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Journal
Journal of Cell Biology, 95(3)

ISSN
0021-9525

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Publication Date
1982-12-01

DOI
10.1083/jcb.95.3.793

Peer reviewed

Association of Myosin Light Chain Kinase with Lymphocyte Membrane—Cytoskeleton Complex

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ABSTRACT A specific antibody against myosin light chain kinase (MLCK) was used to identify the presence of a Ca^{2+}-calmodulin-activated MLCK in mouse T-lymphoma cells. With a double immunofluorescence technique, MLCK was determined to be accumulated directly under Con A-capped structures in a manner similar to that of previously described accumulation of actomyosin. The lymphocyte MLCK was phosphorylated in the uncapped cell and, by immunoprecipitation with a specific MLCK antibody, was shown to possess a M_r of 130,000. The MLCK was also found to constitute a major fraction of the phosphoproteins present in the plasma membrane associated-cytoskeleton. Myosin light chain kinase catalyzed the phosphorylation of both endogenous lymphocyte myosin light chains and those from smooth and skeletal muscle. The enzyme activity was dependent on the presence of Ca^{2+}-calmodulin and was inhibited by the calmodulin-binding drug, trifluoperazine. These data suggest that the membrane-cytoskeleton-associated MLCK activity may be important in regulation of the actin-myosin contraction which is believed to be required for the collection of surface receptors into capped structures.

Lymphocyte surface receptors will aggregate into a cap structure at one pole of the cell in an energy- and temperature-dependent manner upon the addition of external multivalent ligands such as lectins or antibodies (32, 33). Cytochemically, it has been shown that intracellular actin and myosin are always concentrated underneath the cap (4–6). Several research groups have detected biochemically the existence of an actomyosin-containing cytoskeleton in the receptor-cap structures (12, 16, 23). Therefore, it has been proposed that transmembrane linkages occur between the actomyosin filaments and the surface receptors that are required for the clustering of the surface receptors into caps via a sliding filament mechanism analogous to that occurring during muscle contraction (4).

Studies on both smooth muscle and nonmuscle cells indicate that actomyosin-mediated contraction is regulated by myosin light chain kinase which in turn is regulated by Ca^{2+}-calmodulin (3, 14). In addition, the phosphorylation of myosin light chain has been shown to enhance the actin-activated Mg^{++}-ATPase of myosin which produces the energy to drive the sliding filament contraction process (1, 30). In what might be an analogous mechanism, the 20,000-dalton myosin light chain in lymphocytes is phosphorylated during surface receptor capping (9). It has also been shown recently that the capping process can be inhibited (7) by a drug, trifluoperazine (TFP or Stelazine), that binds to calmodulin in a Ca^{2+}-dependent manner. Finally, the process is also directly regulated in vitro by micromolar concentrations of Ca^{2+} ion (10). Together, these observations support the contention that this nonmuscle contractile process is myosin light chain kinase-dependent (3).

Using an immunocytochemical method, we found that myosin light chain kinase (MLCK) is accumulated preferentially under Concanavalin A (Con A)–induced caps. The lymphocyte light chain kinase (MLCK) is associated with the plasma membrane-derived actomyosin-containing cytoskeleton. The identity of this enzyme has been assured by immunoprecipitation of a ^{32}P-labeled 130,000-M_r protein with a specific MLCK antibody and the capability of the enzyme to catalyse the calmodulin-dependent phosphorylation of endogenous lymphocyte, as well as exogenous smooth and skeletal muscle myosin light chains. Consequently, we are proposing that the MLCK-mediated phosphorylation of myosin light chain in response to Ca^{2+}-calmodulin is one mechanism responsible for the activation of contractile elements required for the aggregation of receptors into capped structures.

THE JOURNAL OF CELL BIOLOGY · VOLUME 95 DECEMBER 1982 793–797

MATERIALS AND METHODS

Cells

The mouse T-lymphoma cell line, AKR/J lymphoma cell line BW5147 (gift from Dr. R. Hyman, The Salk Institute), was grown in Dulbecco's modified Eagle's (DME) medium supplemented with 10% heat-inactivated horse serum (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) at 37°C in 5% CO_2/95% air.

Antibodies

Rabbit anti-chicken gizzard MLCK and rabbit anti-human uterus smooth muscle myosin were prepared according to the procedures described previously (19, 31).

Induction of Capping by Fluorescein-conjugated Concanavalin A (Fl-Con A)

Cells stained for their initial surface Con-A distribution were first washed with DME and fixed with 2% paraformaldehyde in 0.1 M PBS (pH 7.4) for 30 min at 0°C. Both prefixed and unfixed cell suspensions (~1 × 10^7 cells/ml) were incubated with Fl-Con A (50 μg/ml) at 0°C for 30 min. Unbound Fl-Con A was subsequently removed by rinsing, and cells were incubated at room temperature for 15 min. In the case of prefixed cells, Con-A receptors were immobilized (Fig. 1 *a*), and unfixed cells displayed a typical cap structure (Fig. 1 *c*).

Drug Effect on Surface Con A Capping

Unfixed cell suspensions (1 × 10^7 cells/ml) were preincubated with 5 × 10^{-5} M of trifluoperazine dihydrochloride (TFP) (Stelazine, Smith Kline and French Laboratories, Philadelphia, PA) (25, 26) for 30 min at room temperature. Drug-treated cells were subjected to the induction of Con-A capping as described above.

Double Immunofluorescence Microscopy

Both capped and uncapped cells were rendered permeable by using either frozen sections (5) or methanol/acetone treatment (11) and stained with rabbit anti-chicken gizzard MLCK (100 μg/ml) followed by rhodamine-conjugated goat antibody against rabbit immunoglobulin. As a control, preabsorbed anti-MLCK-free immunoglobulin was used instead of anti-MLCK antibodies. No MLCK staining was detected in the control samples.

The flourescein- and rhodamine-labeled samples were examined in a Zeiss photomicroscope with a × 40 oil immersion lens and an epiilluminator. Fluorescein and rhodamine fluorescences were excited with an Osram HBO 50-W bulb using filter combination CZ487710 and CZ 487714, respectively. Cells were photographed with Kodak Plus-x film.

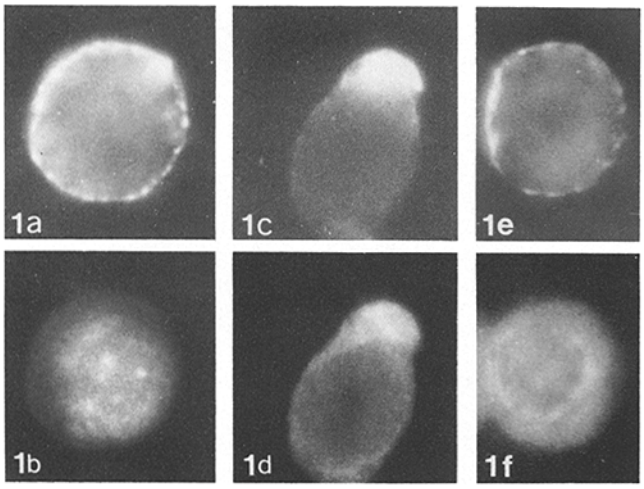

FIGURE 1 Simultaneous localization of surface Con-A receptors and intracellular MLCK. (*a–b*) Prefixed cells stained with Fl-Con A (*a*) followed by either frozen thick sectioning or acetone treatment and rabbit anti-MLCK plus rhodamine (Rh)-goat anti-rabbit immunoglobulin. *a* shows Fl-Con A staining of the same cell stained for MLCK in *b*. (*c–d*) Unfixed cells were induced to form cap by treatment with Fl-Con A (*c*) followed by staining with rabbit anti-MLCK plus Rh-goat anti-rabbit immunoglobulin as described above (*d*). *c* shows the Con A cap on the same cell stained for MLCK as shown in *d*. (*e–f*) In the presence of TFP (Stelazine), cells were treated with Fl-Con A (*e*) followed by labeling with rabbit anti-MLCK and Rh-goat anti-rabbit immunoglobulin (*f*) as mentioned above. *e* represents the Fl-Con A staining on the same cell labeled for MLCK as shown in *f*. (*a–f*) × 1,500.

Radioactive Labeling for Cellular Phosphoproteins

Cell suspensions (1 × 10^6 cells/ml) were washed with phosphate-free DME and labeled with 0.1 mCi ^{32}P ($H_3{}^{32}PO_4$, carrier free, ICN) for 30 min at room temperature. Phosphorylation was terminated by adding an excess (10 vol) of unlabeled 0.1 M PBS at 0°C.

Plasma Membrane Isolation

Plasma membrane fractions were isolated by the method described by Johnson and Bourne (20). ^{32}P-labeled cells were washed twice with Dulbecco's phosphate-buffer medium and resuspended at a density of 10^7 cells/ml in 20 mM HEPES, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.4, at 4°C. Cells were disrupted with a Dounce homogenizer. The lysate was centrifuged at 750 g_{av} for 5 min and the supernatant fluid was centrifuged in an SW50.1 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 43,000 g_{av} for 20 min. The pellet was resuspended in 10% sucrose (wt/wt) in the same buffer and centrifuged in a Beckman SW 50.1 rotor at 100,000 g_{av} for 80 min. The plasma membrane fraction, which sediments between 30% and 40% sucrose, was collected and washed with Dulbecco's PBS. Surface markers such as T200, Thy-1, and glycoprotein (gp) 69/71 have been reported to be preferentially concentrated in this particular plasma membrane fraction by immunoprecipitation techniques (8).

Nonidet P-40 (NP-40) Extraction

The isolated plasma membranes (with or without ^{32}P-labeling) were first resuspended in PPI solution (10 mM sodium phosphate buffer, pH 7.2, containing 1 mM phenylmethylsulphonyl fluoride (PMSF), and 10 mM iodoacetamide). Nonionic detergent extraction was carried out according to the procedures described previously (27) by adding 1% NP-40 to the plasma membrane preparation in PPI solution with vigorous vortexing. Protein concentration was held constant at 0.5 mg/ml. The samples were incubated at 0°C for 20 min with frequent vortexing and then centrifuged for 45 min at 100,000 g_{av}. After centrifugation, the pellets (i.e., NP-40-insoluble fraction) were collected for further biochemical and cytochemical analysis.

Immunoprecipitation

^{32}P-labeled total plasma membranes or cytoskeletal fractions isolated from uncapped cells (1 × 10^9 cells) were incubated with 0.1% NP-40 in 10 mM Tris-HCl, pH 8.0, at 4°C for 30 min. The NP-40-treated membrane or cytoskeletal fractions were then incubated with either rabbit anti-MLCK or anti-MLCK-free (preabsorbed) antisera (100 μg/ml) at 4°C for 30 min. Subsequently, goat anti-rabbit immunoglobulin (200 μg/ml) was added to the samples at 4°C for 30 min to precipitate the immune complex. The immunoprecipitates were obtained by centrifugation at 12,000 g_{av} for 10 min. Each precipitate was washed with PBS three times using the same centrifugation procedure. The polypeptide composition and radioactivity of the immunoprecipitates were determined by SDS PAGE and autoradiography, respectively.

SDS PAGE and Radioactivity Analysis

Electrophoresis was carried out on slab gels using the discontinuous buffer system described by Laemmli (24) and an exponential polyacrylamide gel gradient (7.0–17.5%) (21). All radioactively labeled samples (including total plasma membranes, NP-40 insoluble fractions of plasma membranes isolated from ^{32}P-labeled cells and ^{32}P-labeled immunoprecipitates) were dissolved in a buffer containing 2% SDS, 0.1 M dithiothreitol, 0.003% bromophenol blue, 20 mM Tris-HCl, pH 6.8, and heated at 100°C for 2 min. Approximately 125 μg of protein per sample was applied to the gel. Electrophoresis was carried out at a constant current of 2 mA for 18 h at room temperature, and the polypeptide banding pattern was revealed by staining with Coomassie Blue. The gels were then dried by vacuum, and the ^{32}P-labeled dried gels were exposed to Kodak x-ray (X-Omat, XR-1) film.

Assay of MLCK Activity

The MLCK-assay procedures used in this study were generally the same as those described previously (19). The reaction mixture contained crude enzyme preparations (such as total lymphoma plasma membranes or NP-40 insoluble cytoskeletal material), 20 mM KCl, 1 mM Mg acetate, 10^{-4} M Ca^{2+} (pCa = $-\log_{10}10^{-4} = 4.0$, high Ca^{2+}), with or without the addition of myosin light chains (e.g., 18 mg/ml chicken gizzard myosin light chains or skeletal myosin light chains), 5 μM calmodulin, and 1 mM γ-^{32}P-ATP (100–200 cpm/pmol). As controls, calmodulin was eliminated and 10^{-4} M Ca^{2+} (pCa = 4.0, high Ca^{2+}) was replaced by 10^{-8} M Ca^{2+} (pCa = $-\log_{10}10^{-8} = 8.0$, low Ca^{2+}) in the reaction mixture. In some cases, the enzyme assays were carried out in the presence of 5×10^{-5} M trifluoperazine (TFP, Stelazine). Reactions were initiated by the addition of γ-^{32}P-ATP (ICN Chemical and Radioisotope Division, Irvine, CA) and incubated for 5 min at 30°C. Myosin light chain phosphorylation was assayed by the following three methods: (*a*) spotting 0.005 ml of reaction mixture on Whatman 31 ET chromatography paper followed by 10% trichloroacetic acid (TCA) rinses as described previously (19); (*b*) analyzing the reaction mixture with SDS PAGE, cutting out and determining the radioactive content of the bands corresponding to myosin light chain (20,000 M_r); (*c*) immunoprecipitating the reaction mixture with rabbit anti-human uterus myosin antiserum (31) which has been previously determined to cross-react with lymphocyte myosin light chain (9). Radioactivity was determined in each case by scintillation spectrometry following solubilization of the samples in Aquasol (New England Nuclear, Boston, MA).

Negative Staining Procedures for Electron Microscopy

For negative staining, three successive drops of either a solution of 0.1 M KCl, 0.01 immidazole, pH 7.0, containing intact plasma membranes or a 1% NP-40 insoluble fraction of the plasma membranes from cells were added to 4% Formvar-coated grids. Then three drops of 1% uranyl acetate in water (pH 4.5) were applied to the samples for 30 s and removed with filter paper (18). These negatively stained samples were examined in a Philips 300 electron microscope.

RESULTS

Double immunofluorescence staining of *prefixed* lymphocytes reveals that the surface Con-A receptors are generally distributed uniformly over the surface of the cells (Fig. 1 *a*) while intracellular myosin light chain kinase is diffusely distributed throughout the cytoplasm (Fig. 1 *b*) under the conditions used. However, when *unfixed* cells were induced to form capped structures (Fig. 1 *c*), the intracellular MLCK was found to be concentrated under the Con-A cap (Fig. 1 *d*). If trifluoperazine was added to cells during the capping reaction, then both the Con-A surface receptors (Fig. 1 *e*) and the intracellular MLCK (Fig. 1 *f*) remained uniformly distributed. The results shown in Fig. 1 support the contention that calmodulin may be related to the capping process (10, 22) and suggest that this regulatory molecule and MLCK may be associated with the actomyosin-containing cytoskeleton required for Con-A capping.

Fig. 2 *a* shows a micrograph of the plasma membrane fraction used in this study. The fraction contained vesicles of various sizes but no visual contamination by other membranous components such as those from nuclei, mitochondria, or rough endoplasmic reticulum was apparent. In addition, assays for Na^+/K^+ ATPase and 5′-nucleotidase revealed that the preparation was enriched for plasma membranes by 20-fold. Analysis

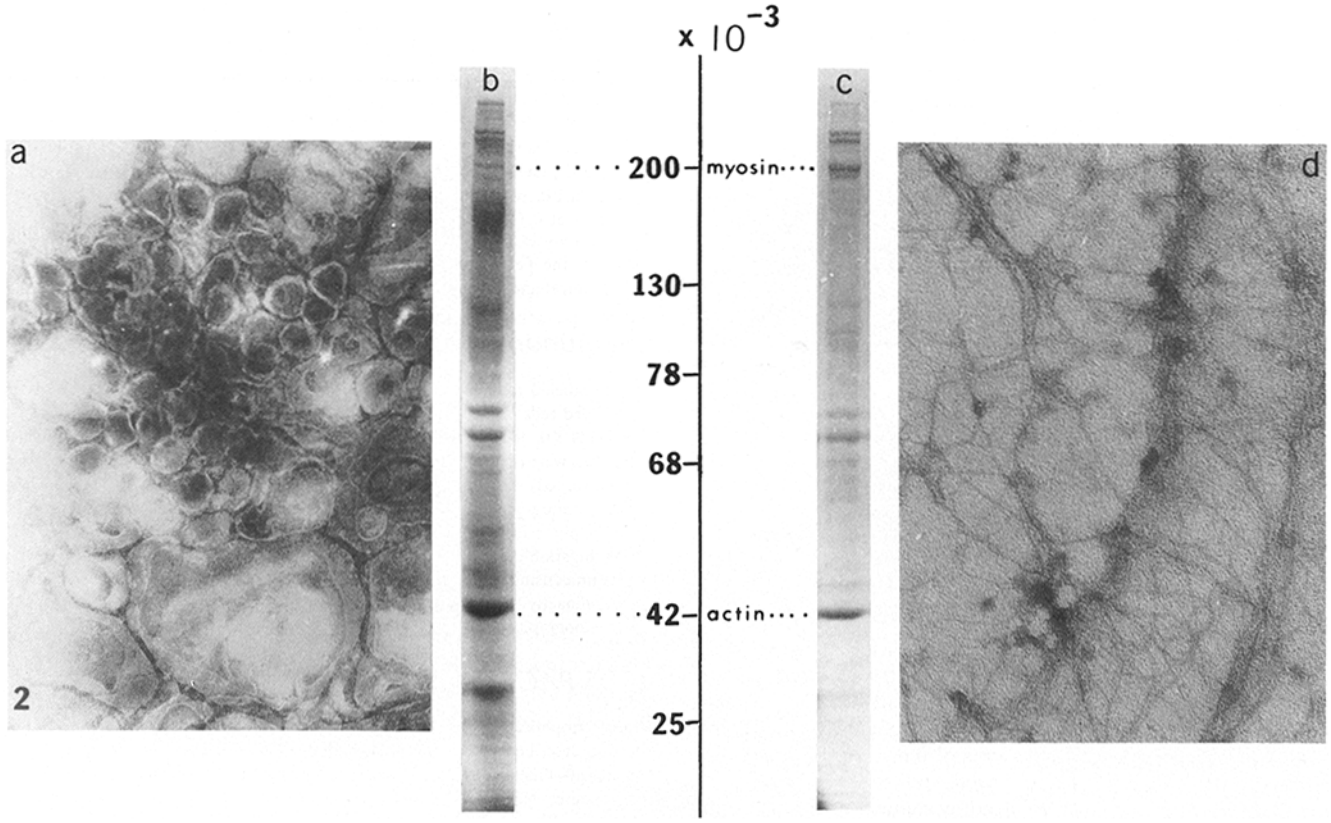

FIGURE 2 Negative staining and SDS PAGE analysis of plasma membranes and NP-40 insoluble fraction of the plasma membranes from mouse T-lymphoma cells. (*a*) Isolated total plasma membranes without NP-40 treatment stained with uranyl acetate. × 20,000. (*b*) Polypeptide composition of total plasma membranes stained by Coomassie Blue. (*c*) Polypeptide composition of NP-40 insoluble (cytoskeleton) fraction of plasma membranes stained by Coomassie-Blue staining (myosin and actin together with a couple of other polypeptides are the major components in this cytoskeleton material). (*d*) NP-40 insoluble fractions cytoskeleton of the plasma membrane stained with uranyl acetate. × 40,000. Molecular weight markers used in the study are in daltons: myosin, 200,000; β-galactosidase, 130,000; lactoperoxidase, 78,000; BSA, 68,000; actin, 42,000; Con A, 25,000.

of this fraction by gel electrophoresis (Fig. 2*b*) revealed at least 30 bands detectable by Coomassie-Blue staining. An electron micrograph of the membrane-associated cytoskeletal complex (prepared by extraction of the plasma membranes with NP-40 followed by centrifugation at 100,000 g_{av}) is shown in Fig. 2*d*. This fraction is composed primarily of 50–70-Å actinlike filament bundles (Fig. 2*d*). Indeed, the polypeptide composition of this fraction appears to be less complex (Fig. 2*c*). In this case, the major proteins are actin (42,000), myosin heavy chain (200,000), and a doublet at 240,000–260,000 which presumably represents the subunits of the spectrinlike proteins described by Glenney et al. (17).

MLCK has been identified as a 130,000-dalton polypeptide in several different type of cells (3, 19) and can be phosphorylated (2). Fig. 3 reveals an analysis of the protein components of both membrane and cytoskeletal fractions by autoradiography after metabolic labeling of lymphocytes with $^{32}PO_4$. At least 12 major phosphorylated polypeptides are evident in the total plasma membrane fraction (Fig. 3*a*), but only four major phosphorylated polypeptides (at 220,000, 180,000, 130,000, and 36,000) are present in the cytoskeletal fraction (Fig. 3*b*). The 130,000 protein in the plasma membrane has been identified as a phosphorylated form of MLCK by immunoprecipitation (Fig. 3*c*) with a specific MLCK antibody. Similar results were obtained with the cytoskeleton fraction (data not shown). The specificity of the immunoprecipitation was established by the fact that an antiserum preabsorbed with MLCK precipitated

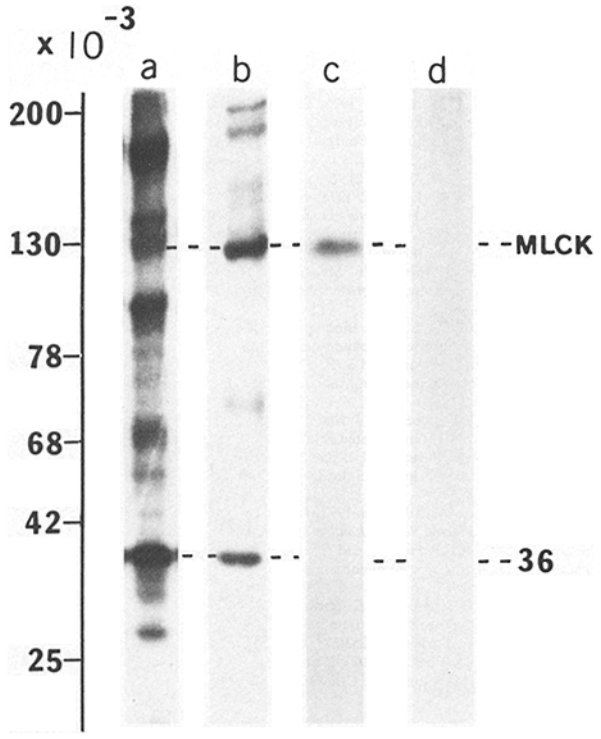

FIGURE 3 Autoradiograms of ^{32}P-labeled proteins from mouse T-lymphoma cells. Cells were metabolically labeled with carrier free $H_3{}^{32}PO_4$ as described in Materials and Methods. (*a*) Total plasma membrane (in the absence of NP-40). (*b*) NP-40 insoluble (cytoskeleton) fraction of plasma membranes. (*c*) Anti-MLCK immunoprecipitable from plasma membrane (similar results were obtained with the cytoskeleton fraction.) (*d*) Anti-MLCK-free (preabsorbed reagent)/(nonspecific) immunoprecipitates from plasma membrane (similar results were obtained with cytoskeleton fraction). Molecular weight markers used in this study were the same as those described in Fig. 2.

TABLE I

Assays for MLCK Activity

Substrates	Conditions	pmol ^{32}P-incorporated/min/mg MLC
Skeletal muscle		
	pCa 8.0	100
	pCa 4.0 + calmodulin	302
	pCa 4.0 + TFP	85
Smooth muscle		
	pCa 8.0	106
	pCa 4.0 + calmodulin	406
	pCa 4.0 + TFP	100
Lymphocytes		
	pCa 8.0	186
	pCa 4.0 + calmodulin	809
	pCa 4.0 + TFP	70

Enzyme reactions were carried out under various conditions such as (*a*) low Ca^{2+} (pCa = 8.0, 10^{-8} M, Ca^{2+}); or (*b*) high Ca^{2+} (pCa = 4.0, 10^{-4} M, Ca^{2+}) plus calomodulin; or (*c*) in the presence of 5×10^{-5} M trifluoperazine (TFP) (Stelazine) and high Ca^{2+} (pCa = 4.0, 10^{-4} M, Ca^{2+}) using NP-40 insoluble (cytoskeleton) fraction from mouse T-lymphoma cells as crude MLCK sources. Reactions were initiated by the addition of γ-^{32}P-ATP and incubated for 5 min at 30°C. The ability of lymphocyte MLCK to phosphorylate skeletal muscle, smooth muscle myosin light chains, and lymphocyte myosin light chain was measured according to the procedures described in Materials and Methods. In this particular case, the level of myosin light chain phosphorylation was assayed by immunoprecipitating the MLCK reaction product with rabbit antimyosin followed by SDS PAGE analysis. The bands corresponding to myosin light chain (20,000 daltons) were cut out and radioactivity was determined using Aquasol and a liquid scintillation counter as described previously (9). Each number represents the average of three experiments with standard deviation less than ±5%.

neither the 130,000 protein nor any of the other phosphorylated proteins (Fig. 3*d*).

Further confirmation of the presence of MLCK was obtained by carrying out functional assays for the enzyme as described in Materials and Methods. Table I shows that membrane-cytoskeletal fractions contain a Ca^{2+}-calmodulin-activated (trifluoperazine-sensitive) MLCK activity. The enzyme is capable of phosphorylation of the endogenous lymphocyte light chains (Table I) as well as that of exogenously added light chains from smooth muscle (Table I) and skeletal muscle (Table I). The MLCK activity appears to be approximately 2–2.5-fold less when assayed with the exogenous myosin light chain as substrate. Two possible explanations for this reduction in activity could be either that the exogenous substrates are phosphorylated and/or immunoprecipitated less efficiently than the endogenous lymphocyte myosin light chains or that the exogenous substrate is competing with the endogenous substrate. However, regardless of the source of substrate, the specific activity of this enzyme is seven- to eightfold greater in the cytoskeletal fraction as compared to the total plasma membrane (data not shown). This enrichment of MLCK activity in the actomyosin complex provides additional evidence for the involvement of this enzyme in the regulation of lymphocyte receptor capping.

DISCUSSION

Both cytochemical and biochemical data now indicate that actomyosin-containing structures are closely associated with cap formation (4–6, 12, 16, 23). Such observations are compatible with the possibility that the force-generating mechanisms involved may be similar to those that occur during other motile events in smooth and nonmuscle cells (14). These mechanisms involve the activation of actomyosin ATPase by the phospho-

rylation of the 20,000 M_r myosin light chain (1, 30). This activation is catalyzed by MLCK in a manner that requires Ca^{2+}-calmodulin (29). Intracellular concentrations of Ca^{2+}, calmodulin and ATP are all required for the capping of Con-A-receptors in lymphocytes (10). Calmodulin has been shown to co-cap with Con-A receptors (28) and myosin light chain phosphorylation occurs during the capping event (9). We report here that the final component of the putative contractile system, the MLCK, also co-caps with Con-A receptors and is, therefore, in the proper locale to catalyze the generation of force.

MLCK was demonstrated to represent a component of the insoluble cytoskeletal network prepared by detergent treatment of lymphocyte plasma membranes. The enzyme was phosphorylated metabolically in uncapped cells and immunoprecipitated by an antibody prepared against chick gizzard MLCK (19). For now the regulatory mechanism involved in the phosphorylation of MLCK in intact lymphocytes is unknown. Consequently, under these conditions, the light chain of myosin does not appear to be phosphorylated as analyzed by autoradiographic method (Fig. 3). These results are consistent with the demonstration that phosphorylation of the kinase lowers the activity of the enzyme (13). The activity of the enzyme was demonstrated using both endogenous lymphocyte myosin light chains and exogenously supplied substrate isolated from both smooth and skeletal muscle. The MLCK activity was both Ca^{2+} and calmodulin dependent and was inhibited by the calmodulin-binding drug, trifluoperazine. By activity measurement, MLCK is enriched in the cytoskeletal preparation seven- to eightfold compared to the plasma membrane. In addition to MLCK, the 50–70-Å filaments in the insoluble cytoskeletal material contained actin, myosin, and two high M_r proteins (240,000–260,000) that presumably represent the spectrinlike molecules described by Glenney et al. (17). These proteins bind both actin and calmodulin and may therefore be involved in the cross-linking of specific components involved in cap formation. The energy required for such interactions could also be derived from actomyosin-catalyzed ATP hydrolysis in response to calmodulin-activated MLCK. Further evidence in support of the regulatory role of MLCK in Con-A cap formation is presented elsewhere (22).

We are grateful for Dr. Gerard J. Bourguignon's assistance in the preparation of the manuscript.

This work was supported by U.S. Public Health grant AI 19188 (L. Y. W. Bourguignon).

Received for publication 29 June 1982, and in revised form 1 September 1982.

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