Manipulation and expression of molecular motors in *Dictyostelium discoideum*

D. J. MANSTEIN

National Institute for Medical Research, London NW7 1AA, UK

K. M. RUPPEL, L. KUBALEK and J. A. SPUDICH

Departments of Cell Biology and Developmental Biology, Stanford University, Stanford CA 94305, USA

Summary

The eukaryote *Dictyostelium discoideum* is an attractive model organism for the study of cytoskeletal proteins and cell motility. The appearance and behavior of this cell closely resembles that of mammalian cells, but unlike mammalian cells, *Dictyostelium* offers the opportunity specifically to alter the cell physiology by molecular genetic approaches.

Key words: *Dictyostelium discoideum*, cytoskeleton, myosin, gene targeting, expression.

We are currently witnessing remarkable advances in our knowledge of the cytoskeleton and its motile elements. This progress can in part be attributed to the fact that the majority of cytoskeletal proteins are highly conserved throughout the eukaryotic kingdom, and to the recent progress made in the application of molecular genetic techniques for the manipulation of lower eukaryotic organisms such as *Saccharomyces cerevisiae*, *Aspergillus nidulans* and *Dictyostelium discoideum*. The simplicity of the genome of these organisms, and the result that transfections lead to a high frequency of homologous recombination events relative to random integration events, allows one to modify the structure and expression of the gene encoding a particular protein in a specific manner. As a subject for study of the cytoskeleton, *Dictyostelium* stands out among these model organisms in that it displays many forms of cell motility. It extends filopodia and pseudopodia, undergoes chemotactic movements and changes shape in response to signals, such as those operating during mitosis. In doing so, the appearance and behavior of this cell closely resembles that of mammalian cells.

The use of *Dictyostelium* to study the cellular function of myosin has been particularly rewarding. Two distinct classes of myosins are known to occur in nonmuscle cells. The first class of nonmuscle myosins are those which, like their muscle-located relatives, consist of two identical heavy chains (about $200\times10^3 M_r$) and two pairs of light chains ($14–24\times10^3 M_r$) and can form bipolar filament assemblies. These are generally referred to as myosin and sometimes as conventional myosin or myosin II. The second class, called myosin I, consist of a single, smaller heavy chain ($110–140\times10^3 M_r$) and one or more light chains and cannot form bipolar filaments. The amino terminal part (approx. $90\times10^3 M_r$) of the heavy chains of both myosin and myosin I form a globular head and it is this region which shows the highest degree of sequence conservation among all classes of myosins. It is also this part of the molecule, often referred to as the myosin head or subfragment-1 (S1), that contains the binding sites for the light chains and that has the catalytic and force generating properties of the myosin molecule (Toyoshima *et al.* 1987).

The high frequency of homologous recombination events in *Dictyostelium* was initially exploited in order to create cells which express a heavy meromyosin (HMM) fragment and only <0.1 % of wild type levels of intact myosin (De Lozanne and Spudich, 1987) and more recently cells in which the entire coding region for the single myosin heavy chain gene (*mhc*A) was deleted (Manstein *et al.* 1989). In complementary experiments antisense RNA was used to reduce the expression of myosin to <1 % of wild type levels (Knecht and Loomis, 1987). It became clear from these studies that in *Dictyostelium*, myosin is required for the furrowing event of cytokinesis, is essential for cell-surface receptor capping but not patching, contributes to the establishment of cell polarity and is necessary for morphogenetic changes associated with development. However, even in the absence of myosin, cells display many forms of movement, including intracellular particle movement, formation of cell-surface extensions, karyokinesis and cell migration, albeit in a less polarized form (Spudich, 1989). Definitive genetic proof that the phenotype attributed to the myosin null mutant is in fact due solely to the elimination of the *mhc*A gene and not to secondary mutations, which might have been induced during their generation, was achieved by the reintroduction of the cloned gene (Egelhoff *et al.* 1990), resulting in complementation of all features of the mutant phenotype.

Another important feature of *Dictyostelium* is that it can be used as an expression system. *Dictyostelium* is biochemically well characterized, can be grown readily in the laboratory on a large scale (200 g of cells are obtained from 30 to 40 liters of shaken culture), and carries out mammalian like post-translational modifications. However, *Dictyostelium* is a phagocytic cell, abundant in lysosomal proteases, and special precautions should be taken to protect proteins against their activity (Spudich, 1987). An effective way to eliminate most of the protease activity is to starve cells for approximately four hours. This prevents further synthesis of proteases and stimu-

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lates their secretion. Secretion can be further stimulated by adding sucrose or other disaccharides to the starvation medium (Dimond *et al.* 1981; North *et al.* 1990). Additionally, cell lysis and protein purification should always be performed in the presence of protective agents such as phenylmethylsulfonyl fluoride (0.5 mM), benzamidine (5 mM), tosyl lysine chloromethyl ketone (0.25 mM) and leupeptin (0.01 mM). The addition of 40 mM sodium pyrophosphate and 30 % (w/v) sucrose to the lysis buffer is also recommended.

The expression and purification of functional myosin head fragments represents a potentially powerful tool for the study of chemo-mechanical coupling. Understanding the mechanism by which myosin catalyzes the transduction of energy stored in chemical bonds into mechanical work requires high resolution structural information. However, obtaining crystals and structural data of S1 has proved to be a difficult task. This is undoubtedly due to heterogeneities in the starting material when S1 is prepared from muscle myosin. Muscle tissue typically contains multiple isoforms of the myosin heavy and light chains and further heterogeneities are introduced during the proteolytic digestion of myosin. Therefore, it was a most promising breakthrough when Rayment and Winkelmann (1984) obtained crystals of S1 from chicken pectoralis muscle. Although these crystals might be sufficiently ordered to diffract X-rays to 0.4 nm resolution, the heterogenious nature of the starting material might still present a problem. One way to avoid all these problems is to express an S1-like fragment in a host like *Dictyostelium*. An additional benefit of working with a recombinant protein is that it is accessible to molecular genetic manipulation. For example, the availability of site-directed mutagenesis might potentially facilitate the analysis of the S1-structure on several levels. One major obstacle in protein crystallography, the trial and error search for suitable heavy atom derivatives, can be overcome by generating S1 molecules with engineered heavy atom binding sites. The ability to alter the protein by site-directed mutagenesis will also provide ways for testing working models for the mechanism of chemo-mechanical coupling.

So far, two functional myosin head fragments have been expressed successfully in *Dictyostelium*. These fragments correspond to the proteolytically defined muscle meromyosin heavy chain (HMM–140), which forms a dimer consisting of two globular heads and 50 nm of alpha-helical, coiled-coil rod, and to a monomeric S1-like myosin head fragment (MHF) of the myosin heavy chain that extends 46 amino acids beyond the proline which marks the region of proteolytic cleavage in muscle myosin. Their expression in a functional form relies in part on the fact that *Dictyostelium* can supply the expressed fragments with its endogenous myosin light chains. Indeed, both recombinant proteins, HMM–140 and MHF, copurify with the essential and regulatory myosin light chain, decorate actin filaments, display actin-activated adenosine triphosphatase activity, and support sliding movement of actin filaments in the *in vitro* motility assay developed by Kron and Spudich (1986). Recombinant fragments are expressed in a wild-type background and not in myosin null cells. The disadvantage of having to purify the myosin fragments away from endogenous myosin is fully compensated for by the ability of the wild-type cells to grow in suspension. The differences in size and and solubility properties make it very easy to separate myosin and myosin head fragments. Typically, one obtains 1–2 mg of purified HMM–140 or MHF from 100 g of cells (Ruppel *et al.* 1990; Manstein *et al.* 1989). Higher expression levels can in principle be achieved. However, cells that produce larger quantities of myosin fragments have phenotypic characteristics similar to myosin null cells and are difficult to grow in suspension.

Crystallization trials using MHF are now in progress. Needle shaped microcrystals can be readily obtained at neutral pH, in the presence of the magnesium salts of either adenosine-5′-diphosphate, adenylyl-imidodiphosphate, or pyrophosphate, and with 8 to 10 % (w/v) polyethylene glycol 4000 as precipitating agent. Electron micrographs of these needles, which are 0.1 to 0.5 μm in width and often exceed 100 μm in length, show extensive order even after preservation in heavy metal stain (uranyl acetate) (Fig. 1). Electron microscopic studies of these needles in the frozen hydrated state should yield more structural information, possibly at higher resolution (Stokes and Green, 1990). However, our efforts are still focused on the generation of crystals suitable for high resolution X-ray analysis. Dramatic differences in the crystallization behavior of a protein are often caused by minor differences in amino acid sequence, as often found among homologous enzymes isolated from different species. Therefore, we are currently exploiting the possibility of expressing different types of myosin head fragments in *Dictyostelium*.

The production of force and movement by S1 is thought to be linked to large conformational changes within the protein, occurring upon binding and interaction with nucleotide and actin. Determining the structure of S1 in these different structural states might therefore prove crucial for the understanding of the mechanism of chemo-mechanical coupling. The information necessary for the modelling of these associated states could be obtained by the crystallization of the myosin head complexed to actin. One way to achieve this is by crystallizing an actin: DNAaseI:S1 complex. Even if the sheer size of this complex creates a formidable crystallographic problem this route should not be ignored, especially since Kabsch, Holmes and coworkers have recently solved the structure

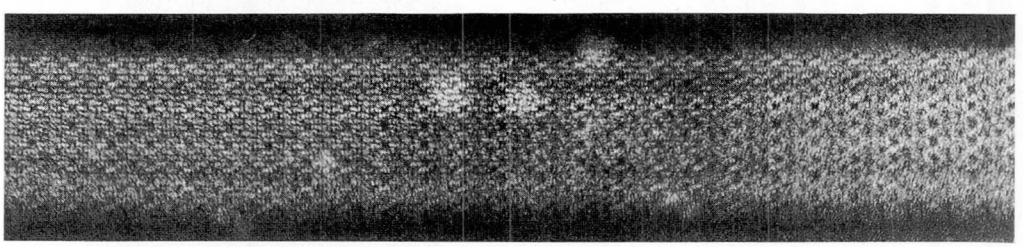

Fig. 1. Electron micrograph of a microcrystal of a *Dictyostelium* myosin head fragment (MHF) negatively stained with uranyl acetate. The microcrystal is about 200 nm in width and shows extensive order even after preservation in 1 % (w/v) uranyl acetate.

of the actin:DNAaseI complex in the ATP and ADP forms at an effective resolution of 0.28 nm and 0.30 nm (Kabsch *et al.* 1990). A second complementary way would be to crystallize a myosin head complexed to a nonpolymerizable monomeric actin. Such a complex may be generated from myosin head fragments and G-actin treated with m-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Bettache *et al.* 1989).

Dictyostelium proved to be an ideal system for the elucidation of the cellular function of the conventional myosin. The application of molecular genetics in this system, directed towards structural studies of the myosin motor, in combination with the recent progress made in the *in vitro* study of this molecular motor (Kron *et al.* 1990) should soon provide us with a better understanding of chemo-mechanical coupling.

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