2. ABSTRACT
The directed movement of cells towards external cues (chemotaxis) is required for multicellular development and full function of the immune system. Starvation initiates a simple developmental program in *Dictyostelium* that requires chemotaxis to cAMP. The actin cytoskeleton and its associated motor proteins, the myosins, power cellular movement and maintain cell polarity. A novel *Dictyostelium* myosin, myoG, has been identified and found to be essential for cell polarization and motility. The myoG null mutants exhibit normal actin-based behaviors (e.g. growth, endocytosis) and motility under basal conditions but fail to increase their rate of translocation and change cell shape (from round to elongated) upon activation of the developmental program. The goal of the proposed work is to establish the contribution of this myosin to cell migration and polarization by determining its subcellular localization in chemotactic cells and establishing the contribution of myosin motor activity by analyzing mutants that cannot move along actin.

3. PRESENT STATUS OF KNOWLEDGE
The directed movement of cells to their correct locations is required for the development of complex organisms (1, 2). For example, the positioning of neurons within the forming layers of the brain requires movement of specified neurons to the correct cortical layer and the formation of a gonad requires the long-distance migration of primordial germ cells (PGCs) to the region of the developing embryo where somatic gonad tissue is specified. These migratory cells follow external chemical cues provided by target tissues, a process known as chemotaxis.

The simple eukaryote *Dictyostelium* is a powerful model organism for dissecting the molecular basis of chemotaxis and cell migration (3). *Dictyostelium* move in an amoeboid fashion, similar to PGCs and lymphocytes, and chemotaxis is mediated by a G-protein coupled receptor, cAR1. *Dictyostelium* appeared early in the animal lineage, making it a valuable system for fundamental studies of cell motility and chemotaxis in development. *Dictyostelium* can exist either as solitary amoebae or a multicellular organism. The transition between the two states is triggered by starvation and results in a fruiting body consisting of a slender stalk tipped by a mass of spores. Completion of this developmental program is dependent on the ability of cells to polarize and chemotax to cAMP shortly after the onset of starvation. Dynamic interactions among the actin cytoskeleton and its associated binding and motor proteins are critical for the generation of both cell polarity and movement.

Myosins are diverse family of ATP-dependent actin-based motor proteins that translocate along and generate force against actin polymers (4). They are best-known for their specialized role in muscle contraction, but recent studies have uncovered an astonishing variety of non-muscle myosins and myosin functions (e.g. organelle transport, endocytosis, transcription) (4, 5). As the majority of myosins do not form filaments and are not processive (i.e. a single molecule moves continuously on the same track instead of diffusing away due to Brownian movement), one cannot simply explain their functions in terms of muscle contraction and a more open-minded approach is required when considering their individual roles.
**A novel myosin required for chemotaxis.** myoG is a unique *Dictyostelium* myosin expressed in both vegetative (i.e. non-starving) cells and all throughout early development. The myoG null express both *carA* and *acaA* (key chemotaxis genes) upon starvation, but fails to polarize and chemotax (Fig. 1). This causes a complete failure in early aggregation and development (Fig. 2). When a large number of wild type cells are mixed with myoG nulls, the mutants do not move towards their polarized, wild type counterparts and wild type cells do not move towards the myoG mutant. The mixture forms a fruiting body, and a disproportionately small number of mutant spores are recovered. Thus, the mutants are capable the necessary developmental transitions to form spores. The small number of spores indicates that a small number of myoG null cells are fortuitously trapped and carried along in the aggregate and this enables them to develop. In summary, myoG has a decisive role in the physical transformation of a starving *Dictyostelium* cell from a rounded, randomly moving cell to a polarized and chemotactically competent cell but is not required for cell fate determination.

The observation that myoG null cells are defective in movement during early development raises the question of whether myoG has a general cytoskeletal role. Preliminary experiments reveal that actin-dependent behaviors of vegetative cells such as translocation, cell growth and fluid uptake are normal in the myoG nulls. However, the dramatic increase in cell speed (~5x) that occurs upon starvation (6) does not occur in the myoG null mutant. Together, these results reveal that myoG is not required for movement per se but rather plays a critical role in generating the increased motility that is necessary for the coordinated and efficient aggregation of starving *Dictyostelium*.

One striking attribute of myoG is its tail structure – the conserved myosin motor domain is followed by a tail that has a tandem repeat of a MyTH4/FERM module separated by an SH3 domain – a domain arrangement identical to that of myosin 7 (M7) (Fig. 2), a conserved myosin with roles in adhesion throughout phylogeny (7) also found in *Dictyostelium*. Thus, although the phylogenetic analysis (carried out by comparing myosin motor sequences) indicates that myoG is unique to *Dictyostelium*, it does have some attributes of a more widely expressed myosin. Since the prevailing view in the field is that distinct myosin tail structures dictate the role of a given myosin by determining its subcellular localization, it is of interest that myoG and M7 have such similar tail structures yet strikingly different functions. This suggests that the motor domain of myoG could make a significant contribution to directing its function.

**4. PLAN OF WORK**

Two fundamentally important questions will be addressed:

1) Is myoG localized at the plasma membrane in regions of dynamic actin (i.e. at the leading edge of extending pseudopodia)?

2) Does myoG acting as a force generator that contributes to enhanced cell migration?

1) **Where is myoG localized?**

Proteins with major roles in cell motility are typically present at either extending or retracting regions of the cell, in the cortex of the cell (that is rich in actin) or on the plasma membrane. Their localization is dynamic, with the appearance of such proteins at the cortex/membrane coinciding with the formation of a protrusion at the front or retraction of the rear of the cell. The ability to follow the dynamic distribution of myoG
during both vegetative and chemotactic motility in living cells will establish whether
myoG is localized to either pseudopodia at the front or the retracting rear of the cell.
An expression plasmid carrying a gene encoding a GFP-tagged myoG will be created,
the fusion expressed in the myoG null mutant and tested for the rescue of the null
mutant phenotype to establish that the fusion protein is fully functional. Once a
rescuing fusion is expressed, the localization of GFP-myG in randomly moving
vegetative and polarized chemotactic cells will be analyzed using confocal microscopy.

The localization of myoG will be directly compared to that of M7 using dual color
imaging. An RFP-tagged myoG will be expressed along with GFP-M7 in either a myoG
or M7 null mutant background. Live cell imaging will be performed as above and the
extent of co-localization (or not) of the two myosins determined. One simple scenario is
that there are two distinct motility machines, a basal one that involves M7, and another
that requires myoG and is solely active in multicellular development. In this case,
myoG may be involved in the adhesion of chemotactic cells and it may be specifically
recruited by a distinct set of binding partners that are expressed upon starvation. Thus,
the two myosins should have strikingly similar localizations in chemotactic cells but
myoG may be localized (or not) differently in vegetative cells. However, if M7’s role is
distinct from that of myoG, they should be differentially localized both in chemotactic
and vegetative cells. In short, a comparison of myoG localization with that of M7 will
provide valuable clues about how myoG contributes to cell migration and its
relationship to M7.

A deletion approach will be used to determine myoG domains that specify subcellular
localization. A GFP-fusion of the tail will be expressed in the null mutant and its
localization and ability to rescue the mutant phenotype compared to that of full-length
myoG. Next, individual or combinations of the major tail domains (MyTH4, FERM,
SH3) will be deleted from the full-length protein and localization and functionality of
the mutant determined. The identity of functionally important regions of the tail will
enable future efforts to isolate myoG binding partners.

Method. Standard Dictyostelium GFP- and RFP-expression plasmids are available in the
laboratory (7, 8). The plasmids contain a cassette conferring resistance to G418, a low
copy number Dictyostelium origin of replication and a strong actin 15 promoter to drive
expression of the GFP fusion. These will be introduced into cells via electroporation
and colonies screened for expression of the fusion by fluorescence microscopy and
immunoblotting. Transformants will be washed into non-nutrient buffer and deposited
on non-nutrient agar plates or buffer-soaked black pads. The plates will be analyzed for
the formation of small aggregates and mounds within 12 hr and mature fruiting bodies
by 24-36 hr. The presence of this structure would indicate that the fusion rescued the
defect. Initial polarization and chemotaxis of cells will be analyzed by depositing
starving cells on a glass slide and observing the movement of a small population of cells
6 hr after the onset of starvation. Confocal microscopy of the rescued strains will be
performed at BIPL (7).

2) What is the contribution of myoG motor activity to its cellular function?
The once simple concept of myosins is being stretched as new functions for this large
class of motors are identified. For example, recent studies have indicated that tension
generated by a class 1 myosin helps to anchor the plasma membrane in microvilli
(specialized actin-filled extensions extended by cells in the intestine) to the underlying actin cytoskeleton. More recent work has revealed that the motor domain can play a significant role in the localization of myosin. In other words, some myosins are force generators that do not move and others may convey nothing but themselves. Detailed studies of several myosins have established a good deal of kinetic diversity between them and this, no doubt, reflects wide functional diversity. Previous work has focused on identifying transport functions and less on other roles. Analysis of cells expressing a kinetically altered myoG should provide insight into its role and, in combination with localization data, this should provide an explanation for how such a unique myosin can have a decisive role in the conversion of single cells to multicellular organism.

Key amino acids that dictate ATP binding and hydrolysis and actin binding are present in the highly conserved motor domains of all myosins. Changes in ATP binding and hydrolysis are tightly linked to actin binding and mutations in myosin ATP binding sequences can dramatically alter the motor function. The effect of two different mutations on the activity of Dictyostelium M2 has been studied in great detail (9, 10). The N233A mutation in the switch I region of the nucleotide binding site results in an almost complete loss of ATP binding, locking the myosin into a “strong actin binding” or rigor state. In contrast, the E476K mutation in the switch II region results in virtually no ATP hydrolysis, and locks the myosin into a permanent weak actin binding state. Both residues are present in these conserved regions of the myoG motor domain and thus we should be able to create both a rigor and weak actin-binding motor by making the same amino acid changes. The mutant myoGs will be independently expressed in the myoG null cells. The impact of the mutation on actin binding will be tested by analyzing the amount of mutant myoG associated with the actin cytoskeleton. The motility of the cells tested and localization of the mutant proteins assessed.

Motors fall broadly into two classes – processive or non-processive (they interact with their track and then immediately release and diffuse away) (4). If myoG is a borderline processive motor (i.e. it maintains contact with an actin filament for a short distance) then conversion to a weak binding (i.e. non-processive) state should disable myoG and the mutant will fail to fully rescue the mutant defect. Conversion into a rigor form should result in accumulation of the mutant on actin and no rescue of the developmental defect. If myoG is, in contrast, a non-processive motor then the weak binding mutant may rescue the null phenotype significantly.

**Method.** The expression plasmid described above will be used to create mutant GFP-myoG expression plasmids. Rescue of the null mutant phenotype and the ability of the expressing cells to move will be analyzed as described above. Actin binding will be determined by western analysis of myoG levels in detergent-extracted cytoskeletons prepared in the absence of ATP (11).

**LITERATURE CITED**


FIGURES

![Graph showing speed and roundness of WT and myoG null cells.](image)

**FIGURE 1.** The myoG null mutants fail to polarize and increase motility after 6 hr of starvation. (left) DIC micrographs of *Dictostelium* cells showing polarized wild type (WT) cells and non-polarized mutant (myoG null) cells. (right) Quantitative analysis of myoG motility (speed) and morphology (roundness, 100% = perfect sphere) (Breshears, Wessels, Titus & Soll, unpublished)

![Diagram of MyoG and M7 protein domains.](image)

**FIGURE 2.** MyoG and its mutant phenotype. (top) Box diagrams of MyoG and M7 indicating major tail domains (c – predicted coil; M – MyTH4; ovals – IQ motifs) and regions rich in a few amino acids (SN; NST; QNT) (bottom) Development of control and myoG null cells on buffer-soaked pads. Note the presence of slender stalks topped by a round spore head in the control sample and the smooth surface in the myoG null.

7. WORD COUNT

Items 2–4: 1996 words