academicJournals

Vol. 5(5), pp. 67-73, July 2014
DOI: 10.5897/JYFR2014.0136
Article Number: 89CFA7446440
ISSN 2141-2413

http://www.academicjournals.org/JYFR

Journal of Yeast and Fungal Research

Full Length Research Paper

Expression of neuronal protein Tau in *Candida albicans*

Kathleen Van Manen, Sara Guariglia, Alejandra del C. Alonso and Elena C. McCoy*

Biology Department, College of Staten Island, 2600 Victory Blvd, Staten Island, NY 10314, United States.

Received 27 May 2014; Accepted 4 July 2014

***Candida albicans* is a dimorphic, opportunist fungal pathogen in which cell adhesion and filamentation contribute to host tissue invasiveness and fungal pathogenicity. Morphogenesis in *C. albicans* which involves yeast to hyphae transition is dependent on temperature, the growth media composition, and is regulated by quorum sensing. In yeasts, filament formation and polarized growth are associated with the actin cytoskeletal network and microtubule interactions. The microtubule associated protein (MAP), Tau (tau), is a neuronal protein in mammals that participates in microtubule binding, microtubule assembly, bundling and stabilization. In the current study, we generated an electroporated tau derivative of a strain of *C. albicans*. We have observed that tau expression accelerates and increases the extent of medium dependent filamentation. We have also noted tau interacts mainly with actin filaments and to some extent, microtubules. We believe that these interactions are involved in the temporal control of filament formation in tau-containing cells.**

Key words: *Candida albicans*, tau, pathogenicity, morphogenesis.

INTRODUCTION

Candida albicans is a dimorphic yeast that has the ability to invade host tissue and initiate superficial and systemic infections through a process which is initiated by cell adhesion and involves filamentation and biofilm formation. Biofilms provide a protective environment for microorganisms, facilitate intercellular communication and allow organisms to remain in a favorable niche with respect to nutrients (Harris et al., 2004). Adherence of yeast cells of *C. albicans* to host tissues and abiotic surfaces is mediated by agglutinin-like sequence proteins (ALS) (Otoo et al., 2008). The ability to transition from yeast to a filamentous state occurs in response to a variety of environmental cues including carbon or nitrogen source depletion, serum addition, pH, temperatures, CO2 levels and phosphate concentration (Chandra et al., 2001). The three pathways that have been described for the regulation of hyphal development which are mediated by specific activation of transcriptional factors include: (i) the Mitogen-Activated Protein Kinase pathway (MAPK), (ii) the cAMP-PKA pathway and (iii) the pH-responsive pathway (Whiteway et al., 2007). In *C.albicans*, cells grown in 2% glucose containing minimal medium do not filament due to the production of quorum sensing molecule, farnesol (Sabine et al., 2009). Although N-acetyl glucosamine and galactose-proline minimal media support filament formation, filamentation

Corresponding Author. E-mail: elena.mccoy@csi.cuny.edu.

in both is delayed. In *C. albicans* filamentous growth is associated with two types of filaments pseudohyphae and hyphae.

Pseudohyphae, are chains of elongated cells with constrictions at septal sites whereas, true hyphae are elongated continuous, segmented, germ tubes (Liu et al., 2001; Shapiro et al., 2012). Hyphal extension in *C. albicans* is mediated by actin and microtubules with their corresponding motor proteins, kinesin, dynein and myosin (Finley et al., 2005; Fischer et al., 2008; Steinberg, 2007). Actin plays a significant role in apical growth, septation and elongation of hyphal filaments and in the localization of vesicles during cell wall extension (Fischer, 2007). Microtubules function in nuclei positioning and regulation of hyphal morphogenesis through the delivery of landmark proteins for the control of growth directionality, the polarization of the actin cytoskeleton (Finley et al., 2005; Fischer, 2007; Fischer et al., 2008; Whiteway et al., 2007) and are associated with virulence in this yeast (Sanchez-Martinez et al., 2001).

The MAP protein tau found mainly in mammalian neuronal cells is a hydrophilic, phosphoprotein, that is reported to play a major role in microtubule assembly, stabilization and bundling (Brandt, 1996; Oyama et al., 2004). Tau is coded for by a single gene located on chromosome 17. In the central nervous system, there are six different isoforms of tau that are generated by alternative splicing of its mRNA. Each isoform differs with respect to amino terminal inserts (0N-2N) and carboxyl terminal repeats (3R or 4R); which affects the protein's length, interactions with cytoskeletal elements, signal transduction and microtubule polymerization and stabilization (Buée et al., 2000; Wang et al., 2008). In microtubule assembly, tau participates in the nucleation of tubulin. Tau also modulates the dynamic instability of tubulin assembly by affecting the rates of polymerization, and the proper formation of microtubules (Brandt, 1996; Weingarten et al., 1975). Phosphorylation of tau protein is developmentally regulated. Normal tau protein contains one to three moles of phosphate per mole of tau protein whereas, in various tauopathies such as Alzheimer's disease (AD), the level of phosphorylation has been reported be three to four times higher than the normal tau protein (Buée et al., 2000; Iqbal et al., 2009; Kopke et al., 1993). The level of tau phosphorylation controls the normal interactions with microtubules. When tau is in a hyperphosphorylated state, it disassociates from microtubules and self assembles into filaments and tangles, as it has been observed in AD and related tauopathies (Alonso et al., 2001; Alonso et al., 2008; Wang et al., 2008).

As for the role of tau-actin interactions in neuronal cells, the literature on it is limited. Fulga et al. (2007) have shown that tau-induced neurodegeneration results in the accumulation of F-actin and the formation of actin-rich rods in *Drosophila* and mouse models. Buée et al. (2000) reported that the N-terminal region of tau binds to actin, and permits microtubules to interconnect with other cytoskeletal elements. Tau flexibility, which is important for neuronal stability in mammalian systems is restricted (Buée et al., 2000; Fulga et al., 2007). These authors also noted that colocalization of tau and the actin cytoskeletal was observed only when tau interacted with src-family kinase, fyn, which activates the tyrosine signaling pathway. This pathway may influence cell shape through interactions with the submembranous actin cytoskeleton (Buée et al., 2000).

The model yeast *Saccharomyces cerevisiae,* does not contain an isoform of tau, however it has been *humanized* to express both 3R and 4R tau isoforms (Braun et al., 2009; Vandebroek et al., 2005). Vandebroek et al. (2005) reported that *S. cerevisiae* has similar mechanisms for phosphorylation of tau. The yeast orthologues of the cyclin dependent kinase 5 (cdk5) and glycogen synthase kinase-3 β (GSK-3 β), Pho85 and Mds-1, can be activated by mammalian cyclins and vice versa in mammalian cells. In yeast cells deficient in Mds-1 and Pho85, hyperphosphorylation of tau was evident (Vandebroek et al., 2005). Tau protein expressed in *S. cerevisiae* does not "*appreciably*" bind yeast microtubules and it is reasonable to assume that the lack of tau binding would exclude this cell system in the analysis of the effects of aberrant tau on the microtubule cytoskeleton (Braun et al., 2009; Vandebroek et al., 2005).

In the current study, *C. albicans* strain CC504, was electroporated with plasmid vector containing the promoter region of CMV and the full length tau (2N, 4R) gene. *C. albicans* was selected as a model organism because of the importance of filamentation in biofilm formation and virulence. We proposed that if tau could be expressed in *C. albicans,* effects on the filamentation capabilities of this yeast might be demonstrable. In this study, we observed filamentation in tau containing cells of *C. albicans* CC504 after 24 h incubation at 37°C whereas the parental CC504 strain and the derivative containing the CMV plasmid required 96 h for filament formation. We also demonstrated that tau interacts with actin filaments as well as microtubules in these cells and have shown that tau interactions with actin were more pronounced in comparison with tau- tubulin interactions. We believe these findings promote the use of *C. albicans* as a model system to investigate tau interactions with cytoskeletal proteins and the influence of the tau-like proteins on the yeast filamentation process.

MATERIALS AND METHODS

Strain and growth conditions

For each experiment, starter cultures of *C. albicans* strain CC504, a clinical isolate (Quality Technologies, Newbury Park, CA) and its derivatives, CC504-CMV (CMV-plasmid), CC504-CMV-Tau (tau-containing) were grown overnight in YEP (1% yeast extract, 2% peptone, 2% glucose) complete medium at 37°C. Overnight culture

cell counts for CC504, CC504-CMV and CC504-CMV-Tau were 10^7-10^8 colony forming units (CFUs). The cells were routinely harvested by centrifugation, resuspended in galactose-proline (gal-pro) minimal medium (0.67% Difco Yeast Nitrogen Base, 2% galactose, 0.02% proline, (Fisher Scientific, Suwanee, GA)). For well plating, 10^{-2} dilutions of the cells were made and resuspended in the same medium and checked daily under stationary conditions, cell counts were performed at 24 h for determination of cell viability. Samples were imaged using the inverted light compound microscope every 24 -72 h to determine aggregation and filament formation. All images were taken with a 20x objective. For immunocytochemistry, cell cultures were prepared in the same manner except that longer time intervals were required to demonstrate filamentation (48-120 h).

Tau expression vector for *C. albicans*

The vector of choice was pFA-CMV (cytomegalovirus vector, Invitrogen, Van Allen Way, Carlsbad, CA), the microtubule associated protein tau (tau) gene was subcloned from pEGFP-N1 plasmid (Alonso et al., 2004).

pFA-CMV was restriction digested with restriction enzymes: xbaI and bgl II and incubated at 37°C overnight, 20 ng/µl was recovered (Promega, Madison, WI). After, it was gel-purified and processed with the Wizard SV gel and PCR clean-up system kits according to manufactures directions.

The tau gene insert (1.3 Kb) was obtained with PCR using infusion primers that matched the cut vector: infusion1: 5'GGGCTGGAATTCTAGAATGGCTGAGCCCCGCCAGGAGTTCG AAGTG-3'; infusion2: 5'TACTTATTCAAGATCTTCACAAACCCTGCTTGGCCAGGGAGG -3' (black sequence, is the original primer and red sequence, for the vector-specific). PCR conditions included 30 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 45 s with a final 72°C extension phase for 10 min, as described (Duff et al., 2000). The PCR product was gel-purified and processed with the Wizard SV gel and PCR clean up system kit.

The plasmid vector, pFA-CMV and PCR-tau, were then ligated using the Infusion-2.0 Dry Down kit following the manufactures guidelines (Clontech, Mountain View, CA) and transformed into *E. coli* DH-5α. For selection of transformed cells, LB-kanamycin plates were prepared at 0.1 mg/ml of kanamycin. Isolated resistant colonies were isolated and re-purified (lysed) with PureYield Plasmid Miniprep system kit (DNA purification) according to manufactures protocols, (Promega, Madison, WI), DNA concentrations were checked with a UV-double beam spectrometry, 3.95 µg/ul of DNA was recovered.

Electroporation of Tau into *C. albicans*

The purified plasmid was electroporated by a modified electroporation method (BIO-RAD Cat.No.165-2100): Cell cultures were grown overnight in YEP, harvested by centrifugation, diluted in the same medium and allowed to grow for 2 h. When cultures were between 0.5 and 1.0 at A600, cells were harvested by centrifugation, washed in ice-cold 1M sorbitol for 15 min, then centrifuged, three times. 200 µl of sorbitol was added to the cell pellets and these were transferred into microcentrifuge tubes on ice with purified vector with and without tau for 5 min. Following the incubation, the cells were pipetted into cold electroporation cuvettes, and electroporated with BioRad *Escherichia coli* Pulser at 1.5 kV for 1 s (BIO-RAD, Hercules, CA). Following electroporation, 450 µl of 1 M sorbitol was added to cuvettes then transferred to microcentrifuge tubes. Yeast cells containing plasmids with and without the tau gene were selected on antibiotic plates with 1000 µg/ml of G418. For practical purposes, the stable electroporated, CC504, *C. albicans* with the empty CMV-vector was called CC504-CMV and with CMV vector containing tau gene, CC504-CMV-Tau.

Immunocytochemistry and confocal scanning

Determination of tau

For analysis of tau protein expression, the cells were harvested by centrifugation for 6 min and resuspended in 1 mM phosphate-buffered saline, pH 7.0. The cells were re-centrifuged, the supernatants decanted and the pellets were fixed in 4% formaldehyde in 1 mM PBS for 60 min at room temperature (RT). The fixed preparations were harvested by centrifugation and blocked for 1 h with blocking buffer composed of 4% Donkey serum and 0.05% Tween-20 in 1 mM PBS. Then the samples were centrifuged and an antibody dilution buffer composed of 2% Donkey serum in 1 mM PBS was added. The primary antibody, DA9 (mouse monoclonal antibody, generous gift of Dr. Peter Davies) was added at the final concentration of 1:200 for 24 h at 4°C. Following the primary antibody exposure, the samples were washed 3 times, for 15 min each, in the antibody dilution buffer and harvested. For secondary antibody labeling, donkey anti-mouse conjugated with a fluorescent (CY5, red, Invitrogen, Carlsbad, CA) was added (1:200 dilution) and incubated at 37°C for 1.5 h in the dark. The samples were centrifuged, resuspended in 1 mM PBS, washed 3 times for 15 min each. After the last wash, 2-3 drops of Slowfade with DAPI was added to each antibody treated sample. 10 µl of each sample was added to a slide and cover slips were used to seal the slides. All samples were imaged with the Leica Confocal Laser Scanning Microscope (CLSM) with Leica software.

For determination of tau distribution with tubulin or actin, the same immunocytochemical protocol was used with some modifications. After 60 min of fixation with 4% formaldehyde, cells are harvested by centrifugation then 100% cold methanol was added to cell preparations for 4.5 min, harvested, then blocked for 1 h with blocking buffer. For primary antibody anti-tubulin, antibody T-3526 (Sigma, St. Louis, MO) was used according to the manufacturer's guidelines (1:80 dilution). Primary labeling of tau was the same as previously noted. For secondary antibody labeling anti-rabbit conjugated with a fluorescent was used according to the manufacturer's guidelines (1:100 dilution, Invitrogen, Carlsbad, CA), or rhodamine-phalloidin stain was added for F-actin labeling (1:100 dilution). Samples were imaged with Leica Confocal Laser Scanning Microscope with Leica Software. All images were taken with 60x objective. All hardware settings were the same for each cell type.

RESULTS

Tau is expressed in *C. albicans*

Tau is a microtubule associated protein that is important in both binding and bundling of microtubules. Tau protein is not normally present in yeast cells. Therefore, we decided to study the effect of tau expression in *C. albicans.* Plasmid vector pFA-CMV, was the chosen vector since, it has the yeast DNA binding domain GAL4. The tau gene was subcloned, into the pfa-CMV vector and was electroporated into *C. albicans,* and electroporated cells were selected for resistant to G418 as described in the material and methods. Tau expression in *C. albicans* was confirmed by immunocytochemistry (Figure 1). In CC504-CMV-Tau,

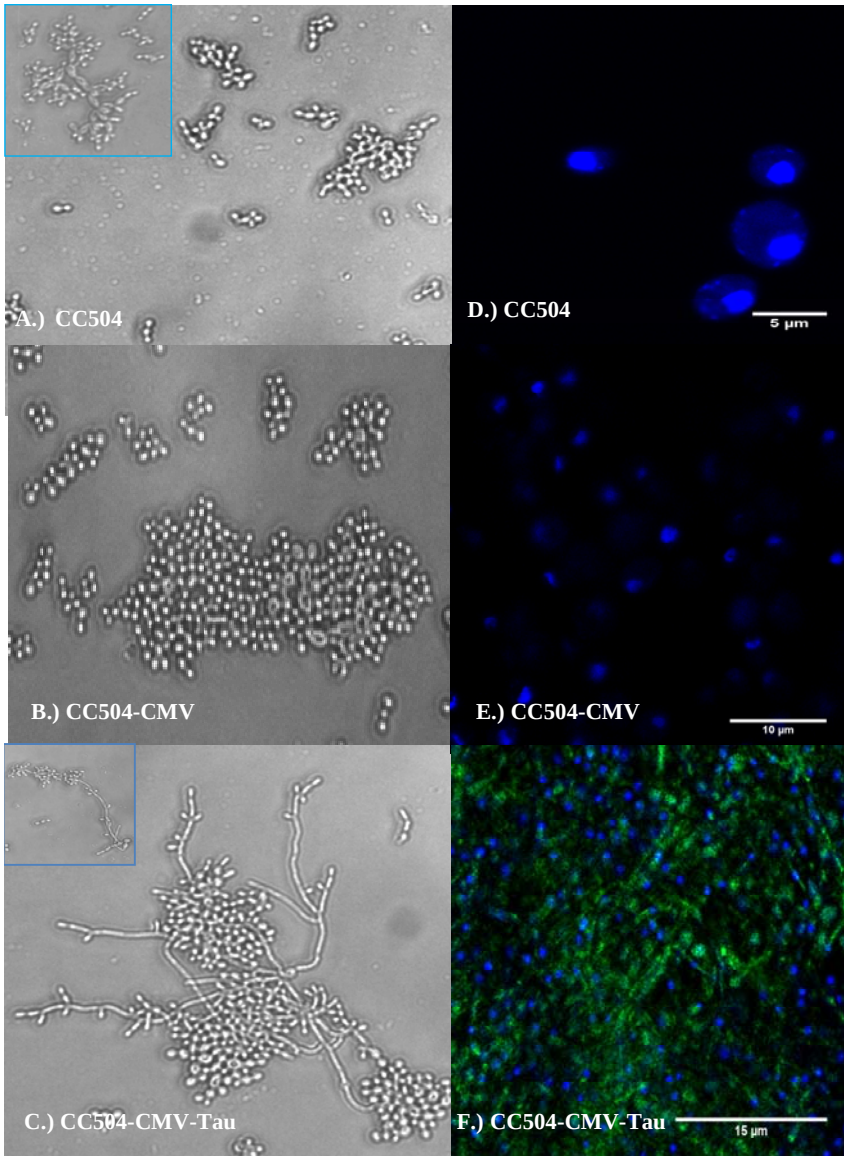

Figure 1. Tau expressing *C. albicans* exhibits filamentation: CC504, CC504-CMV and CC504-CMV-Tau were observed under compound light microscopy between 24-72 h period. Only *C. albicans* cells containing tau exhibited filamentation (C) whereas, without tau expression only yeast cells and aggregation can be noted (Images A, B, C, 200x/20x objective). Tau-expression was verified by immunocytochemistry, cells were fixed, blocked and immunolabeled with anti-tau primary and FITC-conjugated secondary antibody as described in the material and methods, then imaged with confocal laser scanning microscopy (Images D, E, F). CC504 control (D), and CC504-CMV (E) exhibited no tau labeling, and as expected CC504-CMV-Tau (F), exhibited tau expression. The nuclei of the *C. albicans* was stained with DAPI (blue) and tau was stained with green (FITC).

tau expression was observed in the cytoplasm and along the hyphae (Figure 1).

Tau affects filamentation in *C. albicans*

Adhesion and filament formation is important in the ability of *C. albicans* to invade host tissue and to initiate infection (Chandra et al., 2001; Otoo et al., 2008). To determine the effects of tau expression, *C. albicans* cells were grown in gal-pro minimal medium for 24-72 h period in well-plates under stationary conditions and checked daily for adhesion and filament formation. At 24 h, cell counts in gal-pro minimal medium for CC504, CC504-

CMV and CC504-CMV-Tau were 10^6-10^7 CFUs for determination of cell viability. As early as 24 h incubation, CC504-CMV-Tau showed filament formation, whereas the parental strain, CC504 and CC504-CMV did not even after 72 h incubation (Figure 1). This result suggests that tau expression induces early filamentation.

Tau and microtubules in *C. albicans*

Actin and microtubule interactions are important in polarized growth. In polarized growth in *C. albicans*, the delivery of nutrients to the hyphal tip is guided by microtubules, and is essential in hyphal development (Fischer, 2007; Fischer et al., 2008). Microtubule formation in *C. albicans* affects stabilization of hyphae and the polarity of actin. Tau and tubulin distributions were analyzed in CC504-CMV-Tau by immunocytochemistry using anti-tau and anti-tubulin antibodies as described in the materials and methods. It can be noted that tubulin immunoreactivity in yeast cells is more pronounced as compared to filamentous hyphae (Figure 2D). Contrary to our expectations, only a modest co-localization of tau and tubulin was observed (Figure 2F).

Tau and actin in *C. albicans*

F-actin is important in the myosin-based transport processes that participate in membrane trafficking to the growing hyphal tip (Fischer, 2007). Actin is known to be important in polarized growth in filamentous fungi. CC504-CMV-Tau were co-stained for tau and actin (Figure 2). It can be noted that the tau co-localized tightly with actin (Figure 2C). Tau co-localization with actin was observed both in yeast cells and in the hyphae (Figure 2C).

DISCUSSION

The introduction of human tau into strains of *S.cerevisiae* has been performed to study molecular mechanisms associated with tau hyperphosphorylation and aggregation in a eukaryotic cell culture system that is well-defined but less complex than mammalian culture systems (Braun et al., 2009; Vandebroek et al., 2005). In *S. cerevisiae*, the ability to study tau-microtubule interactions has been significantly affected by the lack of sufficient binding of human tau protein to yeast microtubules (Braun et al., 2009). However, studies designed to elucidate the cellular mechanisms that trigger toxicity and cell death have been reported in this yeast system (Braun et al., 2009). In the current investigation, we chose *C. albicans* strain CC504 as a test organism since in previous studies strains of *C. albicans* appeared to be more resistance to oxidative stress induced by different chemicals tested and hyphal filaments were readily demonstrable under defined growth conditions (Chandra et al., 2001; Whiteway et al., 2007; Nikoloau et al., 2009; Jamieson et al., 1996). We have demonstrated in control experiments, that parental cells and the CC504-CMV derivative strain did not exhibit immunolabeling with anti-tau antibody, which suggests that *C. albicans* does not contain a protein with epitopes similar to the human tau protein. However, structural similarities with the yeast cell-wall ALS proteins, and amyloid-forming protein have been described (Otoo et al., 2008). In our studies, CC504-CMV-Tau cells exhibited accelerated and increased filamentation as compared to the control strains. Strong interaction with actin and weak association with microtubules were also noted. There was no evidence of cellular toxicity in *C. albicans* CC504-CMV or CC504-CMV-tau.

The yeast to hyphal transition involves polarized extension of germ tubes and is associated with extensive cell wall remodeling. Actin plays an important role in the maintenance of hyphal polarity in filamentous fungi. Microtubules also function in the morphogenetic transition through protein delivery and polarization of the actin cytoskeleton (Finley et al., 2005; Fischer, 2007; Fischer et al., 2008; Whiteway et al., 2007). In neuronal cells, the formation and stabilization of microtubules and interactions with actin depend on the tau protein and are regulated by tau phosphorylation (Fulga et al., 2007). Although, the role of actin has been well documented in fungi (Harris et al., 2004), actin involvement in various neuronal tauopathies is poorly understood (Fulga et al., 2007).

The initial phases of biofilm formation which requires cell-to-cell contact and adherence are dependent on the expression of ALS proteins in the yeast cell wall. The ALS proteins constitute a family of cell wall bound adhesins with broad binding specificity for mammalian peptide ligands as well as bacterial cells (Otoo et al., 2008). Otoo et al (2008) reported that ALS proteins contain a conserved amyloid- forming T-region with protein aggregation sequences similar to those found in the tau protein of neuronal cells. In a BLAST search, 41% homology between ALS 3 and the tau protein was found (http://blast.ncbi.nlm.gov/Blast.cgi) which does not reflect identity. However, it is possible that ALS proteins and tau may have similar functions, since they have a level of structural similarity. Phosphorylation, the tau modification in mammalian cells that has been extensively studied, has been shown to be involved in the regulation of the C-terminal microtubule binding domains (Vandebroek et al., 2005). *C. albicans* contains a cyclin- dependent kinase, PHO85, an orthologue of the neuronal kinase, cdk5. PHO85 is involved in the regulation of filamentation (Shapiro et al., 2012). Vanhelmont et al. (2010) reported that serine-409 phosphorylation in the proline rich C-terminal domain of tau reduced the binding of tau protein

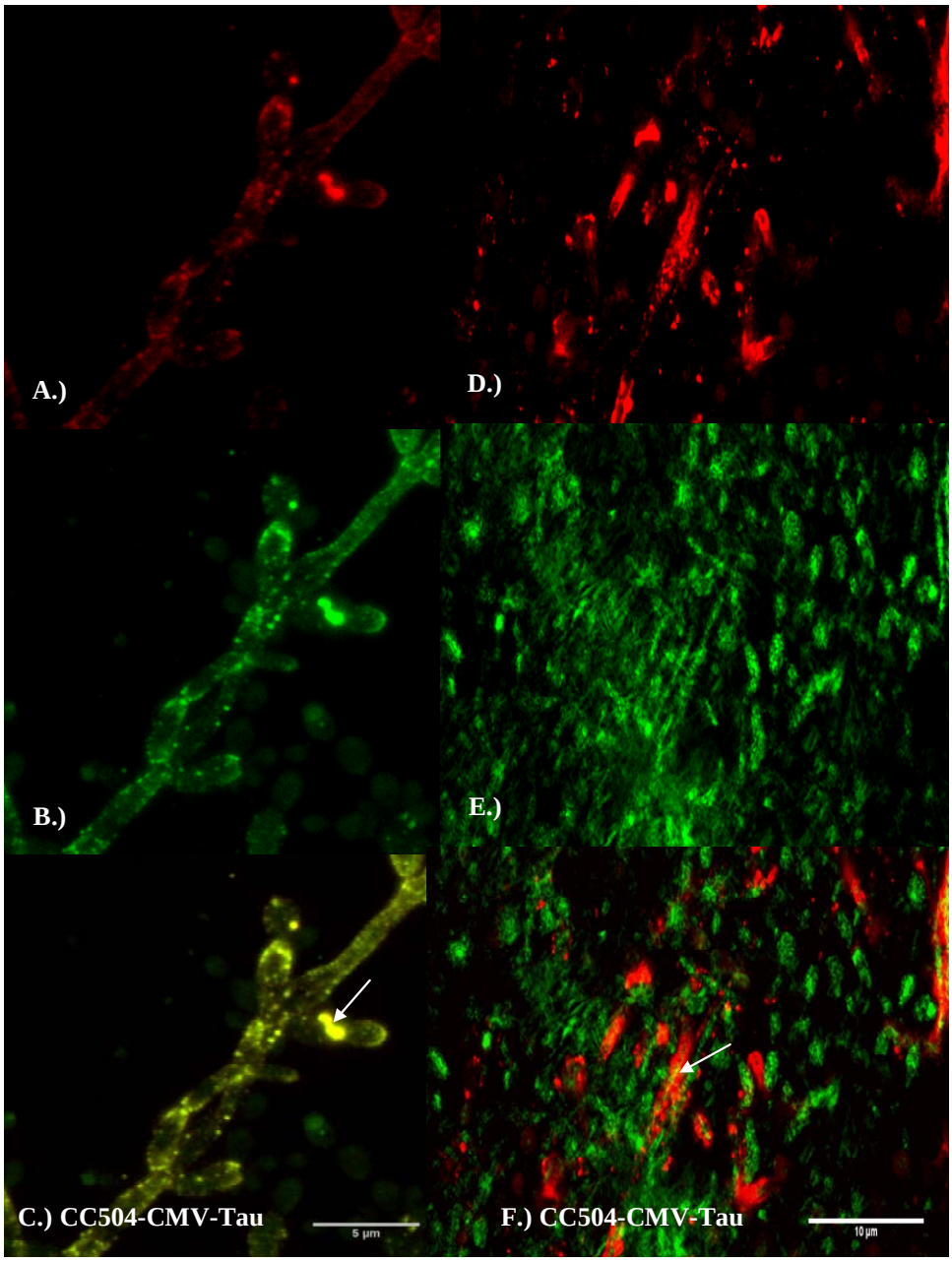

Figure 2. Tau interacts with actin and microtubules in Gal-Pro minimal medium: CC504-CMV-Tau were grown for 72 h in Gal-Pro minimal medium. For determination of tau interaction with actin or microtubules, cells were fixed, blocked and immunolabeled with anti-tau (B, C, E and F, FITC, green), anti-tubulin antibody (D and F, CY3, red) and rhodamine-phalloidin staining for polymerized actin (A and C; red) as described in materials and methods, the regions that are yellow (white arrows) are presumed to be where tau and actin co-localize or tubulin co-localized, scale bars are 5 and 10 µm.

to preformed microtubules in tau expressing yeast cells. Studies on the effect of tau modification which could account for the weak microtubule interaction and the use of an additional tau-containing *C. albicans* laboratory strain which is less efficient in biofilm formation under these growth conditions are currently in progress.

ACKNOWLEDGEMENTS

The authors thank Dr. Chang-Hui Shen, for providing the electroporation method and the BioRad *E. coli* Pulser, and Dr. Jimmie Fata, for providing the inverted-light microscope.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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