Differential effects of myosin–antibody complexes on contractile rings and circumferential belts in epitheloid cells

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Summary

The role of myosin filaments during assembly and activity of microfilament rings was analyzed by microinjecting epitheloid cells (PtK2 and LLC-PK1 kidney cell lines) with specific anti-myosins. Six monoclonal antibodies directed against the light meromyosin (LMM) region of the myosin molecule were characterized with respect to epitope location, and their effects on actin-activated MgATPase as well as on assembly, structural integrity and stability of myosin filaments. All of these antibodies recognized LLC-PK1 myosin, but only three reacted with PtK2 myosin. The remaining three served as matching controls in experiments with this cell line. When injected in amounts sufficient to yield an excess of antibody over myosin, the reactive antibodies significantly delayed formation and constriction of the contractile ring in mitotic cells. These rings contained less myosin, but not less actin, than the controls. This indicates that the recruitment and alignment of actin in the cleavage furrow can occur independently of other components of the contractile ring.

After completion of cytokinesis, the majority of the injected cells was unable to assemble a normal circumferential belt. This resulted in defective epitheloid sheets. Approximately one third of these cells showed grossly distorted cell shapes and an increase in locomotory activity.

All these changes were fully reversible with time, suggesting that the effects of the antibodies were overcome by protein synthesis. The differential sensitivity seen between contractile rings and peripheral belts is discussed with respect to differences in their architecture, stability and proposed function.

Key words: nonmuscle myosin, anti-myosin, microinjection, cytokinesis, epitheloid sheets.

Introduction

Epithelial and endothelial cells are able to organize their microfilaments into two different ring-like bands. One is the peripheral belt seen at the circumference of each member of an epithelial or endothelial sheet, in situ as well as in tissue culture (Owaribe et al. 1981; Connolly et al. 1981; Low et al. 1981; Sanger et al. 1980; Gabbiani et al. 1983; White et al. 1983; Hormia et al. 1985). It is composed of straight or slightly curved microfilaments deposited at the cell periphery, and appears as a polygonal or ring-like structure. These belts are associated with specialized membrane areas involved in cell–cell contact formation. In the case of polarized epithelia, the circumferential microfilaments are part of the terminal web and, together with the belt desmosome, form the zonula adherens (Hull and Staehelin, 1979; Geiger et al. 1984). Cultured cells derived from polarized epithelia may maintain a highly differentiated state (as, for example, the porcine kidney line LLC-PK1). However, dedifferentiation frequently occurs and leads to loss of several epithelial criteria, such as apical microvilli and tight junctions. This is, for example, observed in PtK2, a rat kangaroo epitheloid line.

Even in this case, however, the peripheral belts, together with rudimentary junctions, are sufficient for cell–cell contact formation and growth in epitheloid sheets. Intimate involvement of the circumferential microfilament bundle with cell–cell contact formation is suggested from ultrastructural data (cf. Owaribe et al. 1981) and also from the observations that Ca2+ depletion of the medium leads to splitting of intercellular junctions, and concomitantly to detachment of the circumferential belt from the inner face of the plasma membrane followed by its disintegration (Volberg et al. 1986). Conversely, MgATP-induced contraction of this structure does not disrupt its ties to the plasma membrane, so that the cells acquire a conical shape and the sheets curve (Owaribe et al. 1981; Burgess, 1982).

The second type of ring-like microfilament bundle is the contractile ring of the cleavage furrow (which of course is not a speciality of epithelial or endothelial cells). Here, structural connections must also exist between microfilaments and the plasma membrane because this ring is responsible for membrane constriction, but these have not been identified as morphologically distinct structures.

During the cell cycle, circumferential belts and contrac-
tile rings are mutually exclusive, and it is reasonable to assume that subunits of one structure can serve to assemble the other (cf. Sanger, 1975; Sanger et al. 1980; Kitaniishi-Yumura and Fukui, 1989; Yumura and Fukui, 1985). Both types of structural elements are believed to exert muscle-like contractility. The evidence for this is mainly the following: they consist of the same set of major proteins as myofibrils, i.e. they contain actin, myosin and α-actinin (Schroeder, 1973; Fujiwara and Pollard, 1976; Fujiwara et al. 1978; Bretscher and Weber, 1978), they display actin filaments of opposite polarity (Sanger and Sanger, 1980; Hirokawa and Tilney, 1982), and they contract upon the addition of MgATP (Owaribe et al. 1978; Bretscher and Weber, 1978), they assemble the other (cf. Sanger, 1975; Sanger 1980; Fujiwara et al. 1978; Burgess, 1982). In view of their transient existence, the building blocks of both rings must be able to assemble very precisely upon (so far unknown) signals at specific regions of the cell cortex. Such signals must differ in time and cellular location for both structures. In addition, the mode of their contractility is different: while the circumferential belts predominantly exert tension, the contractile rings perform isotonic contractions to create movement.

We were interested in the presumed role of myosin filaments as building blocks needed for assembly and function of both structures. Therefore, we investigated the effects of specific anti-myosins after microinjection on cytokinesis and sheet formation in two epitheloid cell lines: the rather well differentiated LLC-PK1 and the PtK2 cell. We used a set of six monoclonal antibodies binding to different epitopes all located in the light meromyosin (LMM) region of brain myosin, as well as a polyclonal anti-myosin. All of these antibodies interfered with microfilament rings in epitheloid cells, but the two types of rings considered here responded differently: the anti-myosin-injected cells formed contractile rings, but their myosin content was reduced. Both formation of the contractile ring and cleavage were markedly prolonged. In contrast, assembly of peripheral belts in postmitotic cells was grossly defective, which in many cells led to aberrant morphology, increased locomotory activity and inability to form epitheloid sheets.

Materials and methods

Cells
PtK2 and LLC-PK1 cell lines, originally derived from kidney epithelium of the rat kangaroo and the pig, respectively, were obtained from The American Type Culture Collection and grown in Minimal Essential Medium (MEM, Gibco Laboratories, Eggenstein, FRG), supplemented with 7.5% to 12% fetal calf serum. Under these conditions, both cell lines readily form large sheets where each cell develops prominent circumferential microfilament bundles. For microinjection, cells were seeded onto glass coverslips equipped with diamond loops, to facilitate identification of the injected cells after various time periods.

Antibodies
Monoclonal antibodies were obtained against pig brain myosin that had been purified essentially as described (Pollard et al. 1974). Conventional procedures were used for raising monoclonal antibodies (Köhler and Milstein, 1975; Galfre and Milstein, 1981) from Ag 8/653 (Kearney et al. 1979) and BALB/c spleen cell hybridomas. Eight- to ten-week-old BALB/c mice were immunized intraperitoneally with 20 μg myosin in complete Freund’s adjuvant (ICN Biomedicals, Eschwege, FRG) and reimmunized 3 weeks later with 60 μg myosin in incomplete Freund’s adjuvant. Booster injections with 500–300 μg myosin in 0.9% NaCl without adjuvant were applied at days 4, 3, 2 and 1 before fusion. Culture medium of cells growing in hypoxanthine-aminopterin-thymidine (HAT) medium was screened for the presence of secreted anti-myosins in immunoblotting, using pig brain and PtK2 extracts, and in indirect immunofluorescence (see below) on PtK2 cells and rat fibroblasts. Positive hybridomas were recloned by limited dilution at least three times. Clones and subclones determination of the antibodies was performed by double diffusion against type-specific anti-mouse IgG (Miles mouse monoclonal typing kit, ICN Biomedicals, Eschwege, FRG), and only hybridoma clones secreting IgG antibodies were further cultivated, either in tissue culture, or as ascites in pristane-primed BALB/c mice. Antibodies were purified from tissue culture supernatant of ascites fluid on protein A-Sepharose 4B-CL (Pharmacia-LKB, Freiburg, FRG), and concentrated by pressure dialysis (collodion bags, Sartorius, Göttingen, FRG) in Tris buffer (0.05 M Tris–HCl, pH 7.4, 0.2 M NaCl). Protein concentration was determined as described (Bradford, 1976; Bio-Rad, Munich, FRG), using bovine IgG as a standard.

For microinjection experiments with polyclonal anti-myosins, an antibody against pig brain myosin was raised in rabbits. The animals were injected intraperitoneally with 1 mg purified myosin in complete Freund’s adjuvant and reimunized 3 weeks later with the same amount of protein in incomplete Freund’s adjuvant. Booster injections were given every third week. Immune serum was obtained 7–10 days after each booster injection. Specific anti-myosin was prepared by affinity purification on myosin–nitrocellulose as described (Höner and Jockusch, 1988). The reactivity and specificity of this antibody was tested in immunoblots with crude brain extracts and purified brain myosin, and in immunofluorescence on fixed LLC-PK1 and PtK2 cells.

To localize myosin in contractile rings of injected cells, a polyclonal, affinity-purified antibody against chicken gizzard myosin was used. This antibody stained myosin in stress fibers and contractile rings brilliantly, although it had been found to react only with the light chains of cellular myosin (Höner and Jockusch, 1988).

Second antibodies were rabbit anti-mouse IgG or sheep anti-rabbit IgG, both affinity purified on Sepharose–IgG and coupled to either fluorescein isothiocyanate (FITC) or lyssamine rhodamine (Rh). Rabbit or mouse IgG served as control antibodies.

Characterization of the monoclonal antibodies
After purification, the binding of these antibodies to myosin was analyzed in the following assays.

Immunoblotting was performed with crude brain extracts, with purified brain myosin and with proteolytic fragments obtained by treatment with Staphylococcus aureus V8 protease, pepsin or a-chymotrypsin (Barylko et al. 1989). All six antibodies used in this study recognized specifically the LMM portion of pig brain myosin and also pig epithelial (LLC-PK1) cells, but only three reacted with the PtK2 (rat, kangaroo) protein.

Electron microscopy was employed to characterize antibody binding sites and to determine the influence of the antibodies on geometry and architecture of myosin filaments. Epitope mapping of myosin–antibody complexes after Pt/carbon shadowing was performed as had been described for Dictyostelium (Pagh and Gerisch, 1986), chicken brush border (Citi and Kendrick-Jones, 1988) and turkey gizzard (Trybus and Henry, 1989) myosin. The effects of the monoclonal antibodies on the structure of myosin filaments were analyzed as described by Pagh and Gerisch (1986). Monomeric myosin (1 mg ml−1 in 0.5 M NaCl, 5 mM MgCl2, 0.1 mM DTE (dithioerythritol), 10 mM imidazole, pH 7.2) was incubated with a threefold molar excess of antibodies for 1 h on ice and subsequently induced to polymerize in filament buffer (0.14 M NaCl, 5 mM MgCl2, 0.1 mM DTE, 10 mM imidazole, pH 7.2) for another 60 min on ice. Prepolymerized myosin was incubated with the same molar ratio of antibodies again for 1 h on ice. Portions of both types of samples were diluted 1:10 and subsequently fixed in 0.05% glutaraldehyde. Samples were stained on Formvar-coated copper grid with 1% aqueous uranyl acetate and examined in a Zeiss EM 109 electron microscope. For length determinations of myosin–antibody complexes, the profiles of 100–300 structures were analyzed on photographic negatives.
analyzed in sedimentation analyses as described by Pagh and Gerisch (1986) and Citi et al. (1989). The amount of myosin released by centrifugation at 30,000 g for 15 min (Beckman Airfuge) was quantitated by densitometry of the Coomassie Blue-stained myosin heavy chain after SDS–PAGE.

The influence of the monoclonal antibodies on myosin enzymatic activity was investigated by measuring the actin-activated MgATPase activity as described (Citi and Kendrick-Jones, 1988). The myosin preparation was preincubated in filament buffer with MgATPase activity as described (Citi and Kendrick-Jones, 1988). The myosin preparation was preincubated in filament buffer with myosin light chain kinase and Ca²⁺/calmodulin to optimize phosphorylation. The molar ratio of actin/myosin was 8:1, antibodies were added to myosin in molar ratios 3:1.

Microinjection
Antibodies were injected into the cytoplasm of LLC-PK1 and PtK2 cells in 0.05 M Tris-HCl, pH 7.4, 0.2 M NaCl, as described (Höner and Jockusch, 1989; Höner et al. 1988). Controls included noninjected cells and cells injected with buffer or nonreactive antibodies. All antibody-containing solutions were centrifuged at 15,000 g for 20 min prior to injection. Cells grown within marker loops were injected (30–200 cells per experiment) and returned within 15 min to the incubator. After various time points, they were re-examined under phase contrast and finally fixed and processed for fluorescence microscopy. Alternatively, individual cells were injected and examined continuously by video microscopy (Höner et al. 1988; Sanger et al. 1989).

Fluorescence microscopy
Cells were fixed in 4 % formaldehyde in phosphate-buffered saline (PBS: 0.14 M NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2) at room temperature (RT), subsequently permeabilized with 0.2 % Triton X-100 in PBS for 10 min and washed thoroughly in PBS. Cells were then incubated with each reagent for 30 min at 37°C, washed in PBS and finally mounted in Mowiol 488 (Hoechst AG, Frankfurt, FRG). Staining with a fluorescent (FITC or Rh) probe was used for the following analyses: (1) the injected antibodies (monoclonal and polyclonal antibodies against pig brain myosin) were visualized with fluorochromed rabbit anti-mouse IgG. With this procedure, the injected antibodies were detected up to more than 60 h after injection, even when they had been diluted by two subsequent divisions. (2) The distribution of endogenous myosin was localized in the N terminus. The release of myosin-antibody complexes. In this assay, antibody 9 had the largest effect, while antibodies binding to epitopes located more carboxy-terminal had almost no (antibody 2) or only a slight effect. All antibodies reduced the length of myosin filaments, and again the effects of antibodies 4 and 5 were more pronounced than those of antibodies binding nearer the N terminus. The release of myosin–antibody complexes subjected to high-speed centrifugation was measured as an indication of the stability of such complexes. In this assay, antibody 9 had the largest effect, while antibodies binding to epitopes located more carboxy-terminal had almost no (antibody 2) or only a slight (antibodies 8, 7, 5 and 4) influence on the filament stability under these conditions.

The geometry and architecture of the myosin–antibody complexes were analyzed in detail by examining the same negatively stained preparations used for length determinations (Table 2) at higher magnification. In all cases examined, these complexes were abnormal when compared with controls (Fig. 1). The control filaments showed the typical appearance with a compact, bare central area placed into a depression of a heated, temperature-controlled microscope stage. Medium was applied and could be continuously exchanged using syringes (Höner et al. 1988). In such chambers, the cells could be kept for many hours without adverse effects on morphology or proliferation. (2) The cell-bearing coverslips were mounted on glass slides for microscopy and warmed with a heat curtain as described (Sanger et al. 1989).

Results
Anti-myosins influence myosin filament architecture, stability and actin-activated MgATPase
We first analyzed the binding of the antibodies to myosin in vitro. In immunoblots and in metal-shadowed preparations, all of the monoclonal antibodies considered here reacted exclusively with the LMM region of the myosin molecule, as determined in rotary-shadowed complexes (Barylko et al. 1989; Table 1). Antibody 9 was bound to the N-terminal region of the LMM portion, and antibodies 5 and 4 rather close to the carboxy-terminal end of the myosin tail. Antibodies 9, 2 and 5 recognized epitopes apparently not highly conserved in nonmuscle myosins, since these were not found in myosin from the rat kangaroo cells.

Table 2 shows the antibody-induced effects on structure, stability and enzymatic activity of myosin filaments. With respect to the different ATPase activities displayed by purified myosin, we determined only the actin-activated MgATPase of phosphorylated myosin filaments in the presence of antibodies, since we were interested to compare these values with the effects of the anti-myosins in living cells. It can be seen that this activity is particularly sensitive to the presence of antibodies binding in the last third of the myosin tail (antibodies 4 and 5, reduction to one third to one fourth of the control value), while antibody 9, binding nearer the N terminus, has only a slight effect. All antibodies reduced the length of myosin filaments, and again the effects of antibodies 4 and 5 were more pronounced than those of antibodies binding nearer the N terminus. The release of myosin–antibody complexes subjected to high-speed centrifugation was measured as an indication of the stability of such complexes. In this assay, antibody 9 had the largest effect, while antibodies binding to epitopes located more carboxy-terminal had almost no (antibody 2) or only a slight (antibodies 8, 7, 5 and 4) influence on the filament stability under these conditions.

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**Table 1. Characterization of monoclonal antibodies against the LMM region of pig brain myosin (α-PBM)**

<table>
<thead>
<tr>
<th>a-PBM</th>
<th>Epitope location*</th>
<th>Reactivity with PK4 myosin†</th>
<th>Epitope map</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>33.4±9.5</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>50.4±4.6</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>53.6±5.4</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>67.0±9.0</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>78.7±8.8</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>82.3±8.7</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

* Determined in metal-shadowed complexes (Barylko et al. 1989), expressed as % of the myosin tail length (157±12 nm) with standard deviations.
† Determined by immunoblotting and immunofluorescence.
approximately 160 nm in length and a strictly bipolar arrangement of myosin heads. Anti-myosin-decorated filaments appeared less regular and were frequently branched. Fig. 1 shows individual examples of such structures. In all cases, only small differences were observed between myosin filaments formed in the presence of antibodies and those incubated with antibodies after myosin polymerization. No distinct difference between individual antibodies was found with respect to the appearance of these aggregates. In unfixed, negatively stained preparations, it was seen that the antibodies disrupted myosin filaments and even tightly packed aggregates of myosin rods or paracrystals of rod fragments (Barylko et al. 1989). Periodic striations indicative of regular myosin–antibody packing were not observed.

**Fig. 1.** Panels of pig brain myosin filaments in the presence of a threefold excess of antibodies. C, controls in the presence of control IgG. The numbers refer to the monoclonal antibodies against pig brain myosin as described in Table 1. The anti-myosin-myosin complexes are less regular than control filaments. They branch frequently and show a tandem-like structure, where additional material is added onto a basic bipolar unit (arrowheads). Bar, 0.1 μm.

Anti-myosins lead to formation of contractile rings deficient in myosin

PtK2 or LLC-PK1 cells in interphase or early prophase were injected with antibodies at a concentration of 12 mg ml⁻¹ (which results in an at least a threefold molar excess of antibody as compared to myosin in the cell; cf. Höner et al. 1988). The injected cells were microscopically controlled for mitotic figures, and were fixed at the first visual sign of indentation of the plasma membrane. Localization of actin, myosin and the injected antibodies within the contractile ring was revealed by immunofluorescence (Fig. 2). In noninjected LLC-PK1 controls as well as in cells injected with buffer or non-specific IgG, a concentration of myosin in the contractile ring, as compared with the remaining cytoplasm, was easily
Table 2. Influence of a-PBM on enzymatic activity, size and stability of pig brain myosin filaments

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Actin-activated MgATPase*</th>
<th>Filament length†</th>
<th>a-PBM added to preformed filaments</th>
<th>Myosin release from a-PBM-myosin complexes‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>91.2±5.8</td>
<td>72.2±9.1</td>
<td>71.8±9.7</td>
<td>30±9</td>
</tr>
<tr>
<td>2</td>
<td>78.4±5.8</td>
<td>72.9±7.5</td>
<td>78.4±12.9</td>
<td>4±2</td>
</tr>
<tr>
<td>8</td>
<td>51.3±2.0</td>
<td>61.2±8.4</td>
<td>58.8±9.9</td>
<td>18±5</td>
</tr>
<tr>
<td>7</td>
<td>74.9±7.4</td>
<td>70.3±8.4</td>
<td>71.0±9.3</td>
<td>19±2</td>
</tr>
<tr>
<td>5</td>
<td>33.0±9.9</td>
<td>59.2±4.0</td>
<td>66.4±10.0</td>
<td>18±4</td>
</tr>
<tr>
<td>4</td>
<td>24.0±5.9</td>
<td>57.1±5.7</td>
<td>63.7±11.0</td>
<td>15±6</td>
</tr>
</tbody>
</table>

* Determined in the presence of myosin light chain kinase and Ca²⁺/calmodulin, an eightfold molar excess of actin and a threefold molar excess of a-PBM, in filament buffer. Data are expressed as % of control (myosin incubated with control IgG; actual values 55–93 nmol Pi·mg⁻¹ protein·min⁻¹).
† Determined in negatively stained preparations. Molar ratios of a-PBM–myosin were 3:1. Data are expressed in % of controls (pig brain myosin filaments incubated with a threshold molar excess of control IgG). Total number of filaments analyzed was 100–300.
‡ Determined by quantitating the myosin heavy chain after sedimenting a-PBM–myosin complexes, expressed as % of controls.

detectable with each of the monoclonal anti-myosins used as a marker (Fig. 2A). More than 90% of control LLC1 showed this concentration. In contrast, when cells preinjected with any of the antibodies were examined in anaphase, they showed only weak or no fluorescence in the area of the cleavage ring when the distribution of the injected antibody was revealed by staining with a second antibody (Fig. 2B). Double staining with a rabbit anti-myosin showed that the distribution of the injected antibody was superimposed on the distribution of endogenous myosin in the injected cells, i.e. anti-myosin-injected cells lacked a concentration of myosin in contractile rings. However, the actin distribution, as seen by phalloidin staining, was identical between injected and noninjected cells: filamentous actin was highly concentrated in the contractile ring (Fig. 2C).

Quite similar data were obtained for PtK2 cells (Fig. 3). In this case, antibodies 9, 2 and 8 were used in microinjection experiments as ‘ideal controls’, since it had been shown that these do not react with PtK2 myosin (Table 1). The injection of reactive antibodies (7, 5 and 4) allowed for accumulation of apparently normal concentrations of actin, but led to a marked decrease in the concentration of myosin in the contractile ring; 68% of these cells showed such a decrease, as depicted in Fig. 3A–D. When cells injected with a nonreactive antibody or buffer were examined, 60% displayed either an increase or at least no conspicuous reduction of myosin in the cleavage furrow. An example of these cases is shown in Fig. 3E–H. The small, rounded shapes of mitotic PtK2 cells were more difficult to analyze by fluorescence microscopy than LLC-PK1 cells, but these figures suggest that in both cell types injection of any of the reactive anti-myosins does not prevent the formation of a contractile ring but cause a reduction in its myosin content, without interfering with the actin content.

Fig. 2. The distribution of actin, myosin and injected antibodies during cleavage of LLC-PK1 cells. (A) LLC-PK1 cell fixed during cytokinesis and stained with anti-myosin antibody to reveal myosin concentrated in the cleavage furrow. (B,C) Two views of an LLC-PK1 cell that had been injected with a cocktail of antibodies 7, 4 and 2 (10 mg ml⁻¹, all reacting with this cell type) during prophase and was subsequently fixed near the end of cytokinesis. (B) The distribution of the injected antibody throughout the cytoplasm as revealed by a fluorescein-labeled anti-mouse antibody. (C) The concentration of P-actin in the cleavage furrow and in newly forming stress fibers (arrow) as seen with rhodamine-phalloidin. Bar, 10 µm.
Anti-myosins cause delay in formation and constriction of the contractile ring

The effect of anti-myosin on the duration of mitosis was examined by injecting PtK2 cells with reactive or nonreactive anti-myosins. Approximately 50 cells were injected in each case and continuously observed under phase-contrast optics throughout the remaining interphase and the next mitosis. The time required for the mitotic processes from breakdown of the nuclear envelope to formation of a cleavage furrow (first visible sign of indentation of the plasma membrane) was determined. Fig. 4 shows data obtained by injecting antibody 5 and 2, respectively (both at 12 mg ml\(^{-1}\)). The length of the period defined above differed between the two cell populations: in the controls injected with antibody 2, more than 80% of the cells completed these mitotic stages within 40 min, while only 50% of antibody 5-injected cells reached these scores.

An analogous difference was found between LLC-PK1 cells injected with any of the anti-myosins and controls that had received non-specific IgG (not shown). To investigate the effect of interfering with several epitopes simultaneously in the cell, we also injected antibody cocktails and a polyclonal antibody against brain myosin. Again, a significant delay, but no inhibition of cytokinesis, was observed. Fig. 5 shows a single LLC-PK1 cell that had been injected with a mixture of antibodies 2, 4 and 7 (10 mg ml\(^{-1}\)). The time from anaphase onset (not shown) to

Fig. 3. The distribution of actin, myosin and injected antibodies in dividing PtK2 cells. (A–D) Cells injected with antibody 5 (10 mg ml\(^{-1}\)), double-stained either for actin and antibody 5 (A,B), or for actin and endogenous myosin (C,D). (E–H) Cells injected with antibody 2, double stained either for actin and antibody 2 (E–F), or for actin and endogenous myosin (G,H). Note that in cells injected with the reactive antibody less myosin, and less antibody, is seen in the cleavage furrow as compared with cells injected with the nonreactive control antibody. Actin is concentrated in the cleavage furrow of all cells. Bar, 20 \(\mu\)m.
Fig. 4. Mitotic periods of PtK2 cells injected with anti-myosin (12 mg ml⁻¹) a-PBM, anti-myosin from pig brain. Interphase cells were injected and subsequently observed under phase-contrast optics in filming chambers. The period from the breakdown of the nuclear envelope to the beginning of the constriction of the plasma membrane was determined. Hatched bars: reactive antibody 5 (57 cells); open bars: nonreactive antibody 2 (49 cells).

Fig. 5. Phase-contrast video micrographs of cell division in an LLC-PK1 cell that had been injected during prophase with a mixture of equal amounts of antibodies 7, 4 and 2. The time from anaphase onset (not shown) to the formation of a tight midbody (last frame, lower right) was 55 min. Uninjected cells took an average of 4–10 min to complete this process. The width of each frame is 60 μm.

the formation of a tight midbody was 55 min. A close examination of anti-myosin-injected and control cells by video microscopy revealed that the rate of chromosome movement was identical in both populations: anaphase movement was completed within 5 min in either case. In analogy to these observations, we conclude that the data shown in Fig. 4 for PtK2 cells are also not due to differences in chromosome movement, but reflect differences in the time required for contractile ring assembly. While their chromosome separation was normal, LLC-PK1 cells injected with anti-myosin showed abnormal behavior during cytokinesis: while noninjected or buffer-injected controls, required 4–10 min for the entire period of cytoplasmic constriction, the same process was prolonged and much more variable in anti-myosin-injected LLC-PK1 cells. Cleavage required 15–50 min. Fig. 5 shows an example for an extreme case. Analogous results were seen with a population of PtK2 cells: Fig. 6 shows that almost 75% of PtK2 cells injected with the control antibody 2 completed the constriction of the plasma membrane within 5 min, while only 10% of the cells injected with antibody 5 achieved this value. Cytokinesis in this case was much more variable, and extreme values extended to more than 50 min.

Anti-myosins interfere with assembly of circumferential belts
After completion of mitosis, most of the PtK2 cells injected with antibodies 4, 5 and 7 showed severe defects in reassembly of the circumferential belt and in formation of normal cellular sheets. Many seemed unable to establish cell–cell contacts: abnormal morphologies included cells with thin and variable peripheral belts, poor or absent stress fibers, splitting of cell–cell junctions and loss of a typical polygonal epitheloid cell shape (Zurek et al. 1989). The proportion of these cells depended on the concentration of the injected antibody: with 20–23 mg ml⁻¹, more than 30% showed severely altered cell shapes, and more than 50% showed defective circumferential belts and abnormal cell–cell contacts (Zurek et al. 1989). Lower antibody concentrations reduced the number of aberrant cells accordingly.

Fig. 7A–C shows panels with several extreme examples of the progeny of cells injected 24 h earlier with antibody 5 (12 mg ml⁻¹). As seen by staining with an anti-mouse IgG, the injected anti-myosin was still present and homogeneously distributed throughout the cytoplasm. In contrast to noninjected controls or cells injected with antibody 2, these cells show an extremely aberrant shape and seem unable to grow in sheets (cf. Fig. 7A–C with D). Their actin filaments were neither organized in peripheral belts nor in stress fibers, as revealed by staining with Rh–phalloidin (not shown). Similar changes in cell shape

Myosin in contractile rings and circumferential belts
Fig. 7. The development of altered morphologies in PtK₂ cells as a consequence of injection with antibody 5. (A,B,C) Clones of 4 (A,B) or 2 (C) cells developed from 1 parental cell that had been injected with antibody 5 (12 mg/ml⁻¹) 24 h earlier. As seen by staining with a second antibody, the anti-myosin is still distributed throughout these cells that show grossly altered morphology and have not formed epithelial plaques. Approximately one third of all injected cells adopted such extreme shapes after division. (D) Noninjected controls stained for immunofluorescence with antibody 5 and a second antibody to reveal normal PtK₂ morphology and myosin distribution. Bars: A–C, 50 µm; D, 50 µm.

and epitheloid sheet formation were seen in the progeny of LLC-PK1 cells after injection of monoclonal or polyclonal anti-myosin (not shown).

Anti-myosins stimulate locomotory activity
When such aberrant PtK₂ cells were examined with respect to their locomotory activity, we found that they were much more mobile than controls. Fig. 8 shows data obtained by measuring the position of the cells' nuclei with respect to reference points on the substratum. While control-injected PtK₂ grew in sheets that were mostly immobile (i.e. they migrated between 0 and 2 µm h⁻¹), the cells injected with antibody 5 showed a wide variability in locomotory activity, and an increase in the speed of migration: approximately 30% of them moved as fast as normally observed for migratory fibroblasts (6–10 µm h⁻¹).

With time, all these phenomena were reversible. Since cell division was not inhibited, the cultures of injected cells proliferated and returned to their normal, epitheloid appearance and to normal cleavage times within several days. This is probably due to both dilution of the injected antibodies and protein synthesis.

Discussion
Among six monoclonal antibodies directed against different epitopes located at the tail of pig brain myosin, we
found three that recognized similar epitopes also in myosin from rat kangaroo cells, Ptk2, and three others that did not. The first ones clustered in the carboxy-terminal third while the latter were concentrated in the center portion of the myosin tail (Table 1, filled and open arrowheads). These findings suggest differences in the importance for myosin function between these two regions: apparently, mutations in the central part of the myosin rod do not necessarily lead to malfunctioning molecules and, therefore, can be tolerated throughout evolution.

None of the antibodies prevented myosin filament formation, but the filaments formed in their presence were shorter by 25 (antibody 9) to 40% (antibodies 4 and 5) as compared with controls (Table 2) and showed an abnormal morphology (Fig. 1). They were also less stable than control myosin filaments, as indicated by the sedimentation experiments and direct observation of negatively stained preparations of unfixed antibody–myosin rod complexes (Baryliko et al. 1989). The more irregular packing of myosin molecules induced by antibodies can also be deduced by the lack of obvious striations in antibody-decorated filaments. Instability of myosin filaments as a result of antibodies binding to the LMM region has also been reported for smooth muscle myosin (Trybus and Henry, 1989).

When injected into the cytoplasm of epitheloid cells, all of these antibodies interfered with cell morphology and cell division. Having been injected at high concentrations (12 mg ml\(^{-1}\) and more), the antibodies were detected in the progeny of recipient cells for several days. Thus, it seems plausible that myosin–antibody complexes are continuously formed, and excessive antibody might absorb even newly synthesized myosin. Without detailed knowledge of all the factors controlling and regulating myosin function in the living cell, it seems difficult to correlate the observed changes preferentially with any one of the in vitro effects. However, we suggest that the observed differences between the antibody-induced changes on peripheral belts and contractile rings might be caused by differences in the requirement of these two structures for strictly bipolar, regular myosin filaments.

Contractile rings, which exist only for short periods, may not need a great number of well-organized myosin filaments. Our data show that actin recruitment is independent of myosin, and similar observations have been reported for Drosophila mutants that are homozygous for a ‘myosin null’ allele. In the complete absence of myosin in muscle cells of these organisms, assembly of ordered arrays of thin filaments and Z-bands at the plasma membrane has been observed (Beall et al. 1989). Thus, with the main structural elements arranged properly, a few, even faulty, myosin filaments may suffice for function, i.e. construction of the plasma membrane in epitheloid cells.

In starfish and amphibian blastomeres, cytokinesis could be arrested by the injection of \(N\)-ethylmaleimide–myosin subfragment 1 (NEM-S1; Meeusen et al. 1980) or polyclonal anti-myosin (Mabuchi and Okuno, 1977; Kiehart et al. 1982). A single injection of a crude IgG fraction was even sufficient to inhibit several consecutive cleavages, which may be explained by the low level of protein synthesis in blastomeres. In Ptk2 and LLC-PK1 cells, we could never observe a total inhibition of cleavage, either with individual monoclonal antibodies, with a cocktail of such antibodies, or with an affinity-purified polyclonal anti-myosin. Several possible reasons for this finding can be considered. One may result from differences between the antibodies used, but there may also be differences in the mechanics of the cleavage process itself: the forces that are needed to cleave a large, globular egg have been calculated to be as high as the ones needed for muscle contraction (Rappaport, 1967; Hiramoto, 1975), while the mechanochemical energy for cleavage of much smaller, flat cells derived from vertebratedifferentiated tissue is probably much less. Another explanation is based on the observation that the assembly of the contractile ring in eggs and early blastomeres is subject to endogenous rhythms. If the assembly is suppressed by treatment with cytochalasin B, which is subsequently removed, a new ring cannot be assembled immediately, but awaits the next regular division cycle (Schroeder, 1975). There is no evidence for an analogous clock in somatic tissue cells. For these, the time point of division seems rather unimportant, and, therefore, they may be able to ‘wait’ for the necessary number of functional myosin elements to accumulate in the contractile ring.

On the other hand, the construction of long-lasting circumferential belts probably demands a high degree of structural precision in numerous bipolar myosin filaments, needed to generate tension within the plane of the bundle. Unstable, irregular myosin–antibody complexes would be useless in this context. The observed increase in locomotory activity of cells lacking circumferential belts and a normal cell morphology could reflect the fact that here filamentous actin is not absorbed by the formation of such belts, but available for interaction even with faulty myosin structures. However, it is also conceivable that locomotory activity is based on other types of myosin that are not affected by our antibodies. There is now convincing evidence that not only ‘primitive’ organisms like Dictyostelium (Cote et al. 1986) or Acanthamoeba (Maruta et al. 1979) contain another type of myosin that does not operate via bipolar filaments constructed from aggregated long, a-helical tails, but that such ‘mini-myosins’ also exist in higher organisms like insects and vertebrates (Collins and Borysenko, 1984; Hoshimaru and Nakanishi, 1987; Montell and Rubin, 1988; Adams and Pollard, 1989). Since our antibodies are specific for the tail portion of the conventional large myosin molecule, they can only interfere with motile events requiring conventional myosin.

We thank Dr H. Faulstich, MPI Heidelberg, for rhodamine-phalloidin, Drs S. Citi and J. Kendrick-Jones, MRC, Cambridge, for myosin light chain kinase and stimulating discussions, Ch. Wiegand and J. S. Dome for expert technical assistance, and L. Koch and R. Klocke for typing the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 223). B. Zurek acknowledges a fellowship from the Studienstiftung des Deutschen Volkes. B. M. Jockusch is grateful to the Marine Biological Laboratory, Woods Hole, MA, for the Herbert W. Rand fellowship and to the Pennsylvania Muscle Institute for its support through its Visiting Scientist Program. J. M. Sanger and J. W. Sanger are indebted to the National Institute of Health for their support (GM-25653; HL-13535 to the Pennsylvania Institute).

### References


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