Phosphorylation of Lymphocyte Myosin Catalyzed in Vitro and in Intact Cells

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ABSTRACT Myosin has been isolated from guinea pig B-lymphocytic leukemia cells (L2C). The myosin has been enzymatically phosphorylated and dephosphorylated in vitro using both heterologous and lymphocyte-derived enzymes. Both the heavy chain and 20,000-dalton light chain of lymphocyte myosin are phosphorylated in vitro. Phosphorylation of myosin enhances actin-activated ATPase activity.

Phosphorylation of myosin in murine lymphocytes was analyzed by use of a novel technique for rapid immunoprecipitation of myosin from cell extracts. Both the heavy chain and 20,000-dalton light chain of myosin are phosphorylated in intact cells. Addition of antibody reactive with cell-surface immunoglobulin to lymphocyte populations enriched for B cells stimulates locomotion of these cells and also increases the quantity of $^{32}$P isolated in association with the 20,000-dalton light chain of lymphocyte myosin, when $^{32}$P was present in the medium. In addition, an unidentified, phosphorylated polypeptide with a molecular mass of 22,000 daltons is co-isolated with myosin from cells by rapid immunoprecipitation. These results are consistent with the hypothesis that phosphorylation of myosin may contribute to regulation of movements performed by lymphocytes which are related to their participation in immunologic reactions.

Lymphocytes, the cells of the immune system having the ability to recognize and respond to the presence of foreign macromolecules, perform many movements that are related to their role in immunologic phenomena. To provide a foundation for understanding the molecular basis of these movements, we have isolated actin and myosin from lymphocytes (15). The properties of these proteins are quite similar to those of actin and myosin isolated from other cell types. Additional understanding of the role of actin and myosin in cell motility requires study of their localization in cells, the state of assembly of actin and myosin filaments, and the interaction of actin and myosin with each other and with accessory proteins of the contractile system.

It has been hypothesized that phosphorylation of myosin may contribute to regulation of contraction in a variety of smooth muscle and nonmuscle cells. The ATPase activity of myosin isolated from a number of cell types is enhanced on addition of actin if the 20,000-dalton light chain of that myosin is phosphorylated (1, 36, 37, 40, 41). Further, phosphorylation of the 20,000-dalton light chain of myosin prevents disassociation of thick filaments upon addition of ATP (34, 39). Observations of intact cells are consistent with those made in the in vitro studies. It has been shown that hormonal or electrical stimulation of smooth muscle cells and stimulation of platelets with thrombin results in an increase in phosphorylation of the 20,000-dalton light chain of myosin (4, 5, 12, 13, 21, 27).

More recently, phosphorylation of the heavy chain of myosin has also been described (33). Phosphorylation of the heavy chain of myosin from Dicyostelium discoideum results in a decrease in the ATPase activity of the myosin in the presence of actin and an even more pronounced decrease in its ability to assemble into filaments (23, 30). In contrast, phosphorylation of the heavy chain is required for actin activation of the ATPase activity of the unusual myosin I isolated from Acanthamoeba castellanii (28).

We now report that phosphorylation of lymphocyte myosin results in an increase in its ATPase activity in the presence of actin in vitro, and that stimulation of B lymphocytes with antibody reactive with cell-surface immunoglobulin results in an increase in radiolabeled phosphate isolated in association with the 20,000-dalton light chain of lymphocyte myosin. In addition, phosphorylation of the heavy chain of lymphocyte myosin is observed both in vitro and in intact cells.

MATERIALS AND METHODS

Chemicals

Acrylamide and bis-acrylamide were obtained from Eastman Chemical Co. (Rochester, NY); SDS was from Bio-Rad Laboratories (Richmond, CA); disc-
dium adenosine triphosphate (ATP), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), EGTA, imidazole, HEPES, p-nitrophenyl phosphate (PNP), prostaglandin, and β-mercaptoethanol were obtained from Sigma Chemical Co. (St. Louis, MO). Sepharose 4B was supplied by Pharmacia Fine Chemicals (Piscataway, NJ). Nonidet P-40 (NP-40) was obtained from BDH Chemicals, Ltd. (Poole, England), and 2,5-diphenyloxazole (PPO) was from National Diagnostics (Somerville, NJ). Ham's F-12 nutrient mixture, Iscove's medium, RPMI 1640, and Hank's balanced salt solution were obtained from Grand Island Biological Co. (Grand Island, NY). [3H]Leucine was obtained from Amersham Corp. (Arlington Heights, IL) and had a specific activity of 100 Ci/mmol.

Proteins

Horse serum and fetal calf serum were obtained from Grand Island Biological Co. Bovine serum albumin (BSA), bovine insulin, and human transferrin were supplied by Sigma Chemical Co. Rabbit skeletal muscle actin was purified as previously described (28), except that the actin was pelleted through 0.8 M KCl two times before dialysis to cause its depolymerization. Calmodulin was purified from bovine brain as previously described (14). The 20,000-dalon light chain of myosin was purified from glycinated chicken gizzard (Pel-Freez Biologicals, Rogers, AR), according to published procedures (2). Myosin light-chain kinase was purified from fresh turkey gizzard as described (2) and was the generous gift of R. S. Adelstein and M. A. Conti (National Institutes of Health (NIH), Bethesda, MD). Bovine cardiac phosphoprotein phospha-tase was the generous gift of Emily Noiman (NIH) and was purified as previously described (8). Antibodies with specificity for lymphocyte myosin were elicited by injection of a solution containing 1 mg of myosin, purified from guinea pig β-lymphocyte lymphoma (Lc5) cells (15), in complete Freund's adjuvant into the flanks and foot pads of a rabbit. A second injection of the same preparation was made 4 wk after the first. An IgG-containing fraction was prepared by addition of (NH4)2SO4 to 35% of saturation to the serum derived from blood collected 2–6 wk after the second immunization. Antibody was purified by absorption to a column of lymphocyte myosin coupled to Sepharose 4B (20), and eluted with 0.2 M proptic acid in 0.15 M NaCl. Affinity-purified goat antibody with specificity for the Fab fragment of murine immunoglobulin was prepared by Drs. R. Brunhouse and L. Gooding, and has been tested by immunodiffusion and radioimmunoassay.

Cells

Strains 2 and 13 guinea pigs were bred and raised in our own colonies. The Lc5 cell line was obtained from Dr. Berton Zbar (NIH). The cells were passaged in strain-2 and -13 guinea pigs and prepared for use as previously described (15). CB20 mice were obtained from Drs. E. Mushinski and M. Potter (NIH), and were bred and raised in our own colonies. Lymphocytes were teased from the mesenteric lymph nodes of these mice and washed in Hank's balanced salt solution containing 10% horse serum. Preparations enriched for B lymphocytes were obtained by lysis of T cells using monoclonal IgM antibody directed against Thy 1.2 (cell line HO-13-4-9 from the Salk Institute), and complement. Immunofluorescent staining of the surviving cells with a rhodamine-conjugated goat antibody directed against the Lyt-2 antigen on mouse immunoglobulin revealed that 80–90% of the cells bore surface immunoglobulin.

In preparation for the visual assay of cell motility, 5 × 10³ lymph-node cells, treated with anti-theta and complement as described above, are placed in T flasks (Costar, Cambridge, MA) in a volume of 5 ml of a 1:1 mixture of Ham's F-12 nutrient mixture and Iscove's medium, supplemented to 5 μg/ml bovine insulin, 5 μg/ml human transferrin, 5 × 10⁻⁸ M β-mercaptoethanol, 3 × 10⁻⁸ M prostaglandin, and 25 mM HEPES (Moser's medium). This medium was designed for study of B lymphocytes in vitro in the absence of serum (29). The cells are observed at 37°C Con an inverted phase-contrast microscope (Wild) equipped with an air curta (Sage Instruments Div., Orion Research Inc., Cambridge, MA). An individual cell is defined as motile if it either translocates or changes shape by pseudopod extension or retraction during a 1-min period of observation. In experiments designed to test the ability of a preparation of antibody reactive with cell-surface immunoglobulin to affect the motility of B cell-enriched lymphocyte populations, experimental and control flasks were coded and counted at 10-min intervals after addition of antibody by an individual unaware of their identity. Endogenous incorporation of amino acid into cellular protein was accomplished by culture for 12 h of 1 × 10⁶ cells in 10 ml of RPMI 1640 which contained 5% fetal calf serum that had been dialyzed vs. 0.15 M NaCl. In addition, the normal complement of leucine of the medium was omitted and replaced with 1 mM [3H]leucine. Cells were prepared for incorporation of 3H] by two washes in phosphate-free Mosier's medium, and preincubation at 37°C for 30 min to deplete intracellular phosphate pools. 2.5 × 10⁶ cells were suspended in 1 ml of that medium containing 0.25 or 0.5 mM [3H]leucine, and held at 37°C for 1 h.

Analytical Methods

**PREPARATION OF γ-[32P]ATP**. Labeled ATP was prepared by the enzymatic exchange method (17, 42). Glycerophosphate-dehydrogenase dehydrogenase, 3-phosphoglycerate kinase, and 3-phosphoglyceric acid were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). ATP was isolated from the reaction mixture by absorption to and elution from Norite, and was shown to be >98% radiochemically pure by paper electrophoresis.

**PROTEIN DETERMINATION**: Samples were prepared for protein determination by precipitation with TCA in the presence of deoxycholate (6), washed in 5% TCA at 4°C, and suspended in 1 ml of water. Protein was determined using BSA as the standard as previously described (18).

**ASSAY OF ATPase ACTIVITY**: Myosin ATPase activity was determined at 37°C in 0.5 ml of 0.5 M KCl, 2 mM EDTA, 10 mM imidazole, pH 7.0, and 1 mM γ-[32P]ATP bearing 1,000 cpm/nmol essentially as described (32). Actin-activated ATPase activity was measured at 37°C in a volume of 0.2 ml containing 20 mM KCI, 5 mM MgCl₂, 1 mM DTT, 10 mM Tris, pH 7.5 and 1 mM γ-[32P] ATP bearing 2,000 cpm/nmol. The concentration of myosin was 0.05–0.1 mg/ml, and the actin concentration was 0.5, or 1 mg/ml. Reproducible results were obtained by adding the actin in assay buffer to the myosin in 0.3 M KCl solution (see below), and dialyzing the mixture at 4°C vs. the assay buffer lacking ATP as previously described (40). [3H]Leucine was extracted into the organic phase was counted in Packard Insta-Gel in a Packard turbomitec-N counter. In all ATPase assays, released inorganic phosphate was determined immediately after addition of ATP, and at two subsequent times to ensure the linearity of the reaction.

**ASSAY OF KINASE ACTIVITY**: Myosin light-chain kinase activity was measured using the 20,000-dalon light chain of chicken gizzard myosin as phosphate acceptor (11). Assay mixtures contained 10 mM Tris, pH 7.5, 20 or 150 mM KCl, 0.5 mM DTT, 10 mM MgCl₂, either 0.2 mM CaCl₂ or 2 mM EGTA, 0.5 mM γ-[32P]ATP bearing 100 cpm/nmol, 40 μM 20,000-dalon light chain, and a putative source of kinase in a final volume of 0.1 ml in the presence or absence of 1 μM calmodulin. Phosphate incorporation was measured on duplicate samples with precipitation by TCA followed by Millipore filtration (11) (Millipore Corp., Bedford, MA), or by electrophoresis and autoradiography as described below.

**ASSAY OF PHOSPHATASE ACTIVITY**: Phosphatase activity in fractions obtained by chromatography of lymphocyte actomyosin on Sepharose 4B was determined by measuring the increase in optical density at a wavelength of 410 nm which occurs during hydrolysis of PNPP (16). Each assay mixture contained 0.1 ml of column fraction, and 2.4 ml of 2 mM PNPP, 4 mM MgCl₂, 10 mM Tris, pH 7.5, and was held at 37°C for 1–4 h. The ability of the lymphocyte-derived phosphatase detected by use of the PNPP substrate to cleave phosphate from phosphorylated lymphocyte myosin was determined as described below for measuring dephosphorylation of lymphocyte myosin with bovine heart phosphoprotein phosphatase.

**ELECTROPHORESIS, AUTORADIOGRAPHY, AND DENSITOMETRY**: Proteins were fractionated in gels of 10% polyacrylamide in the presence of SDS as previously described (24). Gels were dried on a slab gel drier (Bio-Rad Laboratories) in preparation for autoradiography. Gels containing proteins labeled with [3H]leucine were subjected to the fluorographic procedure of immersion in PPO before drying (7). Kodak X-Omat film was exposed to a brief pulse of light before autoradiographic exposure to increase the sensitivity of the latter exposure, and to make grain density proportional to the radioactivity present (25).

The quantity of radioactivity present in bands on polyacrylamide gels was determined by measuring the density of exposed silver grains on the film using a Joyce-Loebi microdensitometer. The relative quantity of radioactivity present in myosin samples obtained from cells receiving any experimental treatment is compared after normalization for the amount of myosin actually recovered. The quantity of myosin recovered is estimated by densitometric scanning of the heavy chain of the myosin samples following staining with Coomassie Blue.

Methods for Phosphorylation or Dephosphorylation of Lymphocyte Myosin

**PHOSPHORYLATION OF PURIFIED LYMPHOCYTE MYOSIN CATALYZED BY MYOSIN LIGHT-CHAIN KINASE PURIFIED FROM TURKEY GIZZARD**: Lymphocyte myosin was purified as described (15). A final volume of 4 ml was incubated at 37°C for 1 h in the presence or absence of calmodulin and purified light-chain kinase of turkey gizzard (MLCK). Mixtures contained 0.15 M KCl, 5 mM MgCl₂, 0.2 mM CaCl₂, and 0.33 mM γ-[32P]ATP bearing 850 cpm/nmol, 0.3 mg/ml myosin either in the presence or absence of 1 μM calmodulin and 3.7 μg/ml MLCK in a final volume of 2 ml. Incorporation of [32P] into protein reached a maximum after 1 h and remained at this level.

**PHOSPHORYLATION OF LYMPHOCYTE MYOSIN CATALYZED BY A LYMPHOCYTE KINASE**: Isolation and phosphorylation of myosin from the Lc₅ cell line was performed by a modification of the method employed for lamine
above. Preparation of the extract supernatant, the 35-55% (NH₄)₂SO₄ fraction, the actomyosin fraction, phosphorylation of myosin catalyzed by endogenous kinase present in the actomyosin fraction, and resolution of components comprising lymphocyte actomyosin by gel filtration chromatography on a column of Sepharose 4B were performed essentially as described (40). The eluate from Sepharose 4B was collected in 5-mL fractions and assayed for ATPase activity, radioactivity, kinase activity, and phosphatase activity. Contiguous fractions containing K-EDTA ATPase activity and radioactivity were pooled and simultaneously concentrated and dialyzed vs. 0.3 M KCl, 1 mM EDTA, and 15 mM Tris, pH 7.5.

**DEPHOSPHORYLATION OF LYMPHOCYTE MYOSIN**: Lymphocyte myosin that had been phosphorylated using an endogenous lymphocyte kinase was held at 37°C in 0.3 M KCl, 1 mM EDTA, 1 mM EDTA, and 15 mM Tris, pH 7.5.

**RESULTS**

**Phosphorylation of Lymphocyte Myosin Catalyzed by Myosin Light-chain Kinase from Turkey Gizzard**

Myosin was isolated from Lc cells as previously described (15). In order to determine whether the enzymatic activity of this myosin is affected by phosphorylation, the myosin was held at 22°C with γ-[³²P]ATP in the presence or absence of myosin light-chain kinase from turkey gizzard and calmodulin as described in Materials and Methods. Phosphate incorporation occurred at the rate of 10 nmol/min per mg of kinase, and 1.9 mol of phosphate were incorporated per mole of myosin. Almost all of the covalently bound ³²P was associated with the 20,000-dalton light chain, while a small amount was detected on the heavy chain. No phosphorylation of the 17,000-dalton light chain was found (Fig. 1 B). The incorporation of 0.08 mol of ³²P/mol of myosin in the absence of exogenous kinase indicated contamination of the myosin with endogenous kinase activity. The phosphate incorporated by this endogenous kinase is equally distributed between the heavy chain and the 20,000-dalton light chain of lymphocyte myosin (Fig. 1 A).

The ATPase activity of both the phosphorylated and control preparations of myosin was 260 nmol/min per mg in 0.5 M KCl and 2 mM EDTA. Phosphorylated myosin exhibited an ATPase activity of 58 nmol/min per mg at low ionic strength in the presence of MgCl₂ and 1 mg/mL of rabbit muscle actin. The specific ATPase activity of the control, non-phosphorylated myosin was 8 nmol/min per mg at low ionic strength in the presence of MgCl₂ and was not affected by addition of 1 mg/mL of rabbit skeletal muscle actin.

**Phosphorylation of Lymphocyte Myosin Catalyzed by an Endogenous Lymphocyte Kinase**

Additional information concerning the effect of phosphorylation of myosin on its behavior in vitro was obtained by isolation and characterization of lymphocyte myosin phosphorylated by an endogenous lymphocyte kinase. The procedure for preparation of phosphorylated myosin is described in Materials and Methods.

The elution profile of phosphorylated lymphocyte actomyosin from Sepharose 4B is shown in Fig. 2. Two indicators of the presence of myosin, ATPase activity and radioactivity elute in fairly constant proportion in one set of contiguous fractions.

Myosin obtained in this manner was found to contain 1.3-2.0 mol of ³²P/mol of myosin (average of six experiments was 1.6 mol of ³²P/mol of myosin). In every experiment, the 20,000-dalton light chain bore 80-90% of the phosphate label. The heavy chain of lymphocyte myosin was also phosphorylated in every experiment, while no radiolabel was ever observed to migrate in association with the 17,000-dalton light chain (Fig. 3 A and B).

Analysis of the ATPase activity of a typical preparation of this myosin is shown in Table I, Exp. 1. The activity is relatively high in the presence of 0.5 M KCl and 2 mM EDTA and is quite low at low ionic strength in the presence of MgCl₂. The latter activity is enhanced fivefold to a level of 34 nmol/min per mg in the presence of rabbit skeletal muscle actin.

To correlate the actin-activated ATPase activity of the myosin with the presence of covalently bound phosphate, we treated the phosphorylated myosin with a phosphoprotein phosphatase as described in Materials and Methods. Preliminary experiments indicated that 80-90% of the radiolabeled phosphate was released after 4 h at 37°C. Phosphorylated and dephosphorylated samples derived from the same myosin preparations exhibit similar ATPase activities at high ionic strength in the presence of EDTA and at low ionic strength in the presence of MgCl₂ (Table I, Exp. 2 and 3). In both experiments, the ATPase activity of phosphorylated myosin was...
FIGURE 2  Resolution of molecules comprising phosphorylated lymphocyte actomyosin by chromatography on a column of Sepharose 4B. Procedures used for chromatography and for determination of the quantity of radioactivity, ATPase activity, kinase activity, and phosphatase activity present in the eluate are described in Materials and Methods.

FIGURE 3 A comparison of the subunits of myosin purified from L2C cells with the polypeptides selected from extracts of murine lymphocytes by immunoprecipitation using antibody having specificity for myosin. Polypeptide components of the following samples were resolved by electrophoresis in gels of polyacrylamide in the presence of SDS: lanes A and B: purified myosin, which was labeled with $^{32}$P using a kinase present in the actomyosin fraction of guinea pig lymphocytes (L2C), is stained with Coomassie Blue (lane A), and detected by autoradiography (lane B); lane C: polypeptides were endogenously labeled with $[^3H]$leucine in murine lymphocytes and selected from extracts of these cells by rapid immunoprecipitation using antibody with specificity for guinea pig lymphocyte myosin. These polypeptides were detected by fluorography. Lane D: polypeptides were labeled with $^{32}$P in murine lymphocytes and selected from extracts of these cells by rapid immunoprecipitation using antibody with specificity for lymphocyte myosin. These polypeptides were detected by autoradiography. The abbreviations used to indicate the position of migration of the polypeptides are as follows: H, the heavy chain of myosin; L1 and L2, the 20,000- and 17,000-dalton light chains of myosin, respectively; M, a polypeptide co-isolated with myosin whose identity is not known.

Identification of Lymphocyte Kinase and Phosphatase That Recognize Lymphocyte Myosin as Substrate

The presence of a kinase in lymphocytes that can utilize myosin as a substrate was first noted by measuring phosphorylation of the 20,000-dalton light chain of chicken gizzard myosin that had been added to the actomyosin fraction of lymphocytes. This kinase activity was not affected by addition of 2 mM EGTA, or 2 mM CaCl$_2$ and calmodulin. Moreover, the quantity and distribution of phosphate incorporated into lymphocyte myosin in the actomyosin fraction by the endogenous kinase were not significantly affected by addition of calcium and calmodulin (data not shown). A kinase that recognized the 20,000-dalton light chain of chicken gizzard myosin was also identified in fractions obtained from chromatography of the actomyosin on Sepharose 4B. Kinase activity was found in contiguous fractions that elute with a $K_0$ of 0.67 (Fig. 2). The rate of phosphorylation of 20,000-dalton light chain from chicken gizzard by kinase present in the Sepharose 4B column fractions was also not affected by addition of CaCl$_2$ or EGTA (Fig. 4). A dependence on the presence of calcium and calmodulin was observed, as expected, in control experiments performed with purified myosin light-chain kinase from turkey gizzard, and the 20,000-dalton light chain of gizzard myosin as acceptor (data not shown).

Phosphatase activity present in the actomyosin fraction of lymphocytes, detected by use of the PNPP substrate, elutes from Sepharose 4B in contiguous fractions with a $K_0$ of 0.75 (Fig. 2). The ability of this phosphatase to dephosphorylate myosin previously phosphorylated using the endogenous kinase present in the actomyosin fraction of lymphocytes was measured as described in Materials and Methods. Addition of phosphatase to this substrate resulted in a release of phosphate that was linear with time during the first 8 h of hydrolysis. No release of phosphate was observed in a separate aliquot of the same myosin preparation to which the lymphocyte-derived phosphatase was not added. These results indicate that kinase and phosphatase activities with the potential to regulate the level of phosphorylation of myosin are present in lymphocytes.
with no preclearing step (preclearing refers to a preincubation at 5°C and D). It is likely that these radiolabeled polypeptides are observed only in the extracts which were not precleared (Fig. 5 Band C). Antibody with specificity for lymphocyte myosin was elicited in rabbits, and purified as described in Materials and Methods. Myosin purified in this manner bore radiolabeled phosphate on its heavy chain, on the 20,000-dalton light chain, and on the 22,000-dalton unidentified polypeptide (Fig. 3D). The heavy chain of myosin bears a larger fraction of the total radiolabeled phosphate incorporated in this preparation than does the heavy chain of myosin phosphorylated in the actomyosin fraction of L2C cells (Fig. 3Band D). No phosphorylation of actin, immunoglobulin, or the 17,000-dalton light chain of myosin was observed.

**Phosphorylation of Myosin in Intact Lymphocytes**

To extend and verify results obtained in in-vitro experiments, we examined the phosphorylation of myosin in intact lymphocytes. Antibody specific for the myosin was used to recover it from soluble extracts of cells so that the subunit polypeptides of myosin could be unambiguously identified after PAGE. Antibody with specificity for lymphocyte myosin was elicited in rabbits, and purified as described in Materials and Methods.

Preliminary analysis indicated that this purified antibody could react to form a single line of precipitation, when analyzed by immunodiffusion on plates coated with agarose, if myosin purified from L2C cells, or an extract of guinea pig or mouse lymphocytes, were used as antigen (data not shown). Myosin was successfully recovered by *conventional* immunoprecipitation (22) from cells labeled with [3H]leucine, but absolutely no radiolabeled polypeptides of any kind were present in immunoprecipitates prepared in the same manner from cells labeled with 32P, (data not shown, three experiments).

To test the hypothesis that the failure to observe phosphorylation of myosin in cells was due to dephosphorylation of the myosin during isolation, we employed the method for rapid immunoprecipitation of myosin described in Materials and Methods. The time required from cell lysis to addition of gel sample buffer to the immunoprecipitate was 10–15 min. An autoradiograph of material recovered from lymphocytes labeled with [3H]leucine and subjected to rapid immunoprecipitation is shown in Figs. 3C and 5. Immunoprecipitates prepared with no preclearing step (preclearing refers to a preincubation with staphylococci before addition of antimonyosin antibody) display seven major polypeptides (Fig. 5C). The 200,000-dalton heavy chain and 17,000- and 20,000-dalton light chains of myosin from murine lymphocytes were identified by comigration with lymphocyte myosin purified from L2C cells, and are observed only in precipitates prepared with antimonyosin antibody (Fig. 5B and C). A pair of radiolabeled bands located in positions characteristic of the heavy and light chains of the rabbit antimonyosin antibody, stained with Coomassie Blue, are observed only in the extracts which were not precleared (Fig. 5C and D). It is likely that these radiolabeled polypeptides are from murine immunoglobulin synthesized by the lymphocytes during the culture period and adsorbed onto the staphylococci from the cell lysate. A major band present only in precipitates prepared with antimonyosin antibody comigrates with rabbit muscle actin, and is presumed to be lymphocyte actin present due to incomplete disassociation of actomyosin by the pyrophosphate in the lysis buffer. Another polypeptide of ~22,000 daltons is consistently observed only in precipitates prepared with antimonyosin antibody, and is referred to in subsequent discussion as the unidentified polypeptide isolated in association with myosin (Fig. 5B and C).

This technique for rapid immunoprecipitation of myosin was then applied to lymphocytes enriched for B cells and labeled with 32P in phosphate-free Mosier's medium as described in Materials and Methods. Myosin purified in this manner bore radiolabeled phosphate on its heavy chain, on the 20,000-dalton light chain, and on the 22,000-dalton unidentified polypeptide (Fig. 3D). The heavy chain of myosin bears a larger fraction of the total radiolabeled phosphate incorporated in this preparation than does the heavy chain of myosin phosphorylated in the actomyosin fraction of L2C cells (Fig. 3Band D). No phosphorylation of actin, immunoglobulin, or the 17,000-dalton light chain of myosin was observed.

**FIGURE 4** The effect of calcium on the rate of phosphorylation of myosin light chain from chicken gizzard catalyzed by a partially purified kinase obtained from lymphocytes. The kinase was obtained by separation of the components present in lymphocyte actomyosin on a column of Sepharose 4B (Fig. 2). Phosphorylation of myosin light chain was determined after precipitation with TCA as described in Materials and Methods. Assays were in the presence of either 2 mM EGTA or 0.2 mM CaCl2. Calmodulin purified from bovine brain was present in all assays at a concentration of 1 μM. No incorporation is observed when the substrate, myosin light chain, is omitted. Each point is the average of duplicate determinations.

**FIGURE 5** Polypeptides present in immunoprecipitates derived from murine lymphocytes that had been endogenously labeled with [3H]leucine. The polypeptides were detected by fluorography after resolution in a gel of polyacrylamide in the presence of SDS. The immunoprecipitates whose components are displayed in lanes A–D were all prepared from the same extract of lymphocytes. Staphylococci were added to one-half of the extract, and removed before the actual precipitation (preclearing), whereas the other half of the extract was not absorbed in this way. Immunoprecipitates were selected from the absorbed and nonabsorbed extracts by addition of either affinity-purified antibody having specificity for lymphocyte myosin or an immunoglobulin fraction derived from an animal not deliberately immunized. The immunoprecipitates were prepared as described below: lane A, extract pre cleared, nonimmune IgG; lane B, extract pre cleared, antimonyosin antibody; lane C, extract not pre cleared, antimonyosin antibody; lane D, extract not pre cleared, nonimmune IgG. The position of migration of polypeptides is indicated as follows: H, the heavy chain of myosin; AbH and AbL, the heavy and light chains of immunoglobulin, respectively; A, actin; L1 and L2, the 20,000- and 17,000-dalton light chains of myosin, respectively. The position of migration of a peptide whose identity is not known is indicated (M).
TABLE II

| Relative phosphorylation, % of unstimulated control * |
|-----------------------------------------------------|
| Myosin heavy chain | Unidentified polypeptide | 20,000-dalton myosin light chain |
|---------------------|--------------------------|-------------------------------|
| Exp. 1              | 108                      | 123                           | 149 |
| Exp. 2              | 111                      | 116                           | 114 |
| Exp. 3              | 109                      | 116                           | 137 |
| Exp. 4              | 121                      | 121                           | 134 |
| Avg ± SEM           | 112 ± 3                  | 119 ± 2                       | 133 ± 7 |

Changes in the relative recovery of radiolabeled phosphate associated with polypeptides precipitated by antimonyosin antibody after stimulation of lymphocytes with antibody reactive with cell-surface immunoglobulin.

* Change in phosphorylation in each component precipitated with antimyosin antibody relative to that from unstimulated cell lysates is determined according to the relationship:

\[
\text{Relative phosphorylation} = \frac{3^{2}P}{3^{2}M} \times 100.
\]

The symbols \(3^{2}P\) and \(M\) refer to the quantity of radiolabeled phosphate and the quantity of myosin present, respectively, and are determined as described in Materials and Methods. Subscripts \(s\) and \(c\) refer to stimulated and control cultures, respectively.

To test for any dephosphorylation of myosin during the rapid isolation procedure, we added purified myosin, which had been previously radiophosphorylated by the lymphocyte kinase, to lymphocytes and reisolated it after lysis of cells and immunoprecipitation. The recovery of labeled phosphate on myosin added to cells was 70% as high as that recovered in the absence of cells. Thus, under the conditions employed, the myosin recovered in the immunoprecipitate should retain an appreciable fraction of the phosphate present on the myosin in cells.

Observation of Lymphocyte Motility

The motility of murine lymphocyte populations enriched for B cells was observed in order to confirm the reported stimulation of locomotion of such cells by treatment with antibody reactive with their cell surface immunoglobulin (35). Motility of lymphocytes was determined as described in Materials and Methods. An affinity-purified preparation of goat antibody with specificity for murine immunoglobulin, added to a concentration of 4 \(\mu\)g/ml, consistently increased the fraction of motile lymphocytes about threefold from 10–20% motile before stimulation to 30–60% motile 1 h after addition of antibody. Short-term culture of lymphocytes in medium lacking phosphate had no evident effect upon cell viability, morphology, motility, or stimulation of motility with goat antibody reactive with murine immunoglobulin.

Changes in the Relative Quantity of Radiolabeled Phosphate Isolated in Association with Lymphocyte Myosin

The technique for assessing the state of phosphorylation of myosin in lymphocytes by rapid immunoprecipitation was then applied to populations of lymphocytes enriched for B cells, and treated for 1 h with 4 \(\mu\)g/ml goat antibody reactive with murine immunoglobulin. The relative amount of radiolabeled phosphate recovered on the heavy chain of myosin, the 20,000-dalton light chain, and the unidentified polypeptide isolated in association with myosin was determined, and normalized using the intensity of the Coomassie Blue stain taken up by the myosin heavy chain of the same samples. The results indicate an increase in radiolabeled phosphate associated with the regulatory light chain and unidentified polypeptide obtained from B cells treated with antibody, and a small but reproducible increase in radioactivity migrating in the position of the heavy chain (Table II). The distribution of \(^{32}\)P in polypeptides present in a single immunoprecipitate of the type analyzed in this experiment is illustrated in Fig. 3D.

DISCUSSION

We have observed phosphorylation of both the heavy chain and 20,000-dalton light chain of lymphocyte myosin catalyzed in vitro, and of myosin phosphorylated in intact cells. The results suggest that phosphorylation of myosin observed in vitro may be of physiological significance. Yet, it will be informative to compare the amino acid residues modified on both the heavy chain and 20,000-dalton light chain of myosin catalyzed either in vitro or in intact cells. We have developed a method for rapid immunoprecipitation of myosin from cell extracts, since we were not able to detect phosphorylation of myosin using a conventional procedure for immunoprecipitation (22). This technique is 10 times faster than techniques previously described, and is generally applicable to the study of biosynthesis, processing, and modification of proteins in cells.

Turkey gizzard myosin light-chain kinase and bovine heart phosphoprotein phosphatase have been used to add and release phosphate from myosin in vitro. Analysis of the myosin samples modified by these enzymes indicates that phosphorylation of myosin is important for activation of the ATPase activity of myosin by actin. It is consistent to postulate from these results that the phosphorylation of myosin is a possible mechanism for regulation of contractile activity in lymphocytes, since actin-activated ATPase activity has been correlated with contractile activity for myosin isolated from other cell types (3, 26). However, it is difficult to estimate the quantitative effect of phosphorylation of myosin on the level of actin-activated ATPase activity in cells.

The effects of phosphorylation on the actin-activated ATPase activity of myosin in vitro must be at least partially due to changes in phosphorylation of the 20,000-dalton light chain, since (a) phosphorylation of myosin by turkey gizzard myosin light-chain kinase occurred almost exclusively on this polypeptide, and (b) 80–90% of the radiolabeled phosphate covalently bound to myosin phosphorylated by lymphocyte kinase(s) present in the actomyosin fraction is present on the 20,000-dalton light chain. However, the myosin might also bear unlabeled phosphate of cellular origin. Thus, phosphorylation of the heavy chain could modulate actin-activated ATPase activity, or formation of myosin filaments, as described for myosin isolated from D. discoideum (23). This speculation must be viewed with caution, however, since the site(s) of phosphorylation of the heavy chain of lymphocyte myosin have not been determined.

We have also identified a kinase and phosphatase, present in the actomyosin fraction of lymphocytes, which recognize lymphocyte myosin as substrate. The terms myosin kinase and myosin phosphatase have not been applied to these enzymes, since their catalytic efficiency on other potential substrates has not been tested. The kinase present in lymphocyte actomyosin which phosphorylates lymphocyte myosin does not show de-
pends on calcium or calmodulin for activity. Although the kinases present in smooth and skeletal muscle, platelets, brain, and fibroblasts which recognize myosin as a substrate are regulated by calcium (9, 10, 19, 43), other reports of a calcium-independent kinase have appeared (11, 40). It has been suggested that these calcium-independent enzymes are the products of partial proteolytic digestion of the intact enzyme (3). This issue is not resolved at the present time, since the possibility of proteolysis of the lymphocyte kinase has not been excluded. In addition, preliminary observations indicate that the quantity of radiolabeled phosphate isolated in association with the 20,000-dalton light chain of lymphocyte myosin is decreased by 40% after exposure of lymphocytes to the ionophore, A23187 (0.5 or 5.0 μg/ml) for 5 min (Fechheimer and Cebra, unpublished observation). Additional experiments are required in order to determine whether the phosphorylation of the 20,000-dalton light chain of myosin is stimulated by calcium in intact lymphocytes.

The rapid immunoprecipitation technique has been used to show that the quantity of radiolabeled phosphate found in association with the 20,000-dalton light chain of lymphocyte myosin is increased after stimulation of motility induced by treatment of lymphocytes with antibody reactive with surface immunoglobulin. This alteration in the quantity of radiolabeled phosphate associated with the 20,000-dalton light chain of lymphocyte myosin could be indicative of a similar shift in the stoichiometry of modification. However, a change in specific activity of the cellular phosphate pool, or a change in the rate of turnover of phosphate on myosin, could also affect the quantity of radiolabeled phosphate isolated. Assay of the stoichiometry of phosphorylation by electrophoretic separation of phosphorylated and nonphosphorylated forms of the 20,000-dalton light chain might allow discrimination among the hypotheses presented (13, 31).

If the increase in radiolabeled phosphate associated with the 20,000-dalton light chain which is induced by treatment of lymphocytes with antibody reactive with surface immunoglobulin is due to a shift in the stoichiometry of phosphorylation, then one might presume that the myosin is more competent to form filaments, or to split ATP in the presence of actin. Since treatment of lymphocytes with antibody reactive with immunoglobulin also increases the motility of these cells (35), it would be consistent to postulate that phosphorylation of the 20,000-dalton light chain induced by the same treatment is at least one mechanism by which the motility is regulated.

We have also observed that a phosphorylated polypeptide of 22,000 daltons is specifically selected from cell extracts during rapid immunoprecipitation with antibody reactive with lymphocyte myosin. It is unlikely that this result is due to the presence of antibody molecules with unique specificity for the 22,000-dalton polypeptide, since no evidence supporting this hypothesis was obtained during characterization of the antigen, the antisera, or the antibody purified on an affinity column of myosin lacking this component. It is possible that the unidentified polypeptide is (a) a precursor to one of the myosin light chains, (b) a product of posttranslational modification of one of the light chains of myosin, (c) a polypeptide arising from proteolytic digestion of the heavy chain of myosin, (d) a protein that displays antigenic cross-reactivity with myosin but that is not necessarily the product of a structural gene for one of the subunits of myosin, or (e) a protein that has no antigenic cross-reaction with myosin and is present due to a noncovalent association with myosin in cells. The last hypothesis is consistent with all available evidence if one postulates that this component dissociates from myosin during one of the steps conventionally used in the purification of myosin.

We have described molecular and cellular studies that support the regulation of myosin function by its phosphorylation in lymphocytes. Hypotheses concerning the control of cell motility that are based on molecular analysis in vitro must be tested at the cellular level to determine to the extent to which they do explain the phenomena under investigation. In the future, combined in vitro/in vivo analyses of such processes as the phosphorylation of myosin, the formation of actin and myosin filaments in cells, and the gel-sol transition of the cytoskeleton may contribute to our understanding of the molecular basis of the regulation of the complex movements performed by lymphocytes, as they mediate immune reactions.

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