The Distribution of Myosin II in *Dictyostelium discoideum* Slug Cells

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Abstract. While the role of myosin II in muscle contraction has been well characterized, less is known about the role of myosin II in non-muscle cells. Recent molecular genetic experiments on *Dictyostelium discoideum* show that myosin II is necessary for cytokinesis and multicellular development. Here we use immunofluorescence microscopy with monoclonal and polyclonal antimyosin antibodies to visualize myosin II in cells of the multicellular *D. discoideum* slug. A subpopulation of peripheral and anterior cells label brightly with antimyosin II antibodies, and many of these cells display a polarized intracellular distribution of myosin II. Other cells in the slug label less brightly and their cytoplasm displays a more homogeneous distribution of myosin II. These results provide insight into cell motility within a three-dimensional tissue and they are discussed in relation to the possible roles of myosin II in multicellular development.

MYOSIN II is found in all myocytes and in most eukaryote cells (Korn and Hammer, 1988; Warrick and Spudich, 1987) where it is implicated in cytokinesis (Fujiwara and Pollard, 1976; De Lozanne and Spudich, 1987; Knecht and Loomis, 1987; Kitanishi-Yumura and Fukui, 1989), the control of cell shape (Wessels et al., 1988), the motility of cells (Yumura et al., 1984; Rubino et al., 1984; Spudich and Spudich, 1982; Honer, 1988), the maintenance of cell polarity (Fukui et al., 1990), and the capping of membrane receptor proteins (Pasternak et al., 1989). In multicellular organisms, myosin II may also play an important role in cell–cell interactions. Studies suggest that actomyosin bands are responsible for folding sheets of epithelia during gastrulation (Odell et al., 1981; Lee et al., 1983) and that myosin II is involved in both mouse morula compaction (Sobel, 1983; 1984) and resistance to stress in the embryonic chick area opaca (Monnet-Tschudi and Kucera, 1988). Other studies involving gene disruption indicate that myosin II is necessary for normal multicellular development in *Dictyostelium discoideum* (see below).

D. discoideum is a simple, mobile eukaryote that can exist as an amoeba or as a multicellular aggregate (Bonner, 1967; Raper, 1984). *D. discoideum* thus lends itself to the study of both single cells and three-dimensional tissues. The cytoskeletal proteins of individual amoebae have been widely studied (Spudich and Spudich, 1982; Rubino et al., 1984; Fukui et al., 1987; Condeelis et al., 1987; Knecht and Loomis, 1987; De Lozanne and Spudich, 1987; Gerisch et al., 1989), however, those in multicellular aggregates have not.

Mutant *D. discoideum* cells that lack myosin II survive and undergo (albeit slow) amoeboid movement; such cells differentiate into the two types which normally constitute a slug (prestalk and prespore cells) and they aggregate to form loose mounds of cells (Knecht and Loomis, 1987; De Lozanne and Spudich, 1987; Wessels et al., 1988; Peters et al., 1988; Manstein et al., 1989). At this point, development is blocked: aggregates of myosin-deficient mutants fail to become mobile—they do not form slugs with normal three-dimensional characteristics (Wessels et al., 1988). This developmental block may be a result of diminished control over cell shape (Knecht and Loomis, 1987; Solomon, 1987). Conversely, the developmental block could relate to the lack of myosin II as a powerful contractile protein which enables groups of cells to undergo coordinated movements in three dimensions.

Here we study the role of myosin II in the development and locomotion of wild-type *D. discoideum* slugs. The distribution of myosin II in prestalk cells indicates that cells may be coordinated to effect distortion of the aggregate into its characteristic shape and to direct movement of the slug. We speculate that myosin II is a key molecule for the development of multicellular organisms and three-dimensional tissues.

Materials and Methods

Development of Slugs

A wild-type strain of *D. discoideum* (WS380B; Erdos et al., 1973) was used for all experiments. Slugs were prepared by placing small mounds of amoebae and bacteria (*Klebsiella aerogenes*) on water agar plates (covered Petri dishes containing 1.5% wt/vol agar) (Calbiochem-Behring Corp., San Diego, CA) in water with 250 μg/ml dihydrostreptomycin sulphate. Plates were enclosed in black polyvinyl chloride containers in an illuminated room at 21 ± 1°C and 70–80% relative humidity. Slugs developed and migrated across the agar towards light entering a 3-mm hole in the side of each container.

Preparation of Sections

To fix slugs for myosin II immunostaining, we adapted the method of Fukui et al. (1987) for single amoebae. Cubes of agar (each measuring ~3 mm in length and bearing a single 2–4 d-old slug, which comprised ~10^5 cells)

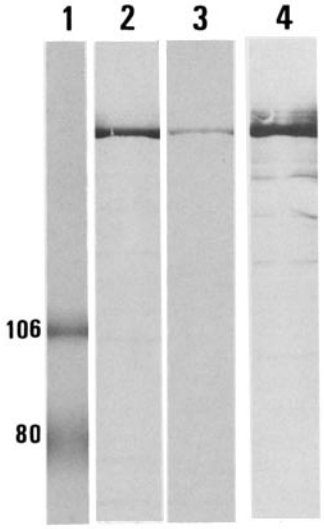

Figure 1. Western blots of WS380B slug cell lysates reacted with antimyosin antibodies. Each lane was loaded with the equivalent of 10^5 slug cells. Lane *1*, molecular weight markers, sizes as indicated in kD; lane *2*, mAb M342; lane *3*, mAb M151; lane *4*, affinity purified polyclonal antibody (lane *4* was taken from a different gel). In both gels rabbit skeletal muscle myosin was run as a standard (not shown). All antibodies revealed a prominent band in a similar position to rabbit skeletal muscle myosin at ~240 kD.

were cut from plates. Three to five cubes were placed on a glass coverslip held by a light aluminium clamp and immersed rapidly in liquid nitrogen for ~10 s. Cubes were then carefully transferred to a solution of 1% formalin in methanol at −15 ± 2°C for 15-20 min, brought to room temperature and air dried for 30 s. Freezing slugs before formalin-methanol fixation results in clear cell images; nuclei are apparent as dark oval structures within the labeled cytoplasm (see Fig. 4). Fixation without prior freezing results in hazy cell images (data not shown). After freezing and fixation, two drops of molten agar (30 ± 5°C) were placed on each cube to encase the slug and to prevent it detaching from the agar. Agar-encased slugs were then washed overnight in PBS (0.015 M potassium phosphate; 0.9% wt/vol NaCl, pH 7.2) and infiltrated for two days at 4°C with a cryotomy embedding compound (OCT; Miles-Tissue Tek II). Sections 4–6-μm-thick were cut at −20 ± 2°C and picked up on slides treated with chrome alum-gelatine (Krefft et al., 1984).

Immunocytochemistry

Slides bearing transverse or longitudinal slug sections were washed overnight in PBS (this was found to remove most formalin-induced fluorescence) and blocked with 5% (wt/vol) skim milk in PBS for 30 min. After three 5-min washes in PBS, slides were incubated for 2–3 h at room temperature with mAbs (M151 or M342 at 5 μg/ml) or affinity-purified polyclonal antibody (5 μg/ml) specific for *D. discoideum* myosin II (see acknowledgments). To distinguish prespore cells within the slug, alternate slides were incubated with MUD62, a mAb specific for a carbohydrate epitope found on several proteins in the sheath, within prespore cells and in the spore coat (Grant and Williams, 1983). After incubation, slides were again washed in PBS, blocked with 5% skim milk in PBS for 20 min and washed in PBS. Sections labeled with mouse mAb were incubated for 60 min at room temperature with FITC-conjugated sheep anti-mouse IgG (Tago) diluted 1:25. Sections labeled with polyclonal antibody were incubated for 60 min at room temperature with FITC-conjugated sheep anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) diluted 1:20. Following the second incubation, slides were washed three times in PBS (10 min each) and mounted with Aquamount (Gurr). Control slides were treated as above except that they were incubated with only the second antibody.

Immunoblotting

Using total cell lysates from disaggregated slugs, SDS-PAGE was performed on 6% polyacrylamide gels. Rabbit skeletal muscle myosin was run as a standard. Proteins were blotted onto nitrocellulose sheets, blocked with 5% wt/vol skim milk in PBS and incubated with M342, M151, or antimyosin polyclonal antibody (5 μg/ml) for 2 h at room temperature and then peroxidase-conjugated second antibody (sheep anti-mouse IgG diluted 1:100 or in the case of polyclonal antibody with antirabbit IgG diluted 1:500) (Sigma Chemical Co.).

Results

Specificity of Antimyosin Antibodies

The antibodies used in this study were directed against myosin II from *D. discoideum* amoebae. Two mAbs were used: M342, which is specific for filamentous myosin II and M151, which recognizes both filamentous and monomeric myosin II (Reines and Clarke, 1985*b*). To confirm the specificity of these antibodies for myosin II in slug cells, we analyzed Western blots. With M342, M151, and polyclonal antibody, a prominent band of ~240 kD was visible (Fig. 1). This band is identical to that observed with vegetative amoebae (data not shown; see Reines and Clarke, 1985*a*,*b*; Carboni and Condeelis, 1985). Both polyclonal and monoclonal antibodies produced similar results in immunofluorescence experiments, although sections treated with the polyclonal antibody were brighter. It is concluded that these antibodies retain their specificity for myosin II in the developing *D. discoideum* slug.

Fixation of Slugs for Myosin II Localization

Fixation of the *D. discoideum* slug for immunocytochemistry of myosin II is more complex than for single amoebae. The main concern is that the fixative penetrates the aggregate sufficiently to preserve myosin in the inner cells. We tried the following fixation techniques commonly used to localize various prespore antigens within the slug: 4% paraformaldehyde in PBS at room temperature for 2 h or more; 3% paraformaldehyde and 0.5% glutaraldehyde in PBS at room temperature for 2 h or more; 100% methanol at −15°C for 15–20 min. None of these techniques preserved myosin II (apparent by the poor signal observed in immunofluorescence experiments). Since the Fukui group has perfected techniques for localizing myosin in single amoebae and 2-dimensional sheets of cells during aggregation (Fukui et al., 1987; Yumura et al., 1984; Yumura and Fukui, 1985), we adapted their technique to visualize myosin in the slug. By tripling the fixation time and adding a snap-freezing step (see methods for full details), we were able to obtain good preservation of myosin throughout the slugs (see below). All cryosections displayed in this report were prepared using this technique.

Myosin II Distribution within Slug Cells

Twenty slugs were sectioned and labeled and results from five slugs are shown in Figs. 2, 3, and 4. As expected, all cells examined were found to contain myosin II. However, cells within the anterior of the slug and those at the periphery reacted more intensely with antimyosin antibodies than cells within the posterior (Fig. 2 *a*; Fig. 3 *a*, *b*, and *c*). This difference is not always readily apparent in low-power micrographs and is best seen at higher magnification (Fig. 3 *d*).

Cells along the ventrum of the slug were always intensely labeled with antimyosin antibodies, although the thickness of this layer varied (1–10 cells) both between slugs and along individual slugs. Sometimes a substantial pillow-shaped group of strongly labeled cells was observed in the midventral region (Fig. 2 *a*). Along the dorsum, cells were usually intensely labeled, forming a (sometimes incomplete) layer one to two cells thick; Fig. 3, *a*, *b*, and *c*). Control sections

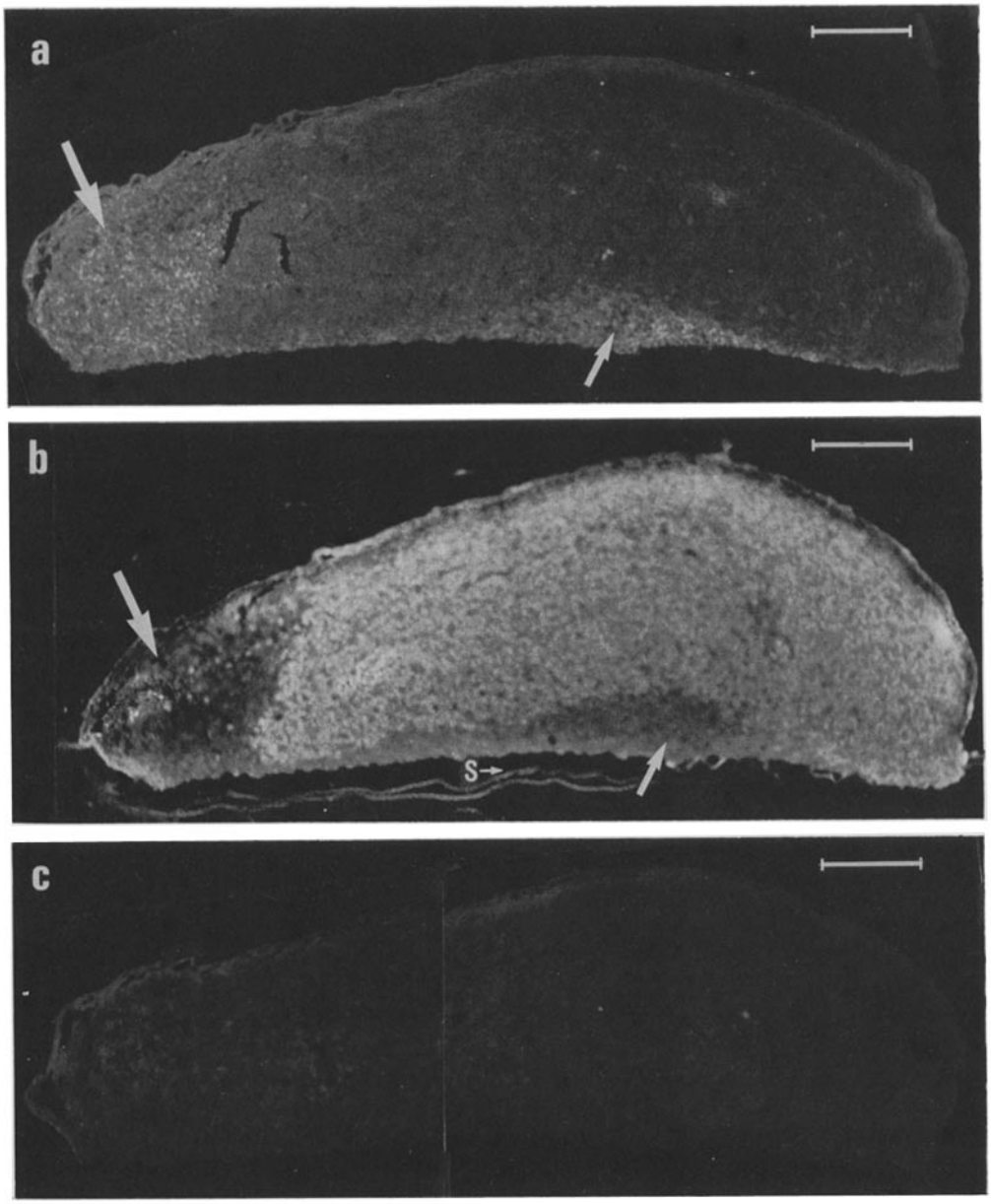

Figure 2. The distribution of myosin II–polarized cells within the slug parallels that of prestalk cells. The fluorescent micrographs shown here are close (but not adjacent) midsaggital frozen sections of the same slug. (*a*) The distribution of myosin II within the slug (mAb M151). Note the strong labeling in the anterior (*large arrow*) and at the periphery, especially along the ventrum (*small arrow*). (*b*) The distribution of prestalk and prespore cells within the slug (mAb MUD62). The prespore region labels more strongly than the prestalk region (*arrows*). Proteins in the sheath are also labeled by MUD62 (*S*). (*c*) Control. Treated with second antibody only. Bars, 90 μm.

showed no difference between anterior (and peripheral) cells and those in the inner posterior of the slug (Fig. 2 *c*). The intense labeling of peripheral cells is not a fixation artifact. Frozen slugs were snapped in half and exposed to the fixative: cells at the snapped edge reacted similarly to their counterparts in whole slugs (Fig. 4 *e*). Cells within the anterior of the slug show strong cortical labeling (see Figs. 2 *a*, 3 *a*, and 4 *a*), indicating that myosin II inside the slug has been well preserved by the fixation technique employed. Therefore the diffuse cytoplasmic staining of cells in the prespore area of the slug is unlikely to be an artifact of poor fixation.

To determine whether the areas of intense antimyosin labeling corresponded with the distribution of prestalk cells in the slug, we labeled adjacent sections with MUD62, a mAb specific to prespore cells (Champion et al., submitted). Antimyosin antibodies and MUD62-labeled complementary areas of the slug (Figs. 2, *a* and *b*), indicating that the intense myosin labeling was restricted to prestalk cells.

Cell Shape

Cells deep within the slug are irregularly spheroidal (Figs. 3 and 4); however cells at the periphery are flattened (almost squamous) and tightly packed, forming an epithelium-like layer (Fig. 4). This is particularly apparent along the dorsum (Fig. 4, *a* and *b*). Frozen sections often tear along the border between the dorsal "epithelium" and underlying cells (as if peripheral cells were more tightly bound to each other than to the inner cells).

Intracellular Localization of Myosin II

We define the orientation of each cell with respect to the slug; thus the posterior cortex of a cell is that part of the cor-

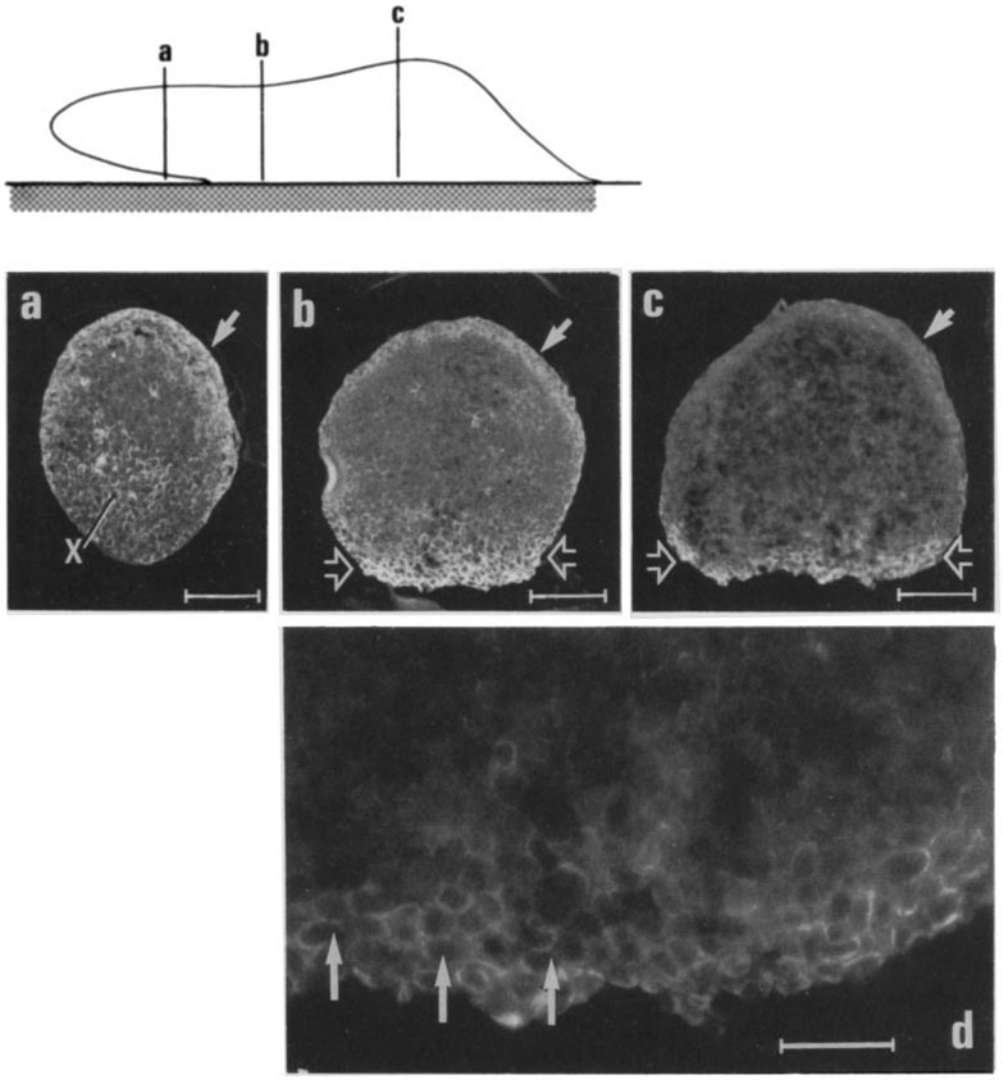

Figure 3. The distribution of myosin II–polarized cells within the slug (mAb M151). The line drawing indicates the source of these fluorescent micrographs (which originate from transverse frozen sections of a single slug); hatching indicates the agar substrate. (*a*) At the anterior of the slug myosin II is localized to the cortex of both peripheral cells (*arrow*) and inner cells (*x*). (*b*) The middle of the slug. In contrast to the inner cells, myosin II is clearly apparent in dorsolateral cells (*closed arrow*) and most clearly apparent in ventral cells (between the open arrows). (*c*) In the posterior region of the slug (anterior of the rump) strongly labeled cells are confined to the ventrum (between open arrows) and are less apparent along the dorsum (*closed arrow*). (*d*) Enlargement of the ventrum of (*c*). The posteroventral layer of myosin II–polarized cells varies in thickness (see also Fig. 2 *b* (*small arrow*). In (longitudinal) cross section, posteroventral cells show a localization of myosin in the cortex (*arrows*). These cells appear annular when sliced through their posterior cortex and C shaped when sliced through their midline. When a cross section is taken through the anterior of such polarized cells they appear unlabeled. Bars: (*a–c*) 50 μm; (*d*) 15 μm.

tex that is furthest from the tip of the slug and the outer lateral cortex is closest to the slug periphery. Myosin is distributed evenly throughout the cytoplasm of cells within the posterior of the slug; however, myosin appears localized to the cortex of cells within the anterior of the slug (such cells are annular in appearance; Figs. 3 *a* and 4 *a*). At the periphery, cells may be flattened and myosin localized around the entire cortex or concentrated in the posterior and/or outer lateral part of the cortex (Fig. 4 *a*, *arrows*).

When the nose of the slug is raised up off the substratum, the cells along the ventral region of the nose appear more weakly labeled and less polarized than cells along the ventral region that are in contact with the substratum (Figs. 3 *a* and 4 *a*).

Degree of Myosin Concentration

Within peripheral cells, there is considerable variation in the degree to which myosin is concentrated in the cortex. Most frequently, myosin can be seen in one half of the cortex. When a cell is cut in the midline, treatment with antimyosin antibodies produces a U-shape; (Fig. 4 *b*, *arrows*). Occasionally, myosin occupies more than one half of the cortex and antibody treatment produces a C shape (Fig. 4 *c*, *arrows*). Often, myosin occupies less than one half of the cortex and the cell appears highly polar; a collection of such (highly polarized) cells is a distinct feature at the rear of all slugs observed, where cells are oriented like a series of fish scales (Fig. 4 *d*). Such polarized cells resemble amoebae in aggregation streams and single amoebae undergoing directed locomotion (Yumura et al., 1984).

Discussion

At different times of its life cycle *D. discoideum* is unicellular or multicellular; the organism may exist as an isolated amoeba or as a mobile aggregate of cells (the slug). This duality implies that there is much in common between the two stages of development, e.g., the isolated cell and the aggre-

gate may use a common locomotory mechanism; slug movement may result from the collective action of individual amoebae within the aggregate (see Odell and Bonner, 1986). Indeed, the polarized distribution of myosin II in some slug cells closely resembles that observed in individual chemotaxing amoebae. The fact that only anterior and peripheral cells display this similarity, however, suggests that the slug is not simply a collection of individually motile amoebae. Peripheral cells differ from inner cells in their shape as well as their distribution of myosin II, implying that the slug is a true tissue and that there may be a division of (locomotory) labor between prestalk and prespore cell groups (Williams et al., 1986; Odell and Bonner, 1986). The concentration of myosin II in prestalk cells suggests that they are supplying the major motive force for slug migration. This is consistent with earlier findings that prestalk cells have greater motive force than prespore cells (Inouye and Takeuchi, 1979) and that prestalk cells diminish in size during slug migration (Voet et al., 1984). To further explore these ideas, it is important to understand the role of myosin II in the movement of isolated *D. discoideum* amoebae.

In an amoeba undergoing directed locomotion, myosin II is localized to the posterior cortex, and myosin I is localized to the leading edge (Yumura et al., 1984; Rubino et al., 1984; Fukui et al., 1989). On the other hand, actin is localized to both the posterior and anterior of cells. This has led researchers (Yumura et al., 1984; Yumura and Fukui, 1985; for review see Fukui and Yumura, 1986) to propose that actin polymerization (possibly together with myosin I) effects the projection of pseudopodia at the leading edge of the cell (Rubino et al., 1984; Newell, 1986; Hall et al., 1988). It has been proposed that the projected leading edge then adheres to the substratum and contraction of actin and myosin II effects the advancement of the cell body (towards the leading edge) (Yumura et al., 1984; Yumura and Fukui, 1985; for review see Fukui and Yumura, 1986). Many researchers have abandoned the latter part of this model because locomotion has been demonstrated in myosin II-deficient cells (Knecht and Loomis, 1987; DeLozanne and Spudich, 1987; Manstein et al., 1989). Several influential cell-biology texts (Alberts et al., 1989; Darnell et al., 1990) no longer include myosin II in discussions of amoeboid movement. However, although cells without myosin II do move, they are morphologically atypical and migrate at less than half the speed of their normal counterparts (Wessels et al., 1988; Peters et al., 1988). That they are capable of movement indicates that cells may possess more than one mechanism for locomotion. Nevertheless, a role for myosin II in cell motility should not be dismissed and the elucidation of this role will undoubtedly lead to a better understanding of myosin II function in multicellular development.

In this study, we have shown that cells at the periphery of the *D. discoideum* slug (with their polarized distribution of myosin II and elongated shape) resemble single motile amoebae. The shape of isolated amoebae has been found to relate to speed of motility: elongated cells are more directed and so move faster than more rounded cells (Futrelle, 1982; Varnum and Soll, 1984). The posterior localization of myosin II in isolated amoebae has been associated with directed movement (Yumura et al., 1984). Thus, the results reported here may indicate that peripheral cells are undergoing active migration and the inner cells are moving passively, although this relies heavily on extrapolation from amoebae in isolation to cells within the slug.

Assuming that myosin II distribution in slug cells reflects their direction of movement, peripheral cells appear as though they are moving anteriorly and inwardly with respect to the slug. Thus these cells could be exerting longitudinal and centripetal forces on the slug interior (see Williams et al., 1986). Similar inwardly directed contractile forces have been proposed by Yumura et al. (1984) for aggregating amoebae. In the mouse morula, cortical myosin is thought to exert a centrally directed force which results in compaction of the cell mass (Sobel, 1983, 1984). During early *Drosophila* embryogenesis a polarized distribution of myosin II within epithelial cells is associated with the coordinated changes in cell shape that occur during gastrulation (Young et al., 1991). Peripheral cells in the *D. discoideum* slug are flattened and tightly adherent, resembling an epithelial sheet. We propose that they are involved in coordinated contractions that result in the movement of the whole organism. How peripheral prestalk cells could move the whole slug is unclear but such movement may involve contraction of actomyosin in longitudinally and/or circumferentially linked cells (Williams et al., 1986; Vardy et al., 1986; Breen et al., 1987).

On the other hand, it may be that peripheral cells act merely to maintain the shape and integrity of the slug rather than to effect its locomotion. This could be like a "shell" of cells under tension which contain the inner cells during slug migration. Myosin-rich cells surrounding the area pellucida in the chick embryo are thought to resist stress generated by migrating cells in the outer area opaca (Monnet-Tschudi and Kucera, 1988). These authors suggest that such cells may protect the inner area pellucida in which morphogenetic movements take place. In the *D. discoideum* slug, the peripheral layer of cells may also have a protective function. These suggestions are not mutually exclusive: peripheral cells may play both protective and locomotory roles. Such roles would explain the behavior of cells lacking myosin II: they both fail to form the "proper" slug shape and migrate (Wessels et al., 1988; Peters et al., 1988).

How is myosin II concentrated in the cortex of anterior and peripheral cells and distributed homogeneously in the cytoplasm of other cells? This difference may be a response to cyclic AMP which is believed to be secreted by the tip of the slug (Schaap, 1986; Darcy and Fisher, 1990). It has been suggested that prestalk cells remain responsive to cAMP throughout development whereas prespore cells lose much of their cAMP sensitivity after aggregation (Garrod, 1974; Mee et al., 1986); thus high levels of Ca^{2+} within prestalk cells would be expected (Abe and Maeda, 1989) resulting in the stimulation of cyclic GMP and the accumulation of cortical myosin (Nachmias et al., 1989; Newell et al., 1990). The polarized distribution of myosin II in peripheral cells may also be related to their proximity to the slime sheath. This could be similar to the way in which extracellular matrices in higher eukaryotes influence the organization of the cytoskeleton of cells with which they are in contact.

We predict that the migration of the *D. discoideum* slug involves the coordinated movement of a specialized subpopulation of cells. A similar division of labor may pertain elsewhere during morphogenesis when sheets of cells undergo complex movements. For a subset of cells to move other

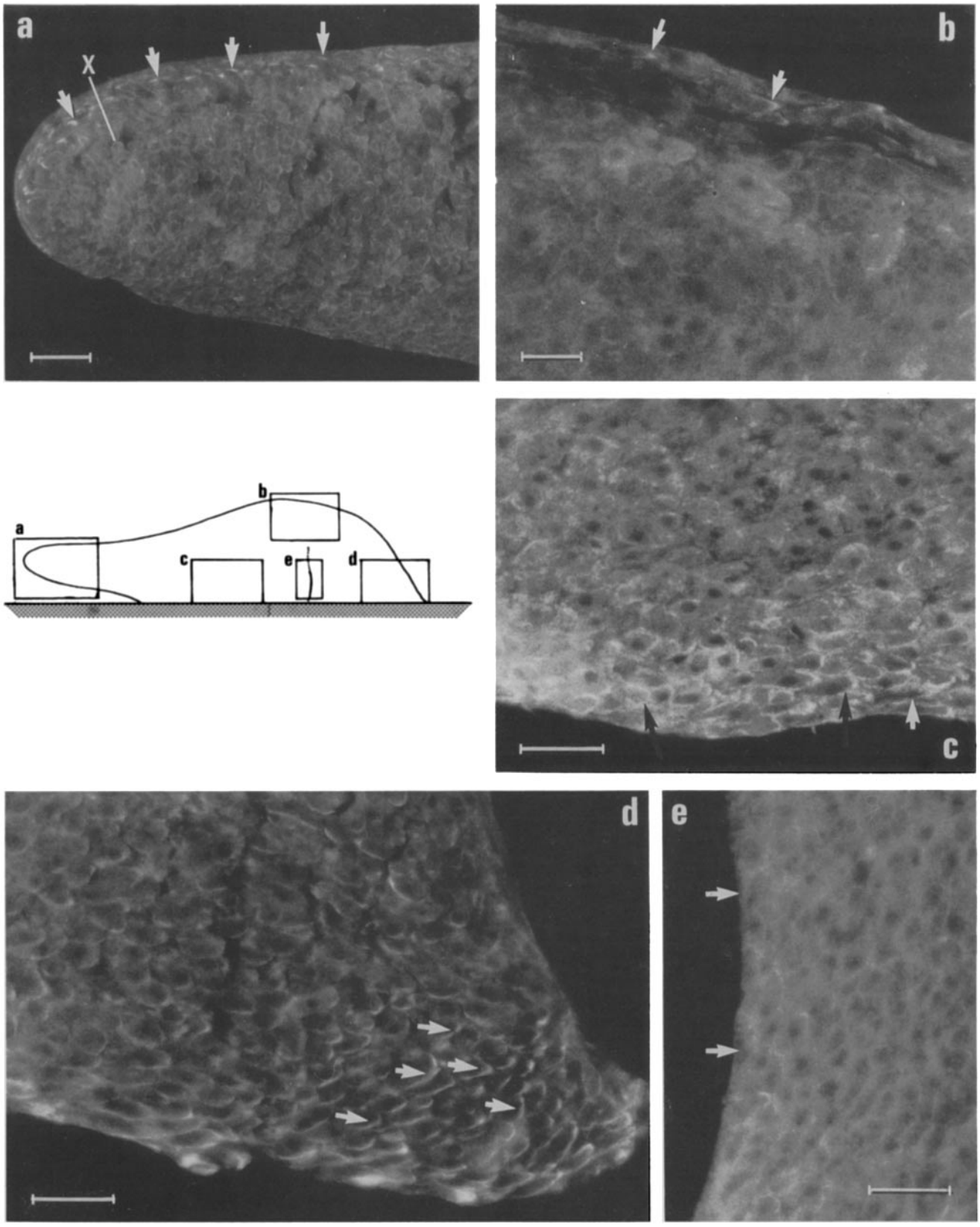

Figure 4. The polarization of myosin II within slug cells. The line drawing indicates the source of the micrographs (which originate from saggital frozen sections of several different slugs). (*a*) The nose of the slug (mAb M342). Peripheral cells are flat and tightly packed as in an epithelium; in these (anterior and dorsal) cells, myosin II is localized to the posterior and outer cortex (*arrows*). Inner cells are less tightly packed and more isodiametric in shape; in these cells, myosin II is localized to the (entire) cortex of each cell (*x*). (*b*) The (postero) dorsum of the slug (*Monoclonal antibody M151*). In the peripheral (epithelium-like) layer, myosin II is localized to the posterior and outer cortex of flat, tightly packed cells (*arrows*); however, in the less tightly packed (and more isodiametric) inner cells, myosin is evenly dis-

cells, a strong motor is required. Myosin II has the ability to form thick filaments in vivo (Yumura and Fukui, 1985) and, together with F-actin, is a powerful force generator (Sheetz and Spudich, 1983). We suggest that cells without myosin II cannot form a migratory slug because they are not strong enough to move other cells (although other non-myosin II based motors may be adequate for the movement of single cells). In any three-dimensional tissue, changes in the shape of individual cells must be coordinated to maintain the integrity of the whole tissue (Odell et al., 1981). We further predict that amoebae may require myosin II to control their shape in order to effectively participate in the coordinated movement of a cellular society. It is interesting to speculate that multicellular organisms capable of coordinated movement did not develop until the evolution of a powerful myosin-based motor.

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tributed throughout the cytoplasm. (*c*) The midventrum of the slug (*M342*). The cytoplasm of peripheral cells shows a localized distribution of myosin II (*arrows*) while inner cells show a more even distribution (as in *b*). (*d*) At the rear of the slug (*M151*) a characteristic group of highly polarized cells is apparent (*arrows*). (*e*) Fixation control. A free edge of (internal) cells was formed by snapping a frozen slug in two prior to fixation. Note that the shape and labeling (*M151*) of cells along the cut surface (*arrows*) matches that of internal cells in intact slugs (see *b*). Bars: (*a*) 40 μm; (*b*) 10 μm; (*c–e*) 15 μm.

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