Electron microscopic mapping of monoclonal antibodies on the tail region of *Dictyostelium* myosin

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Communicated by G.Gerisch,
Received on 10 August 1982

The binding sites of five monoclonal antibodies against myosin of *Dictyostelium discoideum* have been mapped. These antibodies bind to the tail region of the myosin molecule. By rotary shadowing, images of myosin-antibody complexes were obtained in which the mean distance of the midpoint of an antibody molecule from the myosin heads was localized with a precision better than 2 nm (90% confidence limit). Other quantitative data extracted from electron micrographs provided information on the stoichiometry of antibody-myosin interaction. Certain antibodies interacted with myosin molecules only at a ratio of 1:1. Other antibodies formed complexes of two molecules bound to homologous sites on a double-stranded myosin tail. Affinities were estimated and the abilities of different antibodies to cross-connect two myosin molecules were evaluated.

Key words: myosin/monoclonal antibodies/dissociation constants of antigen-antibody complexes/*Dictyostelium*

Introduction

Dictyostelium discoideum is a eucaryotic micro-organism whose cells resemble in size, shape, and amoeboid movement the granulocytes of vertebrates. Like granulocytes, *D. discoideum* cells have the capacity to respond chemotactically to agents specifically bound to cell surface receptors. Chemotaxis is a mechanism by which *D. discoideum* cells assemble into aggregates typically consisting of $\sim 10^5$ cells. The chemoattractant produced and sensed by aggregating cells is cyclic AMP (for review, see van Haastert and Konijn, 1982; Gerisch, 1982).

Chemotactic orientation of an amoeboid cell requires the control of contractility according to the direction of the external concentration gradient of the chemoattractant. This implies a signal processing pathway connecting cell surface receptors with the contractile apparatus in the cytoplasm. Evidence is accumulating for a pathway involving the following steps. Activation of cyclic AMP receptors causes an increased influx of Ca^{2+} into the cell (Wick *et al.*, 1978). Together with calmodulin, Ca^{2+} inactivates a myosin heavy chain kinase present in aggregating cells (Malchow *et al.*, 1981; Maruta *et al.*, 1982). Myosin is dephosphorylated by phosphatase, resulting in an elevated actin-activated Mg^{2+} -ATPase activity and a stronger tendency of the myosin to form filaments (Kuczmarski and Spudich, 1980).

As a tool for analysing myosin functions *in vivo* we have produced monoclonal antibodies against *D. discoideum* myosin. For functional studies using microinjection of these antibodies into cells it is important to select antibodies with high affinity. Predicting the effect an antibody may have re-

quires knowing where it binds on the myosin molecule. Here we report on the results obtained by electron microscopic screening of 25 purified monoclonal IgGs. Five of the antibodies had an affinity high enough for exact mapping of their binding sites on the tail region of the myosin.

Results

Visualization of myosin antibody complexes

Monoclonal IgG reacting with *D. discoideum* myosin was purified from hybridomas derived from spleen cells of three different mice. Two of the mice had been immunized using Al-hydroxide and *Bordetella pertussis* antigen as adjuvants. From each of these two mice, one antibody was obtained whose binding site could be mapped. The two antibodies, mAb 14-26-5 and 15-153-33, bound to the myosin tail very close to each other (Figure 1). The third mouse, immunized with the use of Freund's adjuvant, gave rise to 23 stable hybridoma clones from which the antibodies were purified. All of the antibodies reacted with native myosin as judged from radioimmunoassays (RIAs), but only three formed complexes with myosin that were stable and abundant enough to be clearly recognized in the electron microscope. The binding sites of these three antibodies differed from that of mAb 14-26-5 and 15-153-33: two bound close to the myosin heads and one to the tail near its end (Figure 1).

Precision of mapping

The positions of binding sites for the five antibodies shown in Figure 1 were measured and related to total tail lengths. The measurements revealed well-defined binding sites for four of the antibodies, with negligible background of antibody molecules associated with other parts of the myosin molecule (Figure 2). Also for the fifth antibody, mAb 21-32-3, the binding sites behind the myosin heads were clearly distinguishable in the histogram, but a significant number of antibodies was also distributed over the length of the tail.

Tail lengths were measured in a preparation of unlabelled myosin, and in all preparations used for the determination of antibody binding sites (Figure 2). The unlabelled myosin showed an average length of 188 nm (Table I), which is larger than the 150 nm length reported for myosin from skeletal muscle and some non-muscle sources (Elliott *et al.*, 1976), and much larger than the 90 nm tail length of *Acanthamoeba* myosin II (Pollard *et al.*, 1978). This result is in accord with the high apparent mol. wt. of 215 kd, as determined for *D. discoideum* myosin heavy chains by SDS-polyacrylamide gel electrophoresis.

The average tail lengths in most of the antibody-labelled preparations were significantly shorter than in the control (Table I). This can be explained by assuming that the antibodies cause local shortening of the myosin heavy chains. However, variation from one preparation to the other could also be a reason for the differences seen in Table I. In no case did the decrease in the average length exceed 8% of the control value. We have allowed for these relatively small differences by normalizing the tail lengths. Positions of antibody binding sites were calculated as fractions of the head-tail

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Fig. 1. Myosin molecules of *D. discoideum* (control) and complexes of myosin with five different monoclonal antibodies. For mAb 14-26-5 cross-connection of two myosin molecules is shown. Myosin molecules labelled with two different antibodies are shown on bottom of the right column. One molecule carries two antibody molecules at the binding site of mAb 15-153-33. FD, freeze-dried specimens shadowed unidirectionally. The other molecules were prepared by drying at room temperature and low angle rotary shadowing.

distance measured in each preparation (Table I, right column).

Single and duplicate binding of monoclonal antibodies to the myosin tail

As in most myosins, the tail is double-stranded in *D. discoideum* myosin. Thus, maximally two identical antibody molecules can bind to a myosin tail. Duplicate binding has often been observed for antibodies mAb 14-26-5 and mAb 15-153-33 (Figure 3), but rarely or never for mAb 21-96-3 and 21-51-3, although myosin-antibody complexes were particularly abundant when these antibodies had been used.

Quantitative data were obtained by counting free myosin molecules as well as binary and ternary complexes. The experimental data were compared with theoretical values based on the assumption that both homologous sites on a myosin tail could be occupied independently and with the same probability (Table II). For mAb 15-153-33 the experimental data closely agree with theory. For mAb 21-96-3 and 21-51-3 disagreement is striking. Myosin molecules carrying one antibody molecule are strongly over-represented.

Cross-connection of myosin molecules by antibody

Antibodies 15-153-33 and 14-26-5 sometimes cross-connected two myosin molecules as shown in Figure 1. This is in accord with the duplicate binding often observed using these antibodies, as it also indicates that only one antigen binding site of an antibody molecule is involved in interaction with a single myosin molecule. Cross-connection of two

myosin molecules has also often been observed with mAb 21-51-3, which never bound in duplicates to a single myosin molecule. The number of crosses counted was between 23 and 35 per 1000 myosin molecules, i.e., between 5% and 7% of the myosin molecules were linked by an antibody molecule. In contrast, only a single myosin cross has been seen with mAb 21-96-3 in an even larger population.

Estimates of dissociation constants

Dissociation constants for the interaction of single sites on the antibody and myosin molecules were estimated from the concentrations of myosin and IgG in the incubation mixtures (see Materials and methods) and from the ratio of free myosin molecules and binary and ternary complexes, as shown in Table II, considering also myosin cross-links.

Because for mAb 15-153-33 single and duplicate binding occurred randomly, and also myosin cross-connections were observed, all binding sites on the antibody and myosin molecules could be assumed to interact with the same probability. Accordingly, a defined dissociation constant was calculated (Table III).

It is unclear whether one or two binding sites exist on the double-stranded myosin tail for mAb 21-51-3. Therefore, dissociation constants were calculated assuming either one or two binding sites, the real value probably lying in between (Table III). Antibody 21-32-3 apparently had the lowest affinity among the antibodies that could be mapped. A rough calculation yielded a value between 4×10^{-6} and 1×10^{-5} M

Fig. 2. Histograms showing myosin tail lengths (open areas) and positions of monoclonal antibodies (shaded areas). For the calculation of Table I all data shown here have been used, except mAb 21-32-3 molecules bound at a distance > 30 nm, and mAb 21-96-3 molecules bound at a distance < 50 nm.

for the K_D . We should emphasize here that any calculation of K_D is valid only if the equilibrium does not change drastically during spreading and drying of the specimens, and if myosin and its complexes with antibodies attach with the same efficiency to the mica surface.

Regional bending of the myosin tail with and without antibody

Myosin molecules were often seen to be bent in the middle of the tail (Figure 1). This is similar to local bending of

Table I. Localization of monoclonal antibodies relative to myosin tail lengths

mAb	Tail lengths				Distance of mAb from the heads					per cent of tail length
	in nm				in nm					
	M	CL	SD	N	M	CL	SD	N		
none	187.5	1.2	7.8	119	—	—	—	—	—	
21-32-3	189.6	3.5	14.3	45	6.6	1.0	3.9	38	3.5	
21-51-3	185.2	1.3	9.9	158	22.4	0.7	5.4	161	12	
14-26-5	173.6	2.3	10.8	60	47.7	1.2	6.5	78	27	
15-153-33	172.5	2.6	17.9	125	{	53.5	0.9	5.1	100	31
plus						140.2	1.7	10.5	125	81
21-96-3						147.4	1.2	7.6	110	81
21-96-3	181.0	1.6	10.1	116	147.4	1.2	7.6	110	81	

The right column gives positions of the antibodies relative to the myosin tail length, as determined for the respective myosin-antibody preparation. Second and third column: M, mean value; CL, confidence limit of the mean value for $p = 0.90$; SD, standard deviation of the individual values; N, number of measurements.

Table II. Occupation of binding sites on the myosin tail for three different monoclonal antibodies

mAb	Number of anti-bodies bound per molecule of myosin	Number of myosin molecules counted	Probability	
			Experimental	Theoretical
15-153-33	0	96	0.40	0.39
	1	111	0.46	0.47
	2	35	0.14	0.14
21-96-3	0	33	0.14	0.30
	1	200	0.83	0.50
	2	9	0.04	0.20
21-51-3	0	31	0.16	0.34
	1	161	0.84	0.49
	2	0	0.00	0.18

The theoretical probabilities were calculated for a binomial distribution from the total number of bound antibodies and the number of theoretically available binding sites. It was assumed that there were two binding sites per myosin molecule, that antibodies could bind with the same probability to each, and that binding of an antibody molecule to one site does not change the probability of binding to the second site.

Table III. Dissociation constants for the interaction of two monoclonal antibodies with myosin

mAb	Estimates of dissociation constants (K_D)
15-153-33	$1.6 \times 10^{-6} \text{ M}$
21-51-3	$2 \times 10^{-6} > K_D \geq 3 \times 10^{-7} \text{ M}$

Dissociation constants were calculated for an antigen binding site on the antibody, interacting with one complementary site on the double-stranded myosin tail. Table II showed for mAb 21-51-3 a strong bias towards single binding of antibody molecules to a myosin tail. Therefore the upper and lower limits of the K_D are given. The upper limit would apply if both sites on each myosin tail are accessible to the antibody; and the lower limit if only one site is accessible.

skeletal muscle myosin visualized by negative staining (Takahashi, 1978). In myosin-antibody complexes the myosin tails were often sharply bent at the sites of antibody binding (Figure 3). In order to localize exactly and to quantitate changes in the straightness of the tail, a statistical method has been used to calculate a value (K) which depends upon the angles between successive segments along the myosin tail. For a completely straight, rigid region the angle between segments is always zero, and K equals infinity. For a perfectly flexible region all angles between segments are equally probable and K equals zero (for details, see Materials and methods).

Figure 4 shows the results of the statistical analysis for free myosin molecules and for complexes of myosin with two different antibodies, mAb 15-153-33 and 21-96-3, extreme examples of which are seen in Figure 3. The broad region of reduced straightness in the middle of the myosin tail was extended after antibody binding by new minima at the two antibody binding sites.

Discussion

The electron microscopic data allow a precise localization of antibody binding sites on the myosin molecule. They also provide other information valuable for the application of monoclonal antibodies in modifying myosin functions. Firstly, low-affinity antibodies with a $K_D > 1 \times 10^{-5} \text{ M}$ are easily sorted out. Only 10–20% of the antibodies had an affinity for myosin high enough to enable mapping of the binding sites. Secondly, whether a particular antibody cross-connects individual myosin molecules can be recognized. Antibodies unable to cross-connect can be considered as functionally

Fig. 3. Myosin-antibody complexes with sharp bends at the sites of antibody binding. Preparations as in Figure 1.

Fig. 4. Regional variation of straightness of the myosin tail in the presence or absence of antibody binding. **Top**, control myosin not labelled with antibody; **bottom**, myosin molecules to which antibody molecules were bound at two positions. The sites of binding of the two antibodies were taken from Figure 2 and indicated by shading. Bars represent 90% confidence intervals. The number of myosin molecules measured was 119 for the control, and 40 for the myosin-antibody complexes.

monovalent like an Fab molecule.

Electron micrographs showed that two antibody molecules of the same sort could bind side-by-side to the double-stranded myosin tail. In other cases duplicate binding of antibodies was rare or absent. There are at least four situations in which the latter result could be obtained. (1) Homologous sites on the two polypeptide chains are located so close to each other that an IgG molecule bound to one chain sterically hinders the binding of a second IgG. (2) The two strands forming the myosin tail are associated in an asymmetrical manner, so that a particular antibody binding site is exposed only on one strand. (3) The two strands are different, and only one strand possesses the antibody binding site. (4) The homologous sites are the correct distance apart for an antibody to attach with both its antigen binding sites to the same myosin molecule. The present data do not allow us to distinguish between these possibilities. However, possibility (4) is favoured by the observation that mAb 21-96-3 very rarely cross-connected two myosin molecules. Antibody 21-51-3, by contrast, showed no duplicate binding to a single myosin molecule, but often cross-connected two molecules. In this case possibilities (1), (2), or (3) are applicable.

Electron microscopy also shows distortions of antigen molecules as related to antibody binding. It is likely that these shape changes of the molecules also exist in solution. The antibodies may force the polypeptide chains of the myosin tail to adopt a new configuration, or they may produce a local increase of flexibility. It is also possible, however, that the

changes are caused during specimen preparation by adsorption of the antibodies to the mica, and by shear forces as these are unavoidable when spreading microdroplets on the mica surface. This possibility has to be taken into account, although there was no systematic alignment of molecules due to shear forces in the fields of the preparations analysed.

Materials and methods

Cell culture

D. discoideum strain AX2-214 was grown in fermenters at 23°C in axenic medium containing 1.8% maltose (Watts and Ashworth, 1970) up to a density of $\sim 1 \times 10^7$ cells/ml. The cells were harvested by centrifugation and washed in 17 mM Soerensen phosphate buffer pH 6.0.

Myosin preparation from growth phase cells

Actomyosin was extracted from 500 g of fresh cell sediment with 30% (w/v) sucrose as described by Clarke and Spudich (1974). The actomyosin was precipitated from the sucrose extract with 1.5 M ammonium sulphate. The pellet was resuspended in 100 ml of buffer containing 30 mM Tris-HCl, pH 7.5, 0.5 mM phenylmethyl sulphonyl fluoride, 1 mM dithiothreitol (DTT), 2 mM $MgCl_2$ and 0.1 M KCl, and centrifuged at 100 000 g for 3 h. The interphase, ~ 50 ml, between the clear supernatant and the pellet contained most of the actomyosin. The interphase was supplemented with 1 mM Mg^{2+} -ATP and 0.6 M KI and centrifuged at 100 000 g for 20 min. The supernatant was further fractionated on Bio Gel A15m according to Clarke and Spudich (1974). The slightly turbid myosin fractions were centrifuged at 100 000 g for 3 h. The clear supernatant was precipitated with 3 M ammonium sulphate, dialysed against buffer containing 10 mM Tris-HCl pH 7.5, 1 mM DTT, 0.02% NaN_3 and 15% sucrose, and stored on ice. The yield was ~ 100 mg myosin.

Monoclonal antibody production

Hybridomas were obtained by fusion of mouse spleen cells with myeloma cells (Koehler and Milstein, 1976) using polyethylene glycol MW 4000 (Polysciences, Warrington, PA). The myeloma line Sp2-01 was a gift from Dr. G. Koehler.

BALB/c mice had been immunized with *D. discoideum* myosin using, for fusions no. 14 and 15, Al-hydroxide and *B. pertussis* antigen as adjuvants (Bozzaro *et al.*, 1981). For fusion no. 21, 50 μ g myosin was injected i.p. with complete Freund's adjuvant, followed after 1 month by a booster of 200 μ g myosin with incomplete Freund's adjuvant. Screening was performed by solid-phase RIA, and hybridoma cloning in microtitre plates by examining small droplets under the microscope and selecting those containing one cell.

Antibodies were purified from culture medium containing 10% fetal calf serum by ammonium sulphate precipitation and chromatography on Protein A-Sepharose. The antibodies are assigned by the fusion number, number of the well from which the cell line was isolated, and number of the established clone.

Specimen preparation for electron microscopy

A stock solution containing 3 mg myosin/ml was diluted before use at least 100-fold in a solution containing 0.2–0.5 M ammonium formate, pH 7.3, and 50–100 μ g antibody/ml. Quantities for the experiments of Tables II and III and for counting myosin cross-connections were as follows. Labelling with mAb 21-51-3: 14 μ g myosin and 113 μ g IgG per ml of 0.3 M ammonium formate. Double labelling experiment: 22 μ g myosin, 72 μ g IgG of mAb 15-153-33, and 80 μ g IgG of mAb 21-96-3 per ml of 0.24 M ammonium formate. The mol. wt. of myosin was assumed to be 498 000 (two heavy chains with 215 000, two light chains with 18 000, and two with 16 000 mol. wt.). The protein concentration of the myosin solution was determined according to Lowry and calibrated by quantitative amino acid determination. The mol. wt. of IgG was assumed to be 150 000. The IgG concentration was determined at 280 nm assuming $OD_{1\text{ cm}} = 1.5$ for a solution of 1 mg IgG per ml of phosphate buffered saline, pH 7.2.

After 6–12 h of incubation at -4°C , glycerol was added up to a final concentration of 30% and specimens were prepared according to Elliott *et al.* (1976) and Shotton *et al.* (1979). The solutions were sprayed at room temperature onto freshly cleaved mica and allowed to dry for at least 2 h in the vacuum. Rotating specimens were shadowed with platinum/carbon (95:5 w/w) at an angle of 5° , and coated with a backing film of carbon using electron beam evaporation guns.

For freeze-drying, the suspension was sprayed onto mica mounted with liquid propane on a copperblock cooled with liquid nitrogen. The dried specimens were shadowed unidirectionally with platinum/carbon and coated as above.

Measurements on electron micrographs

Electron micrographs were taken with a Jeol JEM-100 CX electron microscope at a magnification of 19 600. The magnification was calibrated using the 8.75 nm spacing of catalase crystals. To minimize errors, the objective lens current was raised for a short time and then always set to the same value.

The electron micrographic negatives of myosin-antibody preparations or of catalase crystals were projected with 7.5-fold magnification onto a Summagraphics (Fairfield, CT) digitizing tablet connected to a VAX 11 computer. The traces of myosin molecules were followed by a measuring cursor, starting behind the heads and ending either in the middle of an antibody molecule or at the tip of the tail. Length and straightness of the molecules were calculated from the stored x , y coordinates.

Statistical methods

Statistical analysis of the lengths used normal linear statistical methods (Walpole and Myers, 1972). To measure the straightness, we used a FORTRAN program (Fisher *et al.*, in preparation) to divide the digitized images of the molecules into 1–2 mm long segments. The endpoints of each segment were used to calculate the angles between successive segments. The statistical analysis is based on the 'von Mises' or circular normal distribution – an analogue for directional data of the familiar normal or Gaussian distribution (Mardia, 1972). The von Mises distribution is characterized by two parameters – an average direction (μ) and a concentration parameter (K) which measures how concentrated the angles are around the average direction (Fisher *et al.*, 1981). In this work μ is always 0° , and the concentration parameter (K) is used as a measure of the straightness of the myosin molecule at various distances from the heads.

Acknowledgements

We thank G.Geigenberger-Blank, B.Schönbauer, and G.Wagle for cooperation in monoclonal antibody preparation, and D.Grenzendorf and A.Röglin for help in the evaluation of electron micrographs. The work was supported by grant Ge 135-18 from the Deutsche Forschungsgemeinschaft.

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