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The role of actin, actomyosin and microtubules in defining cell shape during the differentiation of *Naegleria* amebae into flagellates

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Abstract

Differentiation of *Naegleria* amebae into flagellates was used to examine the interaction between actin, actomyosin and microtubules in defining cell shape. Amebae, which lack microtubules except during mitosis, differentiate into flagellates with a fixed shape and a complex microtubule cytoskeleton in 120 min. Based on earlier models of ameboid motility it has been suggested that actomyosin is quiescent in flagellates. This hypothesis was tested by following changes in the cytoskeleton using three-dimensional reconstructions prepared by confocal microscopy of individual cells stained with antibodies against actin and tubulin as well as with phalloidin and DNase I. F-actin as defined by phalloidin staining was concentrated in expanding pseudopods. Most phalloidin staining was lost as cells rounded up before the onset of flagellum formation. Actin staining with a *Naegleria*-specific antibody that recognizes both F- and G-actin was confined to the cell cortex of both amebae and flagellates. DNase I demonstrated G-actin throughout all stages. Most of the actin in the cortex was not bound by phalloidin yet was resistant to detergent extraction suggesting that it was polymerized. The microtubule cytoskeleton of flagellates was intimately associated with this actin cortex. Treatment of flagellates with cytochalasin D produced a rapid loss of flagellate shape and the appearance of phalloidin staining while latrunculin A stabilized the flagellate shape. These results suggest that tension produced by an actomyosin network is required to maintain the flagellate shape. The rapid loss of the flagellate shape induced by drugs, which specifically block myosin light chain kinase, supports this hypothesis.

Keywords: *Naegleria*; Actin; Actomyosin; Cytoskeleton; Microtubules; Tubulin

Introduction

The differentiation of *Naegleria* amebae into swimming flagellates presents a dramatic example of changes in both cell shape and cell motility (reviewed in Fulton, 1970, 1977a, b). *Naegleria* amebae, which lack microtubules except during mitosis, have a continuously changing shape. In contrast, flagellates, which move by flagellar beating, have a fixed flattened oval shape and contain an extensive array of cytoplasmic microtubules (Walsh, 1984). The contrast between the ameboid and flagellate forms is all the more striking in that populations of amebae can convert synchronously into swimming flagellates in less than 2 h (Fulton and Dingle, 1967). In some cases flagellate-shaped cells can revert to

Abbreviations: BDM, 2, 3-butanedione monoxime; CD, cytochalasin D; LatA, latrunculin A; MAPs, microtubule-associated proteins; MLC, myosin light chain; MLCK, myosin light chain kinase; MTC, microtubule cytoskeleton; MTOC, microtubule-organizing center; NP-40, Nonidet NP40; 3D, three-dimensional

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ameboid cells with flagella in seconds and may even return to the flagellate shape just as rapidly (Fulton, 1977b). Very little is understood of how these changes in cytoskeletal organization are produced or how they regulate cell shape and function.

Seminal work by Fulton demonstrated that the acquisition of the flagellate shape is dependent on specific changes in the environment (Fulton, 1977b). Fulton's work also identified a number of variables such as divalent cation concentrations and a factor released by the cells themselves that can induce rapid changes in cell shape. This investigation resulted in a working hypothesis for the regulation of cell shape and motility in *Naegleria*. According to this hypothesis the modulation of the intracellular concentration of Ca^{2+} regulates the shift from ameboid locomotion to swimming flagellates. In this model, actomyosin becomes inactive as microtubules form. A subsequent examination demonstrated that the acquisition of the flagellate shape coincides with the development of an extensive array of cytoplasmic microtubules, the microtubule cytoskeleton (MTC); however, the actin cytoskeleton was not analyzed (Walsh, 1984). This work was restricted to cells dried onto slides eliminating any information on the three-dimensional (3D) organization of the actin or MTC.

Our current understanding of the role of Ca^{2+} in the regulation of actomyosin activity (reviewed in Krendel and Mooseker, 2005; Landsverk and Epstein, 2005), the role of actin polymerization in pseudopod extension (Pollard and Borisy, 2003) and the interaction of microtubules with the actin cytoskeleton, e.g. Dehmelt and Halpain (2004), Goode et al. (2000), Ozer and Halpain (2000) and Roger et al. (2004) prompted a reevaluation of the role of actomyosin in *Naegleria* flagellates. This investigation began by carrying out the first 3D analysis of the distribution of actin and microtubules in amebae and flagellates as well as in the transitional stages during the differentiation. This work demonstrated an intimate relationship between the actin and tubulin cytoskeletons in flagellates. It also demonstrated the unexpected formation of microtubules independent of basal bodies in these cells, which are thought to lack any other microtubule-organizing center (MTOC). The effects of drugs, which disrupt the actin cytoskeleton, suggested that an actomyosin complex is important in defining the flagellate shape. This hypothesis is supported by a series of experiments in which myosin II function was selectively disrupted.

Materials and methods

Cell culture and differentiation

Amebae of *Naegleria gruberi* strain NB-1 (reviewed in (Fulton, 1970, 1977a)) (recently renamed *Naegleria pringsheimi* (De Jonckheere, 2004)) were grown on NM agar (0.86 mM K_2HPO_4, 0.73 mM KH_2PO_4, 1.11 mM glucose, 0.2% Difco Bacto-peptone, and 2% Difco Bacto-agar) with a lawn of *Klebsiella pneumoniae* as previously described (Fulton and Dingle, 1967). Amebae were harvested and washed free of bacteria in ice-cold 2 mM Tris–HCl, pH 7.6 (2 mM Tris). Cells were differentiated into flagellates in 2 mM Tris at 25 °C on a shaking water bath and fixed in Lugol's iodine to score for the presence of flagella and to determine cell shape as previously described in detail (Fulton, 1977a).

Fluorescence microscopy

Cytoskeletons were prepared and fixed by gently dropping cell suspensions in 2 mM Tris buffer into an equal volume of ice-cold 0.9% formaldehyde, 0.1% (w/v) NP-40 (Nonidet P40), 0.125 M sucrose in 50 mM sodium phosphate, pH 7.2 (Walsh, 1984). After 30 min on ice, cytoskeletons were further fixed by the addition of 25% glutaraldehyde to 1% and held on ice for an additional 30 min.

Fixed cytoskeletons were embedded in low melting point agarose (Sigma type VII) by mixing with an equal volume of 2% agarose at 42 °C. Aliquots (100–200 μl) of agarose were held in a heating block in small glass tubes. The fixed cells were gently mixed with the warm agarose and a 50–100 μl aliquot was placed on a pre-warmed microscope slide between two pieces of 0.1 mm thick tape (Colored-Label Tape, Fischer Scientific) placed across the slide approximately 1.5 cm apart. The agarose was immediately covered with a second pre-warmed slide. The slides were held tightly together and placed on a metal block held in ice until the agarose had solidified. The slides were separated by gently rotating the top slide. The agarose was cut into 0.5–1 cm^2 squares. The agarose squares were transferred to PBS (0.15 M NaCl, 40 mM sodium phosphate, pH 7.2) in a 2.5 cm diameter by 1 cm deep well machined into a 2.5 cm thick block of Teflon® by gentle rinsing with PBS. All washing and staining was carried out in the wells by removing fluid by gentle suction using a Pasteur pipette equipped with a rubber bulb and a 200 μl micropipettor tip to prevent aspirating the agarose.

Agarose-embedded cytoskeletons were washed twice for 5 min on a reciprocating shaker with PBS, treated twice with 1 mg/ml sodium borohydride in PBS (prepared just before use) for 10 min and washed twice as above. After blocking with 2% ovalbumin in 0.1% Tween 20 in PBS for 30 min, cytoskeletons were incubated with first antibody for 60 min at 37 °C or in some cases overnight at 3 °C. After washing 4 times with PBS, samples were incubated with second antibody for 60 min at 37 °C, and washed 4 times with PBS.

In order to image cells in agarose without distorting the agarose or allowing movement due to hydraulic