Proc. Natl. Acad. Sci. USA
Vol. 78, No. 8, pp. 4738–4742, August 1981
Biochemistry

Characterization of antibodies to smooth muscle myosin kinase and their use in localizing myosin kinase in nonmuscle cells

(antibody/fibroblasts/stress fibers)

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Communicated by Donald S. Fredrickson, April 13, 1981

ABSTRACT **Antibodies to myosin light chain kinase, purified from turkey gizzard smooth muscle, were developed in rabbits and purified by affinity chromatography on a myosin light chain kinase-Sepharose 4B column. The purified antibodies crossreact with purified smooth muscle myosin light chain kinase but not with a variety of contractile or cytoskeletal proteins. The antibodies inhibit the catalytic activity of smooth muscle myosin light chain kinase, and there is an inverse relationship between the kinase activity and the amount of antibody present in an assay. Half-maximal inhibition of myosin kinase activity occurs at an antibody/myosin kinase molar ratio of 10:1. The affinity-purified antibodies to smooth muscle myosin kinase were used to study the location of myosin kinase in a variety of nonmuscle cells. Immunofluorescence studies indicate that myosin light chain kinase is localized on microfilament bundles (stress fibers) in cultured fibroblasts. The stress fiber staining pattern is abolished when the antibodies are incubated with purified smooth muscle myosin light chain kinase prior to staining cells, while the staining pattern is unaffected when the antibodies are incubated with actin, myosin, α-actinin, or tropomyosin prior to staining. Moreover, the stress fiber staining pattern is periodic in well-spread gerbil fibroma cells and experiments have demonstrated that myosin light chain kinase appears to have the same periodic distribution as myosin but an antiperiodic distribution relative to α-actinin. These data indicate that myosin light chain kinase and its substrate, myosin, are in close proximity and are consistent with the hypothesis that myosin light chain kinase regulates actin–myosin interactions in nonmuscle cells.**

The proteins actin and myosin are thought to mediate a variety of contractile events in nonmuscle cells (1). These include general cellular functions such as locomotion (2) and cytokinesis (3) and specialized cell functions such as phagocytosis in macrophages (4) and secretion in platelets (5). Intrinsic to these functions is the ability of myosin to hydrolyze MgATP and generate force when it interacts with actin.

One mechanism for regulating the interaction of actin and myosin in nonmuscle and smooth muscle cells is through the reversible phosphorylation of myosin (for review, see ref. 6). Phosphorylation has been shown to have two effects: (*i*) It is required for, or markedly enhances, the actin-activated Mg^{2+}-ATPase activity of myosin (7–14) and (*ii*) it stabilizes bipolar filaments of myosin (15, 16), which are required for the development of tension.

Experiments with intact smooth muscle strips and with blood platelets have confirmed the relationship between myosin phosphorylation and contractile activity. These studies have shown that myosin is phosphorylated before, or simultaneously with, the contraction of smooth muscle (17–19) and the secretion of granules by platelets (5).

Myosin light chain kinase, the enzyme that catalyzes the phosphorylation of myosin, plays a central role in this regulatory process. Although there is considerable information about the biochemical properties of the enzyme, which has been purified from both smooth muscle (20, 21) and nonmuscle cells (22–25), there is at present little information about its location in these cells. We have used an immunological approach to localize myosin light chain kinase in nonmuscle cells. We purified myosin light chain kinase from turkey gizzard smooth muscle and raised antibodies to this protein in rabbits. These antibodies were purified, tested for their ability to inhibit the catalytic activity of myosin light chain kinase, and then used to determine the intracellular location of myosin light chain kinase in a variety of nonmuscle cells.

METHODS

Antibody Production and Purification. Myosin light chain kinase was purified from turkey gizzard smooth muscle as described (20). Two rabbits were bled prior to immunization (pre-immune serum), and antibodies to smooth muscle myosin light chain kinase were produced by injecting 1 mg of myosin light chain kinase, emulsified in complete Freund's adjuvant, intramuscularly. The rabbits were immunized with 1 mg of myosin kinase at 1-week intervals for 4 weeks. Since both rabbits produced antibodies, they were bled (40 ml per bleed) from an ear vein once a week starting 7 days after the last immunization. Pre-immune sera and immune sera (four separate bleeds from each rabbit) were pooled separately, and IgG fractions were prepared by adding solid $(NH_4)_2SO_4$ to the sera. The protein precipitating at 0–33% $(NH_4)_2SO_4$ saturation was collected by centrifugation, dialyzed exhaustively in 150 mM NaCl/5 mM NaN_3/20 mM Tris·HCl, pH 7.5, and stored at −70°C.

Antibodies to myosin light chain kinase were purified by affinity chromatography of the immune IgG fraction. Turkey gizzard smooth muscle myosin light chain kinase (15 mg) was coupled to 10 ml of CNBr-activated Sepharose 4B (Pharmacia) as recommended by the manufacturer. Immune IgG was applied to myosin light chain kinase-Sepharose 4B beads equilibrated in 150 mM NaCl/20 mM Tris·HCl, pH 7.5 (buffer A). The beads were washed with 10 vol of the same buffer, then with 10 vol of 3 M LiCl/20 mM Tris·HCl, pH 7.5, and eluted with 6 M guanidine·HCl/1 M NaCl/20 mM Tris·HCl, pH 7.5. After dialysis in buffer A, the purified antibodies were concentrated by dialysis in 80% sucrose in buffer A, redialyzed in buffer A/5 mM NaN_3, and stored at −70°C. All steps in the preparation of the IgG fraction and the purification of the antibodies were performed at 10°C.

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Abbreviation: P_i/NaCl, phosphate-buffered saline.

Phosphorylation Assays. An isolated fraction of myosin light chains was prepared from turkey gizzard smooth muscle myosin as described for skeletal muscle myosin (26). Calmodulin was removed from the light chain fraction by ion exchange chromatography on DEAE-Sephacel (20). Porcine brain calmodulin was purified as described (27). Kinase assays were performed at room temperature as described (20). Inhibition of myosin kinase activity was studied by incubating the kinase with the various antibody fractions for 2 min before initiating the phosphorylation reaction.

Cell Culture Methods. Gerbil fibroma cells (CCL 146) and primary chicken embryo fibroblasts were cultured in Dulbecco's modified Eagle's medium/9% fetal calf serum. Human fibroblasts (clone GRC 161D7.9) were the generous gift of J. R. Smith (Alton Jones Center) and were grown in the same medium supplemented with nonessential amino acids.

Indirect Immunofluorescence Studies. Cells grown on glass coverslips were washed with phosphate-buffered saline (P_i/NaCl) pH 7.3 and fixed in 3.5% formalin in P_i/NaCl for 10 min at room temperature. The coverslips were rinsed in P_i/NaCl and then extracted in acetone at −20°C for 5 min. They were rinsed again in P_i/NaCl and then stained with the relevant antibodies. The affinity-purified myosin light chain kinase antibody was used at 90 μg/ml. Rabbit antibodies to myosin were raised by immunization with chicken gizzard myosin and were similar to those described by Gordon (28). The properties of rabbit antibodies to smooth muscle α-actinin and guinea pig antibodies to smooth muscle tropomyosin have been described (29). The myosin light chain kinase antibody was adsorbed with purified myosin light chain kinase, actin, tropomyosin, vimentin, or myosin prior to staining some cells. The antibody fraction was incubated with an equal volume of protein solution (1–2 mg/ml) for 1 hr at 4°C and then centrifuged at 13,000 × *g* for 5 min prior to staining. The coverslips were incubated with the antibodies in a humidified atmosphere at 37°C for 30 min. After washing in P_i/NaCl, the coverslips were stained with fluorescein isothiocyanate-labeled goat anti-rabbit IgG diluted 1:25. Rhodamine isothiocyanate-labeled goat anti-mouse IgG or goat anti-guinea pig IgG [both from Cappel Laboratories (Cochranville, PA)] were used for double-label immunofluorescence studies. After extensive washing in P_i/NaCl, the coverslips were mounted in gelvatol.

Cells were viewed and photographed with a Zeiss epifluorescence photomicroscope 111 using a 63× oil phase lens (NA 1.4). Rhodamine was analyzed by using a Zeiss G456 (narrow-band pass interference filter 546 ± 2 nm) excitation filter and an LP590 barrier filter, and fluorescein was analyzed by using a Zeiss dichroic excitation filter BP485/20 and barrier filter LP520.

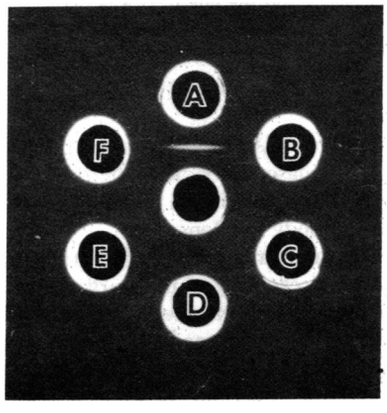

FIG. 1. Immunoprecipitation analysis of myosin kinase antibody in agar. The center well contained the affinity-purified antibody. The outer wells contained, respectively, myosin light chain kinase (A), myosin (B), actin (C), tropomyosin (D), α-actinin (E), and filamin (F). All proteins were purified from gizzard smooth muscle and used at 1.5 mg/ml. Note the single precipitin line between the antibody and the well containing the myosin kinase.

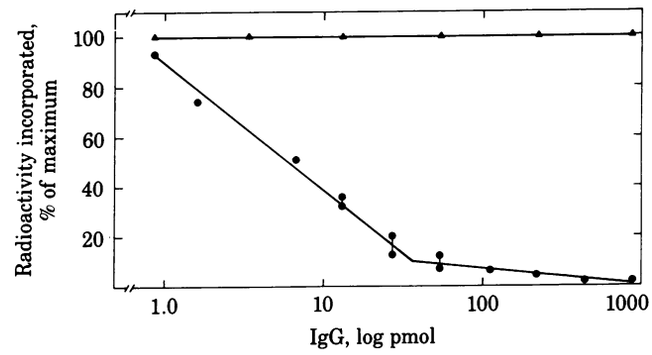

FIG. 2. Inhibition of purified myosin light chain kinase activity by affinity-purified myosin light chain kinase antibodies. A standard amount of purified smooth muscle myosin light chain kinase (0.77 pmol) was incubated with various amounts of purified antibodies (●) or pre-immune IgG (▲). The kinase was incubated with the antibodies for 2 min prior to initiating a reaction that gave linear incorporation of radioactivity into smooth muscle myosin light chains. The reaction was terminated by the addition of trichloroacetic acid at 3 min and the extent of ^{32}P incorporation was determined. Kinase activity is expressed as percent of maximum, using the extent of myosin light chain phosphorylation in the absence of added immunoglobulin as 100%.

Miscellaneous Techniques. Protein concentrations were determined as described by Bradford (30) or by Lowry *et al.* (31) using suitable proteins as standards. Antibody crossreactivity was studied by double-diffusion precipitation reactions on 0.75% agar gels.

RESULTS

Antibodies to myosin light chain kinase were purified by affinity chromatography of the immune IgG fraction on a myosin light chain kinase-Sepharose 4B column. The protein eluting with

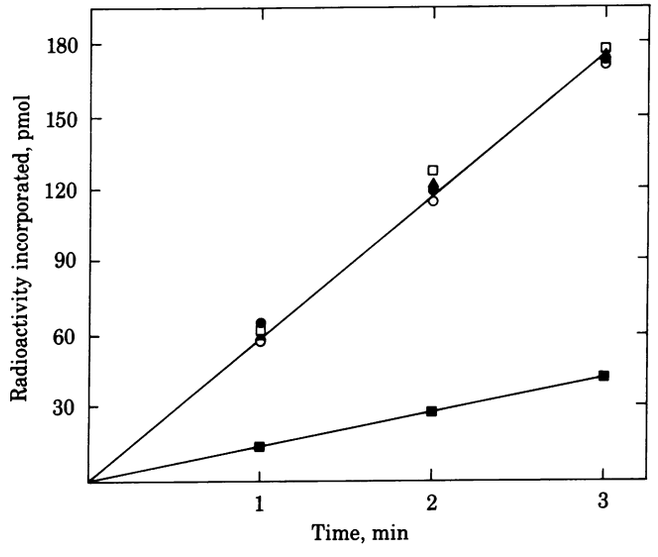

FIG. 3. Time course of inhibition of purified myosin kinase activity by various antibody fractions. A standard amount of smooth muscle myosin light chain kinase (2.31 pmol) was incubated with 81.6 pmol of purified antibody (■) or an equivalent amount of pre-immune (▲) or nonretarded (○) IgG for 2 min. Phosphorylation (final vol 300 μl) was initiated, and aliquots were taken at the times indicated. Phosphorylation of myosin light chains with kinase incubated with 15 mM 2-mercaptoethanol/50 mM morpholinopropanesulfonic acid, pH 7.0, (●) phosphorylation with kinase incubated with antibodies that had been previously incubated with heat-killed formalin-fixed staphylococci.

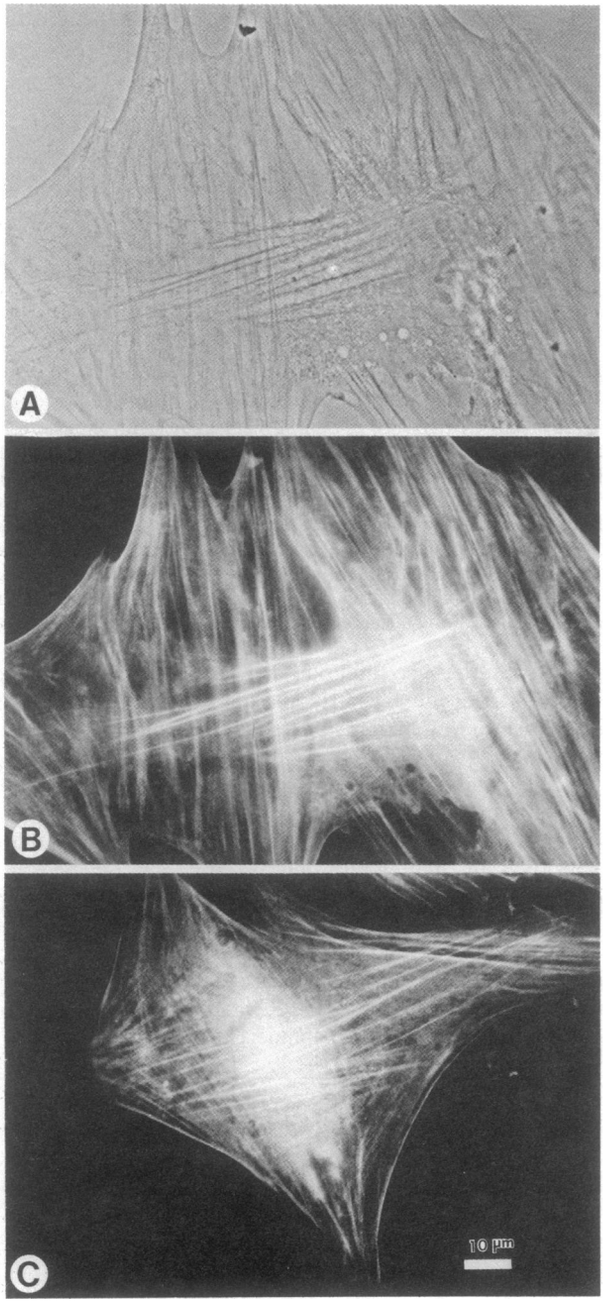

FIG. 4. Phase-contrast (*A*) and fluorescence (*B* and *C*) micrographs of cells stained with affinity-purified rabbit antimyosin light chain kinase antibodies. *A* and *B*, the same human fibroblast cell; *C*, chicken embryo fibroblast. The primary antibodies were visualized by using fluorescein-labeled goat anti-rabbit IgG antibody. Note the prominent staining of the stress fibers in every case. Cells stained with pre-immune IgG did not stain stress fibers but showed some bright fibrillar staining around the nucleus. This perinuclear staining has been shown to be due to a spontaneously occurring antibody to the protein vimentin found in the pre-immune IgG (33).

guanidine·HCl constitutes the affinity-purified antibodies. Care was taken to minimize the length of time the antibodies were exposed to the denaturant. Most of the protein in the IgG fraction did not bind to the affinity column. This protein was pooled and reapplied to the affinity column twice to remove residual myosin light chain kinase antibodies. The protein fraction not binding to the column after the last chromatography step was labeled "nonretarded IgG" and represents a control antibody fraction. It should be noted that washing the column with 3M LiCl before eluting with guanidine·HCl removed a

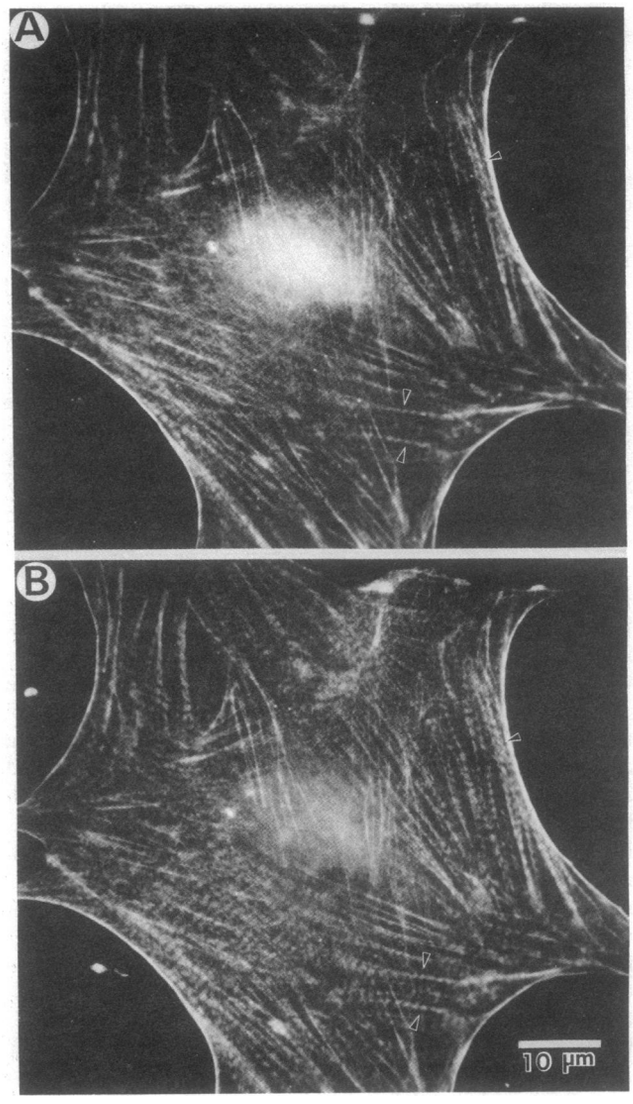

FIG. 5. Fluorescence micrographs of a single gerbil fibroma cell stained with myosin kinase (*A*) and tropomyosin (*B*) antibodies. The cell was stained simultaneously with rabbit myosin kinase antibodies and guinea pig tropomyosin antibodies. Fluorescein-labeled goat anti-rabbit IgG and rhodamine-labeled goat anti-guinea pig IgG were used to visualize the primary antibodies. The micrograph in *A* was taken with fluorescein optics and the one in *B* was taken with rhodamine optics. Note the periodic staining along the stress fibers and the coincidence of the periodicities for the two proteins along stress fibers (arrows).

significant amount of nonspecifically bound protein and a small amount of myosin kinase antibodies. Therefore, the protein in the LiCl wash was not included in either the nonretarded IgG or the purified antibody fractions.

Immunoprecipitation reactions were performed on agar to determine the crossreactivity of the purified antibodies (Fig. 1). The purified antibodies crossreacted with purified smooth muscle myosin light chain kinase but not with a variety of proteins purified from gizzard smooth muscle, including actin, myosin, tropomyosin, α-actinin, filamin, and vinculin. It was also determined that the antibodies do not crossreact with the catalytic subunit of cyclic-AMP-dependent protein kinase or calmodulin. Neither pre-immune IgG nor nonretarded IgG crossreacted with myosin light chain kinase.

The affinity-purified antibodies were tested for their ability to inhibit the activity of purified myosin light chain kinase. The data in Fig. 2 demonstrate an inverse relationship between the

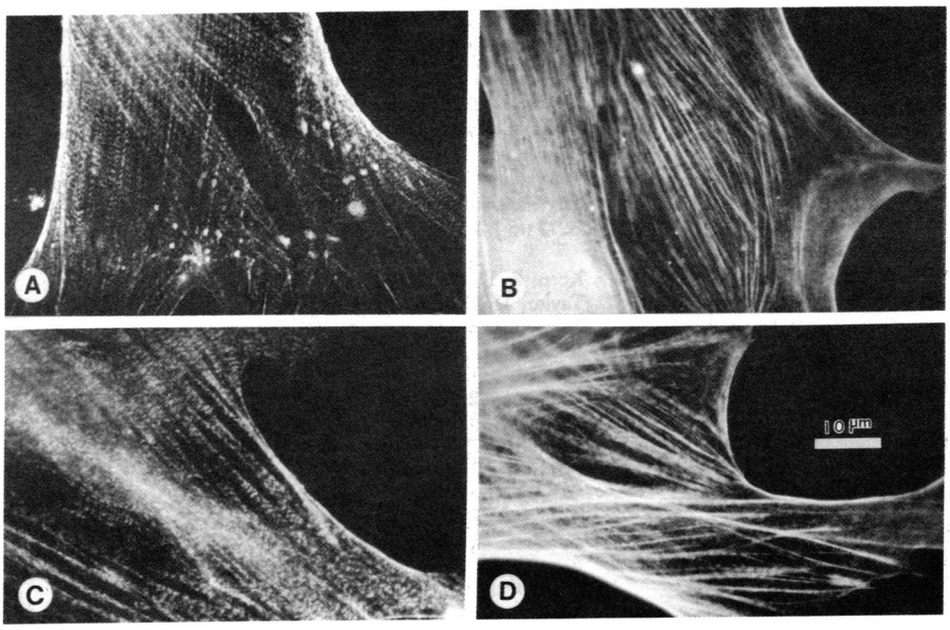

FIG. 6. Fluorescence micrographs of cells stained with various antibodies. Gerbil fibroma cells were stained with rabbit anti-α-actinin alone (*A*), both rabbit anti-α-actinin and rabbit antimyosin light chain kinase (*B*), rabbit antimyosin alone (*C*), and both rabbit antimyosin and rabbit antimyosin light chain kinase (*D*). Fluorescein-labeled goat anti-rabbit IgG was used to visualize the primary antibodies in each case. The periodic staining pattern seen for α-actinin alone (*A*) becomes continuous in the presence of antimyosin light chain kinase antibodies (*B*). On the other hand, the periodic staining pattern seen for myosin staining (*C*) is preserved when cells are stained with both myosin and myosin kinase antibodies (*D*).

extent of myosin light chain phosphorylation and the amount of antibody present in this assay. Fifty percent of the myosin kinase activity was inhibited in the presence of ≈8 pmol of antibody. Because 0.77 pmol of myosin kinase was used per assay, these data indicate that an antibody/myosin kinase molar ratio of ≈10:1 was required for half-maximal inhibition of myosin kinase activity. Incubating the kinase with similar amounts of pre-immune IgG had no effect on myosin light chain phosphorylation. Fig. 3 shows that the rates of myosin light chain phosphorylation are virtually identical for kinase that has been incubated with buffer, pre-immune IgG, or nonretarded IgG. In contrast, there is a significant reduction in the rate of phosphorylation when the kinase is incubated with antibody. Kinase incubated with antibodies that had been treated with excess heat-killed formalin-fixed *Staphylococcus aureus* (Pansorbin, CalBiochem) to remove IgG (32) was fully active. These data show that a gamma globulin specific to the antibody fraction is responsible for inhibiting smooth muscle myosin light chain kinase activity.

After characterizing the purified antibodies to myosin light chain kinase, we used them to localize myosin light chain kinase in a variety of cultured cell types. Fig. 4 shows the distribution of myosin light chain kinase in chicken embryo and human fibroblasts. Fig. 5*A* shows the distribution of myosin kinase in gerbil fibroma cells. In each case, the most prominent distribution is along the stress fibers (bundles of microfilaments). A close examination of Fig. 5*A* suggests periodicities in the staining pattern of the stress fibers in gerbil fibroma cells. No periodicity was detected in the staining of stress fibers in the chicken embryo and human fibroblasts. In addition to stress fiber staining, the chicken embryo cells also show diffuse cytoplasmic staining with the antibodies to myosin kinase (Fig. 4*C*). The staining pattern obtained with the kinase antibodies was unaffected when the myosin light chain kinase antibodies were adsorbed with the smooth muscle proteins actin, myosin, tropomyosin, or α-actinin prior to staining cells. The staining pattern was abolished when the antibodies were adsorbed with purified turkey gizzard myosin light chain kinase prior to staining. These data show that myosin light chain kinase contains the antigenic determinants recognized by the purified antibodies.

The distribution of myosin light chain kinase was also compared with the distribution of actin, tropomyosin, myosin, and α-actinin in gerbil fibroma cells. Double-label immunofluorescence studies confirmed the presence of both actin and myosin light chain kinase in stress fibers (data not shown). The distribution of myosin kinase was also compared with that of tropomyosin as tropomyosin is known to be distributed periodically along stress fibers in a variety of cells (28, 29, 34, 35). Double-label immunofluorescence studies, using fluorescein- and rhodamine-labeled antibodies to visualize the primary antibodies, indicated that tropomyosin and myosin kinase are distributed with the same periodicity along stress fibers (Fig. 5).

We then compared the distribution of myosin kinase with that of α-actinin and myosin as these proteins have an alternating distribution along stress fibers (28, 29, 34, 35). Fig 6*A* shows that rabbit antibodies to smooth muscle α-actinin have a periodic distribution along the stress fibers of gerbil fibroma cells. When these cells were stained simultaneously with myosin light chain kinase and α-actinin antibodies and visualized with fluorescein-labeled goat anti-rabbit IgG, there was continuous staining of the stress fibers (Fig 6*B*). Fig. 6*C* shows that antibodies to myosin also have a periodic distribution along the stress fibers. However, in contrast to the findings with α-actinin antibodies, when gerbil fibroma cells were stained with myosin light chain kinase and myosin antibodies simultaneously, the periodicities along the stress fibers remained prominent (Fig 6*D*). These results are consistent with a close correspondence of distribution for myosin and myosin light chain kinase and an antiperiodic distribution for α-actin and myosin light chain kinase.

DISCUSSION

We have used indirect immunofluorescence to study the distribution of myosin light chain kinase in several different types of fibroblasts. The myosin kinase antibodies used were produced by immunizing rabbits with myosin light chain kinase purified from turkey gizzard smooth muscle. The rationale for using this myosin light chain kinase to produce antibodies was as follows. (*i*) It is possible to obtain a large amount of myosin light chain kinase from turkey gizzards; this permitted us to make a myosin light chain kinase-Sepharose 4B column to affinity purify the kinase antibodies. (*ii*) Structural and functional similarities between myosin kinases in smooth muscle and nonmuscle cells led us to believe that they might share common antigenic determinants (20–25).

The purified antibodies were first characterized before they

were used to perform immunofluorescence experiments. Immunodiffusion experiments demonstrated that the purified antibodies crossreacted with purified myosin light chain kinase but did not crossreact with a variety of other proteins (Fig. 1). The purified antibodies were also found to inhibit the activity of purified myosin light chain kinase (Figs. 2 and 3). The indirect immunofluorescence studies demonstrated that myosin light chain kinase is a component of stress fibers (microfilament bundles) of fibroblasts (Figs. 4 and 5).

It should be noted that, although the immunofluorescence studies reported here demonstrate that the stress fibers represent the most prominent localization of myosin light chain kinase in fibroblasts, they do not exclude the possibility that a significant portion of the myosin light chain kinase may be located on other cellular structures or distributed throughout the cytoplasm. In fact, Fig. 4*C* indicates the presence of diffuse low-intensity fluorescence distributed throughout the cytoplasm of chicken embryo fibroblasts. It is possible that this cytoplasmic distribution is detected most easily in chicken embryo fibroblasts because of the increased crossreactivity between the antibodies produced to an avian antigen (turkey gizzard myosin kinase) and the chicken myosin kinase.

The presence of various proteins on stress fibers makes it important to know whether the myosin light chain kinase is associated directly with the myosin or with some other protein on the stress fiber. We have used gerbil fibroma cells as an experimental model to distinguish between these two possibilities. Double-label immunofluorescence studies demonstrated that tropomyosin and myosin kinase have similar, if not identical, distributions along stress fibers (Fig. 5). Previous work has shown that tropomyosin and myosin have an identical periodic distribution along stress fibers in fibroblasts (35). Thus, the data in Fig. 5 offer indirect evidence for the identical localization of myosin and myosin kinase on stress fibers.

A more direct experiment would be to carry out double-label immunofluorescence studies comparing the distribution of myosin kinase with that of myosin and α-actinin. This was not possible as the antibodies to all these proteins were produced in rabbits. However, staining cells with antibodies to myosin and myosin kinase simultaneously did not eliminate the periodicities (Fig. 6*D*) seen when cells are stained with myosin (Fig. 6*C*) or myosin kinase (Fig. 4*C*) antibodies, individually. In contrast, myosin and α-actinin are distributed with a counterperiodicity in fibroblasts (28), as are myosin kinase and α-actinin (Fig. 6*B*). These findings suggest that myosin and myosin kinase have similar distributions, possibly in register, in these cells. Additional studies, including experiments using double-label immunofluorescence techniques, would be helpful to confirm these results. It will be particularly important to determine whether the periodic distribution of myosin kinase in gerbil fibroma cells can be demonstrated in other cell types. Nevertheless, these data imply that myosin light chain kinase is associated with its substrate, myosin, on stress fibers in gerbil fibroma cells and are consistent with the hypothesis that myosin light chain kinase regulates actin–myosin interactions in nonmuscle cells. The availability of a functional antibody to myosin kinase will also permit studies on the role of myosin phosphorylation in mediating cellular functions of nonmuscle cells.

We wish to thank Drs. Mary D. Pato and James Sellers of the National Institutes of Health for providing us with purified smooth muscle tropomyosin and smooth muscle actin, respectively; Drs. Stephen Blose and James Lin of Cold Spring Harbor Laboratory for providing us with purified vimentin and a mouse monoclonal antibody to actin; and Dr. Edmond Fisher of the University of Washington for providing us with the catalytic subunit of cAMP-dependent protein kinase. We also thank J. D. Watson for his enthusiastic support of this work. Ted Lukralle provided valuable photographic assistance. Part of this work was supported by National Institutes of Health Grants GM 27781 (to K.B.) and GM 28277 (to J.R.F.).

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