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# Ultrastructural immunolocalization of actin in a fungus

## *Rapid communication*

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**Summary.** We have successfully localized fungal actin for the first time using immuno-electron microscopy and hyphal tips of the rice blast pathogen *Magnaporthe grisea*. Following ultrarapid freezing, samples were processed in a novel substitution fluid of 10% acrolein in anhydrous ethanol and embedded in LR White resin. A monoclonal anti-actin antibody, previously shown to recognize *M. grisea* actin, bound specifically to filasomes concentrated in the peripheral cytoplasm of subapical regions, and to the core-region of the Spitzenkörper.

**Keywords:** Actin; Freeze substitution; Fungi; Hyphal tip; Immunocytochemistry; *Magnaporthe grisea*.

**Abbreviations:** IEM immuno-electron microscopy; TEM transmission electron microscopy.

### Introduction

In filamentous fungi actin has been demonstrated using fluorescence microscopy with either phallotoxins or antibodies (for a review, see Heath 1990). In hyphae and germ tubes microfilaments have been most clearly identified with transmission electron microscopy (TEM) following freeze substitution: (1) associated with septum formation (Hoch and Howard 1980, Howard 1981), (2) aligned parallel to microtubules (Hoch and Staples 1983), (3) subtending the spindle pole body (Hoch and Staples 1983), (4) associated with microvesicles to comprise filasomes (Hoch and Howard 1980, Howard 1981, Hoch and Staples 1983, Newhouse et al. 1983, Roberson and Fuller 1988, Bourett and Howard 1990), and located within the Spitzenkörper region (Howard 1981, Roberson and Fuller 1988). There is no direct evidence that any of these microfilaments are composed of actin. Using a monoclonal antibody that successfully labels actin immunofluorescently in a number of filamentous fungi, including *M. grisea* (Bourett and Howard 1991), we have localized fungal actin with immuno-electron microscopy (IEM) for the first time, and show that actin is associated with both filasomes and the Spitzenkörper.

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### Materials and methods

Cultures of *Magnaporthe grisea* (Herbert) Barr [anamorph *Pyricularia grisea* Sacc. (Rossman et al. 1990)] wild type strein O-42 was obtained from Dr. Barbara Valent (Du Pont Co., Wilmington, DE). Vegetative hyphae were generated on 5 × 5 mm pieces of cellophane (193 PUD-O, Du Pont Co.), placed on the surface of oatmeal agar medium (Crawford et al. 1986). A small block of agar medium with actively growing hyphal tips was placed on one edge of each cellophane piece, and incubated for 24–36 h. About 1 h prior to processing, the agar block was removed carefully using a fine scalpel blade.

For TEM, vegetative hyphae were frozen in liquid propane and processed via freeze substitution as previously described (Howard and O'Donnell 1987). For morphological analysis of ultrastructure, hyphae were substituted in 2% osmium tetroxide in acetone followed by embedding in Quetol 651 resin (Howard and O'Donnell 1987). For IEM a modified version of this procedure was employed. Following freezing, hyphae were substituted at − 90 °C for 2.5–3.5 days in 10% acrolein (Tousimis Research Corp., Rockville, MD) in anhydrous ethanol, dried previously over sodium sulfate at room temperature. After 30 min in fresh substitution fluid the samples were transferred to room temperature for 60 min. The remainder of the procedure was carried out at 4 °C. Following 3 rinses in absolute ethanol, samples were infiltrated in a graded series of fresh LR White Hard resin (Ladd Research Industries Inc., Burlington, VT) in ethanol: 25, 50, 75 and two 100% changes for 1–2 h each. The resin was then replaced with freshly prepared LR White resin containing 0.15% LR White accelerator (Dr. Geoffrey Newmann pers. comm.),

and the cellophane pieces were immediately flat-embedded between 2 siliconized microscope slides using plastic coverslip pieces as spacers (Howard and O'Donnell 1987). Polymerization proceeded for 16 h at 4 °C, followed by 2 h at room temperature before separating the slides.

Serial or partial serial thin sections were cut parallel to the hyphal long axis, picked up with empty single slotted grids and transferred to formvar support films (Bourett and Howard 1990). The grids were warmed to 60 °C for 10 min to insure firm attachment of sections to the formvar film and avoid subsequent wrinkling (Abad 1988). Because grids were floated on drops of immunochemicals, labeling occurred on only one face of the sections.

Sections were blocked for 30 min in 20 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl buffer, pH 8.2, plus 1% bovine serum albumin (TBS-B), and stained for 1.0 h with the primary antibody diluted with TBS-B. Actin was localized using monoclonal antibody N.350 (Bourett and Howard 1991, Dr. R. Roberson pers. comm.), obtained from Amersham Corp. (Arlington Heights, IL), at a concentration of 200–400 µg/ml. After rinsing with TBS, grids were transferred to 10 nm gold-conjugated goat anti-mouse IgM (Amersham Corp.) diluted 1 : 50 in TBS-B for 1 h. Following rinses in TBS and then distilled, deionized water, grids were stained with 1% uranyl acetate for 10 min and Reynold's lead citrate for 5 min. Controls included (1) substitution of an anti-clathrin monoclonal antibody (Boehringer Mannheim Corp., Indianapolis, IN) of the same IgM subclass as the anti-actin, used at 100 µg/ml, (2) elimination of the primary antibody from the labeling procedure, and (3) preabsorbtion of the anti-actin antibody with actin protein (Boehringer Mannheim Corp.). The specificity of labeling by this antibody was reported elsewhere (Bourett and Howard 1991). A total of 17 hyphal tip cells in three experiments were labeled via IEM.

## Results

A vesicle-free, core region was prominent in the Spitzenkörper of osmicated, Quetol embedded hyphal tips (Fig. 1). This core region consisted of a filamentous meshwork which, at low magnification, was readily distinguished from ground cytoplasm. Similar but smaller aggregates of filaments, called filasomes (Howard 1981), were prominent in the peripheral cytoplasm of the subapical region, but were thoroughly excluded from the extreme hyphal apex (Fig. 1). Typically, filasomes exhibit a microvesicular center surrounded by a meshwork of microfilaments (Figs. 1 and 2). These microvesicular centers were not well defined in unosmicated, LR White embedded cells (Figs. 3 and 4), nor were apical vesicles, which appeared as electron transparent spots (Figs. 4 and 5). Despite the loss of lipids and vesicle definition, the vesicle-free core region of the Spitzenkörper, apical vesicles and filasomes were all identified in IEM specimens without difficulty.

The monoclonal anti-actin antibody bound to thin sections over filasomes and the Spitzenkörper, especially the core region (Figs. 3 and 4). Serial section analysis demonstrated the highly specific and reproducible character of antibody binding (Fig. 5). The antibody did not appear to label filaments associated with centripetally developing septa, although only three were observed.

Labeling was virtually eliminated when (a) the primary antibody was omitted from the labeling procedure, (b) the anti-actin antibody was preabsorbed with actin protein, or (c) a "nonsense" monoclonal antibody at a similar protein concentration and of the same IgM subclass as the actin antibody was substituted (Figs. 6 and 7).

## Discussion

Using freeze substitution, a method designed to preserve cellular constituents in situ, and an antibody previously shown to recognize actin in *M. grisea* (Bourett and Howard 1991), we have successfully labeled actin in hyphal tip cells. The same antibody has also been used to label actin immunofluorescently in order filamentous fungi (Runeberg et al. 1986, Raudaskowski et al. 1988, Salo et al. 1989, Temperli et al. 1990).

The subapical, peripheral distribution in filasomes in sections of *M. grisea* is very consistent with that observed in other fungi (Hoch and Howard 1980, Howard 1981). Despite the expected lack of definition of the microvesicle center in IEM preparations, the mor-

**Figs. 1–7.** Osmicated, Quetol (Figs. 1 and 2), and unosmicated, LR White (Figs. 3–7) sections of vegetatively growing hyphal tips of *Magnaporthe grisea*. Bars: for Figs. 1, 4, and 5–7 (in Fig. 7), 0.5 µm; Figs. 2 and 3 (in Fig. 3), 100 µm

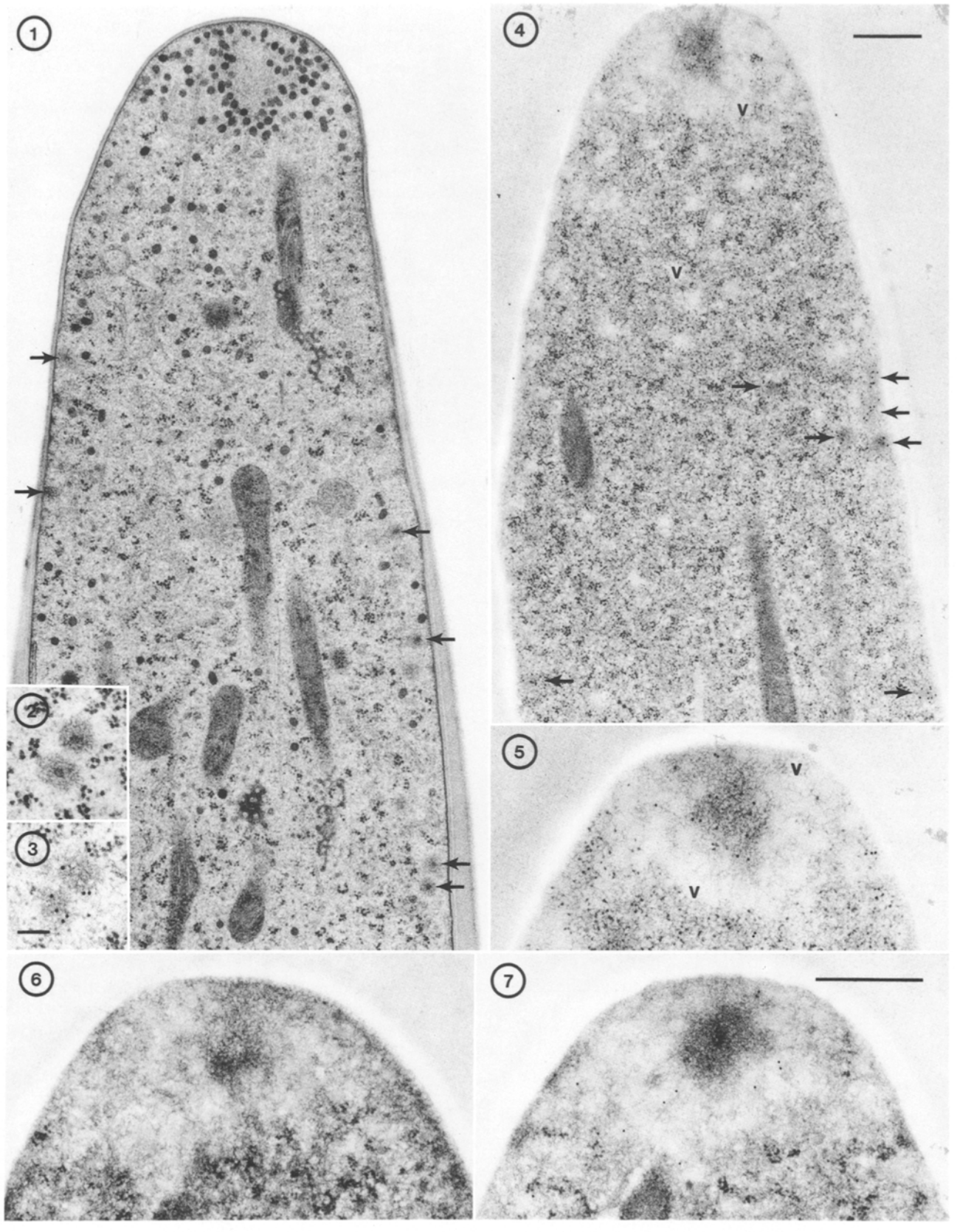
**Fig. 1.** Median, longitudinal section showing the vesicle-free, core region within the Spitzenkörper, and the subapical, peripheral distribution of filasomes (arrows)

**Fig. 2.** The typical microvesicular center of filasomes is visible only in osmicated material

**Fig. 3.** IEM detection of actin component of filasomes

**Figs. 4** and **5.** Adjacent serial sections illustrating consistent IEM anti-actin labeling of the core of the Spitzenkörper, and filasomes (arrows). Apical vesicles (*V*) appear electron transparent

**Figs. 6** and **7.** IEM labeling of the same hyphal tip (with 1 section between) using both a "nonsense" anti-clathrin (Fig. 6) and the anti-actin antibody (Fig. 7)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phology and distribution of the entities labeled with the Amersham monoclonal anti-actin antibody, clearly identify them as filasomes. This finding is consistent with the suggestion that the peripheral plaques seen following immunofluorescence labeling with either phallotoxins plaques seen following immunofluorescence labeling with either phallotoxins or anti-actin are the equivalent of filasomes (Hoch and Staples 1983, Roberson 1991). Others have considered these plaques to be either adhesion plaques because of putative connections between plaques and cytoplasmic filaments (Adams and Pringle 1984), or artefacts of preparation (Heath 1990). The exclusion of filasomes from the apex of freeze substituted hyphae differs from plaque distribution seen with fluorescence microscopy in other higher fungi. The presence of plaques in the extreme apex may well be an artefact of the poor fixation employed for fluorescence localizations.

The Spitzenkörper labeling corroborates the TEM observations of epoxy-embedded material of other fungi (Howard 1981, Roberson and Fuller 1988) and *M. grisea*, where microfilaments appeared most abundant in the vesicle-free, median, core region. This placement suggests that actin may play a role in the trafficking of apical vesicles during tip extension. It is not surprising that Spitzenkörper labeling has not been reported in other fungi using fluorescence microscopy, since it is a extremely labile structure, very sensitive to fixation conditions (Bracker 1971, Grove 1972, Howard and Aist 1979, Hoch and Howard 1980). That the core of the Spitzenkörper, recognized only recently in living cells (Lopez-Franco et al. 1990), was preserved here is directly attributable to the use of freeze substitution. Finally, it should be stressed that this report should not be interpreted to mean that actin is found exclusively within filasomes and the Spitzenkörper. Since the monoclonal probe recognizes but a single epitope, it is easy to envision that lack of additional binding could be due to the masking of this epitope as a result of interaction between actin and specific cellular constituents such as actin-binding proteins.

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