Supramolecular Structures in Mycoplasmas

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Mycoplasma pneumoniae cells treated with Triton X-100 showed a detergent-resistant cytoskeleton. This cytoskeleton consists of microfilaments which seem related to eukaryotic actin filaments, both morphologically and in some chemical properties, including specific staining by anti-actin antibodies and rhodamine-labeled phalloidin. The degree of homology, however, is still unclear. In motile cells the filaments form an irregular network in the cytoplasm of the cell body and a bundle in the frontal projection corresponding to the leading edge of the gliding cells. This particular arrangement may reflect different functions. The microfilaments could be isolated by differential centrifugation. Analysis of the microfilament fraction by SDS gel electrophoresis revealed five major polypeptide bands. One of these proteins, with a molecular weight of 42.5 kd, co-migrated with rabbit muscle actin. No filaments could be found in a nonmotile mutant, M-22.

INTRODUCTION

Motility is one of the most fascinating properties of life and was for a long time regarded as expression of life itself. It occurs in a variety of modalities, including movement of higher organisms by muscle contraction and locomotion of single eukaryotic or prokaryotic cells.

Muscle contractility is based on the interaction of actin and myosin, which appears as thin and thick filaments in ultrathin sections. There is increasing evidence that motility of eukaryotic non-muscle cells also depends on the interaction between these proteins [1].

Prokaryotic organisms, however, exhibit other mechanisms of locomotion: The vast majority of motile bacteria have one or more flagella, which facilitate rapid directional movement in aqueous environments [2]. Spirochetes move in more viscous media by virtue of the possession of an endoflagellum rotating between the protoplasmic cylinder and the outer membrane [3]. A third group of motile bacteria, for example, the myxobacteria, is able to glide on wet solid surfaces, a movement called gliding motility. No external organelles associated with this movement have been identified and the molecular basis of the gliding motility is still not understood [4].

It was rather surprising that motility was detected in mycoplasmas, which have been considered for a long time as the smallest and most primitive prokaryotes. All motile mycoplasmas show gliding motility, in contrast to the related spiroplasmas, where only rotational and flexional movements occur [5]. It is interesting that the motile mycoplasma species, which include Mycoplasma pneumoniae, M. gallise-
ticum, and *M. pulmonis*, are important pathogens of humans, poultry, and small rodents, respectively [5]. In contrast to nonmotile mycoplasmas, all three motile species show specialized structures on the leading edge of the gliding cells, suggesting a possible functional relationship between these specialized structures and the gliding motility [5,6]. The molecular basis for these movements is still unknown, but there are several observations suggesting the presence of a contractile apparatus: (i) contractile waves on the stalk of moving *M. pulmonis* cells [7]; (ii) contractile processes in dividing *M. hominis* and *M. orale* cells [5]; and (iii) demonstration of possibly actin-like proteins in cell extracts of several species, including *Spiroplasma citri* [8,9,10].

The goal of this study was to demonstrate the presence of a cytoskeleton and the *in situ* arrangement of possible contractile material in adherent *M. pneumoniae* cells.

METHODS

All methods have been described previously [11]. Briefly *M. pneumoniae* strain FH cells and a nonmotile mutant M-22 were grown in PPLO broth on parlodion-carbon coated steel or platinum grids and glass coverslips for transmission and scanning electron microscopy, respectively. After 48 hours, both grids and coverslips were washed and treated with various concentrations of Triton X-100 or Nonidet P-40, ranging from 0.1–2.0 percent in buffers containing MgCl₂ and/or KCl. After this treatment the cells were processed for transmission electron microscopy by negative staining with uranylacetate. Cells were either prefixed with 3 percent glutaraldehyde, or no prefixation was undertaken. Other cells were critical-point dried and platinum-replicated or processed for scanning electron microscopy.

In order to obtain more information on the nature of the microfilaments found in detergent-treated mycoplasma, the cells were subsequently incubated with several enzymes and high molar KCl and KI solutions as described elsewhere [11].

In order to detect a possible homology between the mycoplasma filaments and eukaryotic actin, the extracted mycoplasma cells were incubated with anti-chicken muscle actin antibodies, rhodamine-labeled phalloidin, or myosin subfragment 1 from rabbit muscle, respectively [11].

The mycoplasma filaments were isolated by differential centrifugation. Briefly, 

$^{35}$S-methionine labeled *M. pneumoniae* cells were washed and lysed in a buffer containing Triton X-100, phalloidin, and phenylmethylsulphonylfluoride (PMSF). The lysate was then processed as shown in Fig. 1. Each fraction was examined for the presence of microfilaments by transmission electron microscopy of the negatively stained specimens. All fractions were simultaneously analyzed by SDS gel electrophoresis [12].

RESULTS

**Morphology of Intact and Detergent-Treated Cells**

Before starting the experiments, we compared *M. pneumoniae* cells grown on glass coverslips with organisms cultivated on coated platinum or steel grids by scanning and transmission electron microscopy. No morphological differences could be detected and all cells showed the “typical” morphology characterized by a terminal tip structure, a round or oval cell body, and a long filamentous end. The electron microscopic grids did not affect either the growth or the morphology of the organisms [11].
Isolation of Microfilaments

**MpFH 48 h Culture**
- Ultrasonication
  - 3 min, 4°C
- Centrifugation
  - 3500x g, 5 min
  - Pellet I
    - Supernatant I
      - Centrifugation
        - 40000 x g, 30 min
        - Pellet II
          - Supernatant II
            - Buffer + 5 mM ATP
              - 1 h, 4°C
              - Centrifugation
                - 80000 x g, 20 min
                - Pellet III
                  - Supernatant III
                    - Microfilament rich fraction

**FIG. 1.** Isolation of *M. pneumoniae* microfilaments by differential centrifugation.

Cells of the nonmotile mutant M-22 were pleomorphic and often extremely enlarged. No single organism showing the typical morphology of the motile parental cells could be detected.

After membrane solubilization with either Triton X-100 or NP-40, a detergent-insoluble cytoskeleton remained attached to the substrate, consisting of a rodlike structure and a filamentous network, which corresponded to the cytoplasmic area of the cell. The rodlike structure was located at the edge corresponding to the tip structure of intact cells and appeared to consist of a bundle of filaments arranged in parallel. On the distal end, a knoblike extrusion could often be detected, where apparent membraneous material was attached. This suggested that a close contact between the rodlike structure and the membrane existed in this specific area.

Cytoplasmic filaments were noted to rise from a basal thickening of the rod and were about 5 nm wide, forming an irregular network in the cytoplasm of the cell body. As shown in platinum replicas, they seemed to consist of a double-stranded helix composed of globular subunits [11].

**In situ Characterization of the Microfilaments**

The filaments could be readily destroyed by pronase, but were relatively trypsin-stable. Treatment with phospholipase B and DNase I did not affect the cytoskeleton. The structures were stable in high molar KCl solutions, but were destroyed by KI solutions of the same molarity. Following this latter treatment, however, some parts of the rodlike structure remained [11].
FIG. 2. A: Microfilaments of a *Mycoplasma pneumoniae* lysate isolated by differential centrifugation. The size of individual filaments was about 5 nm (arrowhead). No bundles have been found. Negatively stained with 1 percent aqueous uranyl acetate. Bar represents 50 nm. B: Microfilament fraction after 48-hour dialysis against 15 mM MgCl₂ solution. Some of the filaments aggregated to form bundles (arrowheads). Negatively stained with 1 percent aqueous uranyl acetate. Bar represents 100 nm.

**Specific Fluorescence with Anti-Actin Antibodies and Rhodamine-Labeled Phalloidin**

Further evidence for a possible actin-like nature of the *M. pneumoniae* microfilaments was obtained by incubating the fixed and permeabilized cells with highly purified anti-actin antibodies. The resulting fluorescence, however, was weak and mainly restricted to the microcolonies. All controls using non-immune sera were negative [11].

Incubation of permeabilized organisms with rhodamine-labeled phalloidin resulted in a specific fluorescence of a much higher intensity. The whole cells were equally stained and no predominant stain of any part of the cell could be detected. Cells pretreated with unlabeled phalloidin showed no fluorescence. Fluorescence in similarly treated fibroblasts was restricted to the actin-containing stress fibers [11].

**Reaction with Myosin Subfragment 1**

All attempts to decorate the microfilaments with myosin subfragment 1 failed. In none of the preparations was the typical arrowhead formation, that occurs with rabbit muscle actin, detected [11].

FIG. 3. Autoradiograph representing different fractions of a microfilament preparation of metabolically ³⁵S-methionine-labeled *Mycoplasma pneumoniae* cells. To each lane, equal amounts of cpm were applied. Lane A: polypeptides of the 40,000 g supernatant, Lane B: the 80,000 g pellet, Lane C: the 80,000 g supernatant microfilament-rich fraction. The major polypeptide bands are indicated by arrowheads as 61 kd, 50, 42.5, 39 and 33. The 42.5 kd protein co-migrated with rabbit muscle actin (asterisk). RMA = rabbit muscle actin MW = molecular weight standards.
Isolation of the Microfilaments

The microfilaments of motile *M. pneumoniae* cells could be isolated by differential centrifugation and were enriched in the 80,000 g supernatant. In lysates of the nonmotile mutant M-22, microfilaments could not be detected in any of the fractions.

The microfilament-rich fraction contained irregularly crossing filaments obviously contaminated by attached membraneous material. It was impossible to determine the length of individual filaments, but the size was equal to that of the structures found in the *in situ* lysed cells (Fig. 2A) [11]. Even after prolonged dialysis against a high molar MgCl₂ buffer only a few bundles, but no paracrystals, could be detected (Fig. 2B).

Analysis of the microfilament-rich fraction of ³⁵S-methionine labeled *M. pneumoniae* cells by SDS gel electrophoresis and subsequent autoradiography revealed five major polypeptide bands with molecular weights of 61 kilodalton (kd), 50 kd, 42.5 kd, 39 kd, and 33 kd, respectively. The 42.5 kd protein co-migrated with rabbit muscle actin (Fig. 3).

DISCUSSION

The presence of a highly organized cytoskeleton in mycoplasma cells underlines again the unique position that these organisms hold among other prokaryotes, where no such cytoskeleton has been detected. The organization of the detergent-resistant residues showed a different arrangement of the filaments in the cytoplasm versus the tip structure. This, together with the observation that the tip is always the leading edge of the gliding cells, suggests that the two structures may have different functions. Other authors have described a shortening and thickening of the rod structures, indicating some kind of contractile apparatus [10]. Strong evidence for the localization of the locomotor apparatus in the tip structure is given by the fact that the motility of *M. pneumoniae* cells could be inhibited by a monoclonal antibody against a surface protein found exclusively at the tip structure [13].

The morphology of the detergent-resistant microfilaments is very similar to the structure of eukaryotic actin filaments and our results serve to confirm previous reports on actin-like proteins in mycoplasmas [9,14]. These microfilaments differ considerably from the helical structure isolated from different *Acholeplasma* species and from fibrils found in *Spiroplasma citri* [15,16]. The degree of homology between eukaryotic actin filaments and the filaments found in *M. pneumoniae* is still unclear.

Both proteins share several common properties, including polymerization in buffers containing MgCl₂ and/or KCl, relative trypsin resistance, destruction by KI, cross-reaction with anti-actin antibodies, and most interestingly, specific reaction with rhodamine-labeled phalloidin. The latter has been shown to bind specifically only to the filamentous forms of eukaryotic actin [17]. One clear functional difference between the mycoplasmal filaments and F-actin is the failure of the mycoplasma filaments to react with myosin subfragment 1. It is of course possible that molecules other than myosin-like molecules may act as functional counterparts, as indicated by the presence of material remaining after destruction of the filaments by KI.

Further efforts have to be made in order to purify and characterize the different microfilament proteins and establish their role in the mechanism of gliding and the maintenance of the cell shape. Because of their relatively simple structural organiza-
tion, they may also serve as a possible model to study the nature of filament-membrane interactions.

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