temperature, only dispersed cytoplasmic label was seen and little labeling of the cytoplasmic membrane was observed (Fig. 2).

When antibodies prepared against a 17-amino-acid peptide from an internal segment of COT1 (αCOT17) were used, the label was only seen dispersed throughout the cytoplasm and not restricted to certain structures. This was true for wild type and *cot-1* grown at either the permissive or the restrictive temperatures.

As little label was detected in sections treated with the preimmune serum, we conclude that the label observed is due to the detection of COT1 in the fungal cell.

COT1 Kinase Is Present in Different Subcellular Fractions of N. crassa

Two COT1 kinase isoforms (~73 and ~67 kDa, respectively) are expressed in wild-type and *cot-1* strains grown at permissive temperatures. The lower molecular weight isoform was not detected in *cot-1* grown at restrictive temperatures, as determined by immunodetection experiments using either the αCOT17 or the αCOT60 antibodies (Gorovits *et al.*, 1999). In this study, a similar pattern of COT1 expression was observed in subcellular fractions prepared from the wild type and the *cot-1* mutant (Fig. 3).

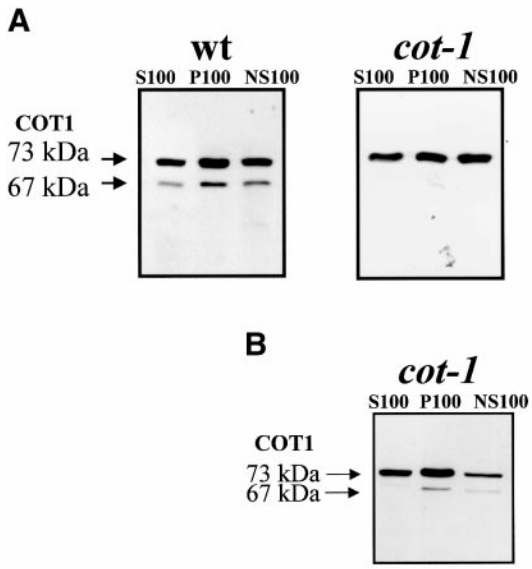

FIG. 3. Detection of COT1 using αCOT60 in subcellular fractions of *N. crassa* by Western blot analyses. (A) Cytoplasmic (S100), membrane (P100), and nuclear (NS100) fractions prepared from wild-type and *cot-1* strains of *N. crassa* grown for 16 h at 25°C and for 4 h at 37°C; (B) *cot-1* grown for 16 h at 25°C, for 4 h at 37°C, and reshifted for 4 h to 25°C.

Whether or not the function of COT1 can be attributed to the cellular distribution of the different COT1 isoforms has yet to be determined.

Western analysis clearly indicates the presence of the 73-kDa COT1 isoform in membrane preparations (P100) from *cot-1* shifted to the restrictive temperature (Fig. 3A). The 67-kDa isoform was almost undetectable by Western blot analysis. As mentioned above, immunostaining failed to detect membrane-associated COT1 in the hyphae of cultures, grown under the same conditions.

Apparently, at least some of the epitopes on COT1 recognized by αCOT17 antibodies were hidden under the conditions used for treating the EM sections but were exposed under conditions of staining the immunoblots.

The fact that the αCOT17 antibodies failed to detect membrane-associated COT1 in immunolocalization experiments could indicate a possible involvement of steric-related interference in the immunolocalization procedure.

Does the subcellular distribution of the different COT1 isoforms have functional significance? Gorovits *et al.* (1999) demonstrated that the ability of the *cot-1* strain to resume normal hyphal elongation following a shift from restrictive to permissive growth conditions was accompanied by the reappearance of the 67-kDa COT1 isoform. Immunoblotting of subcellular fractions prepared from cultures grown at 25°C, shifted to 37°C, and then reshifted to 25°C confirmed the presence of the lower molecular weight COT1 isoform (Fig. 3B). A previous study demonstrated that the level of total Ser/Thr kinase activity (as quantified by MBP phosphorylation) in a *cot-1* mutant was 65–70% of that in the wild type (Gorovits *et al.*, 1999). As the current results suggested that at least some of the COT1 polypeptide is membrane-associated, we determined if a significant change in the level of MBP phosphorylation could be detected in the membrane fraction (P100). Our results indicate that the protein kinase activity, measured in extracts prepared from cultures shifted to the restrictive temperature, was lower (~35%) than that detected in the wild type and in *cot-1* grown at 25°C. The pronounced decrease in the activity of the membrane-associated kinases could be indicative of the functional significance of the 67-kDa isoform of COT1.

DISCUSSION

Ultrastructural studies have demonstrated that the *cot-1* mutant undergoes severe disorganization of the cytoplasm when grown at restrictive temperatures. Vesicle and vesicle-like structures were seen randomly dispersed throughout the cytoplasm; vacuoles and odd-shaped mitochondria and nuclei were also observed. Similar observations have been described in hyphae treated with drugs affecting cytoskeleton integrity (Rupes *et al.*, 1995; Howard and Aist, 1977) suggesting that abnormal activity of COT1 could affect the proper functioning of the cytoskeleton. This is supported by reports of mutations suppressing the *cot-1* phenotype (*ro-1*, *ro-3*, and *ro-4*), which encode structural and regulatory proteins belonging to the cytoplasmic dynein motor complex (Bruno *et al.*, 1996).

Immunodetection experiments have established that a lower molecular weight isoform of COT1 is almost undetectable in *cot-1* when the strain is grown at the restrictive temperature (Gorovits *et al.*, 1999, and this study). Results of the comparative immunolocalization analyses of COT1 indicate that at least a portion of the lower molecular weight isoform of COT1 is associated with the cytoplasmic membrane. This finding is supported by the presence of a significant lower molecular weight COT1 isoform signal in the 100,000*g* pellet as determined by Western blot analyses.

Relocalization of proteins within the cell can induce modulation of their activities directly or through imposed changes in protein–protein associations. As the temporal

and spatial activity of regulators such as protein kinases and phosphatases must itself be regulated, it is highly conceivable that relocalization can play a role in governing the activity of some kinases and phosphatases. One clear example of such a form of regulation has been presented by Lu *et al.* (1997) who studied the significance of relocalization of a kinase belonging to the Pak family (shown to be involved in regulating the organization of the actin cytoskeleton) within the cell in terms of its own activity and its ability to activate downstream signaling pathways. By using a modified Pak1 which resulted in the constitutive targeting of Pak1 to the membrane, Lu *et al.* (1997) demonstrated that membrane localization is sufficient to activate Pak and confer biological changes in 293T cells. It is tempting to speculate that a similar situation occurs with COT1 kinase. On the one hand, the association of COT1 kinase with the membrane fraction was not expected, as COT1 has no clear hydrophobic stretches suggesting membrane anchoring. Nonetheless, the prospect for the presence of a COT1-anchoring protein (similar in function to Nck) is an attractive possibility for explaining the results we have obtained.

To characterize the tissue and subcellular distribution of the human *DM* gene product (DMPK), which is structurally related to COT1, Pham *et al.* (1998) utilized a series of monoclonal antibodies raised against the catalytic and coil domains of DMPK. They detected the presence of several DMPK isoforms (55, 72, and 80 kDa) in skeletal muscle and brain tissues and concluded that a major portion of DMPK is cytosolic. Kameda *et al.* (1998) clearly demonstrated that DMPK could be found as a component of an intramembranous constituent (the endoplasmic or sarcoplasmic reticulum) of human muscle cells. In the light of our analyses in *N. crassa*, and on the basis of the structural similarity between COT1 and DMPK, it is likely that both proteins undergo differential tissue/subcellular localization as part of their regulation.

Based on our results we suggest that most of the higher molecular weight isoform of COT1 is associated with other proteins/organelles. This complexed form of COT1 may be active in cellular functions, which have yet to be determined. We propose that the main COT1 kinase activity required for proper hyphal elongation probably involves the lower molecular weight isoform that is present in the wild type and in *cot-1* grown at 25°C, but not in *cot-1* shifted to 37°C, as the presence of the 67-kDa polypeptide correlates with the normal hyphal elongation of *N. crassa* cells. Abundance of the lower molecular weight isoform is higher in the membrane, but this isoform is also present in other subcellular fractions. Possibly, the complexed 67-kDa COT1 isoform interferes with immunodetection under conditions where extracts are not treated with detergent (as appeared to occur when αCOT17 antibodies were used for transmission electron microscopy). Whether this form of COT1 is active and required for other cellular processes or represents the COT1 kinase pool (perhaps stabilized in complex) has yet to be determined. Even though we suggest that the lower molecular weight isoform may be the one required for hyphal elongation, our findings do not rule out an alternative possibility that this is the role of the 73-kDa isoform (and the presence or absence of the lower molecular weight isoform is just an indication of improper processing or distribution of COT1 in the mutant). Even though *cot-1* has been shown to be involved in regulation of hyphal elongation, the fact that *cot-1* is an essential gene (Yarden *et al.*, 1992) along with the results presented here supports the possibility that COT1 kinase may be involved in several cellular processes and that the spatial and temporal regulation of COT1 activity involves trafficking of the kinase within the fungal cell and its possible interaction with additional proteins.

ACKNOWLEDGMENTS

We thank Michael Plamann for fruitful discussions. This research was supported by BARD, The United States–Israel Binational Agricultural Research and Development Fund, and The Israel Science Foundation.

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